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Mucosal antibody responses in experimental hookworm infection

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SUMMARY

Hookworms are bloodfeeding nematodes that reside in the intestinal mucosa. These parasites secrete proteins that induce robust systemic immune responses in humans and experimental animals. By contrast, mucosal immune responses in and around the site of attachment are not described as well. This paper presents data from studies aimed at examining hookworm-specific mucosal antibody responses in a hamster model of Ancylostoma ceylanicum *infection. Intestinal flush prepared from infected hamsters was analysed by ELISA and shown to be enriched in IgA-specific for* A. ceylanicum *excretory–secretory (ES) products. Evaluation of mucosal IgA responses by immunoblot demonstrated that infected hamsters recognized a broad range of ES proteins. Hamsters repeatedly exposed to drug-terminated infections were shown to have enhanced serum IgG and mucosal IgA responses, as well as a high level of protection from challenge infection. Parasite-specific IgA was also detected in the faeces of hamsters undergoing a primary infection, and increasing faecal IgA responses were coincident with significant reductions in intestinal worm burdens and faecal ES output over time. Together these results suggest that secretory IgA may act in concert with other components of the mucosal and systemic immune response to promote protective immunity against hookworm infection and/or disease.*

Keywords Ancylostoma ceylanicum*, hamster, hookworm, IgA, mucosal immunity*

INTRODUCTION

Hundreds of millions of persons worldwide continue to harbour hookworm infections (1). These bloodfeeding soil transmitted nematodes are a major cause of anaemia and malnutrition, and as such rank among the foremost agents of global morbidity (2), especially among children and women of childbearing age (3–6). It has been suggested that the immunomodulatory influence of hookworms and other helminth infections may exacerbate the clinical course of globally significant infectious diseases such as tuberculosis and HIV-AIDS, and also reduce the efficacy of vaccines (7–10). Although effective anthelminthic agents for hookworm are available, rapid reinfection (11,12), and the possibility of emerging drug resistance (13), suggest that existing control methods may become less effective in the future. Consequently new strategies to control hookworm disease, such as vaccines (14) and novel therapeutics (15) are needed to augment currently available control options. Such efforts will be aided by a thorough understanding of host immune response phenotypes that are germane to protection and susceptibility.

The immune response to hookworms has been the subject of numerous studies in both humans and animals (as reviewed in Refs (16–18)), with considerable attention focusing on the Syrian golden hamster (*Mesocricetus auratus*) model of *Ancylostoma ceylanicum* infection. In contrast to mice, which do not permit the development of *Ancylostoma* spp. hookworms upon larval infection (19,20) and do not support extended parasite survival following adoptive transfer of adult worms (21), immunocompetent hamsters are fully permissive hosts for *A. ceylanicum* (22,23). Furthermore, when infected with a sublethal dose of *A. ceylanicum* larvae, weanling hamsters exhibit the delayed growth and anaemia which typify human disease (24–26). These findings, coupled with the inherent advantages of rodent models as compared to larger species, have made the hamster model of *A. ceylanicum* infection ideal for studies of hookworm pathogenesis and vaccine development (24,27–36). A limitation of the hamster model, however, has been the relative paucity of commercially available immunological reagents, which has hampered the detailed study of immune responses in this species (16).

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When infected with *A. ceylanicum*, hamsters produce vigorous serum IgG responses to soluble hookworm extract and excretory–secretory (ES) products (21,24,31,37). In contrast to serum antibody responses, however, relatively little is known concerning mucosal antibody responses in this model. Based on our previous observation that considerable amounts of highly immunogenic ES products are produced by bloodfeeding adult parasites residing in the intestinal mucosa (38), we hypothesized that ES-specific mucosal antibody responses would be induced by hookworm infection. We further hypothesized that such mucosal antibodies would be largely of the IgA class, which is enriched in hamster intestinal secretions (39,40). Accordingly, this paper presents data from studies aimed at characterizing hookworm-specific antibody responses in intestinal flush and soluble faecal extracts prepared from hamsters following *A. ceylanicum* infection. We demonstrate for the first time that infected hamsters produce a robust parasite-specific IgA response that is largely exclusive to the mucosa. We show that hamsters repeatedly exposed to a protection-inducing regimen of multiple drug-terminated infections have enhanced mucosal IgA responses. Parasite-specific IgA could also be detected in the faeces of hamsters, and increasing faecal IgA responses were found to be coincident with significant reductions in intestinal worm burden and faecal ES output over time. Together these results suggest that secretory IgA may act in concert with other components of the mucosal and systemic immune response to promote protective immunity against hookworm infection and/or disease.

MATERIALS AND METHODS

Parasites and hosts

The *A. ceylanicum* life cycle was maintained in 3–4 week-old male golden Syrian hamsters of the HsdHan : AURA outbred strain (Harlan, Indianapolis IN) as previously described (25). Life cycle hamsters were orally infected with 150–200 third-stage hookworm larvae (L3). Upon development of adult worms (typically 21 days post-infection [dpi]) the animals were euthanized and small intestines removed. The intestines were dissected longitudinally and repeatedly rinsed with room-temperature PBS. This treatment caused most of the worms to detach from the mucosa; with the aid of a dissecting microscope the detached parasites were gently removed from the intestinal debris with fine-tipped forceps and used to prepare ES products as described below. For the multiple truncated infection (MTI) study, hamsters ($n = 6$) were orally infected with 100 L3 on day 0 and the infection terminated on day 7 by oral administration of 1 mg mebendazole (Sigma, St Louis, MO) suspended in 0·2 mL distilled H_2O. Infections were repeated on days 14 and 28, and drug treatment on days 21 and 35. On day 42, the MTI hamsters and an age-matched control group that had received mebendazole by the same schedule without priming infections were challenged with 100 L3. All animals were sacrificed on day 56 for enumeration of adult worm burdens and preparation of intestinal flush. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals and were approved by the Yale University Animal Care and Use Committee.

Parasite antigens

Live hookworms were rinsed with PBS and used to prepare ES products by incubation in sterile PBS (10 worms per millilitres) for 6 h at 37°C. The worms were removed and the raw ES products centrifuged at 3300 ***g*** for 15 min to remove insoluble material. The raw ES was then passed through a 0·45-μm filter and concentrated *c.* 100-fold using a centrifugal concentrator with a 5-kDa molecular weight cut-off (Millipore Corp., Bedford, MA). Protein content of concentrated ES was determined using the BCA system (Pierce Chemical Co., Rockford, IL) with a bovine serum albumin standard curve. Aliquots of concentrated ES were stored at –80°C until use.

Intestinal flush

In order to prepare mucosal antibodies, small intestines were excised from hamsters and flushed with 10 mL PBS using a syringe and 18 gauge needle. Any remaining mucus was then manually extruded into the collection tube. The raw flush was gently agitated for 1 min, centrifuged for 15 min at 16 000 ***g*** (4°C) and the supernatant passed through a 0·45-μm syringe filter. For experiments involving flush prepared from hamsters infected for 20, 41, 70 and 105 days, supernatants from four to six animals were pooled and concentrated *c.* 20-fold using a centrifugal concentrator with a 10-kDa molecular weight cut-off. For the MTI study, intestinal flush preparations were prepared from individual animals by the same method with the exception that the flush was not concentrated. In all cases the protein concentration of intestinal flush preparations was determined using a BCA assay (Bio-Rad Laboratories, Hercules, CA).

Faecal extracts

Following sacrifice, faecal pellets were collected from the large intestines of individual hamsters and frozen at –20°C until use. Soluble faecal extracts were prepared by homogenizing thawed faeces in PBS/0·05% Tween-20 (PBS-T; 2 mL/g wet weight). The homogenate was centrifuged for 15 min at 3300 ***g*** to remove insoluble material and the supernatant

stored at –20°C. Immediately prior to use thawed extracts were centrifuged for 5 min at 12 000 ***g*** to remove any precipitated material.

Western immunoblots

For evaluation of total IgA and IgG in hamster intestinal flush and serum, samples of each (30 μg protein and 0·1 μL, respectively) were diluted in loading buffer (200 mM Tris–Hcl pH 6·8, 40% glycerol, 2% sodium dodecyl sulphate, 0·04% Coomassie Brilliant Blue G-250) and subjected to SDS-PAGE using a 4%–20% acrylamide tricine-buffered gradient gel (Bio-Rad Laboratories). When reduction of samples was necessary, 2-mercaptoethanol was added to the loading buffer at 2% v/v and the samples were heated at 100°C for 5 min. Proteins were blotted to nitrocellulose membranes and the membranes blocked overnight at 4°C with 5% nonfat dry milk in PBS-T. Blocked membranes were probed at room temperature (RT) for 1 h with HRP-conjugated goat antisera to mouse IgA (α heavy chain-specific), mouse IgG or hamster IgG (each raised against the whole IgG molecule) diluted 1 : 1000 (anti-mouse antibodies) or 1 : 5000 (anti-hamster) in 5% milk/PBS-T (all antisera purchased from MP Biomedicals, Inc., Irvine, CA). Blots were washed after each incubation step for 3 × 10 min with PBS-T. Bound HRP-conjugated antibodies were detected by the addition of West-Pico chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL). Chemiluminescence was detected by exposure of blots to BioMax MR autoradiography film (Eastman Kodak Co., Rochester, NY). For analysis of parasite-specific antibodies, samples of ES (2 μg/well) were subjected to SDS-PAGE under nonreducing conditions and transferred to membranes as described above. The membranes were blocked as described, then probed for 3 h at RT with hamster intestinal flush at 50 μg/mL protein or infected hamster serum at 1 : 1000, each diluted in 10% milk/PBS-T. Following wash the membranes were incubated with anti-mouse IgA or anti-hamster IgG and developed as described above.

Enzyme-linked immunosorbent assays (ELISAs)

For assays of parasite-specific antibodies, Immulon-2 microtitre plates (Dynex, Chantilly, VA) were incubated overnight at 4°C with 100 μL/well of ES diluted to 2 μg/mL in sterile PBS. The plates were washed 4× with PBS-T and blocked for 1 h at RT with 1% milk in PBS. Following 4× wash plates were incubated for 3 h at RT with intestinal flush, faecal extract or serum serially diluted in 1% milk/PBS-T to a final volume of 100 μL/well. Plates were then washed 6× and incubated for 2 h at RT with HRP-conjugated goat anti-mouse IgA, anti-mouse IgG (each at 1 : 200) or anti-hamster IgG (1 : 1000) in 1% milk/PBS-T. Following 6× wash bound HRP was visualized by the addition of 100 μL/well substrate solution (1 mg/mL ABTS [2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulphonic acid; Sigma] in 0·1 m citrate buffer, pH 5·0, 0·03% H_2O_2). Plates were incubated at RT for 30 min (serum-based assays) or 1 h (intestinal flush and faecal extract) and the A_{405} was recorded using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). When indicated, antigen-specific serum and mucosal antibody titres of individual animals were calculated by interpolating the sample dilution corresponding to an A_{405} of 0·2 following subtraction of background. All values were normalized to a positive standard which was included in each assay to control for day-to-day variation.

Faecal ES was analysed by a modification of a previously described ELISA protocol (38). Briefly, microtitre plates (Dynex) were coated with rabbit anti-ES capture IgG, blocked as above, and incubated for 3 h at RT with faecal extracts diluted 1 : 50 in PBS-T (four replicates per sample). Captured ES antigens were then detected by incubation with a 1 : 1000 dilution of biotinylated rabbit anti-ES for 1 h at RT, followed by 1 : 1000 horseradish peroxidase (HRP) conjugated streptavidin for 30 min at RT. Bound HRP was visualized by the addition of ABTS substrate solution and after 30 min at RT the A_{405} was recorded. Faecal antigen concentrations were determined using a standard curve generated by addition of purified ES to 1 : 50 diluted uninfected hamster faecal extract.

Statistical analysis of data

Numerical data is presented in the figures as means of duplicate pooled samples ± SD (Figure 2) or scatter plots of individual values with medians (Figure 4) or means (Figure 5) indicated. For the MTI study (Figure 4), two group comparisons of data were conducted using the Mann–Whitney *U*-test. For comparisons of serum IgG and faecal IgA titres between infected and uninfected hamsters at days 20, 31 and 40 (Figure 5a,b, respectively) two-way ANOVA was performed on log-transformed data ($\log_{10}(x + 1)$) with infection status and time as fixed factors. For subsequent comparison of serum IgG and faecal IgA data obtained from infected animals only (Figure 5a,b), and to analyse worm burdens (Figure 5c) and faecal ES (Figure 5d) a one-way ANOVA was performed on log-transformed data with time as a fixed factor followed by a Fisher's Protected Least Significant Difference (PLSD) post-test. In each case complete test statistics are given in the figure legends. The association of faecal ES concentration with faecal IgA titre and serum IgG titre was analysed by Spearman Rank Correlation testing of merged data sets containing values obtained from individual infected animals at 31 and 40 dpi. In all cases *P*-values of $< 0{\cdot}05$ were considered significant.

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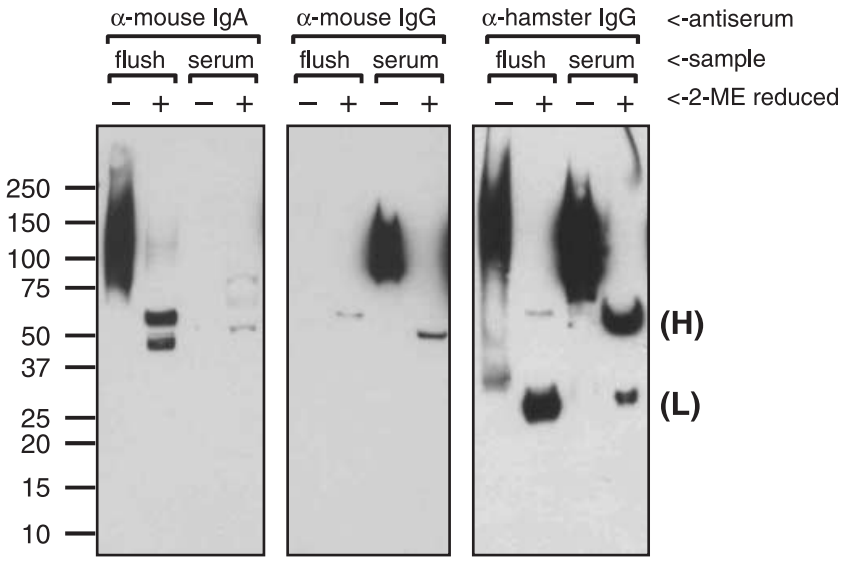

Figure 1 Reactivity of hamster intestinal flush and serum with various goat anti-Ig antisera as analysed by Western immunoblot. Samples of concentrated naive hamster intestinal flush (30 μg protein/well) and naïve serum (0·1 μL/well) were subjected to SDS-PAGE with or without 2-mercaptoethanol (2-ME) reduction, transferred to nitrocellulose membranes, and probed with enzyme labelled anti-mouse IgA (left panel), anti-mouse IgG (centre panel) or anti-hamster IgG (right panel) as described in the Materials and methods. Membranes were exposed to film for 10 min (left and centre panels) or 2 min (right panel). Molecular weight markers, in kDa, are indicated on the left. Putative immunoglobulin heavy (H) and light (L) chains in reduced samples are indicated at right.

RESULTS

Evaluation of anti-mouse reagents for the study of hamster immunoglobulins

Due to the lack of commercially available antisera to hamster IgA, we first sought to determine if reagents raised against mouse immunoglobulins could serve as acceptable substitutes. Western immunoblot analysis was performed on samples of hamster intestinal flush and serum subjected to SDS-PAGE under reducing and nonreducing conditions and probed with goat antisera to mouse IgA, mouse IgG, or hamster IgG. As shown in Figure 1, anti-mouse IgA reacted strongly with a broad high molecular weight region in nonreduced hamster intestinal flush, while under reducing conditions reactivity was largely found in bands of *c.* 55 and 45 kDa (suggestive of hamster Igα heavy chain and a breakdown product, respectively). Little reactivity with anti-mouse IgA was observed in hamster serum under reducing or nonreducing conditions. Probing hamster samples with anti-mouse IgG revealed a contrasting profile, with little signal found in the intestinal flush but strong reactivity in reduced and nonreduced serum (Figure 1). These results are consistent with previous studies that have demonstrated IgA and IgG as the predominant antibody classes in mucosal secretions and serum, respectively (39,40). Probing of the hamster samples with anti-hamster IgG revealed strong immunoreactivity in nonreduced intestinal flush and serum, although data from the reduced samples suggested that the immunoreactivity present in the intestinal flush was in large part due to common immunoglobulin light chains.

Together these findings indicate that the goat anti-mouse IgA is a suitable reagent for the analysis of hamster IgA responses. Furthermore, while the anti-mouse IgG proved to be less sensitive than the corresponding anti-hamster reagent for detection of hamster IgG (as expected), it actually appears to be more specific for this class in the hamster because of its lack of detectable reactivity against hamster Ig light chains. This made it a particularly useful reagent to analyse IgG responses in hamster samples also containing sizeable amounts of IgA (see next section).

Hookworm infection generates mucosal IgA responses against adult ES antigens

In order to characterize mucosal antibody responses to adult *A. ceylanicum* ES during primary hookworm infection, pools of concentrated intestinal flush were prepared from naïve hamsters as well as groups of animals infected for 20, 41, 70 and 105 days. The samples were evaluated for ES-specific IgA and IgG responses by ELISA using the anti-mouse antibodies for each class in order to minimize cross-detection (see Figure 1). As shown in Figure 2(a), anti-ES IgA responses above background were first detected in the 41 dpi intestinal flush. These responses were found to be considerably increased by 70 dpi, and at their highest levels on 105 dpi. ES-specific IgG responses were also detected in the intestinal flush starting with the 41 dpi samples, however, IgG responses increased only modestly in subsequent intervals (Figure 2b). ELISA analysis of ES-specific serum antibodies from 105 dpi hamsters (Figure 2c) demonstrated a strikingly different profile, with a robust IgG response observed but IgA only

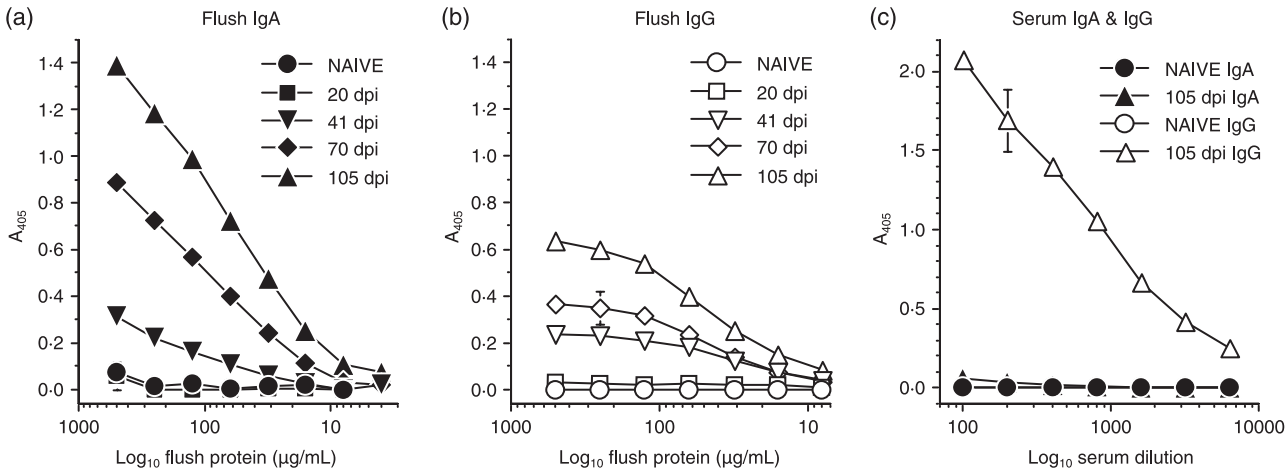

Figure 2 Parasite-specific mucosal and serum antibody responses in hamsters during primary hookworm infection as analysed by ELISA. Microtitre plates were coated with hookworm excretory–secretory (ES) products and incubated with serial dilutions of pooled intestinal flush protein prepared at various times following hookworm infection (a and b) or serially diluted pooled serum (c) followed by detection with goat anti-mouse IgA or IgG as indicated. All data points are means of duplicate pooled samples ± SD.

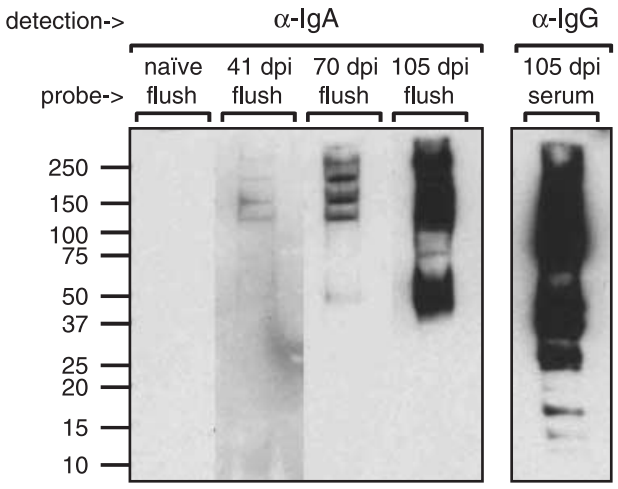

Figure 3 Parasite-specific mucosal and serum antibody responses in hamsters during primary hookworm infection as analysed by Western immunoblot. Samples of adult hookworm excretory/secretory products (ES; 2 µg/lane) were probed with intestinal flush (naïve, 41, 70 and 105 dpi) and 105 dpi serum. Bound intestinal IgA was detected with anti-mouse IgA and serum IgG with anti-hamster IgG as described in the Materials and methods. Membranes were exposed to film for 60 min. Molecular weight markers, in kDa, are indicated on the left.

slightly above background despite being highly enriched in the intestinal flush (Figure 2a). Based on this observed lack of ES-specific serum IgA, subsequent assays of serum IgG responses employed goat anti-hamster IgG in order to achieve maximum sensitivity.

Having demonstrated the presence of mucosal IgA recognizing *A. ceylanicum* ES antigens in the various intestinal flush preparations by ELISA, we next sought to determine the antigen recognition profile of the IgA in these samples by Western immunoblot. ES antigens were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with hamster intestinal flush. As shown in Figure 3, intestinal flush from 41 dpi (the first time point with detectable anti-ES IgA response by ELISA – see Figure 2a) faintly recognized several ES proteins with apparent molecular weight in excess of 100 kDa. This region was also recognized with somewhat greater intensity by IgA in the 70 dpi flush, along with a fainter band of *c.* 50 kDa. The recognition profile was further expanded in the 105 dpi flush, with strong IgA reactivity observed against ES antigens in a broad molecular weight range from *c.* 40 to > 250 kDa. Curiously, there was an apparent lack of detectable mucosal IgA-specific for antigens in the 10–40 kDa range, in marked contrast to serum IgG from this same group of animals (Figure 3). Lack of immunoreactivity in this molecular weight range was confirmed by unsuccessful attempts to detect intestinal IgA responses to *A. ceylanicum* Excretory–Secretory Protein 2 (AceES-2), which is highly immunogenic for serum IgG responses (28) and is known to be secreted by bloodfeeding adult worms residing in the mucosa (38).

Multiple truncated hookworm infection stimulates protective immunity and is associated with enhanced serum and mucosal antibody responses

In an effort to examine the effect of repeated infection on the generation of serum and mucosal immune responses, groups of hamsters were subjected to three drug-terminated infections or left uninfected, then challenged with 100

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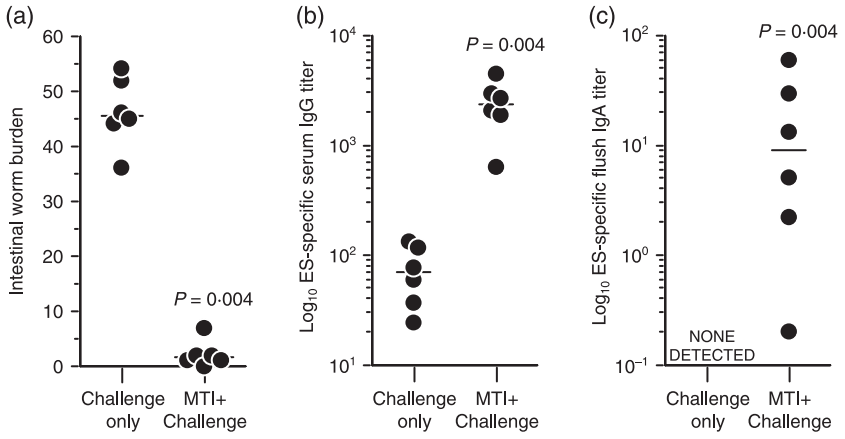

Figure 4 Post-challenge worm burdens, serum IgG and intestinal IgA responses in hamsters previously exposed to multiple truncated hookworm infection (MTI). Hamsters (n = 6 each group) were exposed to three drug-terminated hookworm infections as described in the Materials and methods or left uninfected, with each group then challenged with 100 L3 and sacrificed 14 days later. Intestinal worm burdens are indicated in (a), with each symbol representing data from an individual animal and the group median indicated by a horizontal bar. Individual ES-specific serum IgG titres as determined by ELISA at sacrifice are similarly presented in (b), and ES-specific intestinal IgA titres in (c). Significant comparisons as determined by Mann–Whitney U-test are indicated in each panel (U = 0, U' = 36, n = 12, z = −2·88, P = 0·004 for each analysis).

hookworm larvae and sacrificed after 14 days. Enumeration of intestinal worm burdens (Figure 4a) demonstrated that MTI induced a high level of protection, reducing the median post-challenge parasite burden by 97% when compared to challenge only hamsters. MTI was associated with a 35-fold higher median parasite-specific serum IgG titre at sacrifice (Figure 4b). Furthermore, while no parasite-specific IgA could be detected in the intestinal flush of any of the challenge-only hamsters, all of the MTI animals produced detectable anti-ES IgA (Figure 4c). All of the differences observed between MTI and challenge-only hamsters were found to be significant by Mann–Whitney U-test (see Figure 4).

Hookworm-specific IgA is shed in the faeces of infected hamsters

Having established that parasite-specific IgA responses are present in intestinal flush prepared from hookworm infected hamsters, we next sought to determine if IgA could be similarly detected in soluble extracts prepared from the faeces of animals undergoing a primary infection. Groups of hamsters were infected with 100 larvae or left uninfected and sacrificed at days 20, 31 or 40 for sample recovery. As shown in Figure 5(a), parasite-specific serum IgG titres were strongly induced by infection, increasing from a mean of 9076 on day 20, 22055 on day 31 and to 31022 on day 40. At all time points specific serum IgG responses were below detectable limits in the uninfected controls. Examination of faecal extracts revealed that ES-specific IgA was also induced by infection (Figure 5b). At day 20, the mean faecal IgA titre in the infected and uninfected groups was each below 1·0, however, by day 31 the mean IgA titre had increased in the infected animals to 10·0, and by day 40 it had risen to 24·9. The corresponding mean titres for uninfected hamsters were 0·7, 1·7 and 1·2, respectively. Two-way ANOVA found highly significant effects of infection status, time, and the interaction of infection status and time on both serum IgG and faecal IgA (see Figure 5 legend). Concurrent with the increases in serum IgG and faecal IgA, it was noted that mean parasite burden in infected hamsters exhibited a statistically significant 50% decline from days 20–40 (Figure 5c). A highly significant 79% reduction of mean faecal ES occurred over the same interval in these animals (Figure 5d). Furthermore, Spearman correlation analysis of faecal ES levels with faecal IgA titres from individual animals at days 31 and 40 found a significant negative association between these variables (P = 0·01, R_s = −0·544, 95% CI: –0·801 to –0·120); the association of faecal ES and serum IgG at these time points was not significant (P = 0·47, R_s = −0·171, 95% CI: –0·580 to 0·306).

DISCUSSION

Secretory IgA is a critical component of the mucosal immune system and as such plays an important role as a first defence against a variety of pathogens (41–43). However, despite the fact that adult hookworms reside in the intestinal mucosa and produce considerable quantities of highly immunogenic proteins (38), relatively little attention has been devoted to characterization of secretory IgA responses to hookworm infection in humans or animal models. Although the relevance of hookworm-specific secretory IgA to protection has not been established, it is conceivable that it could act in concert with other components of the mucosal response to hookworm infection such as mastocytosis (35,44,45), goblet cell hyperplasia (35), eosinophilia (35) as well as enteropathological

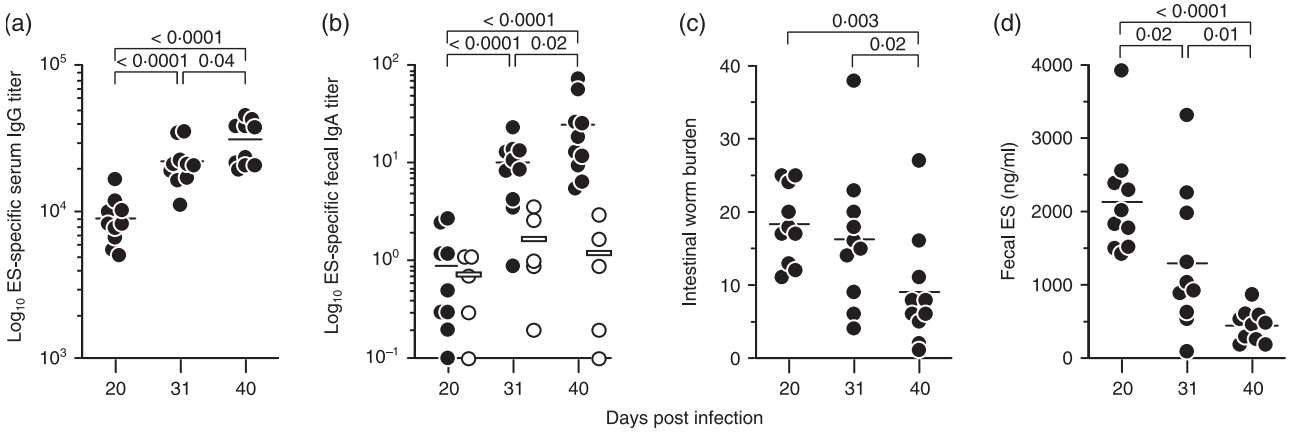

Figure 5 Post-challenge serum IgG, faecal IgA, worm burdens, and faecal ES in hamsters following primary hookworm infection. Hamsters ($n = 30$) were infected with 100 L3 and 10 animals were sacrificed at each time point. Individual ES-specific serum IgG titres as determined by ELISA are presented in (a) and faecal IgA titres in (b), with each closed symbol representing data from an individual infected animal and the group mean indicated by a solid bar. Open circles in (b) indicate values from age-matched uninfected controls ($n = 5$ at each time point), and an open bar indicates the mean of this group (IgG levels in uninfected controls were below detection threshold in every case). Intestinal worm burdens recovered from infected hamsters at each time point are similarly indicated in (c) and faecal ES output, in ng/mL of soluble faecal extract, in (d). (a) Two-way ANOVA was conducted with infection status and time as fixed factors on log-transformed ES-specific serum IgG titres, for main effect of infection status: $F_{1,39} = 11333{\cdot}2$, $P < 0{\cdot}0001$; for main effect of time: $F_{2,39} = 16{\cdot}1$, $P < 0{\cdot}0001$; for the two–way interaction of infection status and time: $F_{2,39} = 16{\cdot}1$, $P < 0{\cdot}0001$. One way-ANOVA was conducted for values from infected animals with time as fixed factor: $F_{2,27} = 33{\cdot}4$, $P < 0{\cdot}0001$; statistically significant pairwise comparisons obtained by Fisher's PLSD post-test indicated in the figure by P-values above the brackets. (b) Two-way ANOVA was conducted with infection status and time as fixed factors on log-transformed ES-specific faecal IgA titres, for main effect of infection status: $F_{1,39} = 37{\cdot}4$, $P < 0{\cdot}0001$; for main effect of time: $F_{2,39} = 15{\cdot}5$, $P < 0{\cdot}0001$; for the two-way interaction of infection status and time: $F_{2,39} = 10{\cdot}4$, $P = 0{\cdot}0002$. One-way ANOVA was conducted for values from infected animals with time as fixed factor: $F_{2,27} = 31{\cdot}7$, $P < 0{\cdot}0001$; statistically significant pairwise comparisons obtained by Fisher's PLSD post-test indicated by P-values above the brackets. One-way ANOVA for ES-specific faecal IgA in uninfected animals with time as fixed factor: $F_{2,12} = 0{\cdot}9$, $P = 0{\cdot}45$. (c) One way-ANOVA was conducted for log-transformed worm burdens from infected animals with time as fixed factor: $F_{2,27} = 5{\cdot}9$, $P = 0{\cdot}008$; statistically significant pairwise comparisons obtained by Fisher's PLSD post-test indicated by P-values above the brackets. (d) One way-ANOVA was conducted for log-transformed faecal ES output from infected animals with time as fixed factor: $F_{2,27} = 14{\cdot}2$, $P < 0{\cdot}0001$; statistically significant pairwise comparisons obtained by Fisher's PLSD post-test indicated by P-values above the brackets.

changes to the mucosal architecture (36) to create an unfavourable environment for the parasites. IgA responses in the mucosa might be hypothesized to target the parasite by mechanisms such as neutralization of secretory molecules (41–43) and/or induction of eosinophil degranulation (46).

Specific antibodies to adult worm somatic extract have previously been detected in gut washings of DSN hamsters exhibiting acquired resistance upon secondary *A. ceylanicum* infection (44). Although the isotype of the gut-associated antibodies was not determined in that study, given the results presented here and the known abundance of IgA in hamster mucosal secretions (39,40), it is likely that IgA was a major constituent of the response. In studies of the canine model of *A. ceylanicum* employing a drug-terminated infection (47) or small primary larval inoculum (48) to induce partial resistance to challenge infection, Carroll and Grove found that post-challenge parasite-specific serum IgA levels were elevated in resistant dogs (secretory IgA was not evaluated). Hookworm-specific serum IgA has also been detected in *Necator americanus*-infected humans (49,50) and serum IgA responses to adult ES (unlike IgG, IgM or IgE) were found to increase in a 2-year period following chemotherapy (50). By contrast, hookworm patients were found to have significantly lower total secretory IgA (but significantly higher IgG and IgM) in intestinal secretions when compared to uninfected controls (51,52). Notably, intestinal IgA levels increased significantly in the hookworm patients following anthelminthic treatment, concurrent with reductions in total IgG and IgM (52). The negative association between intestinal IgA and hookworm infection observed in humans is intriguing given the results presented here for the hamster model, and may be explained by various factors such as host species differences in the capacity to mount secretory IgA responses, variable induction of secretory IgA responses by different hookworm species, relative parasite burdens, length of infection or differences in research methodology. An additional possibility lies in the fact that at least one species of hookworm (*N. americanus*) has been shown to secrete a protease that preferentially degrades human IgA (53). It will

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be of interest to determine if such an activity is present in *A. ceylanicum*, and if so, its relative activity against hamster and human IgA.

The abundance of IgA in the hamster intestine is probably a consequence of local production by plasma cells of the lamina propria of the intestinal mucosa (54) as well as an active hepatobiliary system that transports polymeric IgA from the circulatory system to the bile (55). In contrast to its predominance in secretions, hamster serum contains relatively little IgA relative to IgG (39,40), although serum IgA concentration increases somewhat with age (39). Our results are in general agreement with these findings, demonstrating an enrichment of total IgA in uninfected hamster intestinal flush relative to the serum (Figure 1). Consistent with results for total IgA, evaluation of parasite-specific antibodies in the intestinal flush of hookworm infected animals by ELISA found principally IgA responses that increased over time (Figure 2a). This was in stark contrast to the serum, which was dominated by specific IgG (Figure 2c). Of note, moderate amounts of specific IgG were also detected in intestinal flush from the infected animals (Figure 2b), a rather unexpected finding based on the low amount of total IgG observed in uninfected flush by immunoblot (Figure 1). Further study will be necessary to determine if the IgG found in the intestinal flush is secreted locally (56,57) or is largely serum derived, resulting from gut haemorrhage due to bloodfeeding by the hookworms. Nevertheless, haemorrhage is unlikely to explain the robust IgA responses observed in the intestinal flush, since when these were at maximum (day 105; Figure 2a) very little serum IgA was detected (Figure 2c).

We report here that hamsters infected with *A. ceylanicum* developed intestinal IgA responses against multiple ES antigens over a broad molecular weight range, although when compared to serum IgG a lack of immunoreactivity for antigens < 40 kDa was noted (Figure 3). This is of considerable interest, as many of the adult ES proteins cloned from this species and hypothesized to function in the molecular pathogenesis of hookworm disease (58) are of low molecular weight. These include the anti-coagulant AceAP1 (59), the Kunitz-type serine protease inhibitor AceKI (60), the macrophage migration inhibitory factor AceMIF (61) and *A. ceylanicum* excretory/secretory (AceES) proteins 1 (62) and 2 (28). The example of AceES-2 is particularly notable, as this molecule has been shown to induce robust serum IgG responses in hamsters (28). The inability to detect intestinal IgA-specific to AceES-2 and other low molecular weight ES antigens suggests that the parasite may have evolved a strategy to subvert (or at least delay) the induction of secretory IgA responses to potentially vulnerable proteins. It also suggests that protocols specifically aimed at generating mucosal immune responses, such as oral vaccination (63–65), may be employed to induce or enhance protection in this model. This approach has shown promise using soluble adult worm extracts and recombinant AceES-2 (28).

Hamsters exposed to one or more priming *A. ceylanicum* infections have been previously shown to develop resistance to a subsequent challenge infection (24,44,45,66) and Behnke *et al*. demonstrated that immunity in primed hamsters acted upon L3 and early L4 stages, causing most worms to be cleared within the first week following challenge infection (45). In each of these studies the priming infections were allowed to progress to patency (at least 15 days). Here we show that a regimen of multiple infections truncated by chemotherapy at 7 days (i.e. prior to the onset of bloodfeeding and egg production) also induces a high degree of resistance to challenge infection (Figure 4a), which is associated with enhanced post-challenge serum IgG responses (Figure 4b) and the induction of mucosal IgA responses (Figure 4c) against adult ES products. Similar MTI protocols have been employed to induce protective immunity to other strongylids such as *Nippostrongylus brasiliensis* (67), *Haemonchus contortus* (68,69) and *Trichostrongylus colubriformis* (70,71). Mechanisms of strongylid rapid rejection have been suggested to include mediation by allergic hypersensitivity (72) and antibody responses including IgA (73). Further work will be required to determine the timing and mechanism of worm attrition in this model of multiple truncated hookworm infection, however, it is likely to occur with similar kinetics as previously described by Behnke *et al*. (see above), and based on the data presented here it is conceivable that mucosal IgA responses could play a role in its expression.

In addition to the evaluation of intestinal IgA responses, we report here that hookworm-specific IgA may also be detected in the stool of hamsters undergoing a primary infection (Figure 5b). Like serum IgG (Figure 5a), faecal IgA responses were found to increase in titre over the observation period, and this was concurrent with significant reductions in both worm burden (Figure 5c) and faecal ES output (Figure 5d). Furthermore, faecal IgA titres and faecal ES exhibited a significant negative association that was not observed for serum IgG. This provides support to the hypothesis that intestinal IgA may bind to and neutralize hookworm ES products, thereby attenuating the pathological interaction of these molecules with the host as well as interfering with parasite feeding and survival.

In conclusion, we have characterized for the first time parasite-specific mucosal IgA responses in a hamster model of *A. ceylanicum* hookworm infection. Mucosal IgA responses were observed in primary infection as well as in animals repeatedly exposed to a protection-inducing regimen of MTI. Specific IgA could also be detected in the faeces of infected hamsters, and faecal IgA responses were found to be correlated with reduction in faecal ES output. The study of mucosal IgA responses is expected to provide new insights

into the nature of protective immunity against hookworm infection and disease, and may yield new approaches for vaccination against this globally significant parasite.

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