

Effect of the lactic acid bacterium *Streptococcus thermophilus* on stratum corneum ceramide levels and signs and symptoms of atopic dermatitis patients

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Abstract: A reduced amount of total ceramides could be responsible for functional abnormalities of the skin of atopic dermatitis (AD) patients. The ability of an experimental cream containing sonicated *Streptococcus thermophilus* to increase skin ceramide levels in healthy subjects has been previously reported. The aim of the present work was to investigate the effects of the topical administration of a *S. thermophilus*-containing cream on ceramide levels of stratum corneum from AD patients. A 2-week application of the cream, containing a sonicated preparation of the lactic acid bacterium *S. thermophilus*, in the forearm skin of 11 patients led to a significant and relevant increase of skin ceramide amounts, which could have resulted from the sphingomyelin hydrolysis through the bacterial sphingomyelinase. Moreover, in all patients the topical application of our experimental cream also resulted in the improvement of the signs and symptoms characteristic of AD skin (i.e. erythema, scaling, pruritus).

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Introduction

Atopic dermatitis (AD), a common and potentially debilitating condition that can compromise quality of life, is a chronic, relapsing inflammatory pruritic skin disease (1). The exact pathogenesis is unclear but it appears that it has a complex immunologic and genetic origin (2,3). The non-involved skin of atopic eczema is characterized by severe dryness and an impaired barrier function of the stratum corneum, as indicated by an increased transepidermal water loss (4–7). The chronic, cyclical, pruritic course of AD exacts a huge financial and emotional toll on its sufferers. The keys to successful management of this disease continue to

include accurate diagnosis, hydration of the skin, control of pruritus and infections, and identification and elimination of exacerbating factors. In regards to AD treatment, the use of topical steroids remains the therapeutic standard, even if many drugs are now in development based on improvements in existing therapies or on a better understanding of the cellular and molecular mechanisms involved in AD (8).

Recent studies have demonstrated that AD-associated barrier impairment coincides with marked alterations in the amount and composition of stratum corneum ceramides (9–13). Indeed, a ceramide serves as the major water-holding molecule in the extracellular space of the horny layer. The amount of ceramide in the stratum corneum is regulated by the balance among the ceramide-generating enzymes including serine-palmitoyl-transferase (14), sphingomyelinase (SMase) (15,16),

Abbreviations: AD, Atopic dermatitis; nSMase, neutral sphingomyelinase; S.E.M., standard error mean; SM, sphingomyelin; TLC, thin layer chromatography.

and β -glucocerebrosidase (17), and the degradative enzyme ceramidase (18). Both sphingomyelin and sphingomyelinase, are better able to hydrolyze sphingomyelin into ceramides, are present in the epidermis and are originally contained in lamellar bodies (19,20). An altered SM metabolism in the skin of patients with AD, which has been attributed to a deficient function of SMase and a parallel abnormal expression of glucosylceramide sphingomyelin deacylase, could explain the ceramide deficiency and the marked vulnerability of the atopic skin to irritants or allergens (12,21). Moreover, recent reports suggest that the skin of patients with AD is colonized by ceramidase-secreting bacteria (i.e. *Pseudomonas aeruginosa* and/or related strains), and correlate the deficiency of ceramide in the horny layer of the epidermis and the associated impairing of the barrier permeability with the presence of this ceramide-degradative enzyme (22,23).

Taken together, these findings strongly indicate that skin treatment with exogenous factors capable of increasing the levels of stratum corneum lipids, mainly ceramides, may improve barrier function and stratum corneum flexibility and consequently offer an advantage in patients with AD.

Our group has recently assessed the possibility of increasing ceramide levels either *in vitro* on cultured keratinocytes or *in vivo* on stratum corneum by treatment with a cream containing a sonicated preparation of *Streptococcus salivarius* subspecies *thermophilus* (24). The results showed that *S. thermophilus* treatment led to a relevant increase of ceramide levels both *in vitro* and *in vivo* in healthy subjects. Considering the suggested role of ceramides in maintaining skin integrity, the aim of the present work was to investigate the possibility that the topic administration of *S. thermophilus*, representing a source of exogenous nSMase able to hydrolyze skin SM and consequently to generate ceramide and phosphorylcholine, presumably may lead to an improvement in the clinical signs and symptoms of AD.

Materials and methods

Preparation of bacterium suspensions. *Streptococcus thermophilus* (Strain S244) cultivated in 10% skimmed-milk sterilized at 110°C for 30 min and added to 0.1% yeast extract was obtained from Centro Ricerche YOMO (Milan, Italy) in a pure lyophilized form (10^8 colony-forming units [CFU]/g). Stocks of 1.7-g lyophilized *S. thermophilus* were resuspended in 5 ml of phosphate buffered solution (PBS), sonicated (30 min, alternating 10 s sonication and 10 s pause) with a Vibracell sonicator (Sonic and Materials Inc., Danbury, CT). For the topical applications, the sonicated bacteria

(1.7 g/5 ml) were firstly analyzed for the neutral SMase activity and then mixed with 20 ml of a base cream (Avant Garde, Sigma Tau, Pomezia, Rome, Italy) containing the following components: Sepigel 305, Vitamin E acetate, perfume, glycerol, vaselin oil, Glucam E 20, Fenotan, EDTA, Paracombin, Carbopol ETD 2001, Bensil dm 350, imidazolidinil urea, trietanolamine, Transcutol, demineralized water.

Patients, controls and treatment. The study was conducted on 11 patients (four males and seven females) aged 18–24 years (mean age 20.3 ± 1.8), affected by AD, diagnosed according to the criteria of Rajka and Langeland (25), and 10 healthy Caucasian young volunteer subjects (five males and five females) aged 17–27 years (mean age 21.9 ± 3.21), after informed consent. The trial was conducted during the autumnal season in order to avoid interseasonal variations of the skin ceramide content. During the period of the trial no other drug, topical or systemic, was allowed. Each subject applied twice daily an 'active' formulation (base cream as vehicle containing *S. thermophilus*) to one forearm and the base cream alone to the contralateral forearm, including all eczematous areas there present.

The severity of the dermatitis was evaluated at enrollment and at the end of the 2-week treatment period. All AD patients showed active lesions. Erythema and scaling were assessed both by the doctor and the patient, whereas pruritus was evaluated only by the patient. The patients were asked to treat their lesions with an appropriate quantity of the cream to be tested (experimental or control cream) and to keep a record regarding their signs and symptoms, which was compared with the dermatologist's assessment before and after the 2-week treatment period. In case of discrepancy, the opinion of the patient was considered to predominate. The symptoms and signs were considered absent (score 0), slight (score 1), moderate (score 2) or severe (score 3). We also examined disease activity by using the SCORing Atopic Dermatitis (SCORAD) index, which combines objective (extent and intensity of lesions) and subjective (daytime pruritus and sleep loss) criteria.

Sample collection. To assess the skin ceramide levels, 0.5 g of test product and base cream were applied twice daily to the volar surface of the forearms of both the healthy controls and the AD patients. Stratum corneum sheets were removed from the volar aspect of the forearms (uncleaned before sampling), 10 cm below the antecubital fossa, by a single stripping with 25 cm² of cyanoacrylate resin, before (T0) and after 2 weeks of the application (T1). The stripped stratum corneum sheets were incubated for 18 h with 25 ml of chloroform:methanol (2:1, by vol.), after which cyanoacrylate resin was removed. The samples were concentrated in a rotary evaporator (Savant Instruments Inc., Holbrook, NY) and evaporated to dryness in glass tubes under a stream of nitrogen, and the residues were dissolved in 1 ml of chloroform/methanol (9/1, by vol) and stored at -20°C until use.

Analysis of stratum corneum ceramides. For the lipid extraction, 400 μl of methanol, 500 μl of chloroform and 400 μl of water were added. Samples were stirred for 2 min on a vortex-mixer and centrifuged at $10978 \times g$ for 10 min. The extraction and centrifugation steps were repeated twice. Lipids, previously dried under nitrogen, were then incubated with *Escherichia coli* diacylglycerol kinase (DAG kinase assay kit and ³²P-ATPgamma, spec. act. 3 Ci/mmol, Amersham, Buckinghamshire, UK) to determine the levels of ceramide, according to the manufacturer's instructions, and applied to thin layer chromatography (TLC) silica gel plates using a TLC applicator (Camag; Berlin, Germany). Ceramide phosphate was then resolved using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}$ (65/15/5, v/v/vol) as solvent. Authentic ceramides from bovine brain (Ceramide Type III, non-hydroxy fatty acid ceramides; and Ceramide type IV, hydroxy fatty acid ceramides, Sigma) were identified by autoradiography at $R_f = 0.25$ and $R_f = 0.11$, respectively. Specific radioactivity of ceramides-1-phosphate

was determined by scintillation counting of corresponding spots scraped off the gel. Quantitative results for ceramide production were obtained by comparing the experimental values with a linear curve of the ceramide standards, and are expressed as pmoles of ceramides-1-phosphate/cm².

Statistical analysis. The differences in the analyzed parameters between the control group and the AD patients were tested by using the Student's *t*-test. The *t*-test for paired data was used to analyze the differences between the skin ceramide levels of the controls and the AD patients. The *t*-test for paired data was also used to analyze the differences between the signs and symptoms as well the ceramide levels in the AD patients before (T0) and after (T1) treatment with the experimental cream. The STATPAC Computerized Program was used to perform statistical analysis, and a *P*-value < 0.05 was used as the significance criterion.

Results

Ceramide levels in the normal and AD subjects

The skin ceramide levels of AD patients who showed strong basal subjective variability (range: 1.7–14.9 pmol total ceramides/cm²; mean \pm S.E.M. = 8.17 ± 1.31 pmol total ceramides/cm²) were significantly lower (*P* < 0.001) in comparison with those of healthy donors (range: 10–22 pmol total ceramides/cm²; mean \pm S.E.M. = 16.7 ± 1.42 pmol total ceramides/cm²), thus confirming previously reported findings (9–13) (Fig. 1).

Clinical evaluation

The SCORAD index was evaluated before treatment (mean 39 points). The scores for pruritus, erythema and scaling on the forearms were evaluated before and after treatment with the experimental cream. Mean erythema, scaling and pruritus were rated as moderate/severe by eight or more of the patients at enrolment (patients 1, 2, 3, 4, 5, 6, 7, 9, 10, 11); three subjects (patients 1, 2 and 6) did not show scaling at T0 and one had slight erythema. After treatment, eight of the patients did not show erythema (patients 1, 2, 5, 6, 7, 8, 10, 11) and pruritus (patients 1, 2, 3, 4, 6, 7, 8, 11); three individuals still complained of slight erythema (patients 3, 4, 9), while five patients still had scaling considered as slight in four (patients 5, 7, 8, 10) and moderate in the remaining patients (patient 9). Overall, the clinical evaluation of both the dermatologist and the patients overlapped, and the results confirmed recovery in eight patients and improvement in three individuals; the statistical analysis of these data showed that the effect of the experimental cream was always statistically significant (erythema, *P* = 0.000; scaling, *P* = 0.003; pruritus, *P* = 0.000; vesiculation, *P* = 0.000). Tolerability was considered as very good in nine patients and good in the remaining two patients. In some patients treatment was prolonged for a further 2 weeks and tolerability

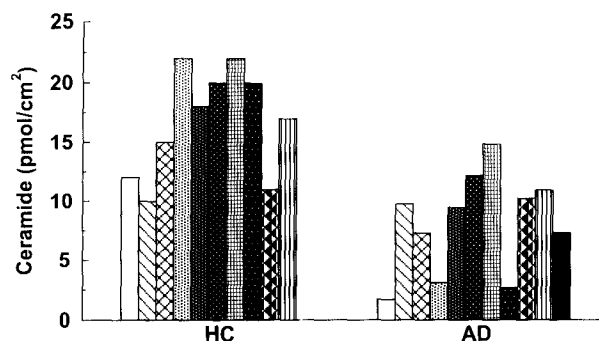


Figure 1. Stratum corneum ceramide levels in healthy subjects and atopic dermatitis (AD) patients. Skin lipid extracts from the forearms of 10 healthy subjects and 11 AD patients were analyzed for ceramide level determination by diacylglycerol (DAG) kinase assay.

was always considered very good. The base cream without *S. thermophilus* did not modify significantly the signs and symptoms, and all patients dismissed the treatment within 4 or 5 days because no appreciable variation was perceived in the AD patients (data not shown). In regards to the ceramide levels, the obtained results indicated that no significant increase was obtained after treatment of the AD patients with the base cream (9.23 ± 1.98). These results suggest that the effects observed after the patients' treatment with the experimental cream could be attributed to the active principle derived from *S. thermophilus*.

Ceramide levels following treatment

Total stratum corneum ceramide levels were increased in all patients following treatment with the experimental cream (mean \pm S.E.M. = 12.86 ± 1.97 pmol ceramides/cm²; T1 vs. T0, *P* = 0.002) (Fig. 2a). Figure 2(b) shows a representative autoradiography of skin ceramide levels obtained before (T0) and after (T1) treatment. The percent increase values ranged between 22.7% and 134.7% (Fig. 2c). The same cream without the *S. thermophilus* preparation did not modify significantly the skin levels of ceramide in patients with AD (data not shown), who interrupted the treatment within the first 5 days. The results of the skin ceramide levels in the healthy subjects before and after treatment with the base or experimental cream were comparable with those previously reported (24). In particular, the total stratum corneum ceramide levels were significantly increased in almost all healthy subjects (22 ± 2.5 pmol ceramides/cm²) following treatment with the base cream alone even if this effect was significantly less relevant when compared with the experimental cream treatment (31% mean increase).

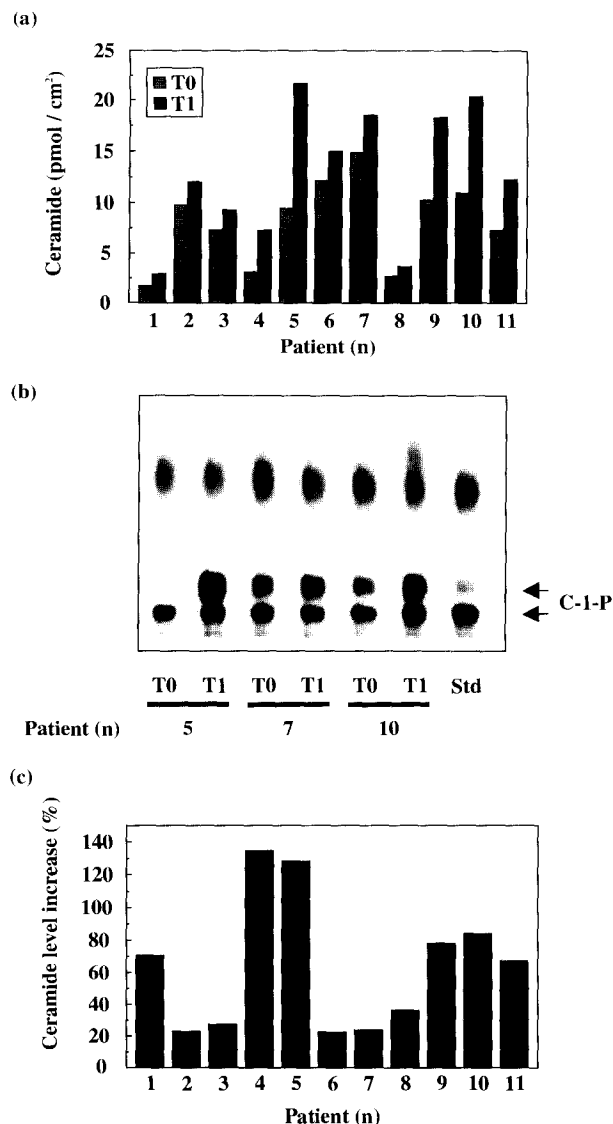


Figure 2. Effect of *Streptococcus thermophilus*-containing cream on the skin ceramide levels in atopic dermatitis (AD) patients. Skin lipid extracts from the forearms of 11 AD patients before (T0) and after (T1) topical treatment for 2 weeks with the experimental cream containing sonicated *S. thermophilus* (a) were analyzed for ceramide level determination by diacylglycerol (DAG) kinase assay. (b) Representative autoradiographies of thin layer chromatography of ceramides extracted from forearm stratum corneum of the indicated patients before (T0) and after (T1) treatment. (c) Percentages of increase in skin ceramide levels resulted after the experimental treatment.

Discussion

Several findings suggest that a decrease in ceramides in the stratum corneum is involved in barrier impairment in AD skin and support the view that altered metabolism of ceramide may be the cause of impaired barrier function in AD, resulting in the characteristic dry and easily antigen-permeable skin of AD (9–13). In addition, AD inflamed skin

may become severely colonized by ceramidase-expressing bacteria further contributing to the deficiency of ceramides in the horny layer of the epidermis (22,23). There is also evidence of involvement of serine-proteases (i.e. stratum corneum chymotryptic and stratum corneum tryptic enzymes) (26,27). In AD patients, although the early phase of barrier recovery, known as cholesterol synthesis, is faster than in controls, the complete restoration of the epidermal barrier is not achievable (28). In fact, to effectively maintain the permeability barrier homeostasis, all three main lipidic components of the stratum corneum, cholesterol, free fatty acids and ceramides, must be present (20,29). The absence of even one of these components in perturbed skin can delay barrier recovery (30,31). From a therapeutic point of view to correct the defect, usage of exogenous cholesterol, ceramides and free fatty acids in 3:1:1 ratio has been suggested (32). However, although this ratio is easily obtainable as a dermatological preparation, it may not be appropriate when applied *in situ*, where all three components are not always needed.

The present study was conducted to assess the possible beneficial effects on AD patients of a topical treatment with a cream containing sonicated *S. thermophilus*, which has been previously reported to increase ceramide levels in the skin of healthy subjects (24). To test the possibility that the use of the *S. thermophilus*-containing cream could improve sphingomyelin dysmetabolism in AD patients, we planned experiments to determine the effect of sonicated *S. thermophilus* on the level of ceramides *in vivo* in the skin of AD patients, and the clinical observation must be considered as a 'feasibility' observation.

Patients with AD had lower levels of ceramides when compared with the control group, confirming previous findings (9–13). Topical treatment with the *S. thermophilus*-containing cream induced a very significant increase of ceramide levels at the end of the treatment period (2 weeks). Ceramides predominantly present in stratum corneum consist of C16–C22 sphingosine and dihydrosphingosine bases amidated to long chain fatty acids, which may or may not be hydroxylated and unsaturated (33). Ceramides generated after treatment with *S. thermophilus* preparation are both hydroxy-fatty acid ceramides and non-hydroxy fatty acid ceramides. Both these types of ceramides exert important functions for epidermal homeostasis, e.g. permeability barrier function of the skin, as previously reported (12,33,34). According to these results and those of normal subjects published by the authors already (24), we assume that the presence of high levels of nSMase in *S. thermophilus*

could be responsible for the observed increase in stratum corneum ceramide levels.

From the clinical point of view, at the end of the 2-week treatment period, topical administration of the cream resulted in a reduction of erythema, scaling and pruritus in all patients. The improvement was steady and constant throughout the observation period. The improvement in the integrity of the barrier also implies a reduced susceptibility to the insults of the environment (e.g. decreased humidity, solvents, and detergents). The control cream (without bacterial preparation) did not influence the AD signs and symptoms (not shown) and all patients interrupted the treatment within 4–5 days. These results suggest that the clinical beneficial effects of our experimental cream could be attributed to the presence of the bacterial preparation and could be associated with the increased levels of skin ceramide. We assume at this point that, having demonstrated the efficacy of this method to increase ceramide levels also in the skin of AD patients, a specifically designed study is necessary to confirm the clinical efficacy of dermatological preparations containing *S. thermophilus* in the long term.

Altogether, our findings have many potential clinical significances. The increase in ceramide content could be beneficial to AD patients as well as to other patients with skin diseases associated with ceramide reduction or deficiency and xerotic skin (i.e. age-related xerosis, ichthyoses).

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