

Antibodies to Interleukin 12 Abrogate Established Experimental Colitis in Mice

By Markus F. Neurath, Ivan Fuss, Brian L. Kelsall, Eckhard Stüber, and Warren Strober

From the Mucosal Immunity Section, National Institutes of Health/National Institute of Allergy and Infectious Diseases/LCI, Bethesda, Maryland 20892-1890

Summary

In this study, we describe a novel murine model of chronic intestinal inflammation induced by the hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS). Rectal application of low doses of TNBS in BALB/c and SJL/J mice resulted in a chronic transmural colitis with severe diarrhea, weight loss, and rectal prolapse, an illness that mimics some characteristics of Crohn's disease in humans. The colon of TNBS-treated mice on day 7 was marked by infiltration of CD4⁺ T cells; furthermore, *in situ* polymerase chain reaction studies revealed high levels of interferon (IFN)- γ mRNA in diseased colons. Isolated lamina propria (LP) CD4⁺ T cells from TNBS-treated mice stimulated with anti-CD3 and anti-CD28 antibodies exhibited a Th1 pattern of cytokine secretion: a 20–50-fold increase in IL-2 and IFN- γ levels and a 5-fold decrease in IL-4 levels as compared with those of stimulated LP CD4⁺ T cells from control BALB/c mice. Administration of monoclonal anti-IL-12 antibodies to the TNBS-treated mice both early (at 5 d) and late (at 20 d) after induction of colitis led to a striking improvement in both the clinical and histopathological aspects of the disease and frequently abrogated the established colitis completely. Furthermore, LP CD4⁺ T cells isolated from anti-IL-12-treated mice failed to secrete IFN- γ upon *in vitro* stimulation. In summary, the data demonstrate the pivotal role of IL-12 and IFN- γ in a TNBS-induced murine model of chronic intestinal inflammation. Furthermore, they suggest the potential utility of anti-IL-12 antibodies in patients with Crohn's disease.

Inflammatory bowel disease (IBD)¹, encompassing Crohn's disease (CD) and ulcerative colitis, are idiopathic chronic diseases occurring with increasing frequency in Western populations (1, 2). Recently, various animal models of chronic intestinal inflammation have been established, which will likely provide new insights into the pathogenesis of IBD (reviewed in 3). These include rats carrying transgenes of HLA-B27 and β 2-microglobulin (4) and mice in which the genes for IL-2 (5), IL-10 (6), and the α or β chain of the TCR (7) have been inactivated by homologous recombination. In addition, a colitis model has been recently established by the adoptive transfer of normal CD45RB^{hi} T cells from BALB/c mice to C.B.-17 scid mice (8). In this case, the transferred T cells manifest a Th1 cytokine response associated with granulomatous inflammation that can be abrogated by systemic treatment with rIL-10, but not with rIL-4 (8).

IL-12 is a recently characterized cytokine with unique structure and pleiotropic effects (9–12). It consists of two disulfide-linked subunits, p40 and p35, that form functionally active p40/p35 heterodimers or inhibitory p40 ho-

modimers. IL-12 is produced mainly by macrophages/monocytes and can be efficiently induced by intracellular parasites, bacteria, and bacterial products. Functional studies showed that IL-12 enhances cytolytic activity of NK cells and macrophages, and that it induces, in synergism with the B7/CD28 interaction, cytokine production and proliferation of activated NK cells and T cells (13). Furthermore, IL-12 plays a pivotal role in Th1 T cell differentiation, and it induces naive T cells to produce IFN- γ . As a result of this ability to drive T cell responses to the Th1 phenotype, IL-12 has been shown to be an effective treatment of established parasitic infections in mice (14, 15) that elicit a Th2 T cell response. In addition, antibodies to IL-12 have been shown to prevent experimental autoimmune encephalitis, a disease mediated by Th1 T cells (16). In the present study, we describe a novel murine model of chronic intestinal inflammation by a single application of the hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS). Furthermore, we demonstrate that successful treatment of established colitis can be achieved by systemic administration of antibodies to IL-12. Since the lamina propria (LP) T cell responses induced by TNBS show similarity to those observed in human CD, the data suggest the potential therapeutic use of anti-IL-12 antibodies in this disease.

¹Abbreviations used in this paper: CD, Crohn's disease; HPF, high power field; IBD, inflammatory bowel disease; LP, lamina propria; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

Materials and Methods

Induction of Colitis. Specific pathogen-free 2–4-mo-old female BALB/c or SJL/J mice were obtained from the National Cancer Institute (Bethesda, MD) and maintained in the Building 10A Animal Facility at the National Institutes of Health. To induce colitis, the mice were lightly anesthetized with metofane (methoxyflurane; Pitman-Moore, Mundelein, IL). A 3.5F catheter was then carefully inserted into the colon such that the tip was 4 cm proximal to the anus. To induce colitis, 0.5 mg of the hapten reagent TNBS (Sigma Chemical Co., St. Louis, MO) in 50% ethanol (to break the intestinal epithelial barrier) was slowly administered into the lumen of the colon via the catheter fitted onto a 1-ml syringe. In control experiments, mice received 50% ethanol alone using the same technique described above. The total injection volume was 100 μ l in both groups allowing TNBS or ethanol to reach the entire colon, including the caecum and appendix. Animals were then kept in a vertical position for 30 s and returned to their cages.

Grading of Histologic Changes. Tissues were removed at indicated time points and embedded in paraffin. Paraffin sections were made and stained with hematoxylin and eosin. The degree of inflammation on microscopic cross-sections of the colon was graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low level; and 2, low level of leukocytic infiltration; 3, high level of leukocytic infiltration, high vascular density, thickening of the colon wall; 4, transmural infiltrations, loss of goblet cells, high vascular density, thickening of the colon wall). Grading was done in a blinded fashion by the same pathologist.

Morphometric Assessment of Colon Wall Thickness. Three or more animals from each treatment group were randomly selected at the indicated time points (see Results), and colon samples were removed and embedded in paraffin. Thickness of the colon wall was determined on cross-sections by measuring the distance from the serosal surface to the luminal surface at 2-mm intervals along the entire length of each section through a calibrated eyepiece using a Vanox S1 microscope (Olympus Corp., Lake Success, NY).

Immunohistochemistry. Samples were put into OCT compound on dry ice, and 7- μ m cryosections were cut according to standard procedures. Sections were then air dried and fixed in cold acetone for 2 min at room temperature. Next, samples were rehydrated in PBS for 15 min, blocked with 5% FCS in PBS for 20 min, and incubated with FITC-conjugated rat anti-mouse CD4 antibody (1:100 dilution; obtained from Pharmingen, San Diego, CA) for 45 min in a dark humid chamber. Sections were then washed for an additional 15 min in PBS. Finally, sections were mounted and analyzed with a fluorescence microscope at an excitation wavelength of 490 nm.

Quantification of CD4⁺ T lymphocytes was performed on cryostat sections for at least three specimens from each time point and each treatment group by examining 10 randomly selected high power fields (HPFs). Under our experimental conditions (magnification of 400), one HPF represented 0.25 mm².

Cell Isolation and Purification of LP CD4⁺ T Cells. LP lymphocytes were isolated from freshly obtained colonic specimens using a modification of the technique described by van der Heijden and Stok (17). After removal of the Peyer's patches, the colon was washed in Ca/Mg-free HBSS, cut into 0.5-cm pieces and incubated twice in HBSS containing EDTA (0.37 mg/ml) and DTT (0.145 mg/ml) at 37°C for 15 min. Next, the tissue was digested further in RPMI containing collagenase D (400 U/ml) and DNase I (0.1 mg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN) in a shaking incubator at 37°C. LP cells were then layered on a 40–100% Percoll gradient (Pharmacia, Uppsala,

Sweden), and lymphocyte-enriched populations were isolated from the cells at the 40–100% interface. Enriched CD4⁺ T cell populations were obtained by negative selection using mouse CD4⁺ T cell isolation columns (Isocell; Pierce Chemical Co., Rockford, IL). The resultant cells when analyzed by flow cytometry (FACSscan®; Becton Dickinson & Co., Mountain View, CA) contained >85% CD4⁺ cells.

Cell Culture of LP CD4⁺ T Cells. Cell cultures of LP CD4⁺ T cells were performed in complete medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 3 mM L-glutamine, 10 mM HEPES buffer, 10 mg/ml gentamycin (Whittaker), 100 U/ml each of penicillin and streptomycin (Whittaker), 0.05 mM 2-ME (Sigma Chemical Co.), and 10% FCS.

Reagents and mAbs. Unconjugated and biotinylated monoclonal rat anti-mouse IL-2 (clones JES6-1A12/JES6-5H4), IL-4 (BVD4-1D11/BVD6-24G2), IL-10 (JES5-2A5/SXC-1), and IFN- γ (R4-6A2/XMG1.2) antibodies and mouse rIL-2 (specific activity = 2.5×10^6 BRMP U/mg), IL-4 (10^7 U/mg by CTLL-2.4 assay), IL-10 (5×10^5 U/mg), and IFN- γ (10^7 U/mg) were purchased from Pharmingen and Genzyme Corp., (Cambridge, MA), respectively. Purified hamster anti-mouse CD3 ϵ (clone 145-2C11) and hamster anti-mouse CD28 (clone 37.51) antibodies were obtained from Pharmingen.

Cytokine Assays. To measure cytokine production, 24-well plates (Costar Corp., Cambridge, MA) were coated with 10 μ g/ml murine anti-CD3 ϵ antibody in carbonate buffer (pH 9.6) overnight at 4°C. 10⁵ LP CD4⁺ T cells were then cultured in 1 ml of complete medium in precoated or uncoated wells, and 1 μ g/ml soluble anti-CD28 antibody was added to the anti-CD3 ϵ -coated wells. Culture supernatants were removed after 48 h and assayed for cytokine concentration. Cytokine concentrations were determined by specific ELISA per the manufacturer's recommendation (Pharmingen) using Immulon 4 96-well microtiter plates (Dynatech Laboratories Inc., Chantilly, VA). ODs were measured on a Dynatech MR 5000 ELISA reader at a wavelength of 490 nm.

Isolation of Spleen CD4⁺ T Cells. Spleens were aseptically removed and subsequently digested with collagenase (400 U/ml) and DNase I (0.1 mg/ml) at 37°C for 15 min. After filtration straining, the resulting splenocyte suspension was depleted of RBC by hypotonic lysis with ACK lysing buffer (B & B Scott, W. Warwick, RI). Cells collected from the 70–90% layer of a Percoll gradient centrifugation underwent further negative selection using mouse CD4⁺ T cell isolation columns as described above. As assessed by FACS® analysis, the resulting cell population contained >85% CD4⁺ cells.

Cell Culture of Spleen CD4⁺ T Cells. 10⁵ spleen CD4⁺ T cells were cultured in 1 ml of complete medium (see above). Culture supernatants were removed after 48 h and assayed for cytokine concentration as described above.

In Situ Reverse Transcriptase PCR (RT-PCR). In situ RT-PCR for IFN- γ mRNA expression was performed as previously described (18). Cryosections were placed on charged glass slides that were cut to fit into 0.5-ml Eppendorf tubes. Samples were then fixed in 10% formaldehyde overnight at 4°C, washed three times in PBS and four times in autoclaved dH₂O for 5 min. Next, sections were permeabilized with 2 mg/ml trypsinogen (Sigma) in 0.01 N HCl for 15 min at 25°C followed by neutralization with buffer A (0.1 M Tris HCl, pH 7.5, 0.1 M NaCl). For DNA degradation, the sections were then incubated in RQ1 RNase-free DNase (8 U/100 ml; obtained from Promega Corp., Madi-

son, WI) in buffer B containing 40 mM Tris HCl, pH 7.9, 10 mM NaCl, 6 mM MgCl₂, and 0.1 mM CaCl₂ at 37°C for 12 min and at 75°C for 10 min. Next, sections were incubated for 60 min at 50°C in a Perkin Elmer thermocycler in 100 µl of the following reaction mixture: 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl₂, 25 µM dATP, 25 µM dTTP, 25 µM dCTP, 25 µM dGTP (Pharmacia Fine Chemicals, Piscataway, NJ), 100 nM of either IFN-γ primer (sense: 5'-GACAATCAGGCCATCAGCAACAAC-3'; and antisense primer: 5'-TCCTGAGGCTG-GATTCCGGCAACA-3' [19]), 10 mM DTT, 75 U RNasin, 400 U M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Slides were washed five times each in sodium citrate buffer (3 M NaCl, 0.3 M Na₃ citrate, pH 7.0, 2× SSC), 1× SSC, 0.5× SSC, and twice in dH₂O.

PCR in situ of either sense- or antisense-primed cDNA was carried out in 100 µl of the following reaction mixture: 25 µM of each the nucleotides dATP, dCTP, dGTP, and 23.7 µM dTTP, 1.25 µM digoxigenin-11-dUTP (dig-11-dUTP; obtained from Boehringer Mannheim), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 5 U Taq polymerase (Boehringer Mannheim), and 10 nM of IFN-γ primers. After denaturation of the samples for 4 min at 95°C, thermocycling was performed for five cycles (94°C 70 s, 62°C 1 min, 72°C 1 min); the final extension was done for 10 min at 72°C. The samples were then washed in SSC solutions (2× SSC, 1× SSC, 0.5× SSC; five times each) and immunodetection was performed using the DIG nucleic acid detection kit (Boehringer Mannheim). Next, sections were dehydrated in graded ethanols, placed in xylene, and mounted on coverslips.

Treatment with Anti-IL-12 Antibodies. The hybridoma cell line (C17.8) producing neutralizing rat anti-mouse IL-12 antibody was kindly donated by G. Trinchieri (The Wistar Institute, Philadelphia, PA [20, 21]). Ascites was prepared in nude mice according to standard procedures, and antibodies were purified using E-Z-SEP purification kits (Middlesex Sciences, Inc., Foxborough, MA). Rat control IgG was obtained from Jackson ImmunoResearch (West Grove, PA). 1 mg of rat anti-mouse IL-12 antibodies or rat control IgG were administered intraperitoneally into mice pretreated with TNBS at indicated time points.

Elispot Assay for IFN-γ. 10⁵ LP CD4⁺ T cells were incubated for 1 d in anti-CD3ε-coated 24-well plates, and 1 µg/ml soluble anti-CD28 antibody was added. Next, cells were incubated in 24-well plates that were coated with rat anti-mouse IFN-γ (Pharmingen). After 12 h, plates were washed in PBS/Tween, biotinylated rat anti-mouse IFN-γ (Pharmingen) (2 µg/ml) was added, and incubation was performed overnight at 4°C. After washing in PBS/Tween streptavidine-alkaline phosphatase (1:1,000 dilution; Zymed Laboratories, San Francisco, CA) was added for 30 min at 37°C. Plates were washed again in PBS/Tween and the AP substrate (Promega Corp.) together with 1% agarose gel was added. Color reaction was allowed to proceed for 24 h before spots were photographed.

Results

Intrarectal Administration of TNBS Induces a Chronic Granulomatous Colitis in BALB/c and SJL/J Mice. Based on previous studies showing that rectal administration of the hapten reagent TNBS induces colitis in rats (22, 23), we explored the possibility that administration of TNBS can induce a chronic inflammation of the murine colon. We found that BALB/c and SJL/J mice subjected to intrarectal administration of TNBS in 50% ethanol reproducibly developed pancolitis with severe diarrhea and rectal prolapse

(Fig. 1 a) accompanied by an extensive wasting disease (Fig. 1 b). The peak of clinical disease occurred at 3 wk, and clinical signs of colitis usually subsided after 2 mo. Control mice treated with 50% ethanol alone failed to develop wasting disease and appeared healthy.

The colons of TNBS-treated BALB/c mice removed 7 d after administration of TNBS revealed striking hyperemia and inflammation (Fig. 2), whereas the colons of control mice treated with 50% ethanol alone showed no macroscopic signs of inflammation. In addition, TNBS-treated mice displayed splenomegaly.

Histologic analysis during the first days after induction of colitis showed infiltrations of neutrophil granulocytes into the colon. On day 7, a transmural inflammation affecting the entire colon (but sparing the small bowel) was found. The colitis was mainly characterized by lymphocytic infiltrates that were associated with thickening of the colon wall, ulcerations, loss of goblet cells, and the presence of granulomas (Fig. 3, a-b).

Immunohistochemical staining showed that on day 7, CD4⁺ T cells were increased in colons of TNBS-treated mice compared to control mice (Fig. 3, c-d). The differences in inflammatory activity were further confirmed by histologic grading of the colon sections (Table 1). As assessed by morphometric analysis of colon wall thickness and number of CD4⁺ T lymphocytes (Table 2), disease in-

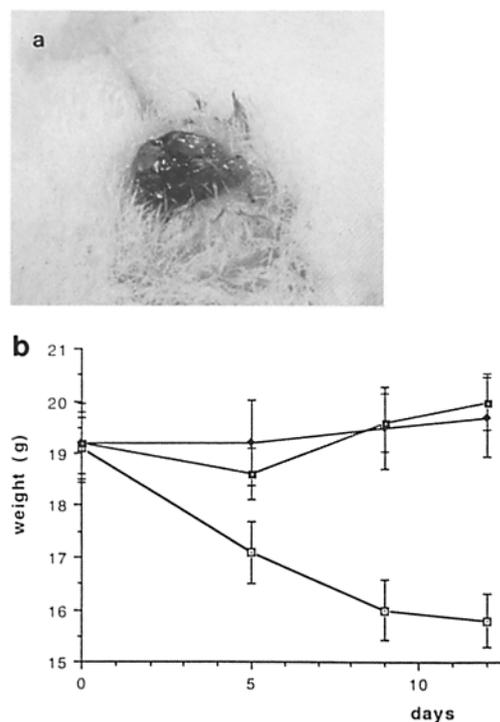


Figure 1. Intrarectal administration of TNBS induces severe diarrhea and wasting disease (a). Rectal prolapse of a BALB/c mouse 7 d after administration of TNBS in 50% ethanol. (b) Weight changes of normal BALB/c mice, control mice treated with 50% ethanol alone, and BALB/c mice were treated with TNBS in 50% ethanol over a 12-d period. Weight data from one representative experiment is shown. Each point represents average weight data pooled from five mice. Standard errors are indicated. —□—, Ethanol group; —◆—, normal BALB/c mice; —○—, TNBS-colitis group.

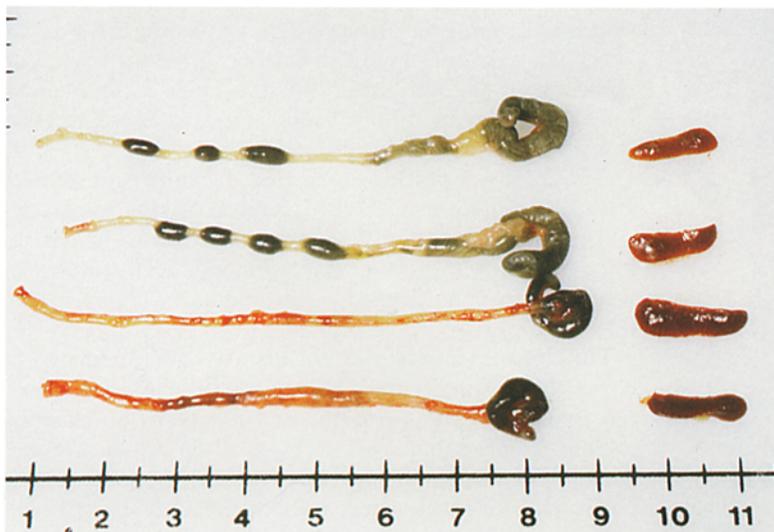


Figure 2. Macroscopic changes of colon and spleen in TNBS-treated mice. Photographs of dissected large intestine and spleen of a normal BALB/c mouse (*top*), a control BALB/c mouse treated with 50% ethanol (*second row*), and two BALB/c mice treated with TNBS in 50% ethanol (*bottom rows*) 7 d after the initial rectal administration. The colons of the TNBS-treated mice are severely inflamed, hyperemic, and they contain less feces due to massive diarrhea.

tensity usually peaked between 2 and 4 wk after administration of TNBS. At later stages of the disease, there was a reduction in the number of granulocytes, but intramural lymphoid aggregates persisted and beginning fibrosis was found (Fig. 3 *e*). These histological signs of inflammation were still detected up to 2 mo after TNBS treatment, but were absent in ethanol-treated mice.

Histologically, the spleens of TNBS-treated mice showed

an increase in the size of the red pulp and the periarteriolar lymphoid sheaths on day 7 when compared with spleens from control mice (Fig. 4, *a–b*). FACS[®] analysis of spleen lymphocytes revealed a twofold increase in the percentage of CD4⁺ and CD8⁺ T cells in TNBS-treated mice compared with control ethanol-treated mice and normal BALB/c mice, along with a reduction in B220⁺ B cells (data not shown).

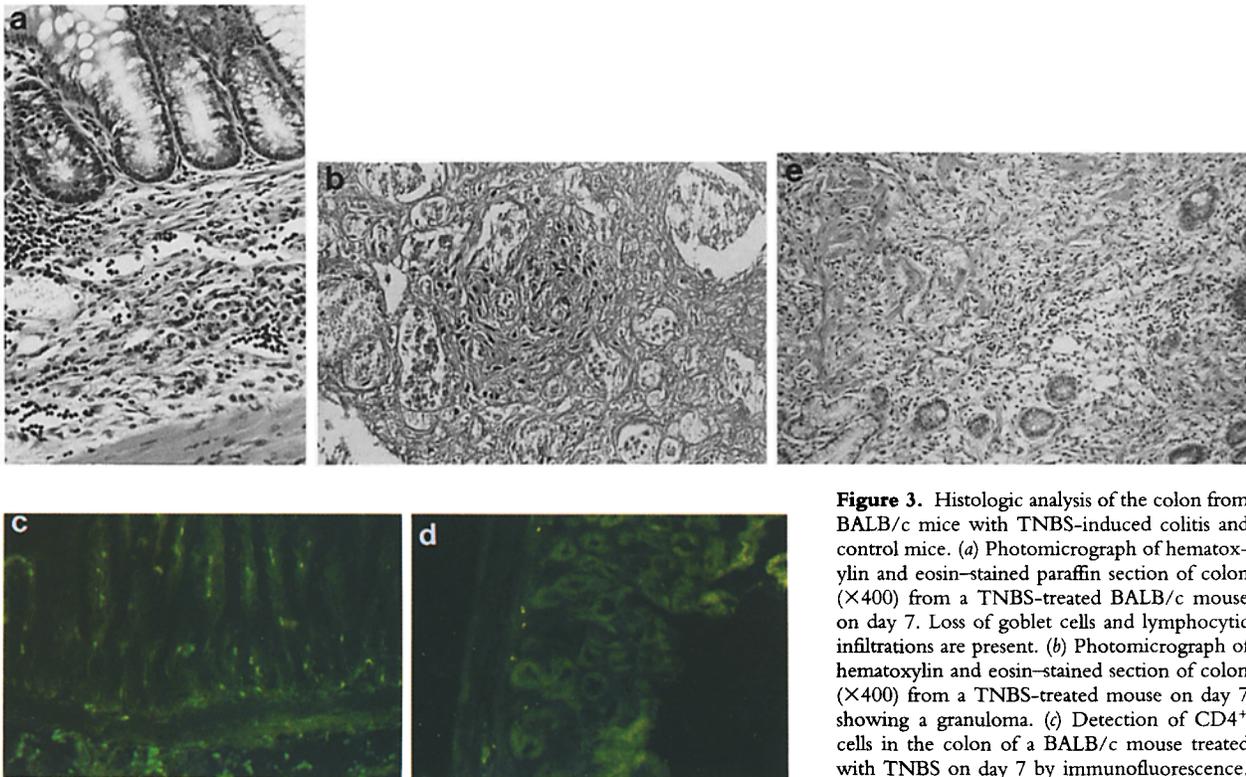
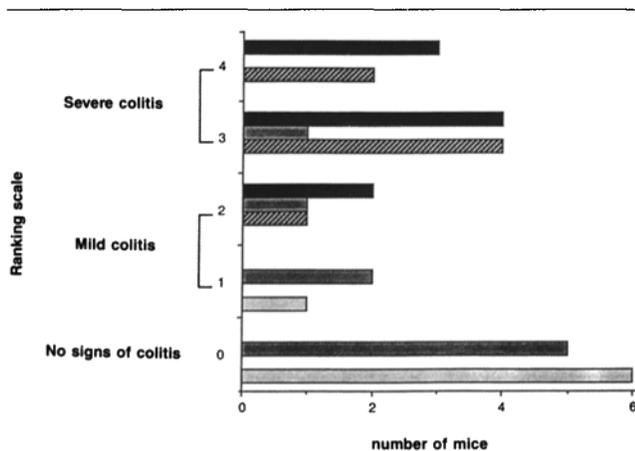


Figure 3. Histologic analysis of the colon from BALB/c mice with TNBS-induced colitis and control mice. (*a*) Photomicrograph of hematoxylin and eosin-stained paraffin section of colon ($\times 400$) from a TNBS-treated BALB/c mouse on day 7. Loss of goblet cells and lymphocytic infiltrations are present. (*b*) Photomicrograph of hematoxylin and eosin-stained section of colon ($\times 400$) showing a granuloma. (*c*) Detection of CD4⁺ cells in the colon of a BALB/c mouse treated with TNBS on day 7 by immunofluorescence. FITC-positive cells were seen in the subepithe-

lial areas and the lamina propria ($\times 100$). (*d*) Immunostaining with FITC-labeled anti-mouse CD4 antibodies in the colon of a control ethanol-treated BALB/c mouse on day 7. Only few positive cells were detected ($\times 100$). (*e*) Photomicrograph of hematoxylin and eosin-stained section of colon ($\times 150$) from a TNBS-treated mouse after 7 wk showing chronic inflammation with intramural lymphoid aggregates and beginning fibrosis.

Table 1. Histologic Grading of Colon Sections from Control BALB/c Mice and from Mice with TNBS-induced Colitis Treated with Anti-IL-12, Control Rat IgG, or with No Additional Reagent



Colon specimens were taken at indicated time points after administration of TNBS or ethanol, and the magnitude of inflammatory changes in the colons was analyzed on hematoxylin and eosin-stained cross-colon sections. Data were pooled from three independent experiments in each group. ■, Ethanol group day 7; ▨, TNBS group day 7; ▩, anti-IL-12 group day 12; ■, rat IgG group day 12.

Stimulated LP CD4⁺ T Lymphocytes of TNBS-treated Mice Secrete Th1 Cytokines. To examine cytokine production by infiltrating LP CD4⁺T cells in the colon of TNBS-treated mice, we purified this population from colonic tissue specimens 7 d after the induction of colitis and compared their cytokine pattern with that of LP CD4⁺ T cells obtained from colonic tissue specimens of control ethanol-treated mice. Cells were cultured for 2 d and culture supernatants were analyzed for concentration of Th1 (IL-2, IFN- γ) and Th2 (IL-4, IL-10) cytokines by specific ELISA. As shown in Fig. 5 *a*, a 10-fold increase in the spontaneous

Table 2. Assessment of Colon Wall Thickness and Number of CD4⁺ T Lymphocytes per HPF in the Colon of TNBS- and Ethanol-treated BALB/c Mice at Different Time Points

Time point	Colon wall thickness (μ m)		CD4 ⁺ T lymphocytes per HPF	
	Ethanol	TNBS	Ethanol	TNBS
0 wk	226.4 \pm 12.5	210.4 \pm 20.8	3.7 \pm 0.4	3.7 \pm 0.4
1 wk	239.8 \pm 6.0	419.8 \pm 38.9	5.1 \pm 0.5	38.9 \pm 4.1
2 wk	213.0 \pm 8.5	522.2 \pm 76.2	4.7 \pm 0.6	58.2 \pm 4.1
4 wk	213.0 \pm 8.6	427.5 \pm 56.3	3.7 \pm 0.4	76.1 \pm 6.4
6 wk	219.3 \pm 16.0	394.2 \pm 45.0	4.3 \pm 0.5	34.4 \pm 3.3
8 wk	238.8 \pm 7.4	412.2 \pm 26.9	5.4 \pm 0.5	29.0 \pm 3.7

Colon wall thickness is expressed in micrometers \pm SEM. The values for CD4⁺ T lymphocytes reported are expressed as positive cells per HPF \pm SEM.

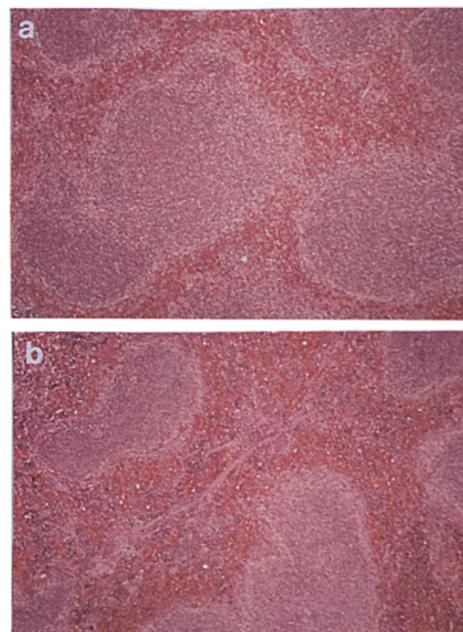


Figure 4. Histologic analysis of the spleen from mice with TNBS-induced colitis and control mice. Photomicrographs of hematoxylin and eosin-stained sections (\times 100) from spleens of a control ethanol-treated BALB/c mouse (*a*) and a TNBS-treated BALB/c mouse (*b*) on day 7. The spleen of the TNBS-treated mouse reveals hypervascularisation and strikingly increased red pulp and periarteriolar lymphoid sheet areas.

IFN- γ production of LP CD4⁺ T cells was found in TNBS-treated mice. Furthermore, LP CD4⁺ T cells from TNBS-treated mice stimulated with anti-CD3 and anti-CD28 produced 20–50-fold higher levels of IL-2 and IFN- γ than LP CD4⁺ T cells from control mice (Fig. 5, *a–b*). Similarly, an increase in the spontaneous (4.5 vs 1.8 U) and induced (46 vs 16.8 U after stimulation with anti-CD3 and anti-CD28) IFN- γ production by spleen CD4⁺ T cells was found in the TNBS-treated animals compared with the ethanol control group at this time point.

In contrast to the above finding, secretion of IL-4 by unstimulated LP CD4⁺ T cells from TNBS-treated mice was identical compared to LP CD4⁺ T cells from ethanol-treated control mice, and in stimulated LP CD4⁺ T cells from TNBS-treated mice, the average secretion of IL-4 was reduced about fivefold compared with ethanol-treated control mice (Fig. 5 *c*). Finally, the secretion of IL-10 by stimulated and unstimulated LP CD4⁺ T cells was similar in TNBS- and ethanol-treated mice (Fig. 5 *d*).

In Situ PCR Studies Show Elevated IFN- γ mRNA Expression in the Colon of TNBS-treated BALB/c Mice. To determine if the observed increase in IFN- γ production was also observed at the mRNA level, we evaluated the mRNA expression of IFN- γ in the colon of TNBS-treated mice by in situ PCR studies. As shown in Fig. 6 *a*, control ethanol-treated animals did not show significant expression of IFN- γ mRNA on day 7. In the TNBS-treated animals, however, we found a dramatic upregulation of IFN- γ mRNA expression at the same time point (Fig. 6 *b*). High staining intensity was seen particularly in the subepithelial areas.

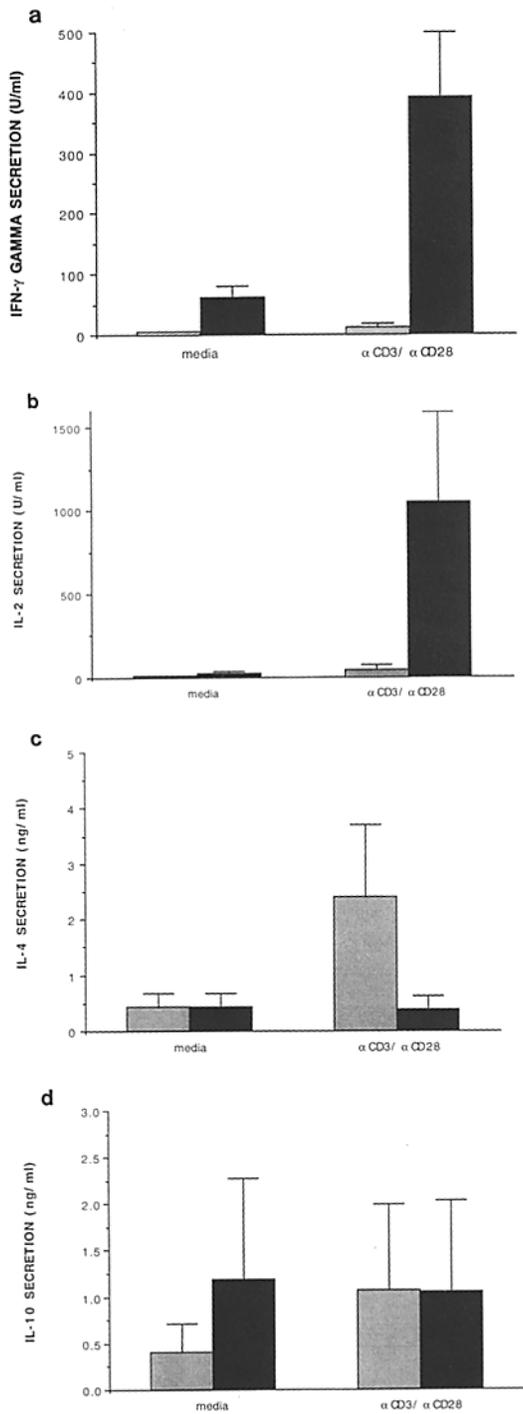


Figure 5. Cytokine production of stimulated and unstimulated LP CD4⁺ T cells in TNBS-induced colitis. LP CD4⁺ T cells were isolated from TNBS- and control ethanol-treated mice on day 7, cultured for 2 d in the absence or presence of anti-CD3 and anti-CD28 (see Materials and Methods) and culture supernatants were analyzed for concentration of IFN- γ (a), IL-2 (b), IL-4 (c), and IL-10 (d). Data represent three independent experiments done in triplicate. Standard errors are indicated. \square , Ethanol group; \blacksquare , TNBS-colitis group.

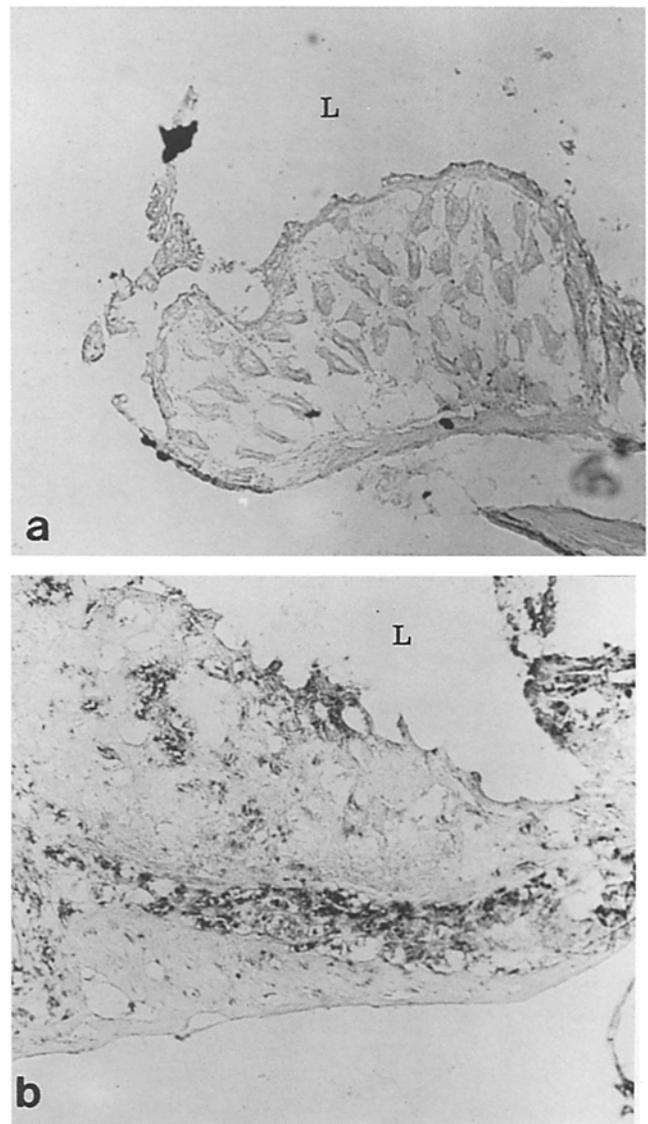


Figure 6. Increased expression of IFN- γ mRNA in the colon of BALB/c mice with TNBS-induced colitis. (a) In situ PCR staining for IFN- γ mRNA expression in the colon of an ethanol-treated mouse. Only very low staining intensity is found using antisense-primed cDNA as template for the PCR reaction. The luminal site (L) of the colon is indicated ($\times 50$). (b) In situ PCR staining for IFN- γ mRNA expression in the colon of a TNBS-treated mouse. High staining intensity is seen in the LP. The luminal site of the colon is indicated. No staining was found with the antidigoxigenin antibody using sense-primed cDNA as template for the PCR reaction (data not shown) ($\times 100$).

Early Administration of Antibodies to IL-12 Represses Colitis and Abolishes Wasting Disease in TNBS-treated Mice. Since the previous data suggested the presence of activated Th1 cells in TNBS-induced colitis, we sought to determine if antibodies to IL-12 might influence disease activity. We therefore treated mice 5 and 9 d after induction of the colitis systemically with anti-IL-12 or control rat IgG (see Materials and Methods). When mice were treated with anti-IL-12, a striking improvement of the wasting disease became apparent. Anti-IL-12-treated mice became more active and lost their ruffled coat appearance when com-

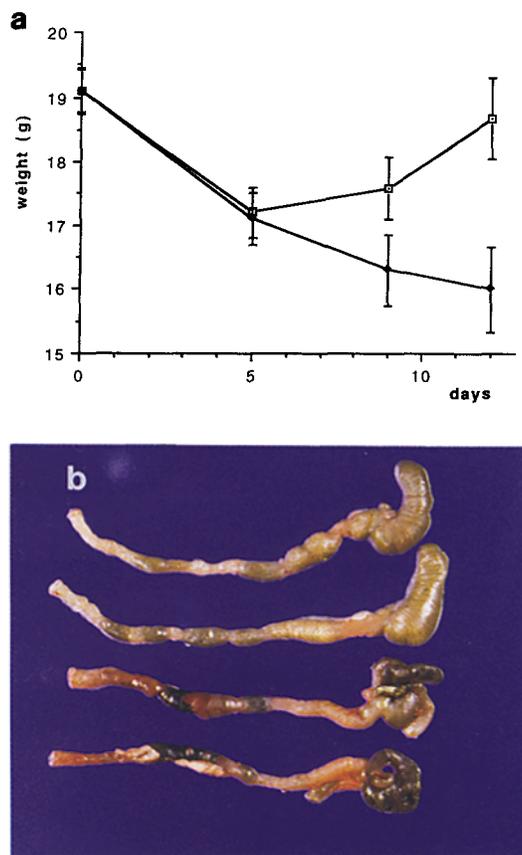


Figure 7. Anti-IL-12 antibodies abrogate colitis present 5 d after administration of TNBS. (a) Weight changes of BALB/c mice with TNBS-induced colitis after early administration (day 5) of anti-IL-12 antibodies or rat control IgG. After initial reduction of the body weight in both TNBS-treated groups, the mice treated with anti-IL-12 showed increase in the average body weight after day 5, whereas mice treated with rat control IgG continuously lost body weight. Each point represents data from five mice. The standard errors are indicated. \square , Anti-IL-12 group; \blacklozenge , rat IgG control group. (b) Gross appearance of the colon from two TNBS-treated mice given anti-IL-12 antibodies (top rows) and two TNBS-treated mice given rat IgG (bottom rows) at day 12 after initial administration of TNBS. There was a reduction in inflammatory activity in the anti-IL-12-treated mice. One representative experiment out of three is shown.

pared with untreated mice or mice given control rat IgG (data not shown); in addition, as depicted in Fig. 7 a, mice that had received anti-IL-12 usually obtained their initial body weight, whereas control IgG-treated mice continued to lose weight. Finally, gross inspection of the colon on day 12 revealed reduction in inflammatory activity in animals administered anti-IL-12 (Fig. 7 b).

Histologic studies showed significantly less inflammatory cells in the colons of anti-IL-12-treated mice (Fig. 8 a–b). In most cases, anti-IL-12 treatment completely abrogated the TNBS-induced inflammation and restored a normal histologic appearance of the colon. This was confirmed by histologic grading of colon sections: pooled data from three independent experiments showed significant reduction in inflammatory activity after anti-IL-12 treatment (Table 1).

IFN- γ Production by Stimulated LP CD4⁺ T Cells Is Reduced in TNBS-treated Mice Given Anti-IL-12. Next, we

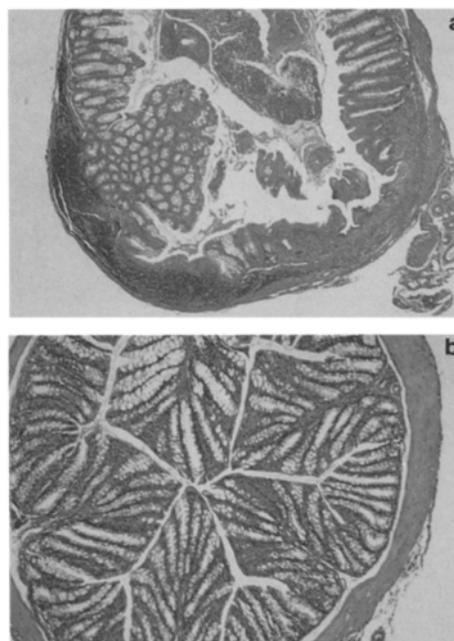


Figure 8. Histologic analysis of the colon in mice with TNBS-induced colitis given anti-IL-12 or rat control IgG. (a) Photomicrograph of HE-stained cross section ($\times 50$) of a colon of a BALB/c mouse with TNBS-induced colitis after treatment with rat control IgG on day 12. There was a severe transmural colitis. (b) Photomicrograph of HE-stained cross-section ($\times 100$) of a colon of a BALB/c mouse with TNBS-induced colitis after treatment with anti-IL-12 antibodies at the same time point. There was a striking reduction in the inflammatory activity of the colon.

analyzed IFN- γ production by LP CD4⁺ T cells in anti-IL-12-treated animals. As shown in Fig. 9 a, we found an abrogation of high level IFN- γ production in TNBS-treated mice that had received anti-IL-12 compared with rat IgG-treated mice suggesting that the anti-IL-12 treatment might act by influencing the Th1-like response of local CD4⁺ T cells. In addition, Elispot assays for IFN- γ secretion by LP CD4⁺ T cells showed a dramatic reduction in the average number of Elispots in the anti-IL-12-treated group compared to the rat control IgG-treated group (Fig. 9 b–c). The size of the Elispots, however, was similar in both groups, indicating that the reduction in IFN- γ secretion by LP CD4⁺ T cells from anti-IL-12-treated mice is mainly due to a reduction in the number of IFN- γ -secreting cells.

Late Administration of Antibodies to IL-12 Abolishes Wasting Disease in Mice with TNBS-induced Colitis. Finally, we wanted to determine if anti-IL-12 treatment would be effective during later phases of the disease when colitis was fully established. We thus started administration of anti-IL-12 or control rat IgG on day 20 and repeated such treatment on days 24 and 28. As shown in Fig. 10 a, a striking increase in the average weight of mice was found after anti-IL-12 treatment but not after rat control IgG treatment. Furthermore, when LP CD4⁺ T cells from such mice were stimulated with anti-CD3 and anti-CD28, we found a reduction of IFN- γ secretion in those mice given anti-IL-12, but not those given rat control IgG (Fig. 10 b).

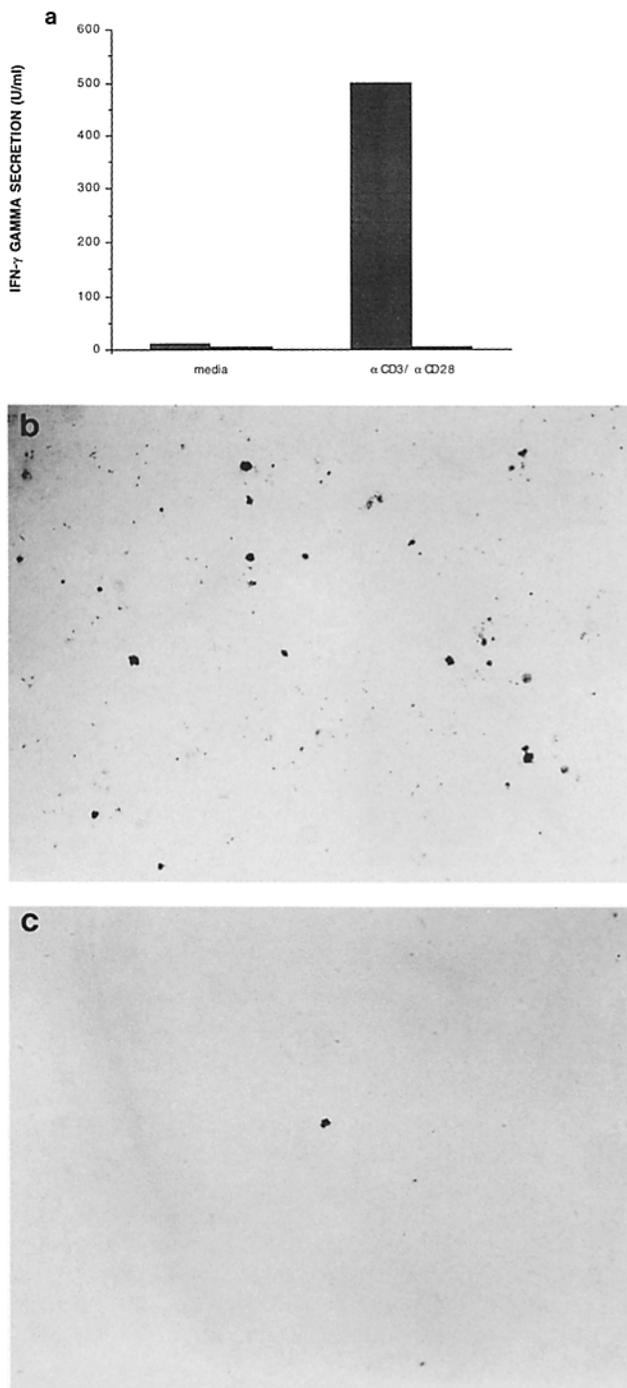


Figure 9. Analysis of IFN- γ production in BALB/c mice with TNBS-induced colitis given anti-IL-12 or rat control IgG. (a) Cytokine production of LP CD4⁺ T cells from TNBS-treated animals on day 12. There was an abrogation of high-level IFN- γ secretion in the anti-IL-12-treated animals. \blacksquare , Rat IgG control group; \boxtimes , anti-IL-12 group. (b) Elispot assay for IFN- γ secretion. LP CD4⁺ T lymphocytes from TNBS-treated mice given rat control IgG were isolated and analyzed as specified in Materials and Methods. A high number of Elispots per high power field was seen. (c) Elispot assay for IFN- γ secretion using LP CD4⁺ T cells from anti-IL-12-treated mice. There was a striking reduction in the average number of Elispots per high power field in the mice treated with anti-IL-12 antibodies compared with those given control IgG.

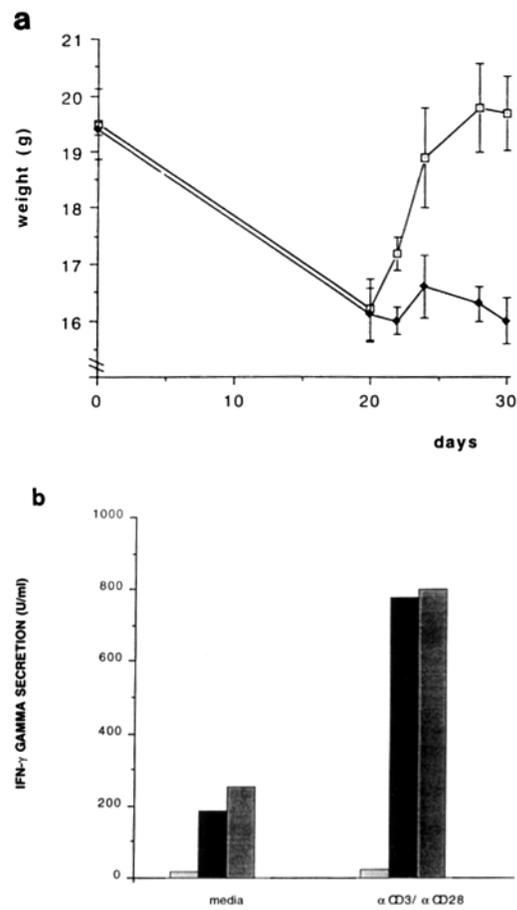


Figure 10. Anti-IL-12 antibodies abrogate established colitis present in BALB/c mice 20 d after TNBS administration. (a) Weight changes in BALB/c mice with TNBS-induced colitis after late administration (day 20) of anti-IL-12 or rat control IgG. After initial reduction of the body weight in both TNBS-treated groups, the mice treated with anti-IL-12 showed increased average body weight. In contrast, no increase was observed in mice given rat control IgG. Each point represents data from five mice. The standard errors are indicated. \square , Anti-IL-12 group; \blacklozenge , rat IgG control group. (b) Cytokine production of LP CD4⁺ T cells from TNBS-treated animals after late administration (day 20) of anti-IL-12 or rat control IgG. LP CD4⁺ T cells from control ethanol-treated mice or TNBS-treated mice on day 30 served as controls. There was a complete abrogation of IFN- γ secretion (<1 U/ml) in the anti-IL-12-treated animals. \square , Ethanol group; \blacksquare , TNBS-colitis group; \blacksquare , rat IgG control group; \boxtimes , anti-IL-12 group.

Discussion

In the present study, we describe a novel murine model of chronic intestinal inflammation induced by a single rectal administration of the hapten reagent TNBS. Furthermore, we demonstrate that the inflammation is associated with a Th1 T cell response and can be abrogated by systemic treatment with antibodies against IL-12, even after inflammation is well established.

The immune responses to hapten determinants such as TNBS are believed to depend on the haptenization of autologous proteins and presentation of MHC class II-fitting peptides to CD4⁺ T cells by antigen presenting cells, ultimately leading to specific CD4⁺ T cell recognition, T cell expansion, and T cell cytokine responses (24). While hap-

ten-induced responses induced in the skin are self-limited reactions, the hapten-induced responses in the colon manifest a striking chronicity, as shown by the fact that histologic signs of inflammation were still present 2 mo after the mice were initially treated with TNBS. A similar chronic colonic inflammation for at least 2 mo after rectal administration of TNBS in rats has been described (22).

The murine TNBS model of chronic intestinal inflammation contains several features that distinguish it from previously described models (4–8): First, the inflammation is rapidly and reliably induced in a mouse with a normal immune system so that it does not require the loss of a major immune capacity. Second, and perhaps most importantly, a chronic colitis is induced that is characterized by a severe, transmural inflammation associated with diarrhea, rectal prolapse, and weight loss. These clinical and histopathological features underscore that TNBS-induced colitis mimics some important characteristics of CD in humans. As such, the formation of granuloma in TNBS-induced colitis is interesting since granuloma can also be found in CD in humans (25) and granulomatous inflammation is considered the most specific histological finding in this disease (26).

Analysis of cytokine production by stimulated LP CD4⁺ T cells derived from mice with TNBS-induced colitis showed strikingly elevated levels of IL-2 and IFN- γ production. This Th1 pattern of cytokine response resembles that obtained in hapten-induced delayed type hypersensitivity in the skin (27, 28). Since recent studies of cytokine patterns from intestinal T cells in IBD have shown a higher number of IL-2- and IFN- γ -secreting cells, as well as increased IFN- γ mRNA and protein levels in patients with CD (reference 29 and Fuss I., M. F. Neurath, M. Boivivant, C. Fiocchi, I. S. Klein, S. A. Strong, and W. Strober, manuscript in preparation), the cytokine pattern of T cells in the murine TNBS-induced colitis is consistent with that found in CD. Furthermore, the levels of IL-4 secreted by stimulated LP CD4⁺ T cells were normal or reduced in TNBS-treated mice resembling the normal or reduced levels secreted by stimulated LP CD4⁺ T cells in CD (Fuss et al., manuscript in preparation). Thus, TNBS-induced colitis in mice has some similarity to human CD at the T cell cytokine level.

Perhaps one of the most striking aspects of the findings reported here is that the TNBS-induced colitis can be successfully treated with antibodies against IL-12, even after the lesion is established. Thus, when we administered anti-IL-12 to mice 5 d after TNBS administration, at a time when they were already losing weight and showing clear evidence of mucosal inflammation, we found an abrogation of the TNBS-induced wasting disease with a dramatic re-

duction of the macroscopic and histologic signs of inflammatory activity. Similarly, TNBS-induced colitis present 20 d after TNBS exposure was also repressed by anti-IL-12 administration. This finding suggests that the presence of IL-12 is essential to maintain TNBS-induced colitis and a persistent local Th1 cytokine response. Therefore, the most likely mechanism by which anti-IL-12 influences TNBS-induced colitis is the prevention of a Th1-like response of intestinal LP T lymphocytes. This hypothesis is supported by the finding that stimulated LP CD4⁺ T cells isolated from mice with TNBS-induced colitis after early or late administration of anti-IL-12 failed to produce high levels of IFN- γ . That this effect is at least caused by changes in the transcriptional regulation of the IFN- γ promoter is supported by electrophoretic mobility shift studies showing that anti-IL-12 causes a striking reduction of inducible nuclear complexes that bind to regulatory sequences of the IFN- γ promoter (Neurath, M. F., I. Fuss, and W. Strober, unpublished data).

The central importance of the pluripotent cytokine IFN- γ in transmural granulomatous colitis has been recently shown by an elegant series of experiments by Powrie et al. (8) in which it was found that the colitis induced in C.B.-17 scid mice by adoptive transfer of CD45RB^{hi} T cells is associated with a Th1-T cell response and responds to systemic treatment with anti-IFN- γ . Similarly, we have recently found that antibodies to IFN- γ partially reverse established TNBS-induced colitis in mice (Neurath, M. F., I. Fuss, and W. Strober, unpublished data). Perhaps even more strikingly, we found in preliminary studies in the murine TNBS-induced colitis model that no significant chronic colitis could be induced in BALB/c mice in which the gene for IFN- γ is inactivated by homologous recombination. One may speculate that IFN- γ functions in experimental colitis via its ability to induce cellular migration into tissues through its effect on the expression of several adhesion molecules (reviewed in 30). Furthermore, it is a key activator of macrophages; in this regard, IFN- γ might influence experimental colitis by facilitating macrophage secretion of inflammatory cytokines.

In summary, the data demonstrate the pivotal role of IL-12 and IFN- γ in a murine Th1 model of chronic intestinal inflammation induced by the hapten reagent TNBS. The fact that this inflammation is abrogated by anti-IL-12 treatment, together with its similarity to CD, suggests that anti-IL-12 antibodies have potential therapeutic utility in patients with this disease. This hypothesis is supported by the recent finding (unpublished data) that there is a striking increase in IL-12 p35/p40 (p70) heterodimer expression, as assessed by immunohistochemistry, in the colon of patients with CD compared to normal colon.

The authors would like to thank Drs. Robert A. Seder, Timothy A. Stewart, Stephen E. Straus, Thomas Wynn, and Alan Sher for helpful discussions and critical reading of the manuscript. In addition, we gratefully acknowledge Dr. G. Trinchieri for providing anti-mouse IL-12 antibodies. Furthermore, the authors would like to thank Dr. Maurice Gately and his colleagues at Hoffmann La Roche for helpful discussions and providing anti-human and anti-mouse IL-12 p70 mAbs.

Address correspondence and reprint requests to Warren Strober, M.D., Mucosal Immunity Section, NIH/NIAID/LCI, Building 10, Room 11N242, Rockville Pike, Bethesda, MD 20892-1890.

Received for publication 23 February 1995 and in revised form 26 May 1995.

References

- Podolsky, D.K. 1991. Inflammatory bowel disease. *N. Engl. J. Med.* 325:928-937.
- Strober, W., and M.F. Neurath. 1995. Immunological Diseases of the Gastrointestinal Tract. In *Clinical Immunology*, Chapter 94. R.R. Rich, editor. Mosby, St. Louis, MO. 1401-1428.
- Strober, W., and R.O. Ehrhardt. 1993. Chronic intestinal inflammation: an unexpected outcome on cytokine or T cell receptor mutant mice. *Cell.* 75:203-205.
- Hammer, R.E., S.D. Maika, J.A. Richardson, Y.P. Tang, and J.D. Taurog. 1990. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human β 2m: an animal model of HLAB-27-associated human disorders. *Cell.* 63:1099-1112.
- Sadlack, B., H. Merz, H. Schorle, A. Schimpl, A.C. Feller, and I. Horvak. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell.* 75:253-261.
- Kühn, R., J. Löhler, D. Rennick, K. Rajewsky, and W. Müller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell.* 75:263-274.
- Mombaerts, P., E. Mizoguchi, M.J. Grusby, L.H. Glimcher, A.K. Bahn, and S. Tonegawa. 1993. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell.* 75:275-282.
- Powrie, F., M.W. Leach, S. Mauze, S. Menon, L.B. Caddle, and R.L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity.* 1:553-562.
- Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. *J. Exp. Med.* 170:827-845.
- Seder, R.A., R. Gazzinelli, A. Sher, and W.E. Paul. 1993. IL-12 acts directly on CD4+ T cells to enhance priming for IFN- γ production and diminishes IL-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA.* 90:10188-10192.
- Ling, P., M.K. Gately, U. Gubler, A.S. Stern, P. Lin, K. Hollfelder, C. Su, Y.-C.E. Pan, and J. Hakimi. 1995. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J. Immunol.* 154:116-127.
- Podlaski, F.J., V.B. Nanduri, J.D. Hulmes, Y.-C.E. Pan, W. Levin, W. Danho, R. Chizzonite, M.K. Gately, and A.S. Stern. 1992. Molecular characterization of interleukin 12. *Arch. Biochem. Biophys.* 294:230-237.
- Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J. Exp. Med.* 180:211-222.
- Wynn, T.A., I. Eltoun, I.P. Oswald, A.W. Cheever, and A. Sher. 1994. Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. *J. Exp. Med.* 179:1551-1561.
- Murray, H.W., and J. Hariprashad. 1995. Interleukin 12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. *J. Exp. Med.* 181:387-391.
- Leonard, J.P., K.E. Waldburger, and S.J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J. Exp. Med.* 181:381-386.
- Van der Heijden, P.J., and W. Stok. 1987. Improved procedure for the isolation of functionally active lymphoid cells from the murine intestine. *J. Immunol. Methods.* 103:161-167.
- Heniford, B.W., A. Shum-Siu, M. Leonberger, and F.J. Hendler. 1993. Variation in cellular EGF receptor mRNA expression demonstrated by in situ reverse transcriptase polymerase chain reaction. *Nucleic Acids Res.* 21:3159-3166.
- Gray, P.W., and D.V. Goeddel. 1983. Cloning and expression of murine immune interferon cDNA. *Proc. Natl. Acad. Sci. USA.* 80:5842-5846.
- Wysocka, M., M. Kubin, L.Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri. 1995. Interleukin-12 is required for interferon- γ production and lethality in LPS-induced shock in mice. *Eur. J. Immunol.* 25:672-676.
- Trinchieri, G. 1994. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood.* 84:4008-4027.
- Morris, G.P., P.L. Beck, M.S. Herridge, W.T. Depew, M.R. Szewczuk, and J.L. Wallace. 1989. Hapten-induced model of colonic inflammation and ulceration in the rat colon. *Gastroenterology.* 96:795-803.
- Yamada, T., S. Marshall, R.D. Specian, and M.B. Grisham. 1992. A comparative analysis of two models of colitis in rats. *Gastroenterology.* 102:1524-1534.
- Cavani, A., C.J. Hackett, K.J. Wilson, J.B. Rothbard, and S.I. Katz. 1995. Characterization of epitopes recognized by hapten-specific CD4+ T cells. *J. Immunol.* 154:1232-1238.
- Sheffield, E.A., and W.J. Williams. 1994. Pathology. In *Sarcoidosis and Other Granulomatous Disorders*. D.G. James, editor. Marcel Dekker, New York. pp. 45-67.
- Haggitt, R.C. 1983. Granulomatous diseases of the gastrointestinal tract. In *Pathology of Granulomas*. H.L. Iachim, editor. Raven Press, New York. pp. 257-305.
- Li, L., J.F. Elliott, and T.R. Mosmann. 1994. IL-10 inhibits cytokine production, vascular leakage, and swelling during T helper 1 cell-induced delayed-type hypersensitivity. *J. Immunol.* 153:3967-3978.
- Mencacci, A., A. Torosantucci, R. Spaccapelo, L. Romani, F. Bistoni, and A. Cassone. 1994. A mannoprotein constituent of *Candida albicans* that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. *Infect. Immun.* 62:5353-5360.
- Breese, E., C.P. Braegger, C.J. Corrigan, J.A. Walker-Smith, and T. MacDonald. 1993. Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. *Immunology.* 78:127-131.
- Trinchieri, G., and B. Perrussia. 1985. Immune interferon: a pleiotropic lymphokine with multiple effects. *Immunol. Today.* 6:131-136.