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Staphylococcus aureus Adherence to Influenza A Virus-Infected and Control Cell Cultures: Evidence for Multiple Adhesins (42230)

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Abstract. During major epidemics with influenza, there is an increased number of pneumonias due to *Staphylococcus aureus* with a subsequent high mortality rate. We have postulated that influenza A virus infection of host cells promotes the adherence of *S. aureus* ultimately resulting in bacterial superinfection. In the present study we compared the adherence of seven strains of ^{3}H-labeled *S. aureus* to Madin–Darby canine kidney (MDCK) cell monolayers, uninfected and infected with influenza A/FM/1/47 virus. Test strains included: Cowan I; a Cowan I protein A-deficient mutant (PA^{-}); EMS, a protein A and clumping factor-deficient mutant; HSmR; 52A5, a teichoic acid-deficient mutant of HSmR; M, an encapsulated strain; and, No. 1071, a clinical isolate. By radioassay, six of the seven strains demonstrated significantly enhanced adherence to virus-infected cell monolayers compared to uninfected controls; only the M strain was adherence negative. Surface hydrophobicity of the staphylococci did not correlate with their ability to adhere. Four strains of labeled staphylococci (Cowan I, PA^{-}, EMS, and No. 1071), untreated or treated with 2.5% trypsin, 1.25% protease, or by autoclaving, were tested in the radioassay. Protease treatment, which was more effective than trypsin treatment, reduced adherence of all four test strains by 74–96%. Results of heat treatment suggested the presence of both thermolabile and thermostable adhesins. Staphylococcal thermal extracts, profiled by anion-exchange HPLC, were used to pretreat monolayers in a blocking radioassay. Adherence was decreased to control cells (9–78%) and to virus-infected cells (56–90%). The data suggest that multiple distinct surface proteins mediate the binding of *S. aureus* to uninfected and influenza A virus-infected cells.

The concept that certain virus infections predispose the host to bacterial infections has long been recognized and accepted by the medical community (1). The classical example of viral–bacterial synergism is influenza in which the most serious and often fatal complication is fulminating staphylococcal pneumonia. Impaired function of lung phagocytes is the reason frequently proposed for the occurrence of secondary staphylococcal pneumonia that occurs after influenza. However, much of the work done to test this proposal has produced results that are inconclusive and in some instances contradictory (2). In one report on the function of phagocytes obtained from volunteers inoculated with influenza A virus, Martin *et al.* (3) reported that the only consistent abnormality in phagocytic function was impaired chemotaxis. It seems reasonable to assume that the mechanism of viral–bacterial synergism is probably complex and multifactorial with impaired host resistance comprising an important but not exclusive explanation for a host's predisposition to bacterial superinfection.

In considering other possible explanations, we originally proposed the hypothesis that influenza A virus infection favors bacterial colonization by promoting the adherence of bacteria to host cells (4). There is now considerable evidence that influenza A virus infection does enhance *in vitro* bacterial adherence, including that of *Staphylococcus aureus* (5–11). Importantly, Musher and Fainstein (12) reported a significant increase in staphylococcal adherence to pharyngeal cells taken from human subjects during a mild experimental influenza virus infection, an observation which is consistent with our original hypothesis.

The present series of experiments was designed to further characterize the adhesin(s) within the mosaic of the staphylococcal cell wall that mediate the binding of radiolabeled strains of *S. aureus* to influenza A virus-infected and control cell monolayers.

Materials and Methods. *Cell cultures and virus inoculation.* Madin–Darby canine kidney cells (MDCK) were routinely maintained in Eagle basal medium (Auto Pow BME; Flow Laboratories, Inc., McLean, Va.) with 10% fe-

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tal calf serum, 0.03% L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Before each experiment, cells were grown in either 6-well Lin Bro plates (Flow) or 24-well Mark II tissue culture clusters (Costar, Cambridge, Mass.) in BME and incubated at 37°C in a 5% CO_2 atmosphere until confluent. Monolayers of MDCK cells were infected with a human strain of influenza A/FM/1/47 (H1N1) virus as previously described (9) using an inoculum of 0.2 ml/well (6-well plates) or 0.1 ml/well (24-well plates) containing 3–4 $\times 10^2$ PFU of virus/0.1 ml of inoculum. Control monolayers were sham inoculated with Hanks' balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N.Y.), pH 7.2.

Labeling of bacteria. Laboratory adapted test strains of *S. aureus,* kindly supplied by David A. Lee (University of Minnesota, Minneapolis, Minn.), included Cowan I, PA^- (a protein A-deficient mutant of Cowan I), EMS (a protein A and clumping factor-deficient mutant of Cowan I) (13), the M (encapsulated) strain (14), HSmR (a spontaneous streptomycin-resistant mutant of strain H) (14), and 52A5 (a teichoic acid (TA)-deficient mutant of HSmR) (15). Also included was No. 1071, a clinical isolate of low passage history (9). Aliquots of bacteria were stored frozen in Todd–Hewitt broth (Difco Laboratories, Inc., Detroit, Mich.) at −70°C. Staphylococci were labeled by inoculating 500 ml of M-199 medium (GIBCO) with 1–2 $\times 10^8$ cfu of bacteria and incubating in stationary culture at 37°C for 18 hr in the presence of 1 μCi of [*methyl*-^{3}H]thymidine (20 Ci/mmole; ICN Chemical and Radioisotope Div., Irvine, Calif.) per milliliter of medium. The ^{3}H-labeled staphylococci were harvested by centrifugation, repeatedly washed with sterile deionized H_2O at 4°C until no more radioactivity could be detected in the washes, and freeze-dried for storage.

Radioassay. Labeled bacteria were weighed, washed in phosphate-buffered saline (PBS; FTA hemagglutination buffer, pH 7.2, Baltimore Biological Laboratory, Cockeysville, Md.), and resuspended in PBS to contain 0.06 to 2.0 mg (dry wt) of bacteria per 0.2 ml inoculum used per well for the 6-well plates or 0.5 mg (dry wt) of bacteria per 0.1 ml inoculum used per well for the 24-well plates. Influenza A virus-infected and control monolayers were washed with PBS, layered with the suspensions of ^{3}H-labeled staphylococci, and incubated for 1 hr at ambient temperature with intermittent agitation. Monolayers were washed free of unbound labeled bacteria by continually adding PBS to the plates while simultaneously aspirating the fluid. To determine the amount of bound ^{3}H-labeled bacteria, the washed monolayers were removed by digestion with 0.5 ml of 0.25% trypsin in 0.02% EDTA at 37°C for 15 min. The digests were transferred to scintillation vials. The wells were rinsed with 0.5 ml of deionized H_2O which was also added to each vial. A 10-ml amount of ScintiVerse I scintillation cocktail (Fisher Scientific Co., Fair Lawn, N.J.) was added to each vial, and radioactivity was measured in a Beckman Mark III liquid scintillation spectrometer. Counts per minute were converted to disintegrations per minute (dpm) because scintillation counters with different efficiencies were used.

To determine the mean number of MDCK cells per well at the end of the procedure, parallel monolayers, not exposed to ^{3}H-labeled bacteria, were removed by trypsin–EDTA digestion and counted in a hemacytometer. Results of the radioassays were expressed as the amount of ^{3}H-labeled bacteria bound to uninfected or virus-infected MDCK cells according to

$$\frac{A}{B} = C \qquad (1)$$

$$\frac{D}{C} \times 1000 = E \qquad (2)$$

where A equals dpms of 5 μl of the ^{3}H-labeled bacterial suspension; B equals μg (dry wt) of bacteria in 5 μl of the ^{3}H-labeled bacterial suspension; C equals dpms per microgram of ^{3}H-labeled bacteria; D equals the mean dpms of bacteria bound in triplicate test wells divided by the mean number of MDCK cells in the triplicate wells; and E equals the nanograms of ^{3}H-labeled bacteria bound per 10^5 MDCK cells.

Treatment of staphylococci. Washed ^{3}H-labeled staphylococci were suspended in 1.0-ml vol of 2.5% trypsin [5 mg (dry wt) of bacteria] for 1 hr at 37°C; 1.25% protease (type VI obtained from *Streptomyces griseus;* Sigma, St.

Louis, Mo.) [3 mg (dry wt) of bacteria] for 1 hr at 37°C; or PBS diluent [5 mg (dry wt) of bacteria] which was heated (121°C, 15 lb/in.^{2}) for 15 min. Control bacterial suspensions in PBS were incubated for 1 hr at 37°C. After each treatment, bacteria were pelleted by centrifugation, rinsed twice with PBS, and resuspended to a concentration of 0.5 mg (dry wt) of bacteria per 100 μl of PBS which was added to uninfected and virus-infected cell monolayers to test for adherence.

Hydrophobicity of staphylococci. The surface hydrophobicity of the seven test strains of *S. aureus* was measured by the procedure of Rosenberg *et al.* (17). Briefly, staphylococci were grown in M-199 at 37°C for 18 hr in static culture. Bacteria were pelleted by centrifugation and washed twice in PUM buffer, pH 7.1 (22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 ml). A turbid suspension of bacteria was prepared in PUM buffer to a concentration of 1.2 to 1.4 at an absorbance of 400 nm. A volume of 1.2 ml of the bacterial suspension was transferred to an acid-washed test tube to which was added 0.2 ml of hexadecane (Sigma); the two phases were vigorously vortexed and allowed to stand for 10 min. The two phases were then mixed under controlled conditions for 2 min and allowed to separate for 15 min. The aqueous phase was removed and measured for light absorbance at 400 nm. Hydrophobicity was expressed as the percentage decrease in absorbance of the bacterial suspension after mixing with hexadecane as compared with that of the original suspension.

Treatment of MDCK cell monolayers. Staphylococci were subcultured on trypticase soy agar for 18 hr at 37°C. The bacteria were harvested, washed repeatedly with deionized H_2O, and freeze-dried. Two grams (dry wt) of the bacteria were suspended in PBS, pelleted by centrifugation, resuspended in 6 ml PBS and autoclaved (121°C, 15 lb/in^2) for 15 min. Following centrifugation, the pellet was discarded and the supernatant fluid (thermal extract) was filtered. The ability of thermal extracts to block bacterial adherence was determined by pretreatment of uninfected and virus-infected cell monolayers (grown in 24-well plates) with 200 μl of the thermal extract (~100 μg total protein) or PBS for 30 min at ambient temperature with intermittent agitation. After aspiration the monolayers were washed with PBS and used in the radioassay.

HPLC. Thermal extracts (~20 μg total protein) were subjected to anion exchange high-performance liquid chromatography (HPLC) on a Protein Pak DEAE-5 PW column (7.5 mm by 7.5 cm; Waters Associates, Milford, Mass.) in conjunction with a DuPont gradient liquid chromatography system (DuPont Co., Wilmington, Del.) including the series 8800 gradient controller, an 860 absorbance detector, and the 8800 chromatographic pump module. The extracts were eluted under the following conditions: 20 min gradient (exponent = −3) from 0.02 *M* Tris–HCl buffer of pH 8.0 to 0.02 *M* Tris–HCl buffer of pH 8.0 containing 0.5 *M* NaCl; flow rate of 1 ml/min; injection volume of 25 μl; and absorbance monitored at 285 nm. An SP4270 chromatography integrator with a 5 × 7 dot matrix printer (Spectra-Physics, San Jose, Calif.) was used to integrate and report data.

Results. *Staphylococcal adherence to virus-infected cells.* Seven strains of ^{3}H-labeled *S. aureus* were tested for their ability to adhere to influenza A virus-infected and control MDCK cells in a dose response fashion. As seen in Fig. 1, all strains were adherence positive except for the encapsulated M strain which did not adhere to either virus-infected or control cell monolayers (Fig. 1F). The adherence of *S. aureus* to virus-infected cell cultures was significantly higher when compared with adherence to uninfected cell cultures and the strains differed quantitatively in their adherence to virus-infected cell cultures with No. 1071 (Fig. 1G) > Cowan I (Fig. 1A) > PA^- (Fig. 1B) > HSmR (Fig. 1D) > 52A5 (Fig. 1E) > EMS (Fig. 1C).

Results of hydrophobicity testing, as seen in Table I, suggest that hydrophobicity was not directly related to the ability of these test bacteria to adhere to normal or virus-infected cell monolayers (coefficient of correlation = <0.26).

Effect of heat and enzyme treatment of staphylococci. Figure 2 summarizes the results obtained when ^{3}H-labeled *S. aureus* Cowan I, PA^-, EMS and No. 1071 were subjected to various treatments before being used in the radioassay. The most effective treatment was protease which reduced adherence of all four test strains to both uninfected cell cultures (~81%; range 74–91%) and to virus-infected

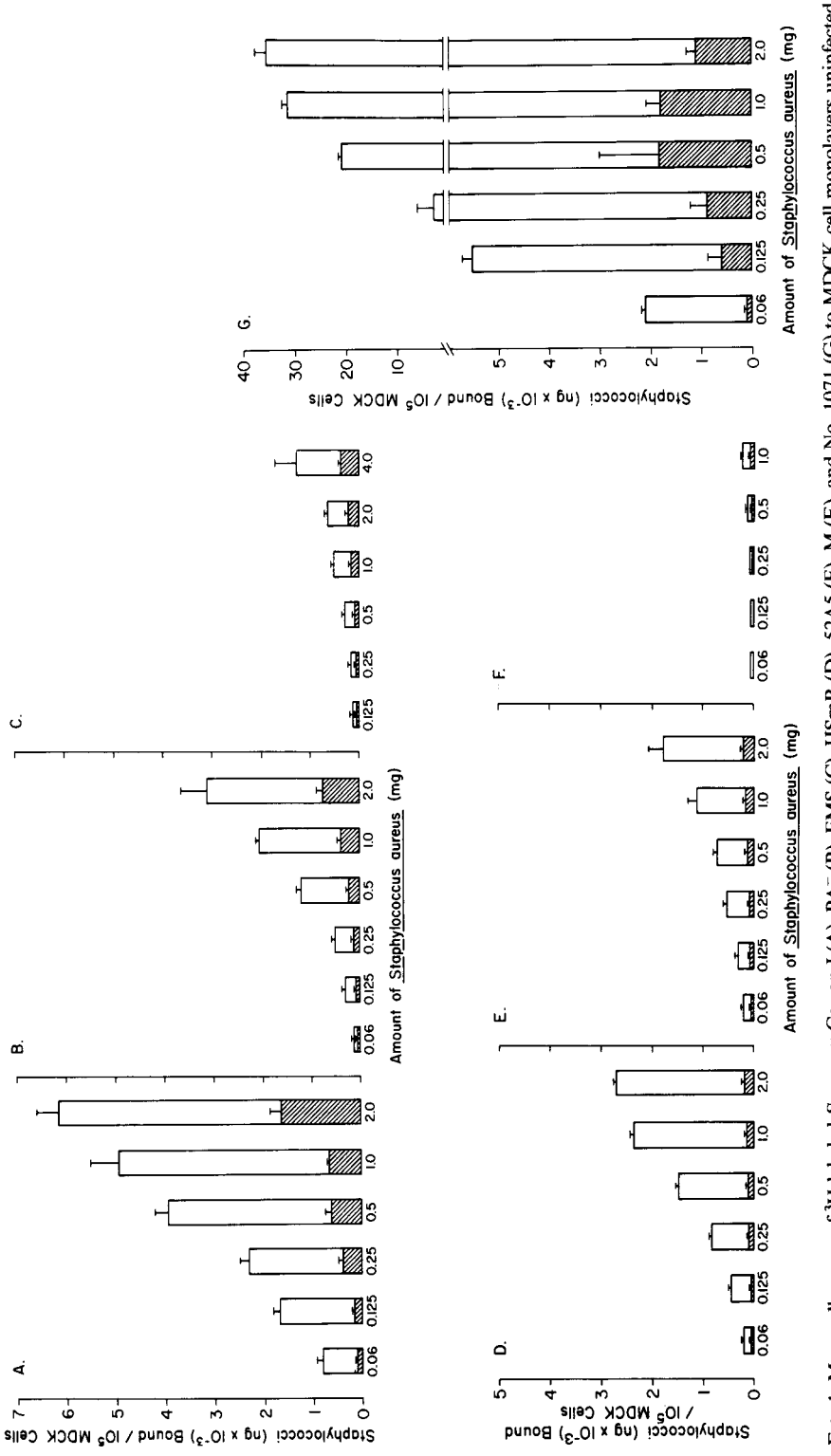

FIG. 1. Mean adherence of ^{3}H-labeled *S. aureus* Cowan I (A), PA$^-$ (B), EMS (C), HSmR (D), 52A5 (E), M (F), and No. 1071 (G) to MDCK cell monolayers uninfected (stripped bars) or infected with influenza A/FM/1/47 virus (open bars). Lines represent the standard error of the mean with triplicate testing.

TABLE I. HYDROPHOBICITY OF THE TEST STRAINS OF *Staphylococcus aureus* EXPRESSED AS THE PERCENTAGE OF ADSORPTION TO HEXADECANE

Strain[a]	%
No. 1071	30
Cowan I	55
PA⁻	0
HSmR	33
52A5	16
EMS	12
M	9

[a] The strains are ranked in order of adherence to uninfected and virus-infected MDCK cells beginning with No. 1071, which was the most adherent strain, and ending with M, which was adherence-negative.

cell cultures (~90%; range 85–96%). The effect of heat treatment varied from no decrease in adherence with either PA⁻ or EMS to a decrease of 76% in adherence of Cowan I to virus-infected cells. Trypsin treatment was most effective in reducing the adherence of: 1) PA⁻ and EMS to control and virus-infected monolayers (≥70%), and 2) Cowan I to virus-infected cells (76%).

Results of blocking tests. Previous studies indicated that autoclaving *S. aureus* No. 1071 resulted in the release of factors into the supernatant fluid which were capable of partially blocking the adherence of No. 1071 to uninfected and virus-infected MDCK cells (10). Thus, we wanted to determine if blocking factors could likewise be heat extracted from the Cowan I, PA⁻, and EMS strains, even though PA⁻ and EMS were shown to remain adherence positive after autoclaving (Fig. 2B and 2C). Figure 3 summarizes the results obtained when monolayers were pretreated with thermal extracts from four test bacteria before being used in the radioassay. Extracts from all four test strains were effective in decreasing the adherence of Cowan I, EMS, and No. 1071

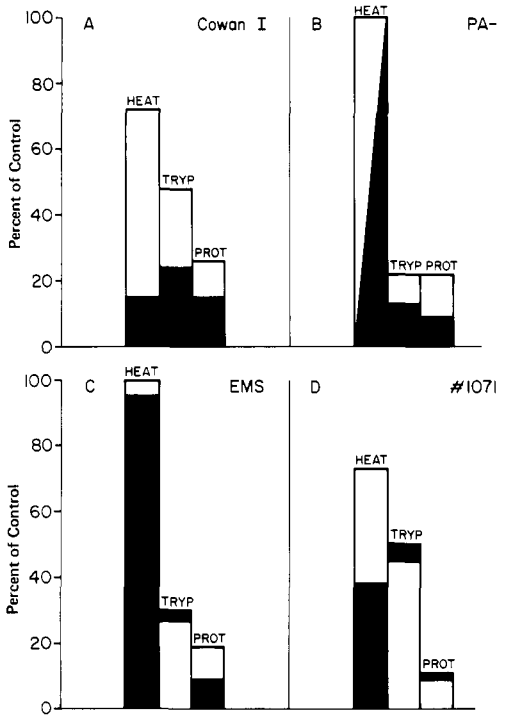

FIG. 2. Effect of heat, trypsin, or protease treatment on the adherence of ^{3}H-labeled *S. aureus* Cowan I (A), PA⁻ (B), EMS (C), and No. 1071 (D) to MDCK cell monolayers uninfected (open bars) or infected with influenza A/FM/1/47 virus (closed bars). The diagonal shading of the bar (B) indicates that heat treatment of PA⁻ had no effect on adherence to uninfected or virus-infected cells. Results are expressed as the percentage of control (adherence of treated staphylococci/adherence of untreated staphylococci) ×100.

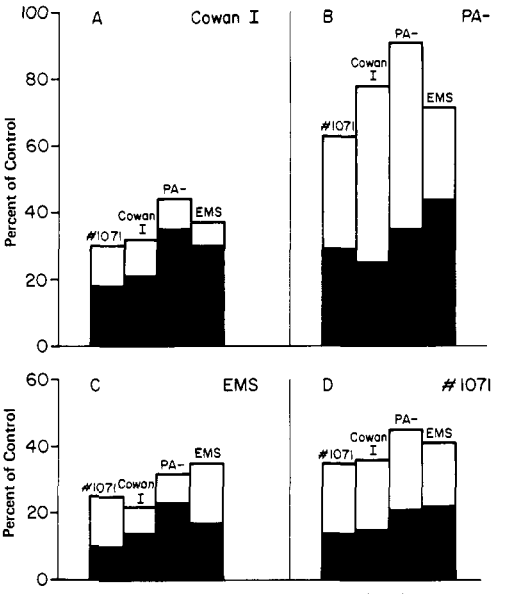

FIG. 3. Supernatant fluids (thermal extracts) obtained from autoclaved *S. aureus* Cowan I, PA⁻, EMS, and No. 1071 were each used to pretreat MDCK cell monolayers uninfected (open bars) or infected with influenza A/FM/1/47 virus (closed bars). ^{3}H-labeled *S. aureus* Cowan I (A), PA⁻ (B), EMS (C), and No. 1071 (D) were then tested for their ability to adhere to the treated monolayers. Results are expressed as the percentage of control (adherence to treated monolayers/adherence to untreated monolayers) ×100.

to control cell monolayers (~66%; range 55–78%) and to virus-infected cell monolayers (~80%; range 65–90%). The extracts were less effective in blocking adherence of PA^- in that adherence was decreased to control cells by only 24% (range 9–37%) and to virus-infected cells by 67% (range 56–75%).

In addition, the thermal extracts were analyzed by anion-exchange chromatography (Fig. 4). The profiles indicate that each thermal extract represents a heterogeneous, but distinct, mixture of acidic polypeptides. All four extracts had a major peak with a retention time of approximately 18.5 min; the area percentage of this peak, relative to the total area of all peaks integrated, was 72.28% for Cowan I, 57.75% for PA^-, 89.76% for EMS, and 81.16% for No. 1071.

Discussion. The diversity of components exposed on the surface of wild-type strains of *S. aureus* makes the study of staphylococcal adherence to host cells very complicated. Teichoic acids, which are major cell-wall components of staphylococci, have been shown by Aly *et al.* (18) to mediate the binding of one strain of *S. aureus* to human nasal epithelial cells *in vitro*. More recently Carruthers and Kabat (19), using a single clinical strain of *S. aureus*, demonstrated that lipoteichoic acid (LTA) mediates adherence of the organism to human buccal mucosal cells. The difference between these two mechanisms of binding is specificity. TA appears to mediate binding to host cell receptors specific for staphylococci; LTA-mediated binding seems likely to be due to a nonspecific hydrophobic interaction. In the present work we were interested in studying the binding of staphylococci not only to normal mammalian cells but also to these cells after they had been altered by infection with influenza A virus. Data obtained with well characterized laboratory-adapted and mutant strains of *S. aureus* indicated that binding to normal or virus-infected cell monolayers was not due simply to TA nor to two major cell surface proteins—protein A and clumping factor (Fig. 1). In contrast to a previous report (9), the Cowan I strain was adherence positive. This difference in results can be explained by the increased sensitivity and objectivity of the radioassay used in the present work over dark-field microscopic examination (9) and to an optimization of assay conditions. We have not eliminated the possibility that LTA might contribute to staphylococcal binding in our test system; however, the lack of correlation between hydrophobicity (Table I) and staphylococcal adherence (Fig. 1) of the test strains suggests that the binding reaction cannot be explained by nonspecific hydrophobic interaction alone. Also, previous work has shown that nonspecific forces of attraction and repulsion, which obviously play a role in any adherence reaction, cannot explain the significantly enhanced adherence of *S. aureus* to virus-infected cells *in vitro* (10). Of the seven strains of *S. aureus* tested, only the M encapsulated strain was adherence negative. This organism, originally described by Scott (20), possesses a large polysaccharide capsule which has been shown to mask protein A (14); the organism lacks clumping factor. The most likely explanation for the inability of the organism to bind to normal or virus-infected cells is that the adhesins, if present, are masked by the capsule.

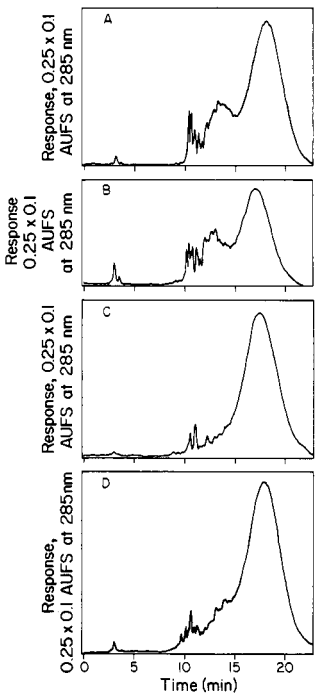

FIG. 4. Anion-exchange HPLC profiles of supernatant fluids (thermal extracts) obtained from autoclaved *S. aureus* Cowan I (A), PA^- (B), EMS (C), and No. 1071 (D).

Beginning with the work of Cowan in 1939 (21), investigators have used the classical serological approach in an effort to define the type-specific antigens present on the surface

of *S. aureus* and thereby make possible a means of determining the similarities or differences between strains (22). Even though no standard method of serotyping staphylococci has emerged, a number of observations have been made that are pertinent to the present study. At least 30 type-specific antigens have been recorded thus far, and most strains of *S. aureus* express multiple antigens that are present in small amounts (22); the antigens detected may be classed as either thermostable or thermolabile depending upon the method of serotyping used (23). Likewise, we determined the effect of heat on staphylococcal surface adhesins (Fig. 2). Results indicated that surface adhesins mediating the adherence of two mutant strains—PA^- and EMS—to both uninfected and virus-infected cells were thermostable. In contrast both thermostable and thermolabile adhesins were detected with the Cowan I and No. 1071 strains of *S. aureus;* adhesins mediating binding to virus-infected cells were significantly more sensitive to heat treatment than adhesins mediating binding to control cells. These data indicate not only a difference between adhesins present on different strains of *S. aureus* but also a difference between adhesins mediating binding to uninfected compared to virus-infected cells. Also one might expect that if the binding phenomenon were strictly a nonspecific interaction, the adherence results would have been more uniform between strains and between the host cells tested.

Investigators have studied the chemical nature of type-specific surface antigens and results, obtained from heating and from proteolytic enzyme treatment of the organisms, indicate that the majority of the surface components are protein (22). Similar results were obtained in the present study where the proteolytic enzyme treatment of Cowan I, PA^-, EMS, and No. 1071 significantly decreased adherence to both uninfected and virus-infected cells. Autoclaving and enzyme treatment of our test strains were not sufficient to render the staphylococci adherence negative. It is possible that adhesins and potential adhesins exist in layers on the surface of the organisms; thus, treatment sufficient to remove or destroy some adhesins might actually expose or unmask potential adhesins in deeper layers. In studying staphylococcal surface antigens, serologists have noted similar results in that autoclaving and enzyme treatment of staphylococci usually made "blocked" antigens accessible to interact with antibody; these results led to the postulation of a surface layer of antigens of protein nature and a deeper layer of carbohydrate antigens (24). Whether any of these surface antigens actually play a role as adhesins in our test system is unknown but the possibility merits further study.

Previous studies in our laboratory (10) showed that thermal extracts obtained from autoclaved No. 1071 were biologically active in blocking the adherence of this strain of *S. aureus* to uninfected and virus-infected cells. Blocking was equally effective with thermal extracts obtained from organisms grown in a chemically defined or on a biologically complex medium, indicating that the blocking factor(s) was not simply a component present from the agar used to grow the cells. In the present study thermal extracts from four of the test strains of *S. aureus* were tested for their effectiveness in blocking adherence of each of the four test strains (Fig. 3). Each of the four extracts contained blocking factors sufficient to decrease adherence of each of the four test strains to virus-infected cells by >55% (range 56–90%). The extracts also significantly blocked adherence to uninfected control cells, except for PA^- where adherence was decreased only 9–37% depending upon the thermal extract used to treat monolayers. Surprisingly the thermal extracts from PA^- and EMS were effective in blocking adherence; heating these two organisms had no effect on their ability to adhere suggesting that adhesins were neither destroyed nor released from the cell surface. It is possible that heat treatment, while releasing adhesins (blocking factors), also unmasks other surface components capable of functioning as adhesins. It is also possible that blocking factors, released by harsh heat treatment, are not surface associated. A comparison of the thermal extracts by anion-exchange HPLC (Fig. 4) indicated that the acidic polypeptides were heterogeneous mixtures. We have compared the anion-exchange HPLC profiles of thermal extracts from strain No. 1071, containing ^{125}I-labeled surface proteins, unabsorbed and absorbed with purified membranes obtained from uninfected and virus-infected cell monolayers (B. A. Sanford, V. L. Thomas, and M. A. Ramsay. Abstracts of the Annual Meeting of the ASM, No. D1, p. 54,

1985). Results showed that most of the labeled proteins had a retention time of 10 to 14 min. Polypeptides in the dominant peak (retention time of 18.5 min) were not labeled suggesting that these polypeptides were not located on the cell surface and therefore were not accessible to labeling with isotope. Furthermore, these preliminary data indicate that only 5–7% of the labeled surface proteins are able to interact with purified cell membranes suggesting that the adhesins are a diverse group of minor proteins that occur in very small amounts in the cell wall.

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