The Effect of Inhibitors of Inducible Nitric Oxide Synthase on Chronic Colitis in the Rhesus Monkey

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ABSTRACT

GI inflammation is associated with an increase in nitric oxide production and expression of the inducible isofom of nitric oxide synthase (iNOS). Using a spontaneous model of chronic colonic inflammation in rhesus monkeys, which shares morphological and clinical features with ulcerative colitis, we assessed the therapeutic benefit of administration of iNOS inhibitors. Sixteen colitic rhesus monkeys underwent an endoscopy procedure before commencement of the trial, and biopsies from three sites of the colon and plasma were collected. Monkeys were randomly assigned to three treatment groups and were administered by oral bolus 60 mg/kg/day L-N 6-(1-iminoethyl) lysine, 60 mg/kg/day aminoguanidine or a placebo (0.9% NaCl) twice daily. Monkeys were sacrificed after 10 days, colonic tissue from multiple sites was dissected and processed for histological and biochemical analysis. In rhesus colitis, diarrhea was characterized by a significant increase in fecal water content and daily fecal output. iNOS was localized immunohistochemically in plasma cells and neutrophils in the colonic mucosa and lamina propria, paralleled by enhanced iNOS gene expression determined by reverse-transcriptase polymerase chain reaction. Only L-N 6-(1-iminoethyl) lysine administration resulted in a significant reduction in systemic nitric oxide production, and neither of the iNOS inhibitors significantly reduced the histological inflammatory score nor ameliorated diarrheal symptoms. From these findings, we conclude that in this chronic, spontaneous model of colonic inflammation, administering iNOS inhibitors with this treatment regimen did not provide any major therapeutic benefit.

There is increasing evidence to suggest that NO, formed enzymatically by NO synthases (NOS), plays a major role in GI health and disease. Under basal conditions, NO is produced by constitutively expressed isofoms of NOS associated with neuronal elements and vascular endothelium (Nichols et al., 1993). These enzymes release small amounts of NO, for a short time, in response to an increase in intracellular calcium. Release of NO exerts a wide range of biological effects in the GI tract and can afford cytoprotection in acute inflammatory conditions by regulating intestinal motility (Caglinano et al., 1992; Stark et al., 1993) and secretion (Tamai and Gaginella, 1993), by maintaining splanchic blood flow (MacNaughton et al., 1989; Pique et al., 1992) and by inhibiting platelet aggregation (Radomski et al., 1992) and leukocyte adhesion to vascular endothelium (Kubes et al., 1991; Kubes et al., 1993).

In contrast, chronic intestinal inflammation is associated with an elevation in NO production above basal levels primarily because of the expression of the inducible isofom, iNOS. This induced enzyme releases much more nitric oxide than its constitutive counterparts over an extended period of time, and it does so in a manner independent of intracellular calcium. In the gut, iNOS has been localized immunohistochemically in a range of cell types, including macrophages, neutrophils, epithelia and neuronal cells (Tracey et al., 1994; Seago et al., 1995; Miller et al., 1995). NO release by iNOS acts as a cytostatic or cytotoxic agent by free radical-mediated mechanisms, thereby providing protection against infectious agents and tumor cells (Nathan and Hibbs, 1991, Hibbs et al., 1988). However, this toxic modality of NO is a nondiscriminating process and may in fact contribute to the pathogenesis of intestinal inflammation by enhancing intestinal

ABBREVIATIONS: iNOS, inducible nitric oxide synthase; NO, nitric oxide; L-NIL, L-N 6-(1-iminoethyl)lysine; AG, aminoguanidine; L-NAME, N-nitro-L-arginine methyl ester; RNI, reactive nitrogen intermediates; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcriptase polymerase chain reaction; VIP, vasoactive intestinal peptide; SP, substance P; IL-1, interleukin-1; TNFα, tumor necrosis factor α; TNBS, trinitrobenzene sulfonic acid.
damage. Indeed, a marked elevation in NO production, iNOS activity and gene expression have been detected in a variety of animal models of intestinal inflammation (Miller et al., 1993; Grisham et al., 1994; Seago et al., 1995, Aiko and Grisham, 1995; Ribbons et al., 1995). Moreover, an exaggeration of NO synthesis has been assessed in inflammatory bowel disease, although the role of NO in the pathogenesis of such conditions remains unclear. Ulcerative colitis is associated with an increase in luminal NO levels (Reynolds et al., 1995a,b), and iNOS enzyme activity has been detected in specimens from patients with active disease (Middleton et al., 1993; Boughton-Smith et al., 1993; Oudkerk-Pool et al., 1995; Rachmilewitz et al., 1995a). In Crohn’s disease, however, there are conflicting reports of both increases and no change in NO production (Boughton-Smith, et al., 1993; Oudkerk et al., 1995; Rachmilewitz et al., 1995a).

A pathogenic role of NO release in intestinal inflammation is supported by findings from experimentally induced rodent models of intestinal inflammation in which administration of inhibitors of NOS, such as L-NAME and aminoguanidine, confers protection that results in amelioration of intestinal damage. A variety of pathological features associated with the induction of inflammation were corrected; these results included a reduction in cellular infiltration, intestinal wall thickening and submucosal fibrosis (Miller et al., 1993; Grisham et al., 1994; Seago et al., 1995; Rachmilewitz et al., 1995b). Although these results suggest that NOS inhibitors have a profound effect on intestinal inflammation, the relevance of these models to human inflammatory bowel disease has yet to be determined.

In all studies where NOS inhibition has protected against gut inflammation, NOS inhibitors were administered at the time of induction of the condition. Hence it has not been possible to determine whether NO inhibitors prevented development of the inflammation or displayed a therapeutic effect, ameliorating established inflammation. To date, no studies have reported a therapeutic effect of NO inhibitors in a chronic model of intestinal inflammation where inhibitor administration began after the disease was fully developed or after iNOS gene expression was noted. Thus the ability of NOS inhibitors to attenuate any established, chronic inflammation remains to be determined.

Recently, we reported a novel model of chronic colitis in captive colonies of rhesus monkeys (Ribbons et al., 1995). This model shares many of the histopathological and clinical symptoms of ulcerative colitis (table 1). Although this form of colitis has not been fully characterized, it bears many of the hallmarks of human inflammatory bowel disease, particularly ulcerative colitis as opposed to Crohn’s disease. In colitic rhesus monkeys, systemic NO levels are markedly elevated above normal levels and are associated with the expression of iNOS; this scenario is comparable to that observed in active ulcerative colitis in the human (Ribbons et al., 1995). Moreover, the level of enzyme activity and the responsiveness of iNOS to inhibitors are comparable in the colitic rhesus monkey and active ulcerative colitis (Ribbons et al., 1995). Using this model to assess the effect of NO inhibitors in chronic inflammation enabled us to test the hypothesis that NOS inhibitors represent a novel therapeutic approach for the treatment of inflammatory bowel disease. This primate model, spontaneous in nature, may have greater predictive value for the human condition than chemical or genetic models induced in rodents. And as far as we know, this is the first report of the effects of chronic administration of NOS inhibitors in a primate inflammatory condition, human or monkey.

To determine the therapeutic benefit of inhibiting NOS activity in chronic inflammation, we selected two inhibitors that display a selectivity for the inducible isoform of NOS: AG (Misko et al., 1993) and L-NIL (Moore et al., 1994). Both inhibitors have been used successfully to reduce systemic NO production and ameliorate inflammation in vivo in a low-dose endotoxin model (Salvemini et al., 1995) and in adjuvant-induced arthritis (Connor et al., 1995). AG has also been reported to attenuate intestinal inflammation in TNBS-induced ileitis in guinea pigs and in chronic granulomatous colitis in rats (Miller et al., 1995; Grisham et al., 1994), but studies investigating the effect of L-NIL in GI inflammation have not been reported. We assessed the therapeutic efficacy of these compounds by comparing the severity of clinical symptoms, histopathological features, systemic NO production and iNOS gene expression in colitic rhesus monkeys before and after treatment.

### Materials and Methods

**Animals and experimental design.** Rhesus macaques (18 months to 5 years of age) were housed at both the California and

| TABLE 1
<p>| <strong>Comparison of rhesus and human colitis</strong> |</p>
<table>
<thead>
<tr>
<th>Profile</th>
<th>Rhesus vs. Human</th>
<th>Comments Relative to Rhesus Colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Comparable</strong></td>
<td><strong>Distinct</strong></td>
<td><strong>Unknown</strong></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pathogen</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Bleeding</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mucosal alteration</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Granulocyte infiltration</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Crypt abscesses</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Disturbed innervation</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Therapeutics</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>iNOS</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Cytokine release</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>X</td>
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</table>
Clearance of orally administered L-NIL into the circulation. In determining the level of nitrite and nitrate in plasma samples collected before and after treatment, nitrite and nitrate levels were quantitated by using a fluorometric assay (Misko et al., 1993), as described by Connor et al. (1995). In brief, plasma was filtered though a 10-kD Ultrafree microcentrifuge filter unit (Millipore, Bedford, MA) at 14,000 rpm for 15 min. Plasma nitrate was converted to nitrite by incubating 5 to 10 µl of filtered plasma with 14 mU of nitrate reductase in 20µM Tris pH 7.6 containing 40 µM NADPH. Fluorescence was measured at 365/450 nm (excitation/emission) by using a fluorescence plate reader (IDEXX Laboratories, Westbrook, ME).

Histological procedures. Zambonis-fixed biopsy specimens and tissue segments collected at necropsy from colitic and from noncolitic rhesus monkeys were dehydrated using a Titerrate automated processor (Miles Scientific, Naperville, IL) and embedded in paraffin. We cut 3-µm transverse sections at two tissue levels, 1 mm apart, using an American Optical 820 microtome (Buffalo, NY), and sections were either stained with hematoxylin and eosin or processed for immunohistochemistry. Using a Nikon DXC-151A video camera and was saved with Image-NIH Shareware software. Saved images were accessed through the Stereology Toolbox software package (Morphometrix, Davis, CA), and a 42-point grid was superimposed over the image. The intersection of each grid point with either colonic epithelial or inflammatory infiltrate cells was then recorded. The inflammatory index was calculated as the mean density of inflammatory infiltrate cells per field.

iNOS was localized in tissue sections from biopsy and necropsy specimens immunohistochemically. Deparaffinized, rehydrated, Zambonis-fixed sections were incubated with antiimmunoreactive iNOS polyclonal antibody (Searle, St. Louis, MO) for 60 min at a final dilution of 1:500 in 0.05 M Tris-buffered saline (TBS) (pH 7.4) containing 1% normal goat serum. Slides were then incubated for 60 min with a 1:200 dilution of biotinylated goat antimouse IgG serum (Vector Laboratories, Burlingame, CA) followed by a 30-min incubation with avidin-biotin peroxidase conjugate (Vector Laboratories, Burlingame, CA). Visualization of bound peroxidase was by the diaminobenzidine H2O2 reaction, and sections were counterstained with hematoxylin. Between incubations, slides were rinsed three times with 0.5 M Tris buffer (pH 7.4) containing 0.01% Tween. Endogenous peroxidase activity was blocked by exposing the tissue sections to Peroxyblock (Zymed, San Francisco, CA) for 2 min. Nonspecific binding of the biotinylated antibody was inhibited by preincubating sections with normal goat serum for 60 min. Negative control slides for primary antibody binding were run for each sample, in which tissues were incubated with a 1:500 dilution of preimmune rabbit serum instead of the iNOS antibody.

iNOS RNA expression. RNA was extracted from colonic mucosa by the guanidine thiocyanate extraction method immediately after collection or from samples stored at −80°C. The integrity of RNA extracts was assessed on a 1.5% agarose gel, and visualization of RNA was by ethidium bromide staining. The presence of iNOS RNA and of GAPDH RNA, a constitutively expressed housekeeping gene, was determined by using the RT-PCR method, as outlined by Ribbons et al., (1995). The oligonucleotide primers used to detect iNOS were based on the sequence of a conserved region of mouse and human iNOS provided by Dr. Charles Rodi (Monsanto/Searle, St. Louis, MO). Sense and antisense primers for GAPDH (Genbank M17701) were the same as those described by Miller et al., (1995).

Assessment of diarrhea status. The percentage water content of the feces and the average daily fecal output were used as parameters of the severity of diarrheal symptoms in colitic rhesus monkeys and were compared to normal values from noncolitic monkeys. From
fecal samples collected on each day over a 10-day period, three subsamples were weighed and dried for 24 hr on a Savant AS160 Speedivac freeze drier (Farmingdale, NY). Samples were reweighed after drying, and the water content was determined as the difference between the wet and dry weights.

**Statistical analysis.** Data are expressed as mean ± S.E.M., unless otherwise stated. Statistical comparisons between treatment groups were made using a one-way analysis of variance, and Tukey’s post-hoc tests and paired comparisons between samples taken before and at the completion of the trial were compared by means of a paired Student’s t test.

### Results

**Systemic effects of AG and L-NIL in colitic rhesus monkeys.** Administration p.o. of 30 mg/kg L-NIL to colitic rhesus monkeys resulted in a systemic L-NIL concentration of 65 to 235 μM within 45 min of administration, which suggests that the compound rapidly crosses the intestine into the circulation. *In vivo* selectivity of both AG and L-NIL for the inducible isofrom of NOS was assessed by monitoring changes in mean arterial pressure. The mean arterial pressure was comparable in colitic rhesus monkeys in both L-NIL and AG treatment groups before the commencement of the trial (58.2 ± 9.3 mm Hg and 53.8 ± 9.2 mm Hg, respectively) and remained unchanged after 10 days of treatment with the NOS inhibitors (60.0 ± 8.9 mm Hg, 63.3 ± 15.8 mm Hg, respectively), which suggests that the constitutive, endothelial isoform of NOS was not affected by administration of L-NIL or AG. These blood pressure values are within the normal range for anesthetized monkeys (colitic or noncolitic) determined via indirect cuff measurements.

**RNI.** In all colitic rhesus monkeys used in this study, plasma RNI levels were elevated above the levels detected in normal, noncolitic monkeys (12 ± 2 μM) and were comparable to the values observed with active rhesus colitis (91 ± 29 μM) previously reported by our group (Ribbons et al., 1995). The plasma RNI levels before and after the iNOS inhibitor trial were compared (table 2). There was a tendency for RNI levels to be reduced in animals that were administered placebo. Although high variation in the RNI levels measured before the start of treatment in this group may account for some of this apparent trend, it is also possible that fasting, daily removal of feces and metabolic cage housing result in a reduction in RNI levels. L-NIL treatment significantly reduced plasma RNI by 72% (P < .05). In monkeys treated with AG, RNI levels decreased by 42%, but this reduction did not achieve significance (table 2).

**Severity of intestinal inflammation.** Morphological characteristics of the colitis condition were assessed in all biopsy specimens collected before the start of the iNOS inhibitor trial. Morphological characteristics of the colitis condition included a diffuse lymphocytic and plasmocytic cellular infiltrate, hyperplasia of the mucosa and a high incidence of crypt abscesses. Because of the limited amount of biopsy material obtained at endoscopy, the number of fields that could be obtained for quantification of the severity of the inflammatory infiltrate was low, so samples from each region biopsied were pooled; this yielded 4 to 6 fields of the colonic mucosa from each animal for cell counting. In all colitic rhesus monkeys, inflammatory cells occupied approximately 50% of the area of the mucosa, and there was no significant difference in the density of inflammatory cells among animals assigned to the placebo (55.5 ± 2.87%), L-NIL (49.6 ± 3.85%) and AG (49.0 ± 1.95%) treatment groups. These data suggest that the severity and extent of colonic inflammation were comparable in all colitic rhesus monkeys before the onset of the trial.

The density of the mucosal inflammatory infiltrate was determined for samples taken 30, 20, and 10 cm proximal to the rectum (termed proximal, mid and distal segments, respectively) collected at necropsy from colitic rhesus monkeys and from normal, noncolitic animals (n = 4) (fig. 1). Rhesus colitis was associated with a significant increase in the number of inflammatory cells in the colonic mucosa (a 40% to 50% increase in the density of inflammatory cells in placebo-treated colitic monkeys compared with noncolitic normal animals) (fig. 1). Treatment with either of the iNOS inhibitors L-NIL and AG failed to reduce the inflammation index below the level observed in placebo-treated animals in each region of the colon. The inflammation index did decline with the iNOS inhibitors, but this trend did not achieve significance, so we conclude that these agents did not improve mucosal inflammation in this treatment regimen (fig. 1).

**Immunohistochemical localization of iNOS in colitic rhesus monkeys.** The cell types expressing iNOS in the colitic rhesus monkey were identified by immunohistochemical detection in biopsy and necropsy tissue samples. Positive iNOS staining was localized in the plasma cells and neutrophils located in the lamina propria and in the lumen of crypt abscesses (fig. 2).

**iNOS gene expression.** Colonic tissue was collected at end
tification of 18S and 28S RNA fragments on a denaturing agarose gel. The amount and quality of RNA derived from all biopsy material collected were insufficient for us to perform any evaluation of iNOS gene expression. Intact RNA samples from necropsy specimens, randomly selected from three monkeys each from the placebo, L-NIL and AG treatment groups and from three normal rhesus monkeys, were used to assess the relative amount of iNOS gene expression in each group. The expression of GADPH was used as an internal standard to indicate the relative amount of RNA used in each RT-PCR reaction. A 907-base pair fragment, corresponding to a conserved region of the iNOS gene, was detected in the colonic mucosa from colitic monkeys in all treatment groups, whereas the complete absence or a relatively small amount of iNOS gene product was detected in noncolitic control monkeys (fig. 3). This result indicates that iNOS gene expression is up-regulated in chronic colitis. Furthermore, the relative intensity of the iNOS gene product was comparable in all samples analyzed from colitic monkeys treated with the placebo, with L-NIL or with AG (fig. 3). This suggests that the administration of inhibitors of iNOS activity did not attenuate iNOS gene expression in chronic colitis.

Assessment of diarrhea status. One of the clinical symptoms observed in chronic colitic rhesus monkeys was a repeated onset of severe diarrhea. Diarrheal symptoms were reflected by a significant increase in the average daily total fecal output and percentage of water content in feces in all colitic monkeys compared with noncolitic controls (table 3). The total fecal output and percentage water content of feces were not significantly different among the placebo, L-NIL and AG treatment groups, which suggests that 10 days of treatment with iNOS inhibitors did not result in a subsidence of diarrhea in chronic colitic monkeys (table 3).

Discussion

This study represents the first trial assessing the therapeutic efficacy of iNOS inhibitors in a model of chronic, spontaneous colitis in a primate. Our results suggest that p.o. administration of either of two NO inhibitors, AG and L-NIL, which both display specificity for the inducible isof orm of NOS, did not confer any major therapeutic benefit on terminally ill animals with active disease. Although L-NIL treatment significantly reduced systemic NO production, neither L-NIL nor AG, administered for 10 days, substantially reduced the number of inflammatory cells in the colonic mucosa or attenuated diarrheal symptoms. Expression of the iNOS gene also remained up-regulated in placebo-treated and iNOS inhibitor-treated animals. We did not anticipate that an iNOS enzyme inhibitor would directly down-regulate

### Table 3

**Diarrhea Status of Rhesus Monkeys**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Output (g/kg body weight)</th>
<th>% Water Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 4)</td>
<td>11.43 ± 0.51</td>
<td>67.16 ± 0.93</td>
</tr>
<tr>
<td>Colitis + placebo (n = 6)</td>
<td>22.7 ± 3.24*</td>
<td>75.88 ± 1.52*</td>
</tr>
<tr>
<td>Colitis + L-NIL (n = 5)</td>
<td>20.12 ± 5.76*</td>
<td>75.82 ± 1.46*</td>
</tr>
<tr>
<td>Colitis + AG (n = 5)</td>
<td>27.78 ± 3.52*</td>
<td>76.97 ± 3.32*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M.

* P ≤ .05 vs. normal monkeys, ANOVA, Tukey’s post-hoc test.
iNOS gene expression, but we hypothesized that attenuation of the colonic inflammation and subsidence of disease symptoms would be reflected by a reduction in iNOS gene expression.

We have previously reported that iNOS expression in rhesus colitis closely mimics iNOS activity in active ulcerative colitis with regard to the level of enzyme activity and responsiveness of the rhesus iNOS to enzyme inhibitors (Ribbons et al., 1995). In the current study, we have localized iNOS activity immunohistochemically to plasma cells and polymorphonuclear cells in the lamina propria and at focal sites of inflammation in the mucosa in the crypt abscesses. A similar profile of iNOS staining in inflammatory cells has been reported in ulcerative colitis (Middleton et al., 1995; Volk et al., 1995), but additional staining in the colonic epithelia of active disease is also detected by some authors (Reynolds et al., 1995). Our group has also observed epithelial iNOS immunoreactivity in biopsy specimens from ulcerative colitis and Crohn’s disease patients (unpublished observations). In the rhesus colitis model, a small degree of iNOS staining is evident in surface epithelia, but it does not appear to be the primary source of iNOS. Rhesus colitis is not characterized by mucosal ulceration; rather, the mucosa is hypertrophied. This is the major morphological difference between this model and clinical ulcerative colitis. The contribution of epithelial iNOS to the difference between the two colitic conditions remains unknown.

It is anticipated that the form of colitis that afflicts rhesus monkeys may provide a means of testing new chemical entities and therapeutic strategies for the treatment of human inflammatory bowel disease. This assumption is based on its presentation in a primate, on the periods of relapse and recovery and on the other clinical presentations outlined in table 1. Although the rhesus model remains to be fully characterized, there are clear points of similarity to the human condition and of disassociation from it. The rhesus colitis bears closer resemblance to ulcerative colitis than to Crohn’s disease, because only the colon is affected and the inflammation is primarily mucosal. However, the mucosal morphology differs from that of ulcerative colitis: crypt abscesses are present in both forms, but in rhesus colitis the mucosa hypertrophies and does not have areas of frank ulceration, although the mucosa is quite friable. Other models of inflammatory bowel disease, both induced and genetically engineered, display mucosal hypertrophy, so this observation is not unique. Therapeutic predictiveness remains to be elucidated because of its limited utilization. In terms of the present study, although we compare our results with those obtained in other experimental models of inflammatory bowel disease, we still do not know whether the model predicts the actions in human disease, because suitable iNOS inhibitors have not yet been tested in colitic patients.

Beyond the issues of the rhesus colitis model’s relevance to the human disease, we note that this is the first report on the efficacy of iNOS inhibitors in established inflammation, i.e., after the induction and expression of iNOS. In this regard, it has yet to be confirmed that NOS inhibition is anti-inflammatory in established, chronic GI or systemic inflammation. Furthermore, this investigation was performed in particularly sick monkeys that would otherwise have been terminated for the severity and intractable nature of their condition. The clinical correlates of this group also tend to be resistant to therapeutics, and that may have a bearing on the lack of effects with L-NIL treatment and AG treatment. Administration of iNOS inhibitors to animals at this stage of the disease may be unable to resolve symptoms. Alternatively, a considerably longer treatment protocol may be required. Subsequent studies need to be performed in animals deemed symptomatic but not terminal.

1-L-NIL treatment was associated with a significant reduction in plasma RNI levels but had no effect on mean arterial pressure, which suggests that 1-L-NIL reduced NO activity in vivo in colitic monkeys and displayed a high selectivity for the inducible isoform. The effects of AG on plasma nitrate/nitrite were not discernably different from those of placebo. This result suggests that 1-L-NIL was a more effective inhibitor of NOS in vivo. Indeed, we have previously observed that 1-L-NIL has a 17-fold greater potency in inhibiting iNOS than AG ex vivo (unpublished observations). Moreover, a more potent effect of 1-L-NIL than of AG has been reported in other models of inflammation (Conner et al., 1995).

Because the colon is the primary site of disease, we believe that the colitic condition was the source of the elevated nitrite/nitrate in plasma. Other organs, including spleen and liver, are potential sources of NO, but their contributions have not been specifically determined. However, they do not appear to be directly involved in the disease process and do not display significant inflammation.

Despite a reduction in systemic RNI levels, iNOS inhibitors did not significantly reduce morphological and clinical symptoms in colitic monkeys. Similar findings have been made in ulcerative colitis patients, where reduced systemic RNI levels were detected after glucocorticoid therapy but were not indicative of the clinical outcome of treatment (Rees et al., 1995).

The dose and time course of AG and 1-L-NIL used (60 mg/kg/day) were within the range of, or in some cases higher than, those used in other models of inflammation in which experimentally induced damage was ameliorated (Conner et al., 1995; Miller et al., 1993; Grisham et al., 1994). However, one major difference between these studies and the treatment regime used in the colitic rhesus model is the time at which iNOS inhibitor therapy was commenced. In both adjuvant-induced arthritis (Connor et al., 1995) and peptidoglycan/polysaccharide-induced colitis (Grisham et al., 1994) p.o. ad libitum administration of AG or 1-L-NIL was begun 3 days before the induction of inflammation and continued for 21 days. Furthermore, in TNBS-induced ileitis, a potent protective effect of iNOS inhibitor treatment was demonstrated when treatment was commenced at the time of induction of inflammation (Miller et al., 1995). From these studies, it is difficult to distinguish between the cytoprotective and the therapeutic modalities of iNOS inhibitors. In contrast, in the rhesus colitis model, we administered iNOS inhibitors to a chronic condition in which inflammation was well established and iNOS already expressed. Comparing our findings with those using iNOS inhibitors in other experimentally induced models of inflammation suggests that many of the protective effects of orally administered AG and 1-L-NIL that have previously been described may be due to a prophylactic modality. Unpublished data from our laboratory generated by using the TNBS model of ileitis in guinea pigs suggest that NOS inhibitors fail to display any anti-inflammatory actions when administered after the inflammation has been estab-
lished and iNOS expressed. This is despite use of the same dose, route and duration of administration. The present results raise the possibility that iNOS inhibition does not ameliorate established bouts of gut inflammation, even though it is very effective in preventing the initiation of injury. Thus iNOS inhibitors may act to maintain the disease in remission, as is the case for the 5-aminosalicylic acid (5-ASA) compounds, but are far less effective in active disease.

A potential explanation for this difference in efficacy between prophylactic and therapeutic treatment regimens is the phenomenon of endotoxin tolerance. Inhibitors of NOS reverse endotoxin tolerance in cell culture (the loss of cytokine secretion in response to repeated administration of endotoxin; Mannick et al., 1996). Using explants of rhesus colon incubated in culture media for 1 to 24 hr, we have determined that the colitic condition is associated with an exaggerated release of IL-1β and TNFα when compared with noncolitic controls (unpublished results). It is important to note that when explants are exposed to endotoxin, explants from noncolitic monkeys increase their release of IL-1β and TNFα, whereas colitic explants fail to respond to endotoxin. Thus the colitic condition has the hallmarks of endotoxin tolerance. If the in vitro findings on NOS inhibition are predictive of the in vivo state, then the administration of NOS inhibitors to colitic monkeys could lead to the reversal of endotoxin tolerance and to a further increase in cytokine release. In terms of the colitic condition, a loss of endotoxin tolerance and the accompanying exaggerated release of cytokines and mediators might counteract the effect of inhibiting NO release. This interpretation would also explain the discrepancy between the prophylactic and therapeutic actions of NOS inhibitors in colitis as well as the comparable observations with 5-ASA compounds.

Several authors have suggested that NO itself is a poor cytotoxic agent and that tissue injury is due to the formation of more toxic nitrogen intermediates, such as iron-nitrosyl complexes (Lancaster and Hibbs, 1990). Furthermore, by interacting with superoxide, NO can form the highly reactive anion peroxynitrite, which can initiate lipid peroxidation (Beckman et al., 1994) and rapidly oxidize sulfhydryl groups (Radi et al., 1991). Production of peroxynitrite in intestinal inflammation is supported by immunohistochemical co-localization of iNOS and nitrotyrosine residues (a marker of peroxynitrite) in TNBS-or adjuvant-induced ileitis (Miller et al., 1995; Seago et al., 1995) although it should be noted that other reactive nitrogen species can nitrate tyrosine residues.

A potent cytotoxic action of peroxynitrite in the GI tract has also been demonstrated after intraluminal administration (Rachmilewitz et al., 1993). Peroxynitrite formation is dependent on the availability of both NO and superoxide. Hence the production of cytotoxic anions from NO is dependent on its interaction with other reactive oxygen species. Treatment with compounds that reduce superoxide levels has been shown to confer benefit in experimentally induced models of intestinal inflammation (Miller et al., 1988; Karmeli et al., 1995). Therefore, the most effective therapeutic approach for inhibiting the cytotoxic effect of NO released by iNOS in chronic inflammation may be not only to reduce NO production but also to limit the amount of reactive oxidant species present.

Although NO released by iNOS has been linked to cytotoxic actions that may contribute to the progression of intestinal disease, our findings do not support the implication of an elevated synthesis of NO in chronic inflammation. Inhibition of systemic NO by L-NIL failed to reflect any therapeutic benefit to the inflamed colon in chronic colitic rhesus monkeys. This finding could be interpreted to mean that NO release in chronic inflammation is an epiphenomenon and does not contribute in a major way to the pathogenesis of chronic colitis. We could also speculate that iNOS expression reflects an up-regulation in host defense in the compromised intestine, in which case long-term inhibition of iNOS might even lead to exacerbation of the disease. Although other reports in experimentally induced models of intestinal inflammation suggest a protective effect of iNOS inhibitors administered prophylactically or during the development of the inflammation, the efficacy of administering iNOS inhibitors therapeutically has to date not been established. Hence, until we can better elucidate the role of NO in chronic inflammation, the therapeutic benefit of iNOS inhibitors in inflammatory bowel disease remains uncertain.

References


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