Transport Characteristics of Ceftibuten, a New Cephalosporin Antibiotic, *via* the Apical H^+/Dipeptide Cotransport System in Human Intestinal Cell Line Caco-2: Regulation by Cell Growth

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Purpose. The intestinal epithelial cell line Caco-2 possesses the H^+/dipeptide cotransport system responsible for uptake of oral cephalosporins. In this study, the transport characteristics of ceftibuten were examined from the viewpoint of cell growth in the Caco-2 cells.
Methods. The uptake of cephalosporins by Caco-2 cell monolayers grown on plastic dishes was measured and analyzed kinetically.
Results. The uptake of ceftibuten was increased by lowering pH of the incubation medium and was inhibited by excess dipeptide. The transport activity of ceftibuten was dependent on the duration of culture, being maximal on the 14th day after inoculation. Kinetic analysis revealed that the development of ceftibuten uptake was due to not only a decrease in *Km* but also to an increase in *Vmax* value.
Conclusions. The uptake of ceftibuten is mediated by the apical H^+/dipeptide cotransport system which is regulated by cell growth and/or differentiation in the Caco-2 cells.

KEY WORDS: cephalosporin antibiotics; H^+/dipeptide cotransporter; Caco-2 cells; cell growth; intestinal absorption.

INTRODUCTION

Di- and tripeptides are actively transported into the intestinal epithelial cells by the H^+/dipeptide cotransport system localized in the brush-border membranes [1]. Using intestinal brush-border membrane vesicles, we found that orally active aminocephalosporin antibiotics are transported *via* the H^+/dipeptide cotransport system [2–3]. Moreover, cefixime and ceftibuten which lack an α-amino group and have two carboxyl groups can be recognized by the H^+/dipeptide cotransport system [3–5]. However, little information has been reported concerning the interactions of these antibiotics with the dipeptide transport system in the human intestine.

Recently, the human colon adenocarcinoma cell line Caco-2 has been used as a model for studying the epithelial functions of the intestine. These cells spontaneously differentiate in culture into polarized cell monolayers possessing microvilli and many enterocyte-like characteristics [6–9]. We [10] and Dantzig and Bergin [11] demonstrated previously that aminocephalosporin antibiotics are transported into Caco-2 cells by an apically localized H^+/dipeptide cotransport system, suggesting a model for studying absorption mechanisms of those antibiotics in the human intestine. Furthermore, we found that both the apically localized H^+/dipeptide cotransport system and the basolaterally localized dipeptide transport system are responsible for the transcellular flux of these antibiotics [12]. Here, we studied the regulation of ceftibuten transport activity by cell growth and/or differentiation in the Caco-2 cells further.

MATERIALS AND METHODS

Materials

Five cephalosporins were used: cephradine (Sankyo Co., Tokyo, Japan), cephalexin and ceftibuten (Shionogi and Co., Osaka, Japan), cefixime (Fujisawa Pharmaceutical Co., Osaka, Japan) and cefotiam (Takeda Chemical Industries, Osaka, Japan). HEPES[a] and MES[b] were obtained from Wako Pure Chemical Co. (Osaka, Japan). Glycyl-L-leucine was purchased from the Peptide Institute Inc. (Osaka, Japan). All other chemicals used were of the highest purity available.

Cell Culture

Caco-2 cells at passage 18 obtained from the American Type Culture Collection (ATCC HTB37) were maintained by serial passage in plastic culture dishes (FALCON, Becton Dickinson and Co., Lincoln Park, New Jersey) as described previously [10,12]. For the transport studies, 60-mm plastic dishes were inoculated with 5×10^5 cells in 5 ml of the complete culture medium. The cells were given fresh medium every 3 or 4 days, and were used between the 14th and 16th day for the transport experiments. In this study, cells between the 29th and 44th passages were used.

Measurement of Cephalosporin Transport

The uptake of cephalosporins was measured in Caco-2 monolayer cultures grown in 60-mm plastic culture dishes. The composition of the incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 5 mM MES (pH 6.0) or HEPES (pH 7.4). After removal of the culture medium, each dish was washed once with 5 ml of incubation medium (pH 7.4), and incubated with 2 ml of the same medium for 10 min at 37°C. Unless otherwise indicated, the cells were then incubated with 2 ml of incubation medium (pH 6.0) containing a test drug (1 mM) for the specific periods of time at 37°C. Thereafter, the medium was aspirated off, and the dishes were rapidly rinsed twice with 5 ml of ice-cold incubation medium (pH 7.4). The cells were scraped off with a rubber policeman into 1 ml of the extraction solution [ceftibuten, 0.05 M ammonium acetate/methanol, 50:50; others, 0.03 M phosphate buffer (pH 7.0)/methanol, 50:50] and maintained for 1 hr at room tem-

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Abbreviations: a HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
b MES, 2-(N-morpholino) ethanesulfonic acid.

perature. The extraction solution was centrifuged at 10,000 r.p.m. (himac CR15D, Hitachi Co., Tokyo, Japan) for 15 min. The supernatant was filtered through a Millipore filter (SJGVL, 0.22 μm) and analyzed by high-performance liquid chromatography (HPLC).

Analytical Methods

Cephalosporins were assayed using a high-performance liquid chromatograph LC-10A (Shimadzu Co., Kyoto, Japan) equipped with a UV spectrophotometric detector SPD-10A (Shimadzu Co.) and an integrator (chromatopac C-R4AX, Shimadzu Co.) as reported previously [10,12]. The protein content of the cell monolayers solubilized in 1.0 ml of 1 N NaOH was determined by the method of Bradford [13], using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, California) with bovine γ-globulin as a standard.

Enzyme Assay

After the indicated culture periods, specific activities of aminopeptidase (EC 3.4.11.2) and Na^+-K^+-ATPase (EC 3.6.1.3) in homogenates of the Caco-2 cells were measured as described previously [14].

RESULTS

Uptake of Various Cephalosporins by Caco-2 Monolayers

As the first step in the characterization of cephalosporin transport, uptake of various cephalosporins by the Caco-2 cell monolayers was examined at pH 6.0. Figure 1 shows the time courses of uptake of various cephalosporins, the order of uptake being ceftibuten > cephradine > cephalexin > cefixime and cefotiam. Assuming a Caco-2 cell volume of 3.66 μl/mg protein [9], the ratios of the intracellular to extracellular concentration of ceftibuten, cephradine and cephalexin were greater than 1 after 60-min incubation, indicating the uphill transport of these cephalosporins.

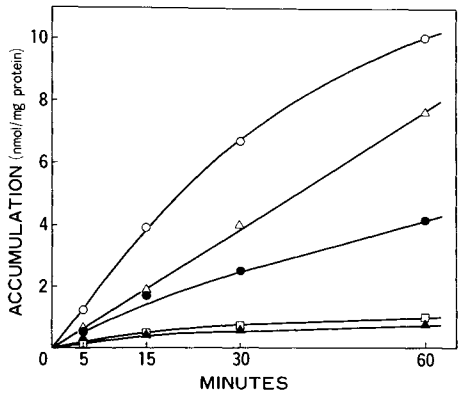

Fig. 1. Time course of cephalosporin uptake by Caco-2 cell monolayers. The monolayers were preincubated for 10 min at 37°C with 2.0 ml of incubation medium (pH 7.4). The medium was then removed, and monolayers were incubated for the designated period at 37°C with 2.0 ml of incubation medium (pH 6.0) containing 1 mM cephalosporin; ○, ceftibuten; △, cephradine; ●, cephalexin; □, cefixime, or ▲, cefotiam. The medium was aspirated off and the monolayers were rapidly washed twice with 5 ml of ice-cold incubation medium (pH 7.4). Cephalosporin extracted from the monolayers was measured by HPLC. Each point represents the mean of two monolayers.

pH-Dependence of Cephalosporin Uptake

We next examined the effects of varying the extracellular pH on the uptake of ceftibuten, cephradine and cefixime in the Caco-2 cell monolayers. As shown in Fig. 2, uptake of both ceftibuten and cefixime was markedly stimulated by lowering pH of the medium. The uptake of ceftibuten and cefixime was almost completely inhibited in the presence of 20 mM glycyl-L-leucine at the acidic pH region, whereas the dipeptide-inhibitable uptake of both antibiotics was not observed at the neutral pH. On the other hand, cephradine uptake was maximal at pH 5.5–6.0, and the uptake of these drugs was suppressed substantially in the presence of glycyl-L-leucine over the pH range studied. Therefore, the acidic pH-stimulated uptake of cephalosporins was suggested to be related to either an increase in the dipeptide transport activity or the affinity of these drugs for the transporter, or both.

Kinetic Analysis of Uptake of Various Cephalosporins

To characterize the transport of cephalosporins in the apical membranes of Caco-2 cells, the kinetic parameters for ceftibuten uptake were compared with those of cefixime and cephradine at pH 6.0. Table I summarizes the kinetic parameters evaluated from the Michaelis-Menten equation using nonlinear least-squares regression analysis [15]. *Vmax*, *Km* and *Kd* are the maximum uptake rate, the Michaelis-Menten constant, and the coefficient of simple diffusion, respectively. The *Km* values for ceftibuten and cefixime were much lower than that of cephradine. In addition, the *Vmax/Km* value, which indicates transport ability, of ceftibuten uptake was higher than those of cefixime and cephradine (ceftibuten, 0.4; cefixime, 0.03; cephradine, 0.1, μl/mg protein/min; values represent means of two independent experiments with three determinations).

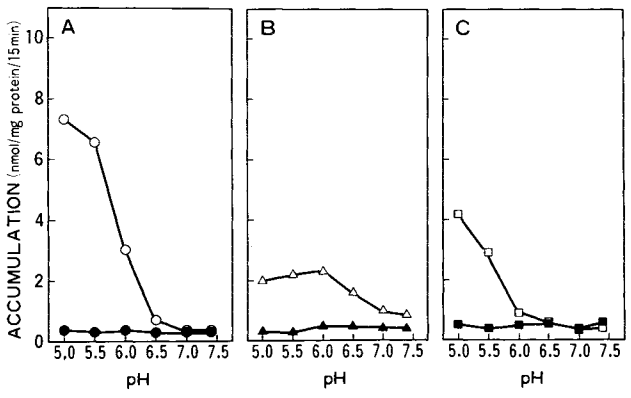

Fig. 2. pH-Dependence of ceftibuten, cephradine and cefixime uptake by Caco-2 cell monolayers. The monolayers were incubated at 37°C for 15 min with 1 mM ceftibuten (A), cephradine (B) or cefixime (C) in the absence (○, △, □) or presence (●, ▲, ■) of 20 mM glycyl-L-leucine at various pHs. The amount of cephalosporin accumulated by the monolayer was then measured. MES (5 mM) was used to buffer the incubation medium between pH 5.0 and 6.0, and HEPES (5 mM) was used as a buffer between pH 6.5 and 7.4. Each point represents the mean of two monolayers.

Table I. Kinetic Parameters for Ceftibuten, Cefixime and Cephradine Uptake by Caco-2 Cell Monolayers.[a]

	Km (mM)	Vmax (nmol/mg protein/min)	Kd (nmol/mg protein/min/mM)
Ceftibuten	1.0	0.40	0.02
Cefixime	1.4	0.04	0.02
Cephradine	8.3	1.20	0.03

[a] The monolayers were incubated at 37°C for 15 min with incubation medium (pH 6.0) containing various concentrations of ceftibuten, cefixime and cephradine. Cephalosporin uptake was then assayed and kinetic parameters were calculated. Each value represents the mean of two independent experiments.

Growth-Dependence of Ceftibuten Uptake and Marker Enzyme Activities in Caco-2 Cells

We reported that the accumulation of bestatin, a dipeptide-like anticancer agent, into the Caco-2 monolayers increased with the duration of culture, and that activity of the H^+/dipeptide cotransporter was expressed in differentiated cells [16]. Thus, the relationships between transport activity of ceftibuten and cell growth and/or differentiation were examined. As shown in Figure 3A, the uptake of ceftibuten was dependent on the duration of culture in the presence of a H^+ gradient, increasing up to the 14th day after inoculation and then decreasing. In the absence of a H^+ gradient (at pH 7.4), the uptake of ceftibuten was independent of the duration of culture, suggesting that the change in ceftibuten transport activity is pH gradient-dependent.

Figure 3B shows marker enzyme activities for the Caco-2 cell apical and basolateral membranes as a function of days in culture. The activity of aminopeptidase, an apical membrane enzyme, was increased with the duration of culture, being maximal at the 17th day, while the activity of (Na^+ + K^+)-ATPase, a basolateral membrane enzyme, was almost independent of the culture period. The present findings suggested that the cell growth-dependent change in the transport activity of ceftibuten was not correlated with either the apical or basolateral membrane enzyme activities.

To clarify the mechanism of the growth-dependent regulation of ceftibuten transport, the concentration dependence of the ceftibuten uptake was measured. Figure 4A shows the curves for ceftibuten uptake by Caco-2 monolayers on the 7th, 14th and 21st days after seeding. The Eadie-Hofstee plots of the data, after correcting the nonsaturable component, showed a linear relationship on each day (Figure 4B), indicating that a single transport system is involved. Table II summarizes the kinetic parameters of ceftibuten uptake represented in Figure 4A. In comparison with the parameters calculated on the 7th day, the apparent *Km* was markedly decreased and the *Vmax* was increased on the 14th day. On the 21st day, *Km* was decreased slightly and *Vmax* showed a marked decrease, compared with those on the 14th day. Thus, the early development of ceftibuten uptake was suggested to be due to a change in both the *Km* and *Vmax* values, whereas the late decline of the uptake was due mainly to a decrease in *Vmax*.

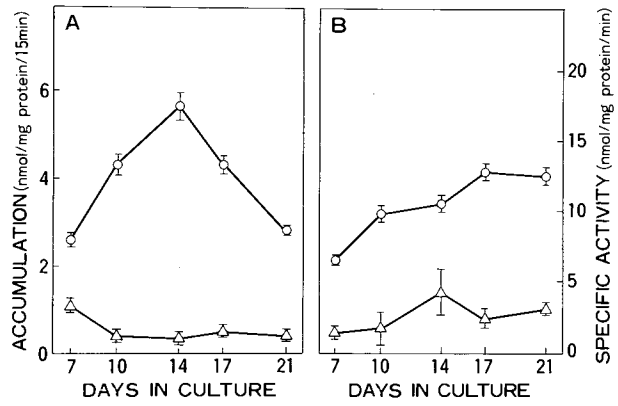

Fig. 3. Growth-dependence of ceftibuten uptake and of marker enzyme activities in Caco-2 monolayers. A. The monolayers, grown for 7, 10, 14, 17 and 21 days, were incubated at 37°C for 15 min with incubation medium (pH 6.0, ○: pH 7.4, △) containing 1 mM ceftibuten. The uptake of ceftibuten was then measured. Each point represents the mean ± S.E. of three monolayers. B. The monolayers, grown for 7, 10, 14, 17 and 21 days, were scraped into the incubation medium and homogenized using a Polytron homogenizer. Aliquots of the homogenates were used for measuring the specific activity of aminopeptidase (○) and (Na^+ + K^+)-ATPase (△). Each point represents the mean ± S.E. of three monolayers.

DISCUSSION

By using human intestinal Caco-2 cell monolayers, we examined the role of the H^+/dipeptide cotransport system in the intestinal absorption of ceftibuten. Our findings showed that ceftibuten can be transported with high affinity by the apical H^+/dipeptide cotransport system which is regulated by cell growth and/or differentiation.

Uptake of ceftibuten was stimulated markedly by acidifying the incubation medium, and was inhibited by an excess of dipeptide. These findings suggest that the H^+/dipeptide cotransport system mediates ceftibuten uptake by Caco-2 cells. The expression of the H^+-coupled dipeptide

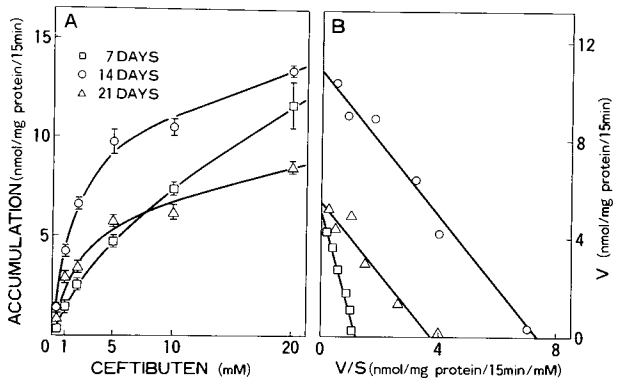

Fig. 4. Concentration-dependence of ceftibuten uptake by Caco-2 monolayers as a function of days in culture. A. The monolayers, grown for 7 (□), 14 (○) and 21 (△) days, were incubated at 37°C for 15 min with incubation medium (pH 6.0) containing various concentrations of ceftibuten. The uptake of ceftibuten was then measured. B. Eadie-Hofstee plots of ceftibuten uptake after correction for the nonsaturable component. V, uptake rate (nmol/mg protein/15 min); S, ceftibuten concentration (mM). Each point represents the mean ± S.E. of three monolayers.

Table II. Kinetic Parameters for Ceftibuten Uptake by Caco-2 Cell Monolayers as a Function of Days in Culture[a]

Days	Km (mM)	Vmax (nmol/mg protein/min)	Kd (nmol/mg protein/min/mM)
7	3.9	0.33	0.03
14	1.5	0.73	0.01
21	1.2	0.36	0.01

[a] The monolayers, grown for 7, 14 and 21 days, were incubated at 37°C for 15 min with incubation medium (pH 6.0) containing various concentrations of ceftibuten. Ceftibuten uptake was then assayed and kinetic parameters were calculated. Each value represents the mean of two independent experiments.

transport system in the Caco-2 cells was demonstrated previously using cephalosporin antibiotics [10–12], bestatin [16] and glycylsarcosine [17] as substrates. The apparent *Km* and *Vmax* values of ceftibuten uptake at pH 6.0 were much lower and greater, respectively, than those values of cephradine uptake. Ceftibuten uptake was stimulated markedly by lowering pH of the medium unlike cephradine uptake, suggesting that the affinity of ceftibuten for the H^+/dipeptide cotransport system increases at acidic pH. Considering that ceftibuten has three ionizable groups (*i.e.*, two carboxyl groups and one aminothiazole group) with the pKa values of 2.3, 3.2 and 4.5 [5], this agent should be mostly in the form of a divalent anion between pH 5.0 and 7.4. Therefore, the pH-dependence of ceftibuten uptake cannot be explained by a change in its ionic species. If charged amino acid residues are localized in recognition and binding sites of the transporter protein, the accessibility of ceftibuten to the transporter may regulate its pH-dependence. The observations that the optimum pH of cephradine uptake is 5.5–6.0 and that the pH profile of cefixime uptake is similar to that of ceftibuten may support this assumption at least partially. Yoshikawa *et al.* [5] found that the uptake of ceftibuten by rat intestinal brush-border membranes was stimulated to the greatest extent at external pH 5.5. This discrepancy might be due to differences in experimental systems or species.

The uptake of ceftibuten was highly dependent on the duration in culture. Kinetical analysis revealed that the early development of ceftibuten transport was due not only to a decrease in *Km* but also to an increase in *Vmax*, and that the late depression of ceftibuten uptake was due to a decrease in *Vmax* value. The change in *Km* value might be due to either cell growth-dependent expression of another dipeptide transporter or functional regulation of the dipeptide transporter. Further studies are needed to elucidate the precise mechanism of the kinetical change in the transport activity of ceftibuten, including multiplicity of the apical membrane dipeptide transporter. Although the physiological implications of up- and down-regulation of the H^+/dipeptide cotransporter are speculative, regulatory processes specific for this transport system may be involved because the development of aminopeptidase, an apical membrane enzyme, did not parallel that of ceftibuten transport activity.

Recently, a complementary DNA encoding the H^+-coupled oligopeptide transporter (PepT1) expressed abundantly in the rabbit small intestine was isolated, and its transport function was characterized using an expression system in *Xenopus* oocytes [18]. However, it has not yet been identified that the PepT1 is responsible for the intestinal absorption of a variety of oral β-lactam antibiotics, and that the transporter protein homologous to the rabbit PepT1 is expressed in the Caco-2 cells. Molecular analysis of the Caco-2 dipeptide transporter will provide useful information for both the expression and function of PepT1 as a peptide-like drug transporter.

In conclusion, the uptake of ceftibuten is mediated by the apical H^+/dipeptide cotransport system which is regulated by cell growth and/or differentiation in Caco-2 cells.

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