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# Identification of the Innate Human Immune Response to Surface-Exposed Proteins of Coagulase-Negative Staphylococci

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**The presumed host defense against coagulase-negative staphylococci (ConS), recognized pathogens in hosts with compromised immunity or indwelling medical devices, is opsonophagocytosis. Targets for opsonization remain unclear. Using radiolabeling techniques, we identified the surface-exposed proteins of ConS and determined the innate humoral immune responses to them among healthy adults. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of surface proteins extrinsically labeled with $^{125}I$ demonstrated 20 to 30 proteins with molecular weights of 15,000 to >130,000. Five to ten of these proteins were immunogenic and recognized by normal human sera, including predominant 18-, 41-, 48-, and 51-kDa proteins. We also evaluated the humoral response of cancer patients with ConS bacteremia. Patients' sera obtained before bacteremic episodes demonstrated a pattern of reactivity similar to that of normal human sera. When patients' sera obtained after bacteremic episodes were used to determine whether an expanded immune response followed infection, only one of seven showed reactivity with more proteins than seen with the innate response. Western blot (immunoblot) analysis and whole-cell enzyme-linked immunosorbent assays were also evaluated. This study identifies (i) the surface-exposed proteins available for host interaction, (ii) the innate human antibody response to these proteins, and (iii) the immune response of cancer patients with ConS bacteremia.**

Coagulase-negative staphylococci (ConS), especially *Staphylococcus epidermidis*, are important components of skin flora (11). Sera from normal individuals contain antibodies directed toward cell structures of ConS as demonstrated by enzyme-linked immunosorbent assay (ELISA) (21), opsonophagocytosis (6, 20), and immunoblotting (1, 18) techniques. Presumably, these antibodies develop because of constant contact between host and organism and are responsible for maintaining a commensal relationship through opsonophagocytosis. Whether or not surface-exposed proteins serve as targets for opsonization is unknown.

Once considered avirulent and nonpathogenic, ConS has recently become recognized as a pathogen in a variety of hosts, including persons with compromised immunity or indwelling medical devices. Insertion of central nervous system ventricular shunts, vascular catheters, prosthetic heart valves, and other devices through the skin provides a portal of entry for organisms such as *S. epidermidis* (8, 13).

This study was designed to (i) identify surface-exposed proteins of ConS, (ii) characterize the innate humoral immune response of normal persons to such proteins, and (iii) determine whether cancer patients with ConS bacteremia develop an expanded immune response following infection.

(This work was presented in part at the Annual Meeting of the American Society for Microbiology, May 1990 [16].)

## MATERIALS AND METHODS

**Bacteria.** *S. epidermidis* 171A (provided by J. T. Parisi, University of Missouri-Columbia) was used as a prototype. In addition, 14 ConS isolates were identified retrospectively in a review of the microbiological records of seven cancer patients with two or more positive cultures. All isolates were from blood cultures and had been stored in skim milk at −70°C. A commercially available identification system (American MicroScan; Baxter, West Sacramento, Calif.) was used to identify to species and to determine the MICs of antimicrobial agents in vitro for each isolate.

**Modified whole-cell lysates.** Modified whole-cell lysates were prepared as described previously (14) and stored at −20°C.

**Patients.** Seven cancer patients with ConS bacteremia (defined as two or more positive blood cultures with acute onset of fever) were retrospectively identified. The median age of the patients was 13 years, with a range of 2 to 15 years. All patients had central venous catheters. Two were diagnosed with acute lymphocytic leukemia and five were diagnosed with acute myelogenous leukemia. Five patients had an absolute neutrophil count of <100/μl at the time of their first positive blood culture.

**Sera.** Sera collected from 11 healthy adults were stored in 0.5-ml aliquots at −20°C. Serum samples from the cancer patients described above were obtained from a serum bank. These sera were obtained an average of 80 days before (range, 22 to 145) and 55 days after (range, 5 to 151) a ConS-positive blood culture and stored at −20°C.

**Iodination of staphylococcal surface proteins.** Staphylococcal surface proteins were iodinated by the method of Fischetti et al. (9). Briefly, an overnight culture of staphylococci was diluted 1:100 into 10 ml of Todd-Hewitt broth and incubated at 37°C until it reached mid-log phase (0.5 optical density [OD] at 650 nm). The cells were pelleted by centrifugation, washed twice in 50 mM phosphate buffer (pH 7.0), and resuspended in 100 μl of the same buffer. $^{125}I$ (10 μl at 0.5-mCi/10 μl specific activity; Amersham Corp., Arlington Heights, Ill.) was added to the cell suspension together with one enzyme-coated nonporous polystyrene bead (Iodo-Bead; Pierce Chemical Co., Rockford, Ill.). The mixture was allowed to stand at room temperature for 15 min. The reaction was terminated by transferring the cells to a clean tube. The cells were freed from the residual $^{125}I$ by several

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washes in phosphate buffer, and modified whole-cell lysates were prepared as described above and stored at −20°C.

**Radioimmunoprecipitation (RIP).** A 10-μl amount of $^{125}$I-labeled whole-cell lysate was combined with 25 μl of sera in a 1.5-ml microcentrifuge tube, which was incubated at 25°C for 1 h with frequent mixing. A 30-μl portion of protein A-Sepharose C1-4B (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) at 70 mg/ml was added to each tube, and tubes were incubated at 4°C for 1 h with frequent mixing. The protein A-antibody-antigen complex was washed five times with 400 μl of a solubilization buffer (10). The final pellet was suspended in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) digestion buffer (12) and heated at 100°C for 5 min to dissociate the antibody-antigen complex. Protein A-Sepharose was removed by centrifugation, and the supernatant was collected and stored at −20°C. The entire sample was subjected to SDS-PAGE by the method of Lugtenberg et al. (12); gels were dried onto filter paper and subjected to autoradiography by exposure to X-ray film (X-OMAT-AR; Eastman Kodak, Rochester, N.Y.).

**Western blot (immunoblot) analysis.** Modified whole-cell lysates were subjected to SDS-PAGE by the method of Lugtenberg et al. (12), using 10% acrylamide gels. The separated polypeptides were transferred to nitrocellulose membranes (pore diameter, 0.45 μm; Bio-Rad Laboratories, Richmond, Calif.) by electrophoretic transfer in 25 mM Tris–192 mM glycine buffer (pH 8.3), containing 20% (vol/vol) methanol (19). Transfer was performed at 4°C with a current of 0.12 A overnight in a transfer cell (Bio-Rad Trans-blot). Free protein sites were saturated by incubation in 3% (wt/vol) bovine serum albumin (Sigma) in PBS (pH 7.4) for 2 h at 25°C or at 4°C overnight. The nitrocellulose membrane was then incubated at 25°C for 2 h with either normal human sera or patient sera diluted 1:100 in 3% bovine serum albumin–0.05% Tween 20. The membrane was washed three times with PBS plus 0.05% Tween 20. It was then incubated sequentially with goat anti-human immunoglobulin G alkaline phosphatase conjugate diluted 1:1,000 (Sigma) and rabbit anti-goat alkaline phosphatase conjugate diluted 1:1,000 (Sigma); both procedures were performed in 3% bovine serum albumin for 1 h at 25°C with three washing steps in between. The membrane was washed again and incubated for 15 min with freshly prepared mixtures of equal volumes of naphthol ASMX phosphate (0.4 mg/ml in distilled water; Sigma) and fast red TR salt (5 mg/ml in 0.2 M Tris, pH 8.2; Sigma) (2). After color development, the membranes were washed extensively with distilled water.

**Slime layer quantitation.** A spectrophotometric technique (5) was used to quantify the slime layer production of ConS isolates from patients. Briefly, overnight cultures in tryptic soy broth were diluted 1:100 in fresh tryptic soy broth, and 200-μl aliquots were pipetted into sterile microtiter tissue culture plates (Costar, Cambridge, Mass.). After overnight incubation at 37°C, the contents of each well were aspirated, and the well was washed four times with 200 μl of PBS, pH 7.2. Adherent organisms were fixed with Bouin fixative for 5 min, rinsed, and stained with Hucker crystal violet for 5 min. Excess stain was then rinsed off with tap water, and the plates were allowed to dry. The OD at 570 nm of stained adherent bacterial films was read with a microplate reader (Titertek Twinreader; Flow Laboratories, McLean, Va.). Measurements were performed in quadruplicate, repeated three times, and averaged. Isolates were grouped into three classes based on ODs: no slime production (OD ≤ 0.120), moderate slime production (OD > 0.120 but ≤ 0.240), and high slime production (OD > 0.240).

TABLE 1. Epidemiologic comparison of patient isolates

| Patient no. | Isolate | Antibiogram[a] | Slime production |
|---|---|---|---|
| 1 | A *S. epidermidis* | Identical | None |
| | B *S. epidermidis* | | None |
| 2 | A *S. epidermidis* | Moderately different | High |
| | B *S. epidermidis* | | None |
| 3 | A *S. epidermidis* | Markedly different | High |
| | B *S. epidermidis* | | None |
| 4 | A *S. epidermidis* | Moderately different | Moderate |
| | B *S. epidermidis* | | High |
| 5 | A *S. epidermidis* | Identical | High |
| | B *S. epidermidis* | | High |
| | C *S. epidermidis* | | High |
| 6 | A *S. epidermidis* | Moderately different | High |
| | B *S. hominis* | | None |
| 7 | A *S. haemolyticus* | | None |
| | B *S. haemolyticus* | Identical | None |

[a] Each isolate was tested for its susceptibility to 16 antimicrobial agents. Identical = Isolates susceptible to the same antimicrobial agents; moderately different = isolates differ in susceptibility to four or fewer antimicrobial agents; and markedly different = isolates differ in susceptibility to more than four antimicrobial agents.

**ELISA.** Whole organisms were used to coat 96-well plates as described by Campbell (3). The ELISA procedure was modified from Robertson et al. (17). Briefly, the plates were washed three times, 5 min each time, with PBS (pH 7.3) plus 0.05% (vol/vol) Tween 20 (PBS-Tween). A 100-μl amount of PBS-Tween with 1% (wt/vol) bovine serum albumin was added to each well, and plates were incubated for 1 h at 25°C. The plates were washed three times with PBS-Tween as above. Test samples were added at appropriate dilutions at 100 μl/well for 2 h at 25°C, and the plates were washed three times in PBS-Tween. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (γ-chain specific; Sigma) at a 1:1,000 dilution in PBS-Tween at 100 μl per well was added for 1 h at 25°C. The plates were then washed three times with PBS-Tween, and 200 μl of an 11-mg/ml concentration of *p*-nitrophenylphosphate (Sigma) in 10% diethanolamine was added per well. The $A_{405}$ was measured after 15 min.

## RESULTS

**Characterization and surface-exposed proteins of ConS.** One prototypic strain of *S. epidermidis* and 15 clinical ConS isolates were used in this study. Species determination, antibiograms, and slime production were used to compare clinical isolates epidemiologically for strain delineation (Table 1). The surface-exposed proteins of ConS were labeled with $^{125}$I, and whole-cell lysates were subjected to SDS-PAGE and autoradiography. Representative profiles of labeled proteins for strain 171A, isolates 1A, 2A, and 2B, are shown in Fig. 1, 2, and 4, respectively. The majority of strains of *S. epidermidis* possessed similar surface proteins; however, several showed marked differences (15). This is best illustrated by the two *S. epidermidis* isolates 2A and 2B

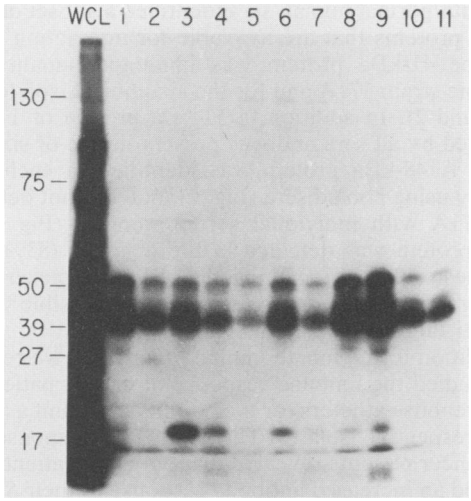


FIG. 1. SDS-PAGE of $^{125}$I-labeled surface-exposed proteins of *S. epidermidis*. WCL, Whole-cell lysate of prototypic strain 171A. Lanes 1 through 11 show labeled proteins precipitated by individual normal human sera by using RIP.

(see Fig. 4, lanes 1 and 5). These differences are supported by the epidemiologic data in Table 1 denoting nonidentical isolates. The surface-exposed proteins of isolates 6B, *S. hominis*, and 7A, *S. haemolyticus*, were markedly different from the *S. epidermidis* strains (data not shown).

**Normal human serologic response to ConS.** To identify surface-exposed epitopes recognized by the normal host, 11 individual sera were used in RIP assays with the prototypic strain 171A. Figure 1 shows that each serum sample recognized proteins of approximately 41 and 51 kDa. Fifty-four percent of the sera also recognized an 18-kDa protein. Due to the uniformity of the response, pooled normal human sera were used to evaluate the innate immune response to a variety of *S. epidermidis* strains. RIP assays were performed with six *S. epidermidis* isolates from patients 1, 2, and 3. Figure 2 compares these paired isolates with the whole-cell lysate of isolate 1A. The major epitope appears to be a 41-kDa protein identified in all isolates. Others identified

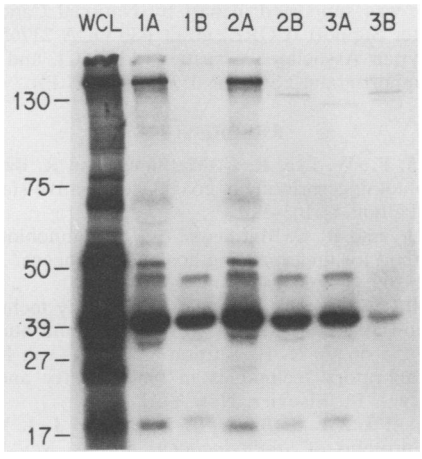


FIG. 2. SDS-PAGE of $^{125}$I-labeled surface-exposed proteins of *S. epidermidis*. WCL, Whole-cell lysate of isolate 1A. Lanes 1A through 3B show labeled proteins of these isolates precipitated by pooled normal human sera, using RIP.

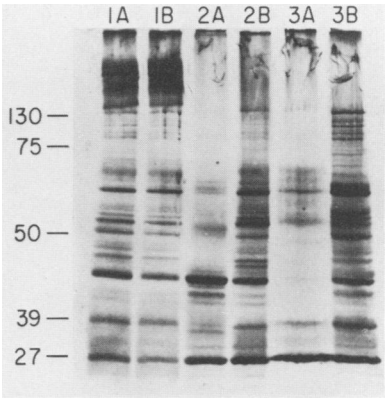


FIG. 3. Western blot analysis showing proteins from isolates 1A through 3B, as detected with normal human sera.

were an approximately 18-kDa protein in all isolates and a 48-kDa protein in 83% of the isolates. Differences between isolates were most noticeable in the very large-molecular-size range (≥130,000) as well as at 36 to 37 and 51 kDa. These differences support findings in Table 1 indicating that the isolates from patients 2 and 3 are different.

The reactivity of normal human sera with these *S. epidermidis* proteins was confirmed by Western blot analysis (Fig. 3). This technique detected several dominant proteins for each isolate and additional proteins of variable molecular weights. With this technique, isolates 1A and 1B appear to be identical, whereas isolates 2A and 2B and isolates 3A and 3B are different. Differences among isolates apparent in the very high-molecular-weight range (>130,000) by RIP were not detectable by Western blot analysis.

**Serologic responses of cancer patients to ConS.** Sera from cancer patients that corresponded to times before and after positive cultures were obtained from a serum bank and compared with normal human sera for responses to surface-exposed epitopes of their respective isolates. Patient 2 was the only one to demonstrate an expanded serologic response to *S. epidermidis* after having multiple positive blood cultures. This result was repeated on three separate occasions. Whole-cell lysates of $^{125}$I-labeled organisms from this patient are shown in Fig. 4, lanes 1 and 5. The profiles clearly indicate that isolates 2A and 2B represent different strains. Lanes 2 and 6 show proteins precipitated from whole-cell lysates by normal human sera, and lanes 3 and 7 show proteins precipitated by patients' sera obtained before cultures were positive. The results are similar to reactions seen with normal human sera in Fig. 1 and 2. Lanes 4 and 8 show the reactivity of a patient's sera, obtained after positive blood cultures, with isolates 2A and 2B. This patient had an expanded serologic response to isolate 2A but not to 2B. Also, sera from patient 2 showed a similar expanded immune response to the prototypic isolate (171A), isolates 5A and 5C, and two other slime-producing *S. epidermidis* isolates from another study (14) (data not shown). Sera obtained before and after positive blood cultures from the other six patients reacted in essentially the same way as normal human sera depicted in Fig. 1 and 2.

Western blot analysis revealed no differences between patient sera and normal human sera (data not shown). Identical proteins as shown in Fig. 3 were identified by this immunoblotting technique. We were unable to confirm the increased reactivity of sera from patient 2 as demonstrated

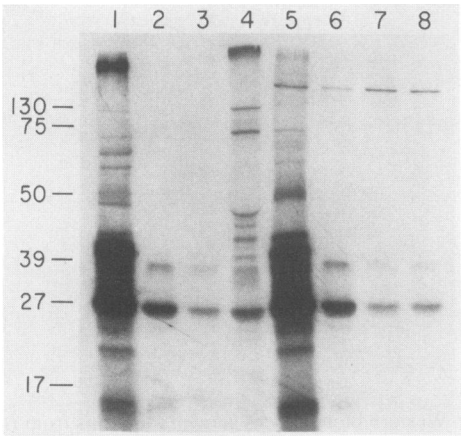


FIG. 4. SDS-PAGE of $^{125}$I-labeled surface-exposed proteins of *S. epidermidis* isolates 2A and 2B (lanes 1 and 5, respectively). RIP was performed with normal human sera and sera from patient 2, obtained before and after presumed *S. epidermidis* bacteremia. Lanes 2 and 6 show proteins precipitated with normal human sera, lanes 3 and 7 show proteins precipitated with presera (before bacteremic episodes), and lanes 4 and 8 show proteins precipitated with postsera (after bacteremic episodes) for isolates 2A and 2B, respectively.

with RIP (Fig. 4, lane 4). With ELISA, normal human sera were shown to react with whole ConS organisms, indicative of an innate response. Approximately the same titer (1:3,200 to 1:6,400) was achieved with sera collected from each of the seven patients before blood cultures were positive; none of the serum samples obtained at later times showed increased reactivity (data not shown).

## DISCUSSION

The role of surface-exposed proteins in the pathogenesis of coagulase-negative staphylococcal infection is unknown. We used radiolabeling analysis to study the protein epitopes that are available for interaction with the host, either as virulence factors or as targets for host defense such as opsonophagocytosis.

To identify specific ConS proteins that were surface exposed, we utilized organisms grown to mid-logarithmic phase and labeled rapidly with $^{125}$I, precluding internal protein labeling (4). Twenty to 30 proteins with molecular sizes of 18 to >130 kDa were observed (Fig. 1 and 2, lane WCL). Previous work has shown that SDS-PAGE analysis of whole-cell lysates of ConS could show differences between different species of ConS but were poor at discriminating within a species (7, 14). With the radiolabeling procedure in this study, marked differences were visualized. This technique also avoids the complications of multiple banding as evidenced in Western blot analysis (1, 18). This study defines surface-exposed proteins which could be virulence factors and contribute to the pathogenesis of this organism.

Immunoblot analysis has been used in two separate studies with normal human and hyperimmune animal sera to investigate the possibility of immunoblots as an epidemiological tool (1, 18). Our study confirms these results by Western blot analysis but extends these observations to show that surface-exposed proteins, as evident by RIP analysis, show a limited repertoire of proteins that can distinguish strains.

RIP with normal human sera identified a subset of staphylococcal proteins that are available for interaction with the host. The 41-kDa protein was immunodominant for the prototypic strain 171A and for the six clinical isolates tested (Fig. 1 and 2). In addition, a 51-kDa protein of 171A was recognized by all sera and was present in five of six clinical isolates. A 48-kDa protein was identified in each clinical isolate by using pooled sera (Fig. 2), but was not detected in strain 171A with individual serum samples (Fig. 1). The 18-kDa protein was detected in the majority (83%) of isolates. These different immunogenic surface-exposed proteins demonstrated in *S. epidermidis* strains are possible virulence factors or targets for host defense.

In addition to the innate immune response, we retrospectively studied the immune response of cancer patients with central venous catheters for their ability to mount a response to their respective isolates. The serological responses of the seven cancer patients revealed that only one patient (patient 2) showed an increased antibody response to their *S. epidermidis* isolates (Fig. 4); this patient's sera also gave an enhanced response to other *S. epidermidis* isolates. Phenotypic data in Table 1, along with the whole-cell lysate profiles and antigen profiles, indicate that the two isolates from this patient represent different strains of *S. epidermidis*, one pathogenic and the other a probable contaminant. Western blot and ELISA techniques were unable to demonstrate this increased antibody response.

The lack of an increased serologic response by the majority of patients in this study may be due to the times the sera was drawn or may indicate that they were not infected with ConS. Alternatively, their malignant disease could have impaired their ability to produce antibody, or perhaps expanded antibody production does not play an important role in opsonophagocytosis and clearing ConS organisms during infection.

In conclusion, 20 to 30 surface-exposed proteins were identified by radiolabeling techniques, with four proteins being immunodominant. Our identification of these specific *S. epidermidis* proteins that induce an antibody response in the normal host completes the first step toward understanding the host defense mechanisms responsible for maintaining a commensal relationship with this organism.

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