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Visualizations of Binding and Internalization of Two Nonlinked Protein Components of Botulinum C_2 Toxin in Tissue Culture Cells

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Binding and internalization of two nonlinked components of botulinum C_2 toxin were visualized in tissue culture cells with components directly labeled with fluorescence. The binding of both untrypsinized and trypsinized component II (UT-II and T-II, respectively) to common specific sites on the cell membrane was evidenced by competitive binding between fluorescence-labeled and unlabeled components. The distribution patterns of fluorescence-labeled T-II and UT-II after binding to cells at 37°C were different; T-II clustered on the cell membrane and entered the cells in endosomes, whereas UT-II entered the cells inefficiently and not in vesicles and was distributed on the nuclear surface. The difference may be due to the multivalent property of T-II, which is not shared with UT-II. Fluorescence-labeled component I, which binds only to cells bound with T-II, entered cells by the same route as T-II did; both colocated on the same clusters on the cell membrane and also in the same vesicles in the cytoplasm. The present results suggest that component I of C_2 toxin, which ADP-ribosylates cytoplasmic actin, directly binds to T-II but not to UT-II on the cell membrane and is internalized into cells together with T-II in the same endosomes.

Botulinum C_2 toxin (C2T) is produced by certain strains of *Clostridium botulinum* types C and D and is composed of two protein components, I and II, which are not linked by covalent or noncovalent bonds (9). These two molecules are functionally different proteins: component II is a binding subunit, and component I is an ADP-ribosyltransferase whose substrate is cytoplasmic actin (7, 11). The toxin has various biological activities, lethality, enterotoxicity, and cytotoxicity, all of which are activated by trypsinization of a mixture of the two components and elicited by their cooperation (5, 6). These activities of C2T are caused by ADP-ribosylation of cytoplasmic actin by component I (11). The activation of the toxin by trypsin is due to the molecular cleavage of component II but not of component I (8). Previous study by indirect immunofluorescence staining has shown that component II, either trypsinized (T-II) or untrypsinized (UT-II), binds to epithelial cells and to the brush borders of mouse intestine, whereas component I alone does not (10). The binding of component I to cells and brush borders is dependent on the presence of T-II but not of UT-II, indicating that the enterotoxic activity of the toxin is initiated by the binding of T-II to the microvillous membranes of intestinal cells and then the subsequent binding to and internalization of component I in cells.

In a previous study, we used indirect immunofluorescence staining to examine the binding of the two components of C2T to tissue culture cells and showed that T-II, UT-II, and also component I in the presence of T-II bound in particle form to tissue culture cells, although the sizes and distributions of the particles were different among the components (5). However, the indirect immunofluorescence staining employed in that study sometimes caused a time lag between binding and detection of the components during which the internalization process might progress, especially when the cells were not fixed. In this study, therefore, we prepared the directly labeled fluorescent derivatives of components I and II and attempted to detect the localization of these two components of C2T during their binding and internalization in tissue culture cells. The results provide visual evidence for the fate of two nonlinked components of the toxin in the cells.

MATERIALS AND METHODS

Chemicals. Dichlorotriazinylaminofluorescein 2HCl (DTAF) and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Research Organics, Inc., Cleveland, Ohio, and Molecular Probes, Inc., Eugene, Oreg., respectively.

Preparation of two components of C2T. The two components of C2T were purified from a culture of *C. botulinum* type C strain 92-13 as described previously (9). T-II was prepared by gel filtration as described previously (8).

Fluorescence labeling of components I and II of C2T. Components I and II were directly labeled with DTAF and TRITC, respectively. To prepare DTAF-labeled component I (DTAF-I), 200 μg of the component was incubated with 40 nmol of DTAF in 100 mM K,Na phosphate buffer, pH 8.0, at 4°C overnight. The reaction mixture was dialyzed three times against 500 ml of 0.2 M NaCl–50 mM K,Na phosphate buffer, pH 7.5, to remove free fluorescence dye and centrifuged at 20,000 × *g* for 15 min. Labeled component I was stored at −80°C. Labeling of UT-II and T-II was done similarly, except that TRITC instead of DTAF was used.

Cell culture. Vero cells were grown in minimal essential medium (MEM) containing 10% fetal calf serum. Cells to be stained with fluorescence-labeled components I and II were grown on glass coverslips cut into squares of about 3 by 3 mm which were placed in wells of a 96-well plastic plate (Corning 25860) and sterilized under UV light for at least 30 min. To stain the fluorescence-labeled components of C2T,

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TABLE 1. Rounding activity of fluorescence-labeled components I and II of C2T

Toxin[a]	Rounding activity (ng)[b]	Ratio[c]
DTAF-I + T-II	0.42	0.82
Component I + TRITC-T-II	0.75	1.47
Component I + TRITC-UT-II[d]	0.66	1.29
Component I + UT-II[d]	0.45	0.88
Component I + T-II	0.51	1.00

[a] Toxin was prepared by combining components I and II on a protein basis of 1:2.
[b] Expressed as nanograms of toxin that caused 50% rounding of cultured cells (5).
[c] Relative to the activity of a mixture of unlabeled component I and T-II.
[d] TRITC-UT-II and UT-II were trypsinized as described previously (8) and combined with component I.

the monolayer culture of the cells was washed twice with Na,K-phosphate-buffered saline (PBS) and incubated with 0.2 ml of fluorescence-labeled toxin component per well (0.16 cm^2 per well) of the 96-well plate at the temperature indicated. The fluorescence derivatives were diluted in MEM containing 1% bovine serum albumin (MEM-BSA). To terminate the reaction, the cells were washed with ice-cold PBS and fixed with 10% formalin–PBS on ice for 30 min. The fixed cells were washed again with PBS and mounted in 50% glycerol–PBS. The cells were viewed in a Nikon Optiphoto microscope (Nippon Kogaku Co., Tokyo, Japan) equipped for epifluorescence. Fluorescence micrographs were photographed with Neopan 400 film (Fuji Photo Film Co., Tokyo, Japan).

Assay for rounding activity. The rounding effect of C2T on Vero cells grown in a 96-well plate was determined as described previously except that the incubation period was 4 h instead of 18 h (5).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with a 10% gel according to the method of Laemmli (4). Gels were stained with 1% Coomassie blue–50% trichloroacetic acid and destained with 7% acetic acid.

Protein determination. Protein concentrations were determined by the protein-dye method (1) with BSA as the standard.

RESULTS

Characterization of fluorescence-labeled components of C2T. To ascertain the biological activities of the fluorescence-labeled components (DTAF-I, TRITC-labeled T-II [TRITC-T-II], and TRITC-UT-II) of C2T, the rounding activities of these fluorescent derivatives were determined. Table 1 shows 50% rounding doses of the fluorescence-labeled and unlabeled components of C2T. All of the fluorescent derivatives retained almost the same activity after being labeled with each type of fluorescence. From the A_{489} of DTAF and the A_{544} of TRITC, the molar ratios of fluorescence to component were calculated as 4.99, 4.59, and 2.72 for DTAF-I, TRITC-T-II, and TRITC-UT-II, respectively.

Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fluorescence-labeled components I and II are shown in Fig. 1. The labeled components were highly homogeneous and have the same mobilities as the corresponding unlabeled components. Without staining the proteins on the electrophoresed gel, reddish pink bands of

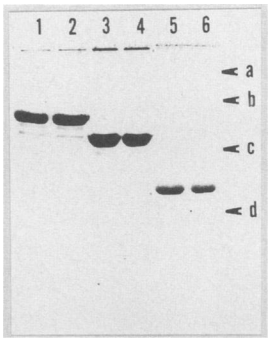

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the two fluorescence-labeled components of C2T. Each 2.5 μg of unlabeled or labeled components I and II was analyzed in a 10% gel, stained with 0.1% Coomassie blue–50% trichloroacetic acid, and destained with 7% acetic acid. Lanes: 1, unlabeled UT-II; 2, TRITC-UT-II; 3, unlabeled T-II; 4, TRITC-T-II; 5, unlabeled component I; 6, DTAF-I. Marker proteins were myosin (200 kDa) (a), β-galactosidase (116 kDa) (b), BSA (66 kDa) (c), and aldolase (42 kDa) (d).

TRITC-UT-II and TRITC-T-II and the yellowish green band of DTAF-I were observed under UV light at 259 nm.

To determine the specific binding of fluorescence-labeled component II, monolayer cells in wells of a 96-well plate were preincubated with 10 μg of unlabeled T-II or 20 μg of unlabeled UT-II per well on ice for 30 min, washed with PBS, and then incubated at the same temperature for the same period with 1 μg of TRITC-T-II or 2 μg of TRITC-UT-II, respectively. Similarly, to determine the specific binding of DTAF-I, cells were preincubated with 10 μg of unlabeled component I in the presence of 10 μg of T-II on ice for 30 min, washed with PBS, and then incubated with 1 μg of DTAF-I at the same temperature for the same period as for component II. In all of these assays, marked decreases in binding of fluorescence-labeled components I and II to cells were observed. Competitive binding between UT-II and T-II was also observed when the cells were incubated with unlabeled T-II prior to incubation with TRITC-UT-II or with unlabeled UT-II prior to incubation with TRITC-T-II as described above; the binding of both types of labeled component II to cells was decreased by preexposing the cells to unlabeled component II. The results indicate that the two fluorescence-labeled components of C2T bind to specific sites on the cell surface and that both T-II and UT-II bind to the same site on the membrane of the cells.

Binding of fluorescence-labeled component II to Vero cells. To examine the localization of membrane-bound T-II, monolayer cultures of Vero cells were incubated with TRITC-T-II at 37°C for various lengths of time. When the cells were incubated with 1 μg of TRITC-T-II per well for 5 min, the fluorescence bound in a "mosaic" or "reticulately" on the cell surface, although some of it formed fine particles (Fig. 2a). At 15 min after incubation, clusters of fluorescence appeared on the cell surface and at cellular borders (Fig. 2b). At 30 min, the number of larger clusters increased (Fig. 2c). Judged from the planes of focus, the clustered fluorescence distributed in the perinuclear region was clearly inside the cells and was interpreted to be inside endocytotic vesicles. At 1 h, these vesicles increased in number and formed larger granules, probably because of the fusion of vesicles (Fig. 2d). At 2 h, fluorescent vesicles were still detected in the perinuclear region, and some of them seemed to be redistributed to the cellular borders (Fig. 2e).

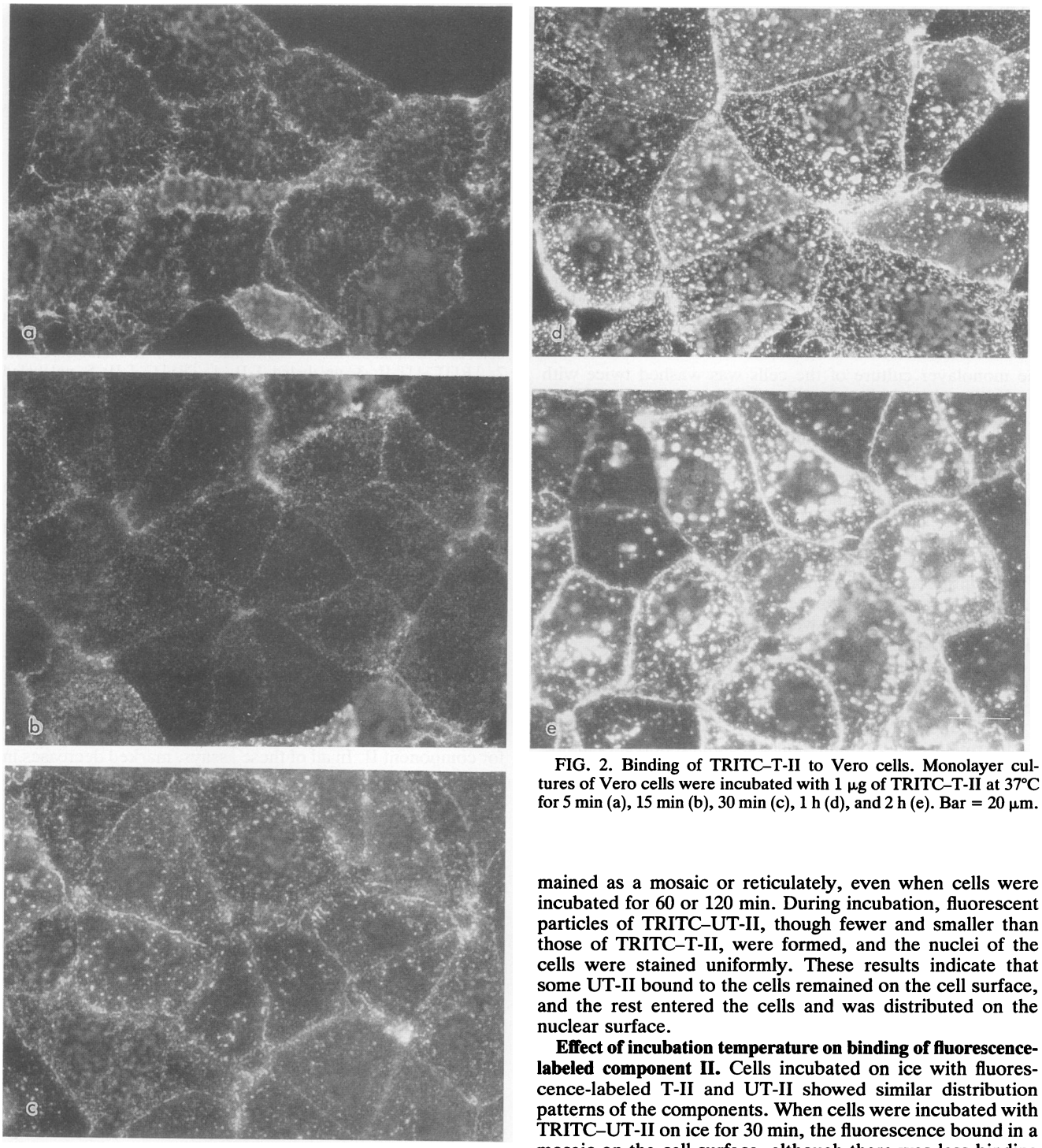

FIG. 2. Binding of TRITC–T-II to Vero cells. Monolayer cultures of Vero cells were incubated with 1 μg of TRITC–T-II at 37°C for 5 min (a), 15 min (b), 30 min (c), 1 h (d), and 2 h (e). Bar = 20 μm.

Figure 3 shows the time course of binding of TRITC–UT-II to cultured cells. The amount of bound TRITC–UT-II was apparently lower than that of TRITC–T-II throughout the incubation period, even if the lower efficiency of fluorescence labeling of UT-II was taken into consideration. At 15 min of incubation, TRITC–UT-II clearly stained the cellular borders. In contrast to the distribution pattern of TRITC–T-II, most TRITC–UT-II bound on the cells remained as a mosaic or reticulately, even when cells were incubated for 60 or 120 min. During incubation, fluorescent particles of TRITC–UT-II, though fewer and smaller than those of TRITC–T-II, were formed, and the nuclei of the cells were stained uniformly. These results indicate that some UT-II bound to the cells remained on the cell surface, and the rest entered the cells and was distributed on the nuclear surface.

Effect of incubation temperature on binding of fluorescence-labeled component II. Cells incubated on ice with fluorescence-labeled T-II and UT-II showed similar distribution patterns of the components. When cells were incubated with TRITC–UT-II on ice for 30 min, the fluorescence bound in a mosaic on the cell surface, although there was less binding than at 37°C (Fig. 4a). When these cells were then incubated at 37°C for 60 min after being freed from unbound TRITC–UT-II, bound fluorescence still remained as a mosaic on the cell surface, while small particles of fluorescence and staining of nuclei were observed (Fig. 4b). This distribution pattern of fluorescence was very similar to that of cells exposed directly to TRITC–UT-II at 37°C for 60 min. The mosaic distribution of fluorescence was also observed when cells were incubated on ice with TRITC–T-II for 30 min (Fig. 4c). When these cells were then incubated at 37°C for 60 min

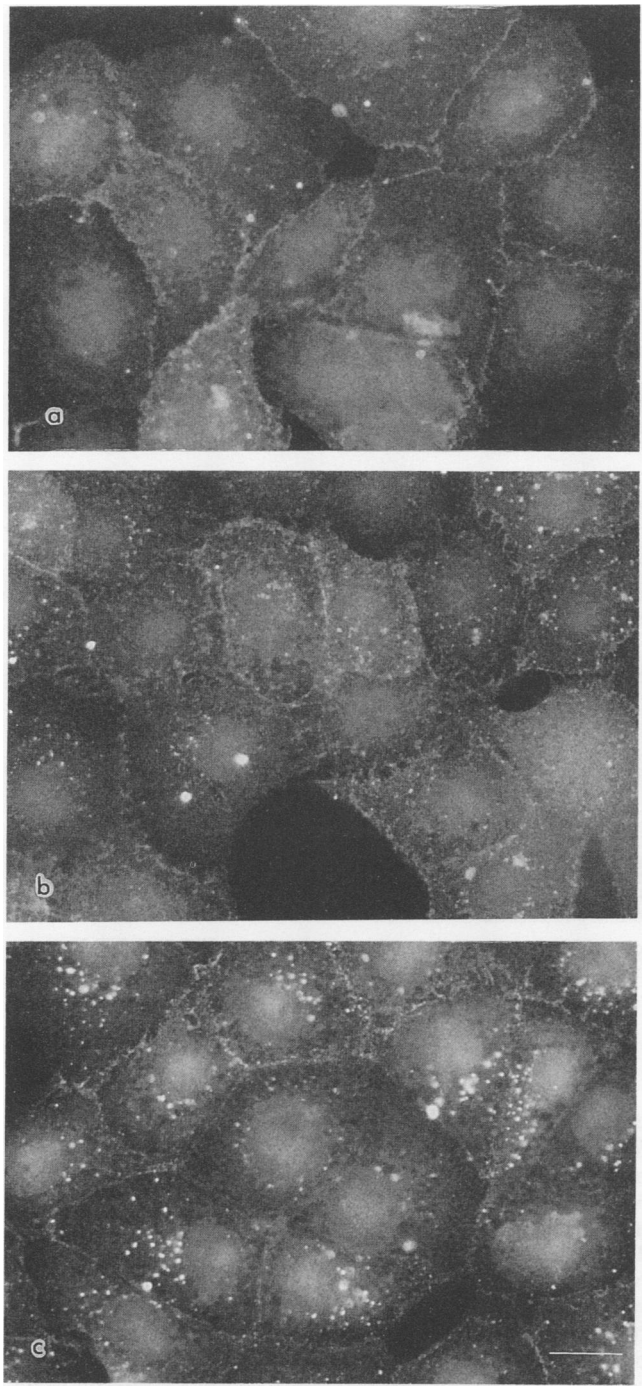

FIG. 3. Binding of TRITC–UT-II to Vero cells. Monolayer cultures of Vero cells were incubated with 2 μg of TRITC–UT-II at 37°C for 15 min (a), 1 h (b), and 2 h (c). Bara = 20 μm.

after being freed from unbound TRITC–T-II, the mosaic fluorescence clustered on the cell membrane and aggregated into vesicles (Fig. 4d). This distribution pattern of TRITC–T-II was again very similar to that in cells exposed directly to the fluorescent derivative for 30 min at 37°C.

Reversible binding of TRITC–T-II on surfaces of cultured cells. When cells were exposed to TRITC–T-II at 37°C for 15 min and then to unlabeled T-II at the same temperature for 60 min, the intensity of the fluorescence bound to the cells was reduced (Fig. 5a and b). On the other hand, almost no decrease in the intensity of fluorescence was observed when the cells were exposed to TRITC–T-II at 37°C for 60 min and then to unlabeled T-II for 60 min (Fig. 5c and d). The decrease in binding of labeled T-II to cells caused by subsequent incubation with unlabeled T-II was more effective when cells and the component were incubated on ice than when they were incubated at 37°C. These results indicate that the component is more efficiently internalized into cytoplasm on longer incubation at 37°C and that the labeled T-II bound on the cell surface is competitively replaceable with unlabeled T-II.

Binding of DTAF-I to Vero cells in the presence of TRITC–T-II. To examine the binding of component I, the monolayer culture of Vero cells was incubated with a mixture of DTAF-I and TRITC–T-II for various lengths of time. At 15 min of incubation, both fluorescence-labeled derivatives bound as a mosaic on the cell surface, although a few clusters were observed, and the distribution patterns of the two labeled components were very similar (Fig. 6a and d). At 30 min, the numbers of clusters of fluorescence-labeled components I and T-II increased and were localized at the same sites; judged from the planes of focus, some of these clusters were apparently endosomes in cytoplasm (Fig. 6b and e). At 60 min, the cells altered from polygonal to actinomorphic forms, and some of the cells rounded because of incubation with a mixture of DTAF-I and TRITC–T-II. Even in these deformed cells, both fluorescence-labeled components were distributed similarly on the cells (Fig. 6c and f).

When cells were incubated with fluorescence-labeled component I and T-II separately, binding of component I to the cells preexposed to T-II was observed. When cells which had been preincubated with TRITC–T-II at 37°C for 30 min and freed from the unbound component were exposed to DTAF-I and immediately washed with PBS, labeled component I bound as a mosaic on the cell surface, while some of the bound TRITC–T-II clustered and was localized in vesicles (Fig. 7a and c) This indicates that component I binds to cells bound with T-II as long as T-II remains on the cell surface. The clustering of DTAF-I was observed when cells which had been pretreated with TRITC–T-II and exposed to labeled component I as described above were incubated at 37°C for 30 min (Fig. 7b to d); the clustered fluorescence of both component I and T-II was localized almost on the same sites. These results indicate that even when the two components of C2T are incubated separately, component I binds to the cell surface bound with T-II and is processed together with T-II during internalization, as was observed with cells exposed simultaneously to both components.

DISCUSSION

By using fluorescence-labeled derivatives of two nonlinked protein components of C2T, we have attempted to visualize (i) the initial binding of the components to the cell surface, (ii) the clustering of the components on the cell membrane, and (iii) the internalization of the membrane-bound toxin components. The derivatives of the two components of C2T that were directly fluorescence labeled retained approximately the same biological activity with each of the corresponding unlabeled components and were competitive with the unlabeled component in binding to the cells. The results indicate that the fluorescence-labeled and unlabeled components bind to the same and specific sites on

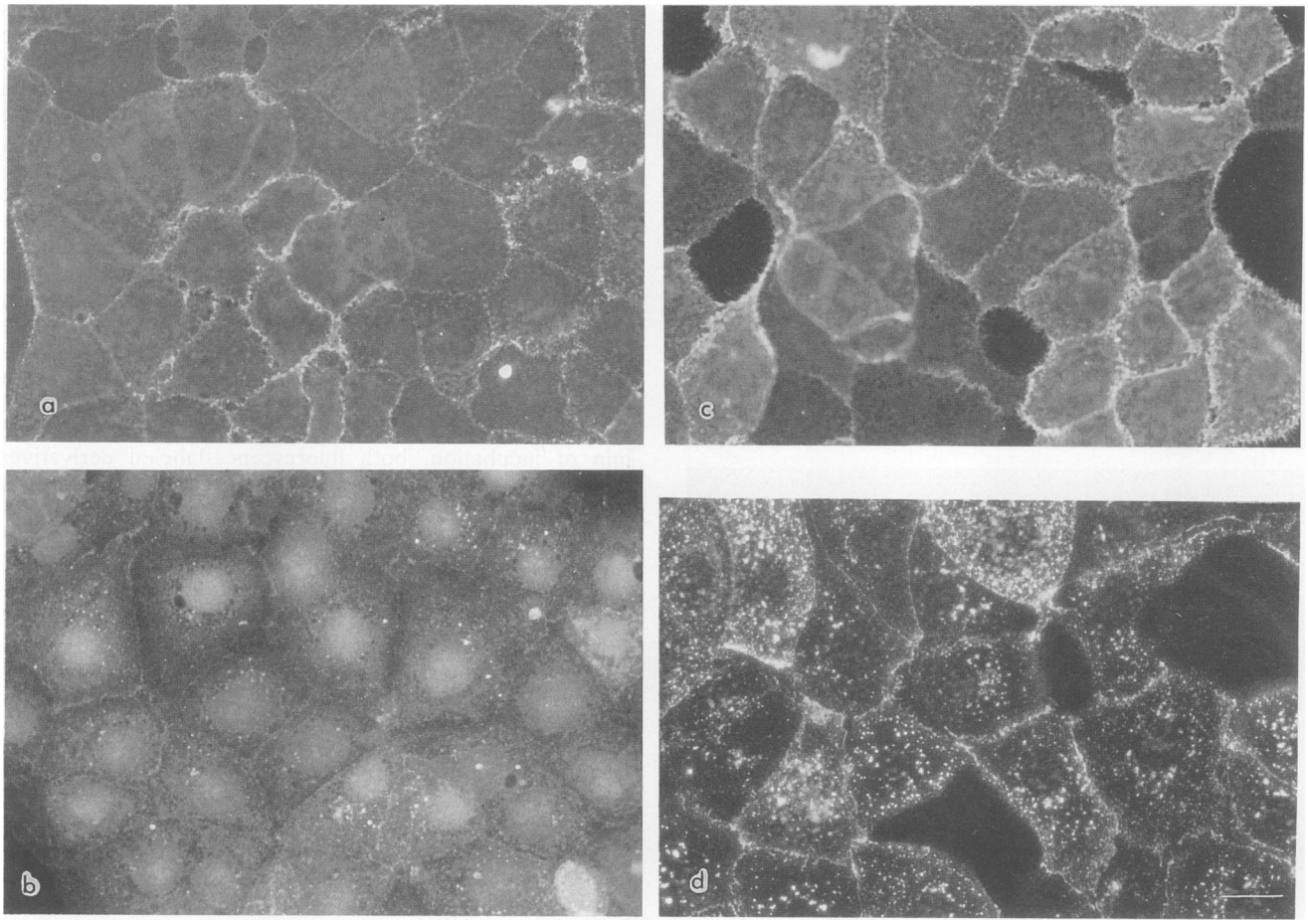

FIG. 4. Binding of TRITC–UT-II and TRITC–UT-II to Vero cells on ice. (a) Vero cells were incubated with 2 μg of TRITC–UT-II on ice for 30 min. (b) After this incubation, cells were washed with PBS and incubated in MEM-BSA at 37°C for 60 min. (c) Cells were incubated with 1 μg of TRITC–T-II on ice for 30 min. (d) Cells exposed to TRITC–T-II were washed with PBS and then incubated in MEM-BSA at 37°C for 60 min. Bar = 20 μm.

the cell membrane. The specific binding of the labeled component was also evidenced by competitive replacement of the labeled component with the corresponding unlabeled component. Thus, the fluorescence-labeled components prepared in the experiment described here allowed direct visualization of the binding of the two components of C2T without amplification and permitted analyses of dynamic processes of the two cell-bound components.

Previous studies by indirect immunofluorescence staining have shown that both UT-II and T-II, the binding component of C2T, bind to isolated epithelial cells and to brush borders of mouse intestine, while component I, the active component of the toxin, binds to cells and brush borders only in the presence of T-II (6). In these experiments, these findings were confirmed with two directly fluorescence-labeled components of C2T in tissue culture cells, and the difference in the dynamic processes of components I and II after binding to the cells was visualized.

Both fluorescence-labeled UT-II and T-II initially bind to the same site on the cell membrane. This was evidenced by the concentration of bound fluorescence of both components on the cellular border and competition between UT-II and T-II for binding to cells. However, the fates of these components after binding to cells differed. At 37°C, the membrane-bound TRITC–T-II redistributed and aggregated to form clusters, while most of TRITC–UT-II remained as a mosaic on the cell membrane. With further incubation at 37°C, the fluorescent vesicles of TRITC–T-II were found inside the cells and later accumulated in a perinuclear region. The endocytosed vesicles in this region became larger, possibly because of fusion with other vesicles. When cells were incubated longer at 37°C, the vesicles in the perinuclear region decreased in number, whereas those near the cellular borders increased in number, implying that the ligand-receptor complex of T-II recycles from the cytoplasm to the cell surface. Thus, T-II of C2T binds to the specific receptor site on the cell membrane, aggregates on coated pits, and enters the cells by receptor-mediated endocytosis.

In contrast to the distribution pattern of T-II, fluorescence-labeled UT-II, which shares the specific binding site of T-II on the cells, was distributed diffusively on the cell surface and formed a few particles even with longer incubations at 37°C. However, membrane-bound UT-II enters the cells in a different way from T-II, because the nuclei of the cells exposed to fluorescent UT-II were always stained with fluorescence even after a short incubation. It is not yet clear whether this staining is due to the incorporation of UT-II by a constitutive endocytosis of the cultured cells used in these

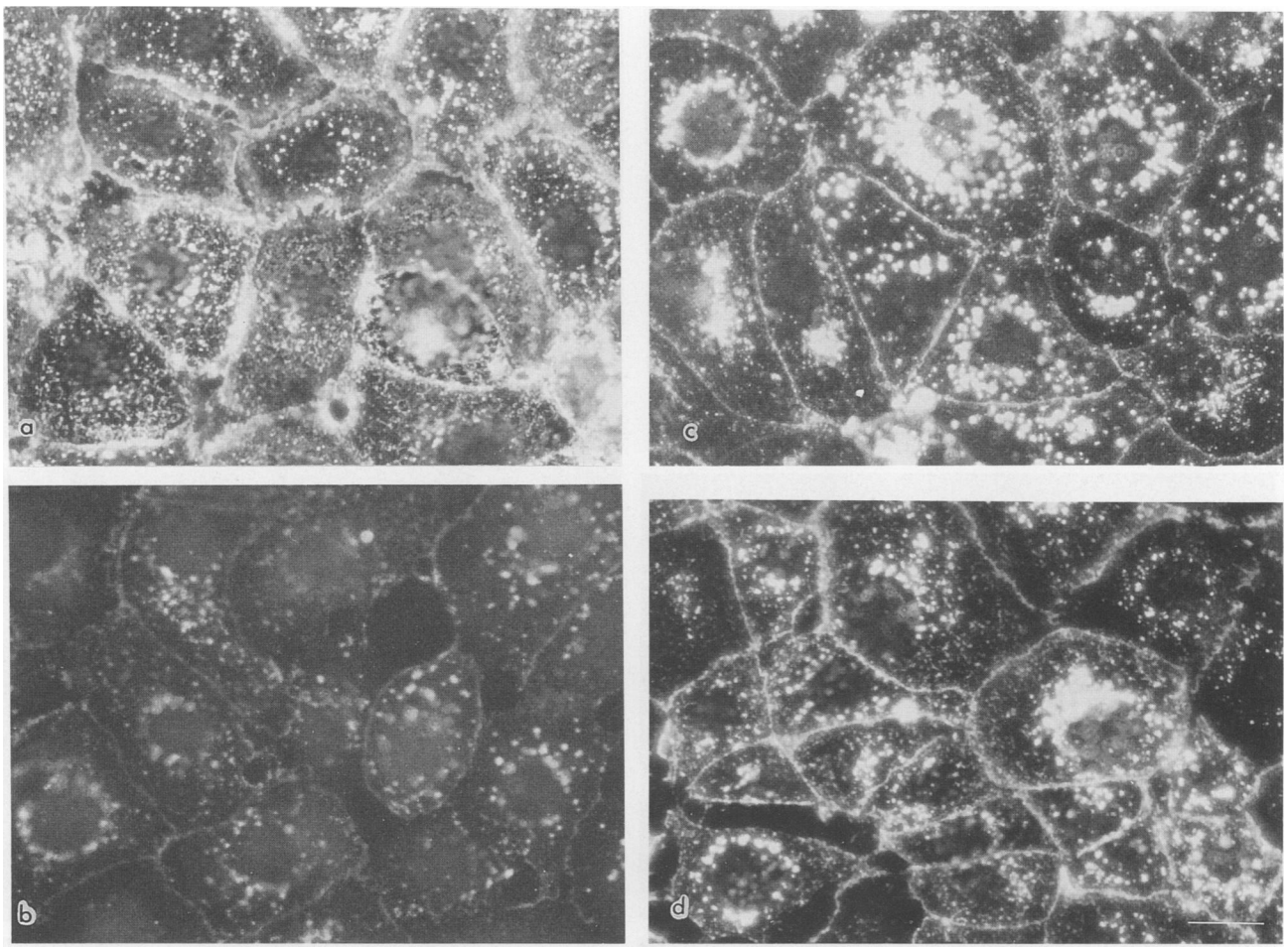

FIG. 5. Reversible binding of TRITC–T-II to Vero cells. Monolayer cultures of Vero cells were incubated with 1 μg of TRITC–T-II at 37°C for 15 min (a, b) or 60 min (c, d) and then with 10 μg of unlabeled T-II at 37°C for 60 min (b, d) or with MEM-BSA for 60 min (a, c). Bar = 20 μm.

experiments. However, the cells apparently have the ability to incorporate the foreign protein if it binds to the cell membrane, though not when it binds in clusters. This probably explains why component I, which alone does not bind to the cell membrane, is not incorporated into cells unless T-II, which induces the binding site for component I on the cell membrane, is present.

In a previous study, we reported that T-II has hemagglutinating activity, whereas UT-II does not (8). This probably causes the difference in processing between UT-II and T-II by the cells after the component binds to the cell surface, because the finding that T-II has hemagglutinating activity indicates that the protein is a multivalent or at least a divalent molecule which possibly cross-links the receptor-ligand complexes and causes them to cluster on the cell surface. There are lines of evidence indicating that multivalent ligands induce clustering on the cell surface prior to endocytosis; divalent antibodies induce patching or capping of immunogloblin receptors in lymphocytes (3, 12), and multivalent lectins induce patch formation in tissue culture cells prior to internalization (2). Therefore, T-II but not UT-II is categorized as one of these proteins. Because T-II (88 kDa) is derived from UT-II (110 kDa) by trypsinization and has exactly the same primary structure as most parts of UT-II (8), the difference in redistribution and internalization of UT-II and T-II provides a useful model for studying the cellular mechanism of internalization of the protein molecule through the cell membrane.

When cells were incubated on ice with fluorescence-labeled T-II and UT-II, the distribution patterns of components bound on the cell membrane were almost identical. When these cells were further incubated at 37°C, the fluorescence of both components distributed similarly, as was observed with cells incubated with labeled components from the beginning at 37°C; T-II clustered on the cell membrane and was internalized in endocytotic vesicles, whereas UT-II entered the cells and was localized on the nuclear surface. These results again show that the initial binding site for both components on the cell membrane is the same, although processing of the components after binding to the cells is different. Moreover, the results suggest that the incorporation of component II, either trypsinized or untrypsinized, is an energy-dependent process, as is incorporation of other protein molecules which enter the cells by endocytosis.

When the cells were exposed to a mixture of fluorescence-labeled component I and T-II at 37°C, double binding of cells to these components was observed, although no binding of component to cells was observed when the cells were incubated with labeled component I alone or with labeled component I in the presence of UT-II. Double binding of

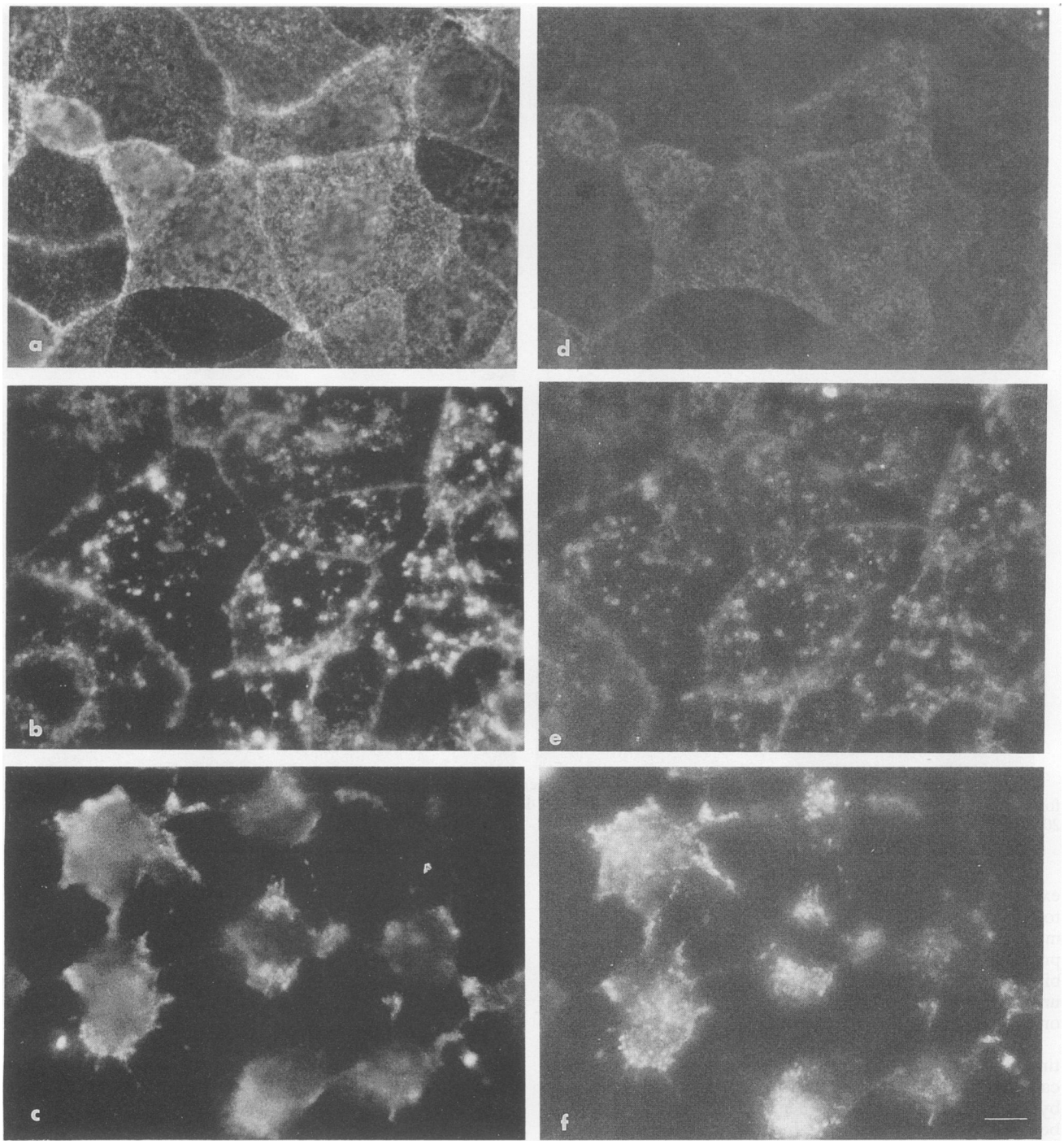

FIG. 6. Binding of DTAF-I and TRITC–T-II to Vero cells. Monolayer cultures of Vero cells were incubated with a mixture of 1 μg of DTAF-I and 1 μg of TRITC–T-II at 37°C for 15 min (a, d), 30 min (b, e), and 60 min (c, f). Photographs were taken with excitation filters for TRITC (a, b, and c) and DTAF (d, e, and f). Bar = 10 μm.

these two components to the cells was also observed when the cells were preexposed to fluorescence-labeled T-II and then to labeled component I. Bound fluorescent component I and T-II clustered on the cell surface and localized in the same vesicles inside the cells. This double binding of the fluorescent components is consistent with the finding that the activity of C2T is elicited only when component I binds to and enters cells bound with T-II but not with UT-II (5). The results indicate two possibilities: that the binding site for component I is induced on the cell membrane by binding of T-II to the cells or that component I binds directly to cell-bound T-II. If the binding site for component I is induced by the binding of T-II to the cell membrane, component I might distribute more diffusively on the cell

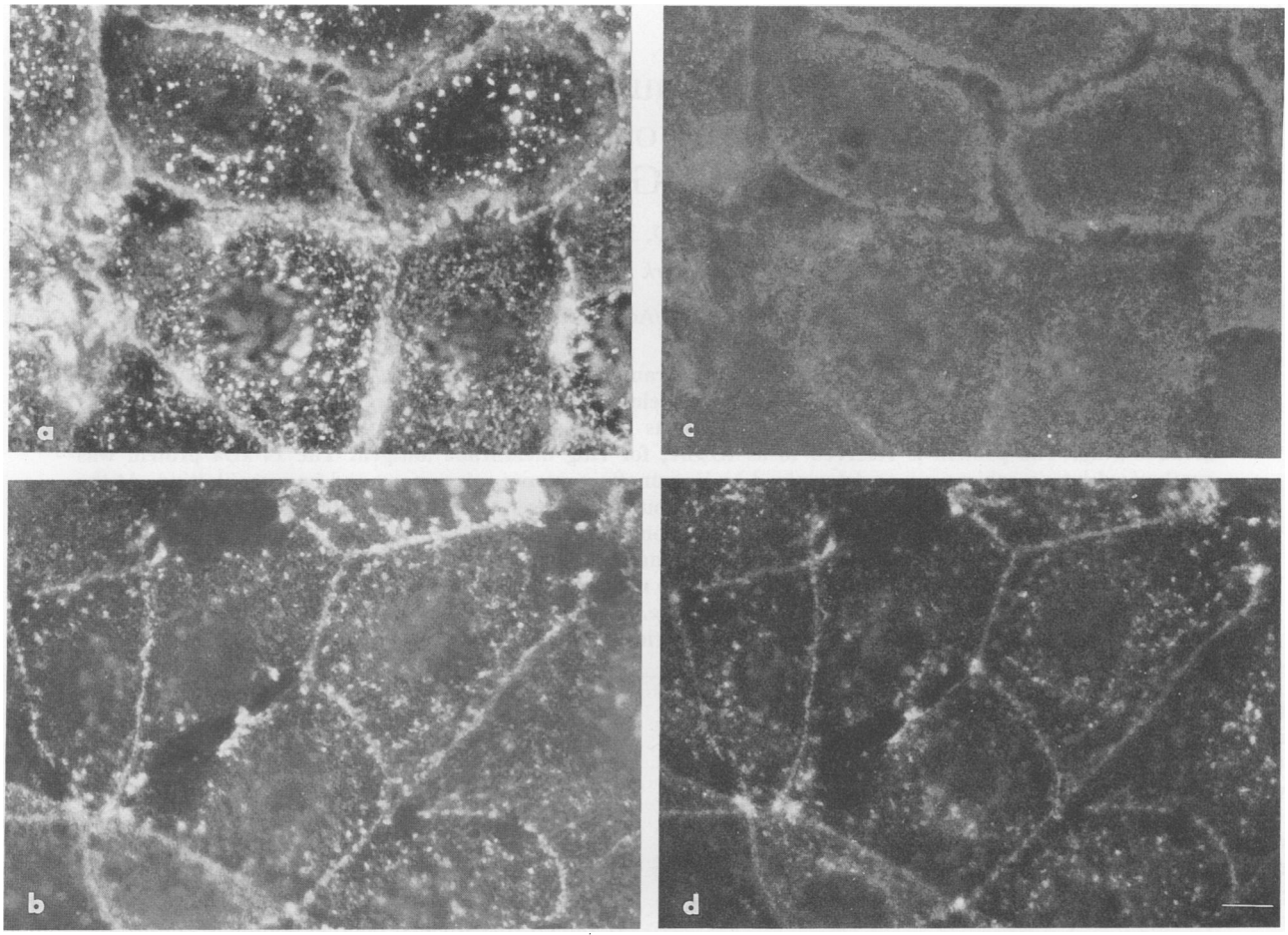

FIG. 7. Binding of DTAF-I to Vero cells bound with TRITC–T-II. Cells preexposed to 1 μg of TRITC–T-II at 37°C for 30 min and freed from unbound T-II by washing with PBS were incubated with 1 μg of DTAF-I at 37°C. Cells were washed with PBS immediately after exposure to DTAF-I (a, c) or after exposure to DTAF-I for 30 min (b, d). Photographs were taken with excitation filters for TRITC (a, c) and DTAF (b, d). Bar = 10 μm.

membrane, and some, but not all, of the clustered component I might localize in different vesicles from the clustered T-II. The fact that component I is distributed almost on the same site of the cell surface as T-II and localizes in the same vesicles in the cytoplasm as T-II suggests that component I binds directly to T-II bound to the cell membrane and enters the cells together with T-II in the same endosomes. The exact mechanism for binding and internalization of these two components is not yet clear, because light microscopy cannot differentiate the exact binding sites for the components. Such differentiation would be accomplished either by ultramicroscopy with colloidal-gold-labeled antibody or by chemical cross-linking to analyze the in vitro and on-cell interactions between the two components of the toxin.

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