Lipid content and lipid type as determinants of the epidermal permeability barrier

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Abstract During terminal differentiation, mammalian epidermal lipids undergo striking changes in both composition and distribution. Phospholipids and neutral lipids are replaced by a mixture of ceramides and neutral lipids organized in intercellular lamellar bilayers. Whether all of these lipids and/or whether specific lipid classes regulate permeability barrier function is not known. When hairless mice were treated with acetone, the degree of barrier perturbation (measured as transepidermal water loss, TEWL) increased linearly with the amount of lipid removed. Moreover, virtually all lipid species appeared to be removed by acetone treatment. In contrast, the nonpolar organic solvent, petroleum ether, while removing greater amounts of lipids, provoked lesser barrier abnormalities. As determined by both quantitative thin-layer chromatography and histochemistry, petroleum ether selectively extracted nonpolar lipids leaving sphingolipids and free sterols in place. In petroleum ether-treated animals, subsequent acetone treatment removed additional sphingolipids and produced a dramatic increase in TEWL. A linear relationship existed for the quantities of sphingolipid removed and degree of barrier disruption in acetone-treated, but not petroleum ether-treated animals. These results support a relationship between the total lipid content of the stratum corneum and barrier function. Secondly, although the results demonstrate the participation of the total lipid mixture in the barrier, removal of nonpolar species alone appears to cause only a modest level of barrier disruption, while removal of sphingolipids and free sterols leads to a more profound level of barrier perturbation.—**Grubauer, G., K. R. Feingold, R. M. Harris, and P. M. Elias.** Lipid content and lipid type as determinants of the epidermal permeability barrier. *J. Lipid Res.* 1989. **30:** 89–96.

Supplementary key words stratum corneum lipids • solvent treatment • sphingolipids

During epidermal terminal differentiation, lipids undergo a dramatic change both in composition and in localization (reviewed in refs. 1 and 2). A mixture of polar and neutral lipids, typical of other tissues, is replaced by a more nonpolar mixture, including ceramides, free sterols, and free fatty acids, as well as variable amounts of triglycerides, sterol esters, and other nonpolar compounds depending on species, age, and sex (1, 2). These lipids are organized in lamellar bilayers in the stratum corneum interstices (3–5), where they are believed to provide the barrier against transcutaneous water loss necessary for terrestrial life (2–4).

Although specific stratum corneum lipid classes (free sterols, polar sterol metabolites, free fatty acids, and glycerolipids) have been implicated in the regulation of desquamation (reviewed in refs. 2 and 6), which lipid species regulate barrier function is not known. Indirect evidence supports a role for sphingolipids in the barrier both because of their emergence in the outer epidermis (7–9) and because of their content of very long-chain, saturated fatty acids (7, 8, 10, 11). Yet, recent work has shown that epidermal sterologenesis also is linked to barrier function (12–14).

As an alternative to a role for specific lipid fractions, the total lipid mixture might regulate barrier function, a possibility supported by: *a*) the direct relationship between the efficiency of the barrier to water loss and the lipid weight percent of different regions of human skin (15); *b*) the direct relationship that exists between the degree of barrier disruption and the extent of epidermal lipogenesis in murine skin (12); and *c*) the fact that barrier disruption with acetone results in a loss of all stainable lipids from stratum corneum, while stimulating a global increase in sterol and fatty acid biosynthesis (14).

In this study, we have addressed the following questions. Are specific stratum corneum lipid classes responsible for barrier function? Or rather is the total lipid mixture responsible? We present data here that show a direct relationship between the extent of barrier disruption and the total amount of lipid removed. Yet, we also found two distinct levels of barrier function in hairless mouse stratum corneum: one level that could be ascribed to removal of highly nonpolar lipids and a second, more profound level, attributable to sphingolipids (16).

Abbreviations: TLC, thin-layer chromatography; TEWL, transepidermal water loss; UV-A, ultraviolet-A; PBS, phosphate-buffered saline; FFA, free fatty acids; SEM, standard error of the mean; TG, triglycerides; FS, free sterols.

MATERIALS AND METHODS

Materials

Chemicals. Trypsin was obtained from Sigma (St. Louis, MO). Nile red was purchased from Polysciences, Inc. (Warrington, PA).

Solvents. Reagent-grade acetone and petroleum ether, from Fisher Scientific (Fairlawn, NJ), were utilized for barrier disruption. Solvents for thin-layer chromatography (methanol, chloroform, glacial acetic acid, diethylether, and hexane) were spectral grade and purchased from Fisher Scientific. Benzene was purchased from Mallinckrodt (Paris, KY). Phospray was obtained from Supelco (Bellefonte, PA).

Thin-layer chromatography. High performance, silica gel thin-layer chromatography plates (TLC) were obtained from Merck (Darmstadt, FRG).

Animals. Male hairless mice (Hr/Hr), purchased from Jackson Laboratories (Bar Harbor, ME), were between 1 and 3 months of age at the time of study. Animals were fed a standard mouse diet (Simonsen Labs., Gilroy, CA), and water ad libitum.

Experimental procedures and lipid biochemistry

Solvent treatment and water loss measurements. Circumscribed areas (≈ 7 cm^2) on the flank of hairless male mice were treated with sequential applications of either acetone or petroleum ether, utilizing pre-cleaned (delipidized) cotton swabs. The contralateral, control side was treated with saline-soaked cotton balls for the same duration and with the same pressure. In most experiments, each swab was saved for quantitative and/or qualitative lipid analysis (see below). After solvent treatments, the mice were allowed to return to normal body temperatures (≈ 10 min), at which point transepidermal water loss (TEWL) was measured, as described previously (12–14, 17).

Lipid extraction and analysis. Pooled swabs were extracted by the Bligh-Dyer method (18). After splitting of the aqueous and organic phases, the samples were dried and weighed. The lipid extracts were spotted on TLC plates and developed in the following solvent system for separation of neutral lipids: petroleum ether–diethylether–glacial acetic acid 80:20:1 (by vol), followed by 100% petroleum ether, as described previously (7). Individual lipid bands were visualized after spraying with 0.2% aqueous 8-anilino-1-naphthalene sulfonic acid under UV-A light, scraped off, extracted in Bligh-Dyer solvents, weighed, and expressed as lipid weight %. For separation of sphingolipids from phospholipids, lipid extracts were spotted on TLC plates and developed in chloroform–methanol–water 90:10:1 (by vol) followed by petroleum ether–diethylether–glacial acetic acid 70:50:1 (by vol) (7), and visualized as described above. Phospray® was applied to visualize any residual phospholipids. A blue color is seen when the quantities of phospholipids exceed 2 μg.

Microchromatography/iatroscan. Since wax esters and sterol esters display the same R_f in our neutral lipid solvent system, in order to determine whether wax esters were present in lipid extracts we utilized a recently described solvent system for microchromatography (Iatroscan®) that separates wax esters from sterol esters (19). After weighing the lipids extracted from the high performance TLC band that comigrates with sterol and wax esters, the lipids were resuspended in chloroform–methanol 2:1 to a final lipid concentration of about 20 μg/μl, and spotted (1 μl) on each Chromarod, which then were developed in hexane–benzene 27:33. After development, the rods were then heated for 30 sec at 100°C to dry off residual solvents, and the rods were charred and quantitated by flame ionization in the THIO-Mark III-TCC Analyzer (Ancal, Inc., Los Osos, CA), using an air flow of 2,000 ml/min atmospheric air, a hydrogen flow of 160 ml/min (high purity hydrogen), and a scanning speed of 3.0 sec/cm (20). Detector response data was collected and integrated with a computing integrator Model # SP 4100 (Spectra Physics, Mountain View, CA). All of the chromarods (Type SII) were pretested with known standards similar to our samples. The sterol/wax ester function was analyzed on at least three separate rods.

We also utilized microchromatography for fractionation of neutral lipids and sphingolipids when only small quantities (< 1 mg) were available. For separation of neutral lipids the following solvent system was used: hexane–diethylether–formic acid 80:20:1 (by vol) followed by hexane alone. For detection of sphingolipids we used tetrahydrofuran–methylal–ammonium hydroxide–methanol–water 60:30:2:10:2 (by vols).

Stratum corneum lipids. Stratum corneum sheets, before and after solvent treatment, were obtained from full-thickness skin of hairless mice by incubation, granular-layer-downward, in 0.5% trypsin in phosphate-buffered saline (PBS) pH 7.4, for 2 hr at 37°C, which yields an intact sheet of stratum corneum plus granular cells. An additional incubation in 0.5% trypsin in PBS for 20 min followed by vortexing removes residual granular cells; the remaining stratum corneum sheet has been shown repeatedly to be devoid of nucleated cells (e.g., 8, 9). The stratum corneum sheets were dried overnight in a Speed-Vac Concentrator (Savant) and weighed. Lipid extraction and subsequent fractionation of lipids were then performed as described above, and lipid weights were calculated as % of total dry stratum corneum weight. For purposes of this report, polar lipids designate phospholipids, sphingolipids, and free sterols, while nonpolar lipids comprise free fatty acids (FFA), triglycerides, sterol esters, *n*-alkanes, and other species with an R_f greater than that of FFA.

Histochemical staining

Nile red, a recently described fluorescent probe that distinguishes between nonpolar and polar lipids in frozen tissue sections (21), was used to depict the distribution of lipids

in murine stratum corneum. A stock solution of nile red (500 μg/ml) in acetone was prepared, stored at − 20°C, and protected from light. A fresh staining solution of nile red was made by the addition of 15–20 μl of the stock solution per 1 ml 75% glycerol followed by brisk vortexing. To stain fresh frozen cryostat sections, each 4 μm thick, a drop of glycerol staining solution was added to each section and the preparation was covered with a glass coverslip. After 10 min at room temperature the sections were examined by fluorescence microscopy, utilizing a Leitz Ortholux II fluorescence microscope equipped for epifluorescence. Nile red fluorescence was then examined at two spectral settings: *1*) yellow-gold fluorescence, using a filter set for fluorescein fluorescence (450–500-nm band pass excitation filter, a 510-nm center wavelength chromatic beam splitter, and a 528-nm-long pass barrier filter), or *2*) red fluorescence, using a filter set for rhodamine fluorescence (515–560-nm band pass excitation filter, a 580-nm center wavelength chromatic beam splitter, and a 590-nm-long pass barrier filter). After staining, neutral lipids are seen with the first spectral setting as yellow-gold fluorescent structures, while the red-orange fluorescent structures comprise phospholipids, other amphipathic lipids, and strongly hydrophobic proteins of cell membranes (21). The variations in neutral versus polar lipid staining are amplified further at the second spectral setting. At both wavelengths, unstained sections (background) revealed very low levels of green fluorescence that did not resemble nile red staining patterns. Depending upon the amount of tissue lipid present and the tissue thickness, 1–5 μg of dye/ml of 75% glycerol was required for optimal fluorescent staining.

TABLE 1. Cutaneous barrier function in various acetone-treated groups

Treatment	Animals per Group	Transepidermal Water Loss[a]	Lipid Weight[b]
Acetone			
Low	10	23 ± 3	0.25
Low	10	30 ± 3	0.26
Low	20	35 ± 2	0.22
Low	10	38 ± 3	0.35
Intermediate	5	85 ± 21	0.53
Intermediate	8	125 ± 32	0.38
Intermediate	10	140 ± 11	0.40
Intermediate	2	183[c]	0.31
Intermediate	2	230[c]	0.44
Intermediate	2	251[c]	0.93
Intermediate	2	260[c]	0.70
High	2	312[c]	0.98
High	2	390[c]	0.88
High	5	391 ± 86	0.76
High	2	570[c]	1.27
High	2	665[c]	1.19
High	4	860 ± 140	1.54
Control	13	6 ± 0.6	

An area (approx. 7 cm^2) of hairless mouse skin was treated with delipidated, acetone-soaked cotton swabs. Cotton swabs were extracted with chloroform–methanol 2:1 (by vol) and the lipids removed per animal were calculated. According to the range of transepidermal water loss, animals were clustered into three groups: Group 1 (Low) TEWL from 23–38 ppm/cm^2 per hr; Group 2 (Intermediate): TEWL from 85–260 ppm/cm^2 per hr; Group 3 (High): TEWL from 312–860 ppm/cm^2 per hr. The wide variations in the High group reflect highly variable changes in barrier function that result from the removal of small amounts of additional lipids in these animals (cf, Fig. 1).

[a]Results are expressed as ppm/cm^2 per hr for each individual animal; mean ± SEM.

[b]As mg removed per animal.

[c]Values are means of two animals.

RESULTS

Role of bulk lipids in barrier function

When the degree of barrier disruption following repeated acetone treatments was correlated with the amount of lipid removed, a linear relationship was apparent (**Table 1**, $r = 0.95$; $P < 0.001$). However, TEWL accelerated somewhat as more lipid was removed during sequential treatments (**Fig. 1**; closed triangles). These results suggest that there is a direct relationship between the amount of lipid removed and the degree of barrier perturbation.

Distribution of lipids in relation to barrier disruption

To determine whether specific lipid species regulate different levels of barrier function, we compared the lipid distribution in acetone extracts from the Low, Intermediate, and High groups, as well as the spectrum of lipids in untreated stratum corneum. In all three acetone-treated groups, disproportinately larger amounts of sterol esters and an unknown lipid class were present in comparison to whole stratum corneum extracts, whereas less triglycerides, free sterols, and sphingolipids were present than in whole stratum corneum extracts (**Table 2**). With increasing barrier disruption (from Low to Intermediate group), there was an increase in the content of both sphingolipids and free sterols in acetone extracts; but even in animals with the most extensive barrier disruption, i.e., TEWL levels in the range of 800–1,000 ppm/cm^2 per hr, the proportions of sphingolipids and free sterols never attained those present in whole stratum corneum (Table 2). Since the quantities of free sterols and sphingolipids never achieved the levels present in whole stratum corneum, this suggests that some of the lipid in acetone extracts derives from other sources, presumably pilosebaceous structures (see below). The principal nonpolar lipid species in the acetone extracts (and in the Bligh-Dyer extracts of whole stratum corneum) were sterol esters, followed, in order, by an unidentified species with an R_f slightly below sterol esters, free fatty acids, triglycerides, and alkanes (Table 2). When the sterol ester region, including the unknown band, was rechromatographed in another solvent system (see Methods) to determine whether wax esters might be present, neither wax es-

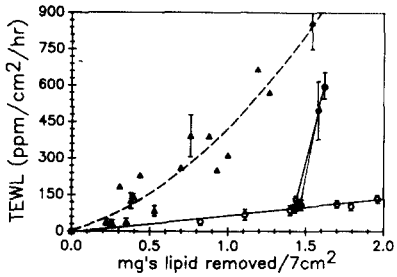

Fig. 1. Changes in transepidermal water loss (TEWL) in relation to total lipid removed (mg) in hairless mice following solvent treatment. Animals were either treated with acetone-soaked cotton swabs (closed triangles), petroleum ether-soaked cotton swabs (open circles), or petroleum ether followed by acetone-soaked cotton swabs (closed circles) to break the barrier. Results are expressed as mean ± SEM, except for data-points where two animals were pooled (cf, Tables 1 and 3). A linear relationship exists for acetone-treated animals ($r = 0.95$; $P < 0.001$ and for petroleum ether-treated animals ($r = 0.94$, $P < 0.05$.

ters nor other co-migrating species were found. Further characterization studies underway on the unknown species have shown that no sterols are present after saponification, and that it is orcein-positive with a molecular weight > 500 (data not shown).

Finally, to eliminate the possibility that some of the lipids in acetone wipes might originate from the living epidermis, TLC plates were treated with a molybdenum salt (Phospray) to detect the presence of phospholipids. Since no phospholipids were detected (data not shown), this suggests that the acetone treatments did not extract significant quantities of lipids from the nucleated cell layers beneath the stratum corneum. In contrast, after tape-stripping off most of the stratum corneum, molybdenum-positive phospholipids appeared in acetone extracts (data not shown).

Role of nonpolar versus polar lipids in barrier function

Since the acetone-induced defects in barrier function were accompanied by linear removal of nonpolar as well as polar lipids, this raised the possibility that nonpolar lipids might participate in barrier function. To address this issue, we compared barrier function after treatment of hairless mice with either acetone alone, a highly nonpolar organic solvent-petroleum ether, or with petroleum ether followed by acetone. Although petroleum ether treatment produced a reproducible, linear defect in barrier function (Fig. 1; $r = 0.94$; $P < 0.001$), even with exhaustive treatment the absolute rates of TEWL plateaued at approximately 150 ppm/cm^2 per hr, at which point little more lipid could be removed (**Table 3**, Fig. 1). Yet, despite its limited effect on the barrier, petroleum ether removed more lipid on a mg lipid/cm^2 per mouse than even the High acetone group (cf, Tables 1 and 3).

As expected, petroleum ether extracts contained a much greater proportion of highly nonpolar species, including sterol esters, as well as the unknown species (see above), than was present in whole stratum corneum (**Table 4**). In contrast, petroleum ether removed almost no sphingolipids and relatively small quantities of free sterols. When the petroleum ether-treated sites were treated subsequently with acetone, a marked increase in TEWL occurred despite removal of only small amounts of additional lipid (Fig. 1; closed circles). The lipid distribution in acetone extracts of skin sites previously treated with petroleum ether did not differ substantially from sites treated with acetone alone (Table 4, cf, Table 2); i.e., in addition to nonpolar lipids, sphingolipids and free sterols also were present.

To address the issue of the role of polar versus nonpolar lipids in the barrier further, we next compared the quanti-

TABLE 2. Lipid content of acetone extracts of murine stratum corneum[a]

Fraction	Degree of Barrier Disruption: Low (n = 4)	Intermediate (n = 3)	High[b] (n = 2)	Whole Stratum Corneum (n = 4)
		lipid weight % ± SEM		
Neutral lipids	86.1	81.4	83.2	75.6
Alkanes	2.2 ± 0.2	2.1 ± 0.4	4.3	3.2 ± 0.3
Sterol esters	45.2 ± 1.3	41.2 ± 0.4	41.8	26.9 ± 1.7
Unknown	14.0 ± 1.1	12.6 ± 1.7	13.6	5.5 ± 1.2
Triglycerides	3.0 ± 0.3	3.8 ± 0.6	2.3	15.0 ± 1.2
Free fatty acids	12.4 ± 1	9.9 ± 0.6	12.5	10.2 ± 0.9
Free sterols	9.3 ± 0.5*	11.8 ± 1.0*	8.7	15.8 ± 1.7
Sphingolipids	14.0 ± 0.7**	18.5 ± 0.7**	16.8	23.5 ± 2.2
Totals	100.1	99.9	100.0	100.1

Lipid composition of whole mouse stratum corneum is compared with the composition in lipid extracts from the stratum corneum of animals with Low, Intermediate, and High rates of transepidermal water loss (cf, Table 1). Data are expressed as lipid weight % ± SEM. Whole stratum corneum lipids are expressed as lipid weight % ± SEM.

[a]Number of groups of animals pooled (n): Low, 20/10/10/10; Intermediate, 10/8/5; High, 5/4. *$P < 0.05$; **$P < 0.01$.

[b]Values are means of two High groups with transepidermal water loss 391 ± 86 and 860 ± 140 ppm/cm^2 per hr (cf, Table 1).

TABLE 3. Cutaneous barrier function in petroleum ether-treated animals

Treatment	Number of Animals	Transepidermal Water Loss[a]	Lipid Weight[b]
Petroleum ether	3	39 ± 7	0.82
	3	67 ± 22	1.11
	7	86 ± 20	1.40
	5	93 ± 17	1.43
	3	102 ± 16	1.79
	4	108 ± 21	1.47
	3	110 ± 14	1.70
	3	129 ± 13	1.44
	3	133 ± 8	1.96
Controls	34	8 ± 2	

An area (approx. 7 cm^2) of hairless mouse skin was treated with precleaned cotton swabs, soaked with petroleum ether. Cotton swabs were extracted in chloroform–methanol 2:1 (by vol) and the lipid removed per animal is shown for animals with various degrees of barrier disruption.

[a]Results are expressed as ppm/cm^2 per hr; mean ± SEM.

[b]As mg removed per animal.

ties of sphingolipids removed with TEWL rates in the acetone and petroleum ether groups. As can be seen in **Fig. 2** (open circles), there is a linear relationship between the extent of barrier disruption and the amount of sphingolipid removed for the acetone-treated group ($r = 0.93$; $P < 0.01$). In contrast, there is no relationship between the quantities of sphingolipids removed and the degree of barrier disruption in the petroleum ether-treated group (i.e., despite increased TEWL rates, little or no sphingolipids are removed; Fig. 2, closed circles).

Origin of lipids in acetone and petroleum ether extracts

To delineate the source(s) of lipids in acetone versus petroleum ether extracts, we stained frozen, unfixed sections of solvent-treated skin with nile red, a fluorophore whose staining properties change in relation to the polarity of the lipid environment; i.e., yellow-gold fluorescence when nonpolar lipids are present versus red or red-orange fluorescence in the presence of lipids with an $R_f \leq$ that of glycosphingolipids (21). Acetone treatment removed virtually all nile red staining of nonpolar lipids from the stratum corneum in the Low (**Fig. 3b**), Intermediate (Fig. 3c), and High (not shown) groups, as shown by depletion of yellow-gold fluorescence (cf, Fig. 3a, control). However, substantial nile red-stainable nonpolar lipids remained in pilosebaceous structures even after exhaustive acetone treatment (Fig. 3c, star). In contrast to the results with acetone, petroleum ether removed nile red-stainable, nonpolar lipids from both the stratum corneum (Fig. 3d, arrow) and pilosebaceous structures (Fig. 3d, star).

When control, acetone-treated, and petroleum ether-treated frozen sections were examined for polar lipids, i.e., orange staining, the entire epidermis and lower stratum corneum appeared to contain polar lipids (**Fig. 4A**). In contrast, little or no polar lipids were seen in pilosebaceous structures, even after petroleum ether treatment (Fig. 4A and 4B, asterisks). Although petroleum ether treatment removed neutral lipids from the stratum corneum, such treatment did not reduce polar lipid staining of the epidermis (Fig. 4B). Finally, acetone treatment removed most polar lipid fluorescence from the stratum corneum (Fig. 4C, above arrow), but did not influence polar lipid staining of the epidermis.

DISCUSSION

These studies were designed to examine whether a relationship exists between the quantity of lipids within the stratum corneum and barrier formation. Although it would have been desirable to correlate lipid removed with lipid remaining in the stratum corneum with the small quantities removed in the Low and Intermediate groups, our chromatographic techniques were not sensitive enough to detect the small differences in residual lipid. However, with more extensive acetone treatment (High group), we detected a substantial reduction in residual stratum corneum lipids, and these lipids displayed the same chromatographic spectrum as acetone extracts (22). Prior studies also have suggested that the quantities of lipids in a particular skin site are more critical for barrier function than other factors, such as the thickness and number of cell layers of the stratum corneum (15). Moreover, the lower quantities of lipids in human palm or sole versus abdomen or facial stratum corneum (11, 23) correlate with the increased transcutaneous water loss of palms and soles (24). Finally, the data in Table 2 also support a quantitative rather than

TABLE 4. Lipid quantities and distribution in petroleum ether-treated hairless mice

Fractions	Petroleum Ether Alone (n = 2)[a] % Distribution	Acetone after Petroleum Ether (n = 2)[a] Lipid Distribution
	lipid weight %	
Neutral lipids	98.9	94.7
Alkanes	1.9	10.6
Sterol esters	67.7	46.8
Unknown	12.9	[b]
Triglycerides	4.4	24.6
Free fatty acids	6.9	4.1
Free sterols	5.1	8.5
Sphingolipids	1.4	5.3
Totals	100.3	100.0

Animals were first treated with precleaned petroleum ether-soaked cotton swabs, followed by treatment with precleaned acetone-soaked cotton swabs (see Table 3). Data are expressed as lipid weight % ± SEM.

[a]Refers to two groups of animals with transepidermal water loss of 86 ± 20 and 110 ± 14 ppm/cm^2 per hr (cf. Table 3).

[b]Triglyceride/unknown area.

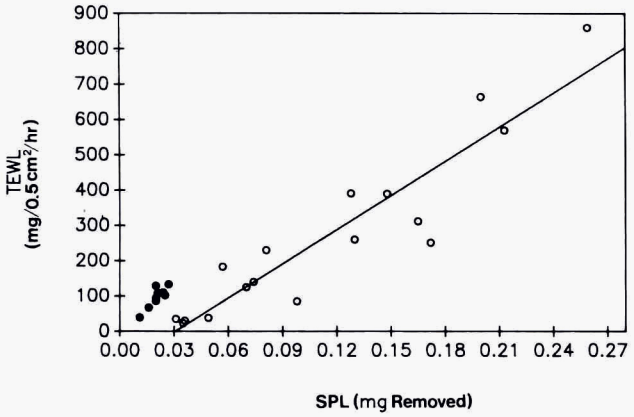

Fig. 2. Relationship of sphingolipid extraction and barrier disruption following sequential treatment with acetone (open circles) or petroleum ether (closed circles). Data represent product of percent sphingolipids removed (from Tables 2 and 4) multiplied by total lipid removed (Tables 1 and 3). Whereas a linear relationship pertains for acetone treatment ($r = 0.93$; $P < 0.05$), no relationship exists between barrier disruption and sphingolipids removed in the petroleum ether-treated group.

qualitative relationship, because the spectrum of lipids removed in animals that demonstrate various degrees of barrier abrogation did not differ substantially.

However, since acetone removes the full spectrum of stratum corneum lipids, the possibility remains that one or more individual lipid species in the acetone extract is critical for barrier function, and that the apparent quantitative relationship demonstrated here is not of paramount importance. To resolve this question definitively would require selective removal of specific classes of lipids (as was done for highly nonpolar species with petroleum ether). However, even the most polar solvents (e.g., methanol and acetonitrile) mobilize substantial neutral lipids from the stratum corneum and no known solvent system selectively removes sphingolipids (P. M. Elias and B. E. Brown, unpublished observations). Yet, the studies reported here support an important role for sphingolipids in barrier function. First, the highly nonpolar organic solvent, petroleum ether, produced only a modest defect in barrier function while removing abundant nonpolar lipid species, but little sphingolipid. Second, when petroleum ether treatment was followed by treatment with the more polar solvent, acetone, additional sphingolipids were removed, resulting in a major defect in barrier function (Fig. 1). Third, a clear relationship was shown between the amount of sphingolipid removed and the extent of barrier disruption (Fig. 2). Prior

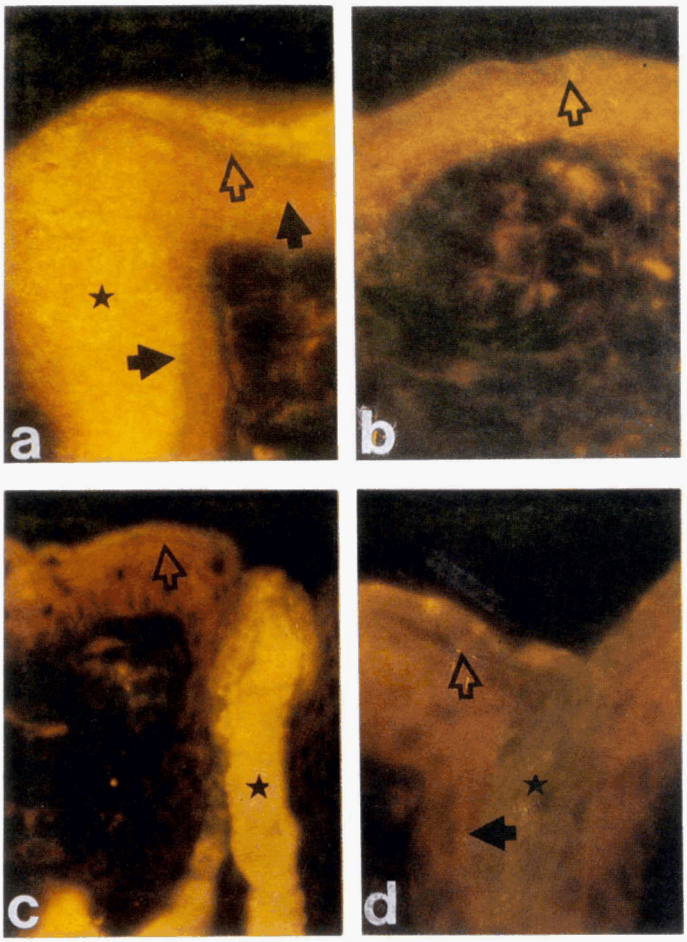

Fig. 3. a) Frozen sections of solvent-treated hairless mouse skin, stained with nile red for nonpolar lipids. Normal control mouse skin was stained with nile red for nonpolar lipids (see Methods). Note the abundance of stainable, nonpolar lipids in both the stratum corneum (open arrow) and the contents of sebaceous glands (star). Stratum corneum appears to be more intensely stained than either the underlying epidermis or follicular epithelium (filled arrows). b) Section of skin from Low transepidermal water loss (TEWL) group (TEWL = 40 ppm/cm^2 per hr). Even minimal acetone treatment removes much of the stainable, nonpolar lipids from the stratum corneum (open arrow). As a result, stratum corneum and epithelial staining (filled arrow) are comparable. c) More extensive acetone treatment (Intermediate group; TEWL = 96 ppm/cm^2 per hr) results in further depletion of virtually all stainable nonpolar lipids from the stratum corneum (open arrow), but abundant nonpolar lipids persist in sebaceous glands (star). d) In petroleum ether-treated skin, neutral lipids are largely removed from both the stratum corneum (open arrow) and sebaceous glands (star) (TEWL = 68 ppm/cm^2 per hr). Note: High group did not differ from Intermediate, and therefore is not shown. Figs. 3b–d are underexposed in comparison to control (Fig. 3a) in order to facilitate comparison of stratum corneum versus pilosebaceous staining; 3a–c, × 400; 3d, × 600.

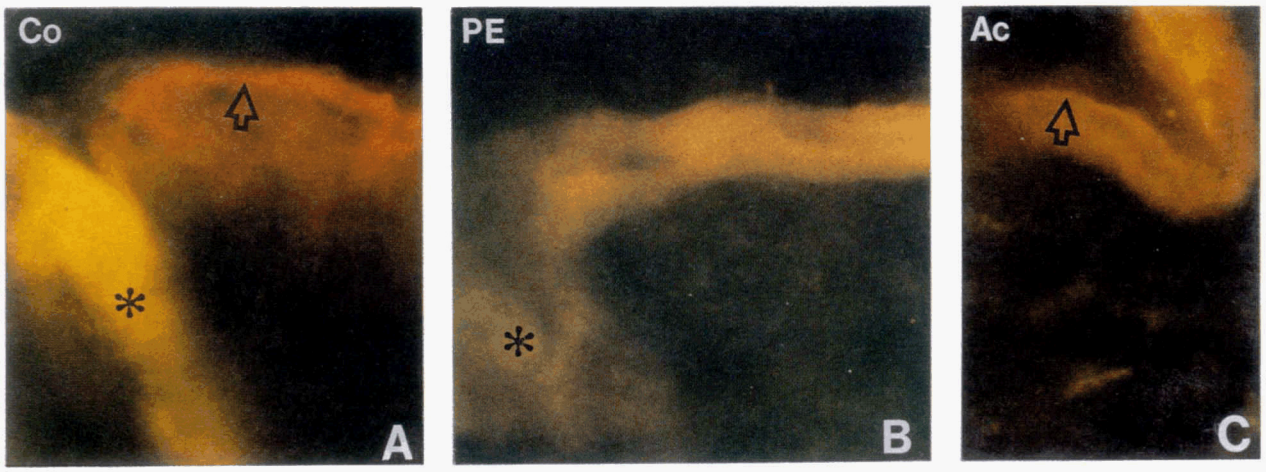

Fig. 4. Frozen sections of solvent-treated hairless mouse skin, stained with nile red for polar lipids. A) In control (Co) skin note prominent orange-red band in lower stratum corneum (arrow), and absence of polar lipids in both pilosebaceous apparatus (asterisk) and outer stratum corneum. B) After petroleum ether (PE) removal of nonpolar lipids, residual polar lipids fluoresce orange rather than orange-red as in control, and are revealed at all levels of the epidermis, with only sparse polar lipids evident in pilosebaceous structures (asterisk). C) After acetone (Ac) treatment, only faint residual fluorescence of polar lipid remains in the stratum corneum (arrow), while the nucleated cell layers continue to display orange fluorescence. Figs. 4a–c, × 600.

studies also have focused on sphingolipids as the principal mediator of barrier function: first, because of the dramatic increase in sphingolipid content of stratum corneum (7–9); second, because of the carriage of saturated, long-chain acylated fatty acids on epidermal sphingolipids (7, 8, 10, 25); and finally, because epidermal sphingolipids are enriched with linoleic acid, a critical ingredient for intact barrier function (8, 10, 25).

Yet, sphingolipids may not be the only lipid of importance for barrier function, since recent studies have shown that both sterol and fatty acid synthesis are stimulated specifically by barrier requirements (12–14).

Moreover, the studies reported here also point to a role for more nonpolar lipid species in barrier function. Petroleum ether, which removed solely nonpolar lipids, was capable of producing modest abnormalities in the barrier, but substantially less than that produced with acetone, which removed both nonpolar and polar species (Figs. 1 and 2). Moreover, the histochemical studies showed that petroleum ether removed nonpolar lipids from the stratum corneum, while leaving polar species in place (Figs. 3 and 4). The fact that surfactant treatment likewise produces only minor defects in barrier function, despite producing extensive damage to the stratum corneum (25–27), may reflect both selective removal of nonpolar lipids and the poor solubility of epidermal sphingolipids in these surface-active agents (28, and P. M. Elias and B. E. Brown, unpublished observations). Although these studies provide strong support for a role for lipids other than sphingolipids in the barrier, the relative importance of each species is still uncertain.

In summary, these results support the existence of two lipid-related barriers in the stratum corneum: a first level of barrier function, subserved largely by highly nonpolar lipids, and a second, more resilient level of barrier integrity, mediated primarily by sphingolipids. The selective effects of petroleum ether versus acetone on barrier function suggest that not all of the lipids in the stratum corneum interstices are uniformly cohesive. Moreover, nonpolar lipids, but not sphingolipids and free sterols, appear in aqueous supernatants following stratum corneum homogenization (28). Thus, sphingolipids and free sterols may be arranged in relatively cohesive lamellar structures, while more nonpolar species may be confined within a separate, relatively loosely bound compartment in the stratum corneum interstices.

This work was supported by NIH grant AR-19098, the Medical Research Service, Veterans Administration, and Fonds zur Forderung der Wissenschaftlichen Forschung (Vienna, Austria). Dr. Mary L. Williams provided many helpful suggestions, and Mr. Bil Chapman capably prepared the manuscript.

Manuscript received 27 April 1988 and in revised form 5 July 1988.

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