Presence of Intact Intercellular Lipid Lamellae in the Upper Layers of the Stratum Corneum

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The epidermal permeability barrier necessary for terrestrial life resides in the intercellular spaces of the stratum corneum and is composed of lipids. Membrane coating granules (MCGs), small intracellular organelles found in the uppermost layers of the living epidermis, contain stacks of membranous disks which are extruded into the intercellular space and undergo both biochemical and physical changes to form the lipid sheets which constitute this barrier. Using ruthenium tetroxide as a secondary fixative, we are able to demonstrate stacks of lamellae filling the intercellular spaces in the uppermost layers of the stratum corneum. The structure of these lipid lamellae is consistent with the proposed derivation of MCG lipid disks and also suggests that the lipid bilayer adjacent to the corneocyte cell envelope may be assembled from lipids not derived from MCGs. *J Invest Dermatol 88:714–718, 1987*

Terrestrial life requires a waterproof covering. The location of this barrier to water permeation has long been known to reside in the stratum corneum, or horny layer, of the epidermis [1], and loss of barrier function following extraction of the epidermis with lipid solvents indicated that it was composed of lipids [2]. The localization of the barrier lipids to the spaces between the flattened keratinocytes of the stratum corneum has been demonstrated histochemically [3], and permeability studies [4–6] indicate that the barrier extends through all but the most superficial (desquamating) layers of the stratum corneum.

A large body of evidence indicates that the barrier lipids are derived from membrane coating granules (MCGs), small intracellular organelles containing stacks of membranous disks [5,7–16], which extrude their lipid contents into the intercellular space between the uppermost layer of the viable epidermis and the stratum corneum [5,9–14]. The extruded disks are believed to undergo biochemical as well as physical changes resulting in the formation of broad intercellular lamellar sheets [5,12–14]. The arrangement of the lipids in these broad sheets has been shown most convincingly by freeze fracture electron microscopy [5,17,18]. Routine transmission electron microscopic techniques using osmium tetroxide as a secondary fixative, while clearly showing the structure of MCGs, have not been as effective in demonstrating the intercellular lamellae of the stratum corneum. Stacked membrane structures have been noted primarily in the intercellular spaces of the lowest layers of the stratum corneum, shortly after extrusion from MCGs, with only fragments visualized in the upper layers [5,6,12–14,19,20]. In a recent report by Menon et al [21] it is stated that intercellular lipid bilayers disappear above the mid stratum corneum in terrestrial mammals.

Since permeability studies suggest that the structure of the barrier lipids should remain intact until desquamation occurs, it was felt that the inability to demonstrate intercellular lamellae in the upper layers of the stratum corneum might be related to suboptimal preservation with standard fixation methods. Using ruthenium tetroxide rather than osmium tetroxide as a secondary fixative, we are able to demonstrate stacks of lamellae filling the intercellular spaces in the uppermost layers of the stratum corneum.

MATERIALS AND METHODS

Full-thickness skins were removed from anesthetized BALB/c neonatal mice. Small pieces measuring approximately 1.5 × 3 mm were fixed in cacodylate-buffered 5% glutaraldehyde for 2 h and postfixed with 0.2% buffered ruthenium tetroxide (Polysciences, Inc., Warrington, Pennsylvania) at pH 6.8 for 1 h at room temperature. Samples were rinsed in 3 changes of buffer and distilled water before dehydrating in a graded series of ethanol and then embedded in Spurr's resin (Polysciences, Inc.). Ultrathin sections were picked up on carbon-stabilized Formvar-coated grids and stained in aqueous uranyl acetate and lead citrate prior to examination in a Hitachi H-600 transmission electron microscope.

In order to determine the number of stratum corneum layers present in mouse skin, samples taken at random from the skins of 4 neonatal mice were india ink–coated and agar-embedded. The samples were then fixed in 2.5% glutaraldehyde, dehydrated in acetone, and embedded in Spurr's resin. One micron-thick sections were cut, stained with Richardson's stain, and examined under a 100× oil immersion lens to count the stratum corneum layers. Carbon particles were clearly present on the surface of the sections. Fifty-eight separate counts were made on multiple sections cut from the 4 samples.

RESULTS

The results are presented in Figs 1–3. Figure 1*a* is a low-power electron micrograph of the stratum corneum. There are approx-

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Abbreviation:

MCG: membrane coating granule

imately 13 stratum corneum layers in this section; this compares favorably with the mean of 12.2 layers (range 10–14) that we determined in the ink-coated and agar-embedded samples (data not shown), and indicates little or no loss of the outer stratum corneum layers during processing. Although fixation is not uniform, there is preservation of large segments of the intercellular space in the outermost layers. The inset, Fig 1*b*, is a higher-magnification view of the area outlined in Fig 1*a*, which demonstrates the presence of multiple lamellae filling the intercellular space. Figure 2*a* and *b* show the characteristic stacked membrane disks of MCGs found in the upper layers of the viable epidermis. Shortly after the extrusion of MCG contents into the intercellular space, the disks appear to reorganize (Fig 2*c*). In Fig 2*d*, it is clear that the lamellae in the outer stratum corneum are closely apposed to the cell surfaces and that there are several layers consisting of alternating electron-dense and electron-lucent bands. It is apparent that there is a narrower dense band within the major lucent band, as is also seen in MCG membrane stacks (Fig 2*a,b*) [9–16,18,19]. In addition, there is a faint, but readily visible, lucent band within the major dense band. This was observed only in the stratum corneum (Fig 2*d*) and has not been reported previously. Also, a lucent band can be seen between the horny cell envelope and the outer half of the first major dense band, as has been noted previously [12–14,19,20]. These features of the intercellular space are illustrated schematically in Fig 3.

Only three previous studies have reported measurements for stratum corneum intercellular lamellae [13,14,16] and in all cases osmium tetroxide, with or without potassium ferrocyanide, was used for postfixation. Table I compares our measurements of MCG and stratum corneum lamellae with those from other studies.

DISCUSSION

Stratum corneum lipids have been well characterized [22–26] and consist of ceramides, fatty acids, cholesterol, and cholesteryl sulfate, with ceramides the dominant component. The aliphatic side chains of the ceramides are largely saturated or monounsaturated [27] as are the free fatty acids of the stratum corneum [19]. This indicates that the use of osmium tetroxide, which reacts with the double bonds of unsaturated lipids, might be less than optimal for the fixation of stratum corneum lipids. Ruthenium tetroxide, a stronger oxidizing agent, reacts well with both saturated and unsaturated molecules and has been shown to react strongly with polar lipids that show no reaction with osmium tetroxide [28]. Excellent membrane fixation has been demonstrated in tissues other than skin, although membranes may appear thicker than with osmium tetroxide [29]. The high reactivity of ruthenium tetroxide results in slow tissue penetration and the uneven, patchy preservation that we and others have noted [28,29].

Based on detailed electron microscopic examination of MCGs, it has been proposed that the lamellar internal structure is formed by the flattening and stacking of unilamellar liposome-like vesicles [15,16,18]. The major dense band thus represents the closely apposed outer polar regions of two adjacent vesicles and the minor dense band represents the compressed inner polar surface of a single vesicle. Landmann [16] found the band widths in the in-

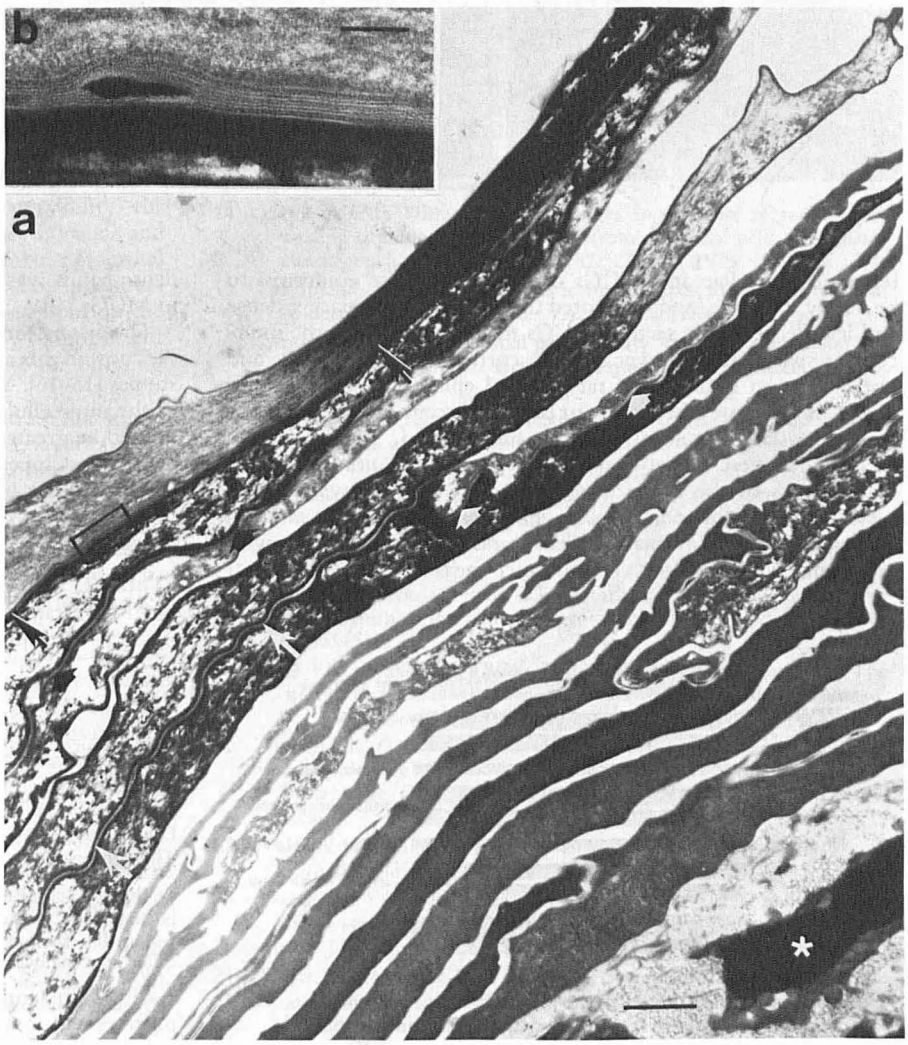

Figure 1. Electron micrograph of neonatal mouse stratum corneum. *a*, *Asterisk*, keratohyaline granule in the upper granular cell layer. *Arrow pairs* enclose areas of intercellular space preservation in the outer stratum corneum. *Bar* = 1.0 μm. *b*, *Inset*, higher magnification of the area outlined in (*a*) showing the detail of the intercellular space. *Bar* = 0.1 μm.

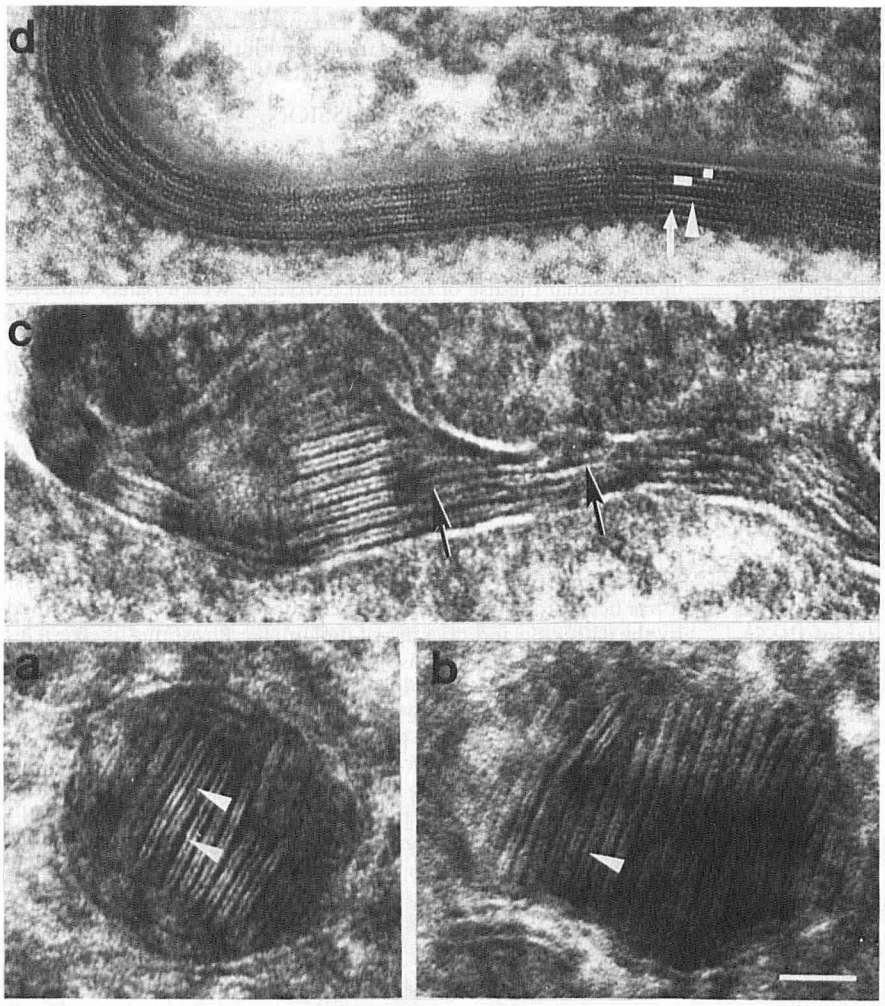

Figure 2. Electron micrographs of lamellar lipid structures in neonatal mouse epidermis. *a and b*, Membrane coating granules in the granular cell layer. Note the "stacked disk" appearance and the pattern of alternating electron-dense and electron-lucent bands. The minor dense band (*arrowheads*) splits the lucent band centrally. *c*, Intercellular space at the junction of the stratum granulosum and the stratum corneum after extrusion of MCG contents. Early fusion of disks is apparent (*area between arrows*). *d*, Intercellular space in the outer stratum corneum. Note that the appearance of the lamellae is different than that seen in the MCGs. Major dense band, *covered by square;* major lucent band, *covered by rectangle;* minor dense band, *arrowhead;* minor lucent band, *arrow*. *Bar* = 50 nm.

tercellular lamellae and MCGs of neonatal mouse epidermis to be nearly the same, and also noted the pattern of alternating dense and less dense bands seen in MCGs to be preserved in the intercellular spaces of the lower stratum corneum. In the middle and upper stratum corneum of rat gingival epithelium, Lavker [13] noted "membrane-like figures or bilayers" composed of two electron-dense lamellae separated by a lucent band. Stacks of these membranes were occasionally observed but variation in density or thickness of the dense bands was not noted. Hayward [14] reported intercellular lamellae in hamster cheek pouch stratum corneum to have alternating dense and less dense bands with intervening lucent bands. The distance measured between major dense bands was somewhat greater in the stratum corneum than in MCGs.

The distance measured from dense band to dense band in MCGs (9.7 nm) in this study agrees very closely with that found in other studies [13–16]. We found the distance between major dense bands in the intercellular lamellae of the upper stratum corneum (12.8 nm) to be greater than that in MCGs and, in addition, the major dense band appears to be split by a narrow lucent area which has not previously been seen.

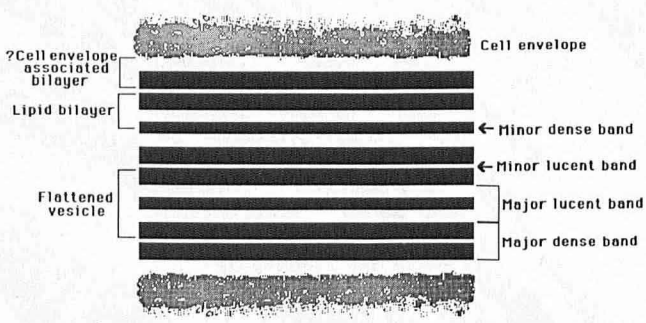

Figure 3. Schematic diagram of the intercellular space in the outer layers of the stratum corneum as seen in Figs 1*a* and 2*d*. Not to scale.

Table I. Comparison of Measurements Previously Reported for Membrane Coating Granule (MCG) and Stratum Corneum Lipid Lamellae With the Present Results

	Center to Center Distance Between Major Dense Bands, (nm)	
Reference	MCG	Stratum Corneum
Lavker [13]	7	12[a]
Hayward [14]	8.5	10.9
Landmann [16]	9.95	9.73
This study	9.6	12.8

Negatives taken at primary magnifications of 80,000, 100,000, or 150,000 were photographically enlarged 2.6×. A magnifying glass (7×) with a 0.1-mm graticule was used for the measurements. The value presented for stratum corneum lamellae is the mean of 7 measurements from various prints. Sixty-eight measurements were made in 11 different MCGs and the mean distance is presented.

[a]Assuming alternating major and minor dense bands with intervening lucent bands.

The differences between the present study and the previous ones [13,14,16] regarding the structural detail of the intercellular lamellae in the stratum corneum may be due to differences in fixation. Since ruthenium tetroxide is chemically more reactive than osmium tetroxide, it is more capable of reaction with the abundant hydroxyl groups of stratum corneum lipids. This may result in a more uniform and distinct deposition of electron-dense material along the polar interfaces of adjoining bilayers.

The differences in appearance between the major and minor dense bands revealed in the present study support the concept of lipid asymmetry between the leaflets of the individual bilayers [16,18], a phenomenon well known in biologic membranes [30]. Also, the relatively narrow width of the minor dense band raises the possibility of head group interdigitation within this region of the membrane system. In either case, the lamellar pattern demonstrated in this study is consistent with the postulate [15,16,18] that membranous disks (stacked and flattened liposome-like vesicles) extruded from MCGs fuse edge to edge to produce the broad lamellae in the extracellular spaces of the stratum corneum (Fig 3). The difference in the appearance of the outer stratum corneum lamellae compared to MCG membrane stacks is consistent with an alteration in lipid composition [23] occurring after the extrusion of MCG contents. The exact mechanism of these alterations and the biochemical basis for the appearance of both MCG and stratum corneum lamellae remain speculative.

Of particular interest are the lucent band surrounding the horny cell envelope and the adjacent half of the first major dense band. The thickness of this region (approximately 6.5 nm) suggests that it should be accounted for by a single lipid bilayer. However, the other intercellular bilayers appear to be paired, as noted above, in accord with the proposal that they are derived from stacked and flattened unilamellar vesicles. This implies that the first bilayer adjacent to the horny cell envelope is not derived from lamellar granules. It may be that the original plasma membrane of the keratinocyte is retained in modified form instead of being completely broken down, as has been believed, during differentiation [31]. This lipid layer may be a covalently attached integral part of the horny cell envelope assembled from intracellular lipoproteins or proteolipids. Consistent with this latter possibility, the presence in stratum corneum of fatty acids, ω-hydroxyacids and ω-hydroxyacylsphingosines covalently attached to epidermal macromolecules has recently been reported [32]. This phenomenon could provide for a layer of covalently bound lipid on the outside of the horny cell envelope. This layer may be important for the proper interaction of the horny cell with the surrounding MCG-derived extracellular lipid lamellae.

The results of the present study clearly demonstrate that intact intercellular lipid lamellae are present in the outer layers of the stratum corneum, as was suggested by epidermal permeability studies. We have recently been able to demonstrate intercellular lamellae in the outer layers of the stratum corneum in both pig and human epidermis (unpublished observations). Poor fixation of these largely saturated membrane structures by standard techniques may account for the lack of adequate visualization in previous transmission electron microscopic studies.

The presence of intercellular lamellae up to the point of desquamation raises the question of their role in stratum corneum cell cohesion. The major difference between the lipid compositions of cohesive and desquamated corneocytes is the disappearance of cholesteryl sulfate [25,26] during desquamation. The genetic inability to hydrolyze cholesteryl sulfate due to a deficiency of steroid sulfatase results in the scaly skin disease, recessive X-linked ichthyosis [33,34]. For these reasons the hydrolysis of cholesteryl sulfate is believed to play a major role in the normal desquamation of corneocytes from the surface of the stratum corneum. It is possible that cholesteryl sulfate is involved in interlamellar adhesion, with its hydrolysis resulting in a loosening of these attachments. Since it has been shown that sheets of lipid bilayers can be made in vitro from stratum corneum lipids without cholesteryl sulfate [35], this hydrolysis need not necessarily result in disruption of the intercellular lamellar structure. It would, however, render the cells on the skin surface susceptible to the external abrasive forces which are ultimately responsible for corneocyte shedding.

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