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• *RAPID COMMUNICATION* •

Favorable response to subcutaneous administration of infliximab in rats with experimental colitis

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Abstract

AIM: To investigate the influence of infliximab (Remicade) on experimental colitis produced by 2,4,6,trinitrobenzene sulfonic acid (TNBS) in rats.

METHODS: Thirty-six Wistar rats were allocated into four groups (three groups of six animals each and a fourth of 12 animals). Six more healthy animals served as normal controls (Group 5). Group 1: colitis was induced by intracolonic installation of 25 mg of TNBS dissolved in 0.25 mL of 50% ethanol and infliximab was subcutaneously administered at a dose of 5 mg/kg BW; Group 2: colitis was induced and infliximab was subcutaneously administered at a dose of 10 mg/kg BW; Group 3: colitis was induced and infliximab was subcutaneously administered at a dose of 15 mg/kg BW; Group 4: colitis was induced without treatment with infliximab. Infliximab was administered on d 2–6. On the 7th d, all animals were killed. The colon was fixed in 10% buffered formalin and examined by light microscopy for the presence and activity of colitis and the extent of tissue damage. Tumor necrosis factor-alpha (TNF-α) and malondialdehyde (MDA) were also measured.

RESULTS: Significant differences concerning the presence of reparable lesions and the extent of bowel mucosa without active inflammation in all groups of animals treated with infliximab compared with controls were found. Significant reduction of the tissue levels of TNF-α in all groups of treated animals as compared with the untreated ones was found (0.47±0.44, 1.09±0.86, 0.43±0.31 *vs* 18.73±10.53 respectively). Significant reduction in the tissue levels of MDA was noticed in group 1 as compared to group 4, as well as between groups 2 and 4.

CONCLUSION: Subcutaneous administration of infliximab reduces the inflammatory activity as well as tissue TNF-α and MDA levels in chemical colitis in rats. Infliximab at a dose of 5 mg/kg BW achieves better histological results and produces higher reduction of the levels of TNF-α than at a dose of 10 mg/kg BW. Infliximab at a dose of 5 mg/kg BW produces higher reduction of tissue MDA levels than at a dose of 15 mg/kg BW.

Key words: Experimental colitis; Infliximab; Inflammatory bowel disease; Tumor necrosis factor-alpha; Malondialdehyde; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis is a chronic relapsing inflammatory condition involving the large bowel of unknown etiology. Clinical manifestations are considered to be the result of an imbalance between proinflammatory and inflammatory cytokines, resulting in inflammation and clinical symptoms. Activated T-lymphocytes release cytokines, thereby recruiting a large number of inflammatory cells in the mucosa. Activation of these cells causes further production of cytokines, cell recruitment and inflammation. In addition to cytokines, leukotrienes, thromboxane, and reactive oxygen species are released from activated mucosal cells[1]. This uncontrolled immune system activation results in the sustained overproduction of reactive metabolites of oxygen and nitrogen[2]. It has been suggested that self-sustaining cycles of oxidant formation may amplify flare-ups of inflammation and mucosal injury in ulcerative colitis[3]. Treatment of ulcerative colitis includes a wide range of anti-inflammatory and immunosuppressant drugs

with satisfactory results.

TNF-α is a pleiotropic cytokine with important proinflammatory and immunomodulatory properties. This cytokine plays a significant role in a number of inflammatory disorders including inflammatory bowel disease[4]. It has been shown that administration of the chimeric anti-TNF-α antibody in patients with active Crohn's disease results in a dramatic improvement of many clinical and laboratory parameters[5,6]. One of the most striking findings of the initially performed clinical trials is the observation that infliximab administered at a dose of 5 mg/kg BW results in better patients' improvement than at the dose of 10 or 15 mg/kg BW. Infliximab has also been administered in severe ulcerative colitis patients with promising results[7-9], though the clinical benefit is not prominent in patients refractory to previous administration of steroids[10,11]. Experimental evidence suggests that TNF-α may also play a role in the pathogenesis of experimental colitis[12].

The aim of this study was to investigate the influence of infliximab on experimental colitis in rats, produced by TNBS and to estimate its influence on the oxidative stress accompanying this model of colitis.

MATERIALS AND METHODS

The experimental procedures described below were approved by the Animal Care Committee according to the European Union Act and Greek Law 160, A-64, May, 1991.

General preparation

Adult male Wistar rats weighing 200-240 g were allowed to adapt to our laboratory conditions 1 wk prior to the experiment. They were housed individually in cages at a constant temperature (22 °C) and in a 12-h d/night cycle with free access to food and water. A total number of 36 rats were used. They were randomly allocated into five groups. *Group 1*: experimental colitis was induced and infliximab was subcutaneously administered at a dose of 5 mg/kg BW ("Infliximab 5"); *Group 2*: experimental colitis was induced and infliximab was subcutaneously administered at a dose of 10 mg/kg BW ("Infliximab 10"); *Group 3*: experimental colitis was induced and infliximab was subcutaneously administered at a dose of 15 mg/kg BW ("Infliximab 15"); *Group 4*: experimental colitis was induced without treatment with infliximab (12 animals) ("Untreated"). Six more healthy animals served as controls (Group 5). On the 7th d, all animals were killed and the colon was removed. The same part of rat's colon was used for histology as well as for MDA and TNF-α estimation.

Induction of experimental colitis

Distal colitis was induced by intracolonic installation of 25 mg of TNBS dissolved in 0.25 mL of 50% ethanol. The solution was injected into the colon 8 cm proximal to the anus with a PE-50 cannula. In order to ensure that TNBS-ethanol solution was not immediately expelled by the rat, the cannula was left in place for 15 s prior to its removal.

Drug administration

Infliximab was administered subcutaneously only in groups 1, 2, and 3 on d 2-6 at the doses of 5, 10 and 15 mg/kg BW, respectively. The subcutaneous administration has not previously been tried in both men and animals. We chose to administer the drug for five consecutive d and not to follow the usual scheme of administration of infliximab in patients with Crohn's disease because we were unable to predict the exact serum levels of the drug following the subcutaneous administration. A second reason was the fact that in this model of experimental colitis, the recommended time to kill the animals was 7 d. Following two or more weeks, the macro- and microscopic lesions were usually not detectable in the survived animals. Healthy control animals were given a subcutaneous dose of normal saline from d 2 to 6.

Histology

Specimens were fixed in 10% buffered formalin and embedded in paraffin blocks (3-4 blocks for each case). Then hematoxylin-eosin stained sections were blindly examined by two pathologists. In each case, the extent of lesions (expressed as a percentage of tissue damage of the whole bowel length) was estimated. The histological lesions such as active ulcers and erosions and reparable lesions (newly re-epithelized lesions or granulation tissue beneath cylindrical epithelium) were estimated. The extent of mucosa without signs of active inflammation was also estimated as previously described[13-16].

Tissue malondialdehyde (MDA) estimation

The MDA measurement was based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole (MPI), with MDA at 45°C. One molecule of MDA reacted with two molecules of MPI to yield a stable chromophore with maximum absorbance at 586 nm. The reagents used included Reagent MPI, 10.3 mmol/L *N*-methyl-2-phenylindole in acetonitrile, MDA standard, 10 mmol/L 1,1,3,3-tetramethoxypropane in 20 mmol/L Tris-HCl, 500 mmol/L butylated-hydroxytoluene, in acetonitrile, 20 mmol/L Tris buffer pH 7.4, 0.9% NaCl, 37% (12 mol/L) HCl, methanol, HPLC grade, acetonitrile and HPLC grade. Before the procedure, three volumes of the MPI reagent were diluted with one volume of 100% methanol. Tissue samples were rinsed with ice-cold isotonic saline before homogenization which was carried-out using Tris buffer 20 mmol/L pH 7.4 and an ULTRA-TURRAX (IKA-Labortecnik) blender. One milliliter buffer was used for 0.1 g of tissue. Ten milliliters of 500 mmol/L BHT was added to 1 mL of tissue homogenate to prevent sample oxidation. The homogenate was centrifuged at 3 000 r/min at 4 °C for 10 min. Then 0.2 mL of sample (plasma or supernatant of tissue homogenate) and 0.65 mL of diluted MPI reagent were added to a polypropylene microcentrifuge tube. The mixture was vortexed and then 0.15 mL of 12 mol/L HCl was added. Tubes were incubated at 45 °C for 60 min and centrifuged at 6 000 r/min.

Table 1 Comparison of histological lesions in treated and untreated groups of animals (mean±SD)

Group	Active ulcers and erosions	*P* value	Reparative lesions	*P* value
1 (Infliximab 5, *n* = 6)	0.07±0.12	0.30	0.38±0.12	0.0001
2 (Infliximab 10, *n* = 6)	3.77±5.07	0.42	0.80±0.14	0.0001
3 (Infliximab 15, *n* = 6)	25.02±38.90	1.00	1.00±0.47	0.0001
4 (Untreated, *n* = 12)	25.00±38.91		15.00±5.48	

Table 2 Percentage of bowel area without active inflammation in treated and untreated groups of animals (mean±SD)

Group	Percentage of mucosa without active inflammation	*P* value
1 (Infliximab 5)	99.55±0.19	0.049
2 (Infliximab 10)	95.43±5.15	0.088
3 (Infliximab 15)	73.98±39.29	0.705
4 (Untreated)	60.00±37.18	

Table 3 Tissue TNF-α levels in treated and untreated groups of animals (mean±SD)

Group	Tissue TNF-α (pg/mL)	*P* value
1 (Infliximab 5)	0.47±0.44	<0.0001
2 (Infliximab 10)	1.09±0.86	<0.0001
3 (Infliximab 15)	0.43±0.31	<0.0001
4 (Untreated)	18.73±10.53	
5 (Healthy animals)	0.00+/–0.0	

Table 4 Mean value of tissue malondialdehyde in treated and untreated groups of animals (mean±SD)

Group	Serum malondialdehyde (μmol/l)	*P* value
1 (Infliximab 5)	1.85±0.20	0.017
2 (Infliximab 10)	1.84±0.37	0.011
3 (Infliximab 15)	2.29±0.56	0.272
4 (Untreated)	2.73±0.46	
5 (Healthy animals)	1.11+/–0.19	

for 15 min. Then 0.8 mL of the supernatant was measured at 586 nm. MDA standards for the standard curve were made by dilutions of the stock 10 mm TMOP solution. The final concentrations were 2.08, 4.16, 8.33, 12.5 and 16.66 μmol/L and the assay procedure was followed as for the samples. The absorbance was 0.059, 0.124, 0.264, 0.4, and 0.545 respectively.

Tissue TNF-α estimation

TNF-α was determined after tissue homogenization by ELISA. In order to avoid errors in the interpretation of results, a specific rat antibody was used (antirat, DIACLONE Research) instead of human antibody against TNF-α.

Statistical analysis

Data were presented as mean±SD. Statistical comparisons between groups were made by one-way ANOVA followed by Dunnett's (two-sided) test. A difference between treated (1-3 groups) and untreated (group 4) animals was considered statistically significant at the level of $P<0.05$. Computations were done using the statistical package SPSS (version 11.0).

RESULTS

Histology

Table 1 shows the percentage of bowel area with the presence of active ulcers and erosions and reparative lesions observed in the treated and untreated groups of animals. Significant differences concerning the presence of reparable lesions between all groups of animals treated with infliximab compared to the untreated ones were found. Though differences were obvious between groups 1 and 2, they did not reach statistical significance (Table 1).

Table 2 shows the extent of mucosa without active inflammation in all groups of animals expressed as a percentage (mean value). Significant differences between the three groups of treated animals compared to the untreated ones were observed.

TNF-α tissue levels (pg/mL) (mean value)

The levels of tissue TNF-α in all groups are shown in Table 3. A significant reduction of the tissue levels of TNF-α was found in all groups of treated animals compared to the untreated ones.

Tissue malondialdehyde levels (μM)

The levels of tissue MDA in the treated and untreated animals are shown in Table 4. A significant reduction was noticed in group 1 compared to group 4, as well as between groups 2 and 4. No significant differences between groups 3 and 4 were noticed.

DISCUSSION

The findings of this experimental study in rats suggested that subcutaneous administration of infliximab was biologically effective; infliximab at doses of 5, 10, and 15 mg/kg BW could reduce the histological changes as observed in this particular experimental model; infliximab could significantly reduce tissue levels of TNF-α and MDA; suggesting that this molecule has probably antioxidant properties though the latter could be the consequence of its anti-inflammatory action.

In more details, subcutaneous administration of infliximab resulted in a significant amelioration of inflammatory histological lesions, a finding which was more prominent in the group of animals receiving 5 mg/kg per d. Administration of the drug resulted in a statistically significantly smaller area of large bowel with reparable lesions compared to the untreated group of animals, although the percentage of bowel area with active ulcers and erosions did not differ significantly between treated and untreated animals. It was also shown that the percentage of bowel area with normal mucosa was

significantly larger only in the group treated with 5 mg/kg BW of infliximab as compared to untreated animals. The percentage of bowel area with normal mucosa did not differ significantly between groups receiving 10 and 15 mg/kg BW and the untreated group of animals. Wooddruff *et al*[17] also showed that a single IV dose of infliximab before the induction of experimental colitis results in a significant reduction of the severity of the lesions.

In our study, all doses of infliximab significantly reduced the tissue levels of TNF-α, suggesting that TNF-α plays a significant pathogenetic role in this model of colitis as well. The IV dose of 5 mg/kg BW is the currently recommended one for the treatment of patients with active Crohn's disease. The beneficial effect of infliximab observed in this experimental model is in accordance with clinical observations showing beneficial clinical effects on some patients with severe ulcerative colitis[7-9]. TNF-α is a 17-ku proinflammatory cytokine produced by monocytes, macrophages, and T cells. The biological actions of this cytokine include induction of acute-phase response, cachexia, and potentially lethal shock[18]. Furthermore, TNF-α stimulates secretion of IL-1 and IL-6, expression of adhesive molecules and fibroblast proliferation. Release of TNF-α is mediated by a specific metalloproteinase (TNF-α convertase). After secretion, TNF-α binds as a soluble ligand to two cell-bound transmembrane TNF receptors, namely TNFR1 and TNFR2[19]. Chronic inflammation in Crohn's disease can be attributed mainly to the production of proinflammatory cytokines, especially TNF-α. This cytokine is considerably increased in the histologically normal as well as inflamed large bowel mucosa of patients with Crohn's disease. It has been described that thalidomide, a drug with well-known anti-TNF-α action, significantly reduces colonic inflammation induced by iodoacetamide, probably via the inhibition of TNF-α[20]. This experiment is another paradigm of amelioration of colitis by a drug with inhibitory influence of TNF-α.

An important finding of this study is the increased tissue levels of tissue MDA in the untreated rats and the reduced tissue levels of MDA in the groups treated with 5 and 10 mg/kg BW. However, the animals treated with 15 mg/kg BW did not show any statistically significant difference from the untreated ones. There is no obvious explanation for that, though it seems that the reduction of MDA levels could not be a phenomenon related to quantity of the reactive elements. We must emphasize that the results of the 5 and 10 mg/kg BW administration of infliximab on MDA levels were quite similar with the results concerning the corresponding histological lesions. We suppose that the reduction in lipid peroxidation and cellular damage originating from oxidative stress following the administration of infliximab is an important factor contributing to amelioration of experimental colitis. A growing number of data suggests that in experimentally-induced colitis, the colon may be subjected to considerable oxidative stress[21]. Oxidative stress leads to the extension and propagation of crypt abscesses either through direct membrane disruption by lipid peroxidation or through generation of secondary toxic oxidants such as chloramines. Subsequently, chemotactic products of lipid peroxidation provide positive feedback to accelerate the inflammatory/oxidative process[22]. Colonic mucosa may be overwhelmed during active inflammation resulting in intestinal inflammation due to the inability of the mucosa to ameliorate the generating stress because of the small amount of antioxidant enzymes contained in it. It could be possible that colonic injury and dysfunction observed in inflammatory bowel disease are due to the elaboration of these reactive species. Infliximab may have antioxidant properties as well, as it can be suggested by the significant reduction of MDA levels observed in all treated groups of animals though this could simply be the consequence of its anti-inflammatory action.

Another point of interest of this study is the fact that subcutaneous administration of the drug resulted in the reduction of inflammation and tissue damage. The recommended route of administration of infliximab is the IV route. The beneficial effect observed in this model suggests that the other routes of administration of the drug could be effective in human beings as well.

In conclusion, the results of the present study suggest that subcutaneous administration of infliximab reduces the inflammatory activity, as well as tissue TNF-α and MDA levels in chemical colitis in rats. Moreover, infliximab at a dose of 5 mg/kg BW achieves better histological results and produces higher reduction of the levels of TNF-α than at a dose 10 mg/kg BW. Finally, infliximab at a dose of 5 mg/kg BW produces higher reduction of tissue MDA levels than at a dose of 15 mg/kg BW. The administration of infliximab in rats with chemical colitis supports the clinical observations that the dose of 5mg/kg BW produces better results than the dose of 10 or 15 mg/kg BW. The possible antioxidant properties of infliximab must be further investigated both from clinical and experimental points of view.

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