

Vaccination with recombinant *Ascaris suum* 24-kilodalton antigen induces a Th1/Th2-mixed type immune response and confers high levels of protection against challenged *Ascaris suum* lung-stage infection in BALB/c mice

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Abstract

Previous studies have shown that antigens from various life-cycle stages of *Ascaris suum* can induce host-protective immunity against challenge infections with infective eggs of *A. suum*. This study evaluated whether *Escherichia coli*-expressed recombinant 24-kDa antigen from *A. suum* (rAs24) was a suitable vaccine candidate for the control of *Ascaris* infections by examining its performance in a mouse model. Immunization of BALB/c mice in three consecutive doses with rAs24 in Freund's Complete Adjuvant (FCA) results in protection against challenge infections as manifested by a 58% reduction ($P < 0.001$) in recovery and stunted development of *A. suum* lung-stage larvae at day 7 post-challenge. Sera obtained from immune protected mice had a significantly increased level of immunoglobulin G (IgG) ($P < 0.0001$) but had no IgE response. Analysis of IgG-subclass profiles revealed that IgG1 ($P < 0.0001$) showed the greatest increase followed by IgG2b ($P < 0.005$), IgG2a ($P < 0.006$) and IgG3 ($P < 0.04$). Splenic T cells from rAs24-FCA immunized mice secreted significantly high levels of both Th1 cytokine gamma-interferon ($P < 0.005$) and Th2 cytokine interleukin-10 ($P < 0.001$) after stimulation with rAs24 in vitro. Interestingly, affinity purified anti-rAs24 IgG was shown to inhibit moulting of *A. suum* lung-stage L3 to L4 in vitro by 26%, indicating an in vivo function of the endogenous As24 in the moulting processes. An intense expression of endogenous As24 in the hypodermis and gut epithelium of *A. suum* lung-stage L3 by immunofluorescence supports a function for endogenous As24. These findings may contribute to the understanding of rAs24-induced Th1/Th2-mediated effector mechanisms required for the protection of *A. suum* lung-stage larval infection. © 2005 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: Roundworm; *Ascaris suum*; Recombinant As24; Protective response; Moulting inhibition

1. Introduction

The nematode parasite *Ascaris lumbricoides* is the commonest geohelminth of humans and is estimated to infect over 1.5 billion people worldwide (Chan, 1997). No vaccines are available for prevention of *Ascaris* infections, and the chemotherapeutic intervention of established infections would eventually fail to reduce the overall prevalence of the disease. This requires discovery of alternative means for the prevention and control ascariasis

in humans and pigs. However, identification and characterization of potential protein antigens from *Ascaris* represents a major challenge in the development of parasite vaccine. Moreover, identification of parasite-specific antigens is critical for the development of safe and successful vaccines, since parasite-specific antigens would not induce antibodies that cross-react with host proteins (Abraham et al., 2001; Tsuji et al., 2003).

Ascaris suum is a very closely related species to *A. lumbricoides*, which can infect human hosts, indicating its zoonotic significance (Anderson et al., 1993; Peng et al., 1998). Recent studies have suggested that *Ascaris*/pig model can be used as a suitable tool for biochemical and

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immunological studies of *A. lumbricoides* since the two species are morphologically and antigenically indistinguishable from each other and possess an identical lifecycle (Crompton, 2001; Tsuji et al., 2001; Abebe et al., 2002). Numerous studies have shown that crude antigens from *A. suum* lifecycle stages can induce host-protective immunity (Urban and Romanowski, 1985; Lukes, 1992; Hill et al., 1994; Serrano et al., 2001), indicating that *A. suum* possess immunogenic molecules. Studies from our laboratory have previously shown that *Escherichia coli*-expressed recombinant protein antigens from *A. suum* can also induce host-protective immunity and confer significant levels of protection against parasite challenge (Tsuji et al., 2001, 2003). However, data to help elucidate *Ascaris*-specific potent immunoreactive protein antigens that could become potential targets for vaccine and/or chemotherapeutic agent is scarce. Furthermore, very little is known about the precise effector mechanisms that trigger and maintain a Th2-biased response to *Ascaris* infections, while results on other gastro-intestinal parasitic infections showing induction of strong Th2-biased responses in various experimental animal models (Mosmann and Coffman, 1989; Mosmann and Sad, 1996; Else and Finkelman, 1998; Hoffmann et al., 2002).

Recently, a nematode-specific 24-kDa antigenic protein from *A. suum* designated as As24 (Islam et al., 2005) was described. The protein has shown to be expressed in all life-cycle stages of *A. suum* with homologues detected in human and dog roundworms *A. lumbricoides* and *Toxocara canis*, respectively, suggesting that As24 homologues can be substituted as a molecule with same potential for human and dog ascariasis. In the present study, we examined the protective efficacy of *E. coli*-expressed recombinant As24 (rAs24) following vaccination in Freund's complete adjuvant (FCA). Our results showed that rAs24-FCA vaccinated mice had a significant level of serum immunoglobulin G (IgG) and conferred high levels of protection as manifested by a 58% reduction in recovery and stunted development of challenged *A. suum* lung-stage larvae. We also observed that stimulated splenic T cells from these mice exhibited elevated levels of both gamma-interferon (γ -IFN) (a Th1 marker) and interleukin-10 (IL-10) (a Th2 marker) cytokines in vitro, suggesting an induction of a Th1/Th2-mixed type of protective response by this vaccination protocol containing rAs24.

2. Materials and methods

2.1. Parasites

Adult *A. suum* were obtained from infected pigs at a slaughterhouse in Shimotsuma, Japan. Unembryonated and embryonated eggs were obtained essentially as described

elsewhere (Tsuji et al., 2001). *Ascaris suum* lung-stage L3 were collected from an infected rabbit as previously described (Islam et al., 2003).

2.2. Animals

Eight-week-old female specific pathogen-free BALB/c mice (SLC, Hamamatsu, Japan) were used in this study. Mice were housed in sterile cages in a barrier environment in the laboratory animal house of the National Institute of Animal Health (NIAH). Mice were fed pelleted food and water ad libitum. All animals were acclimatized to these conditions for 1 week prior to the experiment. Animal experiments were conducted in accordance with the protocols approved by the NIAH Animal Care and Use Committee (Approval no. 382).

2.3. Recombinant As24 and vaccination

Recombinant As24 expressed in his-tagged fusion protein and purified by HPLC equipped with HiTrap™ chelating column (Amersham Biosciences Piscataway, NJ) as previously described (Islam et al., 2005) was used for vaccination of mice. Mice were divided into three groups of 10 animals each. The first group of vaccinated mice was inoculated subcutaneously using a 25-gauge needle in the abdomen region each with 50 μ g rAs24 mixed with FCA (Difco Laboratories, Livonia, MI) in a total volume of $\sim$ 200 μ l under light ether anesthesia. Animals were boosted with same dose of rAs24 mixed with FCA 3 weeks after the primary vaccination. A final booster of rAs24 mixed with same adjuvant was given 2 weeks after the second vaccination. The second group was inoculated with FCA mixed with PBS using the same dose and same route as the first group. The third group was given nothing (non-vaccinated control).

2.4. Challenge infection and measurement of vaccine protection

Five mice from each of the three groups: rAs24-FCA, FCA alone and non-vaccinated controls, were challenged each with 2500 *A. suum* infective embryonated eggs 1 week after the final vaccination. Both vaccinated-challenged and vaccinated-unchallenged animals were sacrificed 1 week post-challenge. The lungs were removed and minced with a surgical knife, and larvae were recovered by Baermann method (Slotved et al., 1996, 1997). The recovered larvae were counted and measured under a light microscope. The vaccinated-unchallenged mice were examined for serum antibody and cytokine analyses. Mice were bled before autopsy and sera were collected and frozen at -20°C until used. Their spleens were removed aseptically for culturing cells.

2.5. Antibody ELISA

Recombinant As24-specific serum IgG, IgE and IgG-subclass antibodies (IgG1, IgG2a, IgG2b and IgG3) in vaccinated mice were measured by ELISA (Tsuiji et al., 2003). IgG and IgG-subclass antibody levels were determined with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgG-subclass antibodies (Bethyl Laboratories Inc, Montgomery, TX). Affinity purified goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 (Bethyl) were used as standards. For rAs24-specific IgE measurement, anti-mouse IgE was used as a capture antibody, and IgE was detected by using biotinylated anti-mouse IgE (Bethyl). Rat monoclonal anti-mouse IgE antibody diluted 1:10,000 was used as the standard (American Research Products, Belmont, MS).

ELISAs were performed in 96-well ELISA plates (MS-8496F; Sumitomo, Tokyo, Japan) in 50 µl reactions, with rAs24 antigen coated at 2 µg/ml of 0.1 M carbonate buffer, pH 9.6. The plates were incubated at 4 °C for 14 h and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Wells were blocked with 100 µl of PBS-1% bovine serum albumin (Sigma) for 1 h at 37 °C. After the wells were washed five times with PBS-T, serial dilutions of the serum were added, followed by incubation at 37 °C for 1 h. After the incubation, the wells were washed five times with PBS-T, and 100 µl of HRP-conjugated anti-mouse IgG, IgE or IgG subclass antibodies were added to the wells. The plates were incubated at 37 °C for 1 h and washed five times with PBS-T. Mouse sera were diluted 1:1000. Conjugates were used at 1:10,000. Both test sera and conjugates were diluted in PBS (pH 7.5). Detection of antibody was performed at 37 °C with 100 µl of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution (ABTS; Kirkegaard and Laboratories, Gaithersburg, MD), and the coloring reaction was terminated with 100 µl of 1% SDS. Plates were read at 405 nm in a microplate reader (Spectrafluor; Wako, Tokyo, Japan). Concentration of antibodies was calculated by reference to standard curve generated by using reference sera (Bethyl).

2.6. Spleen cell culture

Spleen cell suspensions were prepared as described previously (Tsuiji et al., 2003). Briefly, spleens were cut into small pieces and minced through a sterile stainless steel mesh (Sigma) and suspended in cool Hank's balanced salt solution (HBSS) (GIBCO/BRL) supplemented with 5% (v/v) heat-inactivated FCS (Sigma) and antibiotics (penicillin and streptomycin, 100 µg/ml). Erythrocytes in cell suspensions were lysed by 0.2% sodium chloride. The suspended cells were washed three times in HBSS by centrifugation at 500×g at 4 °C for 5 min each. Finally, the spleen cells, mostly T lymphocytes were resuspended in complete RPMI 1640 (GIBCO/BRL), supplemented with heat-inactivated 10%

(v/v) FCS, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 1 mM non-essential amino acid, 1 mg/ml 5-fluorocytosine and 5×10^{-5} M 2-mercaptoethanol. The concentrations of splenic T cells obtained from individual mouse were adjusted to 4×10^6 cells/ml of complete RPMI 1640. For in vitro stimulation, splenic cells were seeded out on 96-well flat-bottomed tissue culture plates (MS-8096F; Sumitomo) in a volume of 100 µl per well. The cells were stimulated alone or with rAs24 antigens (10 µg/ml) for 72 h at 37 °C in a humidified atmosphere containing 5% CO₂, supernatants were removed and frozen at -80 °C until assayed for cytokine content.

2.7. Cytokine ELISA

Cytokines produced by activated spleen cells were measured by standard sandwich ELISA using purified anti-IL-4, IL-10, IL-2 and γ-IFN as capture antibody and the corresponding biotinylated antibody as reporter antibody according to manufacturer's instructions (eBioscience, San Diego, CA). The concentrations of primary capture antibody and biotinylated reporter antibody were used according to protocol (eBioscience). Purified recombinant IL-4, IL-10, IL-2 and γ-IFN were used as standards (eBioscience). TMB microwell peroxidase was used as substrate and the reaction was terminated by 1 M H₃PO₄. Absorbance of wells was read at 450 nm in a microplate reader (Spectrafluor; Wako, Tokyo, Japan). Cytokine levels were calculated by reference to standard curves constructed from supernatants containing known amounts of each molecule.

2.8. In vitro moulting assay for *A. suum* lung-stage larvae

A. suum lung-stage L3 obtained from an infected rabbit were cultured in vitro in the presence of affinity purified anti-rAs24 mouse IgG to evaluate the possible in vivo functions of endogenous As24 proteins in the moulting processes of the parasites (Islam et al., 2003). Briefly, 50–100 *A. suum* lung-stage L3 were cultured in 1 ml RPMI 1640 (GIBCO/BRL), pH 6.8 supplemented with 10% (v/v) fetal bovine serum (Sigma) and antibiotics (penicillin and streptomycin, 100 µg/ml) in 24-well flat-bottomed tissue culture plates. The cultures were incubated at 37 °C, in humidified 5% CO₂ in the presence of increasing concentrations of anti-rAs24 mouse IgG and mouse preimmune IgG (as control) for 10 days. The number of L4 which had moulted from L3 in the culture well was determined as previously described (Islam et al., 2003).

2.9. Localization of endogenous As24 in *A. suum* lung-stage larvae by immunofluorescence

To localize the As24 endogenous proteins, *A. suum* lung-stage L3 obtained from an infected rabbit were washed in

cool PBS containing 0.1% Tween 20 (PBS-T) three times and fixed in cool acetone for 2 min. After washing in PBS-T, the fixed larvae were blocked in 10% goat serum for 1 h and incubated with primary anti-rAs24 antibody (1:20) for 2 h at room temperature. The larvae treated with mouse pre-immune sera (1:20) were served as control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody at a 1:100 dilution. After labeling, larvae were washed in PBS-T and whole larvae were mounted on slides with 70% glycerol. The larvae were analyzed and photographed with an Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany) using appropriate filter sets.

2.10. Statistics

The data are expressed as mean $\pm$ SD for the experimental groups. The statistical significance ($P < 0.05$) was determined by Student's *t* test.

3. Results

3.1. Protective effect of recombinant As24

Protective efficacy of rAs24 molecule against *A. suum* lung-stage infection was investigated in BALB/c mice received one primary vaccination and two boosters followed by an oral challenge 1 week apart with 2500 *A. suum* infective embryonated eggs. Data showed that mice vaccinated with rAs24 in FCA had a significant ($P < 0.001$) reduction in recovery of challenged *A. suum* lung-stage L3 (55.2 ± 20.23) compared with either non-vaccinated controls (132 ± 22.49) or mice vaccinated with FCA alone (112.8 ± 34.43) (Fig. 1). To test whether mice protective immunity could also arrest larval development,

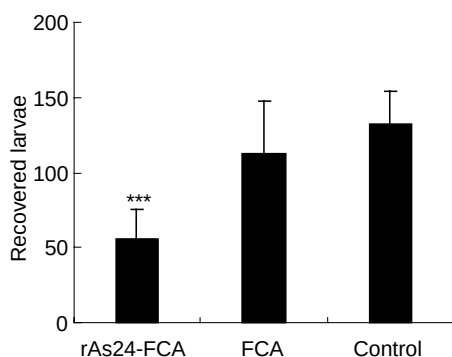


Fig. 1. Number of *Ascaris suum* lung-stage larvae recovered from mice vaccinated with recombinant As24 in Freund's Complete Adjuvant. Mice were vaccinated and then challenged with 2500 *A. suum* infective embryonated eggs 1 week after the final vaccination. Mice were sacrificed 1 week after challenge and the lung-stage larvae were recovered. The results are expressed as mean $\pm$ SD in each group of five mice. Asterisks indicate that the mean value was significantly lower than that of non-vaccinated controls and group vaccinated with FCA alone. *** $P < 0.001$.

we measured 50 challenged *A. suum* lung-stage L3 from mice vaccinated with rAs24-FCA, FCA alone and from non-vaccinated controls for body length and body width. We recorded a stunted development in body length ($897 \pm 114.80 \mu\text{m}$) and body width ($51.60 \pm 6.83 \mu\text{m}$) of challenged *A. suum* larvae recovered from rAs24-FCA-vaccinated mice compared with body length and body width of those grown either in the non-vaccinated controls ($1254.50 \pm 103.96 \mu\text{m} \times 59.65 \pm 8.76 \mu\text{m}$) or in mice vaccinated with FCA alone ($1145.5 \pm 133.70 \mu\text{m} \times 56.65 \pm 6.58 \mu\text{m}$). Lungs removed from vaccinated mice showed significantly reduced verminous pneumonitis (pulmonary haemorrhages) following challenge infections compared with the non-vaccinated controls (data not shown). Moreover, histopathological sections of these lungs (not from non-vaccinated controls) revealed fewer red blood cells and no eosinophils; however, mononuclear cells (for example, lymphocytes, macrophages, etc.) were never seen following infections (data not shown).

3.2. Serum antibody responses to recombinant As24

To assess the potential of rAs24 molecule in eliciting host-protective antibody, we measured rAs24-specific total IgG, IgE and IgG-subclass antibodies in protected mice sera. Results showed that mice vaccinated with rAs24-FCA displayed a significantly ($P < 0.0001$) high level of rAs24-specific IgG compared with either non-vaccinated controls or mice vaccinated with FCA alone (Fig. 2). Antigen-specific IgE responses were not seen in these mice sera (data not shown), indicating that rAs24 may be a non-allergenic molecule for the host. To further assess the efficacy of rAs24 in induction either of an in vivo Th1 or Th2 response, IgG-subclass antibody levels were measured. Our results demonstrate that rAs24-FCA vaccination induced a mixed pattern of IgG1 ($P < 0.0001$), IgG2b ($P < 0.005$), IgG3 ($P < 0.04$) and IgG2a ($P < 0.006$) responses (Fig. 2).

3.3. Cytokine responses to recombinant As24

We examined the cytokine levels in rAs24-stimulated splenic cell culture supernatants to determine the T cell response to rAs24 molecule in vaccinated mice. The in vitro-stimulated splenic T cells from rAs24-FCA-vaccinated mice exhibited a significantly high level of Th2-type cytokine IL-10 ($P < 0.001$); however, only a modest increase of IL-4 ($P < 0.09$) level was observed compared with stimulated T cells from both FCA-vaccinated and non-vaccinated control groups (Fig. 3). Interestingly, stimulated T cells from rAs24-vaccinated mice also showed a significantly increased level of γ -IFN ($P < 0.005$) but not IL-2 ($P < 0.11$) (Th1-type cytokines) compared with those from non-vaccinated controls and FCA-vaccinated animals (Fig. 3). These data suggest that rAs24 molecule induces a Th1/Th2-mixed type immune response in mice.

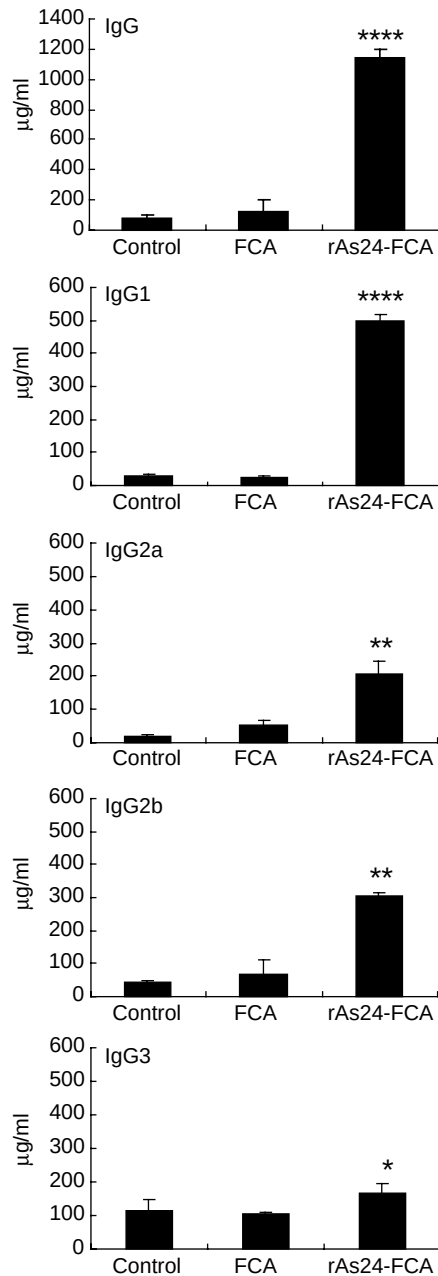


Fig. 2. Serum IgG and IgG-subclass responses in mice vaccinated with recombinant As24 in Freund's Complete Adjuvant. Mice were vaccinated and sacrificed 2 weeks after the final vaccination. The values are expressed as mean $\pm$ SD for each group of five mice. * P < 0.05, ** P < 0.01, **** P < 0.0001.

3.4. In vitro moulting inhibition of *A. suum* lung-stage larvae by anti-rAs24 IgG

We hypothesized that if the endogenous As24 protein plays role in the moulting processes of *Ascaris* parasites, then its neutralization by specific antibody would inhibit larval moulting process. The experimental findings showed that affinity purified anti-mouse IgG inhibited moulting of *A. suum* lung-stage L3–L4 in vitro by 26% at a maximum concentration of 20 μ g/ml culture medium (Fig. 4), larval

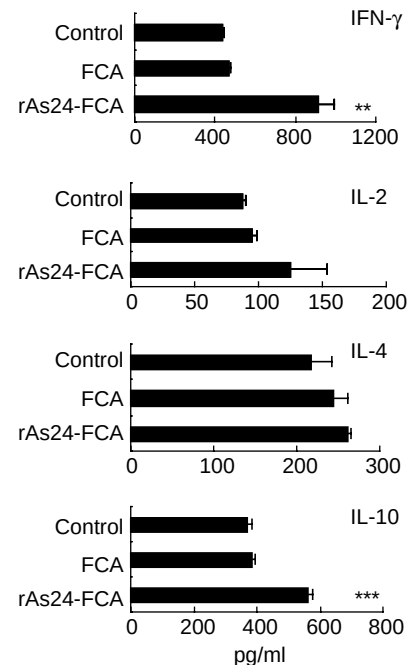


Fig. 3. Th1- and Th2-type immune responses in rAs24-vaccinated mice. Splenic T cells were isolated from mice 2 weeks after the final vaccination. Th1-type (i.e. γ -IFN and IL-2) and Th2-type (i.e. IL-4 and IL-10) cytokines were measured by cytokine-specific ELISA of cell culture supernatants from rAs24-FCA vaccinated, FCA alone and non-vaccinated control at 72 h in vitro stimulation with 10 μ g/ml rAs24. The results are expressed as mean $\pm$ SD for each group of five mice. Each sample was examined in triplicates. ** P < 0.01, *** P < 0.001.

viability was, however, remained unaffected (data not shown). These results suggest a partial role for endogenous As24 proteins in the moulting processes of *Ascaris* larvae. The mean moulting percentage in control culture (in the presence of mouse pre-immune IgG) was recorded as 54.85 ± 6.28 .

3.5. Localization of endogenous As24 in *A. suum* lung-stage larvae by immunofluorescence

To localize the As24 endogenous proteins, *A. suum* lung-stage L3 were reacted with anti-rAs24 primary antibody,

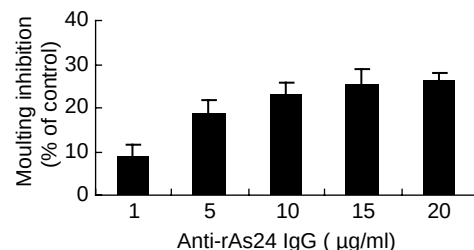


Fig. 4. Moulting inhibition of *Ascaris suum* lung-stage L3–L4 in vitro by anti-mouse rAs24 IgG. *Ascaris suum* lung-stage L3 obtained from an infected rabbit were cultured in serum supplemented RPMI 1640 in the presence of affinity purified anti-mouse rAs24 IgG as described in Materials and methods. Moulting of L3–L4 was evaluated at day 10 post-culture. Moulting percentages are relative to the control in the presence of mouse preimmune IgG (100%). Moulting percentage of control culture was 54.85 ± 6.28 . The results are expressed as mean $\pm$ SD of triplicates.

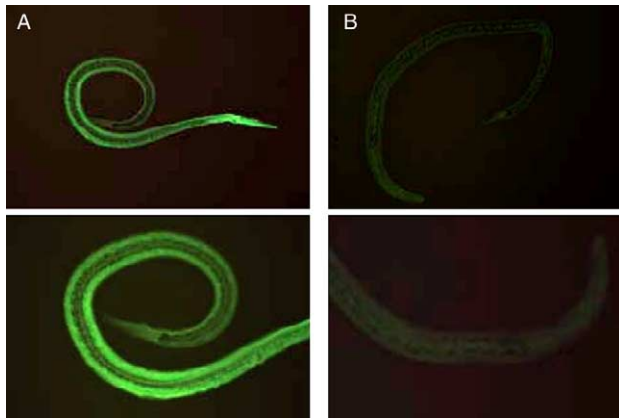


Fig. 5. Localization of endogenous As24 in *Ascaris suum* lung-stage L3 by immunofluorescence. Immunofluorescent staining of whole mounted lung-stage larvae was performed. (A) Lung-stage larvae were washed in cool PBS-Tween 20 (0.1%), fixed in acetone and incubated with anti-rAs24 primary antibody (1:20). FITC-labeled goat anti-mouse IgG was used as a secondary antibody. (B) Lung-stage larvae incubated with mouse preimmune sera (1:20) were regarded as control. Mouse anti-rAs24 antibody bound to the endogenous As24 proteins localized in the hypodermis along the whole body length and in the gut epithelial tissues of *A. suum* lung-stage larvae were observed under an Axiophot fluorescent microscope using appropriate filter sets and photographed (upper panel magnification $\times 80$, lower panel magnification $\times 160$).

and then incubated with FITC-labeled goat anti-mouse IgG as secondary antibody. Immunofluorescence microscopy revealed the bound antibodies in the hypodermis along the whole body length and in the gut epithelial tissues of anti-rAs24 antibody-treated *A. suum* lung-stage L3 (Fig. 5A). In contrast, no reactivity was seen in the larvae treated with mouse pre-immune sera (Fig. 5B).

4. Discussion

Previous studies from our laboratory have shown that mice immunized with *A. suum* L3 are efficiently protected (99%) against challenged *A. suum* migrating larvae (Tsuji et al., 2001). Protection was typically measured by recovering migrating larvae that reach to the lungs after challenge infections. Other studies from the same laboratory have revealed that intranasal immunization of mice with *E. coli*-expressed recombinant 14-kDa and 16-kDa antigens from *A. suum* can induce a significant level of protection (58–64%) against parasite challenge (Tsuji et al., 2001, 2003). In the present study, we evaluated whether a nematode-specific recombinant As24 protein from *A. suum* could be used as a potential vaccine candidate for the control of *Ascaris* roundworm infections in *A. suum*-mouse model. Encouragingly, our data showed that immunization of BALB/c mice (one primary immunization and two boosters) with rAs24 coupled with FCA results in protection against challenged *A. suum* lung-stage L3. Protection was manifested by a significant reduction (58%) in the recovery and stunting of *A. suum* lung-stage L3

at day 7 post-challenge compared with either non-immunized controls or mice immunized with FCA alone (Fig. 1). Recombinant As24-FCA immunization also results in a significant decrease in verminous pneumonitis (data not shown). It was shown that when *A. suum* L3 were administered orally to mice, the larvae penetrate the gastrointestinal tract after approximately 24 h, and the administered larvae reach the lungs, where they cause verminous pneumonitis after 72 h (Slotved et al., 1998). Mice vaccinated orally with *A. suum* L3 were found to be protected against verminous pneumonitis after challenge infections (Guerrero and Silverman, 1972; Tsuji et al., 2001). In a recent report, Girod et al. (2003) showed that mice vaccinated with γ -irradiated larvae of human hookworm *Necator americanus* had reduced pulmonary larval burdens and haemorrhages compared with the non-vaccinated controls following challenge infections. Our results indicated that subcutaneously administered rAs24 in FCA efficiently induced protective immunity to *A. suum* lung-stage larval infection.

Sera obtained from protectively immune mice had high levels of antigen-specific IgG antibody. Further, analysis of IgG-subclass profiles revealed that anti-rAs24 IgG1 showed the greatest increase followed by IgG2b, IgG2a and IgG3, suggesting that rAs24-FCA immunization resulted in a Th1/Th2-mixed type protective immunity. Interestingly, IgE responses were not detected in these mice, suggesting that immune responses to rAs24 are not mediated by hypersensitivity reactions. Although several studies have shown that parasite-specific IgG and IgE responses are associated with protective immunity to human ascariasis (McSharry et al., 1999; Cooper et al., 2000; Geiger et al., 2002), the protective function of these antibodies are yet to be defined. Our data show that protection against challenge by *A. suum* larvae is associated with high levels of all IgG-subclasses examined. However, we did not perform passive immunization with pooled sera from rAs24-FCA immunized mice to examine the potential of antibody in blocking *A. suum* larval migration. Recently, Frontera et al. (2003) reported that acquired resistance against *A. suum* larvae is correlated with the induction of IgG1 and IgM, and not with IgG2; rAs24-specific IgM, however, was not examined in the present study. Our results are in agreement with the report of Ghosh and Hotez (1999) who demonstrated that dog hookworm *Ancylostoma caninum* burden reduction from alum-precipitated recombinant *A. caninum* secreted protein 1 immunization is associated with elevations in all IgG-subclasses but the greatest rise observed in host IgG1 and IgG2b. These authors also showed that *A. caninum* burden reduction is antibody-dependent.

The effector mechanisms that induce stunted development and prevent migration of *A. suum* L3 being reached to the lungs after exit through host's gut wall remain poorly understood. It is reasonable to assume that anti-rAs24 IgG may penetrate the cuticle of *A. suum* larvae during the course

of their liver-lung migration, and mediate neutralization of As24 endogenous protein which has shown to be highly expressed in hypodermis and gut epithelium of *A. suum* lung-stage L3 (Fig. 5), and thus may interfere with larval migration and/or growth and development. Data obtained on moulting inhibition by anti-rAs24 antibody (Fig. 4) support such an assumption. Studies have shown that the nematode cuticle is a dynamic structure with important absorptive, secretory and enzymatic functions (Maizels et al., 1993) and not merely an inert covering as was once believed. The absence of eosinophils and mononuclear cells (e.g. macrophages) in the lung tissues of rAs24-immunized mice (data not shown) where migration by L3/L4 occurs for 2–3 days indicates that damage and/or killing of target *A. suum* larvae may not be induced by macrophages, further supporting our hypothesis of antibody-mediated protection described above. The immune mechanisms that operate against adult stage worms, however, were not examined in the present study. It has been reported that immune responses against tissue-dwelling helminths are different from those against gastro-intestinal parasites (Garside et al., 2000; Hoffmann et al., 2000).

Immunoglobulin isotype-specific responses seen in rAs24-FCA-immunized mice are consistent with cytokine profiles that in vitro stimulation of splenic cells from immunized mice exhibited significant levels of Th1-type cytokine γ -IFN but not IL-2, together with Th2-type cytokine IL-10 levels. However, the level of IL-4, a Th2-type cytokine was only modest. These data suggest that As24 in FCA triggers a Th1/Th2-mixed type of immune response. These findings support the observations with a recombinant 16-kDa antigen from *A. suum* (Tsuiji et al., 2003). It is proposed that a high level of γ -IFN production may suppress IL-4 production by splenic CD4⁺T cells in mice subcutaneously immunized with rAs24-FCA, since γ -IFN and IL-4 affect each other as antagonists (Pene et al., 1988). On the other hand, the reason for a poor release of IL-2 is unclear. It is reasonable to think that a reduced synthesis of IL-4 in rAs24-immunized mice may account for little or no IgE response and tissue eosinophilia (see Finkelman et al., 1991).

To determine the functional significance of anti-rAs24 IgG in the moulting processes of *Ascaris* parasites, a previously developed bio-assay that allows moulting and development of *A. suum* lung-stage L3–L4 in vitro to be observed was employed (Islam et al., 2003). Our data show that neutralization by anti-rAs24 IgG of As24 endogenous proteins results in partial inhibition of moulting (26%) of *A. suum* lung-stage L3–L4 in vitro (Fig. 4). The anatomic location of As24 endogenous proteins in the hypodermis along the whole body length of *A. suum* lung-stage L3 (Fig. 5) supports such a role. Since the hypodermis of parasitic nematodes allows synthesis of components of the new cuticle and thus plays an important role in the moulting processes (Lee, 2002). Recently we observed that *A. suum* inorganic pyrophosphatase is highly expressed in

the hypodermis of both adult and larval worms and is associated with molting and development processes of *A. suum* lung-stage L3–L4 (unpublished data). Furthermore, an intense expression of As24 endogenous proteins in the gut epithelium of *A. suum* larvae (Fig. 5) indicates that the protein may have functional role in the metabolic processes of *Ascaris* parasites.

In summary, the results with this vaccine formulation provides insights into rAs24-induced mechanisms that trigger a Th1/Th2-mixed type of response which may be important in host-protective immunity against *A. suum* larvae in the lung. This may assist in the development of a safe vaccine against ascariasis.

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