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Interaction between corneocytes and stratum corneum lipid liposomes in vitro

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Small unilamellar vesicles were made from a mixture of epidermal ceramides (45%), cholesterol (35%), free fatty acids (15%) and cholesteryl sulfate (5%). Isolated corneocytes prepared from pig epidermis were added to the liposomes and the interaction between corneocytes and liposomes was studied by (1) thin-section electron microscopy and (2) monitoring the release of aqueous contents of the vesicles by following the fluorescence intensity of carboxyfluorescein entrapped in the vesicles. The vesicles adsorbed readily onto the corneocytes and slowly transformed into lamellar sheets. Enhanced fluorescence intensity indicated a corneocyte-induced membrane fusion process that resulted in the release of aqueous contents of the vesicles. The results suggest a cohesive role for the corneocyte cell envelope, which consists of a monomolecular layer of lipids covalently bound to the outside of a cross-linked protein envelope. This may be one of the major factors in the reassembly of extruded membranous disks into lamellar sheets which occurs during the final stages of epidermal differentiation.

Introduction

The stratum corneum (SC) of the mammalian epidermis consists of keratinized cells embedded in an extracellular matrix of multiple lipid lamellae [1]. These intercellular membranous sheets of the SC constitute the epidermal water barrier [2] and are known to originate from the lamellar bodies found in the cells of the granular layer of the epidermis [3,4]. The lamellar bodies contain stacks of membranous disks which appear to be flattened vesicles [4]. After their discharge into the intercellular space, the disks are reassembled to form the intercellular lamellar sheets of the SC. During this process, the lipids undergo drastic biochemical changes as the cells progress from the granular layer to the horny layer, and unlike other biological membranes, the SC lipid lamellae have been shown to be virtually devoid of phospholipids [5]. Recently, we have demonstrated the ability of relatively non-polar lipid mixtures (ceramides, cholesterol, free fatty acids and cholesteryl sulfate) of the SC to form liposomes in vitro [6] and investigated some of the factors that cause the fusion of these vesicles into lamellar sheets [7–9].

Interactions of liposomes with a range of biological membranes and cell types have been widely studied in cell biology to provide models for membrane-mediated biophysical processes such as fusion and cell-cell adhesion [10–13]. The recent discovery in our laboratory of a chemically bound lipid envelope surrounding corneocytes [14] suggested a cohesive interaction between the lipid envelope and the intercellular lipid lamellae. In the present study we investigated whether the interaction between corneocytes and SC lipid liposomes may have a role in promoting transformation of the extruded lamellar disks into the intercellular lamellae of the stratum corneum.

Materials and Methods

Lipids. Epidermal ceramides were isolated by preparative thin-layer chromatography from total lipid extracts of pig epidermis as described previously [15]. A mixture of free fatty acids was prepared by combining carnauba wax fatty acids [16] with palmitic acid to a ratio of 8 : 2 by weight, forming a mixture close to the distribution of free fatty acids in stratum corneum [17]. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically.

Abbreviations: CF, carboxyfluorescein; SC, stratum corneum; SUV, small unilamellar vesicle; TMA, trimethylamine.

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Corneocytes. Corneocytes were prepared from pig SC as described previously [18]. Pieces of epidermis were obtained from freshly killed pigs by heating the skin to 60–65°C for 1 min. The epidermis was then peeled off and digested in 0.5% trypsin (Sigma Chemical Co., St. Louis, MO, Type III) in phosphate-buffered saline (PBS) at pH 7.5 at 4°C overnight. After rinsing in distilled water, the tissue pieces were treated with fresh trypsin/PBS solution for 2 h at room temperature. Pieces of SC thus obtained were washed in distilled water and treated with a detergent solution containing 8 mM *N,N*-dimethyldodecylamine oxide (Procter and Gamble, Cincinnati, OH) and 2 mM sodium dodecyl sulfate (Mallinckrodt, Paris, KY) in PBS at 45°C for 24 h. SC sheets disintegrated into a mixture of individual corneocytes and empty corneocyte envelopes that were separated by centrifugation in cesium chloride solution of density 1.28 [18]. The corneocytes were washed in ethanol to remove the detergents, resuspended in 1 mM sodium azide and stored at 4°C until further use.

Preparation of liposomes. Appropriate volumes of solutions of individual lipids in chloroform/methanol (2:1, by vol.) were combined to obtain a mixture containing 45% by weight of ceramides, 35% cholesterol, 15% free fatty acids and 5% cholesteryl sulfate. Liposomes were prepared from these lipid mixtures in an aqueous buffer containing 100 mM NaCl, 5 mM Tris, 1 mM NaN_3 and 1 mM EDTA by sonication, as described elsewhere [6]. The total amount of lipid in the suspension was 5 mg/ml. The final pH of the dispersions was adjusted to 7.5 by dialysis. In the fusion assay experiments liposomes were prepared in a buffer containing 100 mM carboxyfluorescein (CF), 5 mM trimethylamine (TMA) and 0.5 mM EDTA. Vesicles were separated from nonencapsulated CF by gel filtration on Sephadex G-50. The elution buffer contained 5 mM TMA and 0.5 mM EDTA and was at pH 9, same as that of the liposome suspension. The final pH of the eluted suspensions were adjusted to pH 7.5 by dialysis.

Interaction of liposomes with corneocytes. Corneocytes were added to the liposomes as a suspension in Tris buffer at 37°C to a final concentration of 10 mg/ml. Dry weight of the corneocytes was determined after drying an aliquot of the aqueous cell suspension. The corneocytes were kept in suspension by bubbling nitrogen at the rate of 0.1 ml per min. All the suspensions were maintained at 37°C until analysis by electron microscopy. For fusion assay experiments, corneocytes were added as a suspension in TMA buffer and incubated at 37°C for 1 h in the cell used for fluorescence measurement and the corneocytes were kept in suspension by stirring. The final concentration of the suspension was 1.5 mg of lipid and 1 mg of corneocytes per ml.

Electron microscopy. Control liposomes were analyzed by freeze-fracture electron microscopy as described previously [6]. The corneocyte-containing dispersions were analyzed by thin-section electron microscopy 1 h, 24 h and 1 week after the addition of corneocytes. The dispersions were centrifuged in a microfuge at $10000 \times g$ and the wet pellets were fixed in 0.2% RuO_4 in cacodylate buffer for 15 min at room temperature. The fixed pellets were preembedded in agar, dehydrated in graded acetones, embedded in Spurr's resin and sectioned. Silver-gold sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-7000 electron microscope operating at 75 kV.

Fusion Assay. The release of vesicle contents to the external medium induced by the corneocytes was measured by monitoring fluorescence intensity of carboxyfluorescein (CF), encapsulated initially at a high self-quenching concentration in the vesicles. When the entrapped CF is released to the outside medium, CF is diluted and the fluorescence is directly proportional to the concentration of CF [19,20]. Fluorescence was measured in an Aminco-Bowman fluorometer using excitation and emission wavelengths of 492 nm and 580 nm, respectively. Fluorescence was measured before and after the disruption of the vesicles by the addition of Triton X-100 (0.2% v/v). CF-containing vesicles were incubated at 37°C with corneocytes for 1 h and fluorescence was measured before and after the addition of Triton. The vesicles were also incubated at 37°C with the supernatant buffer from the corneocyte suspension for 1 h and fluorescence was measured before and after the addition of Triton. Relative fluorescence intensities were corrected for dilution after the addition of cell suspensions and TX-100. CF concentrations were determined from calibration curve generated from fluorescence intensity measurements of CF solutions prepared in TMA buffer at pH 7.5.

Results

Fig. 1 shows a freeze-fracture micrograph of control liposomes at pH 7.5. These liposomes were small, unilamellar and ranged in size from 20 to 200 nm. The liposomes remained stable for several weeks. Figs. 2A and 2B show the thin-section electron micrographs (TEMs) of corneocytes prepared from pig epidermis. Fig. 2A shows isolated corneocytes as well as some in contact with each other. Fig. 2B shows two corneocytes in contact with each other. The cell envelope consists of a protein envelope seen as a broad electron-dense band; outside of this is an electron-lucent band corresponding to the covalently linked lipids [14]. Corneocytes in contact are seen to be separated by two electron-lucent bands, representing their respective lipid envelopes in contact with each other. Figs. 3A–3D show the TEMs of corneocytes that were added to the liposome dispersion. Fig. 3A shows small unilamellar vesicles (SUVs) adsorbed on to the corneocyte lipid envelope, while Fig.

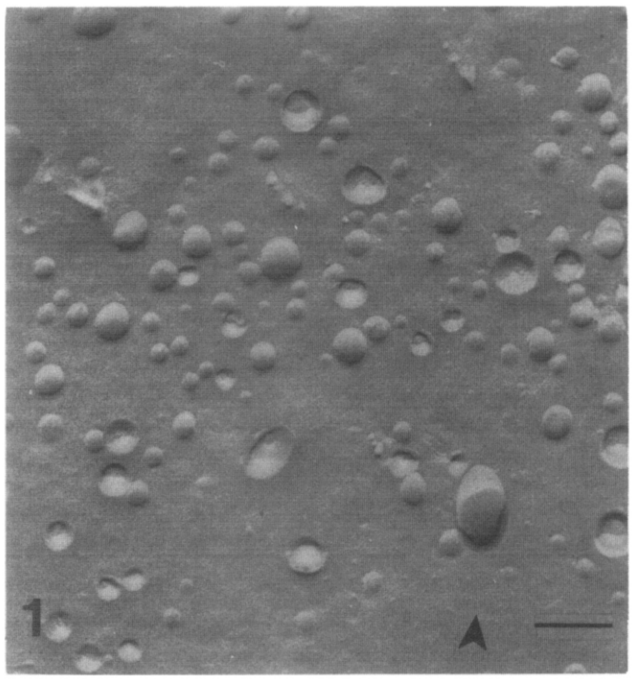
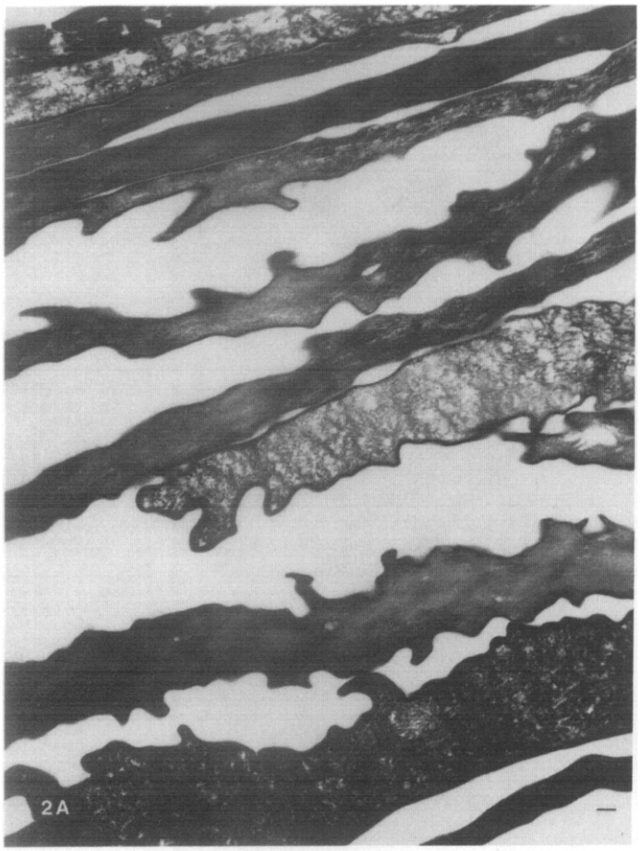


Fig. 1. Freeze-fracture electron micrograph of control liposomes. Arrowhead indicates the direction of shadowing. Bar = 200 nm.

3B shows regions of multiple lamellae similar to those seen in the intercellular region of natural SC. Fig. 3C shows two corneocytes with their intercellular region filled with multiple lamellae formed from the added liposomes. There were regions where the SUVs were adsorbed onto fragments of cell envelope, as seen in Fig. 3D. There were some isolated SUVs that remained stable and unadsorbed even after a week of incubation with corneocytes at 37 °C (not shown).

Release of carboxyfluorescein from SC lipid vesicles under various conditions is shown in Table I. Fluorescence obtained after lysis of the vesicles with Triton X-100 was taken as the value for 100% release. Initial fluorescence of the vesicles was taken as 0% release, which was 50 to 60% of the maximal value, indicating a high initial release of CF from these vesicles. The high initial release of the aqueous contents is due to the history of the vesicles. It was found necessary to use a high pH (≥ 9) during sonication to facilitate the dispersion of these relatively nonpolar lipids in the presence of large quantity (100 mM) of CF. The vesicles were then dialyzed to pH 7.5 after the removal of nonencapsulated CF. Amount of CF encapsulated at pH 9 and 7.5 were found to be 3.77 and 1.28 nmol/μmol of



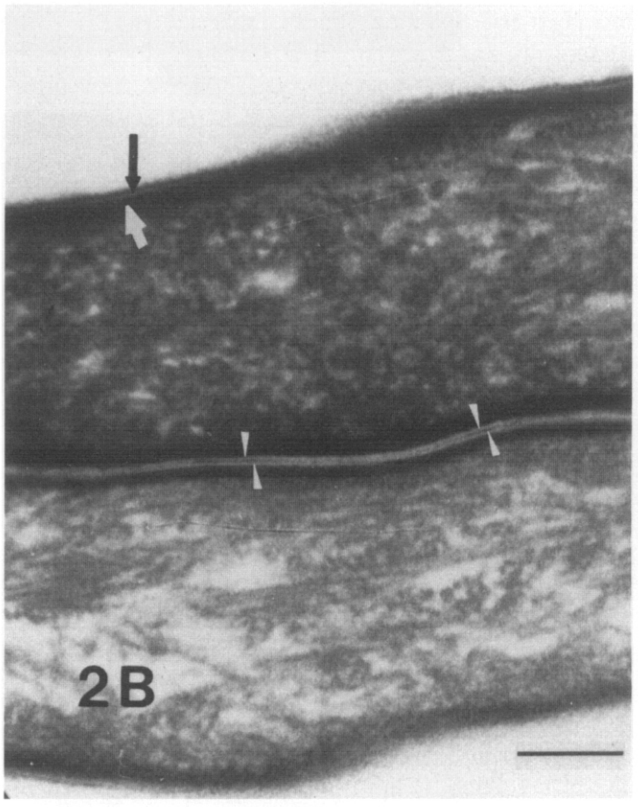
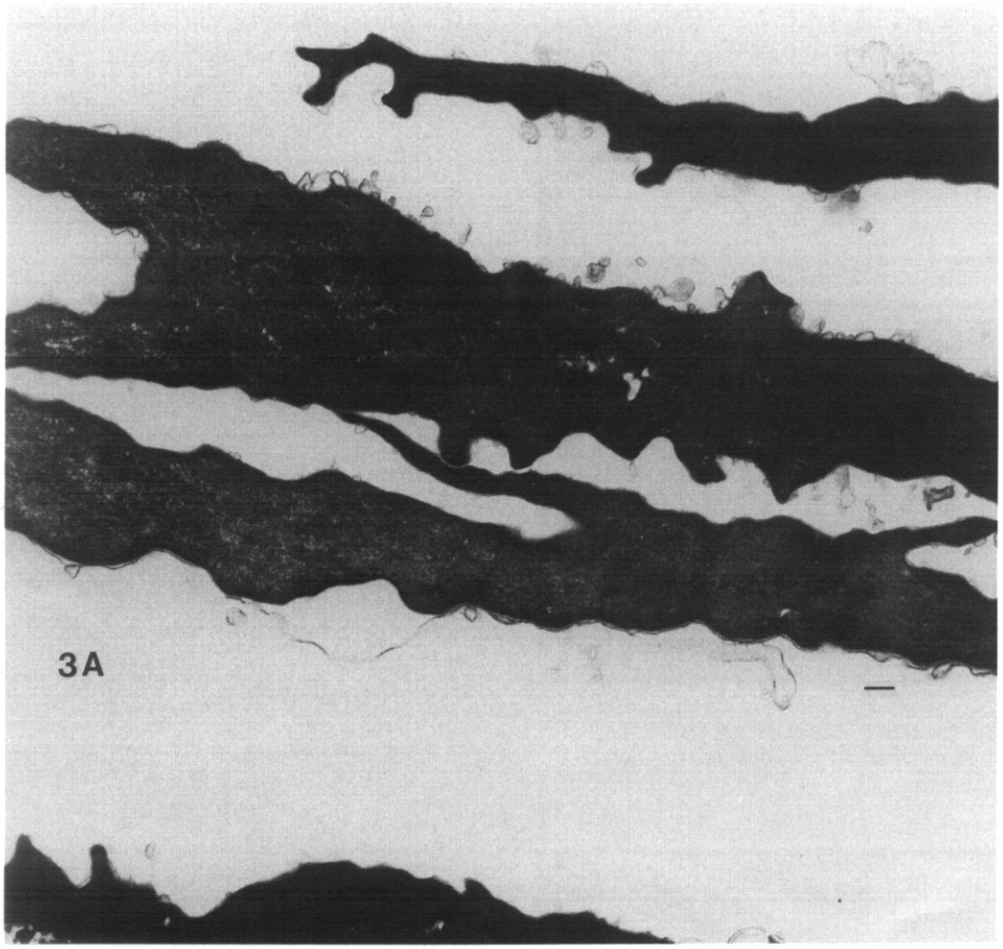
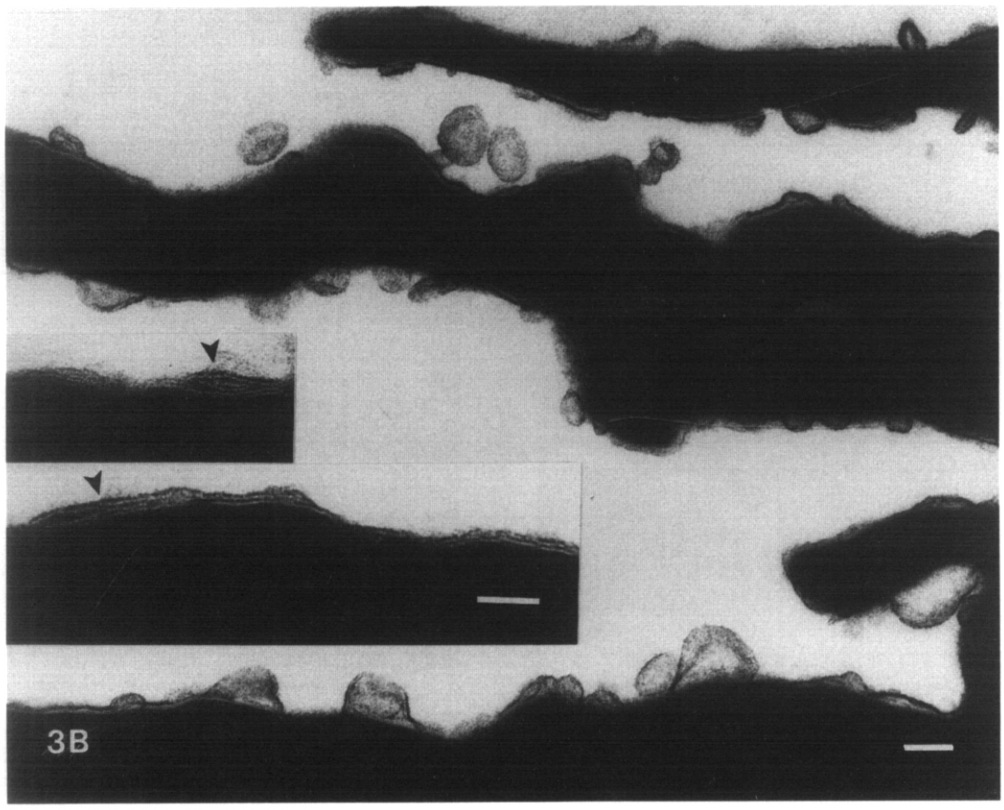


Fig. 2. Thin-section electron micrographs of control corneocytes. The corneocytes were fixed in ruthenium tetroxide and embedded in Spurr's resin. The sections were stained in uranyl acetate and lead citrate. (A) Bar = 200 nm. (B) White arrow shows the protein envelope. Black arrow shows the lipid envelope. Arrowheads show the lipid envelopes from adjacent corneocytes in contact with each other. Bar = 100 nm.


3A


3B

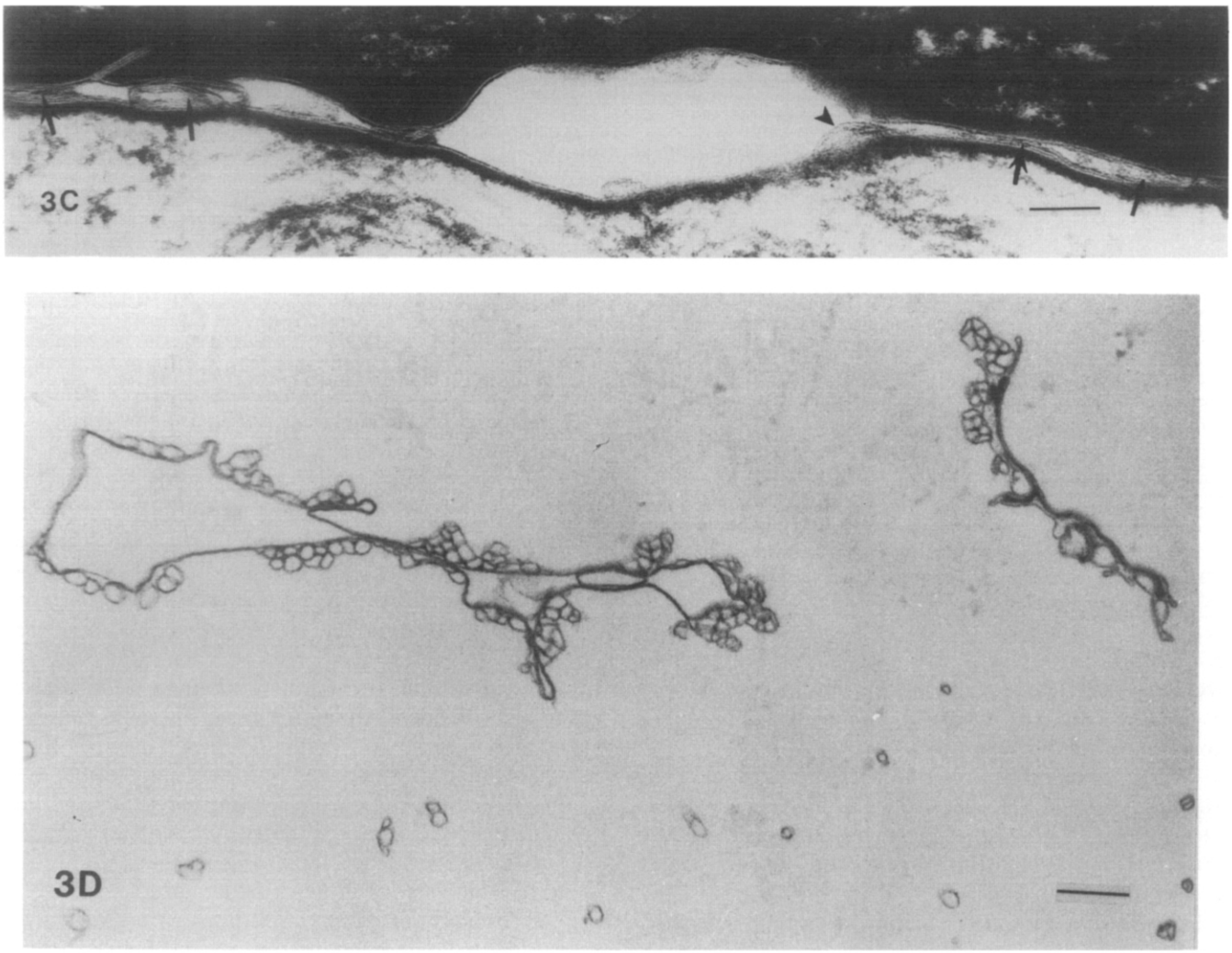


Fig. 3. Thin-section electron micrographs of corneocytes added to the liposomes. Incubation time was 1 h. (A) The liposomes are seen adsorbed onto the corneocytes. Bar = 200 nm. (B) Liposomes are seen at different stages of transformation. Bar = 100 nm. Inset shows multiple lamellae formed from the adsorbed liposomes (arrowheads). Bar = 50 nm. (C) Arrows show the intercellular region filled with multiple lamellae formed from the adsorbed liposomes. Arrowhead shows remnants of a partially formed lamella from the adsorbed liposome. Bar = 100 nm. (D) Liposomes are seen adsorbed onto the exterior of empty cell envelopes. Bar = 500 nm.

lipid, respectively, as shown in Table I. Incubation of the vesicles with supernatant buffer from the corneocytes suspension resulted in some release of entrapped CF (10–12%) while incubation with corneocytes under similar conditions resulted in enhanced release of CF (35–40%) as shown in Table I.

Discussion

Liposomes formed from SC lipids were extremely stable at pH 7.5. The high curvature of these SUVs, the repulsive hydration forces, and the electrostatic forces of the charged headgroups (of the partially ionized fatty acids and the completely ionized cholesteryl sulfate) apparently are sufficient to prevent them from undergoing any fusion or aggregation.

The corneocyte cell envelope has been thought of as a cross-linked protein envelope beneath the horny cell 'plasma membrane' [21,22]. These are usually seen as in Fig. 2B, respectively, as a broad electron-dense band and a narrow electron-lucent band in TEMs. Recently, the electron-lucent band has been shown to consist of a monomolecular layer of lipids, predominantly made up of ω-hydroxyacylsphingosines, that are covalently bound to the protein envelope [14]. These corneocyte lipid envelopes have been proposed to have a cohesive role in regions of cell-cell contact, especially in SC sheets that have been depleted of the intercellular lamellae by extensive extraction with chloroform/methanol [14], as well as in SC that was heated above the 75 °C transition temperature of the SC lipids (unpublished observations). The corneocyte lipid envelopes from contiguous

TABLE I

Release of carboxyfluorescein from SC liposomes at 37°C

	CF released (nmol/μmol of lipid)	
	during incubation with cells/supernatant [a]	after lysis with Triton [b]
Vesicles at pH 7.5		1.28 ± 0.19
Vesicles at pH 9.0		3.77 [c]
Vesicles incubated for 1 h with corneocytes at pH 7.5	0.44 ± 0.06	1.23 ± 0.18
Vesicles incubated for 1 h with supernatant (buffer) at pH 7.5	0.16 ± 0.03	1.45 ± 0.18

[a] Calculated as the difference between the amount of CF released from the vesicles after 1 h of incubation with cells/supernatant and the amount of CF released before the addition of cells/supernatant (initial release).

[b] Calculated as the difference between the amount of CF released after the addition of Triton and the amount of CF released before the addition of cells/supernatant and Triton (initial release).

[c] Mean of two measurements. $n = 4$ for rest of the data.

corneocytes are seen as two lucent bands in regions where isolated corneocytes have come in contact with each other (Fig. 2B), supporting a cohesive role for the corneocyte lipid envelope.

When corneocytes were added to the liposomes, the vesicles adsorbed onto the surface of the corneocytes as seen in Fig. 3A. The liposomes remained adsorbed even after the washing and rinsing procedures during sample preparation for electron microscopy, indicating the stability of the adsorption. Stable adsorption of phospholipid liposomes to different cells has been demonstrated by a variety of techniques [10]. Such adsorption may be mediated either by biochemical forces (surface receptors, antibodies, etc.), by purely physical forces (electrostatic, hydration, hydrophobic, etc.) or by a combination of both types of forces.

SC has until recently been thought to be devoid of surface receptors such as lectins [23,24]. Recently, a 40 kD a glycoprotein has been isolated and has been localized to the corneocyte lipid envelope [25]. Brysk et al. have suggested that this endogenous lectin could play a major role in the cell cohesion in SC [26]. However, the same authors have shown that the membrane glycoproteins are solubilized by the nonionic detergent used in the corneocyte preparation [26]. Thus, in corneocytes prepared by detergent treatment of SC, it is reasonable to assume that it is purely a physical force that causes the cell-cell cohesion, as well as the adsorption of SC lipid liposomes on the surface of the corneocytes.

The amount of CF released after the incubation of vesicles with corneocytes is about three times that caused by the incubation of the vesicles with the buffer alone, suggesting a cell-induced process which results in an enhanced release of the aqueous contents. Adsorption of vesicles onto the corneocytes would not render the vesicles leaky. Enhanced release of CF from vesicles treated with corneocytes suggests a fusion process that renders the vesicles more leaky, presumably resulting from the collapse of the vesicles during or after the fusion process. CF release data along with the appearance of lamellae on corneocytes treated with SC lipid liposomes, as seen in Figs. 3B and 3C, suggests the formation of these lamellae from the adsorbed liposomes by a membrane fusion process. While some of the adsorbed liposomes transform to lamellae within the first hour of incubation, this transformation appears to be an inefficient process judging by the large number of liposomes that remained adsorbed on the corneocytes even a week after the addition of corneocytes to the liposomes. This is not very surprising in the absence of fusogens that are known to promote aggregation and fusion of SC lipid liposomes [7–9], which include calcium [7], epidermal acylceramides and acylglucosylceramides [8,9], low pH (< 6.5) and drying [27]. Presence of large number of adsorbed liposomes and corneocyte surfaces covered with lamellae and time independence of the relative amounts of corneocyte surface with adsorbed liposomes versus cell surface with lamellae suggests an alternative mechanism of formation of lamellar structures by direct transformation of unadsorbed vesicles occurring concomitant to and independent of adsorption process. We do not have any experimental evidence to distinguish between the two mechanisms of formation of the lamellae.

The adsorption of SC lipid liposomes onto corneocytes and their transformation into lamellar sheets in the absence of any of these known fusogens indicates an independent role for the corneocyte lipid envelope in these processes. Two plausible mechanisms can be postulated for the transformation of SUVs into lamellar sheets on the basis of the appearance of these systems in TEM. After initial adsorption of the liposomes, there could be a transfer of lipids from the liposomes to the corneocyte lipid envelope. Such a transfer can be facilitated through transient intermediary structures like mixed micelles that can be formed between partially ionized fatty acids and other lipid components, or through a direct transfer of lipids at the region of adsorption. This would lead to incorporation of part of the vesicle bilayer into the lipid envelope. This may also be accompanied by fusion of the adsorbed liposomes with each other while they are immobilized by adsorption and are in close proximity with each other. This would lead to the collapse of one layer of adsorbed liposomes and their transformation into a bilayer adjacent to the cell envelope, seen as two electron lucent bands, as in Fig. 3B.

In regions where multilamellar structures are seen attached to the corneocyte lipid envelope (Figs. 3B and

3C), the fusion mechanism may involve flattening of the adsorbed and/or unadsorbed liposomes and their subsequent edge-to-edge fusion to form multilamellar structures. Corneocytes, which are flat and elongated, with the molecularly smooth lipid envelope on their surface would act as a template for the initial flattening and for the subsequent transformation of flattened vesicles into extended lamellar structures. It should be informative to see if SC lipid liposomes are adsorbed and/or lamellar structures are formed on delipidized corneocytes. However, the alkaline hydrolysis procedure used for the removal of the ester-linked ω-hydroxyceramides resulted in completely delipidized corneocytes in which the protein envelopes were partially separated from the cell contents [14]. These delipidized corneocytes disintegrated easily and could not be used for any further study.

The observations made in the present study suggest a cohesive role for the corneocyte lipid envelope in the transformation of SC lipid liposomes into lamellar sheets. This role may be one of the major factors in reassembly of the extruded membranous disks into lamellar sheets which occurs in the intercellular space between the upper granular layer and the horny layer during the final stages of epidermal differentiation [3,28].

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