Fibronectin and fibrinogen contribute to the enhanced binding of *Staphylococcus aureus* to atopic skin

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Background: ***Staphylococcus aureus*** **colonizes the skin lesions of more than 90% of patients with atopic dermatitis (AD). The mechanism for increased *S aureus* colonization in AD is unknown. However, the initial event in colonization requires adherence of *S aureus* to the skin.**
Objective: The purpose of this study was to examine the roles of various bacterial adhesins on *S aureus* binding to AD skin.
Methods: In an attempt to delineate the mechanism behind this adherence process, an in vitro bacterial binding assay was developed to quantitate the adherence of various *S aureus* strains to AD, psoriatic, and normal skin sections. *S aureus* strains used in this study were obtained either from cultures of AD skin lesions or from genetically manipulated strains of *S aureus* that lacked specific microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)—namely, fibronectin-binding protein (Fnbp), fibrinogen-binding protein (Clf), collagen-binding protein (Cna), and their parent strains. In addition, *S aureus* strains from patients with AD were pretreated with fibronectin or fibrinogen to block MSCRAMM receptors and interfere with binding.
Results: Under all experimental conditions, binding of *S aureus* was localized primarily to the stratum corneum. Immunocytochemical staining of AD skin sections showed a redistribution of fibronectin to the cornified layer, an observation not seen in normal skin. *S aureus* binding to uninvolved AD skin was significantly greater than the binding to uninvolved psoriatic skin ($P < .0001$) and normal skin ($P < .0005$). The Fnbp-negative *S aureus* showed a significant reduction in binding to the AD skin ($P < .0001$) but not to the psoriatic and normal skin. In the AD skin, a significant reduction in the binding of *S aureus* was also observed in the Clf-negative strain ($P < .0001$) but not in the Cna-negative *S aureus*. Preincubation of *S aureus* with either fibronectin or fibrinogen also inhibited bacterial binding to AD skin ($P < .0001$).
Conclusion: These data suggest that fibronectin and fibrinogen—but not collagen—play a major role in the enhanced binding of *S aureus* to the skin of patients with AD. (J Allergy Clin Immunol 2001;108:269-74.)

Key words: *Atopic dermatitis, collagen, fibrinogen, fibronectin, Staphylococcus aureus*

Atopic dermatitis (AD), a complex chronic inflammatory skin disease affecting more than 10% of children, is a major cause of occupation-related disability.[1,2] More than 90% of patients with AD have *Staphylococcus aureus* colonization of their skin lesions.[3,4] In contrast, only 5% of normal subjects carry *S aureus* on their skin. Numerous reports suggest that *S aureus* plays an important role in the exacerbation and chronicity of AD through the release of toxins, some of which are superantigenic in nature, that deliver strong activating signals to T cells expressing specific T-cell receptor V-β regions and thus augment the skin inflammatory response.[5-9] Reduced skin inflammation after antibiotic treatment in patients with chronic AD strongly suggests that this bacterium can be involved in the development of AD.[10] Because of increasing concerns about the emergence of antibiotic-resistant *S aureus*, it is important to understand the mechanisms by which *S aureus* binds to atopic skin and to identify potential therapeutic targets for reduction of *S aureus* adherence.

The mechanism by which *S aureus* is able to successfully colonize the skin of patients with AD is unknown. However, recent studies have demonstrated that *S aureus* strains express a distinct array of receptors that recognize different human extracellular matrix proteins; these are termed *microbial surface components recognizing adhesive matrix molecules* (MSCRAMMs).[11] This family of microbial cell surface proteins has been shown to specifically bind extracellular matrix proteins such as fibronectin, fibrinogen, and collagen, implicating these proteins as potential ligands for *S aureus* binding to AD skin. In the current study, we therefore examined the potential role of fibronectin-binding protein (Fnbp), collagen-binding protein (Cna), and fibrinogen-binding protein (also referred to as *clumping factor* [Clf]) in the adherence of *S aureus* to atopic skin in comparison with skin sections from patients with psoriasis and from normal controls. Because lesional skin contains an overabundance of *S aureus* that could have interfered with our analyses, we focused our attention on the binding characteristics of uninvolved, normal-appearing skin from these patients.

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Abbreviations used

AD: Atopic dermatitis
Clf: Fibrinogen-binding protein, or clumping factor
Cna: Collagen-binding protein
Fnbp: Fibronectin-binding protein
HSA: Human serum albumin
MSCRAMM: Microbial surface component recognizing adhesive matrix molecules

METHODS

Bacterial strains

Two sources of *S aureus* strains were used in this study. Some strains were cultured from the skin lesions of patients with AD; others were isogenic mutants of *S aureus* that had been generated by allele replacement or transposon mutagenesis to eliminate the expression of specific MSCRAMMs—ie, FnbpA and FnbpB, ClfA and ClfB, and Cna, together with their corresponding wild-type parent strains. The isogenic mutant strains were as follows: (1) strain DU5883, the FnbpA- and FnbpB-negative mutant (8325-4 *fnbA*:Tc^R *fnbB*:Em^R) of *S aureus* strain 8325-4 (provided by Dr Timothy J. Foster, Trinity College, Dublin, Ireland)[12]; (2) strain DU5944, the ClfA- and ClfB-negative mutant (Newman *clfA2*:Tn917 *clfB*:Tc^R) of the Newman *S aureus* strain (provided by Dr Foster)[13]; and (3) strain PH100, a collagen adhesin-negative mutant (Phillips *cna*:Gm^R) made in *S aureus* strain Phillips (generously provided by Dr Magnus Höök, Texas A&M University, Houston, Tex).[14] For each of these mutant strains of *S aureus*, the corresponding *S aureus* parent strain (8325-4 [from Dr Foster]), Newman [also from Dr Foster], or Phillips [from Dr Höök]) was used for comparison of baseline binding to skin sections.

To prepare the *S aureus*, the organisms were streaked onto blood agar plates (Remel, Lenexa, Kan) and incubated overnight at 37°C. One colony from each plate was taken and incubated in 1% human serum albumin (HSA)/tryptic soy broth (Becton Dickinson, Sparks, Md) into early logarithmic growth phase for 4 hours at 37°C with gentle shaking. Bacteria were harvested by 10 minutes of centrifugation at 3000 rpm and washed twice with sterile PBS. The bacteria were resuspended in 1 mL of sterile PBS to a concentration of approximately 10^7 colony-forming units per milliliter before each experiment.

PCR identification of MSCRAMM expression in *S aureus*

S aureus isolated from the skin of patients with AD was analyzed by PCR to assess which MSCRAMM (Fnbp, *clf*, or Cna) genes were encoded by these bacteria. Skin bacterial isolates grown overnight at 37°C under shaking conditions in tryptic soy broth were pelleted by centrifugation, and protoplasts were prepared through use of 50 μg/mL lysostaphin (Sigma, St Louis, Mo), as previously described.[15] Total genomic DNA was extracted from the bacterial pellets through use of DNAzol (Molecular Research Center, Inc, Cincinnati, Ohio), according to the manufacturer's instructions. Bacterial DNA (0.5 μg) was amplified with 2.5 U *Thermus aquaticus* DNA polymerase (Perkin Elmer, Boston, Mass) in 50 μL of 50 mmol KCl, 10 mmol Tris-HCl, 2 mmol MgCl2, 200 μmol of each dNTP, and 125 ng of each primer. PCRs were run for 30 cycles: 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C. The 500–base pair (bp) D-repeat region of the FnBP[16] was amplified with primers (forward) 5′-acacatggatcctcaaaacatcacgctgatg-3′ and (reverse) 5′-tctggtgaattcggcgttggcacg-3′. The 1600-bp DNA fragment containing the A-domain of the fibrinogen-binding adhesin (ClfA) protein[17] was amplified with primers (forward) 5′-cgcggatccagtgaaaatagtgttacgcaatct-3′ and (reverse) 5′-cgcaagcttctctggaattggttcaatttc–3′. Finally, the 1506-bp A domain of the collagen adhesin protein (Cna)[18] was amplified with primers (forward) 5′-gcggatccgcacgagatatttca-3′ and (reverse) 5′-cggtcgacttattcagtattagtaaccac-3′. After amplification of the *FnbB*, *ClfA*, and *Cna* genes, the PCR products were subjected to electrophoresis in 2% (FnbB) or 1% (ClfA and Cna) agarose gels (Boehringer, Mannheim, Germany) and stained with ethidium bromide (Sigma). Bands were visualized with the Eagle Eye II still video system (Stratagene, La Jolla, Calif).

Bacteria binding assay

Skin biopsies were obtained from 6 patients with AD as defined by the criteria of Hanifin and Rajka,[19] from 6 patients with psoriasis, and from 6 normal healthy volunteers with no previous histories of atopy. Biopsies from patients with AD and from patients with psoriasis were excised only from uninvolved areas, inasmuch as skin lesions are colonized with *S aureus* that would have interfered with the bacteria binding assay. Skin biopsies were snap-frozen in OCT (Triangle Biomedical Sciences, Durham, NC), and 10 μm cryostat sections were then prepared. Skin sections were stored at –80°C until used.

Bacteria were washed twice with sterile PBS and then resuspended at a final concentration of 1×10^7 per milliliter, again in sterile PBS. One million bacteria were taken from each prepared stock and washed twice with sterile PBS. One hundred microliters of the bacterial suspension was added to frozen skin sections. The bacteria were then incubated for 60 minutes at room temperature on a rotating table under moist conditions to allow bacterial adherence to the skin sections. After this incubation period, sections were washed twice in PBS to remove unbound bacteria from the skin sections. Sections were then treated with 1% (w/v) paraformaldehyde (Sigma) in PBS for 10 minutes at room temperature, washed twice in PBS, and then air-dried.

The role of extracellular matrix proteins in bacterial adherence to AD skin was further examined by preincubating *S aureus* from patients with AD with 10 μg/mL of fibronectin or fibrinogen (Sigma) or 1 mg/mL of HSA (as a control) for 2 hours at room temperature. Bacteria were harvested by 10 minutes of centrifugation at 3000 rpm. The *S aureus* was washed twice with sterile PBS. Bacteria were resuspended in 1 mL of sterile PBS. One hundred microliters of each bacterial strain was pipetted sequentially on to individual skin sections derived from each subject group. The binding experiment was then done exactly as described in the preceding paragraph.

Bacteria that bound to skin sections were visualized through use of a Gram stain. In brief, slides were fixed by being passed through a flame and then immersed in crystal violet for 1 minute and gently washed in water. The slides were immersed in Gram iodine for 1 minute, washed in water, decolorized with acetone-alcohol, and finally washed in water; they were then stained with safranin for 1 minute, rinsed with water, and blotted dry. The number of bacteria bound to each skin section was quantitated microscopically by a blinded assessor and then expressed as number of bacteria bound per high-power field (×1000); a minimum of 3 fields were counted. Bacterial adherence was also quantitated in relation to the epidermal strata to which they were bound—ie, stratum corneum or subcorneal epithelium.

In selected experiments, labeling of the bacteria with biotin was carried out through use of a previously described method.[20-22] In brief, *S aureus* was washed twice with labeling buffer (8.0 g NaCl, 0.4 g KCl, 1.0 g glucose, 0.2 g $MgSO_4 \times 7\ H_2O$, 0.2 g $CaCl_2 \times 2\ H_2O$, 0.5 g $NaHCO_3$, and distilled water to 1000 mL, pH 7.4; Sigma) to remove any traces of soluble constituents containing amino groups. D-Biotin-N-Hydroxy Succinimide Ester (Sigma) was dissolved in 1 mL DMSO (Sigma) and was added at a concentration of 5 mmol/L to the growth medium. The mixture was incubated for 90 minutes at 37°C. Bacteria were harvested by 10 min-

utes of centrifugation at 3000 rpm and resuspended in PBS. To remove unbound biotin from the suspension, this washing procedure was repeated 3 times. The pelleted bacteria were diluted in PBS, and the colony-forming unit for each specimen was determined to adjust the *S aureus* number for binding assay. After binding experiments and washing with PBS, bound *S aureus* was detected in triplicate with commercially available staining kits (Zymed, San Francisco, Calif). Immunohistochemical staining was carried out, exactly as recommended by the manufacturer, through use of the SA-AP technique. Each specimen was counterstained in Mayer's hematoxylin (Zymed) and mounted with crystal mount (Biomeda, Foster City, Calif).

Immunohistochemical staining for fibronectin

Skin biopsies were washed 3 times in PBS (to remove any residual serum) and snap-frozen; 5-mm cross-sectional frozen sections were then prepared for immunostaining. Sections were blocked for 10 minutes at room temperature with a commercial blocking solution (Superblock, Scytek, Logan, Utah). The blocking solution was then aspirated, and sections were incubated overnight at 4°C with a 1:50 dilution of antihuman fibronectin rabbit polyclonal antibody (Sigma). After this incubation period, sections were washed in 1xPBS and incubated at room temperature for 1 hour with an antirabbit FITC-conjugated antibody (Dako, Carpinteria, Calif) at a final dilution of 1:50 in PBS. Sections were again washed in 1xPBS and then mounted through use of a 50% (v/v) solution of glycerol (Sigma) in 1xPBS and assessed by fluorescence microscopy. Intensity of staining was quantified by image analysis (IP Lab Spectrum, Signal Analytics, Vienna, Va) and expressed in terms of mean fluorescence intensity. To ensure specific quantification of staining within the epithelium, multiple small gates were created through use of the image analysis software. This assured that readings were taken solely within the epithelial layer.

Statistical analysis

The number of bacteria bound per high-power field was expressed as the mean ± SEM. Statistical comparisons between different groups of subjects were performed with ANOVA. Unplanned pairwise contrasts between group means were done through use of the Tukey-Kramer multiple comparison procedure. Differences observed between groups were determined through use of an unpaired, 2-tailed Student *t* test. *P* values of less than .05 were accepted as statistically significant.

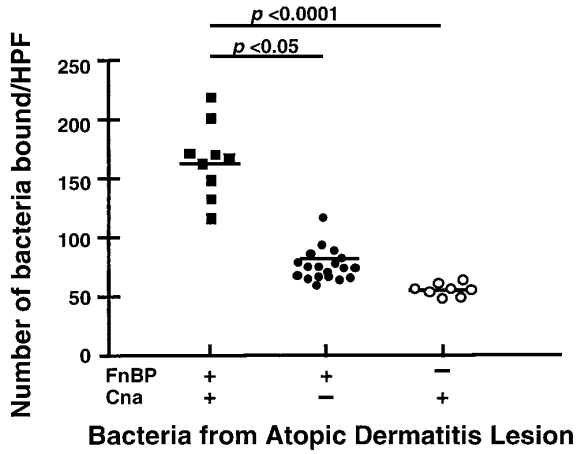

FIG 1. Quantification of *S aureus* adherence to the epidermis of uninvolved AD skin through use of strains isolated from cultures of skin lesions from 37 patients with AD. This experiment is representative of experiments done on 3 different skin biopsies from patients with AD. Differences in bacterial binding between bacterial groups according to MSCRAMM expression were examined statistically through use of an unpaired *t* test.

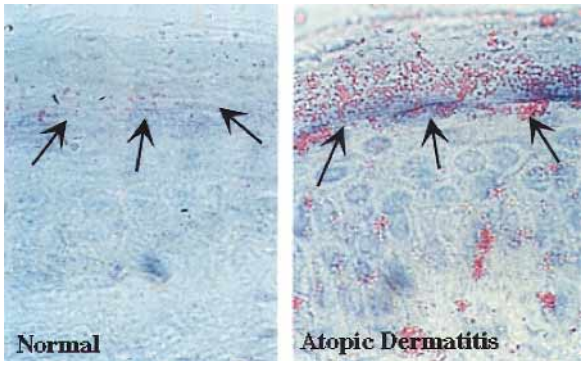

FIG 2. Visualization of *S aureus (arrow)* adherence to normal healthy skin (n = 5; *left panel*) and uninvolved AD skin (n = 5; *right panel*). Binding of *S aureus* was localized mainly to the stratum corneum, with little or no binding to the substrata (×400).

RESULTS

MSCRAMM expression of AD *S aureus*

S aureus was isolated from 37 patients with AD and analyzed by PCR to assess which MSCRAMM (Fnbp, *clf*, or Cna) genes were expressed by these bacteria. All bacterial strains contained the gene for *clf*—ie, the fibrinogen-binding protein—but they varied in their expression of Fnbp and Cna (Fig 1). To determine the potential role of MSCRAMMs in bacterial adherence to AD skin, bacteria from patients with AD were divided into 3 groups according to their MSCRAMM expression: (a) Fnbp-positive, Cna-positive; (b) Fnbp-positive, Cna-negative; and (c) Fnbp-negative, Cna-positive. The groups were then compared for the numbers of *S aureus* adhering to skin sections of a patient with AD.

Under all experimental conditions, the significant difference in binding of *S aureus* between the conditions was observed primarily in the stratum corneum (Fig 2). As shown in Fig 1, the expression of Fnbp seemed to play a decisive role in the amount of bacterial binding to the stratum corneum. The Fnbp-negative strains also have the ability to bind to the stratum corneum—though to a much lesser extent, perhaps because of the residual expression of fibrinogen binding proteins. In contrast, Cna expression had little influence on bacterial adherence to the stratum corneum.

Adherence of AD *S aureus* to AD, psoriasis, and normal epidermis

Our remaining experiments focused on the role of extracellular matrix skin determinants in modulating *S aureus* binding. We used one Fnbp-positive and Cna-positive *S aureus* from a patient with AD to compare the relative

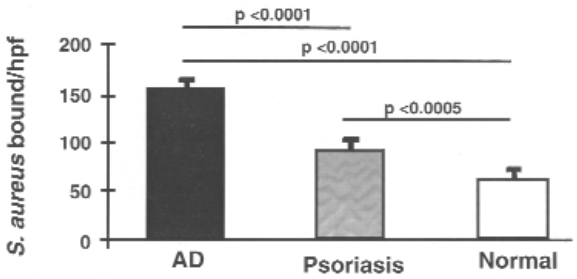

FIG 3. Quantification of *S aureus* adherence to the stratum corneum of uninvolved AD (n = 6), uninvolved psoriatic (n = 6), and normal skin (n = 6) through use of a FbBP-positive, Cna-positive *S aureus* derived from a patient with AD. Differences in bacterial binding among AD, psoriasis, and normal controls were examined statistically through use of an ANOVA; pairwise contrasts between group means were then done through use of the Tukey-Kramer multiple comparison procedure.

binding of *S aureus* with that in uninvolved skin from subjects with AD versus uninvolved skin from subjects with psoriasis versus normal control skin. As shown in Fig 3, the greatest level of binding was to uninvolved AD skin, followed by uninvolved psoriatic skin and normal skin ($P < .0001$ and $P < .0005$, respectively, in comparison with normal; $P < .0001$ for uninvolved AD vs uninvolved psoriatic).

Effect of extracellular matrix proteins on *S aureus* adherence to AD skin

To determine which MSCRAMMs are involved in the binding of *S aureus* to atopic skin, *S aureus* from a patient with AD that expressed all MSCRAMMs was preincubated with fibronectin, fibrinogen, or collagen in an attempt to block the MSCRAMM receptors and assess their ability to inhibit bacterial binding (Fig 4). There was a significant reduction in numbers of *S aureus* binding to the skin after fibronectin or fibrinogen treatment ($P < .0001$ in comparison with untreated controls) but not after collagen treatment. We also used 1% HSA as a control for nonspecific protein blocking of MSCRAMM receptors. *S aureus* incubated with 1 mg/mL of HSA, under the same incubation conditions used with extracellular matrix proteins, adhered to the skin in the same manner as untreated bacteria.

Adherence of *S aureus* mutant strains to AD, psoriasis, and normal skin

To confirm the blocking studies, an independent approach was used to identify the key MCRAMMS involved in the binding of *S aureus* to atopic skin. *S aureus* mutants with selective deficiencies in specific MCRAMMS were examined for their ability to bind AD skin sites. As shown in Fig 5, binding of *S aureus* strain DU5883, which is an FnbpA- and FnbpB-negative mutant, to AD skin was markedly reduced in comparison with its parent 8325-4 strain ($P < .0001$). However, the Fnbp-negative mutant strain of *S aureus* did not show a significant reduction in binding to the psoriatic or normal skin sections. Similarly, binding of *S aureus* strain DU5944, which is a ClfA- and ClfB-negative mutant, was significantly less than the binding of its parent Newman strain ($P < .0001$) in the atopic skin but not in the psoriatic skin or the normal skin. In contrast, the binding to AD skin of *S aureus* strain PH100, which is a Cna-negative mutant, was not significantly different from that of its parent Phillips Strain.

Immunohistochemical localization of fibronectin in the skin

Skin sections both from patients with AD and from normal healthy individuals were immunohistochemically stained for fibronectin (Fig 6). In the normal individuals, fibronectin deposition was observed primarily in epidermal/endothelial basement membranes and diffusely throughout the entire dermal layer. AD skin and normal skin also showed a similar staining pattern for fibronectin. However, fibronectin staining was also observed in the upper strata of the epidermis of AD skin—in particular, the stratum corneum (Fig 6, *left panel*). This pattern of fibronectin deposition was not observed in the epidermis strata of normal skin (Fig 6, *right panel*).

DISCUSSION

S aureus colonization and infection of the skin are thought to play an important role in the pathogenesis of AD.[5-8] This relationship is supported by observations that the number of *S aureus* colonizing the skin of patients with AD correlates with disease severity and that antistaphylococcal antibiotic therapy reduces the severity of skin disease in poorly controlled AD.[3,4] The mechanism by which *S aureus* triggers the exacerbation of AD is an area of active investigation and includes a role for the release of superantigenic toxins that stimulate marked activation of T cells and macrophages.[9]

One aspect of this disease that has not been well studied is the mechanism by which *S aureus* is able to preferentially colonize the skin of patients with AD. It is known that *S aureus* expresses an array of adhesins—MSCRAMMs—that allow it to bind extracellular matrix components and also certain plasma proteins, such as fibrinogen, that can exudate into sites of skin inflammation.[23] Through use of these adhesins, initial adherence to the skin occurs and the colonization process begins.

The current study sought to examine the role of these MSCRAMMs and their ligands on *S aureus* binding to atopic skin in comparison with psoriatic skin and normal skin. Our findings indicate that fibronectin and fibrinogen—but not collagen—are the key MSCRAMMs used in the binding of *S aureus* to AD skin. This is supported by the following observations: First, isogenic mutants of *S aureus* that were selectively deficient in Fnbp or Clf demonstrated reduced binding to AD skin. In contrast, a collagen adhesin-negative mutant did not show decreased binding to atopic skin. The decrement in binding of *S aureus* deficient in either Clf or Fnbp was not observed in normal skin. This supports the concept that fibronectin plays an important role in AD skin. Second,

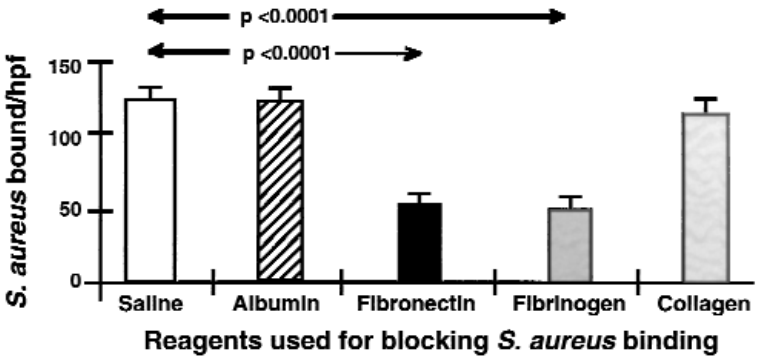

FIG 4. The effect of blocking MSCRAMM receptors with purified fibrinogen and fibronectin on the binding of *S aureus* to the stratum corneum of uninvolved AD through use of an Fnbp-positive, Cna-positive *S aureus* derived from a patient with AD. Fibrinogen and fibronectin had no effects on *S aureus* binding to normal skin (data not shown). Differences in bacterial binding were examined statistically through use of an unpaired *t* test (*P* values are shown).

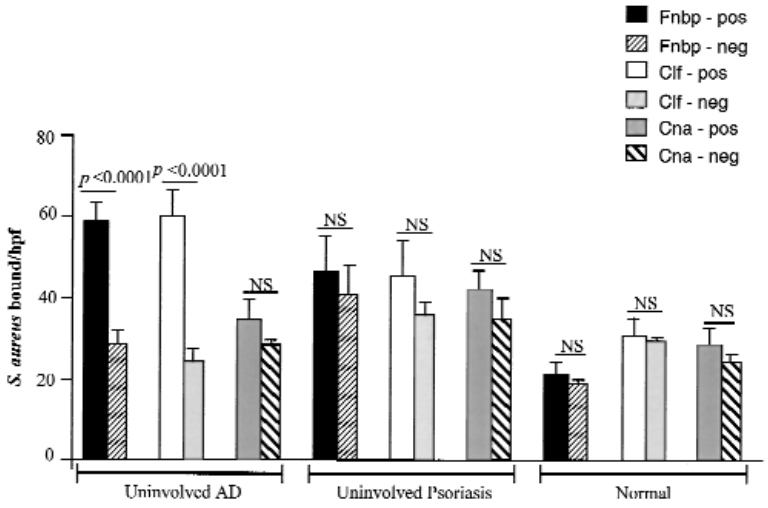

FIG 5. Quantification of MSCRAMM mutant *S aureus* strains (Fnbp-negative, Cna-negative, and Clf-negative) to uninvolved AD skin versus normal skin. Binding of each bacterial mutant strain was compared with that of its parent strain in every case; changes in binding ability were examined statistically through use of an unpaired *t* test (*P* values are shown).

when *S aureus* was preincubated with saline solution, HSA, fibronectin, fibrinogen, or collagen in an attempt to block the MSCRAMM receptors and thus interfere with bacterial adherence, only fibronectin and fibrinogen—and not saline, collagen, or HSA—significantly reduced the level of *S aureus* binding to AD skin. Of note, the bacterial binding sites were confined mainly to the stratum corneum.

The exact mechanism for increased fibronectin deposition in AD skin is unknown. However, it is likely due to the increased expression of T_H2-like cytokines in atopic skin. In this regard, we have previously found that uninvolved skin of patients with AD exclusively expresses IL-4 and IL-13 but not interferon or IL-12.[24,25] In contrast, psoriatic skin, which demonstrated significantly less *S aureus* binding, is characterized by expression of T_H1-like cytokines. This is consistent with previous in vitro studies demonstrating that IL-4—but not IFN-γ—can

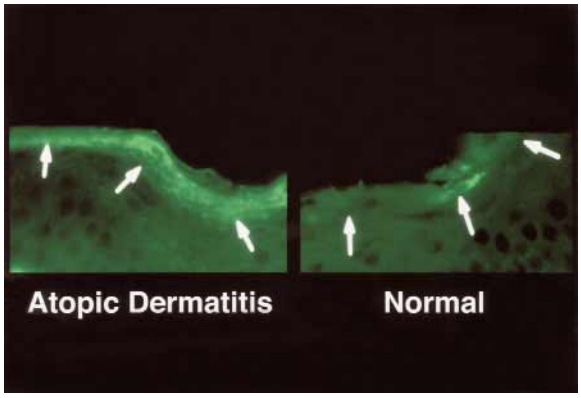

FIG 6. Immunohistochemical staining of skin sections for fibronectin in patients with AD (n = 5) and normal healthy volunteers (n = 5). Fibronectin staining of uninvolved AD skin *(left panel)* was greater than fibronectin staining of normal skin *(right panel)*.

induce fibronectin production by skin fibroblasts.[26,27] Keratinocytes can also act as a source of fibronectin in the skin.[28] Furthermore, allergen challenge in subjects with atopic asthma leads to increased fibronectin in bronchoalveolar lavage fluid, suggesting that an airway T_H2 response can also induce fibronectin.[29] Thus, IL-4–induced fibronectin synthesis, in combination with plasma exudation of fibrinogen, could provide a mechanism by which the atopic/inflammatory environment mediates enhanced *S aureus* attachment to the skin. Interestingly, Wann et al[30] demonstrated that the *S aureus* fibronectin-binding MSCRAMM FnbpA is a bifunctional protein that also binds to fibrinogen. This observation is consistent with our current observations, suggesting that blocking the binding of *S aureus* to fibrinogen and fibronectin might be an important therapeutic target for reduction of *S aureus* colonization in atopic skin.

In summary, using *S aureus* mutant strains deficient in the expression of selective MSCRAMMs, we have demonstrated a link between atopic skin inflammation and increased colonization of the skin by *S aureus*. It should be noted, however, that though fibronectin and fibrinogen provide abundant adherent sites for bacterial adherence, other adhesins could also be involved, inasmuch as both extracellular matrix bacterial incubation and utilization of MSCRAMM mutants of *S aureus* did not totally abrogate the binding of *S aureus*. Nevertheless, our current study provides an approach for further investigation into the interactions between MSCRAMMs and tissue components that initiate bacterial colonization. Additional studies are needed to identify other factors that support this colonization process as well as the further growth of bacteria.

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