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Production of Experimental Endocarditis by Coagulase-Negative Staphylococci: Variability in Species Virulence

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The abilities of 11 coagulase-negative staphylococcal isolates of human origin to establish infective endocarditis in a rat model were compared. Seven of the strains were *Staphylococcus epidermidis*, the species most commonly causing endocarditis in humans. The other four strains were *Staphylococcus hominis*, an infrequent cause of endocarditis. Four of the seven strains of *S. epidermidis* and two of the four strains of *S. hominis* elaborated surface slime. With an inoculum of 10^7 colony-forming units, endocarditis was established in all of 77 rats injected with *S. epidermidis* but in only five (12.5%) of 40 animals injected with *S. hominis* ($P < .001$). Strains of *S. epidermidis* were significantly more resistant to phagocytic killing in vitro than were strains of *S. hominis*. These data indicate that there are major differences in the virulence of various species of coagulase-negative staphylococci, both in vivo and in vitro, and that these differences are reflected in the observed incidence of disease caused in humans by the respective species.

Although *Staphylococcus aureus* has been recognized for many decades as a potentially lethal pathogen in humans, the capabilities of coagulase-negative staphylococci as opportunistic pathogens are just now being fully realized [1–4]. Perhaps the most serious manifestation of infection caused in humans by coagulase-negative staphylococci is bacterial endocarditis superimposed on prosthetic heart valves [5, 6]. Previous studies have examined the prophylaxis and treatment of experimental endocarditis due to coagulase-negative staphylococci [7–9], but no study has focused on the relative pathogenicity of different species and strains in the production of experimental endocarditis. In particular, the comparative virulence of newly described staphylococcal species [10, 11] has not been examined. For example, both *Staphylococcus epidermidis* and *Staphylococcus hominis* are common skin commensals. The former species, however, causes the great majority of endocardial and bacteremic infections in humans [12–14]. Moreover, we recently reported that surface slime is associated with coagulase-negative staphylococcal strains causing iv catheter-associated bacteremia [15].

Using strains from that study (and one additional strain from a patient with endocarditis), we undertook the following investigation. We compared the abilities of pathogenic and commensal strains of *S. epidermidis* and *S. hominis*—both slime-producing and slime-nonproducing—to produce catheter-induced infective endocarditis in a rat model. Our experimental results support the clinical impression that *S. epidermidis* is indeed a more virulent species than is *S. hominis*. The results, however, are inconclusive concerning the importance of slime as an appreciable virulence factor in this model.

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Materials and Methods

Microorganisms. Ten of the 11 strains used in this study were collected as previously described [1, 16]

in 1979 and 1980 during the investigation of an outbreak of coagulase-negative staphylococcal sepsis at the City of Memphis and University of Tennessee hospitals. The bacteriologic evaluation of this collection is reported elsewhere [16]. Because of questions concerning the validity of species identification by the API Staph-Ident system (Analytab Products, Plainview, NY), all strains were examined in blind fashion by Dr. W. E. Kloos (North Carolina State University, Raleigh, NC). Two strains, RP62A and RE19, that had been identified as *S. hominis* by the API Staph-Ident system were reidentified as phosphatase-negative *S. epidermidis* by Dr. Kloos on the basis of colonial morphology, colony size, and anaerobic growth in thioglycollate. The strains were also examined with the DMS Staph-Trac System (DMS Laboratories, Flemington, NJ) according to the manufacturer's instructions; the resulting identifications agreed with those of Dr. Kloos. The remaining strain used in this investigation, strain PC, was a gift from Dr. A. W. Karchmer, (Harvard Medical School, Boston).

Production of endocarditis. We utilized a technique similar to that described by Santoro and Levison [17] for the production of endocarditis in male Wistar rats (Harlan, Sprague-Dawley, Indianapolis). After general anesthetization of rats with a ketamine-xylazine solution and under visualization through a dissecting microscope, a polyethylene catheter (Intramedic PE10; Clay-Adams, Parsippany, NJ) was passed into the left ventricular cavity via the right common carotid artery. Vigorous pulsation of the catheter indicated appropriate positioning of its tip at the left ventricular apex. The catheter was tied in place and the incision closed. After 48 hr the rats were again anesthetized, and 1 ml of the bacterial inoculum was injected through a tongue vein. The rats were killed 96 hr later. At that time 4–5 ml of blood was drawn from the external jugular vein(s) for blood cultures. The anterior rib cage, heart, and proximal portion of the major vessels were resected. The aorta and left ventricle were opened, and both the position of the catheter and the presence or absence of vegetations were noted. Vegetations were excised, weighed, and homogenized with a mortar and pestle in 1 ml of PBS. Catheters were removed and placed in a test tube of trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md) for culture.

Bacteriologic techniques. After rejuvenation from frozen or lyophilized stock cultures, all strains were maintained on trypticase soy agar with 5% sheep blood. Because autoagglutination of the slime-producing strains occurred in most broth media and saline suspensions, all organisms were prepared for inoculation by a modified method. After cultivation on plates of 5% sheep blood agar for ~18 hr, the bacteria were removed from the surface and suspended in sterile PBS (0.005 *M* phosphate–0.15 *M* NaCl, pH 6.8). In this buffer, slime-producing strains formed a smooth suspension. Slime-negative strains were prepared in an identical manner. Gram staining of a sample of suspension from all strains showed the majority of organisms to be in pairs when viewed with the microscope under oil immersion. The isolates were washed once at 1,000 *g* for 10 min and resuspended in PBS at a concentration of 10^8 cfu/ml, as determined by turbidimetry with a Klett-Summerson photoelectric colorimeter; they were then diluted further to the desired final concentration. For confirmation of the inoculum size, quantitative colony counts were performed by dilution in PBS and plating onto blood agar.

Blood cultures were processed in two ways. A 1-ml volume of blood was spread onto the surface of a 150-mm plate containing 70 ml of trypticase soy agar (BBL). The remaining 3–4 ml of blood were cultured in 25 ml of trypticase soy broth. All cultures were maintained for a minimum of five days.

After homogenization of the vegetations in 1 ml of PBS, dilutions for quantitative colony counts were made in PBS and plated onto trypticase soy agar that had been supplemented with 5% sheep blood. Colonies were counted at 48 hr, and numbers of organisms per gram of vegetation were then calculated. The portion of the vegetation suspension that had not been used in the preparation of dilutions was cultured in 5 ml of trypticase soy broth. All staphylococcal strains from all specimens (blood, catheter, or vegetation) were identified as catalase-positive, gram-positive cocci and were confirmed to be the inoculated strain through comparison of antimicrobial susceptibility patterns by the Kirby-Bauer disk-diffusion method [18]. The presence or absence of slime production was checked as previously described [15].

Bactericidal activity of human serum-phagocytic mixtures. Blood samples were obtained from healthy volunteers. A portion was allowed to clot and served as a source of fresh human serum. The remaining 30–40 ml was lightly heparinized (10 units/ml), and leukocytes were separated by sedimentation in 6% Dextran 75 (Travenol Laboratories,

Table 1. Species identification, clinical source, and bacteriologic characteristics of the 11 strains used in this study.

Strain	Staphylococcal species	Clinical source	Slime production	API Staph-Ident system				DMS Staph-Trac system				Routine methods*		
				Phs	Tre	Arg	Code	MDG	NAG	ADH	Code	Gel	D-Man	Phs
RP62A	*epidermidis*	Catheter sepsis	+	−	−	−	2000	−	−	+	6706113	+	−	−
RE19	*epidermidis*	Nasal isolate from nurse	+	−	−	−	2000	−	−	+	6706113	+	−	−
RP12	*epidermidis*	Catheter sepsis	+	+	−	+	3040	−	−	+	6706113	+	−	+
RP23	*epidermidis*	Blood (presumed contaminant)	+	+	−	+	3040	−	−	+	6706113	+	−	+
SP18	*epidermidis*	Blood (presumed contaminant)	−	+	−	+	3140	−	−	+	6726113	+	−	+
SP22	*epidermidis*	Blood (presumed contaminant)	−	+	−	−	3000	−	−	+	6706113	+	−	+
PC	*epidermidis*	Blood from patient with endocarditis	−	+	−	−	3000	−	−	+	6706113	ND	ND	ND
RP7	*hominis*	Catheter sepsis	+	−	−	−	2000	+	+	+	6616173	−	−	−
RP14	*hominis*	Indeterminate	+	−	−	−	2000	+	+	−	6616172	−	−	±
SP2	*hominis*	Blood (presumed contaminant)	−	−	+	−	2400	−	−	−	6616112	−	−	±
RP60	*hominis*	Blood (presumed contaminant)	−	−	+	−	2400	−	−	−	6236112†	−	+	−

NOTE. For simplicity, only the reactions that varied within species are noted in this table. A plus sign denotes a positive reaction, a minus sign a negative reaction, and a plus-or-minus sign a weakly positive reaction. The designation ND indicates that the respective test was not done. Phs = alkaline phosphatase; Tre = aerobic acid formation from trehalose; Arg = aerobic utilization of arginine; MDG = oxidation of α-methylglucoside; NAG = oxidation of N-acetylglucosamine; ADH = anaerobic hydrolysis of arginine; Gel = production of gelatinase; and D-Man = aerobic acid formation from D-mannitol.

* The methods are detailed elsewhere [16].

† Strain RP60 was also negative for lactose and positive for mannitol utilization, unlike the other three strains of *S. hominis* tested.

Deerfield, Ill) at room temperature for 60–90 min. The cells were washed twice with lightly heparinized (1 unit/ml) isotonic saline and resuspended to a concentration of 10^7 PMNLs/ml in Hanks' balanced salt solution (HBSS; Grand Island Biological, Grand Island, NY) with 0.1% gelatin. After overnight growth on fresh blood agar plates (BBL), the test organisms were removed from the agar surface with a sterile cotton swab, suspended in PBS, and adjusted turbidimetrically to a concentration of 10^8 cfu/ml. The phagocytic test mixture consisted of 10^7 cfu of staphylococci, 5×10^6 PMNLs, and 0.2 ml of fresh human serum from the PMNL donor. The final volume was adjusted to 1 ml with HBSS with 0.1% gelatin. The tubes were incubated at 37 C with end-over-end rotation at 8 rpm. Aliquots were removed at 0, 30, 60, and 120 min for quantitative enumeration by serial dilution and plating techniques.

Statistical evaluation. Differences between the infection rates in various groups of rats were analyzed by the χ^2 test. Differences in mean vegetation weights and mean vegetation bacterial concentrations were evaluated by use of Student's *t* test for unpaired data. Differences in mean magnitudes of bacterial killing in vitro were compared by means of the *t* test for paired data. The 50% infective dose (ID_{50}) was calculated from a reference table [19] derived from the method of Reed and Muench [20].

Results

Establishment of endocarditis. Seven of the strains used in this study were *S. epidermidis* and four were *S. hominis* (table 1). All animals receiving 10^7 cfu of any strain of *S. epidermidis* developed infective endocarditis (table 2). In contrast, only 12.5% of animals injected with any of the four strains of *S. hominis* developed endocarditis ($P < .001$). These infection rates were independent of the presence or absence of detectable slime formation.

Mean weights and bacterial concentrations of vegetations. The mean wet weights of the vegetations from rats infected with slime-positive strains of *S. epidermidis* were greater, but not significantly greater ($P = .10$), than those from rats infected with slime-negative strains of *S. epidermidis*. The mean

Table 2. Characteristics of infective endocarditis in rats challenged with 10^7 cfu of various strains of coagulase-negative staphylococci.

Organism	No. of strains	No. of experiments	No. (%) of rats with endocarditis*	Mean weight (mg) of infected vegetation ± SE	Mean (± SE) bacterial concentration (cfu/g of vegatation)	No. (%) of rats with positive catheter cultures	No. (%) of rats with positive blood cultures†
S. epidermidis							
Slime-positive	4	49	49 (100)	15.9 ± 1.48	$7.40 (\pm 9.80) \times 10^8$	48 (98.0)	43 (91.4)
Slime-negative	3	28	28 (100)	12.0 ± 1.94‡	$9.89 (\pm 3.23) \times 10^8$§	24 (85.7)‖	14 (56.0)#
S. hominis							
Slime-positive	2	14	2 (14.3)	13.5 ± 7.24	$4.86 (\pm 4.66) \times 10^4$	1 (7.1)	0 (. . .)
Slime-negative	2	26	3 (11.5)	6.53 ± 2.45	$1.09 (\pm 0.70) \times 10^8$	1 (3.8)	0 (. . .)

* These animals had vegetations that yielded the inoculated strain when cultured. The rate of development of endocarditis after the injection of *S. epidermidis* was significantly higher than that after the injection of *S. hominis* ($P < .001$).

† Blood from two animals infected with slime-positive strains and from three animals infected with slime-negative strains of *S. epidermidis* was not cultured.

‡ $P = .10$ vs. value for slime-positive *S. epidermidis*.

§ $P > .40$ vs. value for slime-positive *S. epidermidis*.

‖ $P > .10$ vs. value for slime-positive *S. epidermidis*.

# $P < .001$ vs. value for slime-positive *S. epidermidis*.

concentrations of staphylococci per gram of vegetation tissue were similar for all seven strains of *S. epidermidis*, regardless of the ability of the strains to elaborate surface slime.

Only five *S. hominis*–challenged rats developed infective endocarditis: two of the 14 rats infected with slime-producing strains and three of the 26 infected with slime-nonproducing organisms. On the basis of the data in table 2 plus additional results from animals challenged with 10^9 cfu (data not shown), it appeared that slime-negative strains of *S. hominis* produced quite small vegetations. In view of the small numbers of infected animals, no definitive comparisons were made between *S. hominis*–infected and *S. epidermidis*–infected rats with regard to vegetation weights or concentrations of microorganisms in cardiac vegetations.

Colonization of intracardiac catheters. Staphylococci were isolated from the intracardiac catheters of 94% of the rats infected with *S. epidermidis* and from those of 5% of the rats infected with *S. hominis* ($P < .001$). Of animals infected with *S. hominis*, the only two with positive catheter-culture results had developed endocarditis.

Bacteremia. In the majority of cases, blood culture specimens were obtained from the great vessels at the time of sacrifice. Ninety-one percent of rats infected with slime-producing *S. epidermidis* had bacteremia at that time, as opposed to only 56% of rats infected with slime-negative *S. epidermidis* ($P < .001$). None of the five animals with endocarditis due to *S. hominis* had demonstrable bacteremia at the time of sacrifice.

Studies of infective dose. As is shown in table 2, infective endocarditis developed in all 77 rats challenged with 10^7 cfu of *S. epidermidis*. In a further assessment of the role of slime as a virulence factor, two slime-positive and two slime-negative strains of this species were tested at lower inocula. When the challenge inoculum was reduced to 10^6 cfu, infective endocarditis still developed in 100% of animals infected with slime-producing strains RP12 and RE19 and slime-nonproducing strain SP22; in contrast, this infection developed in only one (17%) of six rats given slime-nonproducing strain PC. According to data for inocula ranging from 10^4 to 10^7 cfu, the ID_{50} values for strains RP12, RE19, and SP22 were equivalent ($10^{4.5}$–$10^{5.0}$), while the ID_{50} for strain PC was much higher ($10^{6.7}$). Therefore, in these limited studies of infectivity, slime per se did not appear to be a critical determinant of virulence.

Phagocytic killing. The observed differences in rates of blood culture positivity among animals with infective endocarditis (table 2) suggested possible differences in the resistance of coagulase-negative staphylococci to phagocytic killing. We therefore studied the killing of eight strains (two slime-positive and two slime-negative representatives of each species) in rotating tubes containing mixtures of normal human peripheral-blood leukocytes and fresh

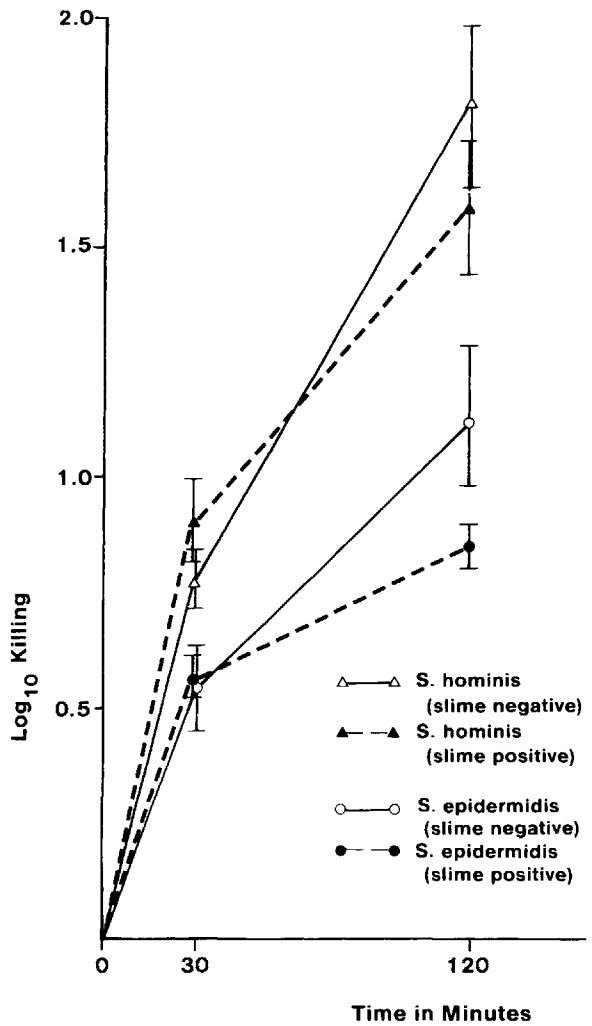

Figure 1. Killing of coagulase-negative staphylococci by normal human leukocytes in fresh human serum. Each point represents the mean result of three experiments. Eight strains were used in this comparison: two slime-positive and two slime-negative strains of each species.

serum (figure 1). Killing of *S. hominis* strains was significantly more pronounced ($P < .001$) than that of *S. epidermidis* strains at both 30 and 120 min. The magnitude of killing of slime-positive *S. hominis* did not differ significantly from that of slime-negative *S. hominis*. At 120 min the magnitude of killing slime-negative *S. epidermidis* was significantly greater than slime-positive *S. epidermidis* ($P < .05$). This difference was due entirely to results with strain PC, which was highly susceptible to killing by PMNLs and also had the highest ID_{50} of all strains of *S. epidermidis* tested in the rat model.

Discussion

Staphylococcus epidermidis is the major species of coagulase-negative staphylococci existing on human skin [21], although *S. hominis* is also a common human-skin commensal that is known to colonize the cutaneous surfaces of arms and legs particularly often [21]. Clinical surveys of coagulase-negative staphylococcal infections have found that most cases are caused by *S. epidermidis*, whereas few are attributable to other species [12–14]. It has been postulated [12] that the predominance of *S. epidermidis* in these surveys merely reflects the relative composition of the cutaneous microflora. If so, given an equal opportunity, the other staphylococcal species should display similar virulence. On the other hand, the frequency of *S. epidermidis* may reflect greater virulence. Information on this question is of importance to an understanding of the pathogenesis of these opportunistic infections. Surprisingly, to our knowledge, no attempts have been made to clarify this point by comparison of the virulence of staphylococcal species in animal models.

We chose to compare *S. epidermidis* and *S. hominis* because both species are human skin commensals and appear to be similar when examined by DNA-DNA hybridization techniques [22]. Nevertheless, major differences exist between the two species in respect to morphological, physiological, and biochemical characteristics [10, 11].

The 11 strains used in this study were isolated from various clinical sources. Isolates of both species that were considered to represent documented infections as well as blood culture contamination were examined. One strain, PC, was isolated from the blood of a patient with native-valve endocarditis superimposed on rheumatic carditis.

The findings in this rat model of experimental endocarditis mirror clinical experience with prosthetic valvular infections in humans. *S. epidermidis*, unlike *S. hominis*, is capable of consistently establishing infective endocarditis in the rat. At the inoculum used in this study (10^7 cfu) all 77 rats challenged with seven strains of *S. epidermidis* developed infective endocarditis. *S. hominis*–challenged rats, on the other hand, rarely developed endocarditis regardless of the strain used. Cultures of blood obtained at the time of sacrifice showed a significant difference in positivity rates that was related to species. The majority (79%) of rats with *S. epidermidis* endocarditis had positive blood-culture results, while none

of the five rats in whom we succeeded in establishing endocarditis with *S. hominis* had positive results.

Demonstrable differences in virulence among coagulase-negative staphylococci were not limited to the animal model. In tests of phagocytic killing by mixtures of normal human leukocytes and fresh human serum, the magnitude of killing of *S. hominis* was significantly greater than that of *S. epidermidis.* The basis for such in vivo and in vitro differences between the virulence of *S. epidermidis* and that of *S. hominis* remains unexplained. Some authors [23, 24] believe that phosphatase is an important virulence factor in both coagulase-positive and coagulase-negative staphylococcal strains. Although the strains of *S. hominis* used in this study were all phosphatase-negative, so were two strains of *S. epidermidis.* In addition, the phosphatase-negative strains of *S. epidermidis* were as virulent as the phosphatase-positive strains.

Our results clearly affirm the biologic significance of the speciation system of Kloos and Schleifer [10, 11]. They may also explain the erratic virulence displayed by individual strains of coagulase-negative staphylococci in similar studies in experimental animals [9, 25]. In future studies of infections caused by coagulase-negative staphylococci in experimental animals, speciation will be required.

Previously, we reported that the majority of coagulase-negative staphylococci obtained from patients with intravascular-line sepsis and associated clinical illness produced a slimy material in vitro that mediated their adherence to smooth surfaces [15]. Blood culture contaminant strains and cutaneous isolates, on the other hand, were much less likely to be slime producers. The rat model of catheter-induced endocarditis studied here seems to be analogous to both prosthetic valve endocarditis and catheter-associated sepsis, involving as it does the presence of a foreign body in the vascular system. Nevertheless, *S. epidermidis* strains consistently caused endocarditis and colonized intracardiac catheters, while *S. hominis* strains generally failed to do so, regardless of whether or not they produced slime. Thus, other virulence factors that are yet undefined seem to be operative in the production of this type of intravascular catheter–associated infection.

Some differences were noted between animals infected with slime-positive strains and those infected with slime-negative strains. Nearly all rats with endocarditis due to slime-producing strains of *S. epidermidis* had positive blood-culture results, whereas only 56% of rats infected with slime-negative strains of this species had positive blood results ($P < .001$). Furthermore, certain slime-negative isolates of *S. epidermidis*, such as strain PC, were highly susceptible to phagocytic killing. Similar (though not statistically significant) differences in resistance to phagocytic killing were noted with slime-positive and slime-negative strains of *S. hominis.*

If slime production is a virulence factor in this model, it clearly is not as important as species designation. Further studies with isogenic slime-positive and slime-negative strains of coagulase-negative staphylococci are in progress and will address the issue of slime as a virulence factor.

In summary, these studies demonstrate marked in vivo and in vitro differences between the virulence of two species of coagulase-negative staphylococci. At the challenge inoculum utilized in our experiments, strains of *S. epidermidis* readily initiate infective endocarditis in the rat and colonize intracardiac catheters, while strains of *S. hominis* only rarely do so. Moreover, *S. epidermidis* isolates of human origin are more resistant to phagocytosis and killing by normal human PMNLs than are *S. hominis* isolates.

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