Spontaneously developing chronic colitis in IL-10/iNOS double-deficient mice

DONNA-MARIE McCAFFERTY;1 ELAINE SIHOTA,1 MARCELO MUSCARA,2 JOHN L. WALLACE,2 KEITH A. SHARKEY,3 AND PAUL KUBES1

1Immunology and 2Neuroscience Research Groups, Department of Physiology and Biophysics, and 3Gastrointestinal Research Group, Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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McCafferty, Donna-Marie, Elaine Sihota, Marcelo Muscara, John L. Wallace, Keith A. Sharkey, and Paul Kubes. Spontaneously developing chronic colitis in IL-10/iNOS double-deficient mice. Am J Physiol Gastrointest Liver Physiol 279: G90–G99, 2000.—Mice deficient in both inducible nitric oxide synthase (iNOS) and interleukin (IL)-10 (iNOS−/−/IL-10−/−) were created to examine the role of iNOS in spontaneously developing intestinal inflammation. IL-10−/−/iNOS−/− mice were compared with IL-10−/− (iNOS−/−) littermates over 6 mo. RT-PCR, Western blot analysis, and immunohistochemistry were performed to measure iNOS message and protein levels. Plasma nitrate/nitrite (NOx) levels were assessed by HPLC. Damage scores (macroscopic and microscopic) and granulocyte infiltration were assessed. At 3–4 wk, IL-10−/− and IL-10−/−/iNOS−/− mice had no signs of colonic inflammation or granulocyte infiltration. Plasma NOx levels were not different from controls. By 3–4 mo, IL-10−/−/iNOS−/− mice had increased damage scores and granulocyte infiltration concurrent with increased mRNA and protein synthesis (restricted to the epithelium) for iNOS in intestinal tissues but not other tissues. Plasma NOx levels increased fivefold. Interestingly, in the absence of iNOS induction or increased plasma NOx, iNOS−/−/IL-10−/− mice had damage and granulocyte infiltration equivalent to those observed in IL-10−/− littermates. These data suggest that iNOS does not impact on the development or severity of spontaneous chronic inflammation in IL-10-deficient mice.

inflammatory bowel disease; nitric oxide; intestine; inflammation; myeloperoxidase

In 1993, Kuhn et al. (20) generated, using embryonic stem cell technology, interleukin (IL)-10-deficient mice that spontaneously develop a chronic enterocolitis within 3 mo of birth. The disease is characterized by weight loss, splenomegaly, and mild to moderate anemia. If kept under specific pathogen-free (SPF) conditions, the mice develop a limited form of colitis predominantly affecting the colon. Histopathological features of the inflammation include abnormal crypt formation, goblet cell depletion, and a marked thickening of the intestinal wall. The inflammatory infiltrate consists of lymphocytes, plasma cells, macrophages, eosinophils, and neutrophils. The development of colitis in IL-10-deficient mice appears to be mediated by CD4+ T cells and an uncontrolled Th1 response (3). In addition to the overproduction of numerous inflammatory mediators (IL-1, IL-6, tumor necrosis factor (TNF)-α) in colonic cultures (3), our own preliminary work revealed an increase in inducible nitric oxide synthase (iNOS) mRNA induction in colonic tissue from IL-10-deficient mice. The latter observation is most intriguing in light of the very significant interest in iNOS in inflammatory bowel disease (IBD) (17, 30, 38) as well as other spontaneously developing autoimmune diseases (1, 5, 9, 12).

Numerous laboratories have proposed that iNOS produces very significant levels of nitric oxide (NO), resulting in the nitrosation of many proteins, causing dysregulation of the inflammatory process and inappropriate tissue injury (2, 16, 26, 41). However, inhibitors of NO have provided both protection as well as exacerbation of experimentally induced inflammation (14, 16, 25, 32, 33, 35). A criticism directed against many of these studies has been the lack of specificity of the inhibitors used; many of the inhibitors block all of the isoforms of NOS (inducible, as well as neuronal and endothelial NOS). It is thought that inhibition of the constitutive forms will cause tissue damage, whereas selective inhibition of the inducible form will prevent tissue injury. However, an NOS inhibitor that is absolutely selective for iNOS and that can be used for many months without bioaccumulation and potential nonselective effects remains unavailable. An alternative approach to inhibitors is to use iNOS-deficient mice that have normal production of the constitutive isoforms of NOS but have no capacity to produce NO from the inducible isoform (22). In this study, we examined the effect of the production of high levels of NO from the iNOS isoform during a spontaneously occurring chronic inflammatory response in IL-10−/− mice, a model that has some features similar to IBD (20).

Address for reprint requests and other correspondence: P. Kubes, Immunology Research Group, Univ. of Calgary, Health Sciences Center, 3330 Hospital Dr. N.W., Calgary, AB, Canada T2N 4N1 (E-mail: pkubes@ucalgary.ca).

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Acute inhibition of NO synthesis has been reported to increase the production of cytokines in inflammatory conditions (10, 39), and iNOS deficiency has been postulated to increase leukocyte recruitment (13) and reduce tissue repair (23). In IL-10-deficient mice, it is possible that there may be a compensatory overproduction of NO in an attempt to reduce the development of inflammation in this model. If this was the case, iNOS-deficient (iNOS−/−) mice bred into an IL-10-deficient (IL-10−/−) background would have increased rates of onset or severity of IBD. Alternatively, iNOS, and the overproduction of NO, may be detrimental in IL-10−/− mice. Indeed, numerous investigators have postulated a role for NO from iNOS in IBD based on levels of this mediator and enzyme in colonic biopsies from patients with IBD (4, 34, 36). With this in mind, our aim was to study the role of iNOS over time (6 mo) in the development of inflammation in IL-10−/− mice. We first fully characterized the profile of iNOS in IL-10−/− mice and then monitored the development of disease from early in life (3 wk) to adulthood (3 and 6 mo) in both iNOS-positive and iNOS-deficient IL-10-deficient (IL-10−/−/iNOS−/−) mice.

MATERIALS AND METHODS

Mice deficient in IL-10 (20) and mice deficient in iNOS (22) were generated by gene targeting in embryonic stem cells as previously described and obtained from Dr. R. N. Fedorak (University of Alberta, Edmonton, AB, Canada) and Dr. J. Mudgett (Merck Research Laboratories, Rahway, NJ), respectively. The iNOS-deficient mice were on a mixed C57Bl6 × 129SvEv background, and the IL-10-deficient mice were on a pure 129SvEv background. All animals were generated in SPF facilities. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and conform to the guidelines established by the Canadian Council for Animal Care.

Generation of IL-10/iNOS double-deficient mice. IL-10/iNOS double-deficient (IL-10−/−/iNOS−/−) mice were generated from IL-10-deficient mice heterozygous for iNOS (IL-10−/−/iNOS+/−) breeding pairs. The double-deficient mice were compared directly with IL-10−/− mice on a pure 129SvEv background. All animals were generated in SPF facilities. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and conform to the guidelines established by the Canadian Council for Animal Care.

A similar PCR protocol was designed to genotype iNOS. The primers used were the following: INOSGT 5′-GCT AGC CCA AAC TCA GGG ATC A-3′, INOSGTWT 5′-GAC TAG GCT ACT CCG TGG AGT GAA CA-3′, and INOSGTO 5′-TCC ATC ATG GAT GCA ATG CG-3′. The PCR reactions used for genotyping both IL-10 and iNOS were set up as outlined previously (23). Analysis of iNOS mRNA expression by RT-PCR. Colon tissue samples were obtained from mice at 3−4 wk, 3−4 mo, and 6 mo of age. In a separate experiment, various tissues (colon, small intestine, stomach, mesentery, liver, lung, and heart) were obtained from 3 to 4-mo-old mice treated with or without lipopolysaccharide (LPS; 50 µg ip) for 4 h to ensure that all organs had the capacity to induce iNOS in IL-10−/− mice. Tissue samples were rinsed in saline, weighed, and placed in guanidinium isothiocyanate to extract total RNA (7). The final RNA concentrations were determined by absorbance using a GeneQuant spectrophotometer (Pharmacia, Piscataway, NJ). The RT and PCR steps were performed as described by Wong et al. (42) and outlined previously (23).

The following primer sequences were used for iNOS: sense 5′-CACTG GGA CAG CAC AGA AT-3′ and antisense 5′-GTG GTC TGC GGA TGT GCT GA-3′ with a final PCR product size of 499 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified as an internal control using the following primer sequences: sense 5′-CGG AGT CAA CGG ATT TGG TGC TAT-3′ and antisense 5′-AGC CTT CTC CAT GGT GGT GAA-3′ (42) with a final PCR product size of 300 bp. PCR cycle numbers were chosen to ensure that the amplification of PCR products was in the exponential range: 26 cycles for iNOS and 22 for GAPDH. Aliquots of PCR products (−10 µl) were electrophoresed through 2% agarose gels (Ultrapure, Pharmacia) containing 0.5 µg/ml of ethidium bromide. Gels were visualized under ultraviolet light and photographed with Kodak film. The optical density of each band for iNOS and GAPDH was quantified using NIH Image 1.6 software, and the results were expressed as a ratio of iNOS to GAPDH.

Western blot analysis for iNOS protein. Intestinal tissue was rinsed with saline, frozen in liquid nitrogen, and stored at −80°C for no more than 1 wk before protein content was determined as follows. Tissue was cut into small pieces and homogenized in 250 µl of buffer [40 mM Tris·HCl, pH 8.0, protease inhibitor cocktail (Calbiochem), 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100] with a hand-held motorized pestle (Kontes). Homogenates were ultracentrifuged in a total volume of 1.25 ml (100,000 g, 4°C, 1 h). Total protein was determined using Bio-Rad Assay Reagent. Homogenization buffer lacking Triton X-100 was added to 100 µg of homogenate total protein to give a final volume of 25 µl. Samples were boiled for 5 min in an equal volume of 2× sample buffer (0.125 M Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue). Samples were resolved by 10% SDS-polyacrylamide gel (Bio-Rad mini gel system) and transblotted to nitrocellulose membrane (Bio-Rad). The membrane was blocked with buffer (PBS containing 0.05% Tween 20 and 5% skim milk powder, 1 h at room temperature) then incubated (2 h, room temperature or overnight at 4°C) with a rabbit polyclonal antibody raised against iNOS (1:500; N32030, Transduction Laboratories, Lexington, KY) before being washed. After incubation (2 h, room temperature) with horseradish peroxidase-conjugated donkey anti-rabbit IgG (NA9340, Amersham) the membrane was washed again. Finally, the membrane was incubated (5 min, room temperature) in SuperSignal substrate (1:1 vol/vol) luminal substrate and stable solution (Pierce). The membrane was
exposed to Hyperfilm (Amersham Life Science) for 1, 2, 5, and 20 min (overexposure) to confirm the absence of bands.

Nitrate/nitrite measurement by HPLC. Blood samples were collected in heparin from mice by cardiac puncture. The samples were centrifuged, and the plasma was frozen at −20°C until assayed. After centrifugation to remove particulate matter (14,000 g, 2 min), plasma samples were subjected to a fivefold dilution with HPLC-grade water and deproteinized by ultracentrifugation through nitrocellulose filters (Centrisart C-4 ultracentrifugation tubes, mol wt cutoff 5,000; Sartorius) by centrifugation (6,000 g, 60 min). One hundred microliters of the ultrafiltrates were analyzed for their nitrite and nitrate content according to the HPLC method previously described by Muscara and de Nucci (29).

Assessment of severity of colitis. Mice were killed by cervical dislocation at 3–4 wk, 3–4 mo, and 6 mo of age. The colon was excised, and the severity of colonic damage was assessed (both the ascending and descending sections were scored separately) using parameters outlined in Table 1. This scoring system includes features of clinical colitis, the presence or absence of adhesions, strictures, and diarrhea (diarrhea was defined as loose, watery stool), and bowel wall thickness in millimeters. The terminal ileum was also assessed for signs of inflammation. After gross macroscopic scoring, samples of colonic tissue were fixed in neutral buffered formalin and processed for subsequent histological examination. In addition, samples of colon were taken for estimation of myeloperoxidase (MPO) activity and immunohistochemical studies as described in Determination of tissue MPO activity and Immunohistochemistry for iNOS protein.

Histological scoring. After overnight fixation in formalin, tissues were dehydrated (graded alcohols) and cleared (xylene) before being embedded in paraffin wax. Sections of tissue were cut and stained with hematoxylin and eosin (H and E) and scored in a blinded manner. Histological scoring was based on a semiquantitative scoring system in which the following features were graded: extent of destruction of normal mucosal architecture (0, normal; 1, 2, and 3, mild, moderate, and extensive damage, respectively), presence and degree of cellular infiltration (0, normal; 1, 2, and 3, mild, moderate, and transmural infiltration, respectively), extent of muscle thickening (0, normal; 1, 2, and 3, mild, moderate, and extensive thickening, respectively), presence or absence of crypt abscesses (0, absent or 1, present), and presence or absence of goblet cell depletion (0, absent or 1, present). The scores for each feature were summed with a maximum possible score of 11.

Determination of tissue MPO activity. Samples of intestinal tissue were weighed, frozen on dry ice, and processed for determination of MPO activity. MPO is an enzyme found in cells of myeloid origin and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into gastrointestinal tissues (18, 27). The samples were stored at −20°C for no more than 1 wk before the MPO assay was performed. MPO activity was determined using an assay described previously (18) but with the volumes of each reagent modified for use in 96-well microtiter plates. The rate of change in absorbance at 450 nm over a 90-s period was determined using a kinetic microplate reader (Molecular Devices). One unit of MPO activity was defined as that degrading 1 µmol hydrogen peroxide/min at 25°C. Values are expressed as units of MPO activity per milligram of tissue sampled.

Immunohistochemistry for iNOS protein. Samples of tissue were fixed overnight in Zamboni’s fixative (pH 7.4) at 4°C, rinsed (3 × 10 min) in PBS, transferred to PBS containing 20% sucrose (pH 7.4), and stored at 4°C overnight. They were embedded in OCT embedding medium (Sakura Finetek, Torrance, CA), cryostat sectioned at 12 µm, and thaw-mounted onto poly-L-lysine-coated slides and dried. To assess the expression of iNOS protein in colonic tissue, frozen sections were rehydrated in PBS containing 0.1% Triton X-100 (PBS-T) and incubated with 2% normal goat serum in PBS for 30 min at room temperature to block nonspecific binding before being placed in primary antibody. Sections were then incubated for 48 h at 4°C with a rabbit polyclonal antibody raised against iNOS (1:500; N32030, Transduction Laboratories, Lexington, KY). The specificity of iNOS antibody was verified by substitution of the same concentration of normal rabbit IgG used for the anti-iNOS IgG. Antibodies were diluted in PBS-T containing 0.1% bovine serum albumin. Immunohistochemical controls routinely performed involved incubation with blocking solution and diluent in place of the primary antibody. Sections were rinsed (3 × 10 min) with PBS-T and incubated for 1 h at room temperature with sheep anti-rabbit IgG conjugated to CY3 (1:100). After a final wash (3 × 10 min) with PBS-T, sections were mounted in bicarbonate-buffered glycerol (pH 8.6) and examined using a Zeiss Axiosplan fluorescence microscope. Sections were photographed using Kodak Ektachrome film.

Statistical analysis. Data are expressed as means ± SE. Groups of data were compared using nonparametric Mann-Whitney U-test or Kruskal-Wallis one-way ANOVA followed by Dunn’s multiple-comparison test. Probabilities (P) of <5% were considered statistically significant.

RESULTS

iNOS mRNA expression. Levels of iNOS mRNA in descending colon samples were measured by RT-PCR in IL-10−/−/iNOS+/+, IL-10−/−/iNOS−/−, and wild-type control mice at 3–4 wk, 3–4 mo, and 6 mo of age. A representative band for each group at each time point is shown in Fig. 1A. The RT-PCR product bands were quantified using nonlinear densitometry, and the ratio of the iNOS to internal standard (GAPDH) was determined. The mean optical density ratios for each group of mice (n = 4) are illustrated in Fig. 1B. In IL-10−/−/iNOS+/+ mice there was a fivefold increase in message for iNOS (P < 0.05) as early as 3–4 wk of age. The level of iNOS message increases at 3–4 mo of age and is still significantly elevated at 6 mo. Our data also reveal that an upregulation of iNOS message could be detected in

Table 1. Parameters used to assess macroscopic damage in IL-10-deficient and IL-10/iNOS double-deficient mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Erythema</th>
<th>Diarrhea</th>
<th>Edema</th>
<th>Stricture</th>
<th>Hemorrhage</th>
<th>Adhesions</th>
<th>Ulceration</th>
<th>Mucosal hyperplasia</th>
<th>Bowel wall thickness, mm</th>
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Each parameter was awarded 1 point if observed upon macroscopic examination of the gastrointestinal tissue, with the exception of the bowel wall thickness (value of which was added in millimeters). IL, interleukin; iNOS, inducible nitric oxide synthase.
the ascending colon and small intestine in a pattern similar to that observed for the descending colon, with the exception of small intestinal tissue from young mice, in which no increase in iNOS mRNA was observed (data not shown). No message for iNOS was detectable in double-deficient (IL-10^−/−/iNOS^−/−) mice at any time point (Fig. 1).

iNOS message was not systemically induced in IL-10-deficient mice because no induction was observed in the stomach, mesentery, lung, and heart (Table 2). A slight increase in message was observed in the liver. As a positive control, tissue was also obtained from IL-10^−/− mice 4 h after LPS (ip) treatment to demonstrate that iNOS message could be induced in the various tissues (Table 2). Messenger RNA could be induced in all tissues tested from IL-10−/− mice4 h after administration of LPS (50 µg ip). * Significant increase from wild-type group; † significant decrease from IL-10-deficient group.

Table 2. Relative mRNA expression for iNOS in IL-10-deficient mice compared with wild-type control mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>IL-10-Deficient</th>
<th>IL-10 Deficient and LPS</th>
</tr>
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<tbody>
<tr>
<td>Colon (total)</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Small intestine</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>Stomach</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Mesentery</td>
<td>−</td>
<td>n.d.</td>
</tr>
<tr>
<td>Lung</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heart</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
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Messenger RNA for iNOS was measured using RT-PCR as outlined in MATERIALS AND METHODS. Total RNA was isolated from various tissues from 3- to 4-mo-old mice. In IL-10-deficient mice, iNOS mRNA was induced in the colon and small intestine and to a much smaller extent in the liver but was not induced in any other tissue tested. However, no iNOS protein could be detected in IL-10−/−/iNOS−/− or in wild-type mice despite the presence of low levels of message in the latter group. Figure 2 also illustrates that no protein for iNOS was observed in lung tissue from any group. The presence of iNOS protein in colonic tissue was confirmed by immunohistochemistry. Figure 3 illustrates fluorescence micrographs from wild-type (Fig. 3A) and IL-10−/− (Fig. 3, B and C) mice stained for iNOS protein. Intense fluorescence was observed in the ascending colon and small intestine in a pattern similar to that observed for the descending colon, with the exception of small intestinal tissue from young mice, in which no increase in iNOS mRNA was observed (data not shown). No message for iNOS was detectable in double-deficient (IL-10^−/−/iNOS^−/−) mice at any time point (Fig. 1).

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cent staining was observed in the epithelial cell border lining the lumen of the gut in IL-10−/− mice (Fig. 3, B and C), localizing iNOS protein to the epithelial cells. No specific staining was noted in tissues from wild-type (Fig. 3A) or IL-10−/−/iNOS−/− (not shown) mice.

Plasma nitrite levels. To ensure that iNOS message was translated into a functional product, plasma nitrites/nitrites were converted to nitrite; the levels are reported in Fig. 4. At 3–4 wk of age plasma nitrite levels in wild-type control, IL-10−/−/iNOS−/−, and double-deficient mice were low (10–25 µM) and not significantly different among the groups, despite an increase in iNOS mRNA as measured semiquantitatively by RT-PCR in IL-10−/−/iNOS−/− mice (Fig. 1). At this point, any iNOS protein present does not increase NO output sufficiently to be detected as an increase in plasma nitrite. However, by 3–4 mo of age plasma nitrite levels had increased significantly in IL-10−/−/iNOS−/− mice (5-fold higher than controls), and a profound increase was observed by 6 mo (425 ± 75 µM). No increase in plasma nitrite levels from control levels was observed in double-deficient mice at any time point. Because only the intestinal tract had signs of iNOS message, the very high plasma nitrite levels suggest very significant NO production within the intestine.

Parameters of inflammation. Figure 5 illustrates the macroscopic damage observed in the descending colon of mice at different ages. At the earliest time point examined (3 wk) there was very little notable damage in any group studied (0.5–0.7). However, by 3–4 mo profound macroscopic damage was observed in IL-10−/−/iNOS−/− mice, with a mean value of 4 ± 0.5 (P < 0.05), which represents hemorrhage ulceration, diarrhea, and increased bowel wall thickness. At this age mice were beginning to prolapse, and by 6 mo of age all mice had rectal prolapse and were underweight. The level of macroscopic damage observed in the double-deficient mice was very similar to that observed in the iNOS-positive, IL-10-deficient mice at all time points. Furthermore, each parameter measured to obtain the macroscopic damage score was very similar for each group of animals, suggesting that the development and progression of the disease was not different between the two groups of animals. Signs of inflammation were also evident in the ascending colon of both the iNOS-positive and iNOS-deficient IL-10-deficient groups, with hyperplasia of the mucosa noted in most animals by 3–4 mo of age. Small intestinal inflammation was rarely observed in any mice studied.

Microscopically similar results were observed. Representative histology (H and E) from transverse sections of the descending colon from wild-type control, IL-10−/−/iNOS−/−, and IL-10−/−/iNOS−/− mice at 3–4 mo of age is illustrated in Fig. 6. Figure 6A (and Fig. 6B, at higher magnification) demonstrates normal mucosal architecture in the mouse colon at 3–4 mo of age. At this time point, IL-10−/−/iNOS−/− (Fig. 6, C and D) had significant mucosal and submucosal cellular infiltration, increased muscle thickness, goblet cell depletion, and crypt abscess formation. In some cases the abscesses had merged together to form ulcers and loss of the epithelial surface layer. The cellular infiltrate was mainly mononuclear, with neutrophils also evident. In the IL-10−/−/iNOS−/− group (Fig. 6, E and F) histological damage similar to that seen in the IL-10−/−/iNOS−/− group was observed. Figure 7 represents the mean microscopic damage scores obtained at 3–4 mo and illustrates that a similar significant increase in microscopic damage over wild-type controls was observed in both IL-10-deficient groups (iNOS−/− and iNOS−/−).

Figure 8 illustrates the level of granulocyte infiltration as measured by MPO activity in the descending colon of mice at various ages. At 3–4 wk of age, levels of MPO activity were similar in all groups. However, by 3–4 mo a very significant elevation in granulocyte levels above control levels was noted in both IL-10−/−/iNOS−/− and IL-10−/−/iNOS−/− mice. At 6 mo the MPO levels had dropped but were still significantly elevated above controls in both IL-10-deficient groups (iNOS−/− and iNOS−/−).

**DISCUSSION**

In this study we demonstrate for the first time that in IL-10-deficient animals there is a profound increase in iNOS mRNA as early as 3 wk of age that persists at least to 6 mo of age. Associated with the increase in iNOS mRNA is also a very substantial increase in iNOS protein at 3–4 mo, resulting in high levels of plasma nitrite/nitrate, the end product of NO metabolism. The increased nitrite levels are likely from iNOS because in the IL-10/iNOS double-deficient mice, no increase in plasma nitrite was noted. Nitrite levels rise in the circulation to >5-fold at 3 mo and 20-fold at 6 mo, remarkably reaching as much as 0.5 mM nitrite at the later stage of the disease. More impressive is the fact that iNOS is not produced by all organs but appears to be restricted to the gastrointestinal tract and more
specifically to the colon (epithelium) and small intestine. This underscores the levels of NO that must be produced within the intestinal tract to achieve such profound levels of nitrite in the circulation. Despite the high level of iNOS activity within the gastrointestinal tract, this enzyme does not appear to be essential for the development or maintenance of colitis, because the IL-10-deficient mice rendered iNOS deficient developed a degree of inflammation, as assessed by microscopic and macroscopic damage, leukocytic infiltration, and intestinal dysfunction (diarrhea and rectal prolapse), commensurate with that of their iNOS-positive littermates.

Fig. 3. Fluorescence micrographs of iNOS immunoreactivity in representative sections from ascending colon. Wild-type mouse colon (A) and IL-10-deficient mouse colon (B) and a higher-magnification image from an IL-10-deficient animal (C) are shown. Tissue was taken from 3- to 4-month-old mice. In IL-10-deficient mice, iNOS immunoreactivity was observed localized to the epithelial cells lining the lumen of the gut. This intense staining was not evident in wild-type (A) or IL-10/iNOS double-deficient mice (not shown). Bar, 100 µm.
The literature to date is equivocal with respect to the effect of IL-10 on iNOS production. Certainly, a number of studies suggest that IL-10 can directly inhibit iNOS production from macrophages stimulated with bacteria or bacterial products (8, 11, 28). However, other studies have proposed that IL-10 increases or has no effect on NOS activity caused by LPS in macrophages (6, 15, 31). The results from our study reveal that in the absence of endogenous IL-10, there is enhanced iNOS production in the small and large intestine, suggesting that under basal conditions IL-10 either directly or indirectly inhibits iNOS synthesis and NO release. It remains unknown whether this observation is caused by a direct regulation of IL-10 on iNOS production, perhaps via nuclear factor-κB as has been proposed for TNF-α and other proinflammatory cytokines. Alternatively, it may be an indirect effect caused by the absence of IL-10 increasing TNF-α and interferon-γ, which are known to induce iNOS production (3). The localized increase in iNOS caused by lack of IL-10 suggests that this cytokine is far more important as a regulator of iNOS production in the intestine than in other tissues, because only the intestinal tract revealed elevated levels of the enzyme. This observation argues against the possibility of a systemic effect such as enhanced levels of circulating proinflammatory cytokines being entirely responsible for the increased iNOS, because this would likely impact on all organs, not just the gastrointestinal tract.

It is conceivable that luminal antigens are responsible for the enhanced iNOS production because the gastrointestinal tract is directly in contact with the external environment. Indeed, animals kept in SPF facilities develop a more limited intestinal inflammation (20). However, other organs in direct contact with the external milieu including the lung did not exhibit increases in iNOS or overt inflammation. Nevertheless, it is conceivable that the gastrointestinal tract is presented with a specific inducer of iNOS not found in other organs or that the load of antigen is only sufficient to induce iNOS in the absence of IL-10, in this and not in other organ systems. Interestingly, preliminary work from our laboratory suggests that the iNOS response to the same concentration of a bacterial product (LPS) is enhanced in IL-10-deficient mice relative to wild-type animals, raising the possibility that in the gastrointestinal tract, normal endogenous levels of antigen may be sufficient to induce iNOS production in IL-10-deficient but not wild-type mice.

In this study, our results reveal that IL-10-deficient mice have increased induction of iNOS mRNA and protein synthesis and very high levels of NO output (nitrite levels in plasma) concomitant with the development of intestinal inflammation. However, intestinal inflammation progressed at the same rate and with the same severity in IL-10-deficient mice deficient in iNOS and in the absence of elevated NO production. Clearly, in this spontaneously developing model of colitis, overproduction of iNOS, which appears to begin after weaning and continues throughout adult life, is neither the initiating factor nor a major contributor to the development of this disease. Although it may appear somewhat surprising that the iNOS-deficient mice are not spared from injury in this chronic autoimmune disease, this observation is not entirely inconsistent with observations from other models of autoimmune inflammation. For example, overproduction of iNOS was not a contributing factor to antigen-induced autoimmune myocarditis (1), Lyme arthritis (5), or bacterial septic arthritis (24). On the other hand, iNOS-deficient MRL-lpr/lpr mice have less vasculitis associated with glomerulonephritis (12), which is a hallmark of this autoimmune disease, whereas Fenyk-Melody et al. (9) reported a more than fourfold increase in incidence and severity of experimental autoimmune encephalomyelitis in iNOS-deficient mice relative to their wild-type littermates. Clearly, the role of iNOS cannot be predicted from these studies and appears to be dependent on the type of autoimmune disease or perhaps the afflicted organ. Our own data for the first time demonstrate that in the colon, the role of iNOS in a spontane-
ously developing model of inflammatory bowel disease is not a critical mediator.

This study has also revealed some new information regarding leukocyte adhesion and infiltration during chronic inflammation. We (19) and others (21) reported previously that constitutive NO synthesis inhibition caused an increase in leukocyte adhesion. Moreover, during an inflammatory response induced by LPS, leukocytes from iNOS-deficient mice adhered in response to concentrations of LPS that did not invoke an adhesive response in leukocytes of wild-type mice (13). In light of this work, it was reasonable to expect increased leukocyte recruitment in the IL-10/iNOS double-deficient mice relative to their IL-10-deficient counterparts that are producing a lot of iNOS. Indeed,

Fig. 6. Histological micrographs (hematoxylin and eosin) of descending colon from wild-type (A and B), IL-10-deficient (C and D), and IL-10/iNOS double-deficient (E and F) mice at 3–4 mo of age. Panel A, and at higher magnification, panel B, demonstrate normal mucosal architecture in the colon. Micrographs from IL-10-deficient mice (C and, at higher magnification, D) show evidence of muscle thickening, cellular infiltration, goblet cell depletion, crypt abscess formation and ulceration. A similar picture is observed in the IL-10−/−/iNOS−/− mice (E and, at higher magnification, F). Bar, 100 µm.

Fig. 7. Mean histology scores for wild-type, IL-10-deficient, and IL-10/iNOS double-deficient mice at 3–4 mo of age; n = 5 in all groups. * Significant increase from wild-type mice.

Fig. 8. Myeloperoxidase (MPO) activity for descending colon from wild-type, IL-10-deficient, and IL-10/iNOS double-deficient mice. Mice were studied at 3–4 wk, 3–4 mo, or 6 mo of age; n = 6 in all groups. * Significant increase from wild-type mice.
in other models of intestinal inflammation involving acute acetic acid-induced colitis (23), the amount of granulocytic infiltrate into the colon was enhanced in animals deficient in iNOS relative to wild-type mice. However, in our IL-10-deficient chronic model of IBD the amount of granulocyte infiltration was enhanced to similar degrees in both types of mutant mice. As a whole, these studies clearly suggest that although iNOS impacts granulocyte recruitment and contributes to the resolution of an acute, rapidly resolving inflammation, in a persistent chronic inflammatory disease the mechanisms are sufficiently different that the presence of iNOS does not affect granulocyte infiltration. It should be noted that the absence of a role for iNOS in reducing the inflammatory response in this study may be specific to this chronic model and that iNOS may play a role in other models of chronic inflammation.

In summary, these are the first data documenting that there is a very significant increase in iNOS mRNA, protein, and product during the lifespan of IL-10-deficient mice and that this event does not contribute to the inflammatory process. This model of colitis allowed us to probe extensively the role of iNOS at early, middle, and late phases of the disease, and the results reveal that the progression of the disease is comparable with or without iNOS. Thus the relation between an autoimmune phenotype and increased systemic NO production is not necessarily correlated with injury. In this study we circumvented any issues of lack of specificity or incomplete inhibition of iNOS by making iNOS/IL-10 double-deficient mice. Although the argument could be levied that this IL-10-deficient model is not a suitable model of colitis, it has certain features that make it an attractive model. It is a spontaneously developing inflammation and therefore does not depend on the addition of toxic chemicals or other exogenous reagents. IL-10-deficient mice do not gain weight to the same extent as wild-type mice; they develop diarrhea and microscopically present with transmural cellular infiltration, increased muscle thickness, goblet cell depletion, and crypt abscess formation, all characteristic features of clinical disease. Interestingly, in this model of colitis we were also able to localize iNOS protein to the epithelial cells lining the gut (by immunohistochemistry), which is in accordance with what has been noted in many clinical studies (17, 30, 38). Most importantly, this mouse model has led to the use of IL-10 as a therapeutic molecule in human IBD with some very promising results (37, 40).

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