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Ultrastructural Changes of Cultured Human Amnion Cells by *Clostridium difficile* Toxin

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The ultrastructure of the surface of primary human amnion monolayer cells undergoing cytopathology induced by *Clostridium difficile* toxin was examined by scanning electron microscopy. Our observations indicated that the type and distribution of cell surface projections were altered dramatically by this toxin. The patterns of such surface changes were specific for the two different types of cells found in this cell culture. Cells with demarcated borders showed rearrangement of microvilli into globular chains or ridges which lined up with the branching membrane. Cells without demarcated borders exhibited studlike microvilli, all arranged into ridges or globular chains. These changes were noted after 1 h of toxin exposure and persisted without further progression, in spite of continued toxin exposure, up to 48 h. These data indicate that *C. difficile* produces a cytolytic toxin and that scanning electron microscopy may be useful in determining toxin-cell interactions.

Previous studies in our laboratories have indicated that antibiotic-induced pseudomembranous colitis is associated etiologically with a toxin produced by *Clostridium difficile* (1, 2, 4). Both the toxin and toxin-producing strains of *C. difficile* have been demonstrated in stools from patients with antibiotic-induced colitis and in an animal model consisting of hamsters given oral clindamycin. *C. difficile* or its toxin isolated from human sources has been shown to reproduce the disease in hamsters.

C. difficile toxin is cytopathic in a variety of tissue culture cells derived from both animals and humans (1, 4, 5, 7, 9). In this report, we describe ultrastructural changes observed by scanning electron microscopy of cultured human amnion cells after exposure to *C. difficile* toxin.

MATERIALS AND METHODS

Primary human amnion cell cultures were prepared by trypsinization of fresh human amnion. Suspension cells were seeded in petri dishes fitted with several glass squares (1 by 1 cm) which were cut from microscope slides. Cells were grown in Eagle minimal essential medium containing 10% fetal calf serum.

C. difficile toxin was prepared by ammonium sulfate precipitation at 60% concentration of hamster fecal contents obtained from a dying hamster which had been fed 1 mg of clindamycin 3 days earlier. The precipitate was dissolved in phosphate-buffered saline at pH 7 and dialyzed against phosphate-buffered saline in a refrigerator overnight. The cytopathic effects of the toxin in tissue culture were completely neutralized by polyvalent gas gangrene antitoxin and by monovalent *C. sordelli* antitoxin, but not by normal equine serum (4). One thousand 50% tissue culture doses were used for the present study.

After toxin inoculation, glass squares from control and toxin-treated petri dishes were removed at 1, 2, 4, 5, 24, and 48 h, washed three times with minimal essential medium, and fixed with a buffered (pH 7.3) 2% glutaraldehyde solution. The cells were dehydrated by a series of ethanol (20 to 100%) followed by two washes in 100% amyl acetate, for 10 min each time. All specimens were dried by the Anderson critical point method with CO_2 as transition fluid and coated with a thin layer (about 20 nm) of gold-palladium (60:40) at 1×10^{-4} to 5×10^{-5} mm of Hg by using a JEOL JEE4B vacuum evaporator. For electron microscopy, a JEOL TSM-U3 scanning electron microscope was operated at 25 kV with 50-s scanning periods.

Controls included normal amnion cell cultures taken at the same intervals (i.e., 1 to 48 h) and cell cultures which had been treated with heated toxin (100°C for 3 min).

RESULTS

Control amnion cells. At low magnification, the monolayer amnion cells examined by scanning electron microscopy had the appearance of epithelium-like cells arranged in a variegated pattern (Fig. 1). They were all polygon-shaped cells with a cobblestone pattern on the surface. Some cells exhibited well-demarcated borders, whereas others showed interdigitating borders with neighboring cells. At high magnification,

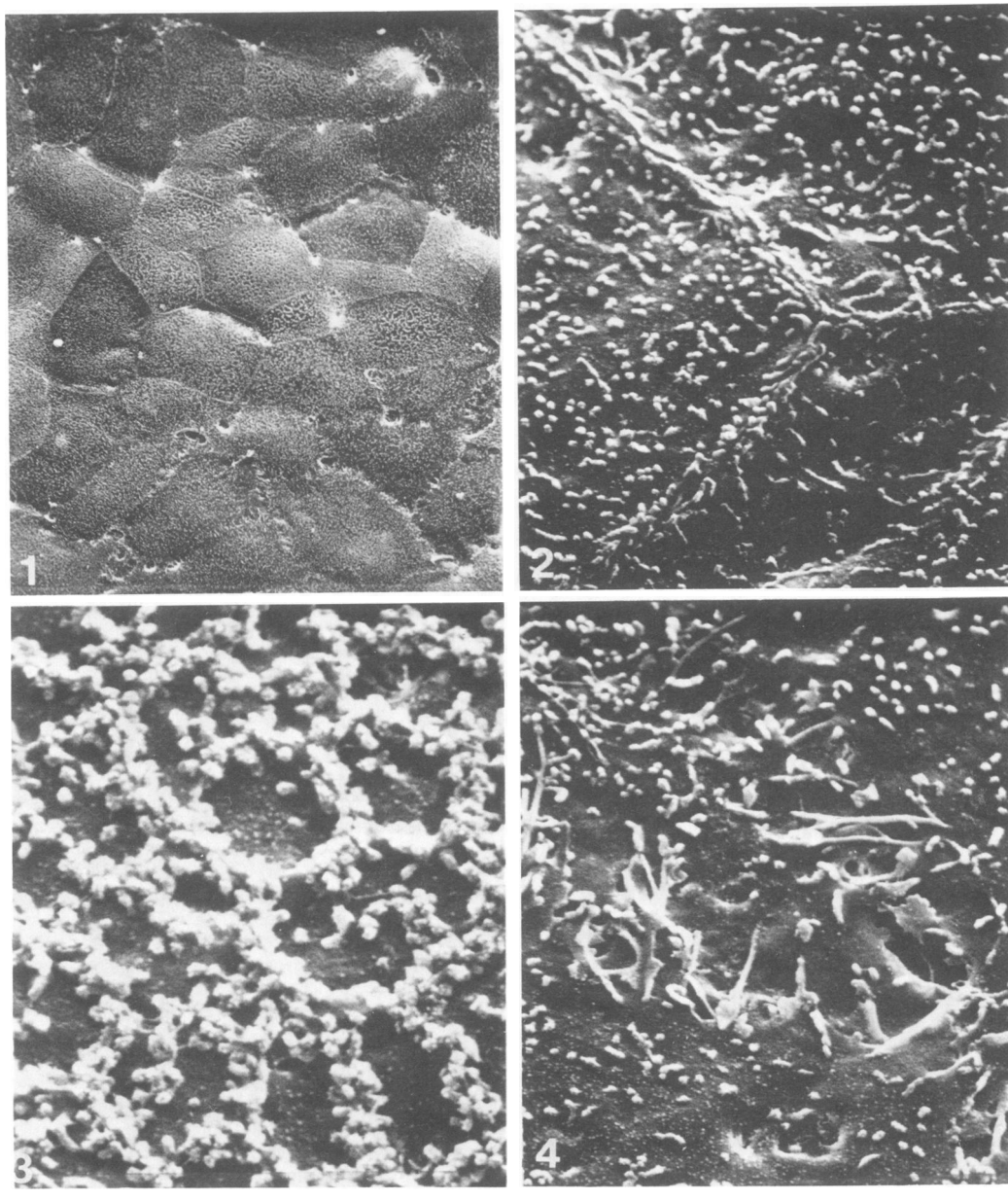


FIG. 1–4. *Control amnion monolayer cells.*

FIG. 1. *Low magnification (×450); cells were flat, polygonal, and interdigitating; some cells showed well-demarcated borders.*

FIG. 2. *High magnification (×5,400): the microvilli exhibited globular shape and some into globular chains; the demarcated cell border was organized by microridges.*

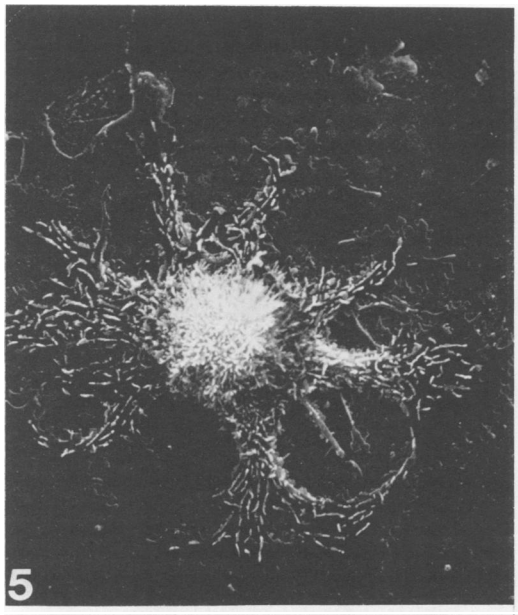
FIG. 3. *Higher magnification (×9,900) view of the central portion of cell surface as in Fig. 2, showing characteristic patterns of microvilli.*

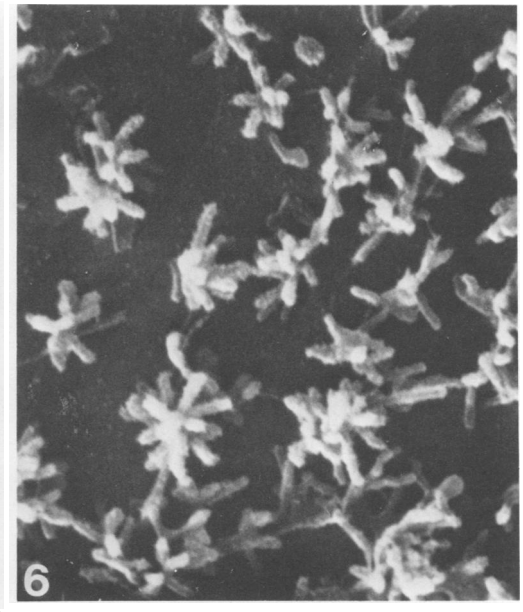
FIG. 4. *High magnification (×5,400) view of the interdigitating cell borders, showing microvilli and filopodia.*

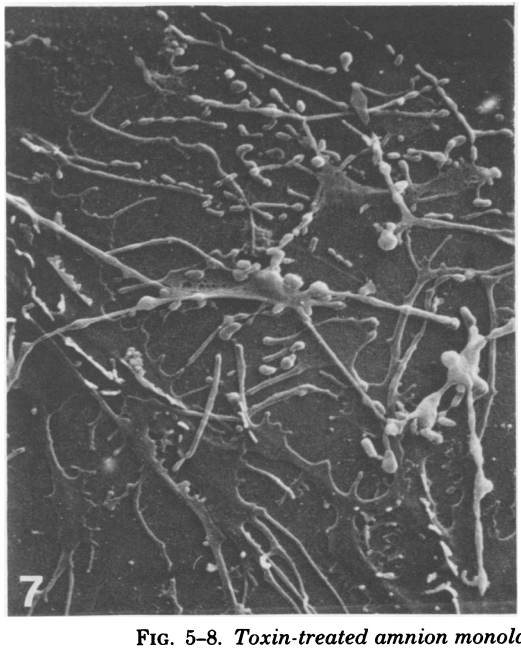
two types of cells could be distinguished. In the first type, the cell surface had well-demarcated borders which were covered with many short microvilli. In areas near the cell borders, these microvilli were arranged in studs or cytoplasmic globular forms. Many of these globules were lined into globular chains or ridges. It appeared that the raised ridges formed the demarcated

borders (Fig. 2). At the central areas of the cell surface, the microvilli were slightly larger and more abundant than those near the cell borders. These studlike microvilli were arranged into a discrete pattern (Fig. 3). The other type of cell did not have a sharply defined border and failed to show any central area with organized microvilli. Instead, the studlike microvilli were more irregularly distributed throughout the cell surface. Interdigitating of microvilli and filopodia was noted at the cell border (Fig. 4).

Toxin-treated cells. Monolayer cells treated with toxin showed dramatic changes, particularly in those cells with demarcated borders. The







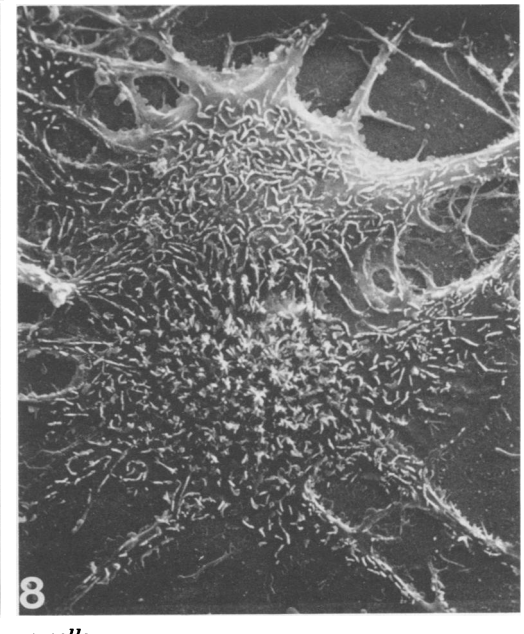


FIG. 5–8. *Toxin-treated amnion monolayer cells.*
FIG. 5. *Low magnification (×440), showing toxin-induced cell surface changes.*
FIG. 6. *High magnification (×9,900), showing bundles of erected microvilli.*
FIG. 7. *Cell surface severed into irregular-sized and -shaped fragments (×2,700).*
FIG. 8. *Two cells with somewhat different toxic changes (×1,800).*

general topology of the cells treated with toxin for 1 h was similar to that of cells treated for 48 h. We assume that the maximum effects on the membrane appeared by 1 h of toxin treatment. The shape of cells was altered from polygonal to actinomorphic (Fig. 5), apparently due to branching of the cell membrane. Most of the globule-like microvilli were merged into globular chains or ridges which lined up with the branching membrane. The microvilli on the central portion of the cell surface appeared elongated, and they aggregated into bundles emanating from the cell surface or the large membrane folds (Fig. 6). All rounded cells showed microvilli which were aggregated into bundles. One interesting finding was that in spaces between the cells there were many irregular membrane fragments, arranged as globules or globular chains which were still attached to the surface of the glass slide (Fig. 7). As for the cells without demarcated borders, the studlike microvilli all changed into ridges or globular chains (Fig. 8).

DISCUSSION

The topological characteristics of human amnion monolayer cells presented in this report were similar to those noted in epithelial cells from various tissues (8, 10). It appears, however, that cultured amnion cells consisted of at least two types of morphologically distinguishable cells, each exhibiting a different type of cytopathic change when exposed to *C. difficile* toxin. Based on morphology, the toxin appears to cause the cell membrane to retract and to cleave. The cells became rounded and they dropped off the glass surface, leaving some membrane fragments still attached. These round cells are unlikely to be associated with mitosis, since cell proliferation activity in primary amnion cell culture is extremely low.

The mechanism of cytopathic effects described in this report is yet to be determined. Our data suggest that modification of the cell membrane is one of the main effects of *C. difficile* toxin; these pathogenic events are seen with a variety of other toxins which bind cell membranes and lead to cell destruction (3, 6). The dramatic effect of this toxin on epithelial cells may have a direct bearing on the disease state in humans and animals.

Ultrastructural changes of the cell membranes were detected within 1 h after toxin exposure, whereas cytopathic effects of the whole cells as detected by conventional light microscopy did not begin until 3 h after toxin exposure (4). Alterations in cell membranes observed by scanning electron microscopy seemed to reach a maximum at 1 h, whereas changes in the cell morphology observed by light microscopy progressed and reached their maximum in 24 to 48 h.

Willingham and Pastan have implicated cyclic adenosine 3′,5′-monophosphate as the key regulator for both microfilament- and microtubule-dependent functions, including cell shape and motility (11). It would be of interest to determine whether there is a change in the intracellular cyclic adenosine 3′,5′-monophosphate level in toxin-treated cells.

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