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Selective Translocation of the A Chain of Diphtheria Toxin across the Membrane of Purified Endosomes*

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Translocation is a necessary and rate-limiting step for diphtheria toxin (DT) cytotoxicity. We have reconstituted DT translocation in a cell-free system using endosomes purified from lymphocytes and have demonstrated this using two different probe/cell systems, which provided identical results: ^{125}I -DT/human CEM cells and ^{125}I -transferrin-DT/mouse BW cells. The cell-free DT translocation process was found to be dependent on the presence of the pH gradient endosome (pH 5.3)/cytosol (pH 7). Among the pH equilibrating agents, nigericin (5 μM) was found to be the most effective, inhibiting DT translocation by 88%. An optimum pH value of 7 on the cytosolic side of the membrane (pH gradient ≈ 1.7) was determined. ATP *per se* is not required for DT translocation. ^{125}I -DT translocation was 3-fold more active from late than from early endosomes, probably because of their slightly more acidic pH. Only the A chain of the toxin was found to escape from either ^{125}I -DT/CEM or ^{125}I -transferrin-DT/BW endosomes. Translocation of control endosome labels (^{125}I -transferrin and ^{125}I -horseradish peroxidase) was never observed. We also show that DT receptors present on resistant (mouse) cells block the translocation of the toxin and are responsible for the resistance of these cells to DT.

Toxins such as ricin and diphtheria toxin (DT)¹ first need to reach the cytosol in order to exert their cytotoxic effect (1). Even though the identity of the cellular compartment from which ricin (at least the A chain) escapes to the cytosol has not yet been clearly identified (1, 2), all recent studies of DT entry into the cell agree that the translocation step is localized in the endosome. This process enables this toxin to gain access to its target, diphthamide, an amino acid only found in elongation factor 2 (3). Its ADP-ribosylation catalyzed by

the A chain of DT (made of two subunits, A and B linked by a disulfide bridge) inactivates protein synthesis leading to cell death (3).

Another action of DT through deoxyribonuclease activity has also been reported, but the contribution of this enzymatic activity in cytotoxicity is still being discussed (4).

Endocytosis is a first and necessary step for DT toxicity (3). After DT binding to a specific receptor (5), the toxin is internalized uniquely via coated pits (2) and reaches the endosome where the acidic pH will induce conformational changes (6). The A chain is then transferred to the cytosol. DT translocation has been studied using whole cells exposed to low pH after DT binding at 4 °C, simulating the endosome environment (3, 7). Incubation at 37 °C induced protection from pronase digestion of the A chain, which could be released from the cells upon permeabilization (3, 8). The presence of a pH gradient and of functional anion transport was required for translocation (3).

Since translocation is the rate-limiting step for toxin cytotoxicity (9), and because we found that the ricin translocation rate (which is pH independent) is much lower from plasma membrane vesicles than from endosomes,² we thought that DT translocation remained to be demonstrated and analyzed at the level of the membrane through which translocation naturally occurs: the endosome membrane.

We show in this paper that DT translocation can be observed in a cell-free system using functional endosomes purified from lymphocytes. A selective translocation of the A chain of DT was demonstrated and found to be dependent upon the pH gradient across the endosome membrane, the temperature, and the time of endocytosis. ATP *per se* was not required for the process. ^{125}I -DT translocation was only observed across the endosome membrane of DT-sensitive cells (CEM human lymphocytes). DT binding sites found on resistant cells (BW mouse lymphocytes) inactivate its translocation ability and protect these cells against intoxication. This resistance could be overcome by the use of an alternative binding system.

MATERIALS AND METHODS

Chemicals and Probes—Chemicals were obtained from Sigma (L'Isle d'Abeau, France) and radioactive products from Amersham (Les Ulis, France). Three different sources of DT were found to give similar results throughout this study. The first batch was obtained from Lisp Laboratories (Campbell, CA), the second was a kind gift of Dr. P. Boquet (Institut Pasteur, Paris), and a third was prepared using established procedures (10) from a suitable supernatant of cultures of *Corynebacterium diphtheriae* purchased from the Institut Mérieux (Marcy l'Etoile, France). Briefly, after ammonium sulfate precipitation and dialysis, DT was purified by ion-exchange chromatography on DEAE-cellulose followed by gel filtration (10). DT was identified by SDS-PAGE in reducing and nonreducing condi-

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¹ The abbreviations used are: DT, diphtheria toxin; Tf-DT, transferrin-DT; Fl-Tf, fluorescein-transferrin; Fl-DT, fluorescein-DT; LDL, low density lipoproteins; PBS, phosphate-buffered saline; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride.

² B. D. Beaumelle and C. R. Hopkins, unpublished data.

tions. It only gave one peak when analysed on a Mono Q column using a linear NaCl gradient and was thus free of contaminating nuclease (11). This preparation was used for transferrin-DT (Tf-DT) conjugation. Labeling of DT, horseradish peroxidase, transferrin, and Tf-DT with ^{125}I was performed using the Iodogen method (12) to a specific activity of 25 $\mu\text{Ci}/\mu\text{g}$ for DT and 8 $\mu\text{Ci}/\mu\text{g}$ for the other proteins. Fluorescein labeling and fluorescence measurements were carried out as described (13). Transferrin was conjugated with horseradish peroxidase using the SPDP method; ricin and DT were labeled with 10-nm diameter gold at pH 7 (13).

Conjugation of Transferrin with DT—Transferrin (15 mg in 1 ml of PBS) was treated with a 2 M excess of SPDP (40 mM in ethanol) for 30 min at room temperature. DT (6 mg in 0.7 ml of PBS) was derivatized in the same conditions with a 2.5 M excess of SPDP. Unreacted SPDP was removed by gel filtration on Sephadex G-25 (PD 10 columns, Pharmacia) eluted with PBS. DT was then concentrated back to its original volume by centrifugation (centricon 10, Amicon) and treated with 50 mM dithiothreitol. The reducing agent was eliminated by passing through a Sephadex G-25 column (1 \times 20 cm) equilibrated with PBS, and the proteins were mixed in a 1:1 molar ratio. After one night at room temperature a 100-fold molar excess (compared with DT) of iodoacetamide was added, and the conjugates were purified on a Sephacryl S-200 column (1 \times 65 cm) eluted with 2 ml of PBS/h. They were identified by SDS-PAGE, and stored sterile at 4 $^{\circ}\text{C}$.

A previously described method for coupling transferrin and DT via SPDP (14) mostly gave high molecular weight conjugates, whereas 1:1 hybrid was the major product of the conjugation when performed as described above (see "Results").

Cell Labeling and Fractionation—The human CCRF-CEM and the mouse BW 5147 leukemic T cell lines were maintained in culture as described previously (13, 15). They were labeled at a concentration of 2×10^7 cells/ml of DMEM supplemented with 0.1 mg BSA/ml (DMEM/BSA) and 150 μg of low density lipoproteins (LDL)/ml for 40 min at 37 $^{\circ}\text{C}$ (13). The following label concentrations were used: ^{125}I -DT and ^{125}I -horseradish peroxidase, 4 $\mu\text{Ci}/\text{ml}$; ^{125}I -transferrin and ^{125}I -Tf-DT, 1 $\mu\text{Ci}/\text{ml}$; fluorescein-transferrin (Fl-Tf) or Fl-DT, 0.1 mg/ml. After cooling to 2 $^{\circ}\text{C}$, washing twice with DMEM/BSA and ricin-gold treatment at 2 $^{\circ}\text{C}$, cells were submitted to the endosome isolation procedure (13). Briefly, cells were lysed (using, after the nitrogen cavitation treatment, 20 and 10 strokes of potter for BW and CEM cells, respectively), the post-nuclear supernatant was treated with 1 μg trypsin/ml for 3 min at 37 $^{\circ}\text{C}$, and crude membranes were separated on a discontinuous sucrose gradient made of 0.5 ml of 2.5 M sucrose and then 3.5 ml of 40, 30, and 20% sucrose prepared in gradient buffer: 1 mM EDTA, 10 mM acetic acid, 10 mM Tris (pH 7.5). Endosomes were collected from the 30%/20% interface. Ricin-gold binding increases the plasma membrane density, enabling pelleting during the gradient (13).

Translocation Assay—Endosomes were resuspended in translocation buffer (110 mM KCl, 15 mM MgCl_2 , 1 μg of penicillin G/ml, 20 mM Pipes (pH 7.1)) \pm 10 mM ATP and 10 mM MgCl_2 . After incubation at 37 $^{\circ}\text{C}$ for 2 h, except when otherwise indicated, translocation was terminated by cooling on ice and spinning through a 17% sucrose cushion (in gradient buffer) for 5 min at 160,000 $\times g$ (Beckman Airfuge centrifuge). Results are expressed as the increase in the ratio $\frac{\text{cpm supernatant}}{\text{cpm membranes}} \times 100$ observed compared with the control value (obtained from tubes kept on ice). When ^{125}I -transferrin- or ^{125}I -horseradish peroxidase-loaded endosomes were examined, no significant variation of this ratio was observed, up to 3 h at 37 $^{\circ}\text{C}$ in the absence or in the presence of ATP (see "Results").

Other Methods—ATP concentrations were assayed using a luciferase kit from Boehringer. Electron microscopy was performed essentially as described (13) except that Epon sections have been observed without uranyl and lead contrast.

Binding experiments were conducted using 0.3% Pronase in PBS for 1 h at 2 $^{\circ}\text{C}$ to scrape off plasma membrane-bound label (13). This procedure proved to be efficient for ^{125}I -transferrin, ^{125}I -DT, or ^{125}I -Tf-DT.

Assays for toxin cytotoxicity were performed as described for these cells (16), except that [^{35}S]methionine was used as labeled amino acid.

ADP-ribosylation activities were tested using purified elongation factor 2 (a gift of Prof. J. P. Reboud, Lyon, France) and [^{32}P]NAD as reported (8), except that 20 $^{\circ}\text{C}$ incubations were used.

At least two independent experiments were performed ($n = 4$ or $n = 6$).

RESULTS

DT Endocytosis by Human and Mouse Lymphocytes—Most studies of DT endocytosis and translocation have been performed using Vero cells because they display $\approx 100,000$ receptors compared with $\approx 4,000$ on most other cell types like lymphocytes (1). Nevertheless, in preliminary internalization studies using DT labeled at $\approx 25 \mu\text{Ci}/\mu\text{g}$, a significant and time-dependent accumulation of ^{125}I -DT inside both human (CEM) and mouse (BW) lymphocytes was observed (Fig. 1). Using continuous labeling, up to 20 or 10% of the label is found inside the cell after 45 min for CEM and BW cells, respectively. When endosomes were isolated from BW cells after Fl-DT labeling (for 40 min at 37 $^{\circ}\text{C}$), a mean pH of 5.20 ± 0.04 ($n = 4$) was observed for the probe, confirming that these cells can endocytose DT into acidic compartments. Significant DT endocytosis by mouse (DT-resistant) cells has been reported previously (17).

Fractionation of Human CEM Lymphocytes—We decided to use CEM lymphocyte endosomes to try to reconstitute DT translocation in a cell-free system. In all fractionation experiments CEM cells were shown to behave like BW cells and, except for a more gentle lysis procedure (see "Materials and Methods"), no adaptation of the endosome isolation method (13) was found necessary. Endosomes from CEM cells were susceptible to LDL treatment used for BW endosome isolation (13), even if their receptor number for LDL is significantly lower (5–7-fold) than the one observed on BW cells (13, 18). The CEM endosome preparations obtained (Table I) show a yield and purity similar to those prepared from BW cells (13).

DT Is Cointernalized with Transferrin by CEM Lymphocytes—The two ligands transferrin and DT enter the cell by the same coated pit pathway (19). Electron microscopic examination of the endosome preparation from CEM cells after labeling at 37 $^{\circ}\text{C}$ with gold-DT and Tf-horseradish peroxidase shows that endocytosis of the latter is more efficient and some peroxidase-positive endosomes do not contain any gold (Fig. 2). DT is mainly found in peroxidase-positive compartments (Fig. 2), demonstrating that these cells cointernalize DT and transferrin. ^{125}I -Transferrin was thus routinely used as a control for ^{125}I -DT translocation experiments. Despite the very low efficiency of fluid phase uptake in lymphocytes, ^{125}I -horseradish peroxidase was also used in this study as a control for endosome lysis.

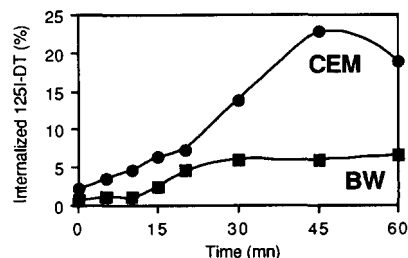


FIG. 1. Endocytosis of ^{125}I -DT by CEM (human) and BW (mouse) lymphocytes. Cells at 2×10^7 cells/ml of DMEM/BSA were incubated with ^{125}I -DT (2 $\mu\text{Ci}/\text{ml}$) at 37 $^{\circ}\text{C}$. After the indicated time they were diluted in ice-cold PBS and washed three times. Plasma membrane-bound and intracellular labels were then determined using a Pronase scraping procedure as described for ^{125}I -transferrin (13). Data are presented as percentages of total cell-associated ^{125}I -DT found resistant to Pronase digestion. The total number of bound DT molecules/cell was in the 1,500–3,000 and 3,000–6,000 range for, respectively, BW and CEM cells.

TABLE I

Yield and purity of endosomes prepared from CEM human lymphocytes

Endosomes were isolated from CEM lymphocytes as described under "Materials and Methods." Data are presented as the percentage of enzymatic activity (or counts) present in the starting homogenate and recovered at the 30%/20% sucrose interface (endosome fraction). Marker enzymes were assayed as described (13).

Subcellular compartment	Yield %
Endosomes	
¹²⁵ I-Transferrin	27 ± 3 ^a
¹²⁵ I-DT	9 ± 3
Plasma membrane	
5'-Nucleotidase	2 ± 3
Lysosomes	
Acid phosphatase	3 ± 2
β-N-Acetylglucosaminidase	1 ± 2
Mitochondria	
Succinate INT reductase	1 ± 2
Golgi apparatus	
Galactosyltransferase	5 ± 3
Endoplasmic reticulum	
NADH cytochrome c reductase	4 ± 2

^a This represents 42% of endosomal transferrin, since after labeling for 30 min at 37 °C, 65% of ¹²⁵I-transferrin was found inside the cell.

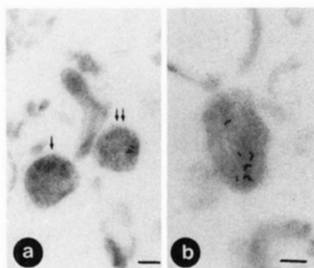


FIG. 2. Transferrin and DT are cointernalized by CEM cells. CEM cells were labeled with Tf-horseradish peroxidase and DT-gold for 20 min at 37 °C. After isolation, endosomes were processed for electron microscopy, using diaminobenzidine as substrate to localize Tf-horseradish peroxidase (13). DT-gold was mainly found in horseradish peroxidase-positive structures (a, double arrow, and b). Tf-horseradish peroxidase was also observed in compartments devoid of DT-gold (a, single arrow). Bar = 0.1 μm.

DT Translocation Can Be Observed in a Cell-free System Using Endosomes Purified from CEM Lymphocytes (It Is ATP-independent)—When ¹²⁵I-DT-labeled CEM endosomes were incubated at 37 °C in the presence of 10 mM ATP a linear time-dependent release of ¹²⁵I-toxin was observed in the medium (Fig. 3a). Cointernalization of DT with ¹²⁵I-transferrin did not induce any release of label from endosomes during the translocation assay (data not shown). As shown in Fig. 3b, DT was able to translocate even without ATP addition. Inclusion of an ATP-depleting system (5 mM glucose, hexokinase 120 units/ml) in the assay to hydrolyse the residual endosomal ATP concentration (always <100 nM) to an undetectable value (<0.1 nM) had no effect on ¹²⁵I-DT translocation rate (89 ± 9% of the rate with 10 mM ATP, n = 6). Thus, DT translocation is ATP-independent.

¹²⁵I-DT Translocation Is Dependent upon the pH Gradient Endosome → Cytosol—The endosomal pH was found to be 5.2 (for both cell types) using FI-DT fluorescence measurements. According to the effect of the agents increasing this endosomal pH up to the buffer value this (5.2 → 7.1) pH gradient is necessary for DT translocation to proceed (shown in the left column of Table II). Among these molecules nigericin (5 μM) was the most efficient, enabling the reduction of DT translocation rate by up to 93%. These agents were the

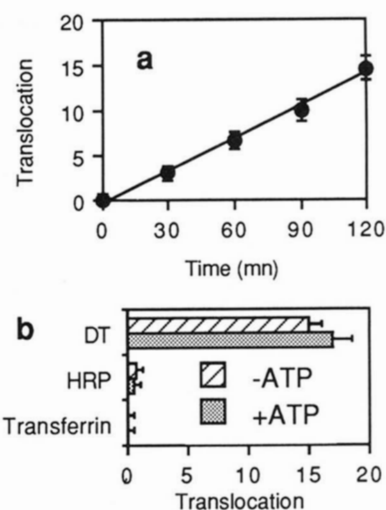


FIG. 3. ATP independence and linearity of the translocation activity of ¹²⁵I-DT from CEM endosomes. a, CEM cells were labeled with ¹²⁵I-DT, endosomes were isolated, and translocation was assayed as described under "Materials and Methods," in the presence of 10 mM ATP. b, translocation of the ¹²⁵I-labeled proteins was assayed in the presence or absence of 10 mM ATP, as indicated.

TABLE II

Effect of agents raising endosome pH on ¹²⁵I-DT translocation rate

CEM and BW cells were labeled with, respectively, ¹²⁵I-DT and ¹²⁵I-Tf-DT. Endosome isolation and translocation assays were performed (with ATP) as described under "Materials and Methods," in the presence of the indicated molecules. Translocation rates are calculated as percent of the control value.

Addition	Label/cell system	
	¹²⁵ I-DT/CEM	¹²⁵ I-Tf-DT/BW
None	100 ± 7	100 ± 3
20 mM NH ₄ Cl and 20 mM methylamine	34 ± 5	20 ± 7
20 μM chloroquine	42 ± 4	55 ± 4
5 μM nigericin	12 ± 5	25 ± 3

only ones able to dissipate the pH gradient across the endosome membrane over the 2 h of the translocation test. Addition of the ATP-depleting system to the assay did not result in a significant increase of endosome pH (as monitored using FI-DT or FI-Tf) after 2 h, and this explains the absence of any effect on the translocation rate. Thus, the pH gradient endosome/cytosol is the driving force for DT translocation, and ATP is only required indirectly by the endosome in order to establish the gradient via the action of the proton ATPase present in its membrane (20). In the absence of ATP, proton leakage of the endosomes was too slow to significantly impair DT translocation over the time of the experiment so that no ATP dependence of the process was observed.

¹²⁵I-DT Translocation Rate Is Increasing with Its Progression in the Endosomal System—Both labeling (Fig. 4a) and chase experiments (Fig. 4b) showed that ¹²⁵I-DT escapes more rapidly from late than from early CEM endosomes. This difference in the behavior of ¹²⁵I-DT between these compartments seems to arise at least partly from acidification, as monitored using FI-DT (and similar labeling procedures as with ¹²⁵I-DT), which is observed along the pathway (Fig. 4c). This acidification observed when probes move from early to late endosomes has been documented earlier (21). Nevertheless, although the translocation rate increases 3-fold between 10 and 40 min of labeling (Fig. 4a), the corresponding acidification is only 0.2 pH unit (Fig. 4c). This is probably because these endosome-pH values are located in the pH 4.9–5.4

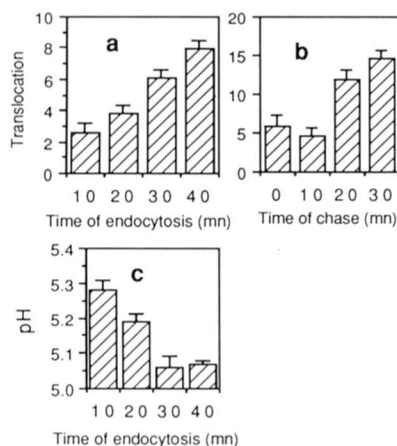


FIG. 4. Translocation rate of ^{125}I -DT as a function of its depth in the endocytic pathway. *a*, CEM cells were labeled with ^{125}I -DT for the indicated period of time before endosome isolation and translocation assay were performed. *b*, cells were labeled with ^{125}I -DT for 10 min at 37°C as indicated under "Materials and Methods"; after washing, a chase was performed in DMEM/BSA at 37°C in the presence of $100\ \mu\text{g/ml}$ of DT for the indicated period of time; endosomes were then isolated and translocation assayed as described under "Materials and Methods." *c*, cells were labeled with DT-FI for the indicated period of time before endosome isolation and pH measurements.

interval where DT translocation is the most pH dependent (on the cis side of the membrane), as judged by the results obtained by exposure of whole cells with bound DT to pH within this interval: a small drop of pH induces a big rise in intoxication (7).

These results on ^{125}I -DT translocation from CEM endosomes concur with data on DT translocation obtained using the cellular system and which indicated a likely ATP independence of the process (8). Since cytotoxicity studies performed on whole cells have also demonstrated a relative ATP independence (22) and an inhibition by weak bases and proton ionophores (1), the data obtained using our cell-free system were thus likely to be relevant in the cellular context, and we decided to study DT translocation in more detail. It has been suggested that it could occur by different mechanisms when directly bound to its receptor or via another binding site, as in the case for immunotoxins or Tf-DT conjugates (23). To examine this problem, we synthesized a Tf-DT conjugate so that we could use it on the BW mouse cells, a cell type already used in many fractionation studies (13, 24–26).

We also intended to take advantage of this system to localize the stage responsible for DT resistance displayed by mouse cells, which has been explained by defects at different steps of the intoxication process, depending on the authors (27).

A Monovalent Tf-DT Conjugate Is Efficiently Endocytosed by Mouse BW Cells—Conjugation of transferrin and DT using SPDP and the procedure described under "Materials and Methods" yields mainly (more than 70%) a 1:1 Tf-DT conjugate (analysis by SDS-PAGE shown in Fig. 5). The ^{125}I -Tf-DT conjugate was about as efficiently endocytosed as ^{125}I -transferrin by BW cells, since the proportion of cell label found inside the cell after 30 min at 37°C reached 65% for ^{125}I -Tf-DT (Fig. 5) and 70% for ^{125}I -transferrin (13). Endocytosis of the conjugate is mainly driven by the transferrin moiety of the hybrid: addition of a 1,000-fold excess of transferrin together with ^{125}I -Tf-DT reduces the proportion of endocytosed label to the one mediated by the DT moiety: 8% (Fig. 5), in agreement with the 10% obtained for unconjugated ^{125}I -DT (Fig. 1).

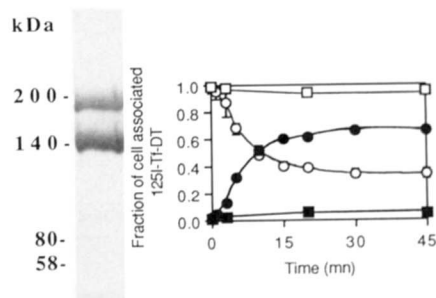


FIG. 5. Purity and endocytosis efficiency of the Tf-DT conjugate. A Tf-DT conjugate was synthesized using SPDP as described under "Materials and Methods." Analysis of the conjugate by SDS-PAGE (nonreducing conditions; molecular masses are indicated in kDa) is shown as well as its endocytosis by BW lymphocytes determined as described (13). \circ , \square , plasma membrane bound label; \bullet , \blacksquare , endocytosed label. \square , \blacksquare , a 1,000 fold excess of transferrin was added together with ^{125}I -Tf-DT.

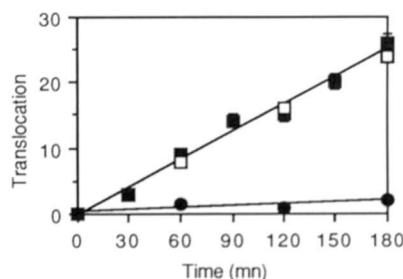


FIG. 6. ^{125}I -Tf-DT and ^{125}I -DT translocation from BW endosomes. BW lymphocytes were allowed to endocytose the indicated label and then endosomes were isolated and translocation was assayed as described under "Materials and Methods," with (closed symbols) or without (open symbols) 10 mM ATP. \blacksquare , \square , ^{125}I -Tf-DT; \bullet , ^{125}I -DT.

^{125}I -DT Is Able to Translocate from ^{125}I -Tf-DT-loaded BW Endosomes—Upon incubation at 37°C in the cell-free system, ^{125}I -Tf-DT-loaded BW endosomes gradually released label (Fig. 6). It can also be seen in Fig. 6 that ^{125}I -DT cannot cross the membrane of BW endosomes and its receptor. These data agree with the results of cytotoxicity assays on BW cells; Tf-DT is more than a 1,000-fold more cytotoxic than DT to these cells ($\text{IC}_{50} = 7.2 \cdot 10^{-11}\ \text{M}$ instead of $7.3 \cdot 10^{-8}\ \text{M}$). The ^{125}I -Tf-DT translocation is linear with time and can be inhibited, as with ^{125}I -DT-loaded CEM endosomes by weak bases and nigericin (Table II). ATP independence was also observed for DT escape from Tf-DT-labeled endosomes (Fig. 6). Hence, DT translocation seems to proceed via similar mechanisms when directly bound to its receptor or by an alternative binding system.

All other experiments were performed using the ^{125}I -Tf-DT/BW murine lymphocyte system.

Temperature Dependence of ^{125}I -DT Translocation—At 15 – 20°C a significant fraction (35%) of the 37°C rate (100%) was already observed. Hence we conclude that temperature is not a very critical factor for DT translocation.

pH Dependence of ^{125}I -DT Translocation—Studies using the ^{125}I -Tf-DT/BW system confirmed that ^{125}I -DT translocation is dependent upon the pH gradient (Fig. 7). Whatever the pH of the external buffer, the endosome pH was found to be constant ($\text{pH} \approx 5.3$, as monitored using FI-Tf) during the experiment. The optimum pH of the buffer (*i.e.* of the cytosolic compartment) for DT translocation is seven corresponding to most cytosolic values (28). Low outside pH ($\text{pH} = 5.5$, no pH gradient) inhibits translocation, but high pH ($\text{pH} = 8$) is inhibitory as well. When the same experiment was repeated

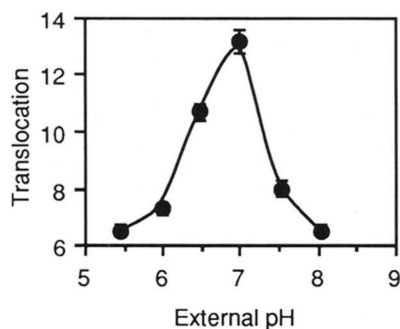


FIG. 7. Dependence of DT translocation upon a pH gradient across the endosome membrane. ^{125}I -Tf-DT-labeled BW endosomes (mean internal pH 5.3) were isolated as described under "Materials and Methods," and translocation was assayed at the indicated pH value using 20 mM buffers (Mes (pH 5.5–6.5), Pipes, (pH 6.5–7.5), Hepes (pH 7–8)) in the presence of ATP.

in the presence of 5 μM nigericin to equilibrate the endosome pH at the buffer value, no significant DT translocation was observed whatever the pH (data not shown), confirming the requirement of the pH gradient for the process.

Analysis of ^{125}I -DT Peptides Liberated into the Medium during the Cell-free Translocation Assay—A mixture of ^{125}I -Tf-DT ($\approx 70\%$), ^{125}I -transferrin ($\approx 20\%$), DT, and its subunits ($\approx 10\%$) was recovered in the endosomes after cell labeling with ^{125}I -Tf-DT for 30 min at 37 $^{\circ}\text{C}$. Analysis by SDS-PAGE of the supernatant obtained from translocation assays using either the ^{125}I -Tf-DT/BW system (Fig. 8a) or the ^{125}I -DT/CEM system (data not shown) shows that only a peptide of 21 kDa, presumably the A chain of ^{125}I -DT is actively translocated from the endosome lumen to the translocation medium during the assay in both systems. Some ^{125}I -DT (58 kDa) constitutes the radioactivity background present in all the supernatants. It is not translocated, but probably liberated, during the last endosome homogenization step (the same phenomenon was observed with ^{125}I -horseradish peroxidase), since the intensity of this spot did not increase with time at 37 $^{\circ}\text{C}$, as shown (Fig. 8) by the densitometric analysis of gels from ^{125}I -Tf-DT/BW translocation assays. Using purified elongation factor 2 as substrate, a considerable ADP-ribosylating activity was found at the level of the 21-kDa band after translocation (Fig. 9). This identifies the translocated peptide as the A chain of the toxin.

No significant proteolysis of the remaining B subunit was observed after translocation, as shown by SDS-PAGE analysis of proteins from the endosome pellet (Fig. 8b). It was not possible to follow the translocation process via the amount of ^{125}I -B-chain remaining in the pellet as it does not only originate from the translocation process (which transports the A chain through the endosome membrane): it is also produced by DT reduction inside the endosome (in that case the A chain produced stays in the endosome, Fig. 8b). Some DT reduction occurs during internalization of the conjugate since both A and B chains are already detected in endosomes at the beginning of the translocation assay (Fig. 8b).

DISCUSSION

The endosome is the doorstep for the entrance into the cell for several pathogens, such as viruses (29) or toxins (19). The involvement of the endosomal compartment in the cytotoxicity of DT was shown by the action of weak bases, which by neutralizing the acidic endosome pH inhibits the intoxication process (1, 3). These results were later complemented by studies in model systems showing that low pH could induce DT insertion into the liposomal membrane and exposure of

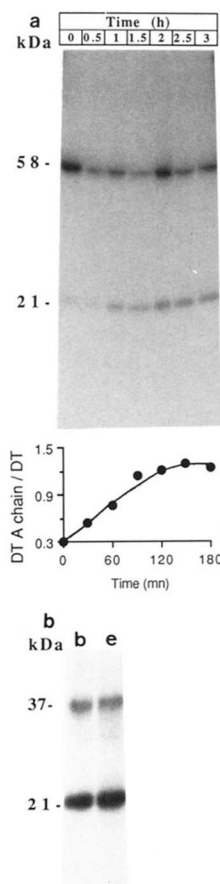


FIG. 8. Analysis by SDS-PAGE of the translocated peptides. Translocation assays of ^{125}I -Tf-DT-labeled BW endosomes were terminated after the indicated time at 37 $^{\circ}\text{C}$ by centrifugation at 160,000 $\times g$. a, supernatants from several assays were concentrated by trichloroacetic acid precipitation, before analysis by nonreducing SDS-PAGE (10–15% acrylamide gradient) and autoradiography. The graph shows the result (expressed as the ratio of the intensity of the bands) of the densitometric analysis of corresponding gels. b, analysis by nonreducing SDS-PAGE of the ^{125}I -DT-chains present in the pellet at the beginning (b) and at the end (e) of the translocation assay. The ^{125}I distribution between DT subunits (A/B) was ≈ 1.3 .

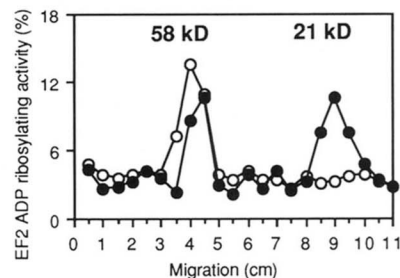


FIG. 9. ADP-ribosylating activity of the translocated peptide. Supernatants from translocation assays were analyzed by SDS-PAGE as described in the legend of Fig. 8. The gel was cut into 0.5-cm slices, and the proteins from each slice were eluted and analysed for ADP-ribosylating activity as described (8), using purified elongation factor 2 and ^{32}P -NAD as substrates. Proteins were then precipitated with 10% trichloroacetic acid (after addition of 300 μg of BSA as a carrier) and collected onto fiberglass filters (Whatman GF/C). Data are presented as percentages of the total ADP ribosylating activity present in the track, for two different translocation times. O, 0 h; ●, 3 h.

part of its polypeptide backbone to the external side of the vesicles (30).

At the present time, selective translocation of DT (at con-

centration $<1 \mu\text{M}$) through pure lipid membranes has never been observed (30–32), and the only system developed for studying DT translocation is based upon the exposure of cells to acidic medium after toxin binding, followed by proteolysis and cell permeabilization to recover the translocated peptides (3). The use of this system nevertheless raises several criticisms, one of them being that the plasma membrane is clearly different from the endosome membrane through which DT translocation naturally occurs (1, 8), according to both protein and lipid composition studies (26, 33).

DT Translocation—The cell-free assay presented in this paper, based on the use of purified endosomes overcomes the major limitations of the cellular system. Endosomes were obtained using a previously detailed technique which yields preparations devoid of significant contamination by other cell compartments (Table I, Ref. 13). Incubation at 37°C of ^{125}I -Tf-DT-labeled BW endosomes or ^{125}I -DT-labeled CEM endosomes in a cell-free system induces the selective release of the ^{125}I -A chain, whereas none of the other endosome labels (^{125}I -horseradish peroxidase or ^{125}I -transferrin) used as controls showed translocation ability. This translocation of only the A chain of DT confirmed the data obtained by Olsnes *et al.* (8) using the cellular model, but in their case another (25-kDa) fragment more tightly bound to the membrane was also released from the cells upon permeabilization (8).

The inhibition of DT translocation across the endosome membrane by weak bases and nigericin is the best demonstration that this movement observed in the cell-free system is similar to the one enabling DT to enter the cytosol. The pH gradient (5.3 \rightarrow 7.1 in our model) endosome \rightarrow cytosol is probably required in order to provide the energy for DT translocation, since ATP was not needed for the process. This ATP independence was also observed using the cellular system and metabolic inhibitors to reduce the ATP concentration by more than 90%, but it could not be ruled out that the residual ATP concentration was sufficient to support translocation (8). In our case, when residual ATP was eliminated ($[\text{ATP}] < 0.1 \text{ nM}$) by the use of an ATP-depleting system, ^{125}I -DT translocation was still observed.

The poor temperature dependence we observed for DT translocation could partly explain why cells kept at 15°C are still DT-sensitive (19).

The optimum cytosolic pH we found for DT translocation (pH = 7) is the same as for most cytosols (28, 34). If the inhibition at low outside pH was reported before (3), the one observed at higher pH is more surprising.

Another example of the sensitivity of DT translocation to minor changes in pH is that a small endosome-pH difference, since it is situated just in the pH interval where DT translocation is the most pH sensitive (on the cis side of the membrane) (7), seems to explain why DT translocation is more active from the more acidic late endosomes.

DT Receptors on Resistant Cells Block the Toxin in a Translocation-incompetent Form—Cells resistant to DT, like rodent cells, are known to have DT binding sites on their membrane (5, 17) and to significantly endocytose DT (17). We found that DT bound to BW mouse cells and was delivered to acidic compartments (pH 5.2). Using LDL density shift experiments (13), we localized DT and Tf-DT in the same endosomes of BW cells (data not shown). The sensitivity of these cells to Tf-DT indicates that the blockage of DT cytotoxicity is located at the translocation step and is not due to a peculiar property of DT-labeled endosomes. This could be directly demonstrated, since ^{125}I -DT was found to be unable to translocate from BW endosomes.

Natural DT binding sites on resistant cells are responsible

for the observed inhibition of its translocation, since when an alternative binding site was provided via transferrin and its receptor, DT was actively transferred through the endosome membrane. This had been suggested previously but not demonstrated in studies showing that the absence of DT cytotoxicity to murine cells could be overcome by the use of Tf-DT (14, 27, 35) (or DT-based immunotoxins (35)). Our data are also fully consistent with results showing that resistant murine (L) cells only have inhibitory DT binding sites on their membrane (which bind DT but inhibit the expression of its cytotoxic effect), whereas sensitive cells also bear a receptor which enables DT to intoxicate cells (5, 36).

DT translocation was found to display similar characteristics when directly bound to its receptor on sensitive cells or to the transferrin receptor by the use of Tf-DT. It is not known why this finding should differ from that of previous studies in which DT bound to cells via the avidin-biotin system failed to produce intoxication (23). One possibility could be the examination of the translocation at the plasma membrane level, since we found using plasma membrane preparations that some ricin conjugates are unable to cross this membrane, whereas they can cross the endosome membrane.²

The cell-free system described in this paper has enabled us to identify some requirements for the DT translocation process (*i.e.* a temperature- and pH gradient-dependent selective transfer of the catalytically active A chain to the cytosol). This agrees with results from studies performed on whole cells (1, 3, 8, 22). Since ricin² and *Pseudomonas* exotoxin³ were found to behave differently in the same cell-free translocation assay, this reinforces the validity of this system for studying toxin translocation. We hope that in the future it will be useful to delineate the sequences of the toxins involved in this activity.

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