Exposure to schistosome eggs protects mice from TNBS-induced colitis

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Submitted 11 February 2002; accepted in final form 7 November 2002

Elliott, David E., Jie Li, Arthur Blum, Ahmed Metwali, Khurram Qadir, Joseph F. Urban, Jr., and Joel V. Weinstock. Exposure to schistosome eggs protects mice from TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol 284: G385–G391, 2003. First published November 13, 2002; 10.1152/ajpgi.00049.2002.—Crohn’s disease results from dysregulated T helper (Th)1-type mucosal inflammation. Crohn’s disease is rare in tropical countries but prevalent in developed countries with temperate climates, in which its incidence rose after 1940. In contrast, exposure to helminthic parasites is common in tropical countries but is rare in developed countries. Helminthic parasites induce immunomodulatory T cell responses in the host. We hypothesize that immunomodulatory responses due to helminths may attenuate excessive Th1-type inflammation. To test that hypothesis, mice were exposed to eggs of the helminth Schistosoma mansoni and then challenged rectally with trinitrobenzesulfonic acid (TNBS) to induce colitis. Schistosome egg exposure attenuated TNBS colitis and protected mice from lethal inflammation. Schistosome egg exposure diminished IFN-γ and enhanced IL-4 production from αCD3-stimulated spleen and mesenteric lymph node cells of TNBS-treated mice. Schistosome egg exposure decreased colonic IFN-γ but increased IL-10 mRNA expression in TNBS-treated mice. Intact signal transducer and activator of transcription 6 was required for attenuation of colitis. Exposure to helminths can decrease murine colonic inflammation.

Schistosoma mansoni

Crohn’s disease (CD), a chronic intestinal inflammation (8), has increased in prevalence over the last 60 yr. Observations on patients and animal models suggest that CD results from a dysregulated T helper (Th)1-type immune response associated with excessive IFN-γ production (11, 20). CD is most common in highly industrialized countries with temperate climates and rare in Asia, Africa, and South America. Individuals raised in the rural southern United States and Europe appear to be at lower risk for CD than those raised in the north (32, 33). This temporal and geographic pattern implies that an alteration in our environment has increased the risk for CD (9).

There is a decreasing frequency of helminthic exposure due to improved hygiene, which appears to correlate with the increasing prevalence of CD (9), raising the possibility that helminths are protective. Historically, helminthic colonization was extremely common, particularly in children living in the Southeastern region of the United States (3, 37). It is probable that, before the 1930s, nearly all children harbored these organisms.

Parasitic helminths induce strong Th2 responses in infected hosts (35). Exposure to helminthic parasites can modulate Th1 responses to unrelated concomitant parasitic, bacterial, and viral infections. For instance, patients infected with Schistosoma mansoni mount more of a Th2-like response to tetanus toxoid immunization than the usual Th1 or Th0 (29). Ethiopian immigrants with a high prevalence of helminthic colonization have eosinophilia and a propensity to respond to the mitogen phytohemagglutinin with Th2, rather than Th1 cytokines (1). Various animal models also show this effect (15, 16, 24, 25).

In schistosome infection, Th1 deviation occurs after the parasites begin to lay eggs (13). Once the parasites lay eggs, animals respond to mitogens with a Th2 cytokine profile. Exposure to schistosome eggs in the absence of infection will also induce Th2 deviation (24, 36).

The trinitrobenzasulfonic acid (TNBS) murine model of colitis is a Th1 inflammation that shares features with CD (10, 18). The colonic inflammation is characterized by infiltrating CD4+ T cells with elevated IFN-γ mRNA expression. Lamina propria T cells from TNBS-treated mice secrete 50-fold more IFN-γ and fivefold less IL-4 than T cells from controls (18). Importantly, TNBS colitis can be prevented or improved by treatment with anti-IL-12 (18), anti-TNF-α (19), or recombinant IL-10 (6).

In the present study, schistosome ova were used to determine whether they could affect the inflammation

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of murine TNBS colitis. The data suggest that exposure to eggs of the tropical helminth *S. mansoni* protects mice from developing TNBS colitis.

**MATERIALS AND METHODS**

Schistosome infection and egg treatment. Experiments used BALB/c (Charles River Laboratories, Frederick, MD) or signal transducer and activator of transcription 6 (STAT6)-deficient mice (Jackson Laboratories, Bar Harbor, ME). Schistosome eggs were aseptically collected from the livers of schistosome-infected hamsters as previously described (7). Washed *S. mansoni* ova were suspended in PBS and then frozen and stored in liquid nitrogen. Normal 6-wk-old BALB/c mice were exposed to 10,000 freeze/thawed schistosome eggs by intraperitoneal injection at day 0. Ten days later, the animals were reexposed to eggs by a second intraperitoneal injection of 10,000 thawed eggs. Four days after the second exposure (day 14), animals were challenged with TNBS. In some experiments, control animals were exposed to sterile Sephadex G-50 microfine beads (Sigma) by intraperitoneal injection of the same number, volume, and timing as used for schistosome eggs. In other experiments, mice were coadministered monoclonal rat anti-mouse IL-4 (11B11, HB-188, American Type Culture Collection [ATCC], Manassas, VA) (21) at 1 mg per mouse at the time of TNBS challenge.

**Induction of TNBS colitis.** Colitis was induced by rectal instillation of TNBS as modified from Neurath et al. (18). Briefly, control or parasite-exposed BALB/c mice were fasted for 30 h and were then anesthetized with methoxyflurane. A 1.2-mm diameter catheter was advanced 4 cm into the colon, and 0.10 or 0.12 ml of TNBS solution (5 mg/ml TNBS [Sigma] in 50% ethanol) was instilled as indicated in RESULTS. The animal was held by the tail for 3 min to ensure uniform contact with colonic mucosa. Animals were euthanized on day 3, 5, 10, or 14 after TNBS challenge. Colitis was maximal at day 3, and this time point was used to analyze the effect of helminthic exposure on intestinal inflammation in most experiments. Investigators were blinded to the animal treatment group at the time of TNBS instillation to remove any possible procedural bias.

**Evaluation of mucosal inflammation.** To grade intestinal inflammation, colons were removed at the indicated time points and then rolled, fixed, and embedded in paraffin. The sections were stained with hematoxylin and eosin. The degree of colonic inflammation was graded from 0 to 4 in a blinded fashion by two investigators. The scoring system was 0 = no inflammation, 1 = low-level inflammation, 2 = intermediate-level inflammation, 3 = high-level inflammation with wall thickening, 4 = transmural infiltration, loss of goblet cells, and wall thickening (18).

**Cell culture.** Splenic and mesenteric lymph node cells were isolated from age-matched control, egg-exposed, and TNBS-treated mice 5 days after TNBS treatment or 9 days after the last egg exposure. Cells were cultured in RPMI 1640 (GIBCO-BRL, Grand Island NY) supplemented with 10% FBS, 10 mM HEPES buffer, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma). Cells were cultured for 48 h at 1 x 10⁶ cells/well in 0.2 ml microtiter wells and stimulated with 5 μg/ml anti-CD3 (145.2C11, CRL-1975; ATCC). IL-4, IL-13, and IL-10 levels in cell culture supernatants were determined by ELISA as previously described (2). Monoclonal antibody R4-6A2 (HB-170; ATCC) was used to capture murine IFN-γ, which then bound rabbit polyclonal anti-mouse IFN-γ antiserum. Bound rabbit immunoglobulin was labeled with biotinylated goat anti-rabbit IgG (Accurate Chemical, Westbury, NY). Monoclonal antibody 11B11 (HB-188; ATCC) was used to capture murine IL-4, which then bound biotinylated monoclonal antibody BV6 (DNAX, Palo Alto, CA). Bound biotinylated antibody was measured with streptavidin-horseradish peroxidase conjugate (Zymed, San Francisco, CA) by using the APTS substrate (Zymed). IL-10 reaction measured at a wavelength of 405 nm on an ELISA plate reader.

**PCR for IFN-γ, IL-4, IL-13, IL-10, and hypoxanthine-guanine phosphoribosyltransferase.** For some experiments, colons were removed from age-matched BALB/c mice that had shamed treatment, egg treatment alone (7 days after last injection), TNBS alone (3 days after exposure), or egg treatment and TNBS exposure (7 days after last egg injection, and 3 days after TNBS treatment) for RNA isolation. The colons were removed, washed in PBS to remove stool, and then flash frozen in liquid nitrogen. Colons from three to five mice were combined for each group. Frozen colons were ground under liquid nitrogen. The frozen powder was extracted with hot acid guanidinium thiocyanate solution (5) to isolate colonic total RNA. Colonic total RNA (5 μg) was reverse transcribed with Moloney murine leukemia virus (400 units) by using an 18-mer of oligo(dT) (0.5 μg) as primer. The first-strand cDNA was diluted to 250 μl, and 10 μl was added to PCR buffer containing 2 units Taq DNA polymerase (1.5 mM MgCl₂, 50 mM KCl, and 100 mM Tris [pH 8.3]) in a total volume of 50 μl. The sense primer to amplify IFN-γ cDNA was 5'-TCTTTGATATCTGGAGGAACGTG-3' and the antisense primer was 5'-TGCGTTCGCTGGTCTGGA-3'. These primers produce a 181-bp amplicon from native IFN-γ transcripts and a 440-bp amplicon from pLoc 2.1. The IL-4, IL-13, and hypoxanthine-guanine phosphoribosyltransferase (HPRT) transcripts were as previously reported (28). The IL-4 primers produce a 240-bp amplicon from native IL-4 cDNA and a 360-bp amplicon from pLoc 2.1. The IL-10 primers produce a 324-bp amplicon from native IL-10 cDNA and a 440-bp amplicon from pLoc 2.1. The HPRT primers produce a 352-bp amplicon from native cDNA and a 450-bp amplicon from pLoc 2.1.

**RESULTS**

**Schistosome egg exposure protects mice from TNBS colitis.** TNBS colitis is characterized by ulcer formation, infiltration of the lamina propria with chronic inflammatory cells, and transmural lymphocytic inflammation. Inhibition of Th1 responses prevents or attenuates TNBS colitis. We tested mice to determine whether exposure to schistosome eggs ameliorates TNBS colitis.

Mice were exposed by intraperitoneal injection of 10,000 dead schistosome eggs. Ten days later, mice were given a second intraperitoneal injection of 10,000 eggs. Four days after the second egg injection, animals were challenged by rectal exposure with 0.10 ml of TNBS solution (5 mg/ml TNBS in 50% ethanol). Colitis was independently scored on a four-point scale by two investigators blinded to the treatment groups. Before TNBS treatment, the animals were coded so that the
investigators did not know which animals had been exposed to parasite eggs.

Exposure to schistosome eggs reproducibly ameliorated TNBS colitis in treated mice (Table 1, Fig. 1). Each experiment used at least 5 mice per group. To ensure that egg exposure didn’t merely alter the kinetic of colitis, histology was evaluated at 3, 5, 10, and 14 days after TNBS treatment. Mice exposed to schistosome eggs had minimal colitis throughout the observation period.

In addition to histological improvement, egg exposure also reduced mortality from TNBS colitis. Mice that received a higher dose (0.12 ml) of TNBS solution often developed fatal colitis within 5 days after treatment. As shown in Fig. 2, cumulative mortality from 11 experiments on BALB/c mice was 65 of 97 mice (68.0 ± 7.6%, mean ± SE) in the TNBS-treated control group that was not exposed to schistosome eggs. The mortality from the same dose of TNBS in the schistosome egg-exposed group was 28.4 ± 5.8%. The two groups differ with a P value of 0.0005. Results are from 11 experiments.

Fig. 1. Trinitrobenzensulfonic acid (TNBS) colitis in unexposed and schistosome egg-exposed mice. Mice were exposed to schistosome eggs as described in MATERIALS AND METHODS and then challenged by rectal instillation of TNBS in 50% ethanol to induce colitis. A: representative field of TNBS colitis in mice unexposed to schistosome eggs showing wall thickening, transmural inflammation, and ulceration. B: representative field of TNBS colitis in mice preexposed to schistosome eggs showing attenuation of colitis. This experiment was performed 3 times with similar results.

Fig. 2. TNBS-associated mortality in unexposed and schistosome egg-exposed mice. Mice were exposed to schistosome eggs as described in MATERIALS AND METHODS and then challenged by rectal instillation of TNBS in 50% ethanol to induce colitis. Increasing the dose of TNBS solution to 0.12 ml resulted in high mortality in control BALB/c mice (68.0 ± 7.6%, mean ± SE) not exposed to helminths. The mortality from the same dose of TNBS in the schistosome egg-exposed group was 28.4 ± 5.8%. The two groups differ with a P value of 0.0005. Results are from 11 experiments.

Table 1. Exposure to schistosome eggs reduces colitis in TNBS-treated mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Histology Score</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.1 ± 0.22</td>
<td>0.0001</td>
</tr>
<tr>
<td>2</td>
<td>3.3 ± 0.43</td>
<td>0.0006</td>
</tr>
<tr>
<td>3</td>
<td>3.5 ± 0.58</td>
<td>0.0061</td>
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</tbody>
</table>

Values are means ± SE; n = at least 5 mice per group. Inflammation was scored by histological examination on a 0-to-4 point scale (see MATERIALS AND METHODS). Scoring was done blinded to treatment groups. TNBS, trinitrobenzensulfonic acid.

Schistosome egg exposure deviates splenic and mesenteric lymph-node cytokine responses to a Th2 profile in TNBS-treated mice. Antigen-stimulated T cell IFN-γ or IL-4 release characterizes Th1 or Th2 responses, respectively. Exposure to schistosome eggs can inhibit IFN-γ secretion and promote IL-4 production in response to unrelated inflammatory stimuli. We therefore determined whether the S. mansoni eggs impeded IFN-γ and promoted IL-4 circuitry in mice with TNBS-induced colitis. As above, mice were exposed by intraperitoneal injection of schistosome eggs. Four days after the second egg injection, mice were challenged by rectal administration of TNBS. Mesenteric lymph nodes (MLNs) and splenic T cells from TNBS-treated, egg-exposed mice displayed decreased IFN-γ and augmented IL-4 release (Table 2) in response to anti-CD3 compared with mice not exposed to schistosome eggs.

Schistosome egg exposure does not protect mice treated with anti-IL-4 monoclonal antibody or lacking STAT6 transcription factor. Schistosome egg exposure augmented splenic and MLN T cell IL-4 production. We treated mice with neutralizing monoclonal anti-IL-4 (11B11) (30) at 1 mg per mouse at the time of TNBS challenge to determine whether blocking endogenous IL-4 would inhibit egg-induced protection from TNBS colitis. Mice given TNBS without egg exposure...
Table 2. Exposure to schistosome eggs inhibits IFN-γ but augments IL-4 production from MLN and splenic T cells of TNBS-treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α-CD3 stimulated</th>
<th>IFN-γ, ng/ml</th>
<th>IL-4, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MLN cells</td>
<td>Spleen cells</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>2.39 ± 0.11</td>
<td>25.5 ± 1.8a</td>
</tr>
<tr>
<td>Egg alone</td>
<td></td>
<td>2.84 ± 1.22</td>
<td>9.8 ± 1.4a</td>
</tr>
<tr>
<td>TNBS alone</td>
<td></td>
<td>4.65 ± 0.02b</td>
<td>47.1 ± 3.0d</td>
</tr>
<tr>
<td>Eggs (ip) and TNBS</td>
<td></td>
<td>2.19 ± 0.11c</td>
<td>21.8 ± 3.4d</td>
</tr>
</tbody>
</table>

Values are means of IFN-γ or IL-4 (ng/ml) ± SD of triplicate determinations measured by ELISA. Results are representative of 3 separate experiments. The lower limit of IL-4 quantification was 0.100 ng/ml in this assay. Cultures contained 5 × 10^6 cells/ml incubated for 48 h in the presence of anti-CD3 (2C11). MLN, mesenteric lymph node. P < 0.05 between lowercase letter and corresponding letter with prime.

developed severe colitis (3.4 ± 0.15, mean ± SE). Mice exposed to eggs, challenged with TNBS, and injected with PBS had attenuated colitis (1.81 ± 0.18). Mice that received monoclonal anti-IL-4 in PBS at the time of TNBS challenge developed severe colitis (3.44 ± 0.08). Results were pooled from duplicate experiments by using five mice per group.

The central cytokines of the Th2 response are IL-4 and IL-13. Receptors for IL-4 and IL-13 each signal by activating tyrosine kinases that phosphorylate a specific transcription factor, STAT6. Mice rendered deficient in STAT6 have disrupted IL-4 and IL-13 signaling. Thus we tested BALB/c STAT6-deficient mice to ascertain whether the STAT6 pathway was important for schistosome egg-induced protection in TNBS colitis. STAT6-deficient or normal BALB/c mice were exposed to schistosome eggs by intraperitoneal injection as stated in MATERIALS AND METHODS. Four days after the second egg injection, mice were challenged with 0.10 ml of TNBS solution by rectal exposure.

Figure 3 shows that normal BALB/c mice developed colitis after TNBS administration (score = 3.22 ± 0.28, mean ± SE), which was attenuated by exposure to dead schistosome eggs (1.25 ± 0.13, P < 0.001). STAT6-deficient BALB/c mice developed colitis after TNBS treatment (3.20 ± 0.19) that was similar to the colitis in wild-type mice. However, unlike wild-type BALB/c mice, schistosome egg exposure did not attenuate TNBS-induced colitis in STAT6-deficient BALB/c mice (3.35 ± 0.20, P = 0.59).

**Schistosome egg exposure alters colonic mRNA expression.** TNBS-induced colonic inflammation is characterized by infiltration of CD4+ T cells with elevated IFN-γ mRNA expression in the lamina propria (18). Experiments examined the effect of schistosome eggs on IFN-γ mRNA expression in TNBS colitis. As shown in Fig. 4, IFN-γ transcripts are not detectable by RT-PCR of RNA isolated from colons of normal mice. Schistosome egg exposure alone did not induce colonic IFN-γ mRNA expression. However, colons removed from mice 3 days after TNBS treatment did contain IFN-γ tran-
scripts detectable by RT-PCR. Schistosome egg exposure reduced the level of TNBS-induced IFN-γ mRNA expression. Quantitative PCR analysis of three experiments using pLoc 2.1 polycompetitor plasmid (28) showed that egg exposure produced a significant ($P < 0.05$) threefold reduction in TNBS-induced colonic IFN-γ mRNA expression.

Transcripts for IL-4 were variably detectable in normal colon by RT-PCR. Colonic IL-4 mRNA was consistently detected in colonic extracts from egg-exposed and TNBS-treated mice. However, we did not detect a statistically significant alteration in total colonic IL-4 mRNA level as determined by quantitative RT-PCR. Similarly, transcripts for IL-13 were consistently detected with or without egg exposure (Fig. 4).

IL-10 is a regulatory cytokine that inhibits Th1-mediated inflammation (17). As shown in Fig. 4, TNBS treatment reduced colonic IL-10 mRNA in mice below the basal level of untreated control animals. Schistosome egg exposure increased colonic IL-10 mRNA expression in TNBS-treated mice by threefold ($P < 0.05$).

**DISCUSSION**

CD is characterized by intestinal inflammation probably resulting from a dysregulated Th1 immune response to luminal contents. CD is common in developed countries with temperate climates. The incidence of CD rose dramatically in the United States during the 1940–1980s. This rise in incidence within one generation (4) suggests that an environmental change increased the risk for this disease.

In the same populations in which CD has become more common, colonization with helminthic parasites are now less prevalent (9). This inverse association might be important, because helminthic parasites promote strong Th2 and inhibit Th1 responses. Th1 and Th2 responses are counterregulatory (31). Thus helminthic parasite exposure may prevent development of excessive Th1 responses (9).

*S. mansoni* larvae (cercariae) infect mammals and mature into worms that lay eggs. The eggs induce a Th2 response and dampen Th1 reactions to other antigens (15, 24). We determined whether exposure to schistosome eggs could downmodulate the Th1 colitis in a murine model of inflammatory bowel disease.

The murine TNBS colitis model is a well-characterized model of intestinal damage that is prevented by inhibiting Th1 cytokines (18). As shown here, exposure to schistosome eggs protected BALB/c mice from TNBS colitis. Reduction in fatal colitis promotes a second measure of the protection afforded by exposure to helminths. Increasing the dose of TNBS solution caused 68% of mice to die from colonic injury. Schistosome egg exposure significantly protected mice from developing fatal colitis after TNBS administration.

In TNBS colitis, exposure to schistosome eggs deviated the cytokine response of dispersed splenic and mesenteric lymph node cells. Egg exposure impeded IFN-γ production from T cells stimulated with anti-CD3. IL-4 production was significantly enhanced. This change in cytokine response to polyclonal T-cell receptor ligation suggests that the egg-exposed animal may respond with a Th2 profile to many different antigens. Other studies have demonstrated that the usual Th1 response to a variety of antigens deviates toward Th2 with helminthic exposure (1, 15, 24, 25, 29, 35).

IL-4 can inhibit inflammatory responses. Treatment of mice with monoclonal anti-IL-4 antibody at the time of TNBS challenge abrogated the protection afforded by egg exposure. IL-4 inhibits macrophage production of TNF-α, IL-12, and IP-10 (12, 34) but induces production of IL-1 receptor antagonist (23). It is likely that egg-induced IL-4 helps limit this intestinal Th1-type inflammation via these regulatory pathways.

Two cytokines central to the Th2 response are IL-4 and IL-13. Both signal through cell surface receptors to phosphorylate STAT6. Phosphorylated STAT6 translocates to the nucleus and initiates transcription of genes to produce the Th2 phenotype. Lymphocytes from mice rendered deficient in STAT6 do not respond to IL-4 or IL-13 as measured by MHC class II display, IL-4 receptor expression, proliferation, immunoglobulin class switching, or IL-4-augmented Th2 cell development (14). Suppression of macrophage proinflammatory mediator production by IL-4 requires intact STAT6 signaling (22).

Exposure to schistosome eggs promotes Th2 responses and protects mice from colitis due to TNBS administration. We used STAT6-deficient BALB/c mice to determine further whether Th2 circuitry was critical for the protection afforded by helminth exposure. Although schistosome egg exposure attenuates TNBS colitis in normal BALB/c mice, STAT6-deficient mice were not protected. Thus STAT6-mediated responses are required for helminth-induced attenuation of colitis in this model.

Schistosome egg exposure significantly inhibited colonic IFN-γ mRNA expression induced by TNBS. Egg treatment also inhibited IFN-γ secretion by splenic and MLN T cells of TNBS-treated mice. Therefore, exposure to schistosome eggs altered immune responses in both the systemic and mucosal compartments.

Colonic IL-4 mRNA expression was variably present in normal, untreated mice. Although these transcripts were consistently detected in colon extracts from egg-exposed animals, this difference did not achieve statistical significance. Likewise, IL-13 transcripts were present in colons from TNBS-treated egg-exposed and -unexposed mice at similar levels. However, inhibiting IL-4 function by blocking antibody or STAT6 deficiency abrogated protection, showing the importance of this mediator in modulating TNBS-induced intestinal inflammation.

Colonic transcripts for IL-10 were increased in mice exposed to schistosome eggs. IL-10 is a potent anti-inflammatory cytokine that limits Th1 effector functions like IFN-γ production (17). IL-10 can inhibit inflammatory pathways not inhibited by IL-4. For example, unlike IL-4, IL-10 inhibits macrophage IL-12 release stimulated by CD40 ligation (34). IL-4 and
IL-10 synergize to inhibit delayed-type hypersensitivity footpad swelling in a recall response to Leishmania major antigen (27). Likewise, IL-4 and IL-10 can synergize to inhibit IFN-γ release by antigen-stimulated CD4+ T cells (26). IL-10 is critically important for mucosal immune homeostasis, because IL-10 knockout mice develop colitis. It thus is likely that egg-mediated mucosal protection also is governed, in part, by stimulating colonic IL-10 production.

Since the 1940s, living conditions in industrialized countries have become increasingly hygienic. We hypothesize that loss of helminths, a previously ubiquitous colonizer, may have allowed genetically predisposed individuals to react with excessive dysregulated Th1 responses. This paper demonstrates that exposure to schistosome eggs, which induce Th2 and inhibit Th1 responses, makes mice resistant to TNBS colitis. This lends support to the hypothesis that eradication of helminths from our environment may have contributed to the increasing prevalence of CD.

This study was supported by the Crohn’s and Colitis Foundation of America; National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-58755, DK-02428, DK-25295, and DK-38327; and the Iowa City Veterans Affairs.

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