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DESMOSOME FORMATION IN NORMAL HUMAN EPIDERMAL CELL CULTURE

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Abstract. Keratinocytes were dissociated from normal human adult epidermis with clostridial collagenase, dithio-erythritol and trypsin, and cultured. Immediately after this, no connection was seen between contiguous cells. Ruptured desmosomes, with masses of tonofilaments and distinct attachment plaques were still left on the cell surface. As culture proceeded, however, they became internalized into cytoplasm. As soon as culture was started and the cells established contact with each other, forming conglomerates, they began to form desmosomes. The cell membranes of a limited area which were in contact with neighbouring cells became thicker and formed attachment plaques. In the intercellular space of the desmosomal portion, fine filaments developed and a midline formed. The progress of desmosome formation was classified into six types.

Key words: Normal human keratinocytes; Desmosome formation

Desmosomes are specific structures of intercellular adhesion between epithelial cells. Numerous studies on the formation and fate of desmosomes have been reported. Most of them were performed on experimental animal materials (5, 7–11, 13, 16), human cancer cells (2), or human amnion cells (12). Few studies have been made on normal human skin (3, 4, 6).

In this study, reproduction of desmosomes was studied. Using normal human epidermal keratinocytes dissociated by enzymes, we studied the relationship between culture period and desmosome formation. During the culture, numerous stages in desmosome formation became evident. We classified these stages into six types, showing the percentage increase in each period.

MATERIALS AND METHODS

A single-cell suspension of human keratinocytes was prepared from normal skin of a middle-aged woman by a method described elsewhere (6). The suspension contained 2×10^4 dispersed keratinocytes per mm^3 without contamination by dendritic cells. 0.1 ml of this suspension was placed in a round area of about 1 cm in diameter on the plastic surface of a Falcon flask (25 ml capacity). Two such areas were placed in each flask. Culture medium was carefully added and incubated at 37°C. The culture medium was prepared from Eagle's minimum essential medium with Earle's salts (GIBCO) 100 ml, fetal calf serum 10 ml, 200 mM L-glutamine 2 ml, penicillin 10 000 IU, and streptomycin 5 mg. The pH of the medium was adjusted to 7.2 with 1 mM Hepes buffer. Keratinocytes were fixed on starting, and at 30 min, 1, 2, 5.5, 15, 16.5, 21, 24, 40.5, 45 hours and 5 weeks after culture start, and prepared for electron microscopy.

For electron microscopy, the material was fixed in 1.5% glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.4, with 7.5% sucrose for one day, and postfixed with 0.5% osmium tetroxide in the same buffer. It was dehydrated in a series of increasing concentrations of alcohols and embedded in Epon 812. Ultrathin sections were cut with a Reichert ultramicrotome 'Ultracut', and stained with uranyl acetate and lead citrate. A JEOL 100CX electron microscope was operated at 80 kV for observation.

Classification and identification of types of cell adhesion were studied on the electron microscopic photographs, of which the final magnifications were ×15000 and ×60000.

RESULTS

During cultivation, cell adhesion showed various appearances. Based on the fine structure, cell adhesion was classified into six different types (Table I). Fig. 1 shows the relation between culture period and types of cell adhesion as well as schematic illustrations of types of cell adhesion.

Type I represents a place where two contiguous cells touch but no desmosomes nor contact organelles are found. *Type II:* increased density of inner leaflets of cell membranes of two neighbouring cells. *Type III:* clear attachment plaques and very fine filamentous structures between the two outer leaflets of the cell membranes. *Type IV:* masses of tonofilaments inserted into the attachment plaques. *Type V:* the middle part of the fine filamentous structure is thickened and appears as a dotted

Table 1. *Periods of cell culture and types of cell adhesion*

Periods of cell culture		Start	0.5 h	1 h	2 h	5.5 h	15 h	16.5 h	21 h	24 h	40.5 h	45 h	5 wks
Type of cell adhesion													
I	No.	49	46	28	66	25	45	58	10	50	15	5	3
	%	100	62.2	43.1	38.2	15.0	16.2	8.3	15.6	9.3	7.3	2.9	2.7
II	No.		28	28	37	74	107	146	12	107	43	27	15
	%		37.8	43.1	21.4	44.3	38.6	20.9	18.8	19.8	20.9	15.6	13.6
III	No.			9	31	35	39	162	8	120	27	18	4
	%			13.8	17.9	21.0	14.1	23.2	12.5	22.3	13.1	10.4	3.7
IV	No.				39	33	86	321	30	245	71	77	33
	%				22.5	19.7	31.1	45.9	46.9	45.0	34.4	44.5	30.0
V	No.							12	4	10	29	25	14
	%							1.7	6.2	1.8	14.1	14.5	12.7
VI	No.									10	21	21	41
	%									1.8	10.2	12.1	37.3
Total number of cell adhesions		49	74	65	173	167	277	699	64	542	206	173	110

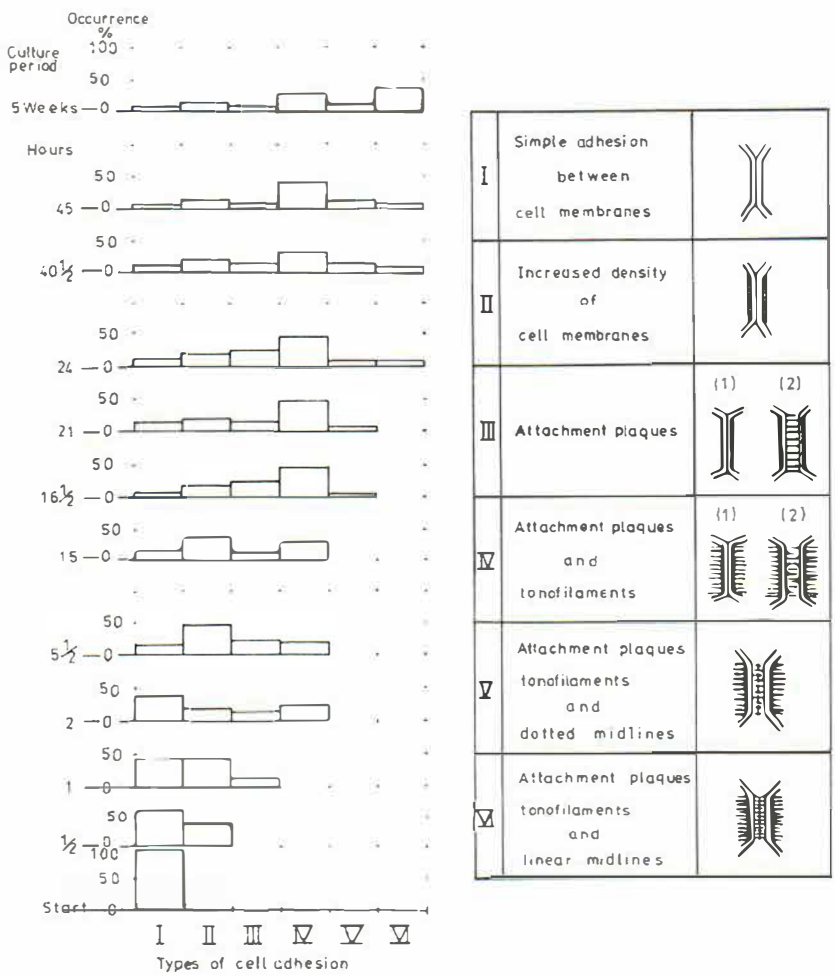

Fig. 1. Periods of cell culture and types of cell adhesion.

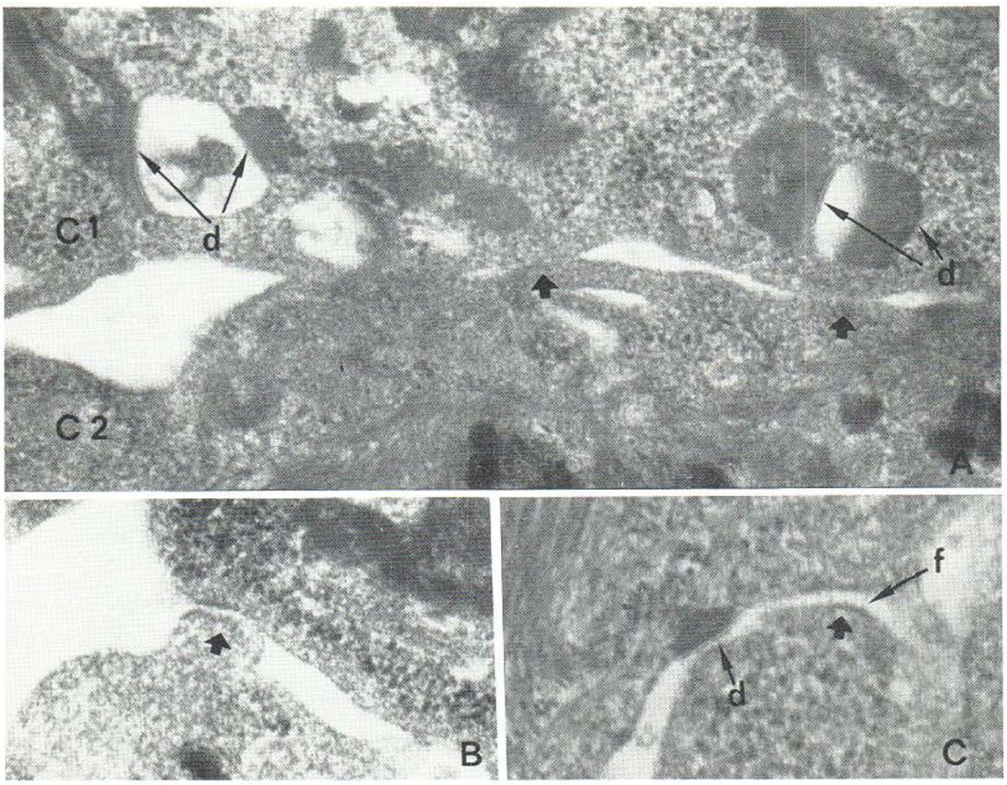

Fig. 2. (A) At start of culture, between two contiguous cells (C1 and C2), only simple adhesions type I (*broad arrows*) are seen. Ruptured desmosomes (*d*) are internalized into cytoplasm. ×54 000. (B) After 30 minutes, an arrow indicates increased density of cell membranes, type II. ×54000. (C) After one hour, type III adhesion (*broad arrow*) with fine filamentous structure (*f*) is observed in intercellular space. A ruptured desmosome (*d*) is seen on the cell surface. ×70200.

midline. Complete desmosomes with attachment plaques, tonofilaments and a linear midline are named *type VI*.

At the start of cell culture, the suspension for implantation contained dispersed keratinocytes. Two contiguous cells had no desmosomes or contact structures (Fig. 2A). Forty-nine adjoining places were studied; all were of type I (Table I and Fig. 1).

At 30 min after culture was started, besides isolated cells, conglomerated cells were found on which no new desmosomes were observed. Where the cells had contact, however, the inner leaflets of the cell membranes started to thicken, and the electron density became increased (Fig. 2B). Seventy-four cell contact points were studied; 62.2% were type I and 37.8% type II.

Type III appeared after a one-hour cultivation (Fig. 2C). Before the tonofilaments inserted into the attachment plaques, fine filamentous structures in the intercellular space were recognized. Type IV was noticed after only 2 hours (Fig. 3B). By 15 hours, there numbers had increased to form up to 31.1%, and their attachment plaques and tonofilaments became more evident and denser (Fig. 4). In the intercellular space, fine filaments, 3–5 nm thick, became more obvious. After 16.5 hours, thickening of the middle part of these filaments was seen, which we called type V (Fig. 5A). After 24 hours had elapsed, type VI adhesion which had a 5–10 nm thick straight midline appeared (Fig. 5B). They amounted to only 1.8% at this time.

After 5 weeks from the start, the cultured cells formed a multiple-layered sheath. Between these cells numerous desmosomes could be seen. They had dense attachment plaques, clear-cell membranes, masses of long tonofilaments and evident straight midlines (Fig. 5C). There were more of

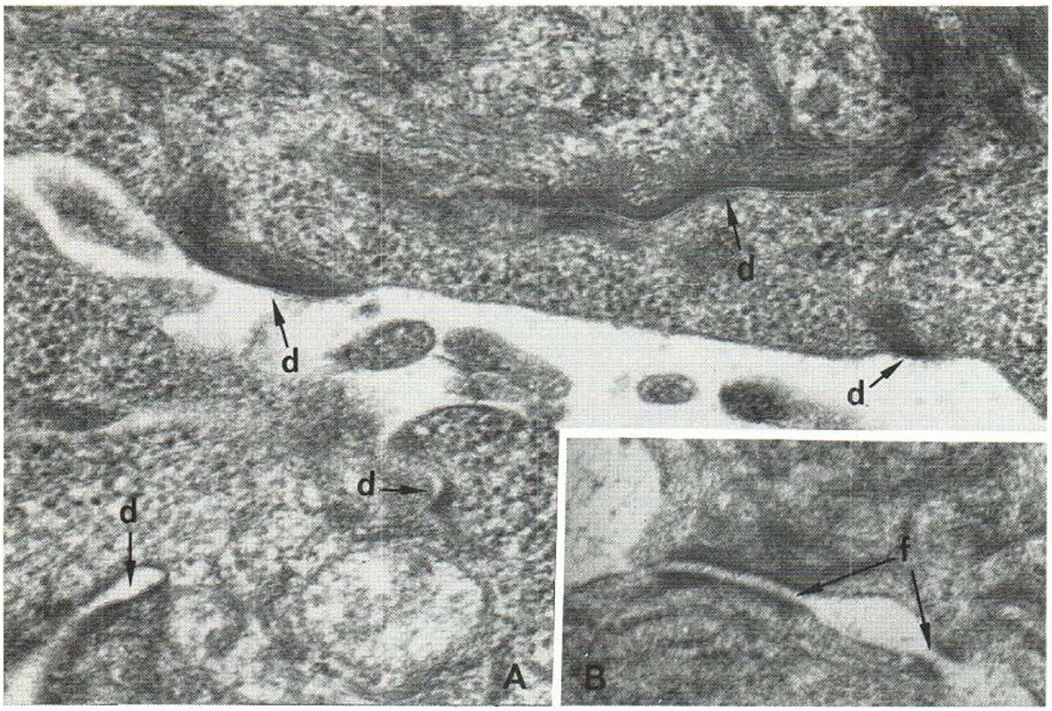

Fig. 3. (A) After 2 hours ruptured desmosomes (*d*) are seen on cell surface and in cytoplasm. ×54 000. (B) Type IV adhesion with attachment plaques, short and small masses of tonofilaments and fine filamentous structures (*f*). ×54 000.

these complete desmosomes than of incomplete desmosomes. Type VI was represented in 37.3%, while type I in only 2.7%.

On the surface of dissociated keratinocytes, at the beginning of cultivation, there were numerous ruptured desmosomes with attachment plaques and tonofilaments. Several split desmosomes surrounding vacuoles were internalized in the cytoplasm (Fig. 2 A). These hemidesmosome-like ruptured desmosomes were situated on the peripheral surface of the cells after 2 hours (Fig. 3 A). At 16.5 hours, in the cytoplasm, the remnants of the internalized desmosomes were still to be found. After 24 hours, however, the fragments of the ruptured desmosomes could no longer be found in the cytoplasm or on the peripheral surface of the cells.

During desmosome formation, the intercellular space became wider. When cell adhesion commenced, the cell membranes of adjacent cells touched each other (Fig. 2 A). Then, after a certain period, they separated, and an interstice between them could be noted. At 15 hours the distance between both outer leaflets of cell membranes of the desmosomal portion was 20 nm (Fig. 4), and at 5 weeks about 30 nm (Fig. 5 C).

DISCUSSION

At the beginning of cultivation, following dissociation with collagenase, trypsin and dithio-erythritol, the epidermal keratinocytes are dispersed, and some of them are coherent. On the periphery of the cells, numerous split desmosomes are observed, as mentioned by previous authors (5, 8–11, 13). These desmosomes are seen not only on the edges but also in the cytoplasm, and resemble the acantholytic cells of pemphigus vulgaris (5).

Overton demonstrated (8, 11) the process of internalization of split desmosomes, following trypsinization. They became engulfed by the cytoplasm and, after 1½–13 hours, structures resembling half-desmosomes were occasionally found. Dembitzer et al. (2) reported that, after 24 hours, desmosomal fragments were extremely rare. At this point, we obtained similar results. Ruptured and internalized desmosomal fragments were seen at 16 hours from

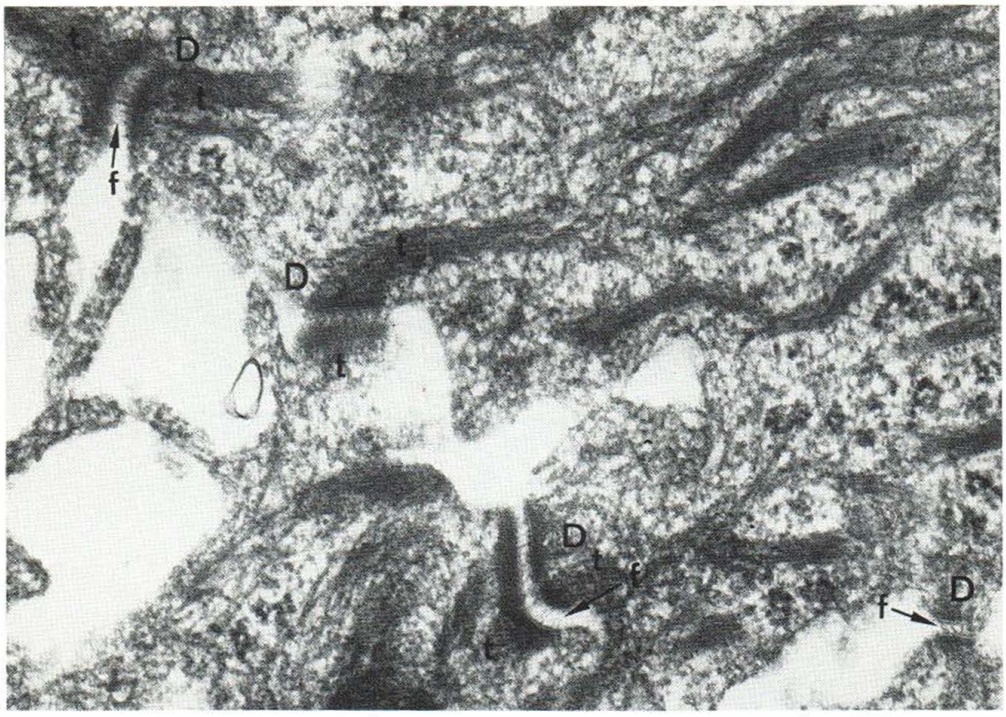

Fig. 4. After 15 hours, type IV adhesions (D) show evident attachment plaques, long and dense tonofilaments (*t*), and filamentous structures in intercellular spaces (*f*). ×54 000.

the start of culture, but after 24 hours, none could be found. In our study, it was observed that these pre-existing halves of ruptured desmosomes on cell surfaces cannot reconstruct new desmosomes between adjacent cells.

After 30 min, type I, the increased electron density of inner leaflets of cell membranes was noticed. Flaxman & Nelson (3), using split-thickness skin culture after 15 min, called them "small specialized junctions, not resembling desmosomes". In this study, it was evident that they represent the primary adhesion which would progress to desmosomes because, following the time sequence, this type of cell adhesion increased.

As regards the time when almost complete desmosomes appeared, we obtained results different from those of previous authors. Krawczyk & Wilgram (7), using young mice, showed desmosome formation between 12 and 18 hours after wounding. Patrizi (12) reported that human amnion cells began to show reconstruction of intercellular attachment zones during the fourth day of tissue culture, and by the 10th day, fully formed desmosomes were apparent. In our study, however, the development of desmosomes started as soon as two cells touched each other and, by 24 hours from culture start, complete desmosomes increased in number. Dembitzer et al. (2), using a human cancer cell line, also reported that the beginning of desmosome formation was observed within a few minutes after start of aggregation.

Wolff & Schreiner (15) reported that the intercellular portion of desmosomes, i.e. filamentous structures and the intercellular contact layer, was not removed by protease treatment. Their results accorded with cytochemical studies showing that they contained mucopolysaccharides (14). Borysenko & Revel (1), treating desmosomes of different tissues with proteolytic enzymes and chelating agents, found that intercellular contact layers contained a trypsin-digestible protein, and that the fine filaments were composed of protein or glycoprotein.

Overton (8) used the term 'desmosome' after the mid-stage of desmosomal formation for a fully recognizable (though not completed) structure, attachment plaques and small quantities of tonofi-

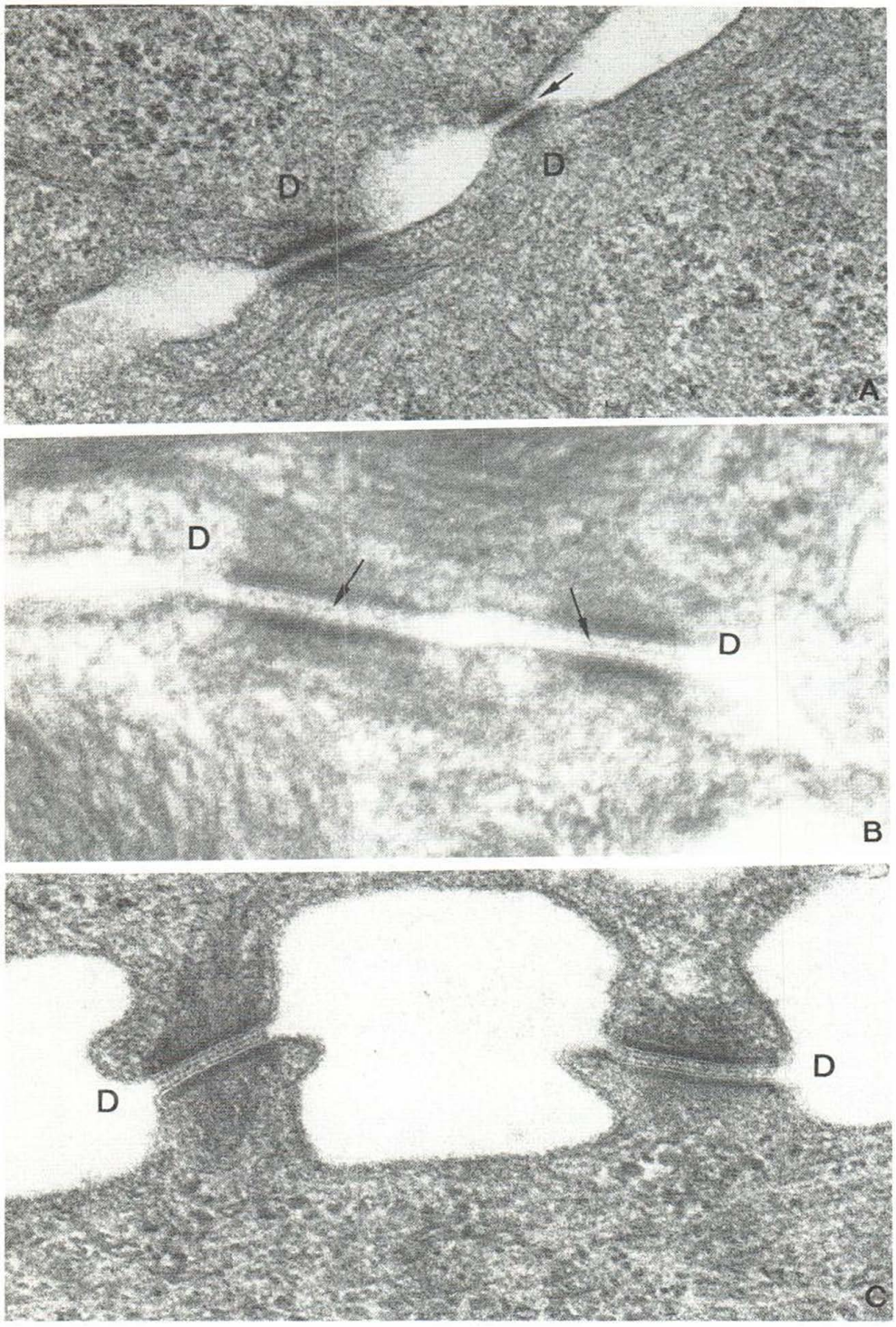

Fig. 5. (A) After 16.5 hours, type V adhesion (D), with thickening of middle part of fine filaments are observed (arrow). ×54 000. (B) After 24 hours, several desmosomes (D) have evident straight midlines (arrows). ×135 000. (C) After 5 weeks, complete desmosomes (D) are seen, with attachment plaques, tonofilament masses, clear-cell membranes and evident midlines. ×89 100.

laments. Zerban & Franke (16) stated that the tonofilament complex could constitute one item of evidence of desmosome formation, in comparison with other kinds of junctions. In this study, type IV adhesion showing tonofilaments, attachment plaques and fine filamentous structures, was also called 'desmosomes'.

Friedman-Kien et al. (4) reported that, after 10 weeks, only a few desmosomes were found among cultured keratinocytes. In vertical sections for electron microscopy, we can confirm that few desmosomes are found after 5 weeks. In horizontal sections, however, numerous desmosomes were observed.

Dembitzer et al. (2) reported that, during desmosome formation, the intercellular space becomes wider. We could confirm this. In the area of desmosomes there was no overlapping of cell membranes, though a certain distance was kept between two outer leaflets of cell membranes.

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