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A natural lipid mixture improves barrier function and hydration in human and murine skin

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Synopsis

Previous studies have demonstrated that three key lipids, cholesterol, free fatty acids, and ceramides, are required for maintenance of the epidermal permeability barrier function in murine and human epidermis. Moreover, it has been shown that all three lipids are required together for barrier function, because only topical applications of complete, equimolar three-component lipid mixtures allow normal barrier recovery in disrupted skin. In contrast, single- or two-component lipid mixtures delay normal barrier recovery. Furthermore, increasing the ratio of any single lipid species, i.e., cholesterol, ceramides, or free fatty acid, to the other two lipids in the three-lipid component mixture actually accelerates barrier recovery in murine and human epidermis. Here we assess whether a natural lipid mixture, containing these three key lipids in addition to a large amount of phospholipids, influences barrier recovery and skin hydration in both murine and human skin. Our results show that this natural lipid mixture enhances barrier recovery significantly in acetone-treated mouse skin. Moreover, this natural lipid mixture also accelerates barrier recovery in acetone-treated and tape-stripped human skin. Finally, this natural lipid mixture increases stratum corneum hydration in both acutely disrupted and normal human skin. These studies show that this naturally occurring lipid mixture can both accelerate permeability barrier recovery and enhance stratum corneum moisturization.

INTRODUCTION

Because of their acknowledged importance for barrier homeostasis, increasing attention has focused on stratum corneum lipids as potential topical therapeutic agents. Prior studies indicated that disruption of barrier function by either tape stripping or organic solvent treatment increases epidermal synthesis of the three major stratum corneum lipids, cholesterol, ceramides, and free fatty acids (1–3). Moreover, each of these three key lipids is required for barrier function, as demonstrated by the ability of pharmaco-

logical inhibitors of each synthetic pathway to alter barrier homeostasis (4–6), ascribable to specific lipid biochemical and membrane structural abnormalities in the stratum corneum (4–7).

Whereas these findings showed the separate requirement for each of the three key lipids for barrier homeostasis, previous studies also have demonstrated that maintenance of normal barrier function requires all three species of stratum corneum lipids together. Topical application of any one or two of the three key lipids delays or worsens barrier recovery following acetone-induced barrier disruption (8). In contrast, topical application of a lipid mixture, containing free fatty acid, cholesterol, and ceramide in an approximately equimolar ratio, allows normal barrier recovery (8). Moreover, optimal ratios of these three lipids accelerate barrier recovery following either acetone treatment or tape stripping, and some types of surfactant treatment of mouse skin, regardless of the extent of barrier disruption (9,10,13). Because the large quantities of the ceramides needed to formulate such optimized mixtures may not be commercially available or affordable, we determined whether a naturally occurring, lipid-enriched mixture of animal origin, containing the three key lipids primarily as complex precursors, could enhance stratum corneum function. Our findings show that a natural lipid mixture, with an approximate lipid ratio of 1:1:3 (cholesterol:ceramides:fatty acids), accelerates barrier recovery following acute barrier disruption of murine and human skin. Moreover, this lipid mixture also enhances stratum corneum moisturization in both normal and damaged murine and human skin.

METHODS AND MATERIALS

MATERIALS

Six- to eight-week-old male hairless mice were purchased from Simonsen Laboratories (Gilroy, CA) and fed Purina mouse diet and water *ad libitum*. Acetone and propylene glycol were purchased from Fisher Scientific (Fairlawn, NJ). Cholesterol, ceramides, and palmitate were purchased from Sigma Chemical Company (St. Louis, MO). The natural lipid mixture, Y2, derived from animal porcine tissue, was purchased from Ocean Pharmaceutical (Weihai, P. R. China).

LIPID BIOCHEMISTRY

The lipid composition of Y2 was quantitated by high-performance thin-layer chromatography (HPTLC) followed by charring and scanning densitometery, as previously published (11). Briefly, 2–5 μg of the lipid extract was applied to the TLC plates for neutral lipid analysis, while 20 or 100 μg were utilized for polar lipid analysis. 0.2 to 1.0 μg of a polar lipid standard and 0.12 to 1.0 μg of a neutral lipid standard were applied to each side of the plates to generate standard curves, as well as to identify the major species. Neutral lipids were fractionated by developing the plates in petroleum ether:diethyl ether:acetic acid (80:20:1, vol), as described previously. Polar lipids were developed to 35 and 55 mm in chloroform:ethyl acetate:ethylmethylketone:2-propanol:ethanol:methanol:glacial acetic acid:hexyl acetate (34:4:4:6:20:28:4:1, vol), to 70 mm in chloroform:ethyl acetate:2-propanol:ethanol:methanol:H_2O (46:4:4:6:28:6, vol), and then to the top of the plates in chloroform:methanol:acetone (80:10:10, vol). Sphin-

golipids were developed to 20 mm in chloroform:methanol:acetone:acetic acid (76:20:4:1, vol), to 50 mm in chloroform:methanol:acetone (80:10:10, vol), and then to the top of the plates in chloroform:ethyl acetate:diethyl ether:methanol (76:20:6:2, vol). The plates then were scanned by scanning densitometry, and the lipid fractions were quantitated using CATS II software, as described previously (11).

PROTOCOL FOR ANIMAL STUDIES

Barrier function was assessed by measurement of transepidermal water loss (TEWL) with an electrolytic water analyzer (Meeco, Warrington, PA) (12). Barrier function was disrupted by repeated treatment with absolute acetone until TEWL rates exceeded 2.0 mg/cm^2/hr. Immediately after treatment, TEWL was measured, and either 1.6% Y2 in propylene glycol:ethanol (7:3, v/v) or vehicle alone was applied to the treated areas (5–7 cm^2). TEWL was measured at the time points indicated. Data are expressed as percentage of recovery from time 0. For stratum corneum hydration studies, either 1.6% Y2 or 1–2% of synthetic lipids were applied to acetone-treated skin. Cholesterol, ceramides, and palmitate were applied individually or as a mixture at the final concentration of 1.1%, and the vehicle (propylene glycol:ethanol, 7:3, v/v) alone served as the control. Stratum corneum hydration, measured as capacitance, was assessed with a corneometer (CM-820, Courage + Khazaka, Germany) both before and two hours after treatment. Data are expressed as percentage of change from levels immediately prior to the applications of lipids or vehicle.

PROTOCOL FOR HUMAN STUDIES

In one group of twenty human volunteers (eight females, 12 males, ages 22 to 64 years), both forearms were treated with either acetone or tape stripping (Scotch type) until TEWL exceeded 1.0 g/m^2/hr. Eighty microliters of vehicle was applied to the treated area of one arm, while the same volume of Y2 was applied at a concentration of 1.6% to the treated area (6–8 cm^2) of the other arm. TEWL was measured with an evaporimeter prior to and at indicated time points after vehicle or lipid applications. Stratum corneum hydration was assessed with a corneometer at the time indicated. In another group of 40 normal human volunteers (20 females, 20 males, ages 17 to 49 years) with no prior history of skin diseases, one shin was treated with the vehicle, while the opposite shin was treated with 1.6% Y2. Hydration studies in all subjects were performed two hours after application under comparable environmental condition and at the same time of the year (autumn). Statistical significances were determined using the Student's two-tailed T test.

RESULTS

LIPID COMPOSITION OF Y2 LIPID MIXTURE

Previous studies have demonstrated that the ability of lipid mixtures to influence the barrier recovery rates is dependent on their composition. Therefore, we first analyzed the lipid composition of Y2. As shown in Table I, the total lipid content is 90.5% by total weight. The major lipids are glycosphingolipids (32%) and phospholipids (20.04%).

Table I
Lipid Composition in Natural Lipid Mixture

	(% of Total weight)
Sphingolipids	
Glycosphingolipid I	15.88
Glycosphingolipid II	14.32
Ceramides III	1.82
Ceramides IV	0.70
Total sphingolipids	32.72
Free sterol	18.49
Free fatty acids	11.51
Triglycerides	7.74
Polar lipids	
Sphingomyelin	2.68
Phosphatidylcholine	3.71
Phosphatidylserine	7.21
Phosphatidyl inositol	0.29
Phosphatidylamine	6.15
Total polar lipids	20.04
Total	90.50

The final molar ratio of these lipids is approximately 3:1:1 (fatty acids:ceramides:cholesterol) if glycosphingolipids (including sphingomyelin), triglycerides, and other phospholipids perform as precursors of ceramides and fatty acids, as demonstrated previously (13).

EFFECT OF Y2 ON BARRIER FUNCTION

Studies in hairless mice. Previous studies have shown that a physiological lipid mixture, with an approximately equimolar ratio of stratum corneum lipids or their precursors, allows normal barrier recovery (8) and that a threefold increase in cholesterol in the lipid mixture accelerates barrier recovery in acetone-treated murine skin (9,10,13). To determine whether the naturally occurring lipid mixture also is effective, we first tested its ability to alter barrier recovery rate in acetone-treated murine skin. Barrier recovery in the Y2-treated animals is significantly faster than in vehicle-treated animals two and four hours after barrier disruption (Figure 1; two-hour data not shown). These results demonstrate that this naturally occurring lipid mixture accelerates barrier recovery in acetone-treated murine skin.

Studies in human volunteers. Although the effect of exogenous lipids on barrier function have been well demonstrated in mice (8–10), little is known about the ability of synthetic or naturally occurring lipid preparations to influence barrier homeostasis in humans. Prior studies have shown that barrier recovery is prolonged in barrier-disrupted human vs mouse skin (14). Therefore, we next examined whether Y2 also accelerates barrier recovery in acetone and tape-stripped human skin. As observed in hairless mice, Y2 significantly accelerates barrier recovery in both tape-stripped and acetone-treated

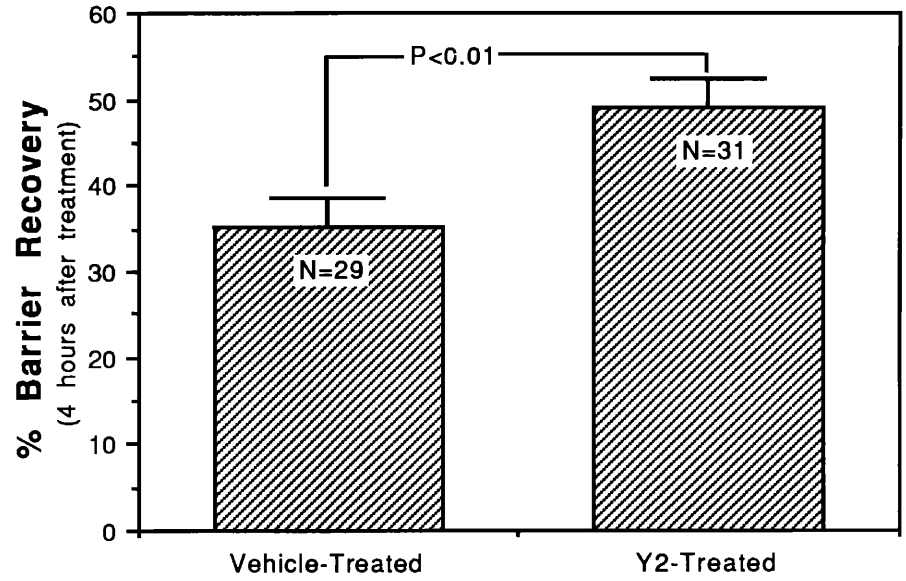

Figure 1. Effect of a natural lipid mixture on barrier recovery in acetone-treated murine skin: 30 μl of Y2 (1.6%) or vehicle was topically applied to acetone-treated mouse skin (2 × 3 cm^2). The barrier function was determined by measuring transepidermal water loss immediately after acetone treatment and four hours after Y2 or vehicle application. Results are mean ± SEM.

human skin two and four hours after barrier disruption (Figure 2; four-hour data not shown). These results suggest, first, that an exogenous mixture of physiological lipids can influence barrier recovery in barrier-disrupted human skin, and second, that com-

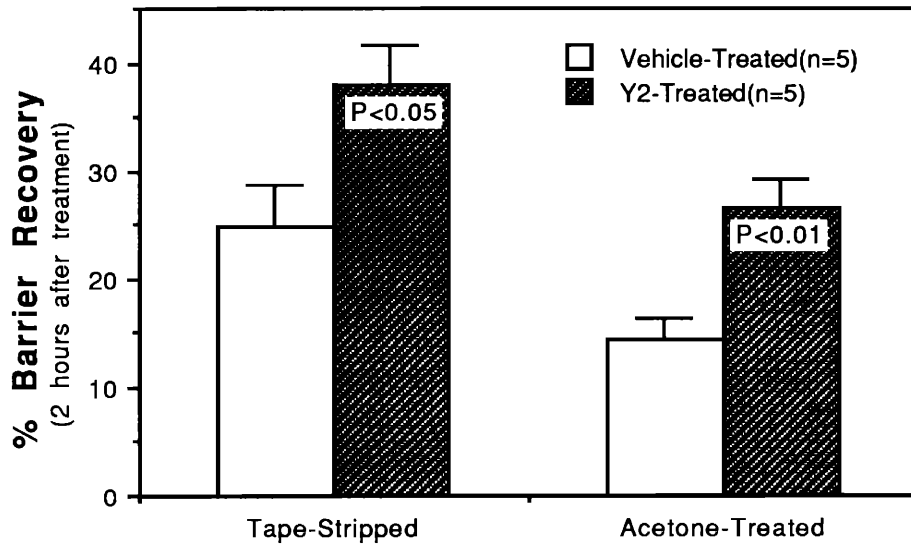

Figure 2. Effect of a natural lipid mixture on barrier recovery in perturbed human skin: 40 μl of Y2 (1.6%) or vehicle was topically applied to acetone-treated or tape-stripped human skin (about 20 cm^2 area). Results are mean ± SEM. Significant differences are for Y2- vs vehicle-treated site.

plex, naturally occurring lipids, with an optimized lipid ratio, can accelerate barrier recovery in human skin, as shown above for murine skin.

EFFECT OF LIPIDS ON SKIN HYDRATION

Studies in hairless mice. Prior studies have demonstrated both that ceramides influence the water-holding capacity of stratum corneum (15–18) and that glucosylceramides stimulate epidermal proliferation (19). There is little information about the effect of other physiological lipids, applied topically, on skin hydration. We next examined the effect of the major individual stratum corneum lipids, as well as Y2, on skin hydration in acetone-treated mouse skin. As shown in Figure 3, both fatty acid (palmitate) and ceramide increase skin capacitance two hours after application. Cholesterol worsens capacitance in comparison to the vehicle. A lipid mixture consisting of cholesterol, ceramide, and palmitate (molar ratio at 1:1:3) also increases skin capacitance. However, the most dramatic increase in skin capacitance occurs following topical application of Y2 (1.6%). These results suggest that certain stratum corneum lipids, i.e., fatty acids and ceramides, improve skin hydration, and that a topical lipid mixture (Y2), enriched in all three stratum corneum lipids, produces the greatest increase in skin hydration.

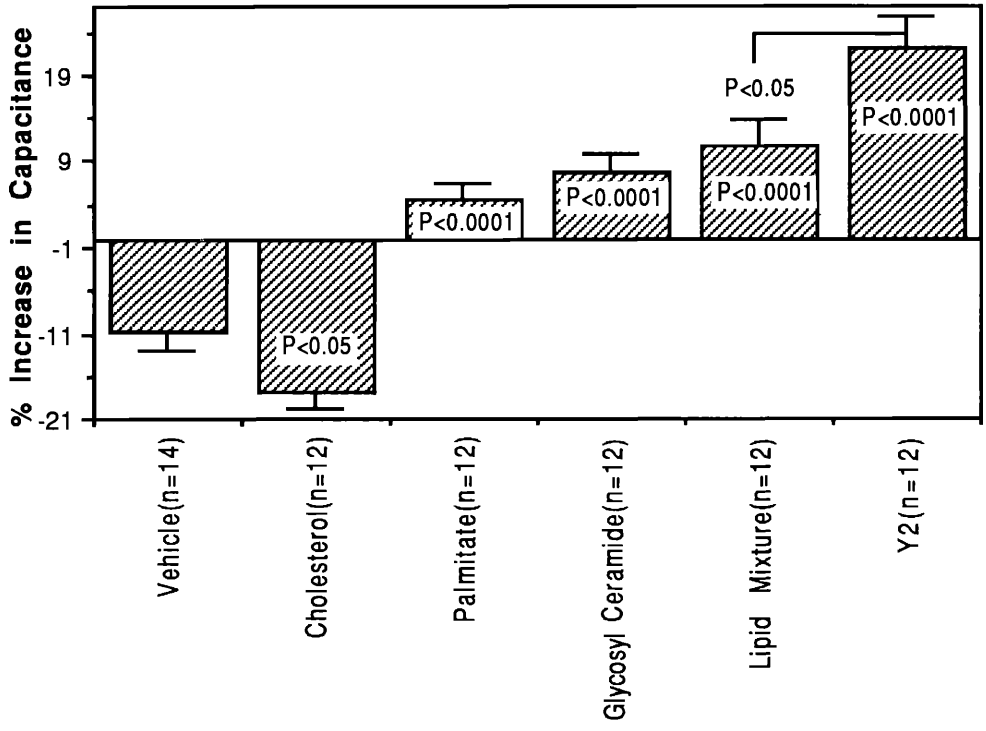

Figure 3. Effect of physiological lipids and a natural lipid mixture on skin capacitance in acetone-treated mouse skin: 40 μl of Y2 (1.6%), cholesterol (2%), galactocerebroside II (1%), palmitate (1%), or vehicle was topically applied to acetone-treated mouse skin (about 2 × 3 cm^2 area). The lipid mixture (1.1%) contains cholesterol, galactocerebroside II, and palmitate (1:1:3; molar ratio). Skin capacitance was measured before and two hours after lipid or vehicle application. The data are expressed as percentage increase after lipid or vehicle application. Results are mean ± SEM. Significant differences are in comparison with vehicle alone.

Study in human volunteers. In order to explore the potential utility of physiologic lipids for improving skin hydration, we next tested whether Y2 influences stratum corneum hydration in acetone-treated vs normal human skin. As shown in Figure 4, Y2 markedly increases stratum corneum water content two hours after acetone treatment in comparison to vehicle treatment alone. Moreover, Y2 also significantly raises stratum corneum water content two hours after treatment of normal skin in comparison to vehicle treatment (Figure 5). These results demonstrate that exogenous, naturally occurring stratum corneum lipids display a moisturizating effect on both damaged and normal human skin.

DISCUSSION

The importance of stratum corneum lipids for barrier homeostasis is firmly established (reviewed in 1–3). Moreover, each of the three key stratum corneum lipids, i.e., cholesterol, fatty acid, and ceramides, is required for barrier homeostasis (4–6). However, recent studies have shown that these lipids are required as a mixture, rather than as individual species (8). Only mixtures of these lipids, in approximately equimolar ratios, allow normal barrier recovery accompanied by formation of normal membrane bilayer structures in stratum corneum (8), and mixtures with optimized ratios of the three stratum corneum lipids further accelerate rates of barrier recovery (9,10,13). In contrast, incomplete mixtures result in formation of abnormal membrane bilayer structures in stratum corneum and actually impede barrier recovery (8). In agreement with these prior results, the natural lipid mixture studied here, Y2, which contains a molar ratio that approximates that in optimized mixtures of physiological lipids (9,10,13),

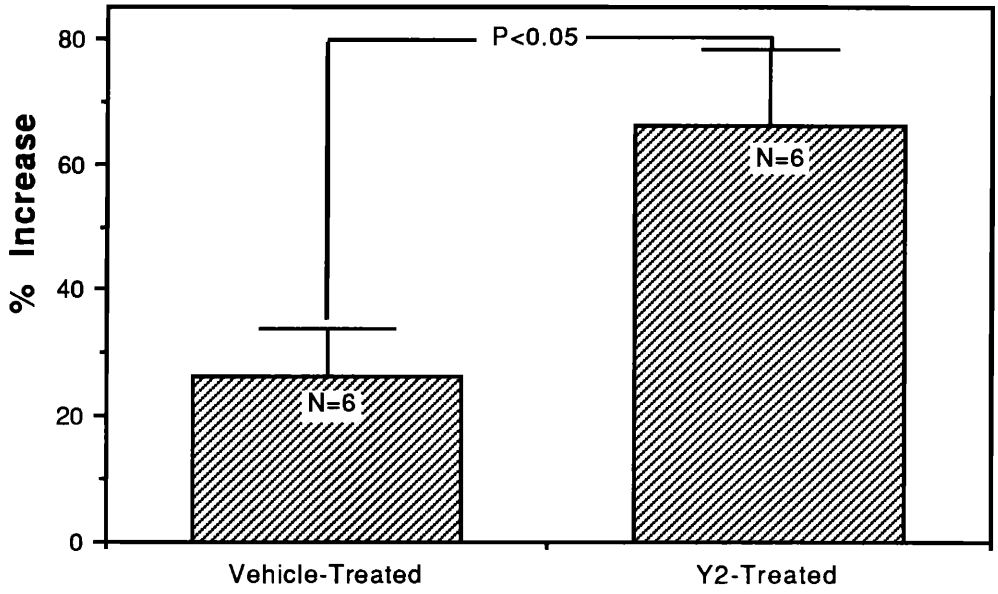

Figure 4. Effect of natural lipid mixture on skin capacitance in acetone-treated human skin: 40 μl of Y2 (1.6%) or vehicle was topically applied to acetone-treated human skin (about 20 cm^2 area). Skin capacitance was measured before and two hours after Y2 or vehicle application. The data are expressed as percentage increase after Y2 or vehicle application. Results are mean ± SEM. Significant difference is in comparison with vehicle alone.

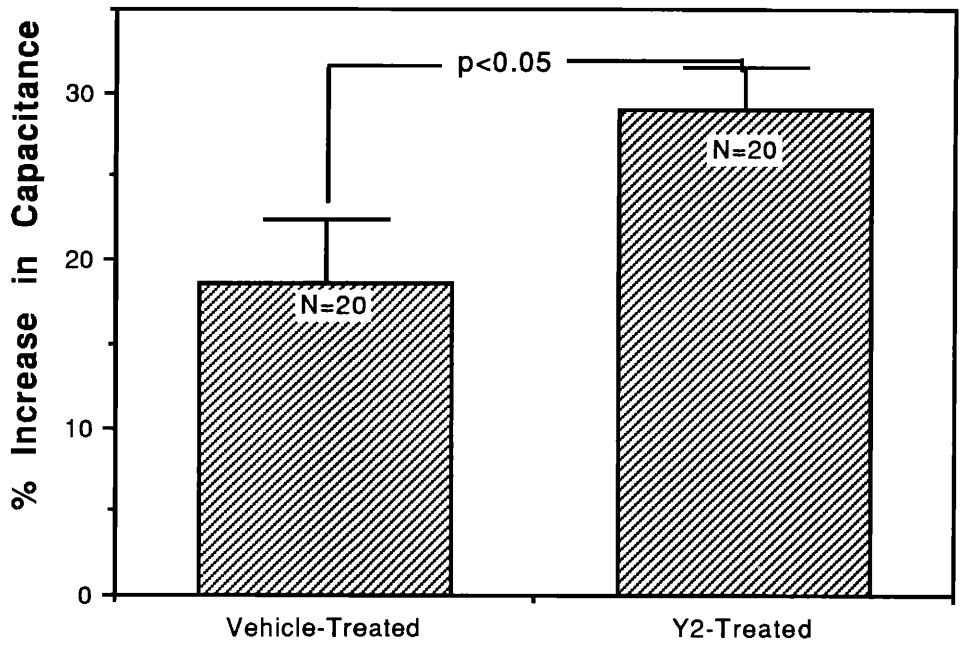

Figure 5. Effect of natural lipid mixture on skin capacitance in normal human skin (n = 20): 40 μl of Y2 (1.6%) or vehicle was topically applied to normal human skin (about 20 cm^2 area). Skin capacitance was measured before and two hours after Y2 or vehicle application. The data are expressed as percentage increase after Y2 or vehicle application. Results are mean ± SEM.

also accelerates barrier recovery after barrier disruption in both human and mouse skin. In contrast to physiologic lipids, nonphysiologic lipids, such as petrolatum, form a nonmembrane domain in stratum corneum, thereby improving barrier function (9). Furthermore, we showed that topical nonphysiologic lipids, such as petrolatum, induce earlier barrier recovery in comparison with physiologic lipids. Thus, it is more likely that the enhancement of barrier recovery by Y2 is due to acceleration of the formation of membrane bilayer structures in the stratum corneum rather than to the formation of a nonbilayer, hydrophobic domain as induced by petrolatum (9). However, more work needs to be performed to confirm this hypothesis. Moreover, further studies are needed to determine whether natural lipids complement or duplicate the properties of other inert species often used in cosmetics, e.g., glycerin, lanolin, and dimethicone. Nevertheless, that the results of our human studies parallel our results in hairless mice further validates the application of the murine assay for assessing the effects of exogenous lipid mixtures on stratum corneum barrier homeostasis.

The Y2 mixture primarily contains complex lipid precursors of the three key lipids, rather than the final products. It has been documented that there are large amounts of hydrolase activity in the upper epidermis (20–23). Moreover, we showed previously that triglyceride could substitute for free fatty acids, and glycosylceramides and sphingomyelin for ceramides in the acetone model (8,13). Thus, we hypothesize that the basis for the efficacy of these complex lipids can be attributed to abundant enzyme activity in the upper epidermis, which would insure that these complex lipids will be catabolized rapidly to their nonpolar products.

Finally, these studies also demonstrate the efficacy of an exogenous lipid mixture on skin moisturization, as assessed by capacitance. An optimal ratio of stratum corneum lipids increases water content in both damaged and normal human skin. Y2 contains 50% of polar lipids, which might retain more water and be superior to physiologic lipids or their mixtures in increasing skin capacitance. The absolute differences in skin hydration between human volunteers and mice are likely due to species differences. Ceramides and fatty acids alone, even in mixtures that disturb barrier recovery (8), will increase skin capacitance in damaged skin (15–18,24). Thus, stratum corneum hydration may be influenced by individual lipids, in contrast to the equimolar or optimized ratios required for barrier homeostasis. Further studies are needed to clarify the relationship of lipids for barrier recovery vs moisturization in this model. In any case, our results suggest that stratum corneum lipids might be useful not only for skin barrier repair, but also as a skin moisturizer. This extract, and presumably other comparable, naturally occurring mixtures, may be a cheaper, alternative source of physiological lipids for cosmetic products.

ACKNOWLEDGMENT

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