Necator americanus in inbred mice: a re-evaluation of primary infection kinetics

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SUMMARY

The course of a primary *Necator americanus* infection was studied in the lungs and small intestines of syngeneic mice. Following percutaneous infection no difference in initial larval establishment in the lungs was found between male BALB/c, NIH or B10.G mice. However, significant differences in the subsequent kinetics of infection were demonstrated between the BALB/c and NIH strains. Lung worm burdens declined more slowly in NIH mice than in BALB/c strain. Surprisingly, however, a greater proportion of larvae remaining in the lungs of BALB/c mice, 9 days p.i., were trapped than in NIH mice. Nevertheless, establishment in the small intestines of the BALB/c strain was consistently greater than in NIH mice. Host immunosuppression resulted in increased larval retention in the lungs of both the BALB/c and NIH strains as well as in the small intestines of BALB/c mice. Treatment with hydrocortisone acetate did not increase intestinal worm burdens in NIH mice. The data presented suggest that, in this complex, dynamic model system, designation of 'susceptible' and 'resistant' strains is inappropriate. The factors underlying the observed strain differences in resistance to infection are discussed.

Key words: *Necator americanus*, hookworm, model, mice, infection kinetics.

INTRODUCTION

Human hookworm infections remain a major cause of morbidity in most tropical and subtropical regions. The two major species affecting man (*Necator americanus* and *Ancylostoma duodenale*) are exquisitely host specific and this has resulted in limited study of the host–parasite relationships. Recent work, however, has highlighted the merits of the mouse as a model host for investigation of the invasive and migratory stages of infection (Wells, 1988; Wells & Behnke, 1988*a*, *b*; Wilkinson, Wells & Behnke, 1990).

Wells & Behnke (1988*b*) described the course of a primary infection in inbred mice and found the route of migration, following percutaneous infection, to be direct and simple. Although adult worms were never observed, L_4 stages were recovered from the small intestines of fully immunocompetent, adult mice and limited growth of these worms occurred. Other experimental models of human necatoriasis have been proposed in which complete development of the parasite occurs, but chronic infections – analogous to those in humans – have only been achieved after infection of neonatal hamsters (Sen & Seth, 1967, 1970; Sen, 1972; Behnke, Paul & Rajasekariah, 1986*a*; Behnke & Pritchard, 1987) and rabbits (Bhopale, Menon & Renapurkar, 1977; Bhopale, Menon & Kulkarni, 1980; Bhopale, Kamath & Menon, 1984) or primates (Orihel, 1971). However, the immunological immaturity of neonates obviates investigation of the host response during the early stages of infection and ethical considerations, as well as the cost of housing and maintenance, prohibit extensive studies involving primates.

In light of the potential value of the mouse–*N. americanus* model, a re-evaluation of the primary infection kinetics was undertaken in the key BALB/c and NIH strains which have been identified as relatively susceptible and resistant respectively (Wells & Behnke, 1988*b*). Differences in susceptibility to infection, between inbred strains of mice, may be multi-factorial in origin. However, it has been suggested that genetically determined differences in the ability to mount an effective immune response may be involved in the observed predisposition of individuals, in human populations, to hookworm infection (Schad & Anderson, 1985). Thus, to investigate the importance of the innate immune response in resistance to infection with *N. americanus* the effects of host immunosuppression were investigated. The data presented in this paper suggest that, in this complex, dynamic model system, the arbitrary designation of 'susceptible' and 'resistant' strains is inappropriate or at least an oversimplification of murine responsiveness to *N. americanus* infection.

MATERIALS AND METHODS

Animals

Specific pathogen-free mice of the inbred BALB/c, NIH and B10.G strains were obtained from Harlan-Olac Ltd, Bicester, Oxon. In Exps 1–4 (see Tables 1

and 2), syngeneic BALB/c and NIH mice were bred and maintained in the Department of Life Science, University of Nottingham. All mice were male and housed under conventional conditions. Animals were approximately 6 to 9-weeks-old when infected.

Parasite

Infective *N. americanus* larvae were obtained by routine passage through neonatal hamsters at the University of Nottingham (Behnke *et al.* 1986*a*). The original isolate was obtained from Dr G. Rajasekariah of Hindustan CIBA-GEIGY Ltd, Bombay, India, in 1983, and had undergone serial passage through 69 generations of neonatal hamster.

Infection of mice and worm recovery

Animals were infected with *N. americanus* L_3 as described by Behnke, Wells & Brown (1986*b*). Briefly, mice were anaesthetized with Sagatal (May and Baker, Veterinary Products), the dorsal thorax shaved and 250 infective larvae applied, to the exposed area, on a gauze and secured with adhesive tape. The gauzes were left in place for 24 h. Enumeration of actively migrating, lung-stage *N. americanus* larvae was carried out according to the method of Behnke *et al.* (1986*a*). After necropsy, the lungs were removed and incubated at 37 °C in Hanks' Balanced Salt Solution (HBSS). The lungs were finely minced with scissors and larvae which had migrated from the tissue were recovered after 2, 6 and 24 h. Since accurate retrieval of all fragments of minced lung tissue from the incubation medium would be impracticable total *N. americanus* burdens were determined separately. Lung tissue, which had been squashed between two microscope slides, was meticulously examined and the total larval burdens determined by counting the worms *in situ* (Wilkinson *et al.* 1990). Intestinal (L_4) stages were recovered using a modified Baermann technique (Behnke *et al.* 1986*a*). The entire small intestine was removed, opened longitudinally and closely inspected for the presence of any 5th-stage worms. Each intestine was then suspended in a 50 ml beaker containing HBSS. Beakers were incubated for 4–6 h at 37 °C and the larvae which had emerged into the saline were counted.

Treatment with hydrocortisone acetate

Where indicated, injections of hydrocortisone 21-acetate (Sigma), suspended at a concentration of 25 mg/ml in sterile distilled water, were administered subcutaneously.

Statistical analysis

Data are presented as group means ± standard error of mean (S.E.M.). Statistical comparisons were made using a non-specific unified rank analysis (Meddis, 1984) and values of $P \leqslant 0{\cdot}05$ were considered significant.

RESULTS

Time-course of N. americanus *infection in the lungs and small intestines of BALB/c and NIH mice*

The time-courses of larval recovery from the lungs and small intestines, of each strain, are shown in Fig. 1 A and B respectively. No significant difference was found between the establishment of larvae in the lungs of BALB/c and NIH mice on days 2, 3 or 4 post-infection (p.i.). On day 2 p.i., the mean recovery from the lungs of BALB/c mice corresponded to 39·8 % of the infective dose and to 38·7 % in the NIH strain. Mean lung worm recovery peaked, in BALB/c mice, on day 3 p.i., at 41·1 % of the infective dose. In the NIH strain, peak numbers of lung worms were recovered 5 days p.i. (46·4 % of the infective dose). There was little difference, however, between the mean numbers of worms recovered from the lungs of NIH mice on days 3 ($114{\cdot}2 \pm 16{\cdot}1$) and 5 ($115{\cdot}4 \pm 6{\cdot}8$) p.i. In BALB/c mice, lung worm burdens had begun to decline by day 5 p.i. and there was a significant difference in lung worm recovery between the two strains at this time-point. This difference persisted, and by day 9 p.i. only 13·9 % of the infective dose could be recovered from the lungs of BALB/c mice in comparison to 27·8 % from NIH mice.

In BALB/c mice, intestinal worm recovery peaked on day 9 p.i. at 5·5 % of the infective dose. In the NIH strain only one intestinal worm was recovered during the course of the experiment, on day 9 p.i. All worms recovered from the small intestine were 4th-stage larvae, no adult worms were observed.

Comparison of larval establishment in the lungs of BALB/c, NIH and B10.G mice

Establishment of *N. americanus* in the lungs of BALB/c and NIH mice was further assessed in 6 subsequent experiments. The results of these comparisons are presented in Table 1.

No significant difference between initial establishment of larvae in the lungs of BALB/c and NIH mice was found in Exps 1, 2, 5 and 6. In Exp. 6, the numbers of larvae recovered from the lungs of both strains had declined by day 5 p.i.; however, significantly fewer larvae were recovered from the BALB/c strain than from NIH mice. These data broadly concur with the time-course of infection depicted in Fig. 1 A and B. In Exp. 5, initial larval establishment in the B10.G strain was not found to differ significantly from that in BALB/c mice.

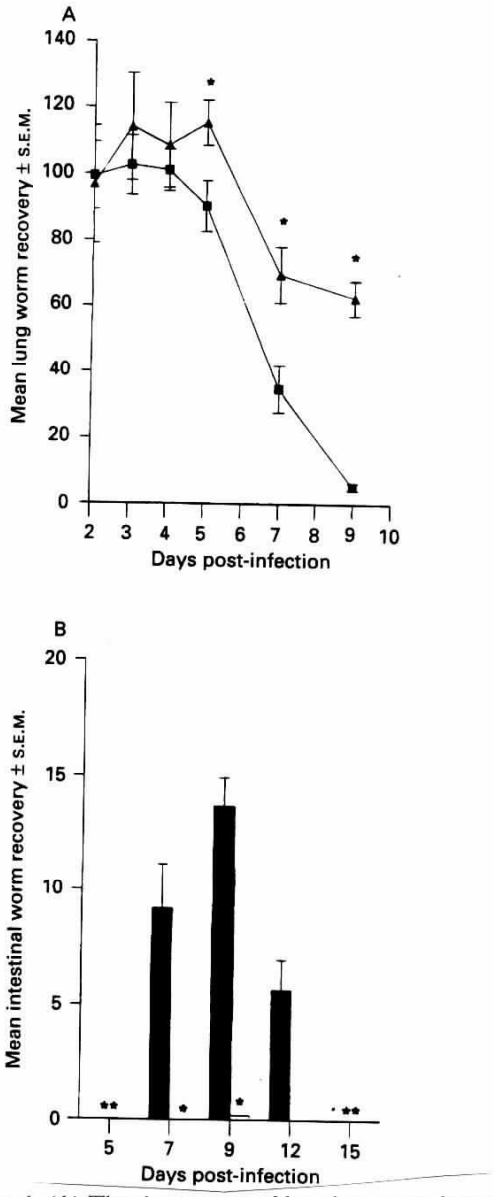

Fig. 1. (A) The time-course of larval recovery from the lungs of male BALB/c (■) and NIH (▲) mice. Worm burdens are expressed as group means ± S.E.M. (n = 4–6). Mice were infected with 250 *Necator americanus* L_3 on day 0. Worm recoveries, at each time-point, were compared according to a non-specific unified rank analysis (Meddis, 1984), $*P \leqslant 0{\cdot}001$. (B) The time-course of larval recovery from the small intestines of male BALB/c (■) and NIH (□) mice. Worm burdens are expressed as group means ± S.E.M. (n = 5–6). Animals correspond to those in (A).
* Fewer than 1 worm/animal recovered.

Only in Exp. 4 was larval establishment in the lungs of BALB/c mice found to be significantly greater than in the NIH strain on day 4 p.i. In Exp. 3, a significantly greater number of larvae were recovered from the lungs of NIH mice, than from BALB/c mice, at this time-point.

Comparison of larval entrapment in the lungs of BALB/c, NIH and B10.G mice

Since no clear patterns of strain susceptibility to infection could be demonstrated, on the basis of initial larval establishment in the lungs, a further parameter was examined. Preliminary experiments have demonstrated bilaterally symmetrical distribution of larvae in the lungs of BALB/c and NIH mice, thus, the number of actively migrating larvae in the right lung was compared to total larval burden in the left lung, of individual animals, on day 9 p.i. and an index of trapping calculated (see Table 2). In Exp. 4 the index of trapping recorded for the NIH strain was lower than that in BALB/c mice, although not significantly different. A further comparison was carried out in Exp. 5 and, on this occasion, significantly less larval entrapment occurred in the lungs of NIH mice than in BALB/c mice. In both these experiments, however, the numbers of larvae counted, in the right and left lungs, were greater in the NIH strain than in BALB/c mice. The index of trapping in B10.G mice was not found to differ significantly from that in BALB/c mice (Exp. 5).

The effect of host immunosuppression on a primary N. americanus *infection*

The effect of host immunosuppression on a primary *N. americanus* infection was evaluated, at various time-points, in the BALB/c and NIH strains. All animals were infected on day 0, untreated groups were naïve when infected and treated groups received 0·1 ml of hydrocortisone acetate suspension on days −3, −1, 1 and 3.

Larval establishment in the lungs of untreated animals, on day 4 p.i., was not found to differ significantly between the BALB/c and NIH strains (Table 3). By day 9, however, a significantly greater number of larvae was recovered from the lungs of the untreated NIH group in comparison to the untreated BALB/c group ($H = 7{\cdot}79$, 1 D.F., $P \leqslant 0{\cdot}0001$). In addition, significantly more 4th-stage larvae were recovered from the intestines of untreated BALB/c mice than from the untreated NIH group at this time point (cf. Fig. 1A and B).

On day 4 p.i., larval establishment in the lungs, of the immunosuppressed BALB/c group, was significantly increased ($H = 4{\cdot}43$, 1 D.F., $P \leqslant 0{\cdot}0001$) in comparison to the untreated control group. There was also a greater mean establishment of larvae in the lungs of the treated NIH mice, when compared to the control NIH group, although this was not significant. By day 9, however, a significantly greater number of worms was harboured in the lungs of both BALB/c ($H = 8{\cdot}67$, 1 D.F., $P \leqslant 0{\cdot}0001$) and NIH ($H = 7{\cdot}50$, 1 D.F., $P \leqslant 0{\cdot}0001$) treated groups in comparison to the respective untreated controls. In the small intestine, at this time-point, a significantly

Table 1. Comparison of the initial establishment of *Necator americanus* in the lungs of male BALB/c and NIH mice

Experiment number	Strain	*n*	Day post-infection	Worm recovery ±S.E.M.	Statistical analysis* ($P <$)
1	BALB/c	3	4	167·0±16·1	
	NIH	4		186·0±13·0	N.S.
2	BALB/c	4	4	114·2±7·6	
	NIH	6		112·0±14·5	N.S.
3	BALB/c	6	4	84·4±12·1	
	NIH	6		111·8±8·4	0·0002
4	BALB/c	5	4	204·8±10·8	
	NIH	6		152·0±10·5	0·0001
5†	BALB/c	7	4	97·3±14·1	
	NIH	6		94·0±7·3	N.S.
	B10.G	6		98·7±9·7	N.S.
6	BALB/c	5	3	134·2±5·4	
	NIH	5		136·6±8·3	N.S.
6	BALB/c	5	5	83·4±11·7	
	NIH	5		111·2±12·0	0·0001

* In each experiment, BALB/c and NIH lung worm burdens were compared using a non-specific unified rank analysis (Meddis, 1984) and values of $P \leqslant 0{\cdot}05$ were considered significant. N.S., No significant difference.
† In Exp. 5, B10.G mice were included as a further comparison. BALB/c and B10.G lung worm burdens were compared as described above.

Table 2. Comparison of the larval entrapment in the lungs of BALB/c, NIH and B10.G mice on day 9 post-infection

			Worm recovery ±S.E.M.			
Experiment*	Strain	*n*	Active migrating larvae (right lung)†	Total larvae (left lung)‡	Index of trapping ±S.E.M.§	Statistical analysis‖ ($P <$)
4	BALB/c	6	12·3±2·0	23·0±6·1	36·8±11·9	
	NIH	6	44·7±4·2	58·5±4·4	22·9±5·9	N.S.
5	BALB/c	6	1·5±0·4	6·7±1·1	74·2±6·2	
	NIH	6	31·5±1·5	33·5±3·5	9·6±5·7	0·0001
	B10.G	5	0·6±0·4	1·4±0·2	60·0±24·5	N.S.

* Experiment numbers correspond to those in Table 1.
† Migrating *Necator americanus* recovered from minced lungs during incubation for 24 h in modified Hanks' Balanced Salt Solution.
‡ Total *N. americanus* burdens of the left lungs were determined by direct examination of squashed tissue.
§ The index of trapping was calculated, for each animal, as:

$$100-\left(\frac{\text{migrating larvae in right lung}}{\text{total larvae in left lung}}\times 100\right).$$

‖ In each experiment the indices of trapping were compared using a non-specific unified rank analysis (Meddis, 1984) and values of $P \leqslant 0{\cdot}05$ were considered significant. In Exp. 5, the indices of trapping for both the NIH and B10.G strains were compared, as described above, with the values for BALB/c mice.

greater number of 4th-stage larvae ($H = 6{\cdot}7$, 1 D.F., $P \leqslant 0{\cdot}0001$) was recovered from the treated BALB/c mice than from the untreated controls. In NIH mice, fewer intestinal worms were found in the treated group than in untreated control animals. It should be noted, however, that only a very small number of intestinal worms were recovered from NIH mice, no more than 4 worms/animal were recovered from the untreated group and no worms were recovered from the intestines of 3 animals in this group. On day 15 p.i., the group mean number of 4th-stage larvae recovered from the small intestines of treated BALB/c mice represented 13·5% of the infective dose. No intestinal worms were recovered from the

Table 3. The effect of treatment with hydrocortisone acetate on a primary *Necator americanus* infection in male BALB/c and NIH mice

Day post-infection	Strain	Lung worm recovery ±S.E.M.		Intestinal worm recovery ±S.E.M.	
		Untreated*	Treated†	Untreated	Treated
4	BALB/c	61·0±5·6	90·0±8·7‡	N.D.	N.D.
	NIH	67·2±8·4	80·0±7·6‡	N.D.	N.D.
9	BALB/c	6·0±0·6	56·3±2·8	6·0±1·6	18·0±2·6
	NIH	26·3±5·7	95·4±2·7‡	1·0±0·6	0‡
15	BALB/c	N.D.	N.D.	0‡	33·8±2·3‡
	NIH	N.D.	N.D.	0‡	0‡

* Untreated animals were percutaneously infected with 250 *N. americanus* on day 0 and were naïve when infected.
† Animals were infected (as in * above) and received 0·1 ml of hydrocortisone acetate suspension on days −3, −1, 1 and 3.
‡ $n = 5$, in all other cases $n = 6$.
N.D., Not done.

control BALB/c group or from either the treated or untreated NIH groups at this time-point. All worms recovered from the small intestines were 4th-stage larvae; no adult (L_5) worms were seen.

DISCUSSION

The rigorous host specificity of *N. americanus* has precluded the development of model systems which mimic the complete disease process. Only recently has the value of discontinuous model systems – in which part of the parasite life-cycle can be studied – been recognized (Carroll, 1990). Overall, the data presented in Fig. 1 confirm that the course of infection in mice is abbreviated and the transient population of 4th-stage larvae, which established in the small intestine, was rapidly expelled. Peak numbers of lung worms were recovered from BALB/c mice on day 3 p.i. confirming previous studies (Wells, 1988; Wells & Behnke, 1988*a*, *b*).

In contrast to earlier studies (Wells, 1988; Wells & Behnke, 1988*b*), however, no significant difference was found between lung worm burdens, in the BALB/c and NIH strains on days 2–4 p.i. Previously, BALB/c and NIH mice had been designated relatively susceptible and resistant, respectively, on the basis of 'initial establishment' of *N. americanus* in the lungs. However, in the majority of comparisons no distinction was made between lung worm burdens assessed on days 4 or 5 p.i. From the data presented here it is evident that, although no difference in establishment could be demonstrated on day 4, a significant difference – in larval recoveries from the lungs – became apparent by day 5 p.i. Since very few active larvae can be recovered from the skin after day 2 and a direct route of migration has been confirmed (Wells, 1988; Wells & Behnke, 1988*b*), it is likely that this reflects variation in the subsequent rate of migration from the lungs and not in establishment at this site.

Indeed, almost all the parameters by which resistance to primary helminth infections can be measured show genetically determined variation (Wakelin, 1985). Clear differences in the kinetics of expulsion have, for example, been found between syngeneic and hybrid strains of mice, following a primary *Heligmosomoides polygyrus* infection (Wahid, Robinson & Behnke, 1989) and variation in acquired resistance to this nematode has been demonstrated between individual, outbred CFW mice (Bartlett & Ball, 1972). Studies such as these have been construed as indirect evidence for the contention that genetically determined factors may be involved in individual predisposition to hookworm infection in humans (Schad & Anderson, 1985). Differences in susceptibility to infection are likely to have their origin in a variety of factors, e.g. ecological, social, behavioural and genetic, either alone or in combination (Schad & Anderson, 1985). However, determination of the relative contributions of these factors, to predisposition in the field, is extremely difficult. Thus, if inbred strains of mice differ in susceptibility to *N. americanus* infection, the range and availability of genetically defined syngeneic and congenic strains might ultimately enable exposition of the genetic basis of innate and acquired resistance to the invasive and migratory stages of hookworm infection.

In light of the potential value of this approach and in an attempt to resolve the conflict between the data presented here and previous work (Wells, 1988; Wells & Behnke, 1988*b*), further comparisons of initial larval establishment in the lungs of BALB/c and NIH mice were carried out. Overall the data confirmed that there was no significant difference between initial establishment of *N. americanus* in the lungs of male BALB/c and NIH mice. In addition,

the results of Exp. 6 clearly confirm that, although no difference in initial establishment could be found, a significant difference in lung worm burdens was evident by day 5 p.i. In only two experiments was a significant difference, on day 4 p.i., found. However, in one of these, establishment was lower in the BALB/c strain and in the other in NIH mice.

The possibility of strain differences in larval entrapment in the lungs of mice, following a primary *N. americanus* infection, has not previously been investigated. However, increased larval entrapment in the lungs of mice, during secondary *N. americanus* infections, has been described and is thought to result from the more rapid onset of an augmented, specific immune response to these parasites (Wilkinson *et al.* 1990; Timothy *et al.* 1992). Surprisingly, comparison of the indices of trapping, calculated for the BALB/c and NIH strains, revealed that a greater proportion of the worms remaining in the lungs of BALB/c mice, at this time-point, were trapped than of those in the lungs of NIH mice. So, although a greater absolute number of larvae were recovered from the lungs of NIH mice, these larvae retained the ability to migrate actively from the tissue, *in vitro*, and therefore presumably to continue migration *in vivo*. If, then, larvae remain viable in the lungs of NIH mice and after prolonged residence, at this site, undergo migration to the intestine, rapid expulsion must follow. On this basis, NIH mice must be considered more resistant to the intestinal stages of infection.

In order to determine whether these manifestations of resistance to infection were immunologically mediated, the effect of host immunosuppression was investigated. Increased larval establishment in the lungs, of both strains, was found on day 4 p.i., suggesting that resistance at a pre-lung (presumably skin) site was immunologically mediated. Interestingly, however, a significantly greater number of active larvae was recovered from the lungs of both immunosuppressed groups, in comparison to the respective untreated controls, as late as day 9 p.i. Comparable findings have been reported in the *Ancylostoma caninum*–mouse system (Sen, Joshi & Seth, 1965; Srilakshmi *et al.* 1982); increased persistence of larvae, in the lungs, was described following immunosuppression. If, however, larval retention in the lungs was the direct result of an innate, local response an increased rate of migration through the lungs, of immunosuppressed animals, would be expected. It is conceivable, therefore, that *N. americanus* requires an appropriate host-derived stimulus to trigger onward migration from the lungs, at least in the mouse, and that treatment with cortisone weakens this signal. In untreated BALB/c mice the signal may be adequate whereas in NIH mice there may be an insufficiency, explaining the extended sojourn of larvae in the lungs of the latter strain.

Alternatively, morphological and/or physiological incompatibility may be responsible for the persistence of larvae in the lungs of NIH mice. Failure of *N. americanus* to establish in the small intestines of NIH mice, even after immunosuppression, may then reflect an inability to migrate beyond the lungs, rather than failure to establish after migration from the lungs. In the BALB/c strain, however, peak intestinal worm burdens – although low in comparison to those in the lungs – were increased following treatment with cortisone. Thus, in BALB/c mice expulsion of intestinal worms from control mice was, at least in part, immunologically mediated.

In conclusion, clear differences were found, between BALB/c and NIH mice, in the kinetics of the pulmonary and intestinal stages of infection with *N. americanus*. The prolonged residence of larvae in the lungs of NIH mice and their failure to accumulate in the gut indicate that genetically determined obstacles may restrict larval migration in hosts of a specific genotype. Whether inherent differences in the capacity of syngeneic mice to respond to infection or in morphological/physiological characteristics, crucial for successful migration and development of larvae, are responsible is not yet clear. However, elucidation of the factors involved may offer an explanation for the rigid host specificity of *N. americanus* and provide a basis for exploring genetic constraints on susceptibility to infection with hookworms.

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