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Cytochalasin B Affects Selectively the Marginal Cells of the Epithelial Sheet in Culture

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ABSTRACT—The epithelial spreading is a fundamental one among morphogenetic movements. To know the mechanism generating force for spreading, the effect of cytochalasin B (cytB) on the corneal epithelium in culture was investigated. Four μg/ml of cytB inhibited completely the epithelial spreading and, at the same time, altered selectively the shapes of marginal cells: they shrinked at leading lamella and retracted quickly leaving fine tails behind, in which the bundles of F-actin were disintegrated to be numbers of fragments, while most of the other cells kept their shapes unchanged, along the cell borders of which F-actin distributed as before the treatment of cytB. The high sensitivity of the marginal cells to cytB led us to consider that F-actin was incessantly polymerized and taken into the intracellular organs generating force which enabled the marginal cells to locomote outwards, and that the outward locomotion, as a main force, spread entire epithelium.

INTRODUCTION

When the body surface of higher animals was injured, the epithelial cells started to migrate and spread quickly over the denuded area as a coherent cell sheet, and ceased the movement after the closure of wound. The locomotion of epithelial cells, as one of fundamental cellular movements in morphogenesis, has attracted the interest of many authors [see 1, 2].

The marginal cells of epithelial sheets in culture were flattened and formed leading lamella at the free end, where they attached to the substratum with both focal and close contacts [1, 3–5]. Lamellipodia, filopodia or microspikes were protruded from the leading edges, accompanying ruffling or blebbing [6], as like as in the fibroblastic cells [7–9]. In the leading lamella of epithelial cells, bundles of microfilaments or of actomyosin were reported to be aligned in a way quite similar with those in fibroblasts [1, 10]. We could therefore assume that the contraction of actomyosin systems in the marginal cells was a main force for the epithelial spreading as in the locomotion of fibroblasts [11]. In order to know more precisely the mechanism of epithelial spreading, cytochalasin B (cytB), the drug known as an inhibitor of actin filament formation since Schroeder [12], was applied to the cultured epithelia. Both shapes of cells and alignment of bundels of F-actin, as well as an inhibition of spreading of epithelial sheets, were altered remarkably. Above all, the marginal cells were affected most conspicuously. The results lead us to accept the hypothesis that the marginal cells are mainly responsible for epithelial spreading. The entire epithelium was pulled outwards with the marginal cells in which the bundles of F-actin were structuralized incessantly for generating force for the outward locomotion. The details of the observation and the discussion on the subject will be presented in this communication.

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MATERIALS AND METHODS

The eggs of white leghorn obtained commercially were incubated for 8 days at 38°C.

Millipore filters (MF), PH or RA (Millipore Co.), pore sizes of which are 0.3 μm and 1.2 μm respectively, were used for substrata. They will be referred as 0.3 MF and 1.2 MF, characterized by numerals expressing pore size.

The cover slip (24×24 mm) cleaned for culture

use (Matunami Glass Inc.) was used as a substratum for an epithelium.

1) *Isolation of the epithelium* More than 200 sheets of corneal epithelium were isolated in the ordinary procedures using 1 mM ethylenediaminetetraacetic acid (EDTA) in calcium and magnesium free Tyrode solution. The details were presented in a previous paper [13].

2) *Culture methods* Each isolated epithelium was combined with a piece of 0.3 MF or 1.2 MF, explanted onto the surface of normal Wolff-Haffen's medium [14] or that containing cytochalasin B (Sigma Co., 4, 0.4, or 0.04 μg/ml in a final concentration, cytB-W-H), gelled in a hollow in a glass block. After covered with glass slide and sealed with melted paraffin, the explant was incubated for 24, 48, or 96 hr at 38°C. CytB-W-H was prepared as follows: 1% agar in Gey's solution, donor horse serum (Flow Lab.), 50% embryonic extract of 9-day chick embryos, which contained cytB in a concentration of 19, 1.9, or 0.19 mg/ml, and Penicillin G-K (Meiji Pharm. Co., 20,000 units/ml) were mixed in a ratio, 7:3:3:1 in volume. CytB was primarily dissolved in dimethylsulfoxide (DMSO) and diluted in embryonic extract. As a result, cytB-W-H contained inevitably 2%, at most, of DMSO in a final concentration. The effect of DMSO was checked in the epithelia cultured on W-H containing 2% DMSO alone.

Fifty or more sheets of corneal epithelia were explanted onto the surface of cover glass with a small drop of L-15 medium (Leibowitz, L15, Gibco Co.) supplemented with 20% of donor horse serum (Flow Lab.), covered with hollow slide, sealed with melted paraffin and incubated at 38°C. After 24 hr of primary culture, only the epithelium started to spread along the glass surface was transferred into filming slide with a L-15 medium contained 4 μg/ml of cytB and 20% of horse serum (cytB-L-15) and observed under a phase-contrast microscope, the stage of which was kept at 38°C. The rest of epithelium attached not firmly enough to the glass surface was cultured for another 24 hr at 38°C in Petri dish with a large amount of L-15 medium. The epithelium which began to spread was served for cytB treatment as described above.

3) *Quantitative assessment* The epithelia cultured on MF for 48 or 96 hr with cytB-W-H were fixed with Bouin's fluid for 2 hr. After a thorough elimination of picric acid with 70% ethanol, they were stained *in toto* with Meyer's hematoxylin and eosin, dehydrated through a series of graded alcohol, cleared with xylene and mounted on slide glass with balsam and cover glass.

A ratio between the area of epithelium before and after the culture was used as the index of migratory activity. The details were presented in the previous report [13].

4) *Scanning electron microscopy (SEM)* More or less than 10 samples were selected at random from each of 8 groups of culture (two types of MF, and four kinds of cytB-W-H in concentration of cytB) at 24, 48, or 96 hr of culture and fixed with 2.5% glutaraldehyde (Taab. Co.) in 0.1 M cacodylate buffer (pH=7.2, c-buffer) for 2 hr at 0°C. Rinsed three times and overnighted in 0.1 M c-buffer at 4°C, they were postfixed with 1% OsO_4 in 0.1 M c-buffer for 2 hr at 0°C, dehydrated through a series of graded ethanol at 0°C and, immediately or after stocked for several days in the refrigerator, dried in a critical point dryer (Hitachi HII) using liquid CO_2 as a medium. After coated with gold in an ion coater (Eiko Co.), the epithelium was observed with Hitachi S430 scanning electron microscope.

5) *NBD-Phallacidin staining* The epithelia cultured on MF for 24, 48, or 96 hr and those on the glass surface, treated or not treated with cytB, were fixed with 3.5% of neutral formalin in phosphate buffered saline (pH=7.2, PBS) for 30 min at 20°C, rinsed several times in PBS, placed on the bottom of Petri dish, and immersed in a drop of NBD-Phallacidin (NBD-Ph, Wako Pure Chem. Indust.) solution, in which 2 units of NBD-Ph were solved in PBS, for 2 hr at 20°C in the moist and dark chamber. After the treatment, they were rinsed several times in PBS to eliminate free NBD-Ph, mounted on slide glass with PBS-glycerine (1:1 in volume) and covered with cover slip.

6) *Optics* A phase-contrast microscope and a microscope equipped with epifluorescent apparatus and with objective lenses free from autofluorescence (UHF ×20, UHF ×40, and UHF

×100, the last two, for oil immersion, Olympus Co.) were used. For microphotography with the fluorescent microscope, Tri-X films (ISO=400, Kodak) were adopted, which were developed with Super prodol (Fuji Co.) for 7 min at 20°C to enhance the sensitivity from ISO 400 to 800. With the phase-contrast microscope, Neopan F (ISO=32, Fuji Co.) was used without a special treatment.

RESULTS

1. Quantitative assessment of the effect of cytB on epithelial spreading

CytB suppressed completely epithelial spreading. In a concentration more than 10 μg/ml, the epithelial cells hardly survived (data not shown). In 4 μg/ml of cytB, in spite of healthy appearance of cells in histological survey, the migratory index (MI) was 1.2±0.2 (an average of 15 cases ± a standard deviation) in the epithelia cultured for 96 hr in combination with 0.3 MF, which was one-sixth of that in the epithelia cultured on normal W-H (7.9±2.3, 10 samples). The value did not differ significantly from that in the epithelia cultured on normal W-H in combination with 1.2 MF (1.1±0.2, 15), showing complete stop of spreading. Even in a low concectration (0.04 μg/ml), cytB still worked inhibitory: the MI was 3.3 ±1.4 [14], approximately as half as that in those cultured on normal W-H. On W-H containing 2% DMSO alone, the MI (6.0±1.2, 10) differed not significantly from that in those on normal W-H in combination with 0.3 MF at 96 hr of culture. These results were summarized in Table 1. As the cytB in the concentration of 4 μg/ml suppressed the epithelial spreading most effectively, and without giving severe damage to cells, the concentration was used in this series of experiments.

2. The early changes of the marginal cells when transferred into the medium containing cytB

CytB was observed to affect at first in the marginal cells within 5 min after the epithelium was transferred into cytB-L-15. They swelled up slightly and their leading lamella were narrowed at their width. Then the cells began to retract but some parts of the distal end still attached firmly to the glass surface. As the result, fine thread-like cytoplasmic bridges were left behind, and the more the cell body retracted, the longer the bridges became (Figs. 1 and 3a). It was noteworthy that the leading lamella never took "arborized" forms as in the fibroblastic cells treated with cytB [15–18]. The retraction of the margin attained to a maximum about 40 min after the application of cytB, and kept the state for several hours (Fig. 1). The retracted marginal cells in cytB-L-15 for 2 hr could restart to develop the leading lamella and to migrate within 30 min after brought back to a normal L-15 medium.

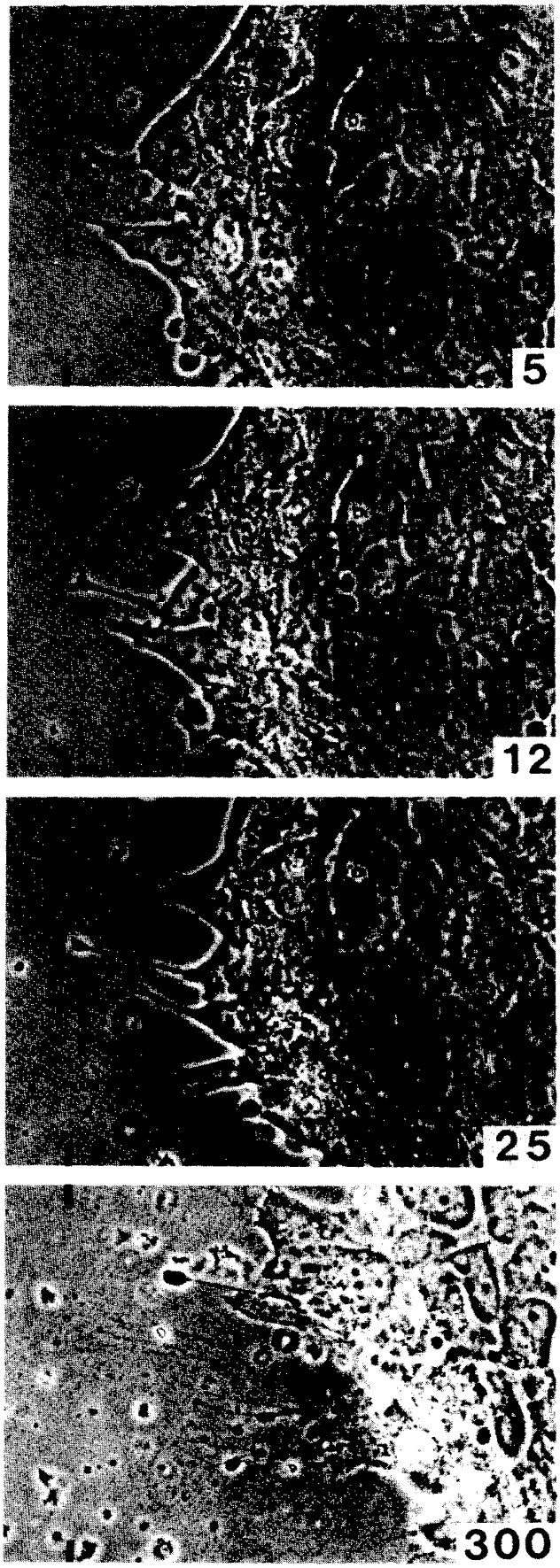
3. SEM observation on the epithelia cultured for more than 24 hr on cytB-W-H

Almost all of the epithelial marginal cells cultured on cytB-W-H in combination with 0.3 MF lost the leading lamella to be a hemisphere in shapes (Fig. 2d). Some had poor leading lamella

TABLE 1. The effect of cytochalasin B on the epithelial spreading

Culture medium	Substratum	No. of samples	Migratory index (Av. ±s.d.)
W-H	0.3 MF	10	7.9±2.3
W-H+DMSO	0.3 MF	10	6.0±1.2
W-H+2% DMSO+0.04 μg/ml cytB	0.3 MF	14	3.3±1.4
W-H+2% DMSO+0.4 μg/ml cytB	0.3 MF	12	1.9±0.6
W-H+2% DMSO+4 μg/ml cytB	0.3 MF	15	1.2±0.2
W-H	1.2 MF	15	1.1±0.1

W-H: Wolff-Haffen's medium. DMSO: dimethylsulfoxide. Av. ±s.d.: Average±standard deviation.



with many blebs at the edges (Fig. 2a) as in the aggregated fibroblasts in a medium containing cytB [19], suggesting no sufficient expansion occurred in the cells [20]. Those on W–H without cytB, in contrast, were flat and had well developed leading lamella, from which filopodia, lamellipodia, micro-filopodial processes, blebs or ruffles were protruded as reported before [21] (Fig. 2c).

The submarginal cells elongated along the direction in parallel with the marginal line and had microvilli on top as in those cultured on normal W–H, suggesting weak or no effect of cytB on them (Fig. 2a).

The inner area differed from place to place in the effect of cytB: Most of places were kept intact. Some still kept regular polygonal shapes, but swelled up slightly, or tended to break contacts with the neighbours to be a fibroblast-like cell on the surface of epithelium (Fig. 2b).

In the epithelium combined with 1.2 MF, the effect of cytB was not clear. Neither the marginal, submarginal nor the inner cells changed conspicuously in their figures on cytB–W–H: The marginal cells scarcely attached to MF and not spread at all, quite similar with those cultured in combination with 1.2 MF on normal W–H (Fig. 2d).

4. The distribution pattern of F-actin in the epithelial cells in the presence of cytB

In the cytoplasmic bridges left behind the retracted marginal cells (Figs. 1 and 3a), there were numbers of small fragments bound with NBD-Ph scattered (Fig. 3b). Similar fragments of fluorescence were recognized also at perinuclear region in the inner cells (Fig. 3d). Most of marginal cells cultured for more than 24 hr on cytB–W–H in combination with 0.3 MF had hemispherical edges, in which the fluorescence of NBD-Ph was recognized faintly only along cell membrane. By contrast, on normal W–H, the

FIG. 1. The process of marginal retraction in the epithelium in cytB–L-15. The numerals at right end in each figure indicate the time after the epithelium was transferred into cytB–L-15. The thick black bar on the top figure, 50 μm. The thin bars in each mark the fixed points on the glass surface.

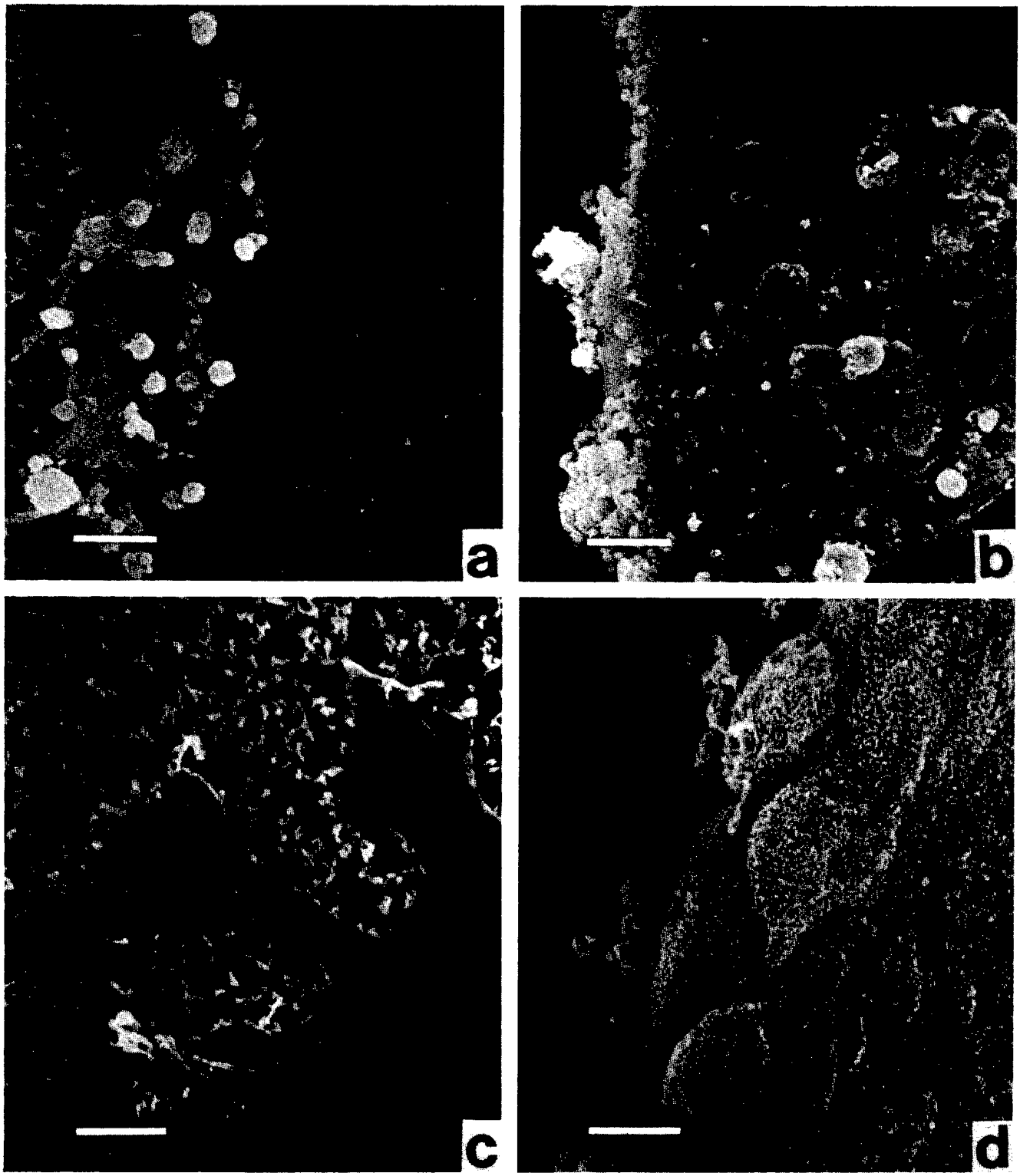


FIG. 2. The epithelial cells observed with an SEM.

a. The marginal cells in the epithelium combined with 0.3 MF and cultured for 12 hr on cytB–W–H. In comparison with Fig. 2c below, the retraction of margin and bleds are marked. The white bar, 10 μm.

b. The submarginal cells and the inner of the epithelium combined with 0.3 MF and cultured for 48 hr on cytB–W–H. The marginal cells retracted completely. Some of inner cells were swelled and loosened connection among them. The white bar, 10 μm.

c. The marginal cells combined with 0.3MF and cultured for 24 hr on normal W–H. Note the leading lamella spread in flat. The white bar, 5 μm.

d. The marginal cells in the epithelium cultured for 24 hr, in association with 1.2 MF, on cytB–W–H. The white bar, 5 μm.

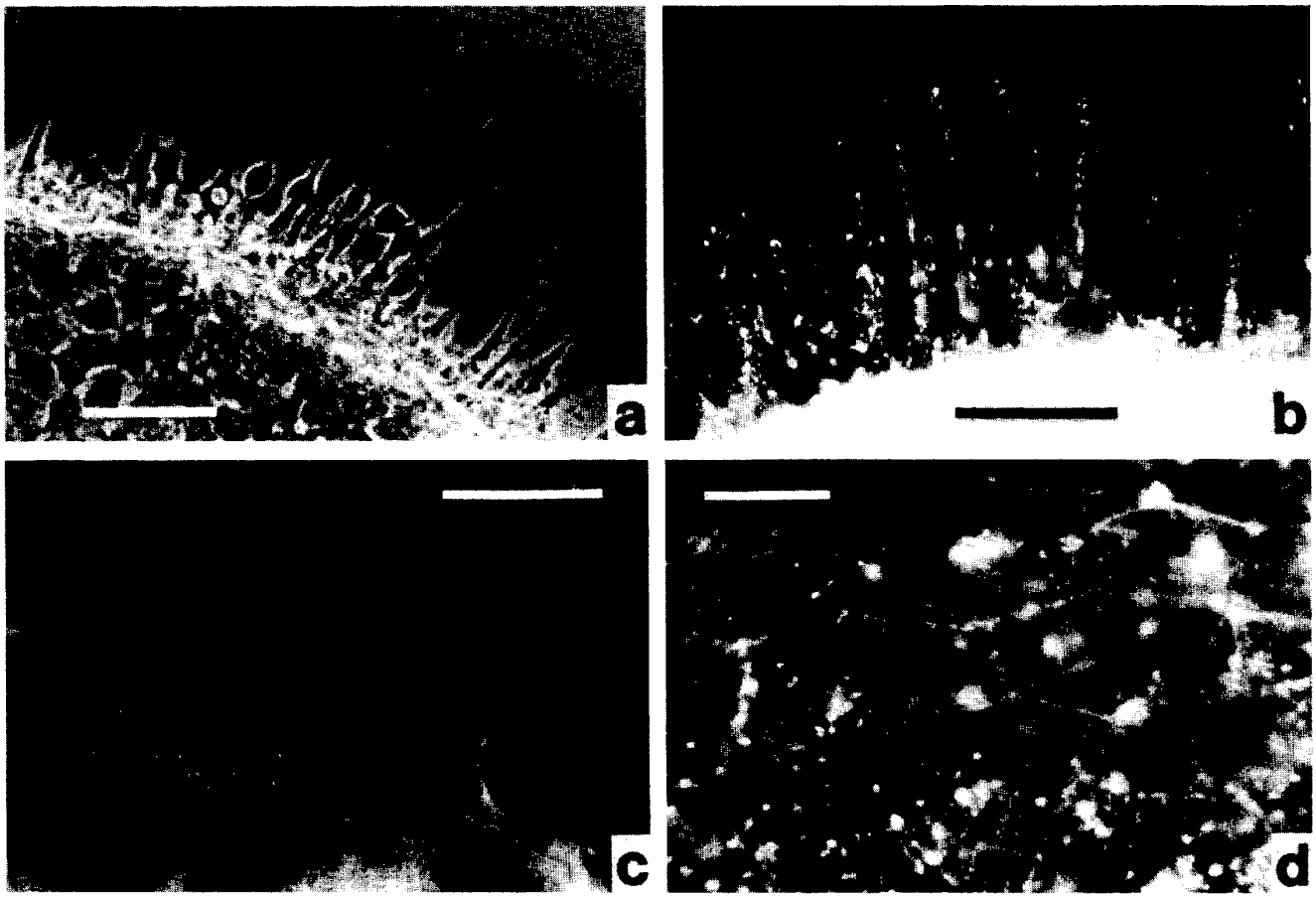


FIG. 3. The epithelial cells cultured on the glass surface.

a. The fine thread-like leading lamella in the epithelial marginal cells after 4 hr of culture in cytB–L-15. The white bar, 50 μm.

b. The small aggregates of F-actin in the thread-like leading lamella in the epithelial cells cultured for 4 hr in cytB–L-15. Compare with the alignment of F-actin bundles in the well developed leading lamella in Fig. 3c below. The black bar, 50 μm.

c. The alignment of bundles of F-actin fibers in the marginal cells cultured for 24 hr in association with 0.3 MF on normal W–H. The white bar, 50 μm.

d. F-actin fibers in the inner cells of the epithelium cultured for 4 hr in cytB–L-15. Note F-actin fibers are aligned along cell borders. The white bar, 20 μm.

bundles of NBD-Ph were observed to run through cell body from a cytocenter to the leading edges. The distribution pattern was similar with that of microfilaments observed with the aid of a transmission electron microscope [1] and also with that of actin filaments observed with the indirect immunofluorescence method using anti-actin antibody ([10] and Takeuchi, unpublished).

In the leading lamella once lost from the marginal cell in the presence of cytB and recovered in a normal medium, the NBD-Ph positive filaments were radially aligned, running through the leading lamella as in the cells not treated with cytB (Fig. 3c). The recovery of NBD-Ph positive filaments was confirmed in both the epithelia cultured on MF on cytB–W–H for 72 hr and those on the glass surface in cytB–L-15 for 2 hr.

In the inner cells, the bundles of NBD-Ph positive filaments were aligned along the polygonal borders as in the intact epithelium [22] without noticeable changes after the application of cytB, suggesting no effect of cytB on them (Fig. 3d). Apart from the border, however, many flakes of NBD-Ph were recognized to be scattered at perinuclear region (Fig. 3d).

DISCUSSION

The effect of cytB on the epithelial spreading

was evident (Table 1). This agreed with the results that the movement of corneal epithelium of newt was arrested with ctyB or cytD [23].

In the epithelia, spreading of which was inhibited, the marginal cells were most severely deformed with cytB, even cultured on a substratum smooth enough to permit spreading: they lost the leading lamella to be hemisphere in shapes as those cultured on the rough surface without cytB [16]. The deformation began soon after the epithelium was transferred into cytB–L-15 (Figs. 2 and 3a). It could, therefore, be said that the inhibition of epithelial spreading was mainly due to the loss of locomotory activity in the marginal cells accomapnied with the deformation. How did cytB act on the cells to bring about deformation or to disrupt the locomotory activity?

The action of cytB on actin filaments

There is a variety of elucidation reporting about the mode of action of cytB: CytB bound to one end of an actin filament to block further association of actin monomers [24–29]. CytB inhibited nucleation of F-actin, so that the formation of actin filaments was delayed [30]. CytB inhibited annealing of F-actin to suppress further elongation [31]. Besides, ctyB was reported to inhibit gelation of actin [26, 30, 32–34]. MacLean-Fletcher and Pollard [30] insisted the inhibition of actin-network formation might be more important in living cells than that of actin elongation. Schliwa [35] pursued the changes in the cytoskeletal system under the presence of cytB. Within minutes, cytB disrupted the cytoskeletal networks by severing the filaments into small fragments. At the next, cytoplasmic contraction, though disorganized and uncontrolled, occurred to form dense foci (aggregates of filamentous materials). A series of changes ended at a state, where the cytoskeletal system was disintegrated completely.

NBD-Ph was reported to associate specifically with F-actin [36]. According to this, the flakes observed in the marginal cells in cytB–L-15, were considered as aggregates of disintegrated F-actin derived from the bundles in the leading lamella as reported by Schliwa [35] (Fig. 3c), or considered as myoid bundles which were assumed to be contracted microfilamentous apparatus at the cell cortex reported by Miranda *et al.* [37]. Whichever the nature of the flakes might be, it was sure that the bundles of F-actin in the cells were disintegrated completely. The problems are how, specifically in the marginal cells, the F-actin bundles were disorganized, especially at early phase, and how the F-actin bundles were reformed and aligned in accopany with restoration of the leading lamella.

The early changes in the marginal cells, when the epithelial sheet was immersed in cytB–L-15

The marginal cells were narrowed very quickly at the leading lamella, when cytB was applied (Fig. 2). This suggested the structure or the machanism keeping the leading lamella flat was most sensitive to cytB.

Heath [38] reported that there were networks called as dorsal cortical microfilament sheath (DCMS) in the leading lamella of fibroblastic cells under locomotion. The DCMS consisting of actin and other contractile proteins were recycled from disintegration to reassociation to spread the leading lamella. The DCMS was moved backwards along dorsal surface of the leading lamella and decomposed to be cytoplasmic sol, which were pushed forwards to the leading edges to be reformed as DCMS. Considering similarity of the epithelial cells in locomotory activities to the fibroblastic cells [1, 6], the DCMS was assumed to work also on the leading lamella of epithelial cells. In the presence of cytB, the DCMS could not be structuralized because F-actin polymerization was inhibited. This might be a reason why the leading lamella was sensitive to cytB and narrowed themselves at first.

There might be another explanation for the mode of action of cytB to the leading lamella. According to Godman *et al.* [39, 40], F-actin was dissociated with actin-binding proteins with cytB and aggregated with myosin as in superprecipitation. This might bring about a general contraction of the leading lamella.

The mode of action of cytB in living cells will be elucidated more comprehensively when the behavior of actin *in situ* is fully understood.

The late changes in the marginal cells, the elongation of cytoplasmic bridges

The initial shrinkage of width in the leading lamella was followed by elongation of it. It was observed to be elongated straight, not so "arborized" as in the fibroblastic cells treated with cytB [15–18]. This might be explained as follows: after the bundles of F-actin were disintegrated with cytB, the marginal cells could not pull any more the rest of epithelial cells outwards but were, in reverse, pulled inwards, too strongly to permit the slack in the cytoplasmic bridge, by elastic force of themselves and/or of neighbouring cells which had counterbalanced tension arisen inevitably in them. Some parts in the leading edge stuck firmly to the substratum worked as supporting points against the recoiling force. Thus, the more the cells retracted, the longer the cytoplasmic bridges became. This hypothesis was agreed with the reports presented previously: there was strong tension at the marginal area of epithelial sheet in culture. The margin retracted to a considerable extent, when the contacts of marginal cells with the glass surface were disrupted mechanically [41]. Furthermore, the tension accelerated the epithelial spreading seemingly by enhancing the transition of the epithelial cells from a stationary state to a motile [42]. The focal contacts [3] were candidates for the supporting points which stuck firmly to the substratum. Indeed, the focal contacts were recognized in the epithelial marginal cells [1, 10]. Some of these contacts remained even in the presence of cytB to work as supporting points against the recoiling force.

Stability of F-actin filaments

It was quite queer that the bundles of F-actin along borders of the inner cells could not be disintegrated with cytB, while those at perinuclear region were broken down to be small flakes (Fig. 3d). Based on the action of cytB discussed above, the intact bundles were considered to be in a stationary state in which neither polymerization nor disintegration occurred. At the perinuclear region of the inner cells or at the leading lamella of marginal cells, in contrast, F-actin filaments were maintained in an equilibrium between incessant association of actin monomers with one end and decomposition at another end as discussed by Brenner and Korn [25]. Füchtbauer *et al.* [43] confirmed this in both the fibroblastic cells and the epithelial cells. Capping proteins for F-actin, when injected into the cells in culture, disintegrated the microfilaments at the site where the focal contacts were formed, causing shrinkage of the cells. According to them, actin monomers were always taken into filaments at the focal contacts.

Summerizing the points, the bundles of F-actin were newly aligned only at such active parts in cells as the leading lamella which generated force for works. In the marginal cells which were responsible for generating force for epithelial spreading, F-actin might be always polymerized and the bundles of them might be aligned along tension in order to work most effectively in accordance with formation of firm contacts between cell and substratum.

These results supported our "two step hypothesis" that tried to elucidate how the stationary epithelial cells acquired motility (Takeuchi, unpublished): the stationary cells were pulled outwards and under tension, at the first step, the bundles of microfilaments along cell border were discomposed completely and, at the second step, the components were reconstructed to be aligned along the direction of tension through cell body to the leading edge which generated force for locomotion. Only cytB-sensitive microfilaments were, therefore, observed in the marginal cells that made the transition from a stationary state to motile.

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