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Effect of epidermal acylglucosylceramides and acylceramides on the morphology of liposomes prepared from stratum corneum lipids

William Abraham, Philip W. Wertz and Donald T. Downing

Marshall Dermatology Research Laboratories, Department of Dermatology, University of Iowa College of Medicine, Iowa City, IA (U.S.A.)

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Epidermal acylglucosylceramides (AGC) and acylceramides (AC) cause aggregation and stacking of stratum corneum lipid liposomes formed from a lipid mixture containing epidermal ceramides (40%), cholesterol (25%), palmitic acid (25%), and cholesteryl sulfate (10%). This demonstrates the ability of these sphingolipids to hold adjacent bilayers in close apposition and their roles in the assembly of lamellar structures in the epidermis. However, AGC and AC in their hydrogenated form also caused aggregation and stacking of the stratum corneum lipid liposomes. This throws into doubt the proposed structural specificity of linoleate in the function of AGC and AC as molecular rivets in the assembly of the epidermal lamellar granules and the stratum corneum intercellular lamellae, respectively.

Introduction

The stratum corneum of mammalian epidermis contains multiple intercellular lipid lamellae [1,2], which consist principally of ceramides (40%), cholesterol (25%), free fatty acids (25%) and cholesteryl sulfate (10%) and trace amounts of unidentified polar components [3,4]. Unlike other biological membranes, those in the stratum corneum have been shown to be virtually devoid of phospholipids. Although the structure and function of the intercellular lamellae are being elucidated, the roles of the individual lipid components in the formation of these bilayer structures remain unknown. The intercellular lamellar sheets of the stratum corneum constitute the epidermal water barrier [5] and are known to originate from the lamellar bodies found in the cells of the granular layer of the epidermis [2,6]. The lamellar bodies discharge their lipid contents as membranous disks into the intercellular space between the granular and the horny layers. These membranous disks are then reassembled to form the intercellular lamellar sheets of the stratum corneum by membrane fusion processes of unknown mechanisms. It has been suggested that acylceramides (AC) found in the stratum corneum have a structure-specific function in anchoring together the adjacent bilayers found in the intercellular space of the stratum corneum [7]. A similar function has been suggested for the structurally similar epidermal acylglucosylceramides (AGC), which are thought to function as molecular rivets in assembly of the epidermal lamellar granules through the stacking of lamellar disks in the granular layer [8]. Both the AC and AGC are unusual in containing a high proportion of linoleic acid esterified to 30- and 32-carbon ω-hydroxy acids that are amide-linked to sphingosine bases [7,9].

In vitro, epidermal AGC have been shown to

Correspondence: W. Abraham, 270 Medical Laboratories, University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A.

cause flattening and stacking of phospholipid liposomes [10]. Furthermore, AGC and AC have been shown to cause aggregation and fusion of preformed liposomes prepared from stratum corneum lipids, with an added impetus from osmotic forces (unpublished data). In the present study we investigated the effect of these two classes of sphingolipids (AGC and AC) on the morphology of stratum-corneum-lipid liposomes. We also investigated the effect of hydrogenating the double bonds in these sphingolipids on the morphology of the stratum-corneum-lipid liposomes in order to determine the specific role of the ester-linked unsaturated fatty acids (linoleic acid in particular) in the assembly of the lamellar structures.

Materials and Methods

Ceramides were isolated by preparative thin layer chromatography from total lipid extracts of full thickness pig epidermis as described previously [7]. Palmitic acid (reagent grade) was obtained from Fisher Scientific Co. (Springfield, NJ) and cholesterol from Sigma Chemical Co. (St. Louis, MO). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically.

Individual lipids were dissolved in chloroform/methanol (2 : 1, by volume), and appropriate volumes were combined to obtain a mixture containing 40% by weight of ceramides, 25% cholesterol, 25% palmitic acid and 10% cholesteryl sulfate. Liposomes were prepared from these lipid mixtures in an aqueous buffer containing 100 mM NaCl, 5 mM Tris, and 1 mM EDTA (disodium salt) at pH 7.5, as described elsewhere [11]. AGC or AC were added to the liposome suspensions as a dispersion in Tris buffer containing 100 mM NaCl and 1 mM EDTA, to a final concentration

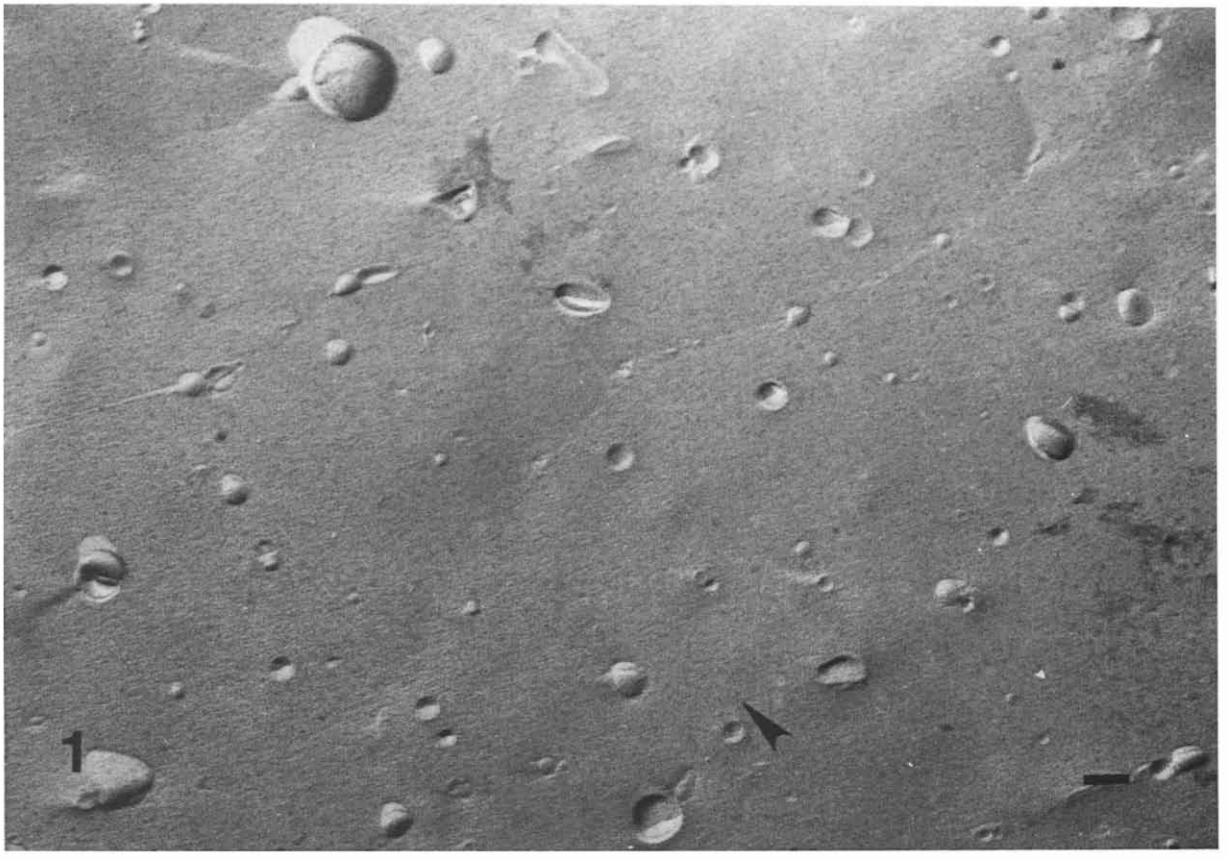

Fig. 1. Freeze-fracture micrograph of control liposomes formed from a mixture of 40% epidermal ceramides, 25% cholesterol, 25% palmitic acid and 10% cholesteryl sulfate. Bar = 100 nm. The arrowhead indicates the direction of shadowing.

of 8% by weight of the total lipid, at 80 °C. AC did not disperse readily in the aqueous buffer, perhaps due to the difficulty in hydration of their less polar headgroup. All the dispersions were incubated at 80 °C for 30 min before lowering the temperature to 37 °C. The preparations were in-

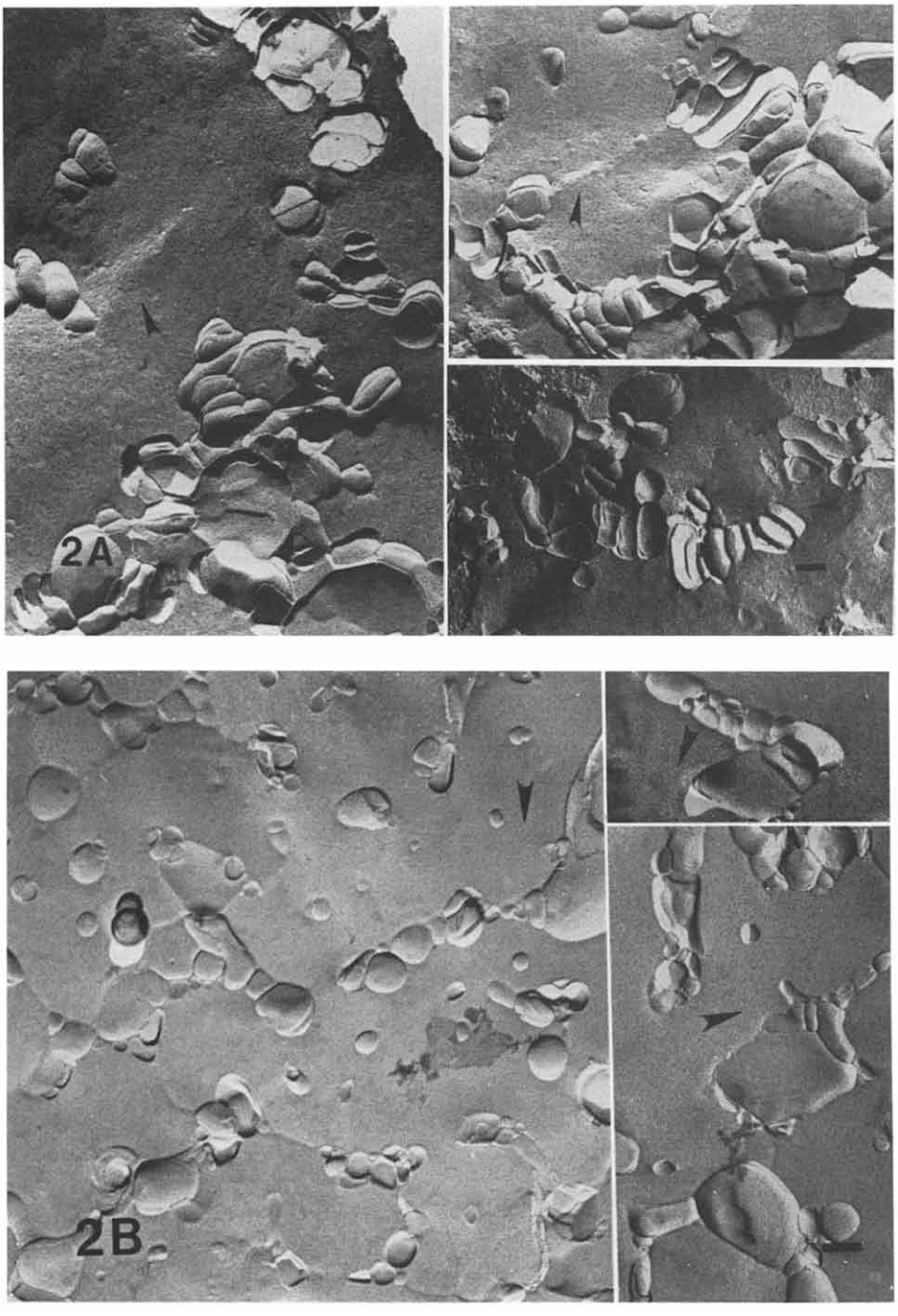

Fig. 2. Freeze-fracture micrographs of liposomes after the addition of AGC in their native (A) and hydrogenated form (B), respectively. Bar = 100 nm.

cubated at 37 °C under nitrogen atmosphere until analyzed by freeze-fracture electron microscopy. In order to observe a representative distribution of liposomes, the dispersions were concentrated by ultrafiltration through a Centricon-30 microconcentrator (30000 M_r cutoff) by centrifuging at 2000 × g. The liposome suspensions were quenched in liquid propane (−190 °C) and

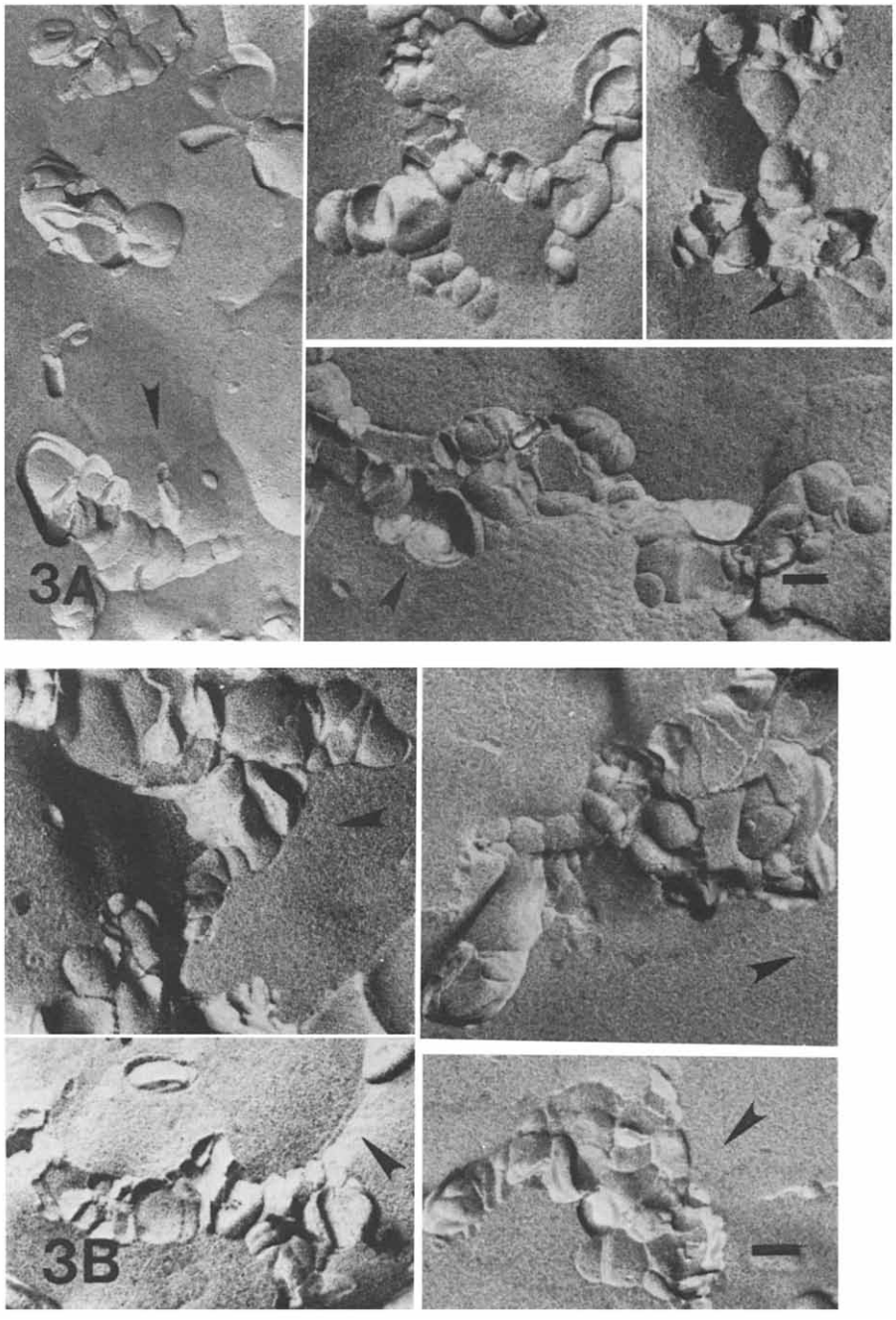

Fig. 3. Freeze-fracture micrographs of liposomes after the addition of AC in their native (A) and hydrogenated form (B), respectively. Bar = 100 nm.

analyzed by freeze-fracture electron microscopy as described previously [11].

Hydrogenation of the double bonds was carried out by bubbling H_2 gas through a solution of the lipid (AGC or AC) in diethyl ether/ethanol/acetic acid (75 : 20 : 5, by volume) over catalytic amounts of PtO_2 (Adam's catalyst) for 30 min. The hydrogenated lipids were purified by preparative thin layer chromatography. The native as well as the hydrogenated AGC and AC were saponified by treatment with chloroform/methanol/10 N NaOH (2 : 7 : 1, by volume) and the liberated fatty acids were analyzed as fatty acid methyl esters by gas chromatography [7]. The major ester-linked fatty acid was linoleic acid (64% in AGC and 38% in AC) and the hydrogenated lipids showed negligible amounts of linoleic acid (less than 1%).

Results

Fig. 1 shows a freeze-fracture micrograph of control liposomes which were small, unilamellar, and range in size from 20 to 200 nm. Figs. 2A and 2B show the liposomes after the addition of AGC and hydrogenated AGC, respectively. Both the native and the hydrogenated AGC caused some fusion of the liposomes as seen by the appearance of large unilamellar vesicles in the size range 30–500 nm, aggregated in several different forms. Liposomes containing native AGC were flattened and stacked together (Fig. 2A). There also were regions showing clusters of partially flattened liposomes and some broad fracture faces indicating lamellar sheet formation. The preparation containing AGC in their native form also showed some non-aggregated unilamellar liposomes (not shown) of varying size. Fig. 2B shows large flattened liposomes clustered together after the addition of hydrogenated AGC. There also were some non-aggregated unilamellar liposomes.

Figs. 3A and 3B show the liposomes after the addition of native and hydrogenated AC, respectively. Many liposomes were larger and were clustered while others were flattened and stacked together in both of the preparations. There also were regions of lamellar sheets resulting from the fusion of these aggregates (Figs. 2A and 3B, for example). Increasing the amount of AGC or AC from 8 to 15% by weight of the total lipid did not cause any significant change in the distribution of the various aggregates as seen by freeze-fracture electron microscopy. In all the preparations containing AGC or AC in their native or hydrogenated form, there were some unilamellar liposomes that remained isolated and non-aggregated even after a week of incubation at 37 °C.

Discussion

The control liposomes made from stratum corneum lipids were extremely stable. Apparently the high curvature of these small unilamellar vesicles and the charged head groups of the component lipids prevent them from undergoing any fusion. When either AGC or AC in their native or hydrogenated form were added to the stratum corneum liposomes, there was an initial fusion, as seen by the increase in the size of the liposomes (Figs. 2A, 2B, 3A and 3B). Thus, there appears to be a fusogenic role common to these sphingolipids independent of their degree of unsaturation.

The observation that AGC or AC in their native form cause stacking of the stratum corneum liposomes (Figs. 2A and 3A) is in accordance with the earlier observations with phospholipid [10]. This supports our earlier hypothesis on the roles of AGC and AC as molecular rivets in the assembly of epidermal lamellar granules and the stratum corneum intercellular lamellae, respectively [7,8]. In the lamellar granules as well as in the intercellular lamellae, the internal surfaces of the bilayers are in close apposition [12]. Such apposition is extremely difficult in our model systems because of (i) the sharp radius of curvature, (ii) the electrostatic repulsion between internal charged head groups, and (iii) the barrier of internal hydration forces that would result from such close apposition.

Both AGC and AC are long enough to span the entire thickness of a bilayer. Thus, when AGC or AC molecules encounter liposomes, the ester-linked fatty acid end of these molecules can anchor in one bilayer while the remaining non-polar region is embedded in an adjacent bilayer in close apposition. This may cause fusion of small unilamellar vesicles to form large unilamellar vesicles. The next step is the stacking and clustering of the

liposomes, brought about by AGC or AC in their native form as well as in their hydrogenated form. This observation is of great significance in view of the fact that the major esterified fatty acid in these sphingolipids is linoleic acid, and throws into doubt the proposed structural specificity of linoleate in the function of AGC or AC as molecular rivets in the assembly of the lamellar granules and the intercellular lamellae, respectively. Linoleic acid, an essential fatty acid, is believed to have a direct role in epidermal barrier function. According to earlier reports, when linoleic acid is absent in the diet, the lamellar granules from mouse skin were found to be empty or only partially filled with membranous disks [13]. Studies performed in our laboratory using essential fatty acid-deficient pigs indicate no such change in the contents of the lamellar granules [14]. However, an increased transepidermal water loss was observed in these linoleate-deficient animals. AGC in the epidermis of linoleate-deficient rats were found to have oleate as the major ester-linked fatty acid [15]. In addition, topical application of linoleic acid has been shown to improve epidermal barrier function locally [16]. These observations, along with the increased epidermal water loss observed in linoleate-deficient animals [17,18], indicate a role for linoleic acid in epidermal barrier function, but the linoleate moiety in the acylsphingolipids does not seem to be essential for the assembly of lamellar structures.

An alternative functional role for these acylsphingolipids has been suggested by Nugteren et al. [19]. These authors suggest a metabolic role rather than a structural role for these linoleic acid-rich lipids, whereby the ability of linoleate to maintain the epidermal barrier function is believed to depend on their conversion to polyoxyacylceramides. Further investigation is necessary to understand the functions of these linoleate-containing lipids in the assembly of the intercellular lamellae that constitute the epidermal barrier.

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