SUCCESSFUL VACCINATION OF BALB/C MICE AGAINST HUMAN HOOKWORM (*NECATOR AMERICANUS*): THE IMMUNOLOGICAL PHENOTYPE OF THE PROTECTIVE RESPONSE

Girod, N.[1], Brown, A.[2], Pritchard, D. I.[2] and Billett, E. E.[1*]

[1] Department of Life Sciences, The Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK.

[2] School of Pharmaceutical Sciences, Boots Science Building, University of Nottingham, Nottingham, NG7 2 RD, UK.

* Correspondence: Dr E Ellen Billett, Department of Life Sciences, Faculty of Science, The Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK, Tel: 0115 848 6356, Facsimile: 0115 848 6355, Email: ellen.billett@ntu.ac.uk

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Abstract

In this murine (BALB/c) model of necatoriasis, high levels of protection against challenge infection by *Necator americanus* larvae (n = 300) were afforded by successive vaccinations at 14-day intervals, either subcutaneously or percutaneously, with γ-irradiated *N. americanus* larvae (n = 300). Percutaneous vaccination was significantly more effective than the subcutaneous route, with pulmonary larval burdens at 3 days post-infection being reduced by 97.8% vs. 89.3%, respectively, after three immunisations ($p < 0.05$). No worms were recovered from the intestines of thrice vaccinated mice. Two percutaneous vaccinations also reduced worm burdens, by 57% in the lungs and 98% in the intestines; $p < 0.05$. In vaccinated animals, lung pathology (mainly haemorrhage) following infection was greatly reduced compared with non-vaccinated animals. In vaccinated mice (but not in non-vaccinated mice) mast cells accumulated in the skin and were degranulated. RT-PCR analyses of mRNAs in the skin of vaccinated animals indicated increased expression of IL-4 relative to γ-IFN. Lymphocytes from the axillary (skin-draining) lymph nodes of vaccinated mice, stimulated *in vitro* with concanavalin A, exhibited enhanced secretion of IL-4 protein and a higher Il-4/γ-IFN protein ratio than lymphocytes from non-vaccinated animals. In vaccinated mice, levels of IgG1 and IgG3 (directed against larval excretory/secretory products) were elevated for the most part compared with those in non-vaccinated animals. These data demonstrate the successful vaccination of BALB/c mice against human hookworm infection and suggest that a localised Th2 response may be important for conferring protection against necatoriasis.

1. Introduction

Despite the wide range of antihelminthic treatments available, the prevalence of hookworm infection remains a major public health concern. The requirement for repeated doses, the development of drug-resistant strains and the risk of re-infection are the main limitations of chemotherapeutic intervention (Quinnell et al., 1993; Hotez and Pritchard, 1995). The development of a vaccine, providing life-long protection, thus represents an attractive and necessary solution to this problem; as such, the Sabin Vaccine Institute's hookworm vaccine initiative has been developed.

Some of the more successful vaccines developed have used attenuated organisms, attenuation providing the immune system with the prolonged antigen exposure necessary for the development of protective immunity. There are several ways of attenuating infective organisms, and irradiation has been successful in animal models against a range of parasites, such as *Plasmodium berghei* (Nussenweig et al., 1998), *Leishmania major* (Rivier et al., 1993), *Toxoplasma gondii* (Dubey et al., 1996), *Schistosoma mansoni* (Smythies et al., 1996) and the nematodes *Heligmosomoides polygyrus* (Hagan et al., 1981), *Strongylus vulgaris* (Clifton et al., 1997) and *Ancylostoma caninum* (Miller, 1971). Despite this success and abundant evidence to indicate that the resistance displayed by mice vaccinated with irradiated parasites is mediated by specific immune mechanisms, few irradiation-attenuated vaccines are commercially available. Vaccines based on attenuated larvae suffer from a number of problems including a short shelf-life and the establishment of a degree of infection. Notwithstanding, useful insights may be learnt from using irradiated larvae as vaccines, including the immunological compartment(s) participating in immunity, the

site of larval attrition and the kinetics of protective responses. Such information will aid the development of successful vaccines and the types of adjuvant which should be administered with these vaccines.

The current paper describes the establishment of a successful vaccine model for *Necator americanus*, exploiting the immunogenicity of irradiated larvae in BALB/c mice. Furthermore, data are presented to indicate the immunological phenotype of the successfully protected vaccine recipient.

2. Materials and Methods

2.1. *Preparation of third stage infective larvae*

Necator americanus was maintained in syngeneic DSN hamsters, as described by Sen and Seth (1967) and the third stage infective larvae cultured from faecal material by a method modified from Harada and Mori (1955), previously described by Kumar and Pritchard (1992). Third stage infective larvae were harvested, concentrated and re-suspended in 50 mM Na_2HPO4, 70 mM NaCl, 15 mM KH_2PO4, pH 7.4 for immediate use. Harvested larvae were exposed to 40 kilorads of γ-radiation from a sealed ^{137}Cs source and used in vaccination experiments within 2 h of irradiation.

2.2. N. americanus *larval excretory/secretory (ES) products*

Freshly collected third stage larvae were exsheathed by bubbling carbon dioxide through the larval suspension for 1 h at room temperature. Exsheathed larvae were

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allowed to settle and then washed extensively with phenol-red free RPMI 1640 containing 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 1 % (w/v) amphotericin B (final concentrations) under sterile conditions. Following sterilisation, the larvae were then cultured in RPMI 1640 containing the above additives for 72 h at 37 ºC, changing the culture medium every 24 h. ES products collected over the 72 h period were pooled, dialysed against distilled water and stored a −20 ºC until required. The protein concentration was estimated using the Bio-Rad protein assay kit, with BSA standards.

2.3. *Vaccination*

Male BALB/c mice (Harlan Ltd UK) were vaccinated three times either s.c. or percutaneously (experiment 1), or two times percutaneously (experiment 2), 14 days apart, with 300 γ-irradiated *N. americanus* larvae (Behnke et al*., *1986). For s.c, immunistaion 0.1ml sterile pyrogen free water containing the larvae was injected using a 25G needle at the nape of the neck. For percutaneous immunisation, mice were anaesthetised by i.p. injection of Sagatal and shaved around the neck. Larvae were applied to the skin on a gauze, held in place for 24 h to allow larval penetration. Gauze without larvae was used to treat control ('non-vaccinated') animals. Two weeks after the final vaccination, mice were challenged ('infected') with 300 normal *N. americanus* larvae. Naïve animals were neither vaccinated nor infected. Each experimental group consisted of six animals.

2.4. *Quantification of parasite burden*

Larvae were recovered post-mortem from the lungs on day 3 p.i. (or days 1 to 10 in the study of larval migration through the lungs) and from the small intestine on days 8 or 9 p.i., as described by Wells and Behnke (1988).

The lungs were finely chopped, placed in pre-warmed Hanks buffered salt solution (HBSS) and incubated for 8 h at 37 °C. Every 2 h, HBSS was collected and replaced with fresh warm HBSS and the lungs were further chopped. The numbers of worms in pooled incubate were counted.

The small intestine was opened along its length on a nylon gauze. The gauze was suspended in pre-warmed HBSS and incubated for 6 h allowing L_4 larvae to sediment. The gauze was then removed, excess HBSS was aspirated and the sedimented L_4 larvae were counted.

2.5. *Serum immunoglobulins*

At time points when pulmonary and intestinal worm burdens were being determined, blood was collected from aortae of sacrificed animals and left to clot for 2 h at 4 ˚C and then centrifuged for 15 min at 13, 000 g. The sera were collected and stored at -20 ˚C until immunoglobulin G subclasses were assayed by ELISA.

For the ELISA, 96-well plates were coated overnight at 4 °C with 0.25 µg larval ES products in 50 µl, washed three times with 0.05 % (v/v) TBS/Tween 20 and then blocked for 1 h at 37 °C with 5 % (v/v) TBS/Marvel milk powder before addition of 50 µl of serum (diluted 1:50 in blocking agent) left incubated overnight at 4 °C. Following washing, 50 µl of secondary antibody labelled with alkaline phosphatase (sheep anti-mouse IgG1, IgG2a, IgG2b or IgG3, Seralab), diluted 1:1,000 in blocking agent were added to individual wells and incubated for a further 1.5 h at 37 °C. Antibody binding was visualised by the addition of 100 µl of p–nitrophenol phosphate substrate (1 mg/ ml in 0.1 M glycine, 1 mM calcium chloride, 1 mM magnesium chloride, pH 10.4). ELISA values were expressed as the absorbance at 405 nm.

2.6. *Analysis of IL4- and γ-IFN- mRNAs in skin samples*

The skin sites of infection were removed and snap frozen in liquid nitrogen until required. Skin samples were homogenised in 1 ml RNA STAT-60™ (Biogenesis) per 100 mg of tissue in a glass-Teflon homogeniser and total RNA was isolated as recommended by the manufacturer. The RNA was resuspended in diethylpyrocarbonate-treated water and quantitated spectrophotometrically.

The levels of IL4- and γ-IFN- mRNAs in extracted RNA from non-vaccinated/infected and vaccinated/infected individual animals were determined in relation to the mRNA of the housekeeping gene β-actin using the Promega Access RT-PCR System (Promega). The PCR products were electrophoresed on 2 % (w/v) agarose gels, stained with ethidium bromide (Sambrook et al., 1989) and photographed under UV light.

2.7. IL4 and γ-IFN production by cultured lymphocytes

Axillary lymph nodes were removed from non-vaccinated/infected and vaccinated/infected animals and individually dissociated in lymphocyte culture medium (LCM) containing RPMI, 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 % L-glutamine. Collected cells were washed into 25 ml LCM and centrifuged for 7 min at 1 300 r.p.m. The cells were then resuspended in 1 ml LCM, counted using trypan blue (Sigma), and adjusted to 2.5×10^6 cells /ml; 200 µl of cell suspension were then aliquoted into 96-well plates and stimulated by the addition of 5 µg/ml concanavalin A (ConA, Sigma). After 3 days incubation at 37 ˚C, 100 µl of supernatant were removed from each well, and stored at –20 ˚C until required for the cytokine assays.

IL4 and γ-IFN were measured using a capture ELISA (DuoSET ELISA Development System kit, R&D Systems) following the manufacturer's instructions, using cytokine standards (0-1000 pg/ml for IL4; 0-2000 pg/ml for γ-IFN) for quantification. Biotin labelled detection antibodies were used, revealed with streptavidin-HRP and a TMB substrate system.

2.8. *Mast cell staining*

The cutaneous responses following infection with *N. americanus* were studied in vaccinated/infected, non-vaccinated/infected and naïve BALB/c mice. The skin sites

of vaccination and/or infection from four mice of each group (or dorsal skin patches in the case of naïve controls) were excised and fixed in 10 % (v/v) neutral buffered formaldehyde. Tissues were processed using the Shandon Hypercentre and then embedded in paraffin wax using conventional techniques. Transverse skin sections of 10 μm thickness were then produced using a rotary Leitz microtome and the sections stained with 0.6 % (v/v) aqueous thionin blue using techniques described by Humason (1979).

2.9. *Lung pathology*

The pulmonary responses following infection with *N. americanus* were studied in vaccinated/infected, non-vaccinated/infected and naïve BALB/c mice. The lungs from four mice of each group were excised, fixed and sectioned as described above (section 2.8). Transverse lungs sections were stained with haematoxylin and eosin.

2.10. *Statistical analysis*

Student's t-test was used to assess potential differences in worm burden, cytokine secretion by activated lymphocytes and serum antibody isotype levels between experimental groups, normal distribution of the data having been established first. Where the direction of a difference between two groups was unknown, a 2-tailed t-test was used; in others cases a 1-tailed analysis was implemented. Where appropriate, when multiple groups are involved, the Bonferroni procedure was used to choose the individual error rate for the null hypothesis (Morrison, 2002).

3. Results

3.1. Protection induced by gamma-irradiated larval vaccine

Table 1, experiment 1, shows the number of actively migrating larvae recovered from the lungs (3 days p.i.) and from the small intestine (8 and 9 days p.i.) in animals vaccinated three times with gamma-irradiated larvae either subcutaneously or percutaneously. Larval numbers in the lungs were reduced in both groups of vaccinated animals, with percutaneous vaccination showing significantly more protection (numbers reduced by 89 % following s.c. vaccination and by 98 % following percutaneous vaccination). No worms were recovered from the intestines of either group of vaccinated animals.

Table 1, experiment 2, shows that significant protection against challenge infection was achieved in BALB/c mice following two percutaneous vaccinations, reducing significantly ($p < 0.01$) the worm burden by 57 % in the lungs and 100 % in the small intestine.

3.2. Effects of infection and vaccination on the skin

Mast cell responses in the skin

Following infection of non-vaccinated animals, a small increase in mast cell number was observed compared with that of the naïve tissue (Fig 1a, b), within which mast cells were sparse. Mast cell granules were observed in both naïve and non-vaccinated tissue. In the vaccinated tissue, an increased number of mast cells (Fig 1c) was

observed compared with the non-vaccinated and naïve animals. These mast cells were accumulated mainly in the innermost part of the dermis and were degranulated.

Mast cell numbers were quantified on days 1, 3 and 8 p.i. (Fig 2). The number of mast cells in the three different groups remained approximately constant from days 1 to 8 following infection. No significant changes in mast cell numbers were observed in the non-vaccinated/infected group compared with the naïve animals. The main difference in mast cell numbers was observed in the vaccinated/infected group, in which the number of mast cells was three and five times that of the non-vaccinated/infected tissue on days 1 and 3 p.i., respectively ($p < 0.0055$, the error rate according to the Bonferroni procedure)

Cytokine profiles

RT-PCR reactions were undertaken for skin samples taken from both non-vaccinated/infected and vaccinated/infected animals. The intensity of housekeeping gene β-actin (170 bp product) was similar in both groups, suggesting that a direct comparison of cytokine mRNA levels between the two different skin samples was possible (Figure 3, lanes 2 and 5). The 279 bp band observed in lanes 3 and 6 corresponds to the IL4 gene product; the intensity of this band was greater in the vaccinated/infected group than in the non-vaccinated/infected group. A feint amplification product of similar intensity of about 400 bp corresponding to γ-IFN mRNA could be detected in both skin samples (lanes 4 and 7).

Analyses of γ-IFN and IL4 production by lymphocytes isolated from skin draining lymph nodes (axillary lymph nodes) of non-vaccinated/infected and vaccinated/infected animals, on days 3, 8 and 15 p.i. are shown in Table 2. Mean levels of secreted IL4 in the vaccinated/infected group were consistently much higher than in the non-vaccinated/infected group and was significantly highest at 8 days p.i. Levels of γ-IFN were generally slightly higher in the vaccinated/infected animals compared with the non-vaccinated/infected group. Overall, the IL4/γ-IFN ratio was higher in the axillary lymph nodes tissues of vaccinated/infected mice compared with the non-vaccinated/infected group, particularly at 8 days p.i.

3.3. *Effects of infection and vaccination on the lungs*

The effect of vaccination on larval migration was studied by monitoring the number of worms recovered from the lungs of non-vaccinated/infected and vaccinated/infected animals on days 1-5, 7-10 following infection (Fig 4). Whilst worms started appearing in the lungs of the non-vaccinated animals 2 days after infection, no worms were detected in the lungs of vaccinated animals until 3 days after infection. Furthermore, the mean number of worms recovered from the vaccinated animals was reduced compared with the number of worms recovered from the lungs of non-vaccinated animals (39 compared with 62 worms).

Fig 5 (a-c) shows images of intact and sectioned lungs from naive, non-vaccinated/infected and vaccinated/infected animals, 3 days p.i.. Intact lungs removed from a healthy mouse (Fig 5a, left panel) were of a uniform pink colour and showed a typical morphology with the bronchioles dividing into several alveolar ducts (ad),

each ending in an alveolar sac (as, Fig 5a, right panel). Lungs from non-vaccinated/infected animals (Fig 5b, left panel) displayed dark red patches, due to an accumulation of red blood cells, demonstrated in the transverse section (Fig 5b, right panel). Lungs from vaccinated/infected animals (Figure 5c) showed little visual haemorrhage compared with the non-vaccinated tissue and transverse sections revealed far fewer red blood cells than in the non-vaccinated/infected tissue.

3.4. *Antibody responses induced in vaccinated animals*

Serum samples from naïve, non-vaccinated/infected and three times percutaneously vaccinated/infected animals were used to examine the IgG subclass response to vaccination and subsequent infection with *N. americanus* (Fig 6). The worm burden data are presented in Table 1, experiment 1. Significantly higher levels of IgG1 were observed in the vaccinated/infected animals compared with naïve and non-vaccinated/infected mice on day 0, 3 and 8 p.i). However, IgG1 levels in the vaccinated animals were statistically lower at days 3 and 8 p.i.. than at day 0 ($P < 0.001$). IgG3 levels were highest in the vaccinated group, rising markedly after infection. Between days 3 and 8 there was also a significant ($P < 0.005$) increase in IgG3 levels in the non vaccinated/infected group. IgG2a levels were also raised, to a lesser extent, in the vaccinated group but IgG2b levels remained low and did not differ significantly between groups throughout.

4. Discussion

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In the fight against infectious diseases, most successful vaccines developed have used attenuated organisms. Attenuated vaccine models have been studied in detail for numerous parasites, including *S. mansoni* (Mountford et al., 1988; Pemberton et al., 1991); however, little is known about responses to such vaccines for *N. americanus* (Ghosh and Hotez, 1999; Sen et al., 2000). It is recognised that attenuated vaccines are unlikely to be the basis for a completely safe and successful human vaccine against *N. americanus*. Notwithstanding, an understanding of the immune response induced by γ-irradiated larvae will assist in the design of an acceptable human vaccine in terms of understanding the protective phenotype which need to be induced, which also has a bearing on the type of adjuvant chosen. The present paper describes the level of protection afforded, and the nature of the immune response induced, by the use of irradiated larvae against subsequent *N. americanus* infection in BALB/c mice.

The data presented demonstrate that complete protection against challenge infection was achieved following three vaccinations with 300 γ-irradiated larvae. It has also been shown that fewer than three immunisations may be sufficient to achieve a high level of protection against challenge infection, as two percutaneous immunisations with irradiated larvae led to 98 % protection in the intestine and 57 % protection in the lungs following infection. It is therefore possible (but not tested here) that a single immunisation with attenuated organisms may also confer significant protection against *N. americanus* infection. It has been reported elsewhere, for example, that a single exposure to 500 attenuated cercariae induces 60-70% protection in C57BL/6 mice against schistosomes (Dean, 1983). Hsu et al. (1983) reported, however, that resistance to schistosomes increased progressively with successive vaccinations with X-irradiated cercariae larvae. Studies in our laboratories have also shown that

vaccination with attenuated *N. americanus* larvae provides higher levels of protection against challenge infections than ES products from irradiated larvae in alum, a Th2 adjuvant (Girod, 2001, Development of a vaccine against the human hookworm, *Necator americanus*, PhD thesis, The Nottingham Trent University). Thus the attenuated organisms, representing a complex mixture of molecules involved in a diverse number of functions, need to be dissected further in order to define the essential components. Nevertheless, it is encouraging to note that a vaccine targeting the infective stage can be successful.

Percutaneous immunisation was shown to give greater protection than s.c. vaccination, implicating the importance of immunity in the skin. Knowing that *N. americanus* larvae infect their host by skin penetration, this first contact between the host and the parasite in the skin may be crucial for the outcome of the infection process and, therefore, an essential prerequisite for the development of a successful vaccine against *N. americanus*. Mast cell accumulation was observed in the skin of vaccinated animals, but not in the non-vaccinated/infected animals. It was also interesting to note that the mast cells in the non-vaccinated tissue were shown to contain granules whereas those in the vaccinated tissue were degranulated. Degranulation may have been caused by the binding of parasite-specific IgE to Fc receptors on the mast cell surface. The released mediators could then stimulate an inflammatory response, protective for the host, as suggested by the level of protection acquired with the irradiated larval vaccine. These results are in agreement with McKean et al. (1989) who suggested that mast cells were major effector cells in the immune response to infection with helminths. For instance, the killing of *S. mansoni*

by eosinophils has been shown to be enhanced by mast cell products (McLaren, 1989).

Mast cell recruitment following vaccination in our model suggested that Th2 cell activation leads to a protective immune response to *N. americanus*, given the IL4 dependence of the IgE response and the IL5 dependence of eosinophilia, both implicated in immunity to Necator in humans (Pritchard et al., 1995; Culley et al.; 2002). To investigate the possible involvement of a Th2 response, γ-IFN (a marker for Th1 cells) and IL4 (a marker for Th2 cells) mRNA expression were monitored in the skin of vaccinated/infected and non-vaccinated/infected animals at the site of challenge, using RT-PCR. As predicted, the results indicated an activation of a Th2 cell subset in the vaccinated animals. It should be noted, however, that mast cells are also able to secrete IL4 (Mota, 1994); thus the elevated Th2 response observed in the vaccinated animals may be partly linked to mast cell activation. Cytokine analyses demonstrated, however, that lymphocytes from the axillary lymph nodes of vaccinated/infected animals, specifically stimulated with ConA, produced high levels of IL4 and less γ-IFN. The difference in the IL4/γ-IFN ratio between non-vaccinated and vaccinated animals was particularly accentuated prior to infection, at which time IL4 levels were 10-times higher in the vaccinated group compared to the non-vaccinated group. The irradiated larval vaccine induced therefore a Th2 response, agreeing with observed recruitment of mast cells and the RT-PCR results. The axillary lymph nodes may play an important role in the development and maintenance of the immune protection against *N. americanus* and may stem from an initial stimulation of skin keratinocytes, mast cells and, probably, antigen presenting cells such as Langerhans cells. These cells could subsequently stimulate T cells in the

axillary lymph nodes which then migrated to the infected tissue, i.e. the lungs, and induced protection. A smaller number of larvae reached the lungs of vaccinated/infected mice, compared to non-vaccinated/infected animals. Indeed there is between 37 % (Figure 4) and 98 % (Table 1, Experiment 1) reduction in worm counts in the lung following infection, suggesting that the skin is an important immune compartment. Since vaccination reduces the number of worms in the gut to virtually zero, indicating that immune surveillance has occurred before the parasite arrives in the gut, a role for lung-draining lymph nodes is suggested. Thus in the future it would be useful to corroborate a shift to the Th2 phenotype, and to check the role of immune responses in the lung, by checking cytokine profiles in lung draining lymph nodes.

The antibody analysis also demonstrated that vaccination induced a Th2 response characterised by the production of IgG1 (a serological marker of the Th2 response). High levels of IL5 (a Th2 cell marker) were also observed in mice vaccinated with attenuated "Necator" larvae (Brown, A., 2000. *Necator americanus*: characterisation of secreted proteinases and vaccine development. PhD, The Nottingham Trent University). This supports the previous analyses done in human populations by Pritchard et al. (1995) on IgE production which showed a negative relationship between IgE (Th2 marker) and parasite weight and fecundity. Another concern regarding the development of a Th2 response was that it could be responsible for immune-mediated pathology (Finkelman and Urban, 1992). However, although the lungs of non-vaccinated/infected animals presented severe generalised haemorrhage, caused by the passage of "Necator" larvae into the lungs, rupturing pulmonary capillaries, little or no pathology was observed in the lungs of vaccinated animals. In

addition, the passage of infective larvae into the lungs of vaccinated/infected animals was delayed compared with that of non-vaccinated/infected animals. These combined data suggest that a Th2 response is responsible for the development of an acquired immunity in the vaccinated/infected animals and not responsible for the development of immuno-pathology. The fact that IgG1 levels were reduced in vaccinated animals three days after infection may indicate that the live infective larvae (unlike attenuated larvae) were moderating the Th2 response. Indeed the increase in IgG3 with time p.i. in both vaccinated and non-vaccinated groups would support this suggestion.

Acknowledgements

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Table 1. **Number of worms recovered from the lungs (3 days post-infection) and small intestine (8 days post-infection) following three subcutaneous or percutaneous immunisations (Experiment 1) and following two percutaneous immunisations (Experiment 2) with 300 irradiated larvae.** The results are expressed as the mean number of worms recovered from the lungs and small intestine ± SD (n = 6). The % protection represents the % reduction of worms recovered from the lungs and small intestine of vaccinated animals vs the non-vaccinated control group. * data significantly different between percutaneous and subcutaneous immunisation, ** data significantly different between vaccinated and non-vaccinated animals.

Group	Lungs (Day 3)		Intestine (Day 8)	
	Worms recovered Mean ± SD (n=6)	Protection (%)	Worms recovered Mean ± SD (n=6)	Protection (%)
Experiment 1				
Non-vaccinated	94 ± 17	0	18 ± 7	0
Vaccinated subcutaneously	10 ± 5	**89**	0	**100**
Vaccinated percutaneously	2 ± 3	**98***	0	**100**
Experiment 2				
Non-vaccinated	79 ± 58	0	20 ± 17	0
Vaccinated twice	34 ± 17	**57****	0	**100**

Table 2. **Levels of IL4 and γ-IFN secreted by *in vitro* ConA stimulated lymphocytes from axillary lymph nodes of vaccinated/infected and non-vaccinated/infected, 0, 3, 8 and 15 days post-infection.** 5×10^5 lymphocytes were stimulated for 3 days with a final concentration of 5 µg/ml of ConA in 200 µl. Values were determined by ELISA (n = 4). * data significantly different between vaccinated/infected and non-vaccinated/infected, $p < 0.006$ (Bonferri procedure, acknowledging 8 data variables).

Days post-infection	Non-vaccinated/infected			Vaccinated/infected		
	IL4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN	IL4 (pg/ml)	γ-IFN (pg/ml)	IL4/γ-IFN
0	1 ± 2	63 ± 31	0.02	6 ± 3	14 ± 20	0.43
3	2 ± 4	57 ± 3	0.03	14 ± 13	58 ± 33	0.24
8	1 ± 1	30 ± 15	0.03	69 ± 21*	52 ± 36	1.33
15	2 ± 3	22 ± 32	0.08	30 ± 39	98 ± 57	0.31

Legend to Figures

Fig 1. Light microscopy showing presence of mast cells in the skin of naïve, vaccinated and non-vaccinated mice 3 days post-infection. 10 μm transverse skin sections stained with thionin blue, (a) from a naïve animal, (b) non-vaccinated/infected animal and (c) vaccinated/infected animal. Skin sections from each group (consisting of 4 individuals) were found to show the same changes with respect to mast cells. A representative skin section (left panel, scale bar = 5 μm) and single mast cell (right panel, scale bar = 2.5 μm) are presented in this figure. E = epidermis, D = dermis, M = mast cell. Mast cells in (c) are degranulated.

Fig 2. Mast cell number (mean ± SD) in the skin of naïve, vaccinated and non-vaccinated animals following infection. Mast cells were detected and counted using thionin blue staining on skin sections of naïve, non-vaccinated/infected and vaccinated/infected animals (n = 4) at various times post-infection (day 1, 3 and 8 post-infection). Each point represents the mean of five different fields randomly selected from sections of four different animals. * = significantly different from the non-vaccinated/infected group, $p < 0.0055$ (Bonferri procedure, acknowledging 9 data points).

Fig 3. RT-PCR analysis of cytokine mRNAs in skin samples from non-vaccinated/infected and vaccinated/infected BALB/c mice three days after challenge. Lane 1: pGEM® DNA molecular weight markers; Lanes 2, 3, and 4 represent products for β-actin, IL4 and γ-IFN, respectively, from skin of non-vaccinated/infected mice; Lane 5, 6 and 7 represent products for β-actin, IL4 and γ-

IFN, respectively, from skin of vaccinated/infected mice. The gel profile is representative of 4 animals analysed.

Fig 4. Larval migration through the lungs of non-vaccinated/infected and vaccinated/infected BALB/c mice. The number of larvae recovered from the lungs of non-vaccinated and twice vaccinated animals (n = 6 in each group) determined on days 1-5 and 7-10 following infection. Results are expressed as the mean (± SD) of the number of worms recovered.

Fig 5. Morphological and histological comparison of the lungs of naïve, non-vaccinated and vaccinated BALB/c mice. (a) Naïve animals, (b) non-vaccinated/infected animals and (c) vaccinated/infected animals. Left panels show representative lungs at post-mortem (scale bar = 0.5 cm). Right panel shows traverse sections of same lungs stained with haematoxylin and eosin (scale bar = 2.5 μm). In lung sections ad = alveolar duct, as = alveolar sac, rb = accumulation of red blood cells, haemorrhage.

Fig 6. IgG subclass of immune response on days 0, 3 and 8 post-infection in non-vaccinated and vaccinated mice compared to naïve animals. IgG subclass levels were determined by ELISA. Results are expressed as the mean absorbance (± 1 SD) of sera obtained from six mice per group (except in naïve group, 3 and 8 days post-infection, where n = 4); assays carried out in triplicate. * = significantly different from the naïve and non-vaccinated/infected groups, $p < 0.0055$ (Bonferri procedure, acknowledging 9 data points for each isotype).

Figure 1.

(a) Naïve tissue
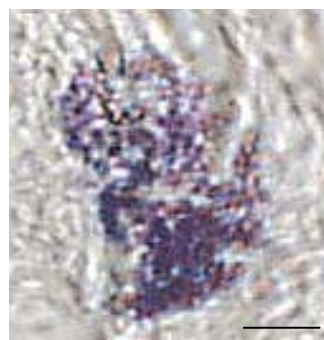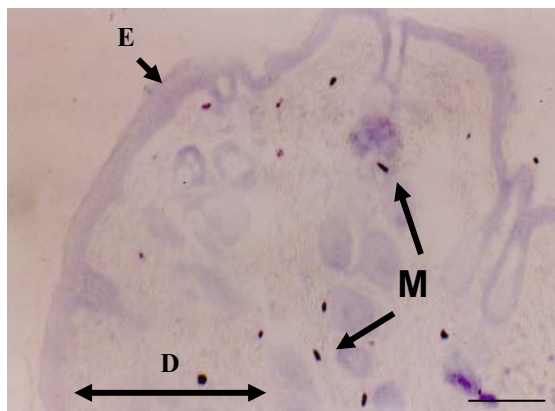
(b) Non-vaccinated/infected tissue
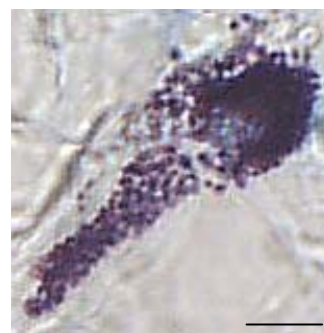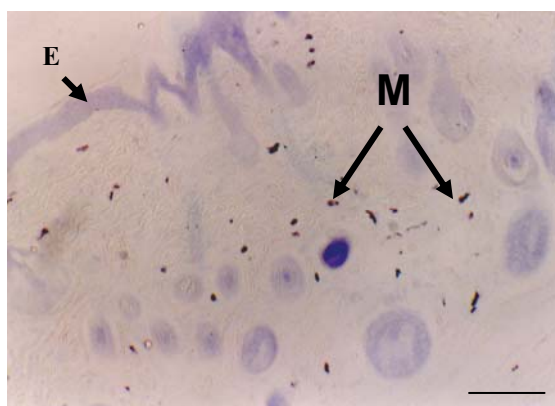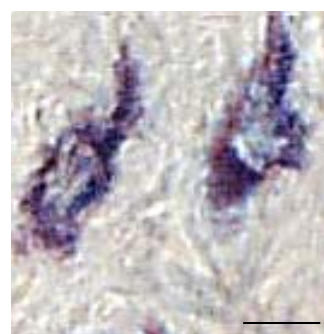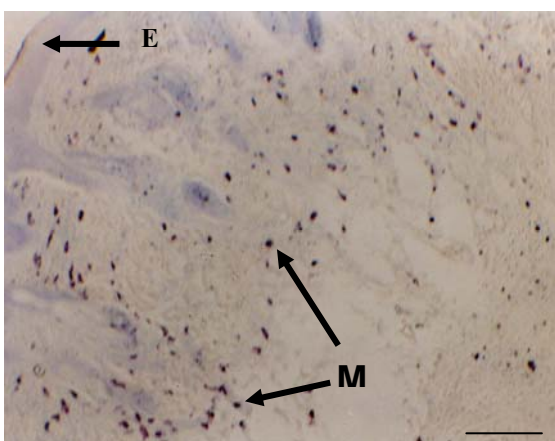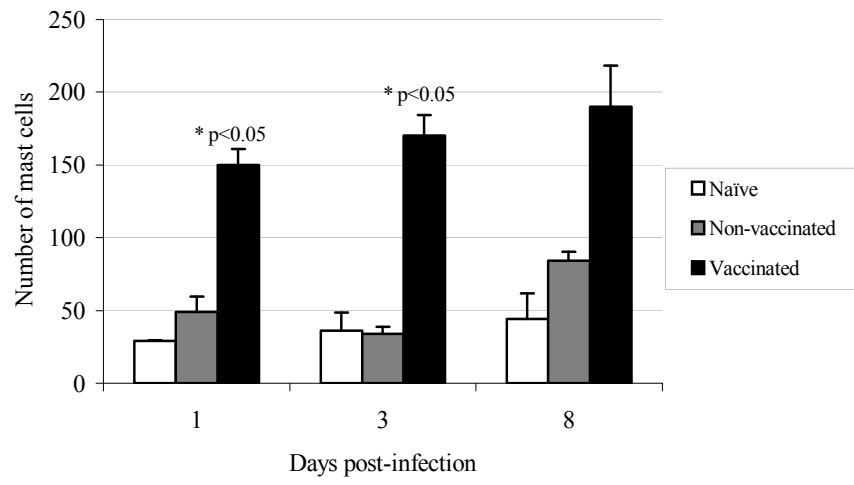

(c) Vaccinated/infected tissue
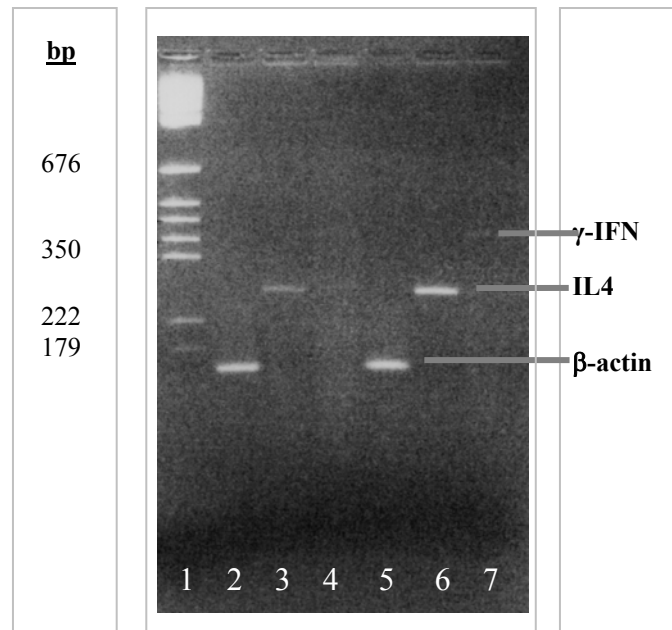
Figure 2.

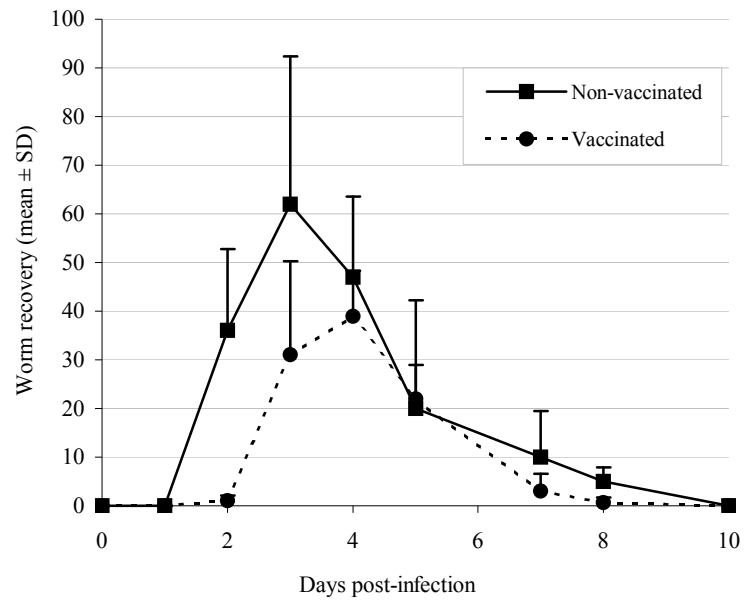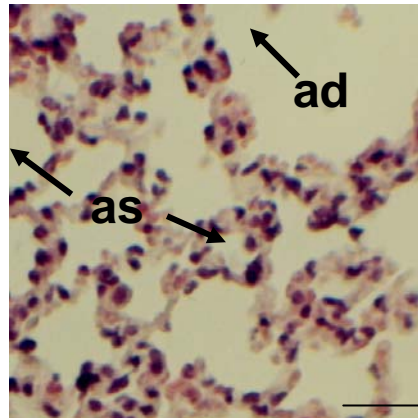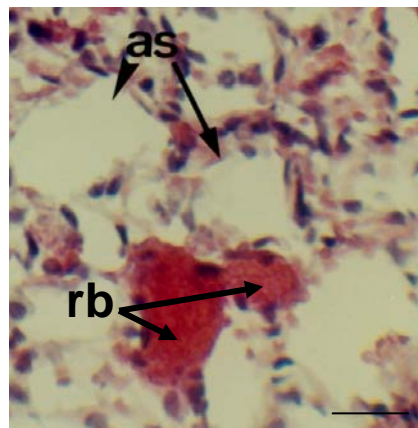**Figure 3.**

Figure 4.

Figure 5.

(a) Naïve mice

(b) Non-vaccinated/infected mice

(c) Vaccinated/infected mice

Figure 6.