

# Optimization of Physiological Lipid Mixtures for Barrier Repair

Man Mao-Qiang,\* Kenneth R. Feingold,\* Carl R. Thornfeldt,† and Peter M. Elias\*

\*Dermatology and Medical Services, Veterans Administration Medical Center; Departments of Dermatology and Medicine, University of California School of Medicine, San Francisco, California 94121, U.S.A.; and †Cellegy Pharmaceutical Corporation, Novato, California 94949, U.S.A.

**Three stratum corneum lipids, ceramides, cholesterol (CHOL), and free fatty acids (FA), are required for permeability barrier homeostasis. Recent studies have shown that application of one or two of these lipids to perturbed skin delays barrier recovery; only equimolar mixtures allow normal recovery. We asked here whether any physiological lipid mixtures improve barrier repair, as assessed by transepidermal water loss. Whereas an equimolar ratio of ceramides, CHOL, and FA (either the essential fatty acid, linoleic acid, or the nonessential FAs, palmitic or stearic acids) allows normal repair, further acceleration of barrier repair occurs as the ratio of any of these ingredients is increased up to 3-fold. Similar preliminary results were obtained in damaged human skin. Likewise, while acylceramides alone delay barrier recovery, acylceramides:CHOL mixtures within a**

**specific range of molar ratios dramatically improve barrier repair. Furthermore, glycosyl ceramides, sphingomyelin, and triglycerides substitute effectively for ceramides and FA, respectively, but neither phospholipids nor cholesterol esters substitute for FA and CHOL, respectively. These studies show the specific requirements of selected stratum corneum lipid mixtures for optimized barrier repair in murine skin, with further validation in human skin. Utilization of physiologic lipids according to these parameters could lead to new forms of topical therapy for dermatoses (e.g., psoriasis, atopic dermatitis, and irritant dermatitis) triggered by abnormal barrier function. Key words: stratum corneum/barrier function/transepidermal water loss/epidermal lipids/epidermal ultrastructure. *J Invest Dermatol* 106:1096-1101, 1996**

**T**he epidermal permeability barrier is mediated by a series of intercellular bilayers in the stratum corneum (SC), which are enriched in cholesterol, ceramides, and free fatty acids (reviewed in Schurer and Elias, 1991). These lipids are delivered to the intercellular spaces as a mixture of precursors by the secretion of epidermal lamellar body (LB) contents. Following their secretion, the lipid precursors are metabolized within the extracellular spaces by colocalized, LB-derived hydrolytic enzymes into the hydrophobic, lamellar basic unit structures, which mediate barrier function (reviewed in Elias and Menon, 1991). Recent metabolic studies have demonstrated a separate requirement for cholesterol, ceramides, and fatty acids for barrier homeostasis (reviewed in Feingold, 1991; Elias and Feingold, 1992). Yet, recent studies have shown that applications of any one or two of these lipid classes to damaged skin impedes rather than facilitates the rates of barrier repair, quantitated by changes in transepidermal water loss (TEWL) (Mao-Qiang *et al*, 1993a). In contrast, when members of all three key lipid classes are supplied together, normal rates of barrier repair

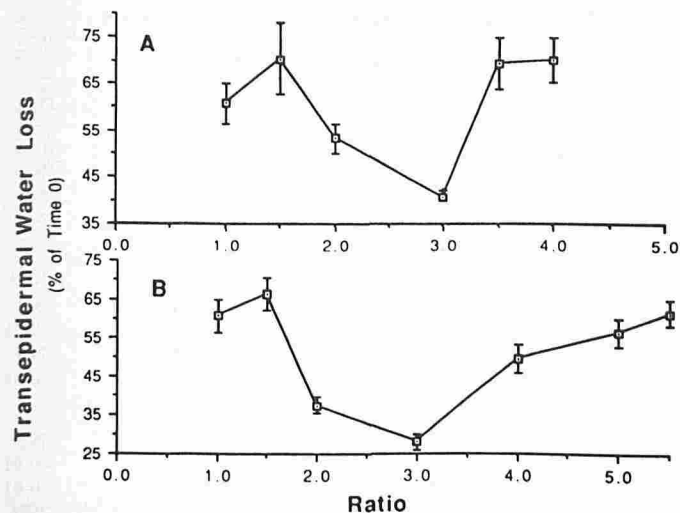
occur. Furthermore, following acute barrier disruption, the exogenous physiologic lipids, whether in complete or incomplete mixtures, quickly traverse the SC, and are taken up by the nucleated layers of the epidermis (Mao-Qiang *et al*, 1993a; Mao-Qiang *et al*, 1995a), and traffic to sites of LB distal to the Golgi apparatus (Mao-Qiang *et al*, 1995a). Then, depending on the lipid composition and proportions, normal or abnormal LB are formed, leading to the formation of either normal or abnormal, lamellar unit structures in the SC interstices (Mao-Qiang *et al*, 1993a; Mao-Qiang *et al*, 1995a). The entire process of lipid transport, uptake, mixing with endogenous lipids in nascent LB, organelle secretion, and reorganization into lamellar unit structures occurs within 2 h in murine epidermis (Menon *et al*, 1992; Mao-Qiang *et al*, 1993a; Mao-Qiang *et al*, 1995a). Moreover, while nonphysiological lipid mixtures (e.g., petrolatum) also repair the barrier, they instead form a bulk hydrophobic phase in the SC interstices (Ghadially *et al*, 1992; Mao-Qiang *et al*, 1995a). Furthermore, intracellular processing of these lipids, in contrast to the physiological lipids is not a prerequisite for barrier repair, because petrolatum normalizes TEWL even when: a) LB secretion is blocked by inhibitors of organellogenesis (Mao-Qiang *et al*, 1995a); and b) LB formation and secretion are blocked by lowering skin temperature (Halkier-Sorensen *et al*, 1995).

In the studies described here, we describe the optimization of complete lipid mixtures for barrier repair, as defined by changes in TEWL, in murine as well as preliminary studies in human skin. Whereas the three component system again allowed normal rates of barrier recovery (Mao-Qiang *et al*, 1993a), we describe here

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Reprint requests to: Dr. Peter M. Elias, Dermatology Service (190), Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121.

Abbreviations: SC, stratum corneum; CER, ceramides; CHOL, cholesterol; FA, fatty acids; LA, linoleic acid; TEWL, transepidermal water loss; LB, lamellar bodies; RuO<sub>4</sub>, ruthenium tetroxide.



**Figure 1. Altering lipid mole ratios changes rates of barrier recovery.** A, ceramides; B, cholesterol. Data shown are rates of barrier recovery 2 h after lipid application. Similar results are seen at 4 h (not shown). In the case of both ceramides and cholesterol, the concentration of each lipid is increased progressively up to a 4- or 5:1:1:1 molar ratio (see text for remaining components). This approach is similar to that shown in Table I, where the concentration of either linoleate or palmitate is increased in the four-component mixture. Data shown are mean  $\pm$  SEM for  $n = 9-11$  animals at each data point. The differences at 2:1:1:1 vs 3:1:1:1 vs 1:1:1:1 are significant at  $p < 0.001$  for both ceramides and cholesterol (by t test).

mixtures of physiological lipids that accelerate normalization of TEWL in both murine and human skin.

#### METHODS AND MATERIALS

**Materials** Male hairless mice (Hr/Hr), 8-12 weeks old, 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). Linoleic acid (10 mg/ml), palmitic acid (10 mg/ml), stearic acid (10 mg/ml), ceramides III and IV (12.5 mg/ml), and cholesterol (12.5 mg/ml) were from Sigma Chemical Co. (St. Louis, MO). Acylceramides (12.5 mg/ml) were isolated from murine epidermis by preparative thin-layer chromatography, using previously described solvent systems (Holleran *et al.*, 1993).

**Experimental Protocols** The permeability barrier, as measured by TEWL, was disrupted in hairless mice by repeated applications of absolute acetone to one flank for approximately 5 min. (Menon *et al.*, 1985). Immediately after TEWL rates exceeded 2.0 mg/cm<sup>2</sup>/h (normal range < 0.2 mg/cm<sup>2</sup>/h), the lipid mixtures shown in Tables 1-4 and Figure 1, solubilized in propylene glycol:ethanol (7:3 v/v), at a final concentration of 1-1.2 lipid weight%, or the propylene glycol:ethanol vehicle alone, were applied topically to a 5-cm<sup>2</sup> area of acetone-treated skin (60- $\mu$ l total volume). This level of barrier disruption produces comparable lipid extraction in different cohorts of animals (Grubauer *et al.*, 1989), and the amount of applied lipid is approximately one order of magnitude greater than the amount removed during barrier disruption (Mao-Qiang *et al.*, 1993a). TEWL was measured with an electrolytic water analyzer (Meeco, Warrington, PA) at serial time points after applications to the treated area (Menon *et al.*, 1985). Because of experiment-to-experiment variations in prelipid application TEWL levels, data are expressed as percent of maximum water loss; i.e., 100% at the beginning of each experiment immediately after acetone treatment. Levels greater than 100% indicate further deterioration in barrier function.

A preliminary trial in human volunteers utilized two cohorts of normal adults (age 30-40), equally divided among males and females. Six subjects were treated with experimental lipid mixtures, while seven received the vehicle alone (protocol approved by University of California, San Francisco Human Research Committee). The six lipid-treated subjects were divided into two groups who received either the cholesterol:ceramide:palmitic acid:linoleic acid (3:1:1:1, A) or 1:1:1:3 (where "3" is palmitic acid, B) mixture (60  $\mu$ l of 1.3% concentration applied immediately after acetone treatment to  $\approx$ 2.5-cm<sup>2</sup> areas on the volar forearm. Water loss data were

obtained from three to four separate treated sites from each subject, both 2 and 4 h after barrier disruption.

Statistical significances were determined using Student's two-tailed t test, as well as for analysis of variance (RM-ANOVA).

**Ultrastructural Methods** Biopsy samples were minced to  $\leq 1$  mm<sup>3</sup> pieces and fixed overnight (approximately 16 h) at 4°C in 2% glutaraldehyde and 2% paraformaldehyde with 0.06% calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.3, washed in 0.1 M sodium cacodylate buffer, and postfixed in either 0.2% ruthenium tetroxide (RuO<sub>4</sub>) (Polysciences, Warrington, PA) with 0.5% potassium ferrocyanide in 0.1 M sodium cacodylate, pH 7.4, at 22°C in the dark for 0.5 h (Hou *et al.*, 1991), or 1% osmium tetroxide with potassium ferrocyanide (1.5%) in 0.1 M sodium cacodylate at 22°C in the dark for 1 h. After postfixation, tissue samples were dehydrated in ethanol, embedded in a low viscosity, epoxy resin (McNutt and Crain, 1981), and examined using a Zeiss 10A electron microscope operating at 60 kV.

#### RESULTS

##### Both Essential and Nonessential Fatty Acids Accelerate Barrier Recovery

In the first group of studies we determined whether barrier recovery requires essential fatty acids. Equivalent, three-lipid component systems, utilizing either linoleic or palmitic acids, normalize barrier repair in acetone-treated skin (Table I, lines 2 and 3 vs line 1), as described previously (Mao-Qiang *et al.*, 1993a). Moreover, the extent of barrier recovery does not increase further when both linoleic acid and palmitic acid are utilized in the same lipid mixture (line 4 vs lines 2 and 3). Increasing the proportions of linoleic acid from a 1:1:1:1 to a 1:1:1:3 ratio causes a decrease in barrier recovery at 2 h, which is followed by a rapid increase in the extent of barrier recovery at 4 h (lines 4-7). In contrast, increasing the proportion of palmitic acid has little effect at 2 h, but increases the extent of barrier recovery at 4 h, with maximum efficacy again at the 1:1:1:3 ratio (lines 11-14). Finally, to determine whether the nonessential fatty acids are required for energy *versus* structural requirements, we next substituted stearic for palmitic acid in the four-component mixture. Stearic acid also increases the extent of barrier recovery (lines 15-18), with a maximum effect seen with the 1:1:1:3 ratio at both 2 and 4 h (line 17). These studies show first, that the structural requirement for free fatty acids in the complete lipid mixture is not restricted to essential fatty acids; second, that increased proportions of palmitic, linoleic, or stearic acids accelerate barrier recovery in a four-component system, with an optimal proportion of three parts of the fatty acid to one part each of the other components. Finally, the effects of nonessential fatty acids can not be attributed to changes in energy production (palmitic acid enters mitochondria much more readily than stearic acid).

##### Optimized Proportions of Other Physiological Lipids Also Accelerate Barrier Recovery

Whereas the studies above suggest that altering the molar ratio of either essential or nonessential fatty acids can accelerate barrier recovery, we next determined the optimum proportions of the other two key lipids (cholesterol and ceramides) for barrier recovery. Increasing the proportion of ceramide or cholesterol to 1:1:1:2 and 1:1:1:3 causes a further, progressive enhancement in the ability of physiologic lipid mixtures to normalize TEWL at both 2 and 4 h (Fig 1A and B; 4-h data are similar, but not shown). However, a further increase in either lipid to 1:1:1:4 or 1:1:1:5 (only cholesterol data are shown) is followed by a progressive decline in barrier recovery. Finally, because equal concentrations of lipids were applied in each case, these effects can not be ascribed to bulk lipid effects of the mixtures alone.

##### Selected Mixtures of Acylceramide:Cholesterol Accelerate Barrier Recovery

Numerous studies have impugned a special role for acylceramides as a key component of the permeability barrier (e.g., Imokawa *et al.*, 1986). Yet, when applied alone, this compound causes a deterioration in barrier recovery, functioning less effectively than cholesterol alone (Table II, compare line 2 with line 1). In contrast, inclusion of cholesterol with acylceramides causes a dramatic increase in the extent of barrier recovery (lines 4-13), which is optimal at a 1:2 ratio of acylceramide to cholesterol (line 9), but recovery rates increase over an extensive range of

**Table I. Optimal Ratios of Essential and Nonessential Fatty Acids Modulate Barrier Recovery Similarly**

Mixture <sup>a</sup>	Molar Ratio	N	Time			
			2 h	Sign	4 h	Sign <sup>b</sup>
1. Vehicle	—	25	77.1 ± 5.1		67.5 ± 3.8	
2. CH+CER+LA	1:1:1	10	78.9 ± 5.2	NS	66.0 ± 4.6	NS
3. CH+CER+PA	1:1:1	11	71.1 ± 4.6	NS	66.8 ± 4.2	NS
4. CH+CER+LA+PA	1:1:1:1	11	80.3 ± 5.1	NS	60.7 ± 4.2	NS
Effect of increased linoleate						
5. PA:CH:CER:LA	1:1:1:2	12	97.6 ± 6.6	NS	71.7 ± 4.6	NS
6. PA:CH:CER:LA	1:1:1:2.5	11	121.7 ± 6.5	<0.01	52.1 ± 4.8	NS
7. PA:CH:CER:LA	1:1:1:3	12	91.9 ± 2.6	NS	46.2 ± 3.0	<0.01
8. PA:CH:CER:LA	1:1:1:3.5	12	110.5 ± 5.8	NS	78.9 ± 5.4	NS
9. PA:CH:CER:LA	1:1:1:4	12	279.6 ± 35.4	<0.01	107.4 ± 5.8	<0.01
Effect of increased palmitate						
10. LA:CH:CER:PA	1:1:1:1.5	11	80.6 ± 6.3	NS	58.6 ± 3.9	NS
11. LA:CH:CER:PA	1:1:1:2	11	82.1 ± 4.7	NS	46.0 ± 5.6	<0.01
12. LA:CH:CER:PA	1:1:1:3	21	73.1 ± 4.8	NS	32.0 ± 2.3	<0.01
13. LA:CH:CER:PA	1:1:1:3.5	11	82.7 ± 5.9	NS	56.0 ± 4.7	NS
14. LA:CH:CER:PA	1:1:1:4	12	101.0 ± 7.3	NS	69.7 ± 4.9	NS
Effect of increased stearate, substituted for palmitate						
15. LA+CH+CER+ST	1:1:1:1	12	62.7 ± 6.6	NS	51.2 ± 7.2	NS
16. LA+CH+CER+ST	1:1:1:2	12	75.3 ± 5.7	NS	50.2 ± 4.3	<0.05
17. LA+CH+CER+ST	1:1:1:3	16	46.7 ± 4.3	<0.05	34.8 ± 4.0	<0.01
18. LA+CH+CER+ST	1:1:1:4	10	61.8 ± 6.5	NS	51.9 ± 6.0	NS

% recovery ± SEM from initial [100%] abnormality. Numbers greater than 100 indicate deterioration to levels even more abnormal than immediately after acetone treatment.

<sup>a</sup> Abbreviations: LA = linoleic acid; CH = cholesterol; CER = ceramides; PA = palmitic acid; ST = stearic acid; NS = not significant; N = number of animals in each cohort.

<sup>b</sup> Sign = significance vs. vehicle (line 1) by ANOVA.

acylceramide:cholesterol ratios (Fig 2). Moreover, the absolute rates of barrier recovery at both 2 and 4 h actually exceed those attainable with any of the four-component mixtures (which all lack acylceramides ( $p < 0.01$  vs 1:1:1:3 [cholesterol]; c.f., Table I). Additional enrichment with cholesterol to a 1:4.5 ratio, however, produces a significant decline in the extent of barrier recovery (Table II, line 13;  $p < 0.001$  for 1:4.5 vs 1:3; Fig 2). These results demonstrate optimal ratios of acylceramide:cholesterol mixtures for barrier recovery.

#### Addition of Other Physiologic Lipids To Acylceramide: Cholesterol Mixtures Does Not Further Enhance Barrier Recovery

Whereas addition of free fatty acids to ceramide- and

cholesterol-containing, complete lipid mixtures either normalizes or accelerates barrier recovery (c.f., Table I and Fig 1), no additional benefit accrues, and in some instances barrier recovery declines significantly (e.g., Table III, compare line 5 with line 2,  $p < 0.05$ ), when linoleic acid or either of the two nonessential fatty acids, palmitic or stearic acids, are added to the acylceramide:cholesterol mixture (Table III, compare lines 3–8 with line 2). Moreover, addition of ceramides to the acylceramide:cholesterol mixture also decreases the extent of barrier recovery (Table III, line 9). These studies show that addition of other physiological lipids to acylceramide:cholesterol mixtures impedes rather than accelerates barrier recovery in comparison to acylceramide:cholesterol mixtures alone.

**Table II. Acylceramide Plus Cholesterol-Containing Mixtures Accelerate Barrier Recovery**

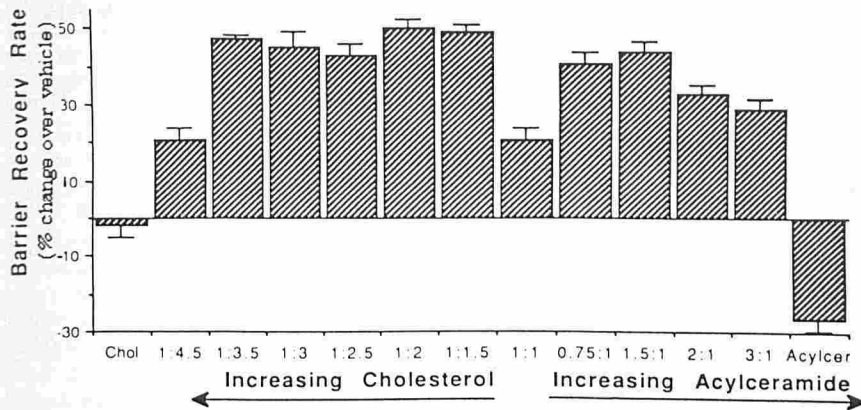
Mixture <sup>a</sup>	Molar Ratio	N	Time			
			2 h	Sign <sup>b</sup>	4 h	Sign <sup>b</sup>
1. Vehicle		25	77.1 ± 5.1		67.5 ± 3.8	
2. AC alone		10	113.3 ± 4.3 <sup>c</sup>	$p < 0.1$	93.8 ± 4.4 <sup>c</sup>	$p < 0.01$
3. CH alone		35	89.5 ± 3.3	NS	69.5 ± 2.8	NS
Increased acylceramides						
4. AC:CH	1:1	11	57.2 ± 4.9	$p < 0.05$	41.1 ± 6.9	$p < 0.01$
5. AC:CH	1.5:1	10			23.6 ± 2.8	$p < 0.01$
6. AC:CH	2:1	11	46.2 ± 4.0	$p < 0.01$	35.0 ± 2.3	$p < 0.01$
7. AC:CH	3:1	11	54.6 ± 3.2	$p < 0.01$	38.8 ± 2.4	$p < 0.01$
Increased cholesterol						
8. AC:CH	1:1.5	11			18.4 ± 1.0	$p < 0.01$
9. AC:CH	1:2	11	25.6 ± 2.2	$p < 0.01$	17.4 ± 2.1	$p < 0.01$
10. AC:CH	1:2.5	11			24.5 ± 3.2	$p < 0.01$
11. AC:CH	1:3	11	44.5 ± 4.3	$p < 0.01$	22.1 ± 4.0	$p < 0.01$
12. AC:CH	1:3.5	10			20.3 ± 1.2	$p < 0.01$
13. AC:CH	1:4.5	10			47.0 ± 3.4	$p < 0.01$

% recovery ± SEM from initial [100%] abnormality.

<sup>a</sup> Abbreviations: AC = acylceramide; CH = cholesterol; N = number of animals in each cohort; NS = not significant.

<sup>b</sup> Sign = significantly better than vehicle by ANOVA.

<sup>c</sup> Sign = significantly worse than vehicle by ANOVA.



**Figure 2.** Changes in acylceramide:cholesterol ratios alter barrier recovery. Horizontal axis represents rate of recovery with vehicle alone at 2 h. Effects of progressively increasing the proportion of either acylceramide or cholesterol are shown (see also Table II). Error bar, SEM.

### Selected Lipid Precursors Can Substitute for the Key Lipids During Barrier Recovery

Prior studies showed that triglycerides and cerebrosides, substituted for free fatty acids and ceramides, respectively, allow normal recovery, but cholesterol esters interfere with barrier recovery (data in Table IV; lines 3 and 5 are from Mao-Qiang *et al.*, 1993a). We next assessed whether other physiological lipid precursors of the free fatty acids and ceramides; i.e., phospholipids and sphingomyelin, respectively, would allow normal recovery. As seen in Table IV, line 4, phospholipids, despite being metabolic precursors of free fatty acids in the SC (Mao-Qiang *et al.*, 1995b; 1996), decreases the extent of barrier recovery significantly when substituted for free fatty acids. Likewise, sphingomyelin, a phospholipid precursor of ceramides present in LB (Elias and Menon, 1991), when substituted for ceramides, slightly decreases barrier recovery at both 2 and 4 h, but the differences do not achieve statistical significance (Table IV, line 6). These results demonstrate that certain, but not all, physiological precursors of the three key lipid classes substitute for their respective lipid product, allowing normal barrier recovery following acetone treatment.

### Optimized Physiologic Lipid Mixtures Also Accelerate Barrier Recovery in Human Skin: Preliminary Studies

To determine whether physiological lipid mixtures also can accelerate barrier recovery in human skin, we performed preliminary studies with two different optimized formulations in six normal volunteers. Both the 3[cholesterol]:1:1:1 and the 1:1:1:3[palmitic acid] formulations significantly increases the extent of barrier recovery both 2 and 4 h after barrier disruption (Fig 3; 4 hr data not shown). These

results suggest that optimized physiological lipid mixtures also accelerate barrier recovery in damaged human skin.

### Different Rates of Barrier Recovery Can Be Ascribed to Changes in Lamellar Body-Derived Membrane Structure

To ascertain the structural basis and cellular mechanisms responsible for accelerated *versus* abnormal rates of barrier recovery, we next examined the LB secretory system, including SC intercellular membranes, in acetone-disrupted sites treated with representative lipid mixtures for 2 h. Prior studies have shown that incomplete lipid mixtures, which delay barrier recovery, generate defective LB and intercellular lamellae (Mao-Qiang *et al.*, 1993a). Conversely, complete equimolar, three- or four-component mixtures yield normal recovery rates and LB products (Mao-Qiang *et al.*, 1993a, 1995a). Two hours after acetone plus vehicle treatment, the intercellular spaces already display substantial replenishment of intercellular lamellae (not shown; see Menon *et al.*, 1992). Likewise, the optimized four-component system (3[cholesterol]:1:1:1) also yields normal numbers of LB and secreted lamellar bilayers, with normal internal structure (not shown). In contrast, the same four-component system, with 5 parts cholesterol, which delays barrier recovery (c.f., Fig 1B), reveals a disproportionately large number of LB with defective internal and secreted contents, as well as abnormal, loosely aggregated intercellular lamellar structures (not shown). A comparison of acylceramide alone (adversely affects barrier recovery; c.f. Table II) *versus* an effective acylceramide:cholesterol (1:2) mixture, reveals that the former produces abnormal, secreted LB contents, while the latter generates normal-

**Table III.** Addition of Other Physiological Lipids To Acylceramide:Cholesterol Mixtures Does Not Further Accelerate Recovery

Mixture <sup>a</sup>	Molar Ratio	N	Time			
			2 h <sup>c</sup>	Sign <sup>b</sup>	4 h <sup>c</sup>	Sign <sup>b</sup>
1. Vehicle		25	77.1 ± 5.1		67.5 ± 3.8	
Plus fatty acids (FA)						
2. AC:CH	1:2	11	25.6 ± 2.2	p < 0.01	17.4 ± 2.1	p < 0.01
3. AC:CH:LA	1:2:1	10	73.3 ± 6.7	NS	26.9 ± 2.1	p < 0.01
4. AC:CH:PA	1:3:1	11	73.3 ± 6.7	NS	26.9 ± 2.1	p < 0.01
5. AC:CH:SA	1:2:1	10	41.8 ± 3.2	p < 0.01	26.8 ± 3.1	p < 0.01
6. AC:CH:SA	1:2:2	10			28.7 ± 2.1	p < 0.01
7. AC:CH:SA	1:2:3	10			17.6 ± 2.0	p < 0.01
8. AC:CH:SA	1:2:4	10			28.1 ± 2.4	p < 0.01
Plus FA and ceramides						
9. AC:CH:PA:CER	1:2:1:1	10	83.3 ± 8.6	NS	48.6 ± 7.0	p < 0.05

<sup>a</sup> % recovery from initial [100%] abnormality.

<sup>b</sup> Abbreviations: AC = acylceramides; CH = cholesterol; PA = palmitic acid; LA = linoleic acid; SA = stearic acid; FA = equal parts PA and LA; CER = ceramides; N = number of animals in each cohort; NS = not significant.

<sup>c</sup> Sign = significance vs. vehicle (line 1) by ANOVA.

<sup>d</sup> mean ± SEM.

**Table IV. Certain Physiological Lipid Precursors Substitute For Lipid Products in Accelerating Barrier Recovery**

Mixture <sup>a</sup>	Molar Ratio	N	Time			
			2 h	Sign <sup>b</sup>	4 h	Sign <sup>b</sup>
1. Vehicle		25	77.1 ± 5.1		67.5 ± 3.8	
Free fatty acids						
2. PA:CH:CER	1:1:1	11	67.8 ± 5.3	NS	45.1 ± 4.0	p < 0.01
3. TG:CH:CER	1:1:1	9	76.8 ± 7.7	NS	71.9 ± 6.6	NS
4. PL:CH:CER	1:1:1	10	101.0 ± 5.1	p < 0.005	82.3 ± 4.9	NS
Ceramides						
5. GSL:CH:PA	1:1:1	11	75.3 ± 5.5	NS	58.1 ± 4.2	NS
6. SPM:CH:PA	1:1:1	9	88.7 ± 12.3	NS	75.6 ± 4.9	NS

% recovery ± SEM from initial [100%] abnormality.

<sup>a</sup> Abbreviations: PA = palmitic acid; CH = cholesterol; CER = ceramide; TG = triglycerides; PL = phospholipids; GSL = glycosphingolipids; SPM = sphingomyelin; N = number of animals in each cohort; NS = not significant.

<sup>b</sup> Sign = significance vs. vehicle (line 1) by ANOVA.

appearing, secreted structures (not shown). These results demonstrate that the ability of the optimized lipids to accelerate barrier recovery can be ascribed to the production of normal LB contents, with their subsequent secretion and formation of normal intercellular lamellar structures.

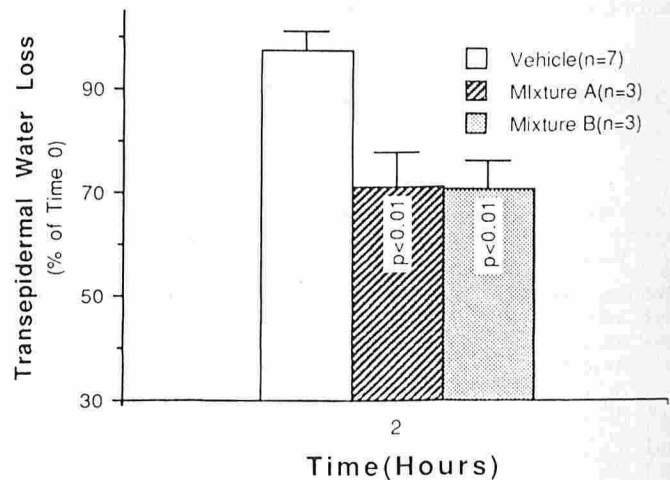
#### DISCUSSION

Whereas prior studies have demonstrated a separate requirement for cholesterol, ceramides, and free fatty acids for permeability barrier homeostasis (reviewed in Feingold, 1991; Elias and Feingold, 1992), when applied individually or as two-component systems to acetone-treated skin, these lipids impede rather than correct barrier recovery (Mao-Qiang *et al.*, 1993a). Normal barrier recovery occurs only when cholesterol, ceramides, and free fatty acids are applied in an equimolar ratio (Mao-Qiang *et al.*, 1993a). These differences are attributable to rapid lipid uptake into the nucleated layers of the epidermis, subsequent incorporation into nascent LB, and formation of intercellular lamellae, a process that requires only 2 h in acetone-treated murine skin (Menon *et al.*, 1992; Mao-Qiang *et al.*, 1993a, 1995a). Moreover, exogenous physiologic lipids also rapidly incorporate into the epidermal nucleated cell layers in intact skin (Mao-Qiang *et al.*, 1995a). In contrast, the inert lipid mixture, petrolatum, repairs the barrier more quickly than the physiological lipids, consistent with the deposition of bulk hydrophobic material within the interstices of the SC (Ghadially *et al.*, 1992; Halkier-Sorensen *et al.*, 1995). Yet, a comparison of these studies with our prior data from petrolatum (Mao-Qiang *et al.*, 1995a) shows that the optimal physiological lipid mixtures perform better than petrolatum at later time points (i.e., 8 h). The delayed impact of the physiological *versus* inert lipids can be explained by the time required for the former to be absorbed, processed within the granular cell, secreted, and remodeled within the SC interstices into competent lamellar bilayer structures (Menon *et al.*, 1992; Mao-Qiang *et al.*, 1993a, 1995a).

The studies described here provide further information about the requirements for optimized barrier recovery with exogenous physiological lipid mixtures. Although the requirement for essential fatty acids (e.g., linoleic acid) or their synthetic analogs (e.g., columbinic acid) for epidermal permeability barrier function is generally accepted (reviewed in Schurer and Elias, 1991), the requirement for nonessential free fatty acids is less well documented. Barrier disruption stimulates epidermal fatty acid synthesis (Grubauer *et al.*, 1987), but such newly synthesized fatty acids could represent lipid destined for more complex lipids; e.g., the *N*-acyl or  $\omega$ -esterified groups of ceramides or the palmitoyl coenzyme A complement of the sphingosine backbone. Moreover, blockade of bulk fatty acid synthesis produces defects in barrier recovery at early time points (Mao-Qiang *et al.*, 1993b), a time at which ceramide biosynthesis remains unaffected (Holleran *et al.*, 1992). Furthermore, biophysical (Friberg *et al.*, 1990) and recent ultrastructural

studies (Mao-Qiang *et al.*, 1996) suggest that free fatty acids can form lamellar membrane structures *in vitro*. Yet, despite the ability of free fatty acids alone to form lamellar structures which display barrier competence *in vitro*, they are ineffective when applied alone to acetone-treated murine skin (Mao-Qiang *et al.*, 1993a). The studies described here clearly demonstrate that nonessential fatty acids facilitate optimal barrier repair, and support our prior studies which showed that nonessential fatty acids, such as palmitic and stearic acids, are at least as effective as linoleic acid in promoting barrier recovery (Mao-Qiang *et al.*, 1993a). Finally, the fact that stearic acid (C18:0) is as effective as palmitic acid (C16:0), strongly suggests that the nonessential fatty acids are required for their structural properties rather than solely as an energy source (palmitic acid enters mitochondria more readily than the larger stearic acid).

The putative requirement for acylceramides for barrier function also is based upon indirect rather than direct evidence. Biochemical studies have shown that these species are enriched in omega-esterified linoleate (Wertz and Downing, 1983; Bowser *et al.*, 1985), and that this moiety is replaced by oleic acid in essential fatty acid deficiency (Wertz *et al.*, 1983). More direct evidence for a role in the barrier has come recently with the demonstration first, that the synthesis of acylceramides, along with other epidermal sphingolip-



**Figure 3. Optimized lipid mixtures accelerate barrier recovery in acetone-treated human skin.** Each lipid mixture 1.2% or the vehicle alone (60  $\mu$ l) was applied to an area of  $\approx 25$  cm<sup>2</sup> on the volar forearm to acetone-treated sites (TEWL  $\geq 4$  mg/cm<sup>2</sup>/h). Formulation A = 3[cholesterol]:1:1:1 molar ratio; formulation B = 1:1:1:3[palmitic acid] molar ratio. Error bar, SEM.

ids, is regulated by permeability barrier function (Holleran *et al.*, 1992), and second, that sphingolipid synthesis is required for permeability barrier homeostasis (Holleran *et al.*, 1991). Yet, despite the capacity of this molecule and its synthetic relatives to enhance SC water content in detergent-damaged skin (Imokawa *et al.*, 1986), acylceramides alone do not ameliorate, but rather aggravate the barrier defect in acetone-treated skin (Table II). In contrast, acylceramide:cholesterol-containing mixtures, at selected molar ratios, are highly potent in accelerating barrier recovery (Table II). The fact that addition of either free fatty acids and/or ceramides to acylceramide-containing mixtures does not further enhance efficacy, probably reflects the fact that the acylceramide molecule already comprises an ester of ceramide and linoleic acid. Moreover, because these animals are neither essential fatty acid-deficient, nor starved (i.e., fatty acid-depleted), an adequate supply of endogenous fatty acids already may exist.

The central issue addressed in this paper concerns the optimal proportions of the key lipids required for barrier repair, assessed by changes in TEWL. Whereas prior studies showed that an equimolar mixture of cholesterol, ceramide, and free fatty acids permits normal recovery (Mao-Qiang *et al.*, 1993a), these studies demonstrate that recovery rates actually accelerate significantly when the proportions of any one of the three species is increased further, with optimal recovery occurring at about a 3-fold increase (Fig 1). Likewise, an acylceramide-cholesterol ratio of 1:2 is optimal. Moreover, we also have shown that the same optimized ratios that accelerate barrier recovery in murine skin (see also Mao-Qiang *et al.*, 1995a; Yang *et al.*, 1995), also accelerate barrier recovery in damaged human skin. Substantially altered proportions of any constituent above or below these limits leads to decreased efficacy. Although the mechanism of the optimal ratio effect is not known, the further boost in efficacy in going from a 1:1:1:1 to a 3:1:1:1 molar ratio can not be attributed solely to bulk hydrophobic effects within the SC, because even further increases in the concentration of any of the key lipid species actually decrease efficacy. The most likely mechanism to account for the increased efficacy of these optimized mixtures would relate to the formation of more compact lamellar structures, leading to decreased transmembrane permeability (Mickel and Hill, 1972; Wiedmann and Salmon, 1991; Thewalt *et al.*, 1992; Lieckfeldt *et al.*, 1993). Our ultrastructural data support this view, because increasing the proportion of any of the key lipid types above a 3:1:1:1 molar ratio, or application of acylceramides in suboptimal proportions, leads to LB with defective internal contents, and with possible phase separation within the extracellular lamellae. Presumably, the excess lipids, which are unable to incorporate into membrane structures, form a separate microdomain within the SC interstices (Mao-Qiang *et al.*, 1993a). We have described the morphological equivalent of such phase separation under a variety of experimental conditions where the critical molar ratio of the SC extracellular lipids is disrupted from either a reduction or an excess of one or more lipid classes (Feingold *et al.*, 1990; Holleran *et al.*, 1991; Mao-Qiang *et al.*, 1993a, 1995a).

Finally, these studies have important practical implications for topical therapeutics, particularly because we have shown here that the optimized mixtures are effective in human subjects. The data in these studies provide the necessary parameters for the development of new forms of treatment for a wide range of diseases characterized by abnormalities in barrier function; e.g., psoriasis, atopic dermatitis, irritant contact dermatitis, and aging skin (Ghadially *et al.*, 1995).

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