BASIC–ALIMENTARY TRACT

Therapeutic Effects of Vasoactive Intestinal Peptide in the Trinitrobenzene Sulfonic Acid Mice Model of Crohn's Disease

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Background & Aims: **Crohn's disease (CD) is a chronic debilitating disease of unknown etiology that is characterized by severe inflammation of the colon. Vasoactive intestinal peptide (VIP) has recently emerged as a promising candidate for treatment of inflammatory Th1-driven diseases. We studied the effect of VIP in trinitrobenzene sulfonic acid (TNBS)-induced colitis, which has clinical and molecular features in common with CD.** ***Methods:*** **A 3-mg enema of TNBS was given to BALB/c mice, and VIP (1 nmol) was given either as a single dose at 12 hours or every other day. Weight loss, histopathology, and chemokine and cytokine levels in serum and colon extracts were assessed. VIP was also tested given 5 days after the onset of TNBS-induced colitis, and its effect was analyzed given a second dose of TNBS.** ***Results:*** **Treatment with VIP reduced the clinical and histopathologic severity of TNBS-induced colitis, abrogating body weight loss, diarrhea, and macroscopic and microscopic intestinal inflammation. The therapeutic effects of VIP were associated with down-regulation of both inflammatory and Th1-driven autoimmune responses, including tumor necrosis factor α, interleukin 1, and interleukin 6 in colon extracts and serum as well as interferon gamma by splenic and lamina propria $CD4^+$ T cells. VIP reduced disease severity when given after disease onset and dramatically reduced disease recurrence given a second dose of TNBS.** ***Conclusions:*** **Our data suggest that VIP has beneficial prophylactic and therapeutic effects in TNBS-induced colitis and is a promising candidate to test for potential benefits in CD.**

Under normal conditions, the intestinal mucosa is exposed to a wide range of agents; some of these agents are toxic, such as pathogens and their toxic components. The main role of the gut-associated lymphoid tissue is to protect the host by neutralizing infectious agents or injurious toxins. In healthy subjects, this protective function is well balanced and does not lead to damage of the intestinal mucosa. In contrast, in diseases such as inflammatory bowel disease, the gut-associated lymphoid tissue–developed immune response is exaggerated, generating a prolonged and severe inflammatory condition of the intestinal mucosa. Human inflammatory bowel disease is a worldwide, chronic, idiopathic inflammatory disease of the distal small intestine and the colonic mucosa. It is of unknown etiology and clinically characterized by 2 overlapping phenotypes: Crohn's disease (CD) and ulcerative colitis. Both CD and ulcerative colitis are inflammatory conditions that primarily affect the digestive tract, although up to 36% of patients with inflammatory bowel disease show extraintestinal manifestations.[1] CD is a chronic incurable disease of the bowel with a prevalence of 0.01%–0.08%; it is marked by periods of exacerbation and remission and characterized by transmural inflammation resulting in abdominal pain, diarrhea, and weight loss.[2] Recent data suggest that CD and a variety of other inflammatory diseases, such as rheumatoid arthritis (RA), reflect an excessive Th1 response. Indeed, it has been shown that agents that block a Th1 response or promote a Th2 profile are beneficial in improving disease symptoms.[3,4] Because the specific cause remains unknown, treatment in many patients is usually symptomatic and aimed at nonspecifically inhibiting inflammation.[5] In many cases, the inefficiency of these treatments results in the need for surgical resection of the colon and ileostomy.[6] Furthermore, there is a great need for the development of new and specific therapies for CD, given that treatment with prednisone, sulfasalazine, antibiotics, or immunosuppressive therapies has been associated with significant adverse effects. New

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inhibitory protein; MPO, myeloperoxidase; PACAP, pituitary adenylate cyclase–activating polypeptide; RA, rheumatoid arthritis; SAA, serum amyloid A; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; VPAC1, VIP receptor type 1; VPAC2, VIP receptor type 2.

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specific and nonspecific approaches to the treatment of CD are under study,[7,8] but additional treatments are desirable.

Chronic intestinal inflammation induced by intrarectal administration of trinitrobenzene sulfonic acid (TNBS) resembles many of the clinical, histopathologic, and immune characteristics of CD in humans,[4,9] inducing chronic colitis characterized by severe transmural inflammation associated with diarrhea, rectal prolapse, and weight loss. This inflammation is characterized by a massive infiltration of neutrophils and macrophages, producing high levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, or IL-6 in the early stages of the disease; this is followed by a T-cell infiltration, mainly of the $CD4^+$ phenotype, producing high levels of interferon (IFN)-γ and reduced amounts of IL-4. As it has been described for CD, this model of inflammation is associated with a Th1 response.[10,11]

Vasoactive intestinal peptide (VIP) is a neuropeptide present in nerve fibers and lymphocytes of the lymphoid microenvironment that, together with the 3 G protein–coupled receptors of the VIP/pituitary adenylate cyclase–activating peptide (PACAP) family, modulates innate and adaptive immunity. This modulation leads to the amelioration or prevention of several inflammatory and autoimmune disorders in animal models, including septic shock and RA.[12–14] CD shares some of the characteristics of RA in terms of the participation of proinflammatory cytokines, oligoclonal expansion, and activation of $CD4^+$ T cells. Given that VIP has recently emerged as an excellent candidate for the treatment of RA, downregulating both the autoimmune and inflammatory components of the disease,[14] we were interested in investigating its potential therapeutic use in other inflammatory disorders, such as CD, and studying its effect in the TNBS-induced model of colitis. In the present study, we show that treatment with VIP has a significant preventive effect at the clinical and pathologic levels in the TNBS-induced experimental model of CD. Data from this study and previous work in our laboratory strongly suggest that VIP could be used as a universal and pleiotropic agent against inflammatory and Th1 disorders.

Materials and Methods

Induction of Colitis

Colitis was induced by intracolonic administration of TNBS (Sigma Chemical Co., St. Louis, MO) as previously described. Briefly, 120 μL of a solution with 3 mg TNBS dissolved in 50% ethanol was slowly administered to lightly anesthetized BALB/c male mice (6–8 weeks old; Jackson Laboratories, Bar Harbor, ME) through a catheter carefully inserted 4 cm from the anus. To study the effect of a second dose of hapten reagent, 1.5 mg TNBS was administered in the same manner. Animals were then kept in a vertical position for 30 seconds and returned to their cages. Using the same technique, control animals received 120 μL of 50% ethanol.

Treatment Protocols and Monitoring of Colitis

A total of 1 nmol VIP (Neosystem, Strasbourg, France) was given intraperitoneally per mouse either 12 hours after the induction of colitis (one pulse) or every other day or at day 5 after TNBS induction of colitis until the collection of samples. Weight was measured every day as an indirect indicator of the progress of colitis, and colitis itself was graded in each mouse from 0 (no colitis) to 4 (severe colitis) by 2 different researchers who were blinded to the treatment conditions. To study the effect of different doses of VIP on the disease, mice were intraperitoneally given 0.5, 1, 5, or 10 nmol VIP every other day. PACAP (Neosystem), secretin (Sigma Chemical Co.), glucagon (Calbiochem, Laufelingen, Switzerland), $VIP_{1–12}$ (Sigma Chemical Co.), $VIP_{10–28}$ (Calbiochem), $[K^{15},R^{16},L^{27}]$VIP(1–7)/GRF(8–27) VIP receptor type 1 (VPAC1) agonist, or Ro25-1553 VIP receptor type 2 (VPAC2) agonist was given intraperitoneally at a dose of 1 nmol per mouse on alternate days.

Histopathology

Colons were collected at the specified times, fixed in Bouin's solution, and embedded in paraffin; 6-μm sections were stained with alcian/hemalum/picroindigocarmine using standard techniques. Inflammation was scored from 0 to 4 as follows by the same pathologist blinded to the treatment conditions: 0, no inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, and goblet loss; and 4, massive loss of goblet cells, extensive fibrosis, and thickening of the colon wall.

Preparation of Colon Extracts

Tissues were removed at the indicated times, washed with phosphate-buffered saline, and cut in small pieces. For myeloperoxidase (MPO) determination, 50 mg/mL of colon tissue was homogenized in 50 mmol/L phosphate buffer, pH 6.0, with 0.5% hexadecyltrimethylammonium bromide using a Polytron (Kinematica, Littau/Luzerne, Switzerland). Samples were frozen and thawed 3 times, centrifuged for 20 minutes at 30,000*g*, and then stored at −20°C until assay. For cytokine and chemokine measurement, 0.5 μg/mL of tissue was homogenized in 50 mmol/L Tris-HCl buffer, pH 7.4, containing 0.5 mmol/L dithiothreitol (Sigma Chemical Co.), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and 10 μg/mL leupeptin (Sigma Chemical Co.). Samples were centrifuged at 30,000*g* for 20 minutes and then stored at −80°C until assay.

Measurement of MPO Activity

MPO activity was determined in 96-well plates using a modification of the method described by Bradley et al.[15] Briefly, samples were diluted 1:30 with assay buffer (50 mmol/L phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine [Sigma Chemical Co.] and 0.0005% H_2O_2) and A_{450nm} was measured in a microtiter reader.

Cell Culture

Lamina propria mononuclear cells were isolated from freshly obtained colons using a modification of the method previously described.[4] Lamina propria $CD4^+$ T-cell lymphocytes were isolated using magnetic-activated cell sorting microbeads (Miltenyi Biotech, Auburn, CA). The purity assessed by flow cytometry (FACScan; Becton Dickinson & Co., San Jose, CA) exceeded 90%.

Spleens were aseptically removed, and cell suspensions were prepared by tapping through a 60-μm wire mesh. Erythrocytes were removed from cell suspensions by hypotonic lysis in ammonium chloride lysis buffer (0.17 mol/L NH_4Cl). $CD4^+$ T-cell percentage was quantified by flow cytometry using a fluorescein isothiocyanate–conjugated rat anti-mouse CD4 antibody (1:100 dilution; Pharmingen, San Diego, CA).

Cells were cultured in 96-well culture plates (Costar Corp., Cambridge, MA) at 1×10^6 cells/mL in complete medium. To stimulate cells to measure cytokine production, wells were precoated with anti-CD3ε clone 145-2C11 (Pharmingen) at 10 μg/mL in carbonate buffer (pH 9.6) overnight at 4°C and 1 μg/mL anti-CD28 clone 37.51 (Pharmingen) was added to the medium. After 48 hours of incubation at 37°C and 5% CO_2, supernatants were collected and frozen until IFN-γ, IL-4, and IL-10 assay by enzyme-linked immunosorbent assay (ELISA).

Cytokine Determination by ELISA

Cytokine and chemokine amounts were measured by sandwich ELISA according to the manufacturer's recommendations as previously described.[14] Capture/biotin TNF-α (MP6-XT22/MP6-Xt3 clones), IL-6 (MP5-20F3/MP5-32C11 clones), IL-10 (JES5-2A5/SXC-1 clones), and IL-12 (C15.6/C17.8) pairs purchased from Pharmingen and growth-regulated oncogene (Gro)α, macrophage inhibitory protein (MIP)-1α, monocyte chemoattractant protein (MCP)-1, MIP-2, and IL-4 pairs purchased from Peprotech (Rocky Hill, NJ) were used. A_{405nm} was measured in a microtiter reader. For IL-1β and IFN-γ determination, murine IL-1β Quantikine M (R&D Systems, Inc., Minneapolis, MN) and murine IFN-γ Eli-pair kit (Diaclone Research, Besançon, France) were respectively used and A_{450nm} was measured.

Measurement of Serum Amyloid A

Serum samples were collected for the detection of serum amyloid A (SAA) levels by a murine ELISA kit (Tridelta Development, Wicklow, Ireland) 3 and 10 days after induction of colitis according to the manufacturer's recommendations and A_{450nm} was measured.

Statistical Analysis

Tests for significance of differences were made with Student *t* test using the program StatGraphics (Manugistics, Inc., Rockville, MD).

Results

Administration of VIP Represses Colitis and Abolishes Wasting Disease

Colitis was induced by intrarectal administration of TNBS, which haptenates autologous colonic proteins with trinitrophenol. Mice treated with TNBS in 50% ethanol developed severe bloody diarrhea and rectal prolapse accompanied by an extensive wasting disease. VIP was administered intraperitoneally at different doses 12 hours after the induction of colitis in a single dose (pulse) or on alternate days for 10 days. As shown in Figure 1*A–E*, mice treated with VIP showed a striking improvement of the wasting disease compared with TNBS-treated mice, as assessed by animal weight loss as well as clinical, macroscopic, and microscopic analysis.

After administration of TNBS, a dramatic and fast decrease in body weight was observed as a result of colitis and was maintained during the 10-day period (Figure 1*A*). Untreated mice and control mice treated with 50% ethanol alone failed to develop wasting disease and had a healthy appearance (Figure 1*A*). However, mice treated with a pulse of VIP or with the same dose every other day significantly recovered the lost body weight (Figure 1*A*). Administration of VIP on alternate days was the most effective treatment compared with mice treated with one pulse of VIP because it led to a faster recovery of the initial body weight. Loss of body weight in TNBS-treated mice was accompanied by massive pancolitis, whereas control ethanol-treated mice showed minor signs of colitis. Both VIP treatments significantly prevented the signs of TNBS-induced colitis (Figure 1*B*).

Macroscopic analysis of colons obtained 3 and 7 days after administration of TNBS (data not shown) showed a striking hyperemia, necrosis, and inflammation compared with control colons from ethanol-treated mice, which only showed a slight inflammation. Treatment with VIP drastically prevented both hyperemia and inflammation in the studied colons (Figure 1*C*).

The histopathologic analysis during the first days after induction of colitis showed infiltration of neutrophils and macrophages into the colonic mucosa and submucosa layers. By day 3, transmural inflammation, characterized by neutrophilic infiltration, was associated with a thickening of the colon wall, ulcerations, loss of goblet cells, and fibrosis found through the colon. By day 7, massive infiltration of lymphocytes

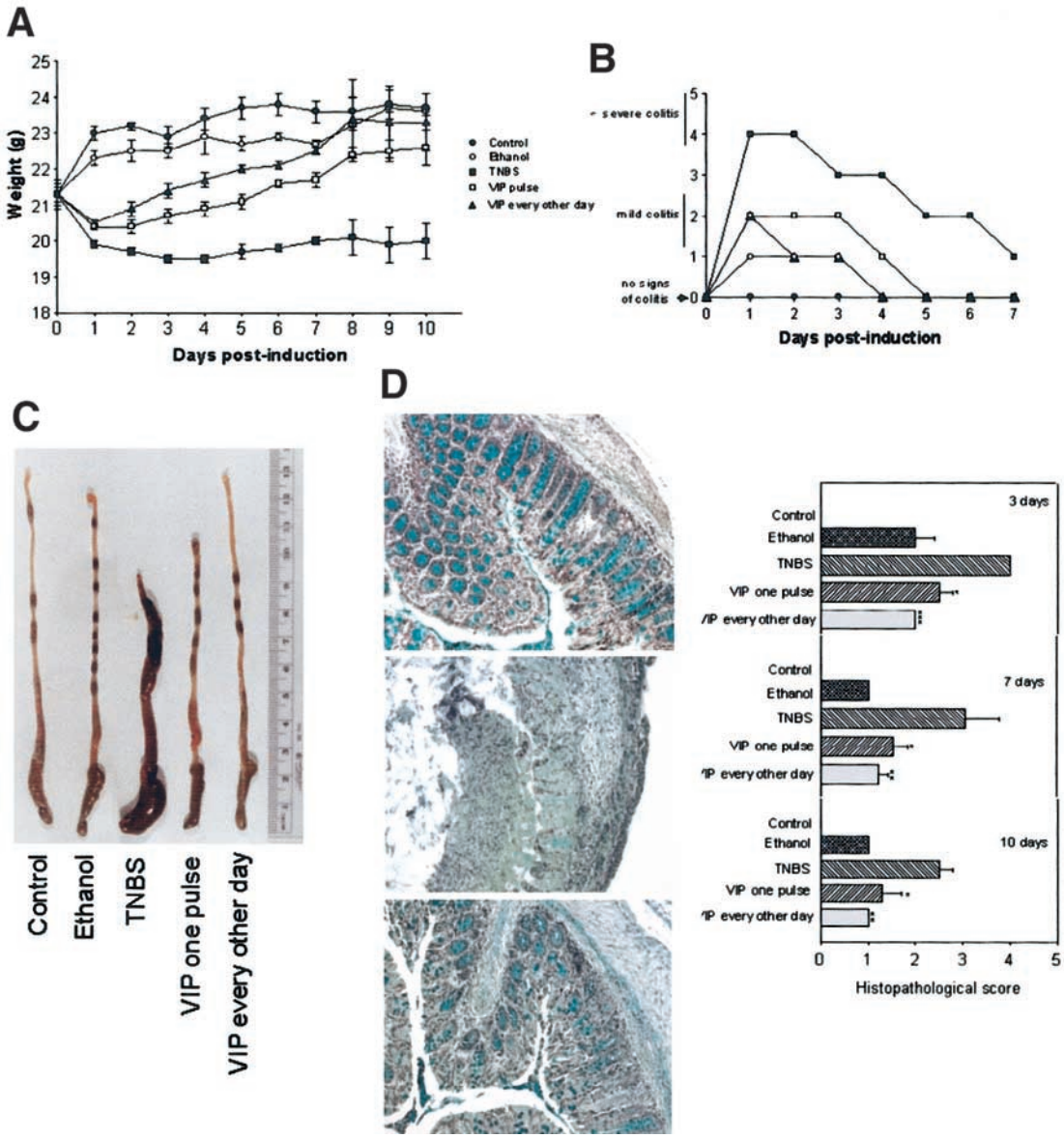

Figure 1. VIP ameliorates the clinical, macroscopic, and microscopic features of TNBS-induced colitis. Colitis was induced by rectal administration of TNBS in 50% ethanol. Mice treated with 50% ethanol alone were used as controls. From 12 hours after administration of TNBS, mice were treated intraperitoneally with either 1 nmol VIP in a single administration (pulse) or on alternate days over 10 days. Clinical evolution and severity were monitored by (*A*) mouse weight changes, (*B*) colitis score, and (*C*) macroscopic and (*D*) histopathologic signs of the disease. (*A*) Each point represents the mean weight ± SE from 10 separate experiments (8 mice/group/experiment). $P < 0.001$ in every treatment vs. TNBS-treated mice after day 2. (*B*) Results show a representative experiment out of 10. $P < 0.001$ in every treatment vs. TNBS-treated mice after day 1. (*C*) A representative photograph of colons from day 7 after the induction of colitis. (*D*) Photomicrographs of colon sections after treatment with ethanol (*top*), TNBS (*middle*), and VIP on alternate days (*bottom*) on day 7 after the induction of colitis with TNBS (original magnification 100×). Histopathologic scoring is shown on the *right*. Results are the mean ± SE from 8 mice per group. $*P < 0.01$, $**P < 0.001$, and $***P < 0.0001$ vs. TNBS-treated animals.

characterized the colon sections studied, which is a major sign of the chronic inflammation in the late stages of this colitis model (Figure 1*D*). Administration of VIP improved these signs, restoring the histologic appearance of the mucosa and submucosa compared with untreated mice (Figure 1*D*), although ethanol-treated mice showed a minor infiltration of neutrophils as a result of a mild inflammation (data not shown). Moreover, from days 3 to 10, the histologic assessment showed significant differences between TNBS-treated and ethanol-treated mice, whereas administration of VIP resulted in a significant decrease in the histologic score (Figure 1*D*).

By assessment of body weight, colitis, and histopathologic score, we observed that the therapeutic effect of VIP was dose dependent (Figure 2*A–C*), with

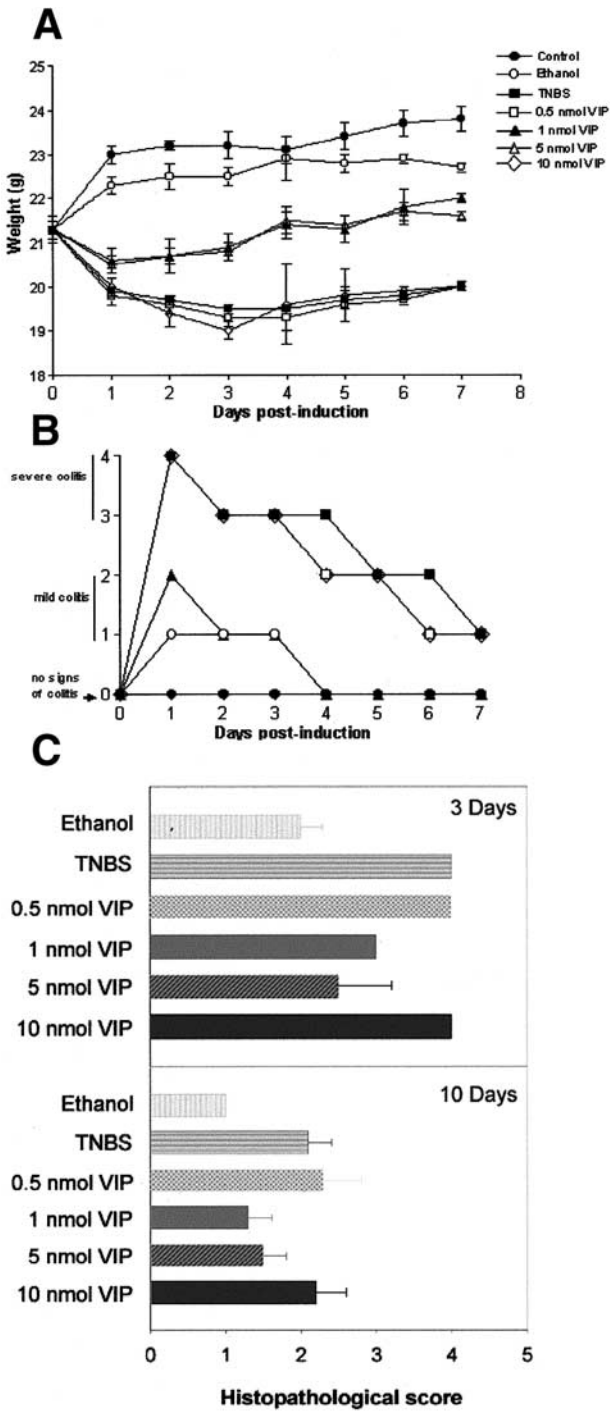

Figure 2. The effect of VIP on TNBS-induced colitis is dose dependent. Colitis was induced as described in the legend to Figure 1, and mice were treated intraperitoneally with 0.5, 1, 5, or 10 nmol VIP on alternate days over 10 days. (*A*) Weight development of TNBS-treated mice with different doses of VIP. Results are expressed as the mean ± SE from 3 separate experiments (8 mice/group/experiment). $P < 0.001$ in every treatment vs. TNBS-treated mice after day 2. (*B*) Colitis score. A representative experiment out of 3 is shown. $P < 0.001$ in every treatment vs. TNBS-treated mice after day 1. (*C*) Histopathologic score. Results are the mean ± SE from 8 mice per group. $*P < 0.01$, $**P < 0.001$ vs. TNBS-treated animals.

maximal effects from 1 to 5 nmol. However, no beneficial effects were observed at 0.5- and 10-nmol doses. Because no differences were observed between both 1- and 5-nmol doses, all further experiments were performed with the 1-nmol dose. Importantly, VIP can prevent and ameliorate already-established disease. When administered 5 days after onset of TNBS-induced colitis, VIP blocked development of disease and led to an improvement as assessed by recovery of lost body weight (Figure 3*A*) and prevention of both necrosis and inflammation signs (Figure 3*B*).

Moreover, VIP prevented reoccurrence of TNBS-induced colitis. Mice treated at day 10 with a second dose of TNBS showed a high rate of mortality (90%), whereas VIP-treated mice survived and recovered the lost body weight (Figure 3C). This shows that treatment with VIP provides an important beneficial effect.

VIP Down-regulates the Inflammatory Response in TNBS-Induced Colitis

Because VIP acts as a potent anti-inflammatory agent, we investigated whether VIP could affect the local production of several inflammatory mediators. We studied the effect of VIP in protein extracts from inflamed colons as well as its implication in the systemic response through study of VIP blood serum levels. First, as a quantitative measure of the neutrophil infiltration in the onset of the disease, we evaluated the effect of VIP on MPO activity in colon extracts. In the acute phase of the colitis (day 3), colon MPO activity values were significantly increased compared with untreated and ethanol-treated control mice (Figure 4). Treatment with VIP, administered either as a pulse and on alternate days, significantly inhibited MPO activity (Figure 4). By day 10, minor differences were observed between controls, mice with TNBS-induced colitis, and mice treated with VIP (data not shown).

The chemokine system plays an important role in the pathogenesis of CD,[16,17] because local chemotactic cytokines are implicated in the recruitment of leukocytes to the intestine. Consequently, we tested whether VIP could affect the production of these proinflammatory agents mainly involved in the acute phase of the disease in the present model of colitis.[17,18] Analysis of colon extracts on day 3 showed high levels of MIP-1α, MCP-1, MIP-2, and Gro-α in mice with TNBS-induced colitis compared with untreated and ethanol-treated animals. Administration of VIP, both on alternate days and as a single dose, inhibited TNBS-induced production of all of these chemokines (Figure 4). Similar to what was previously observed,

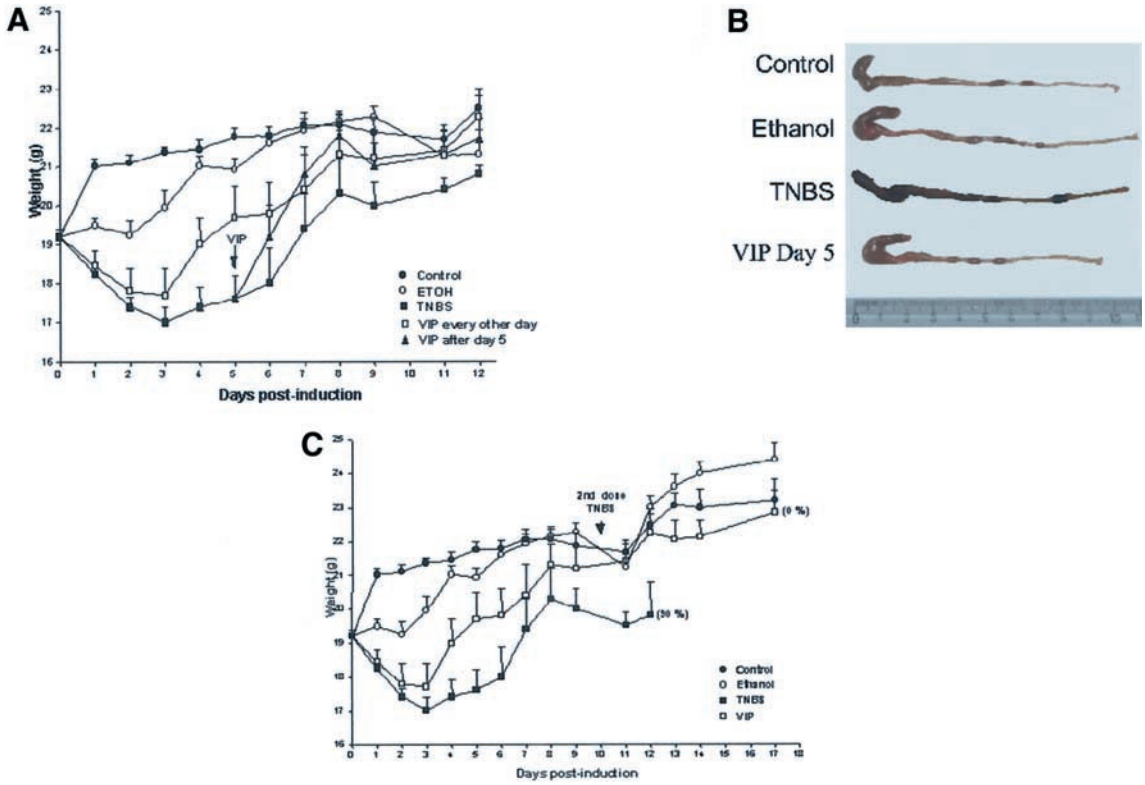

Figure 3. VIP ameliorates the clinical and macroscopic features of established TNBS-induced colitis and protects mice from a second injection of TNBS. Induction of colitis was performed as described in the legend to Figure 1. (*A*) As of day 5 (*arrow*), mice were treated intraperitoneally with VIP (1 nmol) every other day. Severity of clinical signs was assessed by changes in mouse weight and macroscopic appearance. (*B*) A representative photograph of colons from day 12 after the induction of colitis. (*C*) Weight development of mice treated with a second dose of TNBS (1.5 mg) on day 10 (*arrow*); VIP was administered rectally on alternate day 10. Mice injected with a second dose of 50% ethanol were used as controls. Percentage of mortality is represented by numbers in parentheses. Results are expressed as the mean weight ± SE from 8 mice per group.

treatment on alternate days was more effective than a single pulse. Ten days after induction of colitis, no detectable levels of chemokines were found in TNBS-treated or control mice (data not shown). Besides the production of chemokines, in the initial stages of TNBS-induced colitis, cytokines such as TNF-α, IL-6, IL-1β, and IL-12 (involved in inflammation and tissue damage) are secreted. Concomitantly, levels of cytokines with anti-inflammatory effects such as IL-10 decrease. Therefore, we tested whether VIP regulates the production of proinflammatory cytokines in colon extracts. Administration of VIP significantly reduced TNBS-induced production of TNF-α, IL-6, IL-1β, and IL-12 in both the early acute (day 3) (Figure 3) and chronic (day 10) (data not shown) phases of the disease compared with untreated and ethanol-treated controls. Moreover, following treatment with VIP, IL-10 levels in colon extracts in TNBS-treated mice decreased significantly at day 10 (Figure 4), with no effects seen at day 3 (data not shown).

Levels of TNF-α, IL-6, and IL-1β in the blood serum of TNBS- and VIP-treated animals were also measured and compared with controls to assess the involvement of these cytokines in the systemic immune response implicated in colonic inflammation. Similar decreases were observed in serum levels of TNF-α, IL-6, and IL-1β in TNBS- and VIP-treated mice versus animals given TNBS alone on days 3 (Figure 5) and 10 (data not shown).

Finally, one of the most intensively studied systemic responses against an inflammatory stimulus is the hepatic synthesis of acute phase proteins, including the family of SAA. During the first stages of inflammation, these apolipoproteins have a crucial yet undefined protective role in inflammation. However, following a prolonged chronic inflammation, an N-terminal peptide fragment of SAA (the amyloid A) assembles into fibrils, causing extensive damage to the spleen, liver, and kidney.[19] As Figure 4 shows, on day 3 after TNBS-induced colitis, serum SAA levels increased more than 1000-fold compared with the untreated/ethanol levels. Treatment with VIP, either in one pulse or on alternate days, induced a marked decrease in SAA serum levels, reaching control levels.

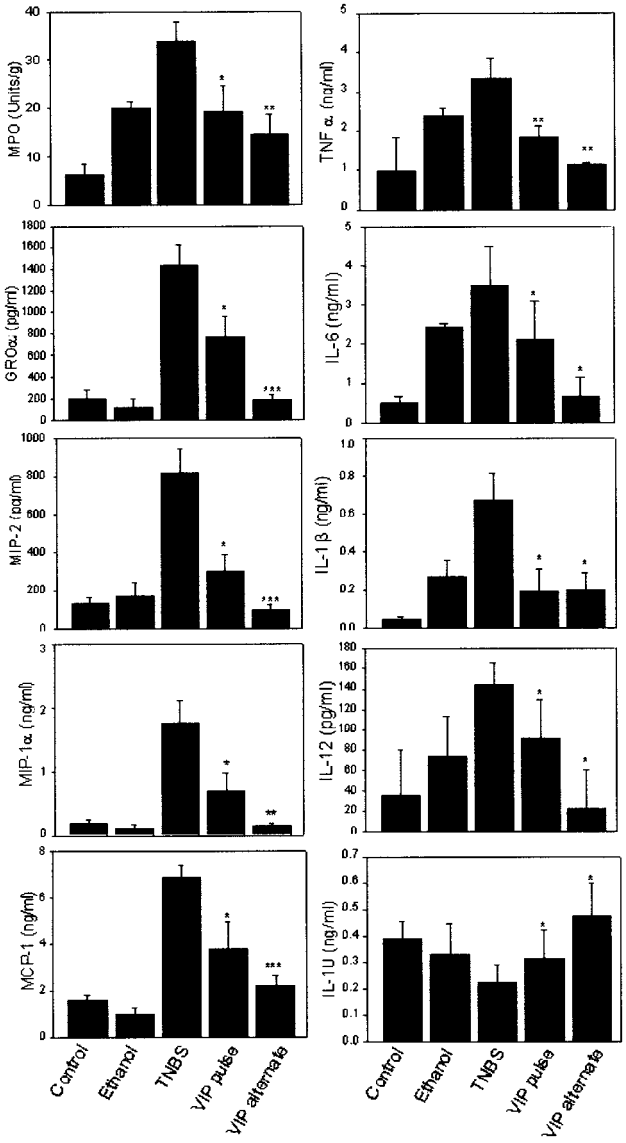

Figure 4. VIP down-regulates the local inflammatory response in mice with TNBS-induced colitis. Mice were treated as described in the legend to Figure 1. Colons were removed and homogenized on day 3 to obtain protein extracts. MPO activity as well as chemokine (MIP-2, Gro-α/KC, MIP-1α, and MCP-1) and cytokine (TNF-α, IL-6 , IL-1α, IL-10) production were determined as described in Materials and Methods. A representative experiment out of 3 with 8 mice per group is shown. Results are the mean ± SE of 8 animals per group. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$ vs. TNBS-treated animals.

VIP Therapy Decreases the T Cell–Mediated Immune Response Regulating Th1/Th2 Cytokine Profiles

Although macrophages and neutrophils are the primary sources of proinflammatory cytokines, $CD4^+$ T cells have a key role in the initiation and perpetuation of CD, producing IFN-γ, a potent inducer of the inflammatory response.[20] To test the involvement of VIP in the regulation of $CD4^+$ T-cell response, we analyzed the spleen and the lamina propria $CD4^+$ T-cell population in mice with TNBS-induced colitis. Fluorescence-activated cell sorter analysis of the splenic lymphocyte population showed a 2-fold increase in the percentage of $CD4^+$ T cells in TNBS-treated mice compared with ethanol-treated and untreated mice (Figure 6*A*). Treatment with VIP on days 3 and 10 partially prevented this TNBS-induced increase in $CD4^+$ spleen cells (Figure 6*A*).

When an immune response is initiated, naive helper T cells secrete IL-2 and proliferate, differentiating into 2 functional subsets and producing distinct effector cytokines. Th1 cells secrete IFN-γ, and Th2 cells secrete IL-4, IL-5, and IL-10. Because TNBS-induced colitis is associated with a Th1 response, similar to the spectrum of cytokines observed in patients with $CD4^+$, we next

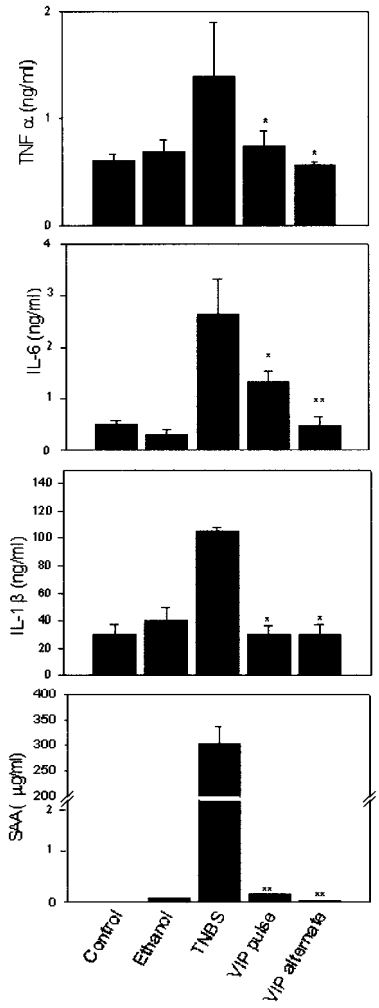

Figure 5. VIP reduces systemic inflammatory response in mice with TNBS-induced colitis. Induction of colitis and treatment with VIP were performed as described in the legend to Figure 1. Sera were collected at the indicated times, and TNF-α, IL-6, IL-1α, and SAA levels were measured by ELISA. Results represent mean ± SE of 3 experiments, each with 8 animals per group. $*P < 0.01$, $**P < 0.001$ vs. TNBS-treated animals.

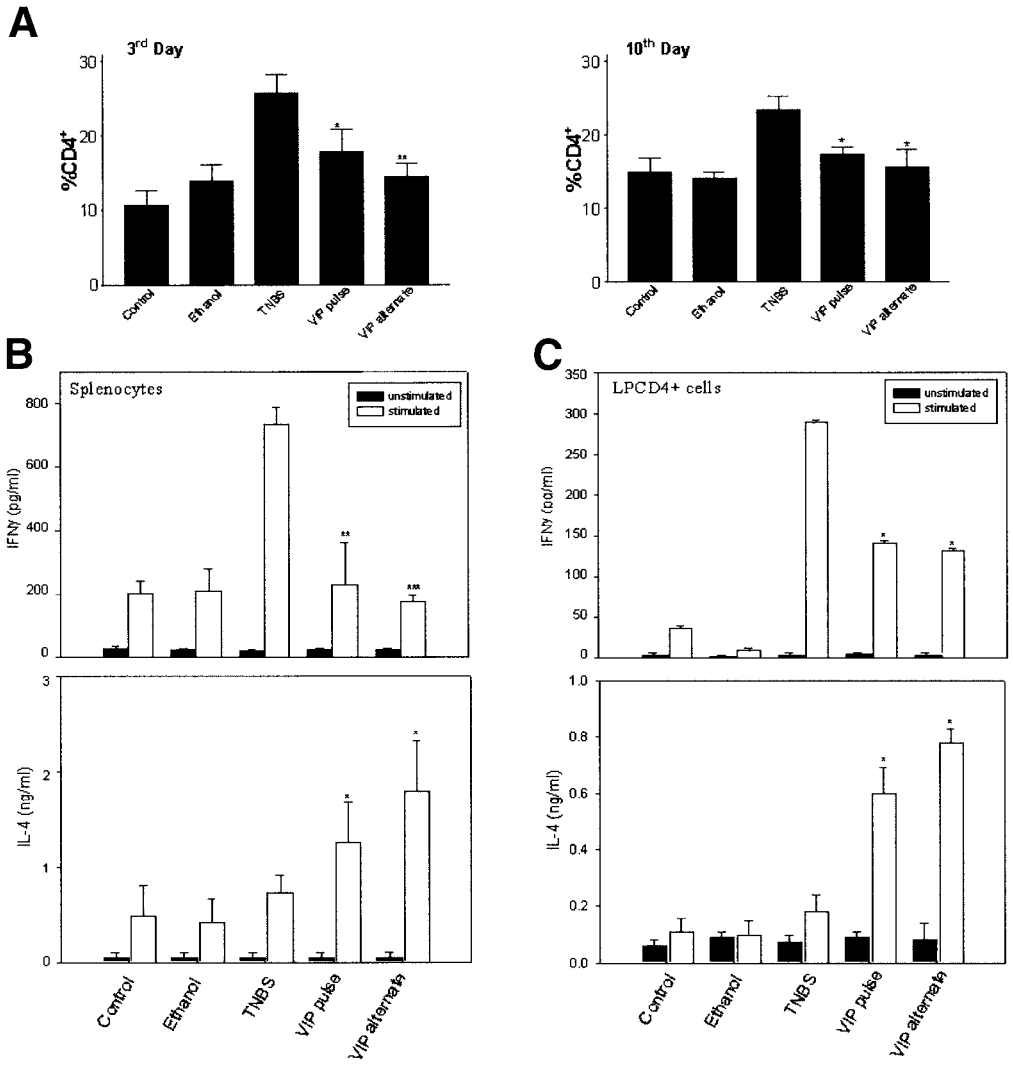

Figure 6. VIP down-modulates the Th1 response generated in TNBS-induced colitis. Mice were treated with 3 mg TNBS and 1 nmol VIP given intraperitoneally in one pulse or every other day. (*A*) VIP reduced the TNBS-induced increase of $CD4^+$ cells. Spleen cells were isolated, and the percentage of $CD4^+$ T cells was determined by fluorescence-activated cell sorter analysis in each experimental group. $*P < 0.01$, $**P < 0.001$. (*B* and *C*) VIP modulated the production of Th1 and Th2 cytokines in mice with TNBS-induced colitis. Spleen cells and lamina propria mononuclear cells were isolated and cultured with medium alone (unstimulated) or stimulated with anti-CD3/CD28 antibodies. Cytokine levels were measured by ELISA in culture supernatants at 48 hours. Data are the mean of 8 animals per group. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$ vs. TNBS-treated animals.

investigated the effect of VIP on Th1/Th2 cytokine production in spleen and lamina propria $CD4^+$ T-cell cultures. TNBS-induced colitis resulted in the development of effector T cells in the spleen culture, producing a 3-fold increase in IFN-γ levels (Figure 6*B*). Treatment with VIP suppressed Th1 cell function, as shown by the decrease in TNBS-induced IFN-γ production in VIP-treated mice. Additionally, Th2 function was enhanced, as seen by the VIP-mediated increase in production of IL-4 (Figure 6*B*) and IL-10 (data not shown). Similar results were observed with stimulated lamina propria $CD4^+$ T-cell lymphocytes (Figure 6*C*), suggesting that VIP modulates the $CD4^+$ T-cell cytokine response to a Th2 secretion profile.

VPAC1 Is the Main Player of the Therapeutic Effect on Colitis

The immunologic effects of VIP are exerted through binding to the family of 3 G protein–coupled receptors: VPAC1, VPAC2, and PACAP receptor 1, which mainly stimulate the adenylate cyclase system. Our in vitro and in vivo studies in several inflammatory models, including RA, have indicated that VPAC1 is the main mediator of the VIP anti-inflammatory action. To evaluate the specificity of the effect of VIP on colitis and to identify the VIP receptor involved, we tested the effects of VPAC1 and VPAC2 agonists. Agonist effects were rated based on animal weight loss, colitis, and microscopic scores as clinical markers of colitis as well as SAA as a disease inflammation marker. Whereas the VPAC1 agonist mimicked VIP effects on all parameters assayed, the VPAC2 agonist showed a weak or no effect on TNBS-induced colitis (Table 1). VIP specificity was probed by the fact that either secretin and glucagon, 2 peptides that belong to the VIP family, or the 2 VIP fragments (VIP_{1-12} and VIP_{10-28}) showed any effect on the studied parameters (Table 1). However, administra-

Table 1. Effect of VIP Agonists and Related Peptides in TNBS-Induced Colitis

	Weight (g)	Colitis score	Histopathologic score	SAA (μg/mL)
Control	22.9 ± 0.3	0 ± 0	0 ± 0	0.08 ± 0.04
TNBS	17.5 ± 0.4	3 ± 0	4 ± 0	301.7 ± 18.2
VIP	21.4 ± 0.3[a]	1 ± 0[a]	1.2 ± 0.2[a]	0.03 ± 0.01[b]
PACAP	21.6 ± 0.4[a]	1 ± 0[a]	1.3 ± 0.3[a]	0.03 ± 0.01[b]
$VIP_{1–12}$	18.8 ± 0.5	3 ± 0	3.7 ± 0.4	298.1 ± 17
$VIP_{10–28}$	18.0 ± 0.5	3 ± 0	3.9 ± 0.1	226.8 ± 51
Secretin	17.0 ± 0.4	3 ± 0	3.7 ± 0.4	310.8 ± 11.8
Glucagon	18.5 ± 0.4	3 ± 0	4 ± 0	237.4 ± 50.8
VPAC1 agonist	21.6 ± 0.3[a]	1 ± 0[a]	1.3 ± 0.4[a]	0.32 ± 0.02[b]
VPAC2 agonist	17.9 ± 0.4	3 ± 0	4 ± 0	234.1 ± 50.1

NOTE. TNBS-administered mice were treated intraperitoneally with the indicated agonist or VIP-related peptide every other day at a dose of 1 nmol. Weight, colitis score, histopathologic score, and SAA levels were assessed as previously described. Data represent mean ± SE of 3 independent experiments (8 mice/group/experiment). [a]$P < 0.01$, [b]$P < 0.001$ vs. TNBS-treated animals.

tion of PACAP, a neuropeptide that shares source, receptors, and function with VIP in the immune system,[21] significantly reduced both clinical and inflammatory signs of colitis. These data show that the therapeutic effect of VIP is mainly mediated through VPAC1.

Discussion

VIP is a neuropeptide with potent modulatory activity on intestinal immunity. Decreased concentrations of VIP and VIP neurons in the gut have been described in previous studies.[22,23] In this study, we showed that the neuropeptide VIP has a highly beneficial effect in TNBS-induced colitis, a murine experimental model of CD. The therapeutic effects of VIP occurred in all phases of the disease (early, acute, and chronic), acting through the inhibition of the inflammatory and T cell–mediated immune responses.

In the early acute phase of bowel inflammation, there is an overlapping of the innate and acquired immune responses, with multiple mediator pathways involved, such as chemokines and cytokines. VIP strongly reduced the inflammatory response by down-regulating the production of different mediators implicated in local and systemic damage as chemokines and proinflammatory cytokines. VIP disrupted the granulocyte and monocyte-macrophage chemokine circuits by down-regulating the production of MIP-1α, MCP-1, MIP-2, and Gro-α, causing at least in part the drastic colon amelioration in the first stages of the disease. Besides, VIP down-regulated the inflammatory response by inhibiting proinflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-12) both in colon extracts and in blood serum and increasing the production of anti-inflammatory agents (IL-10), providing another link in the therapeutic cascade effect of the neuropeptide. We have previously shown the anti-inflammatory effects of VIP in other animal models of disease, such as RA or septic shock, in which VIP also contributed to the down-regulation of these proinflammatory mediators.[14,24–27]

SAA is an acute-phase protein measured for clinical monitoring of CD[28] and is potently induced in response to proinflammatory stimuli that synergize with IL-1 and IL-6 cytokines.[19] Its long-term maintenance is a prerequisite for the development of amyloidosis, a progressive and fatal condition. Although the precise role of SAA in host defense during inflammation has not yet been defined, many potential clinically important functions have been proposed, such as its chemoattractant properties for different leukocyte subpopulations.[29–34] Moreover, the enhancement of SAA production after administration of TNF-α suggests a role of SAA in the inflammatory response.[35] In the present report, we show that treatment with VIP drastically reduced serum SAA levels in the model of TNBS-induced colitis. Although the precise significance of this reduction remains to be elucidated, VIP could represent a regulatory factor for an effective temporal control to avoid damaging consequences of SAA.

The balance of Th1/Th2-type cytokines plays an important role in the establishment of a chronic disease.[36] Th1 cytokines exert potent proinflammatory effects that, when uncontrolled, lead to tissue injury. Conversely, cytokines produced by Th2 cells such as IL-4 and IL-10 have important anti-inflammatory functions. In this study, we showed that treatment with VIP down-regulated the Th1 cytokine profile, decreasing production of IFN-γ and IL-12 (a cytokine that drives to Th1 T-cell differentiation), and up-regulated the Th2 profile, increasing production of IL-4 and IL-10 in stimulated splenocytes and lamina propria mononuclear cell cultures. Similar VIP effects on Th1/Th2 balance have been previously shown for RA[14] and following antigen-specific stimulation.[37] These effects were shown to be associated with the VIP-mediated preferential up-regulation

of the costimulatory molecule B7.2 against B7.1 on antigen-presenting cells as well as the down-regulation of macrophage IL-12 production and subsequent IFN-γ secretion by Th1 cells.[12] Our data show that the therapeutic effect of VIP is mainly mediated through VPAC1, expressed in polymorphonuclear cells, macrophages, and T and B lymphocytes, supporting the involvement of these cell types in the curative effects of VIP, as in other inflammatory/autoimmune diseases such as RA.[14] The fact that the effects of VIP on colitis are mainly mediated through VPAC1 might represent an advantage for the design of more specific and stable therapeutic drugs.

To date, corticosteroids, aminosalicylic acid, and antibiotics have represented the principal approaches for the treatment of inflammatory bowel disease. Novel therapeutic strategies include the blockage of proinflammatory and Th1 cytokines (such as TNF-α,[38] IL-6,[39,40] and IL-12[41]) and the use of recombinant anti-inflammatory cytokines (such as IL-10[42,43] and transforming growth factor β,[44]) while the search for new alternatives, from blocking antibodies to oligonucleotide antisense strategies,[11] continues. The fact that VIP affects a wide range of cytokine mediators of CD is a positive feature with respect to the potential of VIP as a therapeutic agent in humans. We did not observe any adverse effects of the neuropeptide in this murine system at low doses (between 1 and 5 nmol). A higher dose (10 nmol) of VIP led to worsening of the treated mice, with continued diarrhea, probably because of the down-regulation of receptors produced at high doses of VIP.[45] Our study showed that very low pharmacologic doses of VIP produce significant therapeutic effects in intestinal inflammation. Although one pulse of VIP 12 hours after the induction of colitis or on alternate days resulted in the most beneficial effects, injection of VIP even at 5 days after the onset of the disease was enough to exert a significant remission. In view of the clinical application of VIP, some side effects have been described, including a marked decrease in blood pressure.[46,47] Although systemic doses of VIP will need to be tested, local administration of an injectable formulation of VIP is already in use for treatment of erectile dysfunction.[48] The effectiveness of VIP on existing disease and its effect on the prevention of reoccurrence are 2 important characteristics supporting its potential as an anti–inflammatory bowel disease agent.

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