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

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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.

Keywords

Ancylostoma ceylanicum; hookworm; hamster; soil-transmitted nematode

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 *A. ceylanicum* (Bethony, et al., 2006, Ngui, et al., 2012).

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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 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 Jr., et al., 2003), and therefore experimental hookworm infection is typically modeled in hamsters (Bungiro Jr., et al., 2001, Bungiro Jr., et al., 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, Bungiro, et al., 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 3 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 7 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 naïve 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 μ L blood sample was collected via retro-orbital puncture from each animal. Eight μ L 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 twelve-hour period. The pooled fecal pellets were homogenized, and approximately 5g 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 Jr., 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 hours 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.

Soluble larval protein preparations were made as follows: L3 stage *A. ceylanicum* were suspended in 50mM Tris HCl, pH 7.4. The solution was snap frozen in a dry ice ethanol bath and thawed 3 times, and then homogenized using a glass tissue grinder. After centrifugation to remove insoluble debris, aliquots were frozen (-80°C) until use as the capture antigen in IgM, IgG, and IgA ELISAs (Bungiro, et al., 2001, Bungiro, et al., 2008).

2.5 Measurement of antibodies to hookworm proteins

Hookworm antigen-specific antibody responses were measured in hamster serum and FEX samples against both adult and larval proteins. Serum ELISAs were used to detect IgM and IgG, while IgA responses were assessed in FEX. Due to limited quantities of serum from the study animals, IgM and IgG levels were measured using pooled sera from each of the experimental groups. In all cases, Immulon 2HB plates (NUNC) were coated with capture antigen (LEX or ES; $2\mu\text{g/mL}$ diluted in PBS), and incubated overnight at 4°C . The plates were incubated at 37°C for an additional hour, washed with PBS/0.05% tween-20 (PBST), and blocked with 1% milk in PBST. Pooled samples were added in duplicate at a 1:100 starting dilution for sera, and 1:2 starting dilution for FEX samples. Serum IgG and IgM ELISAs were incubated at 37°C for 2 hours. IgA ELISAs were incubated overnight at 4°C to maximize signal from the FEX solutions. Plates were washed and incubated for one hour at 37°C with the appropriate peroxidase-conjugated secondary antibody (anti-hamster IgM (Rockland), IgG (MP Biomedicals), or biotinylated anti-mouse IgA (KPL)) diluted 1:1000 in PBST. IgA ELISAs were washed and then incubated with streptavidin conjugated to horseradish peroxidase (SA-HRP, Pierce) prior to the final detection step. After washing, chromogenic substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS, Sigma, St Louis MO) was added, and the OD at 405nm was measured at 30 and 60 minutes using a Spectramax 190 (Molecular Devices, LLC) plate reader.

2.6 Statistical analysis

Blood hemoglobin levels, fecal egg excretion data, worm burdens, and organ weights were analyzed for statistically significant differences between groups using a one-way ANOVA with a 95% confidence interval, followed by Tukey's post-test, if necessary.

3. Results

3.1 High dose primary challenge with hookworm larvae induces resistance to subsequent infection

Animals exposed to one (2X) or two (3X) truncated hookworm infections prior to challenge exhibited a 79.1–80.2% reduction in intestinal worm burden compared to animals given only a primary challenge (Fig 1A; 3X: 5.7 ± 3.2 worms; 2X: 6.0 ± 2.5 worms; 1X: 28.7 ± 9.7 worms, $p < 0.001$ for 3X and 2X groups vs 1X). Consistent with intestinal worm burdens, hamsters in the 1X group exhibited a statistically significant increase in spleen weight compared to the naïve, 2X, and 3X groups (Fig 1B; 1X: $0.393 \pm 0.092\text{g}$; 2X: $0.235 \pm 0.047\text{g}$; 3X: $0.207 \pm 0.053\text{g}$; naïve: $0.136 \pm 0.016\text{g}$, $p < 0.05$ for naïve, 2X and 3X groups vs 1X). All three hookworm infected groups exhibited an increase in mesenteric lymph node (mLN) weights compared to naïve animals (Fig 1C; 3X: $0.186 \pm 0.016\text{g}$; 2X: $0.186 \pm 0.028\text{g}$; 1X: $0.149 \pm 0.015\text{g}$; naïve $0.034 \pm 0.010\text{g}$; $p < 0.001$ for each infected group vs naïve). In addition, among the infected animals, mLN weights were significantly increased in the 2X and 3X groups compared to the 1X group ($p < 0.05$).

3.2 Clinical phenotype varies based on magnitude and frequency of hookworm exposure

A second study was conducted in order to characterize the effect of larval inoculum and frequency of exposure on susceptibility to *A. ceylanicum* infection. As shown in Figure 2A, the pattern of fecal egg excretion was similar in animals that received a single dose of 100

L3 and those infected with 100 L3 twice a week, despite the 20 fold difference (100 L3 vs 2,000 L3) in larval exposure between the two groups. In contrast, hamsters receiving 10 L3 twice weekly showed a greater magnitude (>350 eggs per gram of feces on 3 consecutive weekly measurements) and prolonged duration of fecal egg excretion compared to animals that received only a single infectious dose of 10 L3 (Fig 2B). This result suggests ongoing accumulation of adult worms in the twice-weekly low dose challenge group.

Bloodfeeding by adult hookworms is a known cause of anemia in both experimental laboratory models and in human infection, and the level of anemia roughly correlates with overall intensity of infection. Both groups of animals given 100 L3 on day 0 experienced a precipitous drop in blood hemoglobin levels from a mean of 16.9 ± 1.19 g/dL on day 7 to 10.8 ± 1.95 g/dL on day 18 post-infection (Fig 2C). Interestingly, however, over the course of the observation period, blood hemoglobin levels in animals that received a single dose of 100 L3 were virtually identical to those that were infected twice weekly (Fig 2C, $p=0.40$). By contrast, the group infected twice-weekly with 10 L3 exhibited lower blood hemoglobin levels than both the singly infected and naïve animals, although these differences were not pronounced enough to reach statistical significance ($p=0.097$; Fig 2D).

At the time of sacrifice (day 83), none of the infected animals harbored more than 5 adult worms in the intestines (Fig 3A), with no statistically significant differences between the study groups. There were also no significant differences in spleen weight, which has been shown to increase in the setting of primary *A. ceylanicum* infection (Dondji, et al., 2008, Dondji, et al., 2010), between naïve animals and each of the infected groups (Fig 3B, $p>0.05$). However, each of the infected groups, ie 100 L3 once (0.1394 ± 0.029 g), 100 L3 twice a week (0.130 ± 0.032 g), 10 L3 once (0.128 ± 0.072 g), and 10 L3 twice a week (0.212 ± 0.030 g), showed increased MLN weights compared to uninfected control animals (0.031 ± 0.003 g; $p<0.05$ vs each of the infected groups) (Fig 3C).

3.3 Intensity, but not frequency, of larval exposure mediates host IgM and IgG antibody responses to hookworm antigens

The magnitude and kinetics of hookworm antigen-specific serum antibody responses over the course of the experiment were measured by ELISA using pooled serum samples. Antigen preparations derived from both adult worms and L3 were used as capture antigens in these assays. As shown in Figures 4A–B, the IgM response to adult hookworm ES antigens was higher in the twice-weekly infected animals than in those receiving a single infection of either 10 or 100 L3. Among the groups that received 100 L3, serum IgM responses to larval proteins (LEX) were more robust in the twice-weekly challenged animals relative to those that were singly infected (Fig 4C). Among the groups that were exposed to 10 L3, serum IgM directed at LEX was highest in the twice-weekly infected group, although overall the IgM response was only modestly elevated above the baseline signal measured in the uninfected animals (Fig 4D).

The anti-ES IgG responses were more robust in animals that received the higher challenge (100 L3); both the single and twice-weekly 100 L3 groups produced antibody levels significantly above background (Fig 5A). In contrast, repeated, but not single exposure to a 10 L3 challenge boosted anti-ES IgG production compared to the naïve controls (Fig 5B). Of note, anti-LEX IgG antibody was detectable only in the animals that received repeated, high-dose larval challenge (Fig 5C).

3.4 The frequency and intensity of larval exposure mediates the host mucosal IgA response to hookworm antigens

Both the 100 L3 and 10 L3 challenge groups exhibited elevated, antigen-specific IgA responses to adult hookworm ES (Figs 6A and 6B) relative to naïve control animals, although there were no differences between the singly and multiply challenged groups. In contrast, mucosal IgA responses to LEX were highest in those groups that received twice weekly exposure to L3 (Figs 6C–D). Furthermore, the magnitude of the IgA response correlated with the total dose of larvae administered to each group; animals receiving twice weekly 100 L3 had the most intense response, followed by those receiving a single inoculum of 100 L3 or 10 L3 twice per week for ten weeks (total of 200 L3), those receiving a single dose of 10 L3, and finally the naïve control animals.

4. Discussion

We have previously demonstrated that animals exposed to primary hookworm infection are resistant to pathology following secondary challenge, whether the initial infection consists of a single, distant exposure or repeated infections truncated with anthelmintic chemotherapy (Bungiro, et al., 2001, Bungiro, et al., 2008). However, detailed studies by Brailsford and Behnke have demonstrated that repeated low dose infection confers “incomplete resistance” to challenge, suggesting that susceptibility can, under defined conditions, be preserved in this model (Brailsford and Behnke, 1992). The data presented here complement those findings, and further suggest that acquired resistance to infection is most likely a function of the size of the inoculum used under experimental conditions.

After first demonstrating that a single dose of 100 L3 induced resistance to *A. ceylanicum* in the hamster model (Fig 1), we then defined a specific threshold inoculum below which susceptibility could be preserved. Throughout the observation period, the two high inoculum (100 L3) challenge groups (once or twice weekly) showed no difference in fecal egg excretion pattern, despite a 20-fold difference in the total exposure to larvae (Fig 2A). By contrast, fecal egg output in the animals receiving the twice-weekly low dose (10 L3) challenge was increased relative to the singly exposed comparison group (Fig 2B). The fecal egg excretion data were supported by measurements of blood hemoglobin levels, which inversely correlate with intensity of infection due to the bloodfeeding of adult worms. The group that received a single infectious dose of 100 L3 demonstrated an identical blood hemoglobin pattern to the group that received 100 L3 twice a week (a total of 2000 larvae per animal). The observed drop in blood hemoglobin levels, beginning at day 10–14 post infection and resolving at approximately day 70, is most consistent with a single, highly synchronized, primary infection (Bungiro, et al., 2001, Bungiro, et al., 2008, Dondji, et al., 2008, Held, et al., 2006), and strongly suggests that only worms from the initial 100 L3 inoculum reached the adult bloodfeeding stage. An alternate explanation of these data is that a local response decreased both the bloodfeeding capacity and fecundity of adult hookworms, a less likely interpretation given the lack of difference in worm burdens between the two groups at the end of the trial.

By contrast, the group that received 10 L3 twice a week (cumulative inoculum of 200 L3 per hamster) showed a more pronounced drop in blood hemoglobin levels than the animals that had received only a single challenge. Although these differences did not reach statistical significance, the data corroborate the fecal egg excretion data and are in stark contrast with the results from those animals in the 100 L3 groups. Taken together, the data suggest that there is a defined threshold of larval exposure below which hamsters remain susceptible to ongoing challenge infection.

These studies also provide insight into the rapidity with which resistance to secondary challenge with *A. ceylanicum* develops in the hamster model. In the first experiment, a single infection terminated with anthelmintic at 1 week was effective at provoking the resistant phenotype. Similar results were noted in the repeated exposure trial, and strongly suggest that the “window” of susceptibility closes quickly, i.e. within just a few days of initial exposure.

Despite the differences in fecal egg excretion and blood hemoglobin levels among the 4 infected experimental groups, there were no significant differences in intestinal worm burden at the time of sacrifice. Consistent with the very low worm burdens, there was also no difference in spleen weight between any of the experimental groups, including the uninfected controls. Prior studies have shown that spleen weights increase with the onset of bloodfeeding in *A. ceylanicum* infection (Dondji, et al., 2008, Dondji, et al., 2010), and decrease with the expulsion of adult worms over time. However, we did find evidence of persistent local inflammation, as the mean mesenteric lymph node (mLN) weights in the three infected groups were each markedly enlarged compared to the naïve controls (Fig 3C). We have demonstrated previously that draining lymph nodes are enlarged in hookworm-infected animals, and exhibit expanded germinal centers, an effect that is CD4+ T cell dependent (Dondji, et al., 2010). Although Mendez et al reported that mLN enlargement is abrogated following establishment of a patent infection (Mendez, et al., 2005), our data are more consistent with the observations of Garside et al, namely that the effect persists for months following primary challenge (Garside, et al., 1989).

Interestingly, the mean mLN weight in the group that received twice weekly low dose (10 L3) challenge was approximately 25% higher than the three other infected groups, which were closely grouped. Although differences in mLN weight between the three infected groups did not reach statistical significance, it supports our hypothesis, like the fecal egg excretion and blood hemoglobin data (Fig 2), that the twice weekly 10 L3 challenge group displays a clinical phenotype that is distinct from the other infected groups. Consistent with previously published reports (Dondji, et al., 2008), we propose that intestinal mediators of local inflammation are highly sensitive to the presence of small numbers of larvae, as evidenced by the persistently increased mLN weights, but that systemic inflammation, as measured by splenic enlargement, requires a threshold intensity of infection with adult worms.

We also analyzed patterns of antibody expression associated with each of the four hookworm infection regimens. While certain patterns of antibody expression correlated with the magnitude of larval inoculum (10 vs 100 L3), others were more closely associated with the interval of exposure (once vs twice weekly). We also found that IgG and IgM responses to adult hookworm-derived antigens were generally more robust than those to LEX, confirming a previous study in hookworm infected hamsters (Mendez, et al., 2005), and perhaps indicating a greater degree of immunogenicity of adult antigens relative to larval proteins.

Interestingly, we also observed that anti-ES IgM in both twice weekly-infected groups continued to rise, while animals that received a single inoculum of larvae exhibited a plateau in this antibody response. These data suggest that chronic larval exposure is capable of inducing *de novo* IgM production over a significant period of time. Although anti-ES IgM has been measured previously in the hamster model of *A. ceylanicum* as part of a vaccine trial (Ghosh, et al., 2006), and in humans with *N. americanus* infection as part of an epidemiological survey (Pritchard, et al., 1992), this is the first analysis of the kinetics of antigen specific IgM over the course of primary and/or repeated infection.

Several studies in hamsters have noted robust IgG titers to adult hookworm antigens that persist for months after primary challenge (Bungiro Jr., et al., 2001, Bungiro Jr., et al., 2008, Garside, et al., 1989), although in humans these responses are thought to decline over time and with successive worm exposures (Loukas and Prociv, 2001). Similarly vigorous ES-specific IgG production was noted here, and the response correlated with infection intensity. Furthermore, the identical level of IgG production between the groups that received 100 L3 as a single primary infection or on a twice-weekly schedule supports our hypothesis that the initial exposure to larvae rendered these animals resistant. This finding is distinct from what we observed in the 10 L3 exposure groups, in which repeated exposure to larvae resulted in a greater intensity of IgG directed at adult ES compared to the singly infected group.

This is the first description of the kinetics of mucosal IgA directed against hookworm antigens during the course of a primary experimental infection. Fecal anti-ES IgA production was independent of the magnitude and the frequency of infection, despite an overall spectrum of larval exposure that ranged from 10 to 2000 L3. This surprising observation differs markedly from the patterns of ES-specific IgM and IgG (Figs 4,5), and suggests that antibody production in the gut is sensitive to even small numbers of adult worms. In contrast, mucosal IgA production to larval antigens correlated more closely with the total inoculum of L3, as the highest levels of antibodies were seen in the group that received 100 L3 twice weekly (Fig 6). In sum, the antibody data reported here suggest distinct mediators of IgM, IgG, and IgA production in the setting of experimental hookworm infection, and point to specific properties of adult and larval antigens as stimuli of the humoral response.

In summary, we propose a model of hookworm pathogenesis in which a “threshold” inoculum of *A. ceylanicum* larvae triggers expression of the resistant phenotype in the hamster, and presumably other experimental models. Moreover, we suggest that this threshold level in the hamster model falls between 10 L3 and 100 L3, based on the data presented here. If the ultimate value of animal models is based on the degree to which they recapitulate the salient features of human disease, then it would seem appropriate to consider adopting the frequent, low inoculum protocol described here for future investigations of hookworm pathogenesis.

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Research 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.

Figure 1.

Measurements of intestinal worm burden (panel A), spleen weights (panel B), and mesenteric lymph node (mLN) weights (panel C) at the time of sacrifice (day 55). Animals in the 3X group received two anthelmintic-truncated hookworm infections prior to the final challenge, 2X animals received one anthelmintic-truncated infection prior to the final challenge, 1X animals received only the final challenge, and UN animals were uninfected controls. Each symbol represents an individual animal, and group means are indicated by horizontal bars. P values were derived by ANOVA followed by Tukey's post-test, and are designated as follows: *p<0.05, **p<0.01, ***p<0.001.

Figure 2.

Clinical phenotype of hookworm infection. Panel A: Fecal egg excretion in animals that received 100 L3 twice a week or as a single primary infection. Panel B: Fecal egg excretion in animals that received 10 L3 twice a week or as a single primary infection. Panel C: Mean blood hemoglobin levels in animals that received 100 L3 twice a week or as a single primary infection. Panel D: Mean blood hemoglobin levels in animals that received 10 L3 twice a week or as a single primary infection. Both groups that received 100 L3 had statistically significant reductions ($p < 0.05$) in their blood hemoglobin levels as compared to naïve controls.

Figure 3.

Measurements of intestinal worm burden (panel A), spleen weights (panel B), and mesenteric lymph node (mLN) weights (panel C) at the time of sacrifice (day 83). Each symbol represents an individual animal, and group means are indicated by horizontal bars. There were no significant differences in adult worm burden or spleen weights between infected groups and uninfected controls. Infected animals had significantly enlarged mLN weights relative to the naive (uninfected) control animals, as measured by ANOVA followed by Tukey's post-test. P values relative to group 5 are designated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.

IgM antibody levels to adult hookworm excretory-secretory proteins (ES) and larval extract (LEX) as measured by ELISA using pooled serum samples diluted 1:100 in PBST. Panel A: Anti-ES IgM antibody levels in hamsters that received 100 L3 twice a week or as a single primary infection. Panel B: Anti-ES IgM antibody levels in hamsters that received 10 L3 twice a week or as a single primary infection. Panel C: Anti-LEX serum IgM antibody levels in hamsters that received 100 L3 twice a week or as a single primary infection. Panel D: Anti-LEX serum IgM antibody levels in hamsters that received 10 L3 twice a week or as a single primary infection. Error bars indicate standard deviation between duplicate wells containing pooled samples.

Figure 5.

IgG antibody levels to adult hookworm excretory-secretory proteins (ES) and larval extract (LEX) as measured by ELISA using pooled serum samples diluted 1:3200 in PBST. Panel A: Anti-ES IgG antibody levels in hamsters that received 100 L3 twice a week or as a single primary infection. Panel B: Anti-ES IgG antibody levels in hamsters that received 10 L3 twice a week or as a single primary infection. Panel C: Anti-LEX serum IgG antibody levels in hamsters that received 100 L3 twice a week or as a single primary infection. Panel D: Anti-LEX serum IgG antibody levels in hamsters that received 10 L3 twice a week or as a single primary infection. Error bars indicate standard deviation between duplicate wells containing pooled samples.

Figure 6.

IgA antibody levels to adult hookworm excretory-secretory proteins (ES) and larval extract (LEX) as measured by ELISA using pooled fecal samples (FEX) diluted 1:2 in PBST. Panel A: Anti-ES IgA antibody levels in hamsters that received 100 L3 twice a week or as a single primary infection. Panel B: Anti-ES IgA antibody levels in hamsters that received 10 L3 twice a week or as a single primary infection. Panel C: Anti-LEX serum IgA antibody levels in hamsters that received 100 L3 twice a week or as a single primary infection. Panel D: Anti-LEX serum IgA antibody levels in hamsters that received 10 L3 twice a week or as a single primary infection. Error bars indicate standard deviation between duplicate wells containing pooled samples.