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Interferon-gamma (IFN-γ)- and tumour necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium (DSS)-induced colitis in mice

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SUMMARY

Excess nitric oxide formation caused by the activity of the inducible nitric oxide synthase has been implicated as a toxic effector molecule in the pathogenesis of experimental colitis and inflammatory bowel disease. It was therefore investigated whether inhibition of this synthase or the cytokines TNF and IFN-γ, inducers of nitric oxide synthase, had effects on chronic colitis in mice. Chronic colitis was induced in mice by repeated feeding of DSS. Cytokines were neutralized by treatment with MoAbs and nitric oxide synthase was inhibited by aminoguanidine. The degree of colonic inflammation was assessed by a histological score and colon length. Aminoguanidine treatment reduced nitric oxide activity by 60% ($P = 0{\cdot}0004$), the histological score by 31% ($P = 0{\cdot}005$) and increased colon length by 1·4 cm ($P = 0{\cdot}002$). Neutralization of TNF and IFN-γ resulted in increased colon length (0·7 cm, $P = 0{\cdot}07$ and 0·8 cm, $P = 0{\cdot}03$), improved histological score (19%, $P = 0{\cdot}045$ and 25%, $P = 0{\cdot}013$), and reduced nitric oxide activity (31%, $P = 0{\cdot}07$ and 54%, $P = 0{\cdot}004$) compared with controls. The combination of anti-cytokine treatments had additive effects. TNF and IFN-γ are involved in perpetuation of chronic DSS-induced colitis, and induction of excessive nitric oxide activity could be their common effector mechanism.

Keywords experimental colitis dextran sulphate sodium tumour necrosis factor interferon-gamma nitric oxide

INTRODUCTION

The pathogenesis of chronic inflammatory bowel disease (IBD) is still unknown. Its aetiology is complex and seems to be multifactorial. There is increasing evidence that the immune system plays a critical role in the development and perpetuation of ulcerative colitis (UC) and Crohn's disease (CD). The available data suggest that a disturbed balance of pro- and anti-inflammatory cytokines is an important mechanism of chronic inflammation in IBD.

Elevated levels of proinflammatory cytokines such as IL-1 [1–3], IL-2 [4], IL-6 [3,4], IL-8 [4–7], IFN-γ [8] and TNF [3,4,8] and impaired responses to anti-inflammatory cytokines such as IL-4 [9,10] have been reported in biopsies from patients. A disturbed balance between IL-1 and its antagonist, IL-1 receptor antagonist (IL-1Ra), has also been found [11,12]. The relevance of cytokines in IBD has been underlined by several animal models (reviewed in [13]). Both IL-2- and IL-10-deficient mice develop chronic colitis [14,15]. Colitis in IL-10-deficient mice could be abrogated by treatment with IL-10 [14]. Neutralization of TNF and IFN-γ after transfer of $CD45RB^{high}$ $CD4^{+}$ T cells to severe combined immunodeficient (SCID) mice prevented the development of colitis [16]. Neutralization of IL-12, an important inducer of IFN-γ production [17], after induction of TNBS-colitis has been shown to reduce inflammation [18]. We have shown in a model of DSS-induced chronic colitis that treatment with neutralizing anti-TNF antibodies led to an improvement of the colitis score [19]. IFN-γ expression has been reported in biopsies from patients with IBD [20–26] and in some animal models of intestinal inflammation [16,18].

There is evidence that the inflammatory cytokines IL-1, TNF and IFN-γ are important inducers of nitric oxide (NO) generation in macrophages, intestinal epithelial and other cells [27–31].

An increased production of the inducible nitric oxide synthase (iNOS) has been found in IBD [32–38] and in models of intestinal inflammation [39–41]. Increased luminal activities of NO have

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also been detected in UC [42] and in the colonic lavage fluid in different animal models of IBD [43,44]. Excessive production of NO in chronic colitis may be detrimental to the integrity of the colonic mucosa [45]. In accordance with this information, inhibition of iNOS has shown positive effects in some animal models of intestinal inflammation [40,46–48] and autoimmune disease [49]. In another rat model, colonic inflammation can be induced by feeding peroxynitrite, a reaction product of the radical NO [50]. Recent results, however, make the role of NO in IBD again controversial (reviewed in [51]), and McCafferty *et al*. described even a protective role for NO in acetic acid-induced colitis using iNOS-deficient mice for experiments [52].

Collectively, these data suggested a role for NO in colitis and the possibility that IFN-γ and TNF exert their effects in colitis via induction of excessive amounts of iNOS. Therefore, we used our model of chronic and acute DSS-induced colitis in mice to study the effect of NO in both and the relationship between NO and TNF/IFN-γ *in vivo*.

MATERIALS AND METHODS

Mice

Female BALB/c mice weighing 20–22 g (Charles River, Sulyfeld, Germany) were used for the experiments. Except for the periods of induction of colitis they had food and water *ad libidum*.

Reagents and cell culture conditions

Hybridoma cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL, Eggenstein, Germany) and 10% HC-3 (PAN Systems, Nürnberg, Germany). Rat anti-mouse TNF MoAb V1q (IgD, κ) [53] was purified from hybridoma culture medium by ammonium sulphate precipitation. The purified material contained > 96% antibody as judged by silver-stained SDS–PAGE analysis. Purified V1q (2 ng) neutralized 0·5 ng TNF *in vitro* in the L929 cytotoxicity assay. The purified material was extensively dialysed against PBS, diluted to a concentration of 1 mg/ml in PBS and stored at –20°C until use. The final material contained 2 pg/ml lipopolysaccharide (LPS) as measured by the limulus assay.

Rat anti-mouse IFN-γ MoAb R4-6A2 (IgG1) [54] was purified from hybridoma culture medium by ammonium sulphate precipitation. The purified material contained > 98% antibody as judged by silver-stained SDS–PAGE. Purified R4-6A2 (1 μg) neutralized 16 U/ml of IFN-γ *in vitro* as measured in a bioassay. In this assay, R4-6A2 antibodies inhibited the protective function of IFN-γ for L929-fibrosarcoma cells against infection with vesicular stomatitis virus. The antibodies were stored at a final concentration of 0·7 mg/ml in PBS at –20°C.

DSS was purchased from ICN (cat. no. 160110, mol. wt 40 000; Eschwege, Germany), rat IgG and aminoguanidine hemisulphate were purchased from Sigma (Deisenhofen, Germany).

Induction of acute and chronic colitis

For induction of acute colitis mice received 5% DSS in drinking water for 7 days. For induction of chronic colitis mice received four cycles of DSS treatment as described [19,55]. Each cycle consisted of 5% DSS in drinking water for 7 days, followed by a 7–10-day interval with normal drinking water. Mice were used for the experimental treatment 4–6 weeks after completion of the last cycle. Mice used for experiments were age-matched and had received DSS treatment simultaneously.

Treatments

Anti-cytokine antibodies were administered intraperitoneally for 5 consecutive days. For neutralization of TNF 100 μg of V1q were injected. As little as 20 μg per mouse had been found to effectively neutralize TNF in a mouse model of experimental peritonitis [53]. For neutralization of IFN-γ antibody R4-6A2 was used at doses of 4 μg, 20 μg and 100 μg per mouse per day. Control groups received 100 μg of rat IgG intraperitoneally in 100 μl of PBS per mouse per day.

For inhibition of iNOS in chronic colitis 1 mg or 10 mg aminoguanidine (AG) was injected intraperitoneally in a volume of 100 μl PBS per mouse per day for 5 days. Control groups in these experiments received 100 μl of PBS per mouse per day.

In the acute form of colitis iNOS was inhibited during the induction phase of colitis from day 0 to day 7 by AG at 10 mg per mouse per day intraperitoneally. Control groups received again 100 μl of PBS.

In acute colitis mice were killed on day 8, in chronic colitis mice were killed on day 6 of the experiment.

Determination of nitrite/nitrate in colonic biopsies

For determination of nitrite/nitrate content 1 cm of the last third of the mechanically cleaned colon was stored in 200 μl of distilled water at –20°C. After thawing, the tissue samples were removed. To 100 μl of the supernatant 14 mU of nitrate reductase (*Aspergillus niger*; Sigma) plus 10 μl of NADPH/H^+ (40 μM; Sigma) were added. The reduction of nitrate was stopped after 5 min by 50 μl of water and 10 μl of diaminonaphthalene (0·05 mg/ml; Sigma) were added. After another 10 min of incubation in the dark at room temperature the reaction was stopped with 5 μl of 2·8 M NaOH. Finally the volume was adjusted to 400 μl with water for evaluation. Fluorescence was measured with a Fluorescence-Spectrophotometer (F-2000; Hitachi, Unterhaching, Germany; excitation 365 nm, emission 405 nm). Nitrite concentrations were calculated from a standard curve using NO_2^- concentrations in a range from 10 μM to 250 nM [56]. To confirm the validity of this method either one, two or three colonic specimens (each in triplicate) were stored in 200 μl of distilled water, followed by the procedure described above. It was found that the means of the measured nitrite/nitrate concentrations correlated with the number of colonic specimens: ('number of biopsies in 200 μl' → 'nitrate/nitrite ± s.e.m.': 3 → 1·066 ± 0·092 μM; 2 → 0·657 ± 0·077 μM; 1 → 0·276 ± 0·064 μM; $r^2 = 0{\cdot}995$). Furthermore, we did not find quantitative differences between measurement immediately after thawing and measurement after another 30 min of incubation, indicating that the diffusion was complete after thawing the samples.

Determination of histological score and colonic length

Since we found a significant reduction of colonic length [19] it was used as a parameter for colonic inflammation, as also described by others [57]. The colon was removed, mechanically cleaned, and was measured to 0·5 cm precision. The distal third of the colon was then cut longitudinally and laid on a filter paper and fixed in 10% formalin overnight. Sections of the paraffin-embedded material were made longitudinally. Three 5-μm sections were cut serially at a distance of 20 μm. The next three sections were cut at a distance of 100 μm. A third set of sections was cut after another 100 μm. The sections were stained with haematoxylin–eosin. Histological analysis was performed in a blinded fashion. Three sections obtained from each of three sites at 100 μm distance were evaluated. Mice were scored individually. Each score represented the mean of nine sections.

Histology was scored as follows: Epithelium (E): 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration (I): 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4, infiltration of the L. submucosa. The total histological score represents the sum of the epithelium and infiltration score (total score = E + I).

Statistical analysis

Statistical analysis was performed by Students-*t* test (nitrite values, body weight) or Mann–Whitney rank sum test (histological score, length of colon). Correlations (r^2) were derived from linear regression analysis.

RESULTS

Characteristics of acute and chronic colitis

Acute colitis induced by a single cycle of DSS feeding was characterized by loose stools from day 3 on and bloody stools beginning on day 4 continuing to days 8–9, and severe weight loss being regained on days 9–12 (not shown). Histological examination on day 8 showed goblet cell and crypt loss and severe inflammatory infiltrate mainly consisting of granulocytes.

The majority of mice with chronic colitis induced by four cycles of DSS feeding had loose stools (60%), a minority had diarrhoea (10%), and bloody stools could hardly be detected macroscopically. These symptoms disappeared gradually after DSS treatment and usually subsided within the first 8 weeks after completion of the induction cycles. Histologically, colitis could still be detected 4 months after completion of the last cycle. The colons of mice with chronic colitis were reduced in length to about 7–8 cm compared with about 11–12 cm of those from healthy mice. Histologically, ulcers were rarely detected, whereas the main effects were epithelial damage and inflammatory cell infiltration, usually restricted to the mucosa and mainly consisting of lymphocytes and macrophages and to a lesser degree (< 15%) of granulocytes [19].

Effects of AG treatment on parameters of acute and chronic colitis

For inhibition of iNOS AG was used because it has a relative specificity for iNOS compared with cNOS [58–62]. Treatment of mice with chronic colitis for 5 days with graded doses of AG led to a significant dose-dependent decrease in nitrite/nitrate concentration of colonic biopsies (Fig. 1). In parallel, as shown in Fig. 2a, AG treatment led to a significant dose-dependent increase in colon length ($P = 0{\cdot}002$). The histological score improved

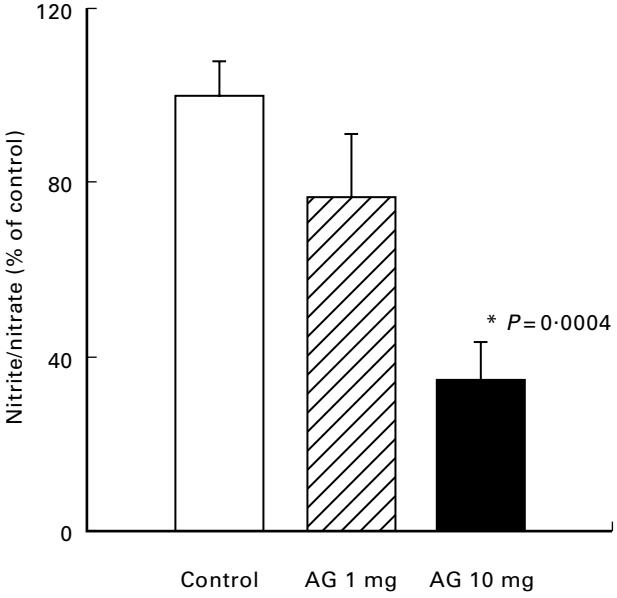

Fig. 1. Effect of aminoguanidine (AG) on colonic NO activity. Mice with chronic DSS-induced colitis received either PBS (control), 1 mg or 10 mg per mouse AG from day 1 to day 5 of the experiment. Nitrite/nitrate contents were determined as described in Materials and Methods. Each group consisted of 9–10 mice. The result is representative of three separate experiments. Bars represent mean ± s.e.m. *Significantly different from control group.

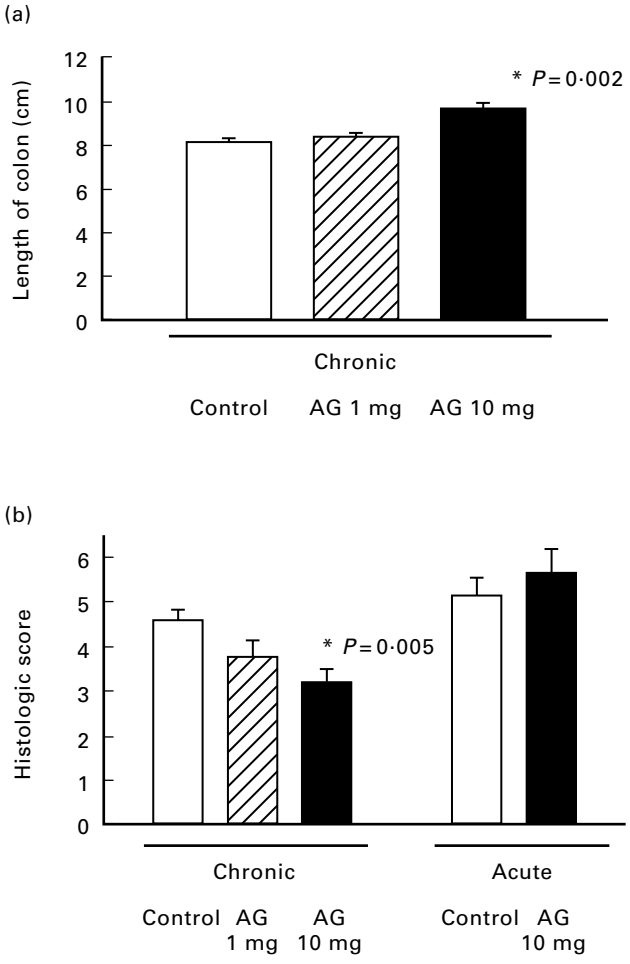

Fig. 2. Effect of aminoguanidine (AG) on inflammatory parameters in chronic and acute colitis. Mice with chronic DSS-induced colitis received either PBS (control), 1 mg or 10 mg per mouse AG from day 1 to day 5 of the experiment or in acute colitis 10 mg AG per mouse per day or PBS (control) from day 0–7 of the induction phase. Colonic length (a) and histological score (b) were determined as described in Materials and Methods. In experiments each group consisted of 9–10 mice. The result is representative of three separate experiments. Bars represent mean ± s.e.m. *Significantly different from control groups.

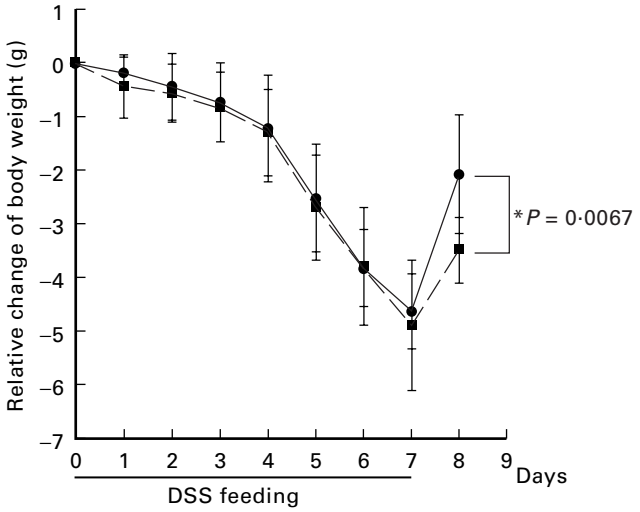

Fig. 3. Kinetics of body weight of mice with acute colitis. During induction of acute colitis (days 0–7) mice were treated daily with aminoguanidine (AG) at 10 mg per mouse (■) or with PBS (control) (●). Body weight of individual mice was monitored daily. In experiments each group consisted of five mice. The result is representative of three separate experiments. Data points represent mean values ± s.d.

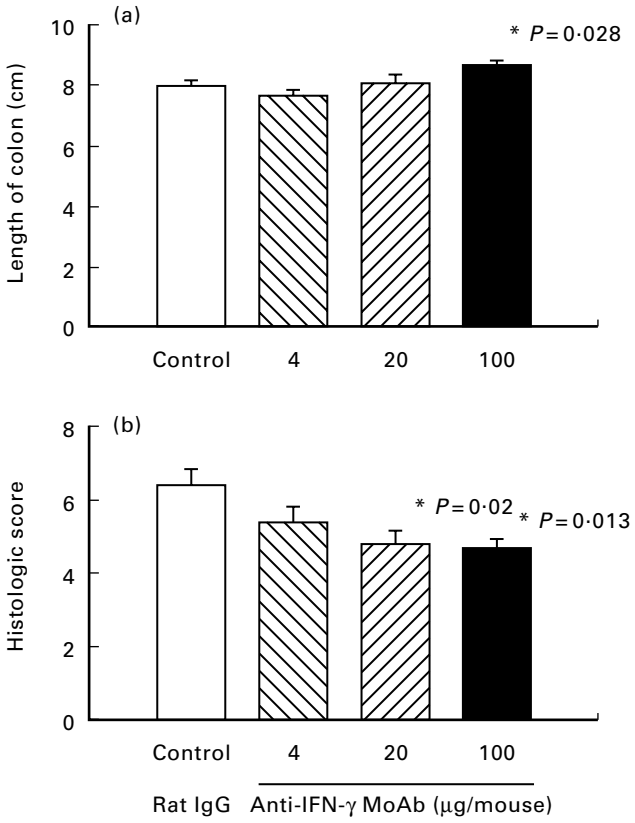

Fig. 4. Effect of IFN-γ neutralization on inflammatory parameters in chronic colitis. Mice with chronic DSS-induced colitis received either rat IgG (control), 4 μg, 20 μg or 100 μg of neutralizing IFN-γ MoAb from day 1 to day 5 of the experiment. Colonic length (a) and histological score (b) were determined as described in Materials and Methods. Each group consisted of 9–10 mice. The result is representative of three separate experiments. Bars represent mean ± s.e.m. *Significantly different from control groups.

significantly in the 10 mg/day AG-treated group ($P = 0{\cdot}005$), but no significant effect was seen at 1 mg/day AG (Fig. 2b). This correlation of nitrite/nitrate content with severity of colitis suggests a causative role of NO in chronic intestinal inflammation. In contrast, AG treatment (10 mg/day) during the acute phase of colitis induction (day 0 to day 7) aggravated colitis compared with PBS-treated control mice. The histological score was altered by 15·1% without reaching statistical significance (Fig. 2b) and in accordance with this finding of aggravation, average weight gain on day 8, 1 day after the end of DSS feeding, was significantly reduced in AG-treated mice compared with the weight gain of PBS-treated control mice on day 8 (Fig. 3).

The cytokines IL-1, TNF and IFN-γ are known to be strong inducers of NO generation. We therefore tested whether neutralization of these cytokines would improve the parameters of chronic colitis and decrease NO activity in colonic biopsies.

Effects of anti-IFN-γ treatment on parameters of chronic colitis

Neutralization of IL-1 activity with antibodies to the IL-1 receptor type I or IL-1Ra had no effect on histological score, as shown before [19], and also no change was found for mucosal nitrite/nitrate concentration or colonic length (data not shown).

Neutralization of IFN-γ with antibodies, however, significantly increased the length of the colon ($P = 0{\cdot}028$) (Fig. 4a) and improved the colitis score ($P = 0{\cdot}013$) (Fig. 4b) in a dose-dependent fashion compared with treatment with rat IgG. As shown in Fig. 5, these effects were paralleled by a dose-dependent decrease in nitrite/nitrate concentrations in the colon which was significant ($P = 0{\cdot}004$) at a dose of 100 μg/mouse.

Effects of combined anti-IFN-γ and anti-TNF treatment on parameters of chronic colitis

We had shown in earlier studies that neutralization of TNF led to improvement of the histological score in chronic DSS-induced colitis [19]. Therefore the effects on colonic length and nitrite/nitrate concentrations were also investigated. When mice were treated with neutralizing anti-TNF antibodies, the histological score was significantly improved (Fig. 6b), but colonic length (Fig. 6a) and nitrite/nitrate contents (Fig. 7) only showed a trend to increase or decrease, respectively, without being significantly different.

When both cytokines, TNF and IFN-γ, were neutralized in combination with 100 μg anti-IFN-γ antibody plus 100 μg anti-TNF antibody, colon length increased significantly ($P = 0{\cdot}03$) and additively (Fig. 6a). The same effect was found for the histological score (Fig. 6b). The most prominent effect of the combined treatment was found for nitrite/nitrate contents in colonic biopsies, which were significantly reduced by 80% ($P < 0{\cdot}0001$) (Fig. 7). As shown in Fig. 8, severity of disease as expressed by the histological score and the measured nitrite/nitrate contents of colonic tissue correlated strongly ($r^2 = 0{\cdot}985$ and 0·980, respectively).

DISCUSSION

We show in this study that decreased NO generation after inhibition of iNOS aggravated acute colitis but improved the condition of chronic colitis in the model of DSS-induced experimental colitis. We also show that inhibition of endogenous TNF and/or IFN-γ by antibodies ameliorated the chronic stage of colitis and

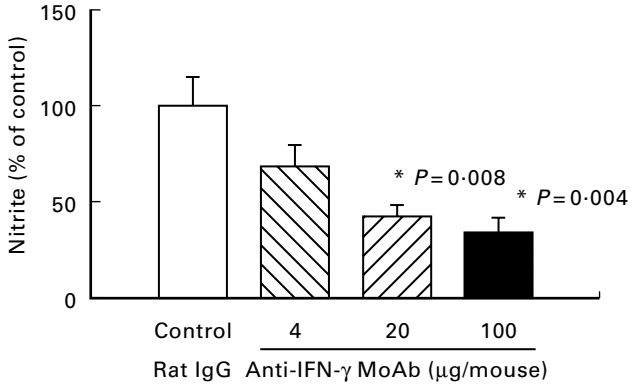

Fig. 5. Effect of neutralization of IFN-γ on colonic nitric oxide (NO) activity. Mice with chronic DSS-induced colitis received either rat IgG (control), 4 μg, 20 μg or 100 μg of neutralizing IFN-γ MoAb from day 1 to day 5 of the experiment. Nitrite/nitrate concentrations were determined as described in Materials and Methods. Each group consisted of 9–10 mice. The result is representative of three separate experiments. Bars represent mean ± s.e.m. *Significantly different from control group.

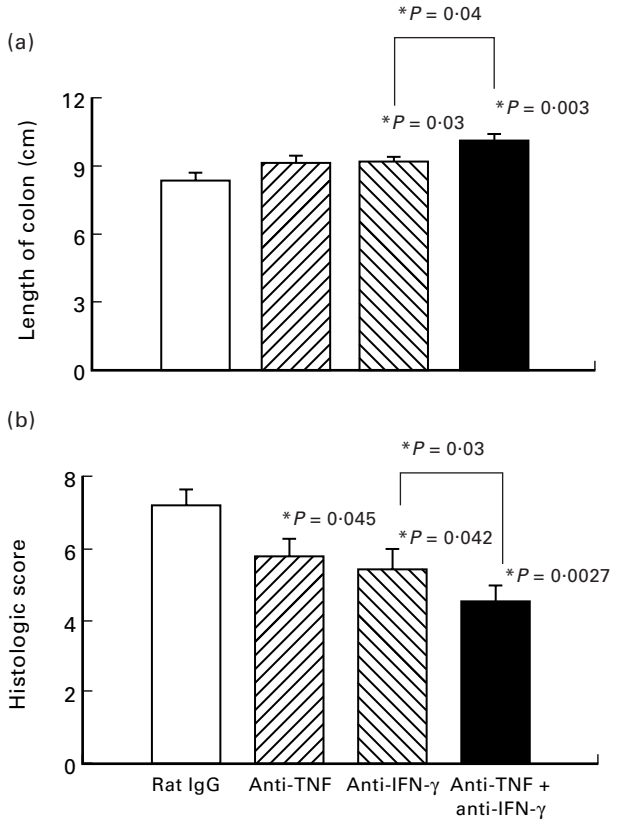

Fig. 6. Effect of neutralization of TNF and IFN-γ in combination on parameters of colitis. Mice with chronic DSS-induced colitis received either rat IgG (control), 100 μg of neutralizing IFN-γ MoAb, 100 μg of neutralizing TNF MoAb or a combination of both MoAbs from day 1 to day 5 of the experiment. Colonic length (a) and histological score (b) were determined as described in Materials and Methods. Each group consisted of 10 mice. The result is representative of three separate experiments. Bars represent mean ± s.e.m. *Significantly different from control groups unless otherwise indicated.

concomitantly decreased NO generation, demonstrating clear correlation. We conclude therefore that in chronic colitis NO contributes to inflammation and tissue damage and that IFN-γ and TNF exert their detrimental role at least partly via the induction of iNOS and the toxic effector molecule NO.

Nitric oxide formation has been implicated in the pathogenesis of several inflammatory diseases including IBD. Evidence that NO is a potent toxic mediator in chronic inflammation was initially demonstrated in animal models of adjuvant-induced arthritis [63] and diabetes [64]. Increased iNOS activity has also been measured in TNBS-induced experimental colitis [65] and in a granulomatous model of chronic colitis, where inhibition of iNOS reduced colonic tissue damage [66,67]. NO activities have been found increased in peripheral blood [34,38], and in the colon of patients with IBD [33,36,42]. Recent results, however, make the role of NO in IBD again controversial. Kubes *et al*. questioned the hypothesis that 'large' amounts of NO cause tissue damage in the gut by demonstrating that NO donors had a protective effect after induction of gut inflammation by platelet-activating factor in cats [68]. When iNOS-deficient mice were used to study the role of NO in colonic inflammation, NO again revealed its protective qualities. After instillation of acetic acid (3%) intrarectally iNOS-deficient mice showed macroscopically a two-fold stronger inflammatory response than wild-type mice after 24 h [52]. Interestingly, these two reports describe the role of NO in an acute phase of inflammation.

Our data provide evidence that in acute inflammation NO indeed tends to be protective, as was previously shown for the cytokines TNF and IL-1 by us [19] and was evidenced in the two reports mentioned above [52,68]. In this line is our result that antibiotics improve acute DSS-induced colitis (Hans *et al*., submitted), arguing for the hypothesis that a competent immune response is necessary to form a barrier against bacteria infiltrating the damaged mucosa during the acute phase.

In the chronic phase of colitis, however, we demonstrate that iNOS blocking significantly ameliorated inflammation and in parallel decreased colonic NO levels, whereas antibiotics (Hans *et al*., submitted) showed no benefit, suggesting an ongoing and dysregulated immune response leading to destruction of the mucosa. These results reveal the different quality of acute and chronic inflammation and help to interpret the contradictory results (reviewed in [51]) concerning the role of NO in intestinal inflammation.

In vitro induction of iNOS and NO formation in macrophages, in a colonic epithelial cell line and other cells by IL-1, TNF and IFN-γ has been demonstrated by several authors [31,69–75]. In addition, a correlation of NO production and expression of TNF and IFN-γ in the course of disease has been demonstrated in a model of cerebral toxoplasmosis [76] and in a model of experimental allergic encephalitis [77,78].

As cited in the Introduction, there is ample evidence in human IBD that the inflammatory cytokines IL-1, TNF, and IFN-γ are over-expressed, and this finding correlates with reports of excessive amounts of NO produced by activated iNOS in lamina propria mononuclear cells and colon epithelial cells [79,80]. This prompted us to investigate whether it was possible in our chronic colitis model to manipulate NO activities by cytokine inhibition and thus decrease mucosal damage.

Neutralization of IFN-γ or TNF was effective in amelioration of chronic DSS-induced colitis and in decreasing colonic NO activity, showing strong correlation. This indicates that IFN-γ is

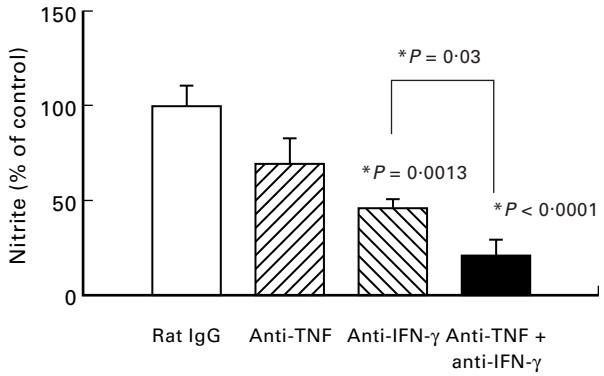

Fig. 7. Effect of neutralization of TNF and IFN-γ in combination on nitric oxide (NO) activity. Mice with chronic DSS-induced colitis received either rat IgG (control), 100 μg of neutralizing IFN-γ MoAb, 100 μg of neutralizing TNF MoAb or a combination of both MoAbs from day 1 to day 5 of the experiment. Nitrite/nitrate concentrations were determined as described in Materials and Methods. Each group consisted of 10 mice. The result is representative of two separate experiments. Bars represent mean $\pm$ s.e.m. *Significantly different from control groups unless otherwise indicated.

involved in the perpetuation of chronic colitis in this model and suggests the possibility that at least in part excessive NO generation is responsible for the deterioration of the mucosa.

Neutralization of IFN-γ was more effective than neutralization of TNF. Both cytokines had an additive effect both on histological score and on colonic NO concentrations, also arguing for a common mechanism of tissue destruction induced by TNF and IFN-γ. Using the hypothesis that NO generation induced by cytokines is the cause for mucosal damage, the differential effectiveness is supported by the fact that IFN-γ is the most potent inducer of iNOS in macrophages [72] and is essential for iNOS induction in colon epithelial cells [31].

It would be interesting to investigate the effect of long-lasting antibody treatment. In our experiments treatment was not extended beyond 5 days to avoid complications due to development of an anti-rat immunoglobulin response. Chronic colitis in this model is a months-long established disease which can be improved by 30–40% by a 5-day treatment and we think that we are only looking at the beginning of a repair and healing process. Receptor antagonists or genetically engineered antibodies are unfortunately not available for these cytokines.

We provide the first *in vivo* evidence that NO concentrations can be down-regulated via neutralization of IFN-γ and TNF in a model of chronic colitis, resulting in a significant amelioration of disease. In conclusion, NO tends to be protective in acute intestinal inflammation, whereas chronic colitis in this model is characterized by the destructive effect of inadequate NO generation induced by a dysregulated TNF and IFN-γ production.

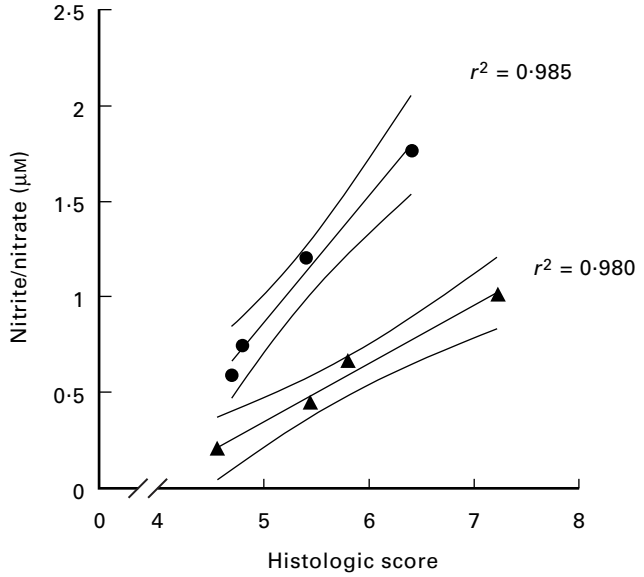

Fig. 8. Correlation of the histological score and nitrite/nitrate concentrations of colonic tissue. The histological scores (abscissa) and nitrite/nitrate (ordinate) concentrations measured in the anti-IFN-γ dose response experiment (●) and in the anti-IFN-γ/anti-TNF combination experiment (▲) were correlated. Results were taken from experiments presented in Fig. 4b and Fig. 5 (anti-IFN-γ dose response) and in Fig. 6b and Fig. 7 (anti-IFN-γ/anti-TNF combination). A confidence interval of 95% is given. r^2 were derived from linear regression analysis.

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