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Bacterial Biofilm Suppression with Antibiotics for Ulcerative and Indeterminate Colitis: Consequences of Aggressive Treatment

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Background. Antibiotics are commonly used in inflammatory bowel disease (IBD). Little is known about their effect on the mucosal flora.

Methods. The mucosal flora was investigated in colonoscopic biopsies from six groups of 20 IBD patients each. Patients were selected with regard to duration of/interval to combined metronidazole and ciprofloxacin therapy: group I patients with 1 day and group II with 7–14 days of antibiotic therapy, group III–V patients evaluated 1–4 weeks, 2–18 weeks, 26–36 weeks after cessation of antibiotic therapy, respectively. The control group VI included patients without antibiotic therapy. Thirty different fluorescent in situ hybridization (FISH) probes representative of the diversity of the human intestinal flora were applied to all specimens.

Results. Bacteria adherent to mucosa could be seen exclusively in DAPI stain and were practically nonamenable to FISH probes in patients on antibiotics $(0.001–3 \pm 0.001–5) \times 10^{10}$/mL. Occurrence and concentrations were significantly reduced in groups I and II as compared to untreated controls. The mucosal bacteria were significantly augmented after cessation of antibiotic therapy in group III (13.2 ± 4.3) and group IV (5.8 ± 2) but not in group V (1.1 ± 0.8) as compared to group VI (0.5 ± 0.4) $\times 10^{10}$/mL. Neither *Bacteroide*s nor *Enterobacteriaceae* groups were permanently suppressed by metronidazole–ciprofloxacin therapy.

Conclusions. The suppressing effects of antibiotics on the mucosal flora are accompanied by massive rebound effects. The concentrations of mucosal bacteria are dramatically increased as soon as 1 week after cessation of antibiotic therapy, remaining at a level that is at least one power higher over a period of 5 months as compared to the group without antibiotic treatment.

Key Words: Inflammatory bowel disease, Mucosal bacterial biofilm, Antibiotics, FISH.

Introduction

Involvement of pathogenic microorganisms in the etiology of inflammatory bowel disease (IBD) has been suspected since the first description of these diseases, but contradicted by the relative lack of success in controlling the diseases with antibiotic treatments (1). Even newer and more potent antibiotic compounds have not improved the situation. Antibiotics are still used frequently in IBD despite relative lack of evidence of efficiency. Indications for antibiotic treatment, choice of antibiotic to be used, dosage and duration of treatment seem to be based more on clinical instinct than documented scientific evidence (2,3). Recent studies also conclude that evidence for efficacy of antibiotic treatment is at best fragmentary and inconclusive (4,5). Clearly

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no specific pathogen has yet been convincingly associated with IBD. Furthermore, it has been shown over and over again that it is impossible to sterilize the gut with antibiotic therapy. A reasonable condition for antibiotic therapy should be that it leads to suppression of enteric bacteria presumably at the sites of the inflammation, which lasts also after the treatment has been discontinued. Such evidence has, however, never been provided. The present study was undertaken in order to fill this gap of information. It compares the concentration and composition of the mucosal flora in colonoscopic biopsies of IBD patients with and without antibiotic therapy, using a wide range of species- and group-specific bacterial fluorescence in situ hybridization (FISH) probes.

Materials and Methods

Inpatients and outpatients with ulcerative colitis (UC) or indeterminate colitis were recruited at the Charité University Hospital in Berlin, Germany. The diagnosis of UC and indeterminate colitis was made according to established criteria (6). Five groups of 20 patients each who had received antibiotics were studied. Oral antibiotic treatment consisted of metronidazole (400 mg twice daily) and ciprofloxacin (500 mg twice daily). Group I patients were treated for 1 day with antibiotics, group II patients were treated for 7−14 days, groups III−V patients were 1−4 weeks (III), 12−18 weeks (IV), and 26−36 weeks (V) after cessation of antibiotic therapy, respectively. Group I consisted of patients who were either hospitalized for acute exacerbation of their disease or received antibiotics on the day prior to colonoscopy. The period of antibiotic treatment in groups II−V lasted for at least 7 days but did not exceed 14 days. The control group VI consisted of patients without antibiotic therapy in the previous 2 or more years. Each patient was investigated only on one occasion. All colonoscopies were performed exclusively for medical indications unrelated to this study. All patients gave informed consent for additional biopsies according to the protocol approved by the ethics commission of the Charité Hospital of the Humboldt University. Patients with *Serpulina* (*Brachyspira*) infections were not included.

Biopsies

Bowel preparation for colonoscopy was performed using senna compounds (75 mL Clean-Prep; Mundipharma, Limburg, FRG) and 2−3 L polyethylene glycol and electrolyte solution (Golytely; Braintree Laboratories, Inc., Braintree, MA) orally.

Biopsies for FISH were taken from the ileum and ascending and sigmoid colon, fixed immediately in non-aqueous Carnoy solution (6/3/1 vol ethanol/glacial acetic acid/chloroform) for 2 h, and then processed and embedded into paraffin blocks using standard techniques. Four-µm sections were placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) for FISH studies.

FISH

Oligonucleotide probes were synthesized with a carbocyanite dye (Cy3), fluorescein isothiocyanate (FITC) or Cy5 fluorescent dye at the 5′ end (MWG Biotech, Ebersberg, Germany). The domain-, group-, and species-specific FISH probes used are listed in Table 1.

Formamide concentration and hybridization temperature were chosen to achieve the optimal stringency as described in the references. Additional hybridizations using a permeation step with lysozyme for 15, 30, and 60 min were performed in parallel for detection of Gram-positive bacteria.

Microscopy was performed with the Nikon e600 fluorescence microscope and photodocumented with a Nikon DXM1200 color camera and software (Nikon, Tokyo, Japan).

Concentrations of Mucosal Bacteria

The concentration of mucosal bacteria was defined as the mean concentration of adherent, mucus-scattered, and

Table 1. FISH probes

Name	Target	Reference
Eub338	Virtually all bacteria, Kingdom *(Eu)Bacteria*	7
Ebac	*Enterobacteriaceae*	8
Erec482	*Clostridium coccoides−Eubacterium rectale* group	9
Lach	Subgroup of EREC (incl. *Lachnospira multipara*)	10
Ehal	Subgroup of EREC (incl. *Eubacterium hallii*)	10
Chis150	*Clostridium histolyticum* group	9
Clit135	*Clostridium lituseburense* group (incl. *C. difficile*)	9
Lab158	*Lactobacillus* and *Enterococcus* group	11
Strc493	*Streptococcus* group	9
Enc131	*Enterococcus* spp and others	12
Efaec	*Enterococcus faecalis*, *Enterococcus sulfuricus*	13
Ato291	*Atopobium*, *Coriobacterium*, *Eggerthella* and *Collinsella* spp	14
Cor653	*Coriobacterium* group	14
Ecyl	*Eubacterium cylindroides* and others	10
Phasco	*Phascolactobacterium faecium* group	9
Veil	*Veillonella* group	10
Rbro, Rfla	*Ruminococcus bromii*, *Ruminococcus flavefaciens* and others	10
UroA, UroB	*Ruminococcus obeum*-like bacteria (subgroup of Erec)	15
Ser1410	Genus *Brachyspira*	16
Bif164	*Bifidobacteriaceae*	17
Bac303	*Bacteroides*/*Prevotella* group	18
Fprau	*Fusobacterium prausnitzii* group	19
Fnuc133	*Fusobacterium nucleatum*	20
Fnec996	*Fusobacterium necrophorum*	20
Pint657	*Prevotella intermedia*	20
Hel274	*Helicobacter* genus	21
Strpyo	*Streptococcus pyogenes*	22
Lis623, 1255	*Listeria*	23
Non338	Nonsense probe used to test for non-specific binding	24

mucus ceiling bacteria in a region of maximal developed biofilm that covered at least 10% of the intact epithelial circumference of the biopsy section. Mucosa-adherent bacteria were defined as bacteria lining 50 μm of the epithelial border (±1 μm of the epithelial border, contained within a 2 × 50 μm field) below the intact mucus layer. Mucus scattered bacteria were calculated within a square field of 10 × 10 μm, which was placed within mucus at the maximal concentration of bacteria next to the epithelial surface. Mucus ceiling bacteria were enumerated within a 5 × 20 μm field placed within the maximal concentration of the mucus ceiling layer but at least 10 μm away from the epithelial surface.

The quantification of bacteria was based on the assumption that a 10 μL sample with a cell concentration of 10^7 cells per mL contains 40 cells per average microscopic field at ×1000 magnification (25).

Amenability of Bacteria

Amenability of mucosal bacteria was defined as the percent of bacteria stained with DAPI, which positively hybridized with the universal Eub338 FISH probe.

Statistical Analysis

Mean values and standard deviations (SD) were calculated from the bacterial counts using Student's *t*-test and chi-square test. A p value of <0.05 was considered significant.

Results

The baseline data for the six groups of patients are presented in Table 2. The composition of the groups did not differ significantly with regard to mean age, gender, disease duration, 5-ASA, or azathioprine therapy. Concomitant prednisolone therapy was more often provided to patients in groups I and II due to the severity of their disease.

Mucosal Bacteria in Patients Treated with Antibiotics

Numerical data characterizing the mucosal flora are summarized in Table 3. The FISH microphotographs with DAPI counter stain of single groups are presented in Figures 1–3. The concentrations of mucosal bacteria were dramatically reduced in group I and II patients treated with antibiotics and were significantly different from groups III–V after antibiotic treatment and with no antibiotic treatment (group VI) ($p < 0.001$). Evaluation using FISH was difficult in patients on antibiotics because of the low amenability of the bacteria. Only 0.1–5% of signals, located within mucus and having typical bacterial morphology with DAPI stain, hybridized positively with the universal bacterial probe Eub338, and the hybridization signals were weak (Figures 1A and 1B). The composition of mucosal bacteria could not be quantitatively accessed because of the low amenability of the bacteria. The number of patients on antibiotics in whom bacteria could be detected using group-specific probes was <40% (Table 3).

Mucosal Bacteria in Patients Postantibiotic Therapy and with No Antibiotic Therapy

Cessation of antibiotic therapy was accompanied by a significant increase in the concentrations of mucosal bacteria. The increase was most pronounced in group III patients 1–4 weeks after cessation of therapy and was significantly different ($p < 0.001$) compared to all other investigated groups (Table 3; Figure 2). Concentrations of the mucosal bacteria did gradually decrease with increasing time since administration of antibiotic therapy. Concentrations of mucosal bacteria in group V, 26–36 weeks after the end of therapy, were still higher than in patients without antibiotic therapy (group VI), but the differences were not statistically significant.

Amenability of bacteria in groups III–VI was about 50% or higher, did not differ significantly between single groups and allowed us to characterize the composition of mucosal bacteria using single group-specific probes.

Composition of Mucosal Bacteria in Groups Postantibiotic Therapy and with No Antibiotic Therapy

Four groups of bacteria contributed 80–95% of amenable bacteria in each IBD patient postantibiotic therapy or with no antibiotic therapy: *Bacteroides* (Bac303), *Eubacterium rectale–Clostridium coccoides* (EREC), *Fusobacterium prausnitzii* (Fprau) and *Enterobacteriaceae* (Ebac). Bacteria that positively hybridized with the Bac303 probe

Table 2. Baseline data for all patients

Group	I	II	III	IV	V	VI
Duration of/interval to antibiotic therapy	Day 1	Day 7–14	1–4 weeks	12–18 weeks	26–36 weeks	No antibiotic
Mean age (years)	41 ± 14	43 ± 14	42 ± 13	45 ± 15	42 ± 11	44 ± 11
UC/indeterminate colitis	12/8	14/6	11/9	12/8	13/7	14/6
Number of patients on prednisolone	15	17	9	6	9	5
Number of patients on 5-ASA	13	18	17	15	17	18
Number of patients on azathioprine	7	8	8	5	7	6

UC, ulcerative colitis.

Table 3. Characteristics of the mucosal flora

	I	II	III	IV	V	VI
	Day 1	Day 7−14	1−4 weeks	12−18 weeks	26−36 weeks	No antibiotic
Group	$n = 20$	$n = 20$	$n = 20$	$n = 20$	$n = 20$	$n = 20$
Concentrations of mucosal bacteria *(Eub 338)* $\times 10^{10}$	0.003 ± 0.005	0.001 ± 0.01	13.2 ± 4.3	5.8 ± 2	1.1 ± 0.8	0.5 ± 0.7
	II NS					
	III−VI $p < 0.001$	III−VI $p < 0.001$	IV−VI $p < 0.001$	V−VI $p < 0.001$	VI $p = 0.07$	
Amenability of bacteria (%)	<1	<5	64	47	52	58
	II NS					
	III−VI $p < 0.001$					
Bacteroides (Bac303)						
A	2	6	40 ± 16	56 ± 13	54 ± 15	60 ± 11
B			17	20	18	20
Eubacterium rectale (EREC)						
A			24 ± 14	15 ± 6.3	12 ± 7.1	9 ± 4.5
B	4	7	17	19	18	19
Fusobacterium prausnitzii (Fprau)						7 ± 8.3
A			1.8 ± 2.6	2.9 ± 4.1	10 ± 5.6	
B	1	2	14 V−VI $p = 0.03$	17	15	16
Enterobacteriaceae (EBAC)						
A			14 ± 8	17 ± 15	29 ± 24	8 ± 11
B	3	8	14	16	16	8
						III−V $p < 0.046$
Bifidobacteriaceae (Bif)						
A			2 ± 1.4	<1	1 ± 1.0	<1
B	1	3	11	13	12	9
Streptococcus (Strep)						
A			<1	<1	<1	1.6 ± 1.9
B	2	3	7	11	6	5
Lactobacillus (Lab158)						
A			<1	<1	1.1 ± 0.9	<1
B	4	4	9	11	10	12
Clostridium lituseburense (Clit)						
A			<1	<1	<1	<1
B	0	0	5	5	6	7
Ruminococcus bromii (Rbro)						
A	0	0	<1	1.9	<1	2
B			7	5	7	9
Veillonella (Veil)						
A			<1	<1	2.4 ± 1.1	<1
B	0	1	7	8	9	9
Atopobium (Ato)						
A			<1	<1	<1	<1
B	0	3	5	6	5	6

NS, not significant.
A, proportion of single bacterial groups within biofilm in percent. Mean ± SD.
B, number of patients with specific bacterial group adjacent to mucosa.

(*Bacteroides* group) were most predominant and most frequently found. They contributed 90% of all bacteria within the mucosa in some patients. The occurrence and proportion of *Bacteroides* within the microbial community did not differ significantly between the different groups (Table 3).

The occurrence of bacteria, which did positively hybridize with the EREC probe, representing *Eubacterium rectale−Clostridium coccoides* cluster, was highest in group III, 1−4 weeks after cessation of antibiotic therapy (Table 3). Concentrations of EREC-positive bacteria continuously declined with time. It was lowest in patients without antibiotic therapy. However, these differences were not statistically significant.

The occurrence of *Fusobacterium prausnitzii* was similar in all groups; however, the proportion of bacteria was significantly lower in groups III (1−4 weeks) and IV (12−18 weeks after cessation of antibiotics) as compared to groups V and VI ($p < 0.01$).

The occurrence and proportion of *Enterobacteriaceae* did not differ in groups III−V (postantibiotic therapy) but were significantly higher as compared to patients with no antibiotic therapy.

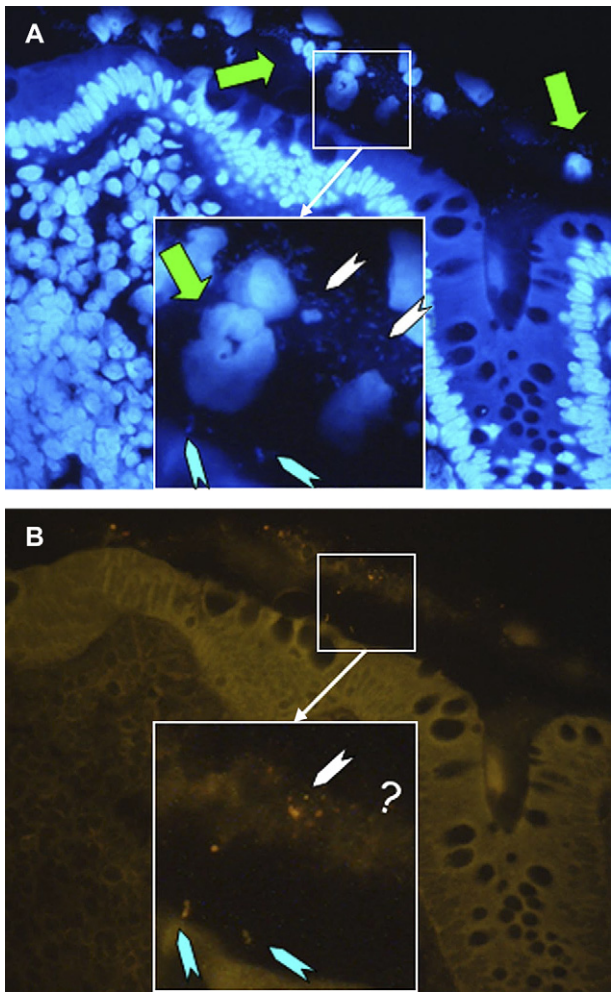

Figure 1. (A) Blue DAPI stain of the sigmoid biopsy section from a patient with ulcerative colitis (UC) treated with ciprofloxacin (500 mg) and metronidazole (400 mg) twice daily for 10 days. Original magnification ×400. The large light-colored dots are nuclei of eukaryotic cells. Epithelial layer with goblet cells, cells of submucosa, and mucus with included leukocytes (green arrows) can be easily recognized. A small number of short rods with typical bacterial morphology can be seen in the mucus ceiling regions (white arrows) and adherent to the epithelial layer (blue arrows). (B) Fluorescence of the same microscopic field as in (A). Hybridization with Eub338 Cy3 probe (universal for all bacteria) performed at low stringency. The background fluorescence of the mucosa allows excellent orientation within anatomic structures. Only 3/25 signals suspicious for bacteria in the DAPI stain hybridized weakly with the universal bacterial probe. Two are adherent (blue arrows) and one is located in the mucus ceiling region (white arrow). Note the lack of a hybridization signal in the regions corresponding to the DAPI signals seen in (A). Color version of this figure available online at www.arcmedres.com

All other bacterial groups investigated contributed cumulatively to about 5–15% of the mucosal bacteria. *Bifidobacteriaceae*, *Streptococcus*, *Atopobium*, and *Veillonella* cluster occurred in >50% of patients, and the mean proportion of these bacteria was mostly 1% or less but reached in single patients up to 15%. The detection of other groups was sporadic. We found no significant difference in the distribution of these bacterial groups in patients postantibiotic

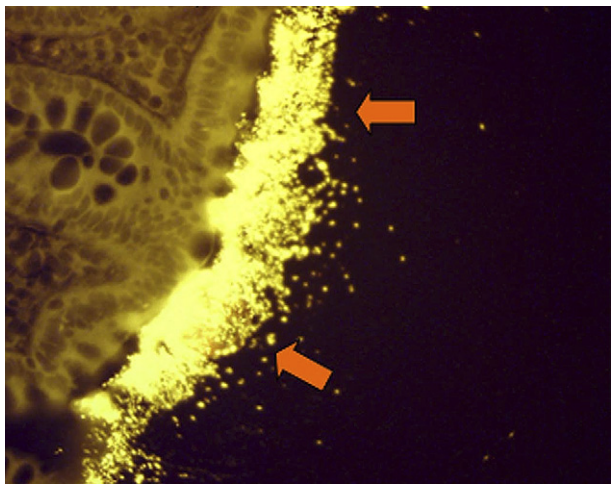

Figure 2. Hybridization with the Eub338 Cy3 probe at magnification ×400. Biopsy is from the sigmoid colon of an UC patient 20 days after antibiotic therapy was discontinued. Prolific adherent highly amenable biofilm can be seen (red arrows, yellow fluorescence). Color version of this figure available online at www.arcmedres.com

therapy or with no antibiotic therapy. For reasons of space, only numerical data for *Bifidobacteriaceae*, *Streptococcus*, *Lactobacillus*, *Ruminococcus*, *Veillonella*, and *Clostridium lituseburense* (including *Clostridium difficile*) cluster are presented in Table 3.

Discussion

Antibiotics are generally assumed to have no significant effect on the overall concentrations of intestinal bacteria (26) but produce significant disarrangements in the flora such as overgrowth of potential pathogens (*C. difficile*, various fungi, etc.), increased numbers of resistant bacteria, and reduced colonization resistance (27). Such observations may be true for luminal but not for mucosal bacteria. Our data clearly demonstrate a high efficiency of orally applied antibiotics in suppression of mucosal bacteria. Concentrations of mucosal bacteria fell below levels that can be reliably detected by FISH already on the first day of antibiotic therapy. Bacteria in the antibiotic-treated groups were still visible in DAPI stain, but concentrations were at least two powers lower than in the groups immediately after or never having received antibiotic therapy. Only 0.1–5% of DAPI-stained bacteria positively hybridized with the universal bacterial FISH probes. FISH detection of bacteria is dependent on metabolic activity of microbes. The number of ribosomes in metabolically inactive bacteria are significantly reduced. Fluorescence signals will fade with falling numbers of targets for 16s RNA-based FISH probes. Although metabolically silent bacteria can still be visualized with unspecific DNA stains such as DAPI, they are no longer amenable to FISH probes. Amenability is therefore an indirect sign of bacterial vitality (28). The low amenability of mucosal

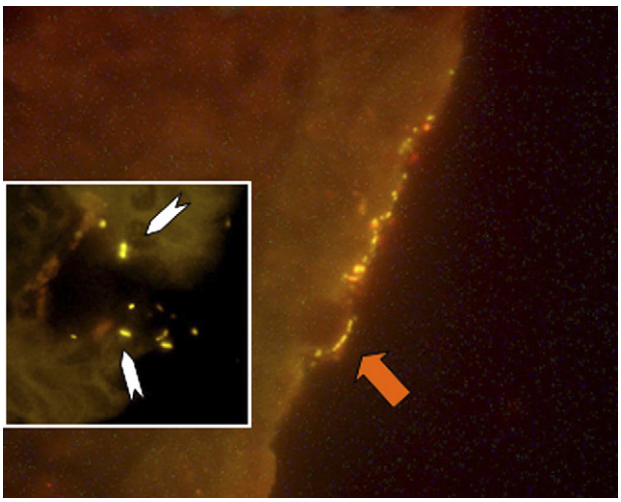

Figure 3. Biopsy from a patient with UC without antibiotic therapy. Bacterial fluorescence of Bac303 (*Bacteroides*) Cy3 probe (yellow fluorescence), Ebac (*Enterobacteriaceae*) Cy5 probe (red fluorescence), and universal Eub338 FITC probe (green fluorescence) are overlaid. A string of adherent bacteria composed mainly of *Bacteroides* and *Enterobacteriaceae* follows the epithelial surface. The bacterial adherence to the outer membrane of the epithelial cell surface can be seen in the region over the vacuole of a goblet cell (red arrow). The insertion within the figure shows *Bacteroides* (white arrows) in the erosion of the epithelial surface. Color version of this figure available online at www.arcmedres.com

bacteria in patients receiving antibiotics indicates that bacteria are either dead or metabolically inactive. In contrast, the amenability of mucosal bacteria in groups of patients after antibiotic therapy was on average between 40% and 60%.

Our data do not allow any conclusions to how long the mucosal flora can be kept suppressed when antibiotics are permanently supplied. However, the ciprofloxacin/metronidazole combination is not usually used in our clinic for longer periods than 2 weeks for indications other than septic complications of IBD. No difference was observed within 14 days of the therapy. It is obvious, however, that the cessation of antibiotic therapy was accompanied by a massive rebound effect. The concentrations of mucosal bacteria in patients 1−4 weeks after cessation of antibiotic therapy were 25 times higher than in patients in group VI who did not receive antibiotic treatment. The rebound effect gradually diminished with time but was still significant in patients 12−18 weeks and 26−36 weeks after cessation of antibiotic therapy.

Antimicrobial agents are still widely used in IBD mostly as additional treatment in patients with severe UC, in patients with fistulas or septic complications, and sporadically as a first line therapy. Use of antibiotic therapy is empiric, based on reported benefits of antibiotic therapy observed in individual patients (1−3). In contrast, most controlled clinical trials evaluating the efficacy of antibiotics in IBD report equivocal or negative results, making evidence-based recommendations impossible (4,5). The discrepancy between impression of positive impact and the negative overall results of controlled clinical studies may have a simple explanation. Antibiotics are highly effective in suppressing bacterial flora at the site of inflammation but are accompanied by extreme rebound effects and increase of mucosal bacterial concentrations far above levels observed prior to therapy. It is also alarming that this rebound effect seems to selectively enhance groups of bacteria that are the primary targets of the antibiotic therapy such as *Bacteroides* (targeted by metronidazole), and *Enterobacteriaceae* (targeted by ciprofloxacin).

Our pilot study has limitations because the changes were not observed longitudinally. However, the marked differences in groups with antibiotic therapy and postantibiotic therapy indicate that future clinical investigations on antibiotic efficiency will need to include studies of the mucosal flora (29).

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