Acquired immunity to *Ancylostoma ceylanicum* in hamsters

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Summary A laboratory model of acquired immunity to human hookworm is described. Significant resistance to challenge infection with *Ancylostoma ceylanicum* was elicited in mature DSN hamsters. The serum and mucosal antibody responses were investigated in both quantitative and qualitative terms and changes associated with immunity were identified. Marked differences in numbers of mast and goblet cells in the small intestine were also recorded and related to the immune status of the host.

Keywords: immunity, hookworm, antibody, mast cell, goblet cell

Introduction

The importance of human hookworm infections to the health of affected populations in endemic regions has been widely emphasized and is now recognized as a significant factor affecting local economy through reduced productivity (Banwell & Schad 1978, Gilles 1985). The chronicity of infections caused by hookworms is not disputed but the existence of acquired immunity in humans is somewhat more controversial (Behnke 1987). In contrast, there is good evidence that dogs develop resistance to hookworms (Carroll & Grove 1985) and there are preliminary data to support the existence of immunity to *Ancylostoma ceylanicum* in hamsters (Gupta & Katiyar 1985, Menon & Bhopale 1985). Whilst the latter is essentially a laboratory model, it offers an opportunity to analyse the components involved in host-protective immunity to hookworms at a level impractical in other systems. Understanding the mechanisms involved should shed light on the crucial elements necessary for the expression of host-protective immunity and in turn should contribute to understanding the enigma of chronic hookworm infection in man.

Materials and methods

ANIMALS AND PARASITE

Inbred male DSN hamsters were bought in at 3–4 weeks of age from Shamrock Farms.

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Animals were kept under standard animal house conditions and food and water were available *ad libitum*. The techniques for infection (Behnke, Wells & Brown 1986), recovery of worms (Garside & Behnke 1989) and faecal egg counts (Behnke & Parish, 1979) have all been described previously. This study was carried out using larvae from the 12th generation of passage through hamsters in Nottingham.

MEASUREMENT OF HOST VARIABLES

Procedures for weighing animals and collecting blood samples have been described previously (Garside & Behnke 1989). At autopsy animals were killed by an overdose of anaesthetic. The small intestine was removed, split longitudinally and a 1-cm piece taken from 15 cm below the ileo-gastric junction to be processed for mucosal mast cell (MMC) (Swieter 1984) and goblet cell counts. Following this, worms were removed from the gut, sexed and counted. Finally, the remaining small intestine was incubated overnight at 4°C in 20 ml phosphate-buffered saline (PBS), in order to encourage complete extrusion of intestinal mucus, and then processed according to Elson, Ealding & Lefkowitz (1984).

MAST CELLS

Tissues were prepared for mast cell counts as described by Sweiter (1984). Briefly, paraffin-embedded sections were hydrated and then placed in Alcian/Astra blue (1:1 v/v), which had been preheated to 56°C, for 1 h. The slides were quickly rinsed in 0·7 N HCl and the tissues counterstained by immersion in prewarmed (56°C) Safranin O for 3 min. The slides were then dehydrated, cleared in xylene and mounted.

GOBLET CELLS

Similar sections to above were hydrated and stained with haematoxylin before dehydration and mounting. Mast cells and goblet cells counts were carried out on five villus crypt units per tissue section and five tissue sections per animal.

ANTIBODY RESPONSES

The parasite-specific antibody response was investigated quantitatively using an ELISA, previously described by Garside, Behnke & Rose (1989a), but the results were expressed as a relative response index. The optical density value for each individual serum sample was expressed as a percentage of the mean value obtained using the day 70 sera from group IC in Experiment 1 and day 56 sera from group 2A in Experiment 2. For observations on qualitative differences in response the Western blotting technique as reported by Carr & Pritchard (1987) was implemented. Briefly, PBS-soluble, adult worm homogenate was electrophoretically transferred to nitrocellulose following separation on 5–20% gradient SDS-PAGE. Strips of the membrane were blocked (10% skimmed milk in PBS-Tween 20) and then incubated with appropriate hamster test serum (1:100). The strips were then developed using a rabbit antiserum raised against saturated ammonium sulphate (SAS) precipitated hamster immunoglobulins followed by horseradish peroxidase (HRP) conjugated protein A.

STATISTICAL ANALYSIS

Where shown the mean ($\bar{x}$) and the standard error of the mean (s.e.m.) have been calculated for data sets. Groups were compared by the Mann–Whitney U-test and by Kruskal-Wallis one-way analysis of variance as appropriate. Changes in the weight of animals from day 0 onwards, were analysed within groups by the Wilcoxon test. For comparison of weight between groups on particular days, the individual data points were expressed as a percentage of the weight on day 0 and were analysed by the Mann-Whitney U-test.

Results

WORM BURDENS

Two experiments were carried out. The first examined the duration of primary infection necessary to elicit acquired immunity, and the second compared the efficacy of intragastrically versus percutaneously administered larvae in the immunizing infection.

The results from Experiment 1 (Table 1) showed that maximum immunity was elicited in animals which had experienced a 21-day primary infection (group 1C *vs* 1D, $P = 0.005$) and therefore all life-cycle stages of the parasite. Groups 1B which would only have experienced a 10 day primary infection also had significantly lower worm recovery (Group 1B *vs* 1D, $P = 0.025$) but group 1A was not significantly resistant in comparison to the challenge control group (1D).

In the second experiment the best protection against challenge was achieved by

Table 1. Worm burdens and percentage protection

Experiment and group (n)	treatment		worm recovery	
	Primary infection	Drug on* day	Mean $\pm$ s.e.m.	Per cent protection
1A (7)	50 L3 i.g.†	Day 4	3.6 ± 0.72	26.5
1B (6)	50 L3 i.g.	Day 10	2.3 ± 0.49	55.1
1C (6)	50 L3 i.g.	Day 21	1.0 ± 0.25	79.6
1D (8)	None	Day 21	4.9 ± 0.81	—
2A (5)	100 L3 i.g.	Day 28	0.8 ± 0.37	98.6
2B (5)	100 L3 perc.‡	Day 28	30.2 ± 8.3	47.8
2C (5)	None	Day 28	57.8 ± 4.8	—

* Pyrantel embonate was given orally in a single dose of 50 mg/kg body-weight which we had previously found to be 100% effective in removing the parasite.

† i.g., Intragastric infection.

‡ perc., Percutaneous infection.

In Experiment 1 all the groups were challenged with 50 L3 on day 28 and were killed 6 weeks later on day 70. In Experiment 2, the challenge infection, comprising 100 L3 was given on day 35 and the animals were killed 3 weeks later on day 56, for worm counts.
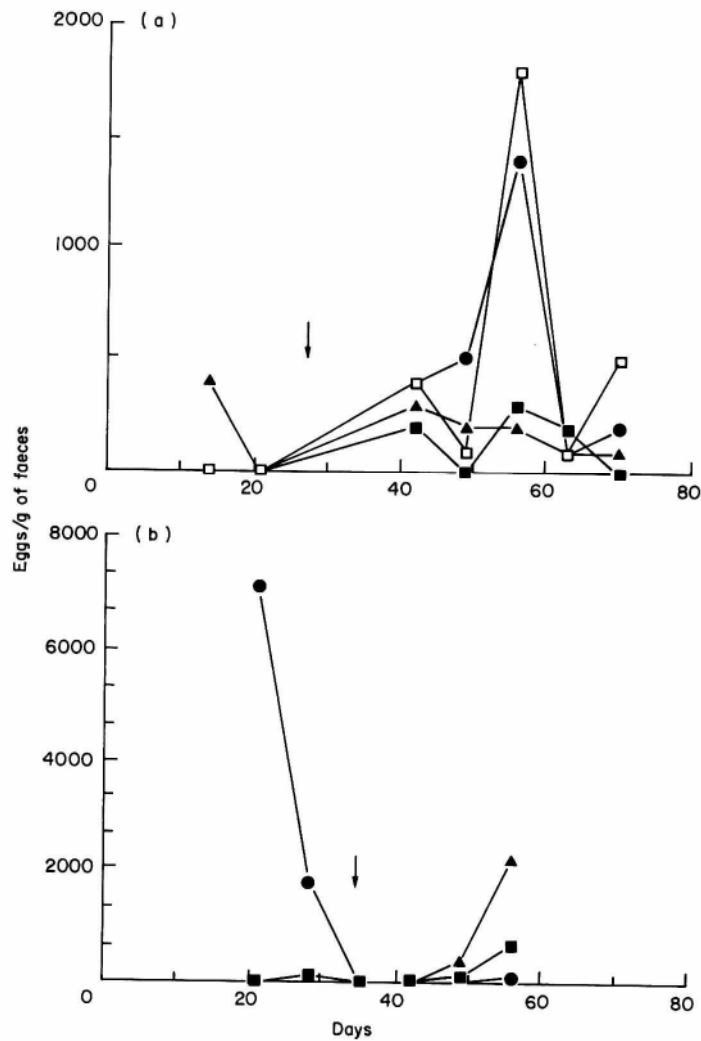
Figure 1. Faecal egg output during the course of Experiments 1 and 2. (a) Experiment 1: ●, Group 1A (4-day primary infection); ■, group 1B (10-day primary infection); ▲, group 1C (21-day primary infection); □ group 1D (challenge control group). (b) Experiment 2: ●, Group 2A (intragastrically immunized); ■, group 2B (percutaneously immunized); ▲, group 2C (challenge control group). Arrows indicate days on which the challenge infections were administered.

intragastric immunization (Group 2A *vs* 2C, $P=0.005$) but the animals given a percutaneous immunizing infection were also significantly protected (Group 2B *vs* 2C, $P<0.05$).

FAECAL EGG COUNTS

Faecal egg counts were used to monitor the infection during both experiments. In the first experiment, only group 1C produced a patent primary infection (Figure 1a) as this was the only group in which worms were allowed to survive to the adult stage. In both

experiments egg counts declined to zero following treatment with anthelmintic, suggesting that all parasites had been cleared. The absence of parasites was further confirmed by autopsy of a representative animal from each group. In Experiment 1, following challenge, the pattern of egg output appeared to be related to final worm burden and generally reached peak values 28 days after challenge. High egg output was only recorded in the challenge control group and the hamsters subjected to a 4-day immunizing infection.

In Experiment 2 faecal egg output was again related to worm burden. Following the primary infection, eggs were detectable on days 21 and 28 post-infection (p.i.) in intragastrically immunized animals and at lower levels in the percutaneously immunized group (Figure 1b). Two representative animals from each of these groups were killed before drug treatment; those from group 2A had 60 and 41 worms respectively, whereas those from group 2B contained 1 and 0 worms. After challenge infection, parasite eggs were detectable in the faeces of all three groups and the intensity of faecal egg counts was related to the final worm burdens.

PACKED CELL VOLUME (PCV)

Relatively stable PCV ranging in value between 50% and 55% were observed in three of the four groups in Experiment 1 (1A, 1B & 1D) during the first 28 days of the experiment (Figure 2). Group 1C, which is the only group in which adult worms were permitted to develop, showed a mean PCV value falling rapidly after day 4. It reached a trough on day 21 and was significantly different from group 1D ($P=0.001$) at this stage. However, by day 28, 1 week after drug treatment, all groups were again at comparable values with no significant differences between them. By 2 weeks after challenge infection there was a gradual decline of mean PCV values in groups 1A, 1B and 1D with the most marked reduction in group 1D, the challenge control group. The mean PCV of group 1D was significantly lower ($P<0.025$) than that of group 1C by day 42 and remained so throughout the rest of the experiment.

The changes in PCV were also marked in Experiment 2 with group 2A, the intragastrically immunized group, having significantly lower mean PCV than group 2C ($P=0.005$) by day 28 of the primary infection. However, 7 days after drug treatment, values had returned to normal and there was no significant difference between the groups. Following challenge infection the PCV of the intragastrically immunized group (2A) was unaffected but hamsters immunized percutaneously (2B) and the challenge control group (2C) sustained declining PCV values which continued to fall until the day of autopsy. Groups 2B and 2C had both significantly lower mean PCV than group 2A on day 49 ($P<0.01$ and $P<0.005$, respectively) and on day 56 ($P<0.025$ and $P=0.005$, respectively).

WEIGHT

In Experiment 1 all 4 groups gained weight during the first 4 weeks. After challenge infection only the control group (1D) lost body weight (5.3%) but this was not significant. At no time was the mean body weight of any of the groups significantly different from the others. This was probably due to the relatively low worm burdens in Experiment 1. The initial establishment of worms was heavier in Experiment 2 (see Fig 1 for egg counts) and there was significant weight loss (13.1%) in the intragastrically infected animals (Day 0 vs day 28, $P<0.04$). Following challenge, only the control group (2C) lost weight (14.7%, $P<0.04$).
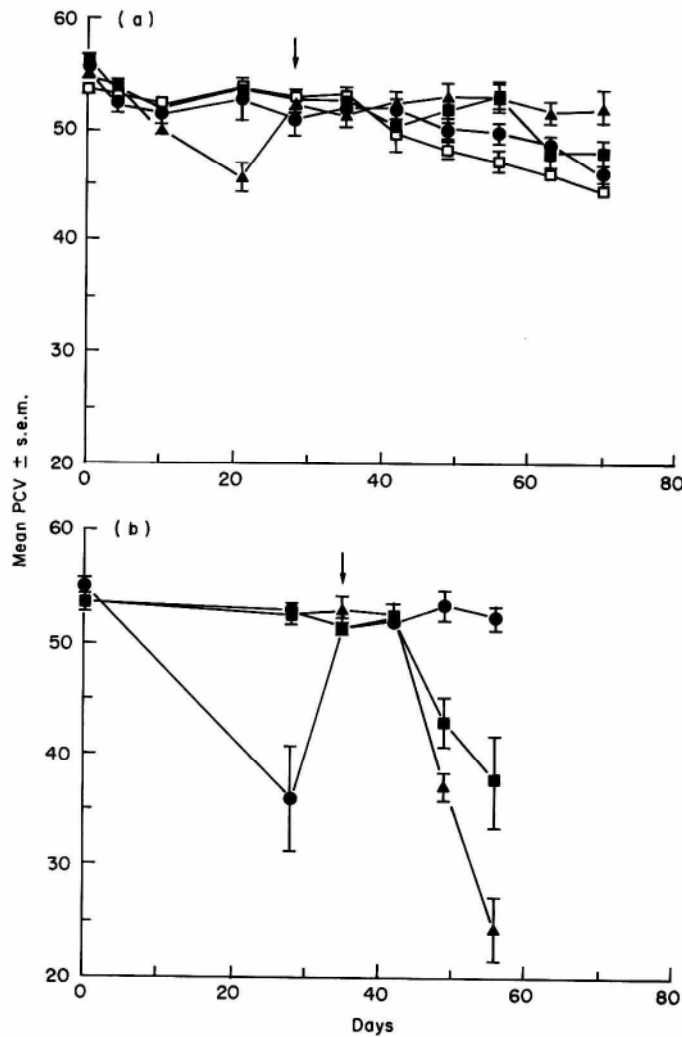
Figure 2. Mean packed cell volume (±s.e.m.) monitored throughout both experiments. (a) Experiment 1: ●, Group 1A; ■, group 1B; ▲, group 1C; □, group 1D. Group 1C had a significantly lower mean PCV than group D on day 21 p.i. ($P = 0.001$). There was then no difference between the groups until day 42 when group 1D had a significantly lower mean PCV than group 1C ($P < 0.025$). By day 56 group 1A was also significantly lower than 1C ($P < 0.025$). On day 70, groups 1A, 1B and 1D were all significantly reduced relative to group 1C ($P < 0.01$, $P < 0.05$ and $P < 0.001$, respectively). (b) Experiment 2: ●, Group 2A; ■, group 2B; ▲, group 2C. Group 2A had significantly lower mean PCV than 2C by day 28 ($P = 0.01$). There was then no difference between the groups until day 49 when groups 2B and 2C both had significantly lower mean PCVs than group 2A ($P < 0.01$ and $P < 0.005$, respectively).

MUCOSAL MAST CELLS

The number of MMC was assessed only in Experiment 2. The background count of naive animals was 1–3 MMC per villus crypt unit (VCU) and in relation to this value all the infected groups had elevated mast cell counts. The intragastrically immunized group (2A)

had $12\cdot7\pm1\cdot65$ MMC per VCU which was significantly higher ($P=0\cdot048$) than the challenge control (2C) group with a value of $8\cdot5\pm0\cdot46$.

GOBLET CELLS

Goblet cell counts were again only assessed for Experiment 2. Counts for all groups were elevated in relation to those for naive animals (6–11 goblet cells per VCU). Intragastrically immunized animals (2A) had $16\cdot25\pm3\cdot28$ goblet cells per VCU (significantly fewer than groups 2B and 2C, $P<0\cdot01$) compared with $37\cdot7\pm3\cdot77$ for the percutaneously immunized group (2B) and $40\cdot86\pm0\cdot98$ for the challenge control group (2C).
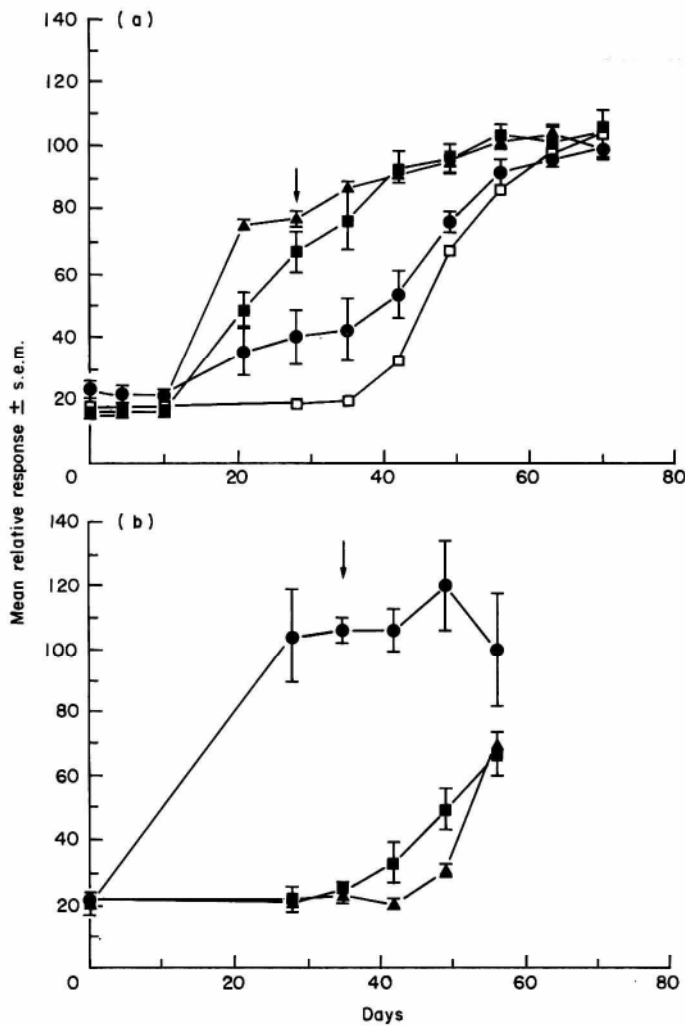

Figure 3. Anti-parasite antibody levels as measured by optical density on ELISA for Experiments 1 and 2. (a) Experiment 1: ●, Group 1A; ■, group 1B; ▲, group 1C; □, group 1D. (b) Experiment 2: ●, group 2A; ■, group 2B; ▲, group 2C.
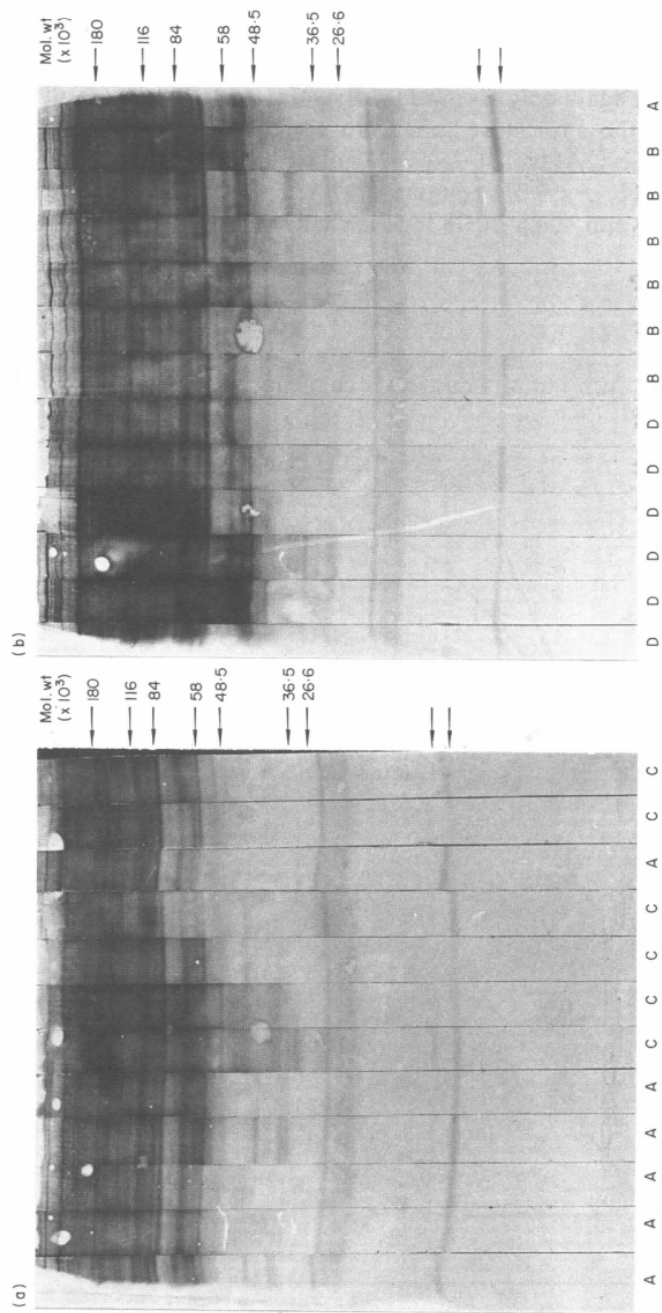
Figure 4 (a & b). Western blot of adult worm homogenate probed with individual sera taken at autopsy during Experiment 1. Lanes are labelled A, B, C and D corresponding to the groups. There was no detectable reaction with naive hamster and rabbit serum or protein A (data not shown).

SERUM ANTIBODY RESPONSE

The results for Experiment 1 (Figure 3) showed that both challenge and control groups attained a similar final antibody titre. Groups 1B and 1C displayed a rapid initial rise in antibody titre when compared with group 1A and the control group (1D). By the time the challenge infection was administered, groups 1A, 1B and 1C had reached 20%, 60% and 70% respectively of the relative response index, calculated by comparison with immune sera from group 1C (day 70).

The ELISA results for Experiment 2 were somewhat different from those of Experiment 1 as a result of the change in experimental design. Again a relative response was calculated with reference to sera from the immune group 2A (day 60). Group 2A showed a large increase in antibody titre by day 28 and this was followed by a period of relative stability interrupted only by a small additional rise 14 days post-challenge and a subsequent decline. In contrast, groups 2B and 2C did not display appreciable increases in anti-parasite antibody until 7 and 14 days after challenge. The initial increase in titre was more rapid in group 2B than group 2C but by the end of the experiment both groups displayed comparable antibody levels.

WESTERN BLOTTING

Sera collected at autopsy in Experiment 1 and the sera and gut washings from Experiment 2 were used to probe Western blots of adult worm homogenate. In Experiment 1 the major difference appeared to be the recognition of an extra band at low mol. wt in immunized animals from groups 1B and 1C. These animals recognized a doublet in this 10–15 kD region whereas challenge controls and the 4-day primary infection group (1A) recognized only one band (Figure 4).

The hamsters in Experiment 2 (Figure 5) did not recognize these bands except for three animals from the intragastrically immunized group 2A. These three individuals were the only animals in this group which still harboured worms, the other two animals being parasite free. Western blots using gut washings from Experiment 2 showed stronger recognition in intragastrically immunized animals relative to the challenge control group but no recognition of the low. mol. wt doublet.

Discussion

Immunity to *A. ceylanicum* has been recorded previously in dogs (Carroll & Grove 1985) and hamsters (Gupta & Katiyar 1985, Menon & Bhopale 1985). In both cases a single, primary, immunizing infection induced significant protection against secondary exposure. The demonstration of acquired resistance to *A. ceylanicum* in hamsters is particularly important because it represents the only rodent model in which mature hosts challenged by hookworm larvae achieve patent infections. This model may be exploited to achieve a detailed analysis of the components necessary for the expression of host protective immunity to hookworm. We have initiated such an analysis in the experiments reported in this paper.

The effects observed on host variables (weight and PCV) during the immunizing infection and in challenge controls were as expected from previous studies (Garside & Behnke 1989), reflecting infection intensity related pathology, particularly in relation to adult worm burden. Changes in pathological parameters, together with those reflecting
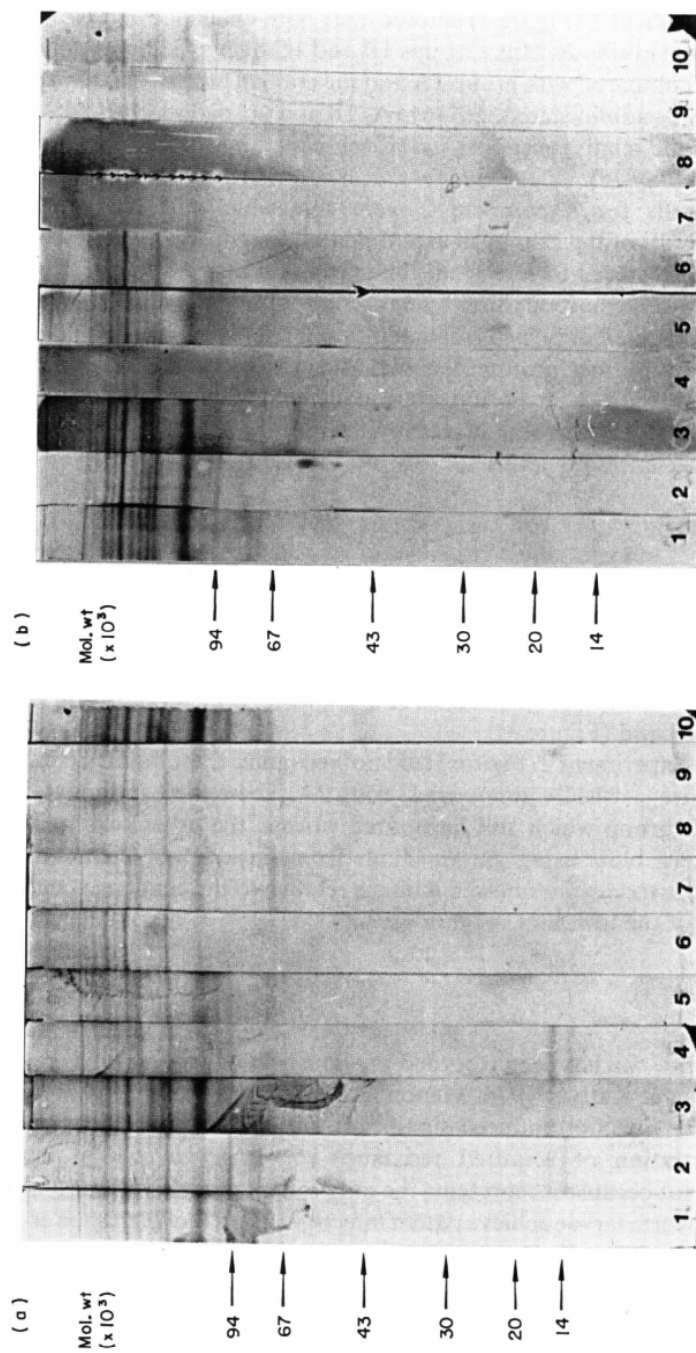
Figure 5. Western blot of adult worm homogenate probed with individual sera (a) and gut washings (b) taken at autopsy during Experiment 2. Lanes 1–5: group 2A; lanes 6–10: group 2C. There was no detectable reaction with naive rabbit and hamster serum or protein A (data not shown).

immunological activity, suggested that the degree of immunity induced was dependent upon the length of primary exposure to the parasite, with longer primary infection resulting in lower secondary worm burden, more intense secondary antibody responses and reduced pathology (Experiment 1).

The influence of the intensity of primary infection on subsequent acquired resistance was not intentionally investigated in the present work, but indirect evidence for a dose-related phenomenon was provided by Experiment 2. Very few percutaneously administered larvae succeeded in establishing a patent infection in group 2B hamsters, in comparison with the intragastrically infected group 2A (see also Garside & Behnke 1989, Gupta & Katiyar 1985); acquired immunity was correspondingly lower in group 2B.

The changes observed in mucosal cell numbers in Experiment 2 were particularly interesting, with a marked mastocytosis evident in the immune challenged group (2A). However, even the challenge control animals, which had heavy primary infection worm burdens, showed a significant mastocytosis relative to naive animals. Whilst it is tempting to propose that enhanced mast cell counts were associated with host protective immunity, the survival of primary infection worms despite intense mastocytosis implies that other mechanisms were also necessary to achieve worm expulsion. Mastocytosis has been documented in a number of gastrointestinal nematode infections and has been correlated with parasite expulsion.

Goblet cells, like mast cells, are believed to be an important component of mucosal immunity (Miller 1984) and it was therefore surprising to find goblet cell counts reduced in the immune challenged hamsters (group 2A). However, unlike mast cells, goblet cells are not bone marrow derived, actually being produced from precursors within the mucosa, but controlled by local factors including T-cells (Miller 1984). As a result, changes within the gut may be more quickly responded to by locally controlled goblet cells which have a faster rate of turnover and a more rapid propensity to decline than the more centrally marshalled mast cells when the source of their induction has been removed. Hence, in our experiments, the low goblet cell counts in immune animals may signify that expulsion took place considerably earlier and that goblet cells were already declining in numbers. This interpretation is supported by the fact that the three animals in the immune group still harbouring worms had higher goblet cell numbers than the remainder.

Hamsters which sustained a mature primary infection responded with a marked serum antibody response to parasite antigens, which appeared to be related to the length and intensity of primary exposure. However, even quite low primary infections, as in group 2B, induced a degree of immunological memory because despite the absence of an overt response to the primary infection there was a more rapid response to the secondary infection when compared with challenge controls. Similar results have been reported in the dog model (Carroll & Grove 1985) where increases in anti-parasite IgG and IgM were recorded in comparison with primary infection controls.

The Western blots showed more intense recognition of parasite antigens by immunized challenged animals in both experiments, corresponding with the increased titres of antibody measured using the ELISA. The association of immunity with the recognition of antigens in the same mol. wt region in both experiments is of particular interest. One of the low molecular bands (10–15 kD) was identified by the challenge control sera from Experiment 1 but not from Experiment 2 and it is possible that this difference is related to the duration of infection. In Experiment 1 the hamsters were killed 6 weeks after challenge whereas this interval was only 3 weeks in Experiment 2. The low mol. wt fraction of adult worm homogenate is currently the subject of a separate investigation which has revealed

that molecules resolving in this mol. wt range have an immunomodulatory function (Garside *et al.* 1986b). Such a role is compatible with the results of the present study since it might be anticipated that an antibody response against molecules with immunomodulatory capacity would inhibit their biological activity and possibly enable host protective immunity to be expressed.

Acknowledgements

References

BANWELL J.G. & SCHAD G.A. (1978) Hookworm. *Clinics in Gastroenterology* **7**, 129

BEHNKE J.M. (1987) Do hookworms elicit immunity in man? *Parasitology Today* **3**, 200

BEHNKE J.M. & PARISH H.A. (1979) *Nematospiroides dubius*: arrested development of larvae in immune mice. *Experimental Parasitology* **47**, 116

BEHNKE J.M., WELLS C. & BROWN J. (1986) An improved technique for experimental infections with skin penetrating nematode larvae. *International Journal of Parasitology* **16**, 461

CARR A. & PRITCHARD D.I. (1987) Antigen expression during development of the human hookworm, *Necator americanus. Parasite Immunology* **9**, 219

CARROLL S.M. & GROVE D.I. (1985) Resistance of dogs to reinfection with *Ancylostoma ceylanicum* following anthelmintic therapy. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 519

ELSON C.O., EALDING W. & LEFKOWITZ J. (1984) A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. *Journal of Immunological Methods* **67**, 101

GARSIDE P. & BEHNKE J.M. (1989) Primary infection of hamsters with *Ancylostoma ceylanicum. Parasitology* **98**, 283

GARSIDE P., BEHNKE J.M. & ROSE R.A. (1989a) The immune response of male DSN hamsters to a primary infection with *Ancylostoma ceylanicum. Journal of Helminthology* **63**, 251

GARSIDE P., MCKEAN P.G., BEHNKE J.M. & PRITCHARD D.I. (1989b) An investigation of the cellular responses to hookworms in the hamster. British Society for Parasitology Spring Meeting 1989, Southampton

GILLES H.M. (1985) Selective primary health care: Strategies for control of disease in the developing world. XVII. Hookworm infection and anaemia. *Reviews of Infectious Diseases* **7**, 111

GUPTA S. & KATIYAR J.C. (1985) Immunological responses of hamsters to *Ancylostoma ceylanicum* infection. *Helminthologia* **22**, 181

MENON S. & BHOPALE M.K. (1985) Efficacy of UV-irradiated larval vaccine of *Ancylostoma ceylanicum* (Looss, 1911) in golden hamsters (*Mesocricetus auratus*). *Journal of Helminthology* **59**, 287

MILLER H.R.P. (1984) The protective mucosal immune response against gastrointestinal nematodes in ruminants and laboratory animals. *Veterinary Immunology and Immunopathology* **6**, 167

SWIETER, M.G. (1984) A histochemical study of mucosal mast cells in the gastrointestinal tracts of four rodent species. PhD thesis, West Virginia University