Escherichia coli and persistent diarrhea
Schultsz, C.

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Chapter 7

The intestinal mucus layer from patients with inflammatory bowel disease harbours high numbers of bacteria when compared to controls

Gastroenterology: in press
Abstract

Background Animal studies have suggested that the intestinal bacterial flora plays an important role in the initiation and perpetuation of both ulcerative colitis and Crohn's disease. Whether the bacterial flora contributes to the pathogenesis of IBD by increased penetration in mucus, increased adherence to epithelial cells or by invasion of the epithelium is unknown. Therefore, we studied the spatial distribution of bacteria in the mucosa of rectal and colonic biopsies from patients with IBD and from controls.

Methods Rectal biopsies from 19 patients with inflammatory bowel disease and from 14 controls were studied by using non-radioactive rRNA in situ hybridization aimed at eubacterial ribosomal RNA. In addition, biopsies from other sites in the colon from 5 of these patients and 2 controls were examined. The total mucosal surface length examined for each patient was measured and the number of bacteria visualized, was estimated semiquantitatively.

Results No bacteria were observed in rectal biopsies from 10 controls (71%) and 6 patients with IBD (32%) (p=0.04; OR 5.42, 95%CI 1.23 - 23.9). Rectal biopsies from patients with IBD contained significantly more bacteria than biopsies from controls (p=0.004). Rectal biopsies from 8 patients with IBD (42%) but none of the biopsies from controls contained >50 bacteria per total mucosal surface length examined. Bacteria were localized in clusters within the mucus layer but did not adhere to the epithelial cells and were not present within the lamina propria. Results of hybridization obtained with rectal biopsies and with biopsies obtained from other sites in the colon were similar for individual patients. There was no correlation between the numbers of bacteria present and either the degree of inflammation in the respective biopsy or the use of anti-inflammatory agents or sulfasalazine compounds.

Conclusions The intestinal mucus in IBD patients is less protective against the endogenous microflora than in controls, resulting in an increased association of luminal bacteria with the mucus layer. The presence of bacteria in the mucus may enhance or sustain the inflammatory process during IBD by enhanced exposure of the mucosal surface, including the mucosal immune system, to bacterial components which otherwise would not have passed the mucus barrier.
The intestinal mucus layer from patients with inflammatory bowel disease harbours high numbers of bacteria when compared to controls

Constance Schultsz¹, Frank M. van den Berg², Fiebo W. ten Kate², Guido N. J. Tytgat³, and Jacob Dankert¹

Departments of Medical Microbiology¹, Pathology² and Gastroenterology³, Academic Medical Centre, Amsterdam, the Netherlands

Introduction

Ulcerative colitis and Crohn's disease are referred to as idiopathic inflammatory bowel disease (IBD). Although the etiology of chronic IBD is unknown, results from animal studies suggest that the endogenous intestinal bacterial flora plays an important role in the initiation and perpetuation of the disease (1). The association between the presence of specific bacterial species, such as pathogenic *Escherichia coli* or *Mycobacterium paratuberculosis*, and IBD has been suggested (2-4). However, the results of numerous studies investigating such an association, are inconclusive (2-4).

Commensal bacteria possess fimbrial and non-fimbrial adhesins, which enable them to adhere to the mucus layer and to the intestinal epithelial cells. *E. coli* strains carrying several types of adhesins, have been isolated from patients with IBD and from controls (2,3). In addition, *E. coli* strains isolated from patients with IBD have been observed to bind to extracellular matrix proteins in high numbers (5). Increased adherence of commensal bacteria to inflamed mucosa may enhance the exposure of the mucosal immune system to intestinal bacteria or bacterial components, resulting in sustained inflammation.

In the absence of identifiable etiologic microorganisms, it has been hypothesized that the intestinal inflammatory response in IBD is the result of an exaggerated intestinal host defense to commensal enteric bacteria or their components (1,6). It is unknown by which
factors and mechanisms the unrestrained inflammatory response is initiated and perpetuated. Although many data have been published about the inflammatory phenomena occurring during IBD, such as recruitment of inflammatory cells and changes in the expression and production of a large variety of inflammatory mediators in the bowel (7), little is known about the events leading to these phenomena.

It has been suggested (8) that in patients with IBD, genetically determined changes in the intestinal mucus, in particular alterations in glycosylation of mucus glycoproteins, result in weakening of the mucus barrier. Therefore, endogenous luminal bacteria or their components, would more easily degrade mucus, penetrate into the mucus layer and exert their effects on the intestinal tissue, leading to enhanced recruitment of inflammatory cells and thus to inflammation (8). While changes in glycosylation of mucin have been shown to occur in ulcerative colitis (9), and various bacteria produce mucin-degrading enzymes \textit{in vitro} (10,11), it is unknown whether luminal enteric bacteria degrade and penetrate into mucus \textit{in vivo}.

In situ hybridization (ISH) provides a means to study the spatial distribution of microorganisms in tissue by light-microscopy. Ribosomal RNA (rRNA) is an attractive target for hybridization since it is present in large quantities in active bacteria and probes can be chosen directed against either conserved or variable regions of the rRNA. To study the distribution of intestinal bacteria in human colonic mucosa, we developed a non-radioactive method for ISH of bacterial rRNA in paraffin embedded tissue. Using this method, we studied the spatial distribution of the bacterial flora in the mucosa of rectal and colonic biopsies from patients with IBD and from controls, in order to determine whether the presence of bacteria might contribute to the pathogenesis of IBD by increased penetration in mucus, increased adherence to epithelial cells or by invasion of the epithelium.
Materials and methods

PATIENTS AND CONTROLS

Nineteen consecutive patients with IBD who visited the Department of Gastroenterology for endoscopy were included. Patients were enrolled in the study if they had clinically active ulcerative colitis (UC), Crohn's disease (CD) or indeterminate colitis. Diagnosis was confirmed by endoscopy and by histology. Controls were 9 patients who required endoscopy for reasons other than IBD (e.g. irritable bowel syndrome) and who did not complain of diarrhoea. Controls were only included if results from endoscopy and histology of biopsies were normal. Patients or controls were not included if they had received antimicrobial therapy during the previous 6 weeks or if they had a history of colon carcinoma.

Four patients with IBD and 6 controls were prepared for colonoscopy by intestinal lavage with balanced electrolyte solution. Fifteen patients with IBD and 3 controls were prepared for sigmoidoscopy with a 500 ml water enema. In order to balance the numbers of patients with IBD and controls who were prepared with an enema, rectal biopsies were included, which had been stored at the Department of Pathology in 1997 and 1998. We included only biopsies which fulfilled the following criteria: 1. biopsies had to be obtained during sigmoidoscopy, by which no abnormalities were seen; 2. sigmoidoscopy was preceded by bowel preparation with an enema; 3. histological abnormalities were not present in the biopsies. Thus included were rectal biopsies from 3 patients with irritable bowel syndrome and 2 patients who underwent sigmoidoscopy to exclude a diagnosis of amyloidosis. No other stored rectal biopsies fulfilled the criteria mentioned.

SAMPLE COLLECTION

From each patient and control, 2-4 rectal biopsies were collected at approximately 10cm from the anal ring for in situ hybridization. Additional biopsies, taken from sites along the large intestine were obtained from 1 patient with UC, 2 patients with CD, 2 patients with indeterminate colitis and 2 controls. All biopsies were fixed in 4% buffered formalin and embedded in paraffin, according to routine procedures, and stored at room temperature.

OLIGONUCLEOTIDE PROBES

Probes were oligonucleotides with 5' and 3' fluorescein groups attached. Probe EUB338 (12) specific to the 16S rRNA of the domain Bacteria was slightly modified and designated
EUB338M (5'-ACTGCTGCCTCCCGTAGGAGTCTGG-3'). This probe was used for detection and localization of any bacterial species in the biopsy sections. An Epstein-Barr virus specific RNA probe (5'-CTAGCAAAACCTCTAGGGCAGCGTAGGTCC-3') designated EB1f, was used as a negative control. A 35-mer oligo dT probe designated dT35f, which detects mRNA of eukaryotic cells, was used for assessment of tissue mRNA integrity of the biopsies.

RNA IN SITU HYBRIDIZATION (RISH).

Paraffin sections (4 μm) were mounted on organosilane coated slides, baked at 60°C for 16-20 hr and stored at room temperature until needed.

For RISH, sections were deparaffinized in xylene and rehydrated to 70% ethanol and, without intermittent washing steps, subsequently immersed in 0.5M EDTA for 15 minutes at 37°C, in 5μg/ml proteinase K (Boehringer Mannheim) in 100mM Tris-HCl, 50mM EDTA, pH 7.5, for 30 minutes at 37°C, and in 70% ethanol for 1 minute.

After dehydration in ethanol, sections were air dried and 13 μl probe mixture containing 1μg/ml of the respective probe in hybridization mixture (0.45 M NaCl, 45mM sodium citrate, pH 7.0, 25% formamid, 10x Denhardts solution, 10% dextran sulphate, 1% Triton X-100 and 0.1% SDS) was applied to the sections and overlaid with a 18x18 mm glass coverslip.

Hybridization was performed for 18 hours at 37°C in a humidified container.

All subsequent steps were performed at room temperature and between steps the slides were rinsed twice with PBS containing 0.05% Tween 20 (PBST), for 5 min. After hybridization, sections were rinsed thoroughly in PBST. Endogenous peroxidase activity was exhausted in 3% hydrogen peroxide in PBST containing 1% sodium azide for 5 minutes. The fluorescein label on the hybridized probe molecules was detected as a hapten by subsequent incubation in PBST with rabbit-anti-FITC for 30 minutes (1:1000; Dakopatts, Glostrup, Denmark), biotin conjugated swine-anti-rabbit for 30 minutes (1:500; Dakopatts) and streptavidin-biotin-HRP complex for 30 minutes (1:200; Dakopatts). Peroxidase activity was developed using DAB chromogen. Nuclei were counterstained with hematoxylin.
RECONSTRUCTION EXPERIMENTS USING SHEEP COLLAGEN-COLON CELL SPECIMENS

Clinical isolates of *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Staphylococcus aureus* were grown on blood agar plates for 16 hours at 37°C. *Helicobacter pylori* was incubated for 72 hours under micro-aerophilic conditions at 37°C. The bacteria were swept from the agar plates and suspended in 1 ml of phosphate buffered saline, pH 7.2 (PBS). HT29-19a colon carcinoma cells were grown on large tissue culture wells in Dulbecco’s MEM (Life Technologies, Scotland, UK), buffered with HEPES (Life Technologies) and containing 10% fetal calf serum, in 5% CO₂ for 72 hours at 37°C. The cells were scraped and suspended in PBS. Bacteria were added to the cell suspensions and the mixtures were pelleted for 10 minutes at 500xg and resuspended in PBS. Aliquots of cell suspensions were gently pipetted onto rectangular blocks (1 x 2 x 5mm) cut from sheep collagen sheets (Johnson & Johnson, Amersfoort, The Netherlands) until the collagen matrix was saturated. The blocks were carefully fixed in 4% buffered formalin and embedded in paraffin according to routine procedures in our diagnostic pathology unit.

EXAMINATION OF BIOPSIES

*Estimation of mucosal surface length*

The length of the apical mucosal surface of each section was estimated microscopically by using a microscopic scale (Stöpler, Germany). The number of sections examined for each patient or control, varied between 2 and 4. The total surface length examined for each patient or control, was calculated by addition of the length of the surfaces of all sections examined by RISH.

*Examination of biopsies after in situ hybridization*

Biopsies were independently examined by two investigators (CS, FvdB), using an Olympus lightmicroscope. The entire mucosal surface length and underlying epithelium of each section was examined for the presence of bacteria. The presence or absence of bacteria in each section was recorded by using the following semiquantitative scale; 0: no bacteria visible; 1: sporadic bacteria visible, 1-10 single bacteria scattered among the entire section; 2: moderate numbers of bacteria visible, 10-50 bacteria per section; 3: large numbers of bacteria visible, > 50
bacteria per section. This semiquantitative scale was used because the clustered distribution of bacteria did not permit accurate assessment of the absolute numbers of bacteria if the number of bacteria was higher than approximately 50. A final assessment was obtained for the total mucosal surface length studied per patient, by combining the results of all sections from each patient. In addition, the location of the bacteria in the biopsy was recorded (associated with epithelium, inside epithelial cells, within the lamina propria). Consensus was reached in case of disagreements between the two investigators. The investigators were blinded as to whether biopsies were obtained from patients or from controls but overt signs of inflammation in the sections were visible, due to counterstaining with hematoxylin.

**Mucin staining**

Sections, adjacent to sections from the same biopsy which were used for RISH, were stained with Alcian Blue at pH 2.5. Colonic mucins are expected to stain light blue at this pH. Co-localization of mucin with bacteria, as detected by RISH, was recorded (no co-localization, probable, evident).

**Assessment of inflammation**

The severity of inflammation was assessed for each biopsy. Sections were stained with hematoxylin-eosin according to routine procedures. Severity of inflammation was recorded by an independent investigator (FK). The presence of ulceration, erosion, crypt abscesses, cryptitis were separately assessed semiquantitatively by assigning scores from 0 (absent) to 3 (severe). A final score was calculated for each biopsy by addition of the individual scores. Thus a maximum score of 12 could be reached. Inflammation was considered to be absent, if final scores were 0, or 1 due to minimal cryptitis (only 1 crypt affected). Active inflammation was considered to be present, if final scores were higher than 0, except in case of a score of 1, due to minimal cryptitis. In addition, the presence or absence of granulomata was assessed.

**STATISTICAL ANALYSES**

The median lengths of the apical mucosal surface in sections from biopsies of patients with IBD and controls were compared using Wilcoxon's rank sum test. The numbers of bacteria, as determined using the semiquantitative score described above, in biopsies from patients with IBD and controls were compared by using Fisher's exact test, and odds ratio's (OR) and 95% confidence intervals (CI) were calculated using Mantel-Haenszel estimates. Correlation
between the numbers of bacteria and the degree of inflammation in biopsies from patients with IBD, and between the numbers of bacteria and the use of anti-inflammatory agents, was analyzed by using Pearson correlation test.

Results

IN SITU HYBRIDIZATION OF SHEEP COLLAGEN-COLON CELL SPECIMENS
Sections of sheep collagen specimens containing HT-29 cells and different species of Enterobacteriaceae, *H. pylori* and *S. aureus* were hybridized with probes Eub338M, dT35f and EB1f. After hybridization with Eub338M, bacteria of all species tested, stained bright brown in contrary to the HT29-19A cells and the collagen in the tissue (fig. 1A). Pre-treatment with RNase abolished the signal completely indicating that the bacterial RNA was responsible for the signal (fig. 1B). Only a faint signal could be detected when sections were not pretreated with Proteinase K before hybridization (fig. 1C). HT29-19A cells stained brown after hybridization with the eukaryotic mRNA-specific probe dT35f, in contrast to the bacteria and the collagen (fig. 1D). No staining was observed after hybridization with EBV-specific probe EB1f. In all subsequent experiments, sections of sheep collagen-colon cell specimens were included and hybridized with probes EUB338M and dT35f, to serve as positive and negative controls, respectively.

EXAMINATION OF BIOPSIES

In situ hybridization of rectal biopsies

Rectal biopsies were obtained from 13 patients with UC, 3 patients with CD, 3 patients with indeterminate colitis and 14 controls. All patients with IBD had a relapse of their disease for which they required endoscopy, except one patient with indeterminate colitis who presented with a first manifestation. The examined surface lengths of patient rectal biopsies (median length 8.6 mm, range 2.6-22.1 mm) and control rectal biopsies (median length 11.6 mm, range 0.9-16.1 mm) were not significantly different (p=0.2).

After hybridization with probe dT35f, the goblet cells showed strong brown staining in all sections from patients and controls, indicating the presence of large amounts of mRNA in these cells. Although the sections were kept for several months before hybridization, none of
the sections had lost their tissue mRNA integrity, indicating that the paraffin embedded biopsies were resistant to RNAse during storage.

The presence of bacteria in sections of rectal biopsies from 19 patients with IBD and from 14 controls is shown in figure 2. Overall, rectal biopsies from patients with IBD contained significantly more bacteria than biopsies from controls (figure 2, p=0.004). High numbers of bacteria (>50/total mucosal surface length examined) were observed in 8 rectal biopsies from 19 patients with IBD (42%) and in none of the 14 controls. No bacteria were observed in rectal biopsies from 10 controls (71%) and 6 patients with IBD (32%) (p=0.04; OR 5.42, 95%CI 1.23 - 23.9). Only 4 of the rectal biopsies from the 14 controls (29%) contained bacteria, but numbers of bacteria were significantly lower than in the biopsies from the patients with IBD.

Figure 1 (opposite page). Results of rRNA in situ hybridization of sheep collagen sections containing HT-29 cells and *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhi* in equal amounts.

A: hybridization with probe Eub338M. B: pretreatment of section with RNA-se before hybridization abolishes the signal of the bacteria. C: omission of pretreatment with Proteinase K decreases antibody penetration resulting in diminished staining of bacteria. D: hybridization with probe dT35f results in staining of eukaryotic mRNA.
Table 1. **Numbers of bacteria observed after in situ hybridization in rectal biopsies from 19 patients with IBD and 14 controls, who were prepared by either intestinal lavage or enema.**

<table>
<thead>
<tr>
<th>Number of bacteria per total mucosal surface</th>
<th>Bowel preparation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lavage</td>
</tr>
<tr>
<td>length examined (%)</td>
<td>(%)</td>
</tr>
<tr>
<td>No bacteria</td>
<td>5 (50)</td>
</tr>
<tr>
<td>1-10</td>
<td>3 (33)</td>
</tr>
<tr>
<td>10-50</td>
<td>1 (10)</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>1 (10)</td>
</tr>
</tbody>
</table>

- Difference between patients with IBD and controls who were prepared with lavage and those who were prepared with enema is not statistically significant (p=0.2, Fisher’s exact test).

Figure 2 (opposite page). **Results of rRNA hybridization with probe Eub338M of rectal biopsies from patients with IBD and healthy controls.**

- ■ patient with ulcerative colitis
- ▲ patient with Crohn's disease
- ◆ patient with indeterminate colitis

Each symbol represents one patient or control

*Note*: Median lengths of total mucosal surface of sections examined from patients and controls were not significantly different.

Biopsies from patients with IBD contained significantly more bacteria than biopsies from controls (p = 0.004, Fisher’s exact test)
<table>
<thead>
<tr>
<th>number of bacteria per total mucosal surface length examined</th>
<th>controls</th>
<th>IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-50</td>
<td><img src="image1" alt="dots" /></td>
<td><img src="image2" alt="shapes" /></td>
</tr>
<tr>
<td>1-10</td>
<td><img src="image1" alt="dots" /></td>
<td><img src="image2" alt="shapes" /></td>
</tr>
<tr>
<td>0</td>
<td><img src="image1" alt="dots" /></td>
<td><img src="image2" alt="shapes" /></td>
</tr>
</tbody>
</table>
Among the 14 controls, stored control biopsies from 5 patients were included, in order to balance the difference in bowel preparation between patients with IBD and controls. The results obtained with these biopsies were similar to those results obtained with the 9 other control biopsies. The numbers of bacteria present in the rectal biopsies from patients and controls who had intestinal lavage did not differ significantly from patients and controls who were prepared with enema before endoscopy (p=0.2, table 1). In addition, separate analysis of the number of rectal biopsies containing no bacteria from patients and controls who had lavage, and from those who were prepared with an enema, showed no significant change in the estimated OR (OR lavage: 6.0, 95%CI 0.34-105.54; OR enema: 6.0, 95%CI 0.91-39.5).

In areas with > 50 bacteria, bacteria were always present in clusters of at least 10 bacteria. Such clusters were located in close vicinity to the epithelial cells. However, direct attachment of the bacteria to the epithelium was not observed (figure 3). In sections which contained less than 10 bacteria, single bacteria were loosely scattered along the epithelial surface. Endocytosed bacteria were not observed in the epithelial cells and no bacteria were found in the lamina propria. The majority of the bacteria was rod-shaped, but coccoid-shaped bacteria were present as well.

Figure 3 (opposite page). Hybridization of rectal biopsy sections from a patient with ulcerative colitis and from a healthy control with probe Eub33M and counterstaining with hematoxylin.

A-D: patient 1; A: 100x, B: 400x, C: 1000x, D: 100x, staining with Alcian Blue
E-F: control; E: 400x, F: 400x, staining with Alcian Blue
[ ] designates area containing bacteria
In situ hybridization of ileac and colonic biopsies

To study whether the number of bacteria in rectal biopsies was similar at other sites in the large intestine, multiple biopsies were taken from the terminal ileum towards the sigmoid. Results of biopsies from 2 patients suffering from Crohn's disease and indeterminate colitis respectively, indicate that the findings obtained from rectal biopsies are representative for the entire large intestine (table 2). Similar results were obtained when comparing biopsies from sigmoid and rectum from a patient with UC; from ileum and rectum from one patient with CD and one patient with indeterminate colitis; and from ileum and rectum, and colon and rectum from 2 controls (data not shown).

Mucin staining

Goblet cells stained bright blue after Alcian Blue staining. In biopsies from 10 (71%) of 14 controls, mucin was visible in a layer at the epithelial surface and in the crypts, and in 4 controls mucin spots were visible at the epithelial surface, in addition to the mucin in the crypts (fig. 3F). In rectal biopsies from 7 (37%) of the 19 patients with IBD, mucin was visible as a layer adherent to the epithelial surface and in the crypts. In 5 additional patients with IBD, mucin spots were visible at the epithelial surface, in addition to the mucin in the crypts. In biopsies from 7 of 12 patients with IBD, bacterial co-localization with mucin in adjacent sections, which were hybridized with probe EUB338M, was evident and biopsies contained > 10 bacteria per total mucosal surface length examined (fig. 3A-D). In biopsies from 4 of the remaining patients co-localization was probable and in 1 patient no co-localization was observed. From 3 patients with IBD, biopsies were obtained in which no bacteria were observed and also no adherent mucus was visible. In biopsies from 2 patients with IBD, no mucus was visible at the epithelial surface although bacteria were detected after ISH. In one patient, bacteria were only observed within the crypts, in which mucin was also visible after staining with Alcian Blue, while no mucus was detected at the epithelial surface. No attempts were made to quantify mucin contents of the biopsies since this can not be done reliably on formalin-fixed tissue.
Table 2. Bacterial counts after rRNA in situ hybridization and degree of inflammation in biopsies from various sites from two patients with inflammatory bowel disease

Patient 14386 (Crohn’s disease)

<table>
<thead>
<tr>
<th>Biopsy site in colon</th>
<th>Number of bacteria per total mucosal surface length examined</th>
<th>Degree of inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal ileum</td>
<td>10-50</td>
<td>0</td>
</tr>
<tr>
<td>Colon – site 1</td>
<td>&gt; 50</td>
<td>3</td>
</tr>
<tr>
<td>Colon – site 2</td>
<td>10-50</td>
<td>2</td>
</tr>
<tr>
<td>Colon – site 3</td>
<td>5-10</td>
<td>2</td>
</tr>
<tr>
<td>Rectum</td>
<td>&gt;50</td>
<td>3</td>
</tr>
</tbody>
</table>

Patient 14030 (indeterminate colitis)

<table>
<thead>
<tr>
<th>Biopsy site in colon</th>
<th>Number of bacteria per total mucosal surface length examined</th>
<th>Degree of inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal ileum</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Colon – site 1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Colon – site 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colon – site 3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Colon – site 4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rectum</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Score of inflammation: the presence of ulceration, erosion, crypt abscesses, cryptitis were separately assessed semiquantitatively by assigning scores from 0 (absent) to 3 (severe). A final score was calculated for each biopsy by addition of the individual scores. Thus, a maximum score of 12 could be reached. Inflammation was considered to be absent, if final scores were 0, or 1 due to minimal cryptitis (only 1 crypt affected). Active inflammation was considered to be present, if final scores were higher than 0, except in case of a score of 1, due to minimal cryptitis.
Assessment of inflammation

There was no correlation between the number of bacteria present in rectal biopsies from patients with IBD and the degree of inflammation (figure 4 and table 2). In addition, no correlation was found between the use of anti-inflammatory agents or sulfasalazine compounds and the number of bacteria present in rectal biopsies (data not shown).

Figure 4 (opposite page). Results of rRNA hybridization with probe Eub338M of rectal biopsies from patients with IBD and relation with inflammation.

■ patient with ulcerative colitis
▲ patient with Crohn's disease
⊙ patient with indeterminate colitis

Each symbol represents one patient or control

Score of inflammation: the presence of ulceration, erosion, crypt abscesses, cryptitis were separately assessed semiquantitatively by assigning scores from 0 (absent) to 3 (severe). A final score was calculated for each biopsy by addition of the individual scores. Thus, a maximum score of 12 could be reached. Inflammation was considered to be absent, if final scores were 0, or 1 due to minimal cryptitis (only 1 crypt affected). Active inflammation or relapse was considered to be present, if final scores were higher than 0, except in case of a score of 1, due to minimal cryptitis.

The number of bacteria per total mucosal surface length examined and the degree of inflammation are not correlated (p=0.4, Pearson correlation test).
Figure 4

Number of bacteria per total mucosal surface length examined:

- > 50
- 10-50
- 1-10
- 0

Inflammation score:

- 0-1
- 2-4
- 5-6
Discussion

In this study we investigated the spatial distribution of intestinal bacteria in the rectal and colonic mucosa in paraffin embedded biopsies from 19 patients with IBD and from 14 controls. For this purpose, we developed a non-radioactive method for ISH which enabled us to study the distribution of intestinal bacteria by light-microscopy. In order to detect as many bacteria as possible, we used a probe which has previously been shown to detect rRNA of eubacteria present in the human large intestine (12,13). In our ISH reconstruction experiments we demonstrated strong and specific staining of various bacteria associated with eukaryotic tissue culture cells. Most studies using ISH for determining the distribution of bacteria in tissue, have been performed with fluorescent probes (14-16). Although the use of fluorescent probes enables quantitative hybridization, it does not permit a detailed view on the exact location of the bacteria in the tissue since only the bacteria are visualized by fluorescence microscopy, but not the surrounding tissue cells. In contrast, using our indirect hapten based method we were able to locate bacteria among cultured cells, by light microscopy.

The biopsies from the majority of the controls contained no bacteria at all. Strikingly significantly more bacteria were observed in 8 biopsies from 19 patients with IBD than in biopsies from the 14 controls. Taking into account the broad specificity of the probe, it is likely that the observed bacteria belong to a variety of species, including aerobic- facultative anaerobic and strictly anaerobic bacteria. Biopsies taken from proximal and distal colon and rectum from the same patient, showed similar numbers of bacteria.

Bacteria in rectal biopsies stained sometimes less strongly after hybridization compared to the bacteria in the sheep collagen-colon cell tissue specimens, and in some biopsies a mixture of strongly and poorly stained bacteria was observed. Since the amount of rRNA is directly related to the growth rate of the bacteria, poor staining may be due to the presence of inactive or dead bacteria in rectal biopsies. Alternatively, it is possible that hybridization occurred, but that staining was weak due to poor penetration of the antibodies of the detection system into the bacteria, as illustrated in figure 1C. This could particularly be the case for Gram-positive bacteria which have previously been shown to perform less well in non-fluorescent ISH experiments than Gram-negative bacteria, probably due to differences in cell-wall composition (17). In addition, the presence of mucus in rectal biopsies may further hinder the penetration of the antibodies and may explain the observed difference in staining intensity between sheep collagen-colon cells tissue sections and rectal biopsies.
Several other investigators studied the mucosa associated flora (MAF) of the colon and rectum from patients with IBD by aerobic and anaerobic culture. Comparison of our data with the results from these studies is not possible since entirely different techniques were used. Hudson et al. (18) performed cultures of the MAF and feces from patients with Crohn's disease before and after antimicrobial therapy. They showed that the total viable counts of bacteria in feces were higher than the bacterial counts of the MAF. Hartley et al. (19), culturing the MAF in rectal biopsies from patients with active ulcerative colitis or quiescent disease, observed a wide variation in counts of intestinal bacteria between individual patients. However, in both studies, no data were available from patients without colitis. Poxton et al. (20) studied cultures of the MAF in colonic and rectal biopsies from patients with UC and from controls. Bacterial counts from proximal colonic and rectal biopsy samples in the same patient were similar. The authors did not observe any differences in the bacterial counts between patients and controls.

Although it is often assumed that commensal bacteria colonizing the human intestine resist removal by the luminal flow through adherence to the epithelial cells, evidence is accumulating that an association of endogenous intestinal bacteria with the intestinal mucus layer is more important for colonization than adhesion to epithelial cells. Poulsen et al. (13), studying the spatial distribution of \textit{E. coli} in the large intestine of streptomycin-treated and conventional mice by using fluorescent ISH of rRNA, found that \textit{E. coli} were not directly attached to the epithelium but were embedded in the mucosal material overlying the epithelial cells. Earlier studies in streptomycin-treated mice showed that human fecal \textit{E. coli} strain F-18 and its colicin deficient derivative F-18\textsuperscript{col\textsuperscript{T}} required growth in mucus, in order to colonize the large intestine (21,22). Recent experiments in streptomycin-treated mice, have demonstrated that avirulent \textit{Salmonella typhimurium} and its LPS-deficient mutant both colonized the large intestine by growth in mucus (16). By using fluorescent ISH, it was shown that the wild type strain was evenly distributed throughout the mucus layer but only initially associated with the epithelium, while the LPS deficient variants were confined to the outer layer of the mucus (16). Electron microscopic studies of human colonic biopsies have shown that resident bacteria in the large intestine are associated with the intestinal mucus layer (23). In our study, bacteria in colonic biopsies from patients with IBD were located in close vicinity of the epithelium and co-localized with mucin. Bacteria adherent to the epithelium were not observed.
It could be argued that the fixation method we used influenced the results obtained after hybridization. Formalin fixation is not the preferred method for preservation of the mucus layer in biopsies (24) and damage to the mucus layer may result in loss of adherent bacteria. However, biopsies from both IBD cases and controls were treated in an identical manner and it is thus unlikely that differences between both groups can be attributed to the fixation method only. Furthermore, since bacteria were associated with mucus, one would expect to find lower numbers of bacteria in biopsies with a disrupted mucus layer than in biopsies with an intact mucus layer. In contrast, we found that the biopsies from the controls contained no or few bacteria while the mucus layer was intact in the majority of biopsies from them.

There are several possible explanations for the difference in the number of bacteria present between patients with IBD and controls. First, subpopulations of fecal anaerobic bacteria have been shown to be able to degrade mucus by producing extracellular glycosidases (10,11). In addition, increased levels of fecal mucinase and sulfatase activity, presumably of bacterial origin, have been found in fecal extracts of patients with IBD (25,26). These data indicate that increased colonization with bacterial populations which are capable of degrading mucin and which easily adhere to or penetrate mucus, may occur during IBD. Second, Rhodes suggested that genetically determined changes in glycosylation of mucin may also contribute to an increased association of bacteria with mucus (8). He hypothesized that a genetic alteration in O-glycosylation may lead to "weak" mucus which has a decreased barrier function and is more susceptible to bacterial degrading enzymes (8). Finally, altered mucus contents in IBD, either due to inflammation or due to genetically determined changes, may result in differences in chemoattractant function of the mucus for intestinal bacteria. This may lead to increased mobility and survival of bacteria within the mucus. However, to our knowledge no studies have been performed addressing this issue.

No correlation was found between the number of bacteria in the rectal biopsy and the degree of inflammation. In addition, the use of anti-inflammatory agents did not appear to influence the degree of bacterial association with mucus. However, we did not study sequential biopsies from individual patients, which might have revealed such a correlation.

We postulate that an altered protective efficacy of the intestinal mucus in IBD results in an increased association of luminal bacteria with the mucus layer. Such an increased association may enhance or sustain the inflammatory process during IBD by exposing the mucosal surface, including the mucosal immune system, to bacterial antigens which otherwise would not have passed the mucus barrier. Bacterial adherence to or invasion of epithelial cells
was not detected by the methods we used, despite the enhanced association of bacteria with the mucus in patients with IBD.
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Reference List


