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Vaccination of mice with a 30 kDa *Schistosoma* antigen with and without human adjuvant induces high protection against *S. mansoni* infection

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Abstract

A 30 kDa antigen was characterized as a hydrophobic polypeptide containing 16 amino acids and evaluated as a potential candidate vaccine against infection by *Schistosoma mansoni*. CD1 albino mice immunized at 0, 14, and 21 days with 25 or 50 μg of the 30 kDa antigen per mouse with and without alum developed high levels of IgG antibodies (predominantly IgG2a and IgG2b isotypes). When immunized mice were infected with 200 *S. mansoni* cercariae, the highest protection levels (61% and 65% reduction in worm burden in two separate experiments) were obtained using the 50-μg antigen without alum adjuvant. The granuloma size decreased to 10%, a non-significant level in mice immunized using alum adjuvant. The results demonstrate the ability of the 30 kDa antigen with and without alum adjuvant to protect mice against *S. mansoni* infection.

Introduction

Over the past decade, several promising candidate vaccine antigens for *Schistosoma mansoni* have been identified and characterized. Six antigens, selected by WHO for protection studies in two independent laboratories, showed resistance against challenge infection but the stated goal of consistent induction of 40% protection or better was not reached with any of these antigen formulations, underlining the difficulty in characterizing and delivering an effective vaccine against schistosomes (Bergquist & Colley, 1998). Various approaches have been used to identify vaccine candidates, e.g. the development of monoclonal antibodies capable of conferring resistance (Capron *et al.*, 1987; Harn *et al.*, 1992). Attallah *et al.* (1999a) produced an IgG2a anti-*S. mansoni* monoclonal antibody, designated BRL4 mAb, which has been shown to reduce worm loads at 51.6%, 41.9% and 53.8% protection levels in three separate passive transfer experiments. The target epitope of the protective BRL4 mAb was identified in three different *Schistosoma* antigenic preparations of cercariae (CAP), adult worms (SWAP), and eggs (SEA) at 74 kDa molecular weight protein antigen (Attallah *et al.*, 1998). The 74 kDa antigen has been shown to protect mice of different strains and to modulate the host immune system (Attallah *et al.*, 1999b). However, a target reactive epitope of the BRL4 mAb was also identified at a lower molecular weight antigen of 30 kDa in urine of infected individuals and α-chymotrypsin and trypsin digestion products of the affinity purified 74 kDa antigen (Attallah *et al.*, 1998). In the present study, we investigated the protection induced against *S. mansoni* infection by immunization of mice with the lower molecular weight antigen, 30 kDa, with and without human adjuvant, alum.

Materials and methods

Host animals, infection and perfusion

Female outbred CD1 albino mice (about 20 g in weight) were used for immunization experiments. A Puerto Rican

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strain of *Schistosoma mansoni* is regularly maintained by laboratory passage through *Biomphlaria glabrata* snails and golden hamsters. Groups of female mice (ten mice per group) were infected percutanously with 200 *S. mansoni* cercariae. Mice were perfused and worms were collected, washed with saline containing heparin, and counted.

Monoclonal antibody and antigenic extract preparation

An IgG2a anti-*S. mansoni* monoclonal antibody designated BRL4 mAb was developed as described by Attallah *et al.* (1998). The cercarial antigen preparation (CAP), soluble worm antigen preparation (SWAP), and soluble egg antigen (SEA) were prepared according to Da Silva & Ferri (1968). The protein content was determined by Lowry's method (Lowry *et al.*, 1951) and stored at −70°C until use.

Isolation of the 30 kDa antigen using SDS–PAGE and gel electroelution

The 74 kDa target antigen of the BRL4 mAb was isolated from CAP according to Attallah *et al.* (1999b). Tryptic digestion products (0.1 mg of trypsin per 1 mg of the purified 74 kDa antigen or CAP incubated for 30 min at room temperature) and urine of *S. mansoni* infected individuals were subjected to 16% vertical slab sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Prestained standard molecular weights, low range (BioRad Laboratories, California, USA) were run in parallel. After silver staining, the band of interest, 30 kDa, was cut and electroeluted from the gel as described by Attallah *et al.* (1999b). The protein content of a sample of the antigen was determined before the remainder was stored at −70°C. The purity of isolated antigen was assessed using reversed-phase high performance liquid chromatography (HPLC; Kontron, Zurich, Switzerland) as described by Attallah *et al.* (1998).

Western blotting

Samples separated on 16% (or 12%) SDS–PAGE were electrotransferred onto nitrocellulose (NC) membrane (0.45 μm pore size, Sigma, St Louis, Missouri, USA). Standard molecular weights, low range (BioRad Laboratories) or high range (Sigma) were run in parallel. The NC filter was blocked using 5% (w/v) non-fat dry milk dissolved in 0.05 M Tris-buffered saline (TBS) containing 200 mM NaCl (pH 7.4), rinsed in TBS and incubated with BRL4 mAb or anti-30 kDa polyclonal antibodies diluted in blocking buffer with constant shaking. Blots were washed three times (30 min each) in TBS, followed by incubation for 2 h with anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1:500 in TBS. After washing three more times with TBS (15 min each), blots were soaked in premixed 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro-blue tetrazolium (NBT) substrate system (Sigma) for 10 min.

Protease and other biochemical treatments

The purified 30 kDa antigen was treated with protease and various chemical reagents and treated samples tested in Western blots against BRL4 mAb to establish whether these treatments were susceptible to the active epitope on the 30 kDa antigen. One mg per ml of the purified antigen was incubated for 1 h with 1 mg per ml of protease type VIIA from *Bacillus amyloliquefaciens* (Sigma) at 37°C; with 20% trichloroacetic acid, TCA (v/v) at 4°C and with pH range 1–14, 25% periodic acid, 35% perchloric acid, 5 mM dithiothreitol (DTT), 7 M B-mercaptoethanol (B-ME), and 5% SDS at room temperature. Periodate oxidation was carried out overnight with 20 mM sodium meta-periodate at room temperature and the reaction was then inhibited by adding an equal volume of 130 mM glycerol. The cercarial antigen preparation and bovine serum albumin were tested in parallel.

Amino acid analysis

One mg of purified 30 kDa antigen was hydrolysed under vacuum for 24 h at 120°C in 6 N HCl containing 1% phenol. The hydrolysed sample was dried and derivatized with phenyl isothiocyanate. The derivatized amino acids were analysed by a reversed phase HPLC system (Kontron Pc Intigrator) equipped with a spherisorb C8 column (250 × 4.6 mm i.d.; Kontron). A blank sample of 6 N HCl containing 1% phenol and a mixture of 17 amino acids were derivatized and analysed in parallel. The injection volume was 10 μl. The mobile phase employed for the separation consisted of two eluents, (0.1 M sodium acetate containing 1 ppm EDTA [pH 5.5]) (eluent A), and organic phase consisting of (CH_3CN:CH_3OH:H_2O [45:40:15]) (eluent B). The flow rate was 1.5 ml per min and detection was performed at 254 nm using a gradient 6% B to 45% B in 60 min.

Immunization of mice using the 30 kDa antigen

Groups of female outbred CD1 albino mice (ten mice per group) were immunized intraperitoneally (i.p.) using 25 or 50 μg of 30 kDa antigen per mouse. All doses were administered with and without alum adjuvant (Pierce, Rockford, USA). Control groups of mice were injected with phosphate buffered saline (PBS, pH 7.2) with or without alum adjuvant. Each dose was injected three times (at Zero, 14 and 21 days). The level of infection was assayed 6 weeks post-infection with 200 *S. mansoni* cercariae by liver perfusion. Sera from immunized and control groups were collected before infection for antibody response measurement using ELISA.

Measurement of antibody response using ELISA

The antibody response of immunized mice was measured three days after the final immunization and tested against the purified 30 kDa antigen and different schistosome antigenic extracts (CAP, SWAP and SEA). Polystyrene flat-bottomed microtitre plates (Costar, Massachusetts, USA) were coated with 1 μg per well of the purified 30 kDa antigen and with 10 μg per well of schistosome antigenic extracts in carbonate/bicarbonate buffer, pH, 9.6 (50 μl per well). After blocking, pooled sera from mice immunized with the antigen and from infected and non-infected mice (as controls); diluted 1:250 in phosphate buffered saline, pH 7.2 containing 0.05% (v/v)

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Tween 20 (PBS-T20) were added (50 μl per well) and incubated at 37°C for 2 h. After washing with PBS-T20, 50 μl per well of anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1:500 in 0.2% (w/v) non-fat milk in PBS-T20 was added and incubated at 37°C for 1 h. One mg per ml of para-nitropheny1 phosphate (Sigma) was used as a substrate, and the absorbance was read at 405 nm using EL311 microplate autoreader (Bio-Tek Instruments, Vermont, USA). The cutoff level of the assay was calculated as the mean optical densities of ten normal mice serum samples plus three × standard deviation (SD).

IgG isotype determination

A microtitre plate, with 96 wells (Costar) was coated with goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Sigma) diluted 1:1000 and incubated 2 h at room temperature. The plate was then washed three times using 0.05% PBS-Tween 20; and the free sites blocked with 0.3% non-fat milk and incubated at room temperature for 1 h. After washing, 50 μl of immunized mice sera diluted 1:100 in PBS-T20, were added. The plate was incubated for 1 h at 37°C. Goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) was added at a dilution of 1:500 in PBS-Tween 20 and incubated for 1 h at 37°C. Para-nitrophenyl phosphate (Sigma) was used as the substrate. The absorbance was read at 405 nm using a microplate autoreader (Bio-Tek Instruments).

Histopathology

Both control and experimental mice were routinely dissected and after perfusion, livers of all groups were removed, fixed in 10% neutral formalin and embedded in paraffin wax. Ten liver sections for each mouse; 4 μm thick and approximately 100 μm apart were stained with haematoxylin and eosin. Measurements of hepatic granulomata size and number were performed blindly according to Ohmae *et al.* (1991). The number of granulomata was calculated in a 0.5 cm^2 area of each liver section using light microscope. The diameters of 150 granulomata per liver were measured (i.e. 15 granulomata per liver section) using an ocular micrometer. Aggregated granulomata were not measured.

Results

Isolation and characterization of the 30 kDa S. mansoni *antigen*

The BRL4 mAb recognized a band of 30 kDa molecular weight in the tryptic digestion products of *S. mansoni* cercariae antigenic preparation (CAP) and of purified 74 kDa as well as in the urine of an *S. mansoni*-infected individual (fig. 1). One mg of the 30 kDa antigen was obtained either from the tryptic digestion of 10 mg purified 74 kDa antigen or from 10 ml urine of the *S. mansoni*-infected individual. Forty gel runs were completed to obtain one mg of the 30 kDa antigen. In each run, 250 μl of urine or 250 μg of tryptic digestion products of purified 74 kDa antigen per preparative gel were electrophoresed. Only one peak was observed by

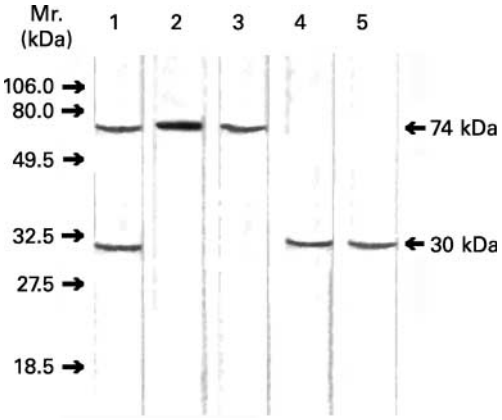

Fig. 1. Identification of the 30 kDa target epitope using BRL4 mAb and Western blot. Samples were resolved on 16% SDS–PAGE, electrotransferred onto nitrocellulose membrane and then immunostained with the BRL4 mAb. Lane 1, urine from *Schistosoma mansoni*-infected individual; lane 2, cercarial antigen preparation (CAP); lane 3, 74 kDa schistosome antigen purified from CAP; lane 4, tryptic digestion products of the purified 74 kDa antigen; and lane 5, the purified 30 kDa antigen from tryptic digestion products. The prestained standard molecular weight protein markers on the left side (Mr.), not shown but indicated by arrows, were phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa). The addition of the dye causes the protein markers to migrate differently than their true molecular weights.

HPLC analysis indicating the purity of the isolated 30 kDa polypeptide (fig. 2).

The biochemical characterization of the purified 30 kDa *S. mansoni* antigen revealed that the reactivity of the target epitope using Western blot was maintained at the pH range 4–10 and was lost at pH ranges 1–3 and 11–14. Periodate treatment did not affect antigen reactivity, while the antigen was sensitive to protease, periodic and

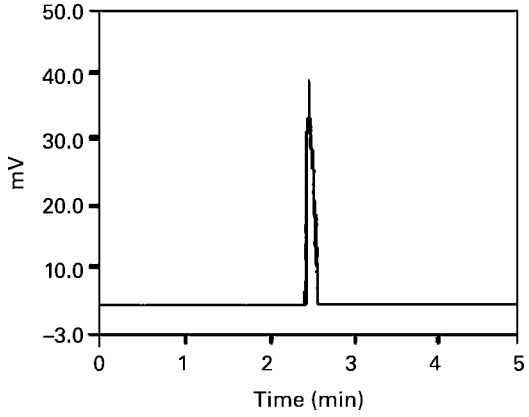

Fig. 2. HPLC chromatogram showing the polypeptide pattern of the purified 30 kDa schistosome antigen. Ten μl was eluted by using a gradient of two eluents: Eluent A (0.1 M sodium acetate containing 1-ppm EDTA, pH 5.5) and eluent B (an organic phase of CH_3CN:CH_3OH:H_2O in ratio of 45:40:15). The gradient from 0% B to 100% B is 60 min. Flow rate was constant at 1.5 ml per min and detection was performed at 254 nm.

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Table 1. Amino acid contents of a 30 kDa purified *Schistosoma mansoni* target antigen.

Classes of amino acids	Name (symbol)	Concentration (nmol mg^{-1} protein)	Total %
1. Hydrophobic	Alanine (Ala)	72.06	54.86
	Isoleucine (Ilu)	42.13	
	Leucine (Leu)	35.39	
	Methionine (Met)	37.25	
	Phenylalanine (Phe)	126.44	
	Proline (Pro)	76.45	
	Valine (Val)	471.02	
2. Hydrophilic	Glycine (Gly)	307.07	25.12
	Serine (Ser)	75.91	
	Threonine (Thr)	50.35	
	Tyrosine (Tyr)	65.97	
3. Basic	Arginine (Arg)	60.58	17.04
	Histidine (His)	104.83	
	Lysine (Lys)	42.64	
4. Acidic	Aspartic acid (Asp)	15.8	2.98
	Glutamic acid (Glu)	25.56	

perchloric treatment. Reducing agents DTT, B-ME, and SDS did not affect the reactivity of the active epitope. The 30 kDa antigen was precipitated with 20% TCA, and reconstituted in PBS, pH 7.2. The reconstituted precipitate showed a higher reactivity towards the BRL4 mAb compared with the untreated sample. In contrast, the supernatant showed no reactivity. The concentrations in nmol per mg protein, and the hydrophobic and hydrophilic natures of the 16 amino acids of the 30 kDa antigen are shown in table 1.

Humoral immune response of immunized mice

Polyclonal antibodies raised in mice against 25 and 50 μg doses of 30 kDa antigen with and without adjuvant showed a high reactivity ($P < 0.001$) towards the 30 kDa antigen, SWAP, CAP and SEA using ELISA. The reactivity of raised polyclonal antibodies injected with adjuvant was higher than that without adjuvant. The IgG2b isotype showed significantly ($P < 0.001$) elevated titres compared with the other IgG isotypes. Western blot revealed that anti-30 kDa mouse polyclonal antibodies reacted with CAP at an apparent molecular weight of 74 kDa and also reacted with the purified 30 kDa antigen. No reactive bands were shown using pooled sera from non-immunized mice (fig. 3).

Effect of antigen dose and adjuvant on resistance to S. mansoni *infection in mice immunized with the 30 kDa antigen*

The 25 and 50 μg doses of antigen produced significant protection ($P < 0.001$) compared with the control group in two separate experiments and the highest protection levels were obtained using the 50 μg dose. Mice immunized with the 30 kDa antigen either without or with alum adjuvant produced a significant resistance ($P < 0.001$) to *S. mansoni* infection in the two separate experiments (table 2).

Histopathology of immunized mice livers

The results of the first and second experiments using 30 kDa antigen without or with alum adjuvant are shown in table 3. The number of granulomata was significantly ($P < 0.001$) reduced with both 25 μg and 50 μg per mouse

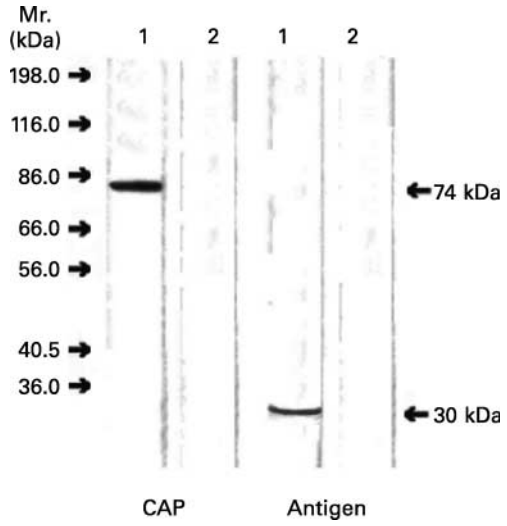

Fig. 3. Reactivity of mouse polyclonal sera anti-30 kDa antigen against CAP and the purified 30 kDa antigen using Western blot. CAP and the purified 30 kDa schistosome antigen were resolved on 12% SDS–PAGE and then electrotransferred onto nitrocellulose membrane. Lane 1, the resolved sample immunostained with sera of CD1 albino mice immunized with 50 μg per mouse of the purified 30 kDa schistosome antigen; lane 2, the resolved sample immunostained with non-immunized mice sera as a control. The molecular weight markers on the left side (Mr.), not shown but indicated by arrows, were α2-macroglobulin (198 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (86 kDa), pyruvate kinase (66 kDa), fumarase (56 kDa), lactic dehydrogenase (40.5 kDa) and triose phosphate isomerase (36 kDa).

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Table 2. Protection against *Schistosoma mansoni* by immunization of CD1 albino mice with different doses of purified 30 kDa antigen without and with alum adjuvant.

		Without adjuvant		With adjuvant	
Experiment no.	Dose (μg)	Worm recovery	% Protection*	Worm recovery	% Protection*
1	PBS	50.2 ± 12.73	–	52.0 ± 9.67	−4
	25	34.4 ± 9.04	32	37.5 ± 2.88	28
	50	19.5 ± 0.57	61	21.5 ± 1.73	58
2	PBS	57.2 ± 8.88	–	57.2 ± 8.52	–
	25	36.6 ± 3.74	36	38.0 ± 3.74	34
	50	20.3 ± 1.71	65	22.2 ± 5.16	61

*Calculated in comparison with number of worms recovered from the control groups.

Table 3. The number and size of liver granulomata following active immunization of CD1 albino mice with different doses of the 30 kDa antigen without and with alum adjuvant.

	Granuloma number		Granuloma size (μm)	
Dose (μg)	Mean ± SD	Reduction%*	Mean ± SD	Reduction%*
Without adjuvant				
PBS	36.5 ± 2.88	–	248.7 ± 49.24	–
25	14.0 ± 4.24	62 (S, $P < 0.001$)	308.58 ± 52.80	−24 (S, $P < 0.05$)
50	15.0 ± 1.41	59 (S, $P < 0.001$)	299.33 ± 57.75	−20 (S, $P < 0.05$)
With adjuvant				
PBS	30.0 ± 3.46	–	215.63 ± 35.53	–
25	14.5 ± 0.71	52 (S, $P < 0.001$)	205.64 ± 43.24	5 (NS)
50	17.5 ± 0.71	42 (S, $P < 0.001$)	194.85 ± 43.63	10 (NS)

*Percent reduction of granuloma numbers or sizes was calculated by comparison with the control group received PBS. P values were not significant (NS, $P > 0.05$) and significant (S, $P < 0.05$).

doses of antigen. The highest levels of reduction was obtained using antigen without adjuvant.

The granuloma size was significantly increased ($P < 0.05$) using both 25 and 50 μg per mouse doses of antigen without alum adjuvant. The granuloma size was decreased but did not reach a significant level ($P > 0.05$) using both 25 μg and 50 μg per mouse doses of the 30 kDa antigen with adjuvant (table 3).

Discussion

The development of an effective vaccine against human schistosomiasis remains a highly desirable yet elusive goal. The reproducible induction of protective immunity with attenuated parasites and with antigenic preparations has led to the agreement that vaccination against schistosomiasis is an achievable goal (Bergquist & Colley, 1998; Eberl *et al.*, 2001; Capron *et al.*, 2002).

The major challenge in the development of anti-schistosome vaccines is to use defined antigens to stimulate an appropriate immune response that leads to resistance. In the present study, the protective anti-*Schistosoma mansoni* monoclonal antibody, BRL4 mAb, recognized a band of 30 kDa molecular weight in the tryptic digestion products of the 74 kDa *S. mansoni* protective antigen and CAP as well as in the urine of an *S. mansoni*-infected individual. The 30 kDa antigen was isolated from these different sources and its partial biochemical characterization revealed similar properties to those previously shown for the higher molecular weight protein antigen, 74 kDa (Attallah *et al.*, 1998). However, the amino acid sequence of the 30 kDa antigen would help in the identification of this molecule.

To determine if the lower molecular weight antigen, 30 kDa would also induce protection against *S. mansoni*, we investigated its potential as a candidate vaccine in two separate experiments using the protective dose of the 74 kDa antigen, 50 μg and a lower dose of 25 μg without and with adjuvant suitable for human use, alum. Immunization of mice with the 25 and 50 μg doses of 30 kDa-target antigen either without or with alum adjuvant stimulated humoral immune responses. Polyclonal antibodies (predominantly IgG2a and IgG2b isotypes) developed in mice to the 30 kDa antigen showed high reactivity toward the 30 kDa antigen and CAP using ELISA in comparison with control sera from non-immunized mice. In addition, anti-30 kDa polyclonal antibodies reacted with CAP at 74 kDa and also reacted with the purified 30 kDa antigen. This confirms the 30 kDa antigen carrying the target reactive epitope identified on high molecular weight antigen, 74 kDa. Mice immunized with the 25 and 50 μg per mouse doses of 30 kDa antigen either without or with alum adjuvant produced significant protection ($P < 0.001$) to *S. mansoni* infection in comparison with control groups in the two separate experiments. However, the highest protection levels were obtained using the 50 μg dose in both experiments. These data suggest the 30 kDa antigen may

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represent a candidate vaccine for immunization against *S. mansoni*.

Besides protection, if granuloma formation could be prevented or suppressed, the development of severe disease might be averted (Boros, 1989). Various experimental approaches have been carried out by several investigators to identify target antigens to provide protective immunity and participate as granulomatous modulating agents in schistosomiasis (Capron *et al.*, 1987; Boros, 1989; Hirsh *et al.*, 1997). In experimental models, many antigens isolated from parasite forms were able to induce moderate levels of protection (Sher *et al.*, 1986; Harn *et al.*, 1987). However, these studies did not analyse any induced granulomatous reactivity to the eggs (Andrade *et al.*, 1992). In the present work, a marked reduction in granuloma number (42% and 59%) was shown using the 30 kDa antigen with and without alum adjuvant; respectively. The most likely explanation of this reduction is the induction of host immune responses to the 30 kDa antigen which, in turn, leads to a decline in the number of eggs produced and parasite mortality (Grzych *et al.*, 1993).

In conclusion, the 30 kDa target antigen has the ability to protect mice and to modulate the host immune system using the human adjuvant, alum. Further studies on the molecular cloning of the target 30 kDa antigen are now required.

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