Effect of the Lactic Acid Bacterium *Streptococcus thermophilus* on Ceramide Levels in Human Keratinocytes *In Vitro* and Stratum Corneum *In Vivo*

Luisa Di Marzio, Benedetta Cinque, Claudio De Simone, and M. Grazia Cifone
Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

The effects of *Streptococcus thermophilus* on ceramide levels either *in vitro* on cultured human keratinocytes or *in vivo* on stratum corneum, have been investigated. *In vitro*, *Streptococcus thermophilus* enhanced the levels of ceramides in keratinocytes in a time-dependent way. The presence of high levels of neutral, glutathione-sensitive, sphingomyelinase in *Streptococcus thermophilus* could be responsible for the observed ceramide increase. The application of a base cream containing sonicated *Streptococcus thermophilus* in the forearm skin of 17 healthy volunteers for 7 d also led to a significant and relevant increase of skin ceramide amounts, which could be due to the sphingomyelin hydrolysis through bacterial neutral sphingomyelinase. Indeed, similar results were obtained with a base cream containing purified bacterial neutral sphingomyelinase. In addition, the inhibition of bacterial neutral sphingomyelinase activity through glutathione blocked the skin ceramide increase observed after the treatment. The topical application of a sonicated *Streptococcus thermophilus* preparation, leading to increased stratum corneum ceramide levels, could thus result in the improvement of lipid barrier and a more effective resistance against xerosis. *Key words: HaCat cells/lactobacillus/skin/sphingomyelinase. J Invest Dermatol 113:98–106, 1999*

The normal morphology of stratum corneum is essential for maintaining the water barrier of the skin (Bowser and White, 1985; Rawlings *et al*, 1994). The protein-enriched corneocytes are embedded in an intercellular lipid matrix which is composed primarily of ceramides (43–46% of total lipids), cholesterol, and fatty acids together with smaller amounts of cholesterol sulfate, glucosylceramides, and phospholipids (Yardley and Summerly, 1981; Elias *et al*, 1988). These lipids form multilamellar sheets within the intercellular spaces of the stratum corneum, the organization of which is essential in maintaining the functionality of the skin as an effective barrier to water loss (Rougier *et al*, 1983; Eckert and Rorke, 1989; Potts and Francoeur, 1991). In addition, they also play an important part in determining the mechanical, cohesive, and desquamatory properties of the stratum corneum, and therefore have a key role to counteract the environmental challenges which can lead to disturbances in skin function (for a review see Rawlings *et al*, 1994). A global decrease in lipid content leads to alterations in lamellar bilayer morphology which appear to underlie the impaired functional abnormalities commonly associated with aging. Indeed, an age-related decline in stratum corneum ceramide, cholesterol, and fatty acid levels has been previously reported (Ghadially *et al*, 1995; Rogers *et al*, 1996). Overall, the total lipid levels decrease by approximately 30%, which may reflect the slower keratinocyte metabolism of the aged (Grove and Kligman, 1983), leading to decreased biosynthetic capacity. Recently, a study demonstrated a seasonal-related reduction in stratum corneum lipid levels probably reflecting decreased epidermal lipid biosynthesis (Rogers *et al*, 1996). Apart from the increased susceptibility of the barrier to damage in the elderly, the reduction in lipid levels could explain the increased incidence of xerosis in winter (Rogers *et al*, 1996). The reduction in lipid levels may in turn reduce the water content of the stratum corneum. This may influence the activity of the stratum corneum proteases thought to be involved in desquamation (Lundstrom and Egullrud, 1990; Egelrud, 1993; Rawlings *et al*, 1994) and interferes with the generation of natural moisturizing factors (Scott and Harding, 1986), leaving the stratum corneum more susceptible to xerosis. The reduction of the stratum corneum ceramide levels have been proposed as a possible etiologic factor in atopic dermatitis and psoriasis (Motta *et al*, 1994; Murata *et al*, 1996). Moreover, topical application of ceramides has been shown to improve directly the barrier function of the stratum corneum (Imokawa *et al*, 1989). Epidermis possesses the capacity to synthesize all lipids required for the barrier formation. Especially unique for the epidermis is the synthesis of large amounts of glucosylceramides and ceramides (Heldberg *et al* 1988), which has also been demonstrated in organotypic keratinocyte cultures (Schurer *et al*, 1989; Madison *et al*, 1990). The amount of ceramide in the stratum corneum is regulated by the balance among the ceramide-generating enzymes including serine-palmitoyltransferase (Holleran *et al*, 1991), sphingomyelinase (SMase) (Menon *et al* 1986; Yamamura and Tezuka, 1990), and β-glucocerebrosidase (Holleran *et al*, 1992) and the degradative enzyme ceramidase (Yada *et al* 1995). Cultured keratinocytes were reported to have a high level of serine palmitoyltransferase activity, which catalyzes the synthesis of the long-chain base precursor of sphingolipids (Holleran *et al*, 1990). Moreover,

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Reprint requests to: Dr. M. Grazia Cifone, Department of Experimental Medicine, University of L'Aquila, Via Vetoio 10, Coppito 2, 67100 L'Aquita, Italy.

Abbreviations: aSMase, acidic sphingomyelinase; DAG, diacylglycerol; GSH, glutathione; nSMase, neutral sphingomyelinase; PC, phosphatidylcholine; SM, sphingomyelin; TLC, thin-layer chromatography.

the activity of ceramide glucosyltransferase, the enzyme responsible for glucosyl-ceramide synthesis in cultured human keratinocytes, has been recently studied and characterized (Sando *et al* 1996). Both sphingomyelin (SM) and SMase, which hydrolyzes SM into ceramides, are present in the epidermis and are originally contained in lamellar bodies (Bowser and Gray, 1978; Yardley and Summerly, 1981). An altered SM metabolism in the skin of patients with atopic dermatitis, which has been attributed to a deficient function of SMase and a parallel abnormal expression of SM acylase, has been reported; this could explain the ceramide deficiency and the marked vulnerability of the atopic skin to irritants or allergens (Murata *et al*, 1996). Moreover, recent reports suggest that the skin of patients with atopic dermatitis 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 epidermis and the associated impairment of the barrier permeability with the presence of this ceramide-degradative enzyme (Okino *et al*, 1998; Ohnishi *et al*, 1999). Interestingly, Rawlings *et al* (1996) have reported the induction of a ceramide level enhancement either in keratinocytes *in vitro* and in skin *in vivo*, which was associated with an increased stratum corneum barrier and an increased effect in resisting skin xerosis. The authors hypothesized that lactic acid could be metabolized to acetate which in turn is used for ceramide biosynthesis in keratinocytes.

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; consequently this may slow down, in healthy subjects, the skin aging process and offer advantages for patients with skin conditions due to a defective synthesis of lipids.

As many bacteria possess high levels of SMase in their membrane, they may be particularly useful in inducing an increase of skin ceramide generation through SM hydrolysis. SM represents one of the most important lipid components of the mammalian plasma membrane and is preferentially localized in the external outleaf thus being readily available for its hydrolysis through an exogenous source of SMase, such as bacterial SMase. Indeed, bacterial SMase often has been used for the depletion of SM in a variety of cellular systems leading to the release of ceramide (Bettaieb *et al*, 1996; Wright *et al* 1996; Flamigni *et al*, 1997; Zhang *et al*, 1997).

Lactic acid bacteria are a group of bacteria belonging to a diverse genera used to bring about milk fermentation, and composed chiefly of bacteria whose primary metabolic end-product of carbohydrate metabolism is lactic acid; this in turn preserves milk by providing the acidity necessary for a tart flavor and for changes in the structure of casein to achieve syneresis and desired functional characteristics (Sanders, 1992; Johnson and Steele, 1997). *Streptococcus salivarium* subspeciem *thermophilus* belongs to this group of bacteria and is used to bring about the fermentation of several dairy products, mainly cheeses and yogurt (Sanders, 1992; Johnson and Steele, 1997) and does not form a constituent of normal skin resident microflora (James and Roth, 1992).

The aim of this work was to assess the possibility of increasing ceramide levels either *in vitro* on cultured keratinocytes or *in vivo* on stratum corneum by treatment with a sonicated preparation of *Streptococcus salivarium* subspeciem *thermophilus*.

Our results indicate that *S. thermophilus* treatment led to a significant increase in ceramide levels both *in vitro and in vivo*. If we consider that ceramides can maintain skin integrity, it is possible that topical administration of *S. thermophilus* (as a source of exogenous SMase able to hydrolyze skin SM) may consequently generate ceramide and phosphorylcholine, possibly leading to an improvement in barrier function and maintenance of stratum corneum flexibility; all these events may help the stratum corneum resist xerosis.

MATERIALS AND METHODS

In vitro studies

Cell culture The spontaneously immortalized human keratinocyte cell line HaCat (Boukamp *et al*, 1988) was a gift from Dr. Diana Boraschi (Dompè Research Center, L'Aquila, Italy). Cells were grown in plastic culture dishes (Nunc, Wiesbaden, Germany) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin per ml, 50 μg streptomycin per ml, and 50 μg gentamycin per ml. Media and culture reagents were obtained from Gibco (Berlin, Germany), penicillin, streptomycin, and gentamycin from Boehringer (Mannheim, Germany). Confluent cells were subcultured every 3 d after detaching the cells with a 0.1% trypsin/0.02% ethylenediamine tetraacetic acid solution. For the duration of the experiments, HaCaT cells were maintained in serum-free keratinocyte basal medium.

Preparation of bacterium suspensions *Streptococcus thermophilus* (strain S244), cultivated in 10% skimmed milk sterilized at 110°C for 30 min with 0.1% yeast extract, was obtained from Centro Ricerche YOMO (Milan, Italy), in a pure lyophilized form (10 cfu per g). Stocks of 1.7 g lyophilized *S. thermophilus* were resuspended in 5 ml phosphate-buffered solution (PBS), sonicated (3 min and 50 s, alternating 10 s sonication and 10 s pause) with a Vibracell sonicator (Sonic and Materials, Danbury, CT). For the *in vitro* experiments, sonicated bacterium suspension was added to keratinocyte cultures at 50 mg per 5×10^6 cells per 10 ml (final concentration). For the topical applications, the sonicated bacteria (1.7 g per 5 ml) were mixed with 20 ml of a base cream (Avant Garde, Sigma Tau, Pomezia, Rome, Italy). A base cream containing purified neutral SMase (nSMase) from *Bacillus cereus* (Sigma) (4 ng per 50 μl PBS per ml base cream) was also prepared. Where indicated, bacterial extracts were preincubated for 60 min with 5 mM glutathione (GSH, Calbiochem) before addition to the cream. The GSH-containing creams were prepared immediately before use.

SMase assay in S. thermophilus Ten milligrams of lyophilized *S. thermophilus* were resuspended in 500 μl HEPES buffer 20 mM pH 7.4 containing 10 mM $MgCl_2$, 2 mM ethylenediamine tetraacetic acid, 5 mM dithiothreitol, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 30 mM p-nitrophenylphosphate, 10 mM β-glycerophosphate, 750 μM adenosine triphosphate (ATP), 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin (Sigma), and 0.2% Triton X-100 (for the assay of nSMase) or 500 μl of 0.2% Triton X-100 [for the assay of acidic SMase (aSMase)] and sonicated as described above. Protein concentration was determined through the Pierce Micro BCA assay kit, with bovine serum albumin standards. For the assay of nSMase, different amounts of bacteria were incubated for 2 h at 37°C in HEPES buffer 20 mM pH 7.4 containing 1 mM $MgCl_2$, and [*N*-methyl-^{14}C]sphingomyelin (SM) (0.28 μCi per ml, specific activity 47 mCi per mmol; Amersham, Bucks., U.K.). The reaction was initiated by the addition of 40 μl of labeled SM, previously dried, resuspended in the assay buffer containing 3% Triton X-100, and solubilized by short bursts of sonication and vortexed. For the assay of aSMase, different amounts of sonicated bacteria were incubated for 2 h at 37°C in a buffer (200 μl final volume) containing 250 mM sodium acetate, 1 mM ethylenediamine tetraacetic acid, pH 5.0, and 40 μl of [*N*-methyl-^{14}C]SM. Where indicated, the bacterial extracts were preincubated for 60 min with 5 mM GSH to inhibit nSMase activity, as previously described (Liu and Hannun, 1997; Liu *et al*, 1998). The reaction was stopped by the addition of 250 μl chloroform/methanol (2/1, by vol). The phospholipids were extracted by the addition of 800 μl chloroform/methanol (2/1, by vol), and 250 μl of H_2O. After centrifugation at 10 978 × ***g*** for 15 min at 4°C, the aqueous phase was extracted again twice more with 500 μl chloroform. The organic phase, obtained in the different extraction steps, were collected and washed once with 1 ml chloroform/methanol/water (3/48/47, by vol), to remove totally free radioactive phosphorylcholine. The aqueous phases were collected, transferred to scintillation vials, and routinely counted by liquid scintillation counting. The counts per min represented the choline phosphate generated from SM hydrolysis. Correspondingly, the organic phase was analyzed on thin-layer chromatography (TLC) plates by using chloroform/methanol/ammonia hydroxide [7 M]/water (85/15/0.5/0.5, by vol). The hydrolysis of SM was quantitated by autoradiography and liquid scintillation.

Ceramide measurement (diacylglycerol kinase assay) Subconfluent keratinocytes were incubated in the presence or absence of sonicated *S. thermophilus* (50 mg per 5×10^6 cells per 10 ml) at different times (0.25–18 h). After incubation, the cells were washed twice with ice-cold PBS (pH 7.2), resuspended in Tris/HCl buffer 20 mM pH 7.4, and harvested using a cell lifter (Costar, Cambridge, MA). The number of cells collected after the culture was not influenced by the presence of sonicated bacteria. After freeze-drying, the cells were sonicated (1 min and 50 s, alternating 10 s sonication and 10 s pause) and the protein concentration was determined using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL),

with bovine serum albumin standards. No significant differences in protein content were observed among control and treated cells. For the lipid extraction, 400 μl of methanol, 500 μl of chloroform, and 200 μl of water were added. Samples were stirred for 2 min on a vortex-mixer and centrifuged at 10 978 × ***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 ^{32}P-ATPγ, specific activity 3 Ci per mmol, Amersham) according to the manufacturer's instructions and applied to silica gel TLC plates using a TLC applicator (Camag, Berlin, Germany). Ceramide phosphate was then resolved using $CHCl_3/CH_3OH/CH_3COOH$ (65/15/5, vol/vol/vol) as solvent. Authentic ceramides from bovine brain (ceramide type III, nonhydroxy fatty acid ceramides; and ceramide type IV, hydroxy fatty acid ceramides; Sigma) were identified by autoradiography at Rf = 0.25 and Rf = 0.11, respectively. Specific radioactivity of ceramide-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 picomoles of ceramide-1-phosphate per 100 μg protein.

[N-Methyl-^{14}C]choline labeling Subconfluent HaCaT cells were incubated with pulse medium (DMEM containing 0.5 μCi [*N*-methyl-^{14}C] choline per ml; Amersham; 55 mCi per mol) for 48 h at 37°C. The cells were then washed twice and cultured (5×10^6 cells per 10 ml) for 18 h with fresh medium in the presence or absence of sonicated *S. thermophilus* (50 mg per 10 ml, final concentration). After incubation, the cells were washed twice with ice-cold PBS (pH 7.2), resuspended in Tris/HCl buffer 20 mM pH 7.4, and harvested as described above. After lipid extraction, the organic phases, obtained from different extraction steps were collected, washed once with 1 ml of the solvent system containing chloroform/methanol/water (3/48/47, by vol), dried under nitrogen, and finally resuspended in 110 μl chloroform. Then, 10 μl aliquots of the chloroform phase were taken for scintillation counting and 100 μl were applied to silica gel TLC plates. Lipids were separated using chloroform/methanol/acetic acid/water (100/60/20/5, by vol) as the solvent system. Unlabeled lyso-phosphatidylcholine, SM, and phosphatidylcholine (Sigma) were used as standards and visualized in iodine vapor (Rf = 0.1, 0.26, and 0.6, respectively). Radioactive spots were visualized by autoradiography, scraped from the plate and counted by liquid scintillation.

Ceramide synthase assay Assay of ceramide synthase activity was performed as previously described (Bose *et al*, 1995). After treatment with sonicated *S. thermophilus* suspension, keratinocytes, collected (as described above), were resuspended in 300 μl of homogenization buffer (25 mM HEPES pH 7.4, 5 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid, 50 mM NaF, 10 μg leupeptin per ml, and 10 μg soybean trypsin inhibitor per ml), disrupted by sonication, and lysates were centrifuged at 800 × *g* for 5 min. Protein concentrations in the postnuclear supernatants were determined through the Micro BCA protein assay reagent kit (Pierce), with bovine serum albumin as the standard. Seventy-five microgram proteins were incubated in a 1 ml reaction mixture containing 2 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 20 μM defatted bovine serum albumin (Sigma), 20 μM dihydrosphingosine, 70 μM unlabeled palmitoyl-coenzyme A, and 3.6 μM (0.2 μCi) [1-^{14}C]palmitoyl-coenzyme A (55 mCi per mmol; Amersham). Dihydrosphingosine was dried under nitrogen from a stock solution in 100% ethanol and dissolved with sonication in the reaction mixture prior to addition of cell extracts. The reaction was started by addition of palmitoyl-coenzyme A, incubated at 37°C for 1 h, and then stopped by extraction of lipids using 2 ml of chloroform/methanol (1/2, by vol). The lower phase was removed, concentrated under nitrogen, and applied to a silica gel 60 TLC plate. Dihydroceramide was resolved from free radiolabeled fatty acid using a solvent system of chloroform/methanol/3.5 M ammonium hydroxide (85/15/1), identified by autoradiography based on comigration with ceramide standards (stained with iodine vapor), and quantitated by liquid scintillation counting. The amount of palmitoyl-CoA consumed did not exceed 5% of total.

Cholesterol level assay To analyze cholesterol levels, lipids were extracted (as described above) from keratinocytes previously incubated in the absence or presence of *S. thermophilus* extract for 1, 6, or 18 h. TLC were developed with chloroform/methanol/acetic acid/water (50/37.5/3.5/2, by vol) to resolve and quantitate cholesterol. Authentic standards were chromatographed in parallel. After solvent development, the chromatograms were air-dried, sprayed with 10% $CuSO_3$, 8% H_3PO_4 aqueous solution and charred on a 180°C hot plate. The charred lipids were quantitated by photodensitometry (BIORAD GS-670, Imaging densitometer).

***In vivo* studies**

Healthy volunteers, treatment and sample collection The study was conducted as a double-blind paired-comparison study, with treatment regimens randomized and balanced to a group of 17 healthy Caucasian volunteer subjects (people from our laboratories; male and female, ages 24–47 y). All the subjects had normal skin as judged by visual assessment. Subjects first completed a 4 d washout phase, during which they were required to use a mild soap bar for general cleansing and apply no moisturizers, or any other products, to their forearms. These requirements persisted throughout the study. During the 1 wk application phase, 0.5 g of test product was applied twice daily to the volar surface of the forearms. Each subject applied an "active" formulation (base cream as vehicle containing *S. thermophilus*) to one forearm and the vehicle alone to the contralateral forearm. Some volunteers were treated in different forearm areas with a base cream containing purified nSMase from *B. cereus* (Sigma) (4 ng per 50 μl PBS per ml base cream). Samples of stratum corneum were collected with a noninvasive procedure for topical lipid extraction from the volar aspect of the forearms, as previously described (Lavrijsen *et al*, 1994), before (T0) or after (T1) the topical application. Briefly, the topical extraction protocol comprised two consecutive extractions with 10 ml solvent solution (acetone/diethylether, 1/1, by vol), the first extraction for 5 min, the second for 25 min. The solution was applied using a glass tube of cross-sectional area 7 cm^2, precleaned in chloroform/methanol and having a simple flat edge without any lining material. The tube was held in position manually on the extraction site with a force sufficient to prevent lateral leakage. To remove contamination with squames the extracts were centrifuged (800 × *g*). Only the second fraction was used for further analysis; the first extract was used to remove sebaceous and exogenous lipids from the stratum corneum. The supernatants were concentrated in a rotary evaporator (Savant Instruments Inc., Holbrook, NY), evaporated to dryness in glass tubes under a stream of nitrogen and the residues were dissolved in 1 ml chloroform/methanol (9/1, by vol) and stored at –20°C until use.

Analysis of stratum corneum ceramides The lipid extracts obtained (as described above) were evaporated under nitrogen and reconstituted in chloroform (200 μl). An aliquot of extracted lipids was incubated with *E. coli* diacylglycerol kinase (DAG kinase assay kit, Amersham) and ^{32}P-ATPγ (specific activity 3 Ci per mmol; Amersham) to determine the levels of ceramide (as described above). To normalize the lipid extracts and to allow interperson comparisons in the *in vivo* studies, the mass of each lipid fraction was normalized to the amount of stratum corneum proteins. Detergent-soluble proteins of the pelleted corneocytes were extracted with protein solubilization buffer (20 mM β-mercaptoethanol in 10 mM sodium phosphate buffer, pH 7.2, with 1% sodium dodecyl sulfate) for 2 h at 60°C with vortexing at 1 and 2 h and then centrifuged at 573 × ***g*** for 5 min to pellet undissolved material. Stratum corneum proteins were assayed using the Pierce Micro BCA assay kit (Pierce). Quantitative results for ceramide production are expressed as picomoles of ceramide-1-phosphate per microgram of stratum corneum proteins.

Statistical analysis For *in vitro* data analysis, the Student's t test was performed by the STATPAC Computerized Program, and $p < 0.05$ was used as the significance criterion. For *in vivo* data analysis, the differences between the results obtained with the control cream treatment and those obtained with the experimental cream treatment were tested by using the t test for paired data.

RESULTS

***In vitro* studies**

nSMase in S. thermophilus To assess the presence of SMase in *S. thermophilus*, the activities of both acidic and nSMase were assayed by using different amounts of sonicated bacteria in appropriate buffers containing [^{14}C]SM (as described in *Materials and Methods*). The results reported in **Fig 1** show the presence of high levels of nSMase in sonicated *S. thermophilus*. The percentage of SM hydrolysis gradually decreased by reducing the amounts of bacteria, which was as expected. On the other hand, no presence of aSMase was detected in bacterial samples (not shown). Considering that nSMase activity could be inhibited by GSH (Liu and Hannun, 1997; Liu *et al*, 1998), we analyzed the effect of GSH on bacterial nSMase *in vitro*. The preincubation for 60 min of the bacterial extracts with GSH (5 mM) strongly reduced (80–90% inhibition) the SM hydrolysis. Similar results were obtained with purified nSMase from *B. cereus*. The SMase activity was also analyzed through the

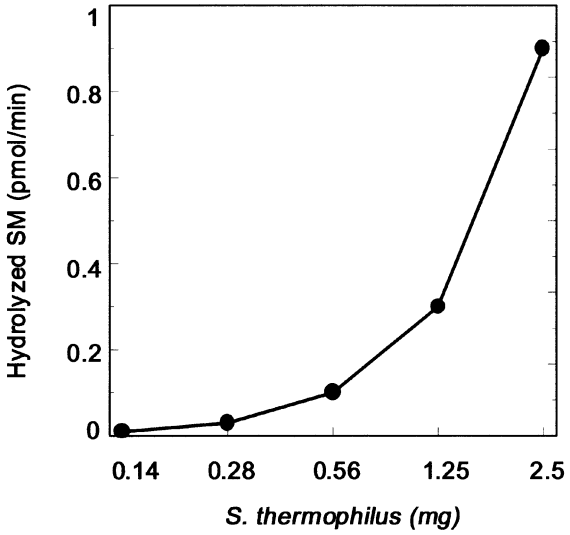

Figure 1. nSMase activity in *S. thermophilus*. Extracts from *S. thermophilus* (at indicated amounts) were reacted at pH 7.4 with labeled SM vesicles in the presence of protease and phosphatase inhibitors, β-glycerophosphate, ATP, and Mg. Hydrolyzed SM was quantitated and expressed as pmol per min. The results are expressed as mean values from three experiments in duplicate. SEM values were ever lower than 5% of mean values.

determination of ceramide levels with the DAG kinase assay. Decreasing amounts of bacteria (2.5, 1.25, and 0.625 mg) were incubated in the presence or absence of a constant amount of unlabeled SM (200 pmol) for 2 h with appropriate buffers to assay nSMase or aSMase. The results reported in **Fig 2**, indicating the ceramide generation from pure SM incubated with *S. thermophilus* confirmed the presence of high levels of nSMase in our bacterial samples. Relevant amounts of both nonhydroxyceramides and hydroxyceramides were generated accordingly with the fatty acid species present in the natural SM molecule. Indeed, even if the predominant fatty acids present in SM are C16:0/C24:0 fatty acids, hydroxylated forms of fatty acids are frequently found (reviewed in Merrill and Jones, 1990; Riboni *et al*, 1997). On the other hand, no ceramide generation was observed in samples that lacked added SM (**Fig 2*A***), in accordance with the lack of SM in prokaryotic plasma membranes (Yeagle, 1993). Also with this experimental approach there was no evidence of aSMase in bacterial sonicates (not shown).

Effects of S. thermophilus *on keratinocyte ceramide levels* To investigate the effect of *S. thermophilus* on ceramide levels of keratinocytes *in vitro*, we treated the cells at different times (0.25–18 h) with a preparation of bacteria, previously sonicated as described in *Materials and Methods*. The treatment of the subconfluent keratinocyte cultures with *S. thermophilus* resulted in a significant increase of ceramide generation, as detected by TLC analysis of phospholipids extracted from cells and subjected to DAG kinase assay; **Fig 3** shows the total ceramide levels expressed as mean values of pmol ceramides per 100 μg protein from three experiments in duplicate. Ceramide generation strongly and gradually increased in the presence of sonicated *S. thermophilus* in a time-dependent manner, being already evident at 15 min and reaching highest levels at 18 h (a 3–5-fold increase with respect to the control value at 18 h). Accordingly, with the previously reported ability of keratinocytes to generate ceramide constitutively (Schurer *et al*, 1989; Holleran *et al*, 1990; Madison *et al*, 1990), the basal level of ceramides present in the untreated cells also increased during the culture, even if to a lesser extent (12–14-fold increase at 18 h with respect to the basal value) with respect to the treated keratinocytes (50–60-fold increase at 18 h, with respect to the basal value). The increase of ceramide levels in keratinocytes (5×10^6 cells per 10 ml) treated with sonicated bacteria interested both hydroxyceramides and

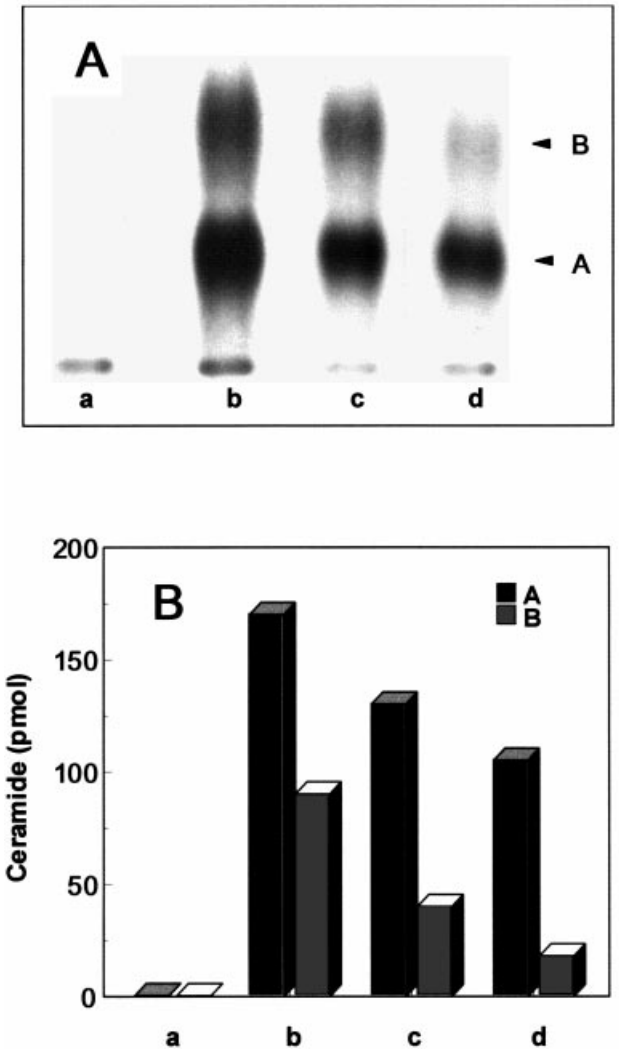

Figure 2. SM-derived ceramide generation through nSMase from *S. thermophilus*. Extracts from *S. thermophilus* at different amounts (a, 2.5 mg; b, 2.5 mg; c, 1.25 mg; d, 0.625 mg) were incubated in Tris/HCl buffer at pH 7.4 with protease and phosphatase inhibitors, β-glycerophosphate, ATP, and Mg, in the absence (a) or presence (b–d) of exogenous unlabeled SM vesicles. Lipids were then extracted and subjected to DAG-kinase assay for ceramide determination. Radioactive spots were visualized by autoradiography, scraped from the plate and counted by scintillation. (*A*) The results from one representative experiment are shown (autoradiography of TLC plate (A, hydroxyceramides; B, nonhydroxyceramides). (*B*) Ceramide generation derived from SM hydrolysis was quantitated and expressed as mean values from three experiments in duplicate. SEM values were ever lower than 10% of the mean value.

nonhydroxyceramides, as shown in the representative experiment reported in **Fig 4(*A*)**. The incubation of sonicated *S. thermophilus* for 18 h led to a significant increase of both hydroxyceramide and nonhydroxyceramide levels**.** Similar results were obtained in two other experiments and the quantitative data (mean values from three experiments in duplicate) are shown in **Fig 4(*B*)**.

Effects of S. thermophilus *on keratinocyte SM levels* To characterize the enzymes responsible for the above reported ceramide generation induced by *S. thermophilus*, we first analyzed the SM hydrolysis in cells after treatment with sonicated bacteria for 18 h. Subconfluent keratinocytes, previously labeled with [*N*-methyl-^{14}C]choline for 48 h, were treated with sonicated *S. thermophilus* for 18 h, as described in *Materials and Methods*. Cellular lipids were then extracted and analyzed by TLC. **Figure 5** shows that the treatment with *S. thermophilus* resulted in an almost total hydrolysis of radiolabeled SM, whereas the other labeled compounds (i.e.,

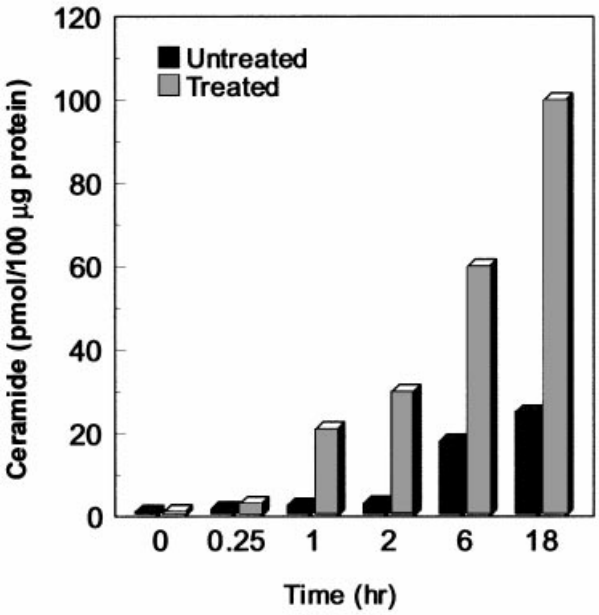

Figure 3. Kinetics of ceramide generation in keratinocytes after treatment *in vitro* with *S. thermophilus*. Cells (5×10^6 per 10 ml) were treated for the indicated times with a sonicated *S. thermophilus* suspension (50 mg per 10 ml, final concentration). Lipids were then extracted and subjected to DAG kinase assay for ceramide determination. Radioactive spots were visualized by autoradiography, scraped from the plate and counted by scintillation counting. Mean values from three experiments in duplicate are shown. SEM values were ever lower than 10% of the mean value.

phosphatidylcholine and lyso-phosphatidylcholine) did not significantly change. Comparable levels of SM hydrolysis were observed by treating the cells for 2 h with exogenous authentic *B. cereus* nSMase, 100 mU per ml (not shown). Thus, ceramide generation observed after treatment with *S. thermophilus* could be attributed, at least partially, to the hydrolysis of keratinocyte SM.

Effects of S. thermophilus *on keratinocyte ceramide synthase activity* The possibility that the *S. thermophilus*-induced ceramide level increase in keratinocytes could also be due to the activation of ceramide synthase, was assessed. This enzyme, also known as sphinganine *N*-acyl transferase (Merrill and Jones, 1990), catalyzes the condensation of sphinganine and fatty acyl-coenzyme A to form dihydroceramide, which is rapidly oxidized to form ceramide. As previously reported (Schurer *et al*, 1989; Holleran *et al*, 1990; Madison *et al*, 1990), this pathway appeared to be constitutively activated in control keratinocytes and could account for the relatively high and gradually increasing basal ceramide levels in our experimental conditions and during the analyzed time interval (0–18 h) (**Fig 6**). As shown, the incubation of keratinocytes in the presence of sonicated *S. thermophilus* did not significantly influence ceramide synthase activity, thus indicating that the *S. thermophilus*-induced ceramide increase described above could not be attributable to a stimulation of this pathway.

Effect of S. thermophilus *on keratinocyte cholesterol levels* The possibility that *S. thermophilus* extracts could also influence cholesterol levels in cultured keratinocytes was assessed. Cholesterol levels from cells previously incubated in the absence or presence of *S. thermophilus* extracts for 1, 6, or 18 h were analyzed and no significant difference between untreated or treated samples was observed.

***In vivo* studies**

Effects of S. thermophilus *on stratum corneum ceramide levels* In order to determine whether the effects of *S. thermophilus* observed *in vitro* on cultured keratinocytes could also be obtained *in vivo*, we analyzed the skin ceramide levels obtained from lipid extracts from the forearms of 17 volunteers, after a 7 d treatment either with the

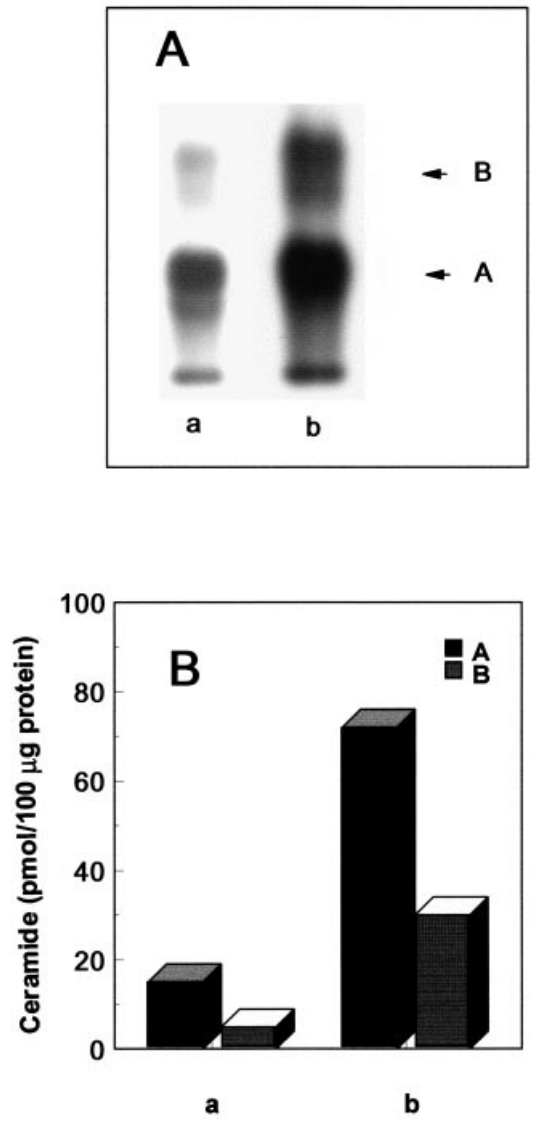

Figure 4. Induction of both hydroxyceramide and non-hydroxyceramide generation in keratinocytes after treatment with *S. thermophilus*. Cells (5×10^6 per 10 ml) were treated for 18 h with a sonicated *S. thermophilus* suspension (50 mg per 10 ml, final concentration). Lipids were then extracted and subjected to DAG kinase assay for ceramide determination. Radioactive spots were visualized by autoradiography, scraped from the plate and counted by scintillation counting. (*A*) The results from one representative experiment are shown (autoradiography of TLC plate (A, hydroxyceramides; B, nonhydroxyceramides). *Lane a*: untreated keratinocytes; *lane b*: keratinocytes treated with *S. thermophilus* for 18 h. (*B*) Mean values from three experiments in duplicate are shown. SEM values were always lower than 10% of the mean value.

base cream or the same cream plus sonicated *S. thermophilus*. Total stratum corneum ceramide levels, which showed a strong basal subjective variability (range: 0.25–198 pmol total ceramides per μg protein; mean ± SEM: 36 ± 14 pmol total ceramides per μg protein), were significantly increased in almost all subjects following treatment with the base cream alone (mean ± SEM: 136 ± 25 pmol ceramides per μg protein; T1 *versus* T0, $p < 0.001$) (**Fig 7*A***). This effect was significantly less relevant when compared with the experimental cream treatment (mean ± SEM: 639 ± 136.21 pmol ceramides per μg protein; T1 *versus* T0, $p < 0.001$) (**Fig 7*B***). Moreover, to show directly that the changes we described in the *in vivo* experiments were caused by the presence of nSMase in *S. thermophilus* extracts, the effects of the topical application of the same base cream containing authentic nSMase from *B. cereus* instead of *S. thermophilus* extracts, were analyzed in four volunteers (2, 7,

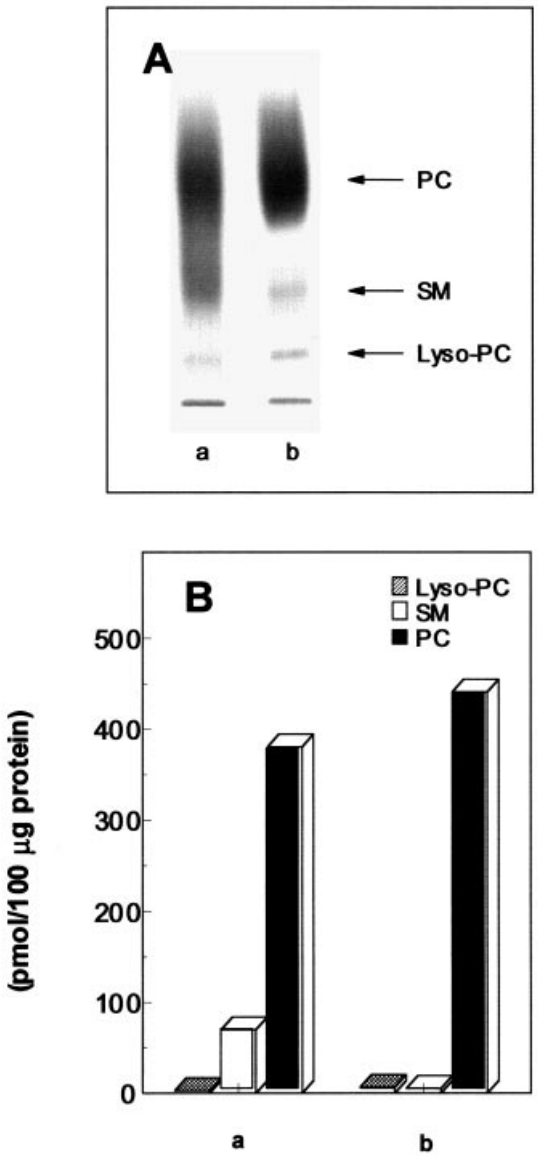

Figure 5. Sphingomyelin hydrolysis induction in keratinocytes after treatment with *S. thermophilus*. Cells (5×10^6 per 10 ml) were labeled for 48 h with [*N*-methyl-C]choline, then treated for 18 h with a sonicated *S. thermophilus* suspension (50 mg per 10 ml, final concentration). Lipids were then extracted and subjected to TLC to resolve lyso-phosphatidylcholine, SM, and phosphatidylcholine. Radioactive spots were visualized by autoradiography, scraped from the plate and counted by scintillation counting. (*A*) Autoradiography of a TLC plate from a representative experiment. *Lane a*, untreated cells; *lane b*, keratinocytes treated with *S. thermophilus* for 18 h. (*B*) Mean values from three experiments in duplicate are shown. SEM values were always lower than 15% of the mean value.

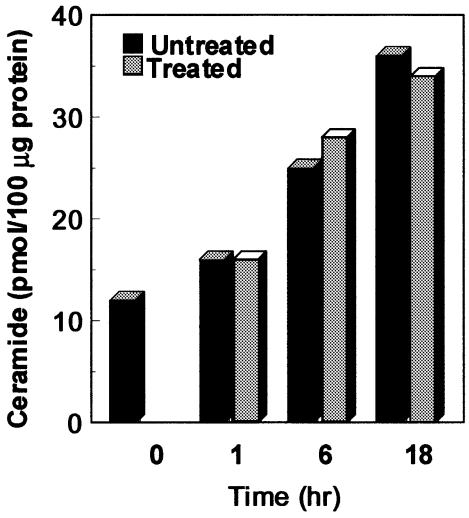

Figure 6. Kinetics of ceramide synthase activity in keratinocytes after treatment with *S. thermophilus*. Extracts from keratinocytes (5×10^6 per 10 ml), treated for the indicated times with a sonicated *S. thermophilus* suspension (50 mg per 10 ml, final concentration) were subjected to ceramide synthase activity assay for 1 h, as described in *Materials and Methods*. Lipids were then extracted using chloroform/methanol (1/2). Ceramides were resolved using TLC, detected by comigration with ceramide standards. Radioactive spots were visualized by autoradiography, scraped from the plate and counted by scintillation counting. Ceramide generation was quantitated and expressed as mean values from two experiments in duplicate. SEM values were always lower than 10% of the mean value.

12, and 17). The results, reported in **Fig 7**(***C***), indicate also that this treatment was able to determine in all subjects a very significant increase of skin ceramide levels, which was comparable with that observed after topical application of *S. thermophilus*-containing cream. **Figure 8** shows some representative autoradiographies of skin ceramide levels obtained before (T0) and after (T1) the base (*lane a*) or experimental cream treatment (*lane b*).

As regards the possibility that the vehicle base cream or the experimental cream contain SM or ceramides, we analyzed the levels of these lipids and no evidence of their presence was obtained (not shown). Therefore, the possibility of such contaminating lipids in the crude homogenates which could substantially alter the SM/ceramide content of cells of the stratum corneum when added exogenously, could be excluded. Interestingly, the inhibition of bacterial nSMase with GSH, almost totally prevented (70%–90% inhibition) the ceramide increase that was induced by topical application of either the *S. thermophilus* extract-containing cream (**Fig 9*A***) or *B. cereus* nSMase-containing cream (**Fig 9*B***). In **Fig 9**(***C***) two representative autoradiographies of skin ceramide levels are reported from one subject (12) before (T0) and after (T1) treatment for 7 d with the experimental creams and with or without GSH.

DISCUSSION

This study was conducted to determine the effect of sonicated *S. thermophilus* on the levels of ceramides either *in vitro* in a human keratinocyte cell line (HaCaT) or *in vivo* on the forearm skin of 17 healthy volunteers.

Our results show a very relevant ceramide increase in keratinocytes *in vitro* after culture with *S. thermophilus*. Both hydroxyceramide and nonhydroxyceramide levels strongly and gradually increased in the presence of sonicated *S. thermophilus* in a time-dependent manner, being evident as early as 15 min and reaching highest levels at 18 h. The increase of ceramide levels could be due to SM hydrolysis observed in keratinocytes previously labeled with an SM precursor. The SM hydrolysis could be correlated with the high levels of a neutral GSH-sensitive SMase detected in *S. thermophilus*. No detectable aSMase activity could be observed in these bacteria. Our results exclude the possibility that the increase of ceramide levels induced in keratinocytes after the treatment with sonicated *S. thermophilus*, was due to a synthesis *de novo* of ceramide through the ceramide synthase activity, as this pathway, basically activated in these cells (Schurer *et al*, 1989; Madison *et al*, 1990; Holleran *et al*, 1990), was not influenced by the presence of sonicated bacteria.

The possibility that *S. thermophilus* extracts could also influence cholesterol levels in cultured keratinocytes was investigated. The obtained results indicate that bacterial extracts present in the cultures for 1, 6, or 18 h did not influence cholesterol levels in these cells.

The *in vitro* results were confirmed by *in vivo* studies in 17 healthy and young volunteers. A relevant increase in stratum corneum ceramide levels was indeed observed in all analyzed

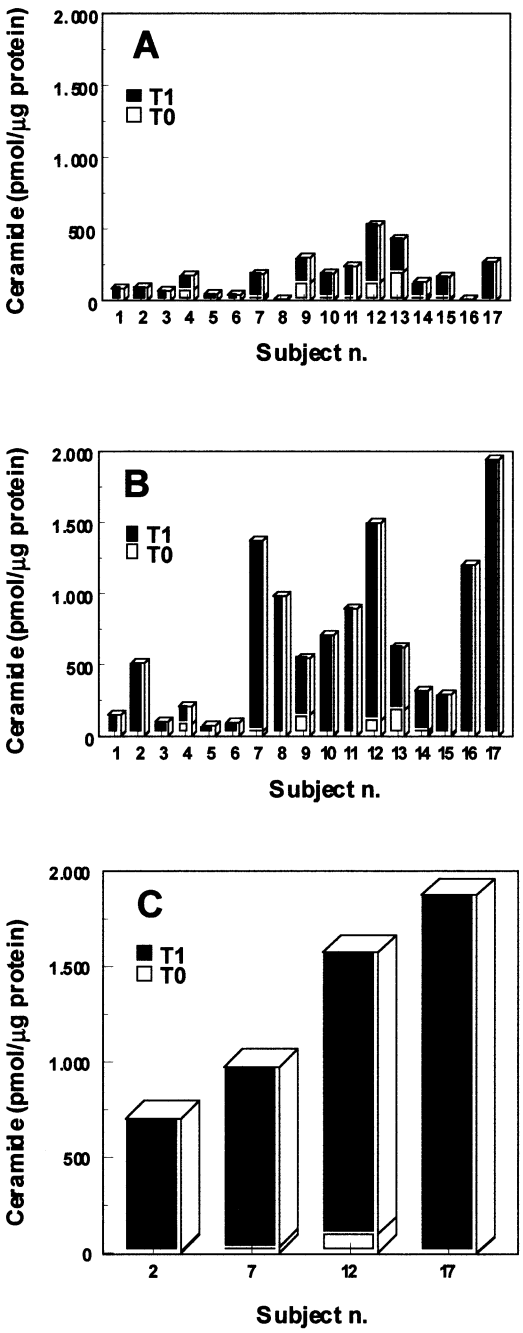

Figure 7. Effects of *S. thermophilus* on stratum corneum ceramide levels. Skin lipid extracts from the forearms of 17 healthy volunteers before (T0) and after (T1) treatment for 7 d with the base cream (*A*) or with the experimental cream containing sonicated *S. thermophilus* (*B*) were analyzed for ceramide level determination through DAG kinase assay. (*C*) The effects of the topical application of a purified *B. cereus* nSMase-containing cream on four volunteers.

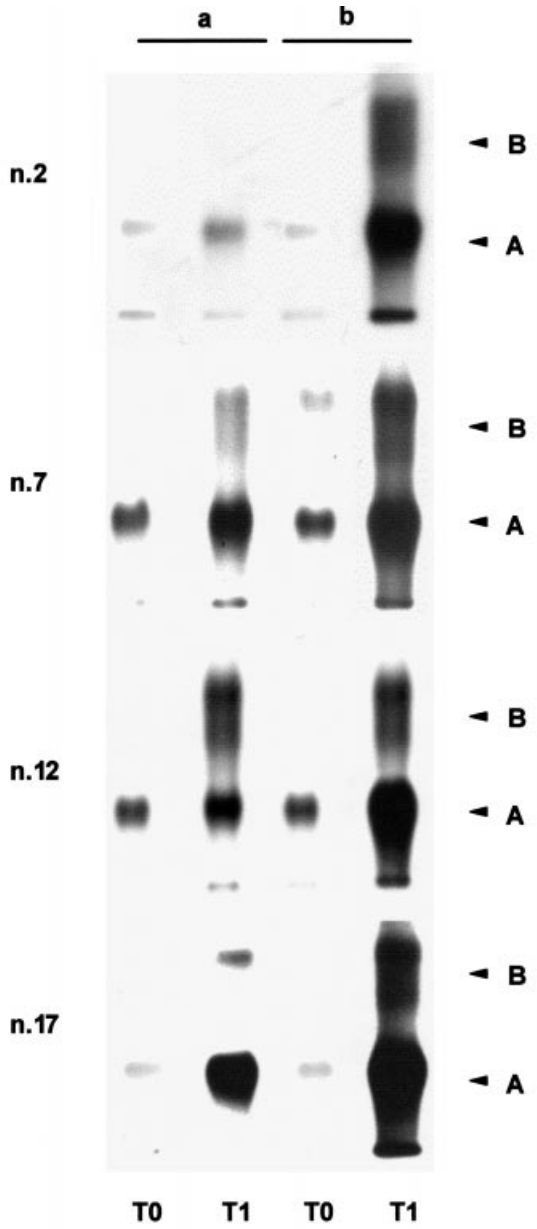

Figure 8. Four representative autoradiographies of TLC. Autoradiographies of TLC of hydroxyceramides (A) and nonhydroxyceramides (B) extracted from forearm stratum corneum of four individuals (relative numbers are indicated) before (T0) and after (T1) treatment for 7 d with the base cream (a) or with the experimental cream containing sonicated *S. thermophilus* (b).

subjects after the 7 d topical application of a vehicle base cream containing lysed *S. termophilus*. Also in these *in vivo* experiments, the topical treatment with *S. thermophilus* induced a very significant increase of ceramide levels. An increase of ceramide content, even if very less relevant, was also observed after treatment with the vehicle base cream. This result could be explained with the presence in the latter of different oils rich in fatty acids which could in turn be utilized for the ceramide biosynthetic pathway (Bose *et al*, 1995). Nevertheless, we cannot exclude other possible mechanisms. We can hypothesize that damage to the barrier caused by lipid solvents used in this work to extract stratum corneum lipids could have led to an increase of epidermal lipidogenesis and fatty acid synthesis proportional to the degree of barrier disruption; also when the hyperpermeability was corrected with the base cream, the lipidogenesis returned to normal values, in accordance with previous investigations which stressed the relationship between barrier function and lipid synthesis (Grubauer *et al* 1985; Menon *et al*, 1985).

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 (Rawlings *et al*, 1994). Ceramides generated after treatment with *S. thermophilus* extracts are both hydroxy-fatty acid ceramides and nonhydroxy 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 (Motta *et al*, 1994; Rawlings *et al*, 1994; Murata *et al*, 1996). The results obtained with keratinocytes *in vitro* strongly suggest that

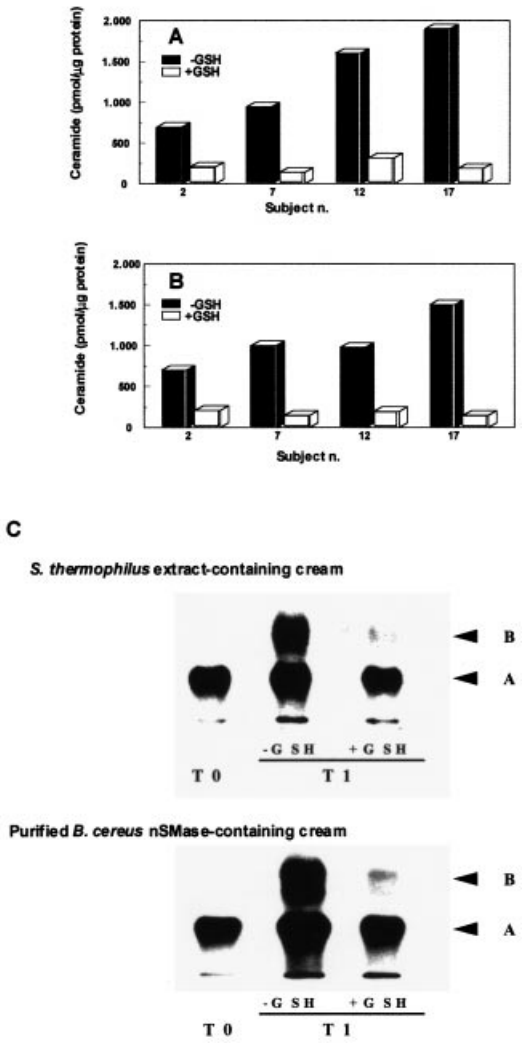

Figure 9. Effect of GSH on the ceramide level increase induced *in vivo* by the treatment with a purified *B. cereus* nSMase-containing or *S. thermophilus* extract-containing creams. Skin lipid extracts from the forearms of four healthy volunteers before (T0) and after (T1) treatment for 7 d with the experimental creams containing sonicated *S. thermophilus* (*A*) or purified *B. cereus* nSMase (*B*) were analyzed for ceramide level determination through DAG kinase. assay. (*C*) Two representative autoradiographies of TLC of (A, hydroxyceramides; B, nonhydroxyceramides) extracted from forearm stratum corneum of one subject (12) before (T0) and after (T1) treatment for 7 d with experimental creams with or without GSH.

S. thermophilus nSMase could be also responsible for the observed ceramide increase *in vivo*. The presence of high levels of nSMase in this micro-organism, indicated by assaying the enzymatic activities *in vitro*, appears to be responsible for the observed increase in stratum corneum ceramide levels. Indeed, a comparable skin ceramide level increase was also observed after the topical application of a cream containing purified *B. cereus* nSMase. In addition, the finding that the inhibition of bacterial nSMase activity through GSH pretreatment, blocked this effect further supports the idea that nSMase is causing the skin ceramide changes observed *in vivo*. It is unlikely that the observed effect was due to contaminating factors potentially present in the bacterial preparations. Bacterial nSMase has been used for many experimental studies on apoptosis in several cell systems (Bettaieb *et al*, 1996; Wright *et al*, 1996; Flamigni *et al*, 1997; Zhang *et al*, 1997), which showed that the enzymatic preparations were able to induce a specific hydrolysis of SM with consequent ceramide generation. Moreover, it seems difficult to hypothesize that the same "putative contaminating factor" could be present either in the *S. thermophilus* or in the *B. cereus* preparations. These bacteria are strongly different: *S. thermophilus* is a non pathogenic gram-positive lactic acid bacterium and *B. cereus* is a pathogenic β-hemolytic gram-positive, aerobe spore-forming rod, able to release several toxins which contribute to its virulence. Finally, it is a remote possibility that a putative contaminant factor present in both bacterial preparations could also be glutathione-sensitive.

The route by which the enzyme is penetrating into viable epidermis and gains access to keratinocytes and intracellular organelles is not clear. The lipids for epidermal permeability barrier function are stored in the epidermal lamellar bodies (Freinkel and Traczyk, 1985; Grayson *et al*, 1985; Elias *et al*, 1988; Holleran *et al*, 1993; Madison and Howard, 1996). Among several other lipids, SM may be also present in the external surface of the lamellar body membranes. These organelles represent the delivery mechanism for most lipid catabolic enzymes and their lipid substrates to the stratum corneum interstices. Moreover, SM is also present in the intercellular domains at the level of the stratum corneum, even if in a small quantity. Exogenous bacterial nSMase could hydrolyze all these different pools of SM thus generating ceramides. The sonication procedure of the bacterial extracts leading to micelle formation together with the presence of absorption enhancers (i.e., polyethylene glycol), oils, and protease inhibitors in the vehicle base cream, capable of reducing the barrier resistance (Verhoef *et al*, 1990; Murakami *et al*, 1998), could favor enzyme access and skin penetration. On the other hand, the transfollicular pathway could constitute a preferential route of penetration for nSMase-containing micelles, as previously reported for the uptake of molecules such as γ-interferon from human skin upon the application of liposomal formulation (Du Plessis *et al*, 1992).

Altogether, our findings have potential clinical significance. First, the increase in ceramide content may be extremely beneficial to the commonly xerotic skin of the aged. Second, the improvement in the integrity of the barrier implies a reduced susceptibility to the insults of the environment (e.g., decreased humidity, solvents, and detergents), as well as an increased capacity to recover from environmental insults. Third, the induction of an increased ceramide level through *S. termophilus*, could represent a useful, simple and inexpensive pharmacologic approach in those pathologic conditions associated with a general reduction in skin lipid content, particularly ceramide. With regard to this, the possible restoration of ceramide content in the skin of patients suffering from desquamatory diseases (i.e., psoriasis), after topical treatment with creams containing sonicated *S. thermophilus*, is already under investigation.

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