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Intestinal absorption of RU 41740, an immunomodulating compound extracted from *Klebsiella pneumoniae*, across duodenal epithelium and Peyer's patches of the rabbit

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Summary

The intestinal transport and degradation of RU 41740, an immunomodulating compound, were tested in vitro across rabbit duodenal epithelium and Peyer's patches mounted in Ussing chambers. Quantification of transport and metabolic behaviour across the intestinal wall were examined using tritiated RU 41740 ([^{3}H]RU 41740). The viability of the tissues was assessed by recording electrical parameters. Transepithelial [^{3}H]RU 41740-equivalent fluxes from mucosa to serosa were determined and the intracellular catabolism was examined by gel filtration of serosal [^{3}H]products on a Sephacryl S-300 column after 2 h of experimentation. Steady-state transport rates were obtained after 60 min; transepithelial fluxes of [^{3}H]RU 41740 equivalents were greater across the duodenum than the Peyer's patches (1647 ± 277 ng $\cdot$ h^{-1} $\cdot$ cm^{-2} versus 357 ± 61 ng $\cdot$ h^{-1} $\cdot$ cm^{-2}). Elution profiles of the tritiated-degraded products in the serosal compartment indicated that the radioactivity was mainly associated with low molecular weight fragments (mol. wt. < 10,000) in the duodenum, whereas higher molecular weight fragments were found in the Peyer's patches. These results indicate that RU 41740, like most macromolecules, is transported across the intestinal epithelium by endocytosis with subsequent extensive degradation probably at the lysosomal level. However, the minor fraction transported intact as well as under high molecular weight products in the Peyer's patches might have an important effect on the underlying immune system.

Introduction

RU 41740 (trade name Biostim) is a glycoprotein extract from *Klebsiella pneumoniae* (K_2 O_1 strain). This heterogeneous compound is composed of two subunits of 350,000 (Eur. Pat. Appl. EP 49, 182) and 95,000 (Ger. Offen. 3,029,111) mol. wt. This product possesses immunomodulation properties both in laboratory animals and in humans, in vivo and in vitro (Wood and Moller, 1984; Weinling et al., 1984; Durant et al., 1985; Wood and Moller, 1985; Martinez-Mazza and Wood, 1985; Guenounou et al., 1984; Andreux et al., 1986; Anthoine et al., 1985; Smets et al., 1986). Murine models have demonstrated that it can reduce mortality resulting from experimental bacterial (Zalisz et al., 1983) or viral (Rudent et

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al., 1985) infections when administered at doses of 10–100 μg/kg i.p. or 10–100 mg/kg orally (Griscelli et al., 1982). Among the known effects, the stimulation of lymphocytes and monocytes has been reported (Guenounou et al., 1985). The question of whether or not the active principle crosses the intestinal epithelium arises, since treatments with RU 41740 are usually administered orally. Proteins have long been shown to be absorbed by an endocytotic process (Warshaw et al., 1974; Silverstein et al., 1977) across columnar epithelial cells and across M cells present over Peyer's patches (Owen, 1977). We have previously demonstrated that proteins may be absorbed across the intestinal epithelium along two main functional pathways: a major pathway involving more than 90% of intracellular catabolism probably at the lysosomal level, and a minor route (< 10%) allowing the absorption of intact proteins (Heyman et al., 1982). When proteins are absorbed across the epithelium covering Peyer's patches, the rate of lysosomal degradation is reduced (Ducroc et al., 1983). This observation is of particular interest considering that Peyer's patches are secondary lymphoid organs which are believed to deliver antigenic information from the luminal content to the underlying lymphocytes (Owen, 1977). The aim of the present study was to quantify the intestinal transport of RU 41740 and to determine the extent of hydrolysis of the product while crossing the epithelium at the duodenal or Peyer's patches level.

Materials and Methods

Rabbits

Male New Zealand white rabbits (2–3 kg) obtained from Lessieux (France) were used in these experiments. They were allowed free access to conventional rabbit chow (UAR).

Preparation of tritiated-RU 41740

RU 41740 was labelled on the carbon-6 of the galactose residues (Van den Eijnden et al., 1977). In brief, oxidation was conducted (4 h, 37 °C) with galactose oxidase in phosphate-buffered 0.1 M saline, pH 7.2, followed by reduction with tritiated sodium borohydrure in 0.05 N ammonia (45 min, 20 °C) and unlabelled sodium borohydrure in excess (45 min, 20 °C). Following neutralization with 4 N acetic acid, the compound was ultrafiltrated on a PM 30 Amicon membrane in the presence of 0.5% acetic acid and purified by gel filtration on Sephacryl S 300 (Pharmacia). Tritiated-RU 41740 (spec. act. 24 μCi/mg) was then lyophilized and stored at −30 °C.

Gel filtration

After a 2 h experiment, the serosal and mucosal compartments of the Ussing chamber were lyophilized until gel filtration.

They were rehydrated in 1.5 ml of bovine serum albumin (BSA; 10 mg/ml) and eluted with Tris-HCl 0.05 M, NaCl 0.15 M, pH 8.0 as 0.75 ml aliquots on a Sephacryl S 300 (1 × 29 cm) column. Molecular weight calibration was performed using Dextran blue (2,000,000), ferritin (440,000), BSA (67,000) or ovalbumin (45,000), Myoglobin (17,800) and Cyanocobalamin (1350).

Measurement of tritiated compounds

The radioactivity was counted by liquid scintillation photometry in a Betamatic II, Kontron counter. After quenching corrections, the radioactivity expressed as disintegrations per minute, was reported as a function of the eluted volume.

Transepithelial fluxes

A modified version of the Ussing apparatus was used for the experimentation (Heyman et al., 1982). Rabbits were killed by an intravenous sodium pentobarbital injection. Part of the duodenum (5 cm from pylorus) and the first two proximal Peyer's patches were removed, rinsed with saline, and the muscular layer was stripped as described previously (Powell et al., 1972). Transepithelial fluxes from mucosa to serosa were obtained as follows: the duodenal fragment or the Peyer's patches (exposed serosal area 1.13 cm^2) were bathed on both sides with 15 ml of a Ringer solution (pH 7.4) containing in mM: Na 140, K 5.2, Ca 1.2, Mg 1.2, Cl 120, HCO_3 25, HPO_4 2.4, and H_2PO_4 0.4. The mucosal compartment was replaced at $t = 0$ by a Ringer solution containing 0.5 mg/ml RU 41740 and [^{3}H]RU 41740 (2.6 μCi/ml) as a tracer.

During 2 h, 8 ml samples were taken from the serosal side at 20 min intervals and replaced by fresh Ringer solution. The viability of the tissue was assessed by constant recording of the electrical parameters (short-circuit current, potential difference and ionic conductance). The rate of [^{3}H]RU 41740 appearance in the serosal compartment was determined by tritium counting of 1 ml aliquots taken from the successive 8 ml samples. The radioactivity was assayed by liquid scintillation photometry. [^{3}H]RU 41740 fluxes ($J_{[^3H]RU41740}$) involved both the intact product as well as the tritiated-metabolites formed during the transport. Consequently, these fluxes are referred as [^{3}H]RU 41740 equivalents and expressed as $ng \cdot h^{-1} \cdot cm^{-2}$. In another set of experiments, samples were taken only at 10 min and 120 min in order firstly, to ensure the integrity of the tissue and secondly, to allow the accumulation of tritiated products on the serosal side. After 120 min, the entire serosal compartment (15 ml) was frozen at $-20\,^{\circ}C$, lyophilized, and the tritiated fragments were characterized by gel chromatography.

Statistical analysis

The results were expressed as means ($\pm$S.E.). Student's *t*-test and non-parametric Mann-Whitney tests were used to compare means and ranges.

Results

Mucosal to serosal fluxes of [^{3}H]RU 41740

[^{3}H]RU 41740 fluxes were first determined according to the rate of tritium appearance in the serosal compartment as a function of time. The steady-state appearance of [^{3}H]RU 41740 on the serosal side was observed after a 60 min period in the duodenum and earlier (40–50 min) in the Peyer's patches (Fig. 1). The rate at which [^{3}H]RU 41740 appeared in the serosal solution remained constant from 60 to 110 min. The steady-state fluxes across the duodenum and the Peyer's patches are given in Table 1. Steady-state fluxes across the Peyer's patches were smaller (by about one-fifth) than across the duodenal epithelium, as has been previously reported for another glycoprotein, horseradish peroxidase (Ducroc et al., 1983). The electrical parameters of the tissues remained stable throughout the experiments and are reported in Table 2. As previously described (Ducroc et al., 1983), there are some differences in

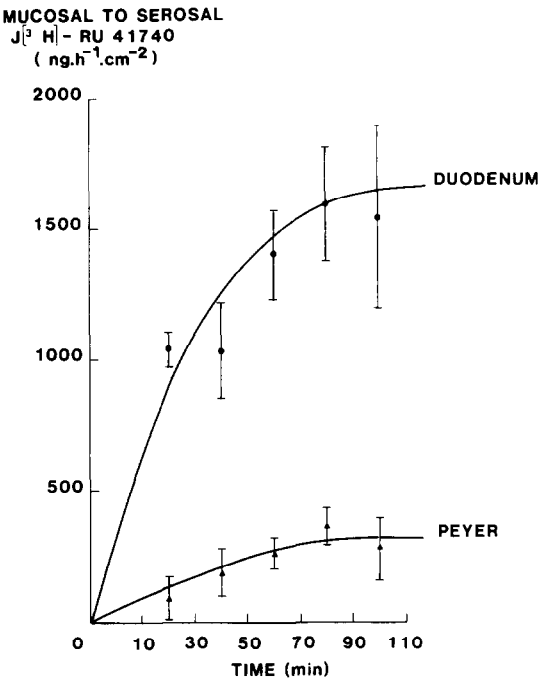

Fig. 1. Mucosal to serosal [^{3}H]RU 41740 fluxes as a function of time. RU 41740 (0.5 mg/ml) was added to the mucosal compartment with [^{3}H]RU 41740 as a tracer (2.6 μCi/ml). Fluxes were measured and expressed as ng of [^{3}H]RU 41740 equivalents $\cdot h^{-1} \cdot cm^{-2}$.

TABLE 1

Steady-state transepithelial fluxes (J ms) of [^{3}H]RU 41740 in duodenal epithelium and Peyer's patches as compared with values obtained for horseradish peroxidase (mol. wt. 40,000), a marker of fluid-phase endocytosis (from ref. Heyman et al., 1982)

	[^{3}H]RU 41740-equiv. J ms ($ng \cdot h^{-1} \cdot cm^{-2}$)	[^{3}H]HRP-equiv. J ms ($ng \cdot h^{-1} \cdot cm^{-2}$)
Intestinal epithelium	1647 $\pm$ 277 (7)	4241 $\pm$ 658 (8)
Peyer's patches	357 $\pm$ 61 * (7)	367 $\pm$ 159 * (7)

Values are means $\pm$ S.E. In parentheses are number of rabbits.
* Significantly different from intestinal epithelium; $P < 0.001$.

TABLE 2

Electrical parameters of the tissues. Values are means ± S.E.

	Isc ($\mu A/cm^2$)	*PD* (mV)	*G* ($mmho/cm^2$)
Duodenum	20.9 ±2.5 (7)	−1.64±0.24 (7)	13.4±1.7
Peyer's patches	5.28±2.13 * (7)	−0.62±0.19 * (7)	7.0±0.8 *

In parentheses are number of rabbits. Short-circuit current (I_{sc}) and potential difference (*PD*) were measured on stripped segments of duodenal epithelium and Peyer's patches. Conductance (*G*) was calculated as the slope of I_{sc} as a function of *PD*.

* Significantly different from duodenum.

the ability of the epithelium of the small intestine and the Peyer's patches to actively transport electrolytes. However, the lack of increase of ionic conductance (*G*), which is an index of the paracellular permeability during the experiment, strongly suggests that the functional integrity of the tissues was maintained. Moreover, the lack of increase in the potential difference and the short-circuit current during the experiment suggests that free galactose is not liberated from RU-41740; such a liberation would have led to the stimulation of Na-sugar co-transport and a short-circuit current rise.

Characterization of [^{3}H]RU 41740 metabolites on Sephacryl S 300

The chromatographic pattern of native RU 41740 on Sephacryl S 300 before the transport studies is presented in Fig. 2A. The eluted fractions are divided in 3 groups of mol. wt. > 440,000; 440,000 < mol. wt. < 10,000; and mol. wt. < 10,000 and the percentages of different mol. wt. groups are presented in Table 3.

An assessment was made of the structural modifications that had occurred to the RU 41740 during the transport process. This was carried out by comparing the elution profile, and hence the molecular weight distribution, of the native RU 41740 with those of the RU 41740 in the mucosal compartment (which had been exposed to the brush border membrane for 2 h) and those of the

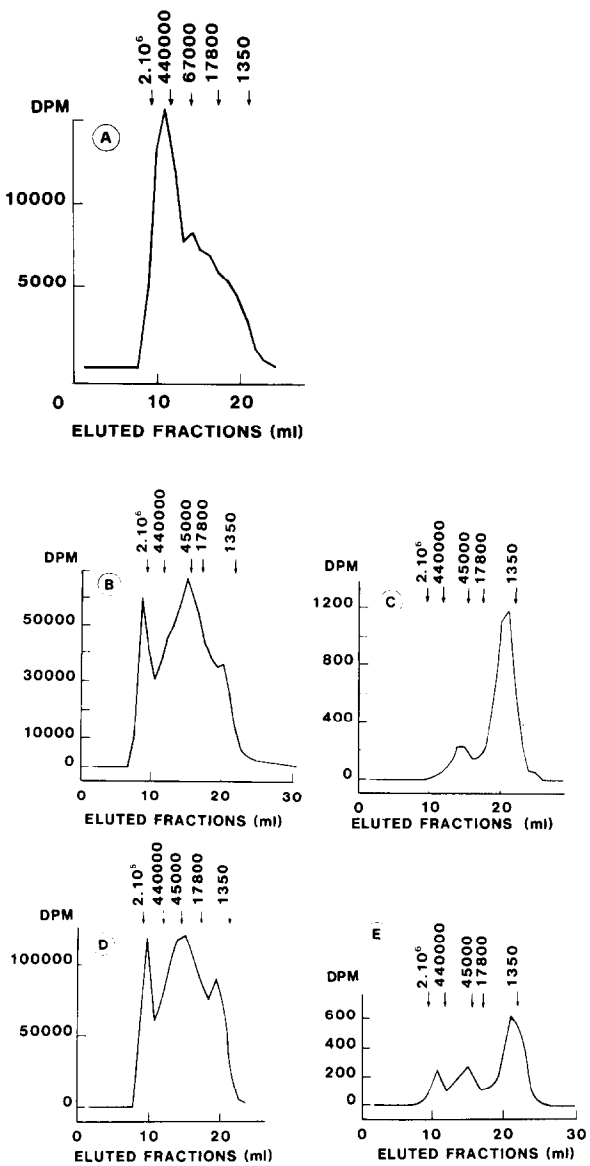

Fig. 2. Typical chromatographic patterns of [^{3}H]RU 41740 on Sephacryl S 300, after 2 h incubation in a Ringer solution (A), after 2 h incubation in the mucosal compartment bathing the duodenal epithelium (B) or the Peyer's patches (D), and after crossing the duodenal epithelial layer (C) or the epithelium overlying the Peyer's patches (E).

RU 41740 in the serosal compartment (which had crossed the tissue).

At the end of the experiment, the RU 41740 present in the mucosal compartment had been

TABLE 3

Comparison of percentages (means and ranges) of different molecular weight fractions of RU 41740 before and after intestinal absorption

		mol. wt. > 440,000	440,000 < mol. wt. < 10,000	mol. wt. < 10,000
Native RU 41740 before transport:		42	46	12
Duodenum	Mucosal side	18.8 (15–24)	58.3 (52–63)	22.8 (22–27)
	Serosal side	4.1 (1–11)	25.4 (9–44)	70.4 (50–90)
Peyer's patches	Mucosal side	21.0 (19–24)	55.5 (52–58)	23.5 (21–25)
	Serosal side	17.3 (2–36) *	25.8 (7–39)	56.8 (35–76)

RU 41740 (with [^{3}H]RU 41740 as a tracer) was placed on the mucosal side of the duodenum or Peyer's patches. After 2 h incubation, the chromatographic pattern of the contents of the mucosal and serosal compartments were determined and the percentage of degraded compounds was measured as the surface areas of the different peaks observed.

* Significantly different from duodenal serosal side; $P = 0.003$.

slightly hydrolyzed by brush border hydrolases or pancreatic enzymes adsorbed at the luminal membrane since a decrease in the tritiated fragments to lower molecular weights was observed. Free monosaccharides, mainly galactose, did not seem to be released from RU 41740, since the short-circuit current, an index of actively transported sodium-coupled sugar, did not rise during the experiment. Most of the radioactivity was still eluted with the high molecular weight products. The elution pattern was similar when RU 41740 was in contact with the mucosal side of either the duodenum or the Peyer's patches.

After crossing the tissue, most of the tritiated products eluted had low molecular weights < 10,000, as expected after endocytosis and lysosomal degradation.

However, the absence of detectable tritiated free galactose and poly-galactosic fragments (molecular weights < 700) was confirmed by gel filtration on Trisacryl GF 05 (IBF-France) (data not shown). Qualitative differences were observed in the elution profile of tritiated products after their transport across the duodenum and Peyer's patches. As reported in Table 3, and in Fig. 2C and E, in the duodenum there were fewer high molecular weight and more low molecular weight products in the serosal than in the mucosal solution, indicating that hydrolysis had occurred during the epithelial transport. In Peyer's patches, the percentage of serosal high molecular weight products was higher than in the duodenum (17.3% vs 4.1%, respectively, $P = 0.003$). Conversely, the percentage of low molecular weight products was smaller, although not significantly, in the Peyer's patches than in the duodenum (56.8% vs 70.4%, respectively).

Discussion

The immunomodulating agent RU 41740 is active when given orally at a dose 10^3 higher than when it is administered intraperitoneally or parenterally (Griscelli et al., 1982; Takada et al., 1982). This suggests that part of the active principle is destroyed in the intestinal lumen or while crossing the intestinal barrier. The aim of the present work was to study in vitro the modifications of RU 41740 during intestinal absorption across the rabbit duodenum and Peyer's patches.

The intestinal epithelium is generally considered as poorly permeable to macromolecular compounds. However, proteins such as immunoglobulins, intrinsic factor, or different hormones are known to be absorbed via a receptor-mediated endocytosis process allowing specific and rapid uptake of a non-negligible fraction of macromolecules (Goldstein et al., 1979). Generally, food or exogenous proteins escaping breakdown at the gastric and pancreatic levels, are absorbed via the non-specific fluid phase endocytosis implicating a high rate of degradation in the epithelial layer, at the lysosomal level. We have previously reported

that the glycoprotein marker, horseradish peroxidase, was 97% degraded while crossing the small intestine (Heyman et al., 1982). The present study clearly indicates that RU 41740 is absorbed across the duodenum and Peyer's patches of isolated epithelium. In order to quantify its transport rate, RU 41740 labelling with tritium was performed: the glycannic fraction which represents 60% of the product was labelled on the galactose residues. As previously described (Heyman et al., 1982) we considered that the rate of appearance of the tritium in the serosal compartment after crossing the tissue was representative of the transport rate of RU 41740, in both intact and degraded forms. The tritium fluxes were then referred to as [^{3}H]RU 41740-equivalent fluxes. The transmural absorption was found to increase with time until a steady-state was obtained after 60 min. This steady-state transport rate, as well as the lack of modification of the ionic conductance during the experiment, suggests that the RU 41740 transport does not occur between cells via paracellular leakage but via a transcellular route, probably by endocytosis. As previously reported with horseradish peroxidase (Ducroc et al., 1983), the transport rate was higher across the intestinal epithelium than across the Peyer's patches (Fig. 1 and Table 1). However, most of the RU 41740 crossing a Peyer's patch was still in a high molecular weight form: this finding could have important consequences since the drug may be transported in an undegraded state into the intercellular space and to the underlying lymphocytes beyond.

The quantification of [^{3}H]RU 41740 transport across the intestine allows us to estimate that about 1500 ng crosses 1 cm^2 tissue in 1 h. Compared to the initial quantity present in the mucosal compartment, i.e. 7.5 mg, the fraction transported after 1 h represents about 1 : 5000. This corresponds well with the ratio 1 : 1000 described for the parenteral versus oral active posology used in murine models.

Taken together, these results suggest that both duodenal and Peyer's patches epithelium are permeable to the immunomodulator RU 41740.

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