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Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice

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SUMMARY

The cytokines TNF and IL-1 have been implicated as mediators of the inflammatory processes in patients with inflammatory bowel disease (IBD). To investigate the role of these cytokines in mucosal inflammation we used anti-cytokine strategies in a mouse model of acute and chronic colitis. Mice which received 5% dextran sulphate sodium (DSS) in their drinking water showed signs of acute colitis on day 4, with severe weight loss and bloody diarrhoea. Chronic colitis was established after four cycles of feeding 5% DSS for 7 days and water for 10 days, with the mice showing diarrhoea but no weight loss. In acute colitis, treatment with anti-IL-1 reagents, anti-TNF MoAb, or dexamethasone (DEX) led to aggravation. By contrast, in chronic colitis, treatment of mice with several IL-1 activity-inhibiting reagents failed to show significant effects, whereas anti-TNF MoAb or DEX significantly reduced the colitis. We conclude that in acute colitis IL-1 and TNF are beneficial, whereas in chronic colitis, TNF but not IL-1 seems to play a major role in perpetuation of chronic inflammation.

Keywords experimental colitis dextran sulphate sodium tumour necrosis factor IL-1 neutralization

INTRODUCTION

The cytokines IL-1 and TNF are important molecules involved in inflammation and regulation of the immune response. One of the relevant factors participating in the initiation, regulation and perpetuation of inflammation in Crohn's disease (CD) or ulcerative colitis (UC) is a disturbed balance of cytokines. It has been hypothesized that the chronic inflammation in inflammatory bowel disease (IBD) may be due to an inappropriate secretion of anti-inflammatory cytokines in response to an initial event, or a disturbed balance of proinflammatory and anti-inflammatory cytokines. Arguments for these hypotheses derive from data obtained from mucosal biopsies of patients with IBD: increased expression of proinflammatory cytokines such as IL-1 [1–3], IL-2 [4], IL-6 [1,4], IL-8 [4–7], interferon-gamma (IFN-γ) [8], and TNF [1,4,8], and impaired response to anti-inflammatory cytokines such as IL-4 [9] have been reported in mononuclear cells isolated from patients. A disturbed balance between IL-1 and its antagonist, IL-1 receptor antagonist (IL-1Ra) has also been found [10,11]. The relevance of cytokines in IBD has been underlined by several animal models (reviewed in [12]). Both IL-2- and IL-10-deficient mice develop chronic colitis [13,14]. Colitis in IL-10-deficient mice could be abrogated by treatment with IL-10 [13]. 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 [15], thus demonstrating that these cytokines are involved in intestinal inflammation. In normal animals, a chronic granulomatous transmural colitis resembling CD can be induced by application of trinitrobenzenesulfonylchloride (TNBS) to the colon. Neutralization of IL-12 after induction of chronic TNBS-colitis has been shown to reduce inflammation and to induce recovery of pretreatment weight [16].

Colitis induced by dextran sulphate sodium (DSS) is characterized by ulceration, epithelial damage, mucosal or transmural inflammatory infiltrate, and lymphoid hyperplasia [17]. As both acute and chronic colitis can be induced in this model, we considered it suitable for the study of cytokines in intestinal inflammation in normal animals. In order to test the hypothesis that TNF and IL-1 are relevant mediators in acute or chronic colitis, we neutralized TNF or IL-1 in DSS-induced acute and chronic colitis and tested their effects in the course of disease.

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MATERIALS AND METHODS

Mice

Female BALB/c mice weighing 20–22 g (Charles River, Sulzfeld, Germany) were used for experiments. They had food and water *ad libitum*.

Reagents and cell culture

Hybridoma cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL,

Eggenstein, Germany) and 5% fetal calf serum (FCS; GIBCO BRL) or 10% HC-3 (PAN Systems, Nürnberg, Germany). Rat anti-mouse TNF MoAb V1q (IgD, κ) [18] was purified from hybridoma culture medium (containing FCS) by ammonium-sulphate precipitation and ion-exchange chromatography (Baker Bond, Gross-Gerau, Germany). The purified material contained > 96% antibody as judged by silver staining of SDS–polyacrylamide gels. Purified V1q (1 ng) neutralized 0·25 ng rTNF *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 LPS as measured by the limulus assay. Mouse anti-mouse IL-1β MoAb (IgG1) [19] was kindly provided by H. Towbin (Ciba-Geigy, Basel, Switzerland), soluble IL-1 receptor type I (sIL-1RI) by R. Kurrle (Behringwerke, Marburg, Germany), and human IL-1 receptor antagonist (hIL-1Ra) by D. Boraschi (Dompe', Aquila, Italy). Neutralizing rat anti-mouse IL-1RI MoAb Reg21 (IgG1, κ) was raised in our laboratory. It was purified from hybridoma culture medium (containing HC-3) by precipitation with ammonium sulphate (50% saturation) at 4°C. The precipitated antibody was dissolved in PBS and dialysed extensively against PBS. After dilution to a concentration of 0·7–1 mg/ml, aliquots were stored at −20°C. Purity was about 99% as estimated from SDS–PAGE analysis. Reg21 (600 ng/ml) completely neutralized 1 U/ml (the concentration needed for half-maximal stimulation) hIL-1α or mIL-1α *in vitro* in a proliferation assay using D10G4.1 cells [20]. DSS was purchased from ICN (Catalog no. 160110, mol. wt 40 000; Eschwege, Germany), rat IgG and dexamethasone (DEX) were purchased from Sigma (Deisenhofen, Germany). DEX was dissolved in dimethylsulfoxide (DMSO) and diluted to the desired concentration with water containing 2·5% DMSO. In acute colitis mice received 3 mg/kg, in chronic colitis 0·25 mg/kg. Control groups received PBS with 2·5% DMSO.

Induction of acute and chronic colitis

For induction of acute colitis mice received 5% DSS dissolved in drinking water for 7 days as described [17]. For induction of chronic colitis mice received four cycles of treatment with DSS. Each cycle consisted of 5% DSS in drinking water for 7 days, followed by a 10-day interval with normal drinking water. Four to 6 weeks after completion of the last DSS cycle, mice were treated as indicated.

Treatments

Mice used for experiments were age-matched and had received DSS treatment simultaneously. For therapeutic purposes in acute colitis, mice were treated by i.p. injection from day 3 to day 7 during DSS administration. In chronic colitis, mice were treated from day 1 to day 5 4–6 weeks after completion of the four cycles of DSS treatment.

For neutralization of TNF in acute DSS-induced colitis, 100 μg of V1q were injected intraperitoneally on days 3–7 of the experiment. For neutralization of TNF in chronic DSS-induced colitis mice received 10 μg or 100 μg V1q by i.p. injection for 5 days 4–6 weeks after completion of the four cycles of DSS treatment. As little as 20 μg of V1q per mouse had been found to effectively neutralize endogenous TNF in a mouse model of experimental peritonitis [18]. Treatment with V1q was restricted to 5 days in order to avoid problems due to the development of mouse anti-rat antibody production in treated mice. PBS (100 μl) or rat IgG (100 μg in 100 μl PBS) were injected intraperitoneally as control treatment. Mice were killed on day 6 or 12.

Several strategies were used to neutralize IL-1 activity. In order to neutralize IL-1β, 50 μg of a mouse anti-mouse IL-1β MoAb in 100 μl PBS were injected intraperitoneally on days 1, 3 and 5 of chronic colitis. IL-1α and IL-1β were neutralized by i.p. injection of 50 μg sIL-1RI in 100 μl PBS on days 1–5 of chronic colitis. Both anti-IL-1β antibody and sIL-1RI have been shown to prevent autoimmune disease *in vivo* using the indicated dosages [19,21]. An attempt was made to neutralize all IL-1 activity by competition for the IL-1RI by either hIL-1Ra or by Reg21. hIL-1Ra (100 μg) was injected intraperitoneally daily from day 0 to day 7 in acute colitis or from day 1 to day 5 in chronic colitis. Anti-IL-1RI MoAb Reg21 (100 μg) was injected intraperitoneally from day 0 to day 7 in acute and from day 1 to day 5 in chronic colitis. In acute colitis, mice were killed on day 8, in chronic colitis on day 6 or day 12 after start of specific treatment.

Determination of histological score

Mice were killed by cervical dislocation. The colon was removed and washed with PBS. The distal third of the colon was cut longitudinally, laid on filter paper and fixed in 10% formalin in PBS 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 blind fashion. Three sections obtained from each of three sites at 100 μm distance were evaluated. Mice were scored individually, and each score represented the mean of nine sections.

Histology was scored as follows: ulceration: 0, no ulcers; 1, one ulcer; 2, two ulcers; 3, three ulcers; 4, > 3 ulcers; epithelium: 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 and/or foci of polypoid regeneration; infiltration: 0, no infiltrate; 1, infiltrate around crypt bases; 2, infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae, thickening of the mucosa with abundant oedema; 4, infiltration of the L. submucosa; lymphoid follicles: 0, no lymphoid follicles; 1, one lymphoid follicle; 2, two lymphoid follicles; 3, three lymphoid follicles; 4, > 3 lymphoid follicles.

The colitis score of individual mice represents the sum of the different histological subscores.

Colonic length was measured immediately after dissection and placement on a paper towel.

RESULTS

Characteristics of acute colitis

Acute colitis was induced by a single cycle of DSS feeding. All treated mice had loose stools from day 3 on, with bloody stools and weight loss beginning at day 4. Bloody stools continued until day 8–9. Pretreatment weight was regained at day 9–12 (not shown). When examined on day 8, colons were shortened in comparison with untreated mice. Histological examination showed ulcerations, goblet cell and crypt loss and severe inflammation, consisting mainly of granulocytes. Hyperplasia of lymph follicles was not a feature of acute colitis (not shown).

Characteristics of chronic colitis

Chronic colitis was induced in all mice by four cycles of DSS treatment. However, the histological scores differed within groups. Most mice (60%) had loose stools, a minority had diarrhoea (10%).

Blood in stools could be detected macroscopically only in a minority of mice (< 1%). These symptoms of colitis subsided within the first 8 weeks after completion of the four cycles. Colons were significantly shorter in DSS-treated mice than in age-matched untreated controls (8·6 ± 0·22 cm *versus* 13·5 ± 0·25 cm (mean ± s.e.m.); $P < 0{\cdot}0001$). DSS-treated colons showed a thickened mucosa and enlargement of lymph follicles macroscopically. Histologically, colitis was still detectable at 4 months after completion of the induction phase. Carcinomas, adenomatous polyps and grossly polypoid foci of regeneration where detected in a small minority of mice (4%). These animals were excluded from the study.

On histology, ulcers were detected in only a few of the animals (Fig. 1a). The predominant epithelial damage was goblet cell loss and crypt loss (Fig. 1b). Crypt abscesses were rarely seen. The infiltrate consisted mainly of lymphocytes and macrophages; granulocytes contributed to a lesser degree (< 15%) to the infiltrate (Fig. 1c). The infiltrate was restricted to the mucosa. Granulomas were scarce in individual sections, and hyperplasia of lymphoid follicles was detected in all mice (Fig. 1d).

Differential effects of treatment with steroids

In order to assess therapeutic efficacy in DSS-induced colitis, mice with acute and chronic colitis were treated with DEX. During induction of acute colitis mice were given DEX from day 3 to day 7. This treatment led to an aggravation of the inflammation, as indicated by an 80% increase in the colitis score shown in Fig. 2. The predominant features of aggravation consisted of an increase of ulcerations and epithelial damage. By contrast, when mice with chronic colitis were treated with DEX for 5 days starting 4 weeks after the last DSS treatment, colitis scores were improved by 40% (Fig. 2).

Effect of neutralization of IL-1 activity

The participation of IL-1 in human IBD was suggested by several clinical studies. IL-1 was also shown to play a prominent role in immune complex-mediated colitis in rabbits [22]. We therefore investigated whether IL-1 activity was also involved in DSS-induced colitis. Strategies were aimed only at the IL-1R type I because the IL-1R type II was shown *in vitro* not to transmit signals [23].

As shown in Fig. 3A, neutralization of IL-1 activity by hIL-1Ra or by Reg21 in acute colitis aggravated colitis, as indicated by a significant increase in colitis score. Also, recovery of pre-DSS treatment weight was delayed compared with PBS-treated controls in this experiment. Rat IgG as control for the antibodies used had no significant effect in acute colitis (data not shown).

No effect on histological score was seen by any of the various anti-IL-1 agents in chronic DSS-induced colitis. Figure 3B,C,D show that anti-IL-1β, sIL-1RI, hIL-1Ra and anti-IL-1RI MoAb had no significant effect on development of colitis. Also, a combination

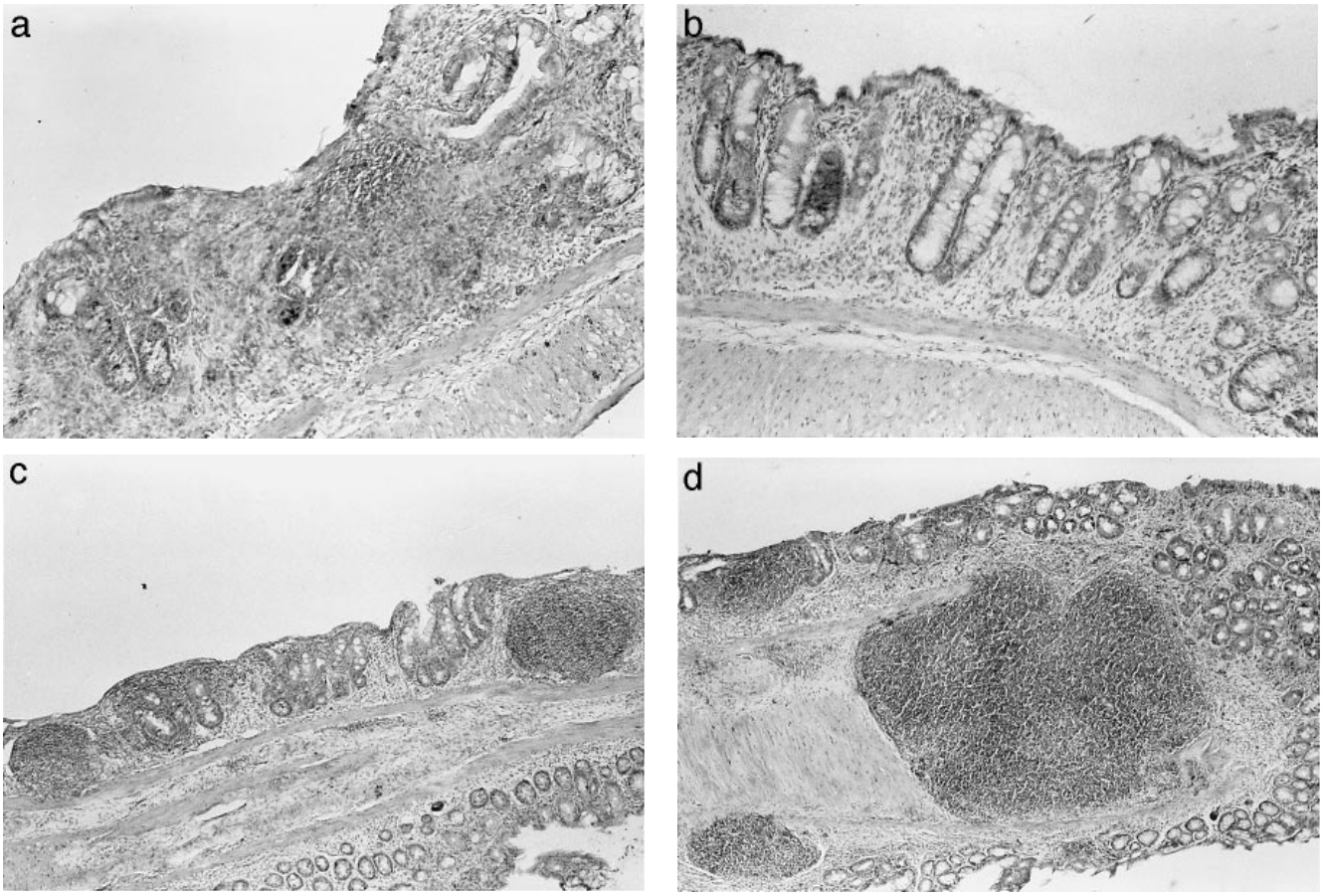

Fig. 1. Histology of chronic dextran sulphate sodium (DSS)-induced colitis. Paraffin sections of the distal third of the colon of mice with chronic DSS-induced colitis were stained with haematoxylin–eosin. (a) Ulceration, accompanying inflammation and crypt loss. (b) Goblet cell loss and crypt loss. (c) Lymphocytic inflammatory infiltrate. (d) Hyperplasia of lymph follicles.

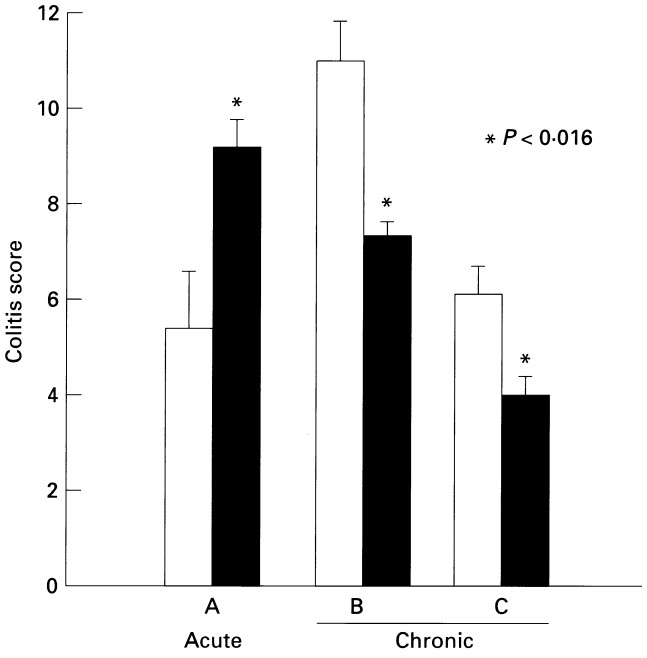

Fig. 2. Effects of steroid treatment in acute and chronic dextran sulphate sodium (DSS)-induced colitis. A, Colitis score of mice (n = 5) with acute colitis treated with dexamethasone (DEX; ■) (3 mg/kg) or 100 μl PBS (□) intraperitoneally. B,C, Colitis score of mice (B, n = 9 PBS, n = 4 DEX; C, n = 8 PBS, n = 4 DEX) with chronic colitis. Four weeks after the last DSS treatment (= day 1), mice were given DEX or 100 μl carrier intraperitoneally. Columns represent mean values of colitis scores, error bars represent s.e.m. *Significantly different from control groups and P values are derived from the Mann–Whitney analysis of ranks.

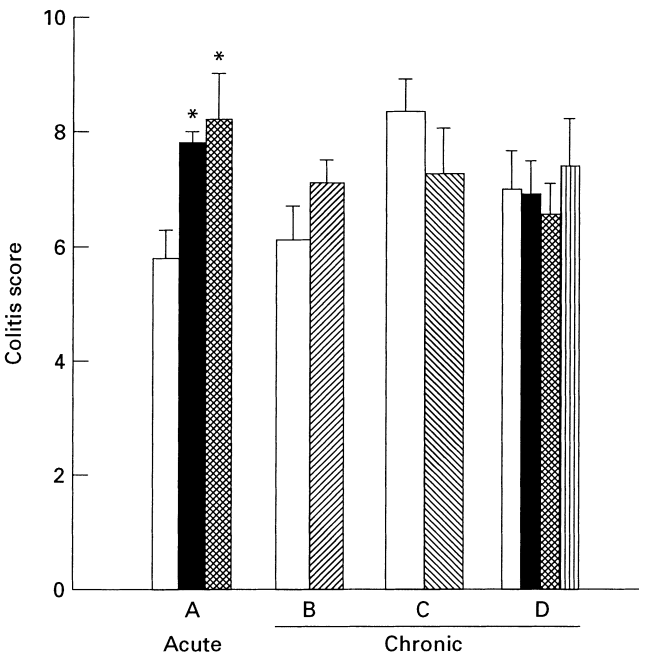

Fig. 3. Neutralization of IL-1 in acute and chronic dextran sulphate sodium (DSS)-induced colitis. A, Colitis scores of mice (n = 5) with acute colitis after treatment with anti-IL-1 receptor antibody Reg21 (100 μg) or 100 μg of hIL-1Ra or 100 μl PBS. B,C,D, Colitis scores of mice with chronic colitis. B, Mice received either 100 μl PBS (n = 10) or 50 μg sIL-1RI (n = 9). C, Mice received 25 μg anti-IL-1β MoAb (n = 9) on days 1, 3, and 5 in 100 μl PBS or 100 μl PBS on the same days. D, Groups of mice received 100 μg hIL-1Ra (n = 9), or 100 μg Reg21 (n = 9), or 100 μl PBS (n = 8) or 100 μg rat immunoglobulin (IgG), respectively. Columns represent mean values of colitis scores, error bars represent s.e.m. *Significantly different from control groups and P values are derived from the Mann–Whitney analysis of ranks. □, PBS; ▨, sIL-1RI; ▧, anti-IL-1β MoAb; ■, anti-IL-1RI MoAb; ▩, IL-1Ra; ▥, rat IgG. *P < 0·05.

of anti-IL-1β MoAb and sIL-1RI was without significant effect (data not shown).

Effects of neutralization of TNF

TNF is a potent mediator of inflammation, and anti-TNF MoAb treatment has been shown to be effective in CD and rheumatoid arthritis (RA) [24,25]. To test whether TNF plays a role in initiating and sustaining colitis we neutralized endogenous TNF with anti-mTNF MoAb V1q. Mice with acute colitis were treated with V1q from day 3 to day 7 of the experiment. When the mice were evaluated on day 8, acute colitis was found to be significantly aggravated by this treatment (Fig. 4A). Increased ulcerations and augmented inflammatory infiltrate were the predominant parameters for the increased scores. In accordance with this finding of aggravation, weight gain after the end of DSS feeding was significantly delayed by anti-TNF treatment (Fig. 5).

In contrast to the findings for acute colitis, neutralization of TNF ameliorated inflammation in chronic DSS-induced colitis. As shown in Fig. 4B, treatment with anti-TNF MoAb reduced colitis score by 30–40%. This effect was dose-dependent (Fig. 4C). A dose of 10 μg/day of the antibody for 5 days did not show significant effect on histological scores. Improvement of histological score by effective doses was most prominent for epithelial damage and inflammatory infiltrate. Scores for lymphoid follicles were lower, but a significant reduction was still achieved (data not shown). Ulcers were too scarce for the determination of statistical significance. This improvement of colitis scores, which was achieved by 5 days of treatment with anti-TNF MoAb, lasted for at least 7 days after termination of treatment (Fig. 4B). As control, non-specific rat IgG had no significant effect on histology. Colonic length increased during the 5-day treatment (rat IgG-treated 8·6 ± 0·22 cm; anti-TNF MoAb-treated 9·35 ± 0·25 cm; P < 0·024). Neutralization of TNF had no effect on weight of the animals (not shown).

DISCUSSION

Increased expression of proinflammatory cytokines has been detected in mucosal biopsies of patients with IBD. Elevated levels of TNF [1,8,26], IL-1 [2,3,10], IL-8 [5,7], decreased ratios of IL-1:IL-1Ra [10,11] have been measured in mucosal biopsies of patients with IBD. In addition, cumulative evidence from the various animal models of intestinal inflammation suggests $CD4^+$ T cells as mediators. These models also support the view of the pathogenic importance of dysregulated expression of inflammatory or regulatory cytokines. Whether elevated levels of cytokines represent ongoing inflammation or whether the cytokines measured play a causative role remains unresolved.

Several animal models of intestinal inflammation have been developed and used in an attempt to analyse the complex situation in intestinal inflammation [12]. IL-1Ra was successfully used for therapy in immune complex-induced colitis in the rabbit [22], and IL-10 had a positive effect in $CD45RB^{high}$ T cell-grafted SCID

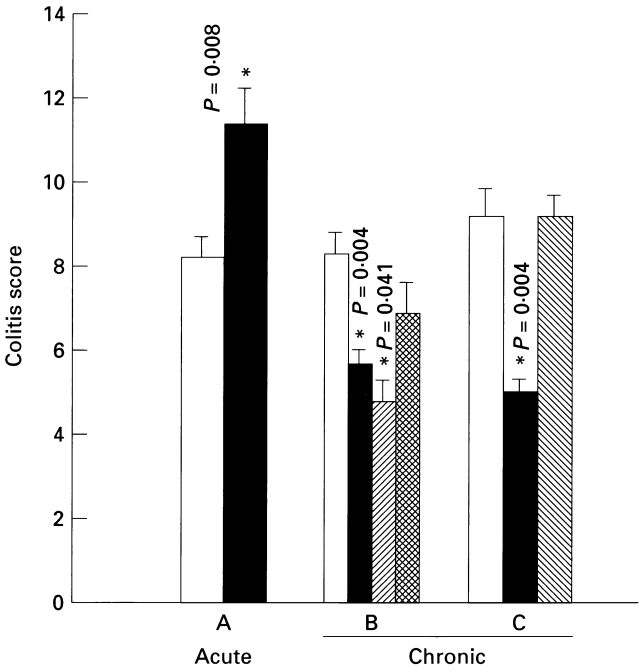

Fig. 4. Neutralization of TNF in acute and chronic dextran sulphate sodium (DSS)-induced colitis. A, Colitis scores of mice with acute colitis (n = 10, V1q; n = 9, PBS). The animals were treated daily with anti-TNF antibody V1q or 100 μl PBS. B, Colitis scores of mice with chronic colitis after treatment with 100 μg V1q, or 100 μl PBS, or 100 μg rat IgG, respectively. Group sizes were: PBS, n = 9; rat IgG, n = 10; V1q 100 μg day 6, n = 9; V1q 100 μg day 12, n = 9. Colitis was evaluated after 6 (day 6) or 12 (day 12) days. C, Mice with chronic colitis were treated with either 100 μl PBS (n = 5), or 100 μg V1q (n = 5), or 10 μg V1q (n = 5) before determination of colitis scores. Columns represent mean values of colitis scores, error bars represent s.e.m. *Significantly different from control groups and P values are derived from the Mann–Whitney analysis of ranks. □, PBS; ■, anti-TNF 100 μg day 6; ▨, anti-TNF 100 μg day 12; ▧, anti-TNF 10 μg day 6; ▩, rat IgG.

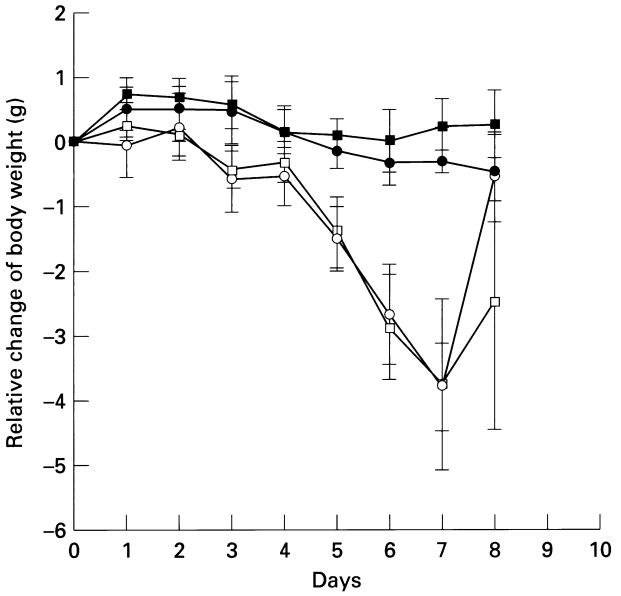

Fig. 5. Kinetics of body weight of mice with acute colitis. During induction of acute colitis mice were treated daily with 100 μg anti-TNF antibody V1q or 100 μl PBS. Body weight of individual mice was monitored daily. Data points represent mean values, error bars represent s.e.m. ●, PBS; ■, anti-TNF MoAb; ○, PBS + dextran sulphate sodium (DSS); □, anti-TNF MoAb + DSS.

mice [15]. In the same model, strategies to neutralize IFN-γ and TNF with antibodies were also successful. Recently it was shown in TNBS-induced colitis in mice that neutralization of IL-12 had beneficial effects [16].

In genetically non-compromised mice, only two models of acute and chronic intestinal inflammation exist which would allow study of the role of different cytokines in detail. TNBS/ethanol-induced colitis induces a transmural inflammation when TNBS is instilled into the colon. The chronic phase in this model lasts about 2–8 weeks [16] and treatment with anti-IL-12 antibodies abrogated colitis [16]. The second model is DSS-induced colitis [17]. Both acute and chronic inflammation can be studied in this model. In our hands, the chronic phase lasted at least 8 weeks after the disease-inducing phase. These criteria make this model suitable for studies addressing the role of cytokines in initiation and perpetuation of chronic IBD.

Treatment of acute and chronic DSS-induced colitis with steroids showed different results. Acute colitis was aggravated, whereas chronic colitis was ameliorated by this treatment, demonstrating the protective role of inflammation and of inflammatory cytokines involved in the acute stage, whereas the sustained inflammatory response in the chronic situation is detrimental. Since steroids inhibit inflammatory cytokine production by inducing the production of IκBα [27], we next investigated whether TNF and IL-1 played a major role in this model of acute and chronic colitis.

The activity of IL-1 is the result of a complex system comprising interactions of IL-1α, IL-1β, IL-1Ra and the IL-1RI. We therefore decided to inhibit IL-1 activity on several levels. Anti-IL-1β MoAb was used to neutralize IL-1β alone, whereas sIL-1RI was used to neutralize both IL-1α and IL-1β. hIL-1Ra was used for neutralizing IL-1 activity by competition with IL-1α and IL-1β for the IL-1RI. Rat anti-mouse IL-1RI MoAb Reg21 neutralized IL-1 activity by blocking accession of the receptor by the various ligands. Neutralization of IL-1 in acute colitis aggravated the symptoms, indicating that IL-1 may play a protective role in acute colitis, similar to the protective effect of TNF in the model of caecal ligation and puncture [18]. On the other hand, neutralization of IL-1 had little or no effect on chronic colitis in our model. IL-1 therefore does not seem to be involved in the maintenance of chronic intestinal inflammation. Alternatively, IL-1 with its pleiotropic properties may be involved in inflammation, but may also be involved in the regeneration of the intestinal epithelium so that, within the time frame of treatment, no net effect could be found. An alternative explanation would assume that expression of IL-1Ra was maximal in this system. This could explain why the other means of IL-1 inhibition had no additional effect. Neutralization of IL-1 had beneficial effects in immune complex-induced colitis in rabbits [22]. However, the main feature of this model of colitis is immune complex-mediated vasculitis. Vasculitis is not a feature of DSS-induced colitis and therefore immune mechanisms inducing the different forms of colitis may not be identical. Immune complex colitis is an acute disease, making extrapolation on chronic inflammatory disease states difficult.

Neutralization of TNF showed different effects when acute colitis and chronic colitis were compared. Acute colitis was aggravated and weight gain after cessation of DSS feeding was delayed, indicating a protective role of TNF during acute colitis. Olson *et al.* [28] could not detect significant effects of an anti-TNF

antiserum on histology and weight gain in acute DSS-induced colitis. However, our results are in line with our earlier [18,29] and other [30] reports showing that neutralization of TNF during acute inflammation had detrimental effects on the course of disease. TNF therefore seems to play a protective role during the initiation of inflammation in acute colitis. In chronic DSS-induced colitis, neutralization of TNF improved histological scores of epithelial damage, infiltrate and lymphoid follicles, arguing strongly for a major role of TNF in the perpetuation of chronic colitis. However, scores were only reduced by 30–40%, suggesting that TNF alone may not be the only relevant factor. We have obtained preliminary evidence that IFN-γ is another candidate cytokine involved in perpetuation of chronic colitis. It is also possible that longer use of anti-TNF treatment would have a more pronounced effect. For our studies we have used a rat anti-mouse MoAb. The development of anti-rat antibodies in mice therefore limited the duration of effective treatment to $\approx$ 5 days. Soluble TNF receptors or genetically engineered antibodies may be a suitable alternative for long-term treatment of DSS-induced colitis.

Neutralization of TNF by MoAb has been studied in patients with CD with success [25]. Neutralization of TNF did not only reduce inflammation, but also granuloma formation. Whether this antibody exerted its effects by neutralization alone or by cytotoxic effects remains to be shown.

We conclude from our data that TNF plays a major role in the perpetuation of DSS-induced colitis. It has to be determined which other factors have a dominant influence in DSS-induced chronic colitis. It might be necessary to modulate more than just a single cytokine for successful therapy.

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