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The relationship between intestinal pathology and immune expulsion of gastrointestinal (GI) nematodes remains controversial. Although immune expulsion of GI helminth parasites is usually associated with Th2 responses, the effector mechanisms directly responsible for parasite loss have not been identified. We have previously shown that while the intestinal pathology accompanying the expulsion of the GI parasite Trichinella spiralis may be dependent on IL-4 and mediated by TNF, parasite loss is independent of TNF. In contrast, intestinal pathology in other disease models has been attributed to Th1 cytokines, although it closely resembles that seen in helminth infections. Whereas production of inducible NO synthase (iNOS) in the gut is important for both homeostasis of the epithelial layer and in protection against pathogenic microorganisms, overproduction of NO has been implicated in the pathogenesis of a number of inflammatory conditions. We therefore investigated the role of NO in T. spiralis infection using iNOS-deficient mice. iNOS−/− and iNOS+/+ mice were infected with T. spiralis, and parasite expulsion and intestinal pathology were followed. Parasite expulsion proceeded similarly in both groups of animals, but significant intestinal pathology was only observed in the heterozygous mice. Thus it appears that, although the protective effects of Th2 responses in GI helminth infection do not require NO, this mediator contributes substantially to the associated enteropathy. NO may therefore be an important mediator of enteropathy in both Th1- and Th2-inducing conditions. The Journal of Immunology, 2000, 164: 4229–4234.

Immune expulsion of nematodes parasitizing the small intestine is often accompanied by a severe T cell-mediated enteropathy, and treatments that suppress this pathology may abrogate the loss of the parasites (1–3). This therefore presents an obstacle to vaccination against such parasites because it might be necessary to induce pathology to be effective. However, we recently demonstrated that TNF-receptor 1-defective mice are able to immunologically expel the highly pathogenic Trichinella spiralis without significant pathology, thereby demonstrating that while pathology accompanies the protective mechanism, it is not required for it (4). This opens up the possibility that immunological interventions could be designed to avoid the induction of pathology. However, interference with cytokine signaling in the intestine as part of an immunization strategy may be difficult or undesirable given its role in other essential processes. To target effective therapy or immunization strategies it will therefore be necessary to extend our understanding of the mediators directly involved in immune expulsion and pathology.

Reactive nitrogen intermediates including NO have been identified as important effector molecules that can restrict pathogen growth in infected hosts while also playing a role in immunoregulation and pathology (5–13). Expression of inducible NO synthase (iNOS, NOS2) is elicited in a wide variety of cell types by pro-inflammatory cytokines or bacterial products such as LPS, and results in high level production of NO from l-arginine substrate (5–8). A number of studies have indicated that NO produced by cytokine-activated macrophages is important in immune responses against bacterial, fungal, helminth, and protozoan infectious agents (9–13). Furthermore, the immunomodulatory activity of NO is manifested in potent immnosuppression and in influencing Th cell development (5, 6, 14, 15). Production of excessive amounts of NO, however, has been implicated in a number of pathological conditions, appearing to play a crucial role in diabetes mellitus, graft-vs-host disease, rheumatoid arthritis, and inflammatory bowel disease (IBD) (5, 16–19). Intestinal pathology in a number of immunologically mediated enteropathies such as graft-vs-host disease, IBD, and the spontaneous gut lesions that develop in certain cytokine knockout mice is usually associated with Th1 type responses and TNF-α (19–22), and iNOS induced by inflammatory cytokines is thought to play an important role in these situations. However, it has recently been demonstrated that other models of IBD could also be associated with Th2 responses (23–25).

Many studies have analyzed the protective role of NO during Th1-inducing infections, but few have analyzed the effects of this mediator in the context of a Th2-stimulating infection such as with helminth parasites. Although the gross enteropathy associated with the immune expulsion of intestinal helminths mirrors that associated with many Th1 responses, both the pathology and parasite loss are Th2-dependent phenomena. Indeed, we have recently shown that expulsion proceeds in the absence of pathology in mice defective for the TNF signaling system, demonstrating that pro-inflammatory cytokines appear to be under the control of Th2 responses in this case, and that pathology is not obligatory for

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3 Abbreviations used in this paper: iNOS, inducible NO synthase; GI, gastrointestinal; IBD, inflammatory bowel disease; MLN, mesenteric lymph node; p.i., postinfection.
parasite loss (4). These findings have implications for vaccine development and the control of idiopathic IBDs, yet the downstream/direct mediators of enteropathy in Th2-mediated events such as in helminth infections remain to be identified.

In this paper we provide evidence that NO is an important mediator of enteropathy in infection with the intestinal nematode *T. spiralis*, and propose that iNOS production is under the control of TNF in such circumstances. Our findings reinforce the concept that enteropathy is not required for the removal of nematode parasites. Furthermore, the identification of NO as a crucial mediator provides a more amenable target for intervention than manipulation of cytokines in the control of enteropathy in infections or idiopathic conditions. If NO is indeed the end point mediator of enteropathy, regardless of aetiology, then the new generation of inhibitors and scavengers of NO will be useful therapeutic agents (26).

Materials and Methods

Animals and infection

The maintenance, infection, and recovery of *T. spiralis* were essentially as described previously (4). Mice were infected with 400 *T. spiralis* larvae on day 0 and killed at various times postinfection (p.i.) as detailed. C57BL/6 mice purchased from Harlan-Olac (Oxford, U.K.) were maintained under standard laboratory conditions. MF-1×129 mice with a homozygous disruption of the iNOS gene (iNOS−/−) and heterozygous littermates (iNOS+/−) were bred and maintained in the Joint Animal Facility, University of Glasgow. The homozygous and heterozygous mice thus generated were backcrossed with MF-1 mice for three generations. Heterozygous mice have previously been shown to be phenotypically identical to wild-type MF-1 mice (13, 27).

Quantitation of intestinal pathology

After weighing the entire small intestine, mucosal architecture, and epithelial cell mitotic activity were assessed in samples of jejunum taken 10 cm from the pylorus, as described previously (4). Samples were fixed in 25% acetic acid/75% ethanol and stained with Schiff reagent (Sigma, Poole, U.K.). Specimens were microdissected and villus and crypt lengths measured using an eyepiece micrometer. The number of mitotic figures per crypt were also counted. Ten villi and crypts were measured for each sample. Consecutive samples of jejunum were fixed in Carnoy's fixative and processed using standard histological techniques. Sections were stained with 0.5% toluidine blue (pH 0.3) for visualization of mast cells. The numbers of mucosal mast cells were counted per 25 villus crypt units (VCU) and data expressed per VCU.

Lymphocyte culture and cytokine responses

Single cell suspensions of mesenteric lymph nodes (MLN) were prepared and incubated in the presence of *T. spiralis* larval homogenate (50 µg/ml protein). Culture supernatants were harvested after 24 h, and IL-2, IL-4, IFN-γ levels measured by ELISA using paired Abs as described previously (4). TNF-α levels were determined in serum by ELISA according to the manufacturer's instructions (PharMingen, Oxford, U.K.).

Measurement of Ab responses

Parasite-specific IgG1 and IgG2a and total IgE levels were determined as described previously (4). *T. spiralis* larval homogenate was used as a target Ag at 10 µg/ml in ELISA, and sera were diluted 1/100. IgG1 and IgG2a were detected using biotinylated anti-mouse IgG1 or IgG2a (PharMingen) at 2 µg/ml each, followed by streptavidin-peroxidase (SAPU, Carlufe, U.K.). Total serum IgE levels were measured by ELISA, anti-mouse IgE was used as capture Ab, and IgE was detected using biotinylated anti-mouse IgE (PharMingen). An IgE mAb specific for TNP (PharMingen) was used as standard.

Measurement of NO

Total nitrate and nitrite concentrations in sera were estimated by the conversion of nitrate into nitrite. Total nitrite content was then measured by the Greiss reaction using NaNO2 as standard with detection limit of 1 µM (28). Briefly, 30 µl of each sample was incubated for 1 h at 37°C with 5 µl Aspergillus nitrate reductase (5 U/ml; Sigma) and 15 µl of NADPH (1.25 mg/ml; Sigma). After incubation, 100 µl of Greiss reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H3PO4) and 100 µl trichloroacetic acid (10% aqueous solution) were added and incu-

FIGURE 1. Parasite burdens in iNOS-deficient mice infected with *T. spiralis*. Mice were infected with 400 *T. spiralis* muscle larvae and worm burdens assessed on day 6 and day 13 p.i. Adult worm burdens are represented as mean ± SEM. * Significant difference between day 6 and day 13 p.i. (p < 0.05). This figure is representative of two experiments.

Results

Our previous work demonstrated that while IL-4 was an absolute requirement for both parasite expulsion and pathology during infection with *T. spiralis*, TNF was only required for expression of the pathological but not the protective responses (4). One possible mechanism by which TNF may exert this effect is through the production of NO via iNOS induction.

NO in *T. spiralis* infection

To investigate whether NO was produced during infection of mice with *T. spiralis*, C57BL/6 mice were infected with 400 muscle larvae, and concentrations of nitrite and nitrate were measured in the serum. Production of NO by *T. spiralis* infected C57BL/6 mice was significantly increased; levels rose from 11.2 ± 1.6 µM to 23.2 ± 1.9 µM (p < 0.05). This confirmed studies of *T. spiralis* infection in rats (29). We next investigated the role of NO in protection and pathology using iNOS-deficient mice.

T. spiralis-associated pathology is diminished in iNOS-deficient mice despite normal expulsion of the parasite

The kinetics of parasite expulsion from heterozygous (iNOS+/−) or homozygous (iNOS−/−) mice infected with *T. spiralis* were identical to those seen in wild-type mice (Fig. 1). There was no significant difference in the establishment (day 6 p.i.) or the expulsion (day 13 p.i.) of the parasite from iNOS−/− mice. To determine whether expulsion may have occurred earlier in iNOS−/− mice, animals were also examined at day 10 p.i., but again there was no significant difference between the two groups at this time (data not shown).

Although there was no significant difference in intestinal architecture between naive iNOS+/− or iNOS−/− animals, only the heterozygous mice displayed severe enteropathy when infected with *T. spiralis*, comprising villus atrophy and crypt hyperplasia with a
concomitant increase in the number of mitotic figures per crypt (Fig. 2). iNOS-deficient mice showed no evidence of villus atrophy, and the crypt hyperplasia observed was significantly less pronounced than that seen in the heterozygous mice. The number of mitotic figures per crypt were also significantly lower in the iNOS-deficient mice compared with the heterozygotes, again indicating reduced crypt hyperplasia. This pattern of reduced enteropathy in iNOS<sup>−/−</sup> mice is similar to that previously seen in TNF-R1-deficient animals infected with <i>T. spiralis</i> (4).

To investigate the role of T helper subsets during infection of iNOS-deficient mice with <i>T. spiralis</i>, we determined levels of IL-2, IFN-γ, IL-4, IL-5, and TNF-α produced by these animals (Table I). There was a significant increase in Th2 cytokines in iNOS<sup>−/−</sup> mice during infection but little increase in IFN-γ. In contrast, iNOS<sup>−/−</sup> mice showed increased production of both IL-2 and IFN-γ with little or no change in the levels of IL-4 or IL-5 following infection. Importantly, while serum TNF-α levels in iNOS<sup>−/−</sup> mice did not increase, those in iNOS<sup>−/−</sup> mice were significantly elevated.

It has been known for some time that NO has an antiproliferative effect on both T and B cells (30, 31), and therefore the proliferative responses of cells from MLN to <i>T. spiralis</i> Ag following infection were determined (Fig. 3). Although MLN cells from both strains of mice proliferated in response to Ag, those from iNOS-deficient infected mice proliferated to a significantly greater extent than those from heterozygous mice.

**FIGURE 2.** Development of intestinal pathology in iNOS-deficient mice infected with <i>T. spiralis</i>. Crypt lengths, villus lengths, and number of mitotic figures/crypt were measured at days 6 and 13 p.i. Uninfected mice are represented at day 0. Results are shown as mean ± SEM. A, iNOS<sup>+/+</sup> crypt and villus lengths (n = 6). B, iNOS<sup>−/−</sup> crypt and villus lengths (n = 7). C. Number of mitotic figures/crypt. *, Significant difference between infected and uninfected animals (p < 0.05); †, significant difference between iNOS<sup>+/+</sup> and iNOS<sup>−/−</sup> (p < 0.05). This figure is representative of two experiments.

**FIGURE 3.** Ag-specific cell proliferation in iNOS-deficient mice infected with <i>T. spiralis</i>. MLN cells were harvested from uninfected or infected iNOS<sup>+/+</sup> and iNOS<sup>−/−</sup> mice 13 days p.i. and stimulated with 50 μg/ml <i>Trichinella</i> muscle Ag. Results are shown as mean ± SEM. *, Significant difference between infected and uninfected animals (p < 0.05); †, significant difference between iNOS<sup>+/+</sup> and iNOS<sup>−/−</sup> (p < 0.05). This figure is representative of two experiments.

| Table I. Cytokine profiles by MLN cells following in vitro Ag stimulation during infection with <i>T. spiralis</i><sup>a</sup> |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|                                                  | iNOS<sup>−/−</sup>                                | iNOS<sup>−/−</sup>                                |
|                                                  | Day 0 | Day 13 p.i. | Day 0 | Day 13 p.i. |
| IL-2 (U/ml)                                      | 0 ± 0 | 6.5 ± 3.1*  | 0 ± 0 | 25.6 ± 5.6†  |
| IFN-γ (U/ml)                                     | 0 ± 0 | 3.5 ± 0.2*  | 32.5 ± 2.0†| 38.1 ± 2.8† |
| IL-4 (U/ml)                                      | 0.5 ± 0.3 | 12.5 ± 3.1* | 0 ± 0 ± 4.1 ± 2.1† |
| IL-5 (U/ml)                                      | 0.9 ± 0.7 | 4.4 ± 1.3*  | 0 ± 0 ± 0 ± 1.0† |
| TNF-α (U/ml)                                     | 0 ± 0 | 11.0 ± 2.9* | 0 ± 0 ± 1.0† |

* Data are shown as mean ± SEM; iNOS<sup>−/−</sup> (n = 6), iNOS<sup>−/−</sup> (n = 7). This table is representative of two experiments.

* †, Significant difference between infected and uninfected animals (p < 0.05).
Th2-associated humoral response is decreased in iNOS-deficient mice

Isotype-specific anti-Trichinella Ag Abs were also measured as an indicator of Th activity. High IgG1 and IgE levels and low IgG2a levels were observed in heterozygous mice (Table II). Although levels of IgG1 and IgE produced by iNOS-deficient mice were still significantly elevated with respect to control animals, they were significantly less than those observed in infected heterozygous animals. Conversely, while levels of IgG2a did not increase significantly in infected heterozygous mice, they were significantly elevated in the iNOS-deficient animals.

Mastocytosis is decreased in iNOS-deficient mice

Numerous studies have indicated that mast cells play an important role in the expulsion of T. spiralis, and they have also been implicated in the mediation of intestinal pathology in other disease models, and NO has been demonstrated to play a role in the activation of mast cells (32–34). Although a mucosal mastocytosis was apparent in iNOS-deficient mice infected with T. spiralis, it was substantially lower than that observed in the heterozygous animals (Fig. 4).

Fluid accumulation is decreased in the intestine of iNOS-deficient mice

It has been suggested that a change in intestinal fluid dynamics that favors fluid accumulation in the gut lumen and an increase in the small intestine smooth muscle contractility results in the expulsion of loosely attached parasites (35). Additionally, NO is involved in the induction of vascular leak syndromes that lead to fluid accumulation in intestinal tissues (36). Although there was a significant increase in the wet weight of the small intestine in both groups of animals, the weights of the intestines from iNOS−/− mice were significantly lower than those from iNOS+/− mice (Fig. 5). Fluid accumulation in the gut lumen of infected mice, mediated by NO, is therefore unlikely to play a role in the expulsion of T. spiralis.

Discussion

The results of this study have two major implications. First, NO is a central mediator of pathology in the context of a Th2-mediated protective immune response. Second, pathology is not required for removal of the parasite. This reinforces our previous findings in animals defective for TNF signaling, but the identification of NO as a downstream mediator allows the development of interventions in certain infections and immunopathologies without potentially deleterious manipulations of the cytokine system.

It has been well documented that protection against Th1-inducing infections is associated with the production of NO via iNOS up-regulation, and NO has also been implicated in many Th1-mediated enteropathies (10–14, 17–19). However, the pathology observed during infection with the highly pathogenic gastrointestinal (GI) parasite T. spiralis is mediated by a Th2 response (4). At present, little work has been conducted on the role of NO in Th2-inducing infections, so we aimed to establish the role of iNOS in the induction of protection and pathology in a Th2-polarizing infection. The observations were consistent with our previous studies in showing that intestinal pathology is not required for the expulsion of GI nematode parasites. Moreover, while NO does not appear to be important in protection against helminths, it is crucial for the pathology induced. Recent studies have also suggested that

Table II. Ab responses during infection with T. spiralis*a

<table>
<thead>
<tr>
<th></th>
<th>iNOS+/+</th>
<th>iNOS−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 13 p.i.</td>
</tr>
<tr>
<td>Parasite-specific IgG2a (A630)</td>
<td>0.193 ± 0.006</td>
<td>0.207 ± 0.010</td>
</tr>
<tr>
<td>Parasite-specific IgG1 (A630)</td>
<td>0.165 ± 0.004</td>
<td>1.071 ± 0.029*</td>
</tr>
<tr>
<td>Total IgE (µg/ml)</td>
<td>9.6 ± 2.5</td>
<td>943.8 ± 23.3*</td>
</tr>
</tbody>
</table>

*a Data are shown as mean ± SEM; iNOS+/+ (n = 6); iNOS−/− (n = 7). This table is representative of two experiments.

†, Significant difference between iNOS+/+ and iNOS−/− (p < 0.05).

* *, Significant difference between infected and uninfected animals (p < 0.05).
NO plays a dual role in inducing protective and pathological responses in other systems. Inhibition of iNOS during a number of infections, including viruses, bacteria, and protozoan parasites, could greatly improve survival despite an increase in pathogen numbers. Importantly, NO may now be considered as an end point mediator of pathology induced by either Th1 or Th2 polarized responses.

Like earlier studies in rats infected with T. spiralis (30), we show an increase NO levels in the serum of T. spiralis-infected mice. Although NO appears to be directly toxic to some helminths in vitro (40–42), our study now shows that NO synthesized by iNOS is not essential for the expulsion of T. spiralis from mice. The results presented here also demonstrate that iNOS plays a crucial role in the induction of the Th2-mediated enteropathy that accompanies GI nematode infections. This contrasts with some models of IBD where NO is associated with excessive production of Th1 cytokines, and where the use of NO inhibitors and iNOS-deficient mice have clearly demonstrated a central role for NO in the induction of enteropathy (17–19). Interestingly, it has recently been shown that Th2-type responses may also mediate intestinal inflammation in other models of enteropathy, although the role of NO in these pathologies is unknown (23–25).

The source of NO in enteropathy has not been identified but a number of cells including epithelial cells, macrophages, and mast cells have been implicated (9, 43–45). Epithelial cells are the first point of host contact for invasive intestinal pathogens and may initiate mucosal inflammatory responses via production of pro-inflammatory cytokines and mediators including NO (8, 43). T. spiralis occupies an intramucosal niche in small intestinal epithelium and infected epithelial cells have been shown to respond by synthesizing cytokines (46). It is therefore possible that the perturbation of the epithelium by invasion of T. spiralis results in the development of an aberrant mucosal immune response mediated by iNOS and the subsequent compromise of intestinal integrity.

A cardinal role for mast cells in host protection against certain GI nematode infections (T. spiralis in particular) has been shown in numerous studies (47, 48). Mast cells have also been shown to induce intestinal pathology by a number of mechanisms including the release of mediators such as cytokines, histamine, and NO (32–34, 49). There is also evidence that mast cells have the ability to synthesize and release NO derivatives through their own expression of iNOS induced by IgE immune complexes (44). Furthermore, iNOS has been shown to be crucial in allergic inflammatory processes by regulating the activation and degranulation of mast cells (50). However, it has also been suggested that mast cells normally serve to inhibit increased mucosal permeability induced by iNOS activity in the gut (33, 34). We find that mast cell numbers were decreased in T. spiralis-infected iNOS-deficient mice, so even if they are involved in parasite expulsion in this context, then they do so without the concomitant pathology.

NO may also exert its effects in enteropathy and parasite infection through its immunomodulatory role in Th cell polarization. It was observed that although iNOS-deficient mice infected with T. spiralis exhibited a decreased Th2 response compared with heterozygous mice, this was nevertheless sufficient to induce expulsion of the parasite. Similarly, iNOS-deficient mice infected with L. major, S. mansoni, or S. aureus have been demonstrated to have enhanced Th1 responses compared with wild-type mice (7, 13, 51). The mechanism by which NO influences immunoregulation is unknown at present but it has been suggested that NO could preferentially regulate the production of Th1/Th2 cytokines through modification of gene transcription, through the induction of apoptosis in Th1 cells, and/or by down-regulating selectin-dependent Th1 cell accumulation at sites of inflammation (14, 15, 30, 52, 53).

The production of TNF-α in T. spiralis-infected iNOS-deficient mice was decreased in comparison to heterozygous mice. In contrast, it has been shown that iNOS-deficient mice infected with S. aureus had increased production of TNF-α compared with heterozygotes, and it was concluded that the increased levels of TNF-α were the result of the exaggerated Th1 polarization induced in these animals (7). However, NO has also been shown to enhance the induction of TNF-α synthesis both in vivo and in vitro (15). The reduced levels of TNF-α in iNOS-deficient mice infected with T. spiralis may instead be due to its induction by a Th2 response rather than a Th1 response. More recently, expulsion of Trichuris muris from TNF-R gene-deficient mice was prevented, and it was proposed that TNF-α mediated its protective effects by regulating the Th2 cytokine response in the intestine (54).

Other NO-dependent functions (including alterations in vasodilation, endothelial integrity, vascular adhesion, emigration of inflammatory cells, increased mucin secretion by goblet cells, altered intestinal smooth muscle function, and intestinal fluid secretion) may also play a role in the enteropathy accompanying parasite expulsion (29, 50, 53, 55–57). It has been suggested that a change in intestinal smooth muscle and fluid dynamics, which favors increased propulsion and fluid accumulation in the gut lumen, leads to the expulsion of loosely attached parasites (35). However, fluid accumulation in the gut lumen of infected mice is unlikely to play a role in the expulsion of T. spiralis because the wet weights of the small intestines from iNOS-deficient mice were significantly lower than those of heterozygous mice. Furthermore, these parasites are embedded in the intestinal epithelium and so would be less susceptible to peristaltic removal.

It is possible that the villus atrophy observed in T. spiralis infection, similar to that described following administration of IL-12, IFN-γ, or TNF-α (36), may be induced by NO either through direct cellular injury via peroxynitrite or indirectly via apoptosis of villus epithelial cells. Although villus atrophy was ablated in iNOS-deficient mice, crypt hyperplasia was also reduced, suggesting that NO is playing a greater role in inducing intestinal inflammation than merely causing the death of epithelial cells. Thus, iNOS may induce intestinal pathology in T. spiralis infection in a number of ways which could include induction of inflammatory cytokines such as TNF, activation of mast cells, immunomodulation, alterations in intestinal physiology, or by the induction of apoptosis of villus epithelial cells.

In conclusion, we have demonstrated that NO is an important component of the pathological response accompanying the expulsion of a GI nematode parasite. In the absence of high output NO synthesis, mice did not develop the severe enteropathy which usually accompanies infection with T. spiralis, but this did not inhibit the ability of the animals to expel the parasite. Thus, iNOS may promote parasite expulsion by down-regulating the induction of Th1 responses, thereby promoting a Th2 response. However, limitation of excessive NO production and the resulting tissue damage is no doubt of general benefit to the host. These results provide insights into the interplay of the protective and pathological responses associated with the expulsion of GI parasites. Importantly, they further suggest that Th2 responses can induce NO-mediated pathology. Hence, selective inhibitors of iNOS, currently under consideration for the treatment of IBDs, may prove beneficial for both Th1 and Th2-mediated enteropathies without compromising Th2 protective responses (26). It is now important to establish the exact mechanism by which iNOS is induced and exerts its pathological influence in intestinal inflammation.
References


