Gut, 1980, **21**, 486-492

Response of the rat small-intestine epithelium to methotrexate*

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SUMMARY We studied jejunal epithelial structure and function in rats 24, 48, 96, and 192 hours after a single intravenous injection of methotrexate (MTX) 30 mg/kg. The acute effect of the drug on the gut at 24 and 48 hours was characterised, as expected, by reduced mitoses in crypts, shortened villi, and depressed activity of thymidine kinase (an enzyme normally confined to intestinal crypt cells). At 96 hours, when MTX was no longer detectable in serum, the intestine had entered a proliferative phase characterised by increased crypt mitoses, accelerated migration of enterocytes along villi, and the presence on villi of epithelial cells with the enzyme profile of crypt cells, decreased disaccharidase, alkaline phosphatase, and Na^+–K^+ ATPase activities and increased thymidine kinase activity. Although the enzyme data suggested that enterocyte maturation was defective during this proliferative phase, glucose-stimulated Na^+ transport, normally a function of fully differentiated villus cells, was normal at 96 hours. Measured both in Ussing chambers and in suspensions of enterocytes isolated from villi, Na^+ transport responded normally to glucose at 96 hours, although the response had been significantly depressed at 24 hours. These findings cannot be attributed to MTX-induced malnutrition, as all comparisons included pair-fed controls. We conclude that, in the small intestine under conditions of altered epithelial renewal, some components of enterocyte function may be affected more than others. Comparing the present experimental model with another intestinal disorder, acute viral enteritis, in which proliferative activity is excessive, it is clear that the nature of the original intestinal injury is a significant determinant of the pattern of enterocyte response.

Methotrexate (MTX), an inhibitor of dihydrofolate reductase and the synthesis of DNA, causes an acute injury to the intestinal epithelium characterised by reduced mitoses in the crypts and shortened villi.[1–4] A morphological study has suggested that, if the recipient survives this acute MTX-induced injury, a period follows in which the small intestinal epithelium proliferates excessively.[2] We gave to rats a single dose of MTX sufficient to cause acute but non-fatal intestinal injury and measured villus cell function at intervals after the drug was injected. Our purpose was to evaluate the impact of this epithelial proliferation on enterocyte differentiation and function.

*These studies were supported by a grant from the Medical Research Council of Canada.

†Dr Taminiau was the recipient of a grant from the Koningin Wilhelmina Fonds.

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Received for publication 4 December 1979

Methods

Male adult Wistar rats weighing 200–300 g were housed in individual cages and assigned at random to three groups. Group 1 were injected with one dose of MTX (30 mg/kg) intravenously in 0·5 ml saline; they were fed *ad libitum*, and their body weight and food intake measured daily. Group 2 (pair-fed controls) were matched with the MTX group by feeding each the amount taken by its MTX-injected counterpart in the previous 24 hours. Group 3 (untreated controls) were fed standard rat chow *ad libitum*. All animals had unlimited access to water, and both groups of controls were injected with 0·5 ml saline intravenously at the time of MTX injection in group 1. The rats were killed with ether immediately before or 24, 48, 96, or 192 hours after MTX was injected, and jejunal segments were taken just distal to the ligament of Treitz. MTX serum levels were measured by enzymatic assay.[5]

For light microscopy, specimens were fixed in

Bouin's solution, sectioned, and stained with haematoxylin and eosin. Measurements were made with a calibrated micrometer.

Enzymatic activities were determined in homogenates of full-thickness mucosa and isolated villus enterocytes. Jejunal segments were flushed with iced saline and opened; the mucosa was scraped off between two glass slides and homogenised with 2·5 mM EDTA, 100 mg/ml. Enterocytes were isolated from jejunal villi in suspension using a vibration technique that excludes crypt cells.[6] Activities of sucrase (Na^+–K^+;–ATPase, alkaline phosphatase, thymidine kinase, total protein, and DNA were assayed in whole mucosal homogenates and isolated villus cells.[7 8]

Unidirectional and net-intestinal sodium fluxes were measured in jejunal segments mounted in Ussing short-circuited chambers that exposed 0·4 cm^2 of mucosa to the bathing solutions.[9] Adjacent segments were incubated in either 1 mM or 30 mM glucose for 24 minutes, allowing for a 15 minute equilibration period and three consecutive 10 minute study periods. Preliminary studies in rat jejunum had shown no advantage of stripped *vs* full-thickness mucosa and a minimum glucose requirement of 1 mM concentration for stability of tissue potential difference (PD), short circuit current (Isc), and ion flux during a 45 minute study. The response of Na^+ flux to glucose was calculated as an increment, comparing the data obtained at the two glucose concentrations, 1 mM and 30 mM.[10 12] In suspensions of enterocytes isolated from jejunal villi, Na^+ transport was studied by measuring the efflux of ^{22}Na from previously loaded cells and calculating the efflux rate constant in the presence of mannitol 10 mM, glucose 10 mM, or ouabain 1 mM.[6]

For autoradiographic studies additional groups (six in each) of MTX-treated rats, pair-fed and normal controls were injected with 100 μ Ci of ^3H-thymidine 84 hours after the MTX, and two from each group were killed one hour, or 12 hours later. Proximal intestine was processed for autoradiography and migration of the foremost labelled cells was measured by light microscopy with a calibrated eye piece.[13]

Results

GENERAL RESPONSE

The MTX-treated rats decreased their food intake within 24 hours of the injection. Anorexia was most severe at 72 hours, intake then gradually increased, becoming normal at 120 hours (Fig. 1). Their mean serum MTX concentration at 24 hours was 10 ng/ml and, at 96 hours, MTX was not detectable in serum. Drug-treated and pair-fed groups had similar weight losses as nutrient consumption diminished, and both regained weight at the same rate. Occasional loose stools were noted on days 2, 3 and 4 after MTX, rarely in pair-fed controls within the first 48 hours, and never in controls fed *ad libitum*.

SMALL INTESTINAL MICROSCOPY

Light microscopy of tissue taken from MTX-treated rats (at 24 hours) showed preservation of the general architecture but shortening of the crypt cells, which contained enlarged, irregular nuclei. At 48 hours villus height in the MTX group was significantly diminished compared with both control groups ($P<0·001$) (Table 1). Villus cells closer to the crypts were more cuboidal and had large nuclei, and crypt cells were cuboidal with enlarged nuclei. At 96 hours the villus epithelium had started to recover; cells were rather cuboidal and those at the villus tips were vacuolated. Villi were elongating and the crypts were significantly deeper than in control group 2 or 3 ($P<0·001$). At 192 hours the only abnormality seen in the MTX group occurred in the crypts, which were still deeper than in either control group ($P<0·025$). Mucosal dimensions in the pair-fed controls at no time differed from controls fed *ad libitum*.

Compared with both control groups, in the MTX group villus cells were decreased in number at 24 hours ($P<0·02$), further decreased at 48 hours ($P<0·001$), and not fully recovered at 96 hours ($P<0·01$;; similarly, the number of cells per crypt was diminished at 24 hours ($P<0·001$), more so at 48 hours ($P<0·01$), and increased at 96 hours ($P<0·001$) and 192 hours ($P<0·01$) (Table 1). After

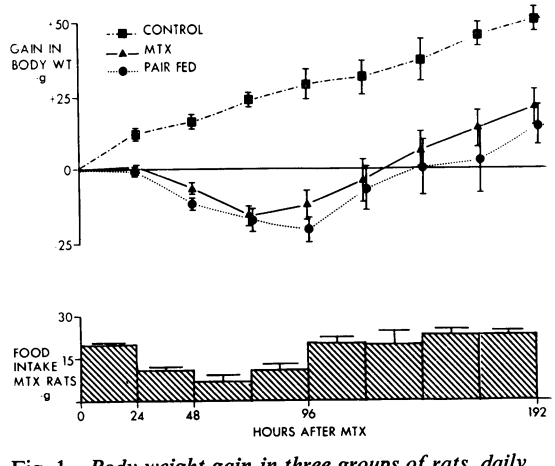

Fig. 1 *Body-weight gain in three groups of rats, daily food intake in MTX group during 192 hour period after MTX. Symbols and bars indicate M±SE.*

Table 1 *Structure of jejunal mucosa in three groups of rats: injected with methotrexate (MTX), 30 mg/kg; untreated but pair-fed (PF); and untreated rats fed* ad libitum *(control) (mean±SE)*

	Control*	Time after injection of methotrexate									
		24 h				48 h				96 h	
		MTX	pa	PF	pb	MTX	pa	PF	pb	MTX	pa
No.	7	8		7		7		7		9	
Villus height (μm)	512	490		510		294		493		407	
	±19	±19	NS	±12	NS	±12	<0·001	±27	<0·001	±46	NS
Crypt depth (μm)	172	191		188		187		203		281	
	±7	±5	<0·05	±3	NS	±14	NS	±12	<0·05	±11	<0·001
Villus cells/column	93	80		96		32		90		58	
	±4	±3	<0·01	±3	<0·001	±3	<0·001	±5	<0·001	±10	<0·01
Crypt cells/column	32	24		36		20		37		40	
	±1	±1	<0·001	±1	<0·001	±4	<0·01	±1	<0·001	±1	<0·001
Mitoses/crypt	2·62	0·05		2·74		1·28		2·58		3·52	
	±0·15	±0·21	<0·001	±0·14	<0·001	±0·5	<0·05	±0·17	<0·05	±0·2	<0·01

* *Ad libitum* fed controls. pa: MTX compared with controls pb: MTX compared with PF NS: not significant

MTX, crypt mitotic figures were decreased significantly at 24 hours ($P<0·001$) and 48 hours ($P<0·05$) and had increased significantly at 96 hours ($P<0·01$) and 192 hours ($P<0·05$) compared with both control groups.

As seen by autoradiography at the 96 hour stage, cell incorporation of ^{3}H-thymidine was greatly increased ($P<0·005$) one hour after injection in MTX rats (12·3±2·3 cells/crypt column; 60% mitotic figures) compared with pair-fed rats (5·2±0·1; no mitoses) and those fed *ad libitum* (7·3±0·4; 10% mitoses). Label incorporation occurred along the entire crypt in the MTX rats, in the basal half in the pair-fed, and in the basal two-thirds in those fed *ad libitum*.

Four hours after label injection in MTX rats, the foremost labelled cells had migrated from the crypt base to the villi (Table 2) and at 12 hours labelled cells covered the villus tips, some obviously having been shed into the lumen. The migration rate of

Table 2 *Autoradiography of rat jejunum 96 hours after injection of methotrexate*

	Distance (μm) of foremost enterocyte labelled with ^{3}H-thymidine from crypt base (mean±SE)		
Time after labelling (h):	1	4	12
Control	140±6	176±10	286±8
Methotrexate (30 mg/kg)	238±10	343±8	392±7*
Pair-fed	90±9	110±4	166±20

*^{3}H-labelled cells had already reached villus tips.

Table 3 *Enzyme activities* in rat jejunal villus enterocytes after injection of methotrexate (MTX) 30 mg/kg*

	Controls	Time after injection of methotrexate									
		24 h				48 h				96 h	
		MTX	pa	PF	pb	MTX	pa	PF	pb	MTX	pa
No.	7	8		7		7		7		12	
Sucrase, U/g protein	72·6	61·6		64·5		23·3		81·1		12·2	
	(63·9–82·5)	(54·9–69·3)	NS	(59·0–70·4)	NS	(17·6–30·7)	<0·01	(73·1–90·1)	<0·001	(8·4–17·8)	<0·001
Alkaline phosphatase, U/g protein	5·3	4·8		3·4		2·3		4·7		1·5	
	(4·6–6·1)	(4·2–5·6)	NS	(2·8–4·1)	NS	(1·9–2·8)	<0·01	(4·3–5·1)	<0·01	(1·2–1·9)	<0·001
$(Na^+–K^+)$-ATPase, U/g protein	2·5	2·3		2·2		0·9		2·9		0·5	
	(2·2–2·9)	(2·0–2·6)	NS	(1·9–2·6)	NS	(0·7–1·3)	<0·05	(2·6–3·2)	<0·01	(0·3–1·1)	<0·05
Thymidine kinase, U/mg protein	6·6	3·2		9·0		5·4		6·6		9·9	
	(4·9–8·9)	(2·8–3·6)	<0·05	(7·8–10·4)	<0·001	(3·6–8·1)	NS	(5·6–7·8)	NS	(7·8–12·7)	NS
Protein/DNA ratio	17·6	22·0		15·6		18·4		16·0		17·3	
	(0·7)	(1·6)	<0·05	(0·9)	<0·01	(4·2)	NS	(0·3)	NS	(1·8)	NS

*Results expressed in antilogs of log mean in U/mg protein, ±SE.
Sucrase, 1U = 1 mmol disaccharide hydrolysed/min.
Alkaline phosphatase, 1U = 1 μmol P nitrophenyl phosphate hydrolysed/h.
$(Na^+–K^+)$-ATPase, 1U = 1 μmol P liberated/h.
Thymidine kinase, 1U = 1 μμmol thymidine phosphate formed/min.
pa = MTX compared with control.
pb = MTX compared with pair-fed rats (PF).

		192 h			
PF	*pb*	*MTX*	*pa*	*PF*	*pb*
6		6		8	
457		529		490	
±29	NS	±29	NS	±14	NS
172		199		181	
±8	<0·001	±7	<0·02	±6	NS
81		91		92	
±3	<0·05	±3	NS	±4	NS
32		36		34	
±2	<0·01	±1	<0·01	±1	<0·05
2·52		3·28		3·20	
±0·33	<0·05	±0·21	<0·05	±0·16	NS

35 μm/h, calculated from one and four hour data, was much greater in MTX rats ($P<0.005$) then in the pair-fed (6 μm/h) or those fed *ad libitum* (12 μm/h).

SMALL INTESTINAL FUNCTION

Sucrase ($P<0.001$), alkaline phosphatase ($P<0.005$), and (Na^+–K^+)-ATPase activities ($P<0.025$), measured in enterocytes isolated from villi, were reduced 48 hours after MTX compared with both control groups. All three activities were decreased further at 96 hours but had returned to control values at 192 hours (Table 3). Thymidine kinase activity was decreased at 24 hours in MTX rats ($P<0.05$) but was significantly higher at 96 hours ($P<0.001$) and 192 hours ($P<0.025$) compared with both control groups. In pair-fed rats compared with control rats fed *ad libitum* alkaline phosphatase and (Na^+–K^+)-ATPase activities were similar but thymidine kinase was decreased ($P<0.05$) at 96 hours and sucrase was increased ($P<0.025$) at 192 hours. Measured in whole mucosal homogenates, or related to DNA content rather than to protein, these enzyme data yielded identical group patterns. The protein-DNA ratio in the isolated enterocytes was similar in all study groups, the only exception being a significantly higher ratio in the MTX rats at 24 hours than in either control group ($P<0.05$).

		120 h			
PF	*pb*	*MTX*	*pa*	*PF*	*pb*
6		6		8	
100·4		79·1		111·3	
(83·8–120·3)	<0·001	(72·0–87·0)	NS	(101·2–122·3)	<0·05
3·4		4·9		4·4	
(2·5–3·3)	<0·05	(4·4–5·5)	NS	(3·7–5·2)	NS
2·9		7·7		2·6	
(2·4–3·4)	<0·05	(1·4–2·1)	NS	(2·2–3·1)	NS
2·6		11·8		6·0	
(2·1–3·3)	<0·001	(9·7–14·5)	NS	(5·3–6·7)	<0·02
18·5		16·3		16·6	
(2·1)	NS	(2·0)	NS	(1·7)	NS

In full thickness jejunal segments studied in short-circuited chambers, mean net Na^+ flux was greater in 30 mM glucose ($P<0.005$) than in 1 mM in both control groups at all stages of the study (Table 4). In the MTX group this response of net Na^+ flux to 30 mM glucose ($J^{Na/net}$) was reduced at 24 hours and 48 hours ($P<0.005$) and normal at 96 hours. Mean unidirectional Na^+ fluxes ($J^{Na/m\rightarrow s}$ and $J^{Na/s\rightarrow m}$) in the presence of 1 mM but not 30 mM glucose were markedly greater in MTX rats than in either control group at 24 and 48 hours ($P<0.005$). As seen in Fig. 2, the increments in PD and Isc in the presence of 39 mM glucose were diminished in MTX rats at 24 and 48 hours ($P<0.001$) but not at 96 hours compared with both groups of controls. Total tissue conductance was raised in the MTX group at 24 hours (1 mM glucose) and 48 hours (1 mM and 30 mM glucose) compared with either control group ($P<0.05$), but at 96 hours it was significantly greater (1 mM glucose) in pair-fed controls than in those fed *ad libitum* or MTX-treated rats ($P<0.01$).

Na^+ efflux rate constants measured in suspensions of villus cells under basal conditions, in the presence of glucose (10 mM), and ouabain (1 mM) are summarised in Table 5. Under basal conditions, the total efflux rate constants in MTX rats at 24 and 48 hours were greater than in the group fed *ad libitum* ($P<0.001$). At all times Na^+ efflux from cells of both MTX and pair-fed groups responded significantly to glucose, but in MTX rats at 24 hours the response was decreased compared with pair-fed controls ($P<0.05$). Passive efflux in MTX-treated cells (1 mM ouabain) increased only at 48 hours compared with controls fed *ad libitum* but never in comparison with cells from pair-fed rats.

Discussion

The early response of rat small intestine to MTX in a dose approximating 50% of the LD_{50}[14] can be attributed to a direct effect of the drug on dividing cells. Previous studies have also noted reduced mitoses in crypt cells and shortened jejunal villi within 24 hours of giving the drug.[1–4] In the present

Table 4 *Sodium flux (μEq cm^{-2}h^{-1}) in short-circuited rat jejunal mucosa after injection of methotrexate (MTX 30 mg/kg (mean±SE))*

	*Controls** (6)	*Time after injection of methotrexate*											
		24 h				*48 h*				*96 h*			
		MTX (6)	*pa*	*PF*	*pb*	*MTX*	*pa*	*PF*	*pb*	*MTX*	*pa*	*PF*	*pb*
1 mM glucose													
$J^{Na}_{m\rightarrow s}$	3·48 (±0·34)	7·86 (±0·30)	<0·001	4·92 (±0·52)	<0·001	11·27 (±1·41)	<0·001	5·21 (±0·52)	<0·01	4·37 (±0·55)	NS	8·41 (±1·26)	<0·01
$J^{Na}_{s\rightarrow m}$	4·41 (±0·41)	7·96 (±0·66)	<0·001	4·17 (±0·66)	<0·001	10·39 (±0·93)	<0·001	5·89 (±0·88)	<0·01	4·93 (±0·48)	NS	8·22 (±1·60)	NS
J^{Na}_{net}	−0·93 (±0·42)	−0·10 (±0·61)	NS	+0·75 (±0·31)	<0·01	+0·88 (±1·27)	NS	−0·68 (±0·64)	NS	−0·57 (±0·26)	NS	+0·19 (±0·75)	NS
30 mM glucose													
$J^{Na}_{m\rightarrow s}$	7·87 (±1·47)	5·72 (±0·66)	NS	8·67 (±1·48)	NS	11·10 ±1·25)	NS	6·99 (±1·15)	<0·05	5·50 (±1·14)	NS	9·16 (±1·45)	NS
$J^{Na}_{s\rightarrow m}$	4·68 (±1·29)	4·22 (±0·81)	NS	3·88 (±0·96)	NS	8·57 (±1·50)	NS	5·24 (±1·60)	NS	3·88 (±0·64)	NS	4·99 (±1·39)	NS
J^{Na}_{net}	+3·19 (±0·46)	+1·50 (±0·30)	<0·02	+4·78 (±0·99)	<0·001	+2·53 (±0·55)	NS	+1·75 (±1·24)	NS	+1·62 (±0·66)	NS	+4·14 (±0·86)	<0·05
J^{Na}_{net}	+4·20 ±0·77	+1·61 ±0·34		+4·30 ±0·83		+1·65 ±1·02		+2·43 ±1·58		+2·18 ±0·69		+3·95 ±1·04	

* = *ad libitum* fed controls.
pa = MTX compared with control.
pb = MTX compared with pair-fed rats (PF).

experiments enzymes normally confined to differentiated villus enterocytes, sucrase, alkaline phosphatase, and Na-K-ATPase were unaffected while the crypt cell enzyme thymidine kinase was significantly diminished in activity at this stage. An exception to this pattern of early damage confined to crypt cells was the unexpected supression of glucose-stimulated Na-transport, observed both in intestinal segments and in suspensions of villus enterocytes and suggesting some impact of the drug on villus cells. Increased unidirectional Na^+ fluxes and high electrical conductances were also part of early response to MTX indicating excessive epithelial permeability.[15] This must have been either intercellular or, if transcellular, confined to crypt cells, as Na^+ efflux in the presence of ouabain was unaffected by the drug when studied in suspensions of enterocytes isolated from villi.

Ninety-six hours after MTX, the jejunal epithelium had clearly entered a highly proliferative state. The increased mitotic figures, deepened crypts, and increased labelling of crypt cells in autoradiographic studies observed in the present experiments have been reported previously in rats four to five days after MTX.[2] Of particular interest are the present data on enterocyte function during this period of

Table 5 *Na^+ efflux from isolated rat jejunal enterocytes after injection of methotrexate (30 mg/kg (mean±SE))*

	Control	*Time after injection of methotrexate*									
		24 h					*48 h*				
		pa	*MTX*	*pa*	*PF*	*pb*	*pa*	*MTX*	*pa*	*PF*	*pb*
Total Na^+ efflux rate constant (h^{-1})											
n	(23)		(30)		(30)			(30)		(30)	
10 mM mannitol	10·6 (±0·4)	<0·01	13·0 (±0·6)	NS	12·1 (±0·6)	NS	<0·001	14·0 (±0·7)	<0·01	13·4 (±0·7)	NS
n	(23)		(30)		(30)			(28)		(30)	
10 mM glucose	15·4 (±0·8)	NS	15·2 (±0·9)	NS	17·1 (±1·1)	NS	<0·05	17·7 (±0·8)	NS	16·9 (±0·9)	NS
pc	<0·001		<0·05		<0·001			<0·001		<0·01	
Passive Na^+ efflux rate constant (h^{-1})											
n	(29)		(18)		(18)			(15)		(15)	
10 mM mannitol +ouabain	7·3 (±0·2)	NS	7·6 (±0·2)	NS	7·1 (±0·4)	NS	<0·001	10·0 (±0·5)	<0·01	8·8 (±0·4)	NS

pa = compared with controls fed *ad libitum*.
pb = MTX compared with pair-fed controls.
pc = mannitol compared with glucose.

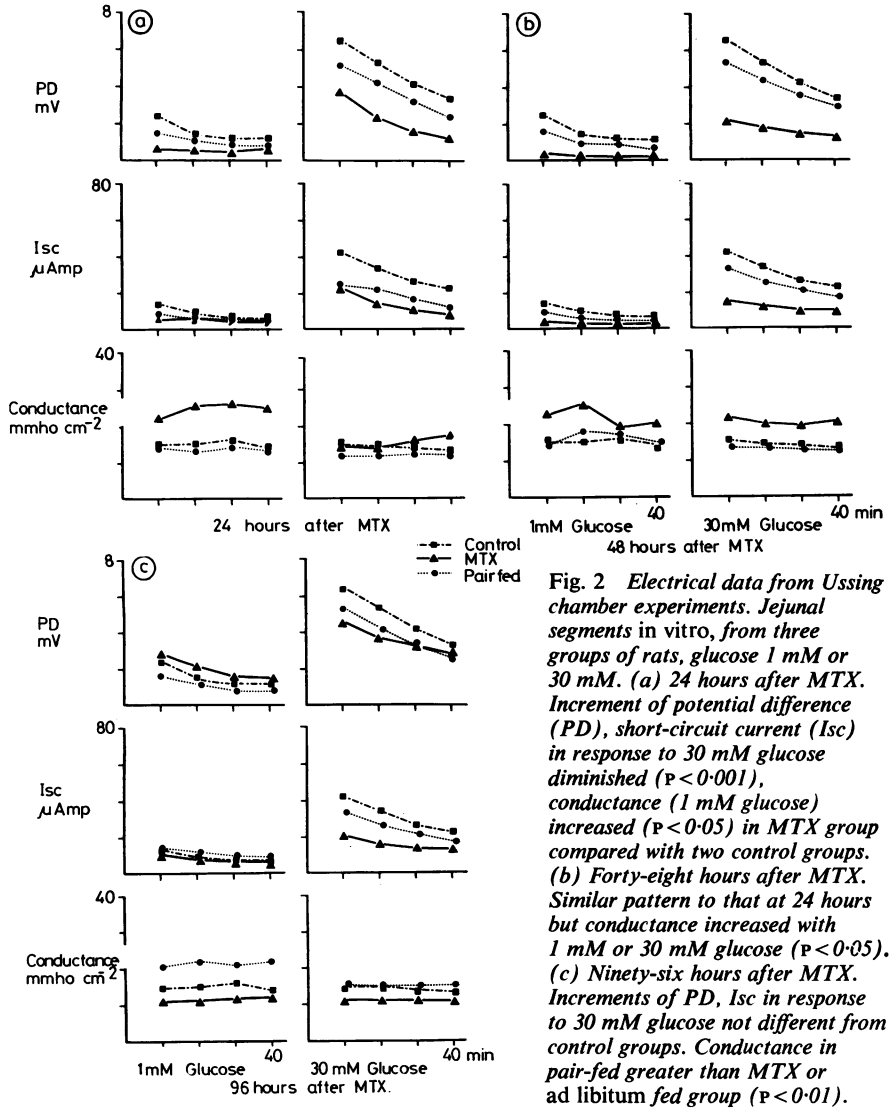

Fig. 2 *Electrical data from Ussing chamber experiments. Jejunal segments* in vitro, *from three groups of rats, glucose 1 mM or 30 mM. (a) 24 hours after MTX. Increment of potential difference (PD), short-circuit current (Isc) in response to 30 mM glucose diminished* ($P < 0.001$), *conductance (1 mM glucose) increased* ($P < 0.05$) *in MTX group compared with two control groups. (b) Forty-eight hours after MTX. Similar pattern to that at 24 hours but conductance increased with 1 mM or 30 mM glucose* ($P < 0.05$). *(c) Ninety-six hours after MTX. Increments of PD, Isc in response to 30 mM glucose not different from control groups. Conductance in pair-fed greater than MTX or* ad libitum *fed group* ($P < 0.01$).

96 h

pa	*MTX*	*pa*	*PF*	*pb*
	(30)		(35)*	
	8·0		14·6	
<0·001	(±0·4)	<0·001	(±0·4)	<0·001
	(27)		(35)	
	15·1		18·2	
NS	(±0·7)	<0·01	(±0·8)	<0·01
	<0·001		<0·001	
	(23)		(21)	
	5·5		7·9	
<0·001	(±0·3)	NS	(±0·3)	<0·001

active enterocyte proliferation. The enzyme profile of cells reaching the villi 96 hours after the drug was one normally seen in crypt cells, not villus cells. Activities of enzymes normally synthesised in enterocytes as they migrate from crypt to villus (disaccharidases, alkaline phosphatase, and Na^+–K^+-APTase) were decreased and the activity of thymidine kinase—normally a crypt cell enzyme—was increased. Evidently cell differentiation did not proceed to completion during this period of rapid cell division and migration. Similar observations have been made on the enzyme activities of enterocytes from piglet jejunum after a very different stimulus to epithelial proliferation, invasive viral infection.[16 17] However, in another important respect, the two models of altered epithelial renewal are very different. In viral enteritis, glucose-stimulated Na

transport, normally a property of differentiated villus cells,[8] is also defective.[9] Glucose-stimulated Na^+ transport, 96 hours after the MTX-induced crypt cell injury, is normal, measured in Ussing chambers and in suspensions of enterocytes isolated from villi, indicating that the glucose-Na^+ carrier, unlike the villus cell enzyme we measured, was adequately synthesised even in these conditions of rapid turnover. The finding of an intact glucose-stimulated Na transport capacity even in the rapidly proliferating and in other respects poorly differentiated epithelium, after MTX, indicates that the control of the differentiation of specific cell components during enterocyte migration is complex. The drug probably had no direct impact during the proliferative phase, as it was not detectable in serum at 96 hours having produced a significant serum concentration at 24 hours.[3] Although the drug had a major impact on the general nutritional status of the rats, our pair-fed control data enable us to exclude malnutrition as a significant determinant of the intestinal abnormalities reported. The anorexia and weight loss documented in the present experiments and noted by others[3 18] do emphasise the importance of evaluating nutritional factors in studying the response of the intestine to this drug.

The authors thank Mrs Mary Perdue, Mrs Angela Chan, Mrs Piya Drew, Mr Mahmood Khan, Mr Bill Wilson, and Mrs Ingrida Leitis for their expert technical assistance, Dr D G Butler for help with the Ussing chamber experiments, and Dr E Cutz for assistance with histological studies, Dr Paul Chello, of the Sloan Kettering Institute, for determining the serum methotrexate levels and Miss Julie Weedmark and Mrs Ene Kopti for secretarial help.

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