

Molecular Characterization of Inflammation and *Staphylococcus aureus* Colonization of Involved Skin of Atopic Dermatitis Patients

A Non-Invasive Approach

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Key Words

Atopic dermatitis · Cytokine markers · Enterotoxins · Protein A · Real-time polymerase chain reaction · *Staphylococcus aureus*

Abstract

Atopic dermatitis (AD) is a multifactorial chronic inflammatory disease mainly stemming from a genetic predisposition that leads to hypersensitivity to environmental factors and a common involvement of *Staphylococcus aureus* (SA) colonization. The aim of this work was to propose a new non-invasive approach to enumerate the genes coding for the toxins of SA in atopic skin samples. In parallel, the study aimed to evaluate the change in AD through 3 markers of the inflammatory response: IL-8, IL-1RA/IL-1 α and IL-18. These methods were tested on 31 patients with AD, and finally on a group of 19 subjects for whom clinical improvement had been reported after various treatments. The study revealed the presence of a large number of genes encoding toxins in atopic samples, indicating a high rate of SA colonization, and also an increase in the level of all cytokine markers in atopic skin compared to the skin of healthy subjects. Finally, we found a positive correlation between increases in the SCORAD (Scoring Atopic Dermatitis Index) value after treatment and

the corresponding evolution of the SA density. These methods provide a means to clinically evaluate the course of AD, and may help in the development of potential treatments.

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Introduction

Atopic dermatitis (AD) is an inflammatory dermatosis involving many likely related factors [1]. These include genetic predispositions, particularly those involving several mutations in the filaggrin gene [2, 3], leading to abnormalities of the cutaneous barrier and/or immune system. The resulting symptoms include hypersensitivity to environmental conditions [4], skin dryness and colonization by *Staphylococcus aureus* (SA) [5], with a possible link between high rates of bacterial colonization and the severity of the lesions [6]. The expression of virulence factors in SA is a predominant element in the pathogenicity of the infection. In particular, toxins produced by certain strains of SA and acting as superantigens may activate the proliferation of T cells and facilitate Th2 polarization of the immune response. This would lead to the production of IgE, the rate of which correlates with the severity of AD in the majority of cases [7].

Staphylococcus superantigen-specific IgE have in fact been identified in subjects presenting with AD [8]. As with lymphocyte activation, these specific IgE may contribute to an activation cascade of other cell types (e.g. keratinocytes, endothelial cells, cutaneous mast cells), leading to the production of inflammatory cytokines and chemokines, as well as the liberation of histamines and other vasoactive substances during the expression phase of eczema [9]. In addition, *Staphylococcus aureus* protein A (Spa) has been shown to induce the liberation of IL-18 in mouse keratinocytes and subsequently trigger an increase in the serum level of IgE [10]. The serum concentration of IL-18 found in patients suffering from AD is linked to the inflammatory lesion score [11], thereby indicating a correlation between the serum concentrations of IgE and IL-18 and the Scoring Atopic Dermatitis Index (SCORAD).

Finally, recent data obtained in murine models demonstrate the major role of IL-18 in the development of AD. Indeed, transgenic mice overexpressing IL-18 in the epidermis developed epidermal lesions similar to those observed in atopic eczema [12]. Furthermore, topical application of Spa on NC/Nga mice, characterized by a ceramide deficit and deficient barrier function exacerbated by the application of detergents, led spontaneously to severe eczema-type lesions and a high IL-18 serum concentration [13].

In this context, we wished to develop non-invasive tools applicable to children that would enable us to characterize, in terms of virulence factors, the colonizing strains of SA found in atopic skin lesions, and establish links between the development of these strains and the appearance of both cutaneous lesions and cytokines such as IL-18.

Here, we present a genotypic real-time polymerase chain reaction (PCR) method of studying the colonization by strains of SA-carrying toxin-encoding DNA. We also describe a second method that has enabled us, from a non-invasive sampling of the skin, to quantify both IL-18 and two markers of the inflammatory response, IL-1RA/IL-1 α and IL-8, used previously in various contexts [14, 15]. Finally, using therapeutic examples that led to clinical improvement, we demonstrate the potential utility of these markers as tools for evaluating the reduction of AD within a given population.

Materials and Methods

Reagents, chemicals and buffers were purchased from Sigma (St. Louis, Mo., USA). The buffer used for skin sampling was prepared from phosphate-buffered saline (PBS) and Triton X100 (0.1% final volume) for better recovery of cutaneous proteins.

Solvents (high-performance liquid chromatography grade) and buffers were filtered with 0.45-mm nylon membranes (Pall Gelman Laboratory, Ann Arbor, Mich., USA). Oligonucleotide primers and TaqMan probes were purchased from Sigma-Proligo. DNA extraction kits were purchased from Qiagen (Hilden, Germany). Plasmid DNA purification kits were purchased from Macherey-Nagel (Hoerdt, France). Synthetic genes were purchased from GeneArt (Regensburg, Germany).

Clinical Cases

This study was conducted according to the principles of the Declaration of Helsinki, and was approved by the French Ethical Committee (CCPPRB) in Toulouse, France, No. CCP 20522. Child volunteers were enrolled in the study after their parents gave written informed consent. These included a population of 31 children, aged from 1 to 6 years and suffering from AD, who were studied alongside a group of age-matched healthy subjects presenting no cutaneous signs or undergoing any pharmacological treatment. The study was carried out under the control and agreement of the required ethical committee. Selected patients refrained from using an emollient on the forearms for 24 h preceding skin sampling. A dermatologist clinically evaluated the severity of the disease using the SCORAD index at the beginning and the end of the study (day 18). SCORAD values were between 8 and 75 for all patients. At the same time, two skin samples were taken from lesional (edemic or inflamed) and xerotic areas of the atopic patients. These areas were localized to the upper or lower limbs. Samples were taken from the same areas of healthy subjects.

Clinical Procedures

During their 18-day visit, patients underwent the daily program offered by the Avène Thermal Spa, including showers and spray baths (running water at 34°C for 20 min), drinking water, and the application of compresses to inflamed areas and emollients to xerotic areas. Patients were also treated with local corticoids in the event of an inflammatory outbreak.

Skin Samples

The applicators used for skin sampling (Copan, Brescia, Italy) were inert and sterile cotton-tipped swabs, which are non-toxic to the skin and designed for diagnostic use. The method of skin sampling outlined below was approved by the French Ethical Committee in Toulouse (CCPPRB No. CCP 20522).

Skin samples were taken by daubing the skin using sterile applicators impregnated with a PBS/0.1% Triton buffer solution. These were then stored in a dry place at -80°C immediately after collection to ensure better stability of the markers. The tip of the applicator was then dipped into PBS containing a protease inhibitor (Complete Mini, Roche Diagnostics, Basel, Switzerland). After vortex agitation, the cutaneous extract was collected, aliquoted, preserved at -80°C and analyzed in sets. Proteins were quantified from the cutaneous extract using the DC-Bio-Rad Kit® (Bio-Rad, Hercules, Calif., USA). This protein concentration was used to counterbalance the cytokine concentration for IL-8, IL-18 and pro-IL-18.

The protein concentration in the cutaneous extracts ranged between 10 and 2,000 μ g/ml depending on the skin area.

DNA Extraction and Purification

Bacterial DNA was extracted from sterile applicators (Copan) using a QIAamp DNA Micro Kit. The nucleic acids were purified according to the manufacturer's instructions (Qiagen), eluted in 40 μ l water and stored at -20°C . Reference plasmids were purified using a NucleoBond Kit from Macherey-Nagel following the manufacturer's recommendations. All DNA was eluted in water and stored at -20°C . Reference DNA was quantified by absorbance at 260 nm on a Beckman DU 800 spectrophotometer (Beckman Coulter, Fullerton, Calif., USA).

Oligonucleotide Design and Sequences

Sequences of primers and TaqMan probes are listed in the appendix. The design was performed using the Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) with the following parameters: primer T_m in the range of $52-57^{\circ}\text{C}$; T_m probe between 62 and 67°C excluding guanine as the first probe base; the size of the amplified region between 75 and 300 bp. Primers and TaqMan probes were selected in regions with high homology between all the variants described. Selected primers and probes were checked against GenBank to exclude potential cross-reacting sequences. All DNA regions corresponding to amplified sequences from each target gene were synthesized and subcloned in the standard cloning vector pGTPb202 (GTP Technology, Labège, France). Serial dilutions of quantified plasmids were used as a standard to calibrate the real-time PCR assay.

Nucleic Acid Detection by qPCR

As a control, at least 1 nucleic acid dilution from reference plasmids and a negative control (containing no DNA) were simultaneously analyzed in each run. All runs were performed using JumpStartTaq polymerase mix (including SYBR green for *sei* and *sek*) with MgCl_2 at a final concentration of 4 mM. The amplification procedure on the Smart Cycler II (Cepheid, Sunnyvale, Calif., USA) was as follows: t1, 2.5 min at 95°C ; t2, 30 s at 94°C ; t3, 30 s at 50°C ; t4, 30 s at 72°C (with t2, t3 and t4 repeated 45 times). The final volume of the PCR mixture was 25 μ l, containing all primers at 500 nM and TaqMan probes at 100 nM. After fluorescence background subtraction, each run was calibrated using the signals of the DNA standard serial dilution and converting fluorescence signals to numbers of gene copies detected. The specificity of the amplification was checked by analysis of the melting curve following CyberGene detection for *sei* and *sek* analysis.

Quantification of Cytokines

Cytokines IL-8, IL-18, IL-1 α and IL-1RA were quantified using the ELISA method [DuoSet Kit, R&D Systems (Minneapolis, Minn., USA) for IL-8, IL-1 α and IL-1RA; MBL Kit (MBL, Woburn, Mass., USA) for IL-18].

Standard curves were prepared according to the manufacturer's instructions for each cytokine quantification experiment. For each experiment, diluted skin samples and standards (in kit buffers) were run on the same ELISA plate to ensure that assay signals were in the standard range. Limits of detection for the cytokines were: IL-1 α : 7.8 pg/ml, IL-1RA: 31.5 pg/ml, IL-8: 31.25 pg/ml, and IL-18: 25.6 pg/ml.

Quantification of pro-IL-18 in the Epidermis of Atopic Skin Compared with Healthy Skin

Cutaneous extracts from 8 individuals (4 patients suffering from atopic dermatitis and 4 healthy volunteers) were denatured, and then loaded onto an SDS-PAGE gel with 3.5 μ g protein per well (4–12% Bis-Tris gel; Invitrogen, Carlsbad, Calif., USA), before being transferred to a nitrocellulose membrane. Membranes were then incubated with a primary antibody (anti-pro-IL-18, R&D Systems) after blocking in 3% BSA/TBS-Tween 20 solution. The secondary antibody and the chemiluminescent substrate used were from Zymed (San Francisco, Calif., USA) and Millipore (Billerica, Mass., USA), respectively. For quantitative comparison, Western blot analysis was digitalized and the band intensity corresponding to each protein determined using an XRS digital camera and Quantity One software (Bio-Rad).

Statistical Analysis

The results of the clinical study are expressed as means \pm SD. Data from all the studies were analyzed using a Wilcoxon test on paired sets for the intragroup analyses and non-paired sets for the intergroup study between healthy subjects and atopic subjects. The correlations of variables before and after treatment was studied using the Spearman coefficient. A probability of $p \leq 0.05$ was considered as statistically significant.

Results

Distribution of Target Genes

Probes and real-time PCR tests were developed to detect the presence of genes coding for SA-produced virulence factors. We verified the linearity of the real-time PCR response for a significant number of these factors. Quantification was confirmed to be applicable for the SEB, SEC, SED, SEG, and SEI-SEU enterotoxins with very good linearity and good sensitivity, with fewer than 200 genes detected in the test sample.

The gene coding for protein A (*spa*) was widespread in the atopic population, and detected in 30 out of 31 samples taken from inflammatory areas (table 1). As this gene is present in the genome of most strains of SA [16], the determination of the number of copies found in the sample gives an estimate of SA density. These densities and the frequencies with which various genes were found are given in table 1. Concerning enterotoxins, the gene coding for enterotoxin G (*seg*) was found to be the most prevalent, detected in 50% of patients. The next most common genes were *sed* found in 11 volunteers, *sem* and *seu* found in 10, *sec* and *seo* in 8, *sen* in 7, and *sel*, *sea*, *seb* and *sei* in 5. Furthermore, in the vast majority of samples we detected combinations of genes, with only *seg* and *sed* genes found in an isolated fashion. The most frequent combination in the population under study was *seg*, *sem* and *seu* ($n = 10$). The combinations comprising *sei*, *sem*,

Table 1. Average frequency and quantity of genes coding for toxins with corresponding SCORAD values

Gene	log(s. + 1) ^a	Frequency ^b	SCORAD ^c
<i>spa</i>	4.15	30 (97)	38
<i>sea</i>	5.36	5 (16)	33.6
<i>seb</i>	3.57	5 (16)	38.1
<i>sec</i>	4.02	8 (26)	38.6
<i>sed</i>	4.82	11 (35)	24.9
<i>seg</i>	5.32	15 (48)	39.6
<i>sei</i>	5.4	5 (16)	55.5
<i>sel</i>	4.35	5 (16)	35.2
<i>sem</i>	4.56	10 (32)	47.5
<i>sen</i>	5.56	7 (23)	49.3
<i>seo</i>	5.27	8 (26)	48.5
<i>seu</i>	5.03	10 (32)	47.5

Values in parentheses are percentages.

^a Number of copies of the given gene quantified, as described in 'Materials and Methods', on a 10-cm² inflammatory area from a population of 31 children suffering from atopic dermatitis.

^b Frequency of the given gene in the studied population.

^c Average SCORAD value for the group of patients with the corresponding gene.

sen, *seo* and *seu* genes were generally found in the most severely affected subjects, as indicated by SCORAD (table 2).

Comparison of Healthy and Atopic Subjects Cytokine Markers and Genomic *spa*

Table 3 shows the mean values of the genomic marker *spa* in the staphylococcal population, alongside the cytokine markers found in non-invasive samples from healthy subjects and patients with AD paired according to age and area. The first remarkable result shown in the table is the increase in the value of all cytokines and *spa* genomic markers in atopic skin compared with that of healthy subjects, not only in inflammatory areas, but also in xerotic areas.

More specifically, table 3 shows the significant increase in cytokine markers in inflammatory areas, by a factor of 7, 4 and 12 for IL-18, IL-8 and IL-1RA/IL-1 α , respectively. Colonization by SA in atopic skin was also higher compared to that in skin of non-atopic subjects, with an average of 10⁵ per sample in the studied population, carried out on 10-cm² samples and reaching 5 \times 10⁶ per cm² in some inflammatory areas. We revealed the presence of *spa* genes in 15% of samples from the control group, but in lower concentrations, reduced by a factor of

Table 2. Frequency of gene combinations coding for enterotoxins and the corresponding average SCORAD value

Combinations	Frequency ^a	SCORAD ^b
<i>seg-sem-seu</i>	10 (32)	47.5
<i>seg-sem-seu-seo</i>	8 (25)	48.5
<i>seg-sem-seu-seo-sen</i>	7 (22)	49.3
<i>seg-sem-seu-seo-sen-sei</i>	5 (16)	55.5
<i>seg-sem-seu-seo-sen-sec</i>	4 (13)	49.1
<i>seg-sem-seu-seo-sen-sei-sec-sel</i>	2 (6)	60

Figures in parentheses are percentages.

^a Frequency of combination of enterotoxin genes in the studied population.

^b Average SCORAD value for the group of patients with the corresponding combination of enterotoxin genes.

roughly 1,000 (data not shown). We also noted a generally higher expression of these markers in inflammatory areas compared to xerotic areas, averaging factors of 2–3 for IL-8 and IL-18, up to 4 for IL-1RA/IL-1 α and 100 for SA.

Concentration of pro-IL-18 in the Stratum Corneum

As an illustrative example, we studied the expression of pro-IL-18 in skin sample extracts of the cornified layer from 8 individuals among the studied population by Western blotting using a specific antibody (fig. 1). Quantification of the blots by densitometry revealed a 4-fold increase in expression levels in inflammatory areas compared to healthy areas. In contrast, we detected no significant difference in expression levels between non-lesional areas of atopic subjects and those of healthy subjects.

Relationship between Improved Clinical Data and Biochemical and Genomic Values

We studied the development of cytokine and genomic markers on 19 atopic subjects for whom clinical improvement was noted after various treatments (local corticotherapy, antihistamines, skin care, etc.).

The results reported in table 4 show variations in SCORAD values estimating the severity of AD and biochemical and genomic markers.

For a significant average decrease of 2 ($p < 0.001$) in the SCORAD value, there was an almost equal significant decrease in cytokine markers including IL-8 ($p < 0.001$) and IL-1RA/IL-1 α ($p < 0.05$), and a decrease close to significance ($p = 0.054$) in IL-18. These results were also accompanied by a significant decrease of 1 and 3

Fig. 1. Comparison of concentrations of pro-IL-18 in the stratum corneum of atopic and healthy subjects. **a** After swabbing, protein extracts from the stratum corneum were loaded onto SDS-PAGE gels, and transferred onto nitrocellulose membranes. Immunoblotting was then performed using anti-pro-IL-18 at 20°C for 2 h. The blots show the strong presence of pro-IL-18 at 26-kDa sizes for the atopic sampling from lesional areas compared with non-lesional and control areas of the healthy population. Equal amounts of protein were loaded onto each blot. For quantitative comparison, the blotting intensity of each band over background staining was assessed using an XRS digital camera and Quantity One software. H = Healthy subject; NLAD = non-lesional AD area; LAD = lesional AD area; SD = standard of pro-IL-18. **b** Quantity of pro-IL-18 in the stratum corneum.

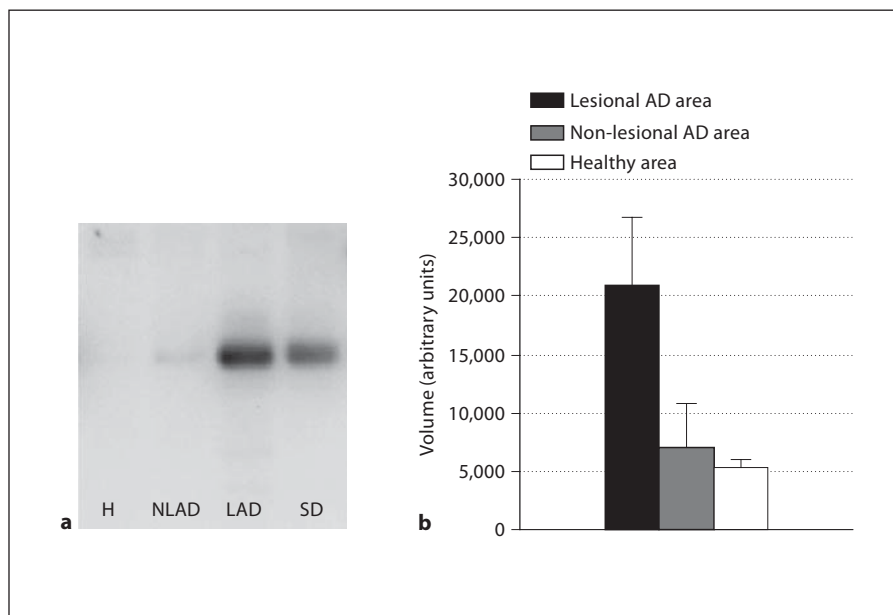


Table 3. Comparison of cytokine and genomic data in atopic and healthy subjects

	Inflammatory area (AD subjects)	Control area (healthy subjects)	Xerotic area (AD subjects)	Control area (healthy subjects)
IL-18, pg/mg protein	10,647 ± 13,861 ^{b, d}	1,498 ± 1,466	3,171 ± 2,734 ^b	1,370 ± 1,406
IL-8, pg/mg protein	2,087 ± 2,240 ^{b, c}	487 ± 525	873 ± 731 ^b	445 ± 525
IL-1RA/IL-1α	1,204 ± 1,431 ^{b, d}	103 ± 104	335 ± 499 ^a	95 ± 96
log (<i>spa</i> + 1), n/10 cm ²	5.07 ± 1.33 ^d	^e	3.08 ± 2.56	^e

Results are expressed as means ± SD. ^a p < 0.005, ^b p < 0.001 AD versus control (non-paired t tests); ^c p < 0.005, ^d p < 0.001 inflammatory area versus xerotic area (paired t tests).

^e 15% of the healthy population demonstrated the presence of *spa* genes with an average concentration roughly 1,000 times lower than that of the inflammatory area of AD subjects.

Table 4. Variations in cytokine and genomic markers relative to clinical improvement of AD

	SCORAD	IL-18 pg/mg protein	IL-8 pg/mg protein	IL-1RA/IL-1α	log(<i>spa</i> + 1) n/10 cm ²	log(<i>seg</i> + 1) n/10 cm ²
Before treatment	36.9 ± 21	6,361 ± 10,238	2,105 ± 1,759	840 ± 777	5.27 ± 0.76	5.55 ± 0.76
After treatment	20.3 ± 16.5 ^d	2,902 ± 3,232 ^a	1,235 ± 1,346 ^d	499 ± 453 ^b	4.09 ± 1.97 ^{d, e}	2.55 ± 2.53 ^c

Results are shown as means ± SD for inflammatory areas of AD samples before and after curative treatment (corticotherapy, antihistamine drugs, etc). ^a p = 0.054, ^b p < 0.05, ^c p < 0.01, ^d p < 0.001 before versus after treatment (paired t tests).

^e The Spearman correlation was used to correlate the changes in the SCORAD value with the changes in SA density [log(*spa* + 1)]; the coefficient was 0.49 (p = 0.0352).

logarithmic units in the number of copies of *spa* ($p < 0.001$) and *seg* ($p < 0.01$) genes, respectively; notably those genes encoding enterotoxins. Moreover, the Spearman coefficient ($R = 0.49$; $p = 0.0352$) indicated a positive correlation between the evolution of the SCORAD value and the log (*spa* + 1) parameter representing SA density, as outlined earlier.

Discussion

Recent work has highlighted not only the aggravating role played by SA colonization in AD, but also the identification of key proteins involved in both antibacterial defense mechanisms and the inflammatory response leading to eczema-type lesions [17, 18].

Dendritic cells, both mast cells and keratinocytes, have the capacity to recognize microbial constituents [19–21] due to specific receptors expressed on their surface, in particular the toll-like receptors. This recognition leads to an increase in the synthesis of mediators of inflammation and immunity and initiates their circulation [22]. In addition, this stimulation induces the maturation of the dendritic cell with increased major histocompatibility complex expression, as well as the activation and the proliferation of T cells after presentation of the epitope [23]. According to the literature, AD is most likely the result of an orientation in response to allergens towards a Th2-predominant-type response and a relative inhibition of Th1-type responses [24]. This immunological dysfunction could be the source of B lymphocyte activation and subsequently of IgE-type antibody synthesis [25].

Staphylococcus toxins are now recognized as playing a major role in inducing an inflammatory response in atopic eczema [26]. As we have mentioned, SA possesses several virulent, membrane and secreted factors such as protein A and enterotoxins, capable of inducing immune and inflammatory responses with additive or synergic effects [27]. Indeed, the enterotoxins commonly expressed by strains responsible for severe infections in AD [28] have a superantigen activity capable of stimulating a large population of T lymphocytes, with a massive liberation of cytokines involved in the inflammatory process [5, 29]. Likewise, the incubation of human epithelial cells from the cornified layer with *Staphylococcus* protein A leads to nuclear translocation of NF- κ B and the production of pro-inflammatory cytokines such as tumor necrosis factor- α and chemokines such as IL-8, without inducing the expression of antimicrobial peptides (hBD2 and LL-37) [30].

In addition, new data suggest that AD development may occur secondarily to the breakdown of the skin barrier [31]. Indeed, genetic defects in the skin barrier, as is the case in filaggrin mutations, represent a major risk factor for the development of AD [2, 3, 32], increasing the penetration of antigenic agents, and subsequently the risk of allergic sensitization.

In this context, the methods presented here seem particularly appropriate for improving our understanding of the mechanisms involved. The application of real-time PCR on non-invasive samples obtained by simple swabbing facilitated the access to a certain number of genes coding for these toxins. Moreover, gene amplification allows increased sensitivity compared with conventional culture techniques, and often enables detection of *Staphylococcus* virulence factors where SA culture and immunological antigen detection would give negative results (data not shown).

This non-invasive method enabled us to enumerate the genes coding for various toxins of SA (protein A and enterotoxins) and to estimate staphylococcal density [33]. Furthermore, we firstly noticed that in most cases, the genes encoding for enterotoxins exist as clusters, and, secondly, that the content of these gene clusters in the study population related to the severity of the atopic dermatitis, particularly for those genes encoding enterotoxins *sei*, *sem*, *sen*, *seo* and *seu* (table 2). These results may relate a particular pathogenic to a given SA genetic profile, with possible significant clinical consequences on AD treatment. Our results do however need confirming on a larger scale.

We have also developed a simple, non-aggressive method of examining inflammatory markers in order to improve our understanding of the immune and inflammatory phenomena associated with infection in AD. ELISA methods which facilitate the detection of inflammation markers from non-invasive samples have been described in recent years, in particular, the quantification of IL-1RA/IL-1 α and IL-8 levels on strippings in various inflammatory situations [14, 15, 34]. We transposed these methods to samples obtained by swabbing, and completed the list of markers with IL-18, a cytokine which is expressed in various cell types including keratinocytes and macrophages, and which, in addition to its pro-inflammatory role, could play a key role in the pathogenesis of AD [35]. Indeed, IL-18 has been shown to induce the production of IgE and Th2 cytokines [12], as well as chemokines by keratinocytes, which in turn promote the infiltration of inflammatory cells into lesions of AD, and thus amplifying the skin inflammation. Our compara-

tive study of control and atopic subjects has underlined considerable disparities. The results show the presence of a generally large number of *spa* genes in the atopic samples, suggesting a high rate of cutaneous colonization by SA, 100 times more in inflammatory compared to non-inflammatory areas. Without resorting to cultures of SA strains, we were able to detect the differences established in previous studies [28]. Moreover, we detected an increase in the level of all cytokine markers in atopic skin compared to healthy skin, in both inflammatory and xerotic areas, that was on average 2 or 3 times higher in inflammatory areas (table 3).

Plasma concentrations of IL-18 have previously been associated with the severity of AD [11]. We found high levels of pro-IL-18 in samples of the cornified layer taken from inflammatory areas of patients suffering from AD (fig. 1). This raises the question of the mechanism of hydrolysis for the liberation of the mature form of IL-18. The mechanism of activation initially described involves caspase 1 proteolytic activity, which to date has not been detected in its active form in the most superficial part of the epidermis [36]. Nevertheless, our results suggest the involvement of a protease in the liberation of IL-18 at the periphery of live cutaneous layers; thus, indicating its potential key role in the closely linked relationship between inflammation and *Staphylococcus* colonization. One possible candidate is caspase 1. A second hypothesis could involve the supply of proteases responsible for the cleavage of pro-IL-18 by SA strains. The combination of the two methods described in this report, both genetic and immunological, could therefore help provide answers to numerous questions surrounding *Staphylococcus* infections in AD.

Of the 19 subjects for whom clinical improvement was noted after various treatments, the latest results indicate that all have shown a concomitant improvement in all cytokine and genomic markers. For a decrease of approximately 50% in SCORAD value, an almost equivalent decrease could be noted in cytokine markers (IL-8, IL-18 and IL-1RA/IL-1 α), alongside a decrease of approximately 1 and 3 logarithmic units in the number of copies of genes coding for protein A and enterotoxin G, respectively. These non-invasive methods could potentially enable us to evaluate the development or reduction of AD and the advantages of potential treatments. Moreover, the correlation (Spearman's coefficient $R = 0.49$) observed between the decrease in SCORAD value and that of log (*spa* + 1) highlights the influence of the density of SA on the clinical severity of the patient. Indeed, the virulence of SA is under the control of the quorum sensing system which induces more particularly the expression of en-

terotoxin genes with increasing bacterial density on the skin [37]. In addition, PCR analysis of skin samples provides a means of distinguishing between *Staphylococcal* latency and reactivation, the latter leading to variations in the bacterial load and consequently an AD outbreak.

In summary, the methods developed and presented here constitute useful additional tools in understanding the role of virulence factors in the etiology of AD. The clear correlation between changes in the level of markers and SCORAD values demonstrates the potential benefit of using these methods in developing future therapeutic strategies.

Although the population of the present study is limited, the data accumulated establish a link between virulence factors and the capacity of AD to evolve, with a sufficiently significant difference to make it indisputable.

A future interest would be in supplementing this approach with other protein players involved at different stages of *Staphylococcus* colonization and in the production of toxins. A better knowledge of the physiopathology of virulence factors involved in AD would not only optimize therapeutic care, but also constitute an advantage in dermatology in the anticipation, and thus prevention, of an outbreak. Finally, knowledge of the molecules playing a role in SA colonization and toxin secretion systems could help the development of new specific therapeutic strategies.

Acknowledgements

We express our gratitude to L. Berkane, MD, L. Berger, MD, A. Gadroy, MD and V. Cibot, MD, for their contributions regarding selection, and their astute observations of the atopic dermatitis subjects. We also thank the staff of the Avène Thermal Spa, in particular C. Pegurier and F. Moha, for their help in taking the samples, and M.A. Martinsic for the interest she has shown in our study.

Appendix

Target genes and sequences of primers and probes used.

Gene	Sequence accession No.	Forward primer	Reverse primer	TaqMan probe
SPA	J01786	GCTGATGCGCAACAAAATAA	GCTTTCGGTGCTTGAGATTC	(FAM)CAACAAAGATCAACAAAGCGCC-TTC(BHQ1)
SEA	AAA26681	TTATCAATGTGCGGGTGGTA	GCCATAAATTGATCGGCACT	(ROX)CAGCTTGTATGTATGGTGGTGTACCGT(BHQ2)
SEB	AAA88550	CGCATCAAACGACAAACGA	CCATCTTCAAATACCCGAACA	(FAM)TGGTGGTGTAACTGAGCATAATGGA(BHQ1)
SEC	CAA29260	CTGCACCAGGCGATAAGTTT	CCATTCTTTGTTGTAAGGTGGA	(Cy3)TGATGTACAACGACAATAAAACGGTTGA(BHQ2)
SED	AAB06195	GCGCGGAAAAATAGAGTTTG	CGGGAAAATCACCCCTTAACA	(ROX)TCTTCTGATGGGTCTAAAGTCTCTTATGA(BHQ2)
SEE	AAA26617	TGCAAAGAGGCTTGATTGTG	ATTGCCCTTGAGCATCAAAC	(FAM)TCATTCTTCTGAAGGGTCCACGGTA(BHQ1)
SEG	AAC26660	CTGAACCGGATATAAACCAA	TGATTGTCTATTGTCGATTGTT	(Cy3)AAGACCACCATACATACAACAACCTCCA(BHQ2)
SEH	AAA19777	CATATGCGAAAGCAGAAGA	TTTTCTTTAATGAATGGGTGA	(ROX)TTGACCATATGCATTAGCTAAAGCT(BHQ2)
SEI	AY920268	TGGATATTTTTGGCATTGAT	AACATCAATTTCTTGAGCTGT	no probe
SEJ	AF053140	TTCTCCCTGACGTTAACT	TTTCAGAGATACCCTTTTCG	(Cy3)CCCCTTAGTTTACAGCGATAGCA(BHQ2)
SEK	AF410775	TTGTTACCGCTCAAGAGATT	AACCCATCATCTCCTGTGTA	no probe
SEL	NC_002758	GCAAGCATCAAACAGTTACA	ACACTCCCCTTATCAAACC	(Cy3)GGTTACCGCACAAGAAATAGATGTCA(BHQ2)
SEM	AF285760	TCGGAGTTTGAATCTTAGG	AATTGATGCGAAAGATGATT	(Cy3)GCTATCCAATTGAAGACCACCAAA(BHQ2)
SEN	AY158703	GGCGTTAATGTATTTAAAGATG	AGATGAGCTAACTGTTCTATTATCA	(Cy3)CCGGTAAACATACAAAAAGGATGCATT(BHQ2)
SEO	AF285760	TGACTAGTGATGTACAAAAAGGTT	TTGATCTGGTAAATTTCCCTTT	(Cy3)CATTCTCATTGGGAGCATAAAGAA-TCA(BHQ2)
SEP	BA000017	TAAATGGTAGCGAGAAAAGC	TGCTGTAAAAATTGATCGTG	(Cy3)TGCAGGGAACAGCTTTAGGCA(BHQ2)
SEQ	AF410775	TCTGGGTTTAACAAAGGAAA	AAAACTCTCTGCTTGACCA	(Cy3)CATACGATTTGTTTACACCGGAA(BHQ2)
SER	AB075606	AAAACGGTTACGGTACAAGA	AGGAAACAAATCGTACCAAA	(Cy3)GGTCGGCATATGAGACGGGT(BHQ2)
SEU	AY205307	ACGTAGATTTGTTGGGACA	ATTGATTTCCATCATGCTC	(Cy3)CGTGTATGTATGGCGGTGTGA(BHQ2)

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