The Presence and Impact of Biofilm-Producing Staphylococci in Atopic Dermatitis

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IMPORTANCE Atopic dermatitis (AD) is thought to be a double-hit phenomenon with an unknown environmental component and a genetic abnormality likely centered on the filaggrin gene. Biologically, the presence of Staphylococcus aureus in AD was reported more than 2 decades ago, but the relationship to AD has been elusive.

OBJECTIVE To explore the bacteria that produce the biofilms in the lesions of AD and the response of the innate immune system to these biofilm occlusions of the sweat ducts by specifically evaluating Toll-like receptor 2.

DESIGN, SETTING, AND PARTICIPANTS University hospital dermatologic clinic study involving the environmental component related to the characterization, correlation, and impact of staphylococci and their biofilms in AD. We processed routine skin swabs from lesional and nonlesional skin from 40 patients with AD and performed scrapings and biopsies. We also obtained 20 samples from controls (10 inflamed skin samples and 10 normal skin samples).

EXPOSURES Gram staining, bright-field microscopy, hematoxylin and eosin, periodic acid–Schiff, Congo red, and light microscopy.

MAIN OUTCOMES AND MEASURES Association of staphylococcal biofilms with AD pathogenesis.

RESULTS All AD-affected samples contained multidrug-resistant staphylococci, with S aureus (42.0%) and Staphylococcus epidermidis (20.0%) as the predominant species. All isolates were positive for extracellular polysaccharide and biofilm (85.0% strong biofilm producers and 15.0% moderately to weakly positive). Polymerase chain reaction revealed the biofilm-mediating icaD (93.0%) and aap (12.5%) genes in the isolates (some contained both). We also examined tissues for microbial identification, extracellular biomass formation, biofilm formation, and staphylococcal biofilm in skin tissues. Occlusion of sweat ducts with periodic acid–Schiff-positive and Congo red-positive material was noted on microscopic tissue examination. Toll-like receptor 2 was shown to be activated in AD lesional skin (immediately proximal to the sweat ducts), which likely led to the initiation of proteinase-activated receptor 2-mediated pruritus and MyD88-mediated spongiosis.

CONCLUSIONS AND RELEVANCE Biofilm formation by AD-associated staphylococci almost certainly plays a major role in the occlusion of sweat ducts and leads to inflammation and pruritus. We believe the environmental hit in AD relates to staphylococci and their biofilms, which occlude sweat ducts.
We recently demonstrated the presence of biofilms in atopic dermatitis (AD) lesions.\textsuperscript{1,2} We have also shown that the eccrine ducts in AD lesions are occluded by what we believe are biofilms.\textsuperscript{3} In the current work, we have explored the bacteria that produce the biofilms in these lesions, as well as Congo red staining in AD lesions. Inasmuch as Congo red stains amyloid, which is part of the “infrastructure” of biofilms,\textsuperscript{4} the presence of Congo red staining conclusively demonstrates that biofilms form the sweat duct occlusions.

Furthermore, we have explored the response of the innate immune system to these biofilm occlusions. Because all the bacteria we recovered with routine cultures were gram positive, we chose to evaluate Toll-like receptor 2 (TLR2) as it is the main “first responder” to gram-positive organisms.\textsuperscript{5} The consequences of TLR2 activation have been documented\textsuperscript{6} and dovetail nicely into the symptoms and signs of AD.\textsuperscript{7,8}

Our hypothesis regarding AD is that “subclinical miliaria” forms the environmental component of a double-hit phenomenon.\textsuperscript{9} Miliaria itself has been shown to arise from sweat ducts occluded by biofilms produced by staphylococci.\textsuperscript{10} The genetic part of AD has been shown to be related to defects in filaggrin or other genes that lead to the production of a faulty stratum corneum.\textsuperscript{11}

What is new in this work is finding conclusive evidence of the presence of biofilms in the eccrine sweat ducts in AD. Also novel is discovering the organisms in AD lesions that make the biofilms that form these ductal occlusions. Those bacteria, along with many others, have been noted previously,\textsuperscript{12} but to our knowledge, they have not been linked to biofilm production or activity in AD. The activity of the biofilms in eliciting TLR2 activation is also novel.

**Methods**

**Ethics Approval**

The study was approved by the institutional review board of Drexel University College of Medicine.

**Sample Collection and Processing**

Forty samples from patients with AD attending the Drexel University College of Medicine Dermatology Clinic were collected for bacteriologic studies, using recommended sterile swabs made for transport. Patients included 21 males and 19 females aged 3 months to 85 years. Samples were taken from (clinically nonimpetiginized) lesions in different locations according to the presentation of the AD, such as antecubitalis in flexural AD (Figure 1) and the face in facial-extensor AD.\textsuperscript{13} Microbial cultures were prepared using routine methods. Samples from 20 control patients were secured and processed similarly to the lesional specimens. These included 10 samples from inflamed skin (psoriasis rosea and others) and 10 from non-inflamed, nonatopic skin.

Skin scrapings and tissue biopsy specimens from AD lesonal and nonlesional areas were processed as per standard procedure. The scrapings were observed under bright-field microscopy after Gram staining. The tissues, stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Congo red, were read by routine light microscopy. The H&E and PAS stains in this disease have been presented elsewhere.\textsuperscript{3}

Ten cases that had the clinical diagnosis of AD and histopathologic findings of both spongiotic dermatitis and acrosyringal blockade with PAS-positive material were selected for immunostaining with phycoerythrin antihuman CD282 (TLR2) antibody (BioLegend) at a titration level of 1:50. Ten samples from inflamed skin (psoriasis, pityriasis rosea, tinea corporis, etc) and 10 samples from normal skin (from surgical tips) were used as controls. Each level of sections of the stained tissue was then evaluated for a positive and negative staining pattern compared with the normal tissue.

**Identification and Speciation of Isolates**

The isolates, which would ordinarily be discarded as “normal flora,” were broadly classified as coagulase-negative staphylococci using the Staphaurex test kit (Thermo Fisher Scientific).\textsuperscript{14} Further identification and speciation was performed using inoculation on Mannitol Salt Agar plates and the API Staph (bioMérieux SA) phenotypic system. The API system was used per the direction of the manufacturer and as described elsewhere.\textsuperscript{15} The isolates were also identified as Staphylococcus by genotyping using the established method of colony-direct species-specific polymerase chain reaction.\textsuperscript{16}

**Antibiotic Susceptibility Testing**

The isolates were tested for their antimicrobial susceptibilities, and minimum inhibitory concentrations were determined with the Sensititer (TREK Diagnostic Systems Inc) plate assay\textsuperscript{17} following the manufacturer’s protocol. The minimum inhibitory concentration values were classified according to the Clinical and Laboratory Standards Institute.\textsuperscript{18} An antibiogram was derived from this information. In addition, methicillin resistance was determined by a cefoxitin screen with a
Biofilm Formation by the Isolates

Concurrently, we used purified staphylococcal isolates recovered from AD lesions for in vitro biofilm detection using a safranin microtiter plate assay\textsuperscript{20} and XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assays.\textsuperscript{21,22} According to the absorbance values, the isolates were characterized as weak, moderate, or strong biofilm producers. Congo red agar testing was performed to demonstrate extracellular slime production by the isolates.\textsuperscript{23} In a parallel test, 10 random samples of tissue scrapings were evaluated for the presence of biofilms, using Gram staining and bright-field microscopy. Ten nonlesional samples were also examined as controls.

Polymerase Chain Reaction Amplification of \textit{aap} and \textit{icaD} Sequences

Polymerase chain reaction was used to identify the presence of the \textit{icaD} and \textit{aap} genes from the isolates.\textsuperscript{23-25} Genomic DNA was isolated from the isolates by using the DNA extraction kit (Qiagen Inc). The extracted DNA was used as a template for polymerase chain reaction.\textsuperscript{26-27} Primers (forward and backward) and further delineation of methods are available on request.

Results

Ninety-three percent of samples from AD lesions (37 of 40) were confirmed as staphylococci using the API Staph identification system, and staphylococci were abundant in nearly pure cultures when grown on blood agar plates (Figure 2). The routine Gram staining and biochemical tests identified the isolates as staphylococci. Ninety-five percent of control samples (19 of 20) were similarly confirmed as staphylococci.

Gram staining of 10 of 10 (100%) tested lesional skin samples showed free and tissue-entangled staphylococci, with many organisms contained within biofilms. Ten of 10 control samples taken from nonlesional skin were negative for biofilms on Gram staining.

Speciation analysis revealed \textit{Staphylococcus aureus} (42.0%) and \textit{Staphylococcus epidermidis} (20.0%) as the predominant species. Other staphylococcal species found in normal skin flora were identified as shown. Results with the Staphaurex kit, used to identify the coagulase-negative and coagulase-positive isolates, matched 100.0% with the species identification. Three isolates were not conclusively identified as staphylococci by the API test. Speciation of controls showed \textit{S aureus} (30.0%) and \textit{S epidermidis} (35.0%), and the remainder showed other staphylococci similar to those found in the lesional skin.

To determine the antibiotic susceptibility of isolates, we performed minimum inhibitory concentration testing. Results indicated multidrug resistance in most isolates. The antibiotics with the highest percentage of isolates that showed resistance were erythromycin (85.7%), clindamycin (80.0%), and levofloxacin (65.7%). Tigecycline was the most effective among the antibiotics tested, with only 37.1% of the isolates resistant to it. Methicillin resistance was observed in 24 of 40 isolates (60.0%) and in 7 of 20 control samples (35.0%).

Biofilm formation was detected using the XTT assay. The isolates were classified according to the literature as strong, moderate, or weak biofilm producers. The results indicated that 85.0% (34 of 40) of the isolates were strong biofilm producers, which included both \textit{S aureus} (100.0% of the isolates) and \textit{S epidermidis} (75.0% of the isolates). Phenotypic testing using Congo red agar demonstrated that all the isolates of staphylococci were strongly or moderately positive for extracellular polysaccharide (biomass). Nineteen of 20 controls showed biofilm production on the XTT assay, and 20 of 20 showed biomass production on Congo red cultures. The presence of specific biofilm-mediating gene(s) (\textit{ica operon type}) was found in 37 of 40 samples (92.5%); the \textit{aap} biofilm-producing gene was detected in 5 cases (12.5%). Thirty-eight of 40 isolates tested were shown to be biofilm positive through either phenotypic or genotypic testing. Samples that showed weaker staining on the XTT assay were positive by Congo red agar testing, polymerase chain reaction, or both. The organisms were present in skin with active lesions and in skin with resolved lesions. Biofilms were noted only on lesional skin.

Thirty-six of 36 lesional specimens (100%) stained with H&E showed occlusion of eccrine ducts. Similarly, 36 of 36 lesional specimens showed Congo red within the ducts (Figure 3). The 10 specimens prepared for immunohistochemical analysis all showed activation of TLR2 in the parakeratotic stratum corneum adjacent to the ductal occlusion (Figure 4A). The controls showed immunostaining in the basal layer of the epidermis and not in the stratum corneum (Figure 4B) (\(P = .001, \chi^2\)). The H&E and PAS findings have been presented previously.\textsuperscript{3}
Discussion

Our findings show that various staphylococci that are components of the normal skin flora have the capability to produce biofilms and extracellular polysaccharide biomass material. Most isolates showed multidrug resistance. A positive correlation between the isolates’ multidrug resistance status and their biofilm formation capabilities and virulence has been reported for other staphylococci.20 These findings support the hypothesis that AD lesional areas have strong biofilm-producing staphylococci and that those biofilms occlude sweat ducts, whereas nonlesional areas do not (or at least do it to a much lesser, nonrecognizable, extent). They also support the concept that subclinical miliaria is an important feature of AD, whereby the biofilms occlude the sweat ducts.3 Our finding that S. aureus is more prevalent in lesions is in accordance with previously presented work.12

Our pathologic findings show occluded eccrine ducts; we reported previously that this is the first time, to our knowledge, this has been noted since Sulzberger’s observation in 1947.3,28 Occluded sweat ducts ordinarily mean miliaria,29 but with the addition of an accompanying genetic defect, we believe that AD also must be considered. We have demonstrated that the pathogenesis of these 2 diseases is similar; the PAS-positive ductal obstruction (representing biofilms) is the common link.10 The finding that Congo red stains the obstructions provides conclusive evidence that biofilms form them, because their infrastructure is made of amyloid, and the Congo red stains amyloid.4 Before this finding, amyloid was found only histopathologically in the dermis in diseases such as macular amyloid. It has not been seen in the stratum corneum of the epidermis. We believe the biofilms form in the eccrine ducts preferentially because of the water and salt found there. Both the salt and water, along with other materials such as ethanol, have been shown to induce biofilm production.30 In nonlesional skin, biofilms form in the sweat ducts (and create miliaria) in a similar fashion. However, without the gene defect (such as filaggrin), the atopic lesion is not created.
The immunopathology of miliaria has received little attention, but occluded sweat ducts are the pathologic hallmark of that disease. Our immunopathologic findings show activation of the innate immune system via TLR2 immediately adjacent to the gram-positive bacteria and their biofilms occluding the sweat ducts in AD. \(^{31}\) (Although our sample size was small, the TLR2 findings are unlikely to have occurred by chance, with \(P = .001).\) Even though we did not evaluate them, the events that occur after TLR2 activation have been well studied. Proteinase-activated receptor 2 is stimulated by increased serine protease,\(^{7}\) and this likely induces the intense pruritus\(^{8}\) that is the main symptom of the disease. The MyD88 pathway is also stimulated, leading to nuclear factor-κB activation and ultimately to tumor necrosis factor, which is the most potent stimulant for spongiosis (the main pathologic finding) in AD.\(^{32}\)

Using a double-hit hypothesis, staphylococci would form the environmental component, whereas the genetic component would be filaggrin (the most common among other genes involved in stratum corneum production) deficiency, leading to an abnormal stratum corneum. The entire pathway would be as follows: obstruction of sweat ducts by bacteria (staphylococci) and biofilms,\(^{33}\) followed by activation of TLR2,\(^{31}\) followed by activation of mediators known to produce pruritus (proteinase-activated receptor 2) and spongiosis (tumor necrosis factor). This would lead to itching, scratching, and the production of a rash. Our findings represent the initial portion of that cascade.

Atopic dermatitis has been associated with skin microflora, especially \(S\) \(aureus.\) \(^{33}\) Clinical evidence demonstrated antibodies with high binding affinity against \(S\) \(aureus\) and hyperimmunoglobulinemia E (to \(S\) \(aureus\)) in these patients.\(^{34,35}\) Although \(S\) \(epidermidis\) was reportedly isolated from these cases and associated with \(S\) \(aureus\) in AD lesions, the latter organism was predominantly thought to be involved in (or at least associated with) the pathogenesis of AD.\(^{35}\) Clinical studies showed that the skin of patients with AD was colonized with greater numbers of \(S\) \(aureus\) than the skin of healthy volunteers, which was colonized with \(S\) \(epidermidis.\)\(^{37}\) Various formulations have been tried to control and disrupt the \(S\) \(aureus\) organisms.\(^{38}\)

Discussion of possible therapies based on these findings is premature, but 2 principles are important. First, when all the bacteria are multidrug resistant and 60% also show methicillin resistance, oral antibiotics are generally not a good option in most patients. Topical antibacterial measures, such as bleach baths or bleach gels, seem more reasonable. Second, while the stratum corneum is compromised, assiduous skin care, including aggressive moisturization, seems appropriate. Further studies are under way.

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NOTABLE NOTES

Mór Cohen, Better Known as Moriz Kaposi
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Today, Moriz Kaposi is remembered for the first description, in 1872, of the entity that bears his name, but he was also one of the founders of the Viennese School of Dermatology.

Kaposi was born in 1837 in a poor Jewish family. The story of his name is curious: his first name is written "Moritz" in the records of the Jewish Community, but he almost always used "Moriz," and in some of his Hungarian publications he used "Moricz" and "Mór." The many versions of his first name simply reflected the multiple languages spoken by the educated classes in the Hapsburg monarchy. Originally his surname was Cohen, but, after his conversion to the Catholic faith, he changed it in 1871 to Kaposi, in reference to his birth town Kaposvár, in the Austro-Hungarian Empire. It is still debated why he changed his surname; it is unlikely to have been due to the pressures of anti-Semitism because Kaposi was not an opportunist, and at that time he was well established in his career. According to his own words, Mór Cohen changed his surname to avoid confusion with 5 other physicians named similarly in the Vienna School of Medicine. ²

In 1886 Kaposi married Martha Hebra, daughter of Ferdinand Ritter von Hebra who was his mentor and with whom he authored the book Textbook of Skin Diseases in 1878. Kaposi's main work, however, was Pathology and Therapy of the Skin Diseases in Lectures for Practical Physicians and Students, which became one of the most significant books in the history of dermatology and was translated into many languages. Kaposi's remarkable skill with languages stood him in good stead; he was fluent in Hungarian, German, and French. In addition, he was versed in English and of course Latin, the official language of the Empire.³

While Hebra is considered the "father of dermatology," Kaposi was one of the first to establish dermatology on its anatomical pathology scientific basis. In his field, Kaposi concerned himself chiefly with syphilis, its clinical presentation, its etiology, and treatment. He wrote with Hebra some of the early descriptions of cutaneous lupus erythematosus and noted the systemic involvement in 1872, and in 1875 he described the rash as "butterfly." Kaposi used his skills of observation and description to first report and delineate many other entities, such as xeroderma pigmentosum, diabetic and leukemic skin changes, syringoma, gangrenous zoster or eczema herpeticum, and pustulosis varioliformis acuta, which later became known as Kaposi varicelliform eruption or eczema herpeticum.

Kaposi died peacefully in his sleep in Vienna at only 65 years of age.

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