ULTRASTRUCTURE OF FREEZE-CLEAVED STRATUM BASALE
AND STRATUM MALPIGHII

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Abstract. The basal and Malpighian layers of the human epidermis were freeze-cleaved in a high vacuum and the cleaved surfaces were replicated with platinum and carbon. Examination of the replicas showed tonofibrils in various patterns of organization and two distinctly different fractured surfaces of plasma membranes. The A-face at a nexus was characterized by patches of aggregated small particles (100–135 Å) and scattered membrane-associated particles, which were never as abundant as shown in other tissue or cells. The B-face at a nexus showed aggregation or cobblestone-like low particles. Pits were occasionally seen on the B surface but not as commonly as described in other organs. Membrane-associated particles were only sparsely distributed on the B surface.

Except for the studies done on the human cervical epithelium by McNutt & Weinstein (9) and McNutt, Hershberg & Weinstein (10), there are no quotable works in the literature on the human keratinizing epithelia as examined by the method of freeze-cleavage or freeze-etching. The major contribution so far made with this technique seems to be the analysis of membrane structure and cell-to-cell junctions at the ultrastructural level.

In this study, en face views of keratinocyte membrane and nexus (gap junction) were examined. In agreement with the observations made by McNutt & Weinstein (9) and McNutt et al. (10) in the normal cervical epithelium and with the findings of other investigators on red blood cells (12, 15), liver cells (4), and on an experimental model of the cell membrane (3), the cleaved surfaces of many keratinocytes were found to be either face A or face B (see below). This report deals with the surface structures of the freeze-cleaved cytomembranes and nexuses of the human epidermis and their correlation with the images observed in thin sections with the aid of lanthanum.

METHODS AND MATERIALS

Normal skin specimens were obtained from the face, arm, hand, leg, and foot of several adults by 2-4 mm punches under local anesthesia with 1% procaine. In addition, thick epidermis from the lateral aspect of a right ring finger was removed when a friction blister was developed by a screwdriver. Some specimens were sliced and immediately frozen in liquid nitrogen at −170°C. Other specimens were fixed for several hours in 5% glutaraldehyde in 1/10 cacodylate buffer, pH 7.4, rinsed in the same buffer overnight and then processed in the same way as non-fixed tissue. A Denton DFE-2 freeze-etch apparatus attached to a DV-502 evaporator was used throughout. Apposed specimen holders were used to mount the specimen. Specimens were fractured at between −170°C and −200°C and shadowed at a 45° angle with a mixture of platinum and carbon and then coated with carbon at between −120°C and −100°C. The fracture surfaces were either etched by sublimation in vacuum (10⁻⁶ – 6 torr) or not, prior to shadowing. The replicas thus produced were detached from the tissue with 30% Clorox and cleaned in three changes of distilled water. The cleaned replicas were placed on 200-mesh copper grids coated with Formvar and examined in a Hitachi HU-12 electron microscope operated at 125 kV. Apart from the epidermal root or the friction blister was permeated with lanthanum after a brief fixation in 2% glutaraldehyde to which 1% lanthanum nitrate was added. Parts of the other skin specimens were permeated with 1% colloidal lanthanum hydroxide. The details of these methods have been reported previously (5, 6, 7) as a modification of the Revel and Karnowsky technique (13).

Interpretation of freeze-cleaved membrane surface

The plane of the membrane cleavage by freeze-fracture procedures has been controversial: (i) the cleavage plane goes over the real cell surface; (ii) the membrane is split in the interior through a lipid bilayer (1, 12) and thus two new faces are created (9, 10); (iii) both real membrane surface and split membrane surfaces are revealed (14); and (iv) the position is undecided. Cell surface labelling with ferritin failed to demonstrate the ferritin particles on the cleaved surface (12). The real cell surface of erythrocytes as revealed by sublimation of water in which they were embedded is smooth, whereas the fractured surfaces are studded with small particles,
Fig. 1. (a) Cell membranes of two apposed keratinocytes (Lm, Lm,) are separated by the intercellular space (Ics). In the nexus the outer surfaces of two membranes are fused intermittently by projected subunits that cover the contact cylinders (C₁, C₂, C₃, C₄, C₅, C₆, C₇). Broken lines indicate the potential cleavage planes which will split the membranes into Lm₁ and Lm₂ (cf. Fig. 1b). In membrane Lm, contact cylinders are broken at various levels within Lm₂ and, when Lm₁ and Lm₂ are separated, project out as A-face particles (cf. Fig. 1b). Although not shown, a more conventional pattern of cleavage may occur: contact cylinders are pulled out completely from Lm₂, giving rise to very high A-face particles. In membrane Lm₂, contact cylinders are fractured within Lm₁ (C₁', C₂'), within Lm₂ (C₃'), or level with the cleavage plane (C₄').

(b) The cleavage of the membranes Lm₁ and Lm₂ creates Face A and Face B. Extracellular surface is designated Face D and juxtaepithelial surface, Face C. The split membrane Lm₁ exposes fractured contact cylinders as A-face particles, all of which are projected out of Face A, and as pits all indented into Face B: thus, classic Face A particles and Face B pits are produced. The fracture of membrane Lm₂ reveals on Face B low profiles of fractured cylinders, namely cobblestone particles (C₁', C₂'), slightly depressed pits (C₃'), and the area level with the cleavage plane (C₄'). This combination actually occurs in some fractured membranes (inset of Fig. 4). Since the counterpart of this type of fracture shows a similar variety of broken cylinders (Face A of Lm₂), it may be difficult to distinguish Face A from Face B.

i.e., the membrane-associated particles (MAP) (12, 15). The double replica technique produced two distinctly different sets of membrane faces that are complementary (2), instead of producing a set of smooth and symmetrical surfaces which one might have expected if the intact cell surfaces of two apposed cells were revealed (11). Although this problem does not seem to have been settled completely, the membrane-splitting hypothesis of Branton (1) as supported by McNutt & Weinstein (9), Chalcoff & Bullivant (2) and others (10, 12, 15) will be adopted and the terminology developed by this group will be followed. According to their assumption, when the intact membrane is split, the cytoplasmic or inner lamella (Lm₁) gives outwardly directed "face A". The true cell surface, i.e., "face D" is said to be revealed at the nexus and the juxtaepithelial surface, i.e., "face C", can be seen.
Freeze-cleaved epidermis

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RESULTS

General description

Membranes, cytoplasm, and various organelles were replicated (Fig. 2). Bundles of tonofibrils were seen projecting out of the cleavage plane, particularly in those specimens etched slightly by sublimation (Fig. 2). An embossed fibrillar pattern of tonofibrils in parallel, weaving or whorl formation could be visualized (Fig. 2). The fracture surfaces of small as well as large vesicles showed various numbers of membrane-associated particles (MAP) (Fig. 2). At the nexus, oval or bizarre-shaped patches or macules studded with small particles were seen on the fractured membranes (Figs. 2, 3). The en face view of the cytomembrane showed undulation. For example, in Fig. 3 an extensive waving as well as mild rippling are clearly visualized.

Nexus (gap junction)

The configuration of the nexus was variable; it consisted of round to oval macules with great variation, ranging from 0.2 to 0.6 μm in long diameter (Figs. 3, 4). Individual macules could coalesce to form large, bizarre-shaped ones. The nexus could extend to a large area and islands of non-nexus areas could be entirely enclosed (Figs. 3, inset). Particulate aggregates elevated to various heights above the plane of fracture (Figs. 3, 4) and less frequently, similarly arranged pits (Fig. 4, inset) were clearly seen. In some areas particles were arranged in lattices of variable regularity (Fig. 3). Orderly register of particles was better recognized in a low magnification (Fig. 3). The number of MAP was variable, depending on the specimen (Fig. 2 vs. Figs. 3, 4). In general, MAPs were not as many as previously reported (9, 10), even on the A face of many specimens.

Face-A at nexus

Since in many preparations MAPs were not abundant on the A face, and the B face did not show many pits but mostly a “cobblestone” pattern, the only criterion to distinguish the A face from the B face was an abundance of high particles at the nexus that were rather regularly elevated from the plane of fracture (Figs. 4, 5a). Although the B face had a fair number of particles, their number was usually less than 1/3 of the cobblestones and their height varied markedly (Figs. 4, 5a, 6a).

The typical face-A particle at the nexus had a long diameter of 100-135 Å and a height of 50-70 Å. They were round or polygonal. In many nexuses, aggregation of face-A particles was partially covered with face-B cobblestone particles of the apposed keratinocyte membrane (Figs. 4, 5a). Face-A particles were individually similar to scattered MAPs which measured about 100 Å in diameter (Figs. 2, 4, 5a).

Face-B at nexus

Face-B surface, particularly that of the finger skin obtained from the roof of a friction blister, showed very few MAP (Figs. 4, 5a, 6a). Nexus macules on the B Face were usually very well demarcated and either slightly depressed (Fig. 2), level with Lm2, or slightly elevated (Fig. 4). The macule was studded with low and high particles. Low particles outnumbered the high particles. The high particles appeared very similar in size (100-135 Å), height (50-70 Å), and configuration to those on the A surface (Figs. 4, 5a). The low particles were round, oval or polygonal, i.e., square to hexagonal, and showed a great variation of size, ranging from 70 Å to 135 Å (Figs. 4, 5a, 6a). Their height also varied from almost level with the fracture surface, up to 25 Å. Crater-like central depressions, measuring about 25-40 Å, were seen in some particles. Those low particles were compatible with the “cobblestone-type” B face nexus structures, as mentioned by McNutt & Weinstein (9). When shadowed at a high angle, pits, measuring 45-50 Å, were occasionally observed (Fig. 4, inset). Lm2, which carries the B surface, often showed a serrated edge in non-nexus areas (Fig. 4, inset), suggesting that Lm2 is composed of globular subunits.

Tight junctions

No tight junctions were found in this study. If present, they should have been recognized as ridges and grooves juxtaposed to the nexus [7]. It is obvious that the lateral tight junction of the stratum granulosum of the normal skin [7] was not fractured in this study.

Desmosomes

Specialized areas on the B face as shown by McNutt et al. (10) were occasionally observed in the fixed specimens. They consisted of a clustering of small
Fig. 3. A large area of the fractured membrane surface of the basal cells exhibits extensive indulation. In one area, the dermal collagen is seen (C). The fracture revealed both A- and B-faces. At nexuses, the A-face (A) shows a cluster of contact cylinders in well-defined macules occasionally in register, whereas the B-face (B) shows either cobblestone-like low particles (c) or pits (p). In many nexuses, Lm2 with B-face cobblestones is partially peeled off (*) and Lm1s of the apposed cell with A-face contact cylinders are revealed. Finger skin. × 50 000. Inset: two large nexuses, probably formed by confluence of smaller ones. Non-junctional B-face (n-j) is enclosed within one of them. Other labels are the same as in the main figure. Finger skin, × 50 000.
Fig. 4. A higher magnification of face A and face B. Face A (A) has some scattered MAPs (small hollow arrows) and clustered contact cylinder particles (C), whereas, face B (B) is smooth with few MAPs and has cobblestone particles (c) admixed with high particles similar to face A contact cylinders. A good correspondence of configuration of nexuses between face A and face B can be seen where face B is partially lifted (*). It must be mentioned here that between the cobblestone membrane, i.e., Lm2, and the membrane on which A-face particles are situated, i.e., Lm1, there is a tightly apposed intercellular space of nexus (at *) and that Lm2 and Lm1 belong to different cells (see Fig. 1a). Lm2 shows globular subunits even in non-nexus areas (thin, solid arrow in the inset) and often reveals a serrated edge. Finger skin. ×75 000. Inset: finger skin. ×112 500.
Fig. 5. (a) A still higher magnification of the A-face (A) showing contact cylinders (C), with dimensions of 100–135 Å in diameter and 50–70 Å in height. The B-face (b) has mainly cobblestone-type particles (c), 70–135 Å in diameter and 0–25 Å in height, but higher particles (*) similar to contact cylinders are also admixed. Finger skin, x 150 000.

(b) Lanthanum-permeated skin of the arm shows lanthanum-filled intercellular spaces. Nexus (N) which follows desmosome (d) shows small, unstained particles measuring about 70 Å. T, tonofibrils. x 150 000.
Fig. 6. (a) A very high magnification of cobblestone particles revealed the round to polygonal contour of individual particles measuring 70–135 Å in diameter. If the correction factor of 2/3 were applied, the actual sizes of these particles would be 45–90 Å. It is possible that some of the low particles were not fully shadowed and, therefore, appear smaller than actual size. Finger skin. x 375 000.

(b) Lanthanum-permeated skin of the arm shows non-stained nexus subunits which measure about 70–75 Å. x 375 000.
particles within an ill-defined area between the
nexuses (Fig. 2, inset). In the human epidermis the
nexuses generally exist juxtaposed to desmosomes
[7] (see below).

_Nexus subunits in thin sections_
The nexus was usually present very closely juxtaposed
to desmosomes (Fig. 5b). Without lanthanum it was
seen clearly only in cross section as 20–30 Å slits
with a central dense layer sandwiched between two
cytomembranes of apposed cells. The sizes of the
nexus were varied greatly, but usually ranged
between 0.1 and 0.6 μm in diameter, thus agreeing
in size with nexus macules that were demonstrated in
the replica. Lanthanum permeated the inter-
cellular spaces and infiltrated 20–30 Å slits
which in lateral view showed small subunits (Figs.
5b, 6b). Lanthanum filled the narrow channels
between subunits, but subunits themselves were not
stained. The subunits were round to polygonal with
a diameter of 70–75 Å (Figs. 5b, 6b).

**DISCUSSION**

Cell surface ultrastructure of freeze-cleaved kera-
tinocytes of the epidermis is not expected to differ
greatly from that of the normal cervical epithelium.
Nevertheless, our observations are at some variance
with those of McNutt & Weinstein (9) and McNutt
et al. (10). Regrettably, only one replica electron
micrograph at a low magnification was included in
their publication (10) and the information extract-
table therefrom is certainly limited. Since they did
not specifically mention in the caption whether the
specimen was derived from a keratinizing part of the
cervix uteri, comparison with the skin is difficult. In
my preparation, the majority of B face structures at
the nexus showed a cobblestone pattern instead of
pits. Although they did not publish the cobblestone
pictures, McNutt & Weinstein (9) gave their dimen-
sions: 90–100 Å in diameter and a height of 35 Å in
the replica. The cobblestones of the epidermal
keratinocytes, as shown in Figs. 5a and 6a, were
found to be more variable in diameter (70–135 Å)
and height (0–25 Å). Also, a variable number of
high particles similar to face A particles at nexus
(contact cylinder) were admixed. These high particles
might actually represent the contact cylinders which
happened to be tightly engaged in pits of Lm2 on
the B surface and, when fractured, were pulled out
from the A surface side of Lm1 where, according to
McNutt & Weinstein (9), they are supposed to stay
(Figs. 1a, 1b). In the other tissues and cells or with
other techniques (2, 4, 9, 10), the contact cylinders of
nexus seem to go with Lm1 and project from the A
face. A possibility that a deep etching produced high
particles was considered. However, in the same B
face the majority of cobblestones remained low,
suggesting that there was a definite difference of
height among these particles at the time of membrane
fracture.

It is certain that cobblestones are present on the
same plane and in the same area as B face pits are
situated, since the pits and cobblestones are simulta-
neously demonstrated within the same nexus (Fig.
4, inset). As mentioned by McNutt & Weinstein (9),
where the B face was shadowed at a high angle and
hence platinum was deposited densely, pits were seen
(Fig. 4, inset).

In the same nexus where the B face was shadowed
at a low angle and hence the deposition of platinum
was light, a cobblestone pattern was revealed (Fig. 4,
inset). There is no explanation, however, of why the
low angle shadow-casting produces cobblestone
patterns. A puzzling problem is that even a high
angle-shadowed B face did not necessarily show pits,
but showed the cobblestone pattern instead (Fig. 3).
It may then be postulated that upon cleavage the
contact cylinders can be (i) pulled out of the B face,
leaving typical pits, (ii) broken at various levels
above the B face plane up to 25 Å, or (iii) left with
the B face as “high particles”. In fact, the height
of contact cylinders on the A face is not uniform in
some nexuses (Fig. 5a); the short ones could repre-
sent the complementary half of high B face particles.
In the nexus of keratinocytes, the second and third
possibilities might actually occur (Figs. 1a, 1b), or it
is possible that this is due to the difference of tech-
nique used in the present study. This problem may
be solved by making double replicas and examining
the complementary set of fractured surfaces.

Slight variations from the previously published
data (9, 10) were noted in calculated dimensions of
some structures such as the diameter of contact
cylinders (Table I). Several technical factors involved
in this method may distort the true dimensions: the
angle of shadowing, thickness of the replica, deforma-
tion of the fractured surface prior to replication
(fluctuation of ambient temperature, etc.), etching by
sublimation, and contamination of vacuum. The
noted variations are very likely due to these uncon-

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Table 1. Measurement of nexus subunit in replica and in thin section

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<thead>
<tr>
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<th>A-face particle</th>
<th>B-face pit</th>
<th>B-face cobblestone</th>
<th>Nexus subunits (with La stain)</th>
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<tbody>
<tr>
<td></td>
<td>Diameter (Å)</td>
<td>Height (Å)</td>
<td>Diameter (Å)</td>
<td>Height (Å)</td>
</tr>
<tr>
<td>McNutt &amp; Weinstein</td>
<td>50-75</td>
<td>50</td>
<td>90-100</td>
<td>35</td>
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<tr>
<td>Revel &amp; Karnowsky</td>
<td></td>
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<td>70-75</td>
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<tr>
<td>Goodenough &amp; Revel</td>
<td>90-100</td>
<td>&lt;90-100</td>
<td>45-50</td>
<td>90-100</td>
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<tr>
<td>Hashimoto (present study)</td>
<td>100-135</td>
<td>50-70</td>
<td>70-135</td>
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<td></td>
<td></td>
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<td>70-75</td>
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*a* Center-to-center measurement.

trollable factors in each investigator's method, and probably not due to a specific difference of the tissue examined.

If an approximate correction factor of 2/3 (8) can be applied to replica dimensions to reduce the actual sizes of structures that are copied on replica, contact cylinders should measure between 65-90 Å and cobblestones between 45-90 Å. The lanthanum-delineated nexus subunits pictured in the same microscope at the same accelerating voltage measured 70-75 Å in diameter. If the reduction of size, due to fixation, dehydration, and lipid extraction by alcohol and propylene oxide is considered, the actual sizes of these subunits could be greater than 70-75 Å. If 20% reduction is a reasonable figure, they should measure approximately 85-90 Å in vivo. Thus, both contact cylinders and cobblestone particles seem to be smaller than these subunits. According to McNutt & Weinstein (9), the nexus subunits may represent the caps covering the contact cylinders on the D face of Lm2 (Figs. 1a, 1b).

In this study, not many specific structures were noted which might correspond to desmosomes. McNutt et al. (10) described an accumulation of small particles as an imprint of a desmosome on the B face in cervical epithelium. In many replicas, careful examination was made in the areas between nexuses where desmosomes are expected to occur in the epidermis (7). Such areas were found to be more often than not smooth on both A and B surfaces. Since MAPs were still demonstrated in these areas (Fig. 4), the possibility that a heavy casting of metal buried desmosomal particles could be ruled out. It may be tentatively stated that, with the present method, desmosomal marks on fractured membranes can be detected only occasionally in the human epidermis.

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**REFERENCES**


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ADDENDUM

After completion of this manuscript, Breathnach et al. (Micron J: 287, 1972; J Anat 114: 65, 1973) described aggregations of small particles found on freeze-fractured human epidermal cell membranes as desmosomal particles. These clustered particles were seen on elevated areas of fractured membrane which is directed toward the cytoplasm. No complementary structures were found at lower levels, but in stratum corneum similar aggregations were seen on slightly depressed areas of outwardly directed fracture surface.