Characterization of Clinical Strains of *Staphylococcus aureus* Associated with Pneumonia

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A total of 5 *Staphylococcus aureus* strains from patients with postinfluenzal staphylococcal pneumonia, 7 from burn patients with staphylococcal pneumonia, and 21 from the nasopharynx of carriers were phenotypically characterized. All or most strains produced coagulase, clumping factor, DNase, thermostable DNase, protease, gelatinase, lipase, and pigment; the strains were low to moderate producers of extracellular protein A, fibrinolysin, and α-hemolysin. All strains were sensitive to mercury, half were sensitive to arsenate and cadmium, and 67 to 92% were resistant to penicillin. Differences between strains were not statistically significant. Cell surface hydrophobicity was determined by measuring percent adsorption to hexadecane. Hydrophobicity of postinfluenzal staphylococcal pneumonia strains was significantly lower than that of pneumonia strains from burn patients and carriers ($P < 0.005$). Immunoblot experiments with sera immune to one clinical test strain allowed the separation of all strains into three groups based on probe-positive reactions with primarily four staphylococcal polypeptides (154,200, 130,000, 77,100, and 64,400 molecular weight). The difference in distribution of clinical and carrier strains was highly significant ($P = 0.007$).

An increased frequency of *Staphylococcus aureus* pneumonia occurs in patients who are immunosuppressed, in patients who acquire infections while hospitalized, and during major epidemics of influenza; mortality rates are high even in patients who receive antimicrobial chemotherapy (27). During the 1968-1969 Hong Kong influenza epidemic, Schwarzmann et al. (23) studied a group of 128 patients who presented with pneumonia; they found a threefold increase in the incidence of staphylococcal pneumonia. They documented a high correlation between staphylococcal pneumonia and influenza infection. In their study group, the case fatality rate for patients with staphylococcal pneumonia was 33%. Between the years 1957 and 1970, Mulder and Hers (19) reported 176 cases of *S. aureus* pneumonia and bronchitis in a total of 478 suspected influenza deaths which were confirmed by histology, virology, and bacteriology. These authors postulated that a prerequisite for the initiation of a superinfection in the air passages was the presence of "virulent" staphylococcus in the nose.

Therefore, a general assumption is that any person who carries or acquires *S. aureus* in the nasopharynx during epidemics of influenza is at risk for a staphylococcal superinfection; however, because such strains of *S. aureus* are not known to have distinguishing characteristics, the more important contributory factors are thought to be virulence of the viral strain and the general condition of the host. Whether or not this assumption is valid is not known, and a comparative study of clinical and carrier strains is needed. In 1984, during the peak months of influenza activity in the community, we obtained clinical isolates from patients with postinfluenzal staphylococcal pneumonia. We are now reporting our study of the phenotypic characteristics of *S. aureus* strains associated with pneumonia in patients with and without a recent history of influenza compared with carrier strains from healthy adults.

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MATERIALS AND METHODS

Bacterial strains. The identification of all *S. aureus* strains isolated from patients and control subjects was confirmed by the API 20GP system (API; Analytab Products, Plainview, N.Y.) and by the production of coagulase as determined by the direct tube method according to the instructions of the manufacturer (BBL Microbiology Systems, Cockeysville, Md.). In January and February 1984, five Mexican-American females (mean age = 42 years, range of 32 to 64 years) admitted to Medical Center Hospital, San Antonio, Tex., were diagnosed as having postinfluenzal staphylococcal pneumonia. Early-passage clinical isolates of *S. aureus* from sputum specimens from four of the patients (strains Ci-1, Ci-2, Ci-3, and Ci-5) and an autopsy specimen of the lung from one patient (strain Ci-6) were obtained from James H. Jorgensen (The University of Texas Health Science Center, San Antonio). Permission for autopsy was obtained in three (strains Ci-1, Ci-3, and Ci-6) of the five cases; influenza A virus antigens were detected in lung tissue from patients with strains Ci-1 and Ci-3 by direct immunofluorescence. During the same period, an additional seven clinical strains of *S. aureus* (Ci-7 through Ci-9 and Ci-11 through Ci-14) isolated from burn patients with staphylococcal pneumonia and without a recent history of influenza were kindly provided by Albert T. McManus, Microbiology Division, U.S. Army Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Tex. The mean age of these patients was 55 years, with a range of 18 to 93 years. Clinical strains were phage typed at the Bacteriology Section, U.S. Air Force Epidemiology Division, Brooks Air Force Base, by using the International Basic Set of 23 typing phages (20). Control strains of *S. aureus* (N1 through N21) were isolated from the anterior nares of 21 normal, healthy, young adult carriers during the same period.

Miscellaneous test strains of *S. aureus* included the following: 1071, a clinical isolate of low-passage history (7); Ci-4, a clinical isolate of low-passage history from a patient

who died of subacute staphylococcal endocarditis; CDC 033, CDC 189, and CDC 587, all toxic shock syndrome (TSS) strains supplied by the Centers for Disease Control, Atlanta, Ga.; Cowan I; PA$^-$, a protein A-deficient mutant of Cowan I; EMS, a protein A- and clumping factor-deficient mutant of Cowan I (8); the M encapsulated strain (18); HSmR, a spontaneous streptomycin-resistant mutant of strain H (18); and 52A5, a teichoic acid-deficient mutant of HSmR (4) kindly supplied by David A. Lee, University of Minnesota, Minneapolis. Stock samples of all test strains were kept stored in 25% sterile glycerol in Todd-Hewitt broth (BBL) at $-20°C$ until testing.

Antimicrobial susceptibility. A standardized disk method was used according to the instructions of the manufacturer (BBL) to determine the susceptibility of test strains to amikacin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, methicillin, oxacillin, tetracycline, tobramycin, and vancomycin.

Production of enzymes and extracellular factors. Susceptibility to heavy metals, $HgCl_2$, $Cd(NO_3)_2$, and $NaHAsO_4$ was determined by the disk method of Barbour (1); a positive reaction was scored if a zone of inhibition was present after a 48-h incubation at 37°C. DNase production was determined by the plate method with DNase test agar with methyl green, following the directions of the manufacturer (Difco Laboratories, Detroit, Mich.); DNase activity was detected by clearing around the streak of inoculation after incubation at 35°C for 24 h and flooding the plate with 0.1 N HCl. The toluidine blue O-DNA agar plate method of Lachica et al. (14) was used to determine whether the DNase produced by test strains of S. aureus was thermostable; a colony, stabilized on an agar block, was boiled, placed on a toluidine blue O-DNA agar plate, incubated for 4 h at 37°C, and read for the presence of a reddish halo of nuclease activity. Lipase activity was ascertained by a plate method using spirit blue agar supplemented with a lipoidal emulsion according to the instructions of the manufacturer (Difco); lipolysis was indicated by haloes surrounding the sites of streak inoculation after incubation at 35°C for 72 h. Protease activity of the test organisms was determined by using three separate plate assays as follows: (i) protease substrate gel tablets (Bio-Rad Laboratories, Richmond, Calif.) dissolved in both water and Mueller-Hinton broth (Difco) to form 1% agar gel plates; (ii) milk agar plates as described by Brown and Foster (2); and (iii) dialyzed brain heart infusion milk agar plates by using the method of Sokol et al. (24). Clearing in the area of stab inoculation during incubation at 37°C for 72 h was indicative of proteolytic activity. Test strains were considered protease positive when a positive reaction occurred with any one of the three assays. A nutrient gelatin agar plate method was used according to the directions of the manufacturer (Difco) to determine the production of gelatinase which was indicated by liquefaction at the site of stab inoculation during incubation at 20°C for 30 days. A modified plate method (1) was used to assess the production of extracellular protein A, which produced a halo of precipitation around the site of inoculation after a 24-h incubation. Fibrinolysin production was determined by the modified fibrin agar method of Barbour (1); an area of fibrin clearing around the site of stab inoculation after incubation at 37°C for 24 h was scored as a positive reaction. To determine hemolytic activity, 5% rabbit blood agar plates were stab-inoculated, incubated at 37°C for 24 h, and kept at 4°C for an additional 24 h. Results were recorded after each incubation period for zones of complete, incomplete, or no hemolysis. The presence of clumping factor on the surface of the staphylococcal test strains was

determined by adding 50 μl of human plasma to a tube containing a heavy suspension of test organisms in 600 μl of H_2O, mixing, and observing for agglutination; the Cadness-Graves slide test (3) was also used. When appropriate, the data were statistically analyzed by Fisher's exact test (two-tailed) by using the computerized Statistical Analysis Systems package.

Hydrophobicity. The surface hydrophobicity of the test strains of S. aureus was measured by the procedure of Rosenberg et al. (21). Briefly, staphylococci were grown in M199 medium (GIBCO Laboratories, Grand Island, N.Y.) or Trypticase soy broth (TSB; BBL) at 37°C for 18 h in static culture. Bacteria were pelleted by centrifugation and washed twice in PUM buffer (pH 7.1; 22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO, 1.8 g of urea, and 0.2 g of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled H_2O). A turbid suspension of bacteria was prepared in PUM buffer to an A_{400} of 1.2 to 1.5. A 1.2-ml volume of the bacterial suspension was transferred to an acid-washed test tube to which was added 0.2 ml of hexadecane (Sigma Chemical Co., St. Louis, Mo.); the two phases were vigorously vortexed and allowed to stand for 10 min. The two phases were then mixed under controlled conditions by using a model 343 Rota-Rack (Fisher Scientific Co., Pittsburgh, Pa.) for 2 min. The mixture was then allowed to separate for 15 min. The aqueous phase was removed, and the A_{400} was measured. Hydrophobicity was expressed as the percent decrease in absorbance of the bacterial suspension after mixing with hexadecane as compared with the A_{400} of the original suspension. Data were statistically analyzed by analysis of variance and by the Student-Newman-Keuls test for variables, by using the computerized Statistical Analysis Systems package.

Antigen pattern analysis. Crude antigen sonicates were prepared from all 44 strains of S. aureus for use in an immunoblot assay by the procedure described by Cohen and Falkow (5) with slight modification. Beginning with stock cultures, each organism was subcultured onto a 5% sheep blood agar plate and incubated for 18 h at 37°C. One colony was transferred to 20 ml of Todd-Hewitt broth and incubated for 18 h at 37°C with constant agitation. The bacteria were pelleted by centrifugation and washed in buffer (50 mM Tris hydrochloride, 10 mM EDTA [pH 8.0]). After centrifugation, bacterial pellets were suspended in 4 ml of sample buffer (0.125 M Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue [Sigma]), the tube was placed in ice, and the cells were sonicated for 90 s with a model W140D Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) with a setting at the microtip limit. Suspensions of broken cells were heated at 100°C for 90 s, sampled, and stored at $-70°C$. When needed for testing, the suspensions were heated at 100°C for 10 min.

A 30-μl volume of each test suspension was subjected to electrophoresis (120-V constant current) on a 5% stacking and 10% resolving SDS-polyacrylamide gel electrophoresis (PAGE) gel according to the method of Laemmli (15). Separated polypeptides were electroeluted out of the gel onto untreated nitrocellulose paper (NCP) by electrophoresis at a constant current of 20 mA for not less than 48 h in buffer (14% glycine, 0.3% Tris, 20% methanol). To quench unbound sites on the filter, the NCP was soaked for 1 h in 1% bovine serum albumin in the TSGAN buffer described by Cohen and Falkow (5), which consisted of 50 mM Tris hydrochloride (pH 7.5), 0.15 M NaCl, 0.25% gelatin, 0.15% sodium azide, and 0.1% Nonidet P-40. The NCP was rinsed three times in TSGAN and overlaid with 30 ml of a 1:100

dilution of either preimmune or hyperimmune convalescent serum in TSGAN for 18 h at ambient temperature. The sera were obtained from a New Zealand White rabbit before and 4 weeks after an experimentally induced osteomyelitis with *S. aureus* strain Ci-1. The NCP was rinsed three times with TSGAN and overlaid with 100 ml of TSGAN containing staphylococcal protein A (Sigma) previously labeled with ^{125}I by the chloramine T method of Daugharty et al. (6); the suspension contained 3×10^6 cpm of labeled protein A which had a specific activity of 1×10^5 cpm/µg of protein. After a 2-h incubation at ambient temperature, the NCP was rinsed, soaked in TSGAN for 1 h, rinsed three times in TSGAN, and dried in air. The NCP was exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with a Du Pont Quanta-III CK intensifying screen (Du Pont Co., Wilmington, Del.) at $-70°C$ for 48 h. Standard molecular weight markers, included in each run, were visualized on the NCP after staining with Ponseau S.

RESULTS

Based on phage typing patterns (Table 1), most of the pneumonia strains showed two or more strong differences, resulting in a likelihood of <5% that they were of common origin (20). Two of the strains had the same phage type (Ci-8 and Ci-12), and two were untypable (Ci-7 and Ci-13). Phenotypically, all 33 clinical and carrier strains were characterized by the production of DNase, gelatinase, and lipase and also by the presence of clumping factor on the cell surface and the appearance of nonwhite pigmented colonies. In addition, most strains produced thermostable DNase (88%) and protease (94%). Less frequent traits included the secretion of extracellular protein A (21%), the production of hemolysin sufficient to produce a zone of complete hemolysis when using sensitive rabbit erythrocytes (61%), and the secretion of fibrinolysin (48%); the staphylococcal pneumonia strains isolated from burn patients more frequently produced fibrinolysin (86%), but the results were of only borderline significance ($P = 0.07$).

A comparison of antibiograms for each of the test strains showed that 11 of 12 (91.6%) clinical isolates were resistant to penicillin compared with 14 of 21 (66.6%) carrier strains. One clinical isolate (C-13) was resistant to all antibiotics tested except vancomycin. Two clinical isolates (Ci-1 and Ci-6) were resistant to tetracycline, and Ci-7 was resistant to erythromycin. One carrier strain was resistant to both chloramphenicol and tetracycline. In testing the sensitivity of staphylococcal strains to heavy metals, no significant difference was noted between the clinical and carrier groups—all

TABLE 1. Phage typing patterns of *S. aureus* strains isolated from patients with staphylococcal pneumonia

Strain	Phage type[a]
Ci-1	29/80
Ci-2	29
Ci-3	(79/94)
Ci-5	(42E/47/52A/54/75/80/81/83A)
Ci-6	47/54/75
Ci-7	NT
Ci-8	95
Ci-9	29/52/52A/53/80/81
Ci-11	96
Ci-12	95
Ci-13	NT
Ci-14	29/52/52A/79/80/81/95

[a] NT, Not typable.

TABLE 2. Hydrophobicity of the test strains of *S. aureus*

Strain	% Hydrophobicity[a] of strains grown in:	
	M199	TSB
Group 1		
Ci-1	23	5
Ci-2	16	10
Ci-3	27	12
Ci-5	30	48
Ci-6	19	35
Mean	23	22
Group 2		
Ci-7	53	59
Ci-8	39	44
Ci-9	24	42
Ci-11	30	49
Ci-12	14	48
Ci-13	41	49
Ci-14	41	29
Mean	35	46
Group 3		
N1	22	56
N2	10	82
N3	3	32
N4	13	71
N5	9	23
N6	10	44
N7	18	59
N8	15	22
N9	16	46
N10	18	49
N11	33	59
N12	28	54
N13	28	49
N14	32	52
N15	31	53
N16	36	53
N17	32	44
N18	36	28
N19	42	44
N20	38	69
N21	54	63
Mean	25	50
Cowan I	55	50
Significance[b]	NS[c]	$P < 0.005$[d]

[a] Expressed as percent adsorption to hexadecane.
[b] Determined by analysis of variance and the Student-Newman-Keuls test for variable.
[c] NS, Not significant ($P > 0.05$).
[d] Group 1 versus groups 2 and 3.

of the organisms were sensitive to mercury, and approximately half the strains were sensitive to arsenate and cadmium.

Since the interaction of *S. aureus* strains with host cells may involve nonspecific hydrophobic interactions, we determined the hydrophobicity of our test strains, reflected by their capacity to adsorb to hexadecane following growth in chemically defined (M199) and biologically complex (TSB) media (Table 2). There was no apparent difference between clinical and carrier strains grown in M199, and hydrophobicity was relatively low. However, when strains were grown in TSB, hydrophobicity of the postinfluenzal staphylococcal pneumonia isolates was significantly lower ($P < 0.005$)
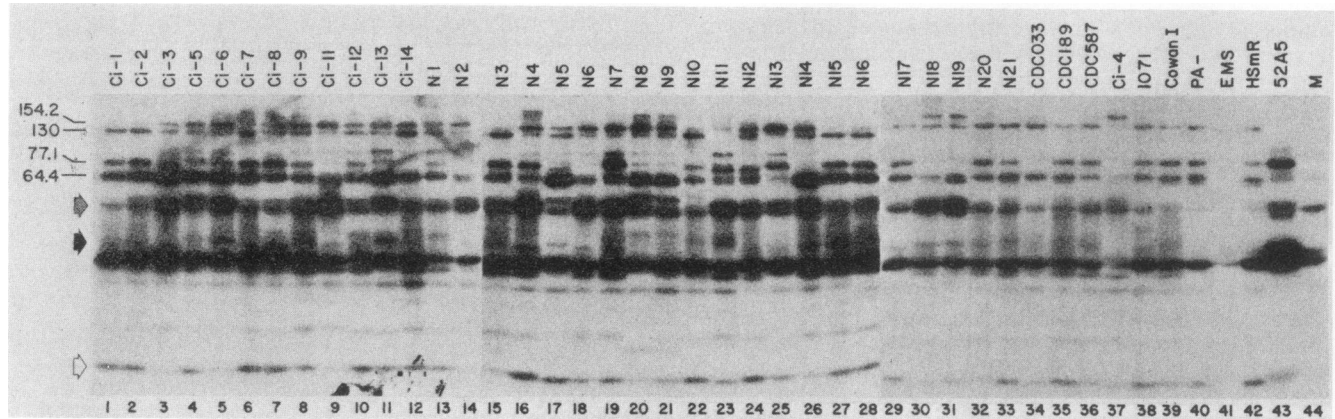
FIG. 1. Identification of polypeptide antigens in sonic extracts of 44 strains of *S. aureus*. The polypeptides were separated by SDS-PAGE, electroeluted on nitrocellulose paper, and reacted with a 1:100 dilution of immune sera from a rabbit infected with Ci-1 (lane 1). Antigenic polypeptides were detected with ^{125}I-labeled protein A and subsequent autoradiography. Molecular weights (10^3) are shown for bands of interest. ✿, Position of protein A, determined by the position occupied by purified protein A in stained SDS-PAGE (data not shown). ➡, Molecular weight position of 41,000 to 42,000 which is considered the correct molecular weight of protein A. ▷, Probable position of clumping factor.

compared with the hydrophobicity of pneumonia isolates from burn patients and carrier strains.

The antigenic relatedness of 44 strains of *S. aureus*, including the 12 pneumonia strains, the 21 carrier strains, 5 miscellaneous clinical strains, and 6 laboratory-adapted strains, was compared by immunoblotting. Polypeptides present in whole-cell sonic homogenates were separated by SDS-PAGE and reacted with either nonimmune rabbit serum or immune serum raised against Ci-1. Antigen-antibody complexes were visualized with radiolabeled protein A. An analysis of the results (Fig. 1) shows that several bands were shared among all the clinical and carrier strains. One band occupied a position at 52,000 to 55,000 molecular weight (stippled arrow) which corresponds to the molecular weight of purified protein A by SDS-PAGE (9, 16); in fact, this band was missing in two mutant strains known to be deficient in protein A, PA⁻ (Fig. 1, lane 40) and EMS (Fig. 1, lane 41). A second band, occupying a position at 14,000 to 15,000 molecular weight (Fig. 1, open arrow), was probably clumping factor, since this band was missing in EMS (Fig. 1, lane 41), which is known to be clumping factor deficient. It is obvious that antigen profiles differed between strains; however, 9 of the 12 pneumonia strains demonstrated one of two patterns based on strong reactions with antigenic polypeptides occupying positions at 154,200, 130,000, 77,100, or 64,400 molecular weight. Pattern A is represented by Ci-1 (Fig. 1, lane 1), which was positive for three of the bands; pattern B is represented by Ci-9 (Fig. 1, lane 8), which was positive for all four bands. Antigenic patterns of the carrier strains were also determined (Table 3). A category of "miscellaneous" was assigned for strains that did not demonstrate either pattern A or B; these strains showed a range of

reactivity. For example, all strains with either pattern A or B were positive for molecular weight bands 77,100 and 64,400, whereas in the miscellaneous category, some strains were positive for only one, as represented by Ci-14, or negative for both, as represented by N18. N7 (miscellaneous) demonstrated still another pattern in which there were three, rather than two, major molecular weight bands between 64,400 and 130,000. Statistical analysis of the distribution of the clinical versus the carrier strains with the two-tailed Fisher's exact test showed a significant difference between patterns A and B ($P = 0.05$) and between pattern B and miscellaneous ($P = 0.007$). The difference between pattern A and miscellaneous was not significant. The immunoblot with homologous nonimmune rabbit serum was negative (data not shown).

DISCUSSION

In the present study we showed that *S. aureus* strains isolated from patients with staphylococcal pneumonia, with or without a recent history of influenza, compared with strains isolated from normal carriers, did not differ significantly in the ability to produce certain enzymes, extracellular protein A, pigment, clumping factor, or α-hemolysin. In addition, the two groups of staphylococci were not notably different in their sensitivity patterns to heavy metals and a range of antibiotics with one exception; more clinical strains were resistant to penicillin (91.6 versus 66.6%). Our data differ in some respects from those reported in other studies. Kondell et al. (13) phenotypically characterized strains from 65 carriers grouped according to distribution of the strains in the oral cavity; although they found no significant differences between groups of strains, 90% of the strains produced α-hemolysin. Only 61% of our strains produced α-hemolysin. A number of investigators have compared and contrasted TSS versus non-TSS strains of *S. aureus* and reported significant phenotypic differences between the two groups (1, 22, 25). Barbour (1) reported that of 15 TSS strains tested, all were resistant to arsenate, cadmium, and mercury, whereas only half of 18 non-TSS strains yielded the same pattern; in our study, all of the 33 test strains were sensitive to mercury, whereas only half of the strains were resistant to arsenate and cadmium. In addition, production

TABLE 3. Summary of immunoblot analysis

Pattern[a]	No. of clinical pneumonia strains (%)	No. of carrier strains (%)	Total no. of strains (%)
A	3 (25)	8 (38)	11 (33.3)
B	6 (50)	1 (5)	7 (21.2)
Miscellaneous	3 (25)	12 (57)	15 (45.5)

[a] Classification of strains based on antigenic patterns after immunoblot analysis.

of fibrinolysin and extracellular protein A in our test strains was considerably lower when compared to the TSS and non-TSS strains in Barbour's study. However, fibrinolysin production in 104 strains of *S. aureus* isolated from animals with bovine mastitis (10) was markedly lower (2 to 6%) than in our test strains (48%).

Because *S. aureus* is so efficient at colonizing the nasopharynx of humans, one might expect cell surface hydrophobicity to be a contributory factor to the interaction between the organism and host tissues. Further, one might predict that the hydrophobicity of clinical isolates would equal, if not exceed, the hydrophobicity of carrier strains. Recently, Ljungh et al. (17) determined the hydrophobicity of 209 clinical isolates and 23 carrier strains of *S. aureus* by the salt aggregation test. A total of 90% of the clinical strains, obtained from patients with septicemia, wounds, and urinary tract infections, and 39% of the carrier strains were hydrophobic. Hydrophobicity was decreased by heat and proteolytic enzyme treatment of the cells, which supports the idea that surface proteins, e.g., protein A and fibronectin-binding protein (11, 12, 26), contribute to high hydrophobicity. By using adsorption to hexadecane as a measure of hydrophobicity, we compared pneumonia and carrier strains (Table 2). Hydrophobicity of all test strains was similar when staphylococci were grown in chemically defined medium (range of mean percent, 23 to 35). However, when the organisms were grown in biologically complex medium, hydrophobicity of the postinfluenzal staphylococcal pneumonia strains was significantly lower ($P < 0.005$) than that of the pneumonia strains isolated from burn patients and normal carrier strains; hydrophobicity of the latter two groups was almost identical and showed an increase after culturing in TSB. These data cannot be explained by a transformation of the organisms during active infection, because all of the clinical strains were isolated from patients with severe pulmonary infections. The data do suggest that high cell surface hydrophobicity, as determined by in vitro testing, is not a prerequisite of strains capable of causing pneumonia in humans.

The antigenic relatedness of the clinical and carrier strains was analyzed by immunoblot by using hyperimmune serum raised specifically against Ci-1 (Fig. 1, lane 1) in the course of an experimentally induced rabbit infection. The immunoglobulins reacted strongly with a number of bands shared between the strains that probably represented group antigens. However, the heterogeneity of the response was surprising, since probe-positive bands were present in heterologous strains that were missing in the homologous Ci-1 strain. Although quantitative differences may serve to partially explain this reactivity, it appears that a considerable amount of molecular heterogeneity exists in these test strains. We know from a recent preliminary experiment that the reactive bands are derived primarily from the surface of the staphylococci, because pretreatment of bacterial pellets with proteolytic enzymes before sonic disruption ablates the reactivity (unpublished data). It would be interesting to know whether these patterns are constant for each organism, or if the organism has the ability to present different faces to the host and thereby gain a survival advantage. By close inspection of the various patterns, we were able to tentatively classify strains based on the presence or absence of four protein antigens (Table 3). A total of 75% of the clinical strains, including all five of the isolates from patients with postinfluenzal staphylococcal pneumonia, were positive for three to four of the antigens, compared with 43% of the carrier strains. The number of test strains from patients with

postinfluenzal staphylococcal pneumonia was not large enough to allow us to conclude that carriers of staphylococci with these antigenic profiles are at higher risk of bacterial superinfection during epidemics of influenza. However, the possibility that surface proteins may contribute to or provide markers for the pathogenicity of *S. aureus* in deep-seated respiratory infections merits further study.

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