A ROLE OF HYDROGEN PEROXIDE PRODUCING COMMENSAL BACTERIA 
PRESENT IN COLON OF ADOLESCENTS WITH INFLAMMATORY BOWEL 
DISEASE IN PERPETUATION OF THE INFLAMMATORY PROCESS

Bacteria in the gut play a central role in the initiation and progress of inflammatory bowel disease (IBD). This study was prepared to elucidate the role in the inflammatory process of the bacterial species which are able to produce hydrogen peroxide, present in samples taken from colon lesions in adolescents with inflammatory bowel disease. Fifty eight adolescents were enrolled into the study from January 2004 to October 2006 in Cracow, Poland. Biopsies and stool samples were collected. Bacteriological examinations and measurements of hydrogen peroxide production by enterococci, streptococci and lactobacilli were performed. For the first time it has been shown here that HP producing bacteria may contribute to increased amounts of hydrogen peroxide in the inflamed mucosa of Crohn’s disease and ulcerative colitis patients. Moreover, we have been able to demonstrate an increase of total populations of aerobic bacteria but not anaerobes in the studied samples of mucosa of adolescents with inflammatory bowel disease which is an indirect evidence of higher oxygen tension present in inflamed tissues in IBD. We have also been able to demonstrate the direct relationship between presence of blood in stools of IBD adolescents and increased populations of Enterobacteriaceae but not streptococci in samples of colon mucosa. It is, therefore, possible that different products of Enterobacteriaceae and especially their lipopolysaccharides may also contribute to perpetuation of the chronic colon inflammation.

Key words: hydrogen peroxide, inflammatory bowel disease, ulcerative colitis, Crohn’s disease, aerobic bacteria, Lactobacillus
n numerous stimulated phagocytes is markedly increased and anti-
oxidative mechanisms are impaired (6, 7). Up to now, it is
commonly considered that increased amounts of ROS present in
colon mucosa of IBD individuals originate from stimulated gut
immune cells (9). This study was devoted to elucidate the role in
the inflammatory process of these bacterial species present in
samples taken from colon lesions in adolescents with IBD,
which are able to produce hydrogen peroxide.

MATERIALS AND METHODS

Patients

Fifty eight adolescents (mean age 15 years; SD±4.13) were
enrolled into the study from January 2004 to October 2006.
There were 12 patients with UC (mean age 14.74 years;
SD±2.9), 22 with CD (mean age 16.22 years; SD±3.8) in the
study group and 24 control subjects (mean age 14.13 years;
SD±4.4) who underwent colonoscopy because of chronic
abdominal pain. All participants were hospitalized in the
Department of Paediatrics, Gastroenterology and Nutrition of the
Jagiellonian University Medical College, Cracow, Poland.
The diagnosis of CD or UC was based on endoscopic,
histopathological, immunological and radiological criteria.
Histology was assessed blindly by an independent
histopathologist. All patients with IBD were in the active phase
of the disease. Presence of frank blood in stools was observed
during sample collection and fecal occult blood in stools was
diagnosed using commercial available quick immunoenzyme
tests (Instalert, Innovacon, USA). The use of antibiotics 30 days
prior to enrolment, infectious diarrhoea, malabsorption,
immunodeficiencies and intestinal enteropathies were the
exclusion criteria. The trial was approved by the Jagiellonian
University Bioethical Committee (No. KBET/236/B/2002) and
informed consent was obtained from all patients’ legal guardians
and/or patients over 16 years of age enrolled into the study.

Sampling of mucosa

Biopsy samples from IBD patients were obtained from the
inflamed colonic mucosa. In the control group, the biopsy
samples were taken from a normal sigmoid colon for the same
assessments. The biopsy samples were transferred directly into
Schaedler Anaerobic Broth (SAB) medium (Difco, BD, Franklin
Lakes, USA) with 10% of glycerol. The samples were
immediately snap frozen on dry ice and kept at –80°C until
analysis. All procedures were performed as quickly as possible,
during sample collection and fecal occult blood in stools was
impossible to use most

Measurement of hydrogen peroxide production by bacteria
from Enterococcus, Streptococcus and Lactobacillus genera

Analytical Merckquant peroxide test strips (Merck) were
used to measure H2O2 production by randomly selected 10
strains of each species belonging to Enterococcus, Streptococcus
and Lactobacillus genera on a detection scale between 0 and
100 mg/l. The tested bacteria were suspended in 2 ml of TSB
broth (Difco) and cultured at 37°C in aerobic conditions. The
measurements of H2O2 were done twice at 4 and 24 hours
according to the procedure provided by the producer. The mean
density of bacteria after 4 h was estimated as approximately
3×106 CFU/ml, but after 24 h this density increased to 1×109
CFU/ml. Uninoculated TSB broth was used as a negative
control. The amounts of H2O2 were given in mM or mg/l.

These data were then used to calculate: (i) the total number
of bacteria belonging to Enterococcus, Streptococcus and
Lactobacillus genera (in CFU per gram of tissue) which
produced hydrogen peroxide in samples of inflamed tissues of
patients with UC or CD vs. control group and (ii) total amounts
of HP produced by bacteria in samples in inflamed tissues of
adolescents with CD in 24 hours in comparison to amounts
produced in control patients. The following formula was used in this
calculation:

\[
\sum \left( \frac{l(i\,\text{strain})}{p(\,\text{strain})} \right) \times \left( \frac{10^7}{1} \right)
\]

m – sample mass (g); l – number of bacteria of i strain; p – mean amount of hydrogen peroxide
produced by 1×107 c.f.u./ml of i bacterial species during 24 hours.

Statistical methods

Due to extremely skewed and far from normal/Gaussian
distribution of the analyzed data, typical parametric statistical tests
could not be used. For this reason comparisons between CD and
control patients were conducted with Wilcoxon test. Moreover,
small numbers of some samples made it impossible to use most
Table 1. Primers used for species identification of selected isolates from biopsy specimens.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Primer codes</th>
<th>Sequences</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>BG1</td>
<td>CTTTGCTGGTTCCGUCACGAAAAACACCCGACGATAGGATATTGG</td>
<td>Kane et al. (25)</td>
</tr>
<tr>
<td></td>
<td>BG2</td>
<td>AACCGGCTGCTCACGTTTCCAAACGTTGGGACCTTTTGGAAGG</td>
<td></td>
</tr>
<tr>
<td><em>Bacteroides-Prevotella group</em></td>
<td>Bac32F</td>
<td>ACGGTCTACGCAAGCCTT</td>
<td>Bernhard et al. (26)</td>
</tr>
<tr>
<td></td>
<td>Bac303R</td>
<td>CCAAATGACGGACACCTCT</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>AcI</td>
<td>AGC TGA ACC AAC AGA TTC AC ACT AGG GGT GCA TCT AAT C</td>
<td>Tilsala-Timsjarvi et al. (27)</td>
</tr>
<tr>
<td></td>
<td>AcI II</td>
<td>AGC TGA ACC AAC AGA TTC AC ACT AGG GGT GCA TCT AAT C</td>
<td>Walter et al. (28)</td>
</tr>
<tr>
<td><em>L. delbrueckii</em></td>
<td>Del I</td>
<td>AGC TGA ACC AAC AGA TTC AC ACT AGG GGT GCA TCT AAT C</td>
<td>Tilsala-Timsjarvi et al. (27)</td>
</tr>
<tr>
<td></td>
<td>Del II</td>
<td>AGC TGA ACC AAC AGA TTC AC ACT AGG GGT GCA TCT AAT C</td>
<td>Walter et al. (28)</td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td>Lfps Fern II</td>
<td>GCC GCC TAA GGT GGG ACA GAT CTG ATG GAT CAG TCA AG</td>
<td>Tilsala-Timsjarvi et al. (27)</td>
</tr>
<tr>
<td></td>
<td>Lfps Plan II</td>
<td>GCC GCC TAA GGT GGG ACA GAT TTA CCT AAC GGT AAA TGC GA</td>
<td>Walter et al. (28)</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>Gasi</td>
<td>GAG TGG GAG AGC ACT AAA G CTG TTT CAA GGT GAG TTT CTC</td>
<td>Tilsala-Timsjarvi et al. (27)</td>
</tr>
<tr>
<td></td>
<td>Gasi II</td>
<td>GAG TGG GAG AGC ACT AAA G CTG TTT CAA GGT GAG TTT CTC</td>
<td>Walter et al. (28)</td>
</tr>
</tbody>
</table>

Table 2. Numbers of bacteria (in CFU/g of tissue) cultured from biopsy and stool samples taken from adolescents with UC, CD and from the control group.

<table>
<thead>
<tr>
<th>Groups (taxons) of bacteria</th>
<th>Adolescents with ulcerative colitis (n=12)</th>
<th>Adolescents with Crohn’s disease (n=22)</th>
<th>Adolescents in the control group (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue samples</td>
<td>Stool samples</td>
<td>Tissue samples</td>
</tr>
<tr>
<td>Global numbers of the cultured bacteria</td>
<td>5.83×10^5</td>
<td>3.41×10^5</td>
<td>3.93×10^5</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td>4.92×10^5</td>
<td>7.35×10^5</td>
<td>2.17×10^5</td>
</tr>
<tr>
<td><em>Enterococcus</em></td>
<td>1.80×10^6</td>
<td>4.55×10^6</td>
<td>1.36×10^6</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>6.83×10^5</td>
<td>5.40×10^5</td>
<td>1.29×10^6**</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>9.28×10^6</td>
<td>1.10×10^6</td>
<td>3.37×10^6</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>5.56×10^5**</td>
<td>1.47×10^5</td>
<td>8.62×10^5*</td>
</tr>
<tr>
<td><em>Bacteroides</em></td>
<td>2.00×10^6</td>
<td>1.00×10^5</td>
<td>1.24×10^5</td>
</tr>
</tbody>
</table>

* significant difference between UC and/or CD vs. control group (p=0.01 to 0.005)
** highly significant difference between UC and/or CD vs. control group (p=0.001 to 0.01).

RESULTS

Numbers of all cultivable bacteria present in homogenates of biopsies taken from mucosal sites with visible inflammation in adolescents with UC were significantly higher than those taken from control group (Table 2). Moreover in patients with UC the total numbers of bacteria of the *Lactobacillus* genus were significantly higher than in control group. Microbiological examination done on the stool samples collected from patients with UC in comparison to control group have shown statistically higher counts of Gram-negative rod populations, especially of *Escherichia coli*.

In the tissue samples collected from inflammatory lesions of adolescents suffering from CD, the numbers of streptococci were significantly higher than in controls. Furthermore in the stool samples obtained from patients with UC in comparison to faecal samples of control adolescents, statistically higher numbers of *Streptococcus* populations and *Escherichia coli* were observed. Both in adolescents with UC and CD, numbers of bacteria of *Bifidobacterium* genus appeared to be significantly lower in inflamed sites than in unchanged sites in controls.

Randomly selected 10 strains of each species belonging to *Enterococcus*, *Streptococcus* and *Lactobacillus* genera which were isolated from biopsies were tested for production of HP in vitro. It appeared that strains of *Lactobacillus acidophilus* produced measurable amounts of peroxide, which differed from 0.3 mM for most of the tested strains up to 1.8 mM liberated in 24 hours by *L. delbrueckii* and *S. mitis* (Table 3).

Numbers of all bacteria belonging to HP producing species of the *Lactobacillus*, *Streptococcus* and *Enterococcus* genera present in samples taken from inflamed sites of adolescents with UC and CD were summarized and compared with corresponding numbers of the same species in samples from control adolescents. As shown in Fig. 1, populations of HP producing bacteria were significantly (p<0.01) more numerous in inflamed tissues of adolescents with UC in comparison to control group. This phenomenon was not observed in adolescents with CD. To elucidate this discrepancy, an attempt was made to calculate the total amounts of HP produced by bacteria of the three genera in tested inflamed sites of all adolescents with CD in 24 hours in comparison to amounts produced by the control group using the formula presented in Materials and Methods section. As shown in Fig. 2, the total amounts of HP produced by all bacteria of the...
Streptococcus genus in inflamed tissues were significantly higher (p<0.048) than those produced in non-inflamed mucosa of the control adolescents. This difference was not significant for bacteria of the remaining, previously listed genera.

Increased numbers of aerobic bacteria, mainly streptococci and lactobacilli present in inflamed mucosa of adolescents with UC and CD and decreased numbers of anaerobic bifidobacteria, as shown in Table 2, may indicate inverse proportions between aerobic and anaerobic bacteria present in inflamed mucosa in comparison to unchanged mucosa in controls. To prove this hypothesis, total numbers of cultivable bacteria belonging to anaerobic and aerobic taxons were compared in samples taken from adolescents with CD and UC and in biopsies from adolescents of the control group. As shown in Fig. 3, populations of aerobic bacteria significantly predominated those of anaerobic bacteria (p<0.001) in samples from inflamed sites of both UC and CD adolescents but not in samples from adolescents of the control group.

Since it was possible to demonstrate significantly increased numbers of aerobic Gram-negative rods of Enterobacteriaceae family in faecal samples of adolescents with CD and UC versus control groups (Table 2), a question was raised if these changes were related to some factors playing a role in pathology of IBD linked to infected mucosa, like blood extravasated from ulcerations. As shown in Fig. 4, presence of blood in stools of the studied adolescents was positively related to elevated numbers of the Gram-negative rods (especially of Escherichia coli) cultured from biopsies and to diminished numbers of

**Table 3. Mean amounts of hydrogen peroxide (in mM) produced in vitro in 4 and 24 hours by 10 randomly selected strains of species belonging to Enterococcus, Streptococcus and Lactobacillus genera isolated from colon mucosal biopsies and faeces obtained from adolescents with UC and CD.**

<table>
<thead>
<tr>
<th>Species of tested bacteria</th>
<th>Mean amount of H₂O₂ produced by 10 strains of the same species in 4 hours (in mM)</th>
<th>Mean amount of H₂O₂ produced by 10 strains of the same species in 24 hours (in mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. faecium</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. avium</td>
<td>0.3±0.047</td>
<td>0.6±0.1414</td>
</tr>
<tr>
<td>E. durans</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>0.3±0.047</td>
<td>0.31±0.057</td>
</tr>
<tr>
<td>S. bovis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. mitis</td>
<td>0.6±0.095</td>
<td>1.8±0.566</td>
</tr>
<tr>
<td>S. oralis</td>
<td>0.3±0.032</td>
<td>0.6±0.1414</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>0.3±0.032</td>
<td>0.9±0</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>0.9±0</td>
<td>1.8±0</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>0.3±0.043</td>
<td>0.6±0.1414</td>
</tr>
<tr>
<td>L. brevis</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
may contribute to increased amounts of HP in the inflamed mucosa has been shown here for the first time that HP-producing bacteria property: ability to produce hydrogen peroxide extracellularly. It of the peptidoglycan in their cell walls but also share similar aerobic members of the commensal gut flora, show similar structure changes strongly resembling those in human CD. Although it is generally accepted that CD and UC are two clinical forms of the IBD in humans, most probably pathomechanisms of these forms are different which influences the composition of the bacterial flora in inflammatory lesions, as described by Frank et al. (13). The review of Weersma et al. (14) on gene mutations leading to inflammation in CD and UC patients also suggests such a possibility. It is of interest that both lactobacilli and streptococci, which are aerobic members of the commensal gut flora, show similar structure of the peptidoglycan in their cells walls but also share similar property: ability to produce hydrogen peroxide extracellularly. It has been shown here for the first time that HP producing bacteria may contribute to increased amounts of HP in the inflamed mucosa of CD and UC patients. It is known that increased concentrations of ROS in gut mucosa are harmful to the integrity of the epithelium (8). Most dangerous to the tissue is the chronically increased ROS flow originating from activated immune cells but also, as shown here, from HP producing bacteria adhering to IBD lesions.

Moreover, we have been able to demonstrate in this study an increase of total populations of aerobic bacteria but not anaerobes in the studied samples of mucosa of IBD adolescents which is an indirect evidence of higher oxygen tension present in inflamed tissues in IBD. This may be a reflection of the imbalanced antioxidative activity of the IBD mucosa (15).

There are many publications on IBD and UC, including somewhat related probiotic applications (16, 17, 18). Our studies done on mice models of IBD have indicated that the process is related to increased numbers of Escherichia coli in the colon (19). Similar increase was reported in IBD patients by two other groups (4, 20). Although we were unable to show more numerous populations of E. coli in tissue samples taken from adolescents with both UC and CD, we demonstrated higher populations of Enterobacteriaceae in faeces of IBD adolescents. It is generally known that IBD lesions cause extravasation of blood from ulcerated tissue and increased amounts of free heme particles present also in colon lumen and content. It is therefore possible that free iron ions may trigger gut populations of E. coli and other members of Enterobacteriaceae family to multiply more intensively. It is known that free iron ions are extensively trapped by E. coli siderophores in more aerobic conditions and cause increased multiplication of the bacterial populations (21-23). We have been able to demonstrate the direct relationship between presence of blood in stools of IBD adolescents and increased populations of Enterobacteriaceae but not streptococci in samples of colon mucosa. It is, therefore, possible that different products of elevated in such way populations of Enterobacteriaceae and especially their lipopolysaccharides may also contribute to perpetuation of the chronic colon inflammation (24).

Acknowledgements: This study was partially supported by a grant no. 2PO5A 094 29 from the Polish Ministry of Research and Higher Education.

Conflict of interests: None declared.

REFERENCES


Received: September 9, 2009
Accepted: November 30, 2009

Author’s address: Prof. Piotr B. Heczko, M.D., Ph.D., Head of the Chair of Microbiology, Jagiellonian University Medical College, 18 Czysta Street, 31-121 Cracow, Poland; Phone: +4812 633 25 67; Fax: +4812 423 39 24; E-mail: mbheczko@cyf-kr.edu.pl