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Influence of *Staphylococcus aureus* Antibody on Experimental Endocarditis in Rabbits

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To evaluate the potential protective benefit of antibody to whole cells of *Staphylococcal aureus* for the prevention of endocarditis, the rabbit endocarditis model was used. Methicillin-sensitive (17A) and methicillin-resistant (173) *S. aureus* strains were evaluated in rabbits with or without indwelling intracardiac catheters. All immunized rabbits developed significant homologous agglutinating antibody titers (the mean reciprocal titers were 15,300 to strain 17A and 1,150 to strain 173). After challenge, virtually no significant differences were observed between immunized and unimmunized animals with respect to (i) incidence of endocarditis, (ii) concentration of bacteria in infected vegetations, (iii) incidence of metastatic renal abscesses, or (iv) concentrations of bacteria in infected kidneys. The clearance of homologous *S. aureus* strains from blood cultures was similar for immunized and unimmunized animals at 10 to 90 min after intravenous challenge. In vivo adherence of homologous *S. aureus* strains to aortic valves and vegetations was similar in immunized and unimmunized animals when evaluated at 30 and 90 min postchallenge. Even without catheterization, the incidence of bacteremia and renal abscesses was the same in immunized and unimmunized rabbits. Whole-cell-induced *S. aureus* antibody did not prevent or modify any stage in the development of endocarditis in rabbits.

Staphylococcus aureus causes 20 to 30% of all cases of infective endocarditis (IE) (11, 26). It is responsible for the majority of acute IE cases, especially left-sided IE (11, 26), and it is the second leading cause of prosthetic valve endocarditis (25, 33). Despite the availability of bactericidal antibiotics for treatment of infection, the morbidity and mortality of *S. aureus* IE remain high. The case fatality rate of acute left-sided *S. aureus* IE is 30 to 40% (6, 31) and reaches 80 to 90% in selected patient populations (9). Permanent valvular damage often remains in those who survive infection, predisposing them to reinfection or to other cardiac complications.

The rabbit endocarditis model has been used in many investigations of the prevention and treatment of endocarditis (19). Several studies have demonstrated protection against development of endocarditis or early septicemia in rabbits actively immunized with whole cells of *Streptococcus mutans* (7), *Streptococcus sanguis* (7, 21), *Streptococcus pneumoniae* (1), and *Pseudomonas aeruginosa* (2). Prevention of staphylococcal IE has been studied in rabbits with *Staphylococcus epidermidis*, in which no protection with whole-cell antibody was observed (27). There have been no studies with *S. aureus*.

To evaluate immunoprophylaxis of staphylococcal IE, we evaluated the potential protective benefit of whole-cell-induced antibody to prevent or modify *S. aureus* endocarditis in rabbits. In the experimental endocarditis model, we evaluated the influence of active immunization on the clearance of bacteremia, adherence of organisms to valvular endothelium in vivo, and development of infected endocardial vegetations and renal abscesses.

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MATERIALS AND METHODS

Organisms. *S. aureus* 17A and methicillin-resistant *S. aureus* (MRSA) 173 were used. Strain 17A was provided by Per Oeding (University of Bergen, Norway), and MRSA 173 was provided by Francisco Sapico (Downey, Calif.). Both strains were originally obtained from patients (3, 16), and they were maintained on Mueller-Hinton agar slants (BBL Microbiology Systems, Cockeysville, Md.) by monthly in vitro passages.

Immunization schedule. Each organism was grown for 18 h in peptone-yeast extract medium (Difco Laboratories, Detroit, Mich.) at 37°C. The culture was then centrifuged, washed twice with Tris buffer, and suspended in 1% Formalin (wt/vol) (Mallinckrodt, Inc., St. Louis, Mo.) for 24 h. After complete killing was confirmed, the killed whole cells were centrifuged, washed three times, and suspended in normal saline to a turbidity of MacFarland standard 10 ($\sim 3.0 \times 10^9$ bacteria per ml). The killed whole cells were administered to New Zealand White rabbits (2.0 to 2.5 kg) as previously described (16). Briefly, the immunization schedule (intravenous) was as follows: on days 1, 2, and 3, 0.1, 0.2, and 0.4 ml, respectively; on days 9, 10, and 11, 0.4, 0.6, and 0.8 ml, respectively; on days 17, 18, and 19, 0.8, 1.0, and 1.0 ml, respectively. On day 24, a 1-ml intraperitoneal booster dose was given. Blood samples were obtained from each rabbit before immunization and 1 week after the booster injection.

Experimental endocarditis. Two protocols were used for the active protection studies. The first, a modification of the method of Perlman and Freedman (19), is referred to as the catheter-placed model. Briefly, rabbits were anesthetized with ketamine hydrochloride (25 mg/kg; Bristol Laboratories, Syracuse, N.Y.) and xylazine (1.0 mg/kg; Miles Labo-

ratories, Inc., Shawnee, Kans.), and a polyethylene catheter (Becton Dickinson, Parsippany, N.J.) was introduced into the left ventricle via the right carotid artery. Dilutions of an overnight culture of the homologous *S. aureus* strain were injected into a marginal ear vein 24 h later. Quantitative blood cultures were obtained at 24 and 48 h after challenge, and all animals were sacrificed at 48 h with pentobarbital sodium (Abbott Laboratories, North Chicago, Ill.). Aortic valve vegetations and kidney tissue were excised by sterile technique and cultured quantitatively (3). Each of 75 rabbits (36 unimmunized and 39 immunized) was challenged with either 10^7, 10^6, 10^5, 10^4, or 10^3 CFU of the homologous strain (MRSA 173).

A second protocol, described by Scheld et al. (21), was used to evaluate protection to minimize the influence of the indwelling intracardiac catheter; this is referred to as the catheter-removed model. Preliminary studies demonstrated that the shortest possible indwelling time of the intracardiac catheter that resulted in consistent rates of IE was 60 min. Thus, 60 min after left heart catheterization, a 1-ml inoculum of an overnight culture of *S. aureus* was administered via the catheter into the left ventricle; 30 min later, the catheter was removed. Quantitative blood cultures were obtained at 30 min and at 24, 48, 72, and 96 h after inoculation. The animals were sacrificed at 96 h after inoculation, and quantitative cultures of cardiac vegetations and kidney tissue were obtained. The catheter-removed model was used for two experiments with strains 17A and MRSA 173 using two challenge doses. Each of 72 rabbits (34 unimmunized and 38 immunized) was challenged with either 10^7 or 10^4 CFU of the homologous strain. These challenge inocula represented approximately 100% and 10% infective doses, respectively, in the catheter-placed model, as determined from pilot studies in this laboratory.

Bacterial clearance and valvular adherence. Six strain 17A-immunized and six unimmunized animals underwent left heart catheterization by the catheter-removed technique. After a challenge dose of 5×10^7 CFU of strain 17A was administered through the catheter, quantitative blood cultures were obtained at 10, 30, 60, and 90 min after inoculation, and all rabbits were sacrificed (at 30 or 90 min). The aortic valve cusps with any vegetations were removed aseptically and were washed with 1 ml of sterile phosphate-buffered saline to remove nonadherent *S. aureus*. The phosphate-buffered saline wash (nonadherent *S. aureus*) and the infected aortic cusps (adherent *S. aureus*) were cultured quantitatively. The percentage of adherent bacteria for immunized and unimmunized rabbits was calculated as follows: (adherent CFU/adherent CFU + nonadherent CFU) × 100.

Renal abscesses. To assess the effect of immunization on the development of renal abscesses, the six unimmunized and six immunized rabbits described in the adherence studies were evaluated. Quantitative kidney cultures were obtained at either 30 or 90 min postchallenge with strain 17A (3).

In addition, to evaluate the effect of immunization on the development of renal infection without the influence of an intracardiac catheter, 16 uncatheterized rabbits (8 unimmunized and 8 immunized with MRSA 173) were studied. Quantitative blood cultures were obtained at 24 and 72 h postchallenge (2×10^7 CFU of MRSA 173). At 72 h postchallenge, both kidneys and the aortic valves were cultured quantitatively (3).

Serologic methods. Bacterial agglutination titers were performed on sera from all animals. Twofold dilutions of serum were made in microtiter wells with 50 μl of phosphate-buffered saline, and equal volumes of a suspension of the homologous killed whole cells were added (~3×10^9 CFU/ml). Negative control wells with phosphate-buffered saline and the whole-cell suspension alone were assayed in parallel. After incubation at 37°C for 1 h and then at 4°C overnight, the highest serum dilution yielding agglutination was recorded.

To confirm that rises in agglutinating antibody titers postimmunization were not due to nonspecific cell clumping, paired sera (pre- and postimmunization) were tested for development of precipitating antibodies to teichoic acid. Whole-cell sonicates of strains 17A and MRSA 173 were prepared as previously described (4); homologous sera were tested for the presence of precipitating antibodies to teichoic acid by the Ouchterlony double-immunodiffusion technique with immune serum gamma globulin (Gammagee; Merck Sharpe & Dohme, West Point, Pa.) to delineate reference lines of identity with teichoic acid-anti-teichoic acid precipitin bands (4).

Serum was also tested for the presence of specific antibody to staphylococcal protein A. Antibody to staphylococcal protein A was detected by the double-immunodiffusion technique of Ouchterlony (18). Pre- and postimmunization sera from 10 rabbits with high agglutinating antibody titers to strain 17A (≥1:16,000) were assayed with the homologous purified protein A (5). After incubation at 4°C for 48 h, the highest serum dilution demonstrating a precipitin line with protein A was recorded. Negative controls (preimmunization sera with undetectable titers) and high-titer positive controls (rabbits immunized with purified protein A; mean titer, 1:32) were tested simultaneously.

Statistical methods. The Fisher exact test was used to compare the incidence of valvular and renal infections and the Student *t* test was used to compare bacterial concentrations in infected vegetations and renal abscesses in immunized and unimmunized animals. All bacterial concentrations were converted to the corresponding logarithms before statistical testing. For each animal, the logarithms of bacterial concentrations in multiply infected vegetations and kidneys were averaged before comparing groups of immunized and unimmunized animals.

RESULTS

Postimmunization antibody responses. All animals immunized with whole cells of strains 17A and MRSA 173 developed significant agglutinating antibody responses. For strain 17A, the geometric mean titer (GMT) rose from 1:11 preimmunization to 1:15,300 postimmunization. For strain MRSA 173, the GMT rose from 1:5 to 1:1,150 postimmunization. By double immunodiffusion, all preimmune sera were negative for precipitating antibodies to teichoic acids of either strain 17A or MRSA 173. Postimmune serum samples contained precipitating antibodies to the teichoic acid of the homologous *S. aureus* strain (at 1:1 or 1:2 dilutions). The postimmunization sera from 10 rabbits with the highest agglutinating antibody titers (GMT, 1:42,000) demonstrated only low-titer antibody responses to homologous protein A by double immunodiffusion (GMT, 1:2). This antibody response to protein A was minimal compared with immune responses after immunization with purified protein A (GMT, 1:32; unpublished data). These postimmunization antibody responses to specific cell-wall antigens suggested that the postimmunization rises in agglutinating antibody titers were probably unrelated to nonspecific clumping factors.

TABLE 1. Effect of immunization on the development of endocarditis and renal abscesses[a]

Immunization status and *S. aureus* challenge (CFU)	No. of animals	Antibody titer[b]	Endocarditis		Renal abscesses	
			No. (%) of animals[c]	Bacterial concn[d]	No. (%) of animals[c]	Bacterial concn[d]
Immunized						
10^7	8	1,800	8 (100)	8.17 ± 0.65	8 (100)	3.95 ± 0.50[e]
10^6	8	830	8 (100)	8.16 ± 0.37	8 (100)	4.55 ± 0.34[e]
10^5	7	1,160	6 (86)	8.11 ± 0.27[e]	6 (86)	4.23 ± 0.25
10^4	8	760	4 (50)	8.06 ± 0.85	4 (50)	4.08 ± 0.68
10^3	8	336	0	0	0	0
Unimmunized						
10^7	7	88	7 (100)	9.11 ± 0.65	7 (100)	5.22 ± 0.42
10^6	6	63	6 (100)	8.95 ± 0.90	6 (100)	6.76 ± 0.97
10^5	7	54	5 (71)	9.41 ± 0.60	5 (71)	4.26 ± 0.77
10^4	8	80	7 (88)	8.45 ± 0.63	7 (88)	3.89 ± 0.62
10^3	8	44	0	0	0	0

[a] Catheter-placed model, MRSA 173.
[b] Geometric mean of the highest reciprocal titer showing agglutination.
[c] Number and percentage of animals with endocarditis or renal abscesses; for each *S. aureus* inoculum, immunized versus unimmunized, $P > 0.10$, the Fisher exact test.
[d] Geometric mean of the number of bacteria in infected vegetations or kidneys ($\log_{10}$ CFU/g) ± the standard error of the mean.
[e] Immunized versus unimmunized, $P = 0.07$ to 0.08, the Student t test; for all other comparisons, $P > 0.30$.

Effect of immunization on development of *S. aureus* endocarditis. Despite the presence of high agglutinating antibody titers, immunized animals developed endocarditis and metastatic renal abscesses at rates similar to those of unimmunized controls in both the catheter-placed and catheter-removed models (Tables 1 and 2). For both models, there were no significant differences between immunized and unimmunized animals with respect to (i) proportion of animals developing endocarditis, (ii) bacterial concentrations in the infected vegetations, (iii) incidence of renal abscesses, and (iv) bacterial concentrations in renal abscesses.

The frequency of positive blood cultures during the postchallenge period was virtually identical between immunized and unimmunized animals for both models (data not shown).

Effect of immunization on the clearance of bacteremia and the adherence of *S. aureus* to aortic valves. Bacterial clearance of *S. aureus* from blood cultures was essentially identical in immunized and unimmunized rabbits at 10 to 90 min postinoculation. Bacterial concentrations in blood were 150 to 200 CFU/ml at 10 min and 10 to 25 CFU/ml at 90 min and were equivalent in both immunized and unimmunized groups.

Although there was slightly less in vivo adherence of *S. aureus* to aortic valves and vegetations in immunized versus unimmunized animals, these differences were not statistically different (58.5 versus 71.2% adherence, respectively).

Effect of immunization on renal infection. In rabbits with catheter-induced aortic valve trauma, the early-postchallenge mean bacterial concentrations in the kidneys were not significantly different in immunized and unimmunized rabbits (mean $\log_{10}$ CFU/gm, 2.56 versus 2.01 at 30 min postchallenge, respectively; 2.32 versus 2.24 at 90 min, respectively; $P > 0.05$ for all comparisons).

In addition, most uncatheterized animals challenged intravenously with 2×10^7 *S. aureus* developed metastatic renal abscesses independent of their immunization statuses (75 to 88%; Table 3). Even in the absence of catheter-induced valvular trauma and endocarditis, persistent bacteremia was noted in 38 to 50% of these latter animals as late as 72 h postchallenge. The mean bacterial densities of *S. aureus* in

TABLE 2. Effect of immunization on the development of endocarditis and renal abscesses[a]

Immunization status and *S. aureus* strain	No. of animals	Antibody titer[b]	Endocarditis		Renal abscesses	
			No. (%) of animals[c]	Bacterial concn[d]	No. (%) of animals[c]	Bacterial concn[d]
Immunized						
17A[e]	21	17,100	20 (95)	7.97 ± 0.48	21 (100)	4.71 ± 0.43
173[e]	7	1,900	7 (100)	9.24 ± 0.91	6 (86)	5.69 ± 0.21
173[f]	7	1,600	0	0	0	0
Unimmunized						
17A[e]	15	24	13 (87)	8.25 ± 0.45	15 (100)	5.48 ± 0.28
173[e]	10	135	10 (100)	8.77 ± 0.51	8 (80)	5.00 ± 0.44
173[f]	9	118	0	0	0	0

[a] Catheter-removed model.
[b] Geometric mean of the highest reciprocal titer showing agglutination.
[c] Number and percentage of animals with endocarditis or renal abscesses.
[d] Geometric mean of the number of bacteria in infected vegetations or kidneys ($\log_{10}$ CFU/gram) ± the standard error of the mean; for each *S. aureus* strain, immunized versus unimmunized, $P > 0.10$, the Student t test.
[e] *S. aureus* challenge dose, 5×10^7 CFU.
[f] *S. aureus* challenge dose, 9×10^4 CFU.

TABLE 3. Effect of immunization on the development of renal abscesses in uncatheterized rabbits

Immunization status	No. of animals	Antibody titer[a]	Blood culture[b]		Renal culture	
			24 h	72 h	No. (%) of animals[c]	Bacterial concn[d]
Immunized	8	2,360	5/8 (63)	4/8 (50)	6/8 (75)	2.45 ± 0.49
Unimmunized	8	ND[e]	5/8 (63)	3/8 (38)	7/8 (88)	3.27 ± 0.69

[a] Geometric mean of the highest reciprocal titer showing agglutination.
[b] Number and percentage of positive blood cultures per number of cultures obtained.
[c] Number and percentage of animals with renal abscesses.
[d] Geometric mean of the number of bacteria in infected kidneys ($\log_{10}$ CFU/g) ± the standard error of the mean; immunized versus unimmunized, $P > 0.30$, the Student t test.
[e] Not done.

the kidneys of these animals were similar in the immunized and unimmunized groups (Table 3).

DISCUSSION

The pathogenesis of endocarditis involves several sequential phases: (i) development of bacteremia initiated from an extracardiac site, (ii) adherence of bacteria to valvular endothelium or a thrombotic lesion (sterile vegetation), (iii) proliferation of bacteria within the vegetation, and (iv) hematogenous dissemination of bacteria resulting in metastatic infection (kidneys, lungs, brain, etc. [32]). The rabbit endocarditis model (19) permits study of the later phases in the pathogenesis of endocarditis, including adherence of bacteria to traumatized valves, development of infected vegetations, and dissemination of bacteria to other organs. However, since organisms are injected intravenously, this model does not permit study of the initiation of bacteremia.

The present study was designed to examine the potential protective effect of antibody induced to *S. aureus* whole cells against the development of endocarditis in the rabbit model. Two standard induction models for experimental endocarditis were used, the catheter-placed model of Perlman and Freedman (19) and the catheter-removed model of Scheld et al. (21). We evaluated both methods because of concern that prolonged use of the indwelling intracardiac catheter would create a lesion susceptible to infection independent of the presence of antibody. We also used two clinically derived *S. aureus* strains to confirm that the experimental results were not strain dependent. Agglutinating antibodies afforded no protection against the development of experimental *S. aureus* endocarditis caused by the homologous strain in either model. With any endpoint of protection, antibody provided no significant protective efficacy. These endpoints included (i) frequency, magnitude, and clearance of bacteremia, (ii) incidence of infected cardiac vegetations, (iii) mean bacterial concentrations in the vegetations, (iv) incidence of renal abscesses, and (v) mean bacterial concentrations in the renal abscesses. A type II statistical error may exist with the 10^4 challenge dose (Table 1); however, this possibility does not change the overall conclusions reached by this study.

Although protection against experimental endocarditis has been demonstrated with antibodies to *S. pneumoniae* (1) and *S. sanguis* (7, 21), we could not show analogous protection for *S. aureus*. To understand this disparity, we examined selected phases involved in the pathogenesis of endocarditis, including (i) initial bacteremia, (ii) adherence of organisms to aortic valves in vivo, and (iii) metastatic infection.

Adler et al. (1) showed that enhanced blood bacterial clearance was the critical factor in the prevention of experimental pneumonococcal endocarditis. Immunized rabbits cleared their bacteremia within 1 h of an intravenous challenge of $>10^8$ CFU of the homologous strain, whereas unimmunized controls remained bacteremic for greater than 4 h postchallenge. In the present study, staphylococcal antibodies had no effect on the course of bacteremia; we found no significant differences between immunized and unimmunized rabbits in the frequency or magnitude of bacteremia or in their ability to clear the bacteremia early (10 to 90 min) or late (24 to 96 h) postchallenge.

Another important step in the induction of endocarditis is the adherence of bacteria to sterile vegetations (13, 22). Scheld et al. were able to correlate the prophylactic efficacy of whole-cell *S. sanguis* antibodies with diminished adherence to vegetations in vitro (21). When homologous organisms were preincubated in immune sera, their adherence to platelet-fibrin matrices was markedly decreased. We found no significant difference between immunized and unimmunized rabbits with regard to adherence of *S. aureus* to aortic valves in vivo.

Another potential means whereby antibody could reduce the level of bacteremia and thereby decrease the risk of developing endocarditis would be to prevent metastatic infections in other organs. We have shown that the kidneys were infected as early as 30 min postchallenge in the presence or absence of staphylococcal antibody. Even in uncatheterized animals, most immunized and unimmunized rabbits had renal abscesses by 72 h after intravenous challenge. Therefore, whole-cell antibodies were unable to prevent early renal lodgment with subsequent abscess formation, and thus renal infection may have served as a focus for persistent bacteremia.

The failure of whole-cell immunization to ultimately prevent renal and aortic valvular infections may relate to the nature of the antibodies induced or to pecularities of the rabbit model. Although the rabbit endocarditis model has been used successfully to evaluate antibody protection of several streptococcal strains, it may not be suitable for studying staphylococcal infections. This model requires the production of gross valvular trauma which is subjected to a large intravenous bacterial inoculum. *S. aureus* adheres well to endothelial cells (17) and fibrin thrombi (28) in vitro, possibly increasing the likelihood of inducing endocarditis in this model. It is possible that under these conditions no quantity of antibody would be sufficient to prevent adherence of *S. aureus* to the sterile aortic valve vegetations.

Although high agglutinating antibody titers were induced by immunization with whole cells, it is possible that antibodies to other antigens potentially important in preventing *S. aureus* endocarditis were not induced by this immunization. In support of this hypothesis are the relatively low antibody levels induced to homologous protein A, a quantitatively dominant cell wall protein present in essentially all *S. aureus* strains (12), including the two study strains (5). The anti-protein A antibody response induced by whole-cell immuni-

zation was less than 10% of that seen after immunization with purified protein A. This suggests that protein A may be hidden by other surface antigens or inaccessible in some whole-cell preparations. Additional cell wall antigens potentially important in the pathogenesis and host defense mechanisms of *S. aureus* infections, including peptidoglycan (14, 20, 24, 30) and fibronectin-binding protein (8, 28), may be inaccessible to the immune system in such whole-cell vaccines.

Lastly, it is likely that, with respect to IE, antibodies to *S. aureus* are not a critical determinant of host protection. All humans possess antibodies to a variety of staphylococcal antigens, including teichoic acid and peptidoglycan (10, 29). Normal human sera contain sufficient quantities of these antibodies to allow efficient opsonization of *S. aureus* in vitro (15). In addition, sera from rabbits immunized with heat-killed or live *S. aureus* fail to further enhance opsonophagocytosis of the homologous organism, as compared with normal rabbit serum (23). Since staphylococci are readily phagocytosed in normal sera, it may be that immunization with any cell wall antigen will not appreciably improve opsonophagocytosis or prevent staphylococcal infections.

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