Detoxification of LPS by alkaline phosphatase
Tuin, Annemarie

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 6

Oral administration of alkaline phosphatase ameliorates colitis

Annemarie Tuin, Alie de Jager-Krikken, Lisette Bok\textsuperscript{1}, Willem Raaben\textsuperscript{2}, Markwin P. Velders\textsuperscript{2}, Dirk K.F. Meijer, Klaas Poelstra and Gerard Dijkstra\textsuperscript{1}

Department of Pharmacokinetics and Drug Delivery, University Centre for Pharmacy, University of Groningen, The Netherlands
\textsuperscript{1} Department of Gastroenterology and Hepatology, University Medical Centre Groningen, The Netherlands
\textsuperscript{2} AM-Pharma, Bunnik, The Netherlands

Submitted
Abstract

Background & Aims: Crohn’s disease (CD) and ulcerative colitis (UC) are chronic multifactorial inflammatory bowel diseases with unknown etiology, but a dysregulated mucosal immune response to gut-derived bacterial antigens is thought to be involved. Toll-like receptor ligands, especially lipopolysaccharide (LPS), seem to contribute in the maintenance of the disease. Previously, we showed that the enzyme alkaline phosphatase (AP) is able to detoxify LPS and the aim of this study was therefore to examine its role in inflammatory bowel diseases.

Methods: We examined intestinal AP (iAP) mRNA expression and LPS-dephosphorylation in intestinal biopsies of control persons and IBD patients, and we studied the effect of orally administered acid-protected enteric coated iAP-tablets on the progression of dextran sodium sulphate-induced colitis in rats.

Results: In healthy persons, iAP mRNA and protein expression was high in the ileum relative to the colon. iAP mRNA expression was not altered in CD patients, but it was markedly reduced in UC patients when inflamed tissue was compared to non-inflamed tissue. Oral administration of iAP-tablets to colitic rats resulted in a significant attenuation of colonic inflammation as reflected by reduced mRNA levels for TNFα, IL-1β, IL-6 and iNOS, a reduced iNOS-staining and inflammatory cell influx, and a significantly improved morphology of the intestinal wall.

Conclusions: The present study shows that epithelial iAP mRNA expression is clearly reduced in UC patients. The rat colitis model showed that oral administration of iAP can not only replenish the intestinal tract with active AP-enzymes, but also results in a significant reduction of gut inflammation. This may provide new opportunities for the treatment of IBD.
**Introduction**

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD) of the digestive tract that are thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of the normal luminal flora (1). The exact causes of IBD are still unclear but environmental factors, genetic predisposition and immunologic disorders are suggested to be involved. Mutations in several genes, like NOD2/CARD15 during CD (2) and TLR4 during CD and UC (3; 4), seem to predispose for IBD. The intracellular protein encoded by the NOD2 gene is thought to interact with bacterial products like peptidoglycans (5; 6) and TLR4 is the signaling receptor for lipopolysaccharide (LPS). Deficiencies in response mechanisms against bacterial products, including LPS, thus seem to be an important factor in IBD.

Alkaline phosphatase (AP) has been found to dephosphorylate LPS (7-10), which results in the formation of a non-toxic lipid A group within the LPS molecule. In general, the lipid A group of LPS harbours 2 phosphate groups that are responsible for the toxicity of LPS and AP was shown to remove at least one of these phosphate groups. This enzyme is abundantly present along the microvilli in the small intestine of all species (11), indicating a possible role in the protection of the host against endotoxins.

As AP is able to detoxify LPS and response mechanisms against LPS are changed during IBD, we wondered whether the levels of AP are changed in the intestines of IBD patients. Therefore, iAP mRNA expression and LPS-dephosphorylation in intestinal biopsies of control persons and IBD patients were determined. Furthermore, we studied the efficacy of orally administrated acid-resistant iAP-tablets on dextran sodium sulphate-induced colitis in rats. In this study, we show that epithelial iAP expression is decreased in UC patients and that oral iAP administration ameliorates LPS-mediated symptoms in colitic rats. In colon biopsies of IBD patients, a response to LPS was only observed when the epithelial layer was affected by ulcerations. These observations provide novel insights and a rationale for new therapeutic strategies against IBD through augmentation of LPS detoxification in the intestinal lumen.
**Materials & Methods**

*Patient characteristics / specimen collection*

Intestinal mucosal biopsy specimens were obtained during endoscopy following informed consent (approved by the Ethics Committee of the University Medical Centre Groningen) from patients with Crohn’s disease (CD), ulcerative colitis (UC) and control subjects. Patient characteristics are described in table 1. Diagnosis of IBD was established by endoscopic and histopathological examination. The group control subjects were referred to our endoscopy centre because of polyp surveillance or changed stool frequency. In control subjects, biopsies were obtained from 4 different intestinal areas (ileum, ascending colon, transverse colon, and rectum). Biopsies from IBD patients were obtained from the rim of ulceration’s or aphthoid lesions if present and from macroscopic non-inflamed areas using a standard biopsy forceps. Intestinal specimens were immediately snap-frozen in liquid nitrogen for mRNA and protein analysis or liquid nitrogen-cooled isopentane for immunohistochemical staining, and stored at –80°C until further processing. For LPS incubation experiments, biopsies from the transverse colon were immediately incubated after endoscopy.

<table>
<thead>
<tr>
<th>Table 1: Patient’s characteristics</th>
<th>Total number (males/females)</th>
<th>Mean age (years)</th>
<th>Medication (without/with)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 10(1/9)</td>
<td>37 (20-80)</td>
<td>7/3</td>
<td>Azathioprine (2), corticosteroids (2) 5-aminosalicylic acid (1)</td>
</tr>
<tr>
<td>UC 10(7/3)</td>
<td>37 (27-59)</td>
<td>4/6</td>
<td>Azathioprine (1), corticosteroids (3) 5-aminosalicylic acid (5)</td>
</tr>
<tr>
<td>Healthy controls 8(4/4)</td>
<td>56 (36-77)</td>
<td>8/0</td>
<td>Polyps (2)</td>
</tr>
</tbody>
</table>

*Enzymehistochemical detection of AP activity*

LPS-dephosphorylation by human iAP was examined in cryostat sections (5 µm) of biopsies of human ileum and colon (ascendens, descendens and rectum) with LPS as a substrate as described previously (12). The specificity of this staining has been demonstrated before using the iAP-inhibitor L-phenylalanine (13). LPS was omitted in control incubations.
Incubation of human intestinal biopsies
Per patient, 8 biopsies were collected and immediately put in 6-well plates containing 2 ml William’s medium E supplemented with glucose (final conc. 25 mM), gentamicin (final conc. 50 µg/ml), amphotericin B (final conc. 2.5 µg/ml) and 1% human serum. Of the 8 biopsies, 2 were incubated in medium only (controls), 2 in medium plus 500U AP, 2 in medium plus 10 µg/ml LPS and 2 in medium plus 500U AP and 10 µg/ml LPS. After 4 hr incubation in a CO₂-incubator, biopsies were snap-frozen in liquid nitrogen and stored at –80°C until RNA isolation.

RNA isolation and real-time PCR
RNA was isolated from incubated human intestinal biopsies using the QIAGEN RNeasy Mini Kit and subsequently converted to cDNA with the Promega Reverse Transcription System. The cDNA was amplified with appropriate primers (Table 2) by quantitative real-time PCR using SYBR Green (Applied Biosystems) and products were detected using the ABI PRISM 7900HT Detection System. Relative quantification of the genes was calculated using the comparative threshold cycle (CT) method as described by Van de Bovenkamp, using GAPDH as a housekeeping gene (14).

Table 2: Primers used for amplification of the listed genes in human cDNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reversed primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCATCACCATCTTCACCAGGAG</td>
<td>CCTGCTTCACCACCTTCTTG</td>
</tr>
<tr>
<td>iAP</td>
<td>ACGCGGCAATGAGGTCATCTT</td>
<td>CGCCAAGGATCACGTCAAT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GGGCCTCAAGGAAAAGAATC</td>
<td>TTCTGCTTGGAGGTCCTGA</td>
</tr>
<tr>
<td>TNFα</td>
<td>CGTCTCCTACCAAGACCAAGG</td>
<td>CCAAAGTAGACCTGCCCAGA</td>
</tr>
<tr>
<td>Villin</td>
<td>TGACCCCTGAGACCCCATC</td>
<td>TCAGCATGATCTGGCCTCA</td>
</tr>
<tr>
<td>CD14</td>
<td>GCAACACAGGAATGGAGAC</td>
<td>CCAAGGAACGACAGATGAG</td>
</tr>
<tr>
<td>TLR4</td>
<td>GGCTTGTCAGTCTCAGAGT</td>
<td>GAGGTCCAGGAAGGTCAAGT</td>
</tr>
</tbody>
</table>
In vivo experiments

To examine whether exogenous iAP affects experimental colitis, a colonic inflammation was induced in male Sprague-Dawley rats by dextran sodium sulphate (DSS). The rats were divided in four groups: 1: normal drinking water and placebo-tablets (n=5), 2: normal drinking water and iAP-tablets (n=5), 3: 5% DSS in drinking water and placebo-tablets (n=10) and 4: 5% DSS in drinking water and iAP-tablets (n=10). Both the iAP- and placebo-tablets had a diameter of 5.3 mm and an enteric coating, consisting of eudragit L, triethylcitraat and talc, to prevent dissolution in the stomach, which would destroy the activity of acid-sensitive AP enzymes. The pH at which the tablets dissolved was determined at 5.5. The AP-tablets contained 1250 glycine units iAP (specific activity: 1035 units/mg protein), as determined by a standard enzyme activity assay.

Table 3: Primers used for the amplification of the listed genes in rat cDNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reversed primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCATCACCATCTTCCAGGAG</td>
<td>CCTGCTTACCCACCTTCTTG</td>
</tr>
<tr>
<td>TNFα</td>
<td>ATGTGGAACCGGAGAGGAG</td>
<td>GGCCATGGAACTGATGAGAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGGCAGTGACTTCTTGTG</td>
<td>GGAGAGCTTTTCAGCTCACAT</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCGGAGAGGAGACTTCAAGAG</td>
<td>ACAGTGATCATCGGTGCTACA</td>
</tr>
<tr>
<td>iNOS</td>
<td>CCTGCCATCTCTCGTCTA</td>
<td>ACCTGGACTTCTACTCTG</td>
</tr>
<tr>
<td>IL-10R</td>
<td>GCCCAGAGACTCTCGTACG</td>
<td>AAGACCCCTCCTTCCCAGA</td>
</tr>
<tr>
<td>villin</td>
<td>TGTGGAACCTGGAGAGGAG</td>
<td>GGGTGTTGGTCTTGGAGTTATT</td>
</tr>
</tbody>
</table>

Oral treatment consisted of daily administration of a tablet, under isofluran/O₂/N₂O anaesthesia, from day 1 to 7 after start of the DSS administration. From day 1 to 8, the rats were daily weighed, their consumption of drinking water was measured and their condition was scored using a standard scoring-procedure. At day 8, the rats were anaesthetized with isofluran/O₂/N₂O and sacrificed by heart puncture. Faeces were collected for measurement of AP activity. The colon was harvested and scored macroscopically by examining whether there was distension.
(score 1), partial distension (score 0.5) or no distension (score 0) and whether the serosa was thickened (score 1), partially thickened (score 0.5) or not thickened at all (score 0). The proximal, middle and distal part of the colon were scored separately and all scores were summed. Thereafter, the colon was weighed, the length was measured and tissues samples of the distal part were stored for RNA isolation and real-time PCR analysis, as described above, for several genes (Table 3). The rest of the colon was filled with Tissue-Tek®, rolled up and frozen in isopentane for histochemical analysis.

**AP activity in faeces**
Homogenates of approximately 1 gram/ml rat faeces in water were centrifuged at 2000 rpm to spin down insoluble materials. The supernatant was removed and centrifuged at 13000 rpm to completely remove insoluble materials. Samples were diluted 0, 2, 4, 8, 16, 32, 64 and 128 times in a 96-wells plate in 0.05 M ammediol buffer containing 2 mM MgCl₂. After addition of 10 µl of 10 mg/ml 4-nitrophenyl phosphate disodium salt, the plate was incubated for 30 min at 37°C. The reaction was stopped by adding 105 µl 1N NaOH. The ODs were measured at 405 nm on a Thermomax microplate reader.

**Immunohistochemistry**
The H&E staining was performed according to standard procedures. Myeloperoxidase (MPO) activity in activated neutrophils was visualized according to Poelstra (15). This staining was inhabitable by catalase. The staining for iNOS was done according to standard indirect immunoperoxidase techniques with a rabbit polyclonal antibody directed against iNOS and GARPO (DAKO) as the secondary antibody. The iNOS antibody was developed in the laboratory of dr. H. Moshage (University of Groningen, The Netherlands) and has been described previously (16). Peroxidase activity was visualized with 3-amino-9-ethylcarbazole. The staining for villin was performed like the iNOS-staining using a goat polyclonal antibody against villin (sc-7672, Santa Cruz) and RAGPO and GARPO as a secondary respectively third antibody (both from DAKO).
Statistical analysis
Statistical analysis of patient data was done by an unpaired two-tailed Student’s t-test, assuming similar variances, and expressed as the mean ± the S.D. The data of the animal experiment were subjected to a non-parametric one-sided Mann-Whitney U test, because these data were not normally distributed. Differences were considered significant at p< 0.05.

Results
LPS-dephosphorylation and iAP mRNA levels in healthy human intestinal biopsies
To investigate whether the human small intestine and colon have LPS-dephosphorylating activity, cryostat sections of biopsies of human terminal ileum, colon ascendens, colon transversum and rectum of healthy persons were examined. Results from the enzymehistochemical analysis showed that the cells in sections of human ileum have a high LPS-dephosphorylating activity as demonstrated by a brown lead sulphate precipitate along the apical side of the microvilli of the enterocyte (Fig. 1A). In contrast, LPS-dephosphorylation was absent in human colon sections; colon ascendens, colon transversum and rectum (Fig. 1B, C and D). These reactions were also negative when a conventional substrate for AP (β-glycerophosphate) was used. Occasional cells stained positive, which probably reflects AP activity in macrophages and endothelial cells of small blood vessels. When LPS was omitted from the incubation medium, no staining was detected (Fig. 1E).

In addition to LPS-dephosphorylation, the biopsies were inspected for their intestinal AP mRNA levels. As previous studies have shown that AP can dephosphorylate LPS (7; 8), AP expression levels may be indicative for the LPS-detoxifying capacity of the intestine. iAP mRNA levels in the human ileum were found to be about 30 times higher than those in the human colon (Fig. 2).
Figure 1: LPS dephosphorylation by sections of the intestine of a normal healthy person (a full color version of this figure is depicted on page 172). A brown staining was clearly visible along the apical side of the microvilli of the enterocyte in the terminal ileum (A). In contrast, biopsies from the colon ascendens (B), colon transversum (C) and rectum (D), showed hardly any LPS-dephosphorylating activity along the enterocytes. Occasional cells stained positive (arrows). Control sections (without LPS) were completely negative (E). Magnification 200*. 
Figure 2: Intestinal iAP expression throughout the human colon and in the ileum. Biopsies were taken from healthy subjects (n=6) and analyzed for iAP mRNA levels by real time RT-PCR. Expression levels of iAP mRNA were related to the average expression level in the colon transversum. Values are expressed as mean ± SD, * = p<0.005 versus ileum.

LPS-dephosphorylation and iAP mRNA levels in colon biopsies of UC and CD patients
After assessing LPS-dephosphorylation and AP-activity in the small intestine and colon of healthy persons, we examined whether these activities had changed during IBD. Similar to normal colon biopsies, no LPS-dephosphorylation could be detected in colon biopsies from CD or UC patients (data not shown).

We subsequently examined mRNA levels of iAP. Real-time PCR revealed clear differences in mRNA levels between inflamed and non-inflamed tissue. A decline in mRNA levels was found in UC patients; a 5-fold decrease of iAP mRNA levels was observed when inflamed and non-inflamed tissue of the same patient were compared (Fig. 3A). To examine whether this decline in mRNA levels was due to the destruction of colonic epithelial cells by ulceration, we measured the amount of villin mRNA (Fig. 3B), which is a marker for intestinal epithelial cells (17; 18). Inflamed mucosa from UC patients displayed a clear decrease in villin expression (p<0.005). In contrast, Crohn patients displayed no significant differences in iAP and villin mRNA levels between inflamed and non-inflamed tissue, but a clear correlation was found between iAP and villin mRNA levels in the ileum of Crohn
Oral administration of alkaline phophatase ameliorates colitis patients ($R^2=0.98$, $p=0.0001$), which links the iAP expression to the intact intestinal epithelium.

Figure 3: iAP (A) and villin (B) mRNA expression in non-inflamed and inflamed intestinal tissue of UC patients ($n=7$). Total RNA was isolated from biopsies of non-inflamed and inflamed intestinal tissue. A decreased iAP expression strongly correlated with a decreased expression of the epithelial marker villin. * = $p<0.005$.

**Responsiveness of human colon biopsies to LPS**

Human colonic biopsies from healthy persons and IBD patients were tested for their responsiveness upon exposure to LPS. Biopsies from healthy volunteers showed at best only a very low induction of inflammation-related genes after incubation with LPS.
It is known that during CD and UC, the LPS receptor TLR4 is upregulated on colon epithelium (3; 19) and therefore it could be envisioned that biopsies of CD and UC patients would react more vigorously upon incubation with LPS. However, also in the biopsies of patients, hardly any inflammatory response was observed. mRNA levels of the inflammatory genes TNFα and IL-1β upon LPS incubation in three UC patients were not significantly induced compared to biopsies incubated without LPS. In two of the three tested CD patients an induction of inflammatory genes after incubation with LPS was observed. However, no effects of iAP could be determined. The two patients that reacted upon LPS had a reduced villin expression and higher expression levels of the LPS receptors TLR4 and CD14. These data indicate that only when the intestinal barrier is damaged due to ulceration, LPS is able to induce an inflammatory response.

**Effect of oral administration of enteric-coated iAP-tablets on DSS-induced colitis in rats**

To examine whether oral administration of iAP affects experimental colitis, inflammation was induced in the colon in male Sprague-Dawley rats by oral intake of dextran sodium sulphate (DSS).

Oral administration of AP in tablets resulted in a higher AP activity within the intestine. AP activity in faeces at 24 hr after treatment had risen from 0.13 units/gram faeces in rats that received normal drinking water plus placebo-tablets to 7.08 units/gram faeces in rats that received normal drinking water plus AP-tablets. The total recovery of AP activity from acid-resistant AP-tablets in the intestine is approximately 30% as determined previously (20).

The daily consumption of drinking water by the rats showed no significant differences, confirming that both treated and untreated rats received the same amount of DSS.

In this study also no difference in weight loss between the different groups that received DSS in their drinking water was found. The rats in the AP-treated and placebo-treated group lost 11.0% and 11.9% of their initial body weight,
Oral administration of alkaline phophatase ameliorates colitis respectively. The control groups, i.e. the groups that received normal drinking water plus either placebo- or AP-tablets, showed an average increase of body weight of 4.5\% respectively 4.6\%.

Macroscopic evaluation of the colons revealed that the colon morphology of AP-treated DSS rats was better than the colon morphology of placebo-treated DSS rats, as illustrated by a significantly lower macroscopical score (Fig. 4, p<0.05). Measurement of the colon length revealed a significant difference between DSS-treated rats receiving placebo-tablets and DSS-treated rats receiving AP-tablets; 8.1 ± 0.8 cm in the placebo-treated group versus 9.5 ± 1.3 cm in the AP-treated group (p<0.05). The shortening of the colon in DSS-treated rats was associated with a clearly visible thickening of the intestinal wall, as reflected by a significant increase in weight per length in DSS-treated rats. AP treatment reduced this thickening (p<0.01).

![Figure 4: Effects of acid-resistant iAP or placebo-tablets on the macroscopic score of the colons in male Sprague-Dawley rats with (n=10 per group) or without (n=5 per group) DSS-induced colitis after 8 days of treatment. Horizontal bars represent the median of each group. The difference between untreated and AP-treated DSS-rats was significant at p<0.05.](image-url)
In the distal part of the colon, relative mRNA expression levels of the following genes were determined: TNFα, IL-1β, IL-6, iNOS, IL-10R and villin. The mRNA levels of the inflammatory genes, TNFα, IL-1β, IL-6, iNOS and IL-10R were all strongly upregulated in DSS-treated rats. Expression levels of all these genes were significantly lower in DSS-rats that received AP-tablets compared to DSS-rats that received placebo-tablets (p<0.05). Only two out of ten rats treated with AP still had elevated levels of TNFα, IL-1β, IL-6, iNOS and IL-10R. In contrast, mRNA levels for the epithelial marker villin, which were profoundly reduced in placebo-treated DSS-rats, were significantly increased in colitic rats receiving AP-tablets (p<0.05). Interestingly, the two rats that displayed aberrant cytokine, iNOS and IL-10R levels, also had reduced villin levels. In other words, the outliers were the same rats for all parameters. These data indicate that the epithelial layer in the colon of rats receiving DSS plus placebo-tablets is severely damaged whereas the epithelial layer in rats receiving DSS plus AP-tablets is significantly less damaged.

Figure 5: Effects of oral treatment with acid-resistant iAP or placebo-tablets on mRNA expression of several genes in male Sprague-Dawley rats with (n=10 per group) or without (n=5 per group) DSS-induced colitis after 7 days of treatment (Figure on page 127). Dot plots show the expression levels of TNFα, IL-1β, IL-6, iNOS, IL-10R and villin mRNA in the distal part of the rat colons. Horizontal bars represent the median of each group. Expression levels were normalized to GAPDH. For each parameter, the difference between untreated and AP-treated DSS-rats was significant at p<0.05.
Oral administration of alkaline phophatase ameliorates colitis.
The colon was also histochemically evaluated by H&E, iNOS, MPO and villin staining. The colons of DSS rats receiving AP-tablets were clearly less damaged than those of DSS rats that received placebo-tablets (Fig. 6C versus Fig. 6B). The epithelial lining of the colon was more intact, confirming the PCR results for villin mRNA levels, and in line with the macroscopical data (Fig. 4), the serosa was less thickened in AP-treated rats. In both groups of rats that received normal drinking water, no staining for the inflammation marker iNOS was observed (Fig. 6D). However, in placebo-treated DSS-rats, all colon segments displayed a profound iNOS staining (Fig. 6E). In contrast, colons from AP-treated DSS-rats showed no or only minor iNOS staining (Fig. 6F). The amount of MPO-positive cells in the colon was highly increased in DSS-treated rats receiving placebo-tablets (Fig. 6H) compared to normal rats (Fig. 6G). Administration of AP-tablets to DSS-treated rats markedly reduced this influx of MPO-positive cells (Fig. 6I). The epithelial marker villin was abundantly present along the epithelial lining of the mucosa in control rats (Fig. 6J), but completely absent in colons of DSS-rats that received placebo-tablets (Fig. 6K). In contrast, DSS-rats that received AP-tablets displayed a clear villin-staining along the epithelial cells which was comparable to control rats (Fig. 6L), which is in line with the PCR data for villin mRNA.

Taken together, all data indicate that the inflammation of the colon and the structural damage after DSS exposure is significantly reduced upon AP treatment.

**Figure 6:** H&E staining (A, B, C), histochemical staining for iNOS (D, E, F) and activated neutrophils (G, H, I) and villin (J, K, L) in male Sprague-Dawley rats with DSS-induced colitis after 7 days of treatment with placebo (mid-panel) or acid-resistant iAP-tablets (right-hand column). Normal rats are depicted in the left-hand column. Note the mucosal ulceration and thickening of the serosa in placebo-treated colitic rats (B) compared to oral iAP-treated colitic rats (C). The inflammation marker iNOS was absent in non-colitic rats (D), enhanced in placebo-treated colitic rats (E), and strongly decreased in oral iAP-treated colitic rats (F). The amount of activated neutrophils was low in non-colitic rats (G), high in placebo-treated colitic rats (H) and clearly reduced in iAP-treated colitic rats (I). The red staining for the epithelial marker villin was visible along the epithelial lining in non-colitic rats (J), absent in placebo-treated colitic rats (K) and retained in iAP-treated DSS rats (L). Magnification of all figures is 100x. A full color version of this article can be found on page 173.)
Oral administration of alkaline phophatase ameliorates colitis

color control
DSS + placebo-tablets
DSS + AP-tablets
Discussion

Inflammatory bowel disease (IBD) is one of the major chronic inflammatory diseases, affecting several millions of people world-wide. It is believed that the inflammation of the damaged gut is the result of an inappropriate ongoing activation of the mucosal immune system triggered by components of the normal luminal flora (1). When the sub-epithelial layers of the intestinal wall are exposed to LPS due to damage, macrophages residing in these layers start to produce cytokines and chemoattractants (21), leading to the migration of high amounts of neutrophils to the site of damage and local activation. Production of high amounts of ROS by accumulated neutrophils will cause local damage and further enhance inflammation. A damaged intestinal barrier sometimes also leads to elevated LPS levels in the blood caused by translocation of luminal LPS and/or bacterial translocation, which in turn may induce elevated levels of circulating cytokines like TNFα, IL-1β, IL-6 and IL-17 (22; 23). This may increase the intestinal permeability even further. Several reports have shown that the enzyme alkaline phosphatase (AP) is able to dephosphorylate and detoxify LPS (7-10). The resulting product, LPS with a monophosphoryl lipid A moiety, is non-toxic (24) and may even antagonize LPS (25). Because inflammatory bowel diseases are associated with a perpetuating inflammatory response towards intestinal bacterial products, in particular LPS, administration of LPS-detoxifying molecules might attenuate the inflammatory response during periods of severe inflammation.

We first studied intestinal AP expression in normal healthy persons with enzymehistochemistry and real-time PCR techniques. The results obtained with both techniques corresponded with each other and showed a strong LPS-dephosphorylation in the human ileum along the lining of the villi whereas the human colon showed almost no LPS-dephosphorylation. This difference was confirmed by real-time PCR; the human ileum showed much higher iAP mRNA levels than the human colon. Previous studies have demonstrated that this LPS-dephosphorylation was caused by AP activity (7; 8) and the localization of this activity here is also in agreement with the reported localization of iAP in the small intestine (11; 26). The reason for the lack of LPS-dephosphorylation in the colon is unknown. Because bacterial titers in the colon are high, the barrier function may be
sufficiently strong under physiological circumstances and LPS receptors are not expressed in the colon epithelium in the normal situation (19; 27; 28), leading to a hyporesponsiveness to LPS within the colon. So, LPS detoxification may not be required in the colon. AP activity within the colon might also be blocked by dephosphorylated LPS, which is a known inhibitor of AP activity (9). In the ileum, where intestinal nutrient uptake is high, LPS-dephosphorylation mechanisms may be more relevant.

Although LPS-dephosphorylation in the colon in the healthy control group was low, we observed decreased mRNA iAP levels in the colon of UC patients. These decreases were paralleled by decreases in the expression levels of the epithelial marker villin, suggesting that the decrease in iAP expression is most likely due to loss of the epithelial cell layer.

LPS-dephosphorylation was shown to be associated with reduced toxicity of this bacterial product (7-10; 29) and this may be relevant in view of the role of LPS in the pathology of IBD. We therefore explored whether administration of calf intestinal AP (ciAP) might affect DSS-induced colitis, a model for human UC in rats (30). The DSS-model is characterized by colonic epithelial damage, diarrhea, bloody faeces, decrease of body weight, colon shortening and neutrophilic infiltration in the intestinal wall (31). Treatment with AP will not affect DSS-induced damage but only the secondary damage caused after destruction of the epithelial layer (32). Intestinal AP appeared to display a profound effect on the colon. All parameters reflecting the colon condition at the macroscopical level showed that the colon was significantly improved in diseased rats receiving AP-tablets.

Measurement of drinking water consumption showed that both treated and untreated groups received equal amounts of DSS, leading to a decreased water-resorption capacity of the colon and diarrhea, and subsequently weight loss in both groups. Nevertheless, significant effects were found on mRNA levels of all genes examined; mRNA levels of inflammatory genes were strongly decreased, indicating a reduced inflammation within the colon wall, and villin mRNA levels were strongly enhanced in the colons of AP-treated rats, reflecting a more intact epithelial cell layer. Also intestinal wall thickness and integrity were nearly normal in all AP-treated colitic rats, and the patchy staining for iNOS found in all DSS-rats
treated with placebo-tablets was completely absent in all rats treated with AP. So, these data demonstrate a significant therapeutic effect of oral administration of iAP-tablets. Although AP activity in the colon is normally low or absent, administration of exogenous iAP seems therefore beneficial. Apparently, during disease, when damage to the colon wall occurs and infiltration of CD14 / TLR4 receptor-positive cells has taken place, shielding mechanisms are affected and LPS-hyperresponsive cells infiltrate in the tissue. So only when the normal barrier is affected, a high concentration of AP may exert a clinical relevant function.

The role of the barrier and the effect of LPS-responsive cells that subsequently infiltrate in the tissue became also clear in human biopsies. Biopsies of normal healthy persons appeared to be quite insensitive to stimulation with LPS. This apparent hyporesponsiveness might be due to the absence of TLR4 in human intestinal epithelial cells (27; 33). After examining the response upon LPS in biopsies from 3 CD and 3 UC patients, we only found induction of mRNA for inflammatory genes in biopsies of 2 CD patients. Biopsies of these two patients also displayed low villin mRNA levels indicating that in these patients the epithelial layer was affected. It has been described that IBD patients have higher expression levels of the LPS receptor TLR4 (3; 19), but in contrast to these reports, we could not detect such differences. Since there was no response upon LPS in most cases, no significant effect of AP on the LPS-response was noted either.

In summary, this study shows for the first time that epithelial iAP expression is decreased in UC patients and that the iAP-mediated endogenous LPS-detoxifying activity is therefore challenged in these patients. Importantly, oral iAP administration was found to reduce LPS-mediated inflammatory effects in colitic rats. These data provide new insights into the role of iAP during IBD and support the notion that oral administration of iAP to UC patients may be therapeutically effective. This prompted us to a phase II proof of concept study in patients with severe UC which is currently ongoing.
Oral administration of alkaline phosphatase ameliorates colitis

Reference List


Oral administration of alkaline phosphatase ameliorates colitis


