The interaction between the mucosal immune system and the commensal microflora of the colon
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Chapter 6

Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells

Chapter 6

Abstract

**Background.** The commensal intestinal microflora has important metabolic and perhaps also immune modulatory functions. Evidence accumulates that the microflora plays a role in the pathogenesis of inflammatory bowel disease. Therefore, there is a growing interest in the intestinal microflora and its interaction with the host. Presumably, this interaction takes place at the mucus layer. In this study we investigated the microflora that is present at the mucus layer and addressed the following questions: (1) does a specific mucus-adherent microflora exist? and (2) is there direct contact between commensal bacteria and epithelial cells?

**Methods.** Snapfrozen biopsies were taken of 5 colon regions and of the terminal ileum of 9 subjects with a normal colon. Faecal samples were also collected. Bacteria were detected in cryosections with fluorescent in situ hybridization (FISH) with 16S rRNA-targeted probes for all bacteria and specific probes for the major representatives (together quantitatively 70% of the bacteria) of the anaerobic microflora (bifidobacteria, *Bacteroides*, clostridia, atopobia) and aerobic microflora (*Enterobacteriaceae*, enterococci, streptococci, lactobacilli).

**Results.** With this sensitive technique bacteria were only observed at the lumenal side of the intestinal mucus layer. Very few microcolonies were present at the mucus layer and the composition of the bacterial microflora present in the faeces was similar to that at the mucus layer of the terminal ileum and colon regions.

**Conclusions.** The equal distribution of bacterial species suggests that intestinal commensal bacteria live in suspension in the lumen and that there is no specific mucus-adherent microflora. We did not observe direct contact between bacteria and epithelial cells.
Introduction

The human intestinal tract harbors a complex microbial ecosystem, usually referred to as the normal commensal microflora. This microflora consists mainly (>99.9 %) of obligatory anaerobic bacteria. The highest bacterial concentrations are found in the colon and terminal ileum: $10^{10} - 10^{12}$ bacteria per gram intestinal content. Each individual has a characteristic commensal colon microflora that is relatively stable over time. The normal microflora has many beneficial functions: synthesis of vitamin K and growth factors for host intestinal cells, prevention of outgrowth of potentially pathogenic bacteria (colonization resistance), stimulation of intestinal motility, promotion of the enterohepatic circulation by deconjugation of bile acids, stimulation and maturation induction of the gut immune system and modulation of the expression of a number of epithelial genes. The intestinal microflora, however, also plays a role in the pathogenesis of inflammatory bowel disease, especially Crohn’s disease. Modulation of the microflora with antibiotics, probiotics or diet may induce a remission in inflammatory bowel disease. Furthermore, there are many animal models that spontaneously develop a colitis, unless they are kept germ free. For all these reasons, there is a growing interest in the intestinal microflora and its interaction with the host.

How this interaction between the host and its commensal intestinal microflora takes place is not known. Theoretically, there are several possible mechanisms: (a) commensal bacteria may influence the mucosal immune system indirectly via uptake of whole bacteria or their fragments by either M cells, dendritic cells that send dendrites into the mucus layer, or via passive penetration of whole bacteria into the mucosa via incidental defects in the epithelial lining and mucus layer (e.g. due to a viral enterocolitis); (b) commensal bacteria may affect epithelial cells directly via epithelial Toll like receptors with affinity for bacterial cell wall components or via small soluble signals that diffuse through the gelatinous mucus layer. Commensal bacteria that are responsible for the interaction with the host are expected to be in close vicinity with the epithelial cells and the mucosal immune system: these bacteria may be present within the mucus layer or be attached to epithelial cells. In contrast to the common belief, the composition of the bacterial microflora within the mucus layer differs from the microflora in the intestinal lumen. An adherent bacterial population, if it exists, needs the capability to attach to the mucus layer or to epithelial cells and multiply within the mucus and, thus, form microcolonies. In contrast, luminal bacteria must be capable to multiply under the strict anaerobic conditions within the lumen and be able to degrade luminal fibers and other remnants and use these as nutrients.

In the present study, we investigated the commensal bacterial microflora that is present at the mucus layer and within the lumen of colon and terminal ileum. For this purpose, we examined biopsies of five regions of the colon and the terminal ileum of 9 patients with a macroscopic and microscopic normal colon. In order to preserve the mucus layer, the biopsies were snapfrozen. The bacteria present in the sections of the biopsies were detected with a sensitive technique: fluorescent in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes directed to all bacteria, in combination with probes specific for the major representatives of the anaerobic and aerobic microflora.

In the present study we show that commensal bacteria are only present at the luminal side of the mucus layer and do not have direct contact with epithelial cells. In each subject, the composition of the bacterial population residing at the mucus layer was identical to that in the lumen and no regional differences were found. In contrast to the common belief, these results suggest that intestinal commensal bacteria live in suspension in the lumen and do not specifically adhere to the mucus layer or to epithelial cells.
Materials and methods

Patients
Patients (aged 18-65 yrs, 6 males and 3 females) that underwent a colonoscopy, with as indication anemia (n=4) or abdominal pain (n=5), were asked to participate in the study. These patients had no comorbidity, did not use antibiotics within the past 4 weeks and had no macroscopic or microscopic abnormalities in colon and terminal ileum. Their bowel was prepared by drinking 4 liters of lavage fluid (Klean-Prep) containing polyethyleneglycol within 16 hours. In order to obtain biopsies from an unprepared colon, the bowel of two additional patients (mean age 55 yr, 1 male) was prepared with an enema and the colonoscope was introduced into the proximal colon, a fecal contaminated region that was not reached by the enema. One healthy subject (male 35 yr) donated a fresh solid fecal sample. The present study was approved by the local medical committee.

Sample collection and fixation
From each of the nine colonoscopy patients twelve biopsies were taken: two biopsies from 5 different colon regions (rectum, sigmoid colon, transverse colon, ascending colon and cecum) and two from the terminal ileum. Furthermore, a fresh fecal sample was collected from each patient and stored at –20° C. From each of the two patients with an unprepared colon 4 biopsies were taken out of a part of the colon with the feces in situ (ascending colon). All biopsies were snapfrozen within 10 minutes. Cryosections (8 µm) were airdried at room temperature and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 16 hours at 4°C, washed in PBS and then fixed 10 min in ethanol 96%. Of each fecal sample 0.5 gram was suspended in 4.5 ml of filtered (0.2 µm pore size filter) PBS and vortexed with glass beads for at least 3 min to homogenize the fecal sample. The suspension was centrifuged at 700 x g for 1 min to remove debris. The supernatant was fixed in 4% paraformaldehyde at 4°C for 16 hours and stored in 50% (vol/vol) ethanol-PBS at –20° C until further use 1,5. The fixed supernatants were diluted in PBS to a concentration at which approximately 100 bacteria could be seen through the microscope per high powerfield. Of these suspensions 10 µl was mounted on eight different slides and dried at room temperature. The solid fecal sample was fixed in 4% paraformaldehyde for 5 days at 4°C followed by ethanol 96% for 24 hours and then embedded in paraffin. Sequentially numbered 8 µm sections were made, deparaffinised with xylol 100% and washed in 96% ethanol before use.

FISH procedure
FISH was performed on biopsy sections, fecal suspensions and sections of the solid fecal sample. Sections and fecal suspensions were hybridised with a mixture of rhodamine-labeled BACT338 probe with specificity for all bacteria (including Helicobacter spp) in combination with one out of eight different fluorescein-labeled group-specific 16S rRNA-targeted oligonucleotide probes: ATO291 for Atopobium –group 17, BIF164 for bifidobacteria 19, ELGC01 for Eubacteria low G+C #2 group 20, EREC482 for Clostridium coccoides and Eubacterium rectale group 18, BAC303 for Bacteroides and Prevotella 21, ENTB1458 for Enterobacteriaceae 22, LAB158 for lactobacilli and enterococci 23, STREP493 for streptococci and lactococci 1. Furthermore, in each experiment appropriately pure cultured bacteria were hybridised as a positive control of the FISH procedure. Gram positive bacteria have an impermeable cell wall. Therefore, some sections were pretreated with lysozyme (2 mg lysozyme / ml 100 mM TRIS-HCl, pH=8.5, 37° C). One section of each biopsy was incubated with lysozyme for 15 minutes which permeates the cell wall of enterococci, streptococci and
lactococci, but not lactobacilli. This section was hybridised with a mixture of LAB158 and STREP493 in addition to the BACT338 probe. A second section of each biopsy was incubated with lysozyme for 75 minutes. This treatment permeates the cell wall of lactobacilli but severely damages enterococci in a way that they do not hybridise with the LAB158 probe. This section was hybridised with LAB158 in addition to the BACT338 probe. Sections and fecal suspensions were incubated with 5 ng oligonucleotide probe / µl hybridisation buffer (20 mM TRIS-HCl, pH=7.2, 0.1% sodium dodecyl sulfate, 0.9 M NaCl) at 50°C for 3 hours (BAC303 and ENTB1458) or 16 hours (all other probes). Sections and fecal suspensions were then subsequently washed with hybridisation buffer without sodium dodecyl sulfate for 20 min at 50 ° C, dipped in distilled water and rapidly dried with a flow of compressed air. The sections were mounted with Vectashield (Vector Laboratories, Burlingame, Calif.).

**Mucus staining**

To determine the intactness and thickness of the mucus layer, a fixed section of each biopsy was stained with alcian blue and evaluated by light microscopy. In order to specifically stain both the mucus layer and bacteria in the same section, some sections (not of all patients) underwent sequential staining with the FISH procedure (rhodamine-BACT338) followed by a wash with PBS and immunofluorescence (affinity-purified FITC-polyclonal goat-F(ab')2- anti-Hu IgA, 1:100, 1% bovine serum albumin, 30 min, 20°C).

**Biopsy sections hybridised with fluorescent probes**

Of each biopsy, eight sections were double hybridised with a mixture of BACT338-rhodamine and one of the eight different fluorescein-labeled group-specific probes. Each section was thoroughly evaluated by epifluorescence microscopy. This microscopic analysis was performed by three different observers. In the biopsies bacteria could be detected by their rhodamine fluorescence due to hybridisation with the BACT338 probe. Eukaryotic cells within the biopsy as well as the mucus layer could be clearly seen without any specific staining procedure due to their autofluorescence. To determine the spatial relation of commensal intestinal bacteria to the host, each section was evaluated for the presence of bacteria in the mucus layer, adhering to the epithelial cells, in crypts or in the lamina propria.

Within the same eight sections of each biopsy the percental and spatial distribution of the different bacterial groups was investigated. In each section if possible at least 200 bacteria (hybridising with BACT338-rhodamine) were evaluated for double hybridisation with a fluorescein-labeled group-specific probe and the percentage of the fluorescein-labeled bacteria was calculated. The whole section was investigated for the spatial distribution of the different bacterial groups in relation to the other bacteria. Clusters of fluorescein-labeled bacteria were regarded as microcolonies.

**Fecal suspensions hybridised with fluorescent probes**

The fecal suspensions that were mounted on eight different slides were hybridised with a mixture of BACT338-rhodamine and one of the eight different fluorescein-labeled group-specific probes. At least 400 bacteria that stained with BACT338-rhodamine were evaluated by epifluorescence microscopy for double hybridisation with the fluorescein-labeled group-specific probe and the percentage of the fluorescein-labeled bacteria was calculated.

**Solid fecal sample hybridised with fluorescent probes**

Sections of the solid fecal sample were hybridised with the eight different fluorescein-labeled group-specific probes. Each section was investigated by epifluorescence microscopy for the presence of microcolonies.
Statistical analysis
Data are presented as mean ± sd. The distribution of the percentages of bacteria that hybridised with the different probes did not significantly deviate from normality. The means were compared with the two-tailed Student’s t test.

Results

Preservation of the mucus layer
The aim of the present study was to determine the spatial relation of the commensal intestinal bacterial microflora to the host. Therefore, we attempted to preserve the fragile mucus layer by making cryosections. Of each biopsy, only the undamaged center part was evaluated. Alcian blue stained sections revealed that all biopsies had an intact mucus layer (fig 1). In both colon and terminal ileum the crypts were completely filled with mucus. In the terminal ileum the sides of the villi facing the lumen were covered by a thin continuous mucus layer (±10 µm) (fig 1a). The colon had a thicker flat mucus layer (79 ± 40 µm) (fig 1b). The thickness of the mucus layer in the present study was similar to that of other studies 24.

Figure 1. Terminal ileum (fig 1a, left picture) and colon (fig 1b) stained with alcian blue. In the terminal ileum the mucus (blue) fills most of the space between the villi. The lumenal side of the villi is covered with a thin mucus layer. The colon wall is covered with a thick flat mucus layer and also the crypts are filled with mucus. Bar = 50 µm

The spatial relation of the commensal intestinal bacterial microflora to the host
In colon and terminal ileum biopsies the intestinal cells and mucus layer could be clearly seen by epifluorescence microscopy without specific staining due to their faint autofluorescence. In
order to detect bacteria we used FISH with a rhodamine- or fluorescein-labeled probe that binds to all species (BACT338). Rhodamine- or fluorescein-stained bacteria showed colours that were different from the autofluorescent intestinal cells. Thus, technically bacteria could be detected within the mucosa. Bacteria were only detected at the luminal side of the mucus layer (fig. 2 and 3). We did not detect bacteria at the epithelial side of the mucus layer, within crypts, adhering to the epithelium or within the mucosa.

**Figure 2.** Colon hybridised with rhodamine-BACT338 and fluorescein-EREC482. The mucus (○) and epithelial cells are visible due to autofluorescence. Bacteria are only present at the luminal side of the mucus layer. EREC482-hybridising bacteria (green and yellow (bright rhodamine and bright fluorescein results in yellow staining)) are randomly dispersed between the other bacteria (orange-red). Lumen = *. Bar = 10 µm

**Figure 3.** Colon hybridised with rhodamine-BACT338 (FISH procedure) and stained with FITC-anti IgA (immunohistochemistry). Large amounts of IgA (green) are present in the mucus (○, fig 3ab), at the apical side of the epithelial cells and in plasma cells that are scattered within the lamina propria (fig 3a, left picture). The rest of the cells of the colon are darkred due to autofluorescence. Bacteria (orange-yellow, arrow) are only present at the luminal side of the mucus layer. Most of them are coated with IgA (fig 3b). Lumen = *. Bar = 50 µm (fig 3a), Bar = 10 µm (fig 3b)
In order to prove that the bacteria were present at the luminal side of the mucus layer, some sections were stained with FISH (rhodamine-BACT338) followed by immunofluorescence (FITC-labeled anti-human IgA). Figure 3 shows a typical example of the IgA containing mucus layer with bacteria at the luminal side. In 7 out of 108 biopsies, lymph follicles were present directly under the epithelial lining. The epithelial cells above these follicles were flattened suggesting the presence of M cells. Bacteria were not seen adhering to or present within these flattened epithelial cells. There was a large interindividual variation, but no regional variation, in the number of bacteria present in each section. One individual had only less than 5 bacteria per section in each biopsy. Three individuals had 100-200 bacteria per section. Whereas the sections of 3 other individuals contained over 200 (often more than 1000) bacteria per section. The biopsies taken from an unprepared colon had more than 1000 bacteria per section.

The composition of the bacterial microflora present at the mucus layer of the colon and terminal ileum in comparison to the fecal microflora.

If the composition of the bacterial microflora present at the mucus layer is different from the luminal flora one would expect (1) microcolonies suggesting local multiplication of bacteria at the mucus layer and (2) a difference in composition of the bacterial microflora at the mucus layer compared to the luminal (fecal) flora. Therefore, eight sections of each biopsy were double hybridised with a mixture of rhodamine-BACT338 and one of the eight different fluorescein-labeled group-specific probes. In nearly all sections the bacteria that hybridised with a group-specific probe, were scattered between the other bacteria (figure 2). Only in 7 out of 108 biopsies small microcolonies were found (figure 4). The microcolonies were found in the terminal ileum (3x), cecum, ascending colon, transverse colon and sigmoid. The microcolonies consisted of bacteria hybridising with EREC482 (3x), LAB158/STREP493 (2x), BAC303 and ATO291. The fact that only rarely microcolonies were detected at the mucus layer was not due to the bowel preparation since also microcolonies were absent within the biopsies taken from an unprepared region of the colon. Within each individual, the percentages of bacteria present at the mucus layer that hybridised with the 8 group-specific probes were identical to the percentages of the corresponding fecal suspensions (paired t-test). Furthermore, within each individual no regional (5 colon regions and terminal ileum) differences were found in the percentages of bacteria hybridising with the 8 group-specific probes (paired t-test) (data not shown). Table 1 shows the data of the 9 individuals for the terminal ileum, the pooled colon regions and the feces. Together these data suggest that there is no specific mucus-associated bacterial microflora.
Table 1. Mean (±sd) percentages of bacteria of the nine individuals that hybridised with group-specific probes. BACT338 = 100%.

<table>
<thead>
<tr>
<th>group-specific probe</th>
<th>terminal ileum</th>
<th>pooled colon regions</th>
<th>feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>anaerobic bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EREC482</td>
<td>21 (±14)</td>
<td>25 (±7)</td>
<td>20 (±4)</td>
</tr>
<tr>
<td>BAC303</td>
<td>19 (±16)</td>
<td>20 (±13)</td>
<td>17 (±11)</td>
</tr>
<tr>
<td>ATO291</td>
<td>6 (±5)</td>
<td>5 (±4)</td>
<td>6 (±3)</td>
</tr>
<tr>
<td>ELGC01</td>
<td>4 (±4)</td>
<td>4 (±4)</td>
<td>6 (±6)</td>
</tr>
<tr>
<td>BIF164</td>
<td>4 (±6)</td>
<td>4 (±7)</td>
<td>4 (±8)</td>
</tr>
<tr>
<td>facultative anaerobic bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAB158/STREP493 15' (*)</td>
<td>0.6 (±0.9)</td>
<td>0.7 (±0.4)</td>
<td>1.4 (±1.6)</td>
</tr>
<tr>
<td>LAB158 75' (**)</td>
<td>0.06 (±0.17)</td>
<td>0.04 (±0.9)</td>
<td>0.08 (±0.13)</td>
</tr>
<tr>
<td>ENTB1458</td>
<td>not detected</td>
<td>0.01 (±0.04)</td>
<td>0.02 (±0.03)</td>
</tr>
</tbody>
</table>

* 15': 15 minutes lysozyme pretreatment: mainly streptococci, lactococci and enterococci
** 75': 75 minutes lysozyme pretreatment: mainly lactobacilli

Solid fecal sample
Since only very few microcolonies were seen at the mucus layer we postulate that the replication of commensal intestinal bacteria takes place in suspension in the intestinal lumen. In the terminal ileum, cecum and ascending colon the lumenal contents is liquid whereas within the sigmoid and rectum the lumenal contents is more solid (feces). In order to determine whether there is local replication of bacteria within solid feces we performed FISH with the 8 group-specific probes on sections of a solid fecal sample.

Fibres of vegetable origin were scattered within the solid fecal sample. Most bacterial groups were diffusely dispersed. However, only with EREC482 microcolonies were seen, especially along vegetable fibers suggesting an interaction between bacteria and fibers (e.g., fermentation). Although only one solid fecal sample was investigated, these data suggest that only few bacterial species can multiply within solid feces. Thus, examination of fecal samples may be representative of the bacterial microflora within the whole colon and terminal ileum.

Figure 5. Section of a solid fecal sample hybridised with fluorescein-EREC482. A band of EREC482 hybridising bacteria (green, small arrow) is present along a vegetable fibre (line). Other EREC482 hybridising bacteria are diffusely scattered throughout the solid fecal sample and do not form microcolonies (*). Bar = 10 µm
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Discussion

In this study we show that (1) in the terminal ileum and colon commensal bacteria are present at the lumenal side of the mucus layer and not in crypts or attached to epithelial cells; (2) the composition of the microflora at the surface of the mucus layer is similar at all anatomical locations (terminal ileum, cecum, ascending colon, transverse colon, sigmoid and rectum) and to feces (lumenal microflora); (3) only few microcolonies are present at the mucus layer which indicates that local multiplication of bacteria at the mucus layer is rare. Together, these data suggest that the commensal microflora lives and multiplies in suspension in the intestinal lumen and that there is no specific mucus-associated microflora. This contrasts with the common belief that there is a mucus-adherent and a non-adherent microflora in the intestine 15, 16.

Thus, our findings suggest that bacterial multiplication occurs in liquid intestinal contents and will, thus, be optimal in ileum, cecum and ascending colon. In the fluid phase bacteria are mixed with nutrients (undigested food remnants, mucus, shed epithelial cells, and metabolites of other bacteria) and can live in symbiotic relation with each other. In the more solid feces of sigmoid and rectum bacteria, nutrients (e.g., fibers) and bacterial metabolites are fixed and contact is more difficult. Local concentrations of nutrients will, therefore, soon decline and concentrations of toxic (for bacteria) metabolites may increase. Both may prevent further outgrowth of most bacteria. In agreement with this we did not observe major clustering of bacteria in solid stool. Only fibers may remain as a nutritional source and only bacteria that can ferment these fibers may be able to multiply here.

In the present study, we show that in each individual, there are no regional differences in the composition of the bacterial microflora. In the colon, the liquid and solid fecal lumen contents are mixed and move back and forth by peristaltic movements 25. This prevents the development of regional differences in bacterial composition. Even reflux through the ileocecal valve may be common since our data show no differences in the composition of the microflora between terminal ileum biopsies and feces. These results indicate that regional differences in intestinal inflammation, as are often present in Crohn’s disease and ulcerative colitis, cannot be explained by regional differences in the composition of the bacterial microflora.

The probes used in this study can identify most of the bacterial groups of which some species are regarded as having probiotic activity: streptococci, lactococci, lactobacilli, enterococci and bifidobacteria. Therefore, our data show that also bacteria with probiotic activity have no direct contact with intestinal epithelial cells and do not specifically adhere to or multiply at the mucus layer.

Our data show that in healthy individuals there is no direct contact between commensal bacteria and epithelial cells. This is in agreement with data of Schultz et al. 26. Our data also suggest that in the healthy colon there is probably no direct contact of bacteria with cellular extensions of dendritic cells in the mucus layer nor with Toll-like receptors in the epithelial cell 9,12. Therefore, the mucus layer, containing large amounts of secretory IgA, lysozyme and defensins 27, appears to present an important layer of defense against direct contact between the mucosa and commensal bacteria. This may be important in the prevention of mucosal inflammation and, thus, inflammatory bowel disease.

Direct contact may take place at M cells. The human colon contains over 10,000 subepithelial lymphoid follicles that are covered by an extra thin mucus layer 26. Some of the epithelial cells above these follicles are M cells: specialised epithelial cells that allow the penetration of particles from the intestinal lumen and bring these in contact with the mucosal immune system. Enteropathogens like Salmonella, Shigella and Yersinia can specifically adhere and endocytose
through M cells. In this way, these pathogenic bacteria use M cells as their main portal of entry. However, M cells can also efficiently take up nonadhering latex particles of bacterial size and, thus, theoretically also nonadhering commensal bacteria. In the present study, we did not observe bacteria adhering to M cells in our biopsies. A possible explanation might be that only few lymphoid follicles were studied.

In conclusion, we have shown that intestinal commensal bacteria live in suspension in the lumen and there is no specific group of bacteria adhering to the mucus layer. We did not observe direct contact between commensal bacteria and epithelial cells. Therefore, the interaction between commensal bacteria and the host may take place via indirect mechanisms. We speculate that two mechanisms may be important for the interaction between the host and its commensal intestinal microflora (or probiotic bacteria): (a) lumenal sampling of bacteria by M cells and (b) soluble signals from bacteria diffusing through the size-selective gelatinous mucus layer that is adherent to the epithelial cells. To pass the mucus layer, these soluble signals must escape a shield of IgA that is abundantly present in the mucus.

Literature