Effect of L-glutamine and n-butyrate on the restitution of rat colonic mucosa after acid induced injury

W Scheppach, G Dusel, T Kuhn, C Loges, H Karch, H-P Bartram, F Richter, S U Christl, H Kasper

Abstract

Background—L-glutamine and n-butyrate are important nutrients for colonocytes affecting both their structure and function. The effect of these epithelial substrates on resealing of rat distal colon after acid induced injury was studied.

Methods—Isolated colonic mucosa of 32 rats was mounted in Ussing chambers and exposed to Krebs-Ringer solution for four hours. Epithelial injury was induced by short-term exposure to luminal hydrochloric acid and resealing was studied with or without added glutamine or butyrate.

Results—Glutamine (luminal and serosal) reduced tissue conductance, mannitol and lactulose permeability, and permeation of enteropathogenic *Escherichia coli*. Glutamine (serosal) diminished conductance and mannitol permeability. Both interventions stimulated bromodeoxyuridine incorporation in nuclei of colonocytes. Luminal butyrate had no measurable effect on these parameters.

Conclusions—These data suggest that L-glutamine stimulates repair mechanisms of rat colonic mucosa after acid injury. This effect on the gut barrier is associated with a stimulation of crypt cell proliferation. The addition of glutamine to parenteral solutions may be beneficial for patients under intensive care whose intestinal barrier is weakened in the course of sepsis and trauma.

(Gut 1996; 38: 878-885)

Keywords: glutamine, butyrate, mucosal repair, colon, Ussing chamber.

In the postprandial state, the colonic mucosa receives its nutrients preferentially from the luminal side and not, as previously assumed, from the vascular side.¹ After a few days of oral starvation mucosal atrophy develops,² probably because of the inability of the vasculature to supply fully the energy needs of the epithelium. An alternative explanation for the finding would be a reduced workload of this tissue (reduced absorption and secretion) in the absence of luminal contents.³ The preferred epithelial nutrients are glutamine in the proximal and n-butyrate in the distal colon,⁴ the second of these being produced by bacteria as an end product of carbohydrate fermentation. These substrates provide energy to the

mucosa and, by unknown mechanisms, stimulate cell proliferation within colonic crypts.^{5,6}

Glutamine and butyrate may be important in supporting epithelial repair mechanisms occurring in several forms of inflammatory bowel disease.⁷ Based on this assumption both substrates have been used empirically to treat patients with pouchitis (glutamine)⁸ or ulcerative colitis (butyrate).^{9,10} To study epithelial injury and repair in experimental colitis, several noxious agents have been used including deoxycholate,¹¹ alcohol,¹² formaldehyde,¹³ acetic acid,¹⁴ and hydrochloric acid.¹⁵ While these models do not reflect all aspects of human colonic disease, they do provide information about mechanisms whereby the colonic epithelium can recover from injury.

When isolated colonic mucosa placed in the Ussing chamber is incubated with luminal HCl the superficial epithelium is desquamated whereas the crypts are left intact. Electron-microscopic studies show that epithelial resealing occurs within several hours, probably as a result of cell migration from the crypts to the flat luminal mucosa.¹⁵ In this study the hypothesis was tested that colonic nutrients (glutamine, butyrate) may accelerate recovery after acid induced injury.

Methods

Preparation of distal colon

Male Wistar rats (250-300 g, Wiga, Sulzfeld, Germany) were fed standard rat chow and water ad libitum before the experiments. Rats were killed by cervical dislocation and, after midline incision, the colon was removed and rinsed clear of its luminal contents with ice cold saline. Before use, tissues were maintained in ice cold saline bubbled with carbogen gas (95% oxygen, 5% carbon dioxide). Within 15 minutes, the serosal and muscular layers were removed by placing the sheet of distal colon, serosal side up, on a rubber plate moistened with ice cold saline. A transverse incision was made with a razor blade through the muscular layer, and the outer layers were gently removed from the mucosa/submucosa with a fine curved forceps. Histological examination showed that a pure mucosal/submucosal preparation of rat colon was obtained (Fig 1).¹⁶⁻¹⁸

Ussing chambers and solutions

Pieces of the mucosa/submucosa preparation were mounted in Ussing chambers exposing

Departments of Medicine

W Scheppach
G Dusel
T Kuhn
C Loges
H-P Bartram
F Richter
S U Christl
H Kasper

and Microbiology

H Karch

University of Wurzburg, Wurzburg, Germany

Correspondence to:
Dr W Scheppach,
Department of Medicine,
University of Wurzburg,
Josef-Schneider-Strasse 2,
D-97080 Wurzburg,
Germany.

Accepted for publication
28 December 1995

0.785 cm² of tissue surface area to 4 ml of appropriate solutions. Control and experimental tissues were always obtained from the same animal and incubated simultaneously. The following solution was used on the serosal and luminal sides of the mucosa: Na⁺ 145 mmol/l, K⁺ 5 mmol/l, Ca⁺⁺ 1.2 mmol/l, Mg⁺⁺ 1.2 mmol/l, Cl⁻ 124.8 mmol/l, HCO₃⁻ 25 mmol/l, PO₄⁻ 4.2 mmol/l. On the luminal side, mannitol (5 mmol/l) and lactulose (5 mmol/l) were added to the electrolyte solution for permeability measurements; this osmotic load to the luminal side was balanced by adding xylose (10 mmol/l) to the serosal side. The pH value of both solutions was 7.55. The chambers were circulated by carbogen gas lift and kept at 37°C in water-jacketed reservoirs. The chemicals were purchased from Sigma (St Louis, MO). It has been shown previously¹⁵ that stripped colonic mucosa bathed in a nutrient free solution can be kept viable for five hours.

Electrical measurements

Transepithelial potential difference (PD) was measured using matched calomel reference electrodes (Ref 401, Radiometer, Copenhagen, Denmark) connected to a high impedance potentiometer (Qualitron, Haarlem, the Netherlands) and immersed in saturated KCl; the electrical apparatus was connected to the chambers through salt-agar bridges. Every five minutes, 5 uA current pulses were applied to chambers through platinum electrodes. The change in PD was recorded and the tissue conductance (mS/cm²) calculated by Ohm's law. Parallel experiments were considered comparable if the conductance of paired tissues differed by less than 25% in the initial equilibration period.

When a stable tissue conductance had been established (usually within 30 minutes), chemical injury was induced by replacing the luminal solution with HCl (15 mmol/l) for five minutes. As shown by other authors, this intervention leads to reversible tissue damage (loss of the superficial epithelium, resealing by cell migration from the colonic crypts).¹⁵ Afterwards, fresh incubation medium (composition as already described) was filled in the chambers and PD/conductance were measured for four hours after tissue injury. The media bathing the serosal and mucosal sides of the mucosa were changed every hour and aliquots frozen at -20°C for subsequent analysis of mannitol, lactulose, and xylose (within three months).

Interventions

In the initial validation experiment, the effect of tissue injury was assessed by adding HCl (15 mmol/l, five minutes) to the luminal side of one Ussing chamber and inducing no injury to the other chamber (n=6). Neither butyrate nor glutamine were used in this experiment.

In further experimental series, chemical injury was induced to both chambers, and the effect of colonic nutrients (glutamine,

butyrate) on mucosal resealing was compared with control conditions (equimolar NaCl added instead of glutamine/butyrate):

(1) L-glutamine (2 mmol/l), added to the serosal and luminal sides after tissue injury with HCl (n=6).

(2) L-glutamine (2 mmol/l), added only to the serosal side after tissue injury with HCl (n=7).

(3) n-butyrate (10 mmol/l), added to the luminal side after tissue injury with HCl (n=6).

(4) n-butyrate (10 mmol/l), added to the luminal side before and after tissue injury with HCl (preincubation period of one hour) (n=7).

Analysis of mannitol and lactulose

The tissue permeability for mannitol/lactulose (luminal to serosal side) was assessed by measuring hourly the concentrations in the incubation media by gas-liquid chromatography.¹⁹ To 200 µl of the incubation medium (contained in disposable injection vials) 250 nmol phenyl-β-D-glucopyranoside was pipetted as internal standard and taken to dryness under nitrogen in a heating block at 75°C. Subsequently, 100 µl oxime solution (25 mg of hydroxylamine hydrochloride per ml of pyridine) was added and the glass vial capped; the sugars were converted to oximes during the following incubation period of 30 minutes at 75°C. After allowing the samples to cool, 100 µl of n-trimethylsilyl imidazole reagent was added and the solution incubated for 15 minutes at 75°C. The silylation reagent was bought from Macherey-Nagel (Düren, Germany) and all other chemicals from Sigma (St Louis, MO).

A stock solution containing mannitol (5 mmol/l) and lactulose (5 mmol/l) was prepared in deionised water. From this solution, 0/10/25/50 µl were pipetted into glass injection vials to which 250 nmol internal standard were added. The conversion to oximes and subsequent silylation was performed as described before. Thus, a standard curve was set up under the same conditions as the unknown samples were analysed.

One µl of derivatised sample was injected into a DB-5 capillary column (15 m×0.53 mm ID, J and W Scientific, Folsom, CA) installed on a HP 5890 A gas chromatograph (Hewlett-Packard, Palo Alto, CA). The chromatographic conditions were as follows:

(1) Gas: helium at a column flow rate of 5 ml/min, make up gas 25 ml/min, splitless injection.

(2) Temperatures: injection port 220°C, flame ionisation detector 300°C, oven temperature programme: 150°C for 0 min, ramp 15°C/min to 300°C (kept for 5 min).

The computerised data analysis made use of an HP 3365 Chemstation (Hewlett-Packard, Palo Alto, CA).

Bacterial strains and bacterial tissue permeation

The *E coli* strain 11-1 (serotype O111:H⁻) was

used for the permeation studies. This strain is resistant to ampicillin and shows localised adherence to HEp-2 cells.²⁰ In addition, strain 11-1 possesses the 90 Kb virulence plasmid and does not produce verotoxins or heat labile or heat stable enterotoxins.²⁰

E. coli O111 cells were grown overnight in Luria broth and centrifuged. The pellet was suspended in the electrolyte solution used for Ussing chamber experiments (composition as before) to obtain 2×10^7 colony forming units (Cfu) per ml. This suspension was added to the luminal half chambers in the fourth hour after tissue injury. The permeation across the mucosal sheet was assessed by measuring the density in the serosal half chambers after 60 minute exposure. From 1 ml of this suspension 100 μ l underwent serial 10-fold dilution (10^5 to 10^2) and were spread on MacConkey agar plates containing 100 μ g/ml ampicillin. Colonies were serogrouped by slide agglutination test with *E. coli* O111 anti-serum (Behringwerke, Marburg, Germany) and by polymerase chain reaction as described.²⁰

Colonic crypt proliferation

In the fourth hour after tissue injury 0.8 μ M bromodeoxyuridine (BrdU) was added both to the luminal and serosal side of the tissue to label proliferating cells within colonic crypts immunohistochemically. Details of this method have been described previously.^{6, 21} In brief, mucosal sheets were fixed in ethanol (95%, v/v) for 12 hours and embedded in Paraplast (Monoject Scientific, Athy, Ireland). The specimens were section cut into 2 μ m slices using a Leitz microtome (Leitz, Wetzlar, Germany). Denaturation of DNA was achieved by incubation (30 min) with 2 N HCl. Mouse anti-BrdUrd monoclonal antibody (no 7580, Becton-Dickinson, San Jose, CA) was applied (1:100 dilution, one hour incubation), followed by biotinylated anti-mouse immunoglobulin (1:100 dilution, 30 minute incubation, RPN 1001, Amersham, Buckinghamshire, UK) as second antibody. After 30 minute incubation with biotinylated streptavidin (1:100 dilution, RPN 1051, Amersham), BrdUrd-labelled cells were visualised using diaminobenzidine solution (0.5 g/l, Serva, Heidelberg, Germany) with 0.15 g/l NiCl_2 and 0.15 g/l CoCl_2 as intensifiers. All reactions were performed at room temperature unless otherwise specified. Finally, the specimens were counterstained with nuclear fast red.

The histological slides were viewed under a Laborlux S microscope (Leitz) at a 625-fold magnification. In every case, proliferation of colonic crypt cells was evaluated by counting the number of BrdUrd-labelled cells and the total number of cells in 40 longitudinally sectioned crypt columns according to the criteria described by Lipkin *et al.*²² An average labelling index (LI) per individual crypt was calculated from the number of labelled cells divided by the total number of cells.

Statistics

Values are given as mean (SEM). Areas under curve (AUC) were calculated using the trapezoidal rule. Significant differences ($p < 0.05$) between interventions were calculated by Wilcoxon's rank sum test for paired data. The statistical software package NCSS (Unisoft, Augsburg, Germany) was used for data analysis.

Results

Validation experiment (n=6)

The model of acid induced injury of stripped rat mucosa maintained in Ussing chambers has been used by Argenzio *et al.*^{11, 14} and Feil *et al.*¹⁵ Based on this previous work we confirmed that a fresh preparation of distal rat mucosa/submucosa stripped of the underlying muscular layer can be kept viable for five hours (histological appearance, maintenance of potential difference, and conductance).

Histological tissue damage was most noticeable one hour after luminal acid exposure (Fig 1A): luminal HCl (15 mmol/l, 5 min) caused uniform mucosal damage characterised by exfoliation of the superficial epithelial layer and denudation of the lamina propria. In contrast, the crypts did not show any histological evidence of damage. Tissue resealing was seen four hours after acid exposure, probably as a result of cell migration from the intact crypts (Fig 1B).

When rat distal colonic mucosa was incubated with HCl in chamber A while no acid injury was induced in chamber B the following data were obtained: after HCl injury the potential difference (PD) dropped significantly

Figure 1: Histological appearance of stripped mucosa after luminal HCl exposure (magnification, $\times 300$). (A) Thirty minutes after injury. Desquamation of the superficial epithelial layer and denudation of the lamina propria; crypt structure intact; S-phase labelling with BrdU (dark cells). (B) Four hours after injury. Epithelial resealing occurring in the flat mucosa; S-phase labelling with BrdU (dark cells).

($p < 0.05$) from 4.4 (0.8) mV to 1.2 (0.2) mV (1 hour) and rose gradually to 2.7 (0.8) mV (4 hours). In the control experiment no significant decrease of PD was obtained. PD was significantly ($p < 0.05$) lower one and two hours after HCl administration than under control conditions.

The conductivity (C) rose significantly ($p < 0.05$) from 8.4 (0.8) to 19.3 (4.5) mS/cm² (1 hour) after HCl, followed by a decline to

14.5 (5.1) mS/cm². In the control run, no increase in C was noted (mean values between 7.0 and 8.1 mS/cm²). C showed a significant ($p < 0.03$) difference between HCl and control at one, two, and three hours.

Markers of mucosal permeability (mannitol, lactulose) were added to the mucosal chamber (5 mmol/l each) and the serosal concentration measured hourly. At one and two hours, the serosal mannitol concentrations were significantly ($p < 0.05$) higher after HCl administration than in the control run (one hour: 22.3 (2.5) v 11.0 (1.4) μ mol/l; two hours: 23.1 (3.0) v 16.2 (2.0) μ mol/l). The same difference between HCl and control was obtained for lactulose (one hour: 12.9 (1.8) v 5.5 (1.1) μ mol/l; two hours: 14.4 (4.2) v 6.8 (0.8) μ mol/l, $p < 0.05$).

In the fourth hour of incubation, the permeation of the enteropathogenic strain 11-1 of *E. coli* O111:H⁻ (EPEC) from the luminal (2×10^7 cfu/ml) to the serosal chamber was assessed. The serosal EPEC density was significantly higher with HCl induced injury (13675 (5479) cfu/ml) than without HCl (1258 (668) cfu/ml, $p < 0.05$).

Cell proliferation within colonic crypts was unaffected by HCl administration: the total crypt labelling index (LI) after HCl (0.16 (0.02)) was not different from LI under control conditions (0.14 (0.01), NS). This provides further evidence of the superficial nature of acid induced mucosal damage.

It was concluded from these data that HCl at the appropriate concentration and exposure time caused reversible injury to the distal colon of rat. A potential modifying effect of colonic epithelial nutrients (L-glutamine, n-butyrate) was investigated in the following experiments.

Glutamine experiments

The effects of L-glutamine (2 mmol/l) added to the serosal and mucosal side ($n=6$) are summarised in Figure 2A and Table 1A. A trend (p values given in Figures and Tables) towards a higher PD in the presence of L-glutamine was found, compared with control conditions (Table 1A). As the conductance is considered a valuable marker for epithelial integrity²³ these data are presented in detail (Fig 2A). As a sign of mucosal leakage tissue conductance increased significantly in the control experiment, reaching its highest values two hours after HCl incubation (216% of the initial value at 0 hours). Thereafter conductance values declined to 120% of the initial value, indicating mucosal resealing. In the L-glutamine experiment a significant rise of conductance did not occur; it differed significantly between L-glutamine and control at one, two, and three hours. L-glutamine (serosal+mucosal) also caused a diminished permeability for mannitol and lactulose (Table 1A). The permeation of EPEC at four hours was significantly decreased in the presence of L-glutamine. L-glutamine stimulated mucosal cell proliferation, raising significantly the total crypt labelling index in the fourth hour of incubation.

Figure 2: Effect of L-glutamine on tissue conductance after HCl exposure (zero hour denotes the time after removal of HCl but before addition of glutamine). (A) L-glutamine (2 mmol/l) added to the serosal and mucosal sides of the mucosa. (B) L-glutamine (2 mmol/l) added to the serosal side of the mucosa. (Filled symbols: L-glutamine incubation, open symbols: control experiment; % iv = per cent of initial value at 0 hours; * shows significant differences with $p < 0.05$).

TABLE 1 Effect of L-glutamine on colonic mucosa after HCl induced injury

	Glutamine	NaCl (control)	p Value
(A) Effect of L-glutamine (2 mmol/l) ($n=6$) added to the serosal and mucosal sides of the mucosa			
Potential difference (AUC: % iv $\times$ h)	278.8 $\pm$ 88.6	194.5 $\pm$ 46.8	0.110
Conductance (AUC: % iv $\times$ h)	420.4 $\pm$ 81.9	642.6 $\pm$ 101.5	0.028
Mannitol _s (AUC: μ mol/l $\times$ h)	42.5 $\pm$ 8.7	146.5 $\pm$ 33.2	0.028
Lactulose _s (AUC: μ mol/l $\times$ h)	35.8 $\pm$ 5.1	87.0 $\pm$ 19.5	0.028
EPEC _s (cfu/ml) 4 h	2022 $\pm$ 1029	33700 $\pm$ 13026	0.028
Mucosal cell proliferation: total crypt labelling index 4 h	0.19 $\pm$ 0.01	0.14 $\pm$ 0.01	0.043
(B) Effect of L-glutamine (2 mmol/l) ($n=7$) added to the serosal side of the mucosa			
Potential difference (AUC: % iv $\times$ h)	250.9 $\pm$ 32.0	134.0 $\pm$ 18.6	0.028
Conductance (AUC: % iv $\times$ h)	462.3 $\pm$ 18.5	569.0 $\pm$ 35.0	0.028
Mannitol _s (AUC: μ mol/l $\times$ h)	154.8 $\pm$ 19.7	238.8 $\pm$ 22.3	0.046
Lactulose _s (AUC: μ mol/l $\times$ h)	83.6 $\pm$ 21.1	109.9 $\pm$ 20.3	NS
EPEC _s (cfu/ml) 4 h	12646 $\pm$ 8383	71743 $\pm$ 59976	NS
Mucosal cell proliferation: total crypt labelling index 4 h	0.15 $\pm$ 0.01	0.10 $\pm$ 0.01	0.036

% iv = per cent of initial value at 0 hours; AUC = area under curve; EPEC_s = enteropathogenic *E. coli* recovered on the serosal side of the mucosa; cfu = colony forming unit; NS = not significant.

Similar data were obtained when L-glutamine (2 mmol/l) was added only to the serosal side ($n=7$) of the chamber (Fig 2B, Table IB). The PD was significantly higher in the presence of L-glutamine than in its absence (Table IB). The conductance (Fig 2B) increased significantly in the control experiment at one hour and remained increased throughout. Also in the

L-glutamine run, conductance rose significantly at one hour, but subsequently returned to the range of the initial value (104–118%, NS). A significant difference between L-glutamine and control experiment was found at two, three, and four hours. L-glutamine diminished the permeability for mannitol, whereas no significant difference was seen for lactulose (Table IB). EPEC permeation was not different between L-glutamine and control run. Serosal L-glutamine raised the total crypt labelling index significantly.

Figure 3: Effect of *n*-butyrate on tissue conductance after HCl exposure (zero hour denotes the time after removal of HCl but before addition of butyrate). (A) Butyrate (20 mmol/l) added to the luminal side of the mucosa without a preincubation period. (B) Butyrate (20 mmol/l) added to the luminal side of the mucosa with a preincubation period (one hour before HCl exposure and four hours thereafter). (Filled symbols: butyrate incubation, open symbols: control experiment; % iv = per cent of initial value at 0 hours).

TABLE II Effect of *n*-butyrate on colonic mucosa after HCl induced injury

	Butyrate	NaCl (control)	p Value
(A) Effect of butyrate (20 mmol/l) ($n=6$) added to the luminal side of the mucosa without a preincubation period			
Potential difference (AUC: % iv $\times$ h)	250.8 $\pm$ 67.8	321.0 $\pm$ 85.7	NS
Conductance (AUC: % iv $\times$ h)	535.3 $\pm$ 41.7	495.2 $\pm$ 15.6	NS
Mannitol _s (AUC: μ mol/l $\times$ h)	204.8 $\pm$ 32.1	177.3 $\pm$ 35.3	NS
Lactulose _s (AUC: μ mol/l $\times$ h)	277.5 $\pm$ 114.2	162.0 $\pm$ 35.4	NS
EPEC _s (cfu/ml) 4 h	3234 $\pm$ 2954	3563 $\pm$ 1173	NS
Mucosal cell proliferation: total crypt labelling index 4 h	0.13 $\pm$ 0.01	0.11 $\pm$ 0.01	NS
(B) Effect of butyrate (20 mmol/l) ($n=7$) added to the luminal side of the mucosa with a preincubation period (one hour before HCl exposure and four hours thereafter)			
Potential difference (AUC: % iv $\times$ h)	249.6 $\pm$ 65.2	282.0 $\pm$ 49.6	NS
Conductance (AUC: % iv $\times$ h)	497.2 $\pm$ 79.3	532.1 $\pm$ 89.1	NS
Mannitol _s (AUC: μ mol/l $\times$ h)	67.4 $\pm$ 5.6	84.3 $\pm$ 14.4	NS
Lactulose _s (AUC: μ mol/l $\times$ h)	64.1 $\pm$ 11.9	73.2 $\pm$ 17.8	0.028
EPEC _s (cfu/ml) 4 h	9200 $\pm$ 3789	25 857 $\pm$ 19 211	NS
Mucosal cell proliferation: total crypt labelling index 4 h	0.15 $\pm$ 0.003	0.16 $\pm$ 0.01	NS

Abbreviations as in Table I.

Butyrate experiments

The data of butyrate experiments (no preincubation with butyrate before HCl incubation, $n=6$) are given in Table IIA and Figure 3A. Butyrate added to the luminal side after acid exposure did not affect PD or tissue conductance. The permeability of mannitol and lactulose was similar in butyrate and control experiments. EPEC permeation did not differ between butyrate and control. In this experimental setting, butyrate had no effect on mucosal cell proliferation.

Following on from experimental data of Loucks and Buell¹² the hypothesis was tested that acid induced tissue damage might be avoided if the mucosa was pretreated with luminal butyrate ($n=7$, Table IIB, Fig 3B). However, PD and conductance were unchanged by butyrate incubation of the mucosa. Epithelial permeability for saccharides was not different between serum and control experiments. EPEC permeation was unaffected by luminal butyrate. No significant differences were detected for epithelial cell proliferation.

Discussion

In this section two issues will be discussed: (a) Is the Ussing chamber technique adequate to study the recovery of colonic mucosa after acid induced injury? (b) What are the implications of the positive glutamine and the negative butyrate data?

Experimental injury and restitution of the colonic mucosa

The technique of mounting stripped colonic mucosa in Ussing chambers and measuring post-injury epithelial recovery has been used previously. Feil *et al*¹⁵ incubated human colonic specimens obtained at surgery with hydrochloric acid (10 mmol/l for 10 min) and observed a 70% drop of PD, which remained low despite morphological restitution of the epithelium. Tissue conductance was not measured in this experiment. Light microscopy of histological sections showed uniform mucosal damage confined to the superficial layer with the crypts left intact (15 minutes post-injury). After two hours, incipient epithelial restitution was noted by light microscopy. After five hours the necrotic layer had lifted off and the denuded basal lamina was covered with flattened epithelial cells that had migrated from the intact crypts. Electron microscopic

analysis showed that non-goblet cells projected lamellipodia and migrated over the denuded basal lamina at a speed of 2 $\mu\text{m}/\text{min}$. Morphometrically, 96% of the surface was judged necrotic, whereas after five hours only 17% of the mucosa remained damaged. In our validation experiment a significant drop of PD was observed and an increase in tissue conductance in the first two hours after injury. Generally, mucosal damage was reflected by a more pronounced change of conductance than PD. Using light microscopy, we found extensive superficial damage one hour and resealing of the mucosa four hours after HCl (Fig 1A/B). We therefore judged that we could assess possible effects of gut nutrients on epithelial restitution within a time frame of four hours.

Mannitol (diameter: 6.7×10^{-10} m) and lactulose (diameter: 9.5×10^{-10} m) are well established markers of intestinal para-cellular permeability.²⁴ Compared with the small intestine, colonic epithelium is considered to be much tighter, having higher epithelial resistance and lower permeability. However, it has recently been shown that the rat colon is permeable even to inulin, a molecule with a diameter of 15×10^{-10} m.²⁵ Sugars like mannitol and lactulose are, therefore, also suitable markers for colonic permeability measurements. Our validation experiment showed a significantly higher permeability for both markers one and two hours after HCl, compared with control conditions. No difference between mannitol and lactulose was detected. This may be explained by the gross superficial damage cause by HCl, which does not select for pore size. Similar data were obtained by Argenzio *et al*¹¹ who perfused the porcine colon in vivo with deoxycholate (15 mmol/l) causing acute mucosal injury similar to that seen in our experiment. This intervention raised the mannitol permeability significantly; however, within 40 minutes mannitol permeability was normalised because of re-epithelialisation with flattened cells migrating from the crypts to the flat mucosa.

Bacterial 'translocation' is defined as invasion of bacteria from the gut lumen to mainly sterile body compartments (liver, spleen, mesenteric lymph nodes). This phenomenon has been seen in animal experiments^{26, 27} but may also contribute to human perioperative morbidity.²⁸ In addition to absorptive and secretory functions the maintenance of the barrier against bacterial entry is an important role of the colonic mucosa. The interaction of enteropathogenic *E coli* O111:H⁻ with rabbit intestinal mucosa has recently been shown in vitro.²⁹ Factors that re-establish the morphological and functional integrity of the epithelium may also contribute to the preservation of the barrier function. In our Ussing chamber study, bacterial 'translocation' was not measured in the strict sense of the definition given above; instead, the term 'permeation' was used. It could be shown in the validation experiment that bacterial permeation from the luminal to the serosal side increased significantly after HCl induced injury, compared with control conditions. Therefore, bacterial

permeation was used as another marker of mucosal damage possibly affected by gut nutrients.

As Figure 1A shows, acid induced injury affects the epithelial surface while it leaves the colonic crypts intact. Cell renewal takes its origin within the crypt where proliferation occurs in the basal compartments. Cells migrate to the upper crypt where they stop proliferating and achieve a state of full differentiation. After a life span of about a week, senescent cells become apoptotic and are shed to the lumen.³⁰ This process is not disturbed by acid induced injury; the validation experiment of our study showed that luminal HCl had no effect on cell proliferation in the crypts. It was hypothesised that gut nutrients stimulate cell proliferation, which may be associated with epithelial restitution after injury.

Effects of gut nutrients (glutamine, butyrate)

L-glutamine is among the most abundant free amino acids in the body and controls many pathways of intermediary metabolism. Windmueller and Spaeth³¹ suggested that glutamine delivered a major portion of the energy required by enterocytes. In colonocytes, similar findings were reported by Ardawi and Newsholme.³² By an unknown signal transduction pathway, glutamine also stimulates DNA synthesis of epithelial cells taken from the human ileum, proximal colon, and distal colon.⁶ Assuming an important role of glutamine for the welfare of the colonic mucosa, we studied effects of this amino acid on epithelial recovery after acid induced injury.

As glutamine is readily taken up both by the apical and basolateral membranes³³ a maximum effect was attempted by adding glutamine to the luminal and serosal sides of the mucosa (Table IA). Glutamine delivered to both sides of the mucosa stimulated DNA synthesis as it was anticipated from previous incubation experiments of human biopsy material.⁶ This proliferative effect was associated with increased functional integrity of the mucosa (conductance, saccharide permeability, EPEC permeation). It is unknown if there is a causal relation between cell proliferation and resealing of the mucosa. Cell migration has been proposed as an alternative mechanism for epithelial restitution; this phenomenon may also be affected by glutamine via unknown pathways.

The data for serosal glutamine (Table IB) were not as clear as for serosal/mucosal glutamine. However, most of the functional parameters were affected (PD, conductance, mannitol permeability). Significant differences were missed for lactulose permeability and EPEC permeation, possibly because of the small sample size. Serosal glutamine had a trophic effect on epithelial cells, as shown by a significantly higher total crypt labelling index. In animal experiments (in vivo) intravenous glutamine has been shown to favour epithelial healing after various forms of injury.^{34, 35} If these findings can be reproduced in the clinical setting, glutamine administration may become

important for patients in the intensive care unit.³⁶ The drawback of free glutamine being unstable during heat sterilisation and storage can be overcome by using the stable and highly soluble dipeptide L-alanyl-L-glutamine. At present, such dipeptide solutions are being marketed.

Under the experimental conditions of this study, luminal butyrate did not affect resealing of the mucosa after acid induced injury. The finding of a lower lactulose permeability in the absence of other significant data (Table IIB) is considered a chance finding. Similarly, negative data were obtained when butyrate was added to the luminal chamber only after acid exposure (no preincubation period) or before and after HCl exposure (butyrate preincubation). This last experiment was performed as Loucks and Buell¹¹ have described a protective effect of butyrate when a preincubation was performed before exposure to a 'barrier breaker' (ethanol). In their study the clearance of ⁵¹Cr-EDTA from blood to lumen was assessed as a marker of microvascular and epithelial permeability, which was significantly reduced in the presence of luminal butyrate (20 mmol/l). It cannot be excluded that the choice of the agent that causes epithelial damage (HCl *v* ethanol) has an impact on results. When monolayers of CaCo-2 colon carcinoma cells were studied³⁷ butyrate (2 mmol/l) reduced paracellular permeability (increased transepithelial resistance, and reduced mannitol flux). Butyrate also improved wound healing of IEC-6 enterocyte monolayers after injury with a razor blade.³⁸ In a rat model, infusion of short chain fatty acids into the proximal colon accelerated the healing of distal colonic anastomosis.³⁹ In view of these data, our negative results are disappointing. However, there is another negative study in which effects of butyrate were tested in the Ussing chamber.⁴⁰ Mucosa taken from distal rat colon was exposed to luminal deoxycholate until the electrical resistance fell by 50%. Luminal butyrate (25 mmol/l) did not influence epithelial recovery (PD, resistance) monitored for five hours after injury. This experimental set up was closest to our procedure and yielded similar data.

In our previous experiments⁵ butyrate stimulated mucosal proliferation in biopsy specimens taken from the human caecum. Other authors^{41,42} have shown this effect in the rat colon (in vivo). It was surprising that butyrate had no effect on DNA synthesis of isolated mucosa placed in Ussing chambers. An explanation for this discrepancy may be that a cell culture medium (containing fetal calf serum) was used in the biopsy studies,⁵ whereas an electrolyte solution was used in the Ussing chamber experiments. It may be that butyrate can stimulate mucosal DNA synthesis only in the presence of a nitrogen source. Glutamine, in contrast, carries two N atoms itself. In addition to providing N, a cell culture medium contains a range of growth factors⁴³ whose presence may be necessary before butyrate can induce cell proliferation.

The pH value of the bathing solutions (luminal and serosal) was 7.55 in this experiment. It

is known that the colonic luminal pH may be as low as 5.5 when there is active fermentation (proximal colon).⁴⁴ It can be speculated that the action of butyrate would have been different in a more acid environment. The amount of butyrate in an un-ionised form may be critical to its action in the colon (pK value of butyrate 4.81).

In conclusion, stripped colonic mucosa placed in Ussing chambers can be used to study epithelial injury and repair in vitro. Under the conditions chosen in this study, glutamine did and butyrate did not affect mucosal resealing after HCl induced injury. The molecular mechanisms underlying the effects of luminal nutrients are unknown. However, it has recently been shown in IEC-6 cells that glutamine activates mitogen activated protein kinases, c-Jun nuclear kinases, and activating protein-1 dependent gene transcription; it also increases the effect of epidermal and insulin-like growth factors on DNA synthesis and may thereby facilitate intestinal repair.⁴⁵

We thank H DeJonge, PhD (Rotterdam) and G Rechkemmer, PhD (Hanover) for valuable advice concerning the Ussing chamber technique. The expert technical assistance of B Plaschke, A Weimer, and E Liebscher is gratefully acknowledged. This work was presented in abstract form (*Gastroenterology* 1995; 108: A752) at the 95th Annual Meeting of the American Gastroenterological Association, San Diego, 17 May 1995.

- 1 Roediger WEW. Metabolic basis of starvation diarrhoea: implications for treatment. *Lancet* 1986; i: 1082-4.
- 2 Janne P, Carpentier Y, Willems G. Colonic mucosal atrophy induced by a liquid elemental diet in rats. *Dig Dis* 1977; 22: 808-12.
- 3 Maxton DG, Cynk EU, Thompson RPH. Small intestinal response to 'elemental' and 'complete' liquid feeds in the rat: effect of dietary bulk. *Gut* 1987; 28: 688-93.
- 4 Roediger WEW. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa. *Gut* 1980; 21: 793-8.
- 5 Scheppach W, Bartram P, Richter A, Richter F, Liepold H, Dusel G, et al. Effect of short-chain fatty acids on the human colonic mucosal in vitro. *J Parenter Enteral Nutr* 1992; 16: 43-8.
- 6 Scheppach W, Loges C, Bartram P, Christl SU, Richter F, Dusel G, et al. Effect of free glutamine and alanyl-glutamine dipeptide on mucosal proliferation of the human ileum and colon. *Gastroenterology* 1994; 107: 429-34.
- 7 Roediger WEW. The starved colon - diminished mucosal nutrition, diminished absorption, and colitis. *Dis Colon Rectum* 1990; 33: 858-62.
- 8 Wischmeyer P, Pemberton JH, Phillips SF. Chronic pouchitis after ileal pouch-anal anastomosis: responses to butyrate and glutamine suppositories in a pilot study. *Mayo Clin Proc* 1993; 68: 978-81.
- 9 Steinhart AH, Brzezinski A, Baker JP. Treatment of refractory ulcerative proctosigmoiditis with butyrate enemas. *Am J Gastroenterol* 1994; 89: 179-83.
- 10 Scheppach W, Sommer H, Kirchner T, Paganelli G-M, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* 1992; 103: 51-6.
- 11 Argenzio RA, Henrikson CK, Liacos JA. Restitution of barrier and transport function of porcine colon after acute mucosal injury. *Am J Physiol* 1988; 255: G62-71.
- 12 Loucks DC, Buell MG. The differing protective effect of short chain fatty acids on ethanol-induced colonic mucosal injury. *Gastroenterology* 1994; 106: A1036.
- 13 Cohen JD, Kao HW, Tan ST, Lechago J, Snape WJ. Effect of acute experimental colitis on rabbit colonic smooth muscle. *Am J Physiol* 1986; 251: G538-45.
- 14 Argenzio RA, Meuten DJ. Short-chain fatty acids induce reversible injury of porcine colon. *Dig Dis Sci* 1991; 36: 1459-68.
- 15 Feil W, Lacy ER, Wong YM, Burger D, Wenzl E, Starlinger M, et al. Rapid epithelial restitution of human and rabbit colonic mucosa. *Gastroenterology* 1989; 97: 685-701.
- 16 Engelhardt W, Burmester M, Hansen K, Becker G, Rechkemmer G. Effects of amiloride and ouabain on short-chain fatty acid transport in guinea-pig large intestine. *J Physiol* 1993; 460: 455-66.
- 17 Field M, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. *Am J Physiol* 1971; 220: 1388-96.
- 18 O'Grady SM, DeJonge HR, Vaandrager AB, Field M. Cyclic nucleotide-dependent protein kinase inhibition by H-8: effects on ion transport. *Am J Physiol* 1988; 254: C115-21.
- 19 Shippee RL, Johnson AA, Cioffi WG, Lasko J, LeVoyer T, Jordan BS. Simultaneous determination of lactulose and mannitol in urine of burn patients by gas-liquid chromatography. *Clin Chem* 1992; 38: 343-5.

- 20 Franke J, Franke S, Schmidt H, Schwarzkopf A, Wieler LH, Baljer G, *et al.* Nucleotide sequence analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor probe and development of PCR for rapid detection of EPEC harboring virulence plasmids. *J Clin Microbiol* 1994; **32**: 2460–3.
- 21 Bartram H-P, Scheppach W, Schmid H, Hofmann A, Dusel G, Richter F, *et al.* Proliferation of human colonic mucosa as an intermediate biomarker of carcinogenesis: effects of butyrate, deoxycholate, calcium, ammonia, and pH. *Cancer Res* 1993; **53**: 3283–8.
- 22 Lipkin M, Blattner WE, Fraumeni JF, Lynch HT, Deschner E, Winawer S. Tritiated thymidine (^3H) labeling distribution as a marker for hereditary predisposition to colon cancer. *Cancer Res* 1983; **43**: 1899–904.
- 23 Moore R, Madri J, Carson S, Madara JL. Collagens facilitate epithelial migration in restitution of native guinea pig intestinal epithelium. *Gastroenterology* 1992; **102**: 119–30.
- 24 Bjarnason I, MacPherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995; **108**: 1566–81.
- 25 Ma TY, Hollander D, Erickson RA, Truong H, Nguyen H, Krugliak P. Mechanism of colonic permeation of inulin: is rat colon more permeable than small intestine? *Gastroenterology* 1995; **108**: 12–20.
- 26 Deitch EA, Winterton J, Berg R. The gut as a portal of entry for bacteremia. *Ann Surg* 1987; **205**: 681–92.
- 27 Wilmore DW, Smith RJ, O'Dwyer ST, Jacobs DO, Ziegler TR, Wang X-D. The gut: a central organ after surgical stress. *Surgery* 1988; **104**: 917–23.
- 28 Sedman PC, Macfie J, Sagar P, Mitchell CJ, May J, Mancey-Jones B, *et al.* The prevalence of gut translocation in humans. *Gastroenterology* 1994; **107**: 643–9.
- 29 Embaye H, Batt RM, Saunders JR, Getty B, Hart CA. Interaction of enteropathogenic *Escherichia coli* O111 with rabbit intestinal mucosa in vitro. *Gastroenterology* 1989; **96**: 1079–86.
- 30 Wright N. *The biology of epithelial cell populations*. Vol 2. Oxford: Clarendon, 1984.
- 31 Windmueller HG, Spaeth AE. Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for postabsorptive rat small intestine. *J Biol Chem* 1978; **253**: 69–76.
- 32 Ardawi MSM, Newsholme EA. Fuel utilization in colonocytes of the rat. *Biochem J* 1985; **231**: 713–9.
- 33 Windmueller H, Spaeth AE. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. *Arch Biochem Biophys* 1975; **171**: 662–72.
- 34 Fox AD, Kripke SA, DePaula J, Berman JM, Settle RG, Rombeau JL. Effect of a glutamine-supplemented enteral diet on methotrexate-induced enterocolitis. *J Parenter Enteral Nutr* 1988; **12**: 325–31.
- 35 Souba WW, Klimberg VS, Hautamaki RD, Mendenhall WH, Bova FC, Howard RJ, *et al.* Oral glutamine reduces bacterial translocation following abdominal radiation. *J Surg Res* 1990; **48**: 1–5.
- 36 Tremel H, Kienle B, Weilemann LS, Stehle P, Fuerst P. Glutamine dipeptide-supplemented parenteral nutrition maintains intestinal function in the critically ill. *Gastroenterology* 1994; **107**: 1595–601.
- 37 Mariadson JM, Gibson PR. The effect of butyrate on paracellular permeability in the CaCo-2 model of colonic epithelium. *Gastroenterology* 1994; **106**: A729.
- 38 Weng V, Purdie B, Smith T, Emerson R, Harnatz P. The effect of short chain fatty acids on intestinal wound healing. *Gastroenterology* 1995; **108**: A763.
- 39 Rolandelli RH, Koruda MJ, Settle RG, Rombeau JL. Effects of intraluminal short-chain fatty acids on the healing of colonic anastomosis in the rat. *Surgery* 1986; **100**: 189–203.
- 40 Prasad M, Goddard PJ, Carter KJ, Milbank AJ, Silen W. In vitro restitution in rat colon: effects of butyrate and bicarbonate. *Gastroenterology* 1993; **104**: A272.
- 41 Kripke SA, Fox AD, Berman JM, Settle RG, Rombeau JL. Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. *J Parenter Enteral Nutr* 1989; **13**: 109–16.
- 42 Sakata T. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors. *Br J Nutr* 1987; **58**: 95–103.
- 43 Dignass AU, Podolsky DK. Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor β . *Gastroenterology* 1993; **105**: 1323–32.
- 44 Cummings JH, Pomare EW, Branch WJ, Naylor CPE, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 1987; **28**: 1221–7.
- 45 Rhoads JM, Chen W, Rippe RA, Westwick JK, Argenzio RA, Guan X, *et al.* Glutamine (GLN) stimulates enterocyte proliferation by activating early response genes. *Gastroenterology* 1995; **108**: A749.