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Protective effects of M40403, a superoxide dismutase mimetic, in a rodent model of colitis

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Abstract

Inflammatory bowel disease is characterised by oxidative and nitrosative stress, leukocyte infiltration, and up-regulation of intercellular adhesion molecule 1 (ICAM-1) expression in the colon. The aim of the present study was to examine the effects of M40403, a superoxide dismutase mimetic, in rats subjected to experimental colitis. Colitis was induced in rats by intracolonic instillation of trinitrobenzene sulfonic acid (TNBS). Rats experienced bloody diarrhoea and significant loss of body weight. At 4 days after TNBS administration, the colon damage was characterised by areas of mucosal necrosis. Neutrophil infiltration (indicated by myeloperoxidase activity in the mucosa) was associated with up-regulation of ICAM-1 and expression of P-selectin and high levels of malondialdehyde. Immunohistochemistry for nitrotyrosine and poly (ADP-ribose) synthetase showed an intense staining in the inflamed colon. Treatment with M40403 (5 mg/kg daily i.p.) significantly reduced the appearance of diarrhoea and the loss of body weight. This was associated with a remarkable amelioration of the disruption of the colonic architecture as well as a significant reduction of colonic myeloperoxidase activity and malondialdehyde levels. M40403 also reduced the appearance of nitrotyrosine and poly (ADP-ribose) synthetase immunoreactivity in the colon as well as reduced the up-regulation of ICAM-1 and the expression of P-selectin. The results of this study suggested that administration of a superoxide dismutase mimetic may be beneficial for treatment of inflammatory bowel disease. © 2001 Published by Elsevier Science B.V.

Keywords: TNBS (Trinitrobenzene sulfonic acid); Superoxide anion (O_2^-); Nitric oxide (NO); Peroxynitrite; Poly (ADP-ribose) polymerase; Colon damage; Free radical

1. Introduction

A growing body of data indicates that oxygen-derived free radicals such as superoxide (O_2^-), nitric oxide (NO) and hydroxyl radicals (OH) have a role in mediating intestinal damage in inflammatory bowel disease. The intestine is well endowed with enzymes capable of producing such free radicals (Parks, 1989). Moreover, when inflammation is present the many phagocytic cells that are attracted and activated can produce large amounts of free radicals. Several studies suggest that peripheral blood monocytes (Kitahora et al., 1988) and isolated intestinal macrophages (Verspaget and Beeken, 1985; Mahida et al., 1989) from patients with inflammatory bowel disease produce of free radicals. Also, high numbers of peripheral neutrophils, which are capable of producing large amounts of oxygen-derived free radicals (Verspaget et al., 1988), migrate into the intestinal wall of such patients (Crama-Bohbouth et al., 1988). Grisham and Granger (1988) hypothesised that in ulcerative colitis, transient ischemic episodes and subsequent reperfusion produce high levels of free radicals. This process initiates a cascade of events leading to the recruitment and activation of leucocytes, resulting ultimately in mucosal ulceration. Recently, Wakefield et al. (1989) presented evidence for multifocal infarctions in the intestine of patients with Crohn's disease, indicating that ischemic episodes may also occur in this disease.

Several studies indicate that sulphasalazine and its active metabolite 5-aminosalicylic acid are efficient scavengers of oxygen radicals in vitro (Auroma et al., 1987; Miyachi et al., 1987). These scavenging potentials may be

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an important mode of action of these drugs in vivo (Auroma et al., 1987; Miyachi et al., 1987; Dull et al., 1987). The pro-inflammatory roles of superoxide are well known: recruitment of neutrophils at sites of inflammation, formation of chemotactic factors (Fantone and Ward, 1982), DNA damage, depolymerization of hyaluronic acid and collagen (Schraufstatter et al., 1986), lipid peroxidation, release of cytokines such tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) and formation of peroxynitrite ($ONOO^-$), which is a highly reactive oxidant produced by the combination of O_2^- and NO at rates approaching the diffusion limit (Beckman et al., 1990; Ischiropoulos et al., 1992). Moreover, peroxynitrite can also cause DNA damage (Inoue and Kawanishi, 1995) resulting in the activation of the nuclear enzyme poly (ADP-ribose) synthetase and poly (ADP-ribose) synthase-driven cell death (Szabo and Dawson, 1998). Furthermore, $ONOO^-$ inhibits the activity of the endogenous superoxide dismutase enzymes, a factor that contributes to increased formation of O_2^- (Yamakura et al., 1998; Macmillan-Crow and Thompson, 1999). Orgotein® (bovine CuZn superoxide dismutase) has been used in preliminary clinical trials in patients with various inflammatory disorders and improvement has been reported in four of four patients with Crohn's disease and three of four patients with ulcerative colitis (Niwa et al., 1985). Emerit et al., 1989 reported a beneficial effect in 82% of patients with Crohn's disease with long-term treatment with bovine superoxide dismutase. These results indicate that removal of O_2^- in humans by a superoxide dismutase enzyme has beneficial outcomes in these disorders. Despite encouraging clinical results in several other areas, Orgotein had to be removed from the market because of its origin (bovine), development of immunogenic responses in some individuals and other properties that made it an inadequate therapeutic agent (Flohe, 1988).

Based on the above, we have developed a series of superoxide dismutase mimetics that catalytically remove O_2^- as potential clinical candidates. The 1,4,7,10,13-pentaazacyclopentadecane containing the added bis(cyclohexylpyridine) functionalities (M40403) is a prototypic example of our low-molecular weight, manganese-containing, nonpeptidic molecule possessing the function and catalytic rate of native superoxide dismutase enzymes, but with the advantage of being a much smaller molecule (MW 483 vs. MW 30,000 for the mimetic and native enzyme, respectively) (Riley et al., 1996). M40403 is cytoprotective and stable in vivo, exerts anti-inflammatory properties in acute inflammation and is not deactivated by $ONOO^-$, an added advantage over the native superoxide dismutase enzyme that is nitrated and deactivated by $ONOO^-$ (Yamakura et al., 1998; Macmillan-Crow and Thompson, 1999). M40403 is not only a highly active catalyst for the dismutation of O_2^-, but it is also highly selective for superoxide. M40403 does not react with hydrogen peroxide, nor does it directly react with other biologically relevant oxidants such as nitric oxide or peroxynitrite (Riley et al., 1996; Salvemini et al., 1999a).

The objectives of the present study were to address whether M40403 exerted protection on the chronic inflammatory response (colitis) caused by injection of trinitrobenzene sulfonic acid (TNBS) in the rat, and if so, highlight possible mechanisms through which M40403 conferred protection.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (300–350 g; Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Induction of experimental colitis

Colitis was induced using a technique of acid-induced colonic inflammation as described previously (Wallace et al., 1992). On day 0, in fasted rats lightly anaesthetised with isoflurane, a 3.5 F catheter was inserted into the colon via the anus until approximately the splenic flexure (8 cm from the anus). 2,4,6-Trinitrobenzene, a sulfonic acid (TNBS; 25 mg/rat), was dissolved in 50% ethanol (total volume, 0.8 ml). Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. Fifteen animals (sham-colitis) received an enema with vehicle alone (50% ethanol, 0.8 ml). After colitis and sham-colitis induction, the animals were observed for 4 days. On day 4, the animals were weighed and anaesthetised with chloral hydrate (400 mg/kg, intraperitoneally). The abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histology and immunohistochemistry. In an additional set of experiments, animals were monitored for 7 days in order to evaluate mortality rate. M40403 (5 mg/kg, $n = 10$) or vehicle (26 mM sodium bicarbonate buffer, pH 8.3, $n = 15$) was given daily as an intraperitoneal bolus at 5 mg/kg starting from day 2. The dose of M40403 used in the present studies was taken from previous studies showing efficacy in models of acute inflammation and ischemia-reperfusion injury (Salvemini et al., 1999a).

2.3. Evaluation of colonic damage

After its removal, the colon was gently rinsed with saline solution, opened by a longitudinal incision, and

immediately examined under a microscope. The visible colonic damage was assessed by a scoring system as previously described (Wallace et al., 1992). All measurements of damage were performed by two observers blinded to the experimental protocol.

2.4. Optical microscopy

After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Thereafter, 7-μm sections were deparaffinized with xylene, stained with trichromic van Gieson's stain, and observed in a Dialux 22 Leitz (Wetziar, Germany) microscope.

2.5. Immunofluorescence localisation of nitrotyrosine, poly (ADP-ribose) synthetase, P-selectin and intercellular adhesion molecule (ICAM-1)

Indirect immunofluorescence staining was performed on 7-μm-thick sections of unfixed rat colon. Sections were cut in with a Slee and London cryostat at −30 °C, transferred onto clean glass slides and dried overnight at room temperature. Sections were permeabilized with acetone at −20 °C for 10 min and rehydrated in PBS (phosphate buffered saline, 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) at room temperature for 45 min. Sections were incubated overnight with (1) rabbit anti-human polyclonal antibody directed at P-selectin (CD62P), which reacts with rat and with mouse anti-rat antibody directed at ICAM-1 (CD54) (1:500 in PBS, v/v) (DBA, Milan, Italy), or (2) with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-ribose) goat polyclonal antibody rat (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit and with fluorescein isothiocyanate (FITC)-conjugated anti-mouse (Jackson, West Grove, PA) or with TRITC-conjugated anti-goat antibody (1:80 in PBS, v/v) for 2 h at room temperature. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a Nikon RCM8000 confocal microscope equipped with a 40× oil objective.

2.6. Measurement of cytokines

TNF-α and IL-1β levels were evaluated in the colon tissues at 4 days after intra-colonic injection of TNBS (Nielsen et al., 1999; Tountas et al., 1999). The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem, USA). The enzyme-linked immunosorbent assay (ELISA) has a lower detection limit of 5 pg/ml.

2.7. Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described (Mullane et al., 1985). At the specified time following the intracolonic injection of TNBS, colon tissues were obtained and weighed. Each piece of tissue was homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37 °C and was expressed in milliunits per gram weight of wet tissue.

2.8. Malondialdehyde measurement

Malondialdehyde levels in the colon tissue were determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). Colon tissue, collected at the specified time, was homogenised in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% sodium dodecyl sulphate, 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid and 700 μl of distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.9. Reagents

Biotin blocking kit, biotin-conjugated goat anti-rabbit immunoglobulin G (IgG) and avidin–biotin peroxidase

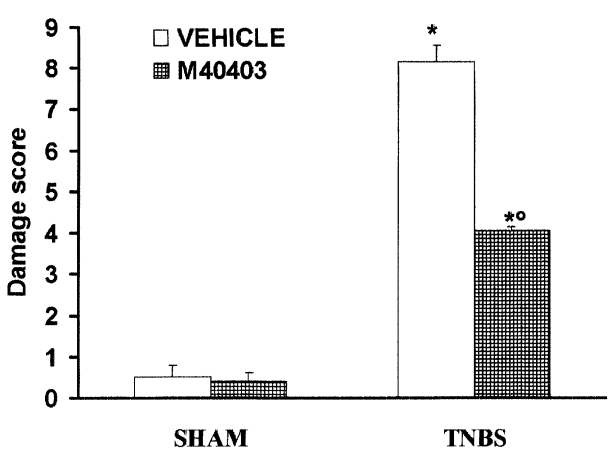

Fig. 1. Effect of M40403 treatment on the damage score. Colonic damage was scored on a 0 (normal) to 10 (severe) scale by two independent observers. Values are means ± S.E.M. of 10 rats for each group. (*) $P < 0.01$ vs. sham; (°) $P < 0.01$ vs. TNBS.

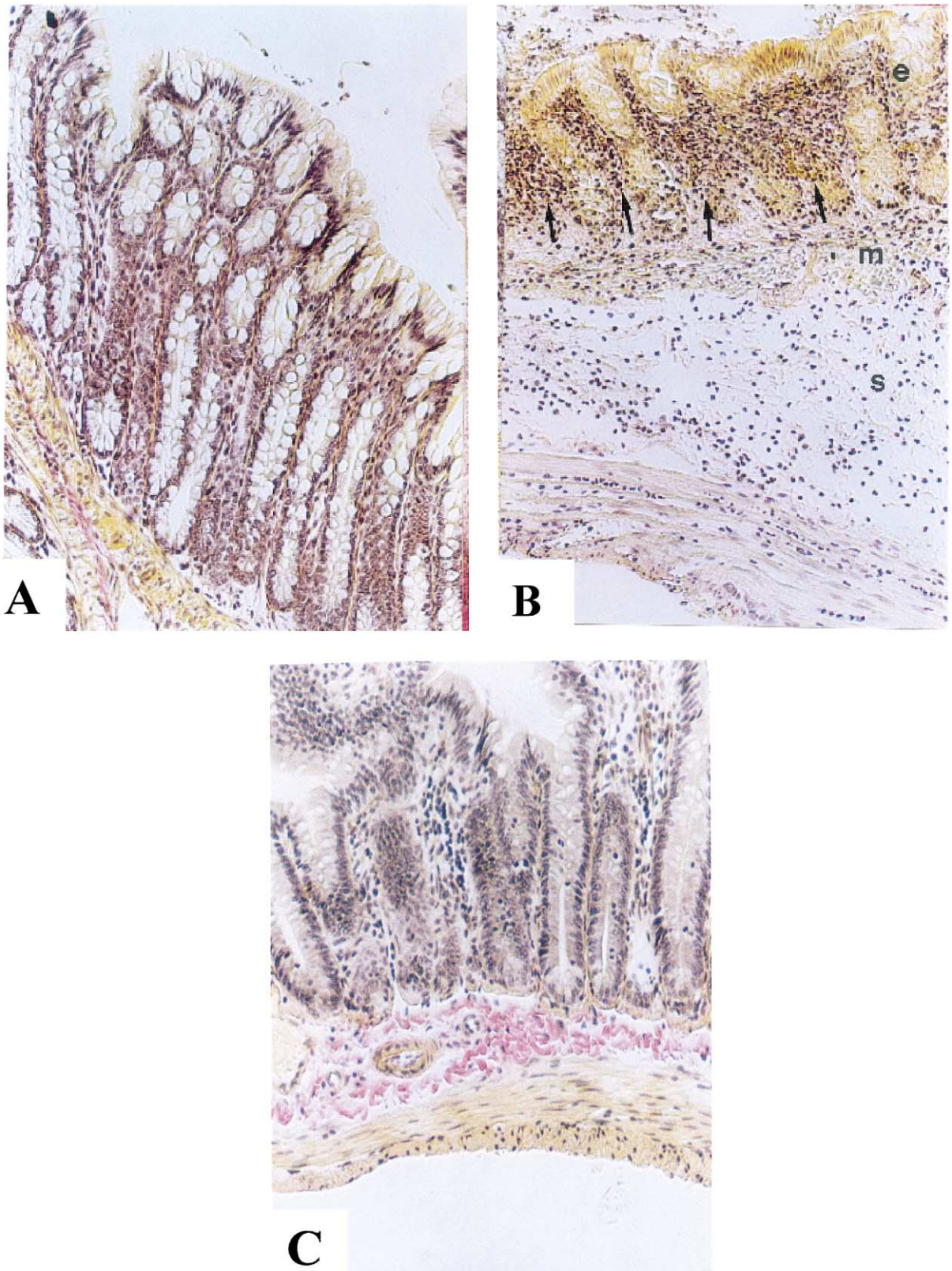

Fig. 2. Effect of M40403 on colon injury. When compared to control (A), histological examination of descending colon from TNBS-treated rats reveals a complete alteration of the epithelial layer (e), muscularis mucosa (m) and submucosal (s) as well as a diffuse inflammatory infiltration (see arrows) by neutrophils, lymphocytes and plasma cells in perilesional area (B). Treatment with M40403 (C) corrected the disturbances in morphology and reduced the inflammatory cells infiltration associated with TNBS administration. Original magnification: ×100. Figure is representative of at least three experiments performed on different experimental days.

Table 1
Effect of M40403 on TBNS-induced increase in colon and spleen weights, myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels in the colon

	Spleen weight (g)	Colon weight (g)	MPO activity (mU/100 mg wet tissue)	MDA levels (μM/mg wet tissue)
Sham + vehicle	0.72 ± 0.06	1.2 ± 0.08	45 ± 3.5	86 ± 3.5
Sham + M40403 (5 mg/kg)	0.84 ± 0.04	1.09 ± 0.05	52 ± 2.5	81 ± 2.8
TNBS + vehicle	1.375 ± 0.1[a]	5.456 ± 0.12[a]	139 ± 4.5[a]	213 ± 6.9[a]
TNBS + M40403 (5 mg/kg)	0.775 ± + 0.08[b]	2.25 ± 0.1[b]	76 ± 3.2[ab]	136 ± 2.9[ab]

Values are means ± S.E.M. of 10 rats for each group. (a) $P < 0.01$ vs. sham; (b) $P < 0.01$ vs. TNBS.

complex were obtained from Vector Laboratories (Burlingame, CA, USA). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, NY). Primary P-selectin (CD62P) and ICAM-1 (CD54) were purchased from Pharmingen (DBA). M40403 was synthetized as described (Salvemini et al., 1999a). All other reagents and compounds used were purchased from Sigma (St. Louis, MO).

2.10. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean (S.E.M.) of *n* observa-

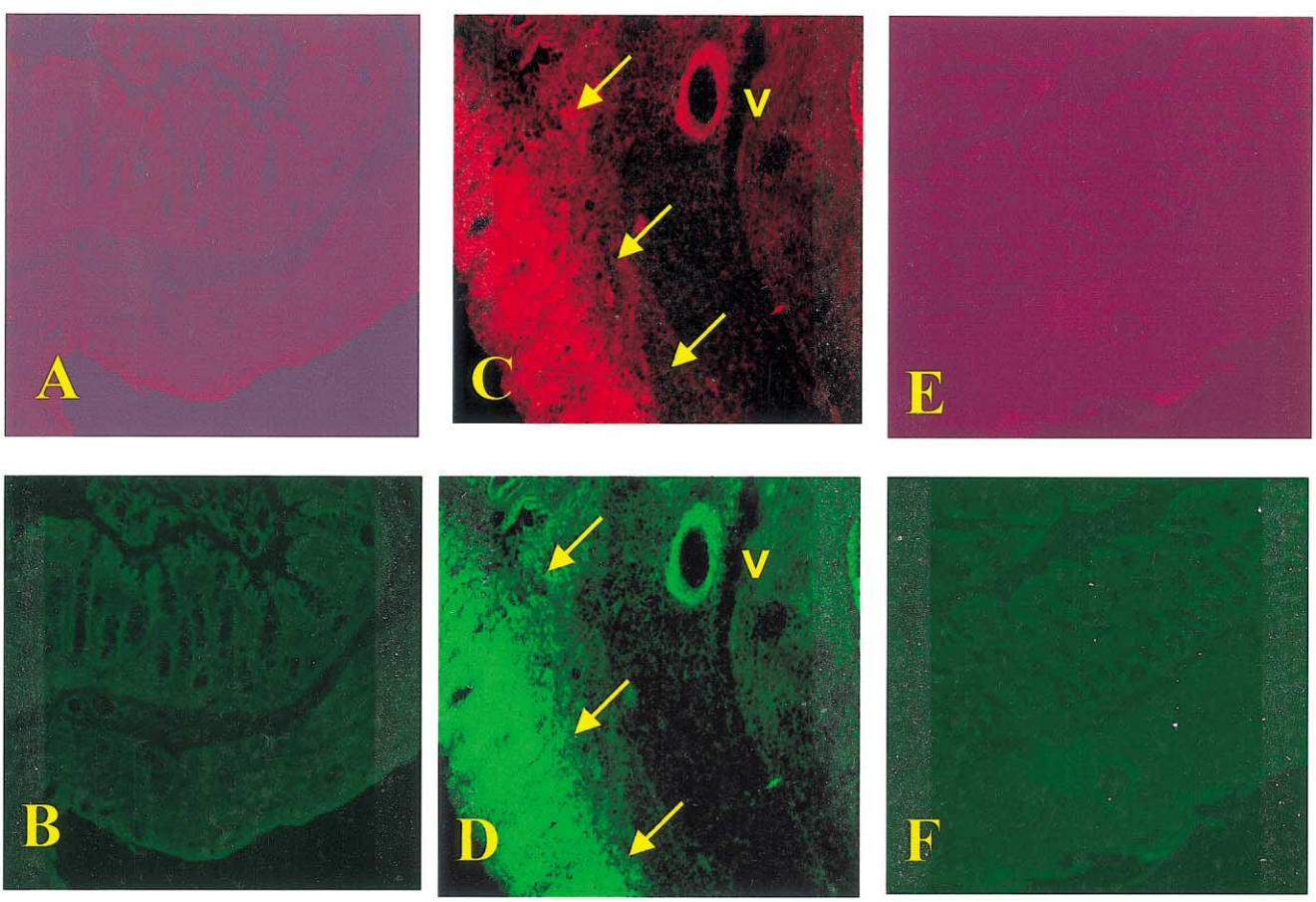

Fig. 3. Immunohistochemical localisation of ICAM-1 and P-selectin in the colon. Staining of colon tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining demonstrating that ICAM-1 is constitutively expressed (A). Ileum section from sham-operated rats revealed no positive for P-selectin staining (B). Section obtained from TNBS-treated rats showed intense positive staining for ICAM-1 (C) and for P-selectin (D) on the vessels (v) as well as in inflammatory cells concentrated below the epithelial layer (arrows). The degree of positive staining for ICAM-1 (E) and for P-selectin (F) was markedly reduced in tissue section obtained from M40403-treated rats. Original magnification: ×100. Figure is representative of at least three experiments performed on different experimental days.

tions. For the in vivo studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analysed by one-way analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons. A *P*-value less than 0.05 was considered significant.

3. Results

3.1. Effects of M40403 on colonic damage

Four days after intracolonic administration of TNBS, the colon appeared flaccid and filled with liquid stool. The cecum, colon and rectum all had evidence of mucosal congestion, erosion and hemorrhagic ulcerations (Fig. 1). Histological examination of lesion area of descending colon sections showed a complete alteration of the epithelial layer, muscularis mucosa and submucosal architecture. Diffuse inflammatory infiltration of neutrophils, lymphocytes and plasma cells was also observed in the perilesional area and concentrated below the epithelial layer at 4 days after TNBS-induced colitis (see arrows; Fig. 2B). The inflammatory changes of the intestinal tract were confirmed by showing a significant increase in both colon and spleen weights (Table 1). Daily treatment with M40403 (5 mg/kg) resulted in a significant decrease in the extent and severity of damage (Figs. 1 and 2C; Table 1).

3.2. Effect of M40403 on colonic myeloperoxidase activity and lipid peroxidation

As expected, colonic injury following TNBS administration was associated with a profound infiltration of neutrophils at the inflamed site (as measured by myeloperoxidase activity, Table 1). Neutrophil infiltration paralleled the increase in tissue malondialdehyde (Table 1), indicative of lipid peroxidation. M40403 significantly attenuated neutrophil infiltration, and malondialdehyde formation (Table 1).

3.3. Effect of M40403 on ICAM-1 and P-selectin expression

To further elucidate the effect of M40403 treatment on neutrophil accumulation in the inflamed colon, we next evaluated the intestinal expression of ICAM-1 and P-selectin. Tissue sections obtained from sham-operated rats with anti-ICAM-1 antibody showed a specific staining, demonstrating that ICAM-1 is expressed constitutively (Fig. 3A). After TNBS administration, the staining intensity substantially increased in the vessels as well as in inflammatory cells concentrated below the epithelial layer from TNBS-treated rats (arrows Fig. 3C). Section from M40403-treated rats did not reveal any up-regulation of the constitutive ICAM-1, which was normally expressed (Fig. 3E). No positive staining was observed in tissue sections obtained from sham-operated rats with anti-P-selectin antibody (Fig. 3). Tissue section of the colon obtained from TNBS-treated rats showed positive staining for P-selectin localised in the vascular wall and in the inflammatory cells (see arrows; Fig. 3D). In tissue obtained from M40403-treated rats, no expression of P-selectin (Fig. 3F) was found. To verify the binding specificity for ICAM-1 or P-selectin, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

3.4. Effects of M40403 on cytokine release

Colonic injury by TNBS administration was also characterised by an increase of pro-inflammatory cytokines (TNF-α and IL-1β) in the colon (Fig. 4A,B). As shown in Fig. 4, M40403 reduced the increase in TNF-α and IL-1β in colonic tissues (Fig. 4).

3.5. Effect of M40403 treatment on nitrotyrosine formation and poly (ADP-ribose) synthetase activation

Nitrotyrosine, a marker of nitrosative stress, and poly (ADP-ribose) synthetase (an enzyme activated following single strand DNA damage) was evaluated by immunohistochemical analysis in the distal colon 4 days after TNBS administration. Sections of colon from sham-administered

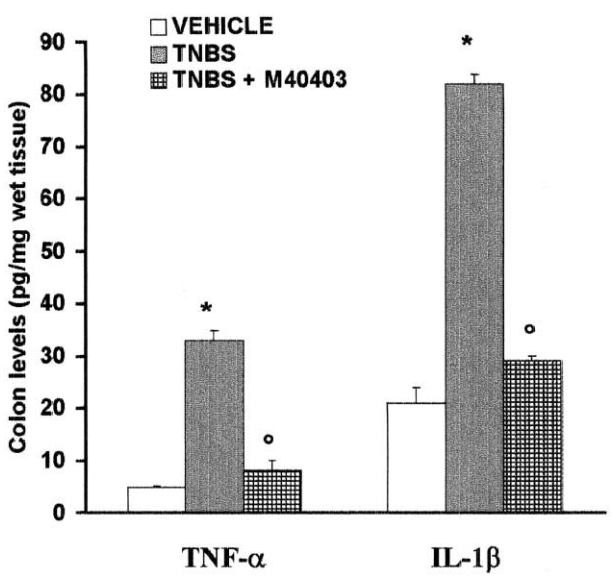

Fig. 4. Effects of M40403 on the levels of cytokines in the colon. Colon levels of TNF-α (A) and IL-1β (B). Cytokine levels were significantly reduced in the colon from M40403-treated rats. Values are means ± S.E.M. of 10 rats for each group. (*) $P < 0.01$ vs. sham; (°) $P < 0.01$ vs. TNBS.

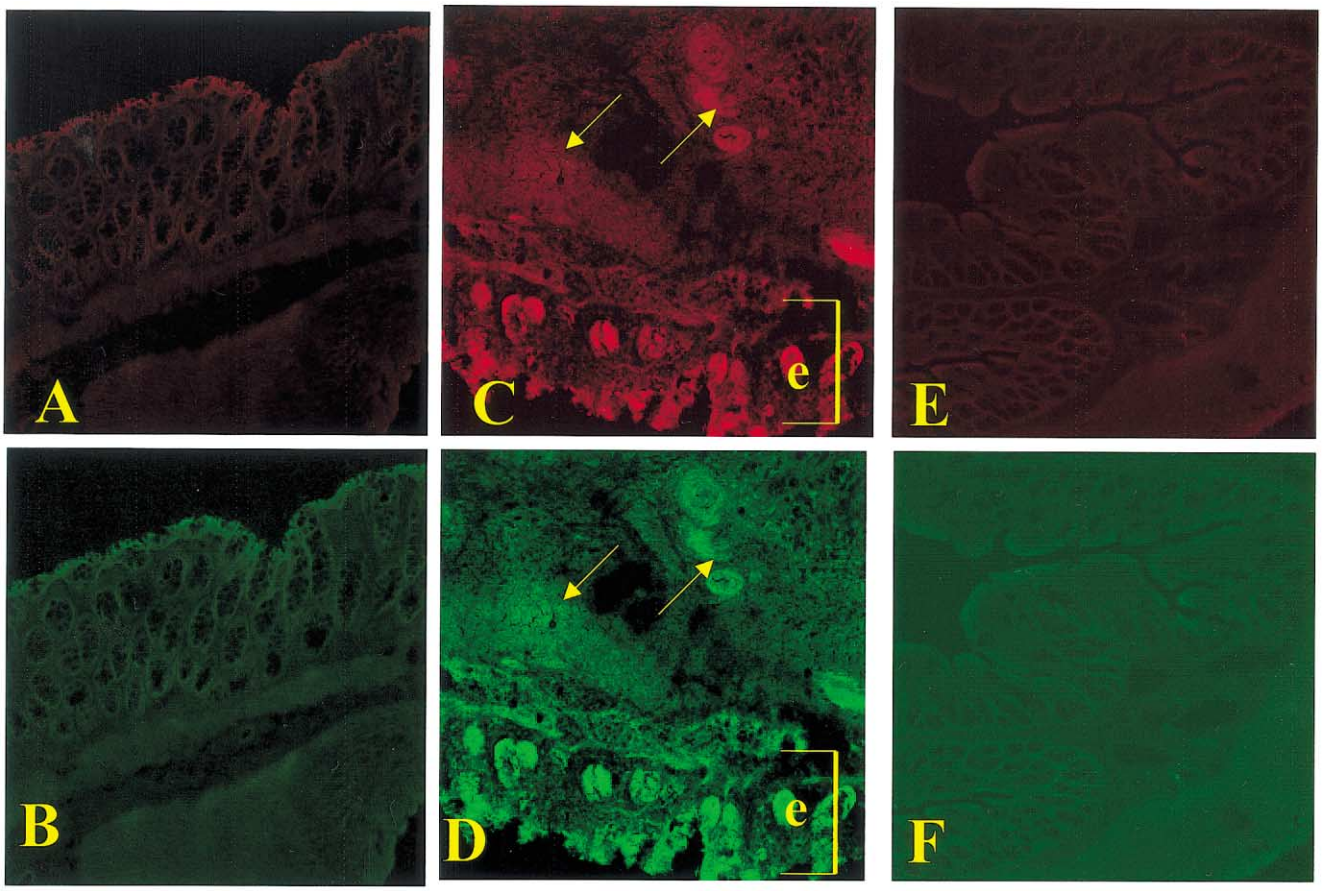

Fig. 5. Immunohistochemical localisation for nitrotyrosine and for poly (ADP-ribose) synthetase in the colon. No positive staining for nitrotyrosine (A) and for poly (ADP-ribose) synthetase (B) was found in the colon section from sham-operated rats. Immunohistochemical for nitrotyrosine (C) and for poly (ADP-ribose) synthetase (D) shows positive staining primarily localised in the infiltrated inflammatory cells (see arrows) and in disrupted epithelial cells (square bracket) from a TNBS-treated rats. The intensity of the positve staining for nitrotyrosine (E) and for poly (ADP-ribose) synthetase (F) was significantly reduced in the colon from M40403-treated rats. Original magnification: ×100. Figure is representative of at least three experiments performed on different experimental days.

rats did not reveal any immunoreactivity for nitrotyrosine and for poly (ADP-ribose) synthetase within the normal architecture of the colon (Fig. 5A,B). A positive staining for nitrotyrosine and for poly (ADP-ribose) synthetase was found primarily localised in the infiltrated inflammatory cells (see arrows) and in disrupted epithelial cells (Fig. 5C,D). M40403 treatment reduced the degree of immunostaining for nitrotyrosine and for poly (ADP-ribose) synthetase in the colon of TNBS-treated rats (Fig. 5E,F). In order to confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for poly (ADP-ribose) synthetase, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

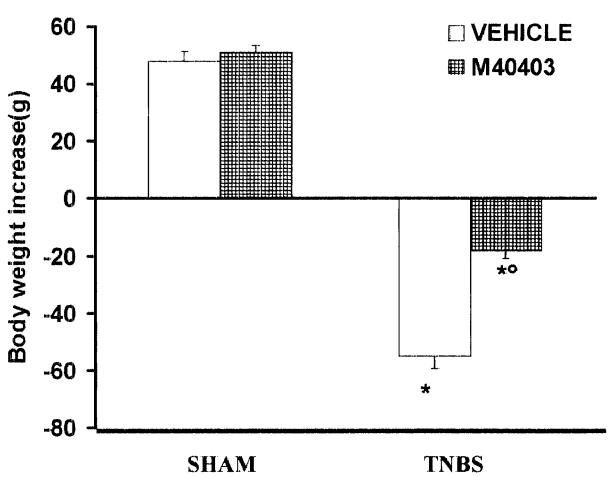

Fig. 6. Effect of M40403 treatment on body weight changes 4 days after TNBS intracolonic administration. Body weight was recorded immediately before TNBS administration and at the end of the experimental period (at 4 days). M40403 treatment significantly prevented the loss of body weight. Values are means ± S.E.M. of 10 rats for each group. (*) $P < 0.01$ vs. sham; (°) $P < 0.01$ vs. TNBS.

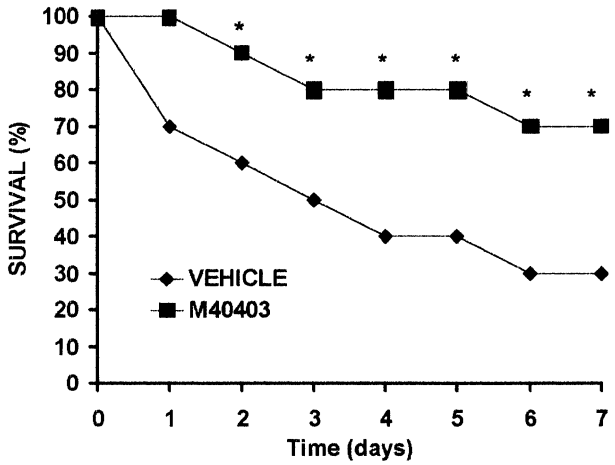

Fig. 7. Effect of M40403 treatment on TNBS-induced mortality. Survival is significantly improved in M40403-treated rats in comparison to the high mortality rate of the TNBS-treated rat. $n = 10$ rats for each group. (*) $P < 0.01$ versus TNBS alone.

3.6. Effects of M40403 on changes of body weight and survival time

At 4 days post TNBS injection, the inflammatory changes of the intestinal tract were accompanied by a significant loss in body weight in comparison to control rats (Fig. 6). Treatment with M40403 significantly reduced the loss in body weight (Fig. 6), which correlated well with the amelioration of colonic injury. Survival time was monitored for up to 7 days. TNBS-treated rats developed bloody diarrhoea, and death occurred early and was substantial (40% and 70% died within 2 and 6 days, respectively, after TNBS administration) (Fig. 7). In contrast, only 30% of M40403-treated rats had a serious diarrhoea and died over a similar time course (Fig. 7). The surviving rats appeared healthy and showed a very mild diarrhoea.

4. Discussion

Our data show that rats treated with M40403, a low molecular weight synthetic enzyme mimetic of superoxide dismutase, are significantly more resistant to death and pathological changes in the colon and rectum associated with TNBS induced colitis.

4.1. Superoxide anion is a requisite of oxidative and nitrosative damage in TNBS induced colitis

Reactive oxygen and nitrogen species play a key role in inflammatory bowel disease (Grisham, 1994; Rachmilewitz et al., 1993). These species are cytotoxic inducing lipid peroxidation and other cellular oxidative stress by cross-linking proteins, lipids, and nucleic acids, which then cause cellular dysfunction, damage, and eventually death. Evidence consistent with damage by reactive radical species is provided by the increase in lipid peroxides in rectal biopsy specimens from patients with ulcerative colitis (Grisham, 1994; Simmonds et al., 1992; McKenzie et al., 1996). In the present study, we found that the mucosal damage induced by intracolonic administration of TNBS was associated with high concentrations of malondialdehyde, which is considered a good indicator of lipid peroxidation (Ohkawa et al., 1979). Recent evidence indicates that nitration of tyrosine can result from a number of chemical actions and can be considered as a global marker of nitrosative stress (Halliwell, 1997). Nitrotyrosine can be formed from the reaction of nitrite with hypochlorous acid or the reaction of nitrite with myeloperoxidase and hydrogen peroxide (Eiserich et al., 1998). In our experiments, we found increased immunohistochemical expression of nitrotyrosine mostly localised on epithelial cells and in the area of infiltrated inflammatory cells, suggesting that peroxynitrite or other nitrogen derivatives and oxidants are formed in vivo and may contribute to tissue injury. These data are consistent with previous findings that immunohistochemical staining for nitrotyrosine was localized on epithelial cells in a TNBS model of guinea pig ileitis (Miller et al., 1995) or rat colitis (Zingarelli et al., 1999a) and in active Crohn's lesions in humans (Inger et al., 1996). The pathogenic role of nitrogen-derived species, such as peroxynitrite (Beckman et al., 1990; Ischiropoulos et al., 1992), in inflammatory bowel disease is further supported by the fact that intracolonic administration of exogenous peroxynitrite induces a severe colonic inflammation, which mimics the features of both ulcerative colitis and Crohn's disease (Rachmilewitz et al., 1993).

In the present study, we observed that epithelial disruption was significant less in rats treated with M40403. Indeed, M40403 treatment prevented the formation of tissue malondialdehyde and nitrotyrosine staining in TNBS treated animals. Furthermore, M40403-rats are more resistant to TNBS-induced lethal disease with a significant resolution of the macroscopic and histological signs of the inflammatory process. Superoxide and peroxynitrite cause DNA single-strand damage, leading to poly (ADP ribose) synthetase activation and cell death (Szabo and Dawson, 1998). Some evidence exists to support the possible role of poly (ADP ribose) synthetase activation in inflammatory bowel disease (Zingarelli et al., 1999b; Szabo et al., 1997). As shown in Fig. 5, M40403 reduced poly (ADP ribose) synthetase immunofluorescence, an effect that might account for the overall protective action of M40403.

4.2. The beneficial effect of M40403 in TNBS-induced colitis is related to an inhibition on cytokines production

Tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β) are clearly involved in the pathogenesis of colitis since these cytokines are present in colon tissues and can be

detected immunohistochemically in the inflamed tissues (Carty et al., 2000; Negoro et al., 1999). Direct evidence that TNF-α and IL-1β play a role in the pathogenesis of experimental colitis has been obtained in animal models, in which blocking of the action of these cytokines has been shown to delay the onset of experimental colitis, suppress inflammation, and ameliorate colon destruction that corresponds to the anti-inflammatory response (Andborn and Hanauer, 1999; Urthy et al., 1999). A role for TNF-α in human disease came from recent studies using Infliximab (Present et al., 1999; Targan et al., 1997; Van Dullemen et al., 1995; Ricart et al., 1999; D'Haens et al., 1999; Baert et al., 1999; Moreland et al., 1996), a chimeric anti-TNF antibody, and CDP571, a humanized monoclonal antibody to TNF-α and membrane-bound TNF without fixing complement nor mediating antibody-dependent cellular cytotoxicity (Stack et al., 1997). In both cases, significant reduction in Crohn's disease activity index (CDAI) as well as attenuation of histopathologic and endoscopic inflammation in Crohn's disease patients were observed.

Since superoxide anions regulate cytokine release (by mechanisms yet to be defined) (Salvemini et al., 1999a, 2001; Volk et al., 1999), we postulate that inhibition of TNF-α and IL-1β in the colon plays an important role in the overall beneficial effects observed with M40403.

4.3. The beneficial effect of M40403 in TNBS-induced colitis is related to an alteration in neutrophil recruitment

Neutrophils play a crucial role in the development and full manifestation of gastrointestinal inflammation, as they represent a major source of free radicals in the inflamed colonic mucosa (Shiratora et al., 1989; Grisham, 1994). Neutrophil infiltration into inflamed tissue plays a crucial role in the destruction of foreign antigens and in the breakdown and remodeling of injured tissue (Lefer and Lefer, 1993). The interactions of polymorphonuclear cells with the endothelium are regulated by various adhesion molecules including the selectins, the β_2 integrins and adhesion molecules of the immunoglobulin superfamily (Geng et al., 1990). Although P-selectin is necessary for early contact of neutrophil with the endothelium, P-selectin-mediated leukocyte–endothelial interaction is not sufficient to allow neutrophil emigration from the vessel. A more firm adherence of the neutrophil to the endothelial surface is required for transendothelial migration (Butcher, 1993). This firm adherence involves the interaction of β_2 integrins (i.e., CD11/CD18) on the polymorphonuclear cells surface and intercellular adhesion molecule 1 (ICAM-1) on the endothelial cell surface (Koizumi et al., 1992).

A major finding of this study was that not only did the M40403-treated rats show a remarkable recovery of the mucosal morphology associated with a reduction in oxidative and nitrosative damage after TNBS administration, but that in M40403-treated rats, infiltration of polymorphonuclear neutrophils was significant reduced in tissue. Furthermore, ICAM-1 and P-selectin were expressed in endothelial and epithelial cells, and neutrophils in the distal colon in TNBS treated rats. This was associated with a significant reduction of ICAM-1 and P-selection expression in endothelial and epithelial cells. Our data suggest that superoxide production contribute to the regulation of neutrophil infiltration and is consistent with previously published data in models of acute inflammation and reperfusion injury (Salvemini et al., 1999a,b, 2001; Cuzzocrea et al., 2001).

4.4. Summary and conclusions

The native superoxide dismutase enzyme, Orgotein, has beneficial and protective effects in pilot clinical trials in patients with Crohn's disease, supporting a role for superoxide in these conditions (Niwa et al., 1985). Recent experience in patients with Crohn's disease with Infliximab and CDP571 teaches that the cytokine TNF-α plays an important role in human disease, and that its removal has a positive impact on disease. TNBS-induced colitis is an experimental model of colitis, which differs somewhat from the pathophysiology of human Crohn's disease or ulcerative colitis, which are spontaneous and heterogeneous disorders with multiple etiological and pathogenic mechanisms. However, this model is frequently used by many investigators since it does reproduce many of the characteristic features of human inflammatory bowel disease, including generation of various inflammatory mediators, generation of oxygen and nitrogen radicals and oxidants, immunological alteration and participation of cytokines such as TNF-α.

Our results demonstrate that a low-molecular weight synthetic of superoxide dismutase, which mimics the effects of the native enzyme, is protective in experimental colitis, and that inhibition of TNF-α formation (amongst other important effects which include inhibition of neutrophils infiltration) in the colon probably accounts for its beneficial effects. Based on the above, and with full recognition that TNBS-induced colitis does not mimic the clinical disease in its full and complicated etiology, it is not unreasonable to propose that superoxide dismutase mimetics such as M40403 may be valuable as clinical candidates for such disorders.

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