

Mucosal Flora in Inflammatory Bowel Disease

ALEXANDER SWIDSINSKI,* AXEL LADHOFF,† ANNELIE PERNTHALER,§ SONJA SWIDSINSKI,||
 VERA LOENING–BAUCKE,* MARIANNE ORTNER,* JUTTA WEBER,* UWE HOFFMANN,†
 STEFAN SCHREIBER,¶ MANFRED DIETEL,† and HERBERT LOCHS*

*Innere Klinik, Gastroenterologie, and †Institut für Pathologie, Charité Humboldt Universität, Berlin; §Max-Planck-Institute, Marine Microbiology, Molecular Ecology Group, Bremen; ||Krankenhaus im Friedrichshain, Mikrobiologisches Labor, Berlin; and ¶Klinik für Allgemeine Innere Medizin, Kiel, Germany

See editorial on page 228.

Background & Aims: Microorganisms that directly interact with the intestinal mucosa are obscured by fecal flora and poorly characterized. **Methods:** We investigated the mucosal flora of washed colonoscopic biopsies of 305 patients with bowel inflammation and 40 controls. The microbial cultures were validated by quantitative polymerase chain reaction with subsequent cloning and sequencing, fluorescence in-situ hybridization, and electron microscopy. **Results:** We found high concentrations of mucosal bacteria in patients with bowel inflammation, but not in controls. The concentrations of mucosal bacteria increased progressively with the severity of disease, both in inflamed and non-inflamed colon. In patients with $>10,000$ cfu/ μL , a thick bacterial band was attached to the intact mucosa without signs of translocation. Patients with inflammatory bowel disease (IBD) and concentrations of mucosal bacteria $>50,000$ cfu/ μL had characteristic inclusions of multiple polymorphic bacteria within solitary enterocytes located next to the lamina propria, without or having no contact with the fecal stream. The identified bacteria were of fecal origin. **Conclusions:** Our findings suggest that the changes in the mucosal flora in IBD are not secondary to inflammation, but a result of a specific host response. We hypothesize that the healthy mucosa is capable of holding back fecal bacteria and that this function is profoundly disturbed in patients with IBD.

Clinical observations¹ and animal experiments² have suggested that intestinal bacteria trigger and perpetuate chronic bowel inflammation.³ Bacterial products and genes have been repeatedly described within the intestinal mucosa of patients with inflammatory bowel disease (IBD) using bacterial culture,⁴ electron microscopy (EM),⁴ immunocytochemistry,⁵ and representational difference analysis.⁶ However, because of the inherent technical limitations of the available methods, bacteria interacting with the mucosa in either healthy

people or IBD patients have not been described adequately.

Culture is the gold standard for identifying bacteria. The sensitivity of the culture is high for most rods. Under optimal conditions, even single bacteria can be detected. In a complex bacterial population, however, rapid growing bacteria overgrow the culture plate, making the quantification and identification of slow growing bacteria impossible. Selective media can help to overcome this problem, but only in cases of known species. Because a large portion of the resident flora in adults is not even characterized, it is not possible to create conditions under which all or at least 500 known constituents can be simultaneously evaluated.

Another difficulty arises from the high concentration of intestinal bacteria. Before mucosa-adhesive and mucosa-invading bacteria can be studied, irrelevant fecal remnants must be removed. Washing biopsies, however, can influence the viability of bacteria. Molecular genetic methods of polymerase chain reaction (PCR) with a universal bacterial primer and comparative sequence analysis of ribosomal RNA (rRNA) genes are independent of culture requirements and circumvent the problem of differential bacterial growth. Unfortunately, the sensitivity of universal 16/23S rRNA PCR is low. Only bacterial concentrations greater than 10^3 cfu/ μL can be reliably detected.

The discussed shortcomings can be overcome by combining different methods in a representative number of patients. Our aims were to study the bacterial culture from washed colonoscopic biopsies from 305 patients and 40 controls according to a previously developed protocol,⁷ and to correlate the results with the results of

Abbreviations used in this paper: DAPI, 4',6'-diamidino-2-phenylindole; EM, electron microscopy; FISH, fluorescence in-situ hybridization; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

© 2002 by the American Gastroenterological Association

0016-5085/02/\$35.00

doi:10.1053/gast.2002.30294

quantitative PCR with subsequent cloning and sequencing, fluorescence in-situ hybridization (FISH), and EM to detect and characterize mucosal and invading bacteria in IBD.

Patients and Methods

Patients

The study was approved by the institutional review board, and the patients gave informed consent. Twenty-eight patients with self-limiting colitis, 104 patients with indeterminate colitis, 119 patients with ulcerative colitis (UC), 54 patients with Crohn's disease (CD), and 40 asymptomatic controls with normal colonoscopic findings were studied at the Charité, the University Hospital of the Humboldt University in Berlin. These patients were referred for evaluation and therapy by family physicians, internists, and gastroenterologists in private practice. The patients' mean age, age range, and gender are summarized in Table 1.

The asymptomatic control group consisted of patients without gastrointestinal complaints, who underwent colonoscopy for cancer surveillance or non-intestinal-related indications. None of the patients in the asymptomatic control group had received antibiotics during the 6 months before the investigation.

Patients who had colitis for the first time and were asymptomatic within 2 months after the colonoscopy were defined to have self-limiting colitis.

The diagnosis of indeterminate colitis was made in patients with chronic colitis of more than 6 months duration, with histologic changes and a clinical presentation suggestive for IBD but not typical for either UC or CD, and in the absence of small bowel involvement.

The diagnosis of IBD was made according to accepted criteria.^{8,9}

Three UC patients were partially colectomized, 13 CD patients had a past history of fistula, and 4 of them had a fistula at the time of the investigation.

Patients with collagenous colitis, eosinophilic colitis, and diverticulitis were excluded from this study.

All patients were of European origin.

Handling of Biopsies

Biopsy excision. Biopsies (3.2–3.6 mm³) were taken with standard forceps from the ileum and ascending and sigmoid colon and processed immediately (biopsy washing and the setting of cultures) in the laboratory next to the endoscopy

room. Additional biopsies were taken from each location for histopathology, EM, and FISH.

Biopsy wash. Each biopsy was first washed in 500 μ L of physiologic saline with 0.016% dithioerythritol to remove the mucus, then washed 3 times in 500 μ L of physiologic saline by shaking for 30 seconds each time. The supernatants from the second and fourth wash were used to evaluate the superficial microflora. After the fourth wash, the biopsy was hypotonically lysed by vortexing for 30 minutes in distilled water. The debris left after the hypotonic lysis was evaluated for mucosal bacteria.

Bacterial Culture

Bacterial culture was obtained from all patients. The second and fourth biopsy washes and the cell debris left after the hypotonic lysis were plated in 10-fold dilution steps on universal and differential media. For the isolation of aerobes, we used Schaedler blood agar, MacConkey agar without supplements, Gram positive selective Columbia blood agar with colistin-nalidixic acid supplement (Heipha-Biotest, Heidelberg, Germany), *Enterococcus* selective Bile-Aesculin Azide agar, and *Pseudomonas* selective EN 511 agar (OXOID GmbH, Wiesel, Germany). For the isolation of anaerobes, we used Schaedler blood agar, Schaedler blood agar with gentamicin and vancomycin (Heipha-Biotest), chromogenic Hackenthaler Bierkowski agar (OXOID GmbH), and *Clostridium difficile* cycloserin-cephoxitin-fructose-agar (OXOID Ltd., Basingstoke, Hampshire, England).

Anaerobic cultures were incubated in Gas Pak jars (AnaerogenTM; OXOID Ltd.). All plates were photo-documented after 48 hours (aerobes) and 96 hours (aerobes and anaerobes) of incubation at 36°C. Colonies were counted and expressed as colony numbers after anaerobic incubation. Although facultative anaerobes, such as *Enterobacteriaceae* and *Enterococcus*, will also grow under anaerobic conditions, their mean counts were negligible compared with the number of true anaerobes, allowing to make this simplification for the presentation of data. Single colonies were picked for further identification. The locations of picked colonies were marked on plate images. Aerobes were identified with an api 20 E, api NE, api Strep, and api Coryne kit and anaerobes with rapid ID 32 A (bioMérieux, Nürtingen, Germany). *Listeria* was cultured on Palcam *Listeria* selective agar after incubation in nutrient broth (Heipha-Biotest).

Fifty anaerobic colonies from the 1:100 dilution after the fourth wash and hypotonic lysis and 20 aerobic colonies from

Table 1. Patients' Clinical Data

	Asymptomatic controls	Self-limiting colitis	Indeterminate colitis	UC	CD
Number of patients	40	28	104	119	54
Mean age	49.2	36.3	45.2	44.1	34.6
Age range	26–77	17–70	19–81	17–86	17–86
Female/male	17/23	12/16	58/46	67/52	29/25

the undiluted aerobic culture after the fourth wash and hypotonic lysis were characterized phenotypically.

Mycobacteria were cultured for 6 weeks on Ogawa and Middlebrook 7H11 agar from hypotonically lysed debris mixed with N-acetyl L-cystein NaOH solution, then incubated for 25 minutes and neutralized with phosphate buffer.

DNA Amplification

Quantitative PCR with subsequent cloning and sequencing was performed in 20 controls and 59 patients (18 self-limiting colitis, 17 indeterminate colitis, 14 UC, and 10 CD). DNA was extracted with 1% sodium dodecyl sulfate and Proteinase K and further purified with Genclean II Kit according to the product protocol (BIO 101 Inc., La Jolla, CA). The 5' half of the 16S rRNA genes (600 base pairs) was amplified with PCR using a primer set universal for bacteria: 5'-AGAGTTTGAT(c/t)(c/a)TGGCTCAG-3', 5'-ACCGC(g/t)(a/g)CTGCTGGCAC-3'. The quantification and identification of bacteria with PCR, cloning, and sequencing were previously described.¹⁰

EM

Biopsy specimens from 14 patients (1 asymptomatic control, 3 indeterminate colitis, 5 UC, 5 CD) were studied with EM. Formalin-fixed biopsies were embedded in epoxy resin. Ultrathin sections were cut from epoxy resin blocks with an LKB ultramicrotome (LKB, Brommer, Sweden) with a diamond knife, mounted on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM10 transmission electron microscope (Zeiss, Oberkochen, Germany) at 60 kV.

FISH

An additional pair of biopsy specimens was taken from each location for in-situ hybridization from 10 asymptomatic controls, 5 patients with self-limiting colitis, 10 patients with indeterminate colitis, 13 patients with UC, and 12 patients with CD (360 biopsies all together). One biopsy was not washed (180 biopsies), and the other biopsy was washed either twice (90 biopsies) or 4 times (90 biopsies). The biopsies were fixed in 500 μ L of 4% buffered formalin without shaking and either embedded in paraffin or cut by cryostat within 2–4 hours after colonoscopy. Two-micrometer paraffin or 6- μ m cryostat sections were mounted on organosilane-coated slides, baked at 60°C for 16–20 hours, and stored at room temperature. Sections of biopsies were hybridized as described previously.¹¹ Selected samples were counterstained with 4',6'-

diamidino-2-phenylindole (DAPI) to visualize DNA. Oligonucleotide probes (Table 2) were synthesized and labeled with either Cy3 or Cy5 fluorochrome at the 5' end (Interactiva, Ulm, Germany).

A confocal laser scanning microscope at 1000 \times and 630 \times magnifications was used (LSM510; Carl Zeiss, Oberkochen, Germany). Hybridized bacterial cells were excited with either a green laser (543 nm) for Cy3- or a red laser (633 nm) for Cy5-labeled oligonucleotide probes. The autofluorescent tissue was excited with a blue laser (488 nm). DAPI-stained DNA was excited with a UV laser (364 nm).

Statistics

Nonparametric data were expressed by median value (range); normal distributed data were expressed as mean \pm standard deviation, and differences between or across groups were evaluated by analysis of variance. Statistical analysis was done using χ^2 and *t* tests. A *P* value of <0.05 was considered significant.

Results

Bacterial Culture

Bacterial concentration. The concentrations of mucosal bacteria were low in asymptomatic controls and high in patients with bowel inflammation, especially in patients with CD (Table 3). The bacterial concentrations in self-limiting and indeterminate colitis were intermediate. The percent of patients with >10,000 cfu/ μ L was significantly higher in IBD (*P* < 0.001) than in the other groups. Figure 1 shows the percent of patients with concentrations of mucosal bacteria of <1000, <10,000, <50,000, and >50,000 cfu/ μ L.

Bacterial species. There was no distinct difference between groups in regard to species isolated from the mucosa. The diversity of isolated bacteria was high. The cultured bacteria were of fecal origin. The mean concentration of anaerobes was 2 powers higher than that of aerobes in all groups. The main anaerobic microorganism was *Bacteroides*. The main aerobes were *Enterobacteriaceae* (primarily *Escherichia coli*). *Bacteroides* made up 50% to 95% of the anaerobic population in all but 3 patients with bacterial concentrations greater than 10³

Table 2. Probes Used in Fluorescence In Situ Hybridization

Probe	Specificity	Sequence (5'-3')	Position	Reference
EUB338	Eubacteria	GCTGCCTCCCGTAGGAGT	338-355 ^a	12
GAM42a	gamma-proteobacteria	GCCTTCCCACATCGTTT	1027-1043 ^b	13
BAC303	<i>Bacteroides</i> spp.	CCAATGTGGGGACCTT	303-319 ^a	14

^aPosition in the 16S rRNA of *E. coli*.

^bPosition in the 23S rRNA of *E. coli*.

Table 3. Mean Concentrations ± Standard Deviation (×10³ cfu/μL) of Mucosal Bacteria After the 4th Wash and Hypotonic Lysis From Ileum and Colon

	Asymptomatic controls (n = 40)	Self-limiting colitis (n = 28)	Indeterminate colitis (n = 104)	UC (n = 119)	CD (n = 54)
Total anaerobes ^a	0.18 ± 0.3	1.8 ± 5.3 NS	3.41 ± 16 P < 0.08	3.8 ± 11 P < 0.01	8.4 ± 18 P < 0.001
<i>Bacteroides</i> ^b	0.02 ± 0.05	0.26 ± 0.6 NS	0.64 ± 2.1 P < 0.01	1.6 ± 10 P < 0.001	2.0 ± 5.5 P < 0.001
Total aerobes	0.003 ± 0.05	0.08 ± 0.4 NS	0.09 ± 0.5 P < 0.005	0.06 ± 0.5 P < 0.05	0.11 ± 0.8 P < 0.001
<i>Enterobacteriaceae</i> ^c	0.002 ± 0.05	0.06 ± 0.5 P < 0.06	0.08 ± 0.3 P < 0.005	0.04 ± 0.5 P < 0.047	0.10 ± 0.8 P < 0.001

NOTE. P as compared with controls.

^aTotal anaerobe counts are expressed as colony numbers after anaerobic incubation. Although facultative anaerobes, such as *Enterobacteriaceae* and *Enterococcus*, will also grow under anaerobic conditions, their mean counts were negligible compared with the number of true anaerobes allowing to make this simplification.

^b*Bacteroides* counts were enumerated on the basis of growth on Schaedler blood agar with vancomycin and gentamicin supplement.

^c*Enterobacteriaceae* are expressed as numbers grown on MacConkey plates.

cfu/μL. The highest mean concentrations of *Bacteroides* were found in patients with UC and CD (Table 3).

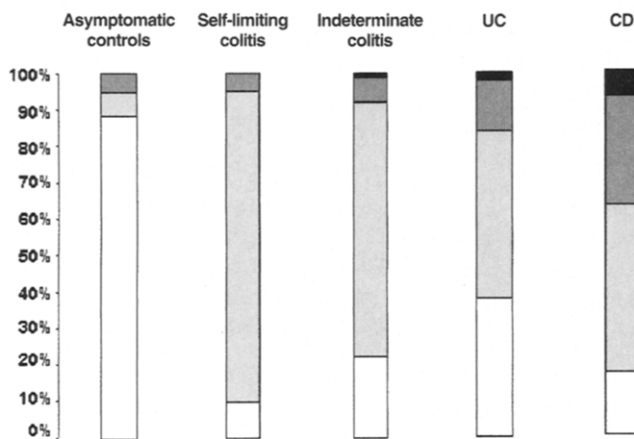
The *Bacteroides* population in each specimen was heterogeneous, composed of at least 3 related species, usually 4 to 7 species. All other rods were less numerous, found in a mix often associated with *Bacteroides* or *Enterobacteriaceae* (Table 4). The second most frequently cultured anaerobe after *Bacteroides* was *Collinsella aerofaciens*, found in 80% of all patients. Other less frequent and less

numerous aerobic and anaerobic organisms are listed in Table 4.

Mycobacteria and *Listeria* were never cultured. *Pseudomonas* species were found in low concentrations (1 to 20 cfu per biopsy) in 11% of patients with bowel inflammation, irrespective of diagnosis.

Yersinia and *Campylobacter* were each cultured twice in patients with self-limiting colitis.

The influence of biopsy location and local inflammation on the concentrations of mucosal bacteria. The concentrations of mucosal bacteria in about half of the patients were the same in all locations within the colon. The mucosal bacterial concentrations were markedly different at different locations in 65% of patients with self-limiting colitis, 27% of patients with indeterminate colitis, and 54% of IBD patients. There was no intra-group difference between the mean concentrations of mucosal bacteria in the ileum, ascending, and sigmoid colon in the asymptomatic control, self-limiting colitis, and indeterminate colitis groups (Table 5-I). The highest



Percent of patients with concentrations of mucosal bacteria:

- 0 - < 1000 cfu/μl
- 1000 - < 10,000 cfu/μl
- 10,000 - < 50,000 cfu/μl
- >50,000 cfu/μl

Figure 1. Percent of patients with high concentrations of mucosal bacteria.

Table 4. Occurrence of Cultured Mucosal Bacteria After the 4th Wash and Hypotonic Lysis

	Percent of patients with isolate of (n = 340)	Mean (maximum) percent of isolates from one biopsy
<i>Bacteroides</i>	95	56 (100)
<i>Collinsella aerofaciens</i>	80	16 (92)
<i>Clostridia</i>	62	9 (35)
<i>Bifidobacteria</i>	34	6 (87)
<i>Enterobacteriaceae</i>	82	4 (100)
<i>Peptostreptococci</i>	32	3 (24)
<i>Eubacteria</i>	23	3 (28)
<i>Propionibacteria</i>	15	<1 (9)
Other	90	3 (89)

Table 5. Mean Concentrations ($\times 10^3$ cfu/ μ L) of Mucosal Bacteria in Different Bowel Segments (I) and Inflamed and Noninflamed Mucosa (II) After the 4th Wash and Hypotonic Lysis

	Asymptomatic controls (n = 40)			Self-limiting colitis (n = 28)			Indeterminate colitis (n = 104)			UC (n = 119)			CD (n = 54)		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
I															
Ileum	0.22	0.03	0.001	1.7	0.05	0.09	2.6	0.88	0.02	8.9	4.6	0.11	10.0	2.9	0.08
Ascending colon	0.04	0.01	0.002	2.2	0.4	0.05	2.1	0.61	0.10	3.0	0.83	0.03	4.9	0.96	0.07
Sigmoid colon	0.27	0.005	0.001	0.8	0.07	0.08	4.8	0.52	0.04	1.8	0.35	0.01	9.1	2.28	1.04
II															
Inflamed mucosa				1.4	0.02	0.08	2.29	0.6	0.09	3.5	1.3	0.03	4.5	1.31	0.03
Noninflamed mucosa				2.1	0.09	0.09	4.48	0.8	0.04	5.5	2.1	0.06	8.5	3.49	0.09

NOTE. A: Total anaerobe counts. B: *Bacteroides* counts from gentamicin and vancomycin agar. C: *Enterobacteriaceae* counts (mostly *E. coli*) from MacConkey agar. Numbers in bold are statistically significant compared with non-bold numbers within a vertical row of the same part of the table, $P < 0.05$.

mean concentrations of mucosal bacteria were found in the ileum of IBD patients. The differences in concentrations of mucosal bacteria were nevertheless individual, differing markedly from patient to patient, rather than group-specific.

In both UC and CD patients, significantly higher mean concentrations of anaerobes, especially *Bacteroides*, were found in the ileum than in other colon segments. The concentrations of mucosal bacteria were higher in apparently normal mucosa than at the sites of inflammation in all patients with bowel inflammation. However, the differences were only statistically significant for total anaerobes in patients with CD (Table 5-II).

The mucosal flora did not differ noticeably between patients with IBD and indeterminate colitis. However, the concentrations of mucosal bacteria were significantly influenced by disease activity, treatment regimen, and other clinical data. If matched for therapy and disease activity, much higher concentrations of mucosal bacteria were found in untreated IBD patients than in untreated patients with indeterminate colitis or patients with self-

limiting colitis. Because of space constraints, the clinical data will be addressed in a separate article.

Quantitative PCR and Sequence Analysis of PCR Products

Quantitative PCR was performed in 20 controls and 59 patients. No bacteria were detected in 18 of 20 asymptomatic controls. In contrast, concentrations between 10^3 and 10^6 cfu/L were found in 83% of patients with self-limiting colitis, 71% of patients with indeterminate colitis, 71% of patients with UC, and 80% of patients with CD (Table 6).

The strongest PCR signal from each patient was cloned, and 120 to 200 clones were sequenced. The most frequent finding was a mix of various sequences differing from biopsy to biopsy and from patient to patient without any sequences appearing in more than 5% of the clones (Figure 2B). In about 70% of patients (Figure 2A, Table 6), sequences of one bacterial genus predominated (more than 30% of all sequences analyzed). These predominant sequences were either that of *Bacteroides* or

Table 6. Percent of Patients With Positive PCR (>1000 cfu/ μ L) and the Predominance of Sequences of a Single Bacterial Group

	Asymptomatic controls (n = 20)	Self-limiting colitis (n = 18)	Indeterminate colitis (n = 17)	UC (n = 14)	CD (n = 10)
Mucosal bacteria (positive PCR)	10%	83%	71%	71%	80%
<i>E. coli</i>	5%	27%	29%	21%	20%
<i>Bacteroides</i>	0%	11%	12%	16%	30%
Mix of sequences ^a	5%	33%	29%	34%	30%
Other (<i>Yersinia</i> and <i>Campylobacter</i>)	0%	11%	0%	0%	0%

^aHigh diversity of sequences, without any sequence appearing in more than 5% of the analyzed clones.

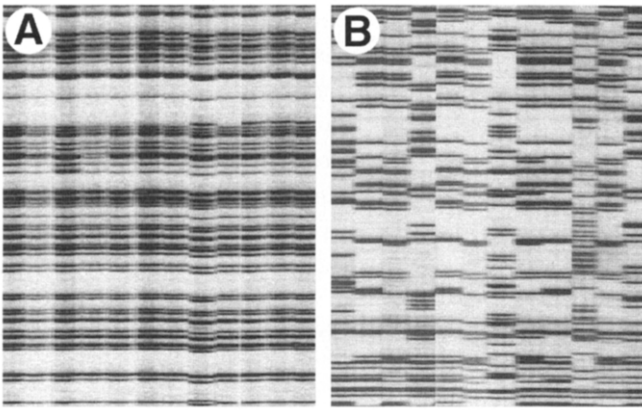


Figure 2. (A) Adenine sequences of 12 clones from a patient with a predominance of an *E. coli* sequence. (B) Adenine sequences from a patient with a mix of different bacterial sequences.

Enterobacteriaceae. Within a biopsy, *Enterobacteriaceae* were represented by a single sequence (usually *E. coli*), and *Bacteroides* by at least 3 related *Bacteroides* sequences. *E. coli* predominance was equally common in patients with IBD and indeterminate colitis (Table 6). The predominance of *Bacteroides* sequences was more common in patients with CD than in patients with UC or indeterminate colitis; the differences, however, were not statistically significant.

Besides these 2 groups, sequences of diverse *Streptococci*, *Peptostreptococci*, and *Clostridium* species were identified, all of them without any system, probably because of the high diversity of mucosal bacteria that is obviously some potencies greater than the number of clones sequenced. None of the sequences had a relevant homology to *Mycobacteria* or *Listeria*.

FISH

No differences were observed in the fluorescent appearance of biopsies from patients of different groups using universal, *Bacteroides*, and gamma proteobacteria fluorescence probes. Marked differences were observed between biopsies with different concentrations of mucosal bacteria in all groups.

Biopsies with low concentrations of mucosal bacteria less than 1000 cfu/ μ L. No bacteria were detected on the surface of biopsies or within the epithelial tissue of 70% of patients with low concentrations of mucosal bacteria. Occasionally, single scattered bacteria were seen in unwashed biopsy sections. These scattered bacteria were not detected in washed biopsies (Figure 3A).

Biopsies with moderate concentrations of mucosal bacteria between 1000 and 10,000 cfu/ μ L. Solitary bacteria were seen scattered over the surface of unwashed biopsies. In 84% of the investigated biopsies, clusters of 10 to 100 bacteria were seen on the surface. These

bacteria were mostly lost after washing. In 3% of biopsies, a thick bacterial layer was seen on the surface. No intraepithelial bacteria were observed (Figure 3B1 and B2).

Biopsies with high concentrations of mucosal bacteria greater than 10,000 cfu/ μ L. All unwashed biopsies with bacterial concentrations greater than 50,000 cfu/ μ L and 83% of biopsies with bacterial concentrations between 10,000 to 50,000 cfu/ μ L had a thick layer of mostly rod-shaped bacteria on the mucosal surface (Figure 3C1/C2). Bacteria were closely attached to the mucosa in freshly made cryostat sections and often loosely attached in previously paraffin-embedded specimens. Using *Bacteroides* and gamma proteobacteria probes (for *Enterobacteriaceae*), both groups of organisms were shown in high concentrations within this layer in accordance with the results of the bacterial culture. A reliable quantification of the superficial bacterial layer and its components was impossible because of the high concentration of bacteria.

Single bacteria were seen within the epithelial cell layer. Their intracellular location was confirmed by confocal laser scanning microscopy (Figure 4).

The intracellular bacteria gave a positive hybridization signal with both the universal (Cy3) and the *Bacteroides* (Cy5) probes. In some cases, in addition to a positive intracellular hybridization with the universal bacterial probe, a positive intracellular hybridization with the gamma proteobacteria probe was seen. This signal was regarded as an error, because single bacteria could not be distinguished. No bacteria were detected in the lamina propria.

The washing procedure disrupted the superficial bacterial layer without eliminating it. Clumps of 10 to 1000 bacteria were seen on the biopsy surface and between the folds and crypts even after the fourth wash. The epithelial cells were damaged by the washing procedure and therefore, rendered useless for the evaluation of intracellular microorganisms.

EM

Biopsy specimens of 14 subjects were studied. Intraepithelial bacteria were seen in 4 patients (indeterminate colitis = 1, UC = 1, CD = 2) with concentrations of mucosal bacteria greater than 50,000 cfu/ μ L (Figure 5A–D), and in 1 of 3 patients with concentrations of mucosal bacteria between 10,000 and 50,000 cfu/ μ L (CD = 1, but not in the patient with indeterminate colitis and not in the patient with UC).

Intraepithelial bacteria were not seen in 7 patients (asymptomatic control = 1, indeterminate colitis = 1,

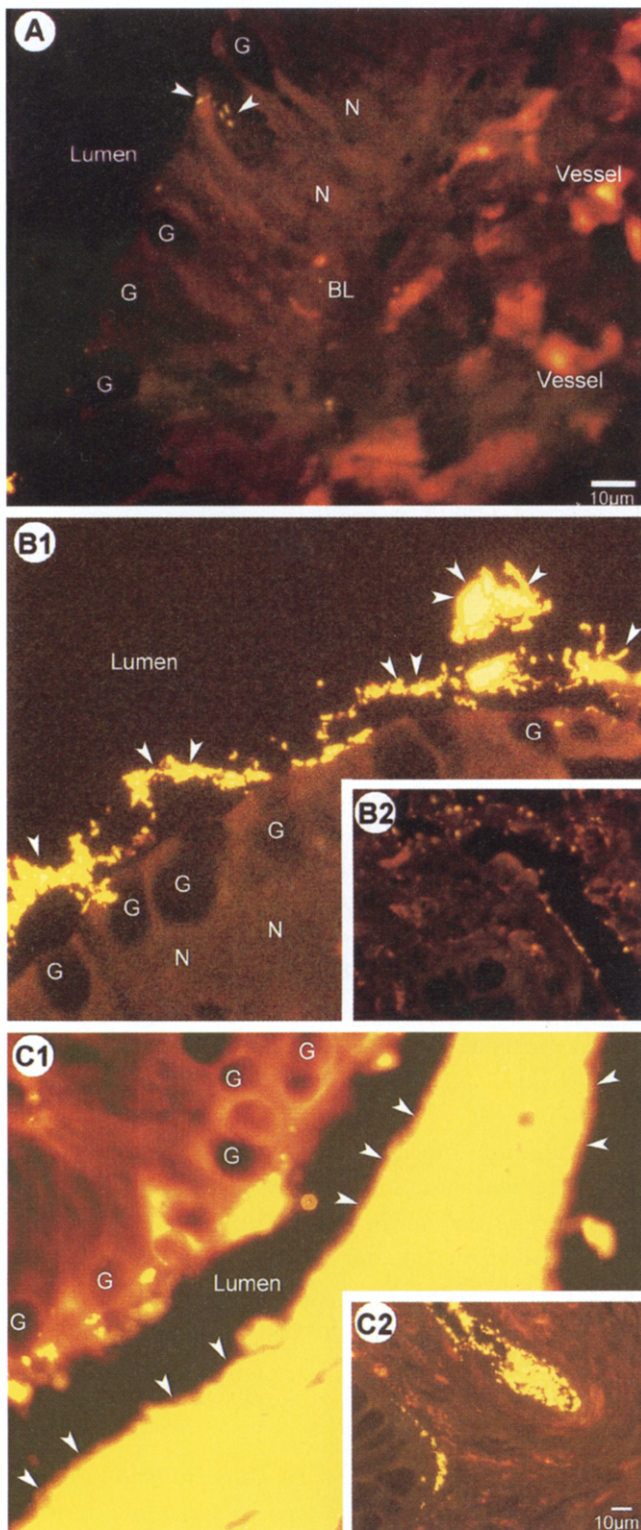


Figure 3. FISH. Superficial bacterial layer (arrows) in unwashed and corresponding washed biopsies visualized with FISH using a universal probe. BL, basal lamina; G, goblet cell; N, nucleus. (A) CD patient with concentration of mucosal bacteria of 300 cfu/ μ L. (B) UC patient with concentration of mucosal bacteria of 1500 cfu/ μ L (B1) unwashed and (B2) washed. (C) CD patient with concentration of mucosal bacteria of 72,000 cfu/ μ L (C1) unwashed and (C2) washed.

UC = 3, CD = 2) with concentrations of mucosal bacteria less than 10,000 cfu/ μ L.

The electron microscopic picture of intraepithelial bacteria was striking and similar in all patients.

Bacterial cells with different morphologic appearance heavily packed the cytoplasm of individual enterocytes located next to the lamina propria and having no direct contact with the fecal stream. There were no signs of apoptosis. No bacteria were seen in adjacent cells (Figure 5). No bacteria were seen between cells or subepithelially. No bacteria were seen in macrophages or M-cells.

Discussion

Using a simple and reliable protocol of biopsy wash followed by hypotonic lysis, we showed that the mucosal surface in healthy controls is basically sterile and that the concentrations of mucosal bacteria increased progressively from self-limiting colitis to indeterminate colitis to IBD, being highest in CD patients.

Similar to previous studies, we found no principal difference in the composition of the mucosal flora in IBD patients and controls.^{4,15-17} Species isolated from the washed mucosa were of fecal origin in all groups. The main anaerobic microorganism isolated from the mucosa was *Bacteroides*. The main aerobes were *Enterobacteriaceae* (primarily *E. coli*). The occurrence and the proportion of *Enterococci/Streptococci*, *Clostridia*, *Peptostreptococci*, and *Eubacteria* were lower and *Collinsella aerofaciens* or *Propionibacteria* higher than usually found in fecal specimens.

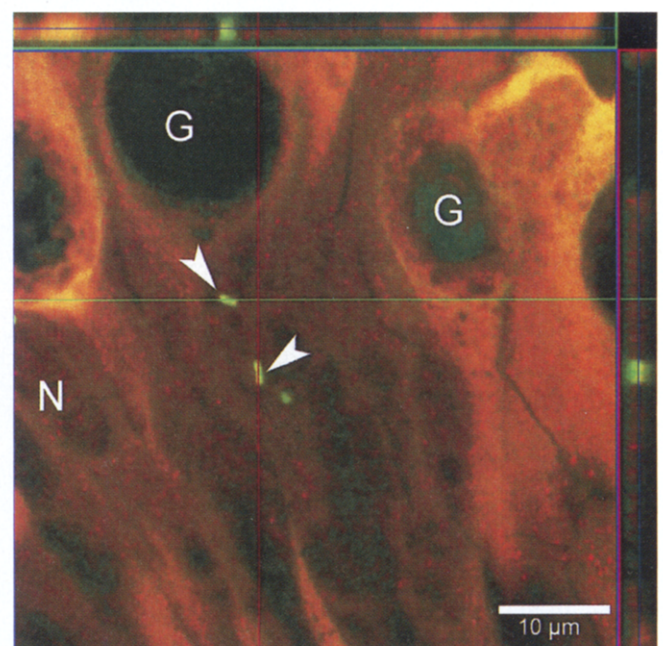


Figure 4. Intracellular bacteria (arrows) visualized with *Bacteroides*-specific probe. G, goblet cell; N, nucleus.

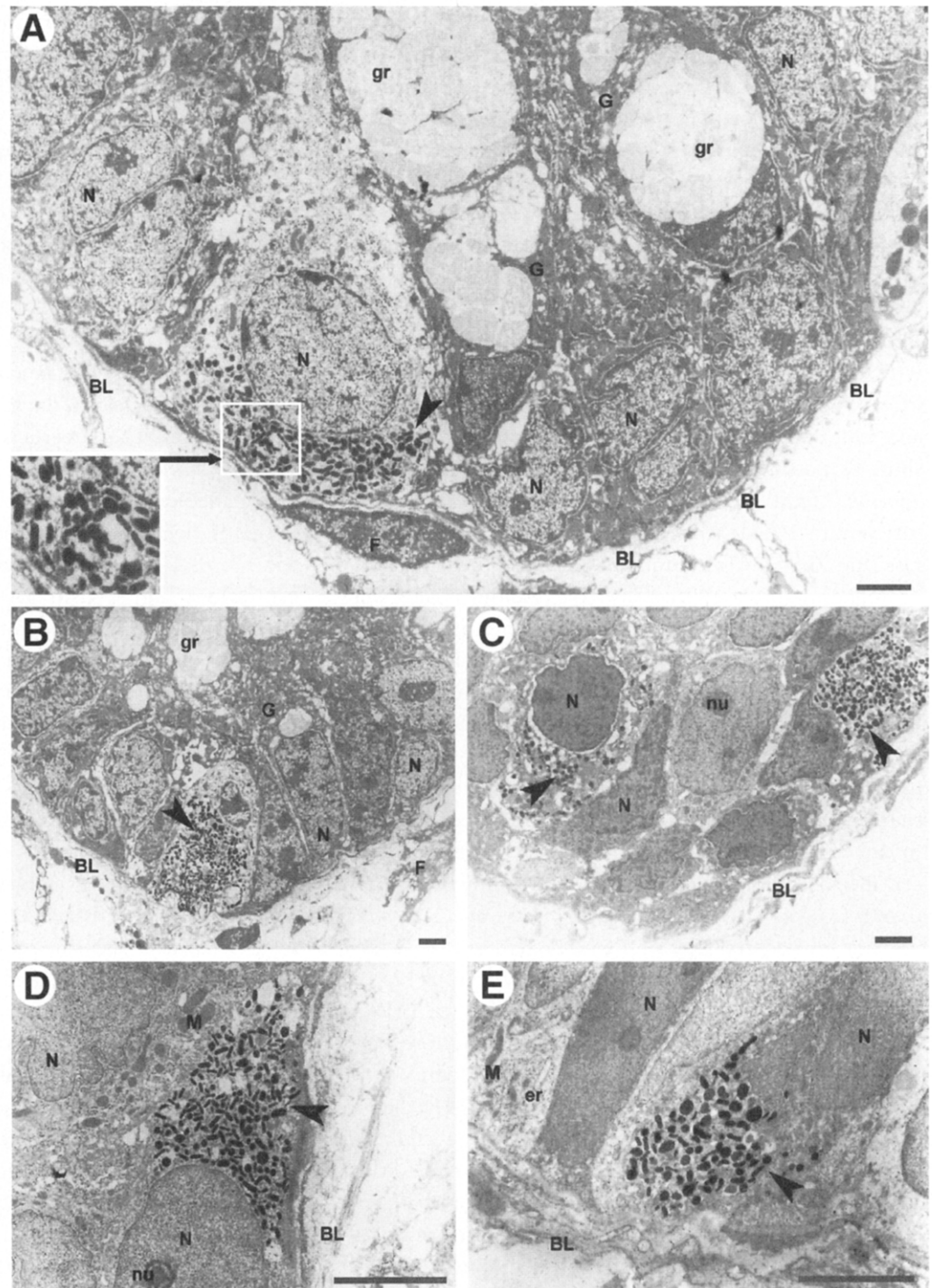


Figure 5. Electron microscopic appearance of intraepithelial bacterial inclusions (arrows). (A–D) Concentrations of mucosal bacteria greater than 50,000 cfu/ μ L. (E) Concentration of mucosal bacteria of 19,000 cfu/ μ L. Bars = 1 μ m. BL, basal lamina; er, endoplasmic reticulum; F, pericyptal fibroblast sheet; G, goblet cell; gr, mucous granules; M, mitochondria; N, nucleus; nu, nucleolus.

We found no *Mycobacteria* and *Listeria* that were previously associated with IBD,^{5,18} either by culture or PCR. The concentrations of *Pseudomonas* were low.

Most microbiologic studies,^{15–17} except one⁴ of isolates from colonoscopic biopsies, reported no difference in bacterial concentrations between IBD patients and controls. The estimated bacterial concentrations between 10^5 and 10^{11} cfu/ μ L were not principally different from fecal concentrations. The difference to our finding of

basically no bacterial concentration in healthy controls, may be a result of the small number of investigated patients, conclusions being drawn exclusively on the basis of bacterial growth without verification by culture independent methods, and a result of contamination by luminal flora, because unwashed biopsies were used and transported under anaerobic conditions to the microbiologic laboratory. The mucosal surface is a boundary between mainly anaerobic intestinal flora and aerobic mu-

cosa. Opposite requirements are needed to maintain the viability of each, making it impossible to create transport and handling conditions, which are adequate for both. The stringent anaerobic conditions for transport improve the recovery of anaerobes, but lead to biopsy necrosis and secondary tissue infiltration by the fecal flora. It is impossible to separate mucosal from fecal flora except if the separation is started immediately. We chose gentle washing conditions for the mucosa, being aware of losses that could occur to the anaerobic organisms. To make the losses calculable, we combined the microbiologic culture with culture-independent methods such as quantitative PCR with subsequent cloning and sequencing, FISH, and EM, and tried to keep the biopsy handling time as short as possible. Animal experiments had shown that "normal" fecal bacteria induced bowel inflammation in interleukin-10-deficient mice and HLA-B27 transgenic rats and that not all luminal gut bacteria have equal proinflammatory activities.^{19–21} *Bacteroides* strains were much more active in inducing colitis in animal experiments than *E. coli*; *E. coli* was more active than *Streptococci* or other fecal species, leading to the assumption that some fecal bacteria can trigger intestinal inflammation.²⁰ On the other hand, it was shown that the appearance and number of mucosal adherent bacteria were altered before the onset of the colitis in interleukin-10 gene-deficient mice.²¹ Our data show the preferential increase in concentrations of *Bacteroides* and *E. coli*, followed by *Collinsella aerofaciens* in IBD patients. However, *Bacteroides* was not represented by a single pathogen, but by at least 3 and often 5 to 7 different species within 1 biopsy. Many investigators studied adhesive *E. coli* in IBD in humans. Based on an in-vitro adherence assay to epithelial cells,^{22–24} adhesive *E. coli* strains were found to be significantly more frequent in patients with IBD than in healthy controls, leading to the assumption that *E. coli* adherence may be of primary importance rather than arising secondary to disease.^{23,24} However, neither serotyping of isolated strains nor investigation of their virulence factors was performed. Our serotyping data and analysis of genetic virulence markers of mucosal *E. coli* (data not presented) showed a wide variability of mucosal *E. coli* strains. This speaks against a single *E. coli* pathovar being responsible.

Surprisingly, concentrations of mucosal bacteria were higher in non-inflamed than inflamed mucosa, which contradicts the hypothesis that microbial pathogens are directly responsible for local lesions in IBD. There was no evidence of bacterial translocation in the lamina propria and in macrophages. The bacteria were not invading denuded mucosal locations, but covering the intact mu-

cosal surface. A secondary invasion of mucosa caused by alterations of the barrier function secondary to inflammation is therefore also unlikely.

A typical finding of FISH was a bacterial film with the integrity and thickness increasing parallel to increasing concentrations of mucosal bacteria. No bacteria were seen on the surface of biopsies with concentrations of less than 1000 cfu/ μ L. Clusters of 10 to 100 bacteria were seen on the surface of biopsies with mucosal bacteria concentrations between 1000 and 10,000 cfu/ μ L. Starting with 10,000 cfu/ μ L, a thick bacterial film was observed tightly attached to the mucosal surface in fresh biopsies and loosely attached to the mucosa in previously paraffin-fixed biopsies. This superficial bacterial film could not be washed away by our washing procedure. The microscopic appearance of the bacterial film did not depend on the patient's diagnosis, but depended on the concentration of the mucosal bacteria. However, mucosal bacterial concentrations higher than 50,000 cfu/ μ L were found in patients with indeterminate colitis, more so in patients with UC, and most often in patients with CD.

Until now, only one other study was published investigating the spatial distribution of bacteria in the rectum using in-situ hybridization.²⁵ Formalin-fixed and paraffin-embedded rectal biopsies from 16 IBD patients and controls were investigated. Similar to our results, bacteria were found within the mucin layer of 12 IBD patients, but not in healthy controls. However, different to our study, no mucosal-attached or intracellular bacteria were seen, and the thickness of the mucosal bacterial layer was moderate compared with our observations (Figure 3C). Differences to our results could be a result of a non-intended wash of biopsies by formalin during the transport and the small number of investigated patients. The moderate number of bacteria located in clusters of 10 to 100 cells is predictable and identical to our result in patients with concentrations of mucosal bacteria between 1000 and 10,000 cfu/ μ L. We could clearly demonstrate intracellular bacteria with FISH in patients with concentrations of mucosal bacteria greater than 10,000 cfu/ μ L using universal and *Bacteroides* specific fluorescence probes.

The EM was superior to FISH in demonstrating the intracellular bacterial inclusions. The electron microscopic picture in different patients was strikingly similar, despite polymorphism of intracellular bacteria. Bacteria were found not in superficial epithelial cells, but in cells close to the basal lamina, not having direct contact with the fecal stream. Bacteria occurred in high numbers within cytoplasm of single cells surrounded by normal, presumably immature epithelial cells without bacterial

inclusions. The nuclei of invaded and adjacent cells showed no signs of apoptosis.

Recently, a series of mutations in the NOD2 gene has been described and studied.^{26–31} It has been suggested that mutations in the leucine-rich region of the NOD2 gene alter activation of nuclear factor κ B in response to bacterial lipopolysaccharides and thus impair the handling and clearance of bacteria. However, it should be pointed out that only about 6% of patients with CD are homozygous and about 18% are heterozygous (in comparison to 6%–8% in UC and normal controls). Therefore, other mechanisms must contribute to the findings reported in our study.

High concentrations of mucosal bacteria in patients with chronic bowel inflammation, but not in healthy controls, location of mucosal bacteria in non-inflamed and morphologically intact parts of the colon, lack of signs of translocation, characteristic appearance of bacteria in epithelial cells located close to the basal lamina without direct contact with the fecal stream, lack of apoptosis in the nucleus of invaded enterocytes, normal electron microscopic appearance of the adjacent cells, and tissue structures all indicate a specific process. However, the high diversity of the mucosal flora without a predominance of a single microorganism is against a specific pathogen being the cause. Much more likely is therefore a specific host response, resulting from peculiarities of immunity, genetic disposition, or symbioses-like interactions.

We hypothesize that the healthy mucosa is capable of holding back fecal bacteria, preventing a close contact of resident microflora with the epithelial surface. This mucosal cleaving function is disturbed in patients with IBD primary to local inflammatory changes.

References

- Campieri M, Gionchetti P. Bacteria as the cause of ulcerative colitis. *Gut* 2001;48:132–135.
- Rath HC, Ikeda JS, Linde HJ, Schölmerich J, Wilson KH, Sartor RB. Varying cecal bacterial loads influences colitis and gastritis in HLA-B27 transgenic rats. *Gastroenterology* 1999;116:310–319.
- Sartor RB. The influence of normal microbial flora on the development of chronic mucosal inflammation. *Res Immunol* 1997;148:567–576.
- Edmiston CE, Avant GR, Wilson FW. Anaerobic bacterial population on normal and diseased human biopsy tissue obtained at colonoscopy. *Appl Environ Microbiol* 1992;43:1173–1181.
- Liu Y, Kruiningen JV, West AB, Cartun RW, Cortot A, Colombel J-F. Immunocytochemical evidence of *Listeria*, *Escherichia coli*, and *Streptococcus* antigens in Crohn's disease. *Gastroenterology* 1995;108:1396–1404.
- Sutton CL, Kim J, Yamane A, Dalwadi H, Wie B, Landers C, Targan SR, Braun J. Identification of a novel bacterial sequence associated with Crohn's disease. *Gastroenterology* 2000;119:23–31.
- Swidsinski A, Khilkin M, Schreiber S, Lehman C, Ortner M, Liebers U, Lochs H. Evaluation of the epithelial barrier in inflammatory bowel disease using 16S rRNA sequences of adherent bacteria (abstr). *Gastroenterology* 1996;110:A1023.
- Malchow H, Ewe K, Brandes JW, Goebell HM, Ehms H, Sommer H, Jesdinsky H. European cooperative Crohn's disease study (ECCDS): results of drug treatment. *Gastroenterology* 1984;86:249–266.
- Chapman RW, Selby WS, Jewell DP. Controlled trial of intravenous metronidazole as an adjunct to corticosteroids in severe ulcerative colitis. *Gut* 1986;27:1210–1212.
- Swidsinski A, Khilkin M, Kerjaschki D, Schreiber S, Ortner M, Weber J, Lochs H. Association between intraepithelial *Escherichia coli* and colorectal cancer. *Gastroenterology* 1998;115:281–286.
- Snaird J, Amann R, Huber I, Ludwig W, Schleifer KH. Phylogenetic analysis and in-situ identification of bacteria in activated sludge. *Appl Environ Microbiol* 1997;63:2884–2896.
- Amann R, Binder IBJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 1990;56:1919–1925.
- Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* 1992;15:593–600.
- Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer KH. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* 1996;142:1097–1106.
- Hudson MJ, Hill MJ, Elliot PJ, Berghouse LM, Burnham WR, Lennard-Jones JE. The microbial flora of the rectal mucosa and feces of patients with Crohn's disease before and during antimicrobial chemotherapy. *J Med Microbiol* 1984;18:335–345.
- Hartley MG, Hudson MJ, Swarbrick ET, Hill MJ, Gent AE, Hellier MD, Grace RH. The rectal mucosa-associated microflora in patients with ulcerative colitis. *J Med Microbiol* 1992;36:96–103.
- Poxton IR, Brown R, Sawyerr A, Ferguson A. Mucosa-associated bacterial flora of the human colon. *J Med Microbiol* 1997;46:85–91.
- Tanaka K, Wilks M, Coates PJ, Farthing MJG, Walker-Smith JA, Tabaqchali S. *Mycobacterium paratuberculosis* and Crohn's disease. *Gut* 1991;32:43–45.
- Rath HC, Wilson KH, Sartor RB. Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with *Bacteroides vulgatus* or *Escherichia coli*. *Infect Immun* 1999;67:2969–2974.
- Rath HC, Herfarth HH, Ikeda JS, Grenther WB, Hamm ThE, Balish E, Taurog JD, Hammer RE, Wilson KH, Sartor RB. Normal luminal bacteria, especially *Bacteroides* species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/Human β 2 microglobulin transgenic rats. *J Clin Invest* 1996;98:945–953.
- Madsen KL, Doyle JS, Tavernini MM, Jewell LD, Rennie RP, Fedorak RN. Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. *Gastroenterology* 2000;118:1094–1105.
- Burke DA, Axon ATR. Adhesive *Escherichia coli* in inflammatory bowel disease and infective diarrhoea. *BMJ* 1988;297:102–104.
- Giaffer MH, Holdsworth CD, Duerden BI. Virulence properties of *Escherichia coli* strains isolated from patients with inflammatory bowel disease. *Gut* 1992;33:646–650.
- Darfeuille-Michaud A, Neut C, Barnich N, Lederman E, Di-Martino P, Desreumaux P, Gambiaez L, Joly B, Cortot A, Colombel JF. Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 1998;115:1405–1413.
- Schultsz C, Van Den Berg FM, Ten Kate FW, Tytgat GNJ, Dankert J. The intestinal mucus layer from patients with inflammatory

- bowel disease harbors high numbers of bacteria compared with controls. *Gastroenterology* 1999;117:1089–1097.
26. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;411:599–603.
 27. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;411:603–606.
 28. Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van Deventer S, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG. An insertion mutation in the NOD2 gene predisposes to Crohn's disease in the German and British populations. *Lancet* 2001;357:1925–1928.
 29. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF(kappa)B. *J Biol Chem* 2001;276:4812–4818.
 30. Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem* 2000;4:2551–2554.
 31. Schreiber S, Nikolaus S, Hampe J. Activation of nuclear factor kappa B in inflammatory bowel disease. *Gut* 1998;42:477–485.

Received January 19, 2001. Accepted September 6, 2001.

Address requests for reprints to: Alexander Swidsinski, M.D., Innere Klinik, Gastroenterologie, Charité, 10098 Berlin, Germany. e-mail: alexander.swidsinski@charite.de; fax: (011) 49 30 450 514 923.

Escherich of *Escherichia coli*



Theodor Escherich (1857–1911) was born in Ansbach, Bavaria, the son of a physician. He studied medicine at Strassburg, Kiel, Berlin, Würzburg, and Vienna, a peripatetic curriculum not unusual for students of his day. At Würzburg he chose to devote his career to pediatrics. Stimulated by the work of Robert Koch (1843–1910), he acquired skills in the burgeoning field of bacteriology. In 1886, he published a monograph on the intestinal bacterial flora of infants in which he described a number of new types of organisms, including the one to which in 1919 his name became attached: *Escherichia coli*. In more recent years this became the single most thoroughly investigated and documented organism on planet Earth. During his tenure at the University of Graz in Austria (1890–1902), he focused his attention on the nutrition of infants. Escherich's mounting reputation was rewarded by his appointment as head of the St. Anna Hospital in Vienna. His career was cut short at its zenith by a fatal stroke at the age of 53.

—Contributed by WILLIAM S. HAUBRICH, M.D.
Scripps Clinic and Research Foundation, La Jolla, California

Copyright holder unknown. Photo obtained from the National Library of Medicine Website (<http://www.nlm.nih.gov>).