Effect of Bacillus subtilis PB6, a natural probiotic on colon mucosal inflammation and plasma cytokines levels in inflammatory bowel disease

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Received 04 August 2008; revised 15 January 2009

The pathophysiology of inflammatory bowel disease (IBD) involves the production of diverse lipid mediators, namely eicosanoid, lysophospholipids, and platelet-activating factor, in which phospholipase A 2 (PLA 2 ) is the key enzyme. Thus, it has been postulated that control of lipid mediators production by inhibition of PLA 2 would be useful for the treatment of IBD. This hypothesis has been tested in the present study by examining the therapeutic effect of a novel natural probiotic Bacillus subtilis PB6 (ATCC- PTA 6737). B. subtilis PB6 is found to secrete surfactins (cyclic lipopeptides) which have anti-bacterial potential. These surfactins inhibit PLA 2 , a rate-limiting enzyme involved in the arachidonic acid associated inflammatory pathway and could downregulate the inflammatory response by regulating the eicosanoid and cytokine pathways. With this concept, an experimental animal trial has been conducted in a rat model of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. The oral administration of PB6 suppresses the colitis as measured by mortality rate, changes in the weight gain, colon morphology and the levels of plasma cytokines. The animals treated orally with PB6 at 1.5 × 10 9 CFU/kg thrice daily from day 4 to 10 significantly improve gross pathology of the colon and regain the colon weight to normal (p < 0.05), compared to TNBS-induced positive control. The plasma levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IFN-γ) are also significantly lowered (p < 0.05) and anti-inflammatory cytokine (IL-10 and TGF-β) significantly (p < 0.05) increased after the oral administration of PB6 on day 11. The present study supports the concept that PB6 inhibits PLA 2 by the secreting surfactins. In a clinical investigation, it is found to be well tolerated by all the healthy volunteers.

Keywords: Inflammatory bowel disease, Bacillus subtilis PB6, TNBS-induced colitis, Wistar rats, Cytokines

Inflammatory bowel disease (IBD) is a chronic disorder of the intestine, relating mainly to ulcerative colitis (UC) and Crohn's disease (CD), associated with abdominal pain, diarrhoea, fever, weight loss, anaemia, arthritic pain, and impairment of liver function. Although the etiology of IBD is not known, its pathophysiology is postulated to have two stages: an insult triggering initial tissue damage, followed by an amplification stage, which propagates the tissue destruction and the duration of the disease. Arachidonic acid (AA) cleaved from membrane phospholipids by the enzyme phospholipase A 2 (PLA 2 ) has been identified as a possible rate-limiting step in one of these inflammatory cascades. Further, AA metabolism produces a wide range of pro-inflammatory eicosanoids (prostaglandins, leukotrienes and thromboxanes). Cytosolic phospholipase (cPLA 2 ) requires phosphorylation by mitogen-activated protein kinase (MAPK) to be fully activated. During the inflammatory cascade, P38 MAPK is activated by its phosphorylation which, in turn, activates NF-κB which plays a vital role in cytokines gene expression (TNF-α, IL-1β, IL-6, IL-10, TGF-β and IFN-γ). It is postulated that the inhibition of cPLA 2 could dampen the inflammatory response by regulating these eicosanoid and cytokine production pathways.
Therapy of IBD is difficult on account of the complex etiology of the disease. Although therapeutic drugs, such as 5-aminosalicylic acid (5-ASA), salazosulfapyridine (SASP) and glucocorticoids could inhibit the inflammatory mediators through different mechanisms which engaged in the down-regulation of the immune and inflammatory responses, their adverse reactions during prolonged treatment and relapse rate-limited their use.

The microbial environment of the intestine plays a major role in the development of IBD and hence targeting of the microbiota presents an option for therapeutic interventions. One potential method to manipulate the intestinal micro biota is by the administration of probiotics. Three distinct cellular and molecular mechanisms have been suggested for probiotic regulation in IBD therapy: (i) probiotics block pathogenic bacterial effects by producing bactericidal substances and competing with pathogens and toxins for adherence to the intestinal epithelium; (ii) they regulate immune responses by enhancing the innate immunity and modulating pathogen-induced inflammation via toll-like receptor-regulated signalling pathways; and (iii) they regulate intestinal epithelial homeostasis by promoting intestinal epithelial cell survival, enhancing barrier function, and stimulating protective responses. Probiotics modulate host cell signalling pathways, including Akt, mitogen-activated protein kinases, and NF-kappaB to mediate these intestinal epithelial functions.

In our earlier study, the probiotic Bacillus subtilis PB6 (Gram-positive bacteria) have been found to secrete the cyclic lipopeptides surfactins through normal metabolism which inhibit PLA2, a rate-limiting enzyme involved in the AA associated inflammatory pathway. Inhibition of PLA2 and subsequent downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines have been used as biomarkers to measure the efficacy of B. subtilis PB6 in this study.

Rat model of TNBS (2, 4, 6 trinitrobenzene sulfonic acid) colitis has the similarity to the human IBD and hence has been adopted in the present study to evaluate B. subtilis PB6 for its efficacy in ameliorating the symptoms of ulcerative colitis. The efficacy of the probiotic B. subtilis PB6 has compared with mesalazine (5-aminosalicylic acid), the active component in Mesacol®, which is used for the treatment of IBD.

**Materials and Methods**

**Animals and treatment**

Forty-two male wistar albino rats, aged 10-12 weeks were obtained from the Sri Ramachandra Medical College & Research Institute, Chennai, India. Animals were housed for 1 week before the onset of the experiment and acclimatized under standard laboratory conditions at a constant temperature of 19-25°C, with a relative humidity of 30-70%, and a 12-h light/dark cycle. Upon their arrival at the test facility, the animals were given a complete clinical examination under the supervision of a veterinarian to ensure that they were in good condition. Each cage contained three animals, with readily available rat pellet food and aquaguard filtered water ad libitum throughout the study, with the exception of the 12 h before the initiation of trial. Veterinary health checks were carried out during acclimatization.

The animals were randomly categorized into seven groups of six animals (1 to 7) and the details are given in Table 1. All experimental protocols were approved by the Institutional Animal Ethics Committee in accordance with the procedures specified by the Committee for the Purpose of Control and Supervision of Experiments on Animals, regarding animal care and handling. All modus operandi was strictly followed the standard operating procedures of the laboratory and good laboratory practice was maintained at all times. Colitis was induced on day 1 in all rats, except the colitis negative control group through intrarectal instillation of TNBS.

Body weight of animals was recorded on days 1, 4 and 10 of the study. The mean weight variation between the groups was minimal and did not exceed ± 20%.

A 5 cm segment of distal colon was removed from each animal and examined for morphological lesions and assigned a score according the scale. The colon samples were weighed, and the wet weight of the segment was determined as a reflection of colonic oedema, the colons were blotted dry and again weighed. The extent of mucosal damage was assessed macroscopically according the scale, which included the area of inflammation and the presence or absence of ulcers. Each colon was assigned a score on this scale ranging from 0 to 5 (Table 2) indicative of areas of mucosal discoloration, erosion, exudation, ulceration, and bowel wall thickening. The degree of intestinal damage was assessed by a naked-eye
Blood samples were collected from treated and control rats on days 4, 7 and 11 after colitis was induced. Plasma cytokines IL1β, IL6, TNF-α, IFN-γ, TGF β and IL10 as inflammatory biomarkers were evaluated using sandwich ELISA development kits and levels were expressed as picogram per ml of plasma. The ELISA development kits were standardized and validated prior to the quantification of the cytokines.

### Results

#### Clinical manifestations of TNBS-colitis and effect of PB6

The intrarectal administration of TNBS resulted in the development of moderate to severe diarrhoea, rectal bleeding and increased mucus within hours of instillation. Anorexia, weight loss and reduced activity ensued, and were most progressive during the first 3 days. The hunchback appearance of the majority of animals for 48 h post TNBS-induction, which was an indication of severe abdominal pain, continued for the TNBS positive control group throughout the study. PB6 at all the dose levels (1.5 × 10⁸ cfu/g and 1.5 × 10⁷ cfu/g) were effective in alleviating the symptoms of diarrhoea in TNBS-induced colitis in rats. The saline controls demonstrated normal bowel function, consistent weight and activity throughout the trial.

#### Body weight and percent body weight gain/loss

Prior to treatment on day 4, all animals administered with TNBS experienced weight loss compared to saline-control rats and PB6 1.5 × 10⁸ cfu/g group showed significant (p<0.05) weight loss (Table 3). On day 10, highly significant changes in body weight were observed between groups (p<0.05). Among the treatment groups, an improvement in body weight was observed only in PB6 1.5 × 10⁸ cfu/g from day 1-10 group, compared to TNBS positive control group. Weight loss was severe in the Mesacol® group, which resulted in two mortalities.
Colon wet weight and morphological score

The degree of oedema determined by the colon weight and degree of macroscopic lesions in the colon as colon score is given in Table 3. The saline and all the other treatment groups demonstrated significantly ($p<0.05$) lower colon weights in comparison to the TNBS positive control groups. There was no significant difference between the treatment groups. The colon of saline control vehicles showed no macroscopic lesions. All treatment groups showed a significant improvement in macroscopic scores, compared to drug-free colitis control rats.

Gross morphology of colon samples

Macroscopic examination of inflamed lesion specimens of the two TNBS-colitis control groups demonstrated widespread damage, with obvious mucosal injury and inflammation, including hyperemia and swollen tissue (Fig. 1A and B). The saline control groups showed no macroscopic lesions in the distal colon (Fig. 1C). The PB6-treated groups (group 4 and 6) showed no visible inflammation or injury in the colonic tissue (Fig. 1D & F). Few colon lesions were seen in PB6-treated group (group 5) (Fig. 1E). The Mesacol® group (group 7) showed no lesions with any visible inflammation or injury in the colonic tissue (Fig. 1G).

Changes in cytokines levels and effect of PB6 in TNBS-induced colitis

Proinflammatory cytokines

The levels of proinflammatory cytokines TNF-$\alpha$, IL-$1\beta$, IL-$6$ and IFN-$\gamma$ were measured in plasma.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Colon wet weight (g)</th>
<th>Colon morphological score</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 1</td>
<td>Day 4</td>
<td>Day 10</td>
<td></td>
</tr>
<tr>
<td>TNBS control sacrificed on day 4</td>
<td>234±7.08</td>
<td>206±8.93</td>
<td>1.32±0.01</td>
</tr>
<tr>
<td>TNBS control sacrificed on day 11</td>
<td>234±7.43</td>
<td>213±8.03</td>
<td>202±4.8</td>
</tr>
<tr>
<td>Saline control</td>
<td>234±7.51</td>
<td>234±7.24</td>
<td>233±7.96</td>
</tr>
<tr>
<td>PB6 $3 \times 1.5 \times 10^8$ from day 1-10</td>
<td>233±7.6</td>
<td>219±7.91</td>
<td>214±10.92</td>
</tr>
<tr>
<td>PB6 $3 \times 1.5 \times 10^9$ from day 4-10</td>
<td>233±7.11</td>
<td>216±7.96</td>
<td>207±6.22</td>
</tr>
<tr>
<td>PB6 $3 \times 1.5 \times 10^9$ from day 4-10</td>
<td>233±5.4</td>
<td>210±5.22</td>
<td>203±7.38</td>
</tr>
<tr>
<td>Mesacol 250 mg/kg from day 4-10</td>
<td>235±6.1</td>
<td>216±3.73</td>
<td>199±5.12</td>
</tr>
</tbody>
</table>

Means showing different superscripts in a column differ significantly ($p<0.05$) by non-parametric Duncan’s One-Way Analysis-of-Variance. $^{abcd}$Show the degree of statistical difference in ascending order.

Fig. 1—Colon morphology
samples and presented in Table 4. TNBS positive control animals showed significant \((p<0.05)\) elevated levels of TNF-α and IL-1β when compared to saline control group on day 4 and these levels maintained significant till day 11. The treatments with PB6 at all dose levels significantly \((p<0.05)\) lowered the plasma levels of TNF-α and IL-1β compared to TNBS positive control on day 11 and were on par to saline control group on day 11. A significant \((p<0.05)\) increase in IL-6 and IFN-γ levels was observed in TNBS positive control group compared to saline control on days 4, 7 and 11 (Table 4). On the other hand, treatments with PB6 at all dose levels significantly \((p<0.05)\) reduced plasma IL-6 and IFN-γ levels in day 11 and were almost equal to saline-treated group. There was no significant difference observed in IL-6 and IFN-γ levels between Mesacol-treated and TNBS positive control groups in day 7 and 11.

**Anti-inflammatory cytokines**

The changes in the anti-inflammatory cytokines IL-10 and TGF-β are given in Table 5. There was no significant change in plasma IL-10 levels between TNBS positive control and saline control groups on day 4. However, a significantly \((p<0.05)\) lower plasma IL-10 level was observed in TNBS control group compared to saline control on day 7 and 11. The groups treated with PB6 and Mesacol showed a significant increase \((p<0.05)\) in IL-10 level compared to TNBS control. A highly significant \((p<0.05)\) decline in TGF-β levels was noticed in TNBS control animals compared to saline on day 4, 7 and 11. Interestingly, only Mesacol-treated groups showed

### Table 4—Effect of PB6 on pro-inflammatory cytokines in TNBS induced colitis in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Pro-inflammatory cytokines (picogram/ml plasma)</th>
<th>Anti-inflammatory cytokines (picogram/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNBS control sacrificed on day 4</td>
<td>TNF-α</td>
<td>IL-10</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
</tr>
<tr>
<td>TNBS control sacrificed on day 11</td>
<td>28±1.3</td>
<td>28±1.2</td>
</tr>
<tr>
<td>Saline control</td>
<td>28±1.3</td>
<td>28±1.2</td>
</tr>
<tr>
<td>PB6 3×1.5×10⁹ from day 1-10</td>
<td>28±1.3</td>
<td>28±1.2</td>
</tr>
<tr>
<td>PB6 3×1.5×10⁹ from day 2-10</td>
<td>28±1.3</td>
<td>28±1.2</td>
</tr>
<tr>
<td>PB6 3×1.5×10⁹ from day 4-10</td>
<td>28±1.3</td>
<td>28±1.2</td>
</tr>
<tr>
<td>Mesacol 250 mg/kg from day 4-10</td>
<td>28±1.3</td>
<td>28±1.2</td>
</tr>
</tbody>
</table>

Means showing different superscripts in a column differ significantly \((p<0.05)\) by non-parametric Duncan’s One-way Analysis-of-Variance. \(a^k\) Show the degree of statistical difference in ascending order.

### Table 5—Effect of PB6 on anti-inflammatory cytokines in TNBS-induced colitis in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-inflammatory cytokines (picogram/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-10</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
</tr>
<tr>
<td>TNBS control sacrificed on day 4</td>
<td>28±1.3</td>
</tr>
<tr>
<td>TNBS control sacrificed on day 11</td>
<td>28±1.3</td>
</tr>
<tr>
<td>Saline control</td>
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</tr>
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Means showing different superscripts in a column differ significantly \((p<0.05)\) by non-parametric Duncan’s One-way Analysis-of-Variance. \(a^k\) Show the degree of statistical difference in ascending order.
significant ($p<0.05$) elevation in TGF-β level, when compared to saline control and PB6 treatment groups on day 11.

**Discussion**

More than 20 animal models of IBD have been used to study the efficacy and mechanisms of probiotics in ameliorating inflammation. The rat model of TNBS-induced colitis has been widely adopted to observe the effects of drugs, due to its similarity to human IBD and the availability of a quantitative scoring system. It is found to be one of the best model to induce macroscopic and microscopic alterations of colonic architecture. The ability of TNBS to induce damage to the colon is reported to be dose-dependent for severity and duration; the induction at dose of 10 mg TNBS completely resolves at day 60 and 30 mg TNBS induces massive necrosis, thickening of the colon, severe histologic changes that are only partially reversed after two months. Based on this, the dosage of 20 mg TNBS is used in the present study to prevent any carcinogenic effect in mice.

In animal models, probiotic treatment can sometimes reduce the development of colitis and treat or attenuate established colitis. Immunomodulation, improvement of epithelial barrier function, competitive exclusion of gastrointestinal pathogens and secretion of antimicrobial compounds which suppress the growth of harmful enteric bacteria are the basic mechanisms by which probiotics exert their anti-inflammatory effect. Studies in animal models indicate that various probiotic species have different effects in selected hosts and inflammatory conditions, suggesting to the involvement of multiple mechanisms of action.

Our results in the present study demonstrate an improvement of TNBS-induced colitis in rats treated with *B. subtilis* PB6, as reflected in the experimental data. PB6 has been effective in alleviating the symptoms of diarrhoea and attenuates morphological signs of inflammation in the colon. The colonic mucosa shows ulcers in the process of healing characterized by mild or no hyperemia compared to TNBS positive control colons showing areas of necrosis, severe ulceration, erosion, edema and adhesions.

Generally, probiotics increase the production of anti-inflammatory cytokines (IL-10 and TGF-β) while reducing the production of proinflammatory cytokines (e.g., TNF, IF, IL 18). Several probiotic bacteria, including *B. breve*, *B. bifidum*, *Ruminococcus gnavus* and *Streptococcus thermophilus* secrete metabolites that reduce LPS-induced TNF-α secretion. AA upregulates P38 MAPK-induced production of TNF-α and associated pro-inflammatory mediators.

TNF-α plays an important role in TNBS-induced colitis and is possibly the key regulator of the inflammatory cascade in IBD. TNF-α signalling is reported to be linked to activated p38 MAPK. Cytokine is one of the best characterized agonist of p38 and JNK pathways, itself regulated by p38. Our results with increased levels of TNF-α also correlate well with the development of colonic inflammation upon TNBS instillation and *B. subtilis* PB6 is significantly effective in bringing down the levels of TNF-α in plasma. The key role for TNF-α and IL-1β in the inflammatory process is supported by the improvement of patients with IBD treated with monoclonal anti-TNF-α antibodies and IL-1 receptor antagonist. In our present study, *B. subtilis* PB6 is found to decrease the plasma IL-1β level.

IFN-γ, another pleuripotent pro-inflammatory cytokine is elevated in TNBS colitic rats, whereas treatment with PB6 has brought down the INF-γ level, showing its anti-inflammatory effect. The central importance of IFN-γ in transmural granulomatous colitis has been shown in an earlier report, wherein colitis has been found to be associated with Th1 response and responds to systemic treatment with anti-IFN-γ. Furthermore, IFN-γ is a key activator of macrophages and it might influence experimental colitis by facilitating macrophage secretion of inflammatory cytokines.

IL-6 stimulates the neutrophil chemotaxis and relates to the presence of necrosis in the colon, leading to the tissue destruction. Our results indicate that the levels of IL-6 in plasma of TNBS colitic rats are elevated than the normal group and decline significantly after treatment with *B. subtilis* PB6 and Mesacol. In the experimental colitis, the treatment with anti-IL-6 receptor mAb blocks the IL-6 signalling pathway and suppresses murine colonic expression of TNF-α, IL-1β and IFN-γ, without changing expression levels of IL-10, TGF-β and IL-4.

In contrast, in our study, the levels of IL-10 and TGF-β are significantly lowered by TNBS-induced ulcerative colitis. However, our results are in agreement with the previous study, wherein a downregulation of IL-10 in experimental colitis is reported. Interestingly, the levels of IL-10 are significantly higher in the animals treated with
B. subtilis PB6 and Mesacol. IL-10 is found to reduce the transcription of and production of TNF-α, IL-1β and IL-6 and increase the release of IL-1 β receptor antagonist. Our results clearly indicate that elevation in plasma levels of proinflammatory cytokines TNF-α, IL-1 β, IFN-γ and IL-6 are associated with decrease in the level of IL-10 and TGF-β. Furthermore, treatment with PB6 has brought back the pro-inflammatory cytokines levels to normal which could be mediated by upregulation of IL-10 and TGF-β.

Although the drugs like Ramicade are effective in the treatment of colitis, but the cost for therapy is very high. Thus, B. subtilis PB6, a natural probiotic is safe and cost-effective and provides an interesting alternative approach for the treatment of IBD. In our study, the plasma levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IFN-γ influenced by synergistic action of cPLA2, p38 MAPK and NF-κB have been obviously decreased, while IL-10, an anti-inflammatory cytokine, is elevated on oral treatment with B. subtilis PB6.

In conclusion, the present results demonstrate that PB6 exerts a beneficial effect in TNBS-induced colitis in rats. Possibly, it decreases the inflammation by downregulating the PLA2-p38 MAPK-NF-κB pathway, as reflected by inhibition of synthesis of proinflammatory cytokines and elevation in the level of anti-inflammatory cytokines, and thereby re-establishing the cytokines balance. Furthermore, B. subtilis PB6 is well tolerated by all the healthy volunteers involved in the study.

References