Preparation of Liposomes from Stratum Corneum Lipids

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Mammalian stratum corneum contains multiple intercellular lipid bilayers that constitute the epidermal water barrier. Unlike all other biologic membranes, the epidermal lamellae do not contain phospholipids, as a result of which the ability of the stratum corneum lipid mixture to form bilayers has been questioned. In the present study, a lipid mixture containing only epidermal ceramides (40%), cholesterol (25%), palmitic acid (25%), and cholesteryl sulfate (10%), approximating the composition of stratum corneum lipids, formed stable, unilamellar liposomes when sonicated at 80°C in buffer containing 100 mM NaCl, 5 mM Tris, and 1 mM EDTA at pH 7.5. The size and form of the liposomes were studied by both freeze fracture and negative staining electron microscopy. Lipid mixtures from which either the palmitic acid or the cholesteryl sulfate were omitted were still capable of forming similar liposomes, but a mixture of ceramides and cholesterol, or ceramides alone, were incapable of forming liposomes. The results indicate that lipid mixtures similar to those found in stratum corneum are capable of forming bilayers at physiologic pH. J Invest Dermatol 87:582–584, 1986

Mammalian stratum corneum contains multiple intercellular lipid membranes that are believed to constitute the epidermal barrier to water permeation [1,2]. The presence of these bilayers implies the presence of amphipathic lipids capable of appropriate orientation in the presence of water [3]. In all cellular and intracellular membranes, such bilayer-forming lipids consist predominantly of phospholipids [4]. However, stratum corneum has been shown to be virtually devoid of phospholipids [1,5], as a result of which its ability to form bilayers has proved somewhat surprising. In order to establish that stratum corneum lipids can indeed form bilayers, Gray and White [6] successfully prepared liposomes from a mixture of cholesterol, ceramides, fatty acid, and glucosylceramides, appropriately dispersed in buffer. This apparently was the first report of stable bilayers formed without phospholipids. Landmann [7] has since confirmed the observation, having reported multiple discrete bilayers from a similar mixture of lipids. However, stratum corneum does not contain glucosylceramides, and being the most polar component of the mixture used by Gray and White, these may have made a major contribution to bilayer formation. In fact, Landmann [7] was unable to prepare lamellar structures when glycolipid was omitted from his lipid mixture. We therefore have reinvestigated the bilayer-forming capability of stratum corneum lipids, using only those lipids whose presence in the fully cornified epidermis has been established. In addition, a variety of lipid mixtures was evaluated for bilayer-forming capacity in order to establish the minimum requirements for this activity.

MATERIALS AND METHODS

Lipids Ceramides were isolated by preparative thin-layer chromatography from total lipid extracts of full-thickness pig epidermis as described previously [8]. Palmitic acid (reagent grade) was obtained from Fisher Scientific Co. (Springfield, New Jersey) and cholesterol from Sigma Chemical Co. (St. Louis, Missouri). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically.

Preparation of Liposomes Individual lipids were dissolved in chloroform:methanol, 2:1, and appropriate volumes were combined to obtain several mixtures, as shown in Table I. Mixture A was chosen as a close approximation to the composition of stratum corneum lipids, without the small proportions of triacylglycerols, cholesteryl esters, and minor polar constituents. The remaining mixtures maintained the relative amount of ceramides to cholesterol as palmitic acid or cholesteryl sulfate or both were omitted. Aliquots of the lipid mixtures were then placed in culture tubes and the solvent was removed with a stream of nitrogen and finally under high vacuum at room temperature.

To determine the lipid phase transition temperature, proton magnetic resonance spectra were obtained at temperatures ranging from 25°–90°C for a sample of mixture A dispersed in 100 mM saline. Spectra obtained above 75°C showed relatively sharp peaks in the aliphatic region (δ = 1.2 ppm), while the spectra obtained at lower temperatures were broad and featureless. This

Table I. Composition of Lipid Mixtures Evaluated for Liposome Formation (wt%)

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Ceramides</th>
<th>Cholesterol</th>
<th>Palmitic Acid</th>
<th>Cholesteryl Sulfate</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>40.0</td>
<td>25.0</td>
<td>25.0</td>
<td>10.0</td>
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<tr>
<td>B</td>
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<tr>
<td>D</td>
<td>61.5</td>
<td>38.5</td>
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</table>
Figure 1. Liposomes from mixture A. A, Freeze-fracturing; B, negative staining. Bar represents 100 nm. The direction of shadowing is indicated by the arrowhead.

Figure 2. Freeze-fracturing (A) and negative staining (B) of liposomes from mixture B.

Figure 3. Freeze-fracturing (A) and negative staining (B) of liposomes from mixture C.
Electron Microscopy

**Freeze fracture:** Liposome suspensions were placed on thin copper specimen carrier plates and frozen in liquid propane at −190°C. Freeze-fracturing was carried out in a Balzers 301 apparatus (Balzers AG, Balzers, Lichtenstein), and the specimen was shadowed with platinum and coated with carbon. The replicas were then coated with a support film of Parlodion applied in amyl acetate and air-dried before the copper carriers were dissolved by floating in an acid mixture (orthophosphoric/sulfuric/glacial acetic, 1:1:1). The replicas were then washed in distilled water, cleaned in Clorex bleach for 2–3 h, and rinsed several times in distilled water before being picked up on Formvar-coated grids. The Parlodion support film was dissolved by standing in methanol for 30 min. The cleaned replicas were examined in a Hitachi H-600 transmission electron microscope operating at 75 kV.

**Negative Staining:** Formvar-coated grids stabilized with carbon were made hydrophilic by glow discharge and floated on the liposome suspension for 30 s. After rinsing with 3 exchanges of Tris (10 mM Tris, 10 mM NaN₃, 1 mM MgSO₄, pH 7.4), the preparations were negatively stained with 2% uranyl acetate.

**RESULTS**

The lipid mixture representing total stratum corneum lipids formed liposomes in the size range 20–150 nm. Both freeze fracture (Fig 1A) and negative staining (Fig 1B) showed these liposomes to be exclusively unilamellar. Similar liposomes were formed by mixture B which contained no cholesteryl sulfate (Fig 2A,B) as well as by mixture C which contained no free fatty acids (Fig 3A,B). These liposome suspensions were very stable and did not form any sediment even after several weeks of incubation at 37°C. The mixture containing neither free fatty acids nor cholesteryl sulfate did not form liposomes and neither did ceramides alone (not shown).

In a separate study, we found that stable unilamellar liposomes can be formed by a mixture similar in composition to mixture A but using commercial ceramides (obtained from Sigma Chemical Co.) instead of epidermal ceramides. This preparation formed very little sediment even after centrifuging at 100,000 g at 0°C for 2 h, indicating the stability of these liposomes.

**DISCUSSION**

The present results establish that stratum corneum lipids can form stable lipid bilayers even in the absence of the additional polarity bestowed by glycosylceramides. The observation is important to the understanding of the epidermal barrier, since existing knowledge did not support the proposal that lipid lamellae could be formed from the array of lipids available in the stratum corneum. Although the ceramides have 1 nitrogen and 3–5 oxygen atoms per molecule, these appear to be insufficient for bilayer formation, perhaps because they are not ionized. It is clear that, under the conditions employed, either free fatty acids or cholesteryl sulfate, which are ionized at physiologic pH, will provide for bilayer formation. However, the pH that obtains in the stratum corneum is probably rather low, and perhaps only cholesteryl sulfate is adequately ionized under those conditions.

In a previous study [9], phosphatidylcholine-cholesterol liposomes were used as a model system for studying the interactions of glycolipids with lipid bilayers. The acylglycosylceramide, which normally is associated with lamellar granules [10], was found to uniquely promote the aggregation of lipid bilayers to form structures resembling the stacks of disks that are seen within the lamellar granules in vivo. This observation strengthened the argument that the acylglycosylceramide functions in assembly of the lamellar granules. The present observations open additional avenues for investigation of the properties of the stratum corneum membrane system. The liposome model should prove useful for elucidating the factors which promote the fusion of the flattened vesicles extruded into the extracellular spaces of the stratum corneum to form the broad sheets that are observed. Also, liposomes could be used to examine the effects of specific lipid substitutions or structural modifications on physical properties of the membrane such as morphology, phase transition temperature, and permeability. This model may provide an in vitro means of screening agents of pharmaceutical or cosmetic interest for their ability to interact with or favorably modify the properties of the permeability barrier.

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**REFERENCES**
