Detection of Anti-Teichoic Acid Immunoglobulin G Antibodies in Experimental *Staphylococcus epidermidis* Endocarditis

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An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of rabbit immunoglobulin G (IgG) antibodies to purified cell wall teichoic acids from the *Staphylococcus aureus* Lafferty strain and three strains of coagulase-negative staphylococci. Significant immunological cross-reactivity occurred only between the teichoic acid of *S. aureus* and one coagulase-negative preparation. The ELISA was used to determine the serum IgG response to *Staphylococcus epidermidis* in a rabbit model of aortic valve endocarditis. Blood samples were drawn before inoculation and then every 5 days until death or sacrifice at 32 to 35 days postinoculation. Valve vegetations were culture positive at autopsy in 16 (59%) of the 27 catheterized rabbits. Antibody titers in this culture-positive group and the culture-negative group began to rise as early as day 6. Although both groups demonstrated an antibody response, the culture-positive group attained a significantly higher titer on days 26 and 31. Antibodies also rose in a control group of rabbits without a heart catheter but which were inoculated with bacteria. Again, the antibody titer was significantly less than that for the culture-positive group. This ELISA may be useful for the diagnosis of coagulase-negative staphylococcal infections in humans.

Cell wall teichoic acids of coagulase-positive staphylococci (*Staphylococcus aureus*) are composed of α- and/or β-N-acetylglucosamine ribitol teichoic acids (13). Elevated serum levels of antibodies to ribitol teichoic acid are associated with endocarditis or other serious infections due to *S. aureus* (2, 11, 20). Coagulase-negative staphylococci are increasingly recognized as pathogens in prosthetic valve endocarditis, cerebrospinal fluid shunts, and intravascular catheter infections (1, 4, 18). The cell wall teichoic acids of coagulase-negative staphylococci contain antigenically distinct glycerol teichoic acids (13). A similar serological assay for anti-glycerol teichoic acid antibodies may prove to be an aid in the confirmation of coagulase-negative staphylococcal infection.

The ability of an enzyme-linked immunosorbent assay (ELISA) to detect anti-teichoic acid antibodies was investigated in a rabbit model of *Staphylococcus epidermidis* endocarditis. Immunological cross-reaction among the glycerol teichoic acids of coagulase-negative staphylococci and the ribitol teichoic acid of *S. aureus* have been described by several investigators (3, 8, 10, 15). Crowder and White (2) noted that two patients with *S. epidermidis* endocarditis possessed precipitins to ribitol teichoic acid of *S. aureus*. Therefore, we also determined cross-reactivity among three separate coagulase-negative cell wall teichoic acid preparations and *S. aureus* teichoic acid by an ELISA inhibition system.

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**MATERIALS AND METHODS**

*Bacteria.* *S. epidermidis* 1254 was used in the production of experimental endocarditis and as one source of teichoic acid antigen in the ELISA. This staphylococcal strain and others used in this study were obtained from Per Oeding (Bergen, Norway) by Arthur White (Indianapolis, Ind.).

In addition to strain 1254, teichoic acid antigens were prepared from reference staphylococcal strains *S. epidermidis* T-2, *S. saprophilicus* 11997, and *S. aureus* Lafferty. The teichoic acids of strains 1254, T-2, and 11997 were previously described by Oeding (13) to comprise the major antigenic types found among coagulase-negative staphylococci. *S. aureus* Lafferty is commonly used in clinical assays for teichoic acid antibodies in patients with *S. aureus* infections (2). All four strains were catalase positive. Strains 1254, T-2, and 11997 were negative for coagulase activity as determined by a tube test with EDTA-rabbit plasma.
whereas the Lafferty strain was positive. Novobiocin susceptibility was determined by swabbing a plate of P. agard with the organisms and then applying a 5-μg novobiocin disk (BBL Microbiology Systems). The zone of inhibition was measured from the edge of the disk to the start of bacterial growth. The zones of inhibition were 10 mm for strain 1254, 10 mm for strain T-2, 6 mm for strain 11997, and 13 mm for the Lafferty strain. The coagulase-negative strains were further characterized by the Staph-Ident system (Analytab Products, Inc.). Strains 1254 and T-2 showed urea utilization but no phosphate activity; both lacked utilization of mannose, mannitol, trehalose, and salicin. Strain 11997 lacked phosphate activity, utilized urea and mannitol, and did not utilize mannose, trehalose, and salicin.

**Antisera.** Antiserum to the individual staphylococcal strains were produced in rabbits (12). The immunization schedule consisted of three series of three successive days of intravenous injections (10⁶ to 10⁷ CFU) of whole bacteria into the marginal ear vein of the rabbit. Each series was separated from the previous by an interval of 5 days. The rabbits were bled 7 days after the last inoculation. Sera from individual immunized rabbits without pooling were used in the immunodiffusion and ELISA inhibition studies.

**Preparation of teichoic acids.** The procedure of Oeding (13) was used to prepare the teichoic acid antigens. Bacteria were cultured in tryptic soy broth (Difco Laboratories) for 18 h at 37°C on a rotary shaker. The bacteria were harvested from the culture broth by continuous-flow centrifugation. The bacterial pellet was shaken with 0.067 M phosphate buffer, pH 6.5, for 24 h at 37°C and then centrifuged at 4,000 × g for 30 min to remove the remaining whole bacteria. After the pH was adjusted to 4.2 with 1.0 N HCl, the supernatant was refrigerated at 4°C for 24 h. The resulting protein precipitate was removed by centrifugation. Crude teichoic acid was precipitated from the supernatant by increasing the pH to 5.2, adding 5 volumes of ethanol, and refrigerating the mixture at 4°C for 24 h. The crude teichoic acid was then collected by centrifugation, dissolved in distilled water, and lyophilized.

The crude teichoic acid was further purified by ion-exchange chromatography on a DEAE-cellulose (DE-52; Whatman Chemical Separation, Inc.) column. A discontinuous gradient of 0.10, 0.20, 0.30, and 0.40 M potassium chloride in 0.01 M phosphate buffer, pH 7.4, was applied to the column to elute the teichoic acid. The fractions were lyophilized and tested by immunodiffusion against rabbit hyperimmune sera to locate the teichoic acid. Precipitin lines were found consistently with the fractions eluted by 0.20 and 0.30 M potassium chloride.

The teichoic acid-positive DE-52 column fractions were pooled and eluted from an LKB 54-5340 cm (LKB Instruments, Inc.) that was equilibrated with 0.10 M phosphate-buffered saline, pH 7.4. The teichoic acid peak was desalted on a Sephadex G-10 column (Pharmacia Fine Chemicals AB) and lyophilized.

Gel immunodiffusion was used to confirm the identity of the preparations. Immunodiffusion was performed in 1.0% agarose in 0.025 M barbital-borate buffer (pH 8.6–4.0%) dextran T-70 (Pharmacia Fine Chemicals AB). Antigen was placed in the outer wells, and rabbit hyperimmune serum was placed in the center well. Dilutions of the α-glucosyl glycerol teichoic acids from strain 1254 were compared for lines of identity with dilutions of a sample kindly provided by P. Oeding. A line of identity existed between the two antigen preparations when precipitated with rabbit hyperimmune serum to whole live bacteria of strain 1254.

Teichoic acid preparations were also analyzed for immunological determinants by crossed immunoelectrophoresis on glass slides (50 by 75 mm). The first phase used a portion of the slide (50 by 25 mm) containing 1.0% agarose and 4.0% dextran T-70 in 0.025 M barbital-borate buffer, pH 8.6. Antigen (1.0 mg/ml) was placed in wells on the cathodal side, and electrophoresis was performed at a constant voltage of 15 V/cm for 5 h. The remaining unused portion of the slide was then filled with agarose as above but with 25 μl of homologous hyperimmune serum per cm². Electrophoresis was performed perpendicular to the first phase at a constant voltage of 5 V/cm² overnight.

Antigen spectral analysis in the UV range (360 to 190 nm) was performed with a Varian series 634 spectrophotometer. A 1.0-cm light path was used, and samples were dissolved in distilled water. The absorbance of antigen was compared with serial dilutions of bovine serum albumin (Sigma Chemical Co.) and yeast nucleic acids (K and K Laboratories).

**Preparation of peptidoglycan.** S. aureus Lafferty peptidoglycan was prepared by the procedure of Verbrugh et al. (23) and Peterson et al. (17). Crude cell walls were obtained by sonication (Branson Heat System, Inc.) of 20% suspensions of whole bacteria and glass beads in 0.1 M phosphate-buffered saline at 4°C. Glass beads and unbroken organisms were removed by centrifugation at 3,000 × g for 10 min, and the walls were pelleted by centrifugation at 25,000 × g for 20 min. The walls were washed, suspended in 2% sodium dodecyl sulfate, stirred for 16 h, washed, and then treated with DNase, RNase, and trypsin. Another wash, the walls were stirred with 40% phenol for 30 min, washed, treated with 20% trichloroacetic acid at 60°C for 90 min, washed a final three times, and lyophilized. The lyophilized preparation was used in the inhibition studies.

**ELISA.** The ELISA for the determination of rabbit immunoglobulin G (IgG) anti-teichoic acid antibodies was performed as originally described by Engvall and Perlmann (6). All procedures were performed with the EIA PR-50 (Gifford Instruments). The disposable Cuvette-Pak (Gifford Instruments), composed of a polystyrene acrylic copolymer, was used as the surface for antigen coating. Each cuvette was coated with 250 μl of a solution containing the antigen in 0.01 M phosphate-buffered saline–0.005% thimerosal, pH 7.4. The antigen concentration for coating was determined by comparing concentrations of antigen 0.1 to 1,000.0 μg/ml) with the level of absorbance obtained with a range of dilute (1:256 to 1:2,048) hyperimmune rabbit serum. The optimal antigen concentration was the lowest concentration (25 μg/ml) yielding a maximal absorbance. Each cuvette was coated with 250 μl of solution containing the antigen in 0.01 M phosphate-buffered saline–0.005% thimerosal, pH 7.4. The antigen was adsorbed to the cuvette by overnight incubation at 37°C. The cuvettes were sealed to minimize evaporation.

The enzyme conjugate was the horseradish peroxi-
dase-conjugated IgG fraction of goat anti-rabbit IgG (Cappel Laboratories). The optimal conjugate dilution was determined by checkerboard titration of conjugate and hyperimmune sera. A conjugate dilution of 1:10,000 provided a low background absorbance and low interwell variability. The conjugate was diluted in 0.01 M phosphate-buffered saline containing 0.05% Tween 20 and 1.0% bovine albumin (Sigma Chemical Co.) before addition to each cuvette. The conjugate was incubated with the test serum-treated, antigen-coated cuvettes for 1 h at 37°C.

Cuvettes were washed before the addition of test sera, conjugate, and substrate. All washes were done with 0.01 M phosphate-buffered saline containing 0.05% Tween 20 and 0.005% thimerosal, pH 7.4. Wash steps consisted of seven washes with no separate soaking time. The cuvettes were incubated at 37°C for 1 h after the addition of rabbit serum or conjugate.

The substrate was 0.003% hydrogen peroxide with a chromagen of 1.0% o-phenylenediamine in a phosphate-citrate buffer, pH 5.0. We allowed the reaction to proceed for 45 min and then stopped it by adding 100 μl of 4.0 N sulfuric acid. Absorbance was detemined at 490 nm in the 1.0-cm optical path of the Gilford ELISA PR-50.

Doubling dilutions of rabbit serum to be tested were made in 0.01 M phosphate-buffered saline containing 0.05% Tween 20 and 1.0% bovine albumin, pH 7.4. Each serum titer was assayed in triplicate, and the mean value was calculated. Dilutions of serum were considered to be positive when their absorbance exceeded that of the buffer by an optical density of greater than 0.150. Titers were expressed as the reciprocal log of the highest positive dilution.

Reproducibility of the ELISA was monitored by including a standard pooled rabbit serum with each test. A dilution of 1:32 was found to provide a significant absorbance range when assayed by all four teichoic acid antigens. Background absorbance was similarly monitored by including a buffer in antigen-coated cuvettes and serum in buffer-coated cuvettes. The standard deviation of the absorbance of the control rabbit serum was found to be 0.120.

**ELISA inhibition studies.** The four separate teichoic acid antigens and the Lafferty peptidoglycan were used as the absorbing antigens in ELISA inhibition studies. Antigen concentrations ranged from 0.001 to 0.250 mg/ml. The antigens were added to fixed dilutions of hyperimmune rabbit serum prepared against the four staphylococcal strains. Serial dilutions of homologous hyperimmune serum were assayed to determine the optimal dilution for inhibition. Serum dilutions which fell within the linear decrease in absorbance were chosen for the inhibition studies. Dilutions were prepared in 0.01 M phosphate-buffered saline-0.005% thimerosal, pH 7.4, and incubated overnight with absorbing antigens at 4°C. The next morning, the dilutions were centrifuged at 25,000 x g at 4°C for 15 min, and the supernatant was added to an equal volume of 0.01 M phosphate-buffered saline-0.10% Tween 20-2% bovine albumin. Portions were added to cuvettes which had been previously coated with antigen, and the ELISA was performed as usual. An unabsoved antiserum control was run simultaneously. An inhibition system thus consisted of the assay of homologous antisera which had been absorbed with various antigens and antigen concentrations. Percent inhibition was expressed as a percentage of unabsoved control. Curves were calculated, and confidence limits were determined by regression and correlation analyses (5).

**Production of endocarditis.** New Zealand white rabbits weighing between 1.4 and 4.0 kg were used for the production of left-sided endocarditis. Initial nonbacterial thrombotic endocarditis was produced by the introduction of an 18.5-gauge polyethylene catheter (I-Cath; CR Bard, Inc.) into the carotid artery and across the aortic valve. The catheter, without the inner metal guide wire, was left in place for the entire study period. The cervical portion of the catheter was tied off and buried within the neck, and the skin was sutured over it. The rabbits were anesthetized for catheter insertion with intravenous pentobarbital or intramuscular ketamine (Ketalar). Seventy-two hours after catheterization, the rabbits were intravenously inoculated in the marginal ear vein with a 1.0- to 2.0-ml suspension of 10^9 CFU of bacteria. A control group of rabbits that had not had catheter insertion was inoculated intravenously.

Blood was drawn for quantitative culture and serology before inoculation, at 24 h after intravenous inoculation, and then every 5 days until sacrifice at 32 and 35 days postinoculation. Rabbits dying before the end of the study period were autopsied, and their valve vegetations were cultured qualitatively. The vegetations were washed with sterile 0.01 M phosphate-buffered saline, homogenized, and suspended in tryptic soy broth (Difco Laboratories). Vegetations from the rabbits sacrificed at the end of the study were weighed, washed, homogenized, and cultured quantitatively in tryptic soy agar pour plates. Rabbits in which the catheter was found at autopsy to be malpositioned were excluded from further study. Culture isolates were Gram stained and checked for catalase production. Coagulase production was determined by a tube coagulase test.

**Culture methods.** *S. epidermidis* 1254 was grown overnight at 37°C in tryptic soy broth before intravenous inoculation. The cultures were centrifuged, washed with sterile 0.01 M phosphate-buffered saline (pH 7.4), and inoculated intravenously into the rabbits as a 1.0- to 2.0-ml suspension. The number of CFU in each inoculum was determined by inoculating tryptic soy agar pour plates with 10-fold dilutions of the bacterial suspension. Blood samples for culture were withdrawn in 1.0-ml amounts from the central ear artery or marginal ear vein of the rabbits. Culture isolates were identified similar to isolates from valve vegetations.

**Statistical analysis.** Means were expressed as the geometric mean. The standard error of the mean was also calculated. Analysis of differences between groups was performed with a two-tailed Student's t test for unpaired data. The significance of differences in reciprocal log antibody titers at intervals postinoculation was determined for: (i) catheterized rabbits with culture-positive vegetations; (ii) catheterized rabbits which were culture negative at autopsy; and (iii) noncatheterized, inoculated rabbits.

**RESULTS**

Antigenic preparation and inhibition studies. UV spectral analysis of the four teichoic acid
antigens (1 mg/ml) were compared at 280 nm and 254 nm with standard dilutions of protein and nucleic acid. The teichoic acid preparations contained at least less than 1.0% protein and nucleic acids. The antigens were also analyzed by crossed immunoelectrophoresis against antiserum to whole bacteria. These studies indicated that the preparations contained a single major precipitin band. ELISA inhibition studies revealed that the teichoic acid preparations were complex, with more than a single immunodeterminant, and that each preparation possessed a distinctly different immunodeterminant. An homologous inhibition system comprised cuvettes coated with teichoic acid prepared from the strain used to raise the antiserum in the system. Thus, the strain 1254 system used 50 μg of strain 1254 teichoic acid per ml to coat the cuvettes, and the strain 1254 antiserum was absorbed with known concentrations of the inhibiting antigens: teichoic acids (strains 1254, T-2, 11997, and Lafferty) and strain Lafferty peptidoglycan. Table 1 shows the antigen concentrations required to achieve 50.0% inhibition in the ELISA system with antiserum to whole bacteria. The primary immunodeterminant for each system was contained in the teichoic acid antigen from the strain used to prepare the antiserum. The teichoic acid from the Lafferty strain was also able to achieve 50.0% inhibition in the strain 1254 system at the maximum concentration (0.250 mg/ml). The primary antigen in each system and strain Lafferty teichoic acid in the strain 1254 system were the only instances in which 50.0% inhibition was achieved. Although the other antigens in each system showed some inhibition, they were much less than 50.0% as shown in the strain 1254 inhibition system (Fig. 1).

Production of endocarditis. A total of 27 rabbits with left heart catheters were inoculated with a suspension containing a mean of 10^9 CFU (10^6.2 to 10^10 CFU) of S. epidermidis 1254. Of the 27 rabbits, 17 died before the end of the study period. Positive vegetation cultures were obtained from 15 of these 17 rabbits. An additional rabbit sacrificed on day 35 was also positive. Thus, a total of 16 (59%) of the rabbits had culture-positive valvular vegetations. The mean period of survival was 20.8 days.

No bacteria were found in quantitative cultures of vegetations from 9 of the 10 rabbits that survived 31 to 35 days and from 2 of the rabbits that died before the completion of the study period. Therefore, 11 (41%) of the 27 rabbits were culture negative at autopsy.

Blood cultures were positive at 24 h in all 27 rabbits. Cultures taken on day 6 or 11 were usually negative or contained less than 4.0 CFU/
ml. Quantities were greater further into the study period, with a mean value of $10^3$ CFU/ml on day 26. Culture-negative rabbits were negative after the first (24-h) postinoculation culture.

The control group of seven rabbits that had not been catheterized but had been inoculated with $10^6$ CFU of S. epidermidis 1254 survived until sacrificed at 32 days postinoculation. Quantitative blood cultures at 24 h were positive for six of the seven rabbits. One rabbit remained positive until day 11. Portions of valvular tissue obtained at autopsy were culture negative.

**Antibody response in noncatheterized rabbits.**

Figure 2 shows the antibody titer in the seven rabbits which received only an intravenous inoculation of strain 1254. A rise in IgG antibody titers to all four of the teichoic acid antigens began by day 6 and continued to rise until a plateau was reached on day 16. The titer rise to the strain Lafferty antigen was greater than that to the antigens of strains 11997 and T-2 but significantly less than that to the homologous strain 1254 antigen.

**Antibody response in culture-negative catheterized rabbits.** There were 11 rabbits in this group. Antibody titers began to rise by day 6, with the highest titers being detected with the strain 1254 antigen (Fig. 3). Antibody titers with strain 1254 teichoic acid were significantly greater than those with strains T-2 and 11997 from day 6 throughout the remainder of the study, but greater than that with strain Lafferty only on day 31. The strain Lafferty antigen gave significantly higher titers than did strain T-2 and 11997 antigens throughout the study period.

**Antibody response in culture-positive catheterized rabbits.** Antibody titers to all four antigens rose by day 6, with antibodies to strain 1254 achieving the highest titers (Fig. 4). Antibodies to strain 1254 were significantly higher than those to Lafferty antigen on days 16, 21, and 26. Strains T-2 and 11997 gave significantly lower titers than did strain Lafferty.

**Antibody response to strain 1254 teichoic acid among all groups.** The IgG antibody level rose sharply by day 6 in all three groups. The titer of the culture-negative and control groups did not increase after day 16, whereas the antibody titers of the culture-positive rabbits continued to rise. The culture-positive group reached a statistically higher level than did the culture-negative and control groups on days 26 and 31. Random blood drawn on a group of 32 rabbits without bacterial inoculation revealed a geometric mean reciprocal titer of log$_2$ 5.4.
with both the cell wall and membrane (lipo-
teichoic acids). Both coagulase-positive and co-
agulase-negative staphylococci contain glycerol
teichoic acid within the cell membrane (23). The
cell wall teichoic acid of coagulase-positive
staphylococci (S. aureus) is α- and/or β-N-ac-
tyglucosamine ribitol teichoic acid. The coagu-
lase-negative staphylococci are a much more
heterogenous group. Oeding (13) considers co-
agulase-negative staphylococci with either α- or
β-glucosyl glycerol teichoic acids as S. epider-
midis and those with N-acetylglucosamine gly-
cerol teichoic acid as S. saprophyticus. Other
types and also nontypable strains of coagulase-
negative staphylococci may contain these
teihoic acids, especially the N-acetylglucosamine
glycerol teichoic acid of strain 11997 (14).

Strain 1254 was originally obtained from the
nasal mucosa of a carrier and was first described
by Losnegard and Oeding (9) in 1963. Although
chemically characterized as containing α-gluco-
syl glycerol teichoic acid (9), strain 1254 also
showed cross-precipitation with a strain of S.
aureus (10). Strain T-2 was characterized by Davison and Baddiley (3) and also Oeding et al.
(13) as containing β-glucosyl glycerol teichoic
acid with minor amounts of glucosamine resi-
dues. Strain 11997 was reported by Johnsen et
al. (7, 8) to contain predominantly β-N-acetyl-
glucosamine glycerol teichoic acid with small
amounts of α-linked residues. The Lafferty
strain of S. aureus is frequently used in clinical
assays for teichoic acid antibodies and contains
both α- and β-N-acetylglicosamine ribitol tei-
choic acids (2). Thus, teichoic acids prepared
from this collection of organisms comprise the
majority of antigenic types found among coagu-
lase-positive and coagulase-negative staphylo-
cocci (13).

As expected, each strain was found to possess
a distinct, antigenic determinant. The prepara-
tion from strain 1254 possessed a separate or
shared determinant with the S. aureus Lafferty
teoichoic acid. This appears to be responsible for
the similarity in anti-teichoic acid antibody titers
detected in the groups of rabbits by both anti-
gens.

Experimental S. epidermidis endocarditis is
well suited to the study of the kinetics of the
serum antibody response because of the rela-
tively long period of survival of infected an-
imals. In contrast, the mean survival of rabbits
with aortic valve endocarditis due to S. aureus is
less than 8 days (16, 22). Wheat et al. (22) used a
rabbit model of S. aureus endocarditis in evaluat-
ing an assay for the detection of serum staphyl-
ococcal antigen and antibody. They reported a
small antibody response in 3 of 12 rabbits. The
mean length of survival of the groups was only
2.7 days.
Our study showed that serum IgG anti-teichoic acid antibody appeared after intravenous inoculation of *S. epidermidis* into rabbits. Antibody titers among catheterized rabbits were greater in culture-negative rabbits than in the culture-negative control groups. There was minimal cross-reactivity with antigens prepared from two other coagulase-negative strains in an ELISA inhibition assay, but major immunological cross-reactivity occurred with *S. aureus* Lafferty teichoic acid.

The ELISA described herein could be used in the diagnosis of human infections caused by both coagulase-positive and coagulase-negative staphylococci. The teichoic acid antigens chosen cover the range of most staphylococcal infections. The next steps in our studies will be to investigate the use of this assay in the confirmation of human staphylococcal infections.

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LITERATURE CITED


