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CHANGES IN D-GLUCOSE UPTAKE BY BRUSH-BORDER VESICLES FROM SMALL INTESTINE OF RATS TREATED WITH MITOMYCIN C

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Abstract—The effect of mitomycin C pre-administration on the D-glucose transport system in the intestinal brush-border membrane of rat small intestine was examined by a rapid filtration technique. Forty-eight hours following the intravenous administration of mitomycin C, there were extensive and severe mucosal derangements. At this time point, membrane vesicles were prepared from the mitomycin-C-pretreated and control rats. Binding studies indicated that D-glucose entered into the intravesicular space of vesicles even in the case of mitomycin-C-pretreated rats. Vesicles obtained from both the mitomycin-C-pretreated and the control rats showed sodium-dependent uptake of D-glucose, but the initial uptake at 15 sec was significantly greater in control rats than in mitomycin-C-pretreated rats. Comparison of kinetic parameters of D-glucose transport indicated that K_m was not significantly different between control and mitomycin-C-pretreated rats. The pretreatment with mitomycin C decreased V_{max} and increased the diffusional permeability to D-glucose considerably. These changes induced by mitomycin C seemed to derive not from a direct effect on mature enterocytes but from an indirect effect secondary to mitotic inhibition in the crypts.

Most anticancer drugs have been shown to induce undesirable gastrointestinal side-effects including anorexia, weight loss, and diarrhoea. A morphological study showed that mitomycin C treatment caused acute injury to the small intestine, characterized by villous atrophy and glandular distortion, in several mammalian species [1].

Whether these morphological alterations in the gastrointestinal tract following mitomycin C administration are associated with alterations in the absorptive function has not been explored.

Malabsorption of nutrients and drugs following treatment with anticancer drugs has been explained in terms of villous atrophy, decrease in the active transport capacity, and loss of surface area of the intestinal mucosa. However, those studies were based on experiments in intact cell preparations *in vivo* and *in vitro* [2–16]. Robinson *et al.* [2] reported that metabolic activity of the intestinal mucosa, as evidenced by respiration, glucose consumption, and lactic acid production, is depressed by the administration of methotrexate in rats. Changes in the absorptive surface area, paracellular shunt permeability and cytoplasmic enzyme activities might make it difficult to evaluate anticancer drug-induced intestinal damage at the level of brush-border membrane where the active transport systems for many solutes are localized.

The purpose of this study was to investigate the effect of treatment with mitomycin C, an anticancer drug, on the intestinal transport of D-glucose by using the small-intestinal brush-border membrane vesicles to further characterize gastrointestinal toxicity.

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MATERIALS AND METHODS

Materials. Mitomycin C was supplied by the Kyowa Hakko Kogyo Co. (Tokyo, Japan). D-[^{3}H]-Glucose (3.5 Ci/mmole) was purchased from Amersham (U.K.). All other reagents used in these experiments were of reagent grade and were used without further purification.

Animals. Male Wistar rats weighing 180–220 g were used. Animals had free access to water and diet for at least 3 days before use to acclimatize them to laboratory conditions. After this period, rats were weighed and, under light ether anesthesia, given a bolus intravenous injection of mitomycin C (3 mg/kg in physiological saline) into the left femoral vein. Control rats received a comparable volume of physiological saline. Animals were maintained on 12-hr light–dark cycles. All injections were given between 12:00 and 13:00 hr Japan Standard Time (JST). Both groups were provided with food and water freely. Body weight and food intake were recorded at 13:00 JST each day.

Preparation of brush-border membrane vesicles. Forty-eight hours following the administration of mitomycin C or saline, brush-border membrane vesicles were prepared from the entire length of the small intestine according to the method of Kessler *et al.* [17]. A detailed procedure for the preparation was reported in our previous paper [18]. For transport studies, the purified brush-border fraction was resuspended in a buffer containing 300 mM mannitol and 10 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)–Tris (pH 7.5) (buffer A). The purity of the membrane preparations obtained from mitomycin-C-pretreated rats was evaluated by the specific activity of the marker enzyme (alkaline phosphatase).

Transport assay. The incubation medium was composed of 300 mM mannitol, 10 mM HEPES–Tris (pH 7.5), 200 mM NaSCN (or KSCN), and D-[^{3}H]-glucose (50 μCi/ml) as a substrate. The uptake of D-glucose was determined by a rapid filtration method [19]. Transport studies were initiated by the addition of 20 μl of the medium to 20 μl of the vesicle suspension (6–12 mg protein/ml) at 25°. At the stated times, 2 ml of an ice-cold stop solution (250 mM NaCl and 1 mM Tris–HCl, pH 7.5) was added to the mixture. The resulting mixture was immediately filtered through pre-wetted 0.45 μm filters (Fuji Photo Film, Tokyo, Japan) and washed twice with 5 ml of ice-cold stop solution. Background value was determined by the addition of substrate medium (20 μl) to 2 ml of ice-cold buffer A containing 20 μl of membrane vesicles followed by filtration. This value was subtracted from the uptake data.

Pretreatment of vesicles with mitomycin C. To investigate the direct action of mitomycin C on the brush-border membrane vesicles, brush-border membrane vesicles isolated from control rats were preincubated with mitomycin C dissolved in buffer A for 10 or 30 min at 25°. The transport experiments were performed, as mentioned above, after this preincubation.

Histological observations. For light microscopy, specimens were fixed in 10% (v/v) formaldehyde, sectioned, and stained with hematoxylin and eosin.

Analytical methods. In D-glucose uptake experiments, radioactivities on the filters were measured by a liquid scintillation counter (Aloka LSC-900, Tokyo, Japan). Protein was assayed by the method of Lowry *et al.* [20] using bovine serum albumin as a standard.

Alkaline phosphatase activities were measured with *p*-nitrophenyl-phosphate as a substrate according to the method of Murer *et al.* [21]. In separate experiments, brush-border membrane lipids were extracted according to the method of Folch *et al.* [22], and total phospholipid contents were calculated from the amount of inorganic phosphate determined with the method of Bartlett [23].

Other methods. For the estimation of kinetic parameters of D-glucose uptake by the brush-border membrane vesicles, uptake data were analyzed by an iterative non-linear least squares method using a personal computer (MZ-80B, SHARP, Tokyo, Japan). The program used was "*MULTI*" [24].

RESULTS

Macroscopically, few outward signs of disturbance were observed during the 48 hr following administration of mitomycin C. Diarrhea was rarely seen. Mitomycin-C-treated rats lost about 5% of their weight, whereas control animals gained about 5%. The small intestine appeared to be somewhat thinner and was often filled with yellowish fluids. The daily food intakes of the mitomycin-C-treated and control rats for the first 2 days were, respectively 11.8 ± 1.3 g/rat/day (mean ± S.E., N = 5) and 19.6 ± 1.3 (N = 5) during the first 24 hr and 6.2 ± 1.3 (N = 5) and 18.6 ± 1.0 (N = 5) during the

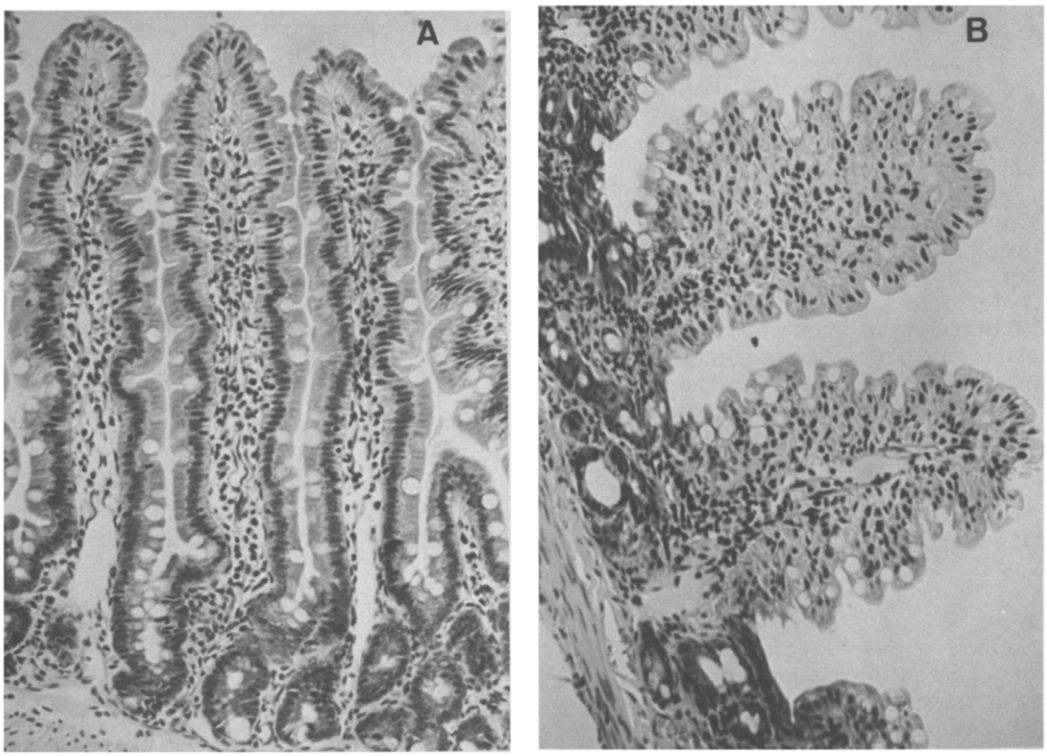

Fig. 1. Intestinal mucosal tissues (×100) 48 hr after the intravenous administration of physiological saline (A) or a single dose of 3 mg/kg mitomcin C (B). Each section was stained with hematoxylin-eosin. In the case of mitomycin C pretreatment, severe villous atrophy and mitotic arrest in the crypts were noted.

second 24 h. The food intake of mitomycin-C-treated rats decreased on each day.

Histological findings are presented in Fig. 1. The mucosal structure and villous population were in good condition in control rats (Fig. 1A), but mitomycin-C induced serious degeneration of the epithelial cells, villous atrophy, and mitotic arrest in the crypts, which were coupled with some disorders in the submucosal tissue (Fig. 1B).

To evaluate the ratio of lipid/protein in the brush-border membrane preparations, phospholipids were determined as a marker of the membrane lipid. Our membrane preparations from control and mitomycin-C-treated rats contained 10.7 ± 1.9 mg phospholipid/100 mg protein (mean ± S.E., N = 3) and 8.7 ± 1.6 mg phospholipid/100 mg protein (N = 3) respectively. These values are not significantly different from each other and are compatible with those previously reported by Forstner *et al.* [25]. The specific activity of alkaline phosphatase was enriched 9.9-fold compared to that found in the initial homogenate of the membrane preparations obtained from mitomycin-C-treated rats. This value is almost identical to that of membrane preparations obtained from control animals reported in our previous paper [18].

Figure 2A shows that D-glucose uptake by the brush-border membrane vesicles isolated from control rats at pH 7.5 transiently reached a value 8.3 times greater than the final value obtained after 30 min (overshoot phenomenon) in the presence of a NaSCN gradient but not in the presence of a KSCN gradient (outside to inside). This agrees well with the data reported previously [18]. In the case of the vesicles prepared from mitomycin-C-pretreated rats, Na^+-dependency of D-glucose uptake was also observed, whereas the initial uptake rate of D-glucose (15 sec) was reduced markedly and transient accumulation above equilibrium level was 3.9-fold (Fig. 2B).

To distinguish between binding of D-glucose to the brush-border membrane and penetration into the intravesicular space, the uptake at 60 min by vesicles from both groups of animals was measured by increasing the osmolarity of the outer medium with mannitol. As is evident from Fig. 3, the amount of D-glucose taken up was proportional to the inverse of the medium osmolarity, suggesting that D-glucose entered an intravesicular space. A positive intercept was obtained by extrapolation to infinite osmolarity (zero space), indicating that some binding components also existed. The binding percentages at the standard osmolarity condition were 10.9% in the case of vesicles from control rats and 15.7% from mitomycin-C-treated ones. The uptake data were not corrected for the binding.

Figure 4 shows the initial uptake of D-glucose as a function of the initial concentration in the presence of NaSCN or KSCN gradient. The relationship between concentration and rate of uptake was nonlinear in the presence of the NaSCN gradient and was linear in the presence of the KSCN gradient. In the selection of model equations, according to Yamaoka *et al.* [26], the equation with minimum Akaike's Information Criterion (AIC) is regarded as the best representation of the experimental data. Uptake in the presence of the NaSCN gradient was assumed to be described by the sum of a saturable term displaying Michaelis–Menten kinetics plus a second non-saturable term, rather than by a simple Michaelis–Menten equation or the sum of two saturable terms, because this equation gave the minimum AIC value. Thus, the rate of uptake (V) can be described by the following equation:

$$V = \frac{V_{max} \cdot [\text{D-glucose}]}{K_m + [\text{D-glucose}]} + P \cdot [\text{D-glucose}]$$

where V_{max} is the maximum velocity of Na^+-dependent uptake, K_m is the D-glucose concentration at which the Na^+-dependent uptake is half-maximal, and P is the permeability coefficient (proportionality constant for the nonsaturable process). The nonlinear least squares fitting to the experimental data

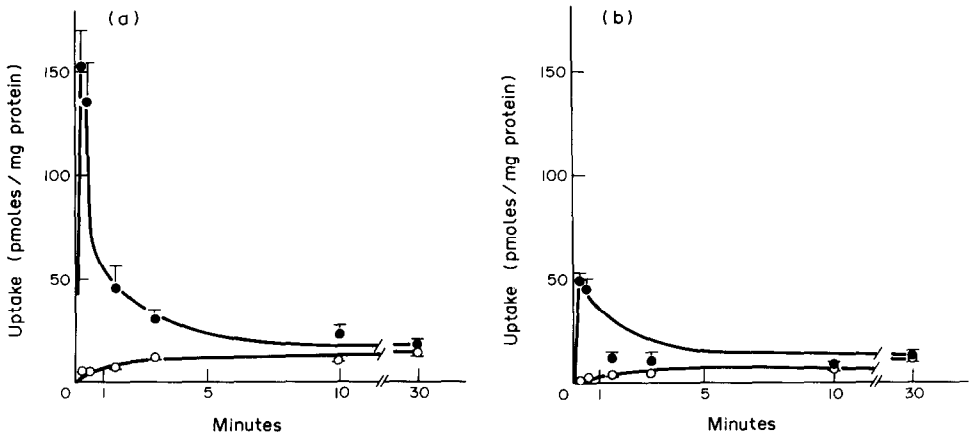

Fig. 2. Time course of D-glucose uptake by brush-border membrane vesicles isolated from control (A) or mitomycin-C-pretreated (B) rats. The final concentration of D-glucose was 0.1 mM. The vesicles were incubated at 25° with two different media prepared in 10 mM HEPES–Tris buffer (pH 7.5) containing 300 mM mannitol and either 200 mM NaSCN (●) or 200 mM KSCN (○). Each point represents the mean ± S.E. of four experiments.

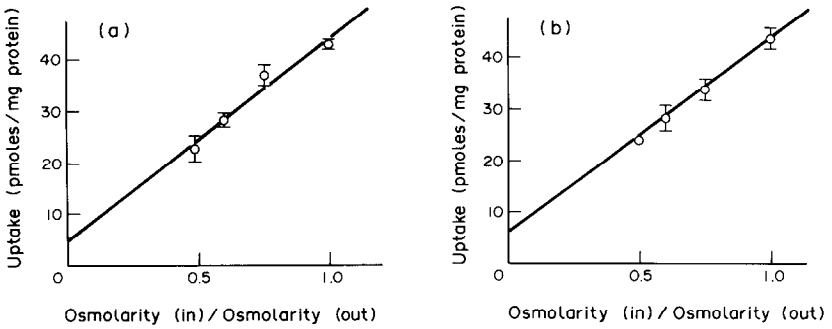

Fig. 3. Effect of osmolarity on the uptake of D-glucose by brush-border membrane vesicles isolated from control (A) or mitomycin-C-pretreated (B) rats. D-Glucose uptake is shown after 60 min. The osmolarity was varied by the addition of mannitol. Each point represents the mean ± S.E. of four experiments.

gave the parameters listed in Table 1. In the control experiment, the apparent K_m value under these conditions, was compatible with the value for rabbit intestinal brush-border membrane vesicles [27].

To investigate whether mitomycin C directly inhibited the D-glucose transport system as do sulfhydryl reagents, brush-border membrane vesicles from control animals were preincubated with mitomycin C dissolved in buffer A, and then transport studies were performed as usual. The results are summarised in Table 2. The uptake of D-glucose by brush-border membrane vesicles was reduced greatly when mitomycin C was administered to the animals *in vivo* 48 hr before experiments, whereas the preincubation of vesicles *in vitro* with mitomycin C, in spite of a relatively high concentration or prolonged preincubation period, had little, or no, inhibitory effect on D-glucose uptake.

DISCUSSION

In an effort to understand mechanisms of anticancer-drug induced disorders in the absorptive function of the gastrointestinal tract, many studies have been carried out on intact cell preparations *in vivo* or *in vitro* [2–16]. Although the anticancer-drug-induced disorders of absorptive function seem to be attributable to a decrease in active transport capacity [2, 7, 9, 15], decrease in the number of epithelial cells [3], diminished water flux through the intestinal membrane [6], loss of mucosal surface area [4, 12], and impaired mucosal integrity [11, 14], there is no information on the effect of treatment with anticancer drugs on the function of brush-border membranes. In the preliminary experiments, we examined the intestinal transport of 3-O-methyl-glucose using everted gut sacs prepared from rats killed at 24, 48, 96 and 120 hr after mitomycin C (3 mg/kg) was injected and obtained results indicating that the active sugar transport system was most strongly depressed at 48 hr after dosing (data not shown). Therefore, only membrane vesicles prepared from rats killed 48 hr after dosing with mitomycin C were tested in the present study.

Studies with isolated plasma membrane vesicles allow one to distinguish clearly phenomena occurring

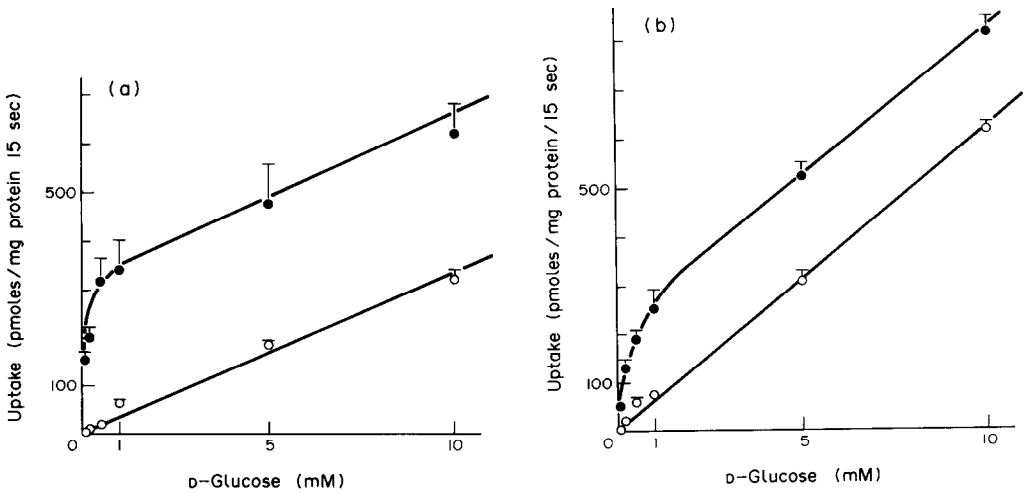

Fig. 4. Concentration dependence of D-glucose uptake by brush-border membrane vesicles isolated from control (A) and mitomycin-C-pretreated (B) rats. D-Glucose uptake for 15 sec at a concentration between 0.1 and 10 mM was determined. The vesicles were incubated at 25° with 10 mM HEPES–Tris buffer (pH 7.5) containing the substrate, 300 mM mannitol and either 200 mM NaSCN (●) or 200 mM KSCN (○). Each point represents the mean ± S.E. of four experiments.

Table 1. Kinetic parameters for the uptake of D-glucose by rat intestinal brush-border membrane vesicles

Vesicles	Michaelis–Menten kinetic parameters		First-order rate constant
	K_m (mM)	V_{max} (pmoles/mg protein/15 sec)	P (μl/mg protein/15 sec)
Control	0.115 ± 0.031	375.8 ± 23.2	0.0238 ± 0.0031
Mitomycin C pretreated	0.293 ± 0.093	253.5 ± 31.6*	0.0568 ± 0.0037†

Results are shown with S.D.
*,† Significantly different from controls: * $P < 0.05$, and † $P < 0.01$ (analysis of variance).

in the brush-border membranes from those occurring in the cell interior or in other membranes. Hence, the anticancer-drug-induced damage in cytoplasm or basolateral membrane can be excluded in the isolated brush-border membrane system.

On the other hand, work with vesicles carries some inherent difficulties. One of them is the binding of solutes to membrane surfaces. As shown in Fig. 3, some binding components existed in the case of both vesicle preparations. However, mitomycin C treatment did not greatly change the binding fraction of D-glucose. Another problem to be solved is whether or not mitomycin C treatment affected the relative amounts of lipids and proteins in the brush-border membrane, because all the uptake data were corrected for the unit content of protein. We showed that the relative amounts of phospholipids and proteins between membrane vesicles isolated from control and mitomycin-C-pretreated animals were not different. These results indicate that brush-border membrane vesicles are a suitable preparation for investigation of membrane phenomena even in the situation where the intestinal mucosa has been damaged by anticancer drugs.

Time courses of D-glucose uptake by brush border membrane vesicles are shown in Fig. 2. D-Glucose uptake was sodium dependent in vesicle preparations prepared from mitomycin-C-pretreated rats as well as those from control rats. Recently, Ghishan and Wilson [28] investigated the developmental maturation of the D-glucose transport system by using rat jejunal brush-border membrane vesicles from several age groups and suggested that the decreased magnitude of the overshoot in sucking rats compared with older ones can be ascribed to the increased Na^+ permeability in sucking animals rather than to the suppressed activity of a glucose-Na^+ cotransporter. But, in our experiments, whether the same mechanisms were involved is uncertain. Table 1 represents the kinetic parameters of D-glucose uptake by brush-border membrane vesicles. Increase P and reduced V_{max} values were noted. Capel *et al.* [9] suggested that pretreatment with 5-flourouracil and methotrexate decreases the efficiency of the barrier to passive penetration and inhibits carriers involved in active transport. From our results, it appears that the capacity of the D-glucose active (saturable) transport system (V_{max}) was reduced; in contrast, the dif-

Table 2. Effect of mitomycin C on D-glucose uptake by brush-border membrane vesicles

	Uptake (pmoles/mg protein/15 sec)	% of Control
Control	152.8 ± 16.1	100
0.1 mM Mitomycin C, 10 min	144.6 ± 7.7*	94.7
0.1 mM Mitomycin C, 30 min	129.1 ± 13.1*	84.5
1.0 mM Mitomycin C, 10 min	159.9 ± 18.4*	104.7
1.0 mM Mitomycin C, 30 min	138.7 ± 13.2*	90.8
Membrane vesicles from mitomycin-C-pretreated rats‡	49.5 ± 4.5†	32.4

The vesicles were pretreated with mitomycin C for 10 or 30 min at 25° prior to the beginning of the uptake. The uptake for 15 sec was determined in the presence of mitomycin C. The incubation medium contained 300 mM mannitol, 10 mM HEPES–Tris (pH 7.5), 100 mM NaSCN and mitomycin C with 0.1 mM D-glucose. Each value represents the mean ± S.E. of four experiments. Statistical significance was determined by means of Student's t-test.

* Not significantly different.

† $P < 0.001$.

‡ Brush-border membrane vesicles were isolated from rats that had received 3 mg/kg mitomycin C intravenously 48 hr before experiments, and the preincubation with mitomycin C was omitted.

fusional permeability to D-glucose (P) was increased significantly. Because the relative purities of the vesicles from the control and the mitomycin-C-pretreated rats were the same, the higher glucose maximal flux in the control vesicles was not due to the vesicles from control rats being purer than the vesicles from mitomycin-C-pretreated rats. But a conclusion that K_m was less affected than V_{max} and P is not possible at present.

Recently, Pinkerton and Milla [16] claimed that methotrexate could exhibit its enterotoxocity as a direct toxic effect on mature enterocytes or as an indirect effect secondary to mitotic inhibition in crypt cells. Mitomycin C is suggested to be active in dividing cells and to behave as an alkylating agent to inhibit DNA synthesis, as shown in *in vitro* studies [29]. Moreover, at a high concentration, RNA synthesis is also affected by this drug. To examine the direct effect of mitomycin C on the D-glucose transport system exclusively shared with mature absorptive cells, membrane vesicles prepared from control animals were exposed to mitomycin C. The observed lack of effect of short-term incubation with mitomycin C on D-glucose uptake suggested that the toxic effect of mitomycin C on brush-border membranes was not due to a direct inhibition of the D-glucose transport system but to an indirect inhibition secondary to the reduction of the mitotic activity in the crypts. Consequently, it is speculated that mitomycin-C-induced intestinal damage is caused by mitotic arrest in the crypts which is followed by stasis of villous cell turnover.

Although isolated membrane vesicles are a useful tool for examining membrane phenomena, even when the anticancer drug has been administered *in vivo*, studies with isolated vesicles do not take into account the complex organization of the cells. Therefore, it is necessary to see the results obtained here in context with studies performed *in vivo*.

In conclusion, by using isolated plasma membrane vesicles, we have shown that the administration of mitomycin C caused a derangement of the D-glucose transport system in the small intestine, which was characterized by reduced capacity and an increase in diffusional permeability to D-glucose. Detailed studies of electrophysiological properties, enzyme and transport activities, protein components and lipid composition of enterocyte plasma membranes in anticancer drug-treated animals should be able to clarify further the mechanisms of anticancer-drug induced gastrointestinal damage.

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