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NOTES

Loss of Surface Fibronectin from Human Lung Fibroblasts Exposed to Cytotoxin from *Clostridium difficile*

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Clostridium difficile cytotoxin caused an irreversible dose- and time-dependent loss of fibronectin from the surfaces of human lung fibroblasts, paralleling the appearance of the cytopathic effect. Fibronectin was not required for the intoxication process. The results lend further support to a transmembrane connective link between fibronectin and the microfilaments.

Clostridium difficile is the major causative agent of antibiotic-associated pseudomembranous colitis (4, 10). A cytotoxin from *C. difficile* has been described (5, 16), and recently an enterotoxin was isolated (2, 3, 23, 24). The cytotoxin affects a variety of cultured mammalian cells, inducing in fibroblasts a characteristic cytopathic effect (CPE) called an actinomorphic change (6, 7) and leading to cell death after some days (9). The enterotoxin causes fluid accumulation in the rabbit ileal loop assay but is almost completely devoid of cytopathic activity (2, 3, 23, 24).

The appearance of the CPE in human lung fibroblasts is paralleled by the disappearance of the microfilament bundles and the condensation of actin around the nuclei, as shown with indirect immunofluorescence with antibodies against actin (25). Whether the microfilaments constitute a primary or secondary target of the toxin is not known. Disorganization of the microfilament system in fibroblasts is generally associated with a loss of the cell surface protein fibronectin, which occurs (for example) during mitosis (26), after treatment with cytochalasins (1, 15), and in most transformed cell lines (12). On the other hand, externally added fibronectin causes the assembly of the microfilament bundles in transformed cells (14). If surface fibronectin is lost by external cleavage with proteases, the microfilament system is disorganized (19, 21). It is, therefore, currently believed that the microfilaments in fibroblasts are connected to surface fibronectin across the plasma membrane (11, 13). This transmembrane association has been demonstrated by electron microscopy (22). The finding that the microfilaments are affected by *C. difficile* toxin prompted us to investigate whether the toxin might also influence the distribution of surface fibronectin.

Toxin from a clinical isolate of *C. difficile* was obtained by repeated extraction of centrifuged bacteria with 1 M NaCl (9). This preparation is here referred to as the cytotoxin since more than 99% of the cytopathic activity of *C. difficile* toxins has been attributed to the cytotoxin (toxin B), even in crude culture filtrates (17). The preparations were devoid of detectable protease, phospholipase C, and hemolytic activities. The unit of toxin activity was the 50% tissue culture dose (TCD_{50}), i.e., the toxin dilution inducing a CPE in 50% of the exposed cells within 20 h (9). One TCD_{50} corresponded to approximately 1 ng of protein in our preparations.

Human lung fibroblasts (MRC-5) cultivated on glass slides in Eagle minimal essential medium were exposed to increasing doses of toxin, and the distribution of fibronectin was studied by an indirect immunofluorescence technique with rabbit immunoglobulin G (IgG) against human plasma fibronectin (8) and fluorescein-conjugated swine anti-rabbit IgG. After 1 h, when no CPE was yet observable, the same fibronectin distribution was seen as for control cells (Fig. 1A). In these cultures dense fibronectin strands formed a continuous network around the cells. Treatment for 2 h with doses of 200 TCD_{50} or higher, resulting in a dose-related development of the CPE, appeared to cause a dose-related loss of fibronectin from the cell surface, although a strict quantification of surface fibronectin is not possible with this method. Treatment for longer times resulted in a higher loss of fibronectin than treatment for 2 h did. After 4 h with 200 TCD_{50}, the CPE had developed in

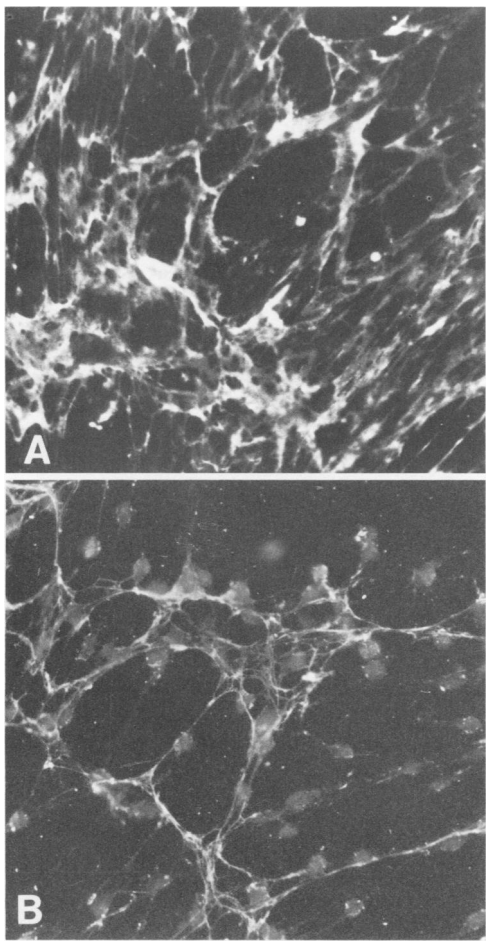

FIG. 1. Distribution of fibronectin on human lung fibroblasts treated with *C. difficile* toxin. The fibronectin was visualized with rabbit anti-fibronectin IgG and fluorescein-conjugated swine anti-rabbit IgG. Cells treated with preimmune IgG instead of anti-fibronectin IgG showed little or no staining. (A) Cells in Eagle medium (B) Cells treated for 4 h with 200 TCD_{50} (in Eagle medium). Magnification, ×450.

almost all of the cells, and the fibronectin network was loose, consisting mainly of long fine fibrils (Fig. 1B). Treatment for 24 h with 200 TCD_{50}, leading to 100% CPE, resulted in a nearly complete loss of fibronectin. Only a few short and coarse strands remained. Thus, the loss of fibronectin appeared to parallel the development of the CPE.

The loss of fibronectin from intoxicated cells was irreversible. If the toxin was removed after 4 h of incubation and replaced with fresh medium, the CPE remained, and the fibronectin network was not restored after 20 h of further incubation. This contrasted with observations on cultures treated with cytochalasin B, in which fibronectin reappeared on the cell surface after 20 h in fresh medium, as has also been shown by others (15).

There are many similarities between cytochalasins and *C. difficile* cytotoxin with respect to their effects on fibroblasts: the arborized actinomorphic shape of exposed cells, the disorganization of microfilament bundles (18, 25), and the loss of surface fibronectin (1, 15). Important differences are that the effects of the *C. difficile* toxin are irreversible (9), whereas those of cytochalasins are completely reversible (15, 18) and also occur more rapidly. There is no competition between *C. difficile* toxin and cytochalasin B, indicating that these agents do not share the same receptor (25).

Human lung fibroblasts stripped from fibronectin with trypsin (1) before toxin treatment, as well as cells coated with anti-fibronectin antibodies, responded with the usual CPE. Two mutant lines of baby hamster kidney fibroblasts deficient in fibronectin (Ric^R14 and Ric^R21) (20) showed the same dose response to the toxin as the wild-type cell line (and as human lung fibroblasts). Thus, surface-bound fibronectin does not seem to play a role in the binding of the toxin to the cells or in its subsequent cytotoxic action.

In conclusion, *C. difficile* cytotoxin induced an irreversible loss of fibronectin from the fibroblast surface, paralleling the CPE caused by a disorganization of the microfilament system. Since the CPE developed independently of the presence of surface fibronectin, the loss is probably a secondary phenomenon in the intoxication process. The fact that the loss of surface fibronectin parallels the disorganization of the microfilaments lends further support to the existence of a transmembrane connective link between surface fibronectin and the microfilament system.

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