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Acquired resistance to the human hookworm *Necator americanus* in mice

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Summary BALB/c mice were exposed to primary or secondary infection with the hamster-adapted strain of *Necator americanus*, and the course of infection was monitored through worm recovery and immunological assays. Significantly fewer viable larvae were recovered from the skin site of reinfected mice on day 2 post-infection, and fewer larvae resided in the lungs of challenged mice 3–5 days after infection, suggesting that the skin was involved in resistance to secondary infection. The serum antibody response to L3 antigen was enhanced during secondary infection, peaking on day 9, and the bronchoalveolar leucocyte (BAL) response was more intense at this stage. Thus the secondary BAL response was initiated more promptly than the primary response, peaking on day 13 at twice the intensity of the primary response and five times above the resting level. Differential counts revealed that by far the most significant changes in cell populations were those observed for eosinophils in lavage fluid. At the peak of the response a 925-fold increase over control levels was detected in mice undergoing a challenge infection. Some cellular and serological components of the secondary response were defined in the present work and it was concluded that reinfected mice have the capacity to trap parasites during their passage through the skin and development in the lungs.

Keywords: hookworms, *Necator americanus*, acquired immunity, bronchoalveolar leucocyte response, antibodies, lungs, skin

Introduction

Hookworms are widespread among human populations living in tropical and subtropical environments, current prevalence being estimated at some 900 million individuals infected worldwide (Peters 1978). Although the epidemiology of the two major species affecting man (*Ancylostoma duodenale* and *Necator americanus*) is still incompletely understood, the parasites are known to be long-lived and people living in endemic regions are believed to acquire worms throughout life (Hoagland & Schad 1978, Banwell & Schad 1978, Roche & Layrisse 1966). Despite the lack of conclusive proof, there is increasing

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evidence that immune mechanisms may be involved in regulating hookworm populations in man (reviewed by Miller 1979, Behnke 1987a). Moreover, acquired immunity is readily demonstrable in experimental animal models of ancylostomiasis, namely *A. caninum* (Miller 1971) and *A. ceylanicum* (Carroll & Grove 1985, 1986a), both in dogs.

Unlike *A. duodenale*, *N. americanus* has no closely related animal species, and the only hosts apart from man in which *N. americanus* has been successfully maintained in the laboratory are the hamster (Sen 1972) and the rabbit (Bhopale, Menon & Renapurkar 1977, Yoshida & Fukutome 1967). However, the necessity for these animals to be infected within 2–4 days of birth imposes severe limitations on the system, particularly in relation to immunological studies (Rajasekariah *et al.* 1985). Thus the development of a suitable animal model is still necessary to enable parasite antigens to be tested for their ability to induce host responses and to facilitate the evaluation of components of potential vaccines for their protective effect against *N. americanus* before trials in human volunteers. We have recently defined the course of primary infection with *N. americanus* in BALB/c mice (Wells & Behnke 1988). In this paper we develop the model further, showing that mice acquire resistance to the invasive and lung stages of *N. americanus* and that the expression of resistance during secondary infection is associated with enhanced immune responses to the larval stages of the parasite.

Materials and methods

ANIMALS

Male BALB/c, CBA and C57BL10 mice were used in this study, and syngeneic DSN hamsters were utilized for passage of *N. americanus*. All animals were bred and maintained under conventional animal house conditions receiving food and water *ad libitum*.

PARASITE

Infective larvae of a hamster-adapted strain of *N. americanus*, originally isolated from man in India, were obtained from Dr G.Rajasekariah of Hindustan Ciba-Geigy Ltd, Bombay, India, in 1983. The parasite was maintained in Nottingham by serial passage through neonatal hamsters (Behnke, Paul & Rajasekariah 1986a).

INFECTION OF MICE AND RECOVERY OF WORMS

Animals were infected percutaneously as described by Behnke, Wells & Brown (1986b). Briefly, mice were anaesthetized with Sagatal (M & B, Veterinary Products). The abdomen was shaved and cleaned and the appropriate dose of larvae was administered on a gauze and secured on the skin with adhesive tape for 24 h. Mice were aged from 6 to 8 weeks at the start of each experiment and experimental groups comprised five to nine animals. After exposure to primary infections of 250 larvae, the mice were challenged 6 weeks later with a further 250 larvae. Control mice were exposed only to the challenge inoculum.

Parasite burdens were subsequently assessed by enumeration of larvae capable of active migration from the tissues as described by Behnke *et al.* (1986b). The animals were killed by the inhalation of chloroform, and the abdominal skin, lungs and small intestine were removed. The skin and lungs were minced with scissors and incubated at 37°C in 5-cm Petri dishes containing Hanks' saline. Larvae migrating out of the tissues were collected and counted at 2 and 6 h when the incubation medium was changed. The dishes were finally inspected at 24 h. The small intestine was split longitudinally and placed on a piece of gauze which was suspended in a 50-ml beaker containing Hanks' saline and incubated for 6 h at 37°C. The larvae were collected after sedimentation.

ANTIBODY RESPONSES

Serum antibody titres to a PBS soluble fraction of an infective L3 larval homogenate were assessed by a standard ELISA technique. The antigen was used at a concentration of 0·5 μg protein per well. Microtitration plates were incubated with serum dilutions of 10^{-1} to 10^{-3} and subsequently with goat anti-mouse polyvalent Ig (G, A, M) or class-specific Ig conjugated with alkaline phosphatase. *N*-nitrophenyl phosphate disodium hexahydrate (Sigma) was used as the substrate. The resulting optical densities were read at 410 nm on a Dynatech MR 700 Microplate Reader.

RECOVERY OF BRONCHOALVEOLAR LEUCOCYTES (BAL)

Mice were killed as before and bled from the thoracic cavity. They were dissected to expose the trachea which was ligated. Phosphate-buffered saline (0·5 ml) was infused into the lungs via a 21G needle inserted through the tracheal walls. The fluid was immediately withdrawn and the process was repeated.

The total cell number was counted separately for each mouse, the cells were pooled and used to prepare cytospin smears. Differential cell counts were made after staining with Wright's stain (a minimum of 500 cells was counted from each smear).

STATISTICAL ANALYSIS OF RESULTS

Worm recovery data are presented as group means $\pm$ s.e.m. and were compared by the non-parametric Mann–Whitney *U*-test (Sokal & Rohlf 1969).

Results

THE TIME COURSE OF PRIMARY AND SECONDARY INFECTIONS WITH *N. AMERICANUS* IN BALB/C MICE

BALB/c mice were selected for this study since previous work had implicated this strain as being consistently susceptible to infection with *N. americanus* (Wells & Behnke 1988). Groups of mice were killed at various times following primary or secondary infection, and mean worm recoveries are illustrated in Figure 1. Significantly fewer viable larvae were recovered from the skin site of reinfected mice on day 2 post-infection (p.i.) ($P < 0{\cdot}05$). In both groups the skin worm burdens declined to less than five per mouse by day 3 p.i., as

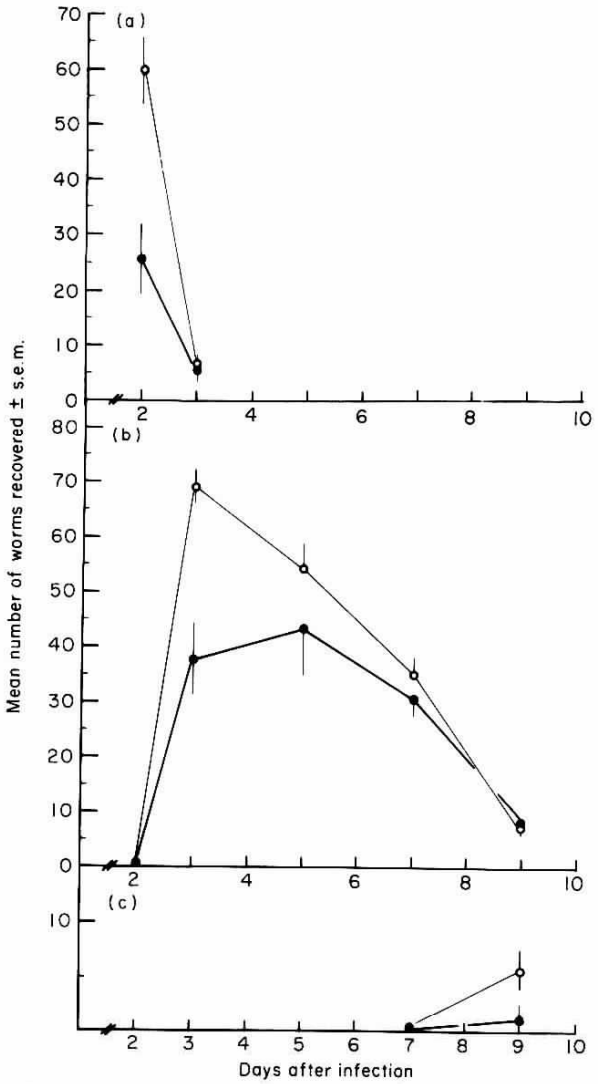

Figure 1. Recovery of *N. americanus* larvae from the skin (a), lungs (b) and intestine (c) of BALB/c mice during primary (○) and secondary (●) infections. Groups of five animals were killed for worm counts on each of the days shown.

larvae appeared in the lungs. On day 3 p.i. significantly fewer larvae had localized in the lungs of mice given secondary infections ($P < 0{\cdot}01$) but subsequently lung worm burdens declined with a similar time course in both groups, larvae first appearing in the intestine 7 days p.i. Although in this experiment worm burdens were not significantly lower in challenged mice on day 5, Table 1 summarizes the results of six further experiments

Table 1. Acquired resistance to *N. americanus* in BALB/c and other syngeneic mouse strains

Experiment	Strain	Mean worm recovery ± s.e.m.* Primary infection	Secondary infection	Percentage protection	*P*†
2	BALB/c	140·0 ± 9·8 (8)	82·9 ± 5·8 (8)	41	0·001
3	BALB/c	77·0 ± 5·8 (5)	32·4 ± 7·9 (5)	58	0·005
4	BALB/c	61·4 ± 9·1 (5)	23·8 ± 6·2 (6)	62	0·005
5	BALB/c	85·0 ± 4·9 (5)	56·0 ± 4·7 (4)	34	0·01
6	BALB/c	69·3 ± 3·2 (4)	37·8 ± 6·2 (4)	45	0·025
7	BALB/c	36·6 ± 5·5 (5)	15·8 ± 1·7 (4)	56·8	0·05
8	CBA	126·8 ± 13·2(5)	15·8 ± 4·9 (5)	87·5	0·001
9	C57 BL10	90·4 ± 5·0 (5)	8·6 ± 2·1 (5)	90·5	0·001

* All the mice in experiments 2–6 were killed 5 days p.i. for lung worm counts. Those in experiments 7–9 were killed on day 4.

† Mean worm burdens were compared by the Mann–Whitney *U*-test.

Table 2. The effect of varying the intensity of the primary infection on acquired resistance to *N. americanus*

Group	No. of larvae in primary infection	Mean worm recovery ± s.e.m.	Percentage protection
A	None	104·8 ± 11·3	—
B	50	61·3 ± 11·2	42
C	100	48·2 ± 3·0	54
D	200	23·4 ± 6·3	78
E	300	39·2 ± 7·8	63
F	400	28·8 ± 7·5	73

All the mice were killed 5 days post-challenge.

Statistical analysis: Group A *vs* group B (Mann–Whitney *U*-test) $P = 0{\cdot}05$; correlation coefficient $r = 0{\cdot}675$ ($P < 0{\cdot}01$).

comparing lung worm burdens on days 4 or 5 p.i. in BALB/c mice and includes experiments in which CBA or C57BL10 mice were investigated. Despite the variation in percentage protection (ranging from 34 to 90·5%) each represents a significant reduction in parasite numbers. After emergence from the lungs, a few larvae established in the

intestine of control mice (Figure 1) peaking in numbers on day 9 ($5{\cdot}8 \pm 4{\cdot}0$) and declining to zero by day 16 (data not shown). Challenged mice only harboured the occasional intestinal worm. In both groups, intestinal parasites, whilst clearly possessing the features of L4 stages, were abnormal, showing little further growth during residence in the gut.

THE EFFECT OF INCREASING THE INTENSITY OF PRIMARY INFECTION ON ACQUIRED RESISTANCE TO *N. AMERICANUS* IN BALB/C MICE

An experiment was carried out to determine whether the degree of protection from challenge infection was dependent on the intensity of the primary infection. Thirty BALB/c mice were divided into six groups which received respectively 0, 50, 100, 200, 300 or 400 infective larvae on day 0. Six weeks later the animals were all exposed to 250 L3 and killed 5 days later for the assessment of lung worm burdens. The results are presented in Table 2, where it can be seen that even 50 L3 induced significant resistance to challenge infection ($P < 0{\cdot}05$). Maximum protection was apparently achieved when an initial dose of 200 larvae was given (77·7%) and this was not improved with higher doses. However, overall there was a significant correlation between the intensity of the primary infection and the degree of protection observed after challenge (correlation coefficient $r = 0{\cdot}675$, significant at $P < 0{\cdot}01$).

THE EFFECT OF VARYING THE SITE OF ADMINISTRATION OF CHALLENGE INFECTION LARVAE

It was of interest to establish whether resistance to challenge infection was influenced by local changes in the skin persisting from the primary exposure to infective larvae. Groups of male BALB/c were exposed to a primary infection given on the back and were challenged by larvae given either on the same site or on the abdomen. The results (Table 3) show that mice were equally susceptible to infection irrespective of the route of larval administration (group A *vs* group C) and that the site of challenge did not influence the degree of protection (group B *vs* group D). This experiment was repeated with similar results.

Table 3. Effect of varying the site of administration of challenge infection larvae

	Site of larval administration		Mean no. worms recovered		
Group	Primary infection†	Secondary infection‡	±s.e.m.	Percentage protection	*P*
A	None	Back	77·0 ± 5·9		
B	Back	Back	32·4 ± 7·9	57·9	*vs* A: 0·01
C	None	Abdomen	61·4 ± 9·1		*vs* A: ns
D	Back	Abdomen	23·8 ± 2·8	61·2	*vs* C: 0·01
					vs B: ns

* All groups were comprised of five male BALB/c mice.
† With 240 L3.
‡ With 200 L3.

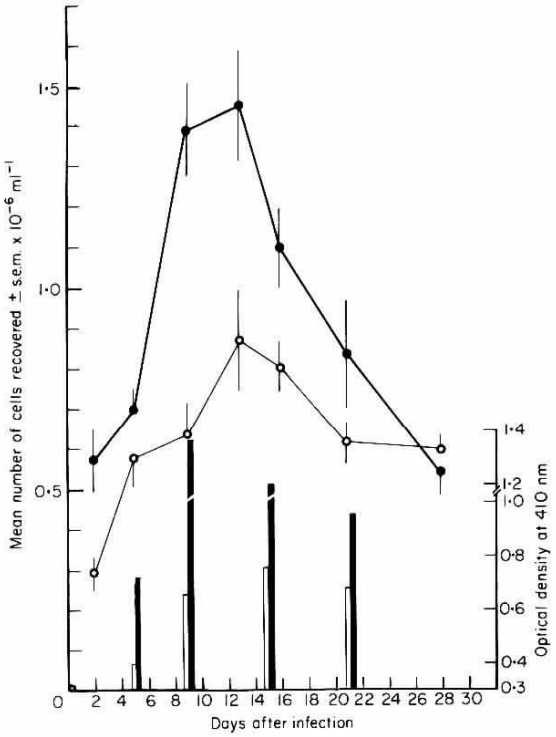

Figure 2. The bronchoalveolar leucocyte and antibody responses during primary (○,□) and secondary (●,■) infection with *N. americanus* in BALB/c mice.

THE BRONCHOALVEOLAR LEUCOCYTE RESPONSE OF BALB/C MICE TO PRIMARY AND SECONDARY INFECTION WITH *N. AMERICANUS*

The passage of larvae through the mouse lungs induced a marked inflammatory reaction. Typical changes observed in the total number of BAL recovered are summarized in Figures 2 and 3. Secondary infections were accompanied by a significantly enhanced BAL response which was initiated more promptly than the primary, peaking on day 13 at twice the intensity of the primary response and five times above the resting level. As expected, the neutrophil response was the most rapid peaking on day 5 in reinfected mice at 38 times the level in naive controls. The most dramatic changes were recorded for eosinophils. At the peak of the response on day 13 a 925-fold increase over control levels was observed in challenged mice, compared to a 415-fold increase during primary infection. Thus eosinophils comprised 58 and 72% of the total BAL in primary and challenge infection mice respectively, compared to less than 1% in naive controls. Although macrophages showed the least relative changes, these cells were the most numerous among BAL at all times except days 9, 13 and 16 when only eosinophils outnumbered them. In naive uninfected mice macrophages represented 98·7% of BAL but at the peak of the

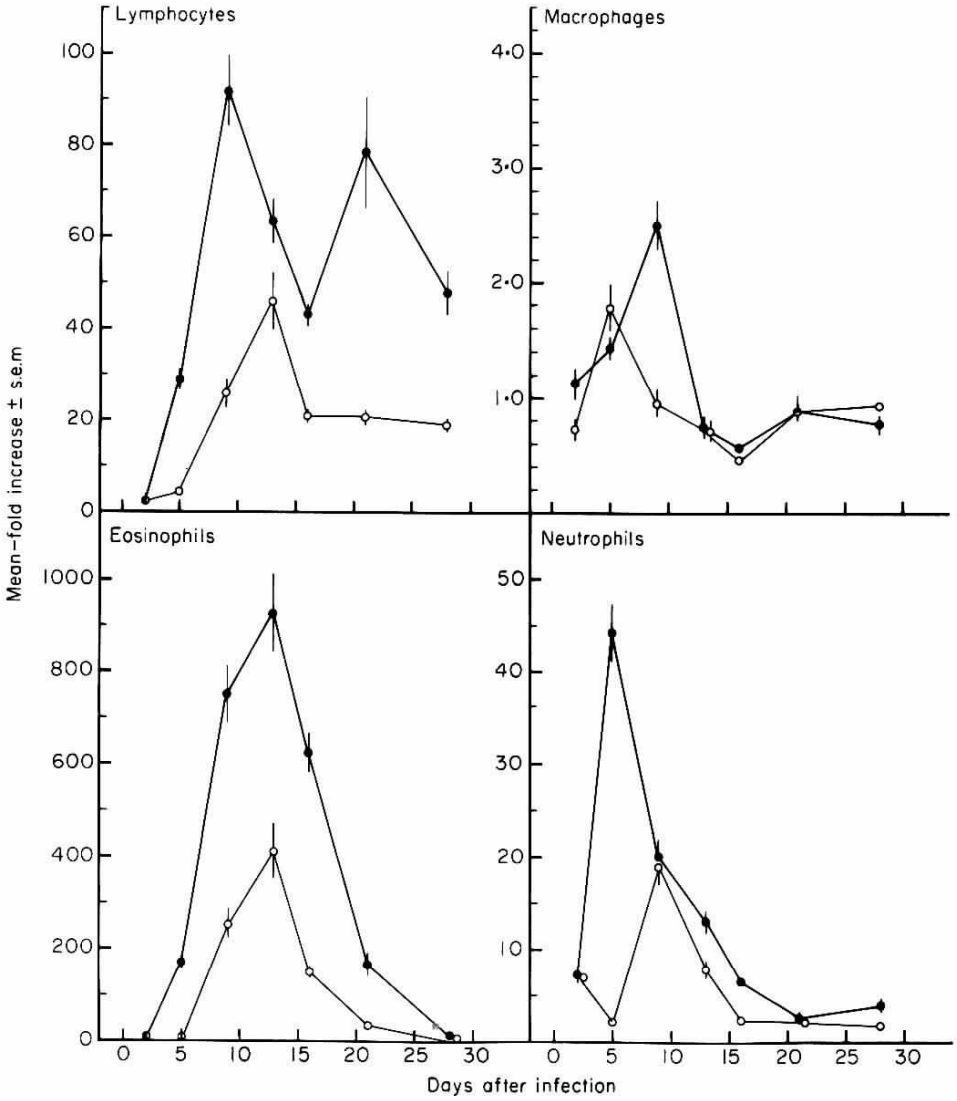

Figure 3. The bronchoalveolar leucocyte response during primary (○) and secondary (●) infection with *N. americanus* in BALB/c mice. The results are expressed in terms of the mean-fold increase over values in naive mice ± s.e.m. The number of cells/ml of bronchoalveolar lavage fluid in naive control mice was as follows: $0{\cdot}16 \pm 0{\cdot}05 \times 10^4$ lymphocytes, $2{\cdot}37 \pm 0{\cdot}68 \times 10^5$ macrophages, $0{\cdot}08 \pm 0{\cdot}02 \times 10^4$ eosinophils and $0{\cdot}08 \pm 0{\cdot}02 \times 10^4$ neutrophils. The total leucocyte count in BAL was $2{\cdot}40 \pm 0{\cdot}69 \times 10^5$.

macrophage response in reinfected mice (day 9) they represented 17% despite showing a 2·5-fold increase over naive controls. The most persistent response was detected in the lymphocyte population where 19-fold and 57-fold increases above control levels were still evident on day 28 in mice given primary and secondary infections respectively. Lymphocytes peaked in reinfected mice on day 9 and in mice given primary infections on day 13, but in the former a second peak was also recorded on day 21.

SPECIFIC ANTIBODY RESPONSE TO PRIMARY AND SECONDARY INFECTIONS

The time course of the antibody response to larval antigens is shown for comparison, beneath the BAL response in Figure 2. It is clear that the specific antibody response was markedly enhanced during the secondary infection, with a twofold increase over the primary infection group on day 9 p.i. Further measurements were made of this time point and the results are presented in Table 4. Challenged mice showed a significantly enhanced antibody response in each experiment.

The antibody isotype responses are given in Figure 4. IgM responses in both primary and secondary infections were transient, peaking on day 9 and subsiding quickly to background levels. The secondary IgA response likewise peaked on day 9 but subsided more slowly. The primary IgA response, although not as intense, was longer lasting. No dramatic changes were seen in IgG antibodies, although the antibody levels were significantly elevated in challenged mice throughout infection.

Table 4. The antibody response of BALB/c mice 9 days following primary or secondary infection with *N. americanus*

Mean optical density ± s.e.m. measured by ELISA in mice given*	
Primary infection	Secondary infection
1·019 ± 0·03	1·95 ± 0·05
0·309 ± 0·01	1·096 ± 0·06
0·502 ± 0·06	1·020 ± 0·08

* The serum of each mouse was measured separately in triplicate and the mean used to derive the overall mean for the group. Sera were tested at a dilution of 10^{-2} using plates coated with 0·5 μg of L3 antigen per well.

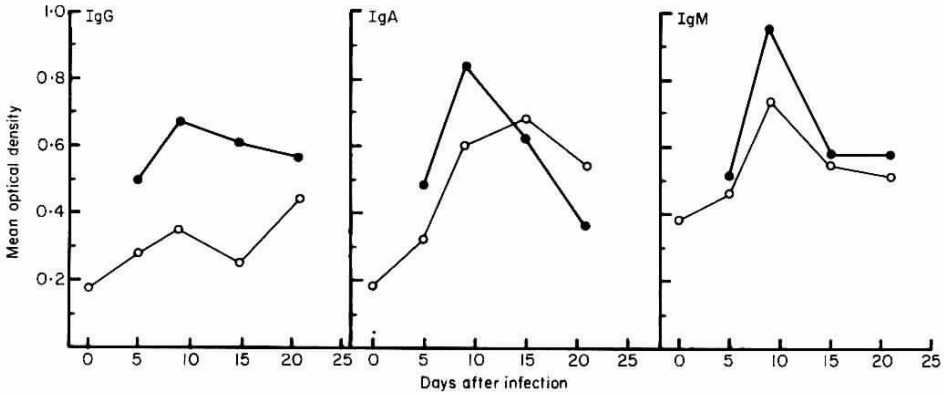

Figure 4. The class-specific antibody response during primary (○) and secondary (●) infection with *N. americanus* in BALB/c mice. The figure shows the mean optical density measured using ELISA. Groups of five mice were killed at each time point and the antibody response was determined in triplicate on pooled serum samples.

Discussion

The characteristic chronic intestinal infections of hookworms in man and the apparent lack of immunity in repeatedly infected volunteers (Ball & Bartlett 1969, Ogilvie *et al.* 1978) have often led to the conclusion that protective immunity in man operates weakly at best. Mechanisms for active evasion of the host's response have been documented in many parasitic infections (reviewed by Behnke 1987b) and it is likely that hookworms also employ strategies for evading or suppressing host protective responses (Hotez, Le Trang & Cerami 1987). However, the fact that immunity in other animal-hookworm systems is universally demonstrable and that the human host does respond immunologically to infection (Miller 1979, Hotez *et al.* 1987), both suggest that man is unlikely to have a unique relationship with these worms. The absence of sterile immunity under field conditions may be a function of the balance between the acquisition of parasites, and the time required to bring about their loss, rather than an innate inability of man to mount the appropriate responses.

From existing studies on experimental animals, it is apparent that hookworms are highly antigenic, inducing specific antibody against the various life-cycle stages (Pritchard *et al.* 1986, Carr & Pritchard 1986, Carroll & Grove 1985). Studies on infected volunteers (Ball & Bartlett 1969, Ogilvie *et al.* 1978, Maxwell *et al.* 1987, Carroll & Grove 1986b) and on naturally infected individuals (Kumar *et al.* 1980, Biroum-Noerjasin 1973, Grove, Burston & Forbes 1974, Villarejos *et al.* 1975) demonstrate marked serological reactivity to hookworms, parasite antigens, eosinophilia and possible cellular involvement (Taylor & Turton 1976, Maxwell *et al.* 1987). However, a strong correlation between these phenomena and host protective immunity has yet to be conclusively established.

Mice fail to support the development of adult *N. americanus*, but nevertheless provide an environment in which the invasive, migratory and pulmonary stages of the parasite develop normally (Wells & Behnke 1988) and in which the accompanying immune

responses can be investigated. These have relevance to man; creeping eruptions, reflecting cutaneous reactivity to hookworms (Ball & Bartlett 1969, Beaver 1945, 1956) and pulmonary symptoms (Areekul, Radomyos & Viravan 1970) are often encountered after exposure to hookworm larvae. Furthermore, there is evidence to suggest that the lung may be a particularly important site for the development and expression of host protective responses in the case of *A. caninum* in dogs (Miller 1965).

Our experiments in mice demonstrated that *N. americanus* larvae elicited acquired resistance in this host and suggested that both the skin and lung sites were involved in parasite attrition. Relatively few live larvae were recovered from the skin of challenged mice 48 h after infection and significantly fewer accumulated in the lungs on days 3–5, when signs of pulmonary trapping were minimal (C. Wells & J.M. Behnke, unpublished observations). Prelung, presumably skin site, resistance is therefore implicated. In support of this conclusion we have also observed that the sites to which challenge infection larvae were applied developed intense erythema and swelling (C. Wells & J.M. Behnke, unpublished observations).

Typical secondary responses were observed in the quantity and quality of BAL recovered from the lungs of mice undergoing challenge infection. Our results were similar to those previously reported by Egwang, Gauldie & Befus (1984a) in response to the pulmonary migration of *Nippostrongylus brasiliensis* in the rat, and may therefore reflect a common response to damage inflicted on the lungs by migrating nematode larvae. Following percutaneous infection, *N. americanus* larvae arrive in the lungs later than *N. brasiliensis* (36–48 h in contrast to 18 h; Wells & Behnke 1988, Gharib 1961a) and do not appear in the intestine until day 7 (compared to 48 h for *N. brasiliensis*; Tuohy 1956, Gharib 1961b). The secondary BAL response to *N. americanus*, which by day 6 was already intense, particularly with respect to neutrophils, may have resulted in some larvae becoming trapped within the lungs during the final stages of their pulmonary development (i.e. days 6–9). Entrapped parasites were observed in lung tissues at this time, both on freshly squashed lung preparations and on histological sections (C. Wells, M. Wilkinson & J.M. Behnke, unpublished observations).

The serum antibody response was elevated in challenged mice. Changes were observed in IgA, IgM and IgG antibodies, bearing some similarity to results reported by Carroll & Grove (1985) albeit on a considerably shorter time scale. Both studies identified transient IgM responses and a generally higher concentration of IgG antibodies in challenged mice, with relatively slow changes in response to either primary or secondary infection. We also detected an IgA response, but this did not persist after challenge in contrast to the canine response to *A. ceylanicum* (Carroll & Grove 1985) possibly because there was no persistent intestinal infection in mice.

At first sight the model described in this paper would appear to be distantly removed from the system of most relevance, namely *N. americanus* in man. However, we believe this to be the only in-vivo model in which immunity to *N. americanus* can be investigated. We have established that BALB/c mice acquire immunity to *N. americanus* and we have defined some aspects of the anamnestic response to challenge, emphasizing those features which resemble the *N. brasiliensis* system and distinguish our model from previous comparable work on hookworms. Several important issues still remain to be resolved, particularly the relative importance of skin versus lung attrition of challenge infection larvae. An acceptable vaccine against hookworms may need to be effective against the larval stages, thus preventing the establishment and development of the more pathogenic

L4 and adult worms. The mouse *N. americanus* model should therefore prove useful in screening candidate antigens and cloned gene products (Carr & Pritchard 1986) for their ability to induce larvicidal responses in an in-vivo system, prior to incorporation into vaccines for human trials.

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