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Anti-inflammatory properties of interleukin-10 administration in hapten-induced colitis

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Abstract

Therapeutic efficacy of interleukin-10 administration in colonic inflammation was assessed in rats. Following intracolonic instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS), subcutaneous administration of 1–1000 μg/kg per day interleukin-10, or a placebo (0.9% NaCl) was commenced and continued for 5 days. Interleukin-10 administered at 1, 10 and 100 μg/kg per day significantly reduced myeloperoxidase activity by 34, 57, and 28%, respectively, compared to the placebo-treated group, which was paralleled by an attenuation of colonic tumor necrosis factor α (TNF-α) content. In contrast, the severity of mucosal necrosis was not affected by interleukin-10 administration at the dose range used. In addition, the 10-fold elevation in nitric oxide release, 5-fold rise in colonic nitrite production and enhanced expression of inducible nitric oxide synthase, associated with TNBS colitis, was not suppressed by interleukin-10. Interleukin-10 gene expression was elevated during the first 14 days of TNBS colitis. We conclude that 5 days administration of interleukin-10 in TNBS colitis displays mild anti-inflammatory properties which were not mediated via a nitric oxide-dependent pathway, but may involve TNF-α.

Keywords: Colitis; Interleukin-10; Inflammation; Nitric oxide (NO); TNF-α (tumor necrosis factor-α)

1. Introduction

Inflammatory bowel disease is associated with an increased production of a range of cytokines including tumor necrosis factor α (TNF-α) and interleukin-1β which display potent pro-inflammatory actions thought to contribute to the pathogenesis of chronic intestinal inflammation (Murch et al., 1993; Olson et al., 1993; Reinecker et al., 1993; Stevens et al., 1992). A novel therapeutic approach for the treatment of such diseases is to down-regulate the expression or action of these cytokines using antibodies or agents which suppress their pro-inflammatory action.

A range of endogenously expressed cytokines or soluble receptors, which display anti-inflammatory actions, have been identified including the interleukin-1 receptor antagonist, which suppresses the action of interleukin-1β (Seitz et al., 1995), and interleukin-10, also known as cytokine synthesis inhibitory factor (Fiorentino et al., 1991b). Interleukin-10 is produced by a range of cell types including macrophages, monocytes, T and B lymphocytes, keratinocytes and tumor cells (see Goldman and Velu, 1995 for review). The anti-inflammatory properties of interleukin-10 have been illustrated in a variety of cell types in vitro: interleukin-10 reduces the transcription and production of interleukin-1β, TNF-α, interleukin-6 and interleukin-8 (De Waal Malefyt et al., 1991; Fiorentino et al., 1991a; Cassatella et al., 1993; Seitz et al., 1995), and increases the release of interleukin-1 receptor antagonist (Cassatella et al., 1994; Seitz et al., 1995). Interleukin-10 also attenuates inflammation by limiting cellular infiltration. B cell aggregation and proliferation are suppressed by IL-10 (Clinchy et al., 1994) as is macrophage infiltration (Richter et al., 1993). This has also been illustrated in vivo, in the murine air pouch model, in which interleukin-10 administration reduces neutrophil accumulation into the air pouch exposed to interleukin-1β (Perretti et al., 1995).

Endogenously expressed interleukin-10 is considered to act as an essential immunoregulator in the intestinal tract.

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In mice rendered deficient in interleukin-10, by gene targeting techniques, a spontaneous enterocolitis develops which shares some of the morphological characteristics of Crohn's disease (Kuhn et al., 1993; Doyle et al., 1996) and appears to involve T helper 1 (TH1)-mediated immune mechanisms (Davidson et al., 1996). However, inflammatory bowel disease is not associated with a quantitative deficiency of interleukin-10 (Schreiber et al., 1995); instead endogenous levels are comparable to, or elevated above those detected in normal patients (Niessner and Volk, 1995). Studies investigating the ability of exogenous interleukin-10 to suppress intestinal inflammation have been limited (Dieleman et al., 1996; Grool et al., 1996; Herfarth et al., 1996; Linevsky et al., 1996; Mitsuyama et al., 1996). Topical administration of exogenous interleukin-10, to a relatively small cohort of ulcerative colitic patients, by enema, has been shown to suppress TNF-α and interleukin-1β production by lamina propria polymorphonuclear cells ex vivo (Schreiber et al., 1995).

In concert to pro-inflammatory cytokine release, chronic intestinal inflammation is associated with an increased production of nitric oxide, paralleled by expression of inducible nitric oxide synthase. This has been demonstrated in inflammatory bowel disease and in a range of experimental models of intestinal inflammation (Boughton-Smith et al., 1993; Ribbons et al., 1995; Miller et al., 1993, 1995). Elevated levels of nitric oxide can induce intestinal injury by enhancing the formation of cytotoxic nitrating species such as peroxynitrite (Miller et al., 1995; Sandoval et al., 1996). Interleukin-10 has been shown to modulate nitric oxide production in vitro and in vivo although the mechanism involved remains unclear. An attenuation of pro-inflammatory cytokines by interleukin-10 administration is paralleled by a reduction in nitric oxide production and inducible nitric oxide synthase gene expression in stimulated keratinocytes (Becherel et al., 1995) and macrophages (Cunha et al., 1992). In vivo, elevated macrophage nitric oxide production in *Candida albicans* infection is suppressed by interleukin-10 administration (Cenci et al., 1993). However, these studies have not been supported by confounding reports of a lack of inhibitory effect of interleukin-10 administration on nitric oxide production (Chesbrown et al., 1994; Perretti et al., 1995). Indeed in murine macrophages, interleukin-10 administration exacerbates nitric oxide release and inducible nitric oxide synthase gene expression (Corradin et al., 1993; Chesbrown et al., 1994). To date, no studies have been undertaken to evaluate the ability of exogenous interleukin-10 to attenuate nitric oxide production in models of chronic gut inflammation.

The objective of this study was to assess the potential therapeutic effect of exogenous interleukin-10 administration in colonic inflammation. Using the trinitrobenzene sulfonic acid (TNBS) model of colitis interleukin-10 was administered at a dose range from 1 to 1000 μg/kg per day. This model was chosen because of its similarities with Crohn's disease and our extensive experience with the role of nitric oxide in this model allowed us to meaningfully address the hypothesis. Therapeutic efficacy was based on an attenuation of mucosal necrosis, cellular infiltration and colonic TNF-α content. The effect of interleukin-10 on nitric oxide production was also determined by measuring nitric oxide and nitrite release ex vivo and colonic inducible nitric oxide synthase gene expression.

2. Materials and methods

2.1. Induction of colitis

Female Sprague-Dawley rats (230–250 g), fasted for 24 h, were anesthetized by an intramuscular injection of ketamine hydrochloride (80 mg/kg) (Ketaset; Fort Dodge Laboratories, Fort Dodge, IO, USA) and Xylazine (4 mg/kg) (Tech-America Veterinary Products, Kansas City, MO, USA). Under aseptic conditions, a mid-line laparotomy was performed and the distal colon was isolated and gently flushed with 2 ml intraluminal saline followed by instillation of 2 ml of air. At the position directly adjacent to the middle colic vein 500 μl of 50% ethanol containing 30 mg 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Fluka Biochemika, Buchs, Switzerland) was injected transmurally into the colonic lumen through a 27 gauge needle. The peritoneum was then closed with silk sutures and 300 μl of sterile saline containing interleukin-10 at the following doses: 0.5 μg/kg ($n = 15$), 5 μg/kg ($n = 11$), 50 μg/kg ($n = 14$), 500 μg/kg ($n = 12$), or 0.9% NaCl alone (placebo, $n = 22$) was injected subcutaneously into the suprascapular region. Six hours later, animals were re-administered a subcutaneous injection of the appropriate interleukin-10 dose or placebo (saline). Sham-operated animals ($n = 12$) underwent the same surgical procedure but were administered saline instead of ethanol and TNBS, and were treated with 0.9% NaCl subcutaneously. Twice daily injections of placebo or interleukin-10 (recombinant human interleukin-10, Schering-Plough, Kenilworth, NJ, USA) was continued for 5 days. A longer treatment period was not assessed because of the limitations of interleukin-10 availability. However, some TNBS-treated rats were allowed to proceed to day 10 or 14 before they were killed, in order to assess interleukin-10 gene expression.

After the treatment period all rats were fasted for 24 h and anesthetized, as described above. The colon was excised from the middle colic vein to the pelvic bone. Before the animals were killed by anesthesia overdose, cardiac blood was drawn for quantification of peripheral leukocyte counts. The excised colonic segment was then slit open longitudinally, rinsed in ice-cold saline and blotted dry. Colonic samples from representative animals from each treatment group were used in one of two collection protocols. In the first protocol, the colon was cut in half longitudinally with one half placed in zinc formalin for 24

h and processed for histological assessment, while the remaining colonic segment was minced and frozen for subsequent myeloperoxidase enzyme analysis. In the second sacrifice procedure, 0.5 cm sections of the proximal and distal ends of the colonic segments were placed, mucosal side up, in a 5.5 cm diameter plastic Petri dish containing RPMI medium (Life Technologies, Grand Island, NY, USA), pre-warmed to 37°C, and used to assess ex vivo nitric oxide release. The remaining colonic segment was cut in half longitudinally. One half was minced and snap frozen in liquid nitrogen for subsequent RNA extraction. The remaining segment was rinsed in sterile saline and transferred to RPMI medium where it was minced into 3 mm diameter cubes (25–30 pieces). Five pieces were randomly selected and frozen in liquid nitrogen for TNF-α analysis, while the remaining pieces were transferred to a 96-well sterile culture plate and used to assess ex vivo nitrite production. To each colonic explant 200 μl RPMI containing 2.5 mmol/l L-glutamine and 5% fetal bovine serum was added. Explants were incubated for 3 h at 37°C in a 95% O_2, 5% CO_2 water jacketed incubator (Forma Scientific, Marietta, OH, USA) after which time explant tissue from 5 wells was harvested, pooled, snap frozen in liquid nitrogen and stored at −80°C. Medium from the 5 explants was collected and stored in a similar manner.

2.2. Morphometry

Colonic segments fixed in zinc formalin were dehydrated using a Titertek automated tissue processor (Miles Scientific, Napervile, IL, USA) and embedded in paraffin. Transverse sections (3 μm) of the entire length of the colonic segment were cut using a Shandon AS-325 retraction microtome (Shandon, Cheshire, UK). Sections were either stained with hematoxylin and eosin or processed for immunohistochemistry.

Using a Nikon AFX-DX microscope (Mellville, NY, USA) at 40 × magnification, hematoxylin and eosin stained sections were viewed and images of each field were transferred to a Macintosh 800 PowerPC via a Sony DXC-151A video camera and MACII frame grabber data acquisition board (Data Translation, Marlboro, MA, USA). Starting at one end of the colonic segment, consecutive fields were captured until the entire length of the segment was retrieved. In each field the total length of the colon, measured along the mucosal luminal surface, and the length of necrotic/ulcerated colonic mucosa were determined using Image NIH-Shareware software. The total length of necrotic colonic mucosa in the segment was determined and expressed as a percentage of the total segment length.

Inducible nitric oxide synthase was localized in tissue sections immunohistochemically using a murine polyclonal antibody as previously described (Miller et al., 1995). Visualization of the bound antibody was by the alkaline phosphatase/anti-alkaline phosphatase chromagen system (Vector Laboratories, Burlingame, CA, USA).

2.3. Cellular infiltration

Colonic myeloperoxidase activity was measured as an index of tissue granulocyte content by a modification of the method of Bradley et al. (1982). In this model of gut inflammation this assay primarily reflects the neutrophil content (resident and infiltrating) as the macrophage content does not dramatically change in contrast to other models of gut inflammation (Seago et al., 1995). Briefly, minced colonic tissue (300–400 mg) collected at necropsy was stored at −80°C for no longer than 7 days. Tissue was thawed and homogenized at 4°C with a Brinkmann polytron (Brinkmann Instruments, Westbury, NY, USA) for 1 min in 50 mmol/l potassium phosphate buffer (pH 6.0). The homogenate was centrifuged at 20 000 × g for 20 min at 4°C, and the resulting pellet was re-homogenized in 50 mmol/l potassium phosphate buffer (pH 6.0) containing 14 mM hexadecyltrimethylammonium bromide. The homogenate was then sonicated for 30 s at 4°C using a Vibra cell sonicator (Sonics and Materials, Danbury, CT, USA), frozen, thawed and re-sonicated. This cycle of freeze-thawing-sonicating was repeated twice. Homogenates were then centrifuged at 20 000 × g for 20 min at 4°C and 100 μl of the supernatant was added to 2.9 ml of 5 mmol/l potassium phosphate buffer (pH 6.0) containing *O*-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (5×10^{-4}% (v/v)). The change in absorbance at 460 nm over 1 min was measured using a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). One unit of myeloperoxidase activity was set as that which degraded 1 μmol/l of H_2O_2 in 1 min at 25°C.

Circulating leukocyte counts were performed from blood collected at necropsy in order to assess potential systemic complications. Whole blood (10 μl) was added to 90 μl of 3% acetic acid and white blood cells were counted using a Neubauer chamber under light microscopy.

2.4. TNF-α tissue content

Colonic tissue was stored at −80°C prior to analysis and tissue wet weights were determined on frozen samples. Colon samples were transferred to a metal tissue grinder, pre-chilled in liquid nitrogen, and pulverized to a fine powder. 0.9% NaCl was then added to ground frozen samples (1 μl/mg) and suspensions were thoroughly mixed and then centrifuged at 10 000 × g for 20 min at 4°C. Aliquots of the resulting supernatant were assayed for TNF-α content using a Cytimmune rat TNF-α enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Biosource, Camarillo, CA, USA). Results were expressed per mg wet weight tissue.

2.5. Nitric oxide production

Two methods were used to estimate the magnitude of nitric oxide release from sham-operated and TNBS-treated rats, including direct measurement of nitric oxide, using an electrochemical microelectrode detection system, and the accumulation of nitrite, a stable end product of nitric oxide metabolism, in colonic explants ex vivo. To detect nitric oxide electrochemically, a Nafion-coated custom microelectrode, sensitive to nitric oxide, was used as the working electrode and was placed to contact the mucosal surface of the colonic segment (Miller et al., 1996). A platinum wire auxiliary electrode and Ag/AgCl reference electrode were positioned in the medium adjacent to the tissue. The current through the working electrode was then recorded with a BAS100B electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN, USA) and the concentration of nitric oxide released was calculated by comparison to current readings from authentic nitric oxide solutions. Readings were taken at three distinct sites in each segment collected with the highest recording used to represent the colonic nitric oxide release from that animal. All ex vivo nitric oxide recordings were taken within 5 min of dissecting the colonic segments and all measurements were performed in a Faraday cage.

Nitrite accumulation in RPMI medium of colonic explants was assessed by the Greiss reaction as described by Miller et al. (1993). In brief, samples of culture medium were added to 100 μl of 2.5% (w/v) H_3PO_4 containing 0.1% (w/v) *N*-1-naphthethylenediamine and 1% (w/v) sulfanidamide (Sigma, St. Louis, MO, USA). Absorbance was measured using a Bio-Rad microplate reader (model 3550; Bio-Rad, Hercules, CA, USA). Concentrations of nitrite were determined by comparison to absorbance readings from solutions of known concentrations of sodium nitrite (J.T. Baker, Phillipsburg, NJ, USA) and were expressed per mg wet weight of colonic explant.

2.6. Inducible nitric oxide synthase gene expression

Total RNA was isolated from colonic samples by the acid guanidine thiocyanate-phenol-chloroform extraction method. Integrity of RNA extracts was assessed on a 1.5% agarose gel and visualization of RNA was by ethidium bromide staining. First-strand complementary DNAs were synthesized from 1 μg RNA using random primers (Boehringer-Mannheim, Indianapolis, IN, USA) and SuperScript II RNAse Reverse Transcriptase (Gibco, Grand Island, NY, USA). The first-strand complementary DNA templates were amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and inducible nitric oxide synthase by polymerase chain reaction (PCR). The oligonucleotide primers used to detect inducible nitric oxide synthase were based on the sequence of a conserved region of mouse and human inducible nitric oxide synthase was provided by Dr. Charles Rodi (G.D. Searle, St. Louis, MO, USA) as described by Miller et al. (1995). Sense and antisense primers for GAPDH (Genbank accession No. M17701) were also the same as those described by Miller et al. (1995). PCR reactions from RNA derived from sham-operated ($n = 4$), and TNBS-colitic rats treated with a placebo ($n = 8$), or with interleukin-10 at 1, 10, 100, 1000 μg/kg per day ($n = 7, 5, 5, 6$, respectively) were performed in addition to PCR analysis of pooled RNA samples from each treatment group, derived from a mix of RNA from animals in each group.

2.7. Interleukin-10 gene expression

With the same RNA extraction conditions outlined above for inducible nitric oxide synthase gene expression, interleukin-10 gene expression was evaluated. First-strand complimentary DNAs were synthesized from 1 μg of total RNA using oligo(dT) (Boehringer-Mannheim) and Superscript II Reverse Transcriptase (Gibco). The first-strand complimentary DNA templates were amplified for glyceraldehyde-3-phoshate dehydrogenase and interleukin-10 by polymerase chain reaction (PCR). The primers for interleukin-10 were as follows:

Sense 5′-CAT CCG GGG TGA CAA TAA CTG C-3′ (a 22-mer oligonucleotide at position 70)

Antisense 5′-ACC TGC TCC ACT GCC TTG CTT T-3′ (a 22-mer oligonucleotide at position 426), giving rise to a 357-bp PCR product.

The primers for glyceraldehyde-3-phosphate dehydrogenase used as an internal standard for rats were as follows:

Sense 5′-ATT CTA CCC ACG GCA AGT TCA ATG G-3′

Antisense 5′-AGG GGC GGA GAT GAT GAC CC-3′

The PCR cycle was an initial step of 95°C for 3 min, followed by 94°C for 30 s; 60°C for 45 s; 72°C for 1 min with 31 cycles and a final cycle of 72°C for 4 min. The negative control was a cDNA reaction using water instead of RNA.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean, unless otherwise stated. Statistical comparisons between treatment groups were made by one-way analysis of variance and Tukey's post-hoc tests using the Instat software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Clinical presentation and morphology

Intracolonic instillation of TNBS into adult female rats induced a 10% reduction in body weight, assessed 5 days after colitis induction, in contrast to sham-operated animals who displayed a 10% gain in body weight over this period.

Table 1
The effect of interleukin-10 administration on the severity of mucosal necrosis in TNBS colitis

	% Mucosal necrosis	Weight change (g)
Placebo	76.5 ± 10.10 (7)	−17.8 ± 4.4 (12)
Interleukin-10 (μg/kg per day)		
1	59.20 ± 15.00 (7)	−28.7 ± 2.6 (15)
10	75.97 ± 19.13 (5)	−19.4 ± 3.0 (11)
100	89.72 ± 3.16 (8)	−27.1 ± 3.8 (14)
1000	80.97 ± 5.47 (6)	−24.2 ± 1.9 (12)

% Mucosal necrosis is the proportion of the colonic segment covered by ulcerated/necrotic mucosa and was determined using quantitative image analysis techniques. Numbers in parentheses show the number of animals in each treatment group measured. The weight change from the pre-surgery value in sham-operated animals was −2.2 ± 3.1 g, which was significantly different from the rats treated with TNBS with or without additional interleukin-10 ($P < 0.05$). There were no statistically significant differences in weight change amongst the colitic groups displayed.

The body weight loss associated with colitis induction was not affected by treatment with interleukin-10 at the dose range assessed (1–1000 μg/kg per day, Table 1). Diarrhea was also associated with TNBS treatment; however, there was no obvious resolution of symptoms in any of the interleukin-10-administered groups.

Morphologically, at 5 days the TNBS-treated colon displayed severe mucosal necrosis and ulceration, transmural inflammation, with the majority of the cellular infiltrate comprised of neutrophils and macrophages, and enlargement of submucosal lymphoid aggregates. The extent of mucosal necrosis, expressed as a percentage length of the entire colonic segment (from the middle colic vein to the pelvic bone) was high in placebo-treated colitic rats (Table 1). Administration of interleukin-10 did not reduce the percentage of mucosal necrosis (Table 1), suggesting that 5 days treatment with interleukin-10 failed to enhance mucosal re-epithelialization or prevent colonic ulceration, induced by TNBS instillation.

3.2. Leukocytes

The severity of granulocyte infiltration was quantitated by assessing colonic myeloperoxidase activity. Associated with the transmural colonic inflammatory response, in TNBS colitis, was a significant increase in colonic myeloperoxidase activity, compared to the sham-operated group (Fig. 1). Interleukin 10 administration induced a significant reduction in colonic myeloperoxidase activity when given at doses of 1, 10 and 100 μg/kg per day for 5 days compared to placebo-treated animals, although the magnitude of myeloperoxidase activity following interleukin-10 treatment was still significantly elevated above that observed for sham-operated controls. In contrast, the highest dose of interleukin-10 administered, 1000 μg/kg per day, failed to attenuate the severity of granulocyte infiltration and produced a colonic myeloperoxidase activity comparable to placebo-treated animals (Fig. 1). These results suggest that interleukin-10 administration displays anti-inflammatory properties in TNBS colitis by attenuating granulocyte infiltration at the lower doses (1–100 μg/kg per day); however, the highest dose assessed (1000 μg/kg per day) was ineffective.

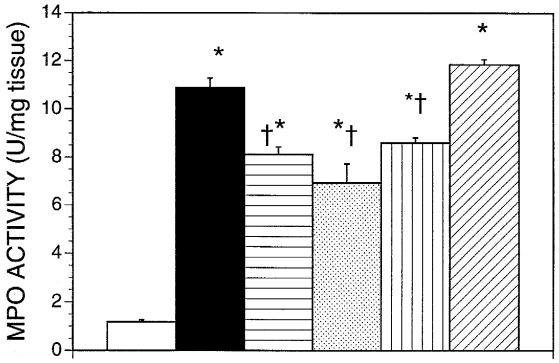

Fig. 1. Colonic myeloperoxidase activity in TNBS-treated rats and the effects of 5 days interleukin-10 administration. Colonic MPO activity for sham-operated (1st column) ($n = 6$), TNBS colitic rats treated with a placebo (2nd column) ($n = 11$), interleukin-10 administered at 1 μg/kg per day (3rd column) ($n = 8$), 10 μg/kg per day (4th column) ($n = 5$), 100 μg/kg per day (5th column) ($n = 7$), 1000 μg/kg per day (6th column) ($n = 6$) are shown.* $P < 0.001$ versus sham-operated group; † $P < 0.001$versus placebo-treated TNBS colitic group and the 1000 μg/kg per day interleukin-10-treated TNBS colitic group.

In contrast to the enhanced colonic inflammatory infiltrate, systemic leukocyte counts were not affected by the induction of colitis by TNBS (Fig. 2). There was, however, a trend, albeit non-significant, for an increase in the systemic leukocyte count in TNBS-treated rats administered 10, 100 and 1000 μg/kg per day interleukin-10 (Fig. 2).

3.3. TNF-α content

Colonic TNF-α content was measured by ELISA specific for rodent TNF-α. TNBS colitis was associated with

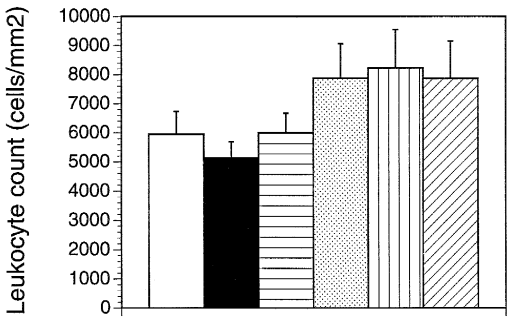

Fig. 2. Total systemic white blood cell count as influenced by TNBS colitis and interleukin-10 administration. Systemic leukocyte count for sham-operated (1st column) ($n = 12$), TNBS colitic rats treated with a placebo (2nd column) ($n = 22$), interleukin-10 administered at 1 μg/kg per day (3rd column) ($n = 15$), 10 μg/kg per day (4th column) ($n = 11$), 100 μg/kg per day (5th column) ($n = 14$), 1000 μg/kg per day (6th column) ($n = 12$) are shown. No statistical significance was detected between the groups.

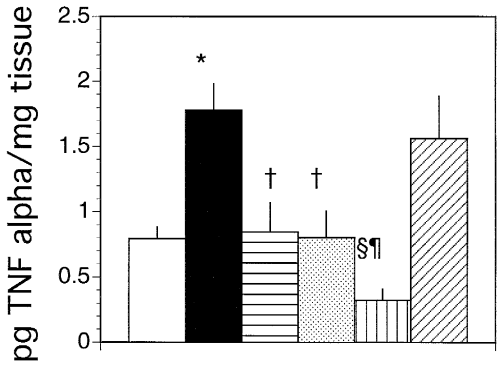

Fig. 3. Colonic TNF-α content following TNBS and interleukin-10 administration. Colonic TNF-α content, expressed as pg/mg tissue wet weight, for sham-operated (1st column) ($n = 5$), TNBS colitic rats treated with a placebo (2nd column) ($n = 11$), interleukin-10 administered at 1 μg/kg per day (3rd column) ($n = 8$), 10 μg/kg per day (4th column) ($n = 6$), 100 μg/kg per day (5th column) ($n = 6$), 1000 μg/kg per day (6th column) ($n = 5$) are shown.* $P < 0.05$ versus sham-operated group; † $P < 0.05$ versus placebo-treated TNBS colitic group; § $P < 0.001$ versus placebo-treated TNBS colitic group; ¶ $P < 0.01$ versus 1000 μg/kg per day interleukin-10-treated TNBS colitic group.

a significant increase in colonic TNF-α content (Fig. 2). Administration of interleukin-10 at 1, 10 and 100 μg/kg per day for 5 days significantly attenuated the enhanced TNF-α production associated with colitis to levels comparable to those detected in sham-operated control animals (Fig. 3). In contrast, the highest dose of IL-10 used, 1000 μg/kg per day, failed to exert any effect on colonic TNF-α levels and resulted in TNF-α content comparable to that observed in TNBS rats treated with a placebo.

3.4. Nitric oxide

The effect of interleukin-10 on nitric oxide release was assessed ex vivo by directly measuring nitric oxide electrochemically and by determining the level of nitrite accumulation in colonic explants. TNBS colitis was associated with a 10-fold increase in ex vivo nitric oxide release compared to sham-operated control animals (Table 2). An enhanced production of nitric oxide was also reflected by a significant 5-fold increase in nitrite accumulation from colonic explants of TNBS placebo-treated animals compared to the sham-operated group (Table 2). Treatment with interleukin-10 did not suppress nitric oxide release or nitrite production at any of the doses used, consequently nitric oxide production remained significantly elevated above normal levels. These data confirm that nitric oxide production is enhanced in TNBS colitis; however, this response is not attenuated by administration of interleukin-10.

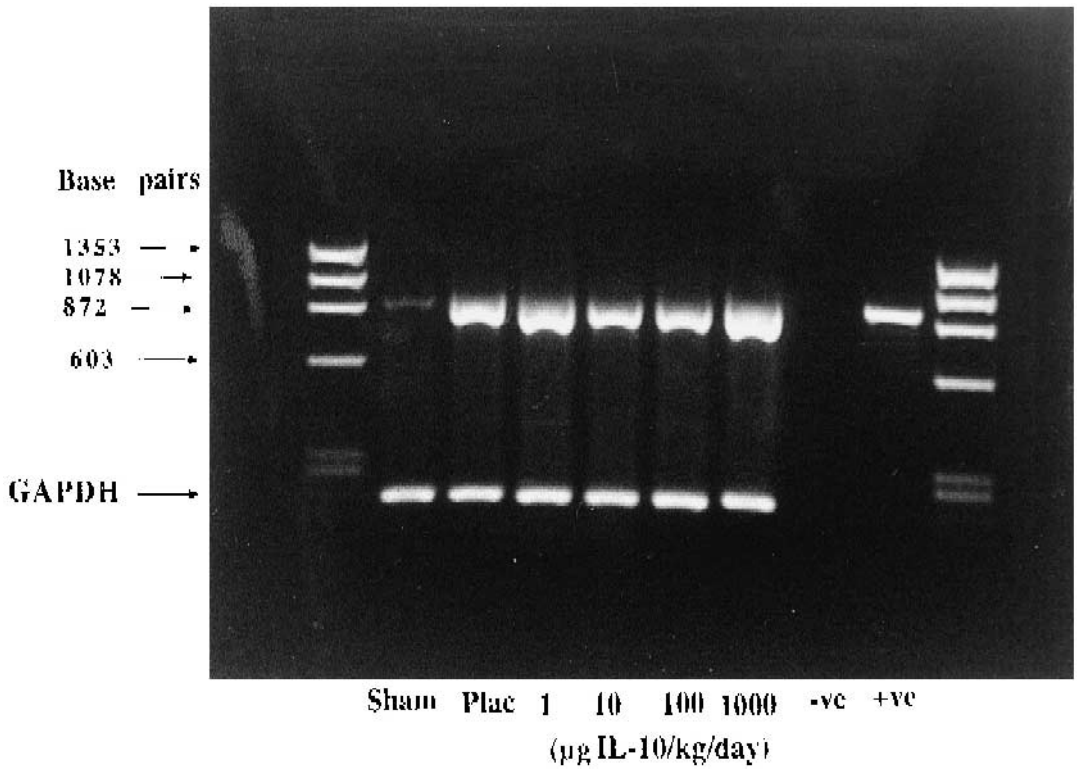

Fig. 4. Effect of 5 days interleukin-10 administration on TNBS-induced inducible nitric oxide synthase gene expression. Reverse transcriptase PCR-amplified complementary DNA was separated on a 2% agarose gel. This gel represents RT-PCR reactions from RNA pooled from representative animals in each treatment group. Base pair markers denoting DNA size are shown in the outside lanes. Inducible nitric oxide synthase (907 bp) and GAPDH (housekeeping gene product for estimation of RNA loading) are shown. The column designated '−ve' contains a non-DNA, water-only control, whereas the column designated '+ve' is an authentic inducible nitric oxide synthase (courtesy Dr. James Cunningham).

Table 2
The effect of interleukin-10 administration on nitric oxide production in TNBS colitis

	Nitric oxide release (μM)	Nitrite production (nmol/mg tissue over 3 h)
Sham-operated	0.73 ± 0.43 (6)	0.05 ± 0.02 (6)
TNBS + placebo	7.05 ± 1.19 (11) [c]	0.27 ± 0.03 (11) [a]
TNBS + interleukin-10 (μg/kg per day)		
1	4.20 ± 0.37 (7) [b]	0.34 ± 0.12 (4) [a]
10	7.10 ± 0.32 (5) [c]	0.35 ± 0.09 (5) [b]
100	7.48 ± 0.36 (8) [c]	0.31 ± 0.05 (6) [a]
1000	NA	0.21 ± 0.08 (5)

Nitric oxide released was assessed directly, using electrochemical detection, and by determining the level of nitrite accumulation in the culture media of colonic explants following a 3 h incubation period. Numbers in parentheses show the number of animals in each treatment group measured. NA, electrochemical detection of nitric oxide release in animals treated with 1000 μg/kg per day was not performed.
[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ versus sham-operated group.

The elevation in nitric oxide release in TNBS-induced colitis was associated with an enhanced expression of inducible nitric oxide synthase (Fig. 4). Inducible nitric oxide synthase expression was present in the colon of sham-operated rats 5 days after performing the laparotomy, seen in Fig. 4 as a weak signal of a 907-bp fragment, identifying inducible nitric oxide synthase gene expression. There was a marked increase in inducible nitric oxide synthase expression associated with administration of TNBS, seen as a strong signal for the RT-PCR product corresponding to inducible nitric oxide synthase (Fig. 4). The intensity of the inducible nitric oxide synthase expression was not substantially altered following treatment with interleukin-10 at the dose range used (1–1000 μg/kg per day), although it is well recognised that this technique is at best semi-quantitative. Taken together, these findings show that TNBS-induced colitis is associated with an increase in the production of nitric oxide which is accompanied by an enhanced expression of inducible nitric oxide synthase. Subcutaneous administration of interleukin-10 for 5 days did not attenuate the enhanced nitric oxide production or inducible nitric oxide synthase gene expression, implying that any anti-inflammatory effects of interleukin-10 that were observed were not mediated by a nitric oxide-dependent mechanism.

Inducible nitric oxide was localized in the TNBS model of colitis immunohistochemically. Positive staining for inducible nitric oxide synthase, indicated by a red reaction product (Fig. 5B) was associated with neutrophils and macrophages in the colonic submucosa 5 days after colitis induction. This staining pattern was shown to be specific for inducible nitric oxide synthase due to a lack of positive staining detected in tissue sections exposed to pre-immune serum instead of the murine inducible nitric oxide synthase polyclonal antibody (Fig. 5C). While a faint RT-PCR signal for inducible nitric oxide synthase gene expression was detected in the sham-operated animals, inducible nitric oxide synthase was not detected immunohistochemically in the control rat colon (Fig. 5A).

Endogenous interleukin-10 production was assessed by RT-PCR evaluation of interleukin-10 gene expression. TNBS colitis was associated with an up-regulation of IL-10 gene expression over sham-operated rats, and this

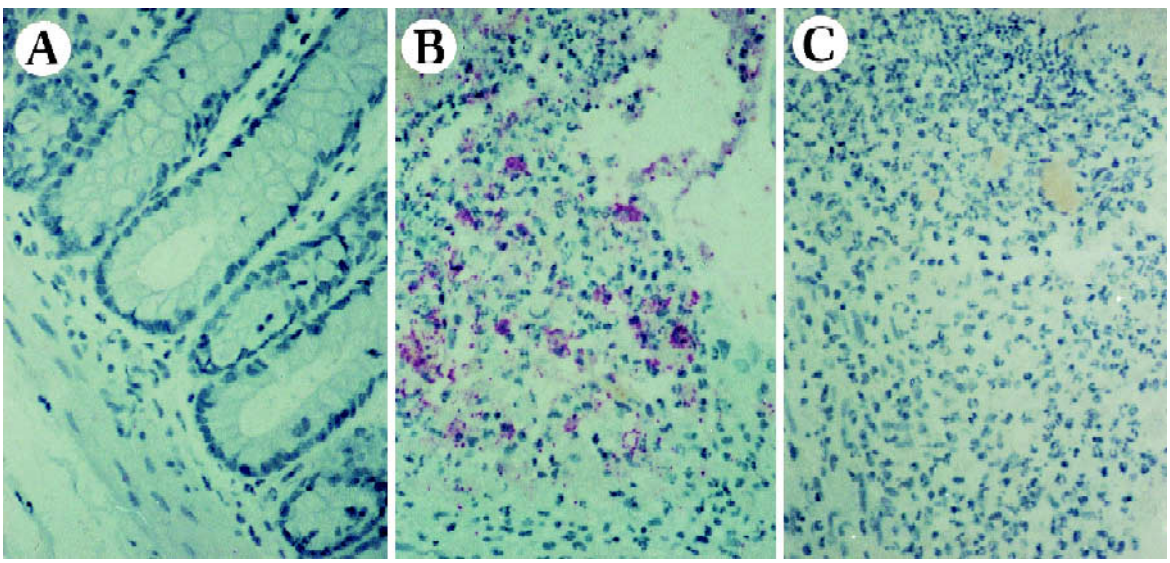

Fig. 5. Immunohistochemical localization of inducible nitric oxide synthase in TNBS colitis 5 days after induction of colitis. Localization of inducible nitric oxide synthase polyclonal antibody binding was by the alkaline phosphatase/anti-alkaline phosphatase chromagen system with positive staining appearing as a red reaction product. (A) Sham-operated rat colon (original magnification ×400); note the absence of positive staining in the colonic mucosa and submucosa. (B) Placebo-treated TNBS colitic rat colon (original magnification ×400); positive inducible nitric oxide synthase staining is localized in macrophages and neutrophils in the colonic submucosa. (C) Placebo-treated TNBS colitic rat colon (original magnification ×400); negative control for inducible nitric oxide synthase staining. Tissue section was exposed to pre-immune serum instead of inducible nitric oxide synthase antibody.

effect persisted until at least 14 days after the administration of TNBS (data not shown).

4. Discussion

The release of cytokines and oxidants is a major component of tissue injury in inflammation. Of these classes of agents, TNF-α and nitric oxide are thought to be key components, particularly in gut inflammation (Murch et al., 1993; Miller et al., 1995; Ribbons et al., 1995). Less is known about endogenous anti-inflammatory regulatory mechanisms although recently the focus has turned to interleukin-10 because of the breadth of its suppressive actions on cytokine release. While in inflammation it is clear that pro-inflammatory forces predominate, it remains possible that enhanced endogenous production or exogenous administration of anti-inflammatory/immunosuppressive cytokines, mediators, or enzymes, could be a viable therapeutic approach. Based on the preliminary evidence by Schreiber et al. (1995), who found that exogenous interleukin-10 reduced TNF-α and interleukin-1β release by lamina propria polymorphonuclear cells in ulcerative colitis patients, we embarked on a study to evaluate the efficacy of interleukin-10 in a hapten-induced model of inflammatory bowel disease, TNBS colitis in rats.

Endogenous interleukin-10 production in TNBS colitis was determined by RT-PCR analysis which indicated that the model was associated with increased formation of interleukin-10. Gene expression analysis indicates that this increased interleukin-10 production is persistent, extending to 14 days after TNBS administration (later time points have not been evaluated). This would suggest that interleukin-10 is up-regulated for an extended period, presumably in an attempt to negate the inflammation or promote mucosal repair.

At the same time as interleukin-10 is increased in the TNBS model pro-inflammatory mediators are also elevated; in this study the focus was TNF-α and nitric oxide, as confirmed in Fig. 2 and Table 2. We hypothesized that if exogenous interleukin-10 was to have anti-inflammatory properties in this model, it would inhibit the production of one or both of these mediators. Results indicate that interleukin-10 did indeed have anti-inflammatory effects in TNBS colitis, albeit mild, and that this effect was associated with a suppression of TNF-α, but not nitric oxide production. This supports the findings of Schreiber et al. (1995) in ulcerative colitis, and suggests that interleukin-10 administration may be useful in the treatment of inflammatory bowel disease.

The current study does raise a number of issues that need further evaluation in order to predict the efficacy of interleukin-10 administration in human disease. One glaring observation was the bell-shaped dose-response curve, where interleukin-10 was effective at low doses but at the highest dose evaluated (1 mg/kg s.c.) interleukin-10 was totally ineffective. The mechanisms for this effect are unknown and initially counter-intuitive; one would predict that in inflammation the higher the dose of an anti-inflammatory agent the greater the efficacy. The results may be due to species differences (human vs. rat) but this does not easily explain the loss of efficacy at higher doses although it may explain the mild nature of the beneficial effects. Clearly we need more information as to the mechanism of action of interleukin-10 and what checks and balances are evoked by exogenous interleukin-10 administration. In the model endogenous formation of interleukin-10 was elevated as indicated by RT-PCR but the interplay between endogenous and exogenous interleukin-10 is unknown. It was interesting to note that the high dose of interleukin-10, which was not effective in the treatment of TNBS colitis, failed to suppress the elevated TNF-α mucosal content associated with colitis, although lower doses were both therapeutic and suppressed TNF-α levels. In a manner that is unknown, it appears that high doses of interleukin-10 fail to regulate TNF-α at the transcriptional level. This association between effects of interleukin-10 on TNF-α formation and anti- inflammatory actions is suggestive of a direct link; however, we have not determined if interventions directed at TNF-α specifically modify the inflammatory process in this model, and therefore these observations remain an association and not a causation.

We have previously observed bell-shaped dose-response curves with therapeutic agents in this model of gut inflammation. Genistein, a tyrosine kinase inhibitor and phytoestrogen, has anti-inflammatory actions in TNBS ileitis in guinea pigs at low doses but with increasing doses the beneficial effects are lost. In contrast to interleukin-10, this dose-response relationship was paralleled by changes in nitric oxide synthesis, low doses of genistein inhibiting nitric oxide release while high doses were without effect on nitric oxide production (Sadowska-Krowicka et al., 1996).

Another proposed pre-transcriptional action of interleukin-10 is the inhibition of inducible nitric oxide synthase-dependent nitric oxide release (Becherel et al., 1995; Cunha et al., 1992; Cenci et al., 1993). In TNBS colitis nitric oxide production is greatly elevated in response to inducible nitric oxide synthase gene expression. Previously we and others have documented that inhibitors of nitric oxide production ameliorate TNBS-induced inflammation (Miller et al., 1993, 1995; Hogaboam et al., 1995) indicating that enhanced nitric oxide production plays a direct role in tissue injury. However, in this study it is clear that interleukin-10 was not affecting nitric oxide production, as determined indirectly by nitrite accumulation or directly by microelectrode detection of nitric oxide levels in mucosal explants. In addition, inducible nitric oxide synthase gene expression was not affected by exogenous interleukin-10. From these results we conclude that in this model of gut inflammation interleukin-10 is not modifying nitric oxide

release, and that any observed anti-inflammatory actions are nitric oxide-independent.

In summary, endogenous interleukin-10 gene expression was elevated in TNBS colitis and exogenous interleukin-10 administration resulted in an attenuation of granulocyte infiltration but did not affect the extent of mucosal necrosis or weight loss associated with this model. The mild anti-inflammatory effects produced by interleukin-10 may be due to the suppression of TNF-α production but were clearly independent of effects on nitric oxide release and inducible nitric oxide synthase gene expression. An unexpected finding was the loss of beneficial effects of interleukin-10 at high doses. These results are supportive of a potential therapeutic application of exogenous interleukin-10 in human inflammatory bowel disease but additional studies are needed to facilitate understanding of the mechanism of action of interleukin-10 and the unusual dose-response relationships.

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