Adsorptive-Mediated Transcytosis of a Synthetic Basic Peptide, 001-C8 in Caco-2 Cells

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INTRODUCTION

The oral administration of pharmacologically active proteins and peptides is limited because of their low membrane permeability and short half-lives in the gastrointestinal tract. Small peptides, which are major digestive products of proteins, and their analogous drugs are absorbed by the oligopeptide transporter PepT1, which transports only for small oligopeptides but not for peptides having more than four amino acid residues (1,2). For larger peptides and proteins, an involvement of receptor-mediated transcytosis (RMT), which demonstrates high substrate specificity and adsorptive-mediated transcytosis (AMT), which is triggered by the binding of cationic ligands to the anionic sites on the cell membranes and shows relatively broad substrate specificity, have been shown in several tissues (3–6). However, very little information is available on AMT in the intestine.

In our previous study, we synthesized a novel cationic peptide 001-C8 (H-MeTyr-Arg-MeArg-D-Leu-$NH_2(CH_2)_8NH_2$) (7), which consists of a partial amino acid sequence of E-2078, a dynorphin-like analgesic peptide (8), and the carboxyl-terminal structure of ebiratide, an ACTH analogue (9); both of these are substrates for AMT at the blood–brain barrier (BBB), with two arginine residues and an octanediamine residue. The peptide has been shown to be transported through the BBB via the AMT mechanism (10). Very recently, we have demonstrated that 001-C8 coupled with 4-nitrobenzo-2-oxa-1,3-diazole (001-C8-NBD) is absorbed in the intestine *in vivo* (12) and it appears to be taken up into enterocyte-like Caco-2 cells via an adsorptive-mediated endocytosis (AME) mechanism (11).

In the present study, we aimed to elucidate and visualize the process of AMT in Caco-2 by measuring the transepithelial transport of fluorescent 001-C8-NBD, as well as by visual three-dimensional analysis of living, nonfixed cells by confocal laser microscopy.

MATERIALS AND METHODS

Materials

[^{3}H]Polyethylene glycol (PEG) 900 (M.W. 800–1000 and S.A. 74–370 MBq/g) and [^{14}C]acetamide (629 GBq/mg) were purchased from New England Nuclear (Boston, MA, U.S.A.), and ARC Co. (St. Louis, MO, U.S.A.), respectively. Salmon roe protamine sulfate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Poly-L-lysine (M.W. ca. 4000), amantadine, and brefeldin A were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 001-C8-NBD (H-MeTyr-Arg-MeArg-D-Leu-$NH(CH_2)_8$NH-NBD) used in the present study was synthesized in this laboratory as described (12). All other chemicals used were commercial products of reagent grade.

Cell Culture and Transport Study

The transport study using Caco-2 cells grown on Transwell clusters (3.0 μm pore size, Costar, Bedford, MA, U.S.A.) was performed as described previously (13). The amount of radiolabeled or nonlabeled compound transported by the cells was determined by radioactivity measurement or HPLC assay and expressed in terms of permeability (μl/cm^2 culture area), which was calculated by dividing the transported amount by the initial concentration in the donor compartment. Radioactivity was determined with a liquid scintillation counter (LSC-1,000, Aloka Co. Ltd., Tokyo, Japan). The HPLC system consisted of a constant-pump (880-PU, Japan Spectroscopic Co., Tokyo), fluorescence detector (RF-550, Shimadzu Co., Kyoto, Japan), and integrator (Chromatopac CR3A, Shimadzu Co.). The analytical column was reversed-phase TSK-gel ODS-80Ts (4.6mm × 15 cm, Tosoh, Tokyo, Japan). The mobile phase was 0.1% trifluoroacetic acid-acetonitrile (85:15) with a flow rate of 1.0 ml/min, and the eluent was detected at excitation and emission wavelengths of 480 nm and 550 nm, respectively. Each result represents the mean ± S.E.M. of three or four experiments using the same culture of Caco-2 cells.

Confocal Microscopy

Confocal microscopy was performed as described (10). In brief, Caco-2 cells were grown on cover glasses equipped with a Flexiperm chamber (Heraeus Instruments GmbH, Germany), mounted on the microscope stage and incubated with 10 μM 001-C8-NBD dissolved in 200 μl of Hank's-balance salt solution (HBSS, pH 6.0).

RESULTS

Time Course of the Transport of 001-C8-NBD and [^{3}H]PEG 900

The transport of 001-C8-NBD was measured in the apical to basal direction and compared with that of [^{3}H]PEG 900,

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ABBREVIATIONS: 001-C8, H-MeTyr-Arg-MeArg-D-Leu-$NH_2(CH_2)_8NH_2$; AME, adsorptive-mediated endocytosis; AMT, adsorptive-mediated transcytosis; BBB, blood–brain barrier; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PEG, polyethylene glycol; RMT, receptor-mediated transcytosis.

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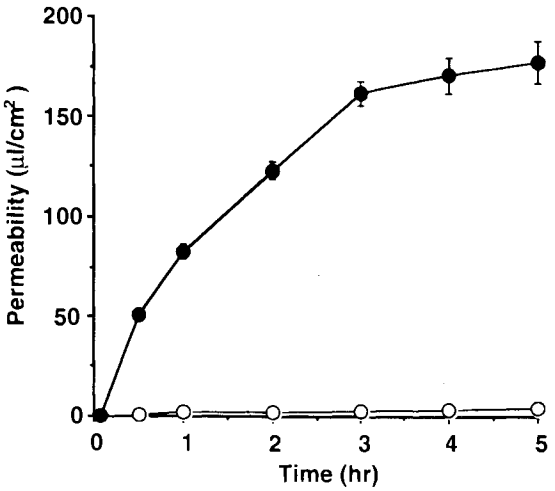


Fig. 1. Time course of transport of 001-C8-NBD and [^{3}H]PEG 900 across cultured monolayers of Caco-2 cells. The permeability of 001-C8-NBD (50 μM: closed circle) or [^{3}H]PEG 900 (250 Bq/μl: open circle) in the presence of 50 μM of 001-C8-NBD from the apical side to the basal side was measured by incubating Caco-2 monolayers in HBSS at 37°C as described in "Materials and Methods". The pH values of the apical and basal solutions were adjusted to 6.0 and 7.4, respectively. Each point represents the mean ± S.E.M. of four experiments.

which reflects the paracellular permeation, in the presence of 001-C8-NBD (Fig. 1). The data were expressed as permeated volume per culture surface area. The transport of 001-C8-NBD proceeded in a time-dependent manner and reached a steady-state value of over 170 μl/cm^2 at 5 hr. The initial permeation rate of 001-C8-NBD (0.900 ± 0.030 μl/min/cm^2, mean ± S.E.M., n = 4) was 60-fold greater than that of [^{3}H]PEG 900 (0.015 ± 0.002 μl/min/cm^2).

Temperature-Dependency and Effects of Various Compounds on the Transport of 001-C8-NBD, [^{3}H]PEG 900, and [^{14}C]acetamide

Table I shows the temperature dependency and the effects of endocytosis inhibitors and several cationic peptides on the transport of 001-C8-NBD (50 μM). At 4°C, the permeability was reduced to 26 % of the control value obtained at 37°C. Endocytosis inhibitors, amantadine (2.5 mM) and brefeldin A (70 μM), significantly decreased the permeability. It was also reduced by polycationic peptides, poly-L-lysine (1 mM) and protamine (1 mM). An excess of nonlabeled 001-C8 also decreased the permeability at 1 mM, suggesting the existence of a saturable mechanism. Such inhibitory effects were not caused by alteration of paracellular permeation, because none of those inhibitors, including 001-C8-NBD, altered the permeation of [^{3}H]PEG 900 (Table I). Furthermore, the transport of [^{14}C]acetamide, which is expected to cross the monolayer by passive diffusion was not affected by these compounds (Table I).

Confocal Microscopic Analysis of Internalization of 001-C8-NBD

Figure 2A shows the subcellular distribution of 001-C8-NBD (10 μM) among representative horizontal optical cross-sections of cultured monolayers of Caco-2 cells, obtained by confocal laser microscopy. Figure 2B shows the cell image obtained by the transdetector. This analysis provides three resolved images from the upper surface to the bottom section of the cultured cells, corresponding to the apical side to the basal side of the cells, respectively. The internalization of 001-C8-NBD into the Caco-2 cells occurred in a time-dependent manner. Fluorescence was detected in the apical section at an incubation time of 5 min, but only a low fluorescence was detected in the basal section. At 20 and 30 min, significant fluorescence extended successively to the medial and the basal sections. The observation of a partial granular staining in each section, excluding the nuclei, indicated the sequestration of 001-C8-NBD within endocytotic vesicles. The fluorescence signal was digitized and quantified as shown in Fig. 2C. It is clear that the increment of the fluorescence in each section was time-dependent and occurred successively from the apical to the basolateral section.

Intracellular Movement of 001-C8-NBD

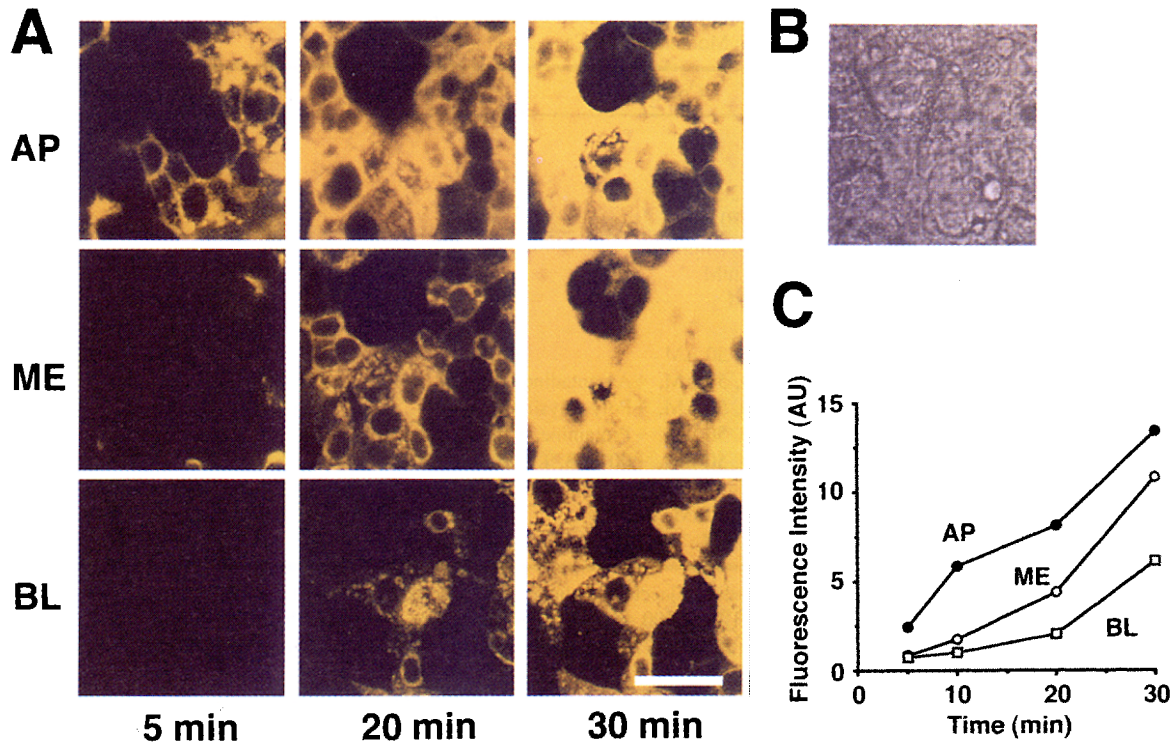
Intracellular movement of 001-C8-NBD was observed using Caco-2 cells which were incubated with 001-C8-NBD (10 μM) for 60 min to internalize the peptide and then were washed out of the incubation medium. Internalized 001-C8-NBD was chased for a further 60 min by confocal microscopy. We observed a decrease and an increase in apical and basal fluorescence, respectively, indicating intersectional movement of 001-C8-NBD from the apical to the basal section (Fig. 3). This shows that 001-C8-NBD was transferred from the apical to the basal side time dependently after internalization.

DISCUSSION

The purpose of the present study was to demonstrate transcytosis of a basic peptide, 001-C8-NBD, in intestinal epithelial cells. Internalization of 001-C8-NBD, which is metabolically stable peptide with negligible degradation when incubated with Caco-2 cells, had been shown to occur by AME in our preceding

Fig. 2. (*opposite, top*) Confocal photomicrographs showing internalization of 001-C8-NBD into cultured monolayers of Caco-2 cells. Caco-2 cells grown on a glass coverslip were loaded with 001-C8-NBD (10 μM), mounted on the microstage and incubated at 37°C. Series of three optical cross-sections were acquired at the apical (AP), medial (ME) and basal (BL) levels of the cells. A: Confocal laser microscopy. Scale bar, 25 μm. B: The cell image obtained by the trans-detector. C: Quantification of digital images obtained by confocal microscopy in A.

Fig. 3. (*opposite, bottom*) Intracellular movement of 001-C8-NBD after internalization into cultured monolayers of Caco-2 cells. Caco-2 cells were incubated with 001-C8-NBD (10 μM) for 60 min and the fluorescent probe in the incubation medium was washed out. Then the internalized 001-C8-NBD was observed by confocal microscopy for a further 60 min. A: Confocal laser microscopy. Scale bar, 25 μm. B: The cell image obtained by the trans-detector. C: Quantification of digital images obtained by confocal microscopy in A.



study using Caco-2 cells (11). In the present study, we evaluated the transcellular transport of 001-C8-NBD both functionally and morphologically. Confocal microscopy allows three-dimensional horizontal optical sectioning through living, nonfixed cells, and thus can provide qualitative morphological information to support the results obtained in transport experiments.

The transport of 001-C8-NBD by monolayers of Caco-2 cells at steady-state (over 170 $\mu l/cm^2$) was significantly higher than that of [^{3}H]PEG 900 (Fig. 1). 001-C8-NBD had no effect on the transport of [^{3}H]PEG 900, so it is likely that a specialized mechanism, rather than paracellular transport or fluid-phase transcytosis, is involved. The transport of 001-C8-NBD was

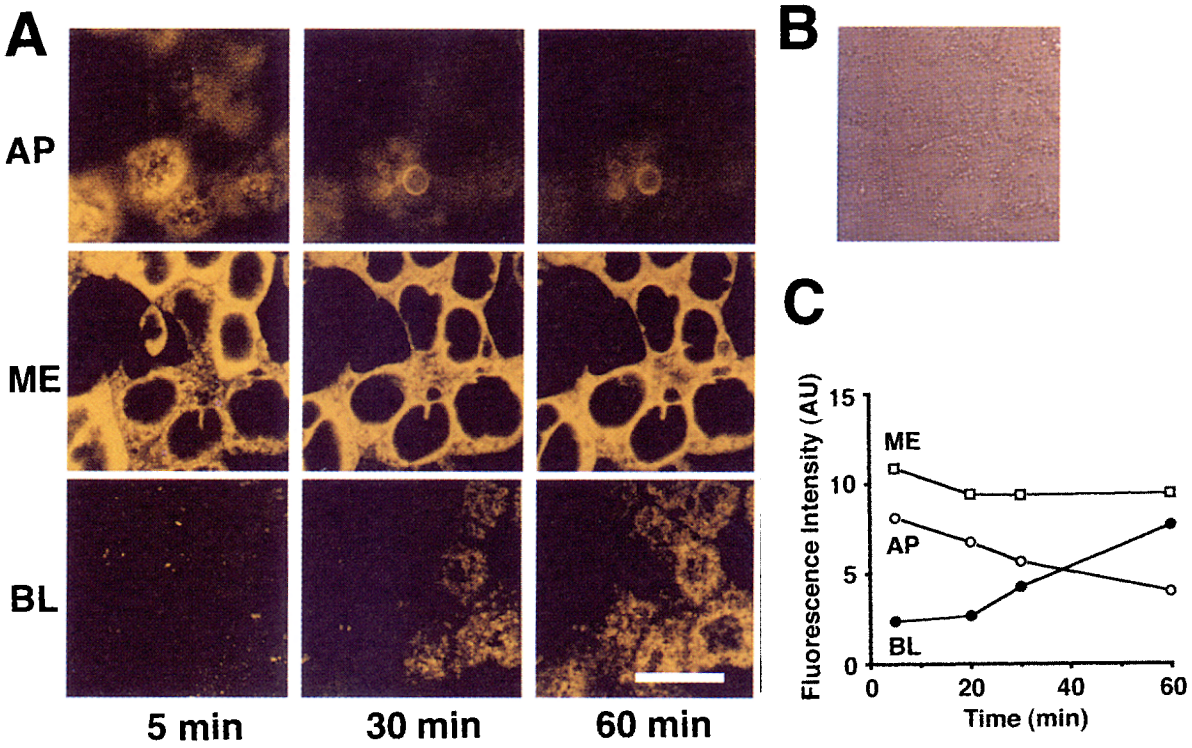


Table I. Effects of Temperature and Various Compounds on Transport of 001-C8-NBD, [^{3}H]PEG900 and [^{14}C]acetamide Across Cultured Monolayers of Caco-2 Cells

Substrate	Condition	Permeability (% of control)
001-C8-NBD	Control	100 ± 3.7
	4°C	25.9 ± 3.0 *
	+Amantadine, 2.5 mM	71.8 ± 2.9 *
	+Brefeldin A, 70 μM	82.5 ± 1.7 *
	+Poly-L-lysine, 1 mM	72.5 ± 2.9 *
	+Protamine, 1 mM	68.5 ± 3.0 *
	+001-C8, 1 mM	60.1 ± 1.6 *
[^{3}H]PEG 900	Control	100 ± 10.8
	+001-C8-NBD, 50 μM	101 ± 3.8
	+001-C8-NBD, 50 μM, +Amantadine, 2.5 mM	105 ± 10.9
	+001-C8-NBD, 50 μM, +Poly-L-lysine, 1 mM	105 ± 6.1
	+001-C8-NBD, 50 μM, +Protamine, 1 mM	113 ± 6.4
	+001-C8-NBD, 50 μM, +001-C8, 1 mM	105 ± 8.6
[^{14}C]Acetamide	Control	100 ± 3.31
	+Poly-L-lysine, 1 mM	106 ± 2.97
	+Protamine, 1 mM	100 ± 6.16

Note: The transport of 001-C8-NBD, [^{3}H]PEG 900 and [^{14}C]acetamide under the indicated conditions was determined as described in the legend to Figure 1. Transport rates of 001-C8-NBD, [^{3}H]PEG 900 and [^{14}C]acetamide were determined from the linear portion of the slope of the time course of transported amount by 30 min, 30 min, and 5 hrs, respectively. Each value represents the mean ± S.E.M. of three or four experiments.

*Statistically significantly different from the control value of each substrate by Student's t test ($p < 0.05$).

inhibited by cationic peptides such as poly-L-lysine and protamine (Table I), while these compounds had no effect on the transport of electroneutral [^{14}C]acetamide (Table I). These results indicate the involvement of electrostatic interactions between the peptide and cell-surface anionic site(s), and suggest that 001-C8-NBD is transported across Caco-2 cells via the AMT.

To confirm the involvement of cytosis for this peptide, we used three-dimensional confocal microscopy. The granular distribution of the staining and the absence of staining in nuclei strongly indicate the sequestration of 001-C8 within endocytotic vesicles (Fig. 2), although a partial involvement of diffusion, paracellular transport, and/or specific mechanism other than the AMT cannot be ruled out. The intracellular disposition of 001-C8-NBD during and after internalization revealed a time-dependent movement of the peptide from the surface to the bottom of monolayers of Caco-2 cells (Fig. 3). Thus, 001-C8 seems to be transferred from the apical to the basal side of the cells by cytosis at least partially. The involvement of cytosis was further confirmed by examining the effects of amantadine and brefeldin A, which selectively inhibit endocytosis of alpha 2-macroglobulin (14) and polymeric immunoglobulin transcytosis (15), respectively (Table I). All of these results indicate that 001-C8 is transported across Caco-2 cells via an AMT mechanism.

The observations in the *in vitro* Caco-2 system, however, may not be necessarily correlated with the physiological situation, and it is important to confirm *in vivo* transcytosis from the lumen of the intestine to the vasculature. In recent studies, we have demonstrated that 001-C8-NBD is absorbed to the extent of about 8% from the rat intestine by using the intestinal vascular perfusion method and the loop method *in vivo* (12). Accordingly, the observed AMT in the present study is likely involved in the intestinal absorption of 001-C8-NBD *in vivo*.

The AMT mechanism is superior to RME as a candidate drug transporter in its relatively broad substrate specificity. It may be possible to utilize AMT as shown in the intestinal transport of 001-C8-NBD in the present and our previous studies for enhanced intestinal absorption of peptides/proteins.

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