THALIDOMIDE TREATMENT REDUCES COLON INJURY INDUCED BY EXPERIMENTAL COLITIS

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ABSTRACT—The immunological and genetic pathogeneses of inflammatory bowel disease (IBD) have been well studied but not well elucidated in the recent years. Accordingly, the pharmacological treatment of IBDs is focusing upon the individual pathologic step (targeting therapy). It has been shown recently that new drugs such as biological immunomodulating agents and anti-inflammatory cytokines have better short-term effects in some respects than the conventional drugs, and they might change the treatment strategy of IBDs in the near future. The aim of the present study was to examine the effects of thalidomide treatment in the development of experimental colitis. To address this question, we used an experimental model of colitis, induced by dinitrobenzene sulfonic acid (DNBS). DNBS-treated mice experienced diarrhea and weight loss. At 4 days after administration of DNBS, the mucosa of the colon exhibited large areas of necrosis. The observed mucosa alteration was associated with the colon production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and vascular endothelial growth factor (VEGF). Neutrophil infiltration (determined by histology as well as an increase in myeloperoxidase activity in the mucosa) was associated with an upregulation of intercellular adhesion molecule-1. Immunohistochemistry for nitrotyrosine and poly (ADP ribose) showed an intense staining in the inflamed colon. When compared with DNBS-treated mice, thalidomide-treated (200 mg/kg orally) mice subjected to DNBS-induced colitis experienced a significantly lower rate in the extent and severity of the histological signs of colon injury. Thalidomide also caused a substantial reduction of the rise in myeloperoxidase activity (mucosa), in the increase in the tissue levels of TNF-α, IL-1β, and VEGF, in the increase in staining (immunohistochemistry) for nitrotyrosine and for poly (ADP ribose), as well as in the upregulation of intercellular adhesion molecule-1 caused by DNBS in the colon. Thus, thalidomide treatment reduces the degree of colitis caused by DNBS. We propose that this evidence may help to clarify the therapeutic actions of thalidomide in patients with Crohn’s disease.

KEYWORDS—Colitis, thalidomide, PARP, inflammatory infiltration, ICAM-1

INTRODUCTION

The inflammatory bowel diseases (IBDs) Crohn’s disease and ulcerative colitis have become important health problems in recent years. With an actual prevalence of 200 to 500 per 100,000 people in Western countries and a world incidence of about 20 per 100,000 people, the prevalence in high-incidence areas almost doubles every 10 years. Indeed, both are diseases of a lifetime, affecting people at a young age. In the last decade, there has been a shift toward more Crohn’s disease in areas with a high incidence of IBD, and IBD has tended to occur at all ages. Substantial progress has been made in characterizing immune cell populations and inflammatory mediators in patients with IBD and murine models. It is well accepted that the mucosa of patients with established Crohn’s disease is dominated by CD4+ lymphocytes with a type 1 helper (Th1) T cell phenotype (1–4), characterized by the production of interferon (IFN)-γ, interleukin (IL)-2, tumor necrosis factor (TNF)-α (5) and activation of nuclear factor (NF)κB (6). In contrast, the mucosa in patients with ulcerative colitis may be dominated by CD4+ lymphocytes with an atypical type 2 helper (Th2) T cell phenotype, characterized by the production of transforming growth factor (TGF)-β and IL-5, but not IL-4 (7). In this particular instance, the characteristics of the immune response and cytokine production are under genetic regulation, and the resultant inflammation is also specific. In contrast, the pattern of cytokines produced in the initial lesion differs from that in the developed chronic lesion. Concerning Th1/Th2 balance, Th1 response is reportedly predominant in patients with Crohn’s disease (8, 9). In addition, it has been demonstrated that macrophages play a main role in the formation of nongaseous epithelioid granuloma in the intestinal mucosa, which is the histopathological characteristic of Crohn’s disease, and is also involved in the mucosal immune response (10). Macrophages are activated by IFN-γ, which is produced by the activated Th1 cells previously mentioned. Activated macrophages produce cytokines such as TNF-α, IL-6, IL-8, etc. The biological action of TNF-α and IL-6 is the main factor in the pathogenesis of Crohn’s disease, and the regulation of this process is very important in controlling the disease (11). In addition, recruitment of inflammatory cells from the circulation is an important process in augmenting inflammatory response (12). TNF-α and IL-6 induce the expression of adhesion molecules in the vascular endothelium, and invasion of inflammatory cells into the mucosal layer subsequently occurs. Selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cellular adhesion molecule-1 (VCAM-1), which are expressed at the surface of the vascular endothelium, are involved in this process (13). Various mediators contribute to the upregulation of endothelial cell and leukocyte adhesion molecules in inflammation. Several

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mediators, including growth factors (e.g., vascular endothelial growth factor, VEGF), cytokines (e.g., TNF-α), and serine proteases (e.g., thrombin), activate gene transcription in endothelial cells, resulting in changes in haemostatic balance, increased leukocyte adhesion, loss of barrier function, increased permeability, migration, proliferation, and successive angiogenesis. The tight control of these processes is essential for homeostasis, endothelial cell activation, and if excessive, sustained, or spatially and temporally misplaced, may result in vasculopathic disease. Under normal conditions, the activation signal may be terminated by negative feedback inhibition of downstream transcriptional networks. Such a mechanism has been well established for TNF-α (14). In contrast, little is known about the major self-regulatory processes involved in VEGF and IBD. VEGF is an endothelial cell-specific mitogen and chemotactic agent that is involved in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, hemostasis, and endothelial cell survival (15, 16). Because ulceration and regeneration of the intestinal epithelium occurs during the course of the disease, angiogenesis and increased cell metabolism are integral to the pathology of IBD. Indeed, some serological studies have suggested that serum concentrations of VEGF, a potent angiogenic factor, are raised in patients with IBD (17, 18).

Increasingly, evidence suggests that TNF-α is an important mediator in the pathogenesis of Crohn’s disease. Recently, the availability of the prototypical anti-TNF agents has offered an important advance in the therapy for patients with Crohn’s disease. Immune response-modulating drugs such as thalidomide, which is capable of inhibiting bacterial lipopolysaccharide (LPS)-stimulated IL-12 and TNF-α synthesis in monocytes (19, 20), may affect neutrophil infiltration, angiogenesis, and the inflammatory process in chronic IBD. In fact, recent pilot trials suggest that thalidomide treatment exerts beneficial effects in different types of colitis, including Crohn’s disease (21, 22).

To verify that thalidomide exerts this beneficial therapeutic effect by interfering with neutrophil infiltration, angiogenesis, and the release of proinflammatory mediators (e.g., reactive oxygen species and TNF-α), we have investigated the effects of thalidomide on the degree of colonic injury, the rise in myeloperoxidase (MPO) activity (mucosa), the production of TNF-α and IL-1β (colon levels), the increase in staining (immunohistochemistry) for nitrotyrosine, the increased expression of ICAM-1, and the release of VEGF caused by dinitrobenzene sulfonic acid (DNBS) in the colon.

**MATERIALS AND METHODS**

**Animals**

The study was carried out in 6-8-week-old (20-25 g) CD1 male mice (Charles River, Calco, Italy). The animals were housed in a controlled environment and were 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 purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

**Experimental groups**

Four groups of mice were used in the experiment. The first group (n = 10) received an equal volume of vehicle (0.5 mL of olive oil) instead of thalidomide (DNBS+ vehicle group). The second group (n = 10) received thalidomide (200 mg/kg) suspended in 0.5 mL of olive oil and administered once daily via intragastric instillation starting 30 min after the administration of DNBS (DNBS+ thalidomide group). In the third group of animals (n = 10), the effect of thalidomide was tested in a group of animals that received an intracolonic administration of 50% ethanol alone (sham + thalidomide group). The fourth group the animals, which received an intracolonic administration of 50% ethanol alone, was treated with the vehicle (0.5 mL of olive oil) instead of thalidomide ( sham + vehicle group). The dosage of thalidomide regimen was chosen in analogy with therapeutic effects in a rat model of colitis (23).

**Induction of experimental colitis**

Colitis was induced with a very low dose of DNBS (4 mg per mouse) by using a modification (24) of the method first described in rats (25). In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized with Enfлуurate. DNBS (4 mg in 100 μL of 50% ethanol) was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Carrier alone (100 μL of 50% ethanol) was administered in control experiments. Thereafter, the animals were kept for 15 min in a Trendelenburg position to avoid reflux. After colitis and sham-colitis induction, the animals were observed for 3 days. On day 4, the animals were weighed and anaesthetized with chloral hydrate, and 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 histological and immunohistochemistry. Colon damage (macroscopic damage score) was evaluated and scored by two independent observers as described previously (24, 26, 27) according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon; and 5-8, one point is added for each centimeter of ulceration beyond an initial 2 cm.

**Light microscopy**

After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, and 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 hematoxylin and eosin and trichrome von Gieson’s stain, and observed in a Leitz 22 Leitz (Wetzlar, Germany) microscope. To have a quantitative estimation of colon damage, sections (n = 6 for each animals) were scored by two independent observers blinded to the experimental protocol. The following morphological criteria were considered: score 0, no damage; score 1 (mild), focal epithelial edema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa; and score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage.

**MPO activity**

MPO activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (28). At 4 days after intracolonic injection of DNBS, the colon was removed and weighed. The colon was homogenized in 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and was centrifuged for 30 min at 20,000g 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 H2O2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μM peroxide per min at 37°C and was expressed in milliunits per gram weight of wet tissue.

**Measurement of TNF-α, IL-1β, and VEGF**

Portions of terminal colon, collected at 4 days after intracolonic injection of DNBS, were homogenized as previously described (29) in phosphate-buffered saline (PBS) containing 2 mM phenyl-methyl sulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and tissue levels of TNF-α, IL-1β, and VEGF were evaluated. The assay was carried out by using a colorimetric, commercial kit (R&D Systems, Milan, Italy) according to the manufacturer’s instructions. All TNF-α, IL-1β, and VEGF determinations were performed in duplicate serial dilutions.

**Localization of nitrotyrosine, ICAM-1, nitrotyrosine TNF-α, and IL-1β by immunohistochemistry**

Four days after the somministration of DNBS, the tissues were fixed in 10% PBS-buffered formaldehyde, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched by incubating the section in 2% normal goat serum in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin- or avidin-binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). Sections were incubated overnight with antinitrotyrosine rabbit polyclonal antibody (1:500 in PBS), with anti-ICAM-1...
polyclonal antibody (CD54; 1:500 in PBS, v/v) (DBA), with anti-TNF-α polyclonal antibody (1:100 in PBS, v/v), or with anti-IL1-β polyclonal antibody (1:100 in PBS, v/v; Santa Cruz Biotechnologies, Santa Cruz, CA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse immunoglobulin (Ig) G and avidin-biotin peroxidase complex (DBA). To verify the binding specificity for ICAM-1, TNF-α, or anti-IL1-β, some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments carried out. To confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (antinitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.

Immunocytochemistry photographs (n = 5) were assessed by densitometry by using Optilab Graflak software on a Macintosh personal computer.

**Reagents**

Thalidomide was obtained from Tocris (Bristol, UK). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA). Primary antinitrotyrosine antibody was purchased from Upstate Biotech (Lake Placid, NY). Primary ICAM-1 (CD54) for immunohistochemistry was purchased from BD PharMingen (San Diego, CA). Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories. All other reagents and compounds used were obtained from Sigma Chemical.

**Statistical analysis**

All values in the figures and text are expressed as mean ± SEM of n observations, where 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. Data sets were examined by one- and two-way analysis of variance, and individual group means were then compared with Student’s unpaired t test. Nonparametric data were analyzed with the Fisher’s exact test. A P value less than 0.05 was considered significant.

**RESULTS**

**Effects of thalidomide treatment on the degree of colitis (histology and general assessment)**

Four days after the intracolonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon, and rectum showed the presence of mucosal congestion, erosion, and hemorrhagic ulcerations (Fig. 1, B and D). The histopathological features included a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon section from DNBS-treated mice (Fig. 2, B and D). The observed inflammatory changes of the large intestine were associated with an increase in the weight of the colon (Fig. 3). The treatment with thalidomide significantly reduced the extent and severity of the histological signs of colon injury (Figs. 1C and 2, C and D) as well as the colon weight (Fig. 3). Four days after colitis induced by DNBS treatment, all mice had diarrhea and a significant reduction in body weight (compared with the control groups of mice; Fig. 4). Thalidomide treatment resulted in a significant reduction of body weight induced by DNBS administration in mice (Fig. 3). No histological alteration was observed in the colon tissue from vehicle-treated mice (Figs. 1, A and C, and 2, A and D).

**Thalidomide treatment modulates production and expression of TNF-α and IL-1β after DNBS administration**

To test whether the treatment with thalidomide may modulate the inflammatory process through the regulation of the secretion of others cytokines, we analyzed the colon levels of proinflammatory cytokines TNF-α and IL-1β. A substantial increase of TNF-α and IL-1β formation was found in colon samples collected from vehicle-treated mice at 4 days after DNBS administration (Fig. 5). In contrast, a significant inhibition of TNF-α and IL-1β levels was observed in the colon tissues collected from of DNBS-treated mice that received thalidomide treatment (C). The macroscopic damage score was made by two independent observers. The figure is representative of all of the animals in each group. Data are means ± SEM of 10 mice for each group. *P < 0.01 versus sham; †P < 0.01 versus DNBS.

**Effects of thalidomide treatment on ICAM-1 expression and PMN infiltration**

The colitis caused by DNBS was also characterized by an increase in MPO activity, an indicator of the neutrophils
accumulation in the colon (Fig. 8). This finding is consistent with the observation made with light microscopy that the colon of vehicle-treated DBNS mice contained a large number of neutrophils. On the contrary, thalidomide treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Fig. 8). To further elucidate the effect of thalidomide treatment on neutrophil accumulation in inflamed colon, we evaluated the intestinal expression of ICAM-1. Tissue sections obtained from sham-operated mice with anti-ICAM-1 antibody showed a specific staining along the vessels, demonstrating that ICAM-1 is expressed constitutively in endothelial cells (Fig. 7). After DNBS administration, the staining intensity substantially increased in the vessels of the lamina propria and submucosa. Immunohistochemical staining for ICAM-1 was also present in epithelial cells and infiltrated inflammatory cells in damaged tissues from DNBS-treated mice (Figs. 7 and 9A). Sections from thalidomide-treated mice did not reveal any upregulation of the constitutive ICAM-1, which was normally expressed in the endothelium along the vascular wall (Figs. 7 and 9B).

Effects of thalidomide treatment on VEGF levels in the colon

To assess whether thalidomide treatment reduces leukocyte adhesion and infiltration through a reduction in VEGF levels,
we measured VEGF colon protein levels via enzyme-linked immunoabsorbant assay. Compared with the colon of sham-treated mice, the colon tissues of DNBS-treated animals demonstrated a 2.3-fold increase in normalized VEGF levels (Fig. 10). The thalidomide treatment significantly reduced the VEGF colon levels (Fig. 10).

**Effects of thalidomide treatment on nitrotyrosine formation**

To determine the localization of “peroxynitrite formation” and/or other nitrogen derivatives produced during colitis, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the distal colon. Sections of colon from sham-administered mice did not stain for nitrotyrosine (Fig. 7). Colon sections obtained from vehicle-treated DNBS-treated mice exhibited positive staining for nitrotyrosine (Figs. 7 and 9C) localized in inflammatory cells and in disrupted epithelial cells. Sections from thalidomide-treated mice did not reveal any positive staining for nitrotyrosine (Figs. 7 and 9D).

**DISCUSSION**

The present findings demonstrate that thalidomide attenuates DNBS-induced colitis in the mice, an established model for human Crohn’s disease (30). In particular, we have demonstrated that thalidomide treatment reduced the degree of diarrhea and weight loss, the degree of colonic injury, the infiltration of the colon PMNs, the positive staining (immunohistochemistry) for nitrotyrosine, the increased expression of ICAM-1, and VEGF production caused by DNBS in the colon. What, then, is the mechanism by which thalidomide inhibits the colon inflammation caused by injection of DNBS?

Thalidomide was introduced into the therapy of Crohn’s disease by Wettstein and Meagher (31) who reported remission in a case of steroid-dependent Crohn’s disease. Thalidomide was developed in the 1950s as a sedative, but was subsequently withdrawn from widespread use in the 1960s because of teratogenicity (32). After the drug was banned for more than two decades, in vitro studies demonstrating that thalidomide inhibits TNF-α production (33) have led to its use in clinical conditions thought to be mediated by increased production of proinflammatory cytokines, such as refractory cutaneous lupus (34), chronic graft versus host disease (35), rheumatoid arthritis (36), and Behcet’s syndrome (37). Thalidomide was effective at a dose of 200 mg/kg daily, which is almost two orders of magnitude greater than the clinically effective dose in man (21, 22), but typical for rodents where the half-life of this type of drug is much shorter (2 ± 3 h compared with 6 ± 8 h in humans) with an almost complete excretion (38). Thus, relatively high doses are required in most animal experiments to observe the immunomodulatory effects of thalidomide. In the rat arthritis model, thalidomide was administered orally at 200 mg/kg once or twice daily (39). There is good evidence that TNF-α and IL-1β are clearly involved in the pathogenesis of colitis because these cytokines are present in colon tissues and can be detected immunohistochemically in the inflamed tissues (40, 41).

Recently, two open label trials on the treatment of refractory Crohn’s disease with thalidomide reported clinical efficacy in patients after a 12-week course (21, 22). Our study showed similar results. As TNF-α is considered to be centrally involved in the inflammatory process in IBD and thalidomide has been

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**FIG. 5.** Effect of thalidomide treatment on colon levels of TNFα (A) and IL1β (B).

Treatment with thalidomide (200 mg/kg day orally) significantly reduced the increase of cytokine production in the colon after DNBS administration. Data are means ± SEM of 10 mice for each group. *P < 0.01 versus sham; †P < 0.01 versus DNBS.

**FIG. 6.** Immunohistochemical localization of TNF-α and IL-1β in the colon. Immunohistochemical analysis for TNF-α (A) and for IL-1β (C) show positive staining localized in the inflammatory cells in the injured area from DNBS-treated mice. The intensity of the positive staining for TNF-α (B) and IL-1β (D) was markedly reduced in tissue section obtained from DNBS-treated mice that were treated with thalidomide (200 mg/kg day orally). The figure is representative of at least three experiments performed on different experimental days.
shown to suppress TNF-α production (33), this mechanism could be responsible for its clinical efficacy. In fact, we found a strong effect of thalidomide on TNF-α production in lamina propria mononuclear cells (LPMC) as well as in peripheral monocytes. However, exclusive suppression of TNF-α may not be sufficient to explain the clinical improvement observed, as a recent study with pentoxifylline, another TNF-α suppressor (42), failed to demonstrate clinical improvement in refractory Crohn’s disease (43). Also, the effect of anti-TNF-α antibody (infliximab) is attributed not only to its direct effect on TNF-α but rather to the combination with other immunomodulating effects (44).

Moreover, Bauditz and colleagues (45) have demonstrated that the thalidomide treatment reduced TNF-α and IL-12 production in patients with active Crohn’s disease and that this effect may explain its clinical efficacy. The cytokine suppressive properties and therapeutic potential of thalidomide and its analogs (46, 47) in Crohn’s disease should be further investigated. In this regard, we report in the present study that thalidomide treatment significantly reduces (among other effects) the biosynthesis and/or the effects of the proinflammatory cytokines TNF-α and IL-1 in an experimental model of IBD.

Endothelial cells appear to be major regulators of PMN trafficking, regulating the process of PMN chemotraction, adhesion, and emigration from the vasculature to the tissue. ICAM-1 is constitutively expressed on the surface of endothelial cells and is involved in PMN adhesion (48–50). Hypoxic or injured endothelial cells synthesize proinflammatory cytokines, which can upregulate endothelial expression of ICAM-1 in an autocrine fashion (51, 52). Significant expression of ICAM-1 in microvessels of previously ischemic tissues occurs within 1 h after reperfusion (53, 54). The upregulation in the expression of ICAM-1 corresponds with the induction of PMN recruitment within the first 4 days after DNBS administration (55, 56). In accordance with these findings, we observed that DNBS (at day 4) induced an upregulation of ICAM-1 on endothelial cells. The thalidomide treatment abolished the upregulation of ICAM-1, but did not affect the constitutive expression of ICAM-1 on endothelial cells. 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 (57). Neutrophil infiltration into inflamed tissue plays a crucial role in the destruction of foreign antigens and in the breakdown and remodeling of injured tissue (58). We have also demonstrated in the present study that thalidomide treatment significantly reduced the infiltration of PMN in tissue. These observations are in agreement with a recent study that have suggest that thalidomide and one of its derivatives impairs Crohn’s disease-like trinitrobenzene sulphonic acid (TNBS)-induced colitis in the rat by down-regulating endothelial adhesion molecule and chemokine expression and, as a consequence, the interaction of these cells with circulating leukocytes (22).

It is well known that reactive oxygen and nitrogen species play a key role in IBD (59, 60). These species are cytotoxic agents, 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. 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 (61). Nitrotyrosine can be formed from the reaction of nitrite with MPO and hydrogen peroxide (62). Recent evidence indicates that nitration of tyrosine can result from the reaction of nitrite with hypochlorous acid 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 and is involved in PMN adhesion (48–50). Hypoxic or injured endothelial cells synthesize proinflammatory cytokines, which can upregulate endothelial expression of ICAM-1 in an autocrine fashion (51, 52). Significant expression of ICAM-1 in microvessels of previously ischemic tissues occurs within 1 h after reperfusion (53, 54). The upregulation in the expression of ICAM-1 corresponds with the induction of PMN recruitment within the first 4 days after DNBS administration (55, 56). In accordance with these findings, we observed that DNBS (at day 4) induced an upregulation of ICAM-1 on endothelial cells. The thalidomide treatment abolished the upregulation of ICAM-1, but did not affect the constitutive expression of ICAM-1 on endothelial cells. 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 (57). Neutrophil infiltration into inflamed tissue plays a crucial role in the destruction of foreign antigens and in the breakdown and remodeling of injured tissue (58). We have also demonstrated in the present study that thalidomide treatment significantly reduced the infiltration of PMN in tissue. These observations are in agreement with a recent study that have suggest that thalidomide and one of its derivatives impairs Crohn’s disease-like trinitrobenzene sulphonic acid (TNBS)-induced colitis in the rat by down-regulating endothelial adhesion molecule and chemokine expression and, as a consequence, the interaction of these cells with circulating leukocytes (22).

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IBD is further supported by the fact that intracolonic administration of exogenous peroxynitrite induces a severe colonic inflammation that mimics the features of ulcerative colitis and Crohn’s disease (60).

In the present study, we observed that epithelial disruption was significantly less in mice treated with thalidomide. Indeed, thalidomide treatment prevented the formation of nitrotyrosine staining in DNBS-treated animals. Reactive oxygen species cause DNA single-strand damage, leading to poly (ADP ribose) (PAR) synthetase activation and cell death (67). Some evidence exists to support the possible role of PAR synthetase activation in IBD (55, 68). As shown in this study, thalidomide treatment reduced PAR synthetase immunoreaction an effect that might account for the overall protective action of thalidomide. Is important to point out that the reduction of PMN infiltration was also paralleled with the inhibition of nitrotyrosine and PAR immunoreactivity. These findings (i.e., that nitrotyrosine and PAR staining is reduced in thalidomide animals) coupled with the protective effects of thalidomide on endothelial cells (22) proving the existence of a self-amplifying suicide cycle in which early oxidant production by endothelium activates PARP; the consequent endothelium injury with activation of PMN-attractive factors (e.g., ICAM-1) and PMN infiltration leads to further production of oxidants, which ultimately are responsible for the colonic injury. Thalidomide treatment would intercept this cycle at the level of endothelial injury. This model would explain the reduction of tissue nitrotyrosine and PAR formation during DNBS-induced colitis in the thalidomide-treated mice: reduced neutrophil infiltration leads to reduced reactive oxygen species. Therefore, to its effect on preserving the cellular energetic status and protecting against oxidant-induced cell necrosis, regulation of neutrophil recruitment may represent a novel important additional anti-inflammatory mode of action of thalidomide.

In addition, recent studies have indicated that serum VEGF concentrations are increased in patients with IBD (17, 18), suggesting that this angiogenic factor is overproduced within the context of an intense angiogenic activity. Recently, it has been shown that no high amounts of VEGF in their immunohistochemical study, where VEGF expression was absent in patients with Crohn’s disease, and only weakly positive in ulcerative colitis, specifically in the epithelial cells, a finding that was also true in normal intestinal tissues. In agreement with this observation, Kanazawa et al. (17), investigating IBD tissues, found that VEGF reactivity was present in endothelial cells but not in epithelial or inflammatory cells. This discrepancy between immunohistochemical and serological findings raises the question of whether serum VEGF may values are informative. Indeed, platelets are the major source of VEGF in the human body, (69, 70), and during ex vivo platelet aggregation, VEGF is released into the supernatant (71). Therefore, serum VEGF concentrations do not reflect VEGF production by the intestinal epithelium or the related inflammation. Plasma concentrations of VEGF are several times lower than those obtained from the sera of patients (72). Because platelet counts are high, especially in active IBD (73), it is possible that serum VEGF concentrations reflect VEGF of platelet origin rather than that produced by the intestine. However, in the present study we demonstrated that Thalidomide treatment significantly reduced the increase of VEGF colon levels (assessed by enzyme-linked immunoabsorbant assay) induced by DNBS in the mice. This finding suggest that the reduced leukocyte adhesion observed with the thalidomide treatment can be attributed to changes in VEGF levels because, in our study, VEGF protein levels were down-regulated with this treatment.
In addition, ICAM-1 levels were reduced with the thalidomide treatment, and previous work has shown that VEGF-induced proinflammatory cytokines, reduced the expression of the adhesion molecule ICAM-1, and reduced the VEGF formation. Finally, these observations may help to clarify the therapeutic actions of thalidomide in patients with Crohn’s disease.

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