A. SWIDSINSKI, V. LOENING-BAUCKE, A. HERBER

MUCOSAL FLORA IN CROHN'S DISEASE AND ULCERATIVE COLITIS - AN OVERVIEW

Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms and Internal Medicine,
Department of Gastroenterology, Hepatology and Endocrinology, Charite Hospital, Berlin, Germany

The intestinal flora harbors varies pathogens. *Clostridium perfringens* (gas gangrene), *Enterococci* (endocarditis), *Enterobacteriaceae* (sepsis), *Bacteroides* (abscesses) are present in the large intestine of every healthy person in high concentrations. These bacteria are, however, separated from the colonic wall by an impenetrable mucus layer and are tolerated by the host. This separation is disturbed in patients with inflammatory bowel disease (IBD), where bacteria adhere to the mucosa and invade epithelial cells with concomitant inflammatory response. This chronic bowel inflammation can not subside as long as the mucus barrier remains defective. The inflammatory response interferes with the state of tolerance to the intestinal bacteria and leads to characteristic changes in the biostructure of the faecal microbiota. These changes in the biostructure of faecal microbiota are specific for active Crohn's disease and ulcerative colitis (UC) and can be longitudinally monitored. The reason for the defect of the mucus barrier in IBD patients is unclear. Epidemiologic studies indicate a negative role of western lifestyle and foods and document the rise in the incidence of IBD in the industrialized countries during the 20th century. In parallel to this, detergents were introduced in households and emulsifiers were increasingly added to food. The cleaning effect of these on the colonic mucus has to be investigated. The present contribution summarizes new data on the biostructure of the intestinal microbiota.

K e y   w o r d s : *mucosal flora, inflammatory bowel disease, mucus barrier, intestinal microbiota, detergents, emulsifiers, fluorescence in situ hybridization, bacterial biofilms, polymicrobial infections, indigenous flora, mucus viscosity, bacterial movements, probiotics, inflammation, stool cylinder*

INTRODUCTION

Until the late 19th century, microbes were the major cause of death in humans. Ironically, the infectious nature of most diseases was not recognized. The treatment, if any, was focused on strengthening the general immunity. This changed with the identification of pathogens by Pasteur and Koch. The knowledge about microbial infections quickly expanded and reduced dramatically their impact on humanity. However these advances referred exclusively to mono-infections. The situation remained unchanged for polymicrobial diseases. A polymicrobial involvement is suspected in caries, pharyngo-tonsillitis, vaginosis, inflammatory bowel disease (IBD) and colon cancer. Research data on coronary heart disease, stroke and autoimmune diseases suggest that pathogens trigger the illness, however positive proof and understanding of causality are lacking. The current medical strategies for these diseases are therefore directed toward managing symptoms, conditioning immunity, and the search for the genetic background.

Most of the polymicrobial infections are probably not recognized. The reason for this unawareness is a lack of appropriate tools. Since Robert Koch and Louis Pasteur, we define a pathogen as a microorganism, which is isolated from a diseased person, absent in a healthy person and causes a disease upon transfection to a healthy person. The value of Koch's principles is however limited in case of polymicrobials. The polymicrobial community can not be grown elsewhere by transfection of single strains and the investigation of isolated microorganisms does not explain how the polymicrobial community functions or why it can flourish under conditions, which are deadly for each of the constituents. Their composite structure in relation to propagation and response to environmental challenges need to be monitored and studied in order to understand polymicrobial infections. Unfortunately, the polymicrobial communities can not be grown in culture. The environmental microbiologists, however, developed different tools to analyze microbiota *in situ*.

MATERIAL AND METHODS

One of the methods to analyze microbiota *in situ* is the ribosomal RNA fluorescence *in situ* hybridization (FISH). Depending on metabolic activity, each bacterial cell contains 10^4-10^8 ribosomes. Each ribosome includes an RNA molecule. Some areas of the ribosomal RNA are strain-specific, other are more universal. Based on sequences of the ribosomal RNA, probes can be synthesized to bind specifically to organisms of

interest. Using probes labelled with different fluorescent dyes, we can simultaneously visualize different types of microbes within complex communities. Over 100 FISH probes are currently available and allow explicit analysis of intestinal bacteria. It is not necessary that the bacteria are alive at the time of the investigation. The FISH investigations can be carried out any time and repeated, if the material is properly fixated (1, 2).

We have investigated biopsies from more than 10000 patients and controls using FISH in order to search for microbial roots of IBD. Human bowel is cleaned before the colonoscopy. To investigate the composition of the mucosa adjacent bacteria throughout the intestine without cleansing, we studied sections of whole mice intestine.

We tested the mobility of intestinal bacteria *in vitro* with a viscous gel layer containing different additives enclosed between two cellulose membranes which were placed on blood agar to attract bacteria (*Fig. 1*). The viscosity of the gel was adjusted by varying the concentration of agarose from 0.2% to 2%. Mixtures of enteric bacteria were overlaid onto the simulated mucus. After 28 hours of anaerobic growth, membranes were fixated, sectioned, and then examined by FISH (3).

We have also investigated stool probes from patients in form of faecal cylinders. These are punched out of the stool by the use of drinking straws, the stool is fixated, embedded in paraffin, cut to slices and hybridised with FISH probes representing 86 different bacterial groups (8).

Microscopy was performed with a Nikon e 600 fluorescence microscope. The images were photo documented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan).

RESULTS

Human

The most striking finding in our studies was a lack of contact between intestinal bacteria and the mucosa in normal subjects. In most healthy controls (84%), the intestinal wall throughout the ileum and colon was covered with mucus, which prevented that bacteria contact the mucosal surface (*Fig. 2*). In contrast to healthy controls, we found a dense coating of bacteria on the intestinal surface in nearly all patients with IBD. Bacteria adhered to epithelial cells, entered crypts and were sporadically found within cells. The intracellular bacteria were located mainly at the bottom of the crypts, which were in most cases empty of bacteria, but not in the columnar epithelium, which directly contacted the dense masses of bacteria (*Fig. 3*). Although adherent bacteria were present in nearly all (94%) IBD patients who had not been treated with antibiotics, the highest concentrations of mucosal bacteria were found in less or macroscopically non-inflamed regions rather than in the inflamed regions of the intestine. In inflamed regions, the bacterial concentrations were reduced due to leukocytes that migrated to the outer regions of the mucus, either preventing access to the mucus layer or exerting antimicrobial effects (*Fig. 4*). Despite high concentrations of leukocytes and reduced numbers of bacteria in the mucus of inflamed gut segments of IBD patients, some of these bacteria reached the intestinal wall leading to development of ulcers, fissures, abscesses and deep tissue infiltrates (*Fig. 5*).

The bacterial adherence to the mucosa was not IBD specific. Bacterial concentrations of 10^9 bacteria/ml or higher were found in nearly all patients with IBD, but also in patients with self-limiting colitis (Sl-colitis), coeliac disease, HIV enteropathy, 62% of patients with acute diarrhoea, 52% of patients with diverticulosis, 45% of patients with carcinoma or polyps, and in 38% of patients with irritable bowel disease (IBS). However, the mean density of mucosal bacteria was significantly lower in groups without intestinal inflammation and the composition of the biofilm was different. Bacteria of the *Bacteroides fragilis* group and *Enterobacteriaceae* were responsible for >60% of the biofilm mass in IBD, but only for 30% in self-limiting colitis. In contrast, bacteria that positively hybridized with the Erec (*Eubacterium rectale*) and Fprau (*Fecalibacterium prausnitzii*) probes accounted for >50% of the biofilm in IBS patients, but only for <30% of the biofilm in IBD. Bacteria other than *Bacteroides, Enterobacteriaceae, Fecalibacterium prausnitzii* or *Eubacterium rectale* were predominant in self-limiting colitis (*Fig. 6, Table 1*).
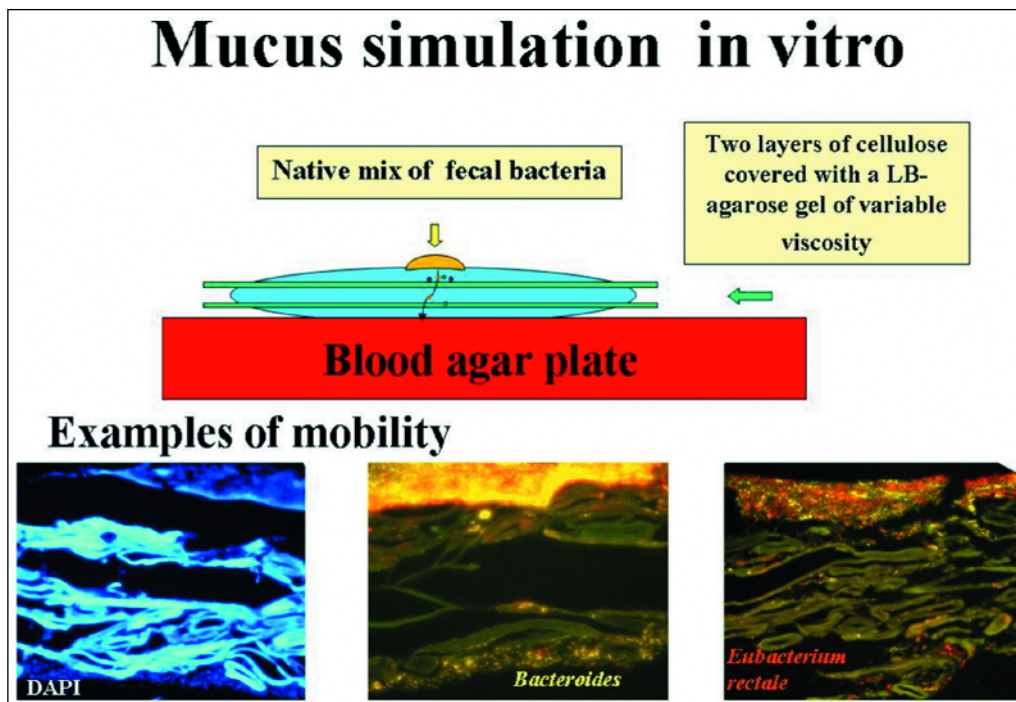

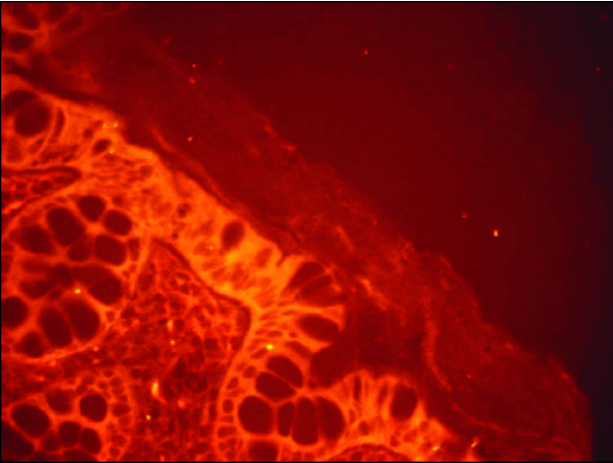*Fig. 1.* Mucus simulation *in vitro*.

Fig. 2. Human colonic wall of healthy controls (84%) is covered with mucus that excludes bacteria from contact with the colonic mucosa
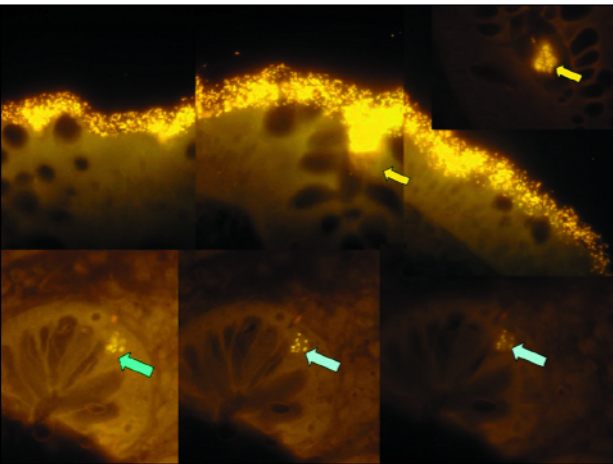

Fig. 3. Prolific *Bacteroides fragilis* biofilm completely covers the mucosal surface and enters crypts in a patient with Crohn's disease (upper part); focusing on the intraepithelial bacterial inclusions in the same patient indicates, that bacteria are located within the tissue and not overlaid (blue arrows-lower part)
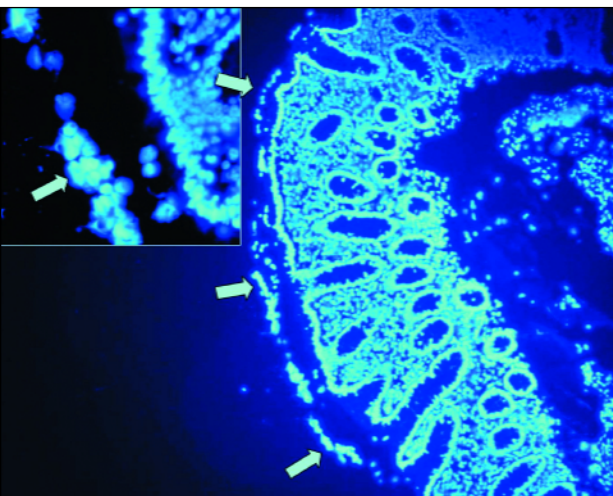

Fig. 4. Leukocytes migrate into the mucus, align in the outer regions and prevent access to the mucosa.
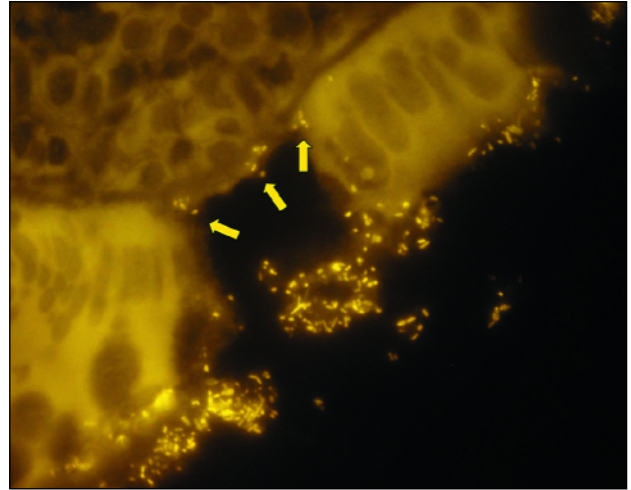

Fig. 5. Ulceration of the epithelial surface in a patient with ulcerative colitis. Bacteria attach to the exposed mucosa. (ulcer ground, arrows)

For better understanding and comparison of the findings, only microphotographs hybridized with the same set of probes are shown throughout this overview. Thus, in the figures *Bacteroides* is Cy3-stained and appears yellow, *Eubacterium rectale* group (EREC probe) is Cy5-stained and has red fluorescence, and all other groups are FITC-stained and appear green. The colours are shown as they appear through the microscope or camera. Micrographs are not manipulated.

Experimental studies

Our tests with agarose (*Fig. 1*) showed that only small coccoid rods of the *Bacteroides* group moved at an agarose concentration of 0.2%. The long rods of the *Eubacterium rectale* group were immobilized (*Fig. 7*). *Bacteroides* was immobilized and only long rods of *Eubacterium rectale* moved at agarose concentration of 0.5% (*Fig. 8*). The movement of all bacterial groups was inhibited at agarose concentrations of 0.7% (*Fig. 9*). The segregation of bacteria in the proximal colon in mice into those bacteria which contact the mucosa and which are separate from the mucosa is therefore not a result of adherence of "probiotic" bacteria but is due to moderate viscosity of the mucus layer in this region, which permits bacteria with a long curly rod shape to move and contact the mucosa but immobilizes the coccoid or short rod shaped bacteria.

Mice

Small intestine of healthy wild type mice contain no bacteria which can be definitively detected by FISH, corresponding to a bacterial concentration of less than 10^6 bacteria/ml. The few microorganisms found were heterogeneously composed, random, and without signs of adhesion or contact with the intestinal wall. All of them were separated from the colonic wall by a mucus layer. The large intestine of healthy wild type mice contain a highly concentrated mass of bacteria. A distinct mucus gap devoid of bacteria completely separates the colonic wall from the highly concentrated bacterial biomass in the distal colon. The width of the mucus layer increases progressively from the middle to the distal colon. No bacteria contact the colonic wall. The same segment stained with alcian blue demonstrates that the gap is indeed filled with mucus (*Fig. 10*). The situation in the distal colon of mice is identical to the situation in human. In the proximal colon of healthy mice, the situation is completely different to that observed in

Table 1. Percent of bacteria within the biofilm in patients with CD, UC, Slc, IBS and in controls.

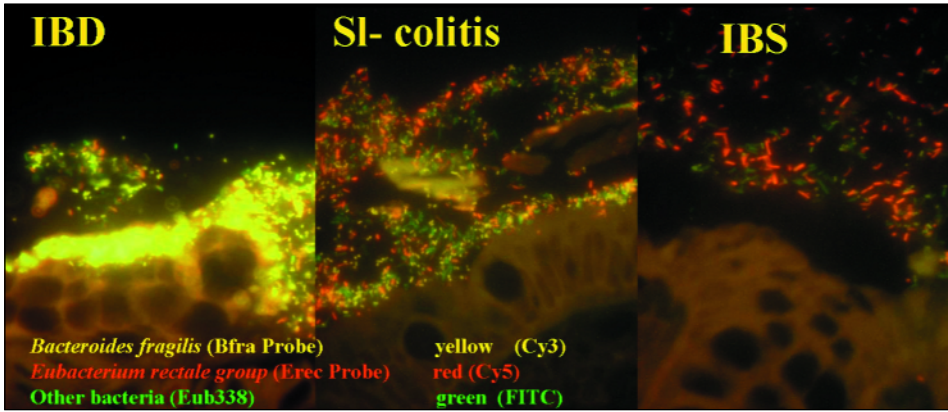| Percent of patients with 10^9 bacteria/ml		CD 98%	UC 94%	Slc 78%	IBS 38%	Contr. 16%
Percent of bacteria within biofilm	Bfra	60%	30%	31%	14%	16%
	Erec	10%	5%	18%	48%	32%

Fig. 6. Bacteroides fragilis (Bfra Probe) *Eubacterium rectale* group (Erec Probe), other bacteria (Eub338) in a patient with inflammatory bowel disease, self-limiting-colitis and irritable bowel disease.
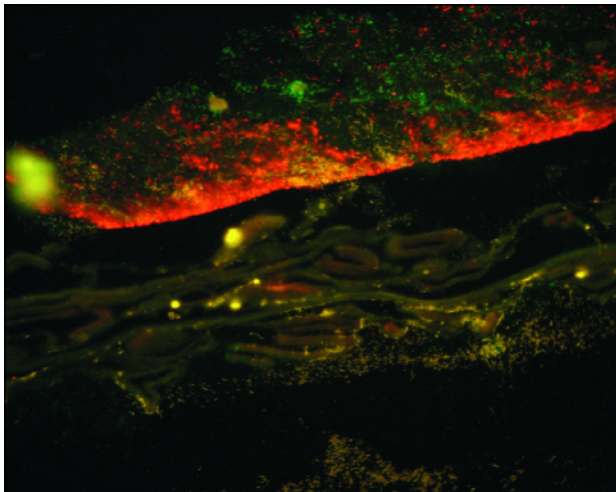

Fig. 7. Bacterial velocity through gels of different viscosity is species specific. Small coccoid rods of the *Bacteroides* group have the highest velocity in gels with low viscosity, here 0.2% agarose
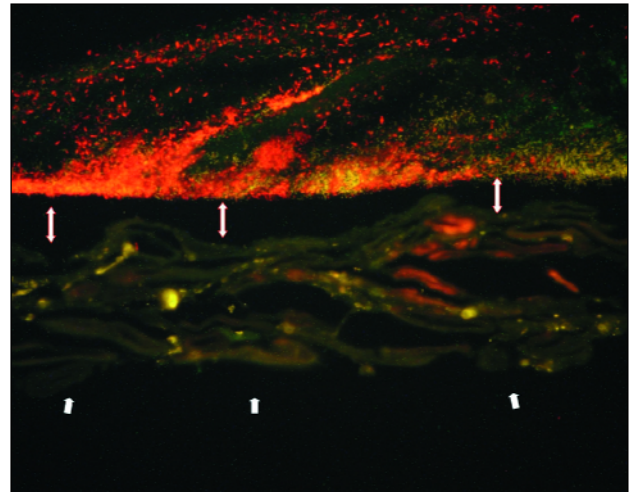

Fig. 9. In 0.7% agarose (arrows); bacteria are absent below the membrane. A gap between the bacteria and the membrane indicates a lack of bacterial movement across the gel layer (double headed arrows).
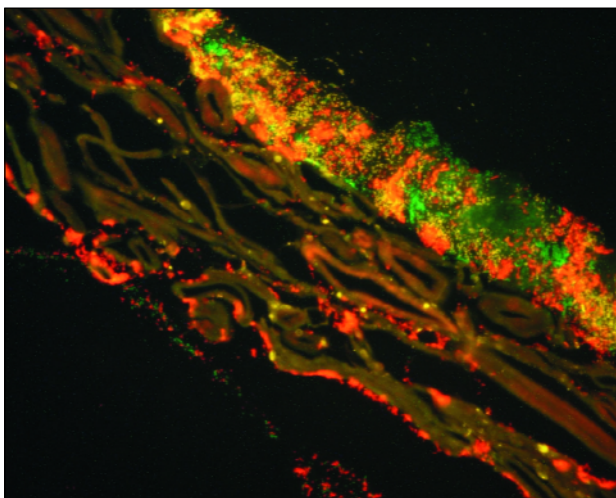

Fig. 8. Long rods of *Eubacterium rectale* group (EREC, red) have the highest velocity in gels with high viscosity, here 0.5% agarose.

healthy human. Luminal bacteria directly contact the colonic wall in the healthy mouse. However, this contact is selective, while *Eubacterium rectale* contacts mucosa and enters crypts to large numbers, *Bacteroides* is separated from the colonic wall. The differences in arrangement of bacterial groups are especially obvious in multi-colour FISH visualizing different species in different colours within the same microscopic field. *Eubacterium rectale* are condensed in extremely dense mats adjacent to the mucosa, which are clearly demarcated from the rest of the faeces and *Bacteroides* (*Fig. 11*). The first impression is that *Eubacterium rectale* prevents *Bacteroides* from contact with the mucosa. This impression is wrong. Bacteria which were separated from the colonic wall were represented by *Bacteroides*, *Enterobacteriaceae*, *Clostridium difficile, Veillonella* and other groups. Typical for these groups was not the biochemistry or phylogenetic relationship, but the bacterial cell morphology of short coccoid rods (*Fig. 12*). Bacteria contacting the proximal colonic wall in mice were also
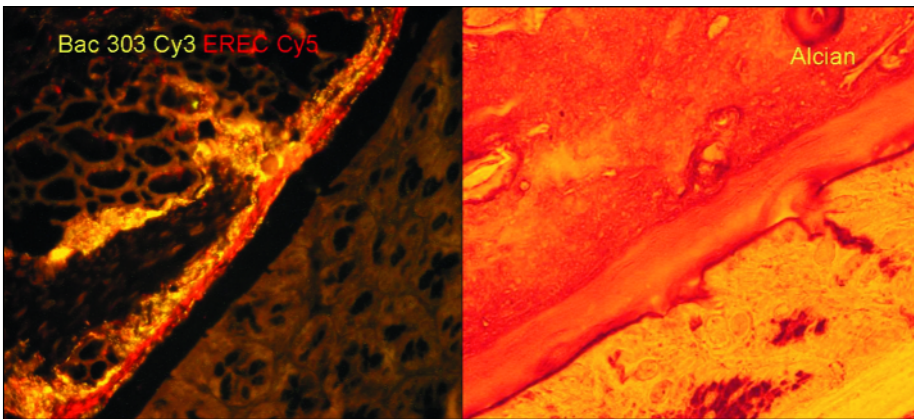
Fig. 10. The mucus completely separates mucosa from faeces in the distal colon of mice similar to the situation in man (as shown in *Fig. 2*).
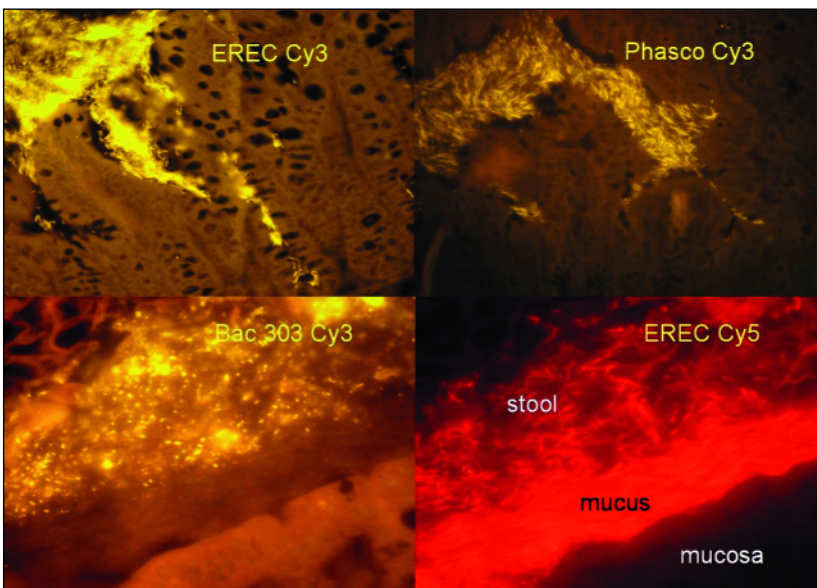

Fig. 11. The separation of bacteria in the proximal colon of mice is selective, *Eubacterium rectale* (EREC) and its subgroup (Physco) enter crypts, *Bacteroides* has no contact with the colonic wall.
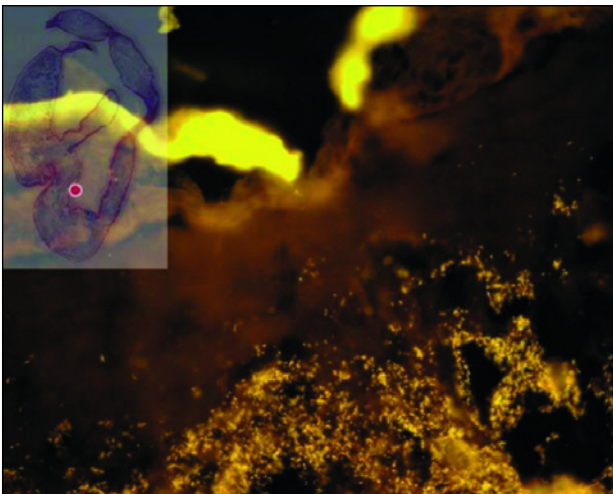

Fig. 12. Short rods of *Bacteroides*, *Enterobacteriaceae*, *Clostridium difficile*, and the *Veillonella* groups have no contact with the colonic wall in mice.

represented by different groups belonging to *Eubacterium rectale* (EREC), *Bifidobacteriaceae* (Bif probe) *Lactobacillius* and other groups. Common for these bacteria was their shape of long often curly rods. The bacterial shape is important for the bacterial movements. Short rods are equipped with multiple pili. Pili enable

movements in a watery environment but not in slime. Short rods have additionally flagella, which like propeller move them through slime. Long curly rods use complex body movements to screw through gels of high viscosity, but are immobile in water (4-6).

The presence of the mucus barrier in the proximal colon of mice can be clearly demonstrated in germ-free mice mono-associated with *Enterobacter cloacae* – a bacterium with a short coccoid form. The distinct mucus layer and separation of bacteria from the colonic wall can be observed in both the distal and proximal colon. Bacteria are perfectly separated in the distal colon, however in the proximal colon some bacteria can be found inside of isolated vacuoles of the goblet cells, especially at the bottom of crypts (*Figs*. 13, 14). The undifferentiated epithelial cells at the base of crypts are primarily mucus-secreting cells, whereas differentiated cells of the columnar epithelium are mainly absorptive cells, removing water and electrolytes from the mucus. The epithelial stem cells at the crypt base proliferate and replace surface cells within 4–8 days. The dissemination of *E. cloacae* in crypt bases and goblet cells outline sones of lower viscosity and confirms independently that during the journey from the crypt base toward the surface epithelium crypt cells become increasingly differentiated and absorptive. The adsorptive cells of the crypt neck and of the epithelial cells of the columnar epithelium dehydrate the mucus layer. Dehydration makes the mucus layer solid and impenetrable for bacteria and protects sites of mucus production and the mucosa from encounters with potential pathogens. The lower viscosity of the mucus at the crypt
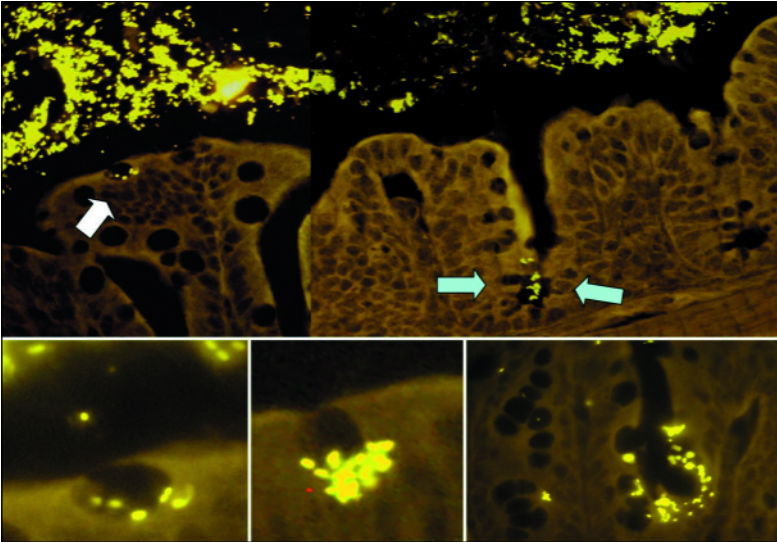
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Fig. 13. Proximal colon of mice mono-associated with *Enterobacter cloacae*. Despite the separation by the mucus layer, bacteria can be found inside of single vacuoles of the goblet cells, especially at the bottom of crypts where the viscosity of the mucus is lower.
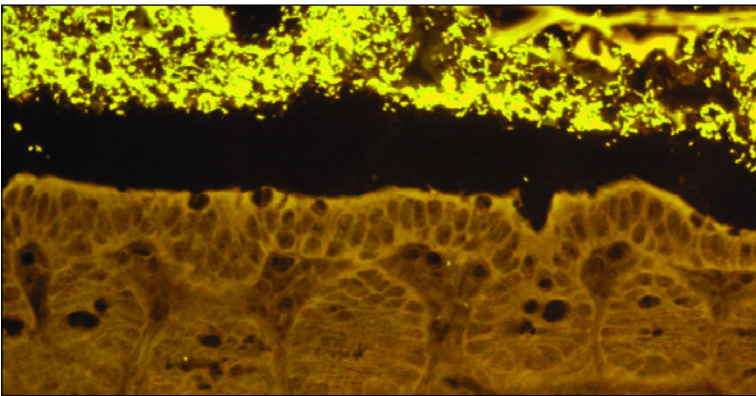

Fig. 14. Distal colon of mice mono-associated with *Enterobacter cloacae*. Bacteria are perfectly separated from the intestinal wall.
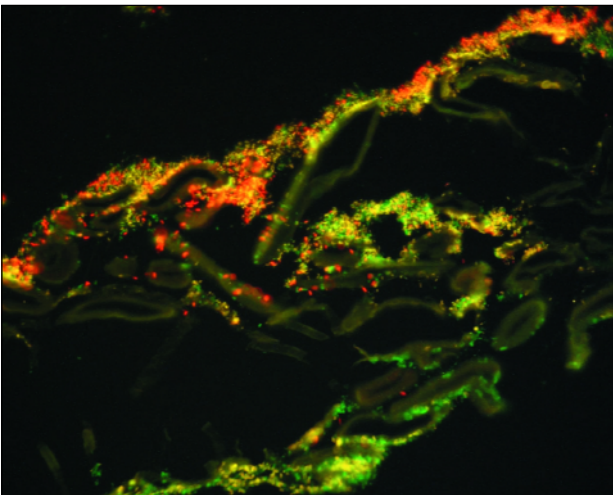

Fig. 15. DSS supplement to gel or to the suspension of faecal bacteria enhances bacterial movements. In DSS supplemented gels, short coccoid bacteria such as *Bacteroides* move up to agarose concentrations of 0,6%. The movements of long rods (EREC) across the mucus can be observed up to concentrations of 0,9%.

Fig. 16. Leukocytes (arrows) migrate into the lumen of the large intestine.

cytoplasm of the columnar epithelial cells in mono-associated mice. Interestingly, crypt abscesses, which are typical histomorphologic findings in human self-limiting colitis and IBD, are also more abundant toward crypt bases.

Effect of dextrane sodium sulphate on the bowel of mice

What happens if the viscosity of the mucus layer is reduced for example by addition of detergents? The addition

base promotes emptying of crypts and prevents obstruction, but as a drawback it may make these types of cells more vulnerable to invasion by potential pathogens. Indeed, invasion of epithelial cells by *E. cloacae* was observed exclusively at the crypt bottom, whereas no *E. cloacae*-containing cells were observed within the
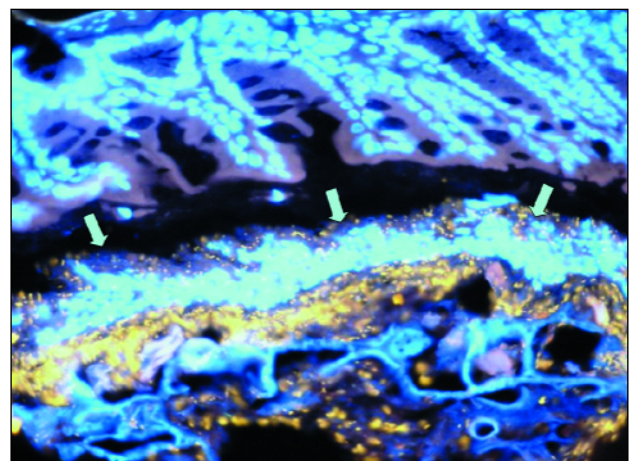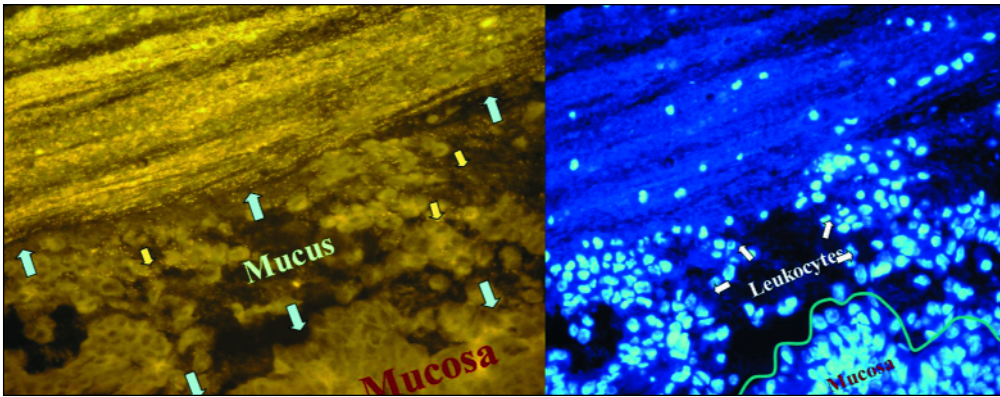
Fig. 17. Bacteroides crosses the mucus (left). The same microscopic field in DAPI (right) shows leukocytes (large blue nuclei) migrate into the mucus and hinder *Bacteroides* from moving towards the mucosa. Normally only single leukocytes are present in the mucus.
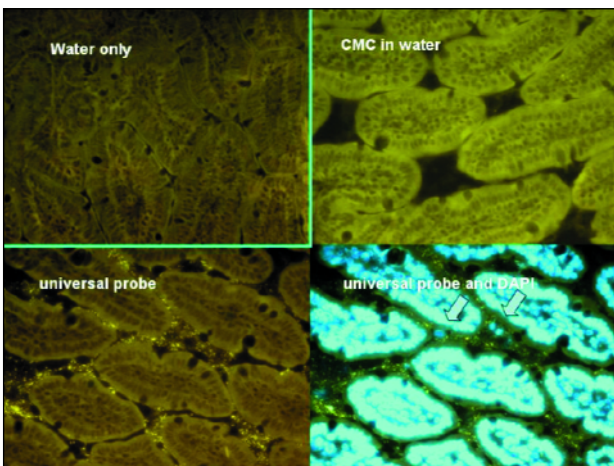
migration of bacteria toward the mucosa. In the distal colon, *Bacteroides* circumvents the leukocytes, passes through mucus, adheres to the mucosa, and causes deep tissue infiltration (*Fig. 17*). The inflammation in the DSS animal model is restricted to the large bowel, although the substance is provided in the drinking water and should have theoretically the same effect throughout the intestine. However bacterial concentrations in the small intestine of mice are extremely low compared to bacterial concentrations in the colon. Mucus barrier failure has therefore fewer consequences in the small intestine than in the large intestine.

Fig. 18. Proximal jejunum, IL-10 KO mice. The arrows indicate bacteria and leukocytes between villi.

Experiments in IL-10 gene-deficient mice treated with carboxymethyl cellulose

Beate Sydora (Alberta University, Canada) has treated IL-10 gene-deficient mice with 2% carboxymethyl cellulose (CMC) dissolved in water. Normally IL-10 knock-out mice develop colitis in adult age. The small intestine is not involved. The pattern of distribution of inflammation is in accordance with murine bacterial colonization. IL-10 knock-out mice have usually no bacteria in the small intestine and high bacterial concentrations in the large intestine. In the CMC experiments of B. Sydora, the controls which consisted of mice treated with water only, had no inflammation and no bacteria between villi in the small intestine. However bacteria and leukocytes were found between villi in the proximal parts of the small intestine in half of the CMC treated mice. (*Fig. 18*) The intensity of changes increased in the distal direction. High bacterial concentrations were found within crypts of Lieberkuhn in the ileum of all CMC treated IL-10 knock-out mice (7). These findings resembled visually the situation, which can be observed in the ileum of patients with Crohn's disease (*Fig. 19*).
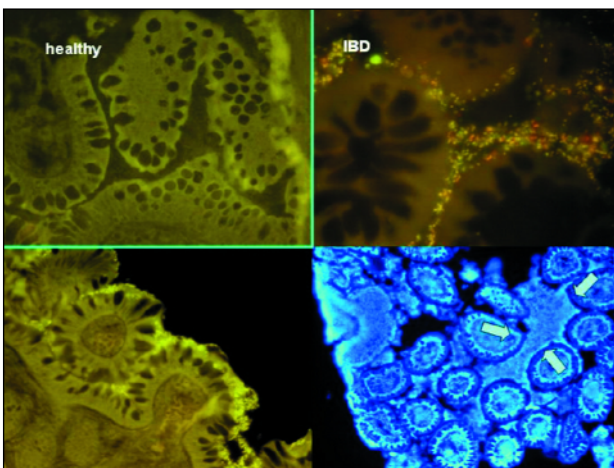

Fig. 19. Ileum of patients with Crohn's disease shows the similarities to the proximal jejunum in the IL-10 KO mouse, treated with carboxymethyl cellulose, the arrows indicate leukocytes between villi.

The biostructure of faecal microbiota in stool samples of human

The evaluation of therapies remodelling the mucus barrier affords simple and effective criteria for efficacy, which are independent of subjective complaints. FISH investigation of bioptic material is an important method, however biopsy can not be performed repeatedly just for the study of the effects of therapy. However the disturbance of the mucus layer leads to changes in the biostructure of faecal microbiota, which can be investigated. We developed a method to investigate the biostructure of faecal microbiota. Faeces proved to be highly spatially organized. Healthy faecal microbiota can be divided into habitual bacterial groups and occasional bacteria, present only in subgroups of patients, either diffusely or locally condensed. With regard to the faecal mucus, bacteria could be divided in faecomucous, mucophob and mucotrop. We found that the stool is covered with mucus which is free of bacteria in healthy persons (*Fig. 20*). The

of dextrane sodium sulphate (DSS) in *in vitro* experiments makes the gels penetrable for bacterial movements at viscosity levels, which normally completely immobilise bacteria (*Fig. 15*). In mice, the addition of DSS to food leads to a colitis. In DSS colitis, leukocytes migrate into the colonic lumen and line up at the border between mucus and faeces (*Fig. 16*). However even this leukocyte response can not stop the

Fig. 20. Stool cylinder, thionin blue staining.

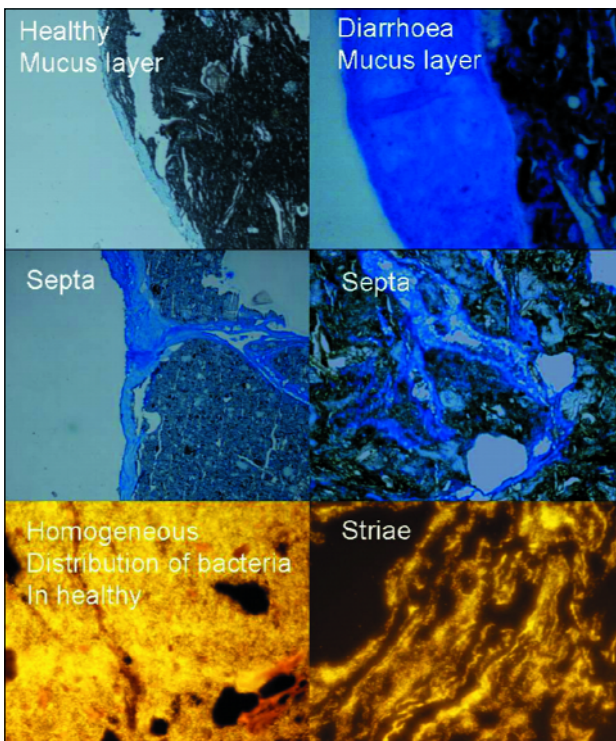*Fig. 22.* Leukocytes covering the surface of the faecal cylinder.

Fig. 21. Distribution of mucus in healthy patients and in patients with diarrhoea.

mucus secretion was increased in patients with diarrhoea. The superficial mucus layer was thicker and mucus could also be found within faeces enclosed in form of broad septa or multiple striae (*Fig. 21*). In ulcerative colitis, the mucus was significantly reduced compared to all disease control groups and to healthy controls. The surface of the faeces was covered with a layer of leukocytes instead. The occurrence of leukocytes stresses the advantages of using stool cylinders over faecal homogenates, since no leukocytes are located within the faecal masses (*Fig. 22*).

So far, we have investigated more than 5000 faecal cylinders. The evaluation of 12 of the most representative bacterial groups in healthy, non-inflammatory disease controls, UC, and CD revealed many characteristic details, which enable the discrimination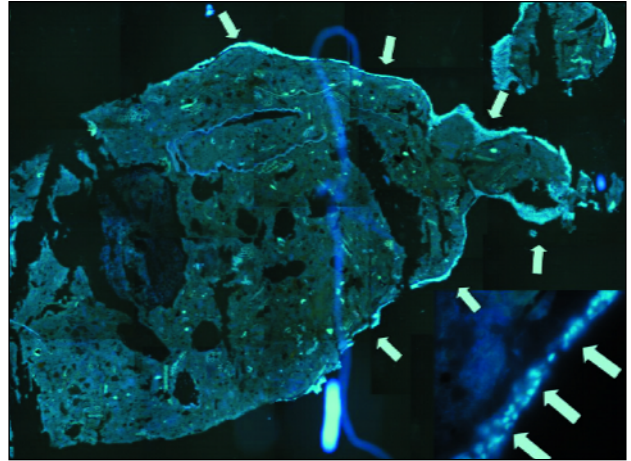 between these conditions. The most prominent features in IBD were: reduction of the mucus thickness especially in UC, progressive decrease in the concentrations of the habitual bacteria and disintegration of their web structure, spheroid precipitation of *Bacteroides* to isolated island in patients with UC, increased concentrations of leukocytes in the mucus and on the surface of faeces in UC, reduction and loss of *Faecalibacterium prausnitzii* in CD, high concentrations by excellent fluorescence of *Faecalibacterium prausnitzii* in UC, increased concentrations and occurrence of mucotrop *Enterobacteriaceae* with decreased concentrations of mucotrop. Verucomicrobiaceae (Hel274) in both CD and UC patients, increased concentrations of faecal cylinders.

Enterobacteriaceae in CD with low concentrations of faecal *Enterobacteriacae* in patients with UC, reduced occurrence of *Eubacterium hallii* and *E. cylindroids* bacteria in CD, and elevated concentrations of *Bifidobacteriaceae* and *Atopobium* in patients with UC. The dynamics in concentrations and/or occurrence of *Faecalibacterium prausnitzii*, faecal *Enterobacteriaceae*, *Bifidobacteria*, *Atopobium, Eubacterium cylindroides, E. hallii* and leukocytes were strikingly opposite in UC and CD, allowing differentiation between both diseases and indicate that these diseases are distinctly different entities and not just different expressions of the same inflammatory process (*Table 2*). However, the quantitative assessment of 2 parameters: leukocytes at the faeces/mucus border and *Faecalibacterium prausnitzii* concentrations were sufficient to diagnose active CD and UC with a 79/80% sensitivity and 98/100% specificity.

The lack of sensitivity in the investigation of faecal cylinders in order to diagnose active CD and UC (79/80%) was due to overlap between Crohn's disease and UC and intermediate colitis, and the lack of specificity (98/100%) was due to overlap between Crohn's disease and coeliac disease/carcinoid of the small bowel. No overlap occurred between IBD and healthy controls, self-limiting colitis, and non-inflammatory disease subjects. In fact, none of the subjects from the healthy or the non-inflammatory control groups matched criteria for IBD.

DISCUSSION

It has been previously assumed that the enormous masses of bacteria present in the intestine directly contact the intestinal wall. The non-pathogenic bacteria are tolerated, while the pathogenic bacteria are responded to. Dysfunction of the immunologic balance would lead to overreaction to normal non-pathogenic faecal components, thus initiating and sustaining

Table 2. Results of investigations of stool cylinders.

	Ulcerative colitis				Crohn's disease				IC	CD coeliac	Slc	Healthy	other
	CAI >9	3≤CAI≤9	Rem. ≤1 year	Rem. >1 yaer	CDAI >200	150≤ CDAI ≤300	Rem. ≤1 year	Rem. >1 yaer					
85 82	N=27	N=32	N=14	N=32	N=19	N=23	N=18	N=22	N=17	N=12	N=9	N=32	N=165
Fprau <1 Leuko ≤30 Crohn pattern		3%	7%	8%	84%	74%	50%	18%	18%	58%			
Fprau >1 Leuko >30 UC pattern	93%	69%	50%	14%	11%	9%	17%	10%	29%				
Fprau <1 Leuko >30 Intermediate IBD pattern	8%	7%	14%	4%	5%	9%			24%				
Fprau >1 Leuko ≤30 Non-inflammatory pattern	0%	21%	29%	78%	0%	9%	33%	72%	29%	42%	100%	100%	100%

chronic inflammation. Unfortunately, the residents of the large bowel can not be clearly divided into good and evil. However, many of indigenous bacteria are pathogenic. *Escherichia coli* causes sepsis, *Bacteroides* causes abscesses, *Enterococci* cause endocarditis, *Clostridium histolyticum* causes gas gangrene. We call these bacterial groups normal inhabitants of the human colon since they can be found in every healthy person. Let us assume that the host can recognize within the faecal mass more or less pathogenic bacteria and specifically hinder them on contact. This response would have to eliminate single bacterial groups from the polymicrobial mixture without affecting all other bacteria – an implication which is difficult to believe.

The FISH analysis of the mucosal flora clearly indicates that the host does not tolerate the indigenous flora or its parts, it ignores it in whole. The bacterial concentrations within the large intestine can reach extremely high concentrations of 10^{11} bacteria/ml, but the mucus barrier efficiently separates colonic bacteria from the colonic wall making any response unnecessary.

Viscous mucus covers the intestinal wall, disables bacterial movements, and protects epithelial cells from contact with bacteria. Leukocytes migrate into and patrol within the mucus layer executing surveillance function without any collateral damage. The sticky outer mucus surface offers the opportunity for probiotic strains to grow and build protective interlaced layers, making it even more difficult for pathogenic strains to reach the mucosa.

The inflammation takes place only after the mucus barrier is broken and the defence is overwhelmed. Since the beginning of the 20th century, there has been a steady increase in reported cases of both Crohn's disease and ulcerative colitis and the peak has obviously not been reached. This increase in IBD is mainly affecting the developed world, especially populations with high living standard and urban areas. Statistically the frequency of the disease correlates with the introduction of tap water, soap and improvement in the living conditions. The hygiene hypothesis argues therefore, that improved hygiene and a lack of exposure to microorganisms of various types have sensitized our immune system, leading to inadequate reaction to harmless bacteria in our environment. Out of this speculation have come recommendations to allow young children a reasonable amount of contact with dirt, pets, and other potential sources of infection as well as therapy with helminths for IBD. The statement that exposure to microbes in the city is lower than in the country

population is basically wrong. The vegetables and fruits on our table are imported from Greece, Portugal, New Zealand, South Africa, and Australia. They import a vast variety of microorganisms that were previously unknown to the consumer. The mobility of the modern society has led to a profound and rapid exchange of bacteria worldwide which was never encountered in the suburban world.

The *in vivo* effects of the dextrane sodium sulphate (DSS) detergent in mice and in the mucus simulation model however reveal other possible potential side effects of cleanliness and urbanisation. Traces of the detergents that make our dishes shine are ingested with our food. The "cleaning" effects of ingested home soaps on colonic mucus have been never investigated. Detergents make the objects clean, they do not sterilize them. Emulsifiers that are added to many foods to achieve a desired consistency may also have effects on the intestinal mucus. The recent data on Il-10 gene-deficient mice support this hypothesis. CMC is extensively used in the food industry, because cellulose is so abundant and cheap and the emulsifying and thickening properties of CMC are useful. The substance is added to food to stabilize emulsions, for instance in ice cream, to dissolve ingredients such as cacao in order to make perfect chocolate and sugar icing, to boost the flavour of the natural aroma and to keep bread fresh and soft. It can be found in toothpaste, chewing gum, a variety of baked goods, candies, sausages, ketchup and other sources. It is a filling and stabilizing component of most pills and it is a main substitute for gluten in manufactured gluten free products. Actually CMC is everywhere in quantities which are larger than those given with the drinking water to the mice in Sydora's experiments. The annual amount of CMC utilized by the food industry is constantly increasing. Presently there are no quantitative restrictions on its use, and its addition to food does not even require to be declared. CMC is, however, not the only emulsifier broadly used by the food industry. The list of emulsifiers which are permitted by the EU is too long to fit in a single page. Emulsifiers are practically everywhere starting with Konjak. Many other factors can influence the mucus barrier (*Table 3*). Bile acids, for example, are natural emulsifiers. Normally they are completely resorbed in the ileum and do not reach the colon. In patients with ileum resection, the resorption is disturbed, bile acids reach the colon and induce diarrhoea. Coeliac disease is regarded as an allergic response although the exact structure within the gluten molecule which is allergic

Table 3. Factors affecting mucus barrier.

Factors affecting mucus barrier		
Exogenic:	Endogenic:	Genet:
Detergents	Bile acid (normally resorbed in ileum but can lead to diarrhoea in large intestine)	NOD 2 Mutation
Bacterial virulence	Defensins	
Glutens (as natural emulsifiers need bacteria to be pathogenetic)	Antibodies	
Smoking	Probiotics, Prebiotics	
	Inflammation	
	Stress	

Table 4. Possible ways to remodel the mucus barrier.

Possible ways to remodel the mucus barrier
▪ Eradication of occasional pathogens which compromise the mucus barrier (Entero-adhesive E. coli, Fusobacterium nucleatum, Serpulina)
▪ Selective control of mucus secretion and dehydration (analogs of cortisol)
▪ Induction of a higher differentiation of epithelial cells, which leads to switch from mainly secretary to adsorptive function (analogs of anti TNF suppressing apoptosis)
▪ Reduction of the burden of detergents and emulsifiers in our foods
▪ Suppression of adherent bacterial biofilms (a possible effect of 5-ASA)
▪ Stimulation of innate immunity (substances like GM-CSF, probiotics as living vaccines)

could not be defined. We do know that symptomatic coeliac disease is always ongoing with bacterial overgrowth in the small bowel. The link between bacteria and glutens is poorly understood. Glutens are however naturally occurring emulsifiers. It could be that bacteria make glutens harmful.

Smoking stimulates mucus secretion but does not increase (probably does diminish) the mucus viscosity. The epidemiologic studies indicate that smoking is beneficial for ulcerative colitis but detrimental for patients with Crohn's disease. A thicker mucus barrier could indeed explain why smoking could be protective in UC patients but have no effect in Crohn's disease, where bacterial suppression is more important, than bacterial separation.

Stress interferes both with mucus production and regulation of the mucus viscosity. It is a known fact that, in IBD patients, stress leads to acute exacerbations of the disease.

Multiple other factors including defensins, probiotics, enteral pathogens, the inflammation itself, genetic background *etc*. interfere with the mucus barrier function. As long as the mucus barrier is compromised, a conflict between the organism and the pathogens inhabiting the colon in large numbers and diversity is inevitable.

What can be done to improve the mucus barrier? There are multiple options to do so (*Table 4*). Prednisolone is a very potent drug. As a glucocorticoid it stimulates the mucus secretion. Its mineral corticoid activity increases the water resorption, thereby increasing the viscosity gradient within the intestinal mucus layer. Development of substances which can selectively control the mucus barrier without the typical side effects of prednisone could be of extreme advantage for IBD treatment.

We have previously mentioned that the columnar epithelial cells are differentiated and mainly resorptive, while crypt cells are immature stem cells and mainly secretory. A balance between both is under tumor necrosis factor (TNF) control. Cell turnover is increased during inflammation. Anti TNF reduces the apoptosis of differentiated epithelial cells and that may explain

why, of many known mediators of inflammation, only anti-TNF antibodies have a clinically proven significance. The development of drugs with an effect on apoptosis regulation of the epithelial turnover should be considered in the future.

Antibiotics can effectively reduce the number of pathogens contacting the mucosa. They have however no direct influence on the mucus barrier and they can not sterilize the polymicrobial colonic microbiota. As soon as antibiotics are withdrawn because of increasing microbial resistance, the situation gets reversed. In the long-term, antibiotics are generally ineffective in IBD. The mucus barrier, however, can be compromised not only by environmental or genetic factors but also by specific pathogens such as *Serpulina, Fusobacteria, Enterobacteriaceae,* or *Gardnerella*. These bacteria can specifically form adherent biofilms on the epithelial surface compromising the mucus barrier and allowing migration of other indigenous bacteria into the mucosa. The specific identification of such a colonization and eradication of it by specific antibiotic treatment could be advantageous.

Mesalazine suppresses bacterial biofilms *in vivo* by mechanisms, which at present are not clear. Different to antibiotic therapy, the suppression with mesalazine does not seem to induce bacterial resistance. It is possible that the suppressive effects of mesalazine could be further expanded, when the mode of action is clarified.

The reduction of the detergent and emulsifier burden in our food was mentioned. We do not know at present which of the substances may reach the colon and accumulate in the human body. These questions still need to be further investigated.

The stimulation of the immune response is an eligible aim. Previous trials with interferon, GM-CSF were half-heated and inconsequent. PEG interferon was for example not tested at all. The therapeutic potential could be enormous.

After all, probiotics may act as some kind of living vaccines using attenuated strains and stimulating mucosal immunity. Actually we do not know how probiotics work. However, since

the influence of antibiotics on polymicrobial microbiota is limited, the use of biologicals as indigenous microbiota is intriguing. At present, all available probiotics use bacterial strains, which work marginally in the human large intestine (less than 0.01%). They were selected mainly for ease of culture, storage, transport and stability within food products. The probiotic potential of anaerobes, which constitute the mass of the indigenous flora of the large intestine, has not been studied.

CONCLUSIONS

The intestinal wall is effectively protected from direct contact with potentially harmful bacterial groups such as *Bacteroides, Enterobacteriaceae, Enterococci,* and *Clostridium histolyticum, which* are indigenous and highly concentrated in the colon. A well-developed mucus barrier and not the epithelial cell layer is the first line of defence against a variety of enteric pathogens. Before bacteria can adhere and invade the mucosa, they must first traverse the mucus barrier. When pathogens penetrate mucus and adhere to epithelial cells, inflammation clears the mucosa from bacterial contact and mucus from the bacteria, thus re-establishing the status quo.

The rising incidence of IBD over the last century may result from a disturbance of the mucus barrier function caused by excessive use of detergents and emulsifiers and from changes in the types and numbers of bacteria in our surroundings.

Against this background, IBD can be viewed as a polymicrobial infection that is characterized by a sustained broken mucus barrier with subsequent bacterial migration toward the mucosa and proliferation of complex bacterial biofilms on the epithelial surface.

As long as the mucus barrier function is impaired, the inflammatory process cannot successfully clear bacteria from the mucosal surface and immunosuppressive therapy remains the main therapeutic option. Other therapeutic principals including regulation of the mucus secretion and viscosity, suppression of bacterial biofilms, eradication of occasional pathogens, probiotics and immunostimulation are however also possible and should be increasingly considered and evaluated in the future.

As a consequence of the inflammatory response, the composition and structure of faecal microbiota is changed. The structural changes can be exactly quantified and used to monitor the disease activity. Based on the biostructure of faecal cylinders, Crohn's disease and ulcerative colitis can be distinguished from each other and other disease controls.

The possibility to monitor the disease activity in faecal samples will allow us to intensify the search for alternative therapeutic strategies aimed at cure of IBD instead at symptom control.

Conflict of interests: None declared.

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Author's address: Dr. A. Swidsinski, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms, Charite Hospital, Berlin, Germany; Phone: +49 30 450 514 003; Fax: +49 30 450 514 039; E-mail: alexander.swidsinski@charite.de