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Ameliorating effects of the immunomodulator 3-(2-ethylphenyl)-5-(3-methoxyphenyl)-1*H*-1,2,4-triazole in an experimental model of colitis in the rat

Maria Antonietta Stasi[a,*], Vito Ruggiero[a], Angela Ursillo[a], Roberto Taurelli[a], Margherita Aglianò[b], Elisabetta Weber[b], Paola Lorenzoni[b], Vincenzo Sorrentino[b], Licia Pacifici[a], Paolo Carminati[a]

[a] *Pharmacology Department-R&D, Sigma-tau S.p.A., Via Pontina km 30.400, 00040 Pomezia (RM), Italy*
[b] *Molecular Medicine Section, Department of Neuroscience, University of Siena, Siena, Italy*

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Abstract

The therapeutic efficacy of the immunomodulator 3-(2-ethylphenyl)-5-(3-methoxyphenyl)-1*H*-1,2,4-triazole (ST1959) in colonic inflammation was assessed in rats. One hour following colonic instillation of ethanolic 2,4,6-trinitrobenzene sulphonic acid (TNBS), intracolonic administration of 0.4 mg/kg ST1959 was started and continued once daily for 1 or 2 weeks. Daily administration of ST1959 for 1 week significantly reduced macroscopic and histological damage, myeloperoxidase activity, and colonic tissue levels of tumour necrosis factor-α and interferon-γ. ST1959 did not affect interleukin-12 levels but significantly enhanced the production of interleukin-10 (sixfold increase). Two weeks of ST1959 treatment reduced the thickness of the colonic wall and myeloperoxidase activity to the same extent, and the histologic appearance of the mucosa was largely restored. The ameliorating effects seem to be ascribable to an impairment of both neutrophil infiltration/activation and tumour necrosis factor-α and interferon-γ production, possibly consequent to the observed increase in the colonic tissue levels of the potent anti-inflammatory cytokine interleukin-10. Similar results were observed with the reference drug 5-aminosalycilic acid.

Keywords: Inflammatory bowel disease; TNBS; Tumour necrosis factor-α; Interferon-γ; Interleukin-10; Myeloperoxidase

1. Introduction

Human inflammatory bowel disease is a chronic, idiopathic inflammatory disorder that primarily affects the digestive tract (Podolsky, 2002). Although the etiology is still poorly defined, inflammatory bowel disease is clinically characterized by two overlapping phenotypes: Crohn's disease and ulcerative colitis. Crohn's disease is clinically characterized by periods of exacerbation and remission. It usually affects segments of the small and large intestine, and it involves a transmural granulomatous inflammation, whereas ulcerative colitis mostly affects the mucosal layer of the colon (Kirsner, 1961; Ward, 1977). Important progress has been made in recent years, and it is becoming clear that these diseases represent the outcome of a combination of interactive cofactors: host susceptibility, enteric microflora, and mucosal immunity (Shanahan, 2001). Normally, the intestinal mucosa is continuously exposed to a plethora of potentially harmful agents derived from environment, food, and bacterial flora, and tightly controlled immune mechanisms operate to balance between responsiveness and tolerance. In healthy subjects, the immune response is well tuned and does not lead to tissue damage. In a few subjects, conversely, a disruption of the regulatory constraints on mucosal immune responses takes place, often conducing to severe damage of the intestinal mucosa.

A crucial role in orchestrating the proper immune response is played by antigen-presenting cells, such as macrophages and dendritic cells, and by $CD4^+$ T lymphocytes.

* Corresponding author. Tel.: +39-6-9139-3718; fax: +39-6-9139-3988.
E-mail address: mariaantonietta.stasi@sigma-tau.it (M.A. Stasi).

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The latter can be further subdivided into functionally distinct subsets defined by the pattern of cytokines they produce upon activation. T-helper 1 (Th1) cells secrete interleukin-2, interferon-γ, and mediate cellular immunity and delayed-type hypersensitivity, whereas the T-helper 2 subset (Th2) secretes interleukin-4, interleukin-5, and interleukin-13, and mediates humoral immune responses (Glimcher and Murphy, 2000). Recently, two additional subsets have been described, namely, T-helper 3 (Th3) and T-regulatory (Tr1), secreting transforming growth factor-β and interleukin-10, respectively, which have been shown to be involved in suppression of mucosal immune responses as well as oral tolerance (Neurath et al., 2002; Bach, 2001). Several drugs have been synthesized for treatment of inflammatory bowel disease by targeting the pathways underlying cytokine production or function such as cyclosporin, azathioprine, and methotrexate (Hanauer and Present, 2003). However, treatment with cyclosporin is often hampered by adverse effects such as hypertension and nephrotoxicity (Rutgeerts, 1998). Treatment with azathioprine may provoke suppression of bone marrow formation and increased susceptibility to infections (Schwab et al., 2002). Methotrexate is teratogenic and is characterized by the length of time required to attain therapeutic effectiveness (Gibson and Anderson, 1998). Therefore, new immunomodulators are under intense scrutiny seeking readiness of treatment and more specific modulation of the pathogenic processes of inflammatory bowel disease, in order to minimize the risk of adverse effects (Podolsky, 2003).

Although the newest avenues of research have drawn deep interest in the use of variably engineered monoclonal antibodies (chimeric, humanized, and fully human) specifically directed against putative crucial mediators of Crohn's disease (Sandborn and Targan, 2002), more data on long-term safety are still being awaited (D'Haens, 2003).

ST1959 (formerly DL111-IT), i.e., 3-(2-ethylphenyl)-5-(3-methoxyphenyl)-1*H*-1,2,4-triazole, originally brought forward as a nonhormonal, nonprostaglandin-like, postimplantation antifertility agent (Galliani et al., 1981), was later found to be endowed with immunomodulatory properties. Actually, Mistrello et al. (1985) showed that this drug was effective at inhibiting the antibody response to both thymus-dependent and thymus-independent antigens, such as sheep red blood cells and lipopolysaccharide, respectively, and it also reduced autoantibody production against autologous erythrocytes. Moreover, ST1959 prolonged skin graft survival in mice, and it significantly reduced the delayed-type hypersensitivity response to sheep red blood cells (Mistrello et al., 1985) even at a dose as low as 1 mg/kg body weight, way below the LD_{50} value (>2000 mg/kg, subcutaneously). As the immunomodulatory effects of ST1959 led to amelioration or prevention of autoimmune disorders in animal models (Mistrello et al., 1985; Ruggiero et al., 2003), we reasoned that it could be a good candidate for the treatment of experimental colitis induced by rectal instillation of trinitrobenzene sulphonic acid (TNBS) in rats (Morris et al., 1989; Elson et al., 1995). TNBS is a skin contactant that induces delayed hypersensitivity reactions when applied to skin by virtue of its ability to haptenate proteins with trinitrophenyl groups, thereby rendering them immunogenic to the immune system. Enema of TNBS into the colons of susceptible animals induces acute and chronic intestinal inflammation (Neurath et al., 2000). The inflammatory processes seem to be the result of delayed-type hypersensitivity immune responses against trinitrophenyl-haptenated autologous colonic proteins. At variance with skin, where the reaction is self-limited, the ensuing mucosal immune response is very intense in that the mucosal immune system continues to be stimulated long after the trinitrophenyl-haptenated proteins have disappeared (Wirtz and Neurath, 2000). The hallmark of TNBS-colitis is a massive mucosal inflammation characterized by dense infiltration of neutrophils, macrophages, and T-cells throughout the wall of the large bowel. This is paralleled by a clinical picture of wasting, bloody diarrhoea, and large bowel wall thickening. This experimental model of colitis shows close histological resemblance to human Crohn's disease, including focal ulceration and transmural inflammation with granulomas and fistulas. Furthermore, it also responds to drugs effectively utilized in human inflammatory bowel disease treatment (Blumberg et al., 1999). Interestingly, colonic lesions and inflammation are accompanied by a dramatic increase in myeloperoxidase activity, a marker of neutrophil infiltration (Kiss et al., 1997).

The aim of this study was to evaluate the activity of ST1959 on the course of TNBS-induced colitis in the rat, and to get further insights into the mechanisms whereby it exerts its beneficial effects.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 250–300 g at the beginning of study were used. The animals were maintained in a room with controlled temperature (23 °C) and light/dark cycle (7 a.m.–7 p.m.), and were housed in standard wire mesh cages. Standard rat chow and water were provided ad libitum. Experiments were performed under an approved protocol in accordance with the animal use guidelines of the Istituto Superiore di Sanità (Rome, Italy). The animals were randomized using a statistical program (Sigma Stat, SPSS, Chicago, IL, USA).

2.2. TNBS induction of experimental colitis

Colitis was induced following the procedure described by Morris et al. (1989) based on a single intracolonic instillation of TNBS dissolved in 50% ethanol. Briefly, rats were fasted for 24 h and lightly anaesthetized with 4% halothane in N_2O/O_2 prior to instillation of 0.25 ml/rat of a

solution containing 120 mg/ml TNBS (Sigma, St. Louis, MO, USA) dissolved in 50% (vol/vol) ethanol (Merck, Darmstadt, Germany) by inserting a rubber cannula fitted onto a 1-ml syringe into the lumen of the colon 8 cm distal from the anus. The total volume was expelled with additional air before removing the cannula. After TNBS instillation, animals were kept in a vertical position for 30 s, and then returned to their cages. A separate group of animals ($n = 10$) received intracolonically 0.25 ml of 50% ethanol only (TNBS vehicle). The rats were sacrificed either 1 or 2 weeks after TNBS injection. The colon was excised, opened longitudinally, rinsed with sterile saline, and the distal 8 cm portion of the colon was photographed (Nikon camera F 301 with macro lens AF micronikkor 55 mm). The area of mucosal damage was assessed by analyzing the photographs by means of an image analysis software (Sigma Scan Pro 5, Aspire Software International, Leesburg, VA, USA) and expressed as a percentage of the total colonic segment area that showed macroscopically visible damage. In addition, the presence or absence of adhesions between the colon and the adjacent organs was annotated, together with the presence or absence of diarrhoea, defined as loose, watery stools.

2.3. Experimental design

After preliminary experiments to find optimal dosage (data not shown), rats were dosed with 0.4 mg/kg ST1959, administered once 1 h after single TNBS rectal instillation, and then once daily either for 7 or 14 days following TNBS initial application. ST1959 (obtained from Dr. A. Assandri, Cross, Switzerland) was dissolved in sesame oil and was administered rectally to halothane-anaesthetized rats (10 animals/group) using a rubber cannula. A matched control group ($n = 10$) received sesame oil alone (ST1959 vehicle). Another group of rats ($n = 10$) was treated with the reference compound 5-aminosalicylic acid (5-ASA, Sigma), administered intracolonically at the dose of 120 mg/kg in 1% carboxymethylcellulose (CMC sodium salt, Sigma), whereas a matched control group ($n = 10$) received carboxymethylcellulose only (5-ASA vehicle). The 5-aminosalicylic acid treatment regimen was the same as ST1959. Five-aminosalicylic acid is known to have beneficial effects in experimental colitis, and for this reason it was used here as a control drug (Martinsson et al., 1999).

2.4. Myeloperoxidase assay

Following 7 and 14 days of ST1959 treatment, myeloperoxidase activity in the colonic tissue homogenate was determined according to the method described by Bradley et al. (1982) with minor modifications. Briefly, the 8-cm-long colonic specimen was homogenized in ice-cold phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyltrimethylammonium-bromide (50 mg of tissue per 1 ml of buffer), freeze-thawed three times and centrifuged ($14{,}000 \times g$, 2 min at 4 °C). Volumes of 100 μl of the supernatant were added to 2.9 ml phosphate buffer (5 mM, pH 6) containing 0.167 mg/ml *O*-dianisidine and 0.0005% hydrogen peroxide. One unit of myeloperoxidase activity is defined as the amount of enzyme activity that degrades one micromole of peroxide per minute at 25 °C. Enzyme activity is expressed in milliunits per milligram wet weight tissue.

2.5. Cytokine assays

After 7 days of treatment with ST1959, colon homogenates were prepared in nine volumes of Greenburger lysis buffer [300 mmol/l NaCl, 15 mmol/l Tris, 2 mmol/l $MgCl_2$, 2 Mol/l Triton X-100, 20 ng/ml of protease inhibitor (Complete™ mini, Boehringer Mannheim Biochemicals, Germany)]. Tissue specimens were lysed for 30 min on ice and centrifuged twice (10 min at $14{,}000 \times g$), and supernatants were stored at −20 °C until assay (Ten Hove et al., 2001). Tumour necrosis factor-α, interferon-γ, interleukin-10 (R&D System, Abingdon, UK), and interleukin-12 (Biosource, Camarillo, CA, USA) levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.6. Histological assessment

The distal colon was removed, cut open longitudinally, pinned out on wax blocks, fixed in Karnovsky (4% paraformaldehyde, 2.5% glutaraldehyde) in 0.1 M cacodylate buffer, pH 7.35 for 24 h at 4 °C, and dehydrated in an ethanol series. Representative samples were taken from each of the following regions: (a) centre of the lesion, (b) upper edge of the lesion, (c) lower edge of the lesion, (d) 0.5 cm above the upper edge, (e) 0.5 cm under the lower edge, and (f) 1 cm above the upper edge. The selected samples were embedded in methacrylate (Technovit 7100, Heraeus), and 3.5-μm-thick sections, obtained with a Reichert Jung microtome, were stained with 0.1% toluidine blue, and then observed under a Nikon Eclipse E600 microscope. From each sample, 2–10 sections were routinely examined at intervals of 100 μm for a total length of 400 μm. Morphometric evaluation of the thickness of the colonic wall was performed with the Nikon program "Lucia M/G".

2.7. Statistical analysis

All data are expressed as the means ± S.E.M. of n independent observations, namely, samples from different animals. After verifying that the values were normally distributed and that variances were homogeneous, statistical evaluation was performed by one-way analysis of variance (ANOVA) followed by Dunnett test. Unpaired t-test with Welch correction was used to analyze the values of interferon-γ. The incidence of diarrhoea and adhesions was compared across the groups using the chi-square test. A P

value < 0.05 was considered statistically significant, irrespective of the statistical test.

3. Results

3.1. Effect of ST1959 on TNBS-induced colitis

The increase in body weight for ethanol-injected rats was 35 ± 2.0 g, the colonic wet weight was 69 ± 3.1 mg/cm, and these rats showed no signs of diarrhoea or bowel adhesions. One week after administration of TNBS, a significant loss ($P < 0.001$) in body weight was observed (Table 1).

In the TNBS group moreover, the colonic wet weight approximately increased fivefold compared to the vehicle-treated group ($P < 0.001$). Macroscopic analysis of colons showed extensive mucosal disruption, linear as well as deep ulcers, and diffuse hemorrhagic necrosis of the mucosa surrounded by hyperemic areas of inflamed tissue typically extending 3–4 cm along the colon. Additionally, diarrhoea and adhesions were present in 70% and 80% of the TNBS group, respectively (Table 1).

Daily intracolonic administration of ST1959 for 1 week significantly ($P < 0.05$) prevented the loss in body weight, inducing an increase of 6 ± 3.9 g, while a marked decrease was observed in the vehicle-treated group (− 13 ± 3.8 g). Furthermore, treatment with ST1959 significantly reduced ($P < 0.05$) colonic wet weight (Table 1) with respect to the vehicle-treated group. Following 1 week of treatment with ST1959, a significantly lower incidence of diarrhoea ($P < 0.01$) and a decrease in the number of adhesions were observed compared to the vehicle-treated group (Table 1). A similar trend was noticed when the animals were treated with the reference drug 5-aminosalicylic acid (Table 1).

Table 1
Changes in body weight, colonic wet weight, diarrhoea, and presence of adhesions between the colon and the adjacent organs 1 week after rectal trinitrobenzene sulphonic acid administration: effect of ST1959 (0.4 mg/kg) and 5-aminosalicylic acid (5-ASA, 120 mg/kg)

	Increase in body weight delta (g)	Colonic wet weight (mg/cm)	Diarrhoea	Adhesions
TNBS vehicle	35 ± 2.0	69 ± 3.1	0/10	0/10
TNBS	− 11 ± 3.6[a]	373 ± 42[a]	7/10[a]	8/10[a]
TNBS + ST1959 vehicle	− 13 ± 3.8	377 ± 28	8/10	7/10
TNBS + ST1959	6 ± 3.9[b]	241 ± 15[c]	1/10[c]	3/10
TNBS + 5-ASA vehicle	− 10 ± 4.0	368 ± 51	8/10	8/10
TNBS + 5-ASA	− 8 ± 8.0	304 ± 35	3/10	4/10

The drugs were administered once daily rectally for 1 week starting 1 h after the induction of colitis with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol). The ST1959 and 5-ASA vehicles were sesame oil and 1% CMC, respectively, while the TNBS vehicle was 50% ethanol. The rats were sacrificed 24 h after the last administration. Values are mean ± S.E.M. (body weight and colonic wet weight), number of rats with diarrhoea, or adhesions/total number of rats in each group. Statistical analysis was conducted using one-way analysis of variance + Dunnett test for body and colonic weight, and chi-square test for diarrhoea and adhesions.

[a] $P \leq 0.001$ vs. TNBS vehicle.
[b] $P \leq 0.05$ vs. TNBS + ST1959 vehicle.
[c] $P \leq 0.01$ vs. TNBS + ST1959 vehicle.

Table 2
Changes in body weight, colonic wet weight, diarrhoea, and presence of adhesions between the colon and the adjacent organs 2 weeks after rectal trinitrobenzene sulphonic acid administration: effect of ST1959 (0.4 mg/kg) and 5-aminosalicylic acid (5-ASA, 120 mg/kg)

	Increase in body weight delta (g)	Colonic wet weight (mg/cm)	Diarrhoea	Adhesions
TNBS vehicle	70 ± 3.0	75 ± 4.4	0/10	0/10
TNBS	50 ± 9.7[b]	302 ± 48[a]	6/10[a]	7/10[a]
TNBS + ST1959 vehicle	43 ± 4.5	300 ± 32	7/10	6/10
TNBS + ST1959	44 ± 6.2	236 ± 30	6/10	5/10
TNBS + 5-ASA vehicle	42 ± 6.0	272 ± 41	7/10	6/10
TNBS + 5-ASA	45 ± 4.0	200 ± 21	6/10	6/10

The drugs were administered once daily rectally for 2 weeks starting 1 h after the induction of colitis with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol). The ST1959 and 5-ASA vehicles were sesame oil and 1% CMC, respectively, while the TNBS vehicle was 50% ethanol. The rats were sacrificed 24 h after the last administration. Values are mean ± S.E.M. (body weight and colonic wet weight), number of rats with diarrhoea, or adhesions/total number of rats in each group. Statistical analysis was conducted using one-way analysis of variance + Dunnett test for body and colonic weight, and chi-square test for diarrhoea and adhesions.

[a] $P \leq 0.05$ vs. TNBS vehicle.
[b] $P \leq 0.001$ vs. TNBS vehicle.

Two weeks after TNBS instillation, almost normal growth rates were resumed within the different experimental groups with TNBS- and vehicle-treated animals showing an increase in body weight of 50 ± 9.7 and 70 ± 3.0, respectively (Table 2). Diarrhoea and adhesions were similar to those observed 1 week after induction of colitis (Table 2), whereas the colonic wet weight was slightly reduced. After 2 weeks of ST1959 treatment, a trend toward a reduction of colonic wet weight (236 ± 30 vs. 300 ± 32 of vehicle-treated group) was noticed, although body weight, diarrhoea, and number of adhesions were not affected (Table 2). Similar results were obtained when the animals were treated with 5-aminosalicylic acid (Table 2).

One week after the induction of colitis with TNBS, the mucosal injury was rather severe ($P < 0.001$ vs. vehicle-treated group), the mucosal-damaged area being approximately 40% of the total analyzed area (Fig. 1A). Treatment with ST1959 or with 5-aminosalicylic acid induced a similar and statistically significant reduction ($P < 0.001$) in the damaged colonic area with respect to the relevant vehicles (Fig. 1A).

Two weeks after colitis induction, the severity and the extension of lesions in the TNBS control group were reduced to approximately 25%. Treatment with ST1959 or with 5-aminosalicylic acid was still effective, the former significantly reducing ($P < 0.001$) the extension of the damaged area down to 9.5%, the latter reducing the damage down to 17%, although not in a statistically significant way (Fig. 1B).

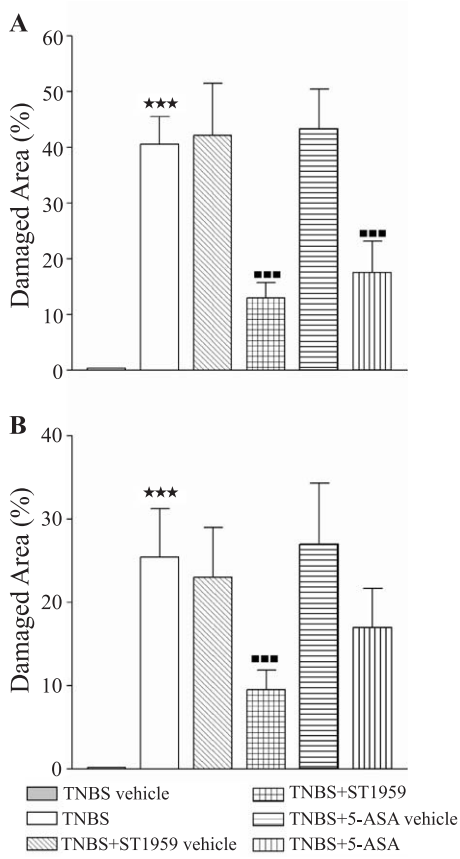

Fig. 1. Effects of ST1959 and 5-aminosalicylic acid (5-ASA) on colitis damage area 1 week (A) and 2 weeks (B) following challenge with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol). The rats were treated once daily intracolonically with ST1959 (0.4 mg/kg in 2 ml/kg of sesame oil) or 5-ASA (120 mg/kg in 2 ml/kg 1% CMC) starting 1 h after TNBS and were sacrificed 24 h after the last administration. The area of mucosal damage was assessed by analyzing the photographs by means of an image analysis software and expressed as a percentage of the total colonic segment area that showed macroscopically visible damage. Statistical analysis was conducted using one-way analysis of variance + Dunnett test: ★★★$P<0.001$ vs. TNBS vehicle and ■■■$P<0.001$ vs. respective vehicles (i.e., TNBS + either ST1959 or 5-ASA vehicle).

3.2. Effect of ST1959 on colonic myeloperoxidase activity

In the acute phase of the colitis (day 7), colon myeloperoxidase activity was significantly enhanced with an approximate 35-fold increase compared with vehicle-treated rats (Fig. 2A). Treatment for 1 week with either ST1959 or 5-aminosalicylic acid induced a significant decrease ($P<0.001$ and $P<0.01$, respectively) of TNBS-induced myeloperoxidase activity (Fig. 2A).

Two weeks after induction of colitis, myeloperoxidase activity values in TNBS-injected rats were still high, and the relative increase compared with the vehicle-treated rats was approximately 17-fold (Fig. 2B). After 2 weeks of treatment, the activity of ST1959 was still persistent and actually showed a significant 56% decrease ($P<0.001$) of TNBS-induced myeloperoxidase activity with respect to the vehicle-treated group (Fig. 2B), whereas treatment with 5-aminosalicylic acid induced a 12% decrease (Fig. 2B), but it was not significant.

3.3. Effect of ST1959 on colonic content of cytokines

In TNBS-administered rats, the content of tumour necrosis factor-α (Fig. 3A), interferon-γ (Fig. 3B), and interleukin-12 (Fig. 3C) in the colon increased, whereas interleukin-10 (Fig. 4) was barely detectable. Administration of ST1959 significantly reduced TNBS-induced tumour necrosis factor-α and interferon-γ (Fig. 3A and B). A similar trend was observed following treatment with 5-aminosalicylic acid, although its effect was not statistically significant (Fig. 3A and B). Conversely, neither ST1959 nor 5-aminosalicylic acid treatment exerted any effect on colonic interleukin-12

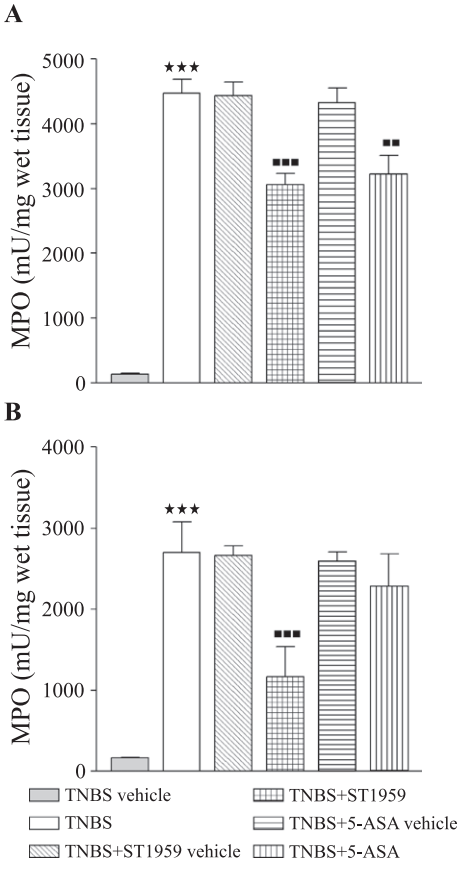

Fig. 2. Myeloperoxidase activity (MPO) following challenge with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol) after 1 week (A) and 2 weeks (B) of a single daily intracolonic treatment with ST1959 (0.4 mg/kg in 2 ml/kg of sesame oil) or 5-ASA (120 mg/kg in 2 ml/kg of 1% CMC) starting 1 h after TNBS. The rats were sacrificed 24 h after the last administration. One unit of MPO activity is defined as the amount of enzyme activity that degrades one micromole of peroxide per minute at 25 °C. Enzyme activity is expressed as milliunits of activity per milligram wet weight tissue. Each bar represents mean ± S.E.M. ($n=10$ rats/group). Statistical analysis was conducted using one-way analysis of variance + Dunnett test: ★★★$P<0.001$ vs. TNBS vehicle and ■■$P<0.01$, ■■■$P<0.001$ vs. respective vehicles (i.e., TNBS + either ST1959 or 5-ASA vehicle).

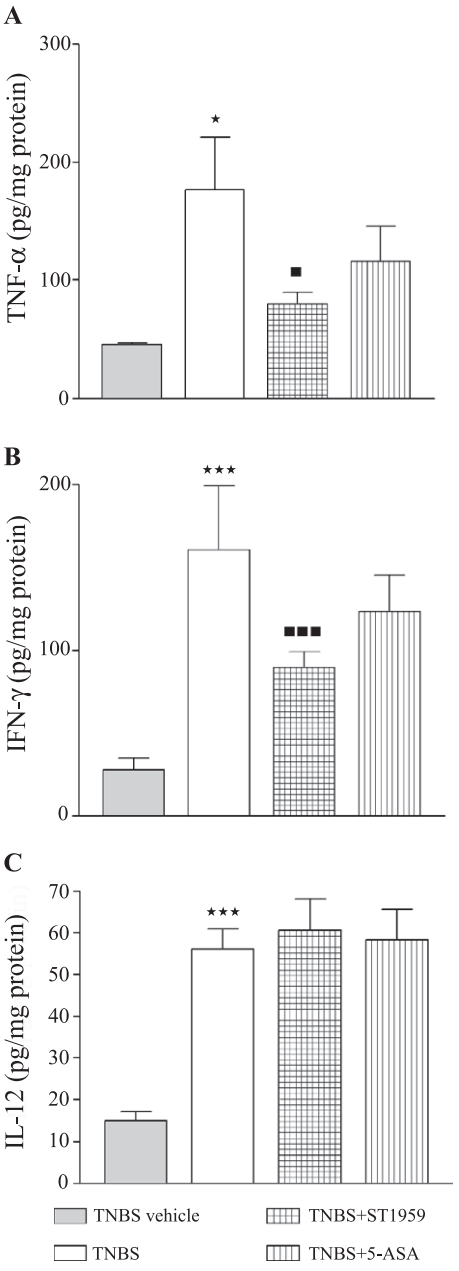

Fig. 3. Colonic levels of (A) tumour necrosis factor-α (TNF-α), (B) interferon-γ (IFN-γ), and (C) interleukin-12 (IL-12) 7 days after TNBS-induced colitis in rats. The drugs ST1959 (0.4 mg/kg in 2 ml/kg of sesame oil) or 5-ASA (120 mg/kg in 2 ml/kg of 1% CMC) was administered once daily rectally for 1 week starting 1 h after the induction of colitis with TNBS (30 mg in 0.25 ml of 50% ethanol). The rats were sacrificed 24 h after the last administration. Each bar represents mean ± S.E.M. ($n = 10$ rats/group). Levels of cytokines were assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Statistical analysis was conducted using one-way analysis of variance + Dunnett test (Fig. 3A) where $^{\star}P < 0.05$ vs. TNBS vehicle and $^{\blacksquare}P < 0.05$ vs. TNBS, and *t*-test (Fig. 3B) where $^{\star\star\star}P < 0.001$ vs. TNBS vehicle and $^{\blacksquare\blacksquare\blacksquare}P < 0.001$ vs. TNBS.

production (Fig. 3C). The colonic content of the anti-inflammatory cytokine interleukin-10 was increased approximately sixfold ($P < 0.05$) following treatment with ST1959 but was not appreciably modulated by 5-aminosalicylic acid (Fig. 4).

3.4. Histopathologic analysis

One week after TNBS-induced colitis, colonic sections taken from the centre of the lesion (Fig. 5) revealed that the colon wall was approximately four times thicker than in ethanol-treated animals (Fig. 5A). The mucosa was necrotic or completely absent. The submucosa accounted for 1/3 of the total wall thickness, and its superficial layer was characterized by a massive neutrophilic infiltration, whereas the deeper layer contained fibrinous exudate. The smooth muscle cells of the muscularis externa were spaced by neutrophils, and the distinction between the inner circular and the outer longitudinal layers was preserved. The serosa was thickened and contained sparse neutrophils, fibroblasts, and connective tissue mast cells. The latter, readily recognizable by the presence of red metachromatic granules, were irregularly gathered at spots.

The upper and lower edges of the lesion did not differ significantly from the centre except for a reduction in the thickness of the entire wall. Away from this region, there was a progressive attenuation of the effect of TNBS, even if the regions beneath the lesion were generally more severely affected.

In ST1959-treated rats, the wall thickness in the centre of the lesion was reduced down to 64% of TNBS-administered animals (Fig. 5C). In the regions located beneath the lesion, the mucosa was necrotic, and not only was the wall nearly

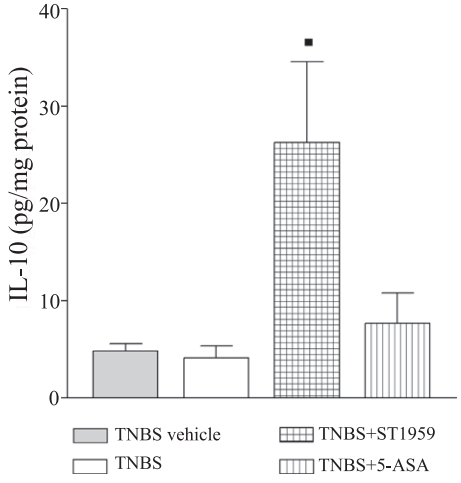

Fig. 4. Colonic levels of interleukin-10 (IL-10) 7 days after TNBS-induced colitis in rats. The drugs ST1959 (0.4 mg/kg in 2 ml/kg of sesame oil) or 5-ASA (120 mg/kg in 2 ml/kg of 1% CMC) was administered once daily rectally for 1 week starting 1 h after the induction of colitis with TNBS (30 mg in 0.25 ml of 50% ethanol). The rats were sacrificed 24 h after the last administration. Each bar represents mean ± S.E.M. ($n = 10$ rats/group). Levels of IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions. Statistical analysis was conducted using one-way analysis of variance + Dunnett test: $^{\blacksquare}P < 0.05$ vs. TNBS.

three times thicker than in control rats but also infiltrated with neutrophils. The regions above the lesion were almost normal, their walls being only twofold thicker than control rats due to a decreased inflammatory infiltration. Overall, administration of ST1959 for 1 week appeared to reduce the extent of the lesion and to ameliorate the histologic appearance of the mucosa and submucosa. At variance with ST1959, the histologic assessment showed that treatment with 5-aminosalicylic acid for 1 week did not appreciably affect the alterations induced by TNBS, and the amelioration of mucosal layer was minimal (Fig. 5B).

Two weeks after induction of colitis by TNBS, the alterations of the colonic wall in all the regions were not substantially different from those observed 1 week earlier, except for the thickness of the colonic wall that was reduced (Fig. 6A). In fact, the mucosa was either necrotic or completely absent, and the submucosa was infiltrated with neutrophils while the serosa was thickened and contained sparse neutrophils and fibroblasts. Treatment with ST1959 for 2 weeks (Fig. 6C) reduced the thickness of the wall almost to the values scored in ethanol-treated animals (Fig. 6D), and attenuated the microscopic damage. This effect was more dramatic in the centre of the lesion where the mucosa was largely restored. A small inflammatory infiltrate persisted in the submucosa. The other layers had a normal histologic appearance (Fig. 6D). In the animals treated with 5-aminosalicylic acid for 2 weeks (Fig. 6B), the appearance of the centre of the lesion was similar to the one observed in TNBS-administered rats (Fig. 6A). Moving away from this region, particularly in the proximal direction, the mucosal integrity was restored and the inflammatory infiltrate was reduced in all the layers (data not shown).

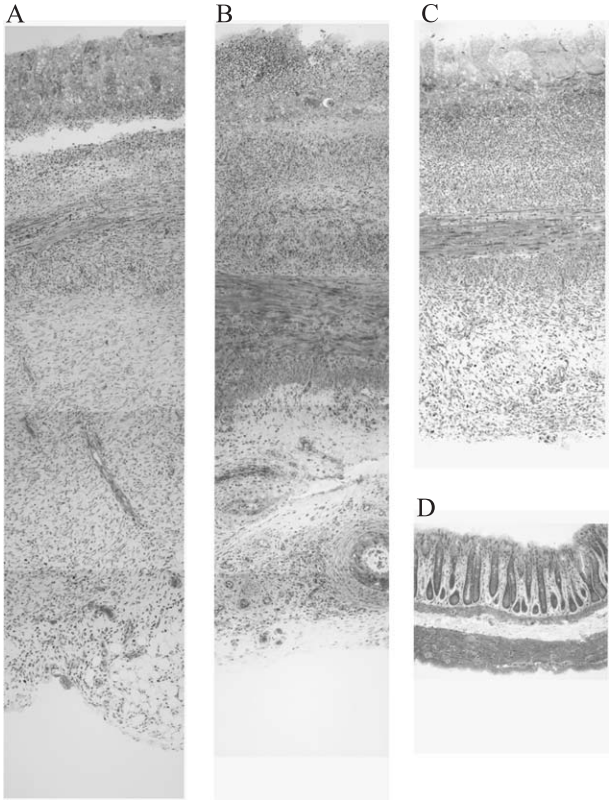

Fig. 5. Histological appearance of normal rat colon and of representative sections taken from the centre of the lesion of the colon 1 week after administration of TNBS. Pictures cover the entire wall of the colon. Thickness differs according to the treatment: (A) TNBS, (B) TNBS + 5-ASA, (C) TNBS + ST1959, and (D) TNBS vehicle (i.e., 50% ethanol). Original magnification × 100.

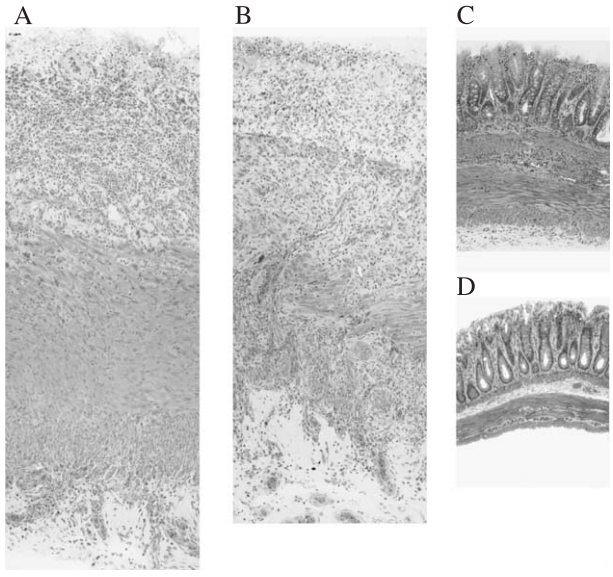

Fig. 6. Histological appearance of normal rat colon and of representative sections taken from the centre of the lesion of the colon 2 weeks after administration of TNBS. Pictures cover the entire wall of the colon. Thickness differs according to the treatment: (A) TNBS, (B) TNBS + 5-ASA, (C) TNBS + ST1959, and (D) TNBS vehicle (i.e., 50% ethanol). Original magnification × 100.

4. Discussion

This study demonstrates that treatment with ST1959 is able to attenuate the severity and the degree of the acute colonic damage induced by ethanolic TNBS in the rat, an experimental model that mimics human Crohn's disease (Morris et al., 1989). After 1 week of treatment, the ST1959-induced decrease in the extent of colitis was paralleled by a reduction of colonic weight, an increase of body weight, and a significant lower incidence of diarrhoea and adhesions. The presence of adhesions is consequent to transmural inflammation and is a common feature of TNBS-colitis (Morris et al., 1989). Therefore, the observed reduced incidence of adhesions is indicative of the beneficial effects of ST1959 in this experimental model of colitis. These beneficial effects were even more evident and significantly greater than those observed with 5-aminosalicylic acid after 2 weeks of treatment.

With regard to tissue damage, 1 week following induction of colitis, the damaged area accounted for approximately 40% of the total area. Histological analysis showed a

fourfold increase in wall thickness, epithelial loss, and submucosal infiltration by neutrophils. Neutrophils have been suggested to contribute significantly to tissue injury and mucosal dysfunction in human inflammatory bowel disease as well as in animal models of colitis (Yamada et al., 1991; Guo et al., 1999) by virtue of their ability to release a panoply of toxic mediators and to disrupt the epithelial barrier when they migrate towards the lumen (Nash et al., 1987). Suppression of neutrophil function was shown to reduce tissue damage (Palmen et al., 1995). Tissue levels of the neutrophil myeloperoxidase are used as a quantitative measure of neutrophil inflammatory response in a variety of clinical and experimental settings (Mullane et al., 1985). Our findings indicate that the level of colonic myeloperoxidase activity was increased in TNBS control animals, and that ST1959 significantly reduced the rise in myeloperoxidase activity after 1 and 2 weeks of treatment, thus suggesting an anti-inflammatory effect of the compound under investigation. These data were also paralleled by the histological findings showing ameliorating effects of ST1959, as evidenced by a restoration of the normal histologic appearance of the mucosa and submucosa, mostly after 2 weeks of treatment. Five-aminosalicylic acid at 120 mg/kg, known to have beneficial effects in inflammatory bowel disease (Martinsson et al., 1999), was used as a control drug. At this dose, 5-aminosalicylic acid slightly affected TNBS-induced myeloperoxidase activity. However, the degree of the inhibitory effect of 5-aminosalicylic acid assessed after 1 week of treatment is in agreement with the one (30%) reported by Martinsson et al. (1999). Unbalanced production of Th1/Th2 cytokines is pivotal in the establishment of mucosal inflammatory diseases (Neurath et al., 2002). Among Th1 cytokines, tumour necrosis factor-α is thought to play a crucial role in the pathogenesis of TNBS-induced colitis (Villegas et al., 2003; Cuzzocrea et al., 2001). Tumour necrosis factor-α is endowed with marked chemotactic activity and induces expression of adhesion molecules and other inflammatory cytokines, such as interferon-γ, which directly or indirectly leads to mucosal injury. Actually, interferon-γ was found to be highly expressed in colonic specimens of patients with Crohn's disease (Camoglio et al., 1998).

Therefore, the significant decrease of local production of tumour necrosis factor-α and interferon-γ following 1 week of treatment with ST1959 may be regarded as a key mechanism involved in the reduction of colonic inflammation. It is of remarkable interest our observation that besides down-modulating these inflammatory cytokines, ST1959 affected the production of the anti-inflammatory cytokine interleukin-10, one of the most important immunomodulatory cytokines. Previous studies convincingly showed that human interleukin-10 was able to down-regulate transcription and secretion of tumour necrosis factor-α by activated monocytes and macrophages (De Waal Malefyt et al., 1992; Schreiber et al., 1995), as well as to strongly inhibit production of interferon-γ by Th1 cells (Fiorentino et al., 1989). Additional studies showed that continuous interleukin-10 administration in hapten-induced colitis in rats was therapeutically efficacious in significantly reducing myeloperoxidase activity, and this effect was paralleled by an attenuation of colonic tumour necrosis factor-α content (Ribbons et al., 1997). Moreover, a very recent study investigated the efficacy of an adenoviral vector encoding interleukin-10 (*AdvmuIL-10*) in TNBS-induced colitis in mice, reporting that the therapeutic efficacy of *AdvmuIL-10* was associated with a decrease in the interferon-γ levels detected in colonic homogenates (Lindsay et al., 2002).

In the light of these results, it would be tempting to speculate that the beneficial effects of ST1959 in TNBS-induced colitis may be consequent to its ability to up-regulate interleukin-10 synthesis, which in turn may be crucial in dampening the production of proinflammatory cytokines, such as tumour necrosis factor-α and interferon-γ, and possibly other inflammatory mediators, such as chemokines in the intestinal mucosa. Actually, ST1959 similarly and strikingly reduced both TNF-α/IL-10 and IFN-γ/IL-10 ratios (values ∼ 3) compared with TNBS alone (values ∼ 40), suggesting a shift toward anti-inflammatory cytokine production by ST1959. A similar effect, although less evident, was observed with 5-aminosalicylic acid (values ∼ 15). Overall, these effects may consecutively lead to a reduced activation and infiltration of neutrophils in the colonic mucosa. To further clarify this issue, future studies are warranted by using an interleukin-10 knockout mouse model recently developed by Feldmann's research team (Scheinin et al., 2003). These knockout mice develop a disease closest to Crohn's disease, while the other resemble more closely ulcerative colitis.

Despite the significant inhibitory effects of ST1959 on tumour necrosis-α and interferon-γ, the compound was not effective at modulating interleukin-12 levels in the colonic mucosa. Interleukin-12 is pivotal in polarizing Th1 response in the lamina propria and plays a crucial role in TNBS-induced colitis in the SJL murine strain, in which massive infiltration of neutrophils is rapidly reverted by systemic administrations of anti-interleukin-12 (Neurath et al., 1995). Moreover, interleukin-12 would seem to directly affect the viability of Th1 cells, because administration of anti-interleukin-12 to mice with TNBS-induced colitis leads to apoptotic cell death of Th1 lymphocytes (Fuss et al., 1999). If this sequence of events holds good in rats, one would anticipate a prominent increase in colonic interleukin-12. In our experiments, conversely, interleukin-12 levels were not greatly increased in TNBS-administered rats, and both ST1959 and 5-aminosalicylic acid were unable to affect interleukin-12 production. These results are reminiscent of the findings reported by Lienenluke et al. (2001) who found that albeit thalidomide and supimide significantly attenuated TNBS-induced colitis in rats, interleukin-12 expression was essentially unaffected by both drugs.

In conclusion, our results indicate that administration of ST1959 at a dose comparable with the ones effective in several experimental settings (Mistrello et al., 1985) is also

protective in experimental colitis. The ameliorating effects seem to be ascribable to an impairment of both neutrophil infiltration/activation and tumour necrosis factor-α, and interferon-γ production, possibly consequent to the observed increase in the colonic tissue levels of the potent anti-inflammatory cytokine interleukin-10. The overall effect of such modulations underlies the accelerated reparative process of colon injury.

The findings reported in this study suggest that ST1959 may be regarded as a key compound in the development of novel drugs that can be beneficial adjuncts in the treatment of inflammatory disorders. Inhibition of chronic intestinal inflammation by specifically blocking one cytokine may be difficult given the redundancy of the inflammatory response (Sartor, 1994). In this regard, the ability of a single pharmacological agent to skew the cytokine response toward an anti-inflammatory profile may provide therapeutic utility to patients suffering from recurrent inflammatory flares and supports the potential use of such a compound for treatment of inflammatory Th1-driven diseases such as Crohn's disease. Although a previous report (Yang et al., 2000) demonstrated that of 0.82 mg/kg ST1959 for four consecutive days was able to terminate early pregnancy in rats when administered by subcutaneous route, it must be underlined that the dosage utilized in this study not only was lower (0.4 mg/kg) but, most importantly, was administered by a different route, namely, rectal instillation.

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