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Curcumin, the major component of food flavour turmeric, reduces mucosal injury in trinitrobenzene sulphonic acid-induced colitis

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1 Inflammmatory bowel disease (IBD) is characterized by oxidative and nitrosative stress, leucocyte infiltration and upregulation of proinflammatory cytokines. In this study, we have investigated the protective effects of curcumin, an anti-inflammatory and antioxidant food derivative, on 2,4,6-trinitrobenzene sulphonic acid-induced colitis in mice, a model for IBD.

2 Intestinal lesions (judged by macroscopic and histological score) were associated with neutrophil infiltration (measured as increase in myeloperoxidase activity in the mucosa), increased serine protease activity (may be involved in the degradation of colonic tissue) and high levels of malondialdehyde (an indicator of lipid peroxidation).

3 Dose–response studies revealed that pretreatment of mice with curcumin (50 mg kg^{-1} daily i.g. for 10 days) significantly ameliorated the appearance of diarrhoea and the disruption of colonic architecture. Higher doses (100 and 300 mg kg^{-1}) had comparable effects.

4 In curcumin-pretreated mice, there was a significant reduction in the degree of both neutrophil infiltration (measured as decrease in myeloperoxidase activity) and lipid peroxidation (measured as decrease in malondialdehyde activity) in the inflamed colon as well as decreased serine protease activity.

5 Curcumin also reduced the levels of nitric oxide (NO) and O_2^- associated with the favourable expression of Th1 and Th2 cytokines and inducible NO synthase. Consistent with these observations, nuclear factor-κB activation in colonic mucosa was suppressed in the curcumin-treated mice.

6 These findings suggest that curcumin or diferuloylmethane, a major component of the food flavour turmeric, exerts beneficial effects in experimental colitis and may, therefore, be useful in the treatment of IBD.

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Keywords: Curcumin; inflammatory bowel disease; colitis; turmeric; cytokines; nitric oxide

Abbreviations: IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TNBS, 2,4,6-trinitrobenzene sulphonic acid; UC, ulcerative colitis

Introduction

Inflammatory bowel disease (IBD), identified and diagnosed by a set of clinical, endoscopic, and histological features (Kirsner & Shorter, 1988), is of still unknown aetiology. Treating IBD while limiting drug-induced toxicity is a continuous challenge. 5-Aminosalicyclic acid and salazosulphapyridine are the drugs of choice for current medical treatment. Corticosteroids, azathioprine, mercaptopurines and cyclosporine are used in more severe forms of the disease (Hanauer, 1996). Owing to the lack of specific, curative treatments with limited toxicity, there is a pressing need for developing effective therapeutic approaches. Various models of IBD have been found to be associated with an overproduction of nitric oxide (NO) because of the expression of the inducible isoform of NO synthase (iNOS) (Nathan, 1996). Increased luminal activities of NO have also been detected in ulcerative colitis (UC) (Lundberg *et al.*, 1994) and in the colonic lavage fluid in different animal models of IBD (Feretti *et al.*, 1997; Gunawardana *et al.*, 1997). Excessive production of NO in chronic colitis may be detrimental to the integrity of the colonic mucosa (McKenzie *et al.*, 1996). In accordance with this information NO-related treatments serve as a promising pharmacological approach in the treatment of these disease states (Neilly *et al.*, 1995; Rachmilewitz *et al.*, 1995a). Interventions, which reduce the generation or the effects of reactive nitrogen, exert beneficial effects in a variety of models of inflammation, including the trinitrobenzene sulphonic acid-induced colitis used here (Miller *et al.*, 1993; Hogaboam *et al.*, 1995; Neilly *et al.*, 1996). In contrast to these findings, however, it has been reported that genetic ablation of iNOS activation may exacerbate intestinal inflammation induced by intraluminal administration of acetic acid in mice (McCafferty *et al.*, 1997). Other experimental studies have shown that only slight pharmacological inhibition of NO formation reduced colonic lesions, while a complete abolition of NO synthesis resulted in increased mucosal damage (Pfeiffer & Qiu, 1995). The critical contribution of iNOS to the pathogenesis of IBD, therefore, has not been clearly delineated.

The use of medicinal plants or their active components is becoming an increasingly attractive approach for the treatment

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of various inflammatory disorders among patients unresponsive to or unwilling to take standard medicines. Among these alternative approaches is the use of food derivatives, which have the advantage of being relatively nontoxic. However, limited scientific evidence regarding the effectiveness of these natural derivatives, in conjunction with a lack of mechanistic understanding of their actions has prevented their incorporation into the mainstream of medical care. During the last decade, a large number of dietary components have been evaluated as potential chemopreventive agents (Sharma *et al.*, 1994). Turmeric, the powdered rhizome of the medicinal plant, *Curcuma longa* Linn, is widely used as a food flavouring and colouring agent in the Asian diet. Its yellow colour is imparted by curcumin (diferuloylmethane), a polyphenolic pigment (Cooper *et al.*, 1994). Curcumin has a long history of medicinal use in India and Southeast Asia and is known to exhibit a variety of pharmacological effects including anti-inflammatory, antitumour, anti-HIV and anti-infectious activities (Mazumder *et al.*, 1996; Allen *et al.*, 1998; Chan *et al.*, 1998; Vlietinck *et al.*, 1998). It is under preclinical evaluation for drug development of cancer prevention and anti-inflammation (Gescher *et al.*, 1998). Interest in curcumin and its promising medicinal value is growing, especially since it does not appear to be significantly toxic (Ammon & Wahl, 1991) and exhibits marked antioxidant and anti-inflammatory properties. Thus, curcumin scavenges active oxygen species including superoxide, hydroxyl radical and NO (Sreejayan & Rao, 1997). It decreases the 12-*O*-tetradecanoylphorbol-13-acetate-induced expression of c-*jun*, c-*fos* and c-*myc* proto-oncogenes (Kakar & Roy, 1994), suppresses activation of nuclear factor-κB (NF-κB) (Singh & Aggarwal, 1995) and elevates the activities of detoxification enzymes of xenobiotic metabolism, such as glutathione transferases and NAD(P)H : quinone reductase (Arbiser *et al.*, 1998).

Of the several animal models of intestinal inflammation, the well-characterized haptene reagent 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis resembles human IBD in terms of its various histological features including infiltration of colonic mucosa by neutrophils and macrophages and increased production of inflammatory mediators including T-helper 1 profile of cytokines (Parronchi *et al.*, 1997). Moreover, various experimental trials using antibodies to IL-12, IL-4 gene transfer, and antisense oligonucleotides against NF-κB have indicated that the TNBS-induced colitis model is useful to test new therapeutic strategies for human (Neurath *et al.*, 1996; Hogaboam *et al.*, 1997; Fuss *et al.*, 1999). Since food flavour turmeric-derived curcumin is a major source of dietary flavonoid having antioxidative and antinitrosative effects and since IBD is associated with a marked upregulation of NO, it was thought worthwhile to investigate the effects of curcumin on the inflammatory response (colitis) caused by intracolonic administration of TNBS.

Methods

Animals and reagents

Female BALB/c mice weighing 25 – 30 g (obtained from National Institute of Nutrition, Hyderabad, India) were used for the experiments. Mice were housed under normal laboratory conditions, that is, at 21 – 24°C and 40 – 60% relative humidity, under a 12 h light/dark cycle with free access to standard rodent food and water. Curcumin was purchased from Sigma Chemical Co., St Louis, MO, U.S.A. For the oral administration experiments, curcumin was emulsified in 2.5% carboxymethyl cellulose and various dosages ranging from 25 mg to 300 mg kg^{-1} were administered by gavage.

Experimental colitis

Colitis was induced in mice by intrarectal administration of 0.1 ml of TNBS (60 mg ml^{-1} in 30% ethanol), through a trochar needle approximately 3 – 4 cm proximal to the anus according to the model described earlier (Neurath *et al.*, 1995). Control mice received 30% ethanol in phosphate-buffered saline (PBS) using the same technique. In the treated group of animals, curcumin was given daily i.g. (25 mg to 300 mg kg^{-1} day^{-1}) for 10 days before subjecting the mice to TNBS-induced colitis and same dose of curcumin was continued till the mice were killed 8 days after the induction of colitis.

Macroscopic assessment of severity of colitis

Mice were killed by cervical dislocation, the colon excised, opened longitudinally, and washed in saline. Macroscopic damage was assessed by the scoring system of Wallace & Keenan (1990), which takes into account the area of inflammation and the presence and absence of ulcers. The criteria for assessing macroscopic damage were based on a semiquantitative scoring system where features were graded as follows: 0, no ulcer, no inflammation; 1, no ulcer, local hyperaemia; 2, ulceration without hyperaemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm. After macroscopic observation, samples of colonic tissue were subsequently excised for microscopic observation of damage, measurement of myeloperoxidase activity, malondialdehyde, NO and O_2^- production, and mRNA expression of cytokines and iNOS.

Microscopic assessment of colitis

The colon was fixed in 10% formalin in PBS for 1 week and the samples were then dehydrated in graded ethanol and embedded in paraffin. Thereafter, 7 μm sections were deparaffinized with xylene, stained with haematoxylin – eosin and examined in a Leitz Ortholux microscope. Histologic changes were graded semiquantitatively from 0 to 4 according to previously described criteria (Neurath *et al.*, 1995) as follows: 0, no leucocyte infiltration; 1, low level of leucocyte infiltration; 2, moderate level of leucocyte infiltration; 3, high vascular density and thickening of colon wall; and 4, transmural leucocyte infiltration, loss of goblet cells, high vascular density and thickening of the colon wall.

Assessment of NO production

Tissues from the proximal third of the colon were homogenized in 40 mM HEPES containing 320 mM sucrose. Nitrite + nitrate production, an indicator of NO synthesis, was measured in the supernatant (10,000 $\times$ *g* for 20 min at 4°C) according to Zingarelli *et al.* (1997). Nitrate in the supernatant

was reduced to nitrite by incubation with nitrate reductase (670 mU ml^{-1}) and NADPH (160 mM) at room temperature for 3 h. A measure of 100 μl of the sample was then mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% *N*-(1-napthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4) and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured. The amount of nitrite released was quantified by comparison with sodium nitrite as standard.

Estimation of O_2^-

Superoxide production was measured as described previously (Markert *et al.*, 1984). In brief, 10^6 extravasated neutrophils were incubated for 30 min at 37°C in the presence of 2 μg ml^{-1} PMA, 1 mg ml^{-1} cytochrome *c*, 30 μg ml^{-1} catalase, $\pm$100 μg ml^{-1} superoxide dismutase in D-glucose PBS. The cells were then removed by centrifugation and the absorbance of reduced cytochrome *c* measured at 550 nm.

Myeloperoxidase activity

Myeloperoxidase is an enzyme found in cells of myeloid origin, and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into gastrointestinal tissues (Morris *et al.*, 1989). Samples of distal colon were homogenized in 10 mM potassium phosphate buffer, pH 7.0 containing 0.5% hexadecyltrimethylammonium bromide and centrifuged for 30 min at 20,000 $\times$ *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. One unit of myeloperoxidase activity was defined as that degrading 1 μmol of H_2O_2 per min at 37°C and was expressed as units per milligram of tissue sampled (U mg^{-1} tissue).

Malondialdehyde measurement

Malondialdehyde levels in the colon were determined as an indicator of lipid peroxidation (Ohkawa *et al.*, 1979). The tissue was homogenized in 1.15% KCl solution. A measure of 0.1 ml of the homogenate was then added to a reaction mixture containing 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid and 0.7 ml of distilled water. Samples were boiled for 1 h at 95°C and centrifuged at 3000 $\times$ *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Analysis of protease activity

A small piece of colon tissue from the centre of the ulcer was homogenized for 10 s in cold PBS. The homogenate was centrifuged (14,000 $\times$ *g* for 5 min at 4°C) and the supernatant was analysed for protease activity on gelatin zymograms according to Hawkins *et al.* (1997). In short, 12% SDS–polyacrylamide gels were prepared containing 0.1% gelatin. An equal amount of protein from each sample (10–25 μg) was applied to the gel in standard SDS-gel loading buffer containing 0.1% SDS but lacking β-mercaptoethanol and samples were not boiled prior to loading. After electrophoresis, gels were soaked in 2% Triton X-100 in distilled water with shaking for 15 min. Gels were then incubated in 50 mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ for 12 h at 37°C, stained in amido black and subsequently destained. Protease activity shows up as clear bands (indicative of cleavage of the gelatin substrate) on a blue background.

RT–PCR analysis of cytokines and iNOS mRNA

RT–PCR was performed in colon tissue samples from mice 4 days post-TNBS to determine the cytokine profile of mRNA for IFN-γ, IL-12 p40, IL-4, iNOS and β-actin. Reverse transcription of 1 μg of RNA was performed according to the manufacturer's protocol for the Superscript One-Step RT–PCR system (Life Technologies, Grand Island, NY, U.S.A.). The primers for all these genes have been published (Kawakami *et al.*, 1997). After the appropriate number of PCR cycles, the amplified cDNA was separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Electrophoretic mobility shift assay

The nuclear extracts were prepared from excised colon according to the method of Yang *et al.* (1998). For electrophoretic mobility shift assay, each 10 μg of nuclear extracts were preincubated with 1 μg of poly(dI-dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol and 50 mM NaCl) for 10 min at room temperature. As a control, a 50-fold molar excess of unlabelled NF-κB competitor oligonucleotide was added. After preincubation, 0.5 ng of ^{32}P end-labelled NF-κB oligonucleotide probe (5′-CGGGGACTTTCCGCTGGGGACTTTCCGCTTGAGCT-3′) was added to the reaction mixture and incubated for 30 min. The DNA–protein complex was then electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5X TBE buffer (0.0445 M Tris, 0.0445 M borate, 0.001 M EDTA).

Statistical analysis

Results are expressed as mean $\pm$ s.d. of *n* observations. We used analysis of variance to determine the statistical significance of intergroup comparisons. $P<0.05$ was considered to be statistically significant. Macroscopic and microscopic scores for colonic erosions for the curcumin-pretreated groups were compared against those for the TNBS-treated group with a two-sided Wilcoxon's rank-sum test.

Results

Macroscopic and histological evaluation

Intracolonic administration of TNBS/ethanol resulted in extensive haemorrhagic and ulcerative damage to the distal colon as observed up to 8 days. Macroscopic examination of the distal colon and rectum from TNBS-treated mice revealed the presence of multiple mucosal erosions and ulcerations. The caecum, colon and rectum showed evidence of mucosal congestion, erosions and haemorrhagic ulcerations evaluated to a peak at 4 days as shown in the damage score (Figure 1). The histological features included a transmural necrosis and oedema and a diffuse leucocyte cellular infiltrate in the

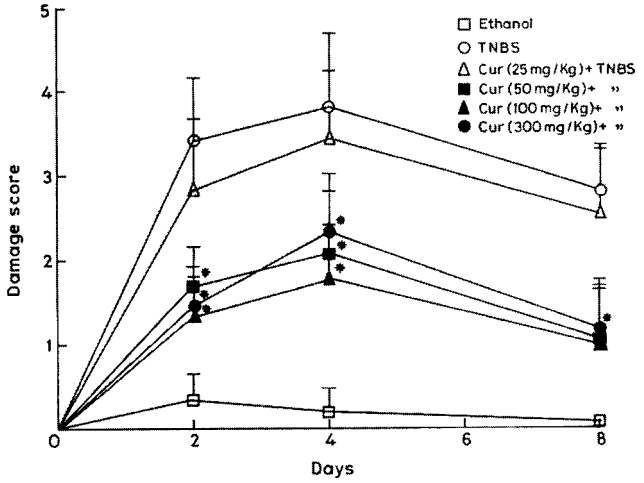

Figure 1 Effect of curcumin pretreatment on the macroscopic damage score of colonic tissues after induction of colitis. Mice were treated with 0.1 ml of TNBS (60 mg ml^{-1}) intracolonically and assessed at various times (2 – 8 days) after treatment. Colonic damage was scored on a scale of 0 (normal) to 5 (severe) by two independent observers. Values are means ± s.d. of 10 rats for each group. Results for the curcumin treatment group were compared against those for the TNBS-treated group with a two-sided Wilcoxon's rank-sum test. *$P<0.001$ vs TNBS.

submucosa (Figure 2b). Control group gave rise to a very mild early damage of the colon. Treatment of mice with curcumin (50, 100 or 300 mg kg^{-1}) resulted in a significant decrease in the extent and severity of the injury of the large intestine as evidenced by macroscopic damage score (Figure 1) as well as histopathological assessment (Figure 2c and Table 1). The observed inflammatory changes of the large intestine were associated with an increase in weight of the colon and spleen as well as a significant decrease in body weight as compared to control mice (Figure 3). In contrast, no significant increase of the weight was found in the colon and spleen of TNBS-treated mice, which has been pretreated with 50, 100 or 300 mg kg^{-1} of curcumin. Moreover, treatment with these dosages of curcumin also significantly reduced the loss in body weight, which correlated well with the amelioration of the colonic injury. However, the altered organ weight and body weight of TNBS-treated mice were not affected by pretreatment of curcumin with a dose of 25 mg kg^{-1} (Figure 3).

Generation of NO and O_2^-

Since infiltration of leucocytes into the mucosa has been suggested to contribute significantly to tissue necrosis and mucosal dysfunction of colitis by generating free radicals and oxidant molecules, both NO and O_2^- were measured. NO was measured in colonic biopsies, whereas O_2^- was measured in extravasated neutrophils (Figure 4). At 4 days after TNBS treatment, both NO and O_2^- levels were significantly elevated compared to controls (8.8 ± 1.0 nmol mg^{-1} tissue and 5.1 ± 0.5 nmol min^{-1} 10^6 cells^{-1} compared to 2.0 ± 0.3 nmol mg^{-1} and 1.3 ± 0.2 nmol min^{-1} 10^6 cells^{-1}, respectively; $P<0.001$). Curcumin pretreatment at dose levels of 50, 100 or 300 mg kg^{-1} resulted in marked decrease of the elevated levels of both NO and O_2^- in the colon of TNBS-treated mice ($P<0.01$). Pretreatment with 25 mg kg^{-1} of curcumin though did not produce any significant effect on either NO or O_2^- production (Figure 4).

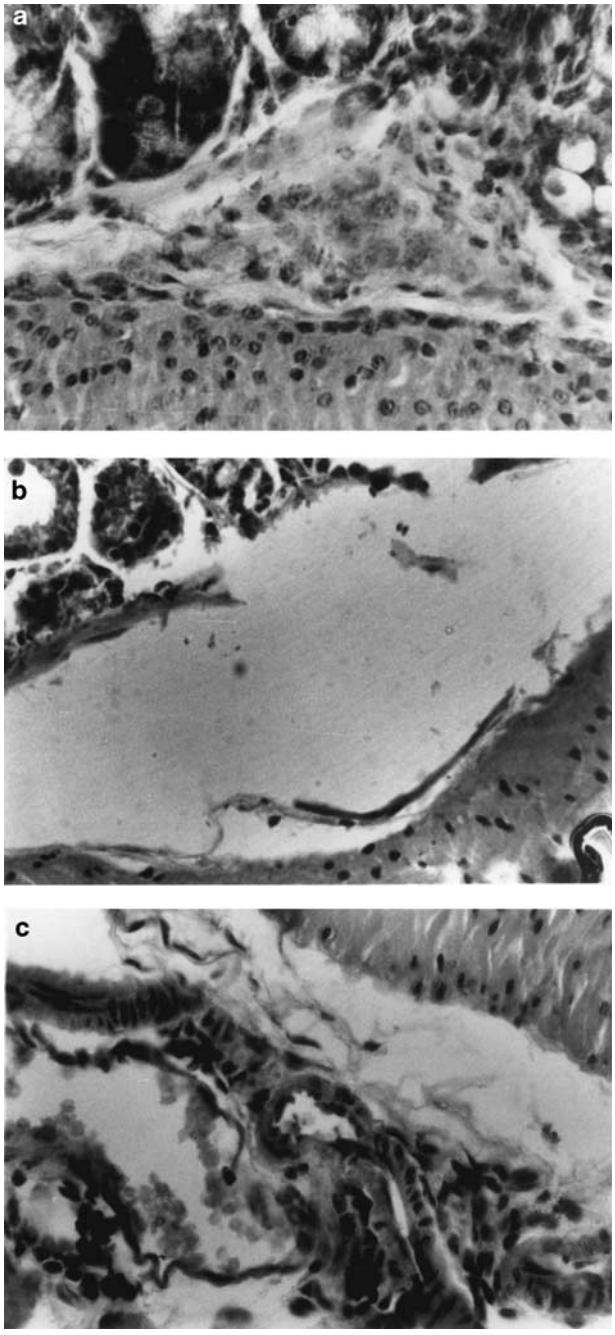

Figure 2 Effect of curcumin on colon injury. (a) Mucosal from control mice did not show any histological modifications, (b) TNBS-induced mucosal injury (at 4 days) associated with transmural necrosis and oedema and submucosal infiltration of inflammatory cells and (c) pretreatment with curcumin prevented the disturbances in morphology associated with TNBS treatment. Original magnification: × 500.

Effect of curcumin on myeloperoxidase and malondialdehyde levels in TNBS-induced IBD

Colonic injury by TNBS administration was also characterized by an increase in myeloperoxidase activity (36.7 ± 3.3 compared with 8.0 ± 1.3 U g^{-1} tissue in controls), indicative of

Table 1 Effect of curcumin pretreatment on colonic cytoprotection following administration of TNBS

	Colonic erosion							
	Scale							
	0	*1*	*2*	*3*	*4*	*n*	*Mean*	*P*[a]
Control	6	4	0	0	0	10	0.40	
TNBS	0	1	3	3	3	10	2.80	
Curcumin (25 mg kg^{-1})	0	2	4	2	2	10	2.40	0.4372
Curcumin (50 mg kg^{-1})	0	7	2	1	0	10	1.40	0.0052
Curcumin (100 mg kg^{-1})	0	7	3	0	0	10	1.30	0.0021
Curcumin (300 mg kg^{-1})	0	6	3	1	0	10	1.50	0.0089

Histological scores graded from 0 to 4 as described in Methods were carried out at 4 days after TNBS administration. [a]Results for the curcumin treatment group were compared against those for the TNBS group with a two-sided Wilcoxon's rank-sum test. The histologic scores for curcumin (50, 100 and 300 mg kg^{-1})-pretreated mice were significantly lower than that of untreated mice with TNBS-induced colitis ($P<0.01$).

neutrophil infiltration in inflamed tissue (Figure 5a) confirming the enhanced leucocyte infiltration seen at histological inspection. In this study, the extent of myeloperoxidase activity closely paralleled the increase of tissue malondialdehyde (40.7 ± 4.1 compared with $9.3\pm1.4\,\mu$M g^{-1} tissue in controls), indicative of a massive lipid peroxidation (Figure 5b). However, curcumin pretreatment of TNBS-treated mice at dose levels of 50, 100 and 300 mg kg^{-1} significantly prevented neutrophil infiltration, as assessed by myeloperoxidase activity ($P<0.01$) and also prevented the increased accumulation of malondialdehyde ($P<0.01$). Nevertheless, curcumin (25 mg kg^{-1}) did not produce any significant change in the elevated levels of either myeloperoxidase or malondialdehyde when compared to TNBS-treated mice.

Colonic serine protease activity

Since protease levels are known to be elevated in IBD and thus may play a role in the extensive tissue damage in IBD, protease activity in colon tissue was analysed. Colon tissue from control mice had little inherent protease activity (Figure 6, lane 1), whereas this was markedly increased in TNBS-treated animals (lane 2). Significantly elevated levels of specific proteases of mass of $\sim$112, 53 and 20 kDa were obtained from colonic mucosa of TNBS-induced colitis. Earlier studies on experimental model of IBD using specific inhibitors of various proteases established that the majority of protease activity observed on the gelatin zymograms is because of serine proteases (Hawkins *et al.*, 1997). However, curcumin pretreatment (50 mg kg^{-1}) greatly reduced the degree of protease activity in the colon of mice, which had received TNBS to cause colitis (Figure 6, lane 3). The suppression of serine protease activity in curcumin-treated mice correlates well with the attenuation of mucosal injury in TNBS-induced colitis.

Cytokine production in treated mice

To gain an insight into the levels of various cytokines and iNOS after curcumin treatment on TNBS-induced colitis, we examined the mRNA expression for a representative Th1 cytokine (e.g. IFN-γ), a Th1 inducer (e.g. IL-12), a Th2 cytokine (IL-4) and iNOS, which catalyses the generation of

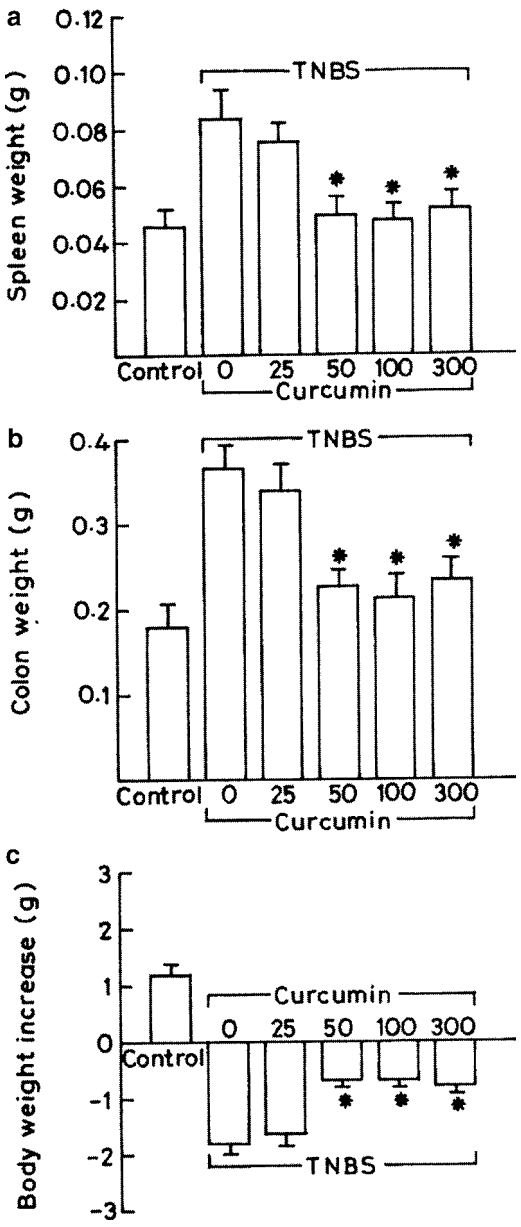

Figure 3 Effect of curcumin pretreatment on (a) spleen, (b) colon and (c) body weight. A significant increase in weight was observed at 4 days after TNBS administration in spleen and colon. Curcumin pretreatment (50, 100 and 300 mg kg^{-1}) significantly prevented the loss in body weight (c) as well as reduced the organ weight (a and b). Values are means$\pm$s.d. of 10 rats for each group. *$P<0.01$ vs TNBS.

NO from L-arginine and play a major role in IBD. RT – PCR analysis of cytokine mRNA levels confirmed that experimental colitic mice treated with curcumin could reverse an established Th1 response into a possible Th2 response (Figure 7). Thus, mucosal cells from mice treated with TNBS contained significantly increased levels of IFN-γ and IL-12 p40 mRNAs than those from control group representing a dominant inflammatory Th1 response. However, curcumin pretreatment resulted in marked suppression of both IFN-γ and IL-12 p40 mRNA levels with a little induction of IL-4 mRNA in TNBS-treated mice. In addition, the iNOS mRNA expression, which was very high in the mucosal cells of TNBS-treated mice, was significantly decreased by curcumin pretreatment. These

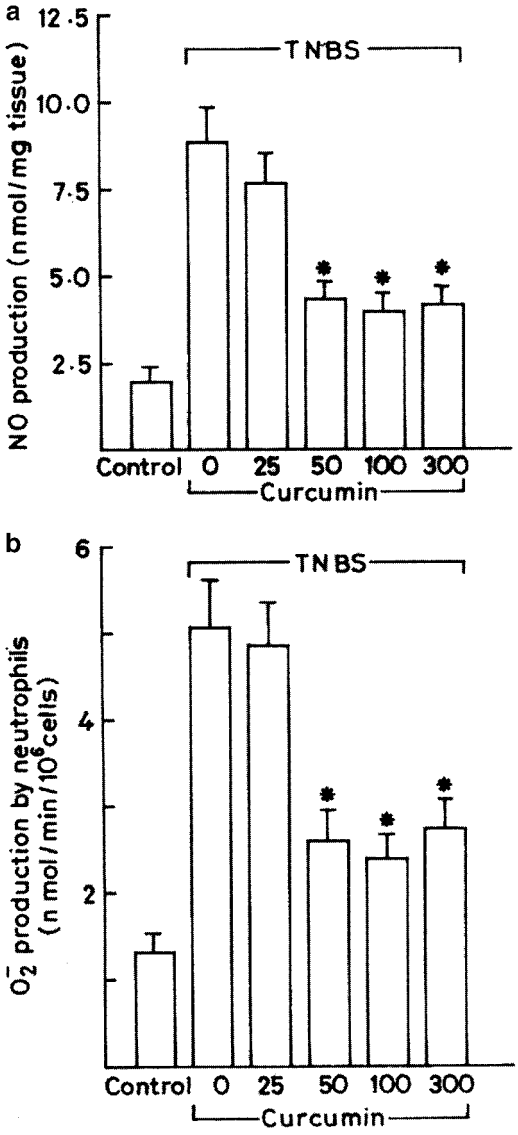

Figure 4 Effect of curcumin on NO and O_2^- production. (a) NO production in colonic tissue and (b) O_2^- production in neutrophils. TNBS treatment caused a significant increase of both NO and O_2^- generation, which were prevented by curcumin pretreatment. Values are means ± s.d. of 10 rats for each group. $^*P<0.01$ vs TNBS.

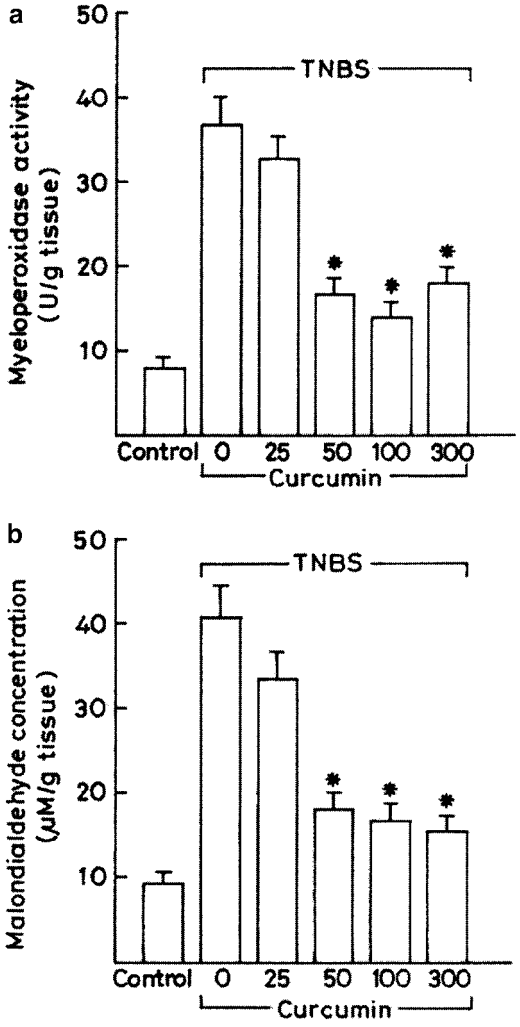

Figure 5 Effect of curcumin on neutrophil infiltration and lipid peroxidation. (a) Myeloperoxidase activity and (b) malondialdehyde levels in the colon. Myeloperoxidase activity and malondialdehyde levels were significantly increased in TNBS-treated mice in comparison to control. Curcumin pretreatment showed a significant reduction of both myeloperoxidase activity and malondialdehyde levels. Values are means ± s.d. of 10 rats for each group. $^*P<0.01$ vs TNBS.

results suggest that inflammatory Th1 functions have been effectively suppressed in BALB/c mice by curcumin treatment so that Th2 functions could possibly be activated to ameliorate mucosal injury in experimental colitis.

NF-κB in colonic mucosa of treated mice

To determine whether the decreased NO as well as iNOS mRNA in colonic mucosa was mediated through inhibition of iNOS transcription by suppression of NF-κB activation, we performed electophoretic mobility shift assay using nuclear extracts of whole colonic tissues from control mice (which received 30% ethanol without TNBS) and curcumin-treated or- untreated TNBS colitis mice (Figure 8). The administration of TNBS alone enhanced NF-κB DNA binding activity of nuclear extracts in the inflamed colonic tissue, which was suppressed by pretreatment with either 50 or 300 mg kg^{-1} curcumin. Excess unlabelled specific oligonucleotides inhibited NF-κB mobility shift indicating thereby the specificity of DNA – protein complex.

Discussion

The use of natural anti-inflammatory products provides an attractive and relatively nontoxic alternative to modulate inflammatory disorders. Curcumin is an anti-inflammatory food product that has been used for centuries (Ammon & Wahl, 1991). However, the lack of information regarding a mechanism of action for curcumin combined with unknown effects on mucosal inflammatory gene expression have precluded the widespread clinical use of curcumin for treatment of intestinal inflammatory disorders. The present study has demonstrated that TNBS causes a substantial degree of inflammation and tissue injury in the mouse colon, which is

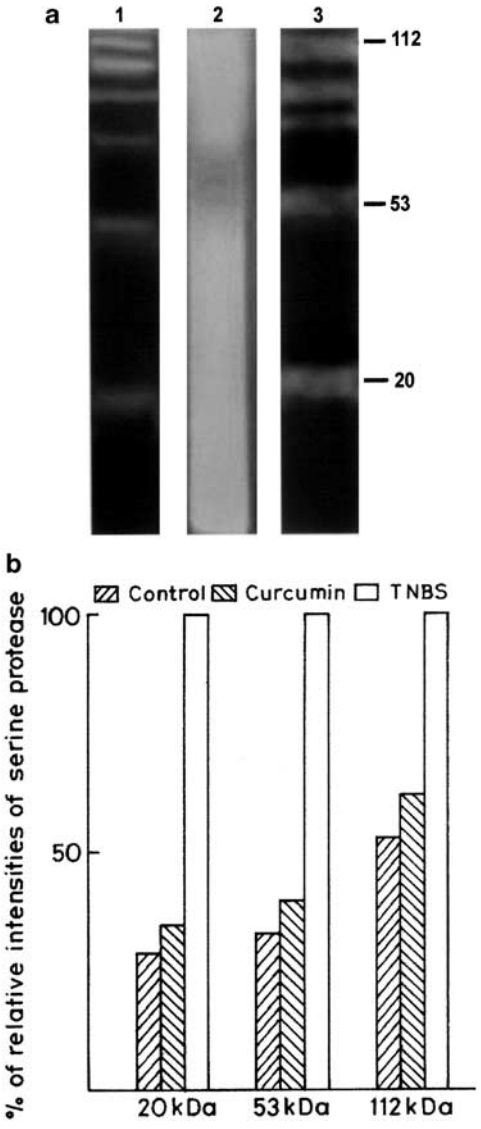

Figure 6 Protease activity in TNBS-treated animals. (a) TNBS treatment showed a several-fold increase in protease activity (lane 2) over the untreated control animals (lane 1). Curcumin-pretreated mice showed a marked reduction in protease activity (lane 3) compared to TNBS-treated mice. Numbers on the right indicate molecular mass in kDa. (b) Band intensities were quantified by densitometry.

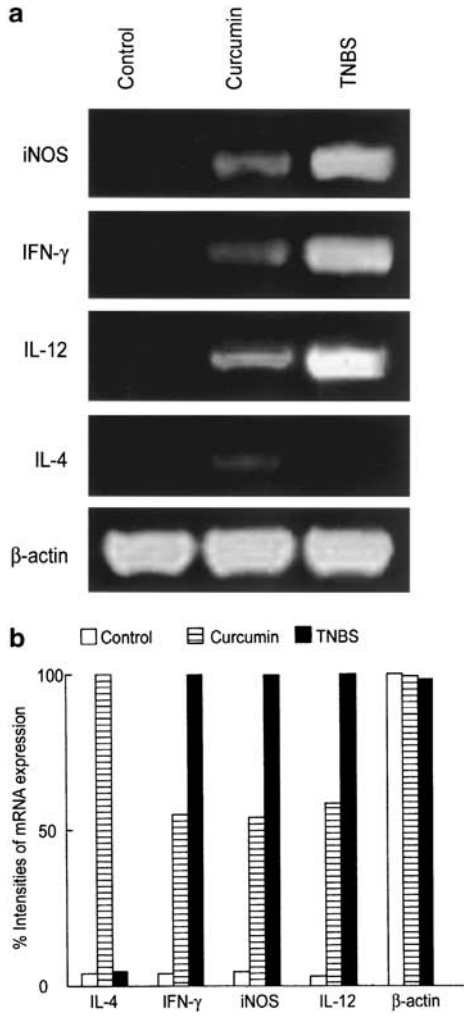

Figure 7 Suppression of Th1 phenotype in TNBS-treated mice subjected to curcumin pretreatment (50 mg kg^{-1}) as analysed by RT – PCR. (a) Expression of IFN-γ, IL-12, IL-4, iNOS and β-actin mRNA by colon tissue samples of control and treated mice. RT – PCR products were visualized by ethidium bromide staining. RNA samples were obtained from six mice in each group. Results are representative of three separate samples. β-actin expression levels were used as controls for RNA content and integrity. (b) Band intensities were quantified by densitometry.

associated with an infiltration of the colon with polymorphonuclear cells (histology and myeloperoxidase activity) as well as lipid peroxidation. The degree of inflammation, tissue injury and lipid peroxidation caused by TNBS was substantially reduced in mice treated with a dose of 50 mg kg^{-1} curcumin, which is lower than the maximal tolerated daily dose in humans (Cheng *et al.*, 2001). The impetus for this natural product therapy was the earlier observations that apart from inhibiting the induction of iNOS in macrophages activated with LPS and IFN-γ (Brouet & Ohshima, 1995), oral administration of curcumin significantly reduced iNOS mRNA expression in the livers of LPS-injected mice (Chan *et al.*, 1998). Moreover, the therapy involving curcumin pretreatment was effective in mice with ongoing disease in which a proinflammatory Th1 response had been established. After treatment, the cytokine profile in these mice indicated a switch from proinflammatory Th1 to anti-inflammatory Th2 pattern.

Reactive NO radical is known to play a central role in human IBD. Increased production of NO, and the presence of iNOS protein and iNOS mRNA have been demonstrated in affected areas of gut in patients suffering from UC or Crohn's disease (Rachmilewitz *et al.*, 1995b; Singer *et al.*, 1996; Kimura *et al.*, 1997). On the other hand, based on data from LPS-induced inflammation, a concentration-dependent dual effect in the gut has been suggested (Laszlo & Whittle, 1995). Low production of NO by cNOS may be protective and inhibitors of this physiological NOS have been reported to enhance intestinal lesions in inflammation (Laszlo & Whittle, 1995; Pfeiffer & Qiu, 1995). Prolonged production of high amounts of NO by iNOS on the other hand is proinflammatory and inhibition of iNOS seems to ameliorate the inflammatory response and tissue injury in experimental colitis (Hogaboam *et al.*, 1995; McCafferty *et al.*, 1997). An *in vivo* study by

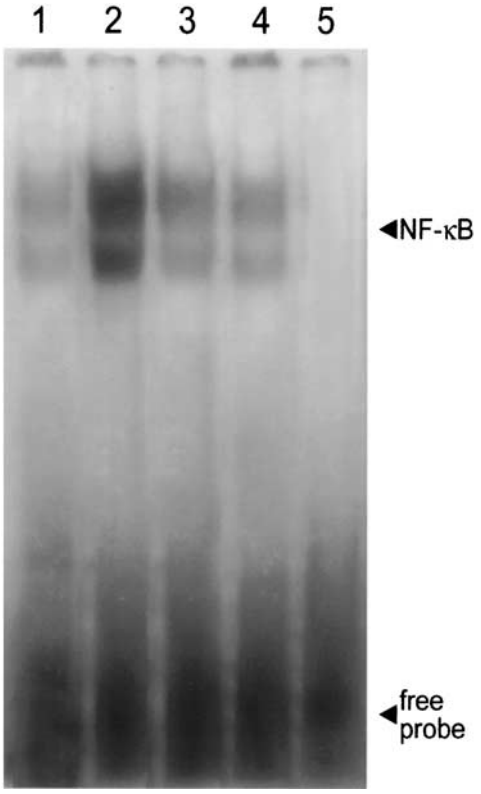

Figure 8 Effect of curcumin pretreatment on NF-κB activation. NF-κB activity was upregulated in nuclear extracts from TNBS-induced colonic mucosa and suppressed by curcumin pretreatment. Nuclear extracts of the colonic tissue from control mice (lane 1), untreated mice with TNBS-induced colitis in absence (lane 2) and presence of 50 molar excess of unlabelled probe (lane 5) and curcumin (300 and 50 mg kg^{-1}) treated mice with TNBS-induced colitis (lane 3 and 4) were analysed.

McKenzie *et al.* (1996) gives direct evidence on NO-induced injury on gut epithelial cells supporting the detrimental role of excessive NO in colitis. There is, therefore, good rationale to suggest that inhibition of excessive NO production by iNOS inhibitors will serve as promising approach in the management of IBD. Enhanced NO generation as well as iNOS mRNA transcripts detected in the inflamed colonic segments may be attributed to the contribution of macrophages and inflammatory neutrophils since colonic NO generation has also been found to be stimulated by LPS and IFN-γ (Rachmilewitz *et al.*, 1995a). In contrast to these observations, a deleterious effect of iNOS deficiency was reported on the ability to resolve a colonic injury in experimental IBD (McCafferty *et al.*, 1997). The discrepancy in these reports may relate to the difference in the stimuli used to induce the injury. However, similar controversial roles of iNOS-derived NO have been ascribed in a variety of pathophysiological conditions. Thus, genetic ablation of iNOS may exert beneficial effects in endotoxic shock, infection by *Toxoplasma gondii* and autoimmune vasculitis (MacMicking *et al.*, 1995; Gilkeson *et al.*, 1997; Khan *et al.*, 1997), whereas other studies have reported that deletion of iNOS gene may exacerbate the inflammatory process in endotoxaemia encephalomyelitis and tuberculosis (Laubach *et al.*, 1995; MacMicking *et al.* 1997; Fenyk-Melody *et al.*, 1998). We provide here the *in vivo* evidence that NO concentrations can be downregulated via suppression of proinflammatory cytokines by curcumin in TNBS-induced colitis, resulting in a significant amelioration of the disease.

There is ample evidence in human IBD that the inflammatory cytokines IL-1, TNF and IFN-γ are overexpressed and this finding correlates with reports of excessive amounts of NO produced by activated iNOS in lamina propria mononuclear cells and colon epithelial cells (Godkin *et al.*, 1996; Singer *et al.*, 1996). This prompted us to investigate whether manipulation of cytokine profile by curcumin would lead to reduced NO activities and thus decrease mucosal damage. Curcumin treatment led to a marked suppression in IL-12 mRNA expression by mucosal cells of TNBS-administered mice, resulting in a reduced ability to induce IFN-γ and perhaps, an increased ability to induce IL-4 in CD4+ T cells. These results suggest that curcumin-mediated inhibition of IL-12 production led to the inhibition of Th1 and a possible enhancement of Th2 cytokine synthesis in CD4+ T cells. The mechanism by which curcumin inhibits IL-12 production seems to be through the downregulation of NF-κB -mediated activation and binding to the p40-κB site, since curcumin is capable of inhibiting NF-κB activity in electrophoretic mobility shift assay using nuclear extracts of whole cells of the colonic tissue. Curcumin has recently been shown to block cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity (Jobin *et al.*, 1999). Since NF-κB activation is believed to play a major role in the regulation of proinflammatory gene transcription, therefore, by suppressing it curcumin may inhibit early steps of inflammation and modulate upregulation of multiple proinflammatory genes.

It may be mentioned that scavengers of reactive oxygen including hydrogen peroxide, superoxide anions and hydroxyl radicals also reduce the tissue injury associated with IBD suggesting that – in addition to reactive nitrogen, reactive oxygen species also play an important role in the pathophysiology associated with this model of inflammation (Cuzzocrea *et al.*, 2000). In addition to reactive oxygen, peroxynitrite ($ONOO^-$ is also generated in IBD (Zingarelli *et al.*, 1998). Reactive oxygen and $ONOO^-$ produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. The curative effect of curcumin accompanied by reduced levels of superoxide anions, nitric oxide, malondialdehyde and serine protease is suggestive of the scavenging capability of both reactive nitrogen and oxygen species by this beverage product. These results are consistent with previous data indicating that curcumin is capable of lowering the generation of both superoxide and nitric oxide from rat peritoneal macrophages (Joe & Lokesh, 1994).

Assessment of the physiological relevance of the findings reported here must take into account the concentrations at which inhibitory or stimulatory effect of turmeric-derived polyphenol on cytokine production was observed. Bioavailability of curcumin is low after oral ingestion (Ammon & Wahl, 1991) but can be sufficiently elevated by coingestion of piperine in both rats and humans (Shoba *et al.*, 1998). Nevertheless, highest concentration of curcumin, regardless of piperine use, is found in the caecum after oral ingestion (Ammon & Wahl, 1991). In addition, luminal curcumin may have a topical activity on colonic epithelial cells independent of systemic absorption. All these observations may have rele-

vance on the beneficial effect of oral administration of curcumin in inflammatory disease of the bowel.

In conclusion, this study demonstrates that the degree of colitis caused by administration of TNBS is significantly attenuated by turmeric-derived radical scavenger curcumin. The anti-inflammatory effects of curcumin are associated with a reduction in (i) upregulation of proinflammatory Th1 cytokine response leading to the suppression of iNOS and attenuation of the recruitment of neutrophils, (ii) lipid peroxidation and (iii) ultimately tissue injury. Being a relatively nontoxic natural product, combined with its excellent anti-inflammatory activity, curcumin could be useful in IBD and other conditions associated with local or systemic inflammation.

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Note added in Proof: While this paper was under editorial review, another study of curcumin in experimental colitis in mice, by Sugimoto *et al*. was published (*Gastroenterology* (2002) 123 1912 – 1922), with similar results to those reported here.