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# Induction of protective immunity against *Schistosoma mansoni* by vaccination with schistosome paramyosin (Sm97), a nonsurface parasite antigen

(schistosomiasis/cell-mediated immunity/myofibrillar protein/γ interferon/recombinant immunogen)

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ABSTRACT **Paramyosin (Sm97), a 97-kDa myofibrillar protein identified by the unusually monospecific antibody response induced by intradermal vaccination of mice with a complex soluble worm antigen preparation (SWAP) of adult *Schistosoma mansoni* administered with bacillus Calmette–Guérin (BCG), was purified and tested for its capacity to protect mice against challenge infection. When administered intradermally with BCG at total doses of only 4–40 μg per mouse, both the native molecule and a recombinant expression product containing approximately 50% of the whole protein were found to confer significant resistance (26–33%) against challenge infection, while 2 mg of unfractionated SWAP was required to induce similar levels of protection. In addition, paramyosin was shown to stimulate T lymphocytes from vaccinated mice to produce lymphokines [e.g., γ interferon (IFN-γ)] that activate macrophages to kill schistosomula. Neither schistosome myosin nor a heterologous paramyosin from a different invertebrate genus were protective, indicating a requirement for specific epitopes in the immunization. That the protection induced by paramyosin involves a T-cell-mediated mechanism was supported by the failure of anti-paramyosin antibodies to passively transfer significant resistance to infection to recipient mice. Lymphocytes from mice vaccinated with paramyosin were found to produce IFN-γ in response to living schistosomula, suggesting that during challenge infection of vaccinated hosts, paramyosin (a nonsurface antigen) may elicit a protective T-cell response as a consequence of its release from migrating parasite larvae. Paramyosin-depleted SWAP was also found to be protective as well as stimulatory for T lymphocytes from SWAP-vaccinated mice, indicating that other antigens in this preparation may have immunoprophylactic potential. In summary, these results (*i*) suggest that the induction of T-cell-dependent cell-mediated immunity against soluble nonsurface antigens may be an effective strategy for immunization against multicellular parasites and (*ii*) in the case of schistosomes, identify paramyosin as a candidate vaccine immunogen in this category.**

Helminth parasites of the genus *Schistosoma* are the causative agents of schistosomiasis, a major medical and veterinary problem in the tropics. Prophylactic vaccination would have a major impact on the control of this infection, and during the last decade considerable progress has been achieved in the development of experimental immunization protocols (1). A vaccine model in which mice are partially protected against challenge infection with *Schistosoma mansoni* by intradermal immunization with either nonliving larval schistosomes or soluble membrane-free schistosome extracts using bacillus Calmette–Guérin (BCG) as adjuvant has been described (2, 3). Data from an ongoing series of experiments strongly indicate that the resistance to infection induced by this procedure results from the stimulation of schistosome-specific T-cell-dependent cell-mediated immune responsiveness—in particular, the production of lymphokines, including γ interferon (IFN-γ), that active macrophages for larval schistosome killing (4–6).

An unusual feature of the intradermal vaccine is that mice protectively immunized with complex worm extracts generate a T-cell-dependent antibody response that is directed primarily against a nonsurface 97-kDa molecule (Sm97) (3, 7) identified recently as the myofibrillar protein paramyosin (8). Though our data indicated no role for antibody in vaccine-induced protection, the unusually monospecific nature of the humoral response suggested that paramyosin, the serologically defined antigen, might also be responsible for inducing protective T-cell-dependent cell-mediated immunity. Here we report the results of a study in which both native and recombinant paramyosin were tested for recognition by IFN-γ-producing helper T cells from vaccinated animals and, more importantly, for their ability to stimulate resistance to challenge infection when administered intradermally with BCG.

## MATERIALS AND METHODS

**Animals and Parasites.** Female C57BL/6J mice were obtained from The Jackson Laboratory at age 6–8 weeks. *S. mansoni* (NMRI strain) cercariae and adult schistosomes were provided by the Biomedical Research Institute (Rockville, MD).

Newly transformed and 3-hr schistosomula were prepared from cercariae, and 7-day-old schistosomula were recovered from heavily infected mice as described (9, 10).

**Antigen Preparation.** A soluble worm antigen preparation (SWAP) was produced from adult schistosomes (which had been snap-frozen in liquid nitrogen at the time of perfusion and subsequently stored at −70°C) by homogenization in phosphate-buffered saline (0.01 M phosphate/0.15 M NaCl), pH 7.4/10 mM EDTA on ice. The use of parasites stored at ultracold temperatures and 10 mM EDTA in the homogenization buffer prevented the breakdown of paramyosin into its two major degradation products of 95 and 78 kDa (7). The antigen was centrifuged at 12,000 × *g* for 30 min, after which the supernatant was respun at $10^5$ × *g* for 4 hr. The antigen was filtered through a 0.45-μm membrane filter (Nalgene) and stored at −70°C. Paramyosin (Sm97) was purified from

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Abbreviations: SWAP, soluble worm antigen preparation; mAb, monoclonal antibody; IFN-γ, γ interferon; BCG, bacillus Calmette–Guérin.

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SWAP by immunoaffinity chromatography with immobilized anti-paramyosin monoclonal antibody (mAb) 4D1 as described (7) except for one modification: after the SWAP had been passed over the immunoabsorbent and the absorbance at 280 nm had returned to baseline but before antigen elution, the column was washed with 1 bed volume of column buffer (100 mM Tris·HCl, pH 8.0/500 mM NaCl/1 mM EDTA) containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS) (Calbiochem). This additional wash reduced nonspecific protein contamination to a level undetectable by silver-stain analysis (see below). SWAP was passed over the anti-paramyosin affinity column until the following radioimmunoassay failed to detect the molecule: 10-$\mu$l samples of the antigen were dotted onto nitrocellulose paper and probed with a 1:1 mixture of culture supernatants from the anti-paramyosin hybridomas 4D1 and 4B1 [which recognize different epitopes (6)] followed by $^{125}$I-labeled protein A adsorption and autoradiography (as described below). At this point the SWAP was considered to be totally depleted of paramyosin. The expression product of the complementary DNA clone MAC 97.3 (8), which encodes a 52-kDa polypeptide bearing the epitope recognized by mAb 4B1, was purified by immunoaffinity chromatography on this mAb as outlined above. Briefly, MAC 97.3 was grown as a lysogen in *Escherichia coli* Y1089, and the bacteria subsequently were solubilized by three cycles of freezing/thawing/sonication in the affinity-column running buffer containing 0.5% Renex (Imperial Chemical Industries, Macclesfield, England) and the protease inhibitors $\alpha_2$-macroglobulin, leupeptin, and phenylmethylsulfonyl fluoride. After centrifugation at $10^5 \times g$ for 4 hr at 4°C, the supernatant was passed over the mAb 4B1 affinity column. Recombinant paramyosin was eluted in 100 mM Tris·HCl, pH 8.0/2 M KSCN in the absence of detergent and dialyzed against phosphate-buffered saline (pH 7.4).

The biochemical purification of schistosome paramyosin and myosin was accomplished by using the salt extraction protocol [or modifications of this technique (8)] of Harris and Epstein (11). Purified *Mercenaria* paramyosin was a gift from C. Cohen (Brandeis University, Waltham, MA).

**Vaccination and Determination of Resistance.** For direct immunizations, mice were injected intradermally in the thoracic region with 100 $\mu$l of schistosome antigen containing $5 \times 10^6$ colony-forming units of *Mycobacterium bovis* (strain BCG, TMC 1012, Trudeau Institute, Saranac Lake, NY) (2). Control injections consisted of BCG alone in phosphate-buffered saline. Two weeks after primary injection and 4 weeks prior to challenge infection, mice received a second intradermal inoculum identical to the first. For passive transfer studies, mice (10 per group) received (*i*) 150 $\mu$g of protein A-purified mAb 4D1/150 $\mu$g of protein A-purified mAb 4B1 or 300 $\mu$g of protein A-purified normal mouse IgG intravenously both 12 hr before and 5 days after challenge infection, or (*ii*) 1.0 ml (0.5 ml intravenously and 0.5 ml intraperitoneally) of serum from a rabbit hyperimmunized with affinity-purified paramyosin (8) or 1.0 ml of normal rabbit serum on either the day before or 5 days after challenge infection. Mice were percutaneously challenged with approximately 100–150 cercariae by exposure of their abdominal skin. Percent resistance was calculated by perfusion of adult worms from the portal vein at 6 weeks after infection and comparing numbers recovered from immunized mice with those recovered from unimmunized control animals within the same experiment. The significance of differences in mean worm recoveries was evaluated by Student's *t* test.

**Immunochemical Analyses.** Antigens for immunization were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and silver staining with Rapid Ag Stain (ICN) or by immunoblotting as described (7). Blots were probed either with undiluted hybridoma culture supernatants or with sera diluted 1:200 in phosphate-buffered saline/0.05% Tween-20/5% fat-free milk powder, and bound antibody was visualized by using $^{125}$I-labeled protein A (Amersham) and autoradiography at −70°C with Kodak X-Omat AR film (Eastman).

**Analysis of Cell-Mediated Immune Responsiveness.** Antigen-specific sensitization of lymphocytes for the production of macrophage-activating lymphokines and/or IFN-$\gamma$ was assayed as described (4). Briefly, culture supernatants of spleen cells from SWAP/BCG-vaccinated mice or from control mice inoculated with BCG only, after 48-hr incubations with or without antigen (at 500 $\mu$g/ml of SWAP or paramyosin-depleted SWAP; or with 5 $\mu$g/ml of paramyosin), were tested for their ability to activate cells of the macrophage line IC-21 to kill 3-hr schistosomula in a standard *in vitro* larvacidal assay (12). Supernatants were quantitated for IFN-$\gamma$, the major macrophage-activating factor for larvacidal activity (13), by using an enzyme-linked immunosorbent assay (14) in which IFN-$\gamma$ in culture supernatants is trapped by immobilized Hb170 rat anti-mouse IFN-$\gamma$ mAb and detected by a polyclonal monospecific rabbit anti-mouse IFN-$\gamma$, followed by a peroxidase-conjugated goat anti-rabbit immunoglobulin reagent. Quantitation was by comparison with known amounts of recombinant mouse IFN-$\gamma$ (a gift from Genentech, South San Francisco, CA).

To assess whether or not paramyosin molecules or relevant epitopes of this molecule are released from living parasites, newly transformed and 7-day-old schistosomula were cocultured for 48 hr with spleen cells (15) from mice vaccinated twice with 10 $\mu$g of purified native paramyosin accompanied by BCG. After this time, culture supernatants were assayed for IFN-$\gamma$ as described above.

## RESULTS

**Antigen Analyses.** After electrophoresis and silver-stain analysis, paramyosin, whether purified by affinity chromatography or salt fractionation, appeared as a single band of 97 kDa, and myosin appeared as a band of 200 kDa with minor contaminating components (Fig. 1). Purified recombinant paramyosin silver-stained as a 52-kDa band with minor contaminants (not shown). Immunoblotted SWAP and paramyosin-depleted SWAP were probed with a mixture of anti-paramyosin mAbs (4B1 and 4D1) or with pooled sera

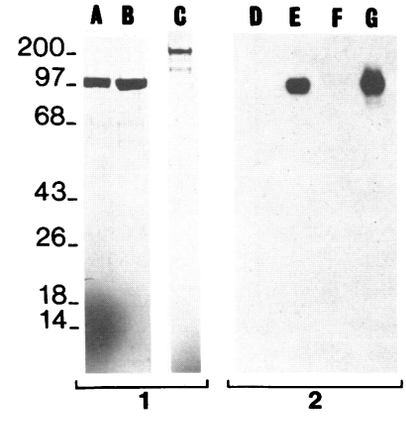


FIG. 1. Immunochemical analyses by sodium dodecyl sulfate/7–15% polyacrylamide gel electrophoresis of antigens used for vaccination studies. Lanes: A–C, silver stain; D–G, immunoblot; A, 1 $\mu$g of affinity-purified paramyosin; B, 1 $\mu$g of salt fractionation-purified paramyosin; C, 1 $\mu$g of salt fractionation-purified myosin; D and F, 50 $\mu$g per lane of paramyosin-depleted SWAP; E and G, 50 $\mu$g per lane of whole SWAP. Lanes D and E were probed with a pool of mAbs 4B1 and 4D1. Lanes F and G were probed with a polyclonal mouse anti-paramyosin reagent. Molecular mass is shown in kDa.

from mice immunized with purified paramyosin emulsified in complete Freund's adjuvant. A 97-kDa band was seen only in the whole SWAP lane (Fig. 1), indicating that multiple passage over the affinity column totally depleted SWAP of paramyosin.

**Assessment of Protective Activity.** In 10 separate experiments, whole SWAP, administered twice at doses of 1 mg per mouse [the dose previously shown to be required for the induction of optimum resistance (16)], was found to induce a mean level of protection of 32.7% (±3.0% SEM; Fig. 2). Native paramyosin, whether purified by immunoaffinity or salt fractionation and administered twice at a dose of only 20 μg per mouse, was as effective as whole SWAP, conferring similar levels of protection (31.0–33.2%, $0.001 < P < 0.05$; Fig. 2). Furthermore, paramyosin was also significantly protective when administered twice at doses of only 10 μg (not shown) or 2–5 μg per mouse (Fig. 2). Recombinant paramyosin, administered twice at doses of 20 μg per mouse, gave levels of protection (26.2% ± 4.1%) that, while somewhat lower than those achieved with similar doses of the native molecule, were also significant.

Maximal levels of protection achieved with SWAP and paramyosin in this study were similar [43% and 39%, respectively (Table 1)]. Nevertheless, in five separate experiments, SWAP depleted of paramyosin was essentially as effective an immunogen as was whole SWAP in inducing immunity. Mice vaccinated twice with 1 mg of this preparation displayed a level of resistance of 29.0% (±2.8%) (Fig. 2). In contrast, neither schistosome myosin nor a heterologous paramyosin from *Mercenaria mercenaria* were protective when administered intradermally with BCG (Fig. 2).

To examine whether the protection conferred by paramyosin was mediated by antibody, passive immunization studies were performed. In these experiments, parasite recoveries from mice that had received control rabbit sera on days −1 and +5 after infection (81 ± 18 and 88 ± 15 worms per mouse, respectively) were not significantly different from recoveries from recipients of rabbit anti-paramyosin antisera administered on the same days (80 ± 20 and 103 ± 16, respectively). Likewise, recipients of a pool of mAbs 4B1 and 4D1 gave a mean challenge worm recovery (56 ± 13) that was statistically indistinguishable from that of control mice (65 ± 9).

Table 1. Induction of resistance to *S. mansoni* infection by vaccination with purified paramyosin (sample experiment)

| Immunization | Mice, no. | Worm recovery per mouse (mean ± SD) | % resistance | Statistical significance* |
|---|---|---|---|---|
| BCG | 9 | 54 ± 6 | | |
| SWAP (2 mg)/BCG | 8 | 31 ± 4 | 43.4 | $P < 0.001$ |
| Affinity-purified paramyosin (4 μg)/BCG | 10 | 33 ± 13 | 39.0 | $P < 0.001$ |

*Statistical significance of reduction in parasite burden as determined by using Student's *t* test.

**Production of Macrophage-Activating Lymphokines in Response to Paramyosin.** Culture supernatants from paramyosin-stimulated spleen cells from SWAP/BCG-vaccinated mice contained significant macrophage-activating lymphokine levels, causing normal macrophages to kill 65% (±21%) of cocultured schistosomula (Table 2). This level of activity was comparable to that found with supernatants from SWAP- or paramyosin-depleted SWAP-stimulated cells. IFN-γ levels in supernatant fluids correlated with these results (Table 2). In addition, recombinant paramyosin was found to stimulate IFN-γ production, although in lower amounts than those induced by the native protein.

**Response of Paramyosin-Sensitized Lymphocytes to Living Schistosomula.** To investigate the mechanism by which paramyosin, an internal antigen, can elicit cell-mediated immunity in vaccinated mice, living newly transformed and

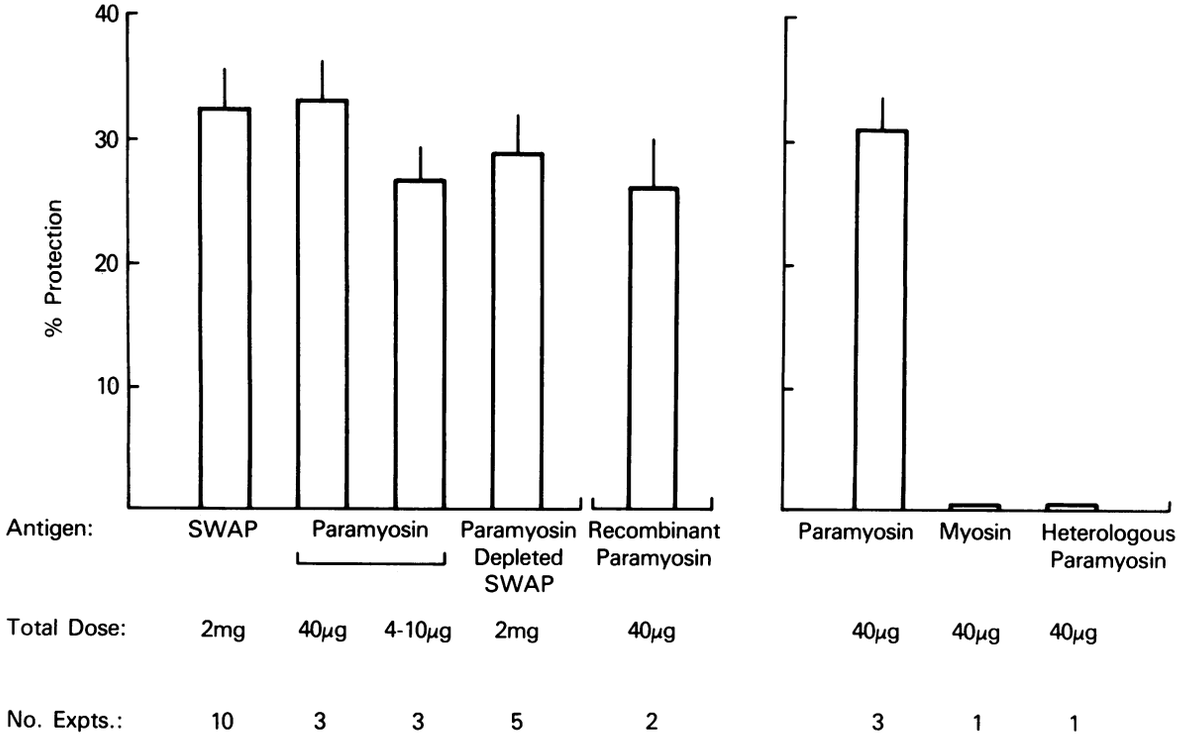


FIG. 2. Induction of resistance to challenge *S. mansoni* infection by intradermal vaccination of antigen in conjunction with BCG. (*Left*) Affinity-purified antigens. (*Right*) Salt-fractionated antigens. Protection data represent mean values (±SEM) of results from the number of replicate experiments indicated. All resistance levels were significant at or above the $P < 0.05$ level. As shown, schistosome myosin and *Mercenaria* paramyosin failed to confer significant protection.

Table 2. Paramyosin-induced lymphokine production by splenocytes from SWAP/BCG-vaccinated mice

| Stimulating antigen | MAF activity* | IFN-γ,† pg/ml |
|---|---|---|
| Medium only | 1 ± 4 | 60 ± 87 |
| SWAP | 80 ± 12 | 7200 ± 400 |
| Paramyosin | 65 ± 21 | 2000 ± 900 |
| Paramyosin-depleted SWAP | 53 ± 32 | 6800 ± 800 |
| Recombinant paramyosin | ND‡ | 900 ± 200 |

*Macrophage-activating factor (MAF) in supernatant fluids from spleen cells of SWAP/BCG-immunized mice or BCG-treated controls was measured by stimulation of macrophage-mediated larvacidal activity. The results shown are the percent schistosomula killed (mean ± SD) for five experiments. Background killing by macrophages exposed to control supernatants from antigen-stimulated cells of mice immunized with BCG alone has been subtracted from each result. The background killing values ranged between 9% and 16%.

†IFN-γ levels were measured by means of an enzyme-linked immunoassay. The results shown have been corrected by subtracting the background IFN-γ levels produced by antigen-stimulated control cells and represent mean (±SD) values from three experiments.

‡ND, not determined.

7-day-old schistosomula were tested for their capacity to stimulate IFN-γ production when cocultured with lymphocytes from paramyosin-vaccinated mice. These parasite stages have been implicated as targets for protective immunity in murine vaccine models (16, 17). Supernatants from cultures containing paramyosin-sensitized cells and either schistosome stage were found to contain significant levels of IFN-γ as measured by an enzyme-linked immunoassay (Table 3).

## DISCUSSION

In this study we show that mice vaccinated intradermally with as little as 4 μg of schistosome paramyosin (Sm97) accompanied by BCG are significantly protected against subsequent infection with *S. mansoni*. The significance of these observations lies in the demonstration that nonsurface schistosome antigens can be protective, suggesting that excreted/secreted or released soluble parasite molecules may represent good candidates for vaccine immunogens. In a limited passive transfer study, the resistance induced by paramyosin was not transferred by antibodies against the protein. However, paramyosin was recognized by T cells from mice immunized intradermally with SWAP/BCG, as assessed by lymphokine production. In conjunction with recently obtained data showing that spleen cells from vaccinated mice can passively protect normal mice (S.L.J., unpublished data), these results support our hypothesis that in the intradermal vaccine model, protection is mediated by T-cell-dependent cell-mediated immune responses. Although neither myosin (another schistosome myofibrillar protein) nor a heterologous paramyosin were protective, SWAP depleted of paramyosin was also found to be as efficient as SWAP as a stimulator of immunity and *in vitro* T-cell-mediated responses, suggesting that other soluble schistosome antigens in addition to paramyosin can also act as protective immunogens when administered appropriately.

Table 3. Stimulation by living schistosomula of IFN-γ production by splenocytes from mice vaccinated with paramyosin/BCG

| Stimulating antigen | Antigen concentration* | IFN-γ,† pg/ml Exp. 1 | Exp. 2 |
|---|---|---|---|
| Paramyosin | 1000 | 680 | ND |
| | 100 | 280 | ND |
| | 10 | 214 | ND |
| Schistosomula‡ | | | |
| Newly transformed | 5000 | 316 | 165 |
| | 500 | 80 | 59 |
| | 50 | 16 | 53 |
| 7-day-old lung stage | 5000 | 215 | 108 |
| | 500 | 20 | ND |
| | 50 | 0 | ND |

ND, not determined.

*Paramyosin was measured by weight (ng/ml), whereas newly transformed and 7-day-old lung-stage schistosomula were measured by number of organisms per ml.

†IFN-γ levels were measured by using an enzyme-linked immunoassay. The results shown have had background IFN-γ levels produced by antigen-stimulated control cells subtracted.

‡Schistosomula were prepared as described and cocultured with spleen cells for 48 hr. Under these conditions, minimal attrition (<5%) of the parasites was observed.

The mechanism by which paramyosin is recognized by the immune system is not clear. Paramyosin, a known myofibrillar protein, is presumably unavailable for interaction with the immune response because of its location (7), unless it is at some point either expressed on the surface of the schistosome or released from the parasite. To date, repeated experiments have failed to detect paramyosin exposed at the schistosome surface (3). However, as shown in the present study, paramyosin epitopes are presented through an as yet uncharacterized mechanism by living schistosomula in culture in a form that stimulates primed T cells to produce IFN-γ. Presumably, *in vivo* antigen recognition in this manner could lead to macrophage activation and direct parasite killing or possibly to local inflammation that might limit parasite migration. Alternatively, it is feasible that *in vivo*, paramyosin released from schistosomula dying as a result of natural attrition induces immune inflammation that could trap and/or kill bystander parasites that normally would survive in an unimmunized mouse.

Specific epitopes in paramyosin appear to be important for vaccination because the related schistosome myofibrillar protein myosin and a heterologous paramyosin (from *Mercenaria*) failed to protect. Moreover, our studies with purified recombinant paramyosin [representing approximately 50% of the native molecule and known to be missing at least one mAb-defined epitope (8)] suggest that multiple epitopes on paramyosin may be involved in the induction of protection, since the recombinant protein is apparently not as protective as the native molecule (Fig. 2) and is less stimulatory than the native protein for paramyosin-specific T cells (Table 2).

The observation that SWAP depleted of paramyosin can induce levels of protective immunity and *in vitro* T-cell responsiveness comparable to whole SWAP or purified paramyosin is of interest because it suggests that soluble antigens in addition to paramyosin function as vaccine antigens. Alternative explanations for the protection observed are that: (*i*) serologically undetectable levels of paramyosin persist in SWAP after multiple passage over the affinity column and that paramyosin is protective at these doses; (*ii*) paramyosin peptides containing relevant T-cell epitopes, but no B-cell epitopes, separate into the flow-through from the affinity column; or (*iii*) there are paramyosin cross-reactive T-cell epitopes on other molecules in SWAP. If indeed molecules in SWAP other than paramyosin are immunoprophylactic, these antigens are likely to be of high molecular weight based on the previously described (18) behavior of the protective activity when separated by gel filtration.

While our data establish beyond doubt that paramyosin, a soluble nonsurface schistosome protein, can induce protective immunity, it should be pointed out that the level of protection achieved with this molecule at present is not high enough to warrant its use in human vaccination. Nevertheless, it is likely that modifications in the mode of antigen

presentation, particularly with respect to dose, timing of booster inoculations, and the use of different immunomodulators, will enhance the level of protection achieved. It is also pertinent that the maximum level of protection obtained with paramyosin (39%) in the mouse is similar to that recently reported for *S. mansoni* glutathione transferase (42%) (19, 20). This immunogen is thought to function by inducing an IgG2a anti-28-kDa molecule response that can direct eosinophil-mediated cytotoxicity against invading schistosomula. It is possible that a combined vaccine consisting of paramyosin and the 28-kDa molecule, two immunogens thought to work through quite different mechanisms, may induce levels of protection high enough to allow consideration for human use.

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