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Alimentary Tract

Alterations in the colonic flora and intestinal permeability and evidence of immune activation in chronic constipation

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Abstract

Background. Disturbances in bowel function in chronic constipation could result in changes in the colonic flora and lead to disordered immunity and to decreased resistance to pathogenic flora.

Aim. To investigate systemic immunity, the faecal flora and intestinal permeability in patients with chronic constipation, under basal conditions and following therapy with the laxative Bisacodyl.

Methods. Intestinal permeability, faecal flora analysis, T- and B-lymphocyte numbers, T-cell subpopulations, lymphocyte proliferation, phagocytosis, intracellular killing of *Staphylococcus aureus* by neutrophils, as well as circulating levels of immunoglobulins, immune complexes and antibacterial antibodies were assessed in 57 patients with functional constipation. In 12 patients with severely delayed transit, investigations were repeated following therapy with Bisacodyl.

Results. Ovalbumin concentrations, in serum, were higher in constipated patients (28.2 ± 4.1 ng/ml versus 1.0 ± 0.4 ng/ml, $p < 0.05$). Elevated counts of CD3+, CD4+, CD25+ cells, increased spontaneous proliferation of lymphocytes, elevated titres of antibodies to *Escherichia coli* and *S. aureus*, diminished counts of CD72+ B cells, diminished lymphocyte proliferation under phytohemagglutinin (PHA) stimulation and a diminished phagocytic index for both neutrophils and monocytes were found in the constipated patients. Concentrations of *Bifidobacterium* and *Lactobacillus* were significantly lower in constipated patients; potentially pathogenic bacteria and/or fungi were increased. Therapy with Bisacodyl resulted in normalisation of the faecal flora, a reduction in ovalbumin concentration and return towards normal for certain immunologic parameters.

Conclusion. Constipation is associated with striking changes in the faecal flora, intestinal permeability and the systemic immune response. Relief of constipation tends to normalise these findings suggesting that these changes are secondary to, rather than a cause of, constipation.

Keywords: Bisacodyl; Chronic constipation; Faecal flora; Immunity; Intestinal inflammation; Intestinal permeability

1. Introduction

In medical practice, physicians are frequently faced with patients whose main concern is constipation. Constipation, however, does not constitute a clearly defined disease entity per se, but is, rather, a symptom of many disorders and, especially, functional disorders of the bowel. Constipation is a disturbance of bowel function manifested either in a prolonged interval between defecations, or in incomplete evacuation of the bowel. In the past, variations in prevalence of chronic constipation (CC) have resulted, in large part, from differences in the definition of the normal range for frequency of defecation [1–3]; most authors nowadays adhere, therefore, to the Rome II criteria for the diagnosis of functional constipation [4].

The large bowel is a natural reservoir for many and varied microorganisms [5]. Patients with chronic constipation may feature changes in the microflora of the large bowel, which are characterised by a relative decrease in obligate bacteria and a parallel increase in potentially pathogenic microorganisms

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and fungi [6–8]. These alterations in the intestinal microflora could alter the metabolic milieu of the colon with resultant changes in the concentration of physiologically active substances that may influence the motor and secretory functions of the bowel [9,10]. Such changes were suggested by the ability of probiotics, administered both to animals with experimentally induced constipation, as well as to patients with CC, to stimulate motility of the large bowel and normalise the intestinal microflora [11,12].

In immunocompetent hosts, the commensal luminal microflora are major determinants of mucosal and systemic immunity. Antigens and toxins derived from microorganisms constantly interact with the mucosal immune system of the large bowel to induce a state of "controlled physiological inflammation" [13–18]. However, in immunodeficient mice and humans, translocation of commensal microorganisms into lymph nodes, spleen, liver and kidney has been documented [19–22]; indeed, systemic gut-derived infections are commonly detected in immunodeficient mice and in patients deficient in phagocytic, bactericidal enzymes [23,24]. These observations suggest that, in certain circumstances, the intestinal barrier to bacterial cells and their products may be impaired. Indeed, a pathogenic role for abnormal intestinal permeability to protein macromolecules and ovalbumin, in particular, has been established in patients with inflammatory bowel disease, chronic enteritis and food allergy [25–28].

Our own clinical trials and findings from others show that patients with functional constipation and other functional gastrointestinal disorders commonly feature extraintestinal disorders, which may also have an inflammatory basis [8,29]. Furthermore, recent studies have demonstrated both low-grade inflammation and immune activation in irritable bowel syndrome, a disorder that frequently features constipation as a symptom and that may overlap with functional constipation [30]. Others have described an inflammatory disorder affecting the colon and ileum in patients with autism and related disorders and have hypothesised that the mucosal inflammation plays a primary pathogenetic role in the neurological features of these disorders [31].

The aims of the present study were, firstly, to determine if constipation is associated with colonic inflammation and, secondly, to define whether any alterations in flora, immune activation or permeability observed in constipation are a primary phenomenon or a secondary consequence of constipation.

2. Patients and methods

2.1. Patient selection

All patients were diagnosed with functional constipation according to the Rome II criteria. Organic lesions of the abdominal cavity and the pelvic floor were excluded in all patients by endoscopic (sigmoidoscopy, colonoscopy, gastroduodenoscopy), radiologic and ultrasonographic evaluation.

2.2. Scintigraphic evaluation of colon transit

To perform scintigraphy, a standard breakfast consisting of a hard-boiled hen's egg injected with a radiolabelled contrast medium (3–5 mBeq198-AU-colloid) was administered. No other dietary changes were allowed; laxatives and cleansing enemas were strictly excluded. Scintiscans were taken by a computerised gamma camera with the subject in the prone position. Successive images were taken at 4, 5, 6, 24, 48, 72 and 96 h following the intake of the radioactive meal [32].

The visual analysis of the scintigraphic images included the identification of the rate of passage of the content along the bowel and the definition of the position of the radiolabel in relation to certain anatomical locations at given points in time. Areas of interest included the distal ileum, the cecum and the ascending colon, the hepatic flexure and proximal half of the transverse colon, the splenic flexure and the distal half of the transverse colon, the descending colon to the rectosigmoid junction, the sigmoid colon and the rectum. Intestinal transit was investigated qualitatively by defining transit in relation to these segments. Normal values for percentage of radioactive content in each segment of the intestine were calculated in 20 healthy persons. The quantity of content evacuated over time and the overall transit time were also calculated. Normal values for percentage evacuation of the radioactive meal at 24, 48 and 96 h were 17.4 ± 5.7, 80.4 ± 4.4 and 94.1 ± 0.9, respectively.

Moderate delay in transit was defined as an increase in transit to between two to four times normal and severe delay as between four to six times that of normal.

For the laxation part of the study, Bisacodyl (Boehringer Ingelheim Corp.) was the laxative employed [33,34].

2.3. Immunological methods

2.3.1. Leukocyte collection

Leukocytes were collected in heparinised venous (20 units of heparin per 1.0 ml of blood) blood and separated by sedimentation of erythrocytes in gelatin (0.4 ml of 3% gelatin upon 1.0 ml of blood). Leukocytes were washed twice in phosphate-buffered saline (PBS, pH 7.4).

2.3.2. Flow cytometry

For flow cytometry analysis, the cells under investigation ($4.0–9.0 \times 10^6$/ml) were resuspended in PBS with 1% foetal calf serum and 0.1% azide. Flow cytometry was performed according to standard procedures using 100 μl cell suspensions per sample. Cells were labelled with a fluorescein isothiocyanate antibody (Becton Dickinson Corp.). Flow cytometry analysis was conducted on FACSCalibur with the use of Cell QUEST analysis program (Becton Dickinson Corp.) [35].

2.3.3. Proliferation assay

Mononuclear cells were developed in growth medium RPM1-1640 (Flow) including 10% inactivated embryonic bovine serum (Flow), 10 mm HEPES buffer (Flow) 2 mM L-glutamine (Institute for Poliomyelitis and Viral Encephalitis, Moscow), and 50 μg/ml gentamicin (Pharmacia). The experimental assay was supplemented with 10 μg/ml phytohemagglutinin (PHA) (Serva). The control assay was supplemented with an adequate quantity of growth medium. The reaction was evaluated by radiometry, using 1 μC ^{3}H-thymidine on the scintillation beta-counter (beta-Track).

2.3.4. Phagocytosis

Absorption of *S. aureus Cowan I* (L.A. Tarasyevitch Institute of Standardization and Monitoring of Biological Products, Moscow) by granulocytes and monocytes was evaluated with flow cytometry. An aliquot of 90 μl leucocytes (2×10^6 /ml) was added to 90 μl killed, FITC-labeled (Sigma Corp.) bacteria concentrated to 10^7 cells/ml, and then incubated for 30 min at 37 °C. Leukocytes were centrifuged for 5 min at $250 \times g$, were then washed with 2 ml of cooled PBS containing 0.02% EDTA and resuspended in 400 μl of PBS-EDTA. Phagocytic index was defined as the percentage of neutrophils and monocytes, which exhibited fluorescence [36].

2.3.5. Intracellular killing of microorganisms

These studies were performed on 96-well round-bottomed plates. A 90 μl suspension comprising live FITC-labeled *S. aureus*, concentrated to 10^7 cells/ml, together with 20 μl pooled serum from 25 donors and a 90 μl suspension of leucocytes (2×10^6/ml) was placed in each test well. A 90 μl suspension of FITC-labelled *S. aureus*, together with 90 μl PBS and 20 μl of pooled serum was placed in each control well. Incubation was carried out for 20 min at 37 °C. The leucocytes were sedimented by centrifugation for 1 min at $200 \times g$ and 4 °C, then washed twice by PBS to remove free microorganisms, then resuspended in 200 μl PBS (pH 7.4), and finally incubated for 1 h at 37 °C. The plate was centrifuged at $200 \times g$ for 1 min and leucocytes destroyed by adding a 200 μl solution of 10 mM carbonate–bicarbonate buffer (pH 9.5) containing 0.2% saponin for 10 min. The bacteria were centrifuged at $1000 \times g$ for 10 min, resuspended in 200 μl PBS containing 2.5 μg/ml propidium iodide (Sigma) to label killed microbial cells. Thirty minutes after this the samples were analysed by flow cytometry. The percentage of killing of staphylococci by leucocytes was calculated by subtracting of values of control wells from those of test wells [36].

2.3.6. Chemiluminescence

Three hundred microlitres PBS, a 100 μl solution of luminol (0.1 mg/ml, final concentration of 5.6×10^{-4} M) and a 100 μl suspension of leucocytes (concentrated to 2×10^6/ml) were placed in a plastic test tube. Chemiluminescence was determined using a scintillation counter (Luminometer-1251, LKB). Following determination of maximal spontaneous chemiluminescence, 10 μl zymozan (concentrated to 20 mg/ml (Sigma)) was added and stimulated chemiluminescence determined [37].

2.3.7. Immunoglobulins and circulating immune complexes

The concentrations, in serum, of immunoglobulins were measured by Mancini's technique of radial immunodiffusion, using sheep serum immunised to human immunoglobulins A, G and M (I.I. Metchnikov Institute of Vaccines and Sera, Moscow). Levels of circulating immune complexes (CIC) were evaluated by sedimentation with 4% polyethylene glycol solution (PEG) followed by spectrophotometry. The thermostable fraction of CIC was evaluated after pre-heating the serum to 56 °C for 30 min.

2.3.8. Antibacterial antibody titres

Antibacterial antibody titres were evaluated by passive haemagglutination. Patient serum was titred against sheep erythrocytes coated with antigens of *E. coli 0–14, S. aureus, Proteus mirabilis* and *Pseudomonas aeruginosa* (bacterial erythrocytic reagents, I.I. Metchnikov Institute of Vaccines and Serums, Moscow).

2.4. Intestinal permeability

The serum concentration of ovalbumin (OVA) was measured by a solid-phase enzyme immunoassay using rabbit antibodies to OVA (Scientific Institute of Nutrition, Moscow). Permeability was assessed by subtracting the OVA concentration in a fasted sample from that obtained 3 h following the ingestion of two raw eggs [38].

2.5. Faecal microflora

Analysis of the faecal microflora was performed using standard microbial culture methods, as follows.

Two- to five-gram aliquots of faeces obtained fresh following a morning bowel movement were placed in sterile containers. In the laboratory, aliquots not exceeding 0.5 g were then dissolved in a dilution of 1:10. Serial dilutions were performed with buffer at a pH of 7.0 until a dilution of 1:109. For isolation of *bifidobacteria* "Bifidum medium" (obtained from the Scientific Center of Applied Microbiology, Moscow, Russia) was implemented. Inoculation was performed from the 1:106, 1:108, 1:109 dilutions on to agar columns containing "Bifidum medium", at a temperature of 37 °C. Colonies of *bifidobacteria* were identified based on their morphologic properties. *Bacteroides* were identified by using Schaedler agar (Himedia, India) with the addition of a non-spore supplement and *Bacteroides* selective supplement reagents. Immediately, after inoculation, agar plates were placed into plastic bags (GENbaganaer, France) containing a gas mixture appropriate for anaerobes (Biomerieux). *Bacteroides* were identified using the Api-systems for anaerobes

(API 20 A, Biomerieux). *Clostridia* were identified using the Wilsson-Blair II (Russia) medium. *Lactobacilli* were identified using the medium "lactobacagar" (Scientific Center for Applied Microbiology, Moscow, Russia).

Facultative anaerobic bacteria of the family Enterobacteriaceae (*E. coli*, *Proteus*, *Klebsiela*, *Enterobacter*, etc.) were inoculated onto Endo-agar and SS agar for 18–24 h (and, in certain cases, for 48 h). Lactose-negative and lactose-positive colonies were identified up to label of genus or species using API 10S or API 20 E (Biomerieux).

Staphylococci were identified in a mannitol-containing medium. Identification of species was made on the Rapidec Staph and Api Staph test systems (Biomerieux).

Enterococci were identified in the agar for enterococci (Himedia, India). Identification was by microscopy, Gram dying catalase testing and determination of growth pattern in a medium containing bile and esculine.

Streptococci were initially identified in agar (Columbia agar) with added 5% sheep blood. Following microscopy, determination of catalase activity, haemolytic properties, and the test system Api Strept (Biomerieux) were used for identification of streptococci; Strepto-Kit was used for identification of the various groups (A, B, C, D, G, F) of β-haemolytic strains of streptococci. Fungi (yeast or mould) were identified in Saburo medium (Scientific Center for Applied Microbiology, Moscow, Russia) from inoculates of dilutions of 1:10 and 1:103. Inoculations were performed at 30 °C for 7 days with daily assessments. *Candida* species were identified using chromogenic agar and Api AUX (Biomerieux). Mould fungi were identified according to their cultural properties and microscopic appearances.

2.6. Study protocol

2.6.1. Baseline studies

Analyses of intestinal microflora, permeability, CIC and antibacterial antibodies were performed in 57 patients with chronic constipation. Studies of cellular immunity, phagocytosis and immunoglobulins were performed in 27 of these patients. Twenty-five normal subjects were used as the healthy controls.

2.6.2. Response to laxation

From the 57 constipated patients, 12 patients with severely delayed transit were selected to receive Bisacodyl for 1 month (10 mg per day). The above investigations were repeated in this subgroup, as follows: 1 week after the first spontaneous bowel movement, 4 weeks after the first spontaneous bowel movement (i.e., at the end of treatment) and 3 months after the end of treatment.

2.7. Statistical analysis

Differences between normal and constipated subjects and between pre- and post-Bisacodyl therapy were determined using Student's *t*-test with Bonferroni's correction for multiple comparisons.

2.8. Ethical approval

The study was performed in accordance with the guidelines of and with the full approval of the local ethics committee.

3. Results

3.1. Patient demographics

The study included 57 CC patients (6 males, 51 females). The CC patients were 42.2 years old on average (range 18–70 years). Mean duration of constipation was 9.95 years (0.5–36 years). The minimal defecation-free period was 3 days. Twenty-seven patients had been avoiding laxatives on a regular basis, seven patients used cleansing enemas and 23 patients achieved defecation by the intermittent use of laxatives. Seventeen patients had allergic disorders (hay fever in six, food allergy in two, drug allergy in nine) and one had bronchial asthma. Colonic scintigraphy revealed moderately delayed transit (×2–4 normal) in 40 subjects, and severely delayed transit (×4–6 normal) in 17 patients.

Following 1-month treatment with Bisacodyl, 11 of the 12 patients reported passing one stool per day; one female patient had an increase to three stools daily. Within 3 months of cessation of Bisacodyl, constipation recurred in all patients. The 45 patients who were not treated with a laxative showed no alteration in frequency of defecation.

3.2. Permeability and bacterial antibodies

All 57 CC patients (Table 1) showed a significant increase in the concentration of serum OVA compared to control subjects. There were no differences in permeability between those with moderate or severe constipation; nor did permeability correlate with the severity of colon transit delay. Serum OVA concentrations were elevated in 71.9% of the CC patients. Antibody titres to *E. coli*, *S. aureus* and *P. aeruginosa* were elevated in 50.9, 36.8, and 38.6% of the CC patients, respectively, and were significantly higher than in controls. Antibacterial antibody titres to *E. Coli* were higher in moderate CC patients than in severe CC. Titres of antibodies against *Proteus* and the concentration of circulating immune complexes (CICs) were also elevated, but these differences were not statistically different from controls. The concentration of the thermostable fraction of CICs was decreased.

3.3. Immune function

Cellular immunity, phagocytosis and immunoglobulins were studied in 27 CC patients. These results are presented in Table 2.

Table 1
Concentrations of ovalbumin (OVA), circulating immune complexes (CIC) and titres of antibacterial antibodies in chronic constipation

Test	Control group ($n = 25$)	Chronic constipation		
		All subjects ($n = 57$)	Moderate ($n = 40$)	Severe ($n = 17$)
OVA (ng/ml)	1.0 ± 0.4	28.2 ± 4.1♦	27.6 ± 4.3♦	29.4 ± 9.5♦
CIC (μ/dl)	148 ± 7.3	163 ± 5	162 ± 6.0	166.5 ± 9.0
Thermostable fraction of CIC (μ/dl)	40 ± 1.0	28.2 ± 3.1♦	29.7 ± 3.8♦	24.8 ± 5.2♦
Antibody titres				
E. Coli	12 ± 3	107.8 ± 23.9♦	140 ± 32.6♦	32.1 ± 9.2♦,*
S. aureus	18 ± 6	79 ± 23.8♦	74.5 ± 26.6♦	89.4 ± 50.6
Proteus	8.5 ± 1.5	15.6 ± 3.5	18.8 ± 4.7♦	8.2 ± 2.6
P. aeruginosa	7.8 ± 1.5	21 ± 6♦	24.8 ± 8.2♦	12.4 ± 5.0

(♦) $p < 0.05$ control group vs. constipation group; (*) $p < 0.05$ moderate vs. severe constipation.

These CC patients, in comparison to the controls, showed elevated proportions of CD3+, CD4+ and CD8+ T lymphocytes and activated CD25+ cells. The absolute counts of CD3+ and CD4+ T lymphocytes were diminished in 14.8 and 22.2% of the patients, respectively. The percentage and absolute counts of CD72+ B cells were also decreased in CC patients.

Among constipated patients, spontaneous proliferation of T lymphocytes was increased; however, the proliferation response to stimulation with PHA, whether measured as the

Table 2
Parameters of cell-mediated immunity and immunoglobulin concentrations in chronic constipation

Test	Control group ($n = 25$) (mean ± S.E.M.)	Chronic constipation (mean ± S.E.M.)		
		All subjects ($n = 27$)	Moderate ($n = 15$)	Severe ($n = 12$)
Leucocytes (10^6/l)	5250 ± 265	4850 ± 240	4720 ± 282.5	5008 ± 448
Lymphocytes				
Percentage	32.2 ± 1.1	34.0 ± 1.0	33.8 ± 1.5	34.4 ± 1.7
Abs	1740 ± 115	1660 ± 107	1583 ± 109	1761 ± 211
CD3+ (T-cells)				
Percentage	67.3 ± 1.3	73.6 ± 1.5♦	73.6 ± 2.55♦	73.6 ± 2.1♦
Abs	1205 ± 76	1227 ± 85	1163 ± 86.7	1307 ± 167
CD4+ (T-cells)				
Percentage	40.5 ± 0.9	47.7 ± 1.4♦	46.5 ± 2.1♦	49.2 ± 2.1♦
Abs	755 ± 57	800 ± 64	741 ± 69.6	876 ± 121
CD8+ (T-cells)				
Percentage	28.2 ± 1.0	31.5 ± 1.25♦	33.1 ± 1.6♦	29.5 ± 2.1
Abs	505 ± 32	520 ± 36	519.5 ± 39.6	521 ± 71.7
CD4+/CD8+	1.4 ± 0.08	1.6 ± 0.09	1.4 ± 0.09	1.8 ± 0.17♦
CD16+ NK-cells (%)	16.4 ± 1.35	15.0 ± 1.15	15.5 ± 2.2	14.4 ± 1.0
CD25+ (T-cells) (%)	5.5 ± 0.6	13.8 ± 0.7♦	13.8 ± 1.2♦	13.8 ± 1.0♦
CD72+ (B-cells)				
Percentage	11.8 ± 1.4	8.6 ± 0.75♦	8.7 ± 1.4♦	8.5 ± 0.67♦
Abs	192.4 ± 15.6	138.5 ± 11.5♦	134.0 ± 18.4♦	144.3 ± 16.2♦
Spontaneous proliferation of lymphocytes (imp/min)	855.4 ± 245	1300 ± 155	922.5 ± 133	1770 ± 264♦,*
PHA-stimulated proliferation (imp/min)	47640 ± 9810	24230 ± 3200♦	26051 ± 5677	21964 ± 3552♦
Stimulation index	50.6 ± 8.0	23.8 ± 3.1♦	31.4 ± 4.8♦	14.4 ± 2.7♦,*
Phagocytic index of neutrophils (%)	89 ± 3.2	79 ± 1.65♦	77.9 ± 2.3♦	80.3 ± 2.7♦
Phagocytic index of monocytes (%)	81 ± 4.6	72 ± 2.2	69.5 ± 3.35♦	74.9 ± 3.3
Spontaneous chemiluminescence (mv/min)	18.0 ± 2.4	17.7 ± 2.0	17.6 ± 1.95	17.9 ± 4.0
Zymozan-induced chemiluminescence (mv/min)	210 ± 19	190 ± 24	206 ± 13.5	170.7 ± 32.5
Stimulation index	47.4 ± 4.0	51.0 ± 2.5	53.3 ± 2.2	47.9 ± 5.0
Intracellular killing of *S. aureus* by neutrophils (%)	35.0 ± 6.0	31.0 ± 0.9	31.3 ± 1.1	30.5 ± 1.7
IgG (g/l)	12.6 ± 0.7	16.0 ± 0.65♦	16.9 ± 1.16♦	14.7 ± 0.6♦
IgM (g/l)	1.5 ± 0.12	2.0 ± 0.1♦	1.95 ± 0.18♦	2.0 ± 0.15♦
IgA (g/l)	2.0 ± 0.18	2.35 ± 0.15	2.3 ± 0.2	2.4 ± 0.3

♦ $p < 0.05$ control group vs. constipation group and * $p < 0.05$ moderate vs. severe constipation.

level of stimulated proliferation or the index of stimulation, was decreased in constipated patients. Similarly, the phagocytic index of neutrophils and monocytes upon absorption of *S. aureus* decreased and the level of Zymozan-stimulated chemiluminescence of neutrophils was lower in the constipated group. IgG and IgM concentrations were higher in CC patients; these levels did not, however, exceed the upper levels of normal (Table 2).

Turning to CC subgroups, spontaneous T-cell proliferation was clearly elevated in severe CC patients in comparison to moderate CC patients and controls (Table 2). Proliferation upon PHA activation was diminished in both subgroups; sig-

Table 3
Response to Bisacodyl therapy

Test	Control group ($n = 25$) (M ± S.E.M.)	Chronic constipation ($n = 12$)			
		Baseline (M ± S.E.M.)	1 week following first stool (M ± S.E.M.)	4 weeks following first stool (M ± S.E.M.)	3 months following therapy (M ± S.E.M.)
OVA (ng/ml)	1.0 ± 0.4	31.1 ± 13.3♦	22.8 ± 8.1♦	18.7 ± 8.15♦	8.2 ± 3.2♦
Leucocytes (10^6/l)	5250 ± 265	5008.5 ± 448.5	5341.7 ± 546.3	4916.7 ± 377.8	5000 ± 184.2
Lymphocytes					
Percentage	32.2 ± 1.1	34.4 ± 1.7	32.7 ± 1.7	37.25 ± 1.8♦	34.1 ± 0.9
Abs	1740 ± 115	1760.8 ± 211.4	1723 ± 145	1831 ± 171.6	1713 ± 97
CD3+(T-cells)					
Percentage	67.3 ± 1.3	73.6 ± 2.1♦	73.3 ± 2.2♦	71.4 ± 1.9	78.1 ± 1.0♦,^
Abs	1205 ± 76	1307 ± 166.6	1267 ± 115.8	1297 ± 110.6	1333 ± 70.6
CD4+(T-cells)					
Percentage	40.5 ± 0.9	49.2 ± 2.1♦	49.3 ± 1.4♦	43.7 ± 1.7*	50.5 ± 1.6♦,^
Abs	755 ± 57	875.6 ± 120.8	856.3 ± 82.1	797 ± 79.2	861.4 ± 53.4
CD8+(T-cells)					
Percentage	28.2 ± 1.0	29.5 ± 2.1	31.9 ± 1.7	30.5 ± 2.6	31 ± 1.8
Abs	505 ± 32	520.8 ± 71.7	548.4 ± 51.5	564 ± 70.8	529.7 ± 41.5
CD4+/CD8+	1.4 ± 0.08	1.8 ± 0.2	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1
CD16+NK-cells (%)	16.4 ± 1.35	14.4 ± 1.0	17.7 ± 1.6	19.6 ± 2.0*	13.1 ± 1.2*
CD25+ (T-cells) (%)	5.5 ± 0.6	13.8 ± 0.95♦	13.6 ± 1.2♦	9.7 ± 1.3♦,^	14.3 ± 0.8♦,^
CD72+ (B-cells)					
Percentage	11.8 ± 1.4	8.5 ± 0.7♦	7.6 ± 0.7♦	5.1 ± 0.8♦,^	7.25 ± 0.7♦,^
Abs	192.4 ± 15.6	144.3 ± 16.2♦	131.6 ± 17.1♦	94.3 ± 17.9♦,^	124.2 ± 13.6♦
Spontaneous proliferation of lymphocytes (imp/min)	855.4 ± 245	1769.2 ± 263.9♦	1667.6 ± 242.1♦	974.7 ± 138.2*	793.3 ± 109.1*
PHA stimulation (imp/min)	47640 ± 9810	21963.7 ± 3551.9♦	19067.7 ± 3950.9♦	19515.4 ± 2532.8♦	41383 ± 7419.7*,^
Stimulation index	50.6 ± 8.0	14.4 ± 2.7♦	14.4 ± 4.4♦	22.1 ± 3.0♦	56.3 ± 11.6*,^
Phagocytic index of neutrophils (%)	89 ± 3.2	80.3 ± 2.75♦	75.5 ± 4.5♦	89.5 ± 1.2*	86.1 ± 1.65
Phagocytic index of monocytes (%)	81 ± 4.6	74.9 ± 3.3	70.8 ± 3.6	83.5 ± 1.7*	77.1 ± 2.0*
Spontaneous chemiluminescence (mv/min)	18.0 ± 2.4	17.9 ± 4.0	15.5 ± 2.1	16.7 ± 2.3	15.8 ± 1.6
Zymozan induced chemiluminescence (mv/min)	210 ± 19	170.7 ± 32.55	233.7 ± 39.8	175.5 ± 15.5	247.1 ± 32.3
Stimulation index	47.4 ± 4.0	47.9 ± 5.0	56.5 ± 3.6	49.6 ± 4.0	60.8 ± 3.0♦,*,^
Intracellular killing *S. aureus* of neutrophils (%)	35.0 ± 6.0	30.5 ± 1.7	28.7 ± 1.1	28.6 ± 0.9	31.4 ± 0.9
IgG (g/l)	12.6 ± 0.7	14.7 ± 0.6♦	–	15.4 ± 0.6♦	16.9 ± 0.7♦,*
IgM (g/l)	1.5 ± 0.12	2.0 ± 0.15♦	–	2.15 ± 0.2♦	1.9 ± 0.2
IgA (g/l)	2.0 ± 0.18	2.4 ± 0.3	–	2.5 ± 0.3	2.4 ± 0.3
CIC (μ/l)	148 ± 7.3	167.5 ± 12.8	151.2 ± 16.6	159.1 ± 14.2	130.6 ± 13.2
Thermostable fraction of CIC (μ/l)	40 ± 1.0	26.2 ± 7.1	53.5 ± 9.0♦,*	48.8 ± 13.7	42.0 ± 6.6
Antibodies titre					
E. coli	12 ± 3	35.8 ± 12.9	66.7 ± 25.9♦	70.0 ± 51.9	70.0 ± 14.5♦
S. aureus	18 ± 6	70.0 ± 52.3	28.3 ± 9.7	30.0 ± 13.8	94.2 ± 26.8♦,*
Proteus	8.5 ± 1.5	10.0 ± 3.5	10.8 ± 3.6	9.2 ± 2.3	16.7 ± 2.6
P. aeruginosa	7.8 ± 1.5	11.7 ± 6.6	165.8 ± 4.5	5.8 ± 3.4	15.0 ± 6.2

Ovalbumin concentration and parameters of immunity in patients with severe chronic constipation in relation to Bisacodyl treatment. Abs: absolute. ♦$p < 0.05$ control vs. treatment group; *$p < 0.05$ baseline vs. post-therapy; ^$p < 0.05$ week 4 vs. 3 months post-therapy.

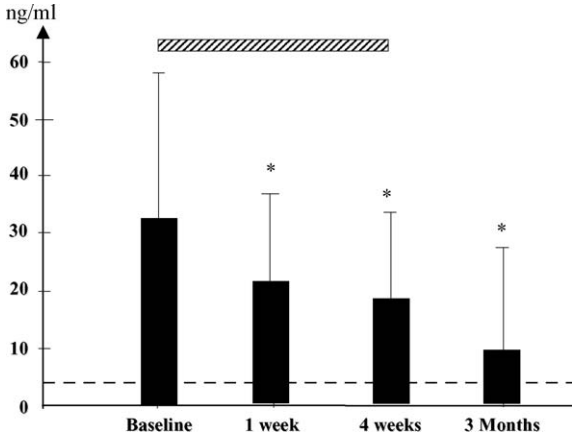

Fig. 1. Intestinal permeability response to laxative therapy: serum ovalbumin concentrations at baseline, 1 week following the first spontaneous bowel movement, 4 weeks following the first spontaneous bowel movement (end of therapy) and 3 months following therapy. Note progressive decrease in intestinal permeability with laxation, which continued for up to 3 months following cessation of therapy. Dashed bar indicates duration of therapy. Dashed line indicates upper limit of normal (≤4 ng/ml). $^{*}p < 0.05$ vs. baseline.

nificant differences, however, were only present in severe CC patients in comparison to controls. The index of stimulation of proliferation was decreased in both subgroups, but in severe CC patients the decrease was more pronounced than in moderate CC patients. Zymozan-stimulated chemiluminescence of neutrophils was decreased in the severe CC group, but not in those who with moderate constipation.

The responses among the 12 patients with severely delayed intestinal transit treated with Bisacodyl are presented in Table 3. With therapy, OVA concentration decreased and continued to fall up to the time of the last evaluation, 3 months after the end of treatment, when a four-fold decrease, in comparison to baseline values, was found (Fig. 1).

While assessment at 1 week after the first induced stool showed no changes in most immunity parameters as compared with the baseline evaluation, evaluation at the end of treatment (4 weeks after the first induced stool) showed favourable changes in some parameters; initially elevated counts of CD3+, CD4+, CD25+ T cells (Fig. 2) were reduced, spontaneous proliferation of lymphocytes (Fig. 3) and the phagocytic index of neutrophils and monocytes reached normal values (Table 3). Moreover, four parameters (CD3+, CD4+ T cells, spontaneous proliferation of lymphocytes (Fig. 3), phagocytic index of neutrophils) which were abnormal at baseline were now no longer different from that of controls. By the end of the treatment, counts of CD16 + NK cells were elevated to control levels (Table 3). In contrast, counts of CD72+ B cells decreased further during the course of treatment, to finally achieve a level which was significantly lower than both the initial level and control levels.

Within 3 months after the end of treatment, the levels of several immune parameters (CD3+, CD4+, CD25+ T cells (Fig. 2), phagocytic index of monocytes had returned to baseline, pre-treatment levels and were significantly differ-

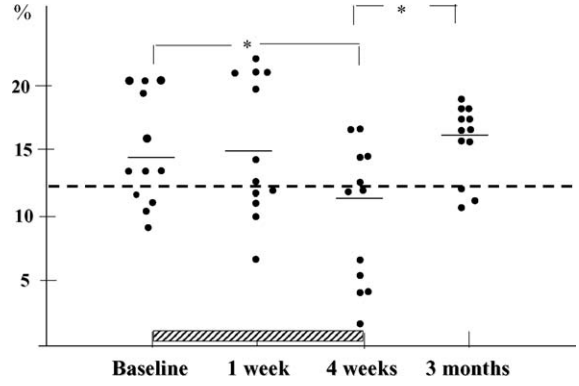

Fig. 2. Response of activated CD25+ lymphocytes to laxative therapy: percentage of CD25+ lymphocytes at baseline, 1 week following the first spontaneous bowel movement, 4 weeks following the first spontaneous bowel movement (end of therapy) and 3 months following therapy. Note elevated percentage of CD25+ cells at baseline in constipated subjects, decrease with laxation with rebound three months following cessation of therapy. Dashed bar indicates duration of therapy. Dashed line indicates upper limit of normal (≤12%). $^{*}p < 0.05$ for the comparisons.

ent from end-of-treatment and control values (Table 3). For other parameters, normal values persisted. Thus, spontaneous T-cell proliferation and proliferation upon PHA stimulation normal at 3 months. (Table 3; Figs. 3 and 4). Chemiluminescence of neutrophils upon Zymozan stimulation had returned to normal at 3 months and the index of stimulation of chemiluminescence showed a significant increase in comparison to control values and also to values at the beginning and end of treatment. Counts of CD72+ B cells were increased in comparison to values obtained at the end of laxative therapy, but remained significantly lower than those of controls. At 3 months, antibody titres against *E. coli* and *S. aureus* were still elevated, being on average, five-fold higher than controls. The concentration of the thermostable fraction of

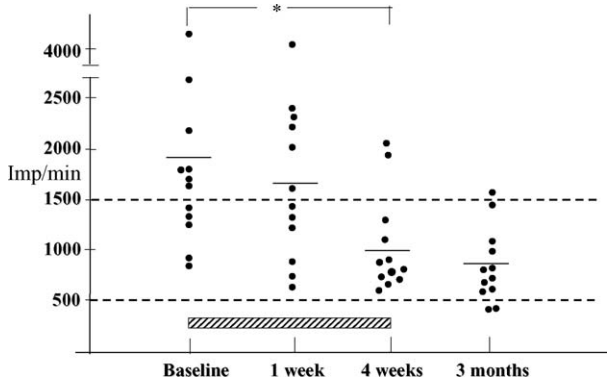

Fig. 3. Response of spontaneous proliferation of lymphocytes to laxative therapy: spontaneous proliferation at baseline, 1 week following the first spontaneous bowel movement, 4 weeks following the first spontaneous bowel movement (end of therapy) and 3 months following therapy. Note increased proliferation at baseline, in constipated subjects, progressive decrease to a normal level with laxation, which continued for up 3 months following cessation of therapy. Dashed bar indicates duration of therapy. Dashed lines delimit normal range (500–1500 imp/min). $p < 0.05$ for the comparison.

Table 4
Faecal microflora in patients with chronic constipation

Microorganisms	Control group ($n = 25$) (number/1 g faeces)	Chronic constipation			
		Number of microorganisms	% of patients		
			All constipation ($n = 57$)	Moderate ($n = 40$)	Severe ($n = 17$)
Bifidobacterium	$10^{8–9}$	$\leq 10^7$	38.6	27.5	64.7*
		Absent	26.3	15.0	52.9
Lactobacillus	$10^{6–7}$	$\leq 10^5$	63.15	65.0	58.8
		Absent	43.8	50.0	29.4
Bacteroides	$10^{8–9}$	$\leq 10^7$	50	50.0	52.9
		Absent	40.35	37.5	47.0
Clostridium	$10^{5–9}$	$\leq 10^4$	56.1	52.5	64.7
		Absent	28.1	25.0	35.5
Streptococcus fecalis	$10^{6–7}$	$\leq 10^5$	50.9	60.0	47.4
		Absent	21.0	22.5	35.3
E. coli (typical)	$10^{6–7}$	$\leq 10^5$	17.5	25.0	0*
		$\geq 10^8$	40.35	32.5	58.8
E. coli (atypical)	10^3	$\geq 10^4$	19.3	20.0	17.6
S. aureus	0	$\geq 10^3$	33.3	32.5	35.3
Enterobacteria (*Citrobacter*, *Klebsiella*, others)	10^3	$\geq 10^4$	21.0	20.0	23.5
Fungi					
Candida	10^3	$\geq 10^4$	40.35	35.0	52.9
Mould	0	$\geq 10^3$	24.5	17.5	41.2

* $p < 0.05$.

CIC, which was reduced before treatment, also returned to normal.

3.4. Faecal flora

Obligate microflora were decreased in CC patients in comparison to controls (Table 4). Individual components of the commensal flora were decreased in between 38.6 and 63.1% of patients and were completely absent in between 21.0 and 43.8%. Meanwhile, potentially pathogenic microorganisms and fungi were increased. Changes in the microflora were most pronounced in severely constipated patients. Counts of *bifidobacteria* were reduced in 64.7% of severely constipated patients in comparison to controls; no growth being found in 52.9% of the cases. Severe CC patients were more likely to demonstrate increases in counts of *E. coli* and fungi, as well as the presence, albeit in low numbers, of *Bacteroides*, *Clostridia* and faecal streptococci. In moderately constipated patients, an absence of growth of lactobacilli and reduced counts of *E. coli* were more frequent and were 1.5- to 3-fold different from the severely constipated subgroup. Both subgroups showed similarly elevated counts of uncommon *E. coli*, *Enterobacteria* and *S. aureus*.

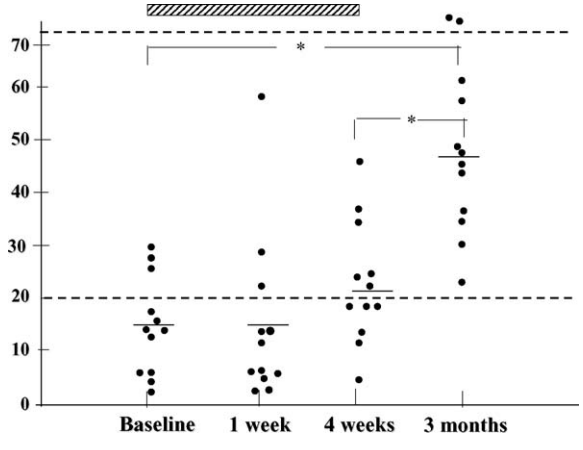

Fig. 4. Response of phytohemagluttinin (PHA)-stimulated proliferation of lympocytes to laxative therapy: stimulation index at baseline, 1 week following the first spontaneous bowel movement, 4 weeks following the first spontaneous bowel movement (end of therapy) and 3 months following therapy. Note decreased proliferation at baseline, progressive increase in proliferation with laxation, which continued for up to 3 months following cessation of therapy. Dashed bar indicates duration of therapy. Dashed lines delimit normal range (20–77). $p < 0.05$ for the comparisons.

Table 5 summarises changes in the microflora in response to Bisacodyl treatment. Before treatment, individual components of the obligate microflora were reduced in 33.3–75% of the patients with a total absence of an individual organism being recorded in between 25.0 and 58.3% of subjects. In contrast, increased growth of an individual potentially pathogenic microorganism was found in between 25.0 and 75.0% of cases.

At the end of the treatment, increased counts of obligate microflora (*Bifidobacterium*, *Bacteroides*, *Streptococcus fecalis*) and decreased counts of potentially pathogenic microorganisms (*E. coli*, fungi) were observed. Three months after the end of treatment, the values for

Table 5
Faecal microflora in patients with chronic constipation following Bisacodyl treatment

Microorganisms	Control group ($n = 25$) (number/1 g faeces)	Chronic constipation ($n = 12$)			
		Number of microorganisms	Number of patients (%)		
			1*	2**	3***
Bifidobacterium	$10^{8–9}$	$\leq 10^7$	7(58.3%)	4(33.3%)	6(50.0%)
		Absent	7(58.3%)	4(33.3%)	6(50.0%)
Lactobacillus	$10^{6–7}$	$\leq 10^5$	6(50.0%)	5(41.6%)	10(83.3%)
		Absent	4(33.3%)	5(41.6%)	5(41.6%)
Bacteroides	$10^{8–9}$	$\leq 10^7$	4(33.3%)	2(16.6%)	10(83.3%)
		Absent	3(25.0%)	2(16.6%)	5(41.6%)
Clostridium	$10^{5–9}$	$\leq 10^4$	9(75.0%)	8(66.6%)	5(41.6%)
		Absent	4(33.3%)	4(33.3%)	2(16.6%)
S. fecalis	$10^{6–7}$	$\leq 10^5$	8(66.6%)	7(58.3%)	8(66.6%)
		Absent	6(50.0%)	4(33.3%)	5(41.6%)
E. coli (typical)	$10^{6–7}$	$\leq 10^5$	0	2(16.6%)	0
		$\geq 10^8$	9(75.0%)	5(41.6%)	8(66.6%)
E. coli (atypical)	10^3	$\geq 10^4$	3(25.0%)	2(16.6%)	1(8.3%)
S. aureus	0	$\geq 10^3$	4(33.3%)	4(33.3%)	7(58.8%)
Enterobacteria (*Citrobacter*, *Klebsiella*, others)	10^3	$\geq 10^4$	3(25.0%)	5(41.6%)	5(41.6%)
Fungi					
Candida	10^3	$\geq 10^4$	5(41.6%)	4(33.3%)	8(66.6%)
Mould	0	$\geq 10^3$	5(41.6%)	2(16.6%)	6(50.0%)

*Before treatment; **after treatment (4 weeks after the first stool); ***3 months after the end of the treatment.

most microorganisms had returned to initial, pre-treatment values.

4. Discussion

In this study, the microbiological studies revealed significant changes in the composition of the faecal microflora among constipated patients. A suppression of major species of the obligate microflora was paralleled by an increased pool of potentially pathogenic microorganisms: common *E. coli* in 40.3% of the cases, atypical *E. coli* in 19.3%, *S. aureus* in 33.3% and enterobacteria in 21%. These changes were most pronounced among those who were most severely constipated and demonstrated the slowest transit through the large bowel; concentrations of *E. coli* and *Candida* were increased by 10- to 100-fold in more than half of the patients in this subgroup. Furthermore, normalisation of evacuatory function by Bisacodyl treatment was accompanied by a relative normalisation of the microflora; the number of patients with normal concentrations of bifidobacteria, *E. coli* and other commensal microorgansims increased 1.5- to 2-fold. Following the discontinuation of therapy, prior abnormalities returned. Taken together, these data suggest that the changes in the microflora are secondary to constipation; this does not discount the possibility that products released by the abnormal flora may contribute to the colonic motility changes that lead to slow-transit constipation [9,10]. The observation that the normalisation of the intestinal microflora, associated with probiotic therapy, is accompanied by a stimulating effect on colonic motility [12,39] further supports the latter concept. We acknowledge, however, the limitations of faecal flora analysis, firstly, in providing and accurate estimate of the composition of the colonic flora and, secondly, in relation to the inability of current culture techniques to detect many species that may well have important functions in health and disease.

Measurements of ovalbumin concentration in serum revealed a significant disturbance in the intestinal permeability to large molecules in CC patients. The degree of transit delay did not influence the OVA blood level. However, the restoration of a regular bowel habit by Bisacodyl resulted in a sustained reduction in OVA concentration, which persisted for up to 3 months after the end of treatment. Elevated serum ovalbumin concentrations have been well documented in inflammatory diseases of the small bowel and among patients with food allergy [26–28]. In ulcerative colitis, a disorder which is, by definition confined to the colon, there appeared to be a direct correlation between the grade of enteric mucosal lymphoplasmocytic infiltration and ovalbumin concentration; furthermore, colitis patients with an associated alteration in the colorectal flora or evidence of food intolerance exhibited four-fold higher levels of ovalbumin that those with uncomplicated ulcerative colitis [25]. There have been no studies to date of mucosal permeability among patients with more subtle inflammatory disorders of the colon, such as lymphocytic colitis. Microscopic inflammation of the colon and terminal ileum and abnormal small

intestinal permeability have been described among patients with autism and related developmental disorders; the contribution of the colon to enhanced permeability in this context is unclear [31]. Most recently, subtle inflammation and evidence of immune activation have been demonstrated in patients with irritable bowel syndrome (IBS) [30]. To date, limited studies of permeability have produced normal findings in this population. The interpretation of correlations between permeability and colonic inflammation is complicated by the potential contributions of the small intestine to any measurement of permeability. This may even be relevant to patients with constipation, given the fact that upper gastrointestinal motility is impaired in constipation [40,41]. Furthermore, in clinical practice, there is considerable overlap between the functional disorders and borders between IBS, a disorder where alterations in the enteric flora have also been mooted, and CC may be, at times, poorly defined.

Perhaps the most striking and original finding in this work was the detection of evidence of immune activation. The detection of elevated levels of CD3+, CD4+, CD8+ T cells, and especially, CD25+ cells (which carry IL-2 receptors), as well as of spontaneous proliferation of lymphocytes, indicates the activation of T-cell immunity. Significantly elevated titres against antigens from *E. coli* and *S. aureus* were also detected; this finding confirms immunisation to these antigens.

T-cell activation, elevated levels of antibacterial antibodies, as well as a tendency toward elevated concentrations of IgG, IgM and circulating immune complexes, when taken together, provide evidence of stimulation of systemic cellular and humoral immunity in CC. These phenomena were paralleled by an increase in intestinal permeability to large molecules, and lead one to speculate that delayed transit through the colon promoted changes in the colonic flora, thereby, leading to an accumulation of bacterial antigenic products in the large bowel which, either through direct interactions with the gut-associated lymphoid tissue, or following translocation across the gut wall, induce hyperactivation of the immune system [42]. Other studies support the concept of immune activation in functional disorders; Salzman et al. documented an increased density of inflammatory cells in biopsies obtained from the proximal colon in constipated patients with IBS [43] and Chadwick et al. revealed non-specific microscopic colitis in 7/11 constipated patients with IBS. In the latter study, counts of immunocompetent cells (intraepithelial lymphocytes, lamina propria CD3+ and CD25+ cells) were found to be elevated in the intestinal mucosa in all IBS patients regardless of the presence or absence of microscopic colitis [30].

The data obtained in this study also suggest a suppression of some immunological functions. Decreased proliferation of T lymphocytes and a lowering of the stimulation index to PHA activation indicate the loss of reserve proliferation potential. Some patients showed reduced absolute values of CD3+ and CD4+ T-cells. A decreased concentration of the thermostable fraction of circulating immune complexes, in constipated subjects, indicated disturbed stability of immune complexes, which can be accounted for by certain changes in intra-complex molecular links as well as a functional insufficiency of antibodies [44]. Reduced relative and absolute pools of CD72+ B lymphocytes indicate a suppression of the cellular component of humoral immunity. The phagocytic index and the level of chemiluminescence of neutrophils showed a tendency towards a decline in their ability to absorb *S. aureus* and also towards a suppression of O_2-dependent bactericidal pathways of phagocytosis.

A comparison of immunological parameters in moderate and severe CC patients revealed a correlation between disturbances in immunological function and the delay in large bowel transit. Compared with moderate CC cases, severe CC patients demonstrated higher levels of spontaneous proliferation of T lymphocytes, a greater suppression of proliferation of PHA, lower titres of antibacterial antibodies, and a tendency towards a greater reduction in Zymozan-stimulated chemiluminescence.

We must acknowledge some limitations here also. These studies did not involve the direct evaluation of the mucosal immune compartment and extrapolations from observation on systemic immune parameters to this compartment must remain guarded until both are studied together in the same context.

What are the implications of these findings? While direct evidence from human studies is lacking, evidence gleaned from experimental models suggests that these immunological changes may have implications for the organism as a whole. Thus, T-cell immunodeficient nude mice and animals depleted of CD4+ and CD8+ T cells have been shown to translocate commensal microorganisms to mesenteric lymph nodes and visceral organs [20–22]; translocation of these microorganisms in animals with a deficiency of phagocyte bactericidal enzymes resulted in fatal infections [23]. Furthermore, systemic gut-derived infections develop in patients with genetic defects of neutrophil and macrophage NADPH-oxidase enzyme complexes [24]. The central role of the luminal flora in the pathogenesis of inflammation is most vividly demonstrated by the observation that colitis develops in genetically modified animal models only in the presence of the gut flora; interestingly, colitis, in these models, is accompanied by extra-intestinal inflammatory changes and is associated with altered intestinal permeability [45,46]. One could speculate, therefore, that the on-going interaction between luminally derived bacterial antigen and the mucosal immune system, in patients with chronic constipation, results, not only in stimulation of the immune system, but also in its suppression, as manifested by depletion of T- and B-cell pools and suppression of phagocytosis and elements of humoral immunity.

The observations in relation on the response to a laxative suggest that these are, in fact, secondary phenomena. Thus, Bisacodyl therapy by restoring evacuatory function, led to a normalisation of the major parameters of cellular immunity, even though titres of antibacterial antibodies remained at a

high level, and had favourable effects on intestinal permeability, the faecal microflora and systemic immunity.

Conflict of interest statement

None declared.

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Editorial Policy on Case Reports

The Journal receives an increasing number of interesting Case Reports, many of which we would like to publish since they often receive a favourable rating from the Referees. There are, however, serious considerations in contrast with this intent, which we would like to share with our readers:

1. Since we firmly believe that the greatest part of the Journal should be reserved for Original Papers and Reviews, it has been decided to limit the publication of Case Reports to one per issue of DLD.
2. As a result of this policy, once a Case Report is accepted it may wait at least 12 months to be published. This is unsatisfactory both for the Authors and for the Journal.
3. Consequently, also bearing in mind that Case Reports, although potentially interesting from a clinical point of view are generally not widely cited, we have decided to restrict acceptance to those that describe really new observations or new associations, excluding those that are a simple, albeit interesting, confirmation of what is already reported in the literature.

As Editors, we must regretfully invite all Authors to bear in mind this note when deciding to send Case Reports or small case series to Digestive and Liver Disease.

Sincerely,

Gabriele Bianchi Porro
Editor-in-Chief

Antonio Craxí
Associate Editor