Mechanisms underlying gut dysfunction in a murine model of chronic parasitic infection

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Motomura Y. Khan WI, El-Sharkawy RT, Verma-Gandhu M, Grencis RK, Collins SM. Mechanisms underlying gut dysfunction in a murine model of chronic parasitic infection. Am J Physiol Gastrointest Liver Physiol 299: G1354–G1360, 2010. First published September 23, 2010; doi:10.1152/ajpgi.00324.2010.—Irritable bowel syndrome (IBS) is common in countries where chronic parasitic infestations are endemic. However, the relationship between parasitic infection and IBS is not clear. The aim of this study was to examine whether chronic parasitic infection is accompanied by gut dysfunction and whether the continued presence of the parasite is required for the maintenance of the dysfunction. We used chronic Trichuris muris infection in Th1-biased susceptible AKR mice to evaluate this relationship. AKR mice were infected with T. muris and were euthanized on various days postinfection (pi) to examine worm burden, muscle function, and immune and inflammatory responses. Mice were treated with the anthelmintic oxantel pamoate to assess the effect of eradication of infection on muscle function. Infection resulted in persistence of the parasite, elevated IFN-γ, and increased MPO activity evident at 45 days pi. This was accompanied by a reduction in muscle contractility and excitatory innervation. Whereas parasite eradication at 7 days pi normalized IFN-γ and muscle contractility, eradication at 28 days pi failed to normalize muscle contractility. Administration of dexamethasone after parasite eradication normalized all parameters. Anthelmintic treatment improved histology except for eosinophils, which were normalized by subsequent dexamethasone therapy. Persistent gut dysfunction is independent of the continued presence of the parasite and is maintained by inflammatory process that includes eosinophils. Thus data in this preclinical model suggest that parasitic infection could be a cause of IBS, and the lack of symptomatic improvement following eradication is insufficient evidence to refute a causal relationship between the infection and IBS.

irritable bowel syndrome; motility; animal model; Th1 cytokine; inflammation

THE IRRITABLE BOWEL SYNDROME (IBS) is the most common disorder seen by gastroenterologists in Western countries, where it imparts a large socioeconomic burden. This condition is also seen in developing countries (18, 20, 21), where its prevalence is similar to that seen in Western countries (24). In addition, the spectrum of clinical presentation of IBS in those regions is similar to that seen in Western countries (16, 21, 25). Despite these similarities, IBS is underdiagnosed in these countries, presumably because of the high incidence of coexisting enteric infection and parasitic infestation (20). The term nondoysterotic amebic colitis has been applied to a condition in which chronic IBS-like symptoms, rather than dysentery, occur in patients known to harbor amebae (7, 23). However, the validity of this entity was questioned, since several studies have shown that successful eradication of the protozoan failed to improve chronic symptoms (1, 27). Previous studies have shown that chronic infection with another protozoan parasite, Blastocystis hominis, occurs more commonly in IBS patients than asymptomatic controls (12, 30), although a more recent study failed to confirm this (29). The finding that serum IgG2 antibody levels were significantly increased in IBS patients compared with asymptomatic controls suggests that IBS may occur within that subset of B. hominis-infected subjects who exhibit a strong immune response to the parasite (15). Thus the relationship of chronic parasitic infection to the development of IBS remains a controversial but important topic from a global perspective (14).

In Western countries, the relationship of infection and IBS has been restricted to the entity of postinfective IBS in which acute bacterial gastroenteritis results in the development of IBS (27). Proof of principle that transient infection leads to persistent gut dysfunction was established in an animal model of nematode infection (4–6). Acute bacterial gastroenteritis is the strongest risk factor identified to date recognized for the development of IBS in Western countries (24). Helminthic infection has been shown to be common among patients with chronic gastrointestinal symptoms in developing countries (26). In an enteric parasitic infection with Trichuris muris, resistant strains (BALB/c, NIH) expel the parasites rapidly through the generation of a Th2 response, whereas susceptible strains (AKR, B10.BR) develop a chronic infection with activation of a Th1 response (11). In this study using susceptible AKR mice, which generate strong Th1 response instead of protective Th2 response in this infection and develop chronic infection, we have investigated the relation between chronic parasitic infection and IBS. We have used contractility of colonic smooth muscle as a marker of gut function to determine whether chronic parasitic infection is accompanied by gut dysfunction and whether the continued presence of the parasite is critical to the maintenance of the dysfunction. We hypothesize that after the induction of an immunological response, the presence of the parasite itself is no longer necessary for the persistence of dysfunction. If correct, this would explain why parasite eradication fails to improve gut dysfunction and would imply that chronic enteric parasitic infection remains a plausible basis for IBS in developing countries.

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MATERIALS AND METHODS

Animals. Male AKR mice were obtained from Harlan (Indianapolis, IN), kept in sterilized, filter-topped cages, and fed autoclaved food in the animal facilities of McMaster University. Only 8- to 10-wk-old male mice were used. All the experiments were approved by the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

Experimental design. The techniques used for T. muris maintenance, infection, and worm burden assessment were described previously (11, 34). Experimental mice were infected with ~300 eggs by oral gavage on day 0 and were euthanized at days 35 and 45 to study worm recovery, number of immune cells, carbachol-induced colonic muscle contractility, tissue myeloperoxidase (MPO) activity, and Th1 cytokines. To determine the effect of anthelmintic on both early stage and chronic infection, mice were given oxaltrazepam (25 mg/kg) (24) orally at day 7 or 28 to eradicate worms and euthanized at days 35 and 45. To assess the involvement of neural component, the effect of tetrodotoxin (TTX) on carbachol- and KCl-induced colonic muscle contractility was investigated. Moreover, to determine the effect of using anti-inflammatory drugs on postinfectious muscle dysfunction, dexamethasone (0.5 mg/kg) was injected intraperitoneally into mice at days 39, 40, and 41, and mice were euthanized at day 45.

Histopathology. Tissue samples were obtained from the proximal colon and cecal tip of infected and control mice at various time points, and mice were euthanized at day 45. Tissue samples were analyzed by hematoxylin and eosin. To determine the number of immune cells, toluidine blue in 0.5 N HCl for 30 min. Sections were examined under light microscope and the number of immune cells was counted in at least five randomly selected fields. Numbers of the cells were expressed per hyper-power field.

Measurement of muscle contraction. The preparation of the colonic longitudinal muscle sections for muscle contractility experiments and the analysis of the carbachol-induced contraction have been described previously (17). Briefly, the colon was removed and placed in oxygenated (95% O2-5% CO2) Krebs solution, and 1-cm sections of whole gut were cut from the proximal colon. The lumen of each segment was flushed with Krebs buffer. Both ends of the strip were ligated with surgical silk, hung in the longitudinal axis, and attached at one end to a Grass FT03C force transducer (Quincy, MA); responses were recorded on a Grass 7D polygraph. Tissues were equilibrated for 30 min at 37°C in Krebs buffer oxygenated with 95% O2-5% CO2 before starting the experiment. The previously identified optimal tension was then applied in carbachol dose-response experiments before the addition of the first dose of carbachol (2). After the application of the tension, gut segments were exposed to different concentrations of carbachol. After the maximal response to each dose was obtained, tissues were rinsed twice and equilibrated in fresh Krebs solution for 15 min before addition of the next dose. Contracit responses to carbachol were expressed as milligrams of tension per cross-sectional area, as described previously (17). For each mouse, the mean tension was calculated from at least three segments.

MPO assay. Myeloperoxidase (MPO) is an enzyme contained in the azurophilic granules of neutrophils as well as other myeloid cells and is commonly used as an index of neutrophil infiltration and inflammation. MPO was measured by a modified version of an assay as previously described (5). After the mice were euthanized, samples of the proximal colon (50–100 mg) were removed for MPO measurement. The samples were snap frozen in liquid nitrogen and stored at 70°C. The activity of MPO is reported as units of MPO per milligram of wet tissue where a unit of MPO is defined as the quantity of enzyme able to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature.

IFN-γ ELISA. The homogenized colonic samples were analyzed by use of a mouse IFN-γ ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Results are corrected for protein concentration that was measured by DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Each experiment was performed at least three times and results were expressed as means ± SE. Data were analyzed by Student’s t-test for comparison of two means or one-way ANOVA for the comparison of more than two means, with a P value <0.05 considered to be significant.

Fig. 1. Characteristics of chronic Trichuris muris infection in AKR mice. Mice were euthanized at days 35 and 45 postinfection (pi). Cecum was removed for investigating worm burden, and proximal colon for MPO activity, IFN-γ production, and carbachol-induced colonic muscle contractility. A: AKR mice failed to expel the worms by day 45 pi. B and C: tissue MPO activity and IFN-γ level were significantly increased until day 45 pi compared with control. D: significantly lower colonic muscle contractility in infected AKR mice compared with uninfected control was observed. Solid bars show infected animals. Values are shown as means ± SE from 4–8 animals, *P < 0.005 vs. control group, **P < 0.01 vs. control group.
RESULTS

Chronic T. muris infection in AKR mice: worm counts, MPO activity, IFN-γ production, and colonic muscle contractility. Mice were euthanized at days 35 and 45 postinfection (pi), and cecum was removed and opened to count worms. AKR mice failed to expel the worms by day 45 pi (Fig. 1A). Tissue MPO activity and IFN-γ level were significantly increased until day 45 pi compared with control (Fig. 1B). Carbachol-induced colonic muscle contractility was measured at day 35 pi. Eradication of worms in early-stage infection prevented the development of colonic muscle hypcontractility as evident on day 35 (Fig. 2C).

Fig. 2. Effect of anthelmintic on early stage of T. muris infection. Infected mice were given anthelmintic at day 7 pi and were examined for MPO activity, IFN-γ production, and carbachol-induced colonic muscle contractility at day 35 pi. A and B: tissue IFN-γ level was returned to normal at day 35, whereas tissue MPO activity was not affected by anthelmintic treatment. C: eradication of worms in early-stage infection prevented colonic muscle hypocontractility as evident on day 35 pi.

Effect of anthelmintic on early stage of T. muris infection. To determine the effect of eradication of worms in early stage of infection on late gut function, we gave an anthelmintic at day 7 pi and examined worm count, MPO activity, IFN-γ production, and muscle contractility at day 35 pi. At day 35, no worms were recovered from anthelmintic-treated animals. Tissue IFN-γ level was returned to normal at day 35 pi, whereas tissue MPO activity was not affected by anthelmintic treatment (Fig. 2, A and B). Carbachol-induced colonic muscle contractility was measured at day 35 pi. Eradication of worms in early-stage infection prevented the development of colonic muscle hypocontractility as evident on day 35 (Fig. 2C).

Fig. 3. Effect of anthelmintic on chronic stage of T. muris infection. Infected mice were given anthelmintic at day 28 pi, and MPO activity was examined, IFN-γ production, and carbachol-induced colonic muscle contractility at days 35 and 45 pi. A and B: tissue IFN-γ level was returned to normal at day 35, whereas tissue MPO activity was not affected by anthelmintic treatment. C: eradication of worms in chronic stage infection did not affect late colonic muscle hypocontractility.
Effect of anthelmintic on chronic stage of T. muris infection.

To determine the effect of eradication of worms in the chronic stage of infection on late gut function, we gave anthelmintic at day 28 pi and examined worm count, MPO activity, IFN-γ production, and muscle contractility at days 35 and 45 pi. At days 35 and 45, no worms were observed in the cecum from anthelmintic-treated animals. Tissue IFN-γ level was returned to normal at day 35, whereas tissue MPO activity was not affected by anthelmintic treatment (Fig. 3, A and B). Carbachol-induced colonic muscle contractility was measured at days 35 and 45 pi. Eradication of worms in chronic-stage infection did not affect late colonic muscle hypocontractility (Fig. 3C). Anthelmintic treatment had no significant effect on muscle contractility in control uninfected mice (data not shown).

Effect of TTX on postinfectious muscle hypocontractility. To understand the underlying mechanisms of persistent colonic muscle hypocontractility following eradication of worms, we examined the effect of TTX on both carbachol- and KCl-induced colonic muscle contractility. The TTX permitted evaluation of smooth muscle contractility in the absence of neural influences. Infected and anthelmintic-treated (at day 28) mice were compared with uninfected control mice. The tissue samples were taken at day 35 and were preincubated with 10⁻⁷ M TTX (Sigma-Aldrich) for 10 min, then stimulated with 10⁻⁶ M carbachol or 5 mM KCl. TTX pretreatment significantly reduced carbachol-stimulated muscle contractility in uninfected control mice but did not affect muscle hypocontractility in infected and anthelmintic-treated animals (Fig. 4A). We have also checked the % difference in contraction between carbachol-stimulated muscle contractility and TTX-pretreated carbachol-stimulated contractility in infected and anthelmintic-treated mice and observed no difference (CCh: 44.0% reduction; CCh+TTX: 57.0% reduction). On the other hand, TTX treatment did not affect KCl-induced muscle contractility in both uninfected control mice and anthelmintic-treated animals (Fig. 4B).

Effect of corticosteroid treatment on postinfectious muscle hypocontractility. To investigate the effect of anti-inflammatory agent on persistent colonic muscle hypocontractility following eradication of worms, we examined the effect of dexamethasone on inflammation and carbachol-induced colonic muscle contractility. Infected and anthelmintic-treated (at day 28) mice were compared with uninfected control mice. Dexamethasone (0.5 mg/kg) was injected intraperitoneally into mice at days 39, 40, and 41. The tissue samples were taken at day 45 and MPO activity and muscle contractility were examined. Tissue MPO activity was returned to normal at day 45 in dexamethasone-treated mice (Fig. 5A). Dexamethasone pretreatment significantly attenuated muscle hypocontractility in infected and eradicating mice (Fig. 5B).
Role of immune cells on postinfectious muscle hypocontractility.

To determine the role of inflammatory cells on postinfectious muscle hypocontractility, we investigated immune cells in the cecum and proximal colon. Numbers of neutrophils, mast cells, and eosinophils significantly increased both in cecum and colon in infected mice compared with control. Neutrophils and mast cells returned to normal level in anthelmintic-treated mice; however, eosinophils in the cecum but not proximal colon remained significantly higher in anthelmintic-treated mice than in control mice (Fig. 6A). The numbers of eosinophils decreased to normal level after dexamethasone treatment (Fig. 6B).

DISCUSSION

The results of this study show that chronic infection of mice with the helminth parasite *T. muris* is accompanied by a low-grade inflammatory response and changes in the contractility of smooth muscle in colon. Importantly, we show that, once the infection is established, elimination of the parasite fails to restore normal muscle function or the low-grade eosinophilia in the colon. In contrast, a subsequent short course of dexamethasone normalized both the eosinophil presence and the muscle dysfunction. Thus this study provides evidence that the continued presence of the parasite is not necessary for the long-term maintenance of gut dysfunction.

The finding of a reduction in muscle contraction differs from the hypercontractile state of muscle found in previous studies involving nematode infections in the small intestine of mice. There are two factors that contribute to this observation. The first is that there are regional differences to muscarinic stimulation of muscle between the small intestine and colon. Using *Trichinella spiralis* as the infective agents, we demonstrated hypocontractility of colonic muscle (13) whereas the same infection produced hypercontractility in the small intestine of the same species (31). The second is that the Th1 response induced by *T. muris* infection in AKR mice may produce hypocontractility of colonic muscle, as has been shown in other Th1 models including hapten-induced colitis (10) as well as following exposure of muscle directly to the Th1 cytokine IFN-γ in vitro (8). In this study, *T. muris* infection was also accompanied by a reduction in TTX-sensitive contractions to carbachol, implicating the involvement of excitatory enteric nerves. Although we have not identified the neural phenotype involved, the loss of neural activity secondary to nematode-induced inflammation is in keeping with previous studies (9).

The ability of successful anthelmintic treatment early in the course of infection to normalize IFN-γ and muscle contractility indicates that the initial immune response to the parasite is critical in the induction of muscle dysfunction. However, we do not know whether this cytokine is critical for the maintenance of the dysfunction, or whether other factors such as tissue eosinophils contributed in the long term. Although marked eosinophilia usually accompanies IL-5 secretion and a strong Th2 response, a previous study has shown that tissue eosinophilia occurs in mice that generate little or no Th1 responsiveness to nematode infection (19). The ability of corticosteroid treatment to normalize both the muscle hypocontractility and the tissue eosinophilia suggests, but does not prove, that these changes may be causally linked. More recently, Marion et al. (22) have reported that transient *Cryptosporidium parvum* infection in rat jejunum resulted in the development of hypersensitivity of the jejunum in the later stage of infection that was accompanied by increased MPO, and treatment by nitazoxanide for 14 days pi normalized later stage hypersensitivity of the jejunum but not MPO. These
results are similar to our results which anthelmintic treatment in early stage of the infection prevented the development of hypocontractility of the muscle but not MPO in later stage of infection.

The basis for the low-grade colonic inflammation that persists after eradication of the parasite is unclear. We propose that this occurs as a result of changes in gut flora toward a proinflammatory profile of commensals, initiated by the parasitic infection and subsequently maintained by the altered colonic physiology. Studies in animals and humans have shown that enteral parasitic infection in mice is accompanied by a change in the composition of commensal bacteria (2, 32). Alterations in flora can also be induced experimentally, resulting in a proinflammatory profile and changes in gut physiology that resemble those seen in IBS (33); correction of the bacterial imbalance in each case normalized gut function. It remains to be determined whether probiotics have a role in the treatment of IBS in developing countries.

The clinical implications of our study are clear. We have established proof of the principle that chronic parasitic infection is accompanied by gut dysfunction, in this case altered muscle contractility, and that the continued presence of the parasite is not necessary for the maintenance of the dysfunction. Thus the demonstration that eradication of enteral parasites failed to improve intestinal muscle function clearly suggests an association between initial infection and the subsequent development of gut dysfunction. We conclude that endemic infections in developing countries may be a basis for at least a subset of the IBS patients in those regions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

