

The assessment of hookworm calreticulin as a potential vaccine for necatoriasis

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SUMMARY

A vaccine against the human hookworm Necator americanus is urgently required to reduce hookworm-induced morbidity in endemic areas. In the present study, recombinant hookworm calreticulin, a nominated vaccine candidate, has been tested in mice. Mice given calreticulin had 43–49% fewer worms in their lungs, compared to non-vaccinated controls, following challenge infection with infective hookworm larvae. These levels of protection were achieved in the absence of adjuvant following intraperitoneal administration of three doses of 15 µg antigen. Antigen was also encapsulated in PLG microparticles. Encapsulated calreticulin elicited higher levels of anti-calreticulin IgG1 than free antigen but failed to induce protective immunity. The protection induced by free calreticulin was associated with low levels of serum IgE and moderate lung eosinophilia whilst administration of calreticulin-loaded microparticles was associated with high levels of serum IgE and higher lung eosinophil activity, suggesting that the classical Th2 phenotype may not always be associated with protective immunity, albeit in experimental necatoriasis.

Keywords calreticulin, hookworm, necatoriasis, vaccine

INTRODUCTION

Necator americanus is a haematophagous parasitic nematode that inhabits the small intestines of humans. Infection causes severe morbidity in the tropical regions of Africa, Asia and the Americas, due to the development of iron deficiency anaemia in malnourished hosts. Although effective anthelmintic chemotherapy is available, people become rapidly re-infected following treatment (1) and acquire very little natural immunity against the worms. The only evidence for any kind of effective immune response is reflected in the fact that infected individuals with high levels of total IgE in their serum appear to harbour smaller and less fecund worms, although worm burden is unaffected (2). This lack of effective immunity is attributed to the ability of *N. americanus* to evade the host immune response and to stimulate an immunological phenotype inappropriate to protection (reviewed by Pritchard & Brown (3), and Pritchard *et al.* (4)).

A vaccine against *N. americanus* would be a highly desirable solution to this serious global health problem. Although infection with irradiated hookworm larvae can elicit effective immunity in mice (5) and dogs (6), such a vaccine would be unsuitable for human use for a number of reasons. Firstly, percutaneous administration of irradiated larvae is unpleasant and multiple doses may be required. Secondly, the larvae are difficult to culture on a large scale and quality control would be problematic. Finally, the widespread use of a 'live' vaccine might be difficult in underdeveloped countries because of the need for a refrigeration chain and the limited shelf life of the larvae. Therefore, an ideal vaccine would be genetically engineered, easy to produce on a large scale and have reasonable stability. It would also be advantageous if the vaccine could be delivered mucosally, to avoid the disease transmission risks and need for specialist health workers associated with needle use, and to induce a protective immunological phenotype.

As IgE responses are equated with a degree of immunity to *Necator americanus*, early research was directed toward the identification of hookworm allergens, and calreticulin

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was identified as one allergenic protein (7). Recombinant *N. americanus* calreticulin has been shown to bind and inhibit the human complement component C1q (8), a biological attribute that suggests its presence as an immune evasion molecule. As a result, calreticulin was nominated as a vaccine candidate (4,8–9) for two reasons: its suggested role as an immune evasion molecule, and its apparent induction of a strong Th2 type immune response (calreticulin-specific IgE) in infected individuals. In the present study, preliminary vaccine testing has been carried out using recombinant *N. americanus* calreticulin and a potential mucosal delivery system has been trialled.

The mucosal delivery system employed in this study utilized antigen encapsulated in poly-lactide-coglycolide (PLG) microparticles. PLG is a well-characterized, biodegradable polymer that has been used in human sutures for many years. Encapsulation of antigen into PLG microparticles has been shown to enhance immune responses compared to free antigen in a number of studies (for example Eldridge *et al.*, Men *et al.* and Rafati *et al.*) (10–12). This is attributed to the microparticles acting as a depot adjuvant, releasing protein slowly and prolonging antigen presentation to the immune system. Furthermore, PLG microparticles have shown potential as mucosal delivery systems because they can protect antigens against degradation in the gut, and targeting moieties may be attached to their surfaces to enhance uptake efficiency to the mucosal immune system (reviewed by Brayden) (13).

In summary, recombinant *N. americanus* calreticulin has been tested in the present study as a vaccine candidate in a mouse model of hookworm infection. The immunological phenotypes of vaccinated animals were studied following administration of free calreticulin or calreticulin encapsulated in PLG microparticles, along with those of non-vaccinated controls. The data obtained are discussed in the context of future vaccine development for necatoriasis.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from Sigma unless otherwise stated. BALB/c mice were obtained from Harlan Olac Ltd, Bicester, Oxon. 'Purified water' refers to chromatography purified water (Elga).

Production and characterization of vaccine formulations

Production and characterization of recombinant N. americanus calreticulin

Recombinant protein was produced following the procedure described by Kasper *et al.* (8). The six histidine-tagged protein was purified from *E. coli* inclusion bodies using metal affinity

chromatography, dialysed against purified water, lyophilized and stored at -20°C until use. Identity of the recombinant protein was confirmed by Western blot and MALDI-TOF MS. Briefly, samples were resolved on 12% SDS-PAGE acrylamide gels under reducing conditions, as described by Laemmli (14). Proteins were transferred to a nitrocellulose membrane (SLS) by the method of Towbin *et al.* (15), and the membrane incubated in 5% Marvel milk powder-TBS for 1 h at room temperature to block non-specific binding. Blots were then probed with 1 : 600 anti-calreticulin rabbit serum (in blocker) and incubated at 4°C overnight. Unbound antibody was washed off using TBS/0.05% Tween and bound antibody detected by incubation for 2 h at room temperature with goat anti-rabbit IgG-alkaline phosphatase. Unbound secondary antibody was washed off using TBS/0.05% Tween and blots were developed using NBT and BCIP in 0.75 M Tris pH 9.6. Purified recombinant protein was also analysed by Matrix Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) following tryptic digestion.

Production of calreticulin loaded and empty microparticles

PLG microparticles were made using a w/o/w double emulsion solvent evaporation technique, as described by O'Hagan *et al.* (16). Briefly, calreticulin (100 μL , 10 mg/mL) solution in 8 M urea was added to a PLG solution in dichloromethane (50 mg/mL, 2 mL). The mixture was emulsified at 19 000 g for 2 min (Ultra Turrax T25 high speed homogenizer) to form a primary emulsion. This was added to a solution of PVA (5% w/v, 20 mL) in purified water and the mixture emulsified at 22 000 g for 2 min. The secondary emulsion formed was stirred gently overnight to allow evaporation of organic solvent. Resultant microparticles were washed four times by centrifugation at 12 000 g for 10 min and re-suspended in purified water, then lyophilized and stored at -20°C until use.

Empty microparticles were made by the same method, except that calreticulin was omitted.

Encapsulation efficiency and protein loading of microparticles

Calreticulin and empty microparticles (2 mg each) were dissolved in NaOH (100 μL , 0.5 M) at room temperature. Samples from the dissolved microparticle solutions were assayed for protein content using a BCA protein assay kit (Pierce). Encapsulation efficiency, i.e. the amount of protein contained in the microparticles as a percentage of the amount of protein initially added to the primary emulsion, was determined. Protein loading was defined as the amount of protein contained in the microparticles as a percentage of the total weight of microparticles (2 mg).

Scanning electron microscopy (SEM) of microparticles

Size and morphology of microparticles were assessed by visualization using a Philips SEM 505 (Eindhoven, Netherlands).

Microparticles were suspended in purified water and a small droplet of the suspension was placed on an aluminium SEM stub. The solution was allowed to dry completely and the dry sample was sputter-coated with gold using a Balzers SCD 050 sputter coater unit (BAL-TEC AG, Liechtenstein). Gold-coated samples were imaged at 2500 magnification using 25 kV tension. Images were retrieved from the SEM using Semicaps 2000 A software, version 8.2.

Calreticulin release from microparticles

Microparticles (2 mg) were incubated in PBS (50 µL) at room temperature for 1 h with shaking. The microparticles were then sedimented by microcentrifugation at 8000 *g* for 3 min and the supernatant was analysed by BCA assay, SDS-PAGE and rabbit anti-calreticulin IgG Western blot as described above. The pellet of microparticles was dissolved completely in NaOH (100 µL, 0.5 M) and the resultant solution was analysed by BCA assay to determine the proportion of protein present near the surface of microparticles (released rapidly into PBS) and the proportion of protein encapsulated in the interior of the microparticles (released after NaOH degradation).

Vaccine testing

Larval culture

Necator americanus was maintained in DSN hamsters by repeated passage as previously described (17).

Immunization protocols and challenge

Vaccination studies were carried out in BALB/c mice. Three groups of five mice (preliminary experiment) or 10 mice (repeat study) were given three fortnightly, intraperitoneal doses of either:

- 15 µg calreticulin (suspended in PBS);
- equivalent mass of microparticles containing 15 µg calreticulin (in PBS);
- equivalent mass of empty microparticles as adjuvant control (in PBS).

Intraperitoneal, rather than mucosal, delivery was utilized in this preliminary study to exclude failed delivery as a reason, should the vaccine fail. In the event of successful intraperitoneal vaccination with calreticulin-loaded microparticles, the vaccine was to be developed further for mucosal delivery.

Blood samples were taken 2, 4 and 6 weeks after the first immunization and blood was allowed to clot at room temperature for 1 h, then microcentrifuged at 13 400 *g* for 10 min to pellet cells. Serum was removed and stored at -20°C until use.

Mice were challenged using 300 infective L3 stage larvae, 2 (preliminary study) or 6 (repeat study) weeks after the third and final immunization (18). Briefly, mice were anaesthetized and the hair removed from the back of the neck using electric hair clippers. Larvae were applied to the bare skin on absorbent gauze and held in place for 24 h with self-binding bandage (International Market Supply, Congleton, Cheshire).

Assessment of worm burdens in the lungs

Half of the mice from each group were killed using approved schedule one methods 3 days after challenge infection. Lungs were excised and worm burdens assessed using the method described by Wells and Behnke (19). The remaining mice from each group were killed 8 days after challenge for the purpose of assessing worm burdens in the intestines. However, due to the failure of *Necator americanus* to complete its life cycle in mice, few worms were recovered from the intestines of mice in any group and the data are not included herein. For example, an average of only 3.6 worms per mouse were isolated from the intestines of mice in the empty microparticle control group.

Immunological phenotyping

Anti-calreticulin IgG

Plates were coated with 50 µL/well recombinant calreticulin (5 µg/mL) or purified mouse IgG1/2a (Pharmingen) standards in the range 0–3 ng/well in 0.05 M carbonate/bicarbonate buffer pH 9.6, sealed and incubated overnight at 4°C. Two hundred µL/well 1% BSA in PBS were added to wells for 1 h at room temperature to block non-specific binding and 50 µL/well serum samples (diluted 1 : 100 in blocker) were added to the wells coated with calreticulin, and blocker alone added to the wells coated with IgG1/2a standards. Plates were sealed and incubated overnight at 4°C. Plates were washed four times with 1 min soaks, using PBS/0.05% Tween-20, and 50 µL/well anti-mouse IgG1/2a-HRP (Pharmingen), diluted 1 : 1000 in blocker, were added to all wells. Plates were sealed and incubated at room temperature for 2 h. Plates were then washed six times with 1 min soaks, using PBS/0.05% Tween-20, and developed using 100 µL/well developer (one 3,3',5,5'-tetramethylbenzidine (TMB) tablet in sodium acetate pH 6.05 (10 mL, 0.1 M), plus 2 µL 30% H₂O₂). The reaction was stopped by adding 50 µL/well H₂SO₄ (2 M) and plates were read at 450 nm using a Dynex MRX microplate reader.

Analysis of total and specific IgE

For total IgE, ELISAs were carried out as above except that plates were coated with 100 µL/well anti-mouse IgE (Pharmingen) (2 µg/mL) in PBS, blocked, and either mouse IgE standards (Pharmingen) in the range 0–0.5 µg/mL or

serum samples diluted 1 : 20 in blocker added at 100 μ L/well overnight at 4°C. Bound antibody was detected using 100 μ L/well biotinylated anti-mouse IgE (Pharmingen) (2 μ g/mL) in blocker for 1 h at room temperature, then 100 μ L/well avidin-HRP, diluted 1 : 1000 in blocker for 30 min room temperature, then TMB reagent as above, with washing between each step.

For specific IgE, ELISAs were carried out as above except that plates were coated with 50 μ L/well of either calreticulin (50 μ g/mL) in PBS or mouse IgE standards in the range 0–0.1 μ g/mL in PBS, blocked, and 50 μ L/well serum samples (1 : 10 in blocker) added to the plates, with blocker only on the IgE standards, overnight at 4°C. Bound antibody was detected using 50 μ L/well biotinylated anti-mouse IgE (2 μ g/mL) in blocker for 1½ hours at room temperature, then 100 μ L/well avidin-HRP, diluted 1 : 1000 in blocker for 30 min at room temperature, then TMB reagent as above, with washing between each step.

Lung eosinophil peroxidase activity

This was assessed using the method described by Das *et al.* (20). Briefly, 5% w/v lung tissue (taken either 3 or 8 days after challenge infection) was homogenized for 1 min at 24 000 g in fresh HBSS using a Yellowline DI 25 Basic homogenizer. Cells were pelleted and erythrocytes removed by hypotonic lysis. Cells were re-suspended in HBSS with 0.5% hexadecyltrimethylammonium bromide (HTAB) and lysed by freeze-thawing in liquid nitrogen. Lung material was then micro-centrifuged and supernatants diluted at 0.313% in HBSS/HTAB.

Eosinophil peroxidase activity was assayed by adding 150 μ L substrate to 75 μ L of each sample (substrate = Sigma FAST 0-phenylenediamine dihydrochloride and buffer tablet set, made up according to manufacturer's instructions) and incubating at 20°C for 30 min. Reactions were stopped by the addition of 75 μ L H₂SO₄ (2 M) and the optical density of each sample read at 492 nm and compared to samples stopped at the start of the incubation period.

Statistical analysis

Data were analysed using Minitab software by one-way analysis of variance (ANOVA).

RESULTS

Production and characterization of vaccine formulations

Recombinant calreticulin characterization

Figure 1 shows positive identification of the recombinant protein as *Necator americanus* calreticulin by Western blot using rabbit anti-calreticulin IgG. The protein resolves at

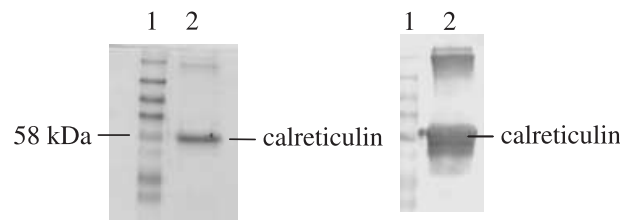


Figure 1 SDS-PAGE gel and rabbit anti-calreticulin IgG Western blot confirming identity of recombinant protein as *Necator americanus* calreticulin. Lane 1, protein standards; lane 2, recombinant calreticulin.

about 56 kDa, as expected, since calreticulin migrates abnormally on SDS-PAGE given its predicted molecular weight of approximately 46.5 kDa (21). The blot used a polyclonal rabbit antibody that has previously been shown to be highly specific to *N. americanus* calreticulin (7). The identity of the protein was also confirmed by MALDI-TOF MS (Peptident score 0.47, 8 peptide matches, most probable match is *Necator americanus* calreticulin in the SWISS-PROT and TrEMBL databases).

Calreticulin loaded microparticles release intact antigen

Figure 2 shows scanning electron micrographs of empty (Figure 2a) and calreticulin loaded (Figure 2b) microparticles. A rabbit anti-calreticulin IgG Western blot showing release of intact calreticulin from microparticles (Figure 2c) is also included. Released protein resolved as expected on SDS-PAGE and reacted with rabbit anti-calreticulin IgG (Figure 2c). Microparticles were smooth and spherical,

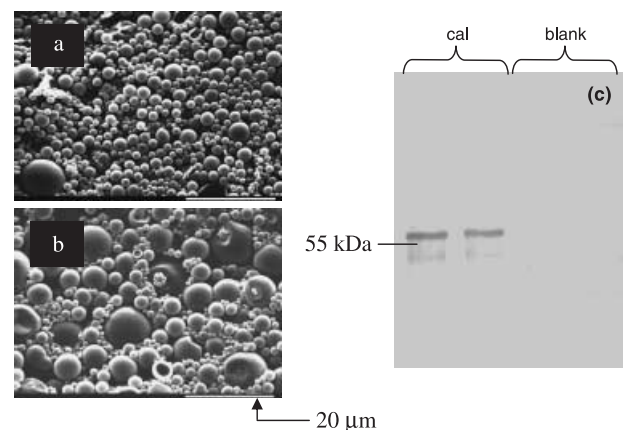


Figure 2 Characterization of calreticulin-loaded and empty microparticles. SEM images show (a) empty and (b) calreticulin-loaded microparticles (bar length = 20 μ m). (c) Rabbit anti-calreticulin Western blot demonstrates the release of intact calreticulin from calreticulin-loaded microparticles with no release of protein from empty microparticles following shaking in PBS. Cal, calreticulin-loaded; blank, empty microparticles.

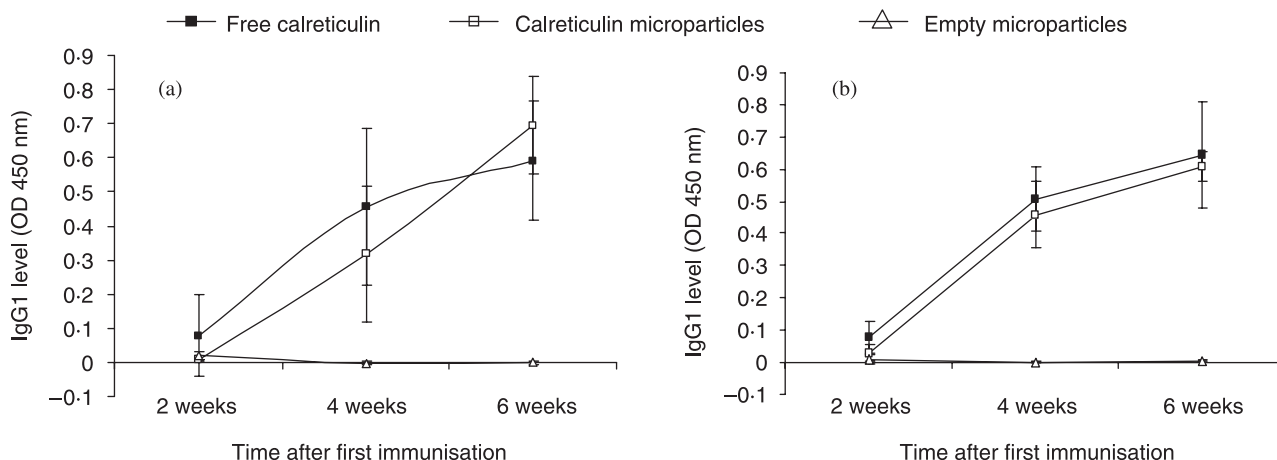


Figure 3 Charts showing mean anti-calreticulin IgG1 levels ($\pm$ standard deviation) in sera of immunized mice 2, 4 and 6 weeks after first immunization in (a) preliminary and (b) repeated studies. Means of 5 (a) and 10 (b) mice per group shown.

without obvious pores or surface imperfections. Calreticulin microparticles had a protein loading of $\sim 0.9\%$. Encapsulation efficiency was good with 45.9% of the protein added at the formulation stage associated with produced microparticles. Protein was present both at the surface (immediate release into PBS) and interior (release upon NaOH digestion) of the microparticles, and microparticles were in the size range 0.1–9 μm (mean 1.7 μm) as measured by SEM.

***Necator americanus* calreticulin elicits strong specific serum IgG1 responses in mice**

Figure 3 shows the levels of anti-calreticulin IgG1 detected in the serum of the immunized mice at three time points before challenge infection, in both the preliminary (Figure 3a) and repeated (Figure 3b) studies. The mice given calreticulin, either free or encapsulated, exhibited strong, specific antibody responses. The antibody levels were very similar between mice given the two different calreticulin vaccines, indicating that the mice were exposed to similar levels of antigen. As expected,

mice given empty microparticles (no antigen) had no anti-calreticulin IgG1 in their sera.

Free, but not encapsulated *Necator americanus* calreticulin can reduce worm burdens in the lungs of challenged mice

The numbers of worms isolated from the lungs of mice following challenge are shown in Figure 4 (a) (preliminary study) and Figure 4 (b) (repeated study). In the first study, administration of free calreticulin reduced the number of worms significantly ($P < 0.05$) compared to the empty microparticle control group (43% reduction in worm burden). However, the number of worms isolated from the lungs of mice given calreticulin microparticles was not reduced, indicating that calreticulin microparticles have no protective effect. Similar trends were seen in the repeated study (free calreticulin reducing worm burdens by 49%), although large variation is seen in the data from microparticle-vaccinated groups in this study, so this reduction is not statistically significant.

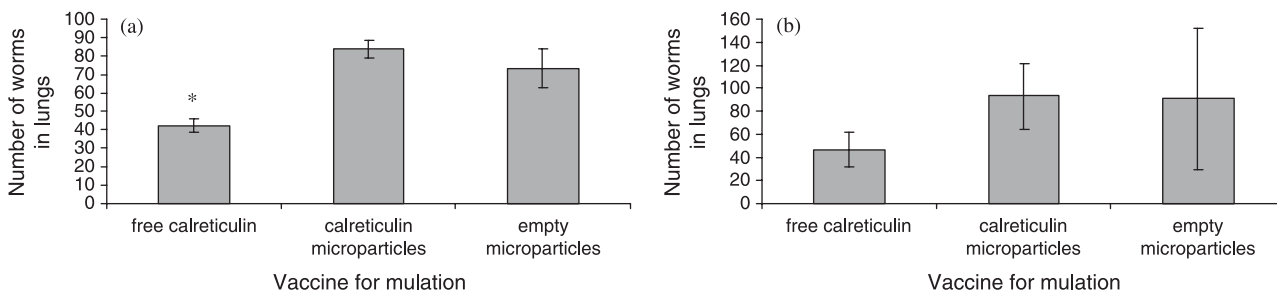


Figure 4 Numbers of worms isolated from lungs of immunized mice 3 days after challenge infection in (a) preliminary and (b) repeated studies. *Significant difference ($P < 0.05$). Means of 3 (a) and 5 (b) mice per group $\pm$ standard deviations shown.

Table 1 Mean serum IgE, IgG1 and IgG2a levels and lung eosinophil peroxidase activities in immunized mice, $\pm$ standard deviations. Means of 10 (IgE) and 5 (eosinophil peroxidase) mice per group are shown. For IgG1/2a titres, titres from pooled endpoint sera are shown

Test	Free calreticulin	Calreticulin microparticles	Empty microparticles
Anti-calreticulin serum IgE (ng/mL)	ND	114.26 $\pm$ 73.54*	ND
Total serum IgE (ng/mL)	89.92 $\pm$ 44.91	246.99 $\pm$ 143.14	313.54 $\pm$ 305.42
Anti-calreticulin IgG1 (titre from pooled endpoint sera)	> 160	> 2560*	< 10
Anti-calreticulin IgG2a (titre from pooled endpoint sera)	> 160	> 640*	< 40
Eosinophil peroxidase activity (OD 492 nm) 3 days post challenge	0.009 $\pm$ 0.007*	0.077 $\pm$ 0.006	0.113 $\pm$ 0.059
Eosinophil peroxidase activity (OD 492 nm) 8 days post challenge	0.107 $\pm$ 0.071	0.058 $\pm$ 0.034	0.085 $\pm$ 0.080

ND, not detectable.

*Significant difference ($P < 0.05$).

Immunological phenotyping of vaccinated mice

Following the repeated study, an investigation was carried out into the immunological phenotypes of animals in the three experimental groups. The main findings are summarized in Table 1. Total serum IgE was higher in the mice given encapsulated calreticulin than in those given free calreticulin. However, serum IgE was also elevated in the adjuvant control group (empty microparticles), suggesting that this response is caused by the hookworm infection rather than by the vaccine. The high standard deviations may reflect the high variability in worm burdens between mice in the microparticle groups (cf. Figure 4b). Encapsulated calreticulin also elicited anti-calreticulin IgE but free calreticulin did not, which is unexpected given that calreticulin is known to be an allergen (7).

In order to determine antibody titres, existing data were tested to confirm normality within each group and sera for each group were pooled. Titrations were then carried out using pooled endpoint sera for each group (Table 1). Analysis of antibody titres rather than ng/mL antibody at a given dilution, as in Figure 3, revealed that mice given encapsulated calreticulin in fact produced higher antibody titres (calreticulin-specific IgG1 and IgG2a) than mice given free calreticulin. This is consistent with previous studies (for example Eldridge *et al.*, Men *et al.* and Rafati *et al.*) (10–12) showing the adjuvant effect of microparticles and their ability to enhance antibody responses. The data also showed that for mice given encapsulated calreticulin, IgG1 responses were stronger than IgG2a responses, suggesting again that these mice were exhibiting a polarized Th2 type immune response. Mice given free calreticulin, on the other hand, produced similar titres of IgG1 and IgG2a suggesting that these mice, although partially immune to challenge, were not exhibiting such a polarized immune response.

Three days after challenge, the mice given microparticle formulations had significantly ($P < 0.05$) higher lung eosinophil activity than the mice given free calreticulin.

Eosinophilia in the lungs 8 days post challenge showed larger standard deviations than at 3 days post challenge and there were no significant differences between the groups – this may reflect a switch from innate (at day 3) to adaptive (at day 8) eosinophilia (22). It is possible that the day-3 data show an early reaction to infection while the highly variable eosinophilia at day 8 follows severe tissue damage after larval migration through the lungs.

DISCUSSION

Recombinant *Necator americanus* calreticulin has been purified from *E. coli* inclusion bodies and successfully incorporated into PLG microparticles. Neither solubilization in 8 M urea nor the encapsulation process caused any detectable damage to the protein as determined by Western blotting. The calreticulin-loaded microparticles produced were of an appropriate size to be taken up by phagocytic cells and elicited a strong, anti-calreticulin immune response in mice. However, it was observed that following challenge infection, calreticulin-loaded microparticles did not confer any protection. This was not due to incomplete antigen release from the microparticles, because similar data were obtained when challenge infection was delayed until 6 weeks after vaccination. In contrast, significant protection was seen following vaccination with naked recombinant calreticulin.

Necator americanus calreticulin is a nominated hookworm vaccine candidate. In the current study, free calreticulin reduced worm burdens in the lungs by 43–49% compared to controls, although a larger sample size will now be required to accurately assess the true level of significance of these data. Nevertheless, this protection has been achieved in the absence of adjuvant, with a recombinant protein. However, the encapsulation of calreticulin in PLG microparticles appears to prevent this protective effect, although calreticulin was shown to be released in an immunologically active form. This finding was surprising because

Table 2 A summary of the key differences between mice given free and encapsulated calreticulin

Calreticulin microparticles	Free calreticulin
Higher total IgE in serum	Lower total IgE in serum
Anti-calreticulin IgE*	No anti-calreticulin IgE
Higher specific IgG1 and IgG2a titres*	Lower specific IgG1 and IgG2a titres
Higher eosinophil activity in lung (soon after challenge)*	Lower eosinophil activity in lung (soon after challenge)

*Significant difference ($P < 0.05$).

microparticles have previously been shown in the literature to greatly enhance immune responses compared to free antigen. Although few studies have gone on to assess the efficacy of these enhanced responses, where protection has been assessed it has been proven (for example, Conway *et al.*) (23). Clearly the protection elicited by free calreticulin in this study is not dependent upon anti-calreticulin IgG1 levels in serum, because mice given encapsulated calreticulin had higher anti-calreticulin IgG1 titres than mice given free calreticulin, but had very different worm burdens in the lungs. Mice given encapsulated calreticulin also produced higher titres of anti-calreticulin IgG2a – so for all antibody classes tested, mice given encapsulated calreticulin responded more strongly than mice given free calreticulin. This suggests that the mechanism of immunity associated with protection in mice given free calreticulin was not antibody-dependent, or that the encapsulated formulation was having an as yet unidentified adverse effect on protective immunological phenotypes.

To begin to elucidate the immunological mechanism(s) underlying the levels of protection observed, the immunological phenotypes of the mice in the second study were examined and some differences were found between mice given free or encapsulated calreticulin (Table 2). This implies that although free calreticulin was a suspension of insoluble protein, and the encapsulated calreticulin was also delivered in a particulate form, the two formulations may have been presented to the immune system in different ways.

Immune responses against nematode worms are typically polarized towards the type 2 phenotype – characterized by high levels of IgE in the serum and by blood eosinophilia (4). The highly effective irradiated larval vaccine is associated with this type of immune response (5), and as mentioned above high levels of IgE in humans are associated with reduced worm size and fecundity (2). However, micro-encapsulated calreticulin elicits a strong, specific type 2 response (as a component of a mixed immunological phenotype), but fails to protect mice against hookworm challenge. Conversely, free calreticulin reduces worm burdens in the lungs of immunized mice but does not induce comparable levels of eosinophilia, IgG1 or IgE production. So in this instance, protection against hookworms has been seen which is not associated with the type 2 phenotype. However, mice given

free calreticulin also fail to produce a strong IgG2a response which would indicate a type 1 bias. Interestingly, naïve mice and mice given an irradiated larval vaccine show elevated non-specific serum IgE responses following challenge, suggesting that this is a response to the challenge infection and that the free calreticulin vaccine suppresses or diverts this response in some way. It has been suggested that by generating high levels of non-specific IgE in the host, hookworms are able to protect themselves from specific IgE (3), so perhaps the effectiveness of the calreticulin vaccine could be attributed in part to the 'low-IgE' phenotype of vaccinated mice.

The present findings therefore suggest that protective immunity against hookworms is complex and may be achievable in different ways, for example by stimulating a non-humoral cell-mediated response. As a result, it may be unrealistic to seek a particular 'protective' immunological phenotype from the point of view of vaccine development. It is also important to note that microparticulate formulations must be carefully tested, since the release of intact protein and the induction of a strong immune response may not necessarily translate into protection against challenge infection. It is possible that encapsulation of calreticulin, whilst leaving the protein whole and antigenic, may degrade certain protective epitopes (24), and this will be the subject of a future study. Furthermore, since use of microparticles as an adjuvant did not result in protective immunity in this study, the use of alternative adjuvants such as alum should be explored in the future to further improve the efficacy of calreticulin as a vaccine against necatoriasis. Nevertheless, the protection seen in this pilot study is worthy of report, and is a sound base on which to build an improved vaccine formulation.

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