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Characterization and Protective Potential of the Immune Response to *Taenia solium* Paramyosin in a Murine Model of Cysticercosis

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Paramyosin has been proposed as a vaccine candidate in schistosomiasis and filariasis. However, limited information is available about its protective potential against cysticercosis and the immune response it induces. Immunization of mice with recombinant full-length paramyosin of *Taenia solium* (TPmy) results in about a 52% reduction in parasite burden after a subsequent challenge by intraperitoneal inoculation of *Taenia crassiceps* cysticerci. Immunization assays using recombinant fragments of TPmy, corresponding approximately to thirds on the amino, central, or carboxyl regions, suggest that protective epitopes are located mostly in the amino-end third. Proliferation assays using T cells obtained from mice immunized with the full-length recombinant TPmy also showed a preferential response to the amino-terminal fragment. In contrast, antibodies in the sera from these mice predominantly recognize epitopes located in the carboxyl-terminal fragment, being the immunoglobulin G1 subclass, the predominant antibody isotype. Characterization of the cellular immune response induced against the protective amino-terminal fragment reveals production of gamma interferon and interleukin-2, but not interleukin-4, suggesting a Th1-like profile.

Paramyosin (Pmy) is a filamentous, α-helical, coiled-coil protein of about 100 kDa, present in some muscles of invertebrates. It is also an antigen during infections by several flatworms that are important parasites of humans and of domestic animals such as *Schistosoma mansoni* (10), *Schistosoma japonicum* (4), *Taenia solium* (10, 12), and *Echinococcus granulosus* (18). The paramyosin of *T. solium* (TPmy) is present in the musculature but has also been found associated with the tegument of the parasite (7). The collagen-binding and complement-inhibitory properties of TPmy have been described previously (8, 9, 11). TPmy is synthesized by the tegumentary cytons and apparently released through the cyst tegument (8). Furthermore, TPmy can be collected in the culture medium in which *T. solium* cysts are maintained (8), suggesting that a similar release to the host tissues might occur in vivo and that TPmy may modulate the host response through diminution of the inflammatory mediators at the host-parasite interface (8, 11).

Paramyosins have been proposed as vaccine candidates in a number of helminthiases including schistosomiasis (3, 20) and filariasis (14, 19). Despite their protective abilities against schistosomiasis and filariasis, limited information is available on their potential as vaccines against cysticercosis. Here we report that immunization of mice with recombinant fragments of TPmy induces significant levels of protection in the murine model of cysticercosis by *Taenia crassiceps.* The profile of cytokine production suggests that the protective amino-terminal fragment of TPmy induces a Th1-like immune response.

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MATERIALS AND METHODS

Animal model. Mice used in all experiments were 4- to 6-week-old female BALB/c AnN strain mice. The ORF strain of *T. crassiceps* was maintained by consecutive passages of cysts in the peritoneal cavities of mice (26). Cysts used to challenge mice in protection studies were obtained from mice after 2 to 3 months of infection, those with diameters of 1 to 2 mm being the ones selected.

Recombinant proteins. A series of constructs derived from the full-length coding sequence of TPmy were designed to express either the full-length protein or fragments that correspond to approximately thirds of TPmy. The full-length paramyosin (VW7-3) is an 863-amino-acid protein as described elsewhere (12); the amino-terminal fragment contains amino acids 1 to 268 (VW2-1), the central fragment contains amino acids 269 to 551 (VW3-3), and the carboxyl-terminal fragment contains amino acids 552 to 863 (VW4-1). All TPmy products were recombinantly expressed and purified by affinity chromatography as described before (J. Vázquez-Talavera et al., submitted for publication). Purified recombinant proteins were exhaustively dialyzed against 0.5 M NaCl, pH 7.3, and the protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Preparation of immunogens. Recombinant fragments (VW2-1, VW3-3, and VW4-1) or full-length rTPmy (VW7-3) were mixed with 1.6% alum [$Al_2(OH)_3$] to a final ratio of 1 to 50 (wt/wt) and incubated at room temperature for 20 min. Alum was sedimented by centrifugation at 8,000 × *g* for 10 min and resuspended in sterile saline. The amount of protein bound to $Al_2(OH)_3$ was determined by quantifying the amount of protein in the supernatant after centrifugation. Binding of protein to the alum was higher than 95%. In all immunizations, one dose corresponded to 20 μg of protein adsorbed to 1 mg of alum.

Protection studies. Mice were immunized two times intraperitoneally (i.p.) at 1-week intervals with one of the recombinant products of TPmy (VW2-1, VW3-3, VW4-1, or VW7-3), prepared as described above. Control mice were injected with 1 mg of alum in saline, following the same procedure as with immunized mice. One week after the last immunization, mice were i.p. challenged with 10 *T. crassiceps* cysts in saline. Mice were bled every week after the last immunization and sacrificed by cervical dislocation at 45 days postinfection, and cysts from the peritoneal cavities were collected and counted.

Antibody recognition of the recombinant fragments of TPmy. To evaluate the antibody recognition of the different regions of TPmy, enzyme-linked immunosorbent assays (ELISA) were performed using pooled sera from four mice that had been immunized i.p. three times at 1-week intervals with VW7-3 that was prepared as described above. Mice were bled 1 week after the last immunization. ELISA was carried out binding equimolar amounts of each recombinant TPmy fragment (VW2-1, VW3-3, and VW4-1) to microtiter plate wells. In brief, 96-well microtiter plates (Immulon 2; Dynatech, Chantilly, Va.) were coated with equal amounts of the immunoglobulin fraction of chicken hyperimmune sera directed

TABLE 1. Protection assays in the murine model of cysticercosis by *T. crassiceps*[a]

Expt no.	Vaccination	No. of mice	Individual burden	Parasite load[b]	Protection[c] (%)
1	Alum	4	48, 38, 22, 36	36 ± 11	
	VW7-3	4	4, 24, 24, 12	16 ± 10	55[d]
2	Alum	6	7, 36, 54, 76, 141, 76	65 ± 45	
	VW7-3	6	19, 24, 52, 37, 43, 9	31 ± 16	52[d]
3	Alum	5	20, 31, 73, 92, 134	70 ± 46	
	VW2-1	5	0, 0, 1, 2, 41	9 ± 18	87[d]
	VW4-1	6	40, 73, 78, 82, 110, 147	88 ± 36	−25
4	Alum	5	38, 44, 54, 76, 110	64 ± 29	
	VW2-1	6	7, 9, 18, 38, 43, 86	34 ± 30	47[d]
	VW4-1	6	0, 0, 10, 20, 66, 150	41 ± 59	36
5	Alum	5	81, 82, 86, 31, 158	88 ± 45	
	VW3-3	5	79, 8, 70, 79, 54	58 ± 30	34
6	Alum	6	107, 300, 134, 55, 170, 64	138 ± 90	
	VW3-3	6	99, 39, 29, 90, 120, 22	67 ± 42	52[d]

[a] Mice were challenged by inoculation of 10 cysts 1 week after the last immunization as described in Materials and Methods.
[b] Values are means ± standard deviations of the parasite loads after 45 days of infection.
[c] Protection was calculated as the reduction of parasite load expressed in percentage, where the alum-injected control mice burden is 100%.
[d] Statistically significant difference between alum-injected control mice and immunized mice ($P \leq 0.05$).

to the 46-amino-acid fusion peptide, located at the amino-terminal end of all recombinant fragments. After being washed four times with phosphate-buffered saline (PBS) containing 0.03% polyoxyethylene sorbitan monolaurate (PBS-Tween 20) and blocking with PBS-Tween 20 plus 1% bovine serum albumin, 100 ng of each recombinant fragment was added per well and incubated at room temperature for 1 h. After being washed, 1:200 dilutions of the pooled sera from the immunized mice were added and incubated at 37°C for 1 h. The amount of antibody bound was quantified using a secondary horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) plus IgM plus IgA plus IgD antibody, following the manufacturer recommendations (Zymed Laboratories, Inc., San Francisco, Calif.). The reaction was developed with *o*-phenylenediamine dihydrochloride plus hydrogen peroxide and stopped with 2.5 N H_2SO_4. Readings of optical density at 490 nm (OD_{490}) were carried out in an ELISA reader (Human Gessellschaft für Biochemica und Diagnostica, Taunusstein, Germany). Isotype analysis of the sera from mice that were immunized with one of the recombinant products of TPmy (VW2-1, VW3-3, VW4-1, or VW7-3) was carried out by ELISA using a crude extract (9) bound to microtiter plate wells and a secondary horseradish peroxidase rabbit anti-mouse IgG1 or IgG2a antibody (Zymed Laboratories, Inc.).

T-cell proliferation assays. A pool was made with spleen cells obtained from four mice immunized with VW7-3 as described above (see "Antibody recognition of the recombinant fragments of TPmy") 1 week after the last immunization. As a control group, a pool of cells was obtained from four naive mice. The T-cell-enriched fractions from naive or immunized mice were obtained by panning the spleen cells in polystyrene tissue culture plates coated with affinity-purified rabbit anti-mouse IgA plus IgG plus IgM antibodies (Sigma Chemical Co., St. Louis, Mo.). The nonadherent cells (including T cells) were recovered from the culture media and used for proliferation assays. Quantification of T cells in this nonadherent fraction, by flow cytometry using fluorescent monoclonal antibodies to surface markers, indicated that T cells ($CD3\varepsilon^+$) represented about 60%, whereas B cells ($CD19^+$) and macrophages ($Mac3^+$) represented less than 0.5%. The remaining 39% of this nonadherent fraction corresponded to cells that were not recognized by the monoclonal antibodies specific for CD3ε, CD19, and Mac3. The T cells were cultured in 96-well microtiter plates (Costar, Cambridge, Mass.) containing 2.5×10^5 cells per well, supplemented with 1.25×10^5 adherent naive cells in a final volume of 200 μl in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (RPMI-sup). The optimal dose and time for culture were determined by stimulation of T cells with 0.6 to 600 pmol of VW7-3 per ml during 48 to 216 h. The T-cell-enriched fraction from immunized mice proliferated optimally (i.e., it had the highest stimulation index, which is the ratio of stimulated cells to unstimulated cells) with a dose of 60 pmol/ml during 72 h. Cultures were maintained at 37°C with 5% CO_2 and humidity at saturation. Eighteen hours before harvesting, cells were pulsed with [*methyl*-^{3}H]thymidine, 1 μCi/well (NEN Life Science Products, Inc., Boston, Mass.), and the amount of incorporated radioactive label was measured in a liquid scintillation counter (Betaplate, Turku, Finland).

Cytokine quantification. Spleen cells from normal mice and from mice that were immunized with VW2-1 (see "Protection studies" above) were cultured in RPMI-sup on flat-bottom 16-well microtiter plates (Costar) at a concentration of 5×10^6 cells per well. Spleen cells were stimulated with 60 pmol of amino-terminal fragment (VW2-1) per ml during 48 h, and the supernatants were collected, aliquoted, and stored at −70°C until used. Basal (nonstimulated) cytokine levels were evaluated in all cultures. The presence of interleukin-2 (IL-2), gamma interferon (IFN-γ), and IL-4 in supernatants was assayed by sandwich ELISA with cytokine-specific kits (PharMingen, San Diego, Calif.) following the instructions from the manufacturer.

Statistical analysis. Student's *t* test was used to compare pairs of data and the analysis of variance test was used for multiple group designs.

RESULTS

Protection assays. The murine model of cysticercosis was used for the identification of potentially protective epitopes in the molecule of TPmy. Protection induced by recombinant fragments of TPmy can be easily evaluated in this model by counting the numbers or determining the volumes of cysts growing in the peritoneal cavities of immunized or control mice after an initial challenge. Susceptible female BALB/c AnN mice were immunized twice with VW7-3 or any of the recombinant fragments of TPmy and challenged by i.p. injection of 10 cysts. The mice were sacrificed 45 days after the challenge, and the numbers of cysts in the peritoneal cavities were evaluated. Mice immunized with VW7-3 showed an ~52% reduction in the number of cysts (Table 1). Protection induced by recombinant fragments of TPmy was highly variable between experiments; however, the higher and significant reduction in the parasite burden was obtained by immunization with the amino-terminal fragment (VW2-1), which induced 47 and 87% protection in two experiments. In contrast, the other fragments showed lower, nonsignificant levels of protection, suggesting that most of the protective epitopes are located in the amino-terminal fragment of TPmy. ELISA tests

TABLE 2. Humoral reactivity against Pmy recombinant fragments after immunization of mice with VW7-3[a]

Antigen	Sera	
	Naive	Immune
VW7-3	0.023 ± 0.015	2.295 ± 0.080[b]
VW2-1	0.076 ± 0.018	0.075 ± 0.006
VW3-3	0.062 ± 0.003	0.515 ± 0.021[b]
VW4-1	0.059 ± 0.010	0.426 ± 0.012[b]

[a] Sera from naive and VW7-3-immunized mice (diluted 1:200) were analyzed by ELISA as described in Materials and Methods. Values are means ± standard deviations of quadruplicates expressed in OD_{490} units.
[b] Values are statistically significantly different from those obtained with naive mice sera ($P < 0.01$).

TABLE 3. Antigen-specific proliferation of T cells from VW7-3-immunized mice

Stimulation[a]	Stimulation index[b] for:	
	Naive mice	Immune mice
RPMI[c]	1	1
RPMI + VW7-3	7.44[d]	15.7[d]
RPMI + VW2-1	3.5[d]	7.1[d]
RPMI + VW3-3	3.22[d]	4.6[d]
RPMI + VW4-1	1.11	1.2

[a] Proliferation was assayed with T cells from immunized and naive mice stimulated with the recombinant fragments of Pmy as described in Materials and Methods.
[b] Reported values are stimulation indexes calculated from the means of radioactivity incorporation (quadruplicates) for each experimental group after an 18-h pulse with [^{3}H]thymidine.
[c] Absolute values were 1.8 ± 0.3 and 1.5 ± 0.3 (kcpm ± standard deviation) for naive and immune medium controls, respectively.
[d] Absolute values for each experimental group were statistically different from absolute values for RPMI-stimulated control cells ($P < 0.05$).

showed that serum samples from mice immunized with the fragments of TPmy, taken before and after challenge infection, had similar levels of IgG1 and IgM antibodies (not shown). Marginal levels of IgG2a were also detected in immunized mice. Experiments of passive transfer of sera from mice immunized with VW2-1 into naive receptors showed no significant differences of cyst burden compared to recipients of sera from naive mice, indicating that protection is not mediated through antibodies (data not shown).

Antibody reactivity against TPmy recombinant fragments. B-cell epitopes were located on the TPmy recombinant fragments by ELISA using sera from mice immunized with VW7-3. This experiment was feasible given the extensive cross-recognition between paramyosins from different flatworms and other invertebrates (11). The sera from mice immunized with the VW7-3 were highly positive against the central (VW3-3) region and the carboxyl end (VW4-1) but negative to the amino end (VW2-1) (Table 2). No antigen-specific antibody reactivity was detected against an unrelated protein containing the same fusion peptide (data not shown).

T-cell response against TPmy recombinant fragments. In order to determine if a specific cellular immune response is generated in mice after immunization with VW7-3, a T-cell-enriched fraction was cultured and stimulated in vitro with VW7-3 and the recombinant fragments (VW 2-1, VW 3-3, and VW4-1). A significant antigen-specific proliferation was observed in the T cells from immune mice stimulated with VW7-3, VW2-1, and VW3-3 but not VW4-1 (Table 3). Unexpectedly, T cells from naive mice were also responsive at lower levels to the full-length VW7-3, VW2-1, and VW3-3, suggesting that some mitogenic activity is associated to the amino and central regions of TPmy. No antigen-specific proliferative response was detected against two control-unrelated proteins containing the same fusion peptide (data not shown).

Cytokine production. To establish the cytokine profile induced by the immunization with the protective amino terminal fragment (VW2-1), spleen cells from immunized and naive mice were stimulated in vitro with the same recombinant product. As shown in Table 4, IL-2 and IFN-γ but not IL-4 are produced in an antigen-specific fashion. No statistically significant differences in cell phenotypes were found in unstimulated spleen cells from control, immunized, and infected mice (data not shown). These results suggest that a Th1 profile is induced by immunization with the amino-terminal fragment of TPmy. The marginal but significant stimulation observed in naive cells cultured with VW2-1 involving IL-2 but not IFN-γ or IL-4 again suggests that a mitogenic activity is associated to the amino-terminal region of TPmy.

DISCUSSION

Paramyosins have been proposed as vaccine candidate antigens against trematodes like *S. mansoni* (3, 22) and *S. japonicum* (20, 21, 23), as well as against nematodes like *Brugia malayi* (14, 19). The feasibility of inducing protection through immunization with heterologous paramyosins has been demonstrated in several parasite models; it was found that vaccination of mice with Pmy from the nematode *Caenorhabditis elegans* induces up to 60% protection against challenge with *B. malayi* (19). Another extreme example is the induction of 35% protection against *S. japonicum* through immunization of mice with a soluble paramyosin-containing fraction from the earthworm *Lumbricus terrestris* (36). In this report, we show that *T. solium* Pmy induced partial heterologous protection against murine cysticercosis by *T. crassiceps* and that most of the protective epitopes are located at the amino-terminal fragment of the protein.

Information on the location of protective epitopes on the Pmy molecule is scarce. Immunization of mice with a recombinant fragment of Pmy from *S. mansoni* corresponding to the central region (amino acids 303 to 742) induces protection against *S. mansoni* (22). In the murine infection by *S. japonicum*, the B-cell epitope IRRA, recognized by a protective monoclonal IgE antibody, is located in residues 359 to 362 of the *S. japonicum* Pmy (21). This sequence is also found at the same position in the *S. mansoni* Pmy. In contrast, although the sequence IRRA is also found in TPmy at positions 743 to 746, it was not associated with protection, as the carboxyl-end fragment was found to be not significantly protective.

Sera from mice immunized with VW7-3 reacted much more against the carboxyl-terminal fragment (VW4-1) and the central fragment (VW3-3) than against the amino terminal (VW2-1) fragment. The sera from neurocysticercotic patients are also strongly reactive against the carboxyl-end region, with poor recognition of the central and amino regions (Vázquez-Talavera et al., submitted). These results are intriguing, con-

TABLE 4. Proliferation and cytokine profile of spleen cells from mice immunized with VW2-1

Immunization status of mice	Treatment[a] of spleen cells	Stimulation index[b]	Mean ± SD of production (pg/ml) of:		
			IL-2	IFN-γ	IL-4
Naive	None	1	713 ± 62	2,683 ± 1,765	≥ 30
	VW2-1	3.25[c]	954 ± 45[c]	4,635 ± 1,260	107 ± 118
Immunized with VW2-1	None	1	813 ± 76	2,032 ± 436	≥ 30
	VW2-1	7.33[c]	1,841 ± 274[c]	7,619 ± 1,019[c]	33 ± 39

[a] Spleen cells from naive mice or from mice immunized with VW2-1 were cultured in vitro with or without VW2-1 as described in Materials and Methods ("Cytokine quantification") to evaluate proliferation and quantify cytokines in the culture supernatants.
[b] Reported values are stimulation indexes calculated from the means of radioactivity incorporation for three individual mice per group after an 18-h pulse with [^{3}H]thymidine.
[c] Statistically significant difference between stimulated cells and nonstimulated cells ($P < 0.01$).

sidering that the secondary structure of paramyosins is highly homogeneous: about 95% of the complete amino acid sequences of these proteins maintains the 7/28 repeat pattern characteristic of α-helical coiled-coil proteins (10, 12). TPmy contains small sequences (15 residues at the amino end and 31 residues at the carboxyl end) that break the helical secondary structure, suggesting that the nonhelical regions have small influence on the asymmetry of the antibody recognition (12). B-cell epitope mapping carried out on *Dirofilaria immitis* paramyosin using sera from patients infected with *Onchocerca volvulus* indicated a preferential recognition of the amino end of the molecule (29). However, sera from patients infected by *S. japonicum* preferentially recognized the carboxyl end (21). These results indicate that paramyosins from helminth parasites have dominant epitopes located in different regions of the molecule.

Mice vaccinated with the recombinant fragments of TPmy produced IgG1 antibodies with marginal levels of IgG2a. This is in agreement with previous studies showing that mice vaccinated with irradiated cercaria from *S. mansoni* show high levels of IgG1 antibodies against recombinant Pmy from *S. mansoni* (24) and that vaccination with DNA encoding *S. japonicum* Pmy also induces production of IgG1 antibodies (37). In contrast to our results, the IgG1 isotype has also been correlated with protection in schistosomiasis (2).

Paramyosins induce proliferative responses of mononuclear cells from patients infected with *S. japonicum* (35) and splenocytes from mice immunized with irradiated cercaria from *S. mansoni* (25). However, no information was available about the region(s) of the molecule inducing the response. Our results indicated that only the amino-terminal (VW2-1) and the central (VW3-3) fragments induced proliferation when a T-cell-enriched fraction was used in the assays.

Information on vaccination against cysticercosis using different antigens is growing rapidly and has been recently reviewed (6, 15, 16, 27). Previous reports suggested that protection against murine cysticercosis by *T. crassiceps* is mediated by a Th1 response (32, 34) featured by $CD4^+$ or $CD8^+$ T cells, implying that T lymphocytes can induce protection irrespective of their phenotypes (17, 31, 33). The in vitro production of IL-2 and IFN-γ by the protective fragment VW2-1 also suggested that protection induced by TPmy is based on a Th1 response. IFN-γ is also produced by immunization with *S. mansoni* Pmy (22), and a delayed-type hypersensitivity response against Pmy is commonly detected in filariasis (19). The failure of a hyperimmune serum raised against the amino-terminal fragment to passively transfer significant resistance to infection in recipient naive mice also supports the idea that protection involves a T-cell-mediated response, as described for *S. mansoni* infection of mice (22).

In contrast to immunized mice, spleen cells from infected mice showed a weak proliferation against TPmy (data not shown). These results suggest that the lower response may result from a long-term infection, as proposed for human (1), porcine (5), and murine cysticercoses (13, 28). This diminution in spleen cell responsiveness might be the consequence of an immunosuppression state induced by the parasite (30).

Our results demonstrate that a moderate protection can be induced by Pmy in a murine model of cysticercosis. This is in agreement with results obtained for other helminth parasites including trematodes and nematodes. Current efforts are being directed to finding out how protection levels are influenced by the profile of the resulting immune response. Although our data provide information on the location of protective epitopes on the protein and on the profile of the protective response, little can be advanced on the mechanism of the TPmy-induced protection. In particular, the accessibility of this protein on the external surfaces of the cysts to become the target of protective immunity deserves further study.

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