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Frequency and intensity of exposure mediate resistance to experimental infection with the hookworm, *Ancylostoma ceylanicum*

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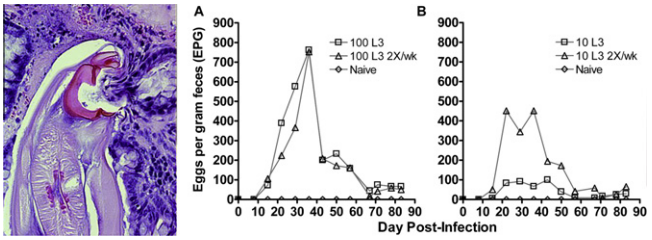
HIGHLIGHTS

- Limited exposure to larvae leads to resistance to subsequent infection with the hookworm *Ancylostoma ceylanicum*.
- Low-dose larval exposure prolongs susceptibility in experimental hookworm infection.
- Anti-hookworm antibody production is independently mediated by larval and adult antigens.
- This is the first description of mucosal IgA kinetics in experimental hookworm infection.

GRAPHICAL ABSTRACT



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ABSTRACT

Hookworms are bloodfeeding intestinal nematodes that are a major cause of anemia in resource-limited countries. Despite repeated exposure beginning in early childhood, humans retain lifelong susceptibility to infection without evidence of sterilizing immunity. In contrast, experimental infection of laboratory animals is typically characterized by varying degrees of resistance following primary infection, although the mechanisms underlying this phenomenon remain unknown. In this study, hamsters subjected to a single drug-terminated infection with 100 third stage hookworm larvae were confirmed to be resistant to pathological effects following a subsequent challenge. In a second experiment, hamsters infected twice-weekly with 10 third stage larvae (low inoculum) exhibited clinical and parasitological evidence of continued susceptibility, while those given 100 L3 (high inoculum) developed apparent resistance within 3 days following the initial exposure. The kinetics of parasite-specific IgA, IgM, and IgG antibody production varied by group, which suggests that the humoral immune response to hookworm infection is stimulated by the nature (frequency and intensity) of larval exposure. These results suggest that intermittent low-inoculum larval exposure, which is characterized by prolonged susceptibility to infection, may serve as a more representative model of human hookworm disease for studies of pathogenesis, as well as drug and vaccine development.

1. Introduction

Hookworm infection, caused by bloodfeeding intestinal nematodes, is a major cause of anemia and malnutrition in resource-limited settings, particularly among pregnant women and young children (Bungiro and Cappello, 2011; Hotez et al., 2008; Humphries et al., 2011). Three hookworm species cause patent infection in humans, *Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum* (Bethony et al., 2006; Ngui et al., 2012). Although hookworm infection is rarely lethal, its geographic overlap with other globally important infectious diseases, e.g. malaria, HIV, and tuberculosis, may be associated with additive or synergistic

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co-morbidity (Borkow et al., 2007; Bundy et al., 2000). Hookworms infect up to 740 million people worldwide (WHO, 2012), including 198 million people in sub-Saharan Africa, making it the most prevalent of the neglected tropical diseases (NTDs) in that region (Hotez and Kamath, 2009).

In general, laboratory research on hookworm pathogenesis has been hindered by the restricted host range of these parasites. Mice are not naturally permissive hosts for *Ancylostoma* or *Necator* (Bungiro et al., 2003), and therefore experimental hookworm infection is typically modeled in hamsters (Bungiro et al., 2001, 2008; Dondji et al., 2008; Garside et al., 1989; Mendez et al., 2005) or dogs (Carroll and Grove, 1986; Fujiwara et al., 2006). Immunologic reagents for these model systems are limited in number, which has hampered detailed investigation of the innate and adaptive immune responses to larval exposure and chronic infection.

In humans, prior infection with hookworm does not appear to induce sterilizing immunity, nor protection against clinical disease upon re-exposure (Anthony et al., 2007; Borkow et al., 2001; Loukas et al., 2006). Therefore, the overall intensity of chronic infection likely results from continued accrual of worms over a prolonged period (Eziefula and Brown, 2008; Hall et al., 2009). However, most experimental systems feature administration of a single inoculum of infective third stage larvae (L3), which in the case of the hamster model of *A. ceylanicum*, may be associated with severe pathology (Held et al., 2006). While effective at inducing anemia, this method is not representative of the likely pattern of human exposure and infection; people living in endemic areas are probably exposed frequently to small numbers of larvae, and accrue adult worms in their intestines gradually over months to years. Moreover, it has been shown that previously infected hamsters do not develop clinical disease following secondary challenge infection (Bungiro et al., 2001, 2008), a resistant phenotype that differs from the preserved susceptibility observed in natural human infection.

In order to develop an experimental animal model that more closely approximates the pathogenesis and immunology of human hookworm infection, we conducted studies to define the nature of the resistant phenotype. The data presented here suggest that resistance to subsequent infection can be triggered within days of a primary infection, and that host immune responses are dependent on both the intensity and frequency of exposure. By mapping the kinetics of host immune responses to adult and larval proteins, we have refined our understanding of the nature of resistance to hookworm *in vivo*, with a goal of establishing a more accurate model of experimental infection with this globally important parasitic helminth.

2. Materials and methods

2.1. Experimental design

We have previously demonstrated that subjecting hamsters to three sequential infections, each truncated with mebendazole, induces robust mucosal IgA responses to adult worm antigens and resistance to subsequent challenge infection with *A. ceylanicum* (Bungiro et al., 2008). In order to determine whether a single truncated infection was sufficient to induce this level of protection, we compared the effect of three (3x), two (2x) or a single (1X) truncated infection(s) on hamsters. A separate group of age matched uninfected animals served as naïve controls ($n = 6$). In this trial, we infected the 3X group of 21-day-old male Golden Syrian hamsters (Harlan Sprague Dawley, Inc.) by oral gavage with 100 third stage *A. ceylanicum* larvae on day 0. On day seven post-infection, all study animals ($n = 24$) were treated with a single oral dose (1 mg) of mebendazole (Sigma) in water. On day 14, a second 100 L3 infection was given to the first group of animals ($n = 6$; 3X), while a separate age matched group ($n = 6$) was given an initial infection with 100 L3 (2X). All study animals ($n = 24$), including the naïve controls, were again treated with mebendazole on day 21, and on day 35, the 3X, 2X, and a previously uninfected group (1X) of hamsters ($n = 18$) were infected with 100 L3. Animals were euthanized on day 55 post-infection and adult worm burdens were determined in all infected animals. Spleen and mesenteric lymph node weights were also measured.

A second experiment was conducted to determine the effect of inoculum size and timing of infection on the induction of resistance. Two groups of hamsters (Groups 1 and 3) received a one-time infectious dose of either 100 L3 or 10 L3, respectively. Two separate treatment groups (Groups 2 and 4) received repeated infections of either 100 L3 or 10L3 twice per week thereafter (day 0, 4, 7, 11, etc.). All animals ($n = 24$) received the initial infection with L3 on day 0 of the experiment. A separate naive control group (Group 5, $n = 6$) was never exposed to larval challenge. Blood hemoglobin (see "Measurement of Blood Hemoglobin Levels" below) and body weight were measured in all animals throughout the experiment. Individual serum samples were obtained and frozen until immunological analysis. Weekly pooled fecal samples were evaluated for egg excretion (see below), and remaining fecal extract was frozen for later immunological analysis. The experiment was terminated 83 days after the initial infection, at which time spleen and mesenteric lymph node weights, intestinal worm burdens, and fecal egg counts were determined. Total larval exposures at the endpoint were as follows: 100 L3 per animal in group 1; 2000 L3 per animal in group 2; 10 L3 per animal in group 3; and 200 L3 per animal in group 4. All animal experiments were conducted in accordance with protocols approved by the Yale University Animal Care and Use Committee.

2.2. Measurement of blood hemoglobin levels

On a weekly basis, a 50 μL blood sample was collected via retro-orbital puncture from each animal. Eight μL of each sample were used to determine the individual hamster's blood hemoglobin level by the Drabkin's method (Austin and Drabkin, 1935). The remaining blood was centrifuged to remove cellular material and the separated serum was frozen for later analysis of antibody levels by enzyme-linked immunosorbent assay (ELISA).

2.3. Fecal egg counts and preparation of fecal extract

Once per week, feces were collected from each group over a 12-h period. The pooled fecal pellets were homogenized, and approximately 5 g of each mixed sample were used to determine the fecal egg count by the McMaster technique. Homogenized samples were inspected multiple times on the McMaster slide such that the detection limit for these assays was 8.33 eggs per gram of feces. Remaining fecal material was used to produce fecal extract (FEX) as previously described (Bungiro and Cappello, 2005) and frozen until analysis.

2.4. Preparation of hookworm antigens

Soluble protein preparations were made from adult and L3 stage *A. ceylanicum* as previously described (Bungiro et al., 2008). Briefly, viable adult worms freshly harvested from the intestines of infected donor hamsters were washed to remove debris and incubated at 37 °C for 6 h in sterile PBS. The resulting excretory–secretory (ES) solution was concentrated using an Amicon Ultra 3000 MW cutoff spin column. The concentration was determined using BCA reagents, and the concentrated ES preparation was frozen (−80 °C) until its use in IgM, IgG, and IgA ELISAs.