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Research Article

Infection with *Hymenolepis diminuta* Is More Effective than Daily Corticosteroids in Blocking Chemically Induced Colitis in Mice

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Purpose. To compare infection with the tapeworm, *Hymenolepis diminuta*, with steroid (dexamethasone) administration in the inhibition of dinitrobenzene sulfonic acid- (DNBS-) induced colitis in mice. *Procedures.* Mice were treated with DNBS ± infected with *H. diminuta* or treated with daily dexamethasone (2 mg/Kg, ip.) and were assessed 72 hours post-DNBS by the calculation of disease activity and histological damage scores, and spleen cell cytokine production. *Results. H. diminuta*-infected mice showed increased IL-4 and IL-10 production by spleen cells compared to other groups and were protected from DNBS-induced colitis. In contrast, there was little benefit of dexamethasone in the treatment of colitis. Collagen deposition in the colon was not different between the groups. *Conclusions. H. diminuta* was superior to dexamethasone in the prevention of DNBS-induced colitis and did not result in additional side effects (i.e., collagen deposition). Comparisons with current therapeutics and long-term followup to studies are essential if "helminth therapy" is to become a viable treatment for specific inflammatory diseases in the gut or other tissues.

1. Introduction

During the last three decades there have been dramatic increases in autoimmune and inflammatory diseases, such as allergy/atopy, diabetes, and inflammatory bowel disease (IBD) that cannot be explained solely on the basis of genetics [1]. In the search for environmental triggers for these conditions, the hygiene hypothesis has arisen that suggests that reduced exposure to infectious agents (via increases in hygiene, sterile drinking water, and use of antibiotics) may result in the generation of greater numbers of autoreactive immune cells in humans, and hence the emergence of autoimmune and idiopathic inflammatory disease [2]. Compatible with this postulate is the geographical divergence in the occurrence of diseases such as IBD and areas of pandemic helminth infection [3]. Epidemiological data must be viewed cautiously when used in support of causation rather than association. They do nevertheless raise the question: could infection with parasitic helminths protect against other concomitant disease?

Representatives of all classes of helminth parasite have been shown to modulate immunity in their hosts, both qualitatively and quantitatively [4–6]. Moreover, infection with helminth parasites evokes stereotypic immune responses in humans and mice that are dominated by T helper 2 (TH2) cytokines. Thus as putative modifiers of disease, the release of immunomodulatory or immunosuppressive molecules from helminths would be expected to impact on concurrent disorders in the host, and the stimulation of TH2 events has the potential to antagonize or inhibit diseases in which the immunopathology is driven by TH1 reactions. We have shown that infection with the rat tapeworm, *Hymenolepis diminuta*, protects mice from the colitic effects of direct instillation of dinitrobenzene sulfonic acid (DNBS) into the lumen of the colon [7]. A substantial amount of data has amassed showing that a variety of species of helminths

(e.g., *H. diminuta*, *Trichinella spiralis*, *Heligosomoides polygyrus*, *Schistosoma mansoni*) reduce the severity of disease in rodent models of IBD, airways inflammation, and multiple sclerosis [8]. In the context of developing new treatments, two major possibilities arise from these proof-of-concept studies: (1) use of the immunological knowledge gleaned from these models to define new targets for pharmacological intervention; and (2) the prescription of a "therapeutic helminth(s)" to defined groups of patients that are refractory to other therapies. The latter is a provocative idea and, despite the spectre of iatrogenic disease [9], studies have been presented in which nematodes, namely, *Trichuris suis* and *Necator americanus*, have been used to treat IBD and as a putative forerunner to use in asthma, respectively [10, 11].

As the concept of a "therapeutic helminth" progresses it is important that appropriate comparisons with current therapies and potential side effects be rigorously assessed. Using the *H. diminuta*-DNBS murine model system, the current study addresses two questions. Will *H. diminuta* be as effective as steroids in the treatment of DNBS-induced colitis? And, is helminth therapy in this acute model associated with increased collagen deposition that could result in fibrosis and stricture formation? Using this acute and spontaneously resolving chemical model of colitis we present data showing that infection with *H. diminuta* is superior to daily steroid therapy and that infected mice, while receiving substantial anti-inflammatory benefit from infection, displayed no greater collagen deposition in their colons than mice receiving DNBS only.

2. Materials and Methods

2.1. Murine Model System. Male 6–8 Balb/c mice (Charles River Animal Supplies, Montreal, QB, Canada) were housed in filter-topped cages with free access to water and rodent chow and on a 12 hour : 12 hour light : dark cycle [7]. Colitis was induced in anesthetized mice via intrarectal (ir.) instillation of DNBS (3.0 or 2.5 mg in 100 μL of 50% ethanol) delivered 3 cm into the colon via a polyethylene catheter [7]. Time-matched control mice received 50% ethanol only. A third group of mice were infected with five *H. diminuta* cysticercoids via oral gavage in 100 μL 0.9% NaCl, eight days prior to receiving DNBS [12]. The final group of animals was treated with the glucocorticoid, dexamethasone (DEX; Sigma Chemical Co., St. Luois, MO) and DNBS (n = 3–8 mice/group). Based on previous reports in which DEX was used to suppress inflammatory disease [13–15] mice was treated with either 1 mg or 2 mg/Kg administered by intraperitoneal (ip.) injection on three consecutive days beginning one hour after DNBS treatment (the effects of 1 and 2 mg/Kg DEX were not significantly different and so the data have been combined and are considered as a single group in the results section). All experiments adhered to the Canadian guidelines for animal welfare and complied with the specific ethical regulations of the University of Calgary.

2.2. Macroscopic Assessment of Colitis. Mice were examined daily, following treatment with DNBS, for signs of ill-health and intestinal dysfunction: weight loss, fur ruffling, decreased activity, and wet or bloody anal area or feces. Upon autopsy, at 72 hours post-DNBS, the colon was excised and inspected for evidence of macroscopic damage. The appearance and length of the colon was recorded to give a Disease Activity Score based on the following criteria: >10% loss of body weight (0 or 1); wet anus, soft stool, or empty colon (0-1); anal bleeding (0 or 1); macroscopic ulcers present in the colon (0 or 1). If an animal deteriorated to a predetermined morbidity endpoint (e.g., rectal prolapse, obvious distress), it was humanely sacrificed and given a score of 5 [7].

Subsequently, the colon was divided based on length (contraction of the colon is characteristic of colitis in this model [7]) and segments preserved for further testing. The distal 20% was snap-frozen in liquid nitrogen for assay of eosinophils peroxidase (EPO) activity, the adjacent 10% segment was fixed in 10% formalin for histological analysis and the next 10% portion of colon was snap-frozen in liquid nitrogen and stored for measurement of collagen levels (the proximal colon was discarded).

2.3. Histological Assessment of Colitis. Formalin-fixed, paraffin-embedded segments of colon were sectioned (3 μm), collected on coded slides, stained with haematoxylin and eosin and histological damage scored by an investigator using the following criteria (max score = 12): loss of architecture (0–3); inflammatory infiltrate (0–3); goblet cell depletion (0-1); ulceration (0-1); edema (0-1); muscle thickening (0–2); presence of crypt abscesses (0-1) [7]. Additional sections were stained with Mason's trichrome stain, which identifies collagen as a blue reaction product.

2.4. Eosinophil Peroxidase Activity. Activity levels of EPO were determined as previously described [16]. Briefly the presence of MPO was assessed using a kinetic assay where H_2O_2 is broken down by the MPO released from the samples of colon. This assay was repeated on duplicate sample aliquots with the addition of 50 mM 3-amino-1,2,4-triazole (AMT; Sigma Chemical Co.) to inhibit EPO. EPO activity was calculated by subtracting the MPO + AMT value from MPO values.

2.5. Collagen Deposition. The amount of collagen in extracts of colon was measured via the Sircol colometric assay (Biocolor Ltd., N. Ireland, UK) following the manufactures instructions.

2.6. Cytokine Production. Spleen cells were isolated following a previously reported protocol [7], and 5×10^6 cells (in 1 mL of RPMI medium) were activated with the T cell mitogen, concanavalin A (ConA: at 2 μg/mL). Culture media were collected 48 hours later and the levels of interleukin (IL)-10, IL-4, interferon (IFN)-γ, or tumour necrosis factor (TNF)-α were determined in duplicate serial dilutions using ELISA protocols stipulated by the manufacture (R&D Systems) [17]. ELISAs detection limits were 9 pg/mL. The bioactivity of the dexamethasone used in these experiments was assessed by its ability to suppress IL-4, IFN-γ, and TNF-α production by in vitro ConA-stimulated murine spleen cells.

2.7. Peripheral Blood Immune Cells. Blood smears were air dried and then stained using the Hema3 differential staining kit following the manufactures instructions (Fisher Scientific, Kalamazoo, MI). Mononuclear cells (T cells, B, cells, monocytes), neutrophils, and eosinophils were counted by a single investigator who was unaware of the treatment groups.

2.8. Statistical Analysis. Data are presented as the mean ± the standard error of the mean (SEM), where n is the number of mice (3–8/group) examined. Statistical comparisons were by one way ANOVA followed by pairwise comparisons using the Student's t-test or Tukey's test for nonparametric data. A statistical difference was set at $P < .05$.

3. Results

In preliminary experiments we confirmed that the dexamethasone used in the in vivo analysis was bioactive. Treatment of spleen cells with ConA for 48 hours resulted in the production of 26 ± 2 pg/mL of IL-4, 508 ± 104 pg/mL of TNFα, and 4412 ± 870 pg/mL of IFNγ ($n = 6$): inclusion of DEX (1 μg/mL) completed blocked ConA-induced production of these cytokines (levels of all 3 were undetectable in ELISA).

In two initial experiments, mice were treated with 3 mg DNBS ir; however, in these studies significant morbidity was observed with 66% (i.e., 6 of 9 (disease activity score = 4.6 ± 0.2)), 12.5% (1 of 8 (disease activity score = 2.6 ± 0.4)), and 25% (i.e., 2 of 8 (disease activity score = 3.8 ± 0.4)) of mice treated with DNBS, *H. diminuta* + DNBS and DNBS + DEX, respectively, reaching an endpoint that necessitated sacrifice prior to completion of the experiment. Despite the severity of the colitis, these data suggest that both infections with *H. diminuta* and daily DEX treatments reduce the effect of DNBS. Subsequent experiments (described below) were conducted with 2.5 mg of DNBS.

3.1. Increased Eosinophils Confirm Successful Infection with H. diminuta. Differential staining of peripheral blood revealed an increase in eosinophils (4.0 ± 0.6% ($n = 3$)) in blood retrieved from *H. diminuta* + DNBS treated mice, confirming successful infection with the helminth. An effective infection was further substantiated by analysis of EPO activity in tissue homogenates that revealed significant increases in tissues excised from *H. diminuta*- + DNBS treated mice (5.7 ± 2.1 U/mg tissue) compared to control (2.1 ± 0.9), DNBS (1.4 ± 0.7), and DNBS + DEX treated mice (0.8 ± 0.2) ($n = 3$), indicating that the mice had received a viable infection of *H. diminuta*.

3.2. H. diminuta Is More Effective than Daily DEX in Inhibiting DNBS-Induced Colitis. As expected [7] mice treated with DNBS developed colitis as gauged by loss of body weight, shortening of the colon and disease activity and histology damage scores (Figure 1). Similarly, and corroborating our earlier report, mice previously infected with *H. diminuta* were protected against the procolitic effects of intrarectal DNBS treatment [7], and, in contrast, no anticolitic effects were observed in the DEX treated group, using the dose and treatment regimen employed in these studies (Figures 1 and 2).

3.3. Infection with H. diminuta Induces IL-10 and IL-4 Production. Only spleen cells isolated from *H. diminuta* + DNBS treated mice ($n = 3$) produced substantial amounts of IL-10 (268 ± 122 pg/mL) and IL-4 (437 ± 192 pg/mL) in response to a 48-hour treatment with ConA. Spleen cells from control mice produced 0–24 pg/mL of IL-10 and IL-4; whereas neither cytokine was detected in culture medium from spleen cells isolated from DNBS- or DNBS + DEX-treated mice ($n = 3$–6). In contrast, lower amounts of IFNγ and TNFα were found in conditioned medium from ConA-stimulated spleen cells isolated from *H. diminuta* + DNBS and DNBS + DEX-treated mice compared to DNBS only treated mice (Figure 3). The latter shows that DEX was having an in vivo effect, despite the lack of inhibition of colitis using the indices of gut form employed here.

3.4. Collagen Deposition Is Not Apparent in this Acute Model of Colitis. Neither histochemical staining (Figure 2) nor biochemical analysis (Figure 4) revealed any significant increases in collagen deposition in the colon in any of the treatment groups (all examined 72 hours post-DNBS) compared to time-matched controls.

4. Discussion

Current medical management of many autoimmune and inflammatory diseases, including IBD, relies heavily on the use of steroids and broad-spectrum immunosuppressive drugs [18]. While effective in reducing disease symptoms, both classes of therapeutic are associated with a range of side effects. In the case of steroids, these include moon-face, fluid retention, insomnia and weight gain, with more serious effects of long-term use being osteoporosis and suppression of the hypothalamic-pituitary-adrenal axis. The use of biologicals (e.g., anti-TNFα antibody) is increasing as these drugs are proving very effective in treating some patients with aggressive disease that have failed other therapies [19]; however, their use comes at considerable financial cost. Thus, in the ongoing search for additional therapeutics, a number of investigators have revived an older notion that infection with helminth parasites can ameliorate concomitant disease [20]. The immunological basis of this postulate is that the immune response mobilized by the host to combat the parasitic helminth, whether it is TH2-immunity or a generalized state of immuno-regulation/suppression, is sufficient to antagonize or inhibit the immunopathological events underlying, for example, atopic disease or IBD.

Substantial proof-of-concept data have accumulated from analyses of rodent model systems to show that infection with helminth parasites either blocks the development of, or significantly reduces the severity of other diseases. With respect to intestinal inflammation, infection with nematodes (*H. polygyrus*, *T. spiralis*), trematodes (*S. mansoni*) and cestodes (*H. diminuta*) can reduce colitis evoked by chemical

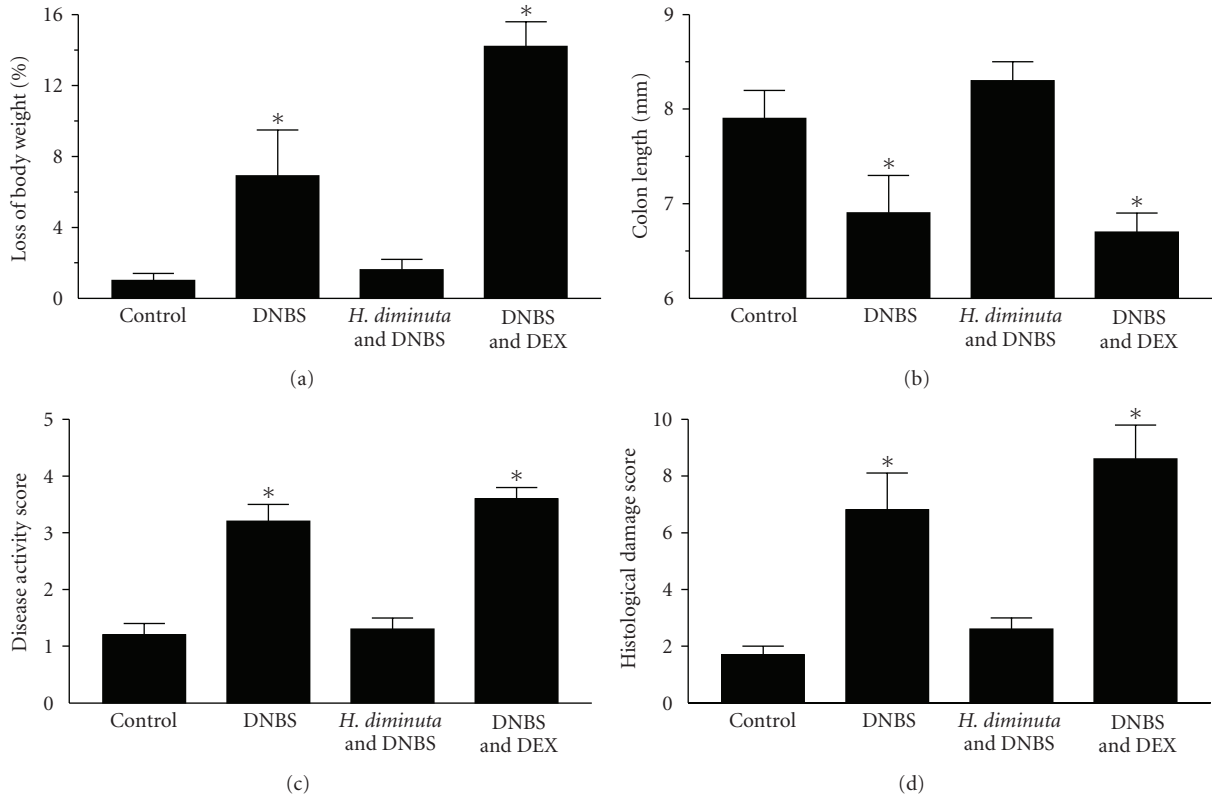

Figure 1: Bar graphs showing that infection with *H. diminuta* 8 days prior to DNBS (ir., 2.5 mg), in contrast to 3 doses of dexamethosone (DEX), reduces the severity of colitis as gauged by (a) body weight, (b) colon length, (c) disease activity scores, and (d) histological damage scores (mean ± SEM; $n = 6$–8 mice from 2 experiments; $^*P < .05$ compared to control).

haptenizing agents, or that which spontaneously develops in the IL-10 deficient mouse (reviewed in [8]). The data from these investigations have been complemented by preliminary and provocative findings showing that "helminth therapy" could be a viable option for the treatment of IBD and possibly asthma [21]. The caveat here is that the introduction of a species into a new niche can have unforeseen consequences, and this needs to be borne in mind.

From the ability of infection with helminth parasites to block inflammatory disease two questions arise: (1) will immunological knowledge gleaned from the helminth-rodent model systems translate into new treatments for human disease? And, (2) are there side effects of "helminth therapy" (and by inference the application of immunological knowledge from the helminth-rodent model systems)? In relation to the former, analyses of infection with various parasitic helminths have implicated IL-10, transforming growth factor (TGF)-β, FoxP3$^+$ regulatory T cells, and the inhibition of TH1 and TH17 events in the anti-colitic effect [4, 8, 22]. These cells and mediators have been implicated in the pathogenesis/pathophysiology of human inflammatory disease, confirming the value of the helminth-rodent models in the development of putative treatments for human disease.

The current study is, to our knowledge, the first to compare infection with parasitic helminths with another therapeutic modality in a model of colitis: dexamethasone (DEX) being selected as the comparator therapy. Consistent with our earlier findings [12], mice infected with *H. diminuta* were significantly protected from the colitic effect of ir. instillation of DNBS. In contrast, and despite the DEX being bioactive (it blocked ConA-induced cytokine production from splenocytes in vitro), the severity of colitis-induced by DNBS was unaffected by three consecutive daily doses of DEX. This treatment regime (dose, route of administration, repeated treatment) was based on studies in which DEX blocked tri-nitrobenzene sulphonic acid- (TNBS-) induced colitis [13–15, 23]. However, only a single study has shown that DEX reduces DNBS-induced colitis in mice, and that study used a tenfold higher dose of steroid than used here [24]. Moreover, while our data are surprising, they are not unprecedented. Atug et al. reported that a 7-day DEX treatment did not alter TNBS-induced histopathology [25, 26] and others have shown that DEX inhibition of TNBS-induced inflammation was not accompanied by reduced local levels of IL-1β and actually increased colonic levels of IFNγ [13]. Thus, while one can speculate on why DEX was ineffective in blocking DNBS-colitis in the present study (e.g., impact of microflora, suppression of a beneficial anti-colitic effect), and despite the reduction in the production in the synthesis of proinflammatory cytokines by spleen cells (e.g., TNFα and IFNγ), the important comparison remains that infection with *H. diminuta* was more effective in blocking DNBS-colitis than a steroid treatment regimen.

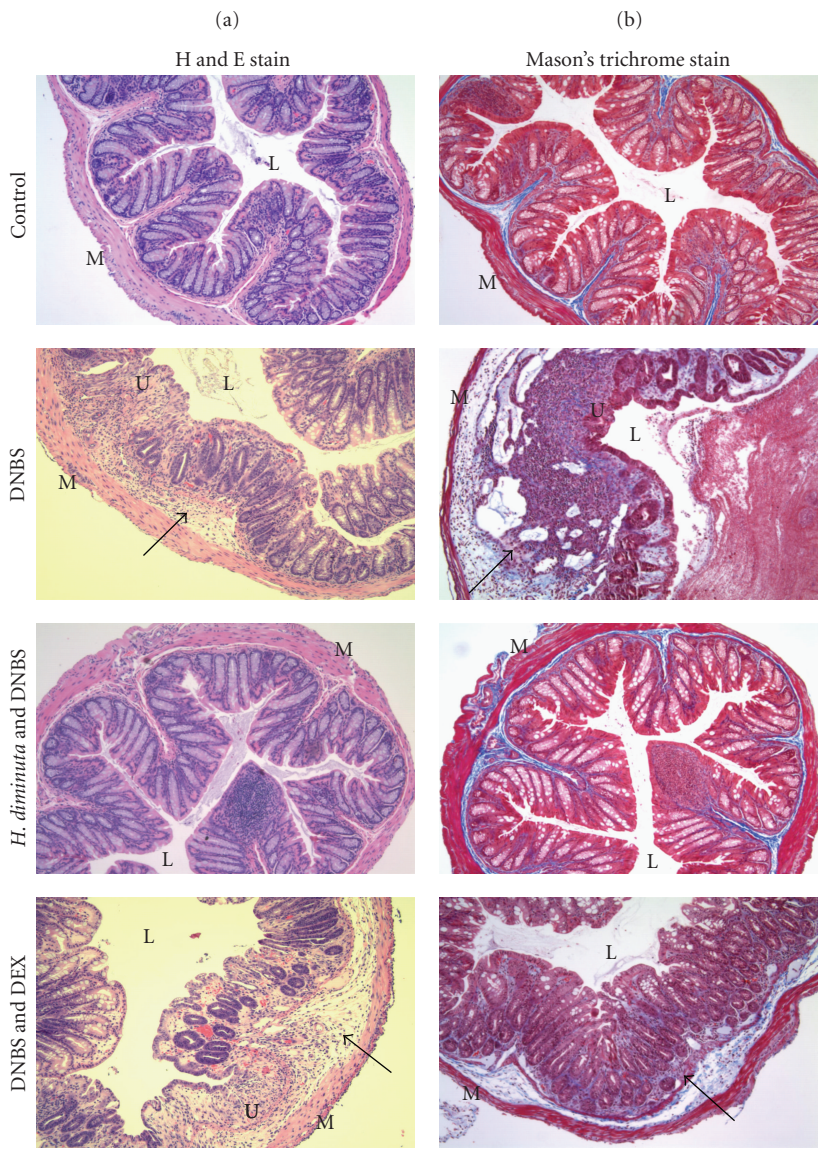

FIGURE 2: Representative images of gut morphology (hematoxylin and eosin (H&E stain); (a)) and collagen deposition ((b); collagen is stained blue) in colonic sections from the 4 treatment groups (M: muscle; L: gut lumen; U: ulcer; arrow, inflammatory infiltrate: original magnification = ×200).

We have previously expressed the concern that "helminth therapy" would be of little value if it predisposed an individual to hypersensitivity/asthmatic conditions or promoted fibrotic disease (both of which can occur in TH2 dominated conditions) [16]. In the present study, we observed no evidence, neither histochemical nor biochemical, in support of additional deposition of collagen in the colon of mice cotreated with *H. diminuta* + DNBS. Furthermore, using the same experimental conditions, we found that the anticolitic effect of infection with *H. diminuta* was not associated with increased sensitivity to bystander protein antigens [7]. Thus, at least in this experimental paradigm with *H. diminuta* in the nonpermissive mouse host and an acute model of chemically induced colitis, significant adverse effects have not been detected in association with the anticolitic effect. However, this does not preclude the possibility of long-term side effects, and studies to address this need to be conducted in this and other model systems.

In conclusion, significant momentum has been generated in the last few years in the assessment of the ability of infection with helminth parasites to block concomitant disease [8, 21]. These studies have the potential to identify one or more "therapeutic helminths" (the caveats of palatability and iatrogenic infection notwithstanding) and, perhaps more pertinently, can identify novel immunological molecules or signaling pathways that can serve as the basis of targets for new therapeutics. As this research area advances, we need to compare and contrast "helminth therapy" with other treatments and exhaustively assess potential side effects. In this context, we found that *H. diminuta* was more effective than

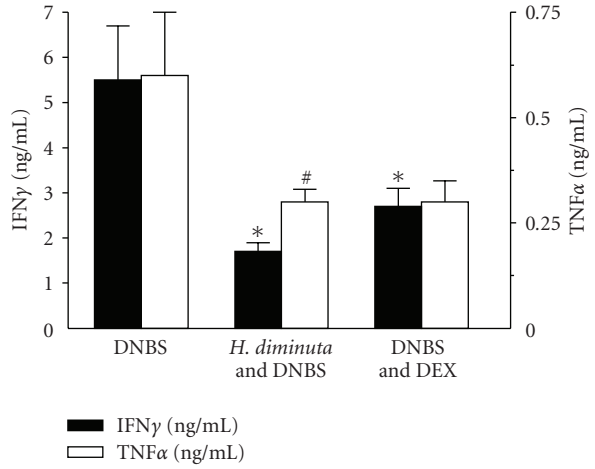

Figure 3: Bar graph showing concanavalin A (2 μg/mL)-stimulated cytokine production from spleen cells (5×10^6), excised from mice 72 hours post-DNBS treatment (mean ± SEM; n = 4-5 mice from 2 experiments; $^*P < .05$ compared to DNBS only treated mice; $\#P = .08$ compared to DNBS only; DEX: dexamethasone).

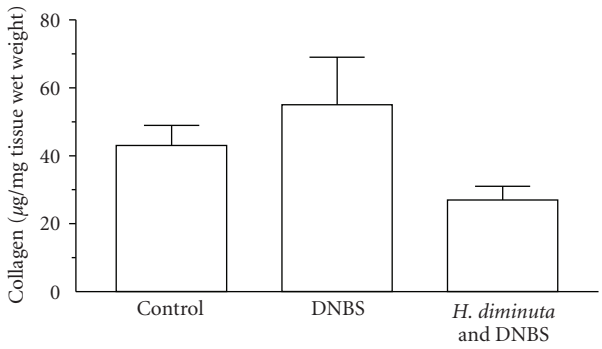

Figure 4: Bar graph showing the amount of collagen deposition in the colon of control, DNBS (ir, 2.5 mg) and *H. diminuta*-infected (5 cysticercoids, 8 days prior to DNBS) + DNBS treated mice (mean ± SEM; n = 3).

the steroid dexamethasone in preventing DNBS-induced colitis and that the benefit of infection with the helminth was not accompanied by increased deposition of collagen in the colon.

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