Azathioprine and Mesalazine-induced Effects on the Mucosal Flora in Patients with IBD Colitis

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Background: The impact of azathioprine and 5-aminosalicylic acid (5-ASA) on the innate immunity and mucosal flora is unknown. The study investigated the influence of IBD treatment on the concentrations and spatial organization of mucosal bacteria using fluorescence in situ hybridization with 16s r-RNA targeting probes.

Methods: We prospectively investigated colonoscopic biopsies from five groups of 20 subjects each: patients with ulcerative or indeterminate colitis treated with azathioprine (group 1), azathioprine and 5-ASA (group 2), 5-ASA (group 3), untreated IBD (group 4), and healthy controls.

Results: The elevated numbers of leukocytes in mucus of IBD patients were reduced nearly to norm in patients treated with azathioprine alone. In contrast, 5-ASA therapy had no influence on mucus leukocyte migration and was associated with the lowest concentrations of mucosal bacteria of all IBD groups. The suppressed migration of leukocytes in azathioprine-treated patients was accompanied by a 28-fold higher concentration of mucosal bacteria when compared with the 5-ASA group or a 1000-fold increase when compared with healthy controls. The percent of the epithelial surface covered with adherent bacteria ($P < 0.001$) and the amenability of mucosal bacteria ($P = 0.01$) were also significantly increased in the azathioprine-treated group compared with all other IBD groups. The patients receiving both 5-ASA and azathioprine did not differ statistically from untreated IBD patients either in mucus leukocyte migration or in bacterial concentrations.

Conclusions: Azathioprine and 5-ASA induce opposite effects on the mucus barrier. Concomitant therapy of 5-ASA and azathioprine mutually neutralizes the effects of both on the mucosal flora and the barrier function.

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Inflammatory bowel disease (IBD) is a common disorder with unclear etiology. In the last 100 years, many therapeutics were introduced that positively effected IBD symptoms; however, none of them is curative. Since the disease is chronic, the life-long effects of these therapies are unclear.[1] Despite increasing therapeutic efforts, the incidence rate as well as the severity of the disease are increasing.[2] Suppression of local mucosal inflammation is the basic principle of IBD therapy.[3] The antiinflammatory 5-aminosalicylic acid (5-ASA) and the immune suppressive azathioprine are the immune modulators most commonly used for control of IBD symptoms. Both are efficient, relatively inexpensive, and associated with acceptable side effects.[3,4] During the last 10 years azathioprine has progressively replaced 5-ASA in the management of IBD. This change is based mainly on data on the superior efficiency of azathioprine for induction and maintenance of clinical remission in patients with steroid-dependent colitis.[4,5] Little is known about the influence of azathioprine or 5-ASA on the innate immunity to the intestinal flora. We have previously shown an impaired mucus barrier in IBD.[6] The epithelial layer in normal controls is covered by mucus that is free of bacteria. The mucus effectively separates highly concentrated luminal bacteria from contact with the intestinal wall in healthy subjects. The separation is impaired in IBD patients, in whom bacteria penetrate mucus, have contact with the epithelial surface, build prolific bacterial biofilms, and invade the submucosa. Leukocytes migrate into the mucus and accumulate especially in the outer mucus layers that border the masses of luminal bacteria, but are incapable of stopping the bacterial migration across the mucus and subsequent bacterial adhesion to the mucosa.[7] The crossing of mucus by bacteria and leukocytes and the mucus production are potentially influenced by azathioprine and 5-ASA. The present study investigated the mucosal flora in IBD patients treated with azathioprine, azathioprine/5-ASA, or 5-ASA using 16s RNA-based fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

All patients were investigated prospectively and gave informed consent for additional biopsies according to the protocol approved by the ethics commission of the Charité Hospital, Humboldt University, Berlin, Germany. Patients

TABLE I. Baseline Data for Patients and Controls

Treatment	Azathioprine ($n = 20$)	Azathioprine/ 5-ASA ($n = 20$)	5-ASA ($n = 20$)	None or Occasional Corticosteroids ($n = 20$)	Controls ($n = 20$)
Mean age (yr)	42 ± 11	46 ± 16	43 ± 14	41 ± 13	48 ± 18
Male/female ratio	8/12	7/13	6/14	10/10	11/9
UC/indeterminate colitis	12/8	15/5	17/3	12/8	0
Number of patients receiving prednisolone	2	3	2	4	0
Mean prednisolone doses (mg)	7.5	15	10	15	0
Mean daily dose of 5-ASA (mg)	0	3200 ± 1524	2800 ± 1000	0	0
Mean daily dose of azathioprine (mg)	132 ± 39	128 ± 34	0	0	0

UC, ulcerative colitis.

with mild to moderate forms of ulcerative colitis (UC) or indeterminate colitis for whom a complete medical history was available and who underwent colonoscopy at the Charité Hospital were enrolled. The diagnosis of UC and indeterminate colitis was made according to established criteria.[8] Groups of patients receiving oral therapy with azathioprine, azathioprine/5-ASA, and 5-ASA were compared with untreated IBD patients and to normal controls who had no abdominal complaints or diseases and were investigated for surveillance purposes. The 5-ASA preparation in our patients was exclusively mesalazine. At the time of the colonoscopy the patients had to be on the same treatment for at least 6 months with no dosage change in the last month. Patients with steroid-dependent disease or other treatments were not included but occasional prednisolone and local 5-ASA therapy were accepted. None of the patients had a history of antibiotic treatment in the last 12 months. Patients with *Serpulina (Brachyspira*) infections were excluded, since these infections are always accompanied with extremely high concentrations of bacteria within specific adherent biofilms. The baseline data of the IBD groups and control group (20 each) are summarized in Table 1.

Biopsies

The biopsies for FISH were taken from the ascending and sigmoid colon and to the extent possible, from macroscopically noninflamed tissues. The biopsies were fixed for 2 hours in nonaqueous Carnoy solution (6/3/1 vol ethanol / glacial acetic acid / chloroform) 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.

Bowel preparation for colonoscopy was performed using 2–3 L polyethylene glycol with electrolytes solution (Golytely).

FISH

Microscopy was performed with the Nikon e600 fluorescence microscope and photo-documented with a Nikon DXM1200 color camera and software (Nikon, Tokyo, Japan). Probes were synthesized with Cy3, FITC, or Cy5 fluorescent dye at the 5′ end (MWG Biotech, Ebersberg, Germany). The hybridization with Eub 338 Cy3 probe universal for Eubacteria[9] was performed at 46°C to visualize all bacteria. Bac 303,[10] EREC,[11] Fprau,[12] Ebac[13] probes representing *Bacteroides, Eubacterium rectale-Clostridium coccoides, Fusobacterium prausnitzii,* and *Enterobacteriaceae* cluster were applied in different combinations including Eub 338 probe to evaluate the bacterial diversity in multicolor analysis. The hybridizations were performed according to standard protocols and always counterstained with DAPI. 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 a magnification of 1000.[7] Additional light microscopic figures of successive sections stained with alcian blue/PAS were used for evaluation of mucus and leukocytes. All microphotographs were made in real colors and not manipulated except for brightness and contrast.

The mucus barrier function for intestinal bacteria was evaluated by the following criteria:

1. Concentrations of mucosal bacteria. The concentration of mucosal bacteria was defined as the mean concentration of adherent, mucus-scattered, and 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) contained within a 2×50 μm field, below the intact mucus layer. Mucus scattered bacteria were counted within a square

TABLE II. Characteristics of the Mucosal Barrier Function

	Azathioprine ($n = 20$) A	**Azathioprine/5-ASA ($n = 20$) B**	**5-ASA ($n = 20$) C**	**None or Occasional Corticosteroids ($n = 20$) D**	**Normal Controls ($n = 20$) E**
1. Concentrations of mucosal bacteria $\times 10^{10}$/mL	10.9 ± 17 *B = 0.013 C = 0.009 D = 0.024 E <0.001	0.77 ± 2.1 C = ns D = ns (0.20) E <0.001	0.18 ± 0.3 D = 0.017 E <0.001	1.49 ± 3.8 E <0.001	0.015 ± 0.02
2. Number of patients with bacteria adherent to at least 10% of the epithelial surface	20	16	12	15	3
3. Percent of epithelium covered with adherent bacteria	74 ± 31 B <0.001 C <0.001 D <0.001 E <0.001	38 ± 33 C = 0.025 D = ns (0.19) E <0.001	22 ± 28 D <0.001 E <0.001	52 ± 36 E <0.001	5.4 ± 12
4. Number of leukocytes per 1 mm	0.89 ± 1.4 B <0.001 C = 0.01 D = 0.03 E <0.001	7.2 ± 11 C = ns D = ns <0.001	10.23 ± 24 D = ns E <0.001	9.4 ± 26 E <0.001	0.048 ± 0.07
5. Amenability of mucosal bacteria(% of bacteria stained with DAPI)	79 ± 19 B = 0.003 C <0.001 D <0.001 E = ns	58 ± 33 C <0.001 D = ns E = 0.002	34 ± 26 D = 0.02 E <0.001	53 ± 37 E <0.001	80.8 ± 25
6. Mucus thickness in μm	37 ± 34 D <0.001	41 ± 47 D <0.001	44 ± 41 D <0.001	34 ± 22 D <0.001	82 ± 42

*P-value when comparing the results in columns A to B; ns, not significant.

field of 10 × 10 μm that was placed within the mucus at the maximal concentration of bacteria next to the epithelial surface. Mucus ceiling bacteria were enumerated within a 5 × 20 μm field that was placed within the maximal concentration of the mucus ceiling layer but at least 10 μm away from the epithelial surface.

2. The number of patients with bacteria adherent to at least 10% of the epithelial surface.
3. The percent of epithelium covered with adherent bacteria.
4. The number of leukocytes per 1 mm surface.
5. Amenability of mucosal bacteria expressed as percent of bacteria stained with DAPI that positively hybridized with the universal Eub 338 FISH probe (FISH-positive bacteria / DAPI-positive bacteria × 100).
6. Mucus thickness in alcian stain.

Statistics

Mean values and standard deviations (SDs) were calculated. Using Student's *t*-test and chi-square test and $P < 0.05$ was considered significant.

RESULTS

Numeric data characterizing the mucus barrier are summarized in Table 2. The FISH microphotographs with DAPI counterstain of single groups are presented in Figures 1–4.

Mucosal bacteria concentrations, bacterial adherence to the mucosa, and the number of leukocytes within mucus were significantly elevated within all groups of patients with IBD colitis when compared with normal controls. The characteristics differed strongly depending on therapy.

Mucosal Bacteria Concentrations and Adherence

In the 5-ASA group the concentrations of mucosal bacteria and percent of the epithelial surface covered by adherent bacteria were the most markedly reduced as compared with the other IBD groups. The differences were significant when compared with IBD patients without treatment and highly significant when compared with IBD patients on azathioprine. In the group receiving azathioprine, bacterial concentrations were increased 28-fold

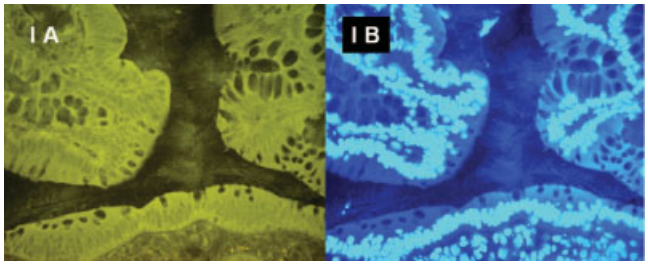

FIGURE 1. A: Hybridization with the universal for all bacteria Eub 338 Cy3 probe at magnification of ×400. The biopsy is from the ascending colon of a healthy control. The background fluorescence of the mucosa allows for excellent orientation within anatomic structures. Signals typical for bacteria are absent within mucus and on the epithelial surface. B: Blue DAPI fluorescence of the same microscopic field as A. The large light colored dots are nuclei of eukaryotic cells. Goblet cells, cells of submucosa, and the epithelial layer can be easily recognized. No bacteria or nuclei of leukocytes are present within mucus.

compared with the 5-ASA group and 1000-fold compared with healthy controls. The adherence of the bacteria was the highest in the azathioprine group, involving all patients, and was significantly different from all other groups. In the group with concomitant azathioprine/5-ASA therapy the bacterial concentrations and adherence were reduced compared with the group without treatment, but the reduction was not statistically significant.

Mucosal Leukocytes

The numbers of leukocytes within mucus were significantly elevated in groups with IBD colitis compared with controls. The leukocyte number in the azathioprine-treated group was still higher than in normal controls, but markedly reduced compared with all other IBD groups ($P = 0.03$ to <0.001). There was no statistical difference in leukocyte numbers within mucus between IBD patients treated with 5-ASA, azathioprine/5-ASA, or not treated at all.

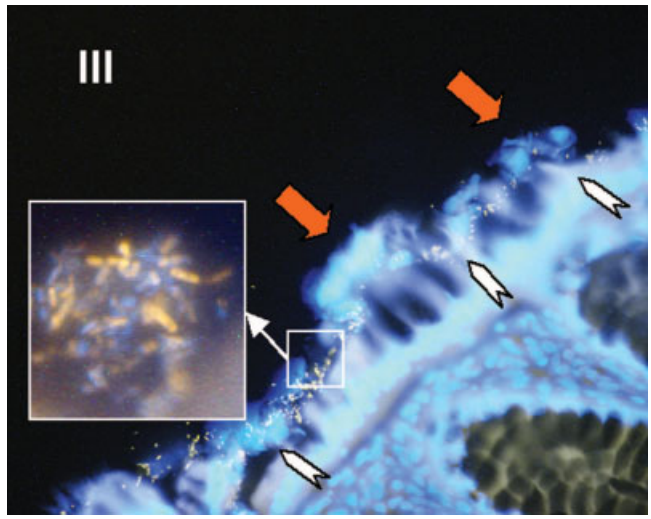

FIGURE 3. Biopsy from a patient with UC treated with 5-ASA alone. Bacterial fluorescence of universal Eub 338 Cy3 labeled probe (yellow fluorescence) and unspecific blue DAPI fluorescence of the DNA structures are overlaid. A large number of leukocytes (red arrows) within mucus and bacteria attached to the mucosa (white arrows) can be seen. The insertion within the figure at high magnification (×1000) shows a region with low leukocytes and high bacterial numbers. Less than 25% of the DAPI-stained bacteria hybridize with the universal FISH probe.

Amenability of Bacteria

The amenability of bacteria in IBD patients without therapy was significantly reduced compared with normal controls. This reduction was not observed in the IBD group treated with azathioprine and more profound in the group treated with 5-ASA. The amenability in the IBD group with

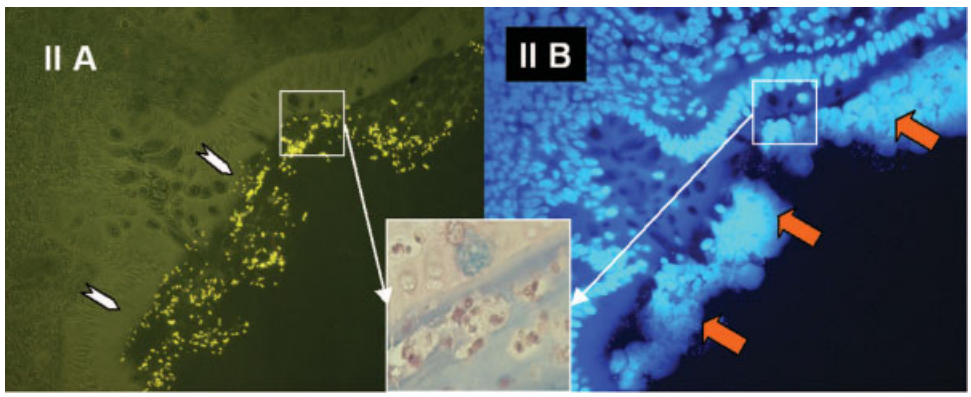

FIGURE 2. A: Hybridization with the Eub 338 Cy3 probe at magnification of ×400. The biopsy is from the ascending colon of an untreated patient with UC. Adherent bacteria and bacteria within mucus can be seen (white arrows, yellow fluorescence). B: Counterstain with DAPI of the same microscopic field as A. Multiple blue nuclei of eukaryotic cells (red arrows) are seen within mucus attached to the mucosa in regions of bacterial adherence. These nuclei can be clearly identified as granulocytes with PAS stain (insertion).

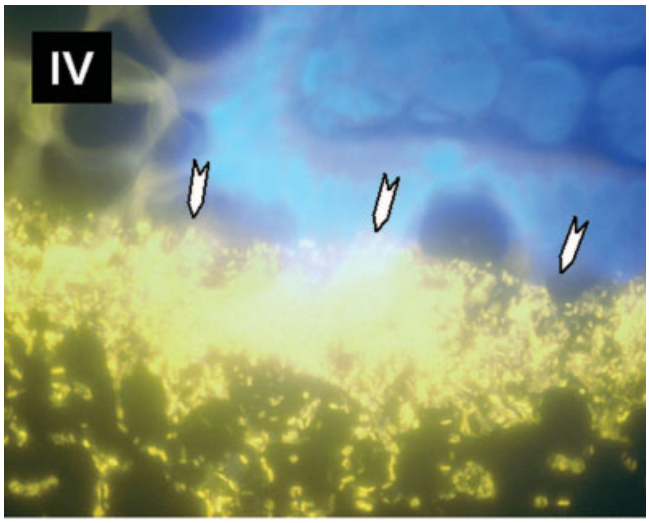

FIGURE 4. Biopsy from a patient with UC treated with azathioprine. Bacterial fluorescence of universal Eub 338 Cy3 probe and unspecific blue DAPI fluorescence of the DNA structures are overlaid. Prolific bacterial biofilm attached to the mucosa can be seen (white arrows). Bacteria are highly amenable. Nearly all DAPI-stained bacteria hybridize positively with the universal Eub 338 bacterial FISH probe. No nuclei of leukocytes can be seen within the mucus.

azathioprine/5-ASA therapy did not differ from the IBD group without therapy.

Mucus Thickness

The mean mucus thickness in all groups with IBD colitis was significantly reduced compared with controls but not different between groups on different therapy regimes.

Composition of the Mucosal Bacteria

In all IBD groups probes specific for *Bacteroides* (Bac 303), *Eubacterium rectale – Clostridium coccoides* (EREC), *Fusobacterium prausnitzii* (Fprau), and *Enterobacteriaceae* (Ebac) clusters identified together 85% of the amenable bacteria. No quantitative differences in composition of mucosal bacteria between the IBD groups were detected. The bacterial concentrations in normal controls were too low for quantitative comparison of single components.

Bacteria of *Bacteroides* and *Enterobacteriaceae* groups were clearly adherent to the mucosa, leading to a coat- or string-like appearance. The contact of other groups with the epithelial surface was less regular, often patchy, and associated with *Bacteroides* or *Enterobacteriaceae* adherence. The higher adherence of bacteria in azathioprine-treated IBD patients was accompanied by a higher adhesion of *Bacteroides* and *Enterobacteriaceae* groups to the mucosal surface.

DISCUSSION

Our data demonstrate that the effects of antiinflammatory substances in IBD are not alike. Both oral azathioprine and 5-ASA proved to be effective in controlling inflammatory activity in IBD colitis.[3–5] However, similar clinical effects of azathioprine and 5-ASA on the symptoms of IBD are obviously accompanied by opposite changes in the mucosal barrier function. 5-ASA significantly reduces the concentrations and adherence of mucosal bacteria as compared with untreated IBD patients. The leukocyte migration in the mucus is unaltered and remains high during 5-ASA treatment. Azathioprine nearly completely abolishes the leukocyte migration into the mucus, while concentrations and adherence of mucosal bacteria dramatically increase. It has been assumed that medications that are effective on their own have additive effects when used in combination. To our surprise, simultaneous azathioprine/5-ASA therapy is not additive. Instead, the drugs seem to neutralize each other's effects on the mucosal barrier, as the numbers of leukocytes, the concentrations of mucosal bacteria, and the adherence do not differ significantly from what is seen in untreated IBD patients. The additive value of 5-ASA to azathioprine therapy has been questioned in the past, both with regard to the rate of remission induction and prednisolone-sparing effects.[14,15] To our knowledge, no blinded, randomized, placebo-controlled clinical trial has specifically addressed this issue previously. The additive or interfering effects of each substance must be addressed specifically in controlled clinical studies. The mechanisms by which antiinflammatory substances interfere with mucus barrier function are generally unknown. The detection of bacteria by FISH is dependent on the metabolic activity of the microbes. The ribosome content of metabolically inactive bacteria is reduced. The fluorescence signals fade with decreasing numbers of targets for 16s RNA-based FISH probes. Metabolically silent bacteria can still be visualized with unspecific DNA stains such as DAPI, but they are no longer amenable to FISH probes. Amenability is therefore an indirect sign of bacterial vitality.[16] The amenability of bacteria in all IBD groups is significantly reduced compared with healthy controls ($P < 0.001$), except in azathioprine-treated patients. This reduction in amenability is probably a manifestation of the antibacterial activity due to ongoing inflammation and leukocyte migration into the mucus layer, as the numbers of leukocytes are significantly elevated in all the studied IBD groups. The observation of similar amenability of mucosal bacteria in healthy controls and in the azathioprine-treated group, where the number of leukocytes within mucus was the lowest, indicates that azathioprine enhances proliferation of mucosal bacteria by suppressing the local inflammatory response. The significant reduction of amenability, adherence, and concentrations of mucosal bacteria in the 5-ASA-treated group, when compared with all, and especially to the untreated IBD group, is more difficult to explain. The mucus thickness was similar in all IBD groups and cannot explain the reduced amenability. The direct antibiotic properties of 5-ASA on intestinal microbiota are un-

known. When we added 5-ASA to suspensions of fecal bacteria and plated them on different growth media, we did not notice any suppression of aerobic or anaerobic bacterial growth (data not shown). It is possible that the antiinflammatory effects of 5-ASA restore the mucus barrier function without altering the innate immunity and suppress the mucosal flora indirectly. However, this does not explain why the combination of 5-ASA with azathioprine antagonizes the azathioprine effects on the leukocyte migration in the mucus, mucosal bacteria proliferation, and bacterial amenability. Does 5-ASA boost the mucosal leukocyte response? We will test this issue in the future.

The basic therapy for IBD has evolved over years. Sulfasalazine (1942)[17] was expanded by corticosteroids (1955),[18] mercaptopurine and precursor azathioprine (1962),[19,20] mesalazine (1977),[21] cyclosporine A, tacrolimus, and infliximab.[1,3] The efficiency of each these medications has been confirmed by randomized clinical studies and recommendations have been made for single indications and groups within IBD.[22,23] However, IBD is an extremely complex disease. In practice, the borders between different groups and courses are fluent. The medications with proven efficiency are usually added in a manner that is just fashionable. Withdrawal of single medications is made mainly under the pressure of costs or adverse effects. We know today that most side effects of sulfasalazine are due to the sulfonamid component. This specific knowledge lead to a massive replacement of sulfasalazine by mesalazine in the therapy of IBD colitis. Presently, less than 1% of the UC patients treated in our clinic receive sulfasalazine. We have simply assumed that the mechanisms of both substances are equivocal since the active component is the same. However, we do not even know how these substances work. The clinical response can be well achieved by several yet unexplored mechanisms. It is important to remember that prednisolone therapy, despite an initially reported excellent response and remission rates, later proved unsuitable due to its long-term effects. Today azathioprine and other immunosuppressive substances are increasingly and successfully utilized to take IBD patients off steroids. It may well be that in the coming years we will search for substances suitable to keep patients off azathioprine and other immunomodulators.

Based exclusively on clinical criteria, no best answers can be given. The general view that UC, like Crohn's disease, is a state of uncontrolled activation of mucosal immunity may prove to be a pitfall for strategic development of new therapy concepts. Uncontrolled activation of the immune system can be observed in any unrestrained infection. IBD is a defect of innate immunity and a polymicrobial infection. No substantial curative progress can be expected as long as the infectious nature of the process and the state of the mucosal immunity is ignored.

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