Necator americanus in inbred mice: evidence in support of genetically determined differences in the cellular immune response to a primary infection

L. M. TIMOTHY* and J. M. BEHNKE

Experimental Parasitology Research Group, Department of Life Science, University of Nottingham, Nottingham NG7 2RD, UK

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SUMMARY

The blood and broncho-alveolar leucocyte (BAL) responses, to a primary *Necator americanus* infection, were studied in male BALB/c and NIH mice. Following percutaneous infection, a significant blood leucocytosis occurred in both the BALB/c and NIH strains. The peak response occurred, in both strains, on day 10 p.i. and this was reflected in the eosinophil response which peaked at the same time-point. The change in blood eosinophil numbers, as a result of infection, was found to be the greatest recorded for any cell type. In BALB/c mice, however, infection elicited a significantly more intense total leucocyte, lymphocyte and eosinophil response than in NIH mice. In contrast, the BAL response was more intense in the NIH strain. Peak BAL responses were observed between days 12 and 16 p.i., in both strains, and the change in broncho-alveolar eosinophil numbers, as a result of infection, was found to be the greatest recorded for any cell type. The relationship between the observed leucocyte responses and resistance to the migrating larvae of a primary infection is discussed.

Key words: *Necator americanus*, hookworm, mouse, broncho-alveolar lavage, leucocytosis.

INTRODUCTION

The anthropophilic hookworm, *Necator americanus*, remains a major cause of morbidity in most tropical and subtropical regions. *N. americanus* has a potential life-span of 17–18 years and is renowned for its longevity in subjects exposed to single pulse primary infections (Beaver, 1988). Despite causing chronic infections, there is little doubt as to the antigenicity of hookworms *in vivo*. Host recognition of hookworms has been demonstrated, in naturally and experimentally infected humans, by the production of specific antibody, by changes in the populations of systemic blood leucocytes and by local, cutaneous and broncho-alveolar leucocyte responses (Yoshida, Okamoto & Chui, 1968; Ball & Bartlett, 1969; Nawalinski & Schad, 1974; Ogilvie *et al.* 1978; Carroll & Grove, 1986; Maxwell *et al.* 1987; Pritchard *et al.* 1992; Walsh, 1994). Moreover, variation in the intensity and duration of these responses has been reported.

However, while it has been suggested that genetically determined differences in the ability to mount an effective immune response to infection may be involved in predisposing individuals, in human

populations, to infection with hookworms (Schad & Anderson, 1985), no clear relationship has been established between resistance to infection and levels of cellular responsiveness. Thus, whilst incontrovertible evidence for the involvement of protective immunity to hookworms in humans is still lacking, highly aggregated frequency distributions of hookworm burdens within human communities (Croll & Ghadirian, 1981; Pritchard *et al.* 1990; Bradley *et al.* 1992) and predisposition to heavy (or light) infection following chemotherapy have been documented (Schad & Anderson, 1985; Haswell-Elkins, Elkins & Anderson, 1987; Haswell-Elkins *et al.* 1988; Quinnell *et al.* 1993). Under field conditions, however, determination of the relative importance of the multitude of predisposing factors is extremely difficult. If, then, consistent and significant differences in murine responsiveness to *N. americanus* infection could be established, the range and availability of genetically defined syngeneic and congenic strains might ultimately enable exposition of the genetic basis of resistance to the invasive and migratory stages of infection in this model.

In previous studies we have shown that mice are susceptible to the skin-penetrating, invasive stages of *N. americanus* and that the subsequent route of migration, though abbreviated, is direct and simple (Wells & Behnke, 1988*b*). In addition, significant differences have been found between the infection kinetics, of this hookworm, in BALB/c and NIH mice. No initial difference in larval establishment in

* Corresponding author. Present address: Centre for Rheumatology/Bloomsbury Rheumatology Unit, University College London, Arthur Stanley House, 40–50 Tottenham Street, London W1P 9PG, UK. Tel: 0171 636 8333 ext. 4366. Fax: 0171 380 9278. E-mail: l.timothy@ucl.ac.uk.

the lungs was found, but, thereafter, lung worm burdens declined more slowly in NIH mice than in the BALB/c strain. Unexpectedly, however, a greater proportion of larvae remaining in the lungs of BALB/c mice, 9 days p.i., were trapped than in NIH mice. In addition, establishment in the small intestines of the BALB/c strain was consistently greater; *N. americanus* larvae may only be recovered rarely from the intestines of NIH mice (Timothy & Behnke, 1993).

It was therefore of interest to determine whether any relationship existed between the contrasting temporal changes in pulmonary (and intestinal) worm burdens, exhibited by inbred mouse strains, and the accompanying cellular responses. In earlier studies, an enhanced broncho-alveolar leucocytic response was found in challenged BALB/c mice when compared to the primary infection group. In the challenged group, impeded migration and development of *N. americanus* were also demonstrated (Wells, 1988). In this paper, we report the results of a comparative study of the blood and broncho-alveolar leucocyte responses, of BALB/c and NIH mice, to a primary *N. americanus* infection. The significance of the observed response phenotypes, in terms of larval migration and development, is discussed.

MATERIALS AND METHODS

Animals

Specific pathogen-free mice of the inbred BALB/c and NIH strains were obtained from Harlan-Olac Ltd, Bicester, Oxon. All mice were male and were housed under conventional conditions. Animals were approximately 6–9 weeks old when infected and experimental groups comprised 4–10 animals.

Parasite

Infective *N. americanus* larvae were obtained by routine passage through neonatal hamsters at the University of Nottingham (Behnke, Paul & Rajasekariah, 1986a). 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

Experimental mice were percutaneously infected with *N. americanus* L₃ as described by Behnke, Wells & Brown (1986b). Briefly, mice were anaesthetized with Sagatal (May & 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.

Haematology

Following terminal anaesthesia, by chloroform inhalation, whole blood was collected from individual animals. In each case, the thoracic cavity was exposed and the aorta severed. Blood was removed and mixed, within 15 sec, with 4.3% (w/v) di-sodium ethylenediaminetetracetic acid (EDTA) at a concentration of 50 μ l/1 ml of whole blood. Total leucocyte numbers were then assessed using an automated haematology analyser (Sysmex, K-1000). Smears were also made from samples of the anticoagulated whole blood. The slides were air dried, fixed in methanol and stained using a standard May-Grünwald Giemsa technique (Dacie & Lewis, 1991). Differential leucocyte numbers were quantified and at least 200 leucocytes were counted/slide. The resulting data were expressed as absolute numbers of each cell type/ml of whole blood. The change in leucocyte numbers, relative to the corresponding group mean control value, was then calculated, for individual experimental animals, at each time-point.

Where peripheral blood eosinophil numbers were enumerated, mice were anaesthetized by inhalation of diethyl ether (May and Baker) and bled from the retro-orbital plexus. From each animal 10 μ l of blood were collected into individual graduated, micro-haematocrit tubes. Immediately after collection, blood was diluted 1:10 in Discombe's fluid containing 3% (w/v) EDTA and the number of eosinophils/ml of blood determined using an improved Neubauer haemocytometer.

Broncho-alveolar lavage

Mice were killed, as already described, and the thoracic cavity dissected such that the lungs and trachea were exposed. Animals were bled, the blood discarded and following ligation of the trachea, 0.5 ml of pre-warmed (to 37 °C) lavage medium (1.4 g lignocaine hydrochloride; 2.4 g HEPES; 5.0 g bovine serum albumin; made up to 500 ml with phosphate-buffered saline; modified from Holt (1979)) was infused into the lungs. The fluid, containing broncho-alveolar leucocytes (BALs), was immediately withdrawn and stored on ice until required. The process was repeated.

Viable cells were enumerated following addition of 10 μ l of a working solution of fluorescein diacetate to 90 μ l of broncho-alveolar lavage fluid. Fluorescing cells were counted, using an improved Neubauer haemocytometer, under a microscope with a reflected fluorescent light attachment. Individual perfusates were then washed ($\times 3$) in lignocaine-free lavage medium and resuspended in a 1:1 (v/v) mixture of lignocaine-free lavage medium and foetal calf serum. Cytospin smears were made from the cell suspensions, air dried and fixed in methcol. Slides were stained, for 15–30 min, with Wright's stain, washed

in running tap water and differential counts were carried out on at least 500 nucleated cells/smear.

Statistical analysis

Data are presented as group means $\pm$ standard error of mean (S.E.M.). Statistical comparisons were made where required and as described in the text.

RESULTS

Time-course of systemic leucocyte responses to a primary N. americanus infection in male BALB/c and NIH mice

Following infection, groups of BALB/c and NIH mice were killed on days 3, 5, 10, 17, 24, 31 and 38 p.i. together with naïve control animals, of both strains. The change in leucocyte numbers, relative to the corresponding control group mean value, was calculated, for individual animals, at each time-point and the resulting data are presented in Fig. 1A–D. Thus, values of 1 reflect no change in the cell population, values greater than 1 indicate an increase in the cell type and values of less than 1 reflect a reduction in cell numbers. Since the data were not normally distributed, $\log_e$ transformation was carried out before analysis. Significant changes were then elucidated by analysis of variance. Lung worm burden data, from this experiment, have been published elsewhere (Timothy & Behnke (1993), Table 1, Exp. 6).

Total leucocytes varied significantly with time in both BALB/c ($F = 5.06$, 6 D.F., $P < 0.001$) and NIH ($F = 4.27$, 6 D.F., $P < 0.001$) mice. Infection had a significant effect on total leucocyte numbers in both the BALB/c ($F = 146.99$, 1 D.F., $P < 0.001$) and NIH ($F = 23.95$, 1 D.F., $P < 0.001$) strains and there was a significant two-way interaction between infection and time (BALB/c, $F = 7.10$, 6 D.F., $P < 0.001$; NIH, $F = 6.30$, 6 D.F., $P < 0.001$). Over the time-course studied, total leucocyte counts from the naïve control BALB/c and NIH groups overlapped and ranged from 5.92 ± 0.62 to 9.15 ± 0.21 and from 5.6 ± 0.48 to 10.8 ± 1.67 respectively (data are expressed as cells $\times 10^{-6}$ /ml of whole blood). The response of the infected groups peaked, in both strains, on day 10 p.i. (Fig. 1A) but there was a significant strain difference in response ($F = 13.26$, 1 D.F., $P < 0.001$). In BALB/c mice the peak response represented a 3.2-fold increase in total leucocytes whereas in the NIH strain, there was a 2.2-fold increase.

There was considerable temporal variation in lymphocyte numbers during the course of the experiment and these changes were significant in both BALB/c ($F = 5.63$, 6 D.F., $P < 0.001$) and NIH ($F = 8.13$, 6 D.F., $P < 0.001$) mice. A significant increase in lymphocytes was also seen in the

experimental groups, relative to the respective controls, as a result of infection (BALB/c, $F = 77.76$, 1 D.F., $P < 0.001$; NIH, $F = 10.15$, 1 D.F., $P = 0.002$) and there was a two-way interaction between infection and time (BALB/c, $F = 6.36$, 6 D.F., $P < 0.001$; NIH, $F = 3.41$, 6 D.F., $P = 0.006$). Over the time-course studied, lymphocyte counts from the naïve control BALB/c and NIH groups overlapped and ranged from 4.21 ± 0.12 to 6.77 ± 0.32 and from 3.56 ± 0.36 to 7.66 ± 1.11 respectively (data are expressed as cells $\times 10^{-6}$ /ml of whole blood). However, there was a significant difference between the strains in their response to infection ($F = 7.09$, 1 D.F., $P = 0.01$). The peak lymphocyte response (Fig. 1B), in BALB/c mice, occurred 17 days p.i., representing a 2.3-fold increase in cells, and declined thereafter. In the NIH strain, an apparently triphasic lymphocyte response occurred peaking on day 10 of the experiment at 1.7 times the control level. A second peak in lymphocytes occurred, in NIH mice, on day 24 and a third on day 38. The apparent triphasic nature of this response may, however, represent a plateau in lymphocyte populations after day 10 of the infection.

A significant difference in eosinophil counts, with respect to time, was found in both BALB/c ($F = 26.53$, 6 D.F., $P < 0.001$) and NIH ($F = 12.55$, 6 D.F., $P < 0.001$) mice. The change in the populations of eosinophils, as a result of infection, was by far the greatest recorded for any cell type and was significant in both the BALB/c ($F = 259.07$, 1 D.F., $P < 0.001$) and NIH ($F = 88.47$, 1 D.F., $P < 0.001$) strains. In addition, a significant two-way interaction between time and infection occurred in both strains (BALB/c, $F = 28.98$, 6 D.F., $P < 0.001$; NIH, $F = 11.65$, 6 D.F., $P < 0.001$). Over the time-course studied, eosinophil counts from the naïve control BALB/c and NIH mice overlapped and ranged from 0.22 ± 0.06 to 0.33 ± 0.06 and from 0.10 ± 0.05 to 0.40 ± 0.07 respectively (data are expressed as cells $\times 10^{-6}$ /ml of whole blood). However, there was a significant difference between the pattern of response to infection in BALB/c and NIH mice ($F = 9.54$, 1 D.F., $P = 0.003$). Eosinophilia peaked, in both strains, 10 days after infection (Fig. 1C). In BALB/c mice, the peak response represented a 37-fold increase over the control value and declined rapidly thereafter having decreased to 5.2 times the control level by day 17 p.i. In the infected NIH group, eosinophils peaked at only 10.3 times the control value but the rate of decline in eosinophil numbers was less rapid than in the BALB/c strain, and by day 17 of the experiment eosinophil counts were still 5.7 times above the control value.

Neutrophil levels varied greatly in the infected and control groups, of both strains, throughout the experiment. Nevertheless, neutrophil counts from the naïve control BALB/c and NIH mice overlapped and ranged from 0.60 ± 0.14 to 2.06 ± 0.17 and

Fig. 1. The change in blood leucocyte numbers, following a primary infection with *Necator americanus*, in male BALB/c (■) and NIH (▲) mice. At each time-point, the change in leucocyte numbers, relative to the corresponding group mean control value, was calculated, for individuals, and is presented as the group mean value $\pm$ S.E.M. ($n = 4-5$). (A) Change in total leucocytes; (B) change in lymphocytes; (C) change in eosinophils; (D) change in neutrophils.

from 0.88 ± 0.06 to 4.20 ± 1.15 respectively (data are expressed as cells $\times 10^{-6}$ /ml of whole blood). Variation, with respect to time, was significant only in the BALB/c strain ($F = 18.22$, 6 D.F., $P < 0.001$). Infection induced a significant neutrophilia in BALB/c mice ($F = 42.60$, 1 D.F., $P < 0.001$) and there was a

significant two-way interaction between infection and time ($F = 6.70$, 6 D.F., $P < 0.001$). In the NIH strain, infection alone did not induce a significant change in neutrophil numbers, however, there was a significant two-way interaction between infection and time ($F = 6.70$, 6 D.F., $P < 0.001$). Although the

Fig. 2. Time-course of peripheral blood eosinophilia in male (A) BALB/c and (B) NIH mice following a primary infection with *Necator americanus*. Naïve BALB/c (□), infected BALB/c (■), naïve NIH (△), infected NIH (▲). Eosinophils are expressed as cells $\times 10^{-6}$ /ml of peripheral blood and data are presented as group means $\pm$ S.E.M. ($n = 4-5$). In a further experiment (C), the peripheral blood eosinophil response, of male BALB/c and NIH mice, was assessed 5 and 10 days after a primary infection with *N. americanus*. Naïve BALB/c (□), infected BALB/c (■), naïve NIH (▨), infected NIH (▩). Data are expressed as in (A) and (B). In all cases $n = 5$.

overall pattern of the neutrophil response was similar in both strains (Fig. 1D), there was a slight, but significant, strain difference in response ($F = 4.52$, 1 D.F., $P = 0.032$). It is likely that this variation is attributable to the clear difference in response seen on day 3 p.i. At this time-point the neutrophil response, in infected BALB/c mice, was 2.4 times that in the control group whilst, in the NIH strain, the neutrophil response represented only 0.82 times the control value. In both strains, however, neutro-

philia peaked on day 10 of the experiment and in BALB/c mice this represented a 2.6-fold increase over the control value. In NIH mice, at day 10 p.i., the infected group exhibited a 3-fold increase in neutrophils. When the indices for change, in neutrophils/ml, were compared, using a non-specific unified rank analysis (Meddis, 1984), no significant strain difference was found on day 10 p.i.

Variation in the number of monocytes, with time, was significant in both the BALB/c ($F = 11.46$,

6 D.F., $P < 0.001$) and NIH ($F = 6.48$, 6 D.F., $P < 0.001$) strains (data not shown). Over the time-course studied, monocyte counts from the naïve control BALB/c and NIH mice overlapped and ranged from 0.01 ± 0.01 to 0.27 ± 0.06 and from 0.03 ± 0.01 to 0.03 ± 0.11 respectively (data are expressed as cells $\times 10^{-6}$ /ml of whole blood). Following infection, monocyte populations increased significantly in BALB/c ($F = 48.56$, 1 D.F., $P < 0.001$) and NIH ($F = 26.40$, 1 D.F., $P < 0.001$) mice and, in the BALB/c strain, there was a significant two-way interaction between infection and time ($F = 7.52$, 6 D.F., $P < 0.001$). Overall, however, there was no significant strain difference in the monocyte response.

Time-course of the peripheral blood eosinophil response to a primary N. americanus infection in male BALB/c and NIH mice

In the previous experiment, the change in blood eosinophil numbers was found to be greater than that in any other cell type and 2 further experiments were carried out to confirm and extend this finding. In Exp. 2, male BALB/c and NIH mice were infected on day 0 and serially bled on days 1, 6, 8, 10, 13, 16, 23, 30 and 35 p.i. Naïve control animals, from each strain, were bled at corresponding time-points. The results are presented in Fig. 2A and B and cell counts are expressed as cells $\times 10^{-6}$ /ml of peripheral blood. Lung worm burden data have been published elsewhere (Timothy & Behnke (1993), Tables 1 and 2, Exp. 5). In the third experiment, male BALB/c and NIH mice were infected on day 0 and the peripheral blood eosinophil response assessed, in independent groups, on days 5 and 10 p.i. Baseline data were obtained from naïve animals, of both strains, at corresponding time-points. Lung and intestinal worm burdens were assessed and have been published previously (Timothy & Behnke (1993), Table 3). Eosinophil data are presented in Fig. 2C and are expressed as cells $\times 10^{-6}$ /ml of peripheral blood. Statistical comparisons were made according to a specific, unified rank analysis (Meddis, 1984).

From Fig. 2A and B, it is evident that the overall pattern of eosinophilia, in both the BALB/c and NIH strains, was similar to that depicted in Fig. 1C. The peak responses occurred on day 10 p.i. and the absolute number of eosinophils/ml of peripheral blood, observed in the infected BALB/c mice ($1.02 \times 10^6 \pm 0.34 \times 10^6$) was greater than in NIH mice ($0.54 \times 10^6 \pm 0.12 \times 10^6$) at this time-point. In this experiment, however, the peak response in the BALB/c strain represented a 17.1-fold increase over the control value, whilst in the NIH strain the response, on day 10 p.i., was 36 times greater than the group mean control value. Thereafter, the rapid decline in numbers of eosinophils, observed in the

BALB/c strain, and the more gradual waning of the response in NIH mice were similar to that shown in Fig. 1C. In the third experiment illustrated in Fig. 2C, it is clear that no significant increase in eosinophil numbers, in either infected group, had occurred by day 5 p.i. On day 10 p.i., however, a significant eosinophilia had developed in both the infected BALB/c ($Z = 2.63$, $P = 0.0045$) and NIH ($Z = 1.70$, $P = 0.0446$) groups. The day 10 response represented a 20-fold increase over the control group mean value, in BALB/c mice, and a 6-fold increase in the NIH strain.

Time-course of broncho-alveolar leucocyte responses to a primary N. americanus infection in male BALB/c and NIH mice

Groups of mice, of both strains, were infected and the animals were killed on days 2, 5, 9, 13, 16, 21 and 27 p.i. Naïve control animals, of both strains, were killed 3 days prior to infection. BAL numbers/ml of lavage fluid were calculated for individual animals, at each time-point, and data are presented in Fig. 3A–E. Lung and intestinal worm burdens were assessed and have been presented previously (Timothy & Behnke (1993), Fig. 1).

It is evident that, whilst both strains began to respond by day 5 p.i., the intensity of the BAL response was considerably greater in the NIH than in the BALB/c strain (Fig. 3A). On day 13 p.i., NIH mice yielded 20.0×10^5 cells/ml whilst BALB/c mice yielded only 4.5×10^5 cells/ml. By day 27 p.i., the response had run its course and BAL numbers had returned to base levels. It is also clear from Fig. 3 that the more intense BAL response of NIH mice comprised higher lymphocyte, neutrophil and, in particular, eosinophil counts. On day 13 p.i., NIH mice yielded 101.0×10^4 eosinophils/ml compared with 13.1×10^4 /ml recovered from BALB/c mice.

A further experiment was carried out to confirm the surprising findings of the previous experiment and the BAL response was assayed at crucial time-points only. Groups of male BALB/c and NIH mice were infected and killed on days 12 and 16 p.i. Naïve control groups, of both strains, were killed on day 17 of the experiment. BAL numbers were calculated as before and the data are presented in Fig. 4A–E. Statistical comparisons, between infected and control groups of animals, were made using a specific unified rank analysis and a non-specific unified rank analysis was used for comparison of the BALB/c and NIH responses (Meddis, 1984).

There was a significant increase in total BALs in both mouse strains (Fig. 4A), relative to the control, on both days (BALB/c, $Z = 2.45$, $P = 0.0073$; NIH, $Z = 2.61$, $P = 0.047$) and 16 (BALB/c, $Z = 1.96$, $P = 0.0248$; NIH, $Z = 2.61$, $P = 0.047$) p.i. Whilst there was no significant difference between the strains on day 12, by day 16 p.i., the magnitude

Fig. 3. Time-course of the broncho-alveolar leucocyte response, of male BALB/c (■) and NIH (▲) mice, after a primary infection with *Necator americanus*. Data are expressed as cells $\times 10^{-5}$ (A and E) or 10^{-4} (B–D) and presented as group mean values $\pm$ S.E.M. Ten naïve control mice of each strain were killed on day -3 and for all other time points $n = 5$.

Fig. 4. The broncho-alveolar leucocyte response, of male BALB/c (■) and NIH (▨) mice, 12 and 16 days after a primary infection with *Necator americanus*. Data are expressed as cells $\times 10^{-4}$ /ml and presented as group means $\pm$ S.E.M. Naïve control groups were killed on day 17 of the experiment ($n = 4-5$). (A) Total leucocytes; (B) lymphocytes; (C) eosinophils; (D) neutrophils; (E) macrophages.

of the response in NIH mice was significantly greater than that in the BALB/c strain ($H = 6.00$, $P < 0.0001$).

A significantly greater number of lymphocytes (Fig. 4B) was recovered from infected compared with the naïve control groups of both strains, on both

days 12 (BALB/c = 2.45, $P = 0.0073$; NIH, $Z = 2.19$, $P = 0.0141$) and 16 p.i. (BALB/c, $Z = 2.45$, $P = 0.0073$; NIH, $Z = 2.61$, $P = 0.0047$). The strain differences in total BALs were not, however, reflected in the lymphocyte response and no significant difference was found, between the BALB/c and NIH strains, at either time-point.

No eosinophils (Fig. 4C) were observed in either of the naïve control groups. On day 12 p.i. an eosinophilia was recorded in the lungs of both strains and the numbers of eosinophils recovered from NIH mice were significantly greater than those recovered from BALB/c mice ($H = 2.16$, 1 D.F., $P = 0.0019$). This strain difference in response had increased by day 16 p.i. ($H = 6.0$, 1 D.F., $P < 0.0001$). Similarly, too few neutrophils were recovered from the lungs of the naïve BALB/c ($0.004 \times 10^4 \pm 0.004 \times 10^4$) and NIH ($0.012 \times 10^4 \pm 0.007 \times 10^4$) control groups to be depicted (Fig. 4D). By day 12 p.i., however, there was a significant neutrophilia in the lungs of BALB/c ($Z = 2.56$, $P = 0.0054$) and NIH ($Z = 2.64$, $P = 0.0043$) mice. Pulmonary neutrophilia had declined in the BALB/c strain by day 16 p.i. but was still significantly above control levels ($Z = 2.56$, $P = 0.0054$). In NIH mice, the numbers of neutrophils recovered from the lungs had increased by day 16 and were thus significantly greater than in the control animals ($Z = 2.64$, $P = 0.0043$). There was a clear strain difference in neutrophil response to infection, although this was only found to be significant on day 16 p.i. ($H = 6.0$, 1 D.F., $P < 0.0001$).

A significantly greater number of pulmonary macrophages (Fig. 4E) were recovered from the lungs of infected BALB/c mice, in comparison to the naïve controls, on day 12 p.i. ($Z = 2.20$, $P = 0.0137$). By day 16, however, macrophage numbers had declined, in the lungs of BALB/c mice, and were not significantly different from those in the controls. In NIH mice, there was a significant increase in macrophages, relative to the control, on both days 12 ($Z = 2.61$, $P = 0.0047$) and 16 ($Z = 2.61$, $P = 0.0047$) p.i. There was no significant strain difference in response on day 12 p.i., however, by day 16 a significantly greater number of pulmonary macrophages was observed in the lungs of NIH mice compared with BALB/c mice ($H = 4.86$, 1 D.F., $P < 0.0001$).

DISCUSSION

Differences between individuals in the magnitude and persistence of leucocyte responses, following exposure to hookworm larvae, have been reported previously from case studies and observations on volunteers exposed to *N. americanus*, but the underlying mechanisms have not been investigated (reviewed by Behnke, 1991). In particular, the role of

genetically based differences between hosts in facilitating or resisting infection and in determining the pattern of accompanying cellular responses has not been examined in human infections and only the former has been tackled in animal studies (Wells & Behnke, 1988a).

Although murine *N. americanus* infections do not survive to patency, the early stages of infection – specifically the skin penetrating and tissue migratory phases – occur much as in the human host (Wells & Behnke, 1988b). Since parasite burdens can readily be quantified in mice, comparisons between mouse strains of known genotypes enable genetic influences on host resistance to be identified and correlated with the accompanying cellular/humoral responses. Our earlier studies revealed that murine strains did indeed differ in the course of infection with *N. americanus*, following exposure to a uniform inoculum of infective larvae (Timothy & Behnke, 1993). BALB/c and NIH mice exhibited a contrasting pattern with pulmonary infections in the former being cleared considerably earlier than in the latter strain, and only the former strain tolerating a brief intestinal phase of infection. The present study has shown that there are also significant differences, between these strains, in the pattern, magnitude and duration of the blood leucocyte response and the BAL response in the lungs where the larval stages reside. Interestingly, infection in BALB/c mice elicited a less intense pulmonary response compared with the NIH strain, but a considerably more intense systemic response. When specific cell types were quantified, the most striking changes occurred in the numbers of blood and broncho-alveolar eosinophils.

The blood leucocyte response followed a very predictable pattern with a detectable increase being apparent from day 5 p.i. onwards and a peak in both strains on day 10 p.i., after which a decline to base levels ensued, or marginally above base levels, usually by week 6 p.i. Increased numbers of all the myeloid cell types were evident, with neutrophils and eosinophils dominating the initial response and lymphocyte numbers peaking a few days later. However, by the time this systemic leucocytosis was first evident, on day 5 p.i., lung worm counts were already declining and at the time of the peak leucocytosis, the lungs would have harboured very few parasites (Timothy & Behnke, 1993). Thus, the peak blood leucocytosis was evident almost a week after peak parasite numbers resided in the lungs (days 3–4 p.i.). Moreover, in both murine strains, the pulmonary response, as reflected by BAL numbers, peaked later still, i.e. between days 12 and 16 p.i. and, at these time-points, pulmonary worm numbers would have been relatively low. In the BALB/c strain, there would have been almost no worms present beyond day 12 p.i., perhaps explaining the lower local sequestration of eosinophils. In contrast, worm numbers declined much more slowly in NIH mice,

and substantial lung worm burdens persisted at least until day 9 p.i. (Timothy & Behnke, 1993).

Thus, the peak blood leucocyte response of the NIH mice was consistently lower than that in the BALB/c strain and the local pulmonary response was consistently of a greater magnitude. NIH mice are known to be high responders to several other nematode parasites (Wakelin, 1980; Behnke & Robinson, 1985) and it is likely that, in this case, NIH mice responded to the pulmonary stages of infection more rapidly than the BALB/c strain. Hence, the onset of eosinophilia was swiftly followed by sequestration of eosinophils into the lungs, where the persisting larval burden would have acted as a continuing stimulus for the eosinophil response and a target for localization in the pulmonary tissues. It is also conceivable that the transient establishment of an intestinal population of worms experienced by BALB/c, but not NIH, mice elicited an enhanced circulating eosinophilia. It is known from other murine model systems that stimuli from the gut provide potent signals for mobilization of eosinophils from the bone marrow (Lammas *et al.* 1992). There is no direct evidence, however, that the greater intensity of response in the NIH strain affords heightened resistance to infection. Indeed the converse is true, a significantly greater proportion of larvae remaining in the lungs of BALB/c mice, 9 days p.i., have been shown to be trapped than in NIH mice (Timothy & Behnke, 1993). Moreover, Wells (1988), using an *in vitro* assay, investigated the adherence of BALs to *N. americanus* L₃ and found that cells from both naïve and infected BALB/c mice adhered to larvae in greater numbers than those from NIH mice. Subsequent studies revealed that the degree of adherence was primarily determined by the source of the cells used, rather than the presence of specific antibodies. Adherence in the absence of complement was minimal. Studies using human eosinophils have produced similar results (Desakorn *et al.* 1987). Thus, appropriate cellular activation may be more important than proliferation *per se* in limiting infections.

In both strains, however, the peak cellular response in the pulmonary airways occurred after the majority of larvae would have been expelled from the small intestine. A similar phenomenon has been described in the *Nippostrongylus brasiliensis*-rat model (Egwang, Gaudie & Befus, 1984; Ramaswamy & Befus, 1989; Ramaswamy *et al.* 1991). In both these systems, the timing of the pulmonary responses leaves open the possibilities that inflammation is generated in response to exogenous larval products remaining in the lungs, following onward migration, or to effect clearance of degenerating larvae which for non-immunological reasons were unable to undergo further migration.

In conclusion, our study has established clearly that in this murine model system, based on the

human hookworm *N. americanus*, genetic differences between hosts play a significant role in influencing the cellular immune responses which follow exposure to invasive larvae. Indeed, NIH and BALB/c mice differ in their MHC haplotype (H-2q and H-2d respectively) and in many of their non-MHC background genes which control components of the immune response. However, genetic differences between murine strains may operate by mechanisms other than by simply influencing the capacity of hosts to mount a protective immune response. For example, innate resistance may be attributable to differences in local pulmonary anatomy with possible consequences for onward larval migration (Elsaghier *et al.* 1989; Elsaghier & McLaren, 1989). Further analysis of this system, incorporating other mouse strains including H-2 congenic and recombinant mice, will shed more light on the factors involved.

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