

Development of a Model of Hookworm Infection Exhibiting Salient Characteristics of Human Infection

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Abstract. Patent and pathologic infections of the human hookworm *Necator americanus* were established in the common marmoset (*Callithrix jacchus*). In a pilot study, a laboratory strain of *N. americanus* was compared with a fresh field isolate. Pathology was more severe in animals infected with a fresh isolate. In all animals, infection was associated with increased total plasma IgE and production of IgG specific to adult worm excretory/secretory (ES) products. Histamine was released by basophils in response to IgE, ES products, and a recombinant hookworm allergen, calreticulin. The pilot study indicated the potential of this animal model of hookworm infection and led us to investigate the consequences of infecting a further cohort with the fresh field isolate. This second study confirmed our initial findings, that it is possible to investigate the human hookworm *N. americanus* in a model exhibiting many of the characteristics of the immunology of hookworm infection in its definitive host.

INTRODUCTION

Approximately 740 million people worldwide are estimated to harbor hematophagous hookworm infections.¹ Thus, hookworms are a leading cause of anemia and malnutrition, particularly in children and women of child-bearing age in developing countries.^{2–4}

Consequently, two major initiatives were recently announced, with the intention of increasing our knowledge of the molecular genetics of hookworms (The Wellcome Trust Beowulf Initiative) and to develop rationally designed vaccines for hookworm infection (The Hookworm Vaccine Initiative, Sabin Vaccine Institute, and George Washington University).

Furthering these initiatives will be partially dependent on the use of models of hookworm infection and disease. However, many of the models used historically to investigate the immunobiology of hookworm infection have been biologically deficient. For example murine models, in which few parasites reach the gut and thus cannot exhibit either patency or gut pathology, may have provided valuable information on the protective inflammatory responses to hookworm challenge but cannot support the full life cycle.^{5–9} Canine models infected with the dog hookworm *Ancylostoma caninum* could also be of value in proof of principle studies with trial vaccines but these studies use infection with an inappropriate species. Therefore, it would be scientifically valuable to establish a model of human hookworm infection that maintained the full life cycle and exhibited many of the pathologic and immunologic features of human hookworm disease. In a pilot study, patent and pathologic infections were established in marmosets using a laboratory maintained isolate (passaged at Nottingham since 1983) or a fresh field isolate (PNG) collected in October 2001 from Papua New Guinea (Haven, Madang Province). This was followed by a more extensive study of the PNG isolate in a larger group of animals.

From these studies, it would appear that patent infections can be established in adult marmosets by using laboratory and fresh field isolates. In addition, our study may have detected key physiologic and biochemical differences between the two parasite isolates, with the fresh field isolate inducing a profile of pathology more comparable to infection in humans, which suggested passage-induced attenuation of the laboratory strain.

MATERIALS AND METHODS

Maintenance of the *Necator americanus* laboratory isolate and preparation of parasite antigens. The laboratory strain of *N. americanus* used in this study was maintained in syngeneic DSN hamsters (*Mesocricetus aureus*) at the University of Nottingham using a method described previously.¹⁰ This hamster-adapted strain of human hookworm was originally obtained from G. Rajasekariah (CIBA-GEIGY Ltd., Bombay, India) and has been passaged in hamsters since 1983.^{11–13} This represents approximately 460 individual passages through DSN hamsters over a 20-year period.

To maintain the laboratory isolate, 2–4-day-old hamsters were infected percutaneously with 100 infective third-stage larvae and the infection was allowed to proceed until adult worms in the small intestine became fecund, approximately 42 days post-infection. Fecal material containing *N. americanus* eggs was cultured by a method described previously.¹⁴

Acquisition of a fresh *N. americanus* isolate from Papua New Guinea. A fresh field isolate of *N. americanus* was obtained from Papua New Guinea in October 2001. Fecal material obtained from a hookworm-infected person living in Haven village on the Bogia Coast Road of Madang Province was cultured as previously described.¹⁵ Freshly cultured larvae were transported back to the United Kingdom and used to infect neonate hamsters in Nottingham and three marmosets at the Defence Science and Technology Laboratory in Porton Down 15 days after arrival in the United Kingdom and 22 days after collection in Papua New Guinea.

Collection of *N. americanus* ES products. Excretory/secretory (ES) products from adult worms were collected as described previously.¹⁶ *Necator americanus*-infected ham-

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sters were killed 35 days post-infection and the small intestines were removed, cut along their length, and placed in Petri dishes containing Hanks' balanced salt solution. The Petri dishes were incubated at 37°C to enable adult worms to detach voluntarily from the intestines, thus minimizing the possibility of host tissue contaminating subsequent ES cultures. Once detached, adult worms were washed over a period of two hours in RPMI 1640 medium containing 100 IU/mL of penicillin and 100 µg/mL of streptomycin and cultured in RPMI 1640 medium for 24 hours. The ES products obtained after 24 hours were stored at -20°C until required.

Proteolytic activity and inhibition of factor Xa by ES products. The proteolytic activity of normal and heat-inactivated (100°C for 30 minutes) ES products was determined using fluorescein isothiocyanate-labeled casein (FITC-casein).¹⁷ The ES products (12 µg in 20 µL) were mixed with FITC-casein (10 µL of a 0.5 mg/mL stock) and 170 µL of 50 mM phosphate buffer, pH 6.5, containing 5 mM cysteine, and incubated at 37°C for two hours. To stop the reaction and precipitate undigested protein, trichloroacetic acid (120 µL of 5% [w/v]) was added and allowed to stand at room temperature for one hour. Precipitated protein was removed by centrifugation at 13,000 × g for 10 minutes. Aliquots of the supernatant (20 µL) were added to 80 µL of 0.5 M Tris, pH 8.5 and the fluorescence was measured (excitation at 490 nm and emission detection at 525 nm) by using an MFX microplate fluorimeter (Dynes Technologies, Chantilly, VA). Under these conditions, untreated ES products released 2,439 ± 66 fluorescence units from FITC-labeled casein over two hours. No activity was detected in heat-inactivated ES products.

Inhibition of human factor Xa activity was measured by monitoring the release of 7-amino-4-methylcoumarin (AMC) by Factor Xa (Calbiochem) from the fluorogenic substrate Boc-Ile-Glu-Gly-Arg-AMC.¹⁸ Ten micrograms of ES products were pre-incubated for 15 minutes with 0.03 units of factor Xa in 0.05 M Tris, 0.05 M NaCl, pH 7.4, prior to the addition of substrate to a final concentration of 5 µM. The release of AMC was measured over a 15-minute period (excitation at 365 nm and emission detection at 465 nm) using a microplate fluorimeter (Dynes Technologies). Uninhibited factor Xa released 1,536 fluorescence units over 15 minutes. The ES products from the fresh field isolate reduced this to 446 units, a reduction of 71%. The ES products from the laboratory strain failed to inhibit human factor Xa.

Expression and purification of recombinant calreticulin. Recombinant calreticulin was expressed as described previously¹⁹ and purified using a combination of Bugbuster protein extraction reagent (Novagen, Madison, WI) and a His Bind Purification kit (Novagen). Harvested cells were resuspended in Bugbuster reagent (5 mL per g of cell pellet) containing 25 units benzonase/mL of Bugbuster reagent and incubated at room temperature for 20 minutes. Insoluble cell debris was removed by centrifugation at 16,000 × g for 20 minutes at 4°C, and the supernatant was loaded directly onto a 5-mL His bind resin column previously equilibrated with 5 column volumes of 50 mM NiSO₄ and three column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). After application, the column was washed with 10 volumes of binding buffer and 6 volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9) prior to elution. Bound calreticulin was eluted with six column volumes of elution

buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). Fractions containing calreticulin, as determined by protein estimation (Bio-Rad, Hercules, CA), were pooled, dialyzed against phosphate-buffered saline (PBS), and stored at -20°C until required. Purified recombinant protein was sequenced by matrix assisted laser desorption/ionization time-of-flight mass spectrometry to confirm its identity.

Animal husbandry. Common marmosets (three males and three females in the pilot study, and five males and five females in the follow-up study) bred at the Defence Science and Technology Laboratory in Porton Down and weighing 325–430 g were used. The animals were housed as mixed sex pairs, the males having previously been vasectomized. Each pair had access to four cage units (each with a height of 72 cm, a width of 47 cm, and a length of 60 cm) linked by one vertical and two rigid extensions.

Animals were fed daily in the afternoon and each received primate pellets (Special Dietary Services, Witham, Essex, United Kingdom) as well as fruit, eggs, and other supplements, including access to forage mixture, over the course of the week. Water was freely available at all times. Various items of cage furniture, including hanging wooden dowels, buckets, and other playthings, were also placed in the cages. Illumination was provided by sodium lighting at a level of 350–400 lux 1 meter from the ground by using a 12-hour light/dark cycle with dusk and dawn effects.

Infection of marmosets with *N. americanus*. Marmosets were anesthetized with ketamine (40 mg/kg) and an area of skin approximately 2 cm² below the scapulae was close clipped. Infective larvae were placed on a gauze fixed to a self-adhesive bandage (International Market Supply, Congleton, Cheshire, United Kingdom), which was wrapped around the thorax and held in place with a Tubigrip jacketTM (Seton Products Ltd., Oldham, U.K.). The jacket and bandage were removed after 24 hours.

The pilot study used six marmosets that were divided into three treatment groups. Treatment group 1 (marmosets 1–3) received 300 laboratory strain infective larvae on two occasions. Treatment group 2 (marmoset 4) received 300 laboratory strain infective larvae followed by re-infection with 300 larvae from the PNG fresh isolate. Treatment group 3 (marmosets 5 and 6) were infected on one occasion with the PNG fresh isolate (300 and 600 larvae, respectively). The infection protocols are summarized in Figure 1. In the second, more extensive study, 10 marmosets were infected with one dose of 300 larvae from the PNG isolate. This dose was chosen to represent the lower end of the infectivity scale shown to induce a degree of pathology in the model during the pilot study. To ensure that the PNG strain had no opportunity to adapt to rodents, for the pilot study, marmosets were infected with larvae cultured from a person in Papua New Guinea. These larvae were also used to infect a human volunteer in the United Kingdom previously shown to be negative for human immunodeficiency virus and hepatitis B and C viruses. The larvae used in the second study were freshly cultured from the volunteer in the United Kingdom.

Post-infection monitoring. In the pilot study, animals in treatment group 1 were monitored for 330 days post-infection, animals in treatment group 2 for 220 days, and animals in treatment group 3 for 120 days. During this time, observations were made for any signs of ill health such as

Pilot Study

Animal	Treatment Group	1st Infection D 0	2nd Infection D 98-103				
		D50	D150	D200	D250	D300	
1	1	LS (300 Larvae)	LS (300 Larvae)				PM (D330)
2	1	LS (300 Larvae)	LS (300 Larvae)				PM (D330)
3	1	LS (300 Larvae)	LS (300 Larvae)		PM (D220)		
4	2	LS (300 Larvae)	PNG (300 Larvae)		PM (D220)		
5	3	PNG (300 Larvae)		PM (D120)			
6	3	PNG (600 Larvae)		PM (D120)			

FIGURE 1. Study overview outlining infection protocols for the pilot and secondary study. During the pilot study, treatment group 1 (marmosets 1–3) received 300 laboratory strain infective larvae on two occasions. Treatment group 2 (marmoset 4) received 300 laboratory strain larvae followed by re-infection with 300 larvae from the Papua New Guinea (PNG) fresh isolate. Treatment group 3 were infected on one occasion with 300 (marmoset 5) or 600 (marmoset 6) larvae from the PNG fresh field isolate. In the second study, all animals were infected with 300 larvae from the PNG fresh field isolate and the infection was monitored for 70 days. LS = laboratory strain larvae; PNG = fresh larvae from Papua New Guinea; PM = post mortem; D = day.

lethargy or respiratory problems, which might have been expected because in the human disease, the larvae migrate from the skin to the gastrointestinal tract via the lungs. Blood samples were taken at two weekly intervals for approximately three months and monthly thereafter to monitor specific antibody formation, erythrocyte count, hemoglobin level, and packed cell volume (PCV). In treatment group 3, mean cell volume was also monitored. Erythrocyte count, hemoglobin level, and PCV were monitored to ensure that the animals did not have adverse side effects caused by blood loss as the infection progressed. Blood samples were taken in accordance with recommended animal welfare guidelines,²⁰ and the study was licensed under the United Kingdom Animal (Scientific Procedures) Act 1986.

Samples of feces were collected at regular intervals. Fecal matter was weighed, and eggs harvested by salt flotation and counted.²¹ Egg counts were expressed as eggs per gram of feces (epg). In the second study, all blood samples were taken at 14-day intervals and fecal samples were taken at 7-day intervals. The infection was terminated 70 days post-infection; this time point corresponded to the peak of anemia found in the pilot study.

Immunologic and pathologic analysis. *Total IgE determination.* Ninety-six well polystyrene plates were coated overnight at 4°C with mouse anti-human IgE (50 µL, 5 µg/mL diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, clone no. G7-18; BD Pharmingen, San Diego, CA). The plates were washed with PBS/Tween 20 (0.05% [v/v]), pH 7.2 (PBS/Tween) and blocked with bovine serum albumin (BSA) (200 µL of 1% [w/v]) in PBS (BSA/PBS) for one hour at room temperature. After blocking, the plates were washed and 50 µL of marmoset serum (diluted 1:5 in 1% BSA/PBS) added to each well and incubated overnight at 4°C. In addition 50 µL of human IgE standards (doubling dilutions from 100 IU/mL or 100 µg/mL) were included on each plate. All assays were carried out in duplicate. After overnight incubation, the plates were washed

and 50 µL of biotinylated mouse anti-human IgE (2 µg/mL diluted in 1% BSA/PBS, clone no. G7-26, BD Pharmingen) added to each well and incubated at room temperature for two hours. After two hours, the plates were washed and 50 µL of streptavidin conjugated to horseradish peroxidase (diluted 1:1,000 in 1% BSA/PBS) added to each well and incubated for one hour at room temperature. The plates were washed and developed with 100 µL of 0.1 mg/mL 3,3',5,5'-tetramethylbenzidine (TMB) containing 2 µL of hydrogen peroxide/10 mL of TMB. The reaction was stopped by adding 20 µL of 2.5 M sulfuric acid and the absorbance of each well was measured at 450 nm.

Specific IgG determination. Fifty microliters of *N. americanus* ES products (5 µg/mL in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6) were used to coat the wells of a 96-well polystyrene plate overnight at 4°C. The plate was washed with PBS/Tween and the wells were blocked with 200 µL of 5% skimmed milk powder/PBS (blocking agent) for one hour at 37°C. The plate was washed as before and 50 µL of marmoset serum (diluted 1:100 in skimmed milk powder/PBS) was added to individual wells and the plate incubated overnight at 4°C. The plate was washed, 50 µL of sheep anti-human IgG conjugated to horseradish peroxidase (Binding Site, San Diego, CA) diluted 1:1,000 in blocking agent was added to individual wells, and the plate incubated for two hours at room temperature. The plates were washed and antibody binding was visualized by the addition of 100 µL of TMB prepared as described above. The reaction was stopped by adding 20 µL of 2.5 M sulfuric acid, and the absorbance of each well was measured at 450 nm. All assays were carried out in duplicate. Enzyme-linked immunosorbent (ELISA) values are expressed as the absorbance at 450 nm after the subtraction of a negative control value.

Western blotting. *N. americanus* ES products (10 µg/lane) were separated, under reducing conditions, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 12% gel²²

and transferred onto a nitrocellulose membrane.²³ Western blots were blocked for one hour in 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS) at room temperature. Marmoset serum (diluted 1:200 in 5% [w/v] skimmed milk powder/TBS) was added to the blots and incubated overnight at 4°C. Blots were washed with TBS/0.05% (v/v) Tween 20 and incubated in sheep anti-human IgG (Binding Site) diluted 1:1,000 in 5% (w/v) skimmed milk powder/TBS for two hours at room temperature. After washing, blots were developed in 4-chloro-1-naphthol (10 mg/mL) containing 0.02% hydrogen peroxide. The validity of these anti-human reagents in ELISA and Western blotting has previously been confirmed.²⁴

Whole blood basophil histamine release. Whole blood was collected from marmosets into heparinized tubes. One hundred microliters of blood was added to give a total volume of 500 μ L in PIPES buffer (0.01 M Piperazine-N'N-bis[2-ethaneculfonic acid], 0.14 M sodium acetate, 5 mM potassium acetate, 0.1% glucose, 1 mM CaCl₂, and 0.03% human serum albumin, pH 7.4). Spontaneous histamine release was assessed after incubation for one hour at 37°C, and total histamine release was assessed when 50 μ L of whole blood in 450 μ L of double-distilled water was freeze-thawed three times. Standard histamine calibrators of 0, 10, 25, 50, 100, and 250 ng/mL (Hycor Biomedical Ltd., Penicuik, United Kingdom) were included with each set of whole blood challenges, mediated by anti-IgE, ES products, or recombinant calreticulin. Histamine released in each whole blood challenge was detected using a Histamine Assay Kit (Hycor Biomedical Ltd.). Fifty microliters of challenged whole blood was added to histamine-coated 96-well plates followed by 50 μ L of mouse anti-histamine monoclonal antibody conjugated to alkaline phosphatase. After incubating for one hour at room temperature, wells were washed three times with the provided wash solution. Antibody binding was visualized by the addition of 100 μ L (1 mg/mL) of *p*-nitrophenyl phosphate substrate. The plates were developed for one hour at 37°C, and the absorbance was measured at 405 nm using an MRX absorbance microplate reader (Dynex Technologies). Histamine levels were determined against a standard curve.

Pathologic analysis. At the end of the study, animals were killed with an overdose of sodium pentobarbitone and intestinal and lung tissue were fixed for a minimum of 48 hours in

10% (v/v) neutral (phosphate)-buffered formaldehyde. Tissues were resected into standard infiltration cassettes. Intestinal tissue was first enveloped in Whatman (Maidstone, United Kingdom) no. 1 filter paper. Tissues in cassettes were processed in a Sakura (Torrance, CA) Tissue Tek VIP processor, incorporating a pressure/vacuum and agitation cycle throughout with a nominal solvent chamber temperature of 40°C. Paraffin wax was introduced at 60°C. Tissues were dehydrated through three changes each of graded alcohols (80%, 90%, and absolute ethanol) followed by chloroform (one hour), chloroform (two hours, two changes), chloroform and xylene (50:50; 15 minutes), xylene (10 minutes), and paraffin wax (Paramat pastillated at 58°C; two hours, three changes). Embedding was performed using a Sakura Tissue Tek embedder with reservoir set at 65°C. Wax-embedded tissues were cooled on a freezing plate prior to storage at 4°C while awaiting microtomy. Sections were cut at a nominal thickness of 5 μ m by using a Leitz base sledge microtome (Leica, Wetzlar, Germany). Sections were floated out on water at 52–55°C, collected onto pre-cleaned glass microscope slides, and dried in an oven overnight at 40°C prior to staining. Hematoxylin and eosin stain was applied to tissue sections by using an automated Sakura Linear Stainer II with a routine staining method²⁵ based on the instrument Technical Manual Code 1419/1423 supplied through Bayer Plc (Newbury, United Kingdom).

RESULTS

Pilot study. A summary of infection parameters for the pilot study is shown in Table 1. In the pilot study, all animals in treatment groups 1 and 3 showed evidence of infection, as indicated by the appearance of eggs in feces. All animals reacted immunologically to infection but only the two animals in treatment group 3, which were exposed to one challenge with 300 or 600 larvae from the PNG fresh field isolate demonstrated infection-associated pathology, as shown by a dramatic reduction in hemoglobin 48–62 days post-infection (Figure 2C) and PCV (Table 1). The animal infected with 600 larvae showed a peak decrease in hemoglobin levels approximately 14 days earlier than the animal infected with 300 lar-

TABLE 1
Overall infection parameters in marmosets infected with *N. americanus* during the pilot study and study 2*

Fecundity (day)	Peak eggs per gram of feces	Maximum hemoglobin level (g/dL)	Minimum hemoglobin level (g/dL)	Maximum PCV, %	Minimum PCV, %	Maximum total plasma IgE	Maximum IgG level vs. ES products (absorbance at 450 nm)
Pilot study							
Treatment group 1 infected with 300 laboratory isolate larvae, re-infected with 300 laboratory isolate larvae							
67	269	18.2	14.3	56	41	16.5 ng/mL, (baseline 4 ng/mL)	0.66
Treatment Group 2 infected with 300 laboratory isolate larvae, re-infected with 300 PNG isolate larvae							
ND	ND	20.3	17.2	61	43	124.5	0.64
Treatment Group 3 infected with 300 or 600 PNG isolate larvae							
48	365	17.6	8.2	48	23	64	0.65
Study 2. All animals infected with 300 PNG fresh field isolate larvae							
44.8 $\pm$ 2.13	726.1 $\pm$ 99.5	19.1 $\pm$ 0.45	14.2 $\pm$ 1.2	51 $\pm$ 1	39 $\pm$ 3.1	325.8 $\pm$ 43.1 IU/mL, (baseline 154.8 $\pm$ 21.2 IU/mL)	0.77 $\pm$ 0.16

* Data are fecundity (first day of detection of eggs in feces), Day of peak egg count, pathologic effect of infection as indicated by maximum and minimum packed cell volumes (PCVs) and hemoglobin levels, and antigenicity of infection as shown by parasite-specific IgE and IgG. Some values are mean $\pm$ SD. ES = excretory/secretory; PNG = Papua New Guinea; ND = not detected.

vae. Treatment group 2, which received the laboratory isolate prior to the PNG fresh isolate, did not show either pathology (Figure 2B) or egg production (Table 1). Similarly, erythrocyte numbers were only reduced during infection in treatment group 3, which were infected with the fresh field isolate and reached their lowest levels 40–60 days post-infection, and corresponded to peak egg output (Figure 3C). This effect was not seen in treatment groups 1 and 2 (Figure 3A and B). In treatment group 3, where it was also possible to measure mean cell volume, microcytic anemia was observed.

In the pilot study, antigenicity of infection in all animals was confirmed by the appearance of specific IgG antibodies in the ELISA to *N. americanus* ES products (Table 1). Antibod-

ies recognized a typical antigen profile on Western blots, which included the major 33-kD antigen (Figure 4) in all animals except for the animal in treatment group 2.²⁵ Although the response was stronger in some animals than others, the peak response on Western blots corresponded in most cases with peak egg output.

The characteristic increase in total IgE levels in five of six animals involved in the pilot study by hookworm infection, which is shown in Table 1, led us to investigate the sensitization of basophils with hookworm-specific IgE. Results in Figure 5 showed that all treatment groups had significant histamine release to ES products and a recombinant hookworm allergen calreticulin. Those animals exposed to the fresh field

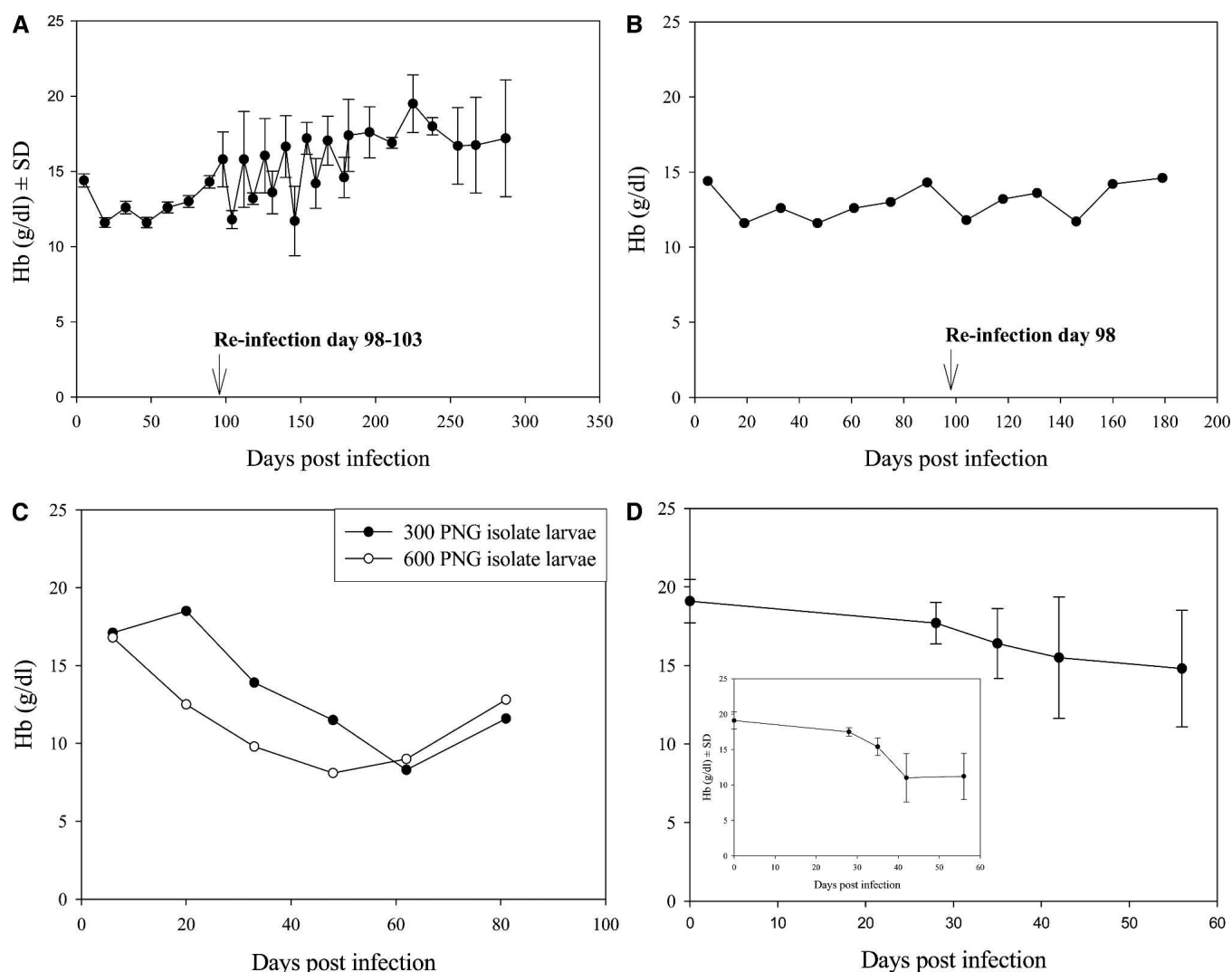


FIGURE 2. Hemoglobin (Hb) levels in animals infected with *N. americanus* during the pilot study and secondary study. For the pilot study, six marmosets were infected with *N. americanus* as described in the Materials and Methods. **A**, Treatment group 1 ($n = 3$) were infected with 300 laboratory strain larvae and re-infected with 300 laboratory strain larvae between days 98 and 103. **B**, Treatment group 2 ($n = 1$) was infected with 300 laboratory strain larvae and re-infected with 300 new isolate larvae on day 98. **C**, Treatment group 3 were infected with 300 ($n = 1$) and 600 ($n = 1$) new isolate larvae, respectively. Hemoglobin levels (normal range = 14.9–17.9 g/dL) were measured over the time course of infection using a Baker 9000 hematology analyzer (Serono Baker Diagnostics, Allentown, PA). Only animals in treatment group 3 demonstrated a decrease in Hb levels. **D**, In the second study, 10 marmosets were infected with the Papua New Guinea isolate of *N. americanus*. All 10 animals demonstrated a decrease in Hb levels, with four animals showing worms in the small intestine at autopsy and having the most significant decrease in Hb levels (inset). Where group number allowed, data are expressed as mean $\pm$ SD Hb levels. In all other cases, data are expressed per animal.

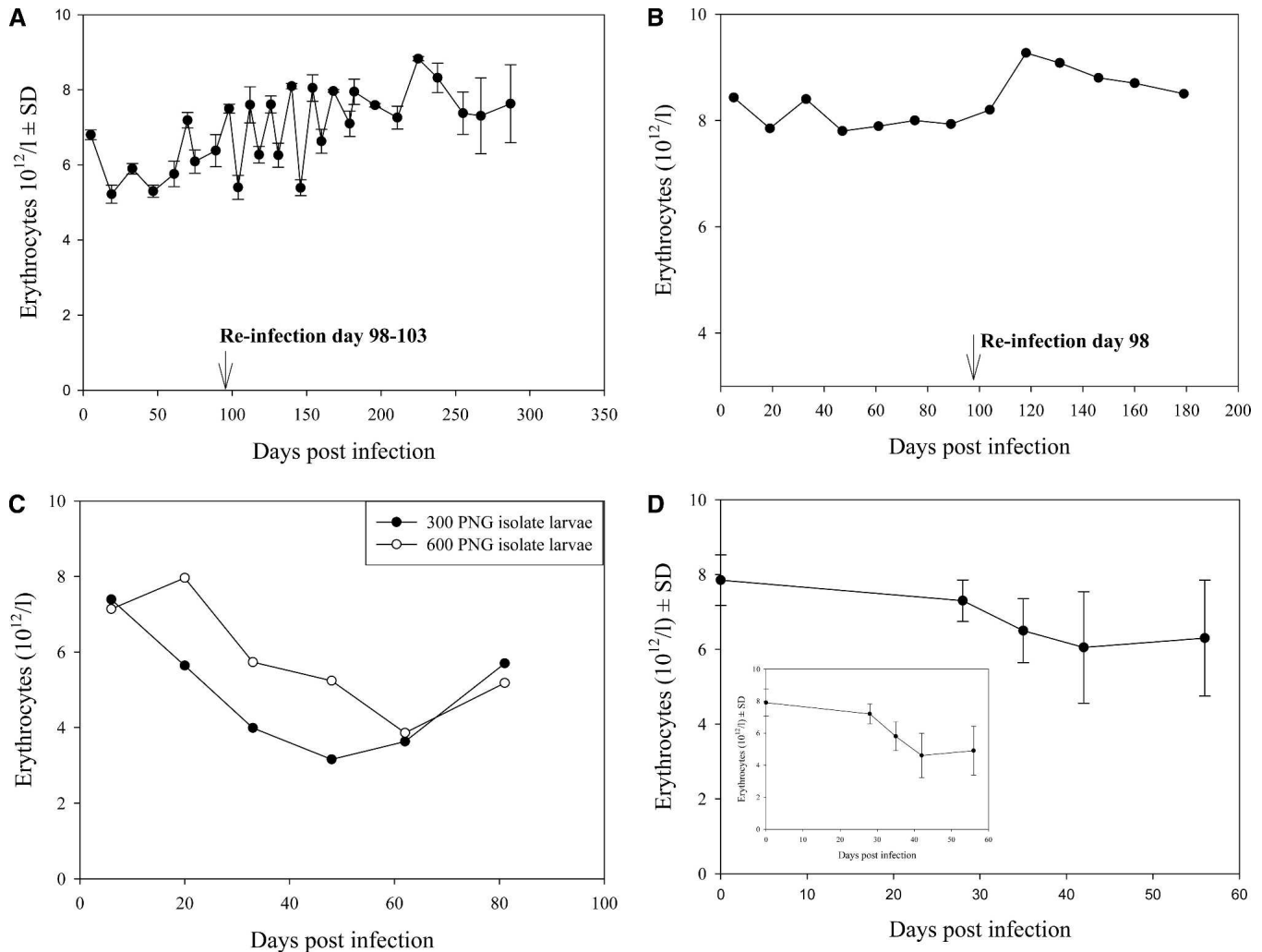


FIGURE 3. Changes in erythrocyte numbers after infection with *N. americanus* during the pilot study. Treatment groups 1–3 were infected as described previously and monitored for changes in peripheral blood erythrocytes (normal range = $5.7\text{--}6.95 \times 10^{12}$ cells/L) during the time course of infection using a Baker 9000 hematology analyzer (Serono Baker Diagnostics, Allentown, PA). **A**, Treatment group 1 ($n = 3$); **B**, Treatment group 2 ($n = 1$); **C**, Treatment group 3. Only those animals infected with either 300 ($n = 1$) or 600 ($n = 1$) larvae of the fresh field Papua New Guinea (PNG) isolate demonstrated a reduction in erythrocyte numbers. **D**, In the second study, 10 marmosets were infected with the PNG isolate of *N. americanus*. All animals demonstrated a decrease in erythrocyte levels, with four animals showing worms in the small intestine at autopsy and having the most significant decrease in hemoglobin (Hb) levels (inset). Where group number allowed, data are expressed as mean $\pm$ SD Hb levels. In all other cases, data are expressed per animal.

PNG isolate showed increased histamine release (Figure 5B–D) than animals only exposed to the laboratory isolate (Figure 5A). To control for possible non-specific release of histamine by enzymes in ES products,²⁶ some preparations were heat inactivated by boiling for 30 minutes to neutralize activity. Heat-inactivated ES products produced a similar level of release, which indicated the presence of non-proteolytic, allergenic material in heat-inactivated ES products. To control for the ability of basophils to release histamine, cells were also challenged with antibodies to IgG and IgE. Three of six animals (treatment groups 2 and 3) released histamine to antibodies to IgE to a greater extent than that induced by antibodies to IgG. The failure of treatment group 1 to release histamine suggested that the cells were not sufficiently sensitized with hookworm-specific IgE when the old laboratory isolate was used.

At autopsy, the small intestines were removed from each

marmoset in the pilot study, opened along their length, and placed in Hanks' balanced salt solution at 37°C to enable any remaining worms to detach. However, residual worms were observed in the marmosets in treatment group 3. The animal infected with 300 fresh isolate larvae showed four males, two females, and three worms of undetermined sex. These worms were fixed *in situ* for histologic analysis. Eleven worms (five males, two females, and four of undetermined sex) were observed in the marmoset infected with 600 PNG fresh isolate larvae. These worms were also fixed *in situ* for histologic analysis.

At *post mortem*, pulmonary pathology, characterized by peripheral alveolar hemorrhagic and fluid accumulation, was evident in animals that received the fresh PNG isolate, an example of which is shown in Figure 6A. Figure 6B shows a transverse section of the mouth end of a hookworm, located in the gut of the animal that had received 600 fresh *N. ameri-*

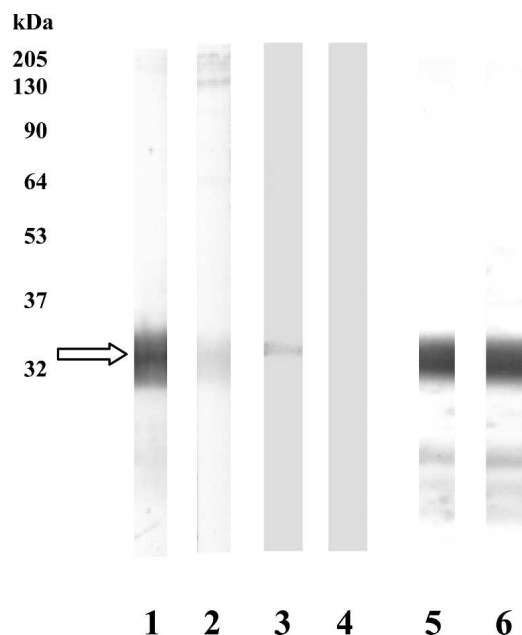


FIGURE 4. Antigenicity of ES products after infection with laboratory and field isolates of *N. americanus* during the pilot study. Western blot analysis of the antigenicity of the ES products of *N. americanus* probed with post-infection marmoset plasma followed by peroxidase-conjugated anti-human IgG. In most cases the peak day of antigenicity, as assessed by band intensity on Western blot, coincided with peak egg output. Lanes 1–3, treatment group 1; lane 4, treatment group 2; lanes 5 and 6, treatment group 3 infected with 300 and 600 larvae, respectively. **Arrow** indicates 33-kDa band.

canus larvae approximately four months earlier. The buccal cavity, cutting plates, esophagus, and amphidial glands of the worm can be seen.

Secondary study. The pilot study indicated the potential relevance of this model to human necatoriasis. In the second study, we were able to confirm the infection parameters observed with the PNG isolate in the pilot study (Table 1). All 10 animals demonstrated a reduction in PCV and hemoglobin levels (Table 1 and Figure 2D), corresponding approximately with peak egg output. However, the most significant reductions were observed in the four animals that harbored worms at autopsy (Figure 2D, inset). Animal 7 harbored 18 worms (12 males and 6 females), animal 12 harbored 1 worm (1 male), animal 13 harbored 29 worms (17 males and 12 females), and animal 15 harbored 9 worms (6 males and 3 females). Similarly, erythrocyte levels decreased in all animals (Figure 3D), with the most significant decreases again observed in the four animals with worms at autopsy (Figure 3D, inset). All animals showed an increase in total IgE and parasite-specific IgG post-infection (Table 1). In addition, histopathologic examination of the lungs confirmed the presence of fibrosis (alveolar thickening) associated with evidence of debris left by worms in transit. The animal with the greatest number of adults found in the intestine (animal 13) also showed the greatest preponderance of these localized changes.

DISCUSSION

Hookworm infection is regarded by many to represent a significant threat to the health and well-being of afflicted

communities. Consequently, efforts are being concentrated on developing a full understanding of the molecular biology of the host-pathogen interface, with a view to fully understand the pathobiology of infection and develop efficacious vaccines to protect against hookworm disease. However, the hookworm lifecycle is difficult to maintain in animal models because of the subtle adaptation of human hookworms to life in their definitive host. This renders most available hookworm models deficient.

Our report describes pilot and follow-up studies in which patent and pathologic hookworm (*N. americanus*) infections were established in non-human primates. These studies had the aim of developing an improved and biologically relevant animal model in a species closely allied to humans, thus reducing the use of inappropriate animal species to study necatoriasis.

The pilot was successful in that patency was observed in most animals, whether given laboratory or PNG isolates of the parasite. However, it is significant that blood loss was only seen in treatment group 3 (infected with 300 and 600 PNG larvae), which indicated that attenuation may have occurred in the laboratory isolate. This attenuation may have been a result of repeated passage (approximately 460) through hamsters during the laboratory maintenance of this strain since 1983, in which they seem to have lost their full anti-hemostatic repertoire. The blood loss caused by the PNG isolate was demonstrated by a dramatic decrease in hemoglobin and PCV levels in treatment group 3, accompanied by evidence of a microcytic anemia, presumably as a direct result of blood loss in the lungs during transit⁹ by infective larvae, and feeding by adult worms in the gut.

Infection in all animals was associated with an immune response reminiscent of that seen in humans, where antibodies were readily detectable against hookworm secretions, accompanied by (with the exception of the animal in treatment group 2) a typical Western blot profile, with a 33-kDa antigen predominant.²⁷ The animal in treatment group 2 received 300 larvae from the laboratory strain followed by 300 larvae from the PNG isolate. After secondary infection, this animal did not show the patency or pathology associated with infection with the PNG isolate. This finding may have been caused by a potential protective effect induced by infection with the laboratory strain. Basophil sensitization with parasite IgE was a salient feature of this model, which is indicative of activation of the T-helper 2 immunologic subsystem and deemed important in immunity to *N. americanus*.²⁸ Basophil histamine release to multiple agonists was most consistent in animals exposed to the PNG isolate. This finding probably reflected more efficient loading of Fc ϵ RI on basophils with parasite-specific IgE, which was not detectable serologically because of the low sensitivity of anti-human IgE reagents in the ELISA. Histamine release by calreticulin confirmed the allergenic properties of this molecule.²⁹

The follow-up study, in which all animals were infected with the PNG isolate, confirmed our pilot study findings of patent infections with a relatively consistent onset of fecundity approximately 42 days post-infection. In addition, both the epg level and the day of peak epg are comparable with data obtained from humans.³⁰ All 10 animals demonstrated a considerable immune response to infection and a decrease in hemoglobin levels and PCV, although this was more noticeable in some animals than others. However, worm recoveries

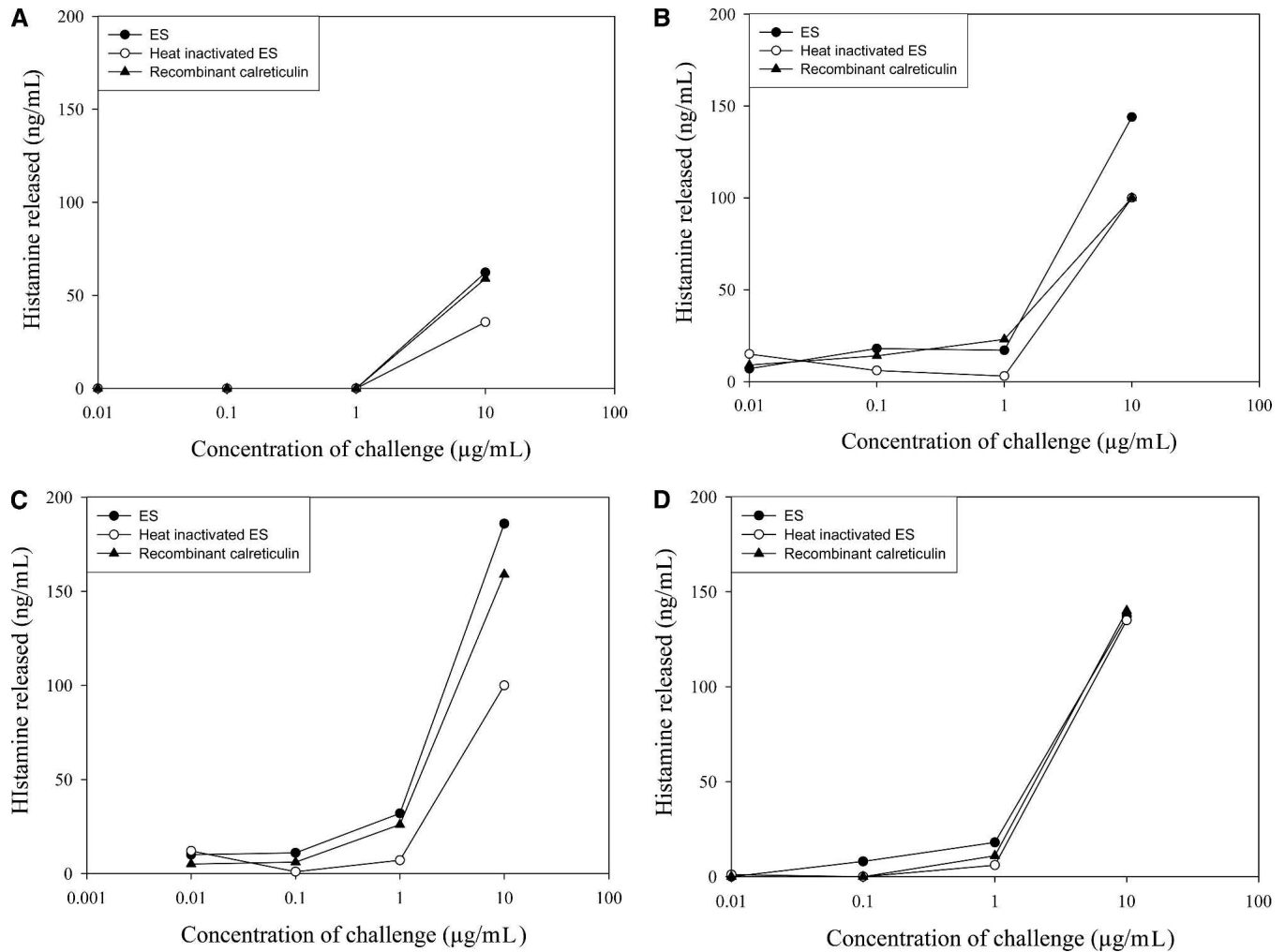


FIGURE 5. Basophil histamine release in infected animals from the pilot study after challenge with *N. americanus* ES products (intact or heat-inactivated to neutralize enzymatic activity) and a recombinant hookworm allergen, calreticulin (rCAL). Basophils from animals in treatment groups 1–3 were challenged with ES products, heat-inactivated ES products, and recombinant calreticulin as described in the Materials and Methods. Histamine release was measured using a Histamine Assay Kit (Hycor Biomedical Ltd., Edinburgh, United Kingdom). **A**, Treatment group 1 (n = 3); **B**, Treatment group 2 (n = 1); **C** and **D**, Treatment group 3 (n = 2), infected with 300 and 600 Papua New Guinea fresh field isolate larvae, respectively.

were inconsistent, with only four animals still harboring worms at autopsy.

It can be concluded at this stage that further development of this model will add to our knowledge of the pathobiology

of necatoriasis *per se*, and support the investigation of a number of related immunologic issues. For example, given the increasing availability of reagents for immunologic studies in the marmoset²⁴ the primate model will also provide an op-

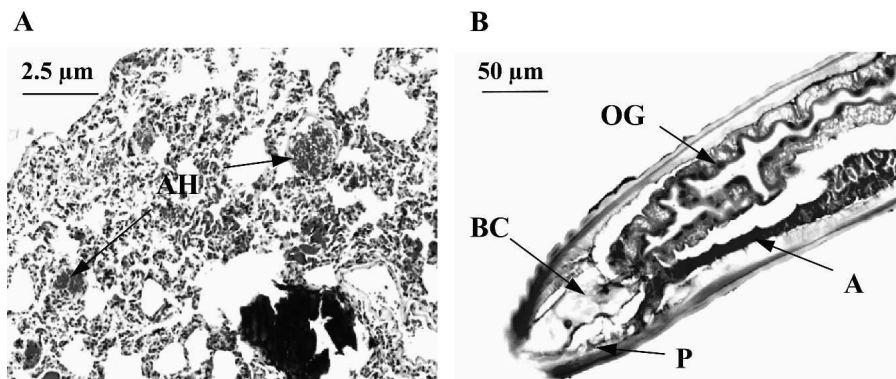


FIGURE 6. Lung pathology associated with infection with a fresh field isolate of *N. americanus*, and a section of an adult worm recovered from the gut *post mortem*. AH = alveolar haemorrhage; BC = buccal cavity; P = cutting plates; OG = oesophagus; A = amphidial gland.

portunity to assess the relationship between hookworm infection and the development of allergic³¹ and autoimmune diseases and the impact of hookworm infection on co-current infection with malaria and simian immunodeficiency virus.

Finally, further development of the model may ultimately provide a unique opportunity to develop a hookworm vaccine in a system where vaccine safety, delivery, and efficacy can be assessed against worm establishment, parasite patency, infection-associated pathology, and cognitive development.^{32,33} Furthermore, the comparatively close evolutionary relationships between marmosets and humans, the strong degree of similarity between their MHC and T cell receptor genes, their immunologic characterization, and the availability of defined reagents combined with their suitability for cognitive studies, and their acceptance by the Food and Drug Administration as being important to preclinical evaluation of biotechnology derived pharmaceuticals,³⁴ lend support to the choice of this species as a model for hookworm vaccination development, in preference to the less suitable mouse, hamster, and canine models.

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