Leucocyte endothelial cell adhesion in indomethacin induced intestinal inflammation is correlated with faecal pH

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Abstract

Background—Recent studies indicate that faecal pH is acidified in patients with inflammatory bowel disease compared with healthy controls. In healthy volunteers, stool pH, faecal flora, and bile acid concentration could be affected by means of elemental diets.

Aims—To assess the role of variations of faecal pH on leucocyte endothelial cell adhesion in indomethacin induced long lasting ileitis in rats.

Methods—Indomethacin (7.5 mg/kg subcutaneously) was injected twice, 24 hours apart. Rats were either fed with the identical diet before and 10 days after the induction of inflammation until the experiment, or the diet was changed at the time of induction. Ten postcapillary mesenteric venules (30 µm diameter) per animal were observed using intravital microscopy. Macrosopic visible intestinal ulceration was scored and faecal pH of different sections of the small bowel was determined.

Results—Small intestinal faecal pH was 8.5 in controls and 8.0 in indomethacin treated animals. Indomethacin significantly changed microcirculatory parameters: there was a 2.3-fold increase in leucocyte adherence, a 3.2-fold increase in leucocyte emigration, and a 20% reduction in shear rate. Application of various diets or diet combinations resulted in variations in faecal pH ranging from 7.8 to 8.8 which were inversely correlated with macroscopic ulcerations (\( r = -0.67 \)). Leucocyte adherence was attenuated with increased pH and augmented with decreased pH (\( r = -0.55 \)). Venular wall shear rate was positively correlated with faecal pH (\( r = 0.48 \)) while leucocyte emigration showed no correlation. Leucocyte rolling velocity was not significantly altered. Normalisation of faecal pH by different alkalising drugs induced a significant decrease in leucocyte adherence in standard fed, indomethacin treated rats.

Conclusions—Faecal pH is lowered in the indomethacin model of long lasting ileitis in rats, which is similar to human inflammatory bowel disease. Alkalisation of faecal pH due to different diets or alkalising drugs reduces indomethacin induced leucocyte endothelial cell adhesion and macroscopic intestinal damage. These results may provide a rationale for the therapeutic effect of enteral diets in Crohn’s disease.

Keywords: faecal pH, indomethacin, inflammatory bowel disease, diet, intravital microscopy

Several luminal factors have been implicated in the pathogenesis of inflammatory bowel disease (IBD). Among these, bacteria and bacterial products, nutritional metabolites, and faecal bile acid pattern are under investigation.

The important role of enteric bacteria in initiating and/or perpetuating intestinal inflammation in IBD and animal models of IBD is illustrated by the attenuation of inflammation in germ free rats and the beneficial effect of antimicrobial agents like metronidazole, on inflammatory activity in patients and experimental animals. The influence of nutrition has been shown by the effect of bowel rest using total parenteral nutrition or elemental diets. Furthermore, dietary habits seem to be different between patients with IBD and healthy subjects.

Little is known about a possible effect of the intraluminal intestinal pH on inflammatory activity in IBD. In a clinical study the faeces of patients with IBD had a lower pH than faeces of healthy subjects. Faecal pH may be affected by faecal bile acid pattern, luminal bacteria, and nutritional metabolites, parameters which can also affect each other. Intraluminal pH may be shifted by the bacterial profile. Enss et al showed that colonic mucins are acidified after polyvalent bacterial colonisation of germ free rats. Certain species may be favoured by the application of different diets. Bacterial conversion from primary to secondary bile acids might be altered by variations in intestinal flora. Alternatively intestinal pH may be changed by administration of antagonists of bicarbonate secretion.

The objective of this study was to assess the role of small bowel faecal pH on leucocyte endothelial cell interaction in postcapillary mesenteric venules in indomethacin induced long lasting ileitis in rats. Sublethal doses of indomethacin produce chronic inflammation of the distal jejunum and proximal ileum that persists for more than 11 weeks in genetically susceptible rats. The clinical, histological, and pathophysiological features of indomethacin induced lesions are similar to those in Crohn’s disease, and are characterised by thickening of the small intestine and mesentery, adhesions, obstructions, linear mucosal ulcerations, acute...
and chronic transmural granulomatous inflammation, crypt abscesses, and fibrosis.\textsuperscript{20,21}

In our experiments intestinal pH was varied by feeding with different diets (standard laboratory rat chow or combinations of commercially available isoenergetic special diets). In some groups ion exchangers were used to increase faecal pH. The effect of various diet combinations and ion exchangers on intestinal pH was determined 10 days after indomethacin administration by intravital microscopy in order to characterise the influence of pH modification on leucocyte endothelial cell interaction.

Methods

EXPERIMENTAL PROTOCOL

One hundred and fifty male Sprague-Dawley rats (160–240 g) were housed for two weeks in a room with a constant temperature of 25°C and a 12:12 hour light:dark cycle. They were given water and standard laboratory rat chow (ST) or different diets ad libitum (rich in carbohydrates, CH; rich in fatty acids, FR; poor in fatty acids, FP; rich in cholesterol, CR; poor in cholesterol, CP; poor in saturated fatty acids, SA; poor in unsaturated fatty acids, US; commercial available isoenergetic special diets). Animals from each group were tested, but no significant differences in microcirculatory parameters between these groups were obtained. Animals were immediately refed after the injection and fasted 12–18 hours prior to the experiments. Previous experiments had shown that different diet combinations without induction of inflammation by indomethacin did not alter leucocyte endothelial cell interaction (data not shown). To check whether the appearance of pathological bacteria could be responsible for the microcirculatory differences measured, the small intestinal contents of two animals from each group were tested, but no pathogens could be detected.

ANIMAL PREPARATION

After anaesthesia with pentobarbital (40 mg/100 g body weight) tracheotomy was performed, and the left carotid artery was cannulated for continuous measurement and recording of systemic blood pressure and heart rate (Statham P23A Transducer, Oxnard, California; Grass Recorder, Grass Instruments, Quincy, Massachusetts).

INTRAVITAL MICROSCOPY

Rats were placed in a supine position on an adjustable Plexiglas microscope stage and the exteriorised segment of the mid-jejunum was draped over an optically clear viewing pedestal, allowing transillumination of a 2 cm$^2$ section, as described previously.\textsuperscript{22,23} The exposed bowel wall was draped with saline soaked gauze, and the mesentery was continuously superfused with warm bicarbonate buffered salt solution (BBS, pH 7.4) bubbled with 5% O$_2$/95% N$_2$. The temperature of the pedestal was maintained at 37°C by a constant temperature circulator (Fisher Scientific, model 80). Single unbranched mesenteric venules 25–35 µm in diameter and approximately 150 µm long were transilluminated with a 12V, 100W light source and observed through an intravital videomicroscope (Nikon Diaphot, Japan) with a ×40 objective lens (Nikon UD 40/0.65, Japan) and a ×10 eyepiece. A videocamera (Hitachi WK-C150, Japan) mounted on the microscope, projected the image onto a colour monitor (Sony PVM-2030, Japan). Venular diameter (D) was measured using a video image measuring monitor (Microcirculation Research Institute, Texas A&M University). Red
blood cell centreline velocity was measured on line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Mean red blood cell velocity was calculated assuming \( V_{cc} = \text{centreline velocity}/1.6^{2} \). Venular shear rate (\( \gamma \)) was calculated based on the Newtonian definition:\(^{2}\)
\[ \gamma = 8(V_{cws}/D_{v}) \]. When all parameters measured on line (arterial pressure, erythrocyte velocity, venular diameter) were in a steady state during superfusion with BBS, images from the mesenteric preparations were videotaped using a videocassette recorder (Panasonic NV8950, Japan) for five minutes. Approximately 10 venules per animal were monitored for playback analysis.

The numbers of adherent and emigrated leucocytes were determined during playback of videotaped images. A leucocyte was defined as adherent to venular endothelium if it was stationary for at least for 30 seconds. Leucocyte adhesion was expressed as the number per 100 µm length of the venule. Leucocyte emigration was expressed as the number of white blood cells per microscopic field (1.7 \( \times \) \( 10^{-5} \) mm²). Rolling leucocytes were defined as white blood cells moving at a slower velocity than erythrocytes in the same vessel. Leucocyte rolling velocity (\( V_{cws} \)) was determined from the time a leucocyte took to move along 100 µm of the microvessel. A mean of 10 estimates of transit time was used to calculate \( V_{cws} \).

**FAecal pH Measurement**

Following intravital microscopy the animals were sacrificed with an overdose of pentobarbital and the intestines were excised and opened longitudinally. Gross findings were ranked using the following criteria: 0—no change in serosa or mucosa; 1—hyperaemic lesions and/or petechial bleeding; 2—single mucosal erosion or ulceration; 3—multiple erosions or ulcerations without any lesions in the serosa and mesentery, or single mucosal ulceration or ulcer with hyperaemic, adhesive, or haemorrhagic lesions in the serosa; 4—multiple erosions or ulcerations (on less than 10 cm of bowel length) with hyperaemic, adhesive, or haemorrhagic lesions in the serosa; 5—multiple erosions or ulcerations (more than 10 cm of bowel length) with hyperaemic, adhesive, or haemorrhagic lesions in the serosa.\(^{28}\)

**STATISTICS**

Data were analysed using analysis of variance with the Scheffé (post hoc) test. All values are expressed as mean (SEM). Statistical significance was set at \( p < 0.05 \). Regression analysis was performed according to the method of least squares. The confidence interval was set at 95%.

| Table 2 Effect of the various diets and combinations on faecal pH and on parameters of microcirculation |
|---|---|---|---|---|---|
| Diet | Faecal pH | Adherence (per 100 µm venule) | Emigration (per microscopic field) | Leucocyte rolling velocity (µm/s) | Shear rate (s⁻¹) | Macroporous score (arbitrary unit) |
| FP/FR | 7.76 (0.14)* | 5.95 (0.53)* | 4.15 (0.53)* | 61.2 (3.3) | 415 (19) | 3.9 (0.6) |
| US/FR | 7.85 (0.09) | 4.48 (0.44) | 1.13 (0.19)* | 75.8 (3.2) | 420 (18) | 2.5 (0.7) |
| ST/CR | 7.98 (0.24) | 3.59 (0.42)* | 1.49 (0.19)* | 70.7 (3.7) | 409 (18) | 3.8 (0.3) |
| ST/ST | 7.99 (0.05) | 4.71 (0.36) | 4.57 (0.43) | 69.8 (2.4) | 429 (15) | 3.5 (0.4) |
| US/US | 8.00 (0.17) | 4.40 (0.44) | 2.19 (0.28)* | 69.4 (3.4) | 391 (17) | 2.8 (0.8) |
| CP/FR | 8.01 (0.19) | 3.48 (0.31)* | 3.36 (0.33)* | 73.8 (4.0) | 400 (13) | 3.7 (0.9) |
| FP/FR | 8.11 (0.09) | 4.29 (0.38) | 2.93 (0.47)* | 59.1 (3.4) | 403 (17) | 2.3 (0.2)* |
| FP/ST | 8.14 (0.25) | 3.92 (0.38) | 4.97 (0.81) | 68.5 (3.3) | 440 (21) | 2.9 (0.5) |
| CR/ST | 8.16 (0.16) | 3.67 (0.48)* | 1.55 (0.32)* | 59.3 (3.5) | 434 (25) | 2.0 (1.0)* |
| ST/ST | 8.24 (0.35) | 3.15 (0.32) | 1.65 (0.28)* | 73.3 (3.5) | 452 (19) | 3.0 (0.3) |
| ST/US | 8.25 (0.9)* | 3.54 (0.44) | 3.51 (0.45)* | 68.1 (3.0) | 470 (19) | 2.3 (0.7) |
| SA/SA | 8.33 (0.03)* | 2.29 (0.23) | 1.56 (0.22)* | 81.5 (2.8) | 500 (19)* | 1.1 (0.5)* |
| ST/SA | 8.34 (0.02)* | 3.56 (0.40)* | 3.32 (0.47)* | 69.1 (2.4) | 411 (14) | 2.4 (0.7) |
| C1 | 8.34 (0.12)* | 2.33 (0.27)* | 2.00 (0.23)* | 70.6 (2.7) | 493 (20)* | 0.8 (0.3)* |
| C2 | 8.39 (0.03)* | 3.37 (0.54) | 0.68 (0.20)* | 72.8 (4.5) | 487 (26)* | 2.3 (0.5) |
| SA/FR | 8.42 (0.21)* | 2.52 (0.22)* | 1.86 (0.27)* | 55.6 (2.8) | 564 (17)* | 0.3 (0.2) |
| CP/ST | 8.43 (0.19)* | 3.33 (0.46)* | 2.12 (0.33)* | 71.7 (3.3) | 445 (23) | 1.8 (0.6)* |
| ST/CH | 8.46 (0.16) | 3.56 (0.26) | 3.03 (0.24) | 75.6 (2.5) | 429 (11) | 0.7 (0.2)* |
| ST/FR | 8.50 (0.07)* | 3.48 (0.27) | 3.95 (0.40) | 82.2 (2.3) | 439 (11) | 1.5 (0.5)* |
| CR/CR | 8.52 (0.14) | 1.80 (0.25)* | 1.06 (0.32) | 65.9 (2.8) | 475 (13) | 0.6 (0.1)* |
| Control | 8.54 (0.06) | 2.07 (0.23) | 1.43 (0.19) | 77.0 (2.5) | 526 (17)* | 0.6 (0.2)* |
| PS1 | 8.50 (0.05)* | 2.13 (0.21) | 0.54 (0.10) | 64.6 (3.3) | 495 (24)* | 0.3 (0.2)* |
| HC2 | 8.61 (0.18) | 3.10 (0.41) | 1.62 (0.27) | 61.9 (2.9) | 485 (20)* | 1.1 (0.3)* |
| PS2 | 8.64 (0.03)* | 1.60 (0.20) | 1.60 (0.17) | 65.4 (3.0) | 440 (19) | 1.3 (0.8)* |
| CP/CF | 8.83 (0.14) | 2.12 (0.21) | 1.89 (0.37) | 62.5 (3.0) | 435 (17) | 0.8 (0.3)* |
| HCI1 | 8.83 (0.15)* | 1.82 (0.23)* | 0.92 (0.18)* | 72.8 (2.7) | 537 (27)* | 0.3 (0.1)* |

Data are expressed as mean (SEM). \( * p < 0.05 \) vs. ST/ST.
Results

Faecal pH of small intestinal contents decreased approaching the ligament of Treitz by approximately 1 pH unit per 70 cm of bowel. Faecal pH was also affected by the different diet combinations. Figure 1 shows the pH shift in the small bowel from the caecum to the ligament of Treitz for four groups of differently treated rats: control (negative control without indomethacin), ST/ST (positive control with indomethacin (ST/ST)), and treated with potassium hydrogen citrate without indomethacin (HC1). Mean values are shown.

Table 2 summarises the effects of various diet combinations and of treatment with the various alkalisising drugs (sodium hydrogen carbonate, polysulphonic acid, cholestyramine) on faecal pH and microcirculatory parameters. Ten days after induction of indomethacin induced inflammation there was a decrease in faecal pH compared with the identical fed control group without indomethacin (ST/ST versus control); at the same time leucocyte adherence and emigration were increased significantly, venular wall shear rate was decreased, and leucocyte rolling velocity was not affected. Faecal pH was mainly increased by the different diet combinations compared with the positive control (ST/ST); only the use of the combinations poor in fat/rich in fat, poor in unsaturated acids/rich in fat, and standard/rich in cholesterol resulted in lowering of faecal pH versus the positive control. The reduction or increase in pH was accompanied by a proportional increase or decrease (correlation coefficient $r = -0.55$) in leucocyte adherence (fig 2). Faecal pH and shear rate were positively correlated ($r = 0.48$) (fig 3), although venular diameter and erythrocyte velocity—from which the shear rate is calculated—were not significantly correlated with faecal pH. There was no correlation between pH, leucocyte emigration ($r = 0.13$) and leucocyte rolling velocity ($r = 0.05$).

Alkalisising substances kept faecal pH in the range of the negative control group (without indomethacin). There was no significant difference in pH between indomethacin and vehicle treated animals (potassium hydrogen citrate with indomethacin 8.61 (0.18), without indomethacin, 8.83 (0.15); polysulphonic acid with indomethacin 8.64 (0.03), without indomethacin 8.56 (0.05); cholestyramine with indomethacin 8.39 (0.03), without indomethacin 8.34 (0.12)). Blood pH was not affected by this treatment (data not shown). Leucocyte adherence was significantly reduced in animals treated with cholestyramine or potassium hydrogen citrate plus indomethacin, and it was normalised to control levels in animals treated with polysulphonic acid plus indomethacin. Administration of alkalisising drugs without
indomethacin had no effect on leucocyte adherence.

Macroscopic visible intestinal damage, scored 0–5 according to the degree of inflammation, was also influenced by the various diet combinations. There was a significant inverse correlation between the degree of macroscopic ulceration and faecal pH ($r = -0.67$) (fig 4).

**Discussion**

Variations in faecal pH are caused by differences in nutrition, intestinal bacterial colonisation, and faecal bile acid pattern.\(^{10-15}\) Previous experiments have shown that artificial variations in diet, alterations in bacterial colonisation by antimicrobial agents, and changes in faecal bile acid pattern may reduce inflammatory activity in the indomethacin model of a long lasting experimental ileitis.\(^{3-29}\)

The aim of this study was to assess whether there is a comparable decrease in faecal pH in this model of IBD according to the findings in Crohn’s disease, whether the pH shift is proportional to the intestinal inflammatory activity, and whether the artificial variation in faecal pH may affect the inflammatory response. Experiments were done in the chronic phase of indomethacin induced ileitis 10 days after application. As the maximum inflammation in the indomethacin model is usually 10 to 20 cm proximal to the caecum we measured the pH of the small intestinal contents of this section, which is approximately one pH unit higher than in the duodenum and intracolonically near the anus.\(^{30}\) Inflammatory activity was determined microscopically by measuring the leucocyte endothelial cell interaction in post-capillary mesenteric venules of the same region and macroscopically by scoring intestinal epithelial lesions of the whole small bowel.

As in patients with IBD, indomethacin treated rats fed with a standard diet had a lower pH and a higher inflammatory activity, shown by an increase in leucocyte adherence and emigration compared with control rats without indomethacin. The pH shift in the indomethacin model might be due to inhibition of the duodenal bicarbonate response to luminal acidification.\(^{31}\) The use of various diet combinations in indomethacin treated rats resulted in an increase or even a further decrease in faecal pH. With some diets pH was nearly normalised to the level of untreated controls. The fact that pH variations were not determined by the second diet alone (fed from the onset of inflammation for 10 days until the experiment), but also by the first diet, suggests that very early inflammatory processes which are affected by luminal metabolites before indomethacin administration or long lasting effects of the diets on the bacterial contents are at least partly responsible for the faecal pH measured.

The pH variations were accompanied by an inverse proportional effect on leucocyte adherence. Although there was also a direct proportional increase or decrease in shear rate, correlations between pH and venular diameter or erythrocyte velocity, respectively, from which shear rate is calculated, were not significant due to inconstant changes of both parameters due to pH shifts. As already shown,\(^{32}\) leucocyte rolling velocity in the chronic phase (more than seven days after indomethacin administration)
of inflammation is not significantly affected by indomethacin treatment.

In order to elucidate the question of whether variations in faecal pH are a consequence or the cause of alterations in leucocyte endothelial cell adhesion we administered different alkalisising substances to standard fed rats with or without indomethacin treatment. While the pH increasing effect of the anion exchange polymer cholestyramine might be partially due to its additional binding capacity for bile acids and fatty acids, the observed increases in faecal pH by potassium hydrogen citrate and polysulphonic acid are probably exclusively due to their alkalisising properties. Application to animals not treated with indomethacin induced no further increase in faecal pH compared with control, untreated rats. In indomethacin treated animals all three substances induced alkalisisation of faecal pH to the same level as in the rats with the same alkalisiser but without indomethacin, respectively. At the same time, leucocyte adherence was significantly attenuated or even normalised to the level of untreated control rats. This fact favours the idea that faecal pH may influence the degree of leucocyte endothelial cell interaction. The lack of correlation of faecal pH with leucocyte emigration could be due to differences in activation of the various adhesion molecules responsible for leucocyte adherence to and transmigration through the microvascular endothelium, respectively. This has been already described for the differential effect of P selectin on leucocyte adherence and emigra-
tion in chronic indomethacin induced inflammation.

Although the reduced mucosal accumulation of granulocytes was not accompanied by protection against indomethacin induced intestinal mucosal ulcerations (there was no correlation between macroscopic ulceration score and leucocyte emigration), there was an inverse correlation between the extent of mucosal ulcerations and faecal pH. It has been shown previously that in the chronic phase of indomethacin induced inflammation granulocyte infiltration into the mucosa is a conse-
quence of intestinal lesions. Loss of the normal mucosal barrier to the uptake of macromolecules, bacteria, and bacterial anti-
gens is thought to be an important aspect of Crohn’s disease. An increase in mucosal permeability has also been shown for indomethacin induced ileitis.

A direct effect of low pH on the mucosal barrier, thereby increasing or reducing the extent of inflammation, seems unlikely. Intrarectal administration of HCl did not produce colonic inflammation and variations in pH per se did not induce intestinal inflammation. A role of colonic pH in the production of hydrogen from carbohydrates by colonic bacte-
rial flora was described by Pernan et al.

Glucose metabolism and hydrogen production was pH dependent and inhibited at acidic pH. Another possibility is an effect of faecal pH on bacterial colonisation which might in turn affect mucosal permeability. We did not detect pathogens on microbiological examin-

ation of small intestinal contents of differently fed rats in our experiments, but the intestinal flora present was not further differentiated.

The most important effects of variations in pH on inflammatory activity could include alterations in the faecal bile acid pattern and direct or, due to alterations in bile composition, indirect changes in enterohepatic circulation of indomethacin. Approximately 50% of parenterally given indomethacin undergoes biliary recycling. In vitro experiments showed that bile of indomethacin treated rats has a concentration dependent higher cytotoxicity in rat epithelial cells than bile of vehicle treated rats or those treated with indomethacin alone. The increasing hydrophilicity of indomethacin with increasing pH would suggest that alkalisisation of faeces would reduce the solubility of indomethacin in the bile, resulting in a lower uptake through the lipophilic membranes of the epithelial cells. Furthermore, a shift in faecal pH might change the composition of bile by altering the state of ionisation of the various bile acids and bacterial conversion of primary to secondary bile acids.

Other ways in which enteric pH influences the adhesion of white blood cells in mesenteric venules distant from the circulation of the intestinal mucosa remain speculative. A direct pH effect could be excluded by normal blood pH and by the lack of effect of pH modulation of superfusion buffer directly applied to the mesenteric preparations (unpublished results). Further experimentation is needed to elucidate whether systemic effects of proinflammatory mediators such as leukotriene B4, or tumour necrosis factor α—both raised in peripheral blood of patients with IBD—are responsible for the observed effects of faecal pH on mesenteric leucocyte endothelial cell adhesion, or whether there are local nervous reflex controls for proinflammatory or anti-
inflammatory mediators similar to those de-
scribed for postprandial vasodilatation.

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