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## Deficiency of Dermcidin-Derived Antimicrobial Peptides in Sweat of Patients with Atopic Dermatitis Correlates with an Impaired Innate Defense of Human Skin In Vivo<sup>1</sup> ✓

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# Deficiency of Dermcidin-Derived Antimicrobial Peptides in Sweat of Patients with Atopic Dermatitis Correlates with an Impaired Innate Defense of Human Skin In Vivo<sup>1</sup>

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Antimicrobial peptides are an integral part of the epithelial innate defense system. Dermcidin (DCD) is a recently discovered antimicrobial peptide with a broad spectrum of activity. It is constitutively expressed in human eccrine sweat glands and secreted into sweat. Patients with atopic dermatitis (AD) have recurrent bacterial or viral skin infections and pronounced colonization with *Staphylococcus aureus*. We hypothesized that patients with AD have a reduced amount of DCD peptides in sweat contributing to the compromised constitutive innate skin defense. Therefore, we performed semiquantitative and quantitative analyses of DCD peptides in sweat of AD patients and healthy subjects using surface-enhanced laser desorption ionization time-of-flight mass spectrometry and ELISA. The data indicate that the amount of several DCD-derived peptides in sweat of patients with AD is significantly reduced. Furthermore, compared with atopic patients without previous infectious complications, AD patients with a history of bacterial and viral skin infections were found to have significantly less DCD-1 and DCD-1L in their sweat. To analyze whether the reduced amount of DCD in sweat of AD patients correlates with a decreased innate defense, we determined the antimicrobial activity of sweat in vivo. We showed that in healthy subjects, sweating leads to a reduction of viable bacteria on the skin surface, but this does not occur in patients with AD. These data indicate that reduced expression of DCD in sweat of patients with AD may contribute to the high susceptibility of these patients to skin infections and altered skin colonization. *The Journal of Immunology*, 2005, 174: 8003–8010.

Antimicrobial peptides (AMP)<sup>3</sup> are an integral part of the innate defense (1). They are expressed by cells within the epithelial lining or are produced by adjacent cells and secreted onto the surface (2). In mammalian skin, two major classes of AMPs, the cathelicidins and the  $\beta$ -defensins, have been described. They are produced in keratinocytes and primarily function in response to injury and inflammation (3, 4). Dermcidin (DCD) was recently discovered by our group to be an AMP with a broad spectrum of activity and no homology to other known AMPs. DCD is constitutively expressed in eccrine sweat glands, secreted into sweat, and transported to the epidermal surface. Constantly secreted, DCD takes part in the constitutive defense mechanisms of human skin (5, 6). Recently, it has been reported that a small percentage of breast cancer cells express DCD-RNA (7). Furthermore, it was shown that after oxidative stress induction,

different types of tumor cells produce proteolytically processed DCD peptides with diverse functional capabilities (8–12).

Full-length DCD consists of 110-aa residues with an N-terminal 19-aa signal peptide characteristic of secreted proteins. In eccrine sweat, different processed DCD-derived C-terminal peptides of 48-aa residues (DCD-1L), 47-aa residues (DCD-1), and shorter fragments can be detected by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (13). DCD-1L and DCD-1 show antimicrobial activity against pathogenic microorganisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Enterococcus faecalis*, and *Candida albicans* under in vitro conditions resembling human sweat (5, 14, 15). In human sweat, 1–10  $\mu\text{g/ml}$  DCD-1 is found, a concentration that is toxic to most microorganisms tested (13).

Atopic dermatitis (AD) is a chronic inflammatory skin disease complicated by frequent skin infections, particularly those due to *S. aureus*. The microbial flora of atopic skin shows striking differences, with >90% of inflammatory lesions and 76% of normal skin being colonized by *S. aureus* compared with <10% in healthy individuals (16, 17). To date, the underlying immunological mechanisms of the susceptibility of atopic skin to *S. aureus* infection and colonization are still poorly understood.

Recent evidence suggests that impaired innate immune mechanisms in AD contribute to the propensity of atopic patients to skin infections. The inducible antimicrobial peptides, human cathelicidin LL-37, human  $\beta$ -defensin (HBD) 2, and HBD-3, were shown to be expressed at a significantly lower level in lesional atopic skin compared with lesional skin in psoriasis (18, 19). These findings point to the lack of an adequate inducible innate response in lesional atopic skin. However, because in the absence of inflammation, colonization with *S. aureus* is also a prominent feature of nonlesional atopic skin,

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<sup>3</sup> Abbreviations used in this paper: AMP, antimicrobial peptide; AD, atopic dermatitis; BCA, bichinchonic acid; SELDI-TOF-MS, surface-enhanced laser desorption ionization time of flight mass spectrometry; HBD, human  $\beta$ -defensin.

we hypothesized deficiencies in the constitutive innate defense of human skin. We assumed a reduced expression of the antimicrobial peptide DCD in sweat of patients with AD contributing to the high susceptibility of these patients to skin infections and altered skin colonization. Therefore, we investigated the expression of DCD in sweat of atopic subjects using SELDI-TOF-MS protein chip technology and an ELISA. We also analyzed the antimicrobial activity of sweat in healthy individuals and patients with AD *in vivo*.

## Materials and Methods

### Collection of sweat

Human sweat was collected from 17 healthy volunteers (7 women and 10 men; mean age, 28.6 years) and 17 patients (8 women and 9 men; mean age, 27.0 years) with AD. The diagnosis of AD was confirmed by the diagnostic criteria of AD defined by Hanifin and Rajka (20). Systemic or topical corticosteroid treatments in the preceding 4 wk were considered exclusion criteria. Participants were asked not to wash or apply topical treatment for 6 h before investigation. To determine the severity of AD, the scoring AD index in each patient was evaluated. The study was approved by the ethics committee of the medical faculty of Tubingen University, and all study participants gave written informed consent. Sweating was induced by physical exercise on a bicycle ergometer. From each donor, 5  $\mu$ l of the initial portion of sweat from the forehead was collected and frozen immediately at  $-20^{\circ}\text{C}$  until analysis.

### Semiquantitative SELDI analysis of sweat

The sweat samples were analyzed on a reverse phase (H4) Protein Chip Array (Ciphergen Biosystems). Sweat samples were centrifuged for 1 min at 13,400 rpm, 1  $\mu$ l of the supernatant was added to 4  $\mu$ l of 50 mM sodium phosphate buffer (pH 6.5). Protein Chip Arrays were pretreated with 3  $\mu$ l of 50% (v/v) acetonitrile/water for 1 min; after 2 min of drying, 1  $\mu$ l of the diluted sweat solution was added. After drying (8 min), spots were washed four times with 3  $\mu$ l of  $\text{H}_2\text{O}$  (by flushing each spot three times). After 5 min, 1  $\mu$ l of matrix consisting of a saturated solution of sinapinic acid in 50% acetonitrile/water (v/v) and 0.5% trifluoroacetic acid with 10 pmol/ $\mu$ l bovine insulin ( $M_r$ , 5,733.6 Da) was added. SELDI analysis of each sweat sample was performed in triplicates. Chips were read with the following instrument settings: laser intensity, 230; detector sensitivity, 8; and detector voltage, 19 kV; positions 18–78 were read with an increment of 5, resulting in 12 different spot positions. Ten laser shots were collected on each position (total shots collected and averaged, 120/sample), two warming shots were fired at each position, which were not included in the collection. The acquired mass range included 0–12,500 Da with a focus mass at 4,800 Da. Calibration was performed externally with a bovine insulin peak ( $M_r$ , 5,733.6 Da). The spectra were analyzed by Protein Chip software 3.1 (Ciphergen Biosystems). After baseline subtraction, all spectra were normalized to the bovine insulin peak for semiquantification.

### ELISA

Sweat from 9 healthy volunteers (3 women and 6 men; mean age, 30 years) and 12 patients with AD (5 women and 7 men; mean age, 24.2 years) was analyzed. The protein concentration in human sweat was determined by bicinchoninic acid (BCA) test (Pierce) as described by the manufacturer. The wells of microtiter plates (Nunc Brand Products, MaxiSorb surface) were coated with serial dilutions of 1/100 diluted native sweat in PBS in a final volume of 50  $\mu$ l/well at  $4^{\circ}\text{C}$  overnight. The plates were washed twice with 200  $\mu$ l of wash buffer (PBS/0.05% Tween 20, pH 7.0) and blocked with blocking buffer (PBS/0.05% Tween 20, pH 7.0, containing 2% BSA) for 1 h at  $37^{\circ}\text{C}$ . After washing, the plates were treated for 1 h at  $37^{\circ}\text{C}$  with anti-DCD mAb G-81 (1/4000 diluted in PBS/0.05% Tween 20, pH 7.0, containing 0.5% BSA; provided by Dr. Sagawa, Wakayama Medical University, Wakayama, Japan) (21). After washing, the plates were incubated with biotin-conjugated goat anti-mouse Ig (DakoCytomation; 1/1500 diluted in PBS/0.05% Tween 20/0.5% BSA), then incubated for 1.5 h at  $37^{\circ}\text{C}$  with HRP-conjugated streptavidin (Roche; 1/1000 dilution in 100 mM Tris-HCl and 150 mM NaCl, pH 7.5). Color was developed with 100  $\mu$ l/well *p*-nitrophenyl phosphate disodium (0.03 mg/ml) in 0.1 M glycine buffer, 1 mM  $\text{MgCl}_2$ , and 1 mM  $\text{ZnCl}_2$ , pH 10.4. Absorbance at 405 nm was measured with a microplate reader (Molecular Devices). HPLC-purified synthetic DCD-1L peptide in concentrations between 1 and 80  $\mu\text{g}/\text{ml}$  served in the assays as a standard for quantification of the amount of DCD-derived peptides in each sweat sample.

### Peptide synthesis and antimicrobial assay

Peptides were synthesized using standard F-moc/tBu chemistry on a multiple peptide synthesizer (Syro II; MultiSynTech). The cleaved products were purified by gradient reverse phase HPLC to a purity of  $>97\%$ . Antimicrobial assays were performed using the CFU assay as previously described (5). *S. aureus* (American Type Culture Collection; ATCC 25923) cultures were grown to midexponential growth phase and washed twice with 10 mM sodium phosphate buffer, pH 7.0. Bacterial concentration was estimated photometrically at 600 nm. Absorbance of 1.0 corresponded to  $1.97 \times 10^7$  cells. After dilution to a concentration of  $10^6$  CFU/ml, 10  $\mu$ l of the dilutions were incubated at  $37^{\circ}\text{C}$  for 2 h with various peptide concentrations in a total volume of 30  $\mu$ l in 10 mM sodium phosphate buffer, pH 7.0/10 mM NaCl. After incubation, cells were diluted 1/100 in 10 mM sodium phosphate buffer, pH 7.0, and 90  $\mu$ l of the diluted bacterial suspension was plated in triplicates on blood agar. The  $\text{LD}_{50}$  describes the concentration of the respective synthetic peptide in micrograms per milliliter that leads to 50% reduction of CFU.

### *In vivo* activity of antimicrobial activity of sweat

A total of 15 healthy volunteers (7 women and 8 men; mean age, 29.7 years) and 11 patients with AD (5 women and 6 men; mean age, 28.1 years) participated in this *in vivo* investigation. Secondary infection or either systemic or topical antibiotic treatments in the preceding 4 wk were considered exclusion criteria. Participants were asked not to wash or apply topical treatment for 6 h before the investigation.

Quantitative examination of viable bacteria on the skin surface was undertaken before and after sweating. Using the scrub technique described by the German Society for Hygiene and Microbiology, skin colonization of the forearm was investigated. An area of 5  $\text{cm}^2$  was scrubbed in a standardized manner with a tryptone soy broth-soaked cotton swab (NOBA). The swab was immediately placed in a tube with 5 ml of tryptone soy broth and vortexed for 30 s. One and 100  $\mu$ l of the vortexed fluid were plated in duplicate onto blood agar and incubated aerobically at  $37^{\circ}\text{C}$ . CFU per square centimeter were calculated by standard methods. Bacteria were identified by Gram's stain, coagulase positivity, and the Slidex Staph Plus (bioMerieux) agglutination kit and differentiated into *S. aureus*, coagulase-negative staphylococci/micrococci, and diptheroids. Bacterial identification was confirmed using a BBL Crystal Identification System (BD Biosciences). After the initial sampling (referred to as "before sweating"), participants exercised on a bicycle ergometer until moderate perspiration (resulting in a shiny skin surface) could be observed. Exercise was stopped before profound sweating with droplet formation occurred. After the skin surface was allowed to dry for 10 min, a second set of samples was taken from the respective body sites (referred to as "after sweating").

### Statistical analysis

Statistical analysis was performed with JMP software version 5.0.1.2 (SAS Institute). SELDI data were analyzed using two-sample *t* tests, and the means of relative units of the respective DCD peptides were compared. For analysis of C- and N-terminal peptides,  $\log_{10}$  values of the sum of the respective peptides were calculated and compared. DCD and total protein concentrations, determined by ELISA and BCA assay, were compared using two-sample *t* tests. For analysis of the results of the *in vivo* assay of antimicrobial activity, CFU per square centimeters were subtracted (after

Table I. DCD-derived peptides identified in human sweat<sup>a</sup>

Name	Amino Acid Positions	Peptide Length (Amino Acids)	Mass (Da)	$\text{LD}_{50}$ ( $\mu\text{g}/\text{ml}$ ) <i>S. aureus</i>
DCD-1L	63–110	48	4818.5	4
DCD-1	63–109	47	4705.3	6
SSL-46	63–108	46	4606.2	10
LEK-45	66–110	45	4531.2	$>200$
LEK-44	66–109	44	4418.0	$>200$
LEK-43	66–108	43	4318.9	ND
YDP-42	20–61	42	4302.6/8605.2	$>200$

<sup>a</sup> The sequence of full-length DCD is: *MRFMTLLFLTALAGALVCAYPDPEAA* SAPGSGNPCHESAAQKENAGEDPGLARQAPKPRKQR **SSLLEKGLDGA** *AVGGLGKLGKDAVEDES***VGKGA***VHDVKDVLDSVL*. DCD-1L (amino acid position 63–110) is marked in bold, the signal peptide (amino acid position 1–19) is marked in italics. The  $\text{LD}_{50}$  describes the concentration of the respective synthetic peptide in micrograms per milliliter which leads to 50% reduction of CFU of *S. aureus* ATCC 25923 in a standard antimicrobial assay.

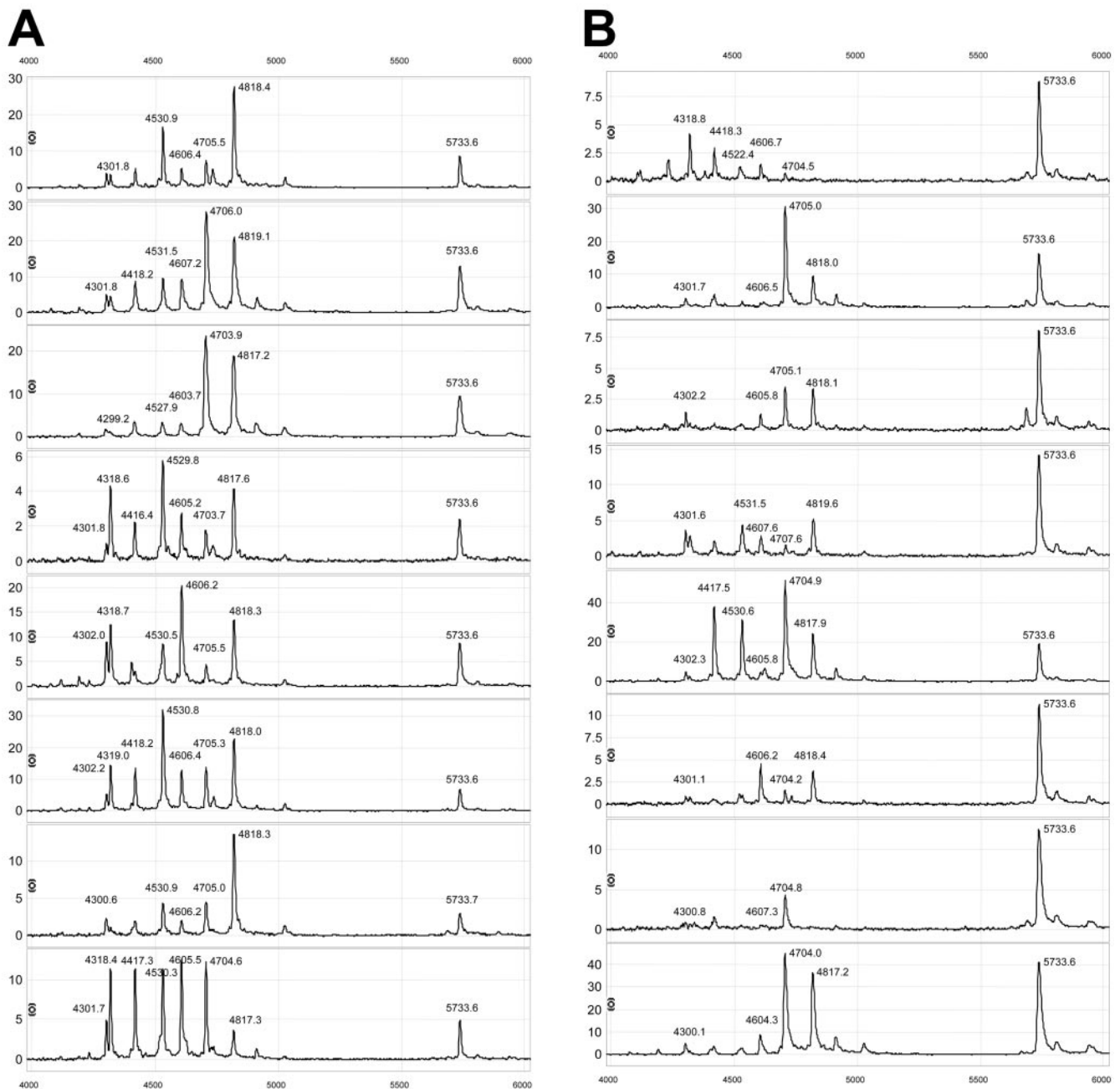
sweating – before sweating). The  $\log_{10}$  of the resulting values were compared using two-sample *t* tests. Significance was assumed for  $p < 0.05$ .

## Results

### Peptide profiling of DCD-derived peptides in sweat from patients with AD

In a previous study we identified several proteolytically processed peptides derived from DCD-1L in human sweat from healthy subjects, using SELDI-TOF-MS (13). In Table I, a list of the masses, peptide lengths, and amino acid positions of the most prominent DCD-1L-derived peptides are given. Among those are six peptides derived from the C-terminal end of the precursor DCD peptide with a length of 43–48 aa (named DCD-1 and DCD-1L, SSL-46,

and LEK-45 to -43); a peptide named YDP-42, derived from the N terminus, with a length of 42 aa; and its dimeric form. DCD-1 and DCD-1L show antimicrobial activity against various microorganisms, among those *E. coli*, *E. faecalis*, *S. aureus*, and *C. albicans* (5). The other C-terminal DCD-derived peptide, SSL-46, is also toxic to *S. aureus*, whereas the N-terminal peptide, YDP-42, and the C-terminal-derived DCD peptide lacking the first three amino acids, SSL, did not show antimicrobial activity against *S. aureus* (Table I). To assess whether the processing of DCD in sweat is different in patients with AD compared with control subjects, we performed SELDI analysis of individual sweat samples from 17 controls and 17 AD patients. As shown in Fig. 1, the proteolytic patterns of DCD-derived peptides were similar between healthy



**FIGURE 1.** Spectra of SELDI analysis of human sweat of eight control subjects (A) and eight patients with AD (B) using a reverse phase (H4) protein chip. After baseline subtraction, spectra were normalized to the bovine insulin peak (5733.6 Da). In both groups, similar DCD-derived peaks were detected: DCD-1L (4818.5 Da), DCD-1 (4705.3 Da), SSL-46 (4606.2 Da), LEK-45 (4531.2 Da), LEK-44 (4418.0 Da), LEK-43 (4318.9 Da), YDP-42 (4302.6 Da), and its dimer (8605.2 Da; not shown).

and atopic subjects. Although the processing of DCD was individually different, in both groups the different C-terminal-derived DCD peptides, LEK-43 to -45, SSL-46, DCD-1, and DCD-1L, as well as the N-terminal peptide, YDP-42, and the dimeric form can be found in sweat. This indicates that the processing of full-length DCD is not different in patients with AD.

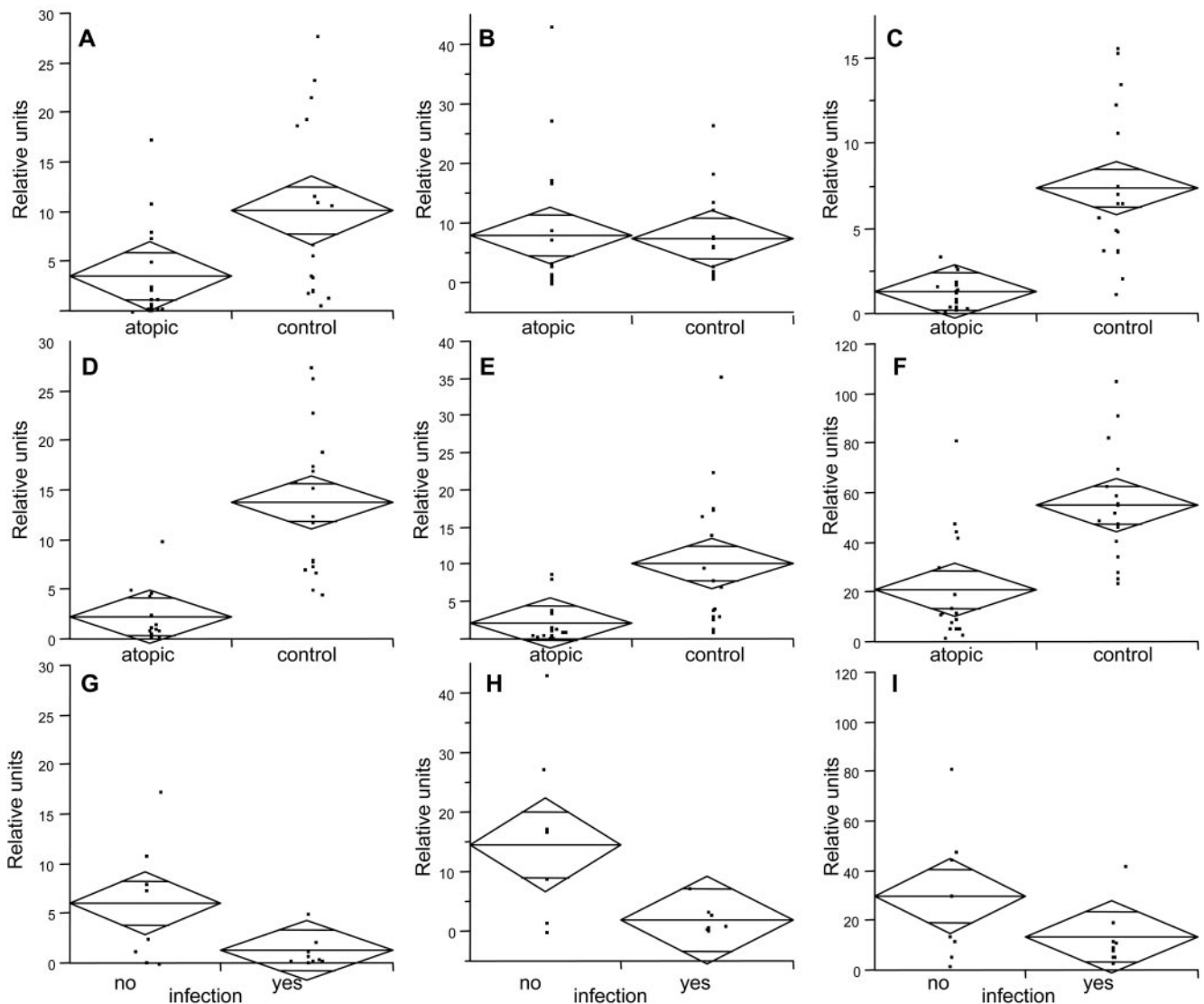
#### Semiquantification of DCD-derived peptides in sweat of patients with AD

Sweat samples from 17 healthy volunteers and 17 patients with AD were applied together with a given amount of bovine insulin ( $M_r$ , 5733.6 Da) on a reverse phase (H4) Protein Chip Array (Ciphergen Biosystems). The spectra were analyzed using Protein Chip software 3.1 (Ciphergen Biosystems). After baseline subtraction, all spectra were normalized to the bovine insulin peak for semiquantification of the DCD-derived peptides.

Although the proteolytic patterns of DCD-1L-derived peptides were similar in healthy and atopic subjects, the amount of DCD-1L-derived peptides in the sweat of patients with AD was signif-

icantly reduced (Figs. 1 and 2, A–F). The DCD precursor protein is first processed to the peptides YDP-42 and DCD-1L; subsequently, the other truncated DCD peptides are generated from the DCD-1L precursor (data not shown). Therefore, one would also expect a reduced amount of the other DCD-1L-derived peptides. Indeed, by semiquantitative analysis, sweat of patients with AD contained significantly less DCD-1L peptide (4818.5 Da); the truncated peptides SSL-46 (4606.2 Da), LEK-45 (4531.2 Da), LEK-43 (4318.9 Da), and YDP-42 (4302.6); and its dimer (8605.2), whereas LEK-44 (4418.0) and the DCD-1 peptide (4705.3) were detected in approximately equal amounts in both groups. Accordingly, semiquantification of the summation of C-terminal-derived DCD peptides revealed significantly less appearance of these peptides in atopic sweat (Fig. 2F). The same held true for the summation of peptides DCD-1L, DCD-1, and SSL-46, which are antimicrobially active against *S. aureus* ( $p < 0.039$ ).

Next, we analyzed whether there is a difference with respect to the amount of DCD-derived peptides in sweat of patients with AD depending on bacterial skin infections. Among the 17 patients with



**FIGURE 2.** Semiquantitative analysis of DCD-derived peptides in 17 patients with AD and 17 healthy volunteers, as detected by SELDI (A–F). A, DCD-1L (4818.5 Da;  $p = 0.011$ ); B, DCD-1 (4705.3 Da;  $p = 0.88$ ); C, SSL-46 (4606.2 Da;  $p < 0.0001$ ); D, LEK-45 (4531.2 Da;  $p < 0.0001$ ); E, LEK-43 (4318.9 Da;  $p = 0.0017$ ); F, Sum of C-terminal DCD peptides ( $p < 0.0001$ ). G–I, Semiquantitative analysis of DCD-1L ( $p < 0.04$ ); G, DCD-1 ( $p < 0.03$ ); H, and the sum of C-terminal DCD peptides ( $p = 0.11$ ); I) in a subgroup of AD patients with a history of infectious complications (infection: yes) compared with those patients with AD lacking these complications (infection: no). All samples were analyzed in triplicate, and means were compared using two-sample  $t$  tests. The means and 95% confidence limits (mean  $\pm$  double SE) are displayed by diamonds;  $p$  values are indicated above.

AD, 9 had a history of bacterial superinfection (requiring systemic antibiotic treatment) or a history of eczema herpeticum. Compared with atopic patients without previous infectious complications, this subgroup had significantly less DCD-1L (Fig. 2*G*), DCD-1 (Fig. 2*H*), and the summation of DCD-1L, DCD-1, and SSL-46 ( $p < 0.035$ ); however, the subgroup did not have less of the sum of all C-terminal-derived DCD peptides (Fig. 2*I*). An effect on the expression of DCD-derived peptides by age, sex, and scoring AD index was not observed (data not shown). By SELDI analysis with a reverse phase (H4) chip (see Fig. 1) and a cation exchange chip (data not shown), detectable amounts of the antimicrobial peptides LL-37 (4493.2 Da) or the further processed forms (22), human  $\beta$ -defensins HBD-1 (3934.6 Da), HBD-2 (4334.2 Da), and HBD-3 (5161.2 Da) could not be identified, indicating that in human sweat these peptides are not present in sufficient amount to account for a relevant antimicrobial activity.

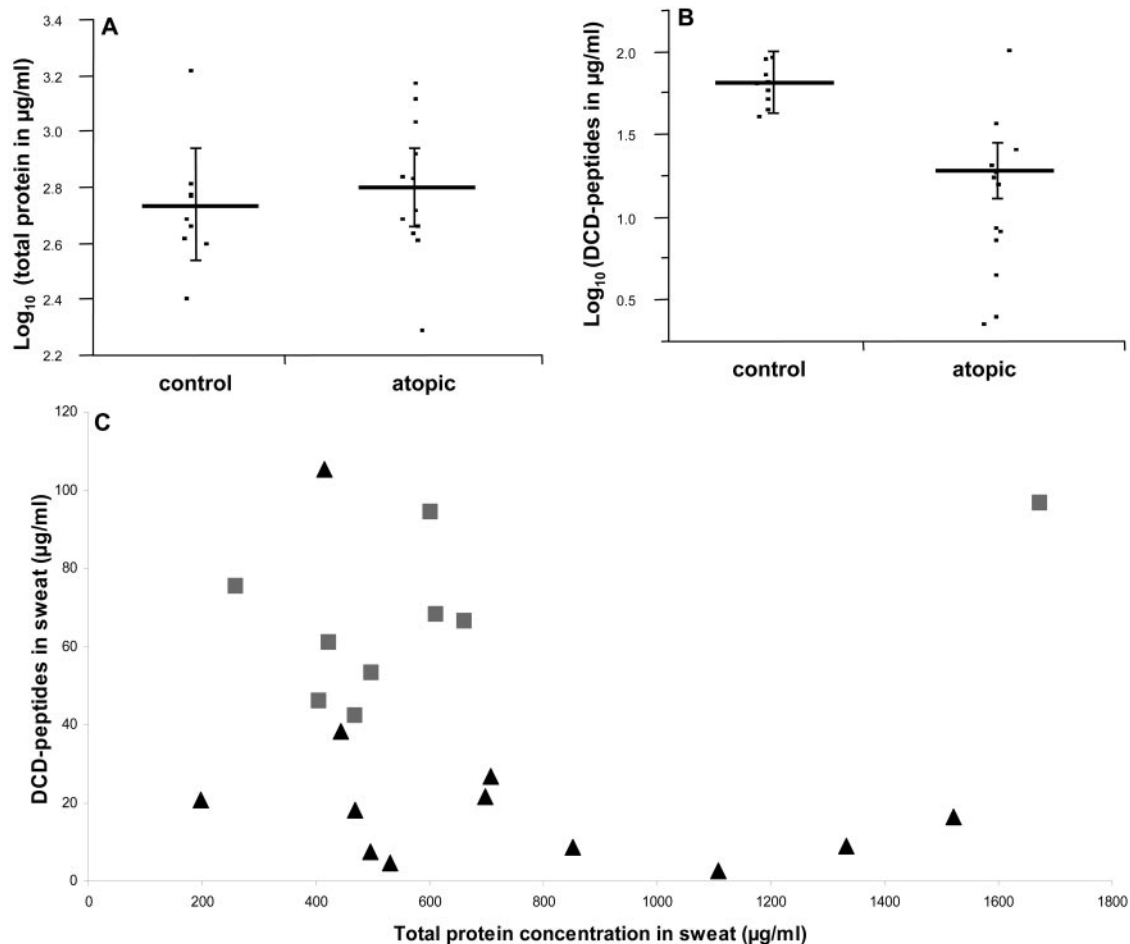
#### *DCD-derived peptides in human sweat are specifically reduced in AD patients*

The mAb G-81 (21) recognizes all the above-described C-terminal-derived DCD peptides present in human sweat. Using this Ab

in an ELISA, we compared the amounts of C-terminal-derived DCD peptides in human sweat of controls with those in AD patients (Fig. 3*B*). Using serial dilutions of individual sweat samples from 9 controls and 11 AD patients, we detected a significant difference in the amount of DCD-derived peptides in sweat of AD patients ( $p < 0.001$ ). This contrasts with the total protein concentration in sweat samples measured by BCA test, which does not differ between controls and AD patients ( $p = 0.743$ ; Fig. 3*A*). Furthermore, we could show that the amount of DCD peptides is independent of the total protein concentration in human sweat in both groups (Fig. 3*C*). This indicates that in sweat of patients with AD, DCD-derived peptides are specifically reduced.

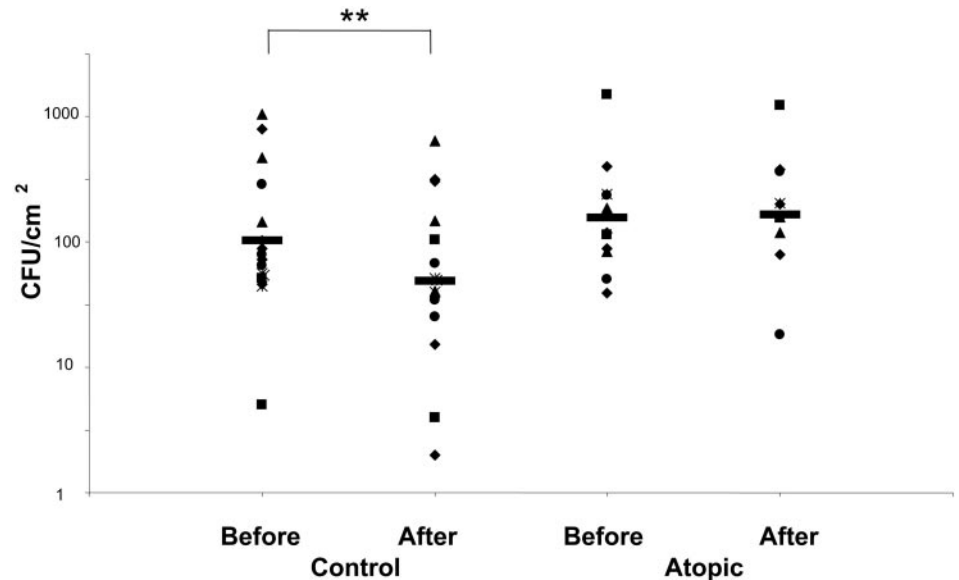
#### *Sweat of patients with AD shows reduced antimicrobial activity in vivo*

Next we analyzed whether there are functional differences in the antimicrobial activity of sweat from controls and AD patients. Removal of DCD peptides from sweat by immune adsorption could not be performed, because the available Abs do not recognize all antimicrobial DCD peptides in human sweat. However, because DCD-derived peptides are the predominant constituent of human



**FIGURE 3.** Quantitative analysis of the concentration of total proteins (*A*) or DCD peptides (*B*) in sweat of healthy volunteers (control) and AD patients (atopic). *A*, The total protein concentration in sweat was determined using a BCA test. Shown are individual values of the  $\log_{10}$  total peptide concentration in sweat. Means were compared using two-sample *t* tests; the means and 95% confidence limits are displayed. Differences in the protein concentration between patient groups were not statistically significant ( $p = 0.743$ ). *B*, The concentration of C-terminal-derived DCD peptides in sweat (1/100 diluted) was determined by ELISA, using the G-81 mAb. Shown are individual values for the  $\log_{10}$  DCD peptide concentration in sweat. Means were compared using two-sample *t* tests; the means and 95% confidence limits are displayed. The difference in the DCD peptide concentration between both patients groups was highly significant ( $p = 0.0008$ ). *C*, Shown are individual values for the concentration of DCD peptides in sweat vs the total protein concentration in sweat from control subjects (■) and AD patients (▲). The graph shows that the amount of DCD peptides in sweat is independent of the total protein concentration.

**FIGURE 4.** Analysis of the in vivo antimicrobial activity of human sweat. Shown is the  $\log_{10}$  CFU per square centimeter of skin before and after sweating in individuals in the control group (15 subjects) and in the AD group (11 patients). Mean values are indicated by a bar. The average reduction in bacterial counts (total CFU per square centimeter) in healthy individuals was 46.2% compared with 3.1% in AD patients. The differences in total CFU per square centimeter before and after sweating were significant (\*\*,  $p = 0.018$ ) in healthy individuals, but not in AD patients ( $p = 0.728$ ). The identity of individuals before and after sweating is indicated by assigning them the same markers.



sweat, and we could not detect other known antimicrobial proteins or peptides in sweat (Fig. 1 and data not shown), we analyzed directly the in vivo antimicrobial activity of sweat. Using a standardized scrubbing technique, the number of viable bacterial cells on the skin surface was determined before and after an episode of sweating.

Moderate sweating induced by physical exercise resulted in an overall reduction of viable bacteria on the skin surface in 13 of 15 healthy volunteers, whereas in only 6 of 11 patients with AD a reduction was observed. The average reduction in bacterial counts (total CFU per square centimeter) in healthy individuals was 46.2% compared with 3.1% in atopic patients. The differences in total CFU per square centimeter before and after sweating proved to be significant ( $p = 0.018$ , by paired  $t$  test) in healthy individuals, but not in AD patients ( $p = 0.728$ ; Fig. 4). Differentiation of bacteria in groups of coagulase-negative staphylococci/micrococci, *S. aureus*, and diptheroids revealed stronger antimicrobial activity in healthy individuals than in AD subjects. In healthy subjects, both the number of patients in whom a reduction in bacterial counts was observed as well as the average reduction in CFU per square centimeter were higher for all three subgroups of bacteria compared with those in the AD group.

## Discussion

DCD is a recently described AMP that is constitutively expressed in eccrine sweat glands and is transported via sweat to the epidermal surface. DCD-1, DCD-1L, and the shorter C-terminal-truncated peptide, SSL-46, display antimicrobial activity against *S. aureus* and a variety of other microorganisms (5, 14, 15). In our investigation we demonstrate a decreased expression of the antimicrobial DCD peptides in patients with AD. By SELDI technology, we found DCD-1L (48-mer) and several proteolytically derived DCD peptides in significantly reduced levels in sweat of patients with AD compared with those in healthy individuals. Our investigations by semiquantitative SELDI technology were paralleled by a quantitative ELISA. Using an mAb directed against the C-terminal region of DCD, reduced expression of the C-terminal-derived DCD peptides could be confirmed. Our investigations revealed a specifically reduced expression of DCD-derived peptides, because the total protein concentration, as determined by BCA assay, did not differ in sweat of AD vs healthy individuals. Furthermore, we observed a significant reduction in viable bacterial

cells on the skin surface after sweating in healthy individuals, but not in patients with AD. This indicates that sweat of patients with AD shows a decreased antimicrobial activity in vivo. Together with the facts that 1) sweat of AD patients contains a reduced amount of DCD-derived antimicrobial peptides; and b) we did not identify other antimicrobial peptides in significant amounts in human sweat, we suggest that a reduction of DCD in sweat may be an important factor for the reduced innate defense of skin in patients with AD.

Recently, it was reported that the processed active form of the antimicrobial peptide LL-37 is present in human sweat at a concentration of  $\sim 0.1 \mu\text{M}$ , using Western blot analysis (23). In addition, several shortened processed LL-37 peptides were identified in human sweat with enhanced antimicrobial activity (22). However, in our own experiments using SELDI-TOF-MS (Fig. 1), HPLC analysis, or gel filtration and peptide sequencing (data not shown), we could not identify or isolate LL-37, processed LL-37-derived peptides, or other antimicrobial peptides (i.e.,  $\beta$ -defensins) or proteins (i.e., lysozyme or phospholipase A2) in human sweat in significant quantity. One reason for not detecting LL-37 in human sweat might be that we used crude sweat in our analysis, whereas Murakami et al. used sweat concentrated 20/1 (23) or 50/1 (22). Therefore, it might be that the amount of LL-37 in sweat was overestimated in previous studies. Concerning the presence of the  $\beta$ -defensin HBD-2 in eccrine sweat, we detected in a previous study HBD-2 protein expression in eccrine sweat glands using immunohistology (6). However, our subsequent extensive studies of the protein constituents of eccrine sweat revealed that the  $\beta$ -defensins are not present in sweat in significant quantity (see Fig. 1 and data not shown). Because we could not isolate other AMPs in several independent experiments and using different methods, we conclude that in human sweat isolated ex vivo without further manipulations, DCD-derived peptides are the main antimicrobial peptides present in significant functional amounts.

The full-length DCD precursor protein is first proteolytically processed in sweat to the peptides YDP-42 and DCD-1L. Subsequently, the C-terminal peptides are further processed to the truncated peptides lacking either single amino acids at the C terminus (i.e., SSL-46) or the first three amino acids at the N terminus (LEK-45, LEK-44, and LEK-43). Furthermore, antimicrobial DCD peptides with a length shorter than 30 aa are processed from

DCD-1L in human sweat (data not shown). Using SELDI-TOF-MS, we demonstrate that in sweat of AD patients, the overall amount of N- and C-terminal processed DCD peptides is reduced, suggesting that also the precursor DCD protein is present in sweat in lower amounts. A reduced amount of C-terminal-derived DCD peptides was verified by ELISA. Analysis of the antimicrobial activity of several DCD-derived peptides revealed that the C-terminal-derived peptides, DCD-1L, DCD-1, and SSL-46, are antimicrobially active against *S. aureus* at concentrations present in sweat. Interestingly, the N-terminal-derived peptide, YDP-42, and the C-terminal-derived peptides, LEK-45 and LEK-44, lacking the first three amino acids of SSL from the DCD-1L sequence, show no antimicrobial activity against *S. aureus* in micromolar concentrations. It might be either that the first three amino acids from the DCD-1L sequence are important for antimicrobial function or that the LEK-45 and LEK-44 peptides have an antimicrobial spectrum different from that of DCD-1L. Additional investigations will clarify the antimicrobial spectrum of the different proteolytically processed DCD peptides and whether they act synergistically. Although Murakami et al. (23) reported only a limited antimicrobial activity of the synthetic DCD-1L peptide against various pathogens (at a peptide concentration of 80  $\mu\text{M}$ , 40% of *S. aureus* were killed), other groups, including ours, did not confirm these data. In accordance with other groups we show that DCD-1L possesses an LD<sub>50</sub> against *S. aureus* at peptide concentrations of 1–2  $\mu\text{M}$  (5, 15), and an LD<sub>50</sub> against *S. epidermidis* at peptide concentrations of 4  $\mu\text{M}$  (14).

AD is a chronic inflammatory skin disease that is frequently associated with allergic rhinitis or asthma (24). Recurrent bacterial and viral infections are typical complications in patients with AD and often necessitate in-patient treatment (25). Altered skin colonization with *S. aureus* as a dominant pathogen is another characteristic feature of AD, also indicating a defective epithelial barrier (16, 17). In recent years there has been considerable interest in the underlying mechanisms that make patients with AD prone to microbial skin infection and pronounced colonization.

Recent evidence suggests that impaired innate immune mechanisms in AD contribute to the propensity of AD patients to skin infections. The inducible antimicrobial peptides, LL-37, HBD-2, and HBD-3, were shown to be expressed at significantly lower levels in lesional AD skin compared with lesional skin in psoriasis (18, 19). Accordingly, the expression of the innate immune response genes, inducible NO synthetase and IL-8, was found to be decreased in AD due to the overexpression of Th-2 cytokines (IL-4 and IL-13) and low levels of proinflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$ , and IL-1 $\beta$ ) (19). *S. aureus* is the predominant pathogen in AD, with densities exceeding 10<sup>7</sup> organisms/cm<sup>2</sup> in lesional skin. Even in nonlesional AD skin, *S. aureus* is present in densities of 10<sup>3</sup>–10<sup>4</sup> organisms/cm<sup>2</sup> (16, 17). Because in AD patients, colonization with *S. aureus* is also a prominent feature of nonlesional AD skin in the absence of inflammation, we hypothesized deficiencies in the constitutive innate defense of human skin. Indeed, our data strongly suggest that a reduced amount of the antimicrobial DCD peptides in sweat of patients with AD contributes to the high susceptibility of these patients to skin infections and to altered skin colonization. Our results show for the first time that a deficiency in the expression of a constitutively expressed antimicrobial peptide in human skin correlates with compromised innate skin defense.

In addition to a deficiency in DCD-derived peptides in eccrine sweat, other factors may influence the differential colonization of AD skin. In AD, a slight alkalization in the average pH of skin from pH 5.0 to pH 5.5 can be observed (26). Decreased levels of sphingosine in lesional and nonlesional stratum corneum were reported to be involved in the vulnerability of AD skin to coloniza-

tion by *S. aureus* (27). Moreover, distinct functional variations in the skin of patients with AD were described. Among these, differences in sweating patterns are well established. Impaired sweating in AD subjects has been evaluated in thermal and physical stress experiments. Healthy individuals perspired nearly three times as much as did patients with AD after induction through physical exercise (28). Accordingly, AD patients show significantly lower sweat rates upon moderate thermal stress (29). Differences in the composition of sweat, with reduced secretion of IgA (30) and altered sweat electrolyte concentrations (31) in children with AD, have been described. Therefore, our findings underline specific differences in sweating that may be related to the high incidence of skin infections. In addition to a specifically reduced DCD expression in atopic sweat, the overall reduced amount of sweat contributes to the impaired innate defense mechanism in AD patients.

We propose that sweat plays an important role in modulating the microbial flora of the skin. Several features make it a good candidate. First, being secreted onto the outermost surface, pathogens are exposed to a biofilm of sweat even before contact with keratinocytes occurs. Second, the dermal localization of the eccrine glands on the epithelial lining does not allow suppression by microbial counterstrategies or even the host's own cytokine milieu, as observed with LL-37 and HBD-2/3 (18, 32). Third, the increased sweat secretion in stress situations and the highest density of eccrine glands on palms and soles, which are most often exposed to pathogens and minor trauma, also support our hypothesis.

In conclusion, we found that sweat of AD patients showed a significantly reduced amount of DCD-derived peptides compared with sweat of healthy individuals. Furthermore, decreased DCD expression correlated with clinical infectious complications and may therefore contribute to the propensity of AD skin to recurrent bacterial and viral skin infections and altered skin colonization.

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## Disclosures

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