

Pouchitis: result of microbial imbalance?

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Abstract

To elucidate the role of microbiological factors in pouchitis, this study investigated the composition of ileal reservoir microflora, the mucus degrading capacity of bacterial enzymes as well as the pH and the proteolytic activity of pouch effluent. Stool samples were collected from five patients with pouchitis and nine patients without pouchitis. The flora of patients with pouchitis had an increased number of aerobes, a decreased ratio anaerobes to aerobes, less bifidobacteria and anaerobic lactobacilli, more *Clostridium perfringens*, and several species that were not found in control patients (for example, fungi). Furthermore the pH was significantly higher in patients with pouchitis (median value 6.5) than in control patients (5.4). To find out if the pH might influence the breakdown of intestinal mucus glycoproteins, the activity of glycosidases and proteases, and the degradation of hog gastric mucin by the pouch flora was tested at pH 5.2-7.6. Some glycosidases were inhibited, others were stimulated by a low pH, however, in each sample the proteolytic activity was inhibited for 75% at pH 5.2 compared with pH 6.8 and 7.6. Degradation of hog gastric mucin by the pouch flora was an active process at pH 7.2: within two to four hours of incubation more than half of the mucin was degraded. At pH 5.2 it took twice as long. It is concluded that pouchitis possibly results from instability of the flora in the pouch, which causes homeostasis to disappear (dysbiosis), and the protection of the pouch epithelium by the mucus layer becomes affected by increased activity of bacterial and host derived enzymes.

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In the past, a permanent Brooke ileostomy was inevitable for patients requiring a proctocolectomy for either ulcerative colitis or familial adenomatous polyposis. During the past two decades, the continent ileostomy, devised by Kock, and the ileoanal anastomosis, introduced by Parks and Utsunomiya, have evolved into attractive alternatives. Both procedures have the advantage of removing all diseased mucosa while avoiding a conventional and incontinent ileostomy. The construction of an ileal reservoir, however, often results in mucosal changes. Although most of these changes remain subclinical, some patients will develop a clinical syndrome known as pouchitis.^{1,2} To define pouchitis different diagnostic criteria have been used, which makes it hard to interpret the reported data on pouchitis. Recently, it had been advocated that an unequivocal diagnosis should be based on a diagnostic triad, consisting of the following components: clinical symptoms, endoscopic features of acute inflammation,

and histological evidence of a prominent polymorphonuclear cell exudate.³ Pouchitis occurs in 10-20% of the patients who have surgical treatment for chronic ulcerative colitis (only a few cases have been described in patients operated for familial adenomatous polyposis). The incidence has risen, however, with a longer period of follow up to 40%.⁴⁻¹⁰ Most patients with pouchitis respond well to metronidazole, but chronic pouchitis patients require continuous treatment with sulphasalazine or corticosteroids.¹¹

The cause of pouchitis is not clear. The immediate response to antibiotic treatment suggests a role for the flora in the pouch. There are few indications of the participation of intestinal pathogens and it has become evident that the flora is not directly responsible for pouchitis.¹²⁻¹⁸ The ileum reservoir is colonised with large numbers of bacteria that closely resemble the flora found in the large intestine and outnumber the flora of the normal ileum.^{19,20} Micro-organisms are known to be active producers of metabolites and toxins and when localised to the ileal reservoir bacterial products could injure mucosal epithelial cells. Bacterial enzymes with the capacity to degrade intestinal mucus are also potentially dangerous to the ileal mucosa. Enzymes participating in the breakdown of oligosaccharide side chains of mucin glycoproteins are glycosidases. These enzymes are produced by several representatives of the human colonic flora²¹⁻²⁴ and were present in ileal pouches of experimental colectomised dogs.²⁵ Breakdown of the polysaccharide moiety of the glycoprotein may result in impairment of the protective function of the mucus, which may become more permeable to toxic bacterial metabolites and host derived proteolytic enzymes.

This might be the situation in patients with pouchitis: luminal components, such as bacterial antigens, may cause a local inflammatory reaction when the integrity of the mucosa is affected by a combined action of bacterial glycosidases and pancreatic proteases.

To examine the factor that might control this process, we compared several parameters (composition of the pouch flora, activity of bacterial mucus degrading enzymes, pH, proteolytic activity) in faeces from patients with and patients without pouchitis.

Methods

PATIENTS

Fourteen patients (six males, eight females) with a median age of 27 years were studied. The patients had a restorative proctocolectomy for ulcerative colitis (n=12) or familial adenomatous polyposis (n=2). An S pouch was constructed in

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12 patients, whereas in two patients a W pouch was created. This study was performed at least one year after the restorative proctocolectomy. The diagnosis pouchitis was based on the triad: (a) clinical symptoms (watery and sometimes bloody diarrhoea, abdominal discomfort, urgency, incontinence, and sometimes fever and malaise); (b) endoscopic features of acute non-specific inflammation (granularity, oedema, erythema, friability, petechiae, hypersecretion, and multiple superficial erosive defects), (c) histological evidence of an acute inflammatory cell infiltrate, consisting of polymorphic granulocytes, associated with crypt abscesses and ulcerations. Using these criteria five patients presented with pouchitis and nine did not (controls). Two patients were investigated during an episode of pouchitis and during a disease free period. Four patients had pouchitis that resolved promptly after treatment with metronidazole (flagyl); one patient did not respond to metronidazole and required continuous treatment with 5-aminosalicylate (5-ASA) to remain asymptomatic.

Stool samples were transferred to the laboratory immediately after defecation. Two samples of faeces were obtained from each patient without pouchitis, with at least a two month interval and were examined for flora composition and enzymatic activity. Faecal samples from patients with pouchitis were examined before treatment.

BACTERIOLOGICAL TECHNIQUES

Within one to two hours after collection, the stools were processed. The samples were thoroughly mixed and 10-fold dilutions were prepared in anaerobic dilution solution.²⁶ Samples of appropriate dilutions were plated aerobically on MacConkey (Oxoid), Sabouraud (Oxoid), Rogosa (Oxoid), azide blood (Oxoid), and blood agar plates. Anaerobes were cultured in anaerobic culture flasks, filled with a 90% N₂ and 10% CO₂ mixture as described before,^{27,28} on Schaedler broth (Oxoid) supplemented with 2% agar (Difco), 0.002% resazurin (BDH), and 0.025% dithiothreitol (Sigma), and azide blood agar with 0.05% cysteine (Sigma) and 0.025% dithiothreitol. After two days of incubation at 37°C, colonies on the various media were counted. All colonies grown on the anaerobic flasks were tested for aerobic growth on blood agar plates. The aerobes were identified by conventional methods. Gram stain, morphology, carbohydrate fermentations, and gas chromatographically estimated end products of glucose fermentation were used to identify the isolates to genus or species level. Anaerobic bacteria were classified according to Holdeman *et al.*²⁹

pH MEASUREMENT IN FAECES

The pH was determined with a pH meter (28, Radiometer, Copenhagen) by inserting the electrode (GK2402C, Radiometer) directly in fresh undiluted faecal samples of at least 25 g; collected in a small container. Direct measurement without previous mixing of the samples prevents as much escape of gas as is possible, which constitutes more than 50 per cent of the

total volume of the ileal reservoir.³⁰ After the direct measurements, the samples were carefully mixed with a spatula and the pH was estimated again. Apart from the samples used for bacteriological and enzymatic investigation, at least 10 faecal samples (obtained over a month at various times of the day) from each patient without pouchitis were examined. Stools from patients with pouchitis were examined before treatment with metronidazole.

ENZYME ASSAYS

Within two hours of collection, the stool samples were stored at -20°C. Preliminary studies showed no changes in enzyme activities after storage for at least one month. Samples were diluted and homogenised (Stomacher Lab Blender 400) for 10 minutes. Coarse particles were removed by gauze filtration.

The activities of α L-fucosidase, α and β D-NAc galactosaminidase, α and β D-galactosidase, β D-NAc glucosaminidase, and α D-mannosidase were estimated by using their *p*-nitrophenyl glycoside substrates.³¹ One unit of enzyme activity was defined as the amount that released 1 μ mol of *p*-nitrophenol/min at 37°C. Neuraminidase activity was measured with NAc-neuramin-lactose (Sigma) as substrate by the method of Warren.³² One unit of neuraminidase released 1 μ mol of neuraminic acid/min at 37°C. The limit of detection in these assays was 0.01 U.

Proteolytic activity was determined with azocasein (Sigma) as substrate.³³ Proteolytic activity was expressed in milligrams of azocasein hydrolysed in one hour at 37°C. All enzyme activities were described per gram of faeces.

In these standard enzyme assays dilutions of the faecal samples and enzyme substrates were made in 0.1 M acetate buffer pH 6.0 (neuraminidase), 0.1 M phosphate buffer pH 6.8 (the other seven glycosidases), and 0.1 M phosphate buffer pH 7.6 (proteases). To test the effect of pH on the enzyme activities, all faecal samples (from nine control patients) were diluted in citric acid-phosphate buffer (0.1 M Na₂ HPO₄/2H₂O, 0.1 M citric acid/H₂O) pH 5.2, 5.8, 6.8, and 7.6. Additionally the substrate solutions were made in the appropriate buffers.

MUCIN DEGRADATION

About 100 g of fresh faeces was mixed carefully, the pH was measured, and 35 g faeces was diluted 1:1 with citric acid-phosphate buffer with a pH in the range 4.6 to 5.2 to obtain pH 5.2 in the mixture. Another 35 g of faeces was mixed with citric acid-phosphate buffer with pH in the range 7.2 to 8.0 to adjust the mixtures to pH 7.2. The mixtures were homogenised (Stomacher Lab Blender 400) and coarse particles were removed by gauze filtration.

Hog gastric mucin (Sigma) was purified by repeated ethanol precipitation.³⁴ A 1.2% solution was made in citric acid-phosphate buffer pH 5.2 and 7.2 and was added to the faecal suspensions. The two mixtures with pH 5.2 and 7.2, and a final composition of 1 part faeces, 1.7 part buffer, and 0.3% mucin, were incubated for

TABLE I Flora, enzymes, pH, and dry weight of pouch effluent*

	Patients		Pouchitis		p†
	No pouchitis				
Total number of micro-organisms‡	9.41 (9-10)	8.80 (8-10)			NS
Aerobes	7.00 (3-8)	8.52 (7-8)			0.03
Anaerobes	9.41 (9-10)	8.48 (7-10)			NS
Aerobes					
<i>Enterobacteriaceae</i>	5.18 (ND-8)	7.70 (5-8)			NS
<i>Streptococcaceae</i>	6.58 (ND-8)	7.56 (5-9)			NS
<i>Lactobacillaceae</i>	3.78 (ND-7)	3.90 (ND-7)			NS
<i>Candida albicans</i>	3.30 (ND-5)	4.30 (ND-7)			NS
Anaerobes					
<i>Bacteroidaceae</i>	8.88 (ND-10)	8.15 (ND-9)			NS
<i>Peptococcaceae</i> and <i>Streptococcus</i> sp	8.07 (ND-8)	8.7 (ND-9)			NS
<i>Eubacterium</i> sp	8.30 (ND-9)	7.60 (ND-7)			NS
<i>Bifidobacterium</i> sp and <i>Lactobacillus</i> sp	8.48 (ND-9)	ND (ND-8)			0.01
<i>Clostridium perfringens</i>	ND (ND-7)	5.60 (ND-8)			0.03
Ratio anaerobes/aerobes	143 (5-10 ⁴)	2 (0.3-900)			0.01
Glycosidases§					
α D-galactosidase	0.07 (0.01-0.16)	0.03 (ND-0.11)			NS
β D-galactosidase	0.15 (ND-0.30)	0.04 (ND-0.11)			NS
α L-fucosidase	ND (ND-0.04)	ND (ND-0.02)			NS
α D-mannosidase	ND (ND-0.02)	ND (ND-0.14)			NS
β D-NAc glucosaminidase	0.02 (ND-0.13)	0.01 (ND-0.02)			NS
α D-NAc galactosaminidase	0.01 (ND-0.09)	ND (ND-0.02)			NS
β D-NAc galactosaminidase	0.01 (ND-0.09)	ND (ND-0.02)			NS
Neuraminidase	0.03 (ND-0.03)	0.02 (0.01-0.02)			NS
Proteolytic activity 	14.0 (5.5-23.5)	14.5 (7.1-17.3)			NS
pH	5.4 (4.7-6.1)	6.5 (6.0-7.8)			<0.01
Dry weight, mg/g faeces	84 (57-103)	52 (30-110)			NS

*All values are given as medians and ranges (in parentheses); ND, not detected; † value of difference *v* controls; NS, not significant; ‡ numbers of bacteria are given as log₁₀/gram wet faeces and the ranges are rounded off to the nearest log₁₀. The limits of detection were as follows: for *Enterobacteriaceae*, aerobic cocci, anaerobic bacteria 4.3; for aerobic lactobacilli and yeasts 2.3. Not included in the Table are species that were found only once or one to three times but in small amounts: *Staphylococcus epidermidis*, *Bacillus* sp, *Candida* sp, fungi, *Veillonella* sp, and *Clostridium* sp. § all glycosidase activities were measured as micromoles of *p*-nitrophenol released/minute (units)/gram wet faeces, apart from neuraminidase, which was measured as micromoles of neuramic acid/minute (units)/gram wet faeces. The limit of detection was 0.01 U/gram; || proteolytic activity is expressed as milligrams of hydrolysed azocasein/hour/g wet faeces. The limit of detection was 0.02 mg hydrolysed azocasein/hour/g.

eight hours at 37°C; a sample was removed every two hours and placed in a waterbath at 80°C for 15 minutes to stop bacterial and enzymatic activity. After centrifuging, the remaining mucin was recovered by ethanol precipitation. Control experiments were performed by incubating faecal homogenates in buffer without mucin (pH 5.2 and 7.2), mucin solutions (0.3%) in buffer (pH 5.2 and 7.2), and autoclave sterilised mixtures of faeces and mucin, under identical conditions. Degradation of mucin was determined by estimation of mucin protein³⁵ using a modification described by Miller and Hoskins,³⁴ hexosamines by the method of Ghuysen³⁶ after hydrolysis in 2.5 N HCl at 90°C, hexoses (corrected for fucose) according to Mokrasch,³⁷ and fucose by the method of Dische and Shettles.³⁸ Mucin bloodgroup A and H antigenicity was determined in haemagglutination inhibition tests³⁹; the reciprocal value of the highest dilution of antigen glycoprotein that completely inhibited haemagglutination was defined as the antigen titre. Faecal samples from four control patients and the patient who required daily 5-ASA were used for these experiments.

STATISTICS

The Mann-Whitney U test was used to compare numbers of bacteria, glycosidases, and proteolytic activity and pH in faeces obtained from control patients with those obtained from patients with pouchitis. The Wilcoxon signed rank test was used to compare the activities of

proteases at different pH values⁴⁰ and the carbohydrate and protein fractions after incubation of mucin with a pouch flora at pH 5.2 and 7.2.

Results

FLORA, ENZYMES, AND pH IN POUCH OUTPUT

There were no differences found in total numbers of bacteria when pouch effluent from controls and patients was compared (Table I). Although the median value of the total counts of anaerobes was lower in patients with pouchitis, this difference was not statistically significant ($p=0.08$). Statistically higher numbers of aerobes were cultured from faeces taken from pouchitis patients. This is reflected in the proportion anaerobes to aerobes: patients without pouchitis harboured more than hundred times more anaerobes than aerobes, pouchitis patients only two times more anaerobes.

Bifidobacterium and anaerobic *Lactobacillus* species were found only once in pouchitis effluent, but in 84% of the control samples. Furthermore *Clostridium perfringens* was found in more than 75% of the pouchitis samples, but only in 25% of the samples from patients without pouchitis. No significant differences in the composition of the aerobic flora were found.

From the pouch of each control patient two samples were cultured with at least a two month interval. We noticed large differences in anaerobic bacterial composition between two samples of one subject. In the first sample more than 10 different species were found but just a single species in the second sample. Furthermore from the pouchitis group we cultured fungi and yeasts, other than *Candida albicans*; these were not found in the control group.

All tested glycosidases were shown, α and β D-galactosidase in nearly every sample, but α L-fucosidase in only five samples. Their activity, measured on *p*-nitrophenyl substrates at pH 6.8 (neuraminlactate pH 6.0, for neuraminidase) was similar in both groups. The same applied to the proteolytic activity, the standard measured with azocasein substrate, at pH 7.6.

The most striking difference between the control and the pouchitis group was the pH. In faeces from the control patients the pH was 5.4 (median value), which is significantly lower than the pouchitis group (pH 6.5). The time of the day the faeces was collected did not influence the results. To find out if the pH is (one of) the controlling mechanism(s) in the breakdown of intestinal mucusglycoproteins, we tested the influence of the pH on the activity glycosidases and proteases present in pouch effluent, and on the degradation of hog gastric mucin by the pouch flora.

EFFECT OF pH ON ENZYMATIC ACTIVITY

Table II shows that in pouch effluent from nine control patients the activities of four glycosidases were tested at four different pH values: α D-galactosidase and β D-NAc glucosaminidase were found to have 2-4 times higher activity at a low pH; neuraminidase and β D-galactosidase were mainly active at pH 6.8 and 7.6. Apart from α

TABLE II Activity of four glycosidases in faeces from nine patients without pouchitis

	pH			
	5.2	5.8	6.8	7.2
α D-galactosidase	0.21* (9/9)†	0.14 (0/9)	0.07 (0/9)	0.07 (0/9)
β D-galactosidase	0.11 (1/9)	0.13 (0/9)	0.15 (3/9)	0.17 (5/9)
β D-NAc glucosaminidase	0.08 (5/9)	0.05 (2/9)	0.02 (2/9)	0.02 (0/9)
Neuraminidase	0.01 (0/9)	0.03 (1/9)	0.05 (8/9)	0.02 (0/9)

* Glycosidase activity was measured as micromoles of *p*-nitrophenol released/minute (units)/g wet faeces, apart from neuraminidase, which was measured as micromoles of neuramic acid/minute (units)/gram wet faeces (mean values); † number of samples with their highest activity at that specific pH/number of tested samples.

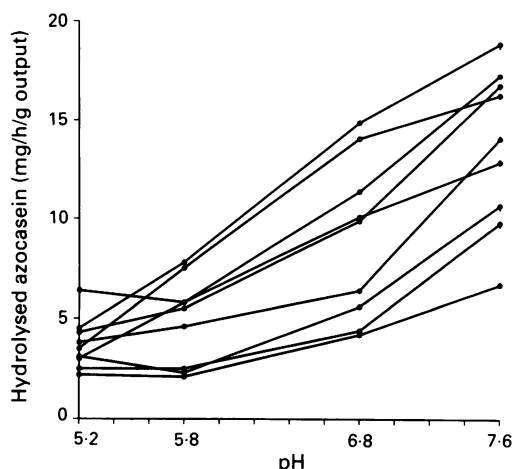


Figure 1: Proteolytic activity at pH 5.2-7.6 in faeces from nine patients without pouchitis.

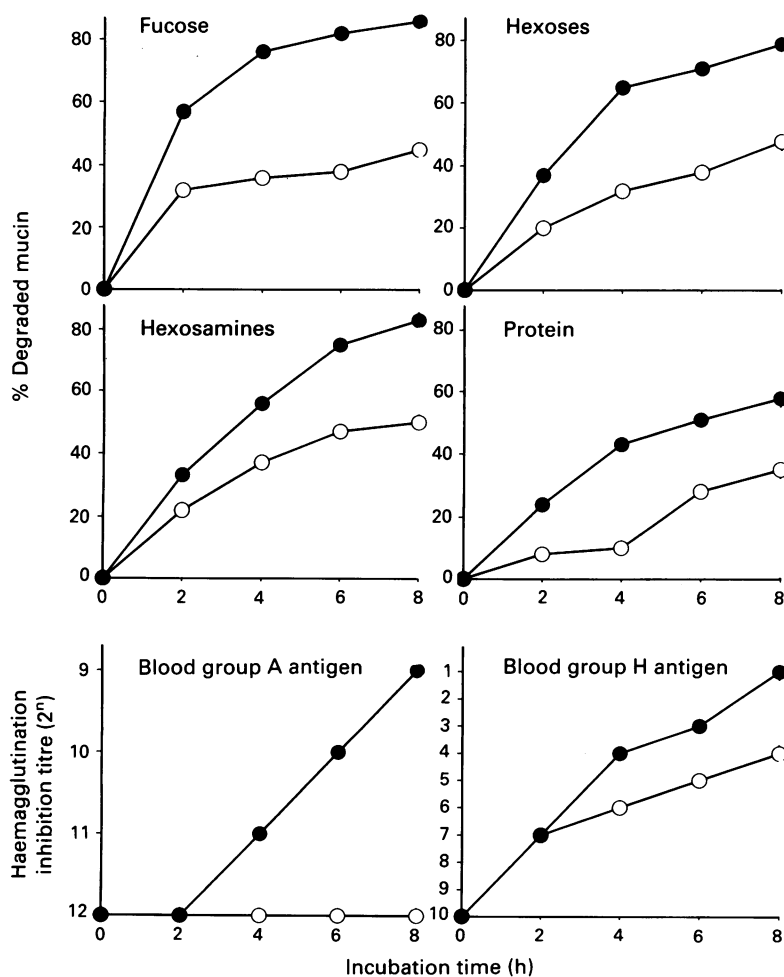


Figure 2: Degradation of mucin by the pouch flora during incubation at pH 5.2 (○-○) and pH 7.2 (●-●); medians of five values.

D-galactosidase, however, there were several individual differences, as shown in Table II.

Figure 1 shows that the pH dependence of the proteolytic activity was similar in each tested sample. At pH 6.8 and 7.6 the activities were respectively three and four times higher than at pH 5.2 ($p < 0.001$ for both comparisons).

EFFECT OF pH ON MUCIN DEGRADATION

The incubation of the pouch flora with hog gastric mucin at pH 7.2 resulted in an active breakdown of the mucin for each of the tested parameters (Fig 2). At pH 7.2 the fucose part of the mucin had disappeared for nearly 60% within two hours of incubation, compared with 30% at pH 5.2. The removal of 60% of the hexoses and hexosamines from the mucin took four hours of incubation at pH 7.2, but only 35% was degraded at pH 5.2. In addition blood group antigens A and H were much more actively removed from the mucin at a pH 7.2 than at pH 5.2. The degradation of the protein part of the mucin was not as fast as the degradation of the carbohydrate part. After four hours incubation nearly half of the proteins (43%) were found to be cleaved at pH 7.2, but only 10% at pH 5.2.

Mucin degradation was determined in five experiments with faeces from five different subjects (four control patients and one patient who required daily 5-ASA). In each experiment mucin degradation was inhibited at a low pH (5.2) compared with pH 7.2; $p = 0.01-0.05$ for the carbohydrate and the protein compounds.

The flora from three patients (one of them was the patient who required daily 5-ASA) caused a similar rate of mucin degradation (reflected by the median values in Fig 2). The flora of patient 4 degraded the hexoses part of the mucin slowly and gradually until a 60% loss after eight hours of incubation at pH 7.2, but hardly any protein or hexosamines were cleaved from the mucin. Patient 5, however, showed an extremely fast degradation rate: within two hours of incubation 90-95% of the mucin, as well as the carbohydrates as the protein fraction was broken down at pH 7.2. For both patients the degradation at pH 5.2 was slower.

In summary, it was found that mucin is broken down at a pH 7.2 twice as fast as at pH 5.2.

Discussion

The cause of pouchitis is still unknown. The good response to antibiotics supports the hypothesis that faecal stasis in the reservoir, with, consequently an increase in bacterial numbers compared with the normal ileum, plays an important part in the aetiology. Several flora related parameters, such as bacterial metabolism of bile acids and volatile acid concentration, have been associated with pouch inflammation and pouchitis. By bacterial dehydroxylation of primary bile acids the secondary bile acid deoxycholic acid is formed, which may reduce the water and salt transport through the epithelial cells.¹¹ Reduced values of short chain fatty acids in pouch effluent have found to be

correlated with mucosal villous atrophy and pouchitis.¹⁶⁻⁴³ Bacterial volatile acids, especially butyric acid, are important energy substrates for colonic epithelial cells⁴⁴ and a lack of this acid can make them more susceptible to inflammation. Recently, the response to metronidazole has been attributed to its capacity to remove oxygen radicals, which are increased in ischaemia.⁴⁵ It is still not possible for a definite conclusion to be stated.

Since the construction of the first ileal reservoirs there has been a great interest in the development of the flora. The results of studies, however, on the composition of the pouch flora from both patients without pouchitis and patients with pouchitis are contradictory.¹³⁻¹⁸

This study shows that the total number of bacteria in patients with and without pouchitis (about 1.5×10^9 bacteria/g wet faeces) is 10 times higher than in ileostomy effluent.¹⁹⁻⁴⁶⁻⁴⁷ This is not surprising for instead of free passage of the ileum contents in patients with a conventional ileostomy, the contents accumulates in the pouch for several hours, dependent on the stool frequency of the patients, which is 4-8 times/24 h.⁴⁸ In faeces of healthy subjects the total number of bacteria is about 10 times higher than in pouch effluent.⁴⁹⁻⁵⁰ Faeces is the final product of a 20-140 hour stay in the large intestine and of many complicated biochemical events in the different regions. The ecological significance of the ileum reservoir is probably best compared with the caecum and the ascending colon. In this part of the intestine the largest amounts of substrates for bacterial metabolism are available, including dietary residues and host produced substances like mucus glycoproteins, exfoliated epithelial cells, and pancreatic secretions.⁵¹ Only few data are available for bacterial numbers and fermentation in the human caecum. Mcfarland⁵² cultured from two sudden death victims 1 and 4×10^{10} bacteria/g dry caecal contents. These are about the same numbers, after conversion to dry weight, that we found in the ileum reservoirs. Furthermore the pH in caecum and colon ascendens was 5.9 and 5.8 respectively (subject 1), and 5.1 and 4.9 respectively (subject 2). This is in line with the low pH measured in the pouch output from the control patients. The low pH in both the caecum and ileum reservoir results from the high metabolic activity of the anaerobic flora and is probably the combined result of the bacterial production of short chain fatty acids (butyric, propionic, and acetic acid) and other organic acids (lactic acid, succinic acid) and the buffering capacity of the ileum contents (by bicarbonate secretions of pancreas, liver, and epithelial cells).

The delicate mucosal epithelial cells from the ileum are protected by a layer of mucus glycoproteins from mechanical injury, the action of antigens and toxins, and the invasion of enteric bacteria. The chief constituent of the intestinal mucus, secreted by goblet cells, consists of a glycoprotein that contains up to 85% carbohydrates. Common parts of the carbohydrate are fucose, galactose, NAc-glucosamine, NAc-galactosamine, NAc-neuraminic acid, and mannose in α or β glycoside linkage.⁵³⁻⁵⁵ Because it is not dependent on oral intake and is always

available in roughly the same amounts, mucus serves as an important energy source for the bacterial flora in the intestine. The degradation depends on the action of bacterial glycosidases, releasing monosaccharides from the polysaccharide chains that surround the protein core of the glycoprotein.²¹ This study shows that glycosidase activity is present in the pouch and it is probable that the carbohydrate part of the mucus glycoprotein is removed by their action. Degradation of the mucus may be continued, when the protein core of the glycoprotein is no longer protected after the removal of the oligosaccharide side chains, by proteolytic enzymes from the liver, pancreas, and brush border.⁵⁶

Our experiments show that on artificial substrates the proteolytic activity is strongly inhibited at a low pH (5.2-5.8). This pH resembles the pH in the pouch of the control patients (pH 5.4). On their *p*-nitriphenyl glycoside substrates some bacterial glycosidases were inhibited, but others were stimulated in their activity. Nevertheless the pouch flora degraded two times more of a natural substrate, such as hog gastric mucin (the carbohydrate part as well as the protein part), at a neutral pH compared with a low pH. The individual differences in degradation rate found is probably attributed to differences in flora composition. The significance of these data is that in a healthy pouch a low pH maintained by the flora might delay the mucin breakdown.

This might be the situation in a healthy pouch. The pouch flora degrades active mucus glycoproteins, but there is a balance between glycoprotein secretion by the goblet cells and the degradation by the flora. The low pH in the pouch, which is created by the bacterial metabolites, might be an important factor in inhibiting mucus breakdown. The homeostasis in the pouch might be dependent on the stability of the flora. In the case of pouchitis homeostasis is not maintained: we found that the flora composition had changed, a part of the anaerobes had disappeared in favour of the aerobes, the ratio anaerobes to aerobes moved from 143 to 2. This is in agreement with the findings of Onderdonk *et al*¹⁵ who cultured significantly more aerobes from tissue biopsy samples from patients with pouchitis than from control patients. The instability of the flora of patients with pouchitis was evident as several species that we had not cultured from the faeces of control patients occurred, such as fungi, *Candida* species apart from *Candida albicans* and *Bacillus* species. Furthermore *Clostridium perfringens* was found in nearly every pouchitis sample, sometimes in very high numbers (up to 2×10^8 /g output), but only in a few samples from the control patients. The same phenomenon was seen by Brandi.¹⁴ We cannot exclude the possible role of *C. perfringens* in the cause of pouchitis as enterotoxigenic strains of *C. perfringens* have been defined as an important cause of infections and antibiotic associated diarrhoea.⁵⁷⁻⁵⁹ The screening of the patients' faeces for toxins might give a definite answer. The large differences in flora composition between two samples from the control patients suggests that in addition the microbial balance in their pouches is unstable. Potential

pouchitis patients possibly have flora that degrades mucin quickly (as patient 5) and it is possible that patients with flora that does not degrades much mucin do not develop pouchitis (see patient 4) for the period their flora remains stable.

Because anaerobes are largely responsible for the production of fatty acids, it is possible that in pouchitis the fermentation patterns have been changed. Claussen⁴² and also Wishmeyer⁴³ found decreased amounts of volatile fatty acids in output from patients with pouchitis. A consequence might be a pH that is no longer acid, but more neutral. The comparatively high pH in patients with pouchitis might be regarded as a symptom of the instability of the flora. In this situation mucus degradation is possibly faster than production. The pouch content is a large reservoir of bacterial antigens, which may stimulate the immune system, and cause a local inflammatory reaction, when the integrity of the mucosa is affected. After the loss of (parts of) the mucus layer, the epithelial cells may become accessible to the action of host derived proteases. It has been postulated that pancreatic proteolytic enzymes have an inhibitory effect on intestinal healing processes⁶⁰ and promote progress of haemorrhagic intestinal necrosis.⁶¹ Impairment of the protective function of the ileal pouch mucosa makes the pouch epithelium more susceptible to accumulating bacterial toxins, deoxycholic acid, oxygen radicals, and shortage of butyric acid. Furthermore, the invasion of enteropathogenic micro-organisms is no longer inhibited when the pH become more neutral (loss of colonisation resistance).

Patients that have an operation for ulcerative colitis in principle develop pouchitis. There is evidence that one particular species of mucin, which was defined by oligosaccharide side chain composition, is deficient in the colon of patients with ulcerative colitis.⁶² In the pouch there is a change from small bowel type mucin (high degree of sialylation) to colonic type mucin (more sulphated).⁶³ It is possible that the colonic mucus and consequently the changed pouch mucus of patients operated for ulcerative colitis, is more susceptible to degradation by the combined action of bacterial glycosidases and pancreatic proteases, than pouch mucus from patients operated for familial adenomatous polyposis.

Our conclusion is that pouchitis is possibly the result of instability of the flora in the pouch of susceptible patients (ulcerative colitis), which causes homeostasis to disappear (dysbiosis), and the protection of the pouch epithelium by the mucus layer becomes affected by increased activity of bacterial and host derived enzymes.

The pouch flora in general is probably very susceptible to influences from outside, such as dietary variation and bacterial contamination, which is expressed by instability that may lead to pouchitis. After treatment of pouchitis with metronidazole, a state of great imbalance is again created and new flora have to colonise the pouch. We think that a stable flora with a normal fermentation pattern (including a low pH) might prevent pouchitis. This might be realised by oral ingestion of lactobacilli (probiotics), which has

been proved to be successful in the treatment and prevention of intestinal infections and antibiotic associated diarrhoea.^{64 65}

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