

Oral treatment with Hev b 13 ameliorates experimental colitis in mice

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Accepted for publication 19 March 2012

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Summary

Hev b 13 is an allergenic esterase obtained from the rubber tree *Hevea brasiliensis*, which has been shown recently to induce human mononuclear cells to release interleukin (IL)-10 *in vitro*. This immunoregulatory cytokine appears to play an important role in preventing inflammation and mucosal damage in animal models of colitis and in Crohn's disease patients. The aim of this study was to evaluate the therapeutic effect of Hev b 13 in mice with 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis. Two hours following colonic instillation of the haptenizing agent, and daily thereafter for 5 days, Hev b 13 was administered by oral gavage. In mice treated with daily doses of either 0.5 mg/kg or 5.0 mg/kg of Hev b 13, the clinical signs of diarrhoea, rectal prolapse and body weight loss and also histological damage of the distal colon, were reduced significantly, in comparison with water-treated diseased mice. These findings suggest a potent anti-inflammatory activity of Hev b 13; this activity is speculated to be related to its interaction with cells from the immune system.

Keywords: colitis, Hev b 13, interleukin-10, mice, trinitrobenzene sulphonic acid

Introduction

Inflammatory bowel disease (IBD) refers to a family of chronic, relapsing and tissue-destructive disorders, which are marked by cellular inflammation and abnormal cytokine production that leads ultimately to damage of the distal small intestine and colonic mucosa, and is associated with an increased risk for colon cancer [1]. Although its aetiology remains unknown, the current hypothesis suggests that IBD results from an uncontrolled immune response against intraluminal bacterial antigens in genetically and environmentally predisposed individuals [2].

IBD is clinically subdivided into two main phenotypes: ulcerative colitis, characterized by superficial inflammation of the colon, and Crohn's disease (CD), in which the inflammation can be present in any part of the gastrointestinal tract, and is complicated by deep ulcerations that have the potential to form fistulae and abscesses. CD is an incurable condition, with a prevalence of 0.05% whose symptoms include abdominal pain, diarrhoea, rectal bleeding, weight loss, fever, fatigue, skin and eye disorders and delayed growth and sexual maturation in children [3,4].

Recently, several animal models of CD have been developed. The murine model of colonic inflammation

induced by intrarectal administration of 2,4,6-trinitrobenzene sulphonic acid (TNBS) resembles many of the clinical, histopathological and immunological features of human CD [5]. In the TNBS-induced colitis model, intestinal inflammation results from covalent binding of the haptenizing agent to autologous host proteins, with subsequent stimulation of a delayed-type hypersensitivity to TNBS-modified self-antigens [6]. This model consistently exhibits uncontrolled production of T helper type 1 (Th1) inflammatory cytokines, and has been used extensively for the evaluation of a wide range of therapeutic approaches and potential anti-colitic agents [7].

Current therapies for IBD include 5-aminosalicylic acid compounds, corticosteroids, immunomodulators and biological [mainly anti-tumour necrosis factor (TNF)] agents. Some of them, however, are not entirely effective, are somewhat non-specific and have multiple adverse effects. Some patients are refractory even to the combined use of these agents; this can result in the need for surgical resection of the colon or ileostomy [3,8]. Therefore, there is a great need for the development of novel and specific therapies for IBD.

Hev b 13, an allergenic esterase from the rubber tree *Hevea brasiliensis* [9,10], has been shown to strongly induce human mononuclear cells to release interleukin (IL)-10 *in vitro* [11].

This immunoregulatory cytokine appears to play an important role in preventing the onset of IBD, as animals deficient in IL-10 develop colitis spontaneously [12,13], and low levels of IL-10 are correlated positively with recurrences of CD [14]. The clinical significance of IL-10 expression is also supported by studies showing that immune augmentation of the cytokine prevents inflammation and mucosal damage in animal models of colitis and in human disease [15–21].

Due to its potent stimulation of IL-10 production, we hypothesized that Hev b 13 is preventative for IBD and may improve colonic inflammation. The aim of this study, therefore, was to investigate the potential anti-inflammatory action of Hev b 13 and its potential therapeutic use in the TNBS-induced model of colitis.

Materials and methods

Obtaining Hev b 13

The Hev b 13 was obtained in a highly homogeneous form, as described previously [11]. Briefly, latex extracted from *H. brasiliensis* trees (clone RRIM 600) was coagulated, and the serum obtained in this way was applied to a diethylaminoethyl (DEAE)-cellulose column. Two more chromatographic steps (on Asahipak ES-502NP and Sephadex G-50 columns) were necessary to purify the protein, whose identity was then determined by peptide mass fingerprinting and amino-terminal protein sequencing.

Animals

Pathogen-free female BALB/c mice, weighing approximately 20 g, were obtained from the Animal Center of University of São Paulo. They were housed under controlled temperature (25°C), humidity (55%) and a 12-h light/dark cycle, and were given free access to a diet of regular laboratory chow and water. The study protocols (no. 066/2008) were approved by the Commission of Ethics in Animal Research of College of Medicine of Ribeirão Preto, University of São Paulo, according to governmental guidelines for animal care and experimentation.

Induction of colitis and study protocol

Colitis was induced in BALB/c mice according to an adaptation of a published method [5]. Briefly, 12-h fasting mice were anaesthetized by an intraperitoneal injection of ketamine (20 mg/kg) and xylazine (8 mg/kg). A 3.5-F catheter was then inserted carefully into the rectum so that the tip was 4 cm proximal to the anal verge. To induce colitis, 5.0 mg of the hapten reagent TNBS (Sigma-Aldrich Brasil Ltda, São Paulo, Brazil), dissolved in 0.1 ml of 50% ethanol (to break the intestinal epithelial barrier), was administered slowly into the lumen via a 1-ml syringe fitted to the catheter. In control experiments, mice received 50% ethanol alone, fol-

lowing the same technique. To ensure distribution of the TNBS throughout the entire colon, mice were held in a vertical position for 60 s, before being returned to their cages.

To assess whether early administration of Hev b 13 protects against development of colitis, mice receiving TNBS were randomized to receive water or Hev b 13. Purified Hev b 13 was administered using oral gavage 2 h following the TNBS enema instillation, and daily thereafter for 5 days, at doses of either 0.5 or 5.0 mg/kg, dissolved in 0.1 ml of water. To assess whether these treatments led to clinical alterations in signs of colitis, mice were weighed and inspected visually for diarrhoea and rectal prolapse during the experimental period. Mice were then killed by cervical dislocation at day 5, and 2 cm of the distal colon of each mouse was removed for histological analysis.

Histological analysis of the colon

Colonic segments were fixed in 3.7% buffered formaldehyde and embedded in paraffin for histological assessment of colitis. Six-micrometer sections were deparaffinized with xylene and stained with haematoxylin and eosin, using routine methods. Tissues were examined in a blinded fashion.

Statistical analysis

A mixed-effects linear regression model, followed by an orthogonal contrast *post-hoc* test, was used to compare body weight changes over time for the different treatments. The log-rank test was used to compare the survival curves between groups. Statistical analyses were performed with SAS version 9 software (SAS Institute Inc., Cary, NC, USA).

Results

Hev b 13 improves clinical signs of inflammation in TNBS-induced colitis

Mice subjected to intrarectal administration of TNBS in 50% ethanol developed a severe condition, characterized by bloody diarrhoea and rectal prolapse, accompanied by extensive wasting syndrome. The daily oral treatment with both the lower and higher doses of Hev b 13 improved the clinical signs of inflammation, as assessed by body weight change and the overall appearance of the mice. The protein doses were chosen after a similar preliminary study, in which a dose of 5.0 mg/kg of FrHb 3, the fraction derived from the first step in the production of purified Hev b 13, had been administered successfully (results not shown).

As shown in Fig. 1, the incidence of diarrhoea (Fig. 1a) and rectal prolapse (Fig. 1b) was markedly higher among water-treated TNBS-control mice than among Hev b 13-treated mice.

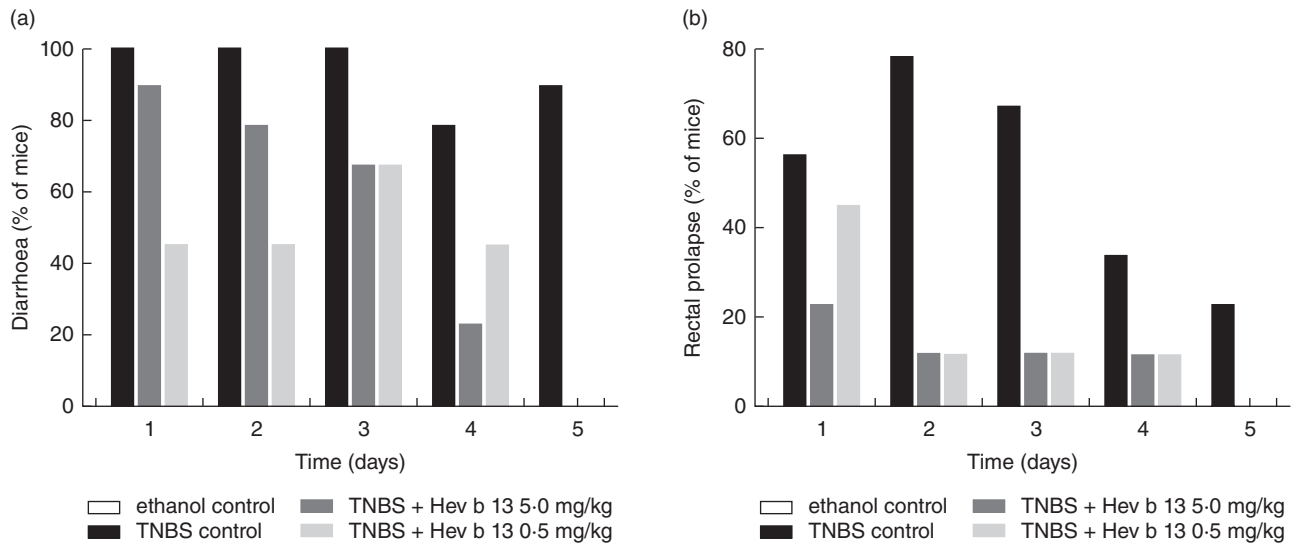


Fig. 1. Graphical representation of the visual inspection of mice with 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis. The daily oral Hev b 13 or water treatments were initiated 2 h after colitis induction at day 0. (a) Percentage of mice with diarrhoea during the experimental period. (b) Percentage of mice with rectal prolapse during the experimental period; $n = 9$ mice per group.

The body weight changes during the experimental period are seen in Fig. 2. Control mice, which received only 50% ethanol, gained body weight after the initial fasting period. In the water-treated group with TNBS-induced colitis, body weight decreased until the third day, but recovered partially at the end of the experiment. Conversely, body weight in mice treated with 0.5 or 5.0 mg/kg of Hev b 13 stabilized

after 1 day, and recovered significantly in a dose-independent manner compared to the water-treated diseased mice.

The induced colitis was severe enough to result in some deaths in all experimental groups. The survival rate was slightly increased by Hev b 13 oral treatments, although no statistical difference was found (data not shown).

Hev b 13 improves histological outcome in TNBS-induced colitis

Histological examination of the distal colon of mice administered TNBS revealed transmural inflammation involving all layers of the bowel wall. As shown in Fig. 3, tissue sections from this group showed disruption and disorganization of crypts, reduction in the density of tubular glands and pronounced depletion of mucin-producing goblet cells. Moreover, there was a marked increase in the thickness of the lamina propria and submucosa, accompanied by intense leucocyte infiltration, increased amount of blood vessels and haemorrhagic areas. When mice were treated with Hev b 13, a striking improvement in these histological signs became apparent, with a significant reduction in the inflammatory activity and leucocyte infiltration (Fig. 3).

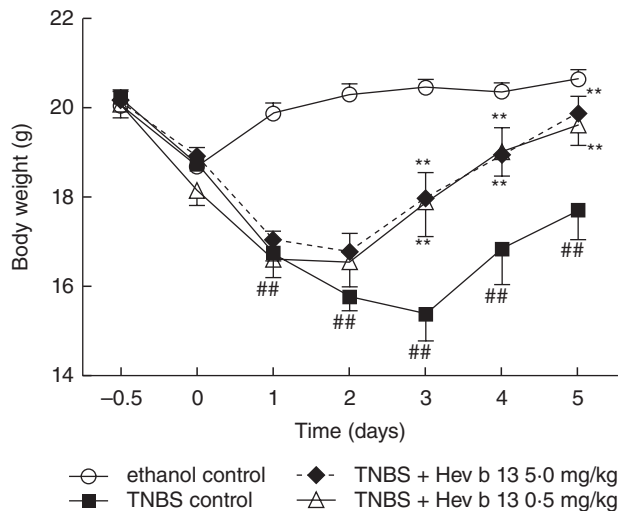


Fig. 2. Effects of Hev b 13 on changes in body weight in mice with 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis. Mice that received only 50% ethanol were used as controls. The daily oral treatments were initiated 2 h after colitis induction at day 0. The range between -0.5 and 0 corresponds to the fasting period prior to the experiment. Data are expressed as means $\pm$ standard error of the mean ($n = 9$ mice per group). $##P < 0.01$ versus ethanol control mice; $**P < 0.01$ versus TNBS-control mice.

Discussion

In this study we show, for the first time, that the early oral administration of Hev b 13 protects mice effectively against the development of inflammation in the acute TNBS-induced colitis model, a widely used experimental model that mimics some features of human Crohn's disease. In this setting, with the immune response being induced by a single

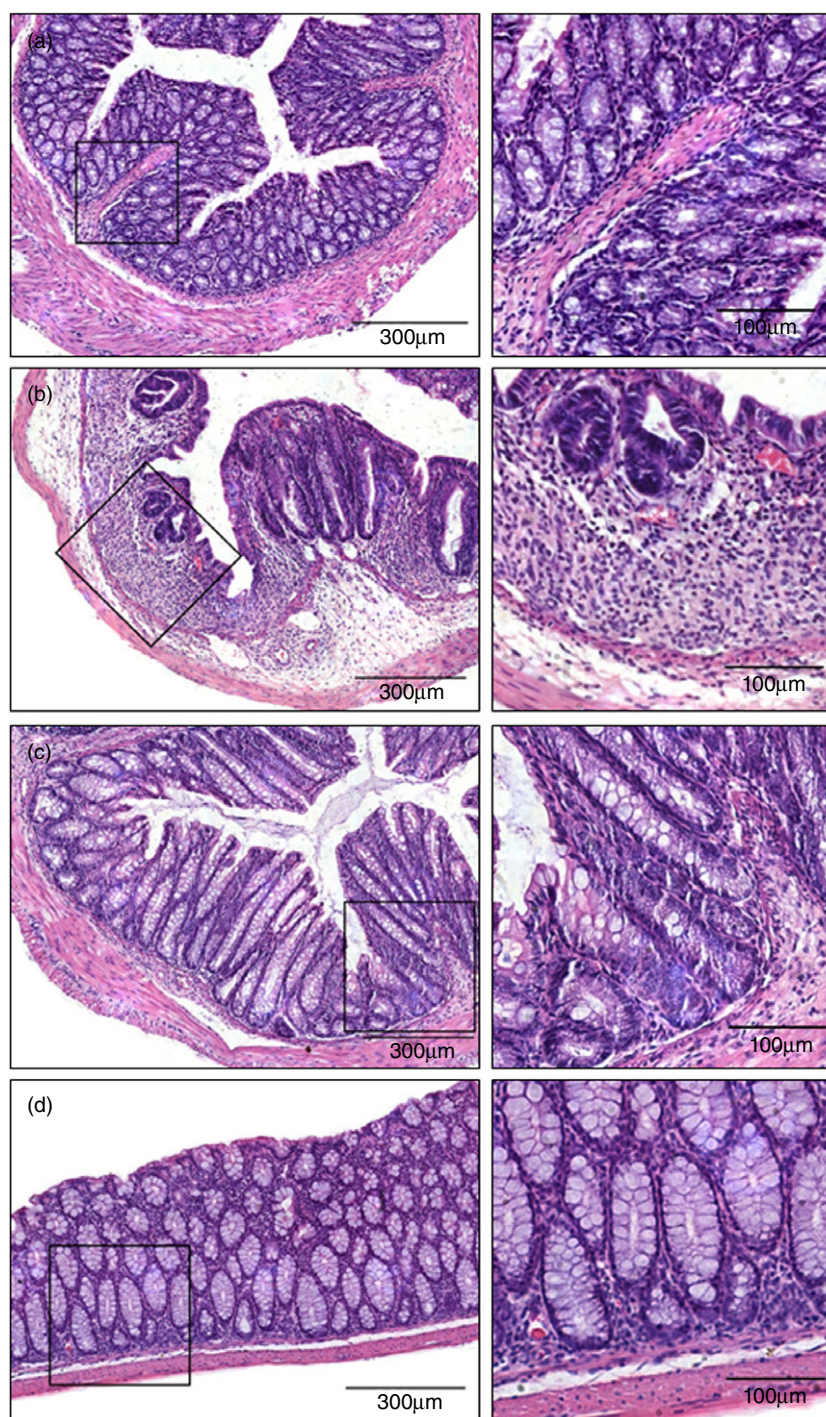


Fig. 3. Photomicrographs of representative histological sections from the distal colon of mice subjected to induction of colitis with 2,4,6-trinitrobenzene sulphonic acid (TNBS)-treatment. The daily oral treatments were initiated 2 h after colitis induction at day 0, and euthanasia was performed on day 5. (a) Control mice, which received only 50% ethanol. (b) Mice with TNBS-induced colitis, which received only water treatment. (c) Mice with TNBS-induced colitis treated with 5.0 mg/kg of Hev b 13. (d) Mice with TNBS-induced colitis treated with 0.5 mg/kg of Hev b 13. All slides were haematoxylin and eosin-stained (original magnification, $\times 144$).

relatively large dose of the haptenizing agent, colitis takes the form of an IL-12-driven, Th1-mediated transmural colonic inflammation that is dependent on the activation of the transcription nuclear factor (NF)- κ B. This short-term model is, however, limited, as it cannot be used to assess tissue fibrosis, a frequent result from the chronic phase of IBD [22].

After 6 days of treatment with Hev b 13 at a daily dose of 0.5 or of 5.0 mg/kg, mice showed significantly reduced diar-

rhoea, rectal prolapse, body weight loss and microscopic tissue damage. These doses are equivalent to doses of 12 and 120 nmol kg⁻¹ day⁻¹, respectively. These findings suggest a potent anti-inflammatory activity of this allergenic esterase. Hev b 13 has been classified as major allergen in natural rubber latex hypersensitivity [9]; however, when evaluated in serological assays in a highly purified form, the sensitization rates were generally low [10]. Up to 12 sets of sequential immunoglobulin E-binding peptides have been identified

along the amino acid sequence of the glycoprotein, including one containing a putative N-glycosylation site [23].

The mechanism of action for Hev b 13 is speculated to be related to its interaction with immune cells. In a previous report, we showed that Hev b 13 stimulates human monocytes to release high levels of IL-10 *in vitro* [11]; this prompted us to investigate a possible pharmacological effect for this agent in the TNBS murine model. However, whether IL-10 is the key immune mediator for the anti-inflammatory effect of Hev b 13 treatment remains to be determined; this would require further experiments with neutralizing anti-IL-10 antibodies.

It is also noteworthy that this 391-amino acid protein exerts its actions even when administered via an oral route, despite the typical poor stability of such macromolecules in the gastric and intestinal environments, and their limited uptake across the intestinal epithelium. Conversely, the high level of peristalsis and inflammatory events associated with this particular pathology (such as modification of the mucus layer, destruction of the extracellular matrix and alteration of local proteolytic activities and transport functions) would be able to circumvent this difficulty, enhancing the transport capacity for such a molecule, although these events could also result in more severe limitations to macromolecular drug delivery [24]. Therefore, the *in-vivo* distribution of Hev b 13 after its oral administration and the final structure of the active compound are now being investigated.

This work indicates conclusively that Hev b 13 has potential as an anti-Crohn's disease agent. In this therapeutic setting, the aim is to gain rapid control of inflammation, thus preventing tissue damage and improving patients' quality of life. The means to this end is ultimately to target specific immune and biochemical abnormalities at the molecular and cellular level.

Acknowledgements

The authors thank Professor Amilton Antunes Barreira (FMRP-USP) for his permission to use the facilities and equipment of the Laboratory of Neurosciences, CEMEQ for statistical analysis, and FAEPa for providing financial support. The authors are grateful to Sílvia Helena Epifânio Netto, José Seminate Filho, Izilda Rodrigues Violante and Antônio Renato Meirelles Silva for their excellent technical assistance. L.B. Teixeira also thanks CAPES for a PhD fellowship.

Disclosure

None of the authors has conflicts of interest to declare.

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