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Comparison of the Protective Efficacy on Mekongi Schistosomiasis in Mice Induced by Antigens Derived from Cercariae, Schistosomulae and Adult Worms

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SUMMARY **Protective efficacy of the extracts of cercariae, schistosomulae and adult worms of *S. mekongi* was studied in mice receiving immunizations with these extracts emulsified with Freund's complete adjuvant initially and incomplete adjuvant subsequently, and compared with mice receiving physiological saline with or without adjuvants as controls. After challenge with cercariae, the animals were sacrificed and the larvae or adult worms harvested by lung recovery and perfusion techniques on day 5 and weeks 6-8, respectively. Worm reduction rates were significantly higher in mice receiving extracts of schistosomula (59%) and adult worms (51%) than in those receiving the cercarial extracts (31%). Similar findings were obtained with the perfusion technique showing worm reduction rates of 57%, 53% and 30% in mice receiving extracts of schistosomulae, adult worms and cercariae, respectively. ELISA antibody titers were correspondingly increased in mice receiving extracts of schistosomulae and adult worms, but not in those receiving cercariae. This apparent association may be inadequate to suggest that the increase in ELISA titer be used as an indicator for resistance in mekongi schistosomiasis.**

Like most other heiminthic infections, schistosomiasis initiates immune responses which exert a state of concomitant immunity of the host.[22] Such naturally acquired immunity is quite efficient against reinfection in spite of the fact that the host still harbors the worms of the primary infection. In view of this finding, extensive research has been conducted to determine which stage specific antigen(s) is(are) responsible for initiating immunity in the host.[5] Our experiments were designed to compare the protective efficacy induced by antigens prepared from cercariae, schistosomulae and adult worms of *Schistosoma mekongi* in experimental mice.

MATERIALS AND METHODS

Preparation of cercariae

Tricula aperta (β-race) snails were collected from the Mun River, Ubon Rachathanee Province, Thailand. They were examined for natural *S. mekongi* infection in the laboratory for 2 months.[10] The non-infected snails were then individually exposed for 2 hours under incandescent light at 25-27°C to 5 newly-hatched miracidia of *S. mekongi* in a glass vial (12×15 mm) containing about 0.3 ml of dechlorinated water.[25] The exposed snails were transferred into a 3"×7" glass bowl aquaria containing about 1,000 ml of dechlorinated tap water and were fed regularly with diatoms and soil substrate. Thirty-five days after exposure to the miracidia, the snails were examined at weekly intervals, by placing each of them in a glass vial (12×15 mm) containing 1 ml of dechlorinated water and kept under an incandescent light. The initial shedding of cercariae was observed under a dissecting microscope. The positive snails were transferred to another aquarium and kept in the dark to prevent sheeding. The negative snails were examined once a week until the 8th week after an initial exposure to the miracidia. The snails which remained negative were then discarded.[11]

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Preparation of schistosomulae

The schistosomulae of *S.mekongi* were prepared by the method modified from Ramatho-Pinto *et al,*[18] Yasuraoka *et al,*[26] and Torio.[23] The cercariae were collected, counted and immobilized by chilling in an ice bath for 15 minutes. They were then concentrated by centrifugation at 175 × g for 3 minutes. The packed cercariae were washed twice by centrifugation with RPMI 1640 medium containing 400 units/ml of penicillin, 200 μg/ml of streptomycin and 50% fresh human type A or B serum. The preparation was incubated in a candle jar at 37°C for 3 hours. The worms were agitated by a Vortex mixer and then the preparation was centrifuged at 175 × g for 3 minutes. The supernate was discarded. The packed worms were resuspended in about 15 ml of fresh RPMI 1640 medium without antibiotics or serum. The worm suspension was cooled in an ice bath for 10 minutes. The schistosomular bodies were allowed to settle down while the tail-rich supernate was pipetted out. The schistosomulae were lyophilized and kept at -70°C for antigen preparation.

Preparationof adult *S. mekongi*

Albino Swiss mice were used for maintaining the life cycle of *S. mekongi.* Six week old female mice were infected with 20-30 freshly shed, active cercariae by looping method. The shaved and wet abdominal skin of each anesthetized mouse was exposed to the cercariae. The mice were returned to their domestic cages for 6-8 weeks after infection. Finally, the positive mice were killed and the adult worms collected by perfusion technique of Duvall and Dewitt.[4]

Preparation of antigens

Antigens were prepared from cercariae, schistosomulae and adult worms according to the method previously described by Maddison *et al,*[13] with modifications as follows : Approximately 5,000-10,000 worms (cercariae, schistosomulae or adults) were homogenized in a Potter-Elvehjem tissue grinder in a small volume of physiological saline. The preparation was then subjected to ultrasonic treatment in a ultrasonic disintegrator (full speed; MSE, UK) at 8 microns between peaks 5 minutes 3-5 times. The homogenate was extracted with cold extraction medium containing 0.5 M HCl, 1.0 M NaCl, 2 mM EDTA and 1 mM DL-cysteine HCl in 0.05 M Tris/HCl, pH 3.0. The preparation was stirred at 4°C for 2-3 hours, then centrifuged at 12,500 × g for 30 minutes. The supernate was collected while the pellet was reextracted as above twice more. All supernates were pooled and dialysed against physiological saline and distilled water. Protein contents of the preparations were determined by a modified Lowry's method.[15]

Immunization of mice

Six week old female outbred Swiss mice reared in the Faculty of Tropical Medicine were used. They were divided into 5 groups. Group 1 comprising 14 mice were immunized with cercarial extract, half of which were used for the lung recovery technique and the remaining half were used for the perfusion technique. All animals survived the infection. Group 2 comprising 12 mice received immunization with schistosomular extract, 5 of which were used for the lung recovery technique and the remaining 7 for the perfusion technique. After infection, only 3 and 5 mice survived the infections to be used for lung recovery and perfusion techniques. Group 3 comprising 12 mice received immunization with adult worm extract, 5 of which were used for lung recovery and the remaining 7 for the perfusion technique. All survived the infection. Group 4 and 5 each comprising 12 mice were given either saline plus adjuvant (group 4) or saline alone (group 5). Five mice were used for lung recovery and the remaining 7 for the perfusion technique. All mice survived the infection.

Antigens derived from any stage of schistosoma worms were emulsified in an equal volume of complete Freund's adjuvant (CFA) to give a final concentration of 1 mg/ml, then 0.2 ml aliquot was injected intramuscularly into each thigh of a mouse. Mice of two control groups were injected with 0.2 ml of adjuvant-saline mixture and saline alone, respectively. Two weeks later, mice in the experimental group received an intramuscular booster of 400 μg antigen mixed with incomplete Freund's adjuvant (IFA). Appropriate boosters were also given to the mice in the control groups. Two weeks thereafter, immunized and control animals were bled from the retro-orbital plexi. The sera were collected and stored at -20°C until assay for antibody titers against homologous antigens by an enzyme-linked immunosorbent assay (ELISA). Three days after the bleeding, each mouse was challenged with 100 cercariae. Five days after the challenge, schistosomulae were recovered from lungs of some mice of each group[17,20] with modifications (see below) while others were rechallenged with 30 cercariae and adult worm recovery was performed 6-8 weeks thereafter by perfusion technique.[4]

Lung recovery technique

Schistosomulae were recovered from the lungs of mice 5 days after rechallenge with 30 cercariae. Each mouse was injected (IV) with 10-15 ml of Hank's balanced salt solution (HBSS) pH 7.2 containing 10 units/ml of heparin. When the animals did not die from the injection, they were killed by ether euthanasia. Immediately after death, the lungs were removed, washed with heparinized HBSS and then chopped into small pieces in a sterile test tubes. The lung pieces were suspended in 10-15 ml of heparinized minimum essen-

tial medium (MEM) pH 6.8 and the preparation was incubated at 37°C for 3 hours. After incubation, the preparation was filtered through a 30-mesh stainless steel sieve which allowed the schistosomulae to pass through while retaining the bigger pieces of lung. The filtrate was centrifuged at 700×g for 10 minutes at room temperature. The red blood cells were lysed by rapidly adding 2 ml of distilled water, for 2-3 minutes and then 8 ml of MEM, followed by centrifugation as above. The pellet was resuspended in MEM supplemented with 0.5% fetal calf serum and the number of live schistosomulae determined.

Statistical analysis

Differences on immunogenicities of antigens from 3 stages of the schistosomes were analysed by Kruskal-Wallis, while Mann-Whitney U test was used to determine the differences between experimental groups and control groups. P value of < 0.05 were considered significant.

The percentage of worm reduction was calculated from the formula:

$$\%\text{Reduction} = \frac{A-B}{A} \times 100$$

where A = mean of worm recovery from control mice injected with saline alone

B = mean of worm recovery from immunized mice.

Indirect enzyme linked immunosorbent assay (ELISA)

ELISA antibody titers of the mouse sera against various stage derived antigens of worms were determined. Wells of polystyrene microtiter plates (Nunc, Denmark) were coated with 100 µl of 5 µg/ml of appropriate antigens in a carbonate buffer pH 9.6 then incubated at 37°C for 1 hour and at 4°C overnight. After decanting the non-adsorbed antigens, the wells were washed extensively with phosphate buffer saline pH 7.4 (PBS, pH 7.4) containing 0.05% polysorbate 20 (PBST). Blocking of the empty sites on plates was accomplished by incubating with 100 µl of PBS containing 1% bovine serum albumin (BSA). After incubating at 37°C for 1 hour, excess BSA was washed away and the wells were filled with 100 µl of appropriate dilutions of sera. Control wells (blanks) to which no serum but PBS was added were included in the plates. The plates were incubated and washed. After washing, 100 µl of peroxidase-labelled rabbit-anti-mouse immunoglobulins (Dakopatts, Denmark) diluted 1:1,000 in PBST containing 1% BSA was filled to each well. After incubation for 1 hour, the excess conjugate was removed by washing and 100 µl of freshly prepared phenylendiamine dihydrochloride substrate solution added into each well. The reaction was allowed to proceed for 30 minutes then stopped with 50 µl of 1 N NaOH. The optical density of each test well was determined against the blank by Uniskan II Labsystem ELISA reader at the O.D. 405 nm.

RESULTS

Aeverage numbers (means ± SD), percentage recovery and percentage reduction of schistosomulae from immune mice and controls at 5 days after challenged with 100 cercariae are shown in Table 1. Statistical analysis indicated that the mice which were immune to schistosomulae and adult worm antigens (group 2 and 3) had significantly lower percentage recovery of schistosomulae than those of the two groups (groups 4 and 5). The percentage reduction of schistosomulae of the group 2 and 3 were inversely correlated with the percentage recovery and thus were significantly higher than those of groups 4 and 5. The cercarial antigens, on the contrary, conferred neither significant reduction of the percentage schistosomulae recovery nor significant increase in the percentage of worm reduction from mice of group 1 when compared to those of the controls.

Table 2 presents the average number, percentage recovery and percentage reduction of adult schistosomes from mice of various treatment groups at 6-8 weeks after the second challenge with 30 cercariae. The overall results were similar to those data on schistosomulae recovery. Mice immunized with schis-

Table 1 Average numbers (mean ± SD), percentage recovery and percentage reduction of schistosomulae from immunized and control mice 5 days after challenge with 100 cercariae

Group	Immunogen	Number per mouse (mean ± SD)	Percentage recovery	Percentage reduction	*P* value
1	Cercariae	5.6 ± 1.6	5.6	31	>0.05
2	Schistosomulae	3.3 ± 1.5	3.3	59	<0.01
3	Adult worms	4.0 ± 0.6	4.0	51	<0.01
4	Adjuvant + NSS	8.1 ± 3.0	8.1	0	—
5	NSS	8.1 ± 2.7	8.1	0	—

Table 2 Average numbers (mean ± SD), percentage recovery and percentage reduction of adult worms 6-8 weeks after the second challenge with 30 cercariae

Group	Source of immunogen	Number per mouse (mean ± SD)	Percentage recovery	Percentage reduction	*P* value
1	Cercariae	6.3 ± 2.3	21	30	0.29
2	Schistosomulae	4.3 ± 1.3	14	53	<0.05
3	Adult worms	3.8 ± 0.8	13	57	<0.05
4	Adjuvant + NSS	8.0 ± 3.5	26	11	0.52
5	NSS	9.0 ± 4.9	29	—	—

tosomular and adult worm antigens had significantly lower adult worm reduction than those of control mice in groups 4 and 5. The mice immune to the cercarial antigens had only modest degrees of the immune signals which was not significantly different from the controls.

Reciprocal mean ln ELISA titers in mice of all treatment groups are presented in Fig. 1. The serum titers in mice of groups 2 and 3 were significantly higher than those of the two control groups, while those of mice immunized with cercarial antigens were not. When comparison was made between the titers elicited by the adult worm antigens and schistosomular antigens, the former were significantly higher.

Figure 2 shows a correlation between antibodies titer and schistosomulae reduction by lung recovery technique. The percentage reduction of schistosomulae in the mice that were immune to schistosomulae antigens was correlated with antibodies titer ($p = 0.00001$), whereas the percentage reduction of schistosomulae in the mice immunized with cercarial antigens and adult worm antigens was not statistically correlated with antibodies titer ($p = 0.36044$), $p = 0.21236$), respectively.

Correlation between antibody titer and worm reduction by perfusion technique is shown in Fig. 3. The percentage reduction of worm in mice which were immunized with cercarial antigens and adult worm antigens, respectively, correlated with antibody titer ($p = 0.06341$, $p = 0.09430$). However, percentage reduction of worms in mice immunized with schistosomular antigens did not correlate with antibody titer ($p = 0.21236$).

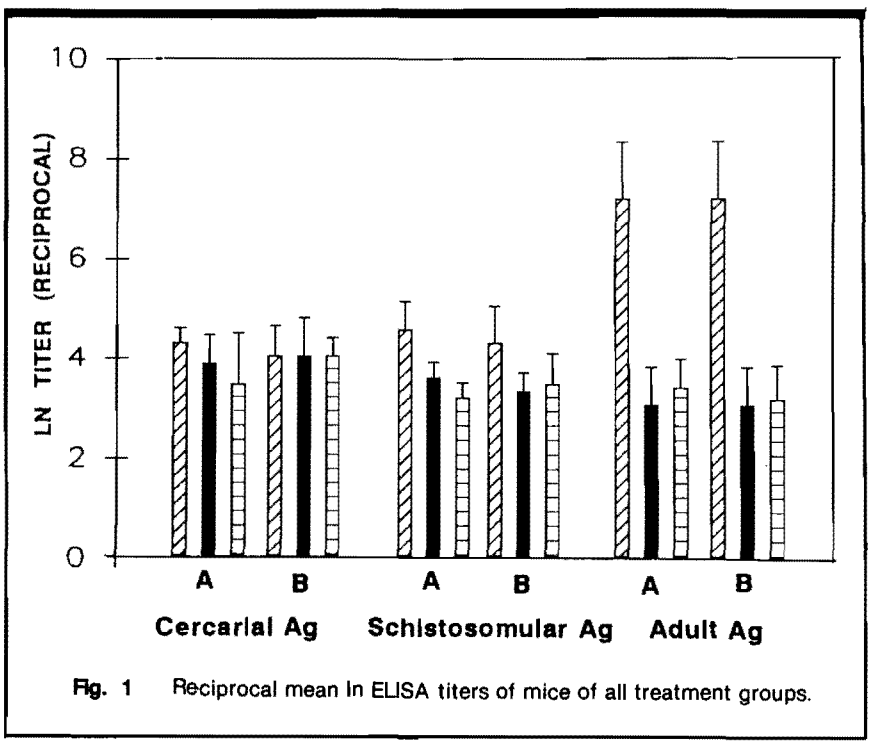

Fig. 1 Reciprocal mean ln ELISA titers of mice of all treatment groups.

DISCUSSION

In the present study, protective efficacies of immunization with cercaria, schistosomulae and adult worm antigens against challenge with *S. mekongi* infection were assessed by two different techniques comprising lung recovery and perfusion. We agree with Doenhoff *et al*[3] and others[12] that the lung recovery technique is more advantageous than perfusion techniques for the following reasons: 1) it allows early termination of the experiment on day 5 instead of 6-8 weeks after infection; 2) collection of larvae from the lung is less labor-

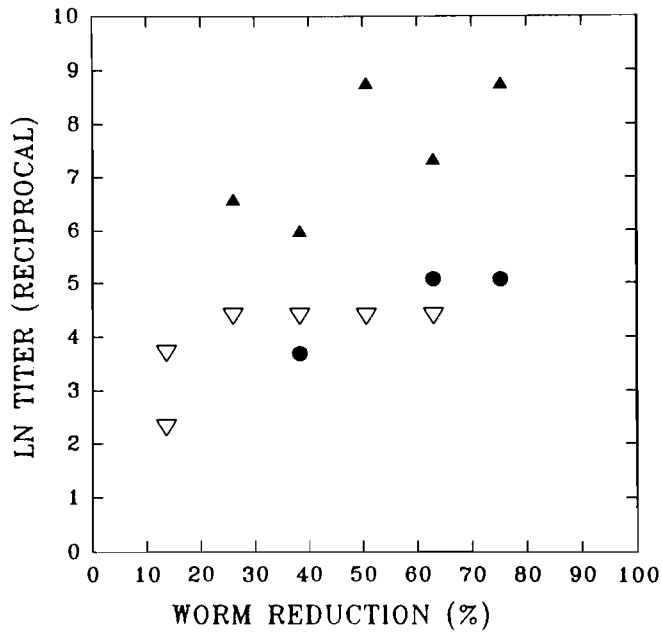

Fig. 2 Correlation between antibodies titer and schistosomulae reduction by lung recovery technique.

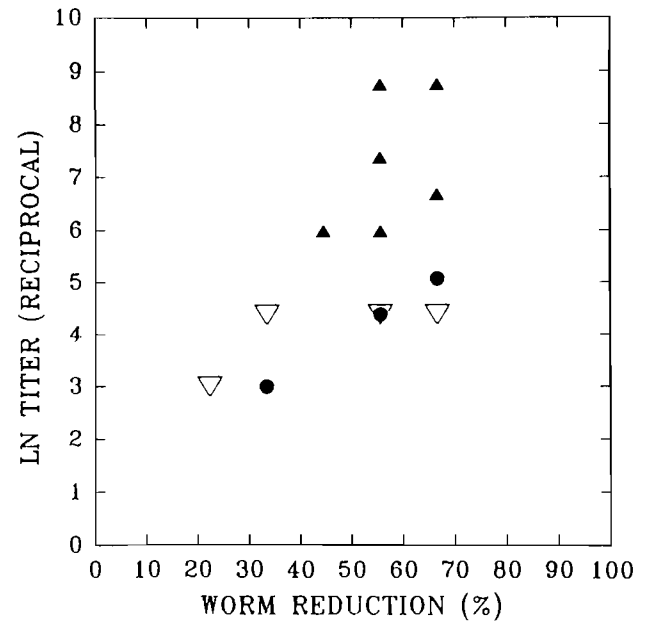

Fig. 3 Correlation between antibodies titer and worm reduction by perfusion technique.

intensive. Nevertheless, the lung recovery technique also has limitations. Because of smaller size of the larvae obtained by lung recovery than the adult worms collected by perfusion, counting of the larvae is less accurate. The larvae that have passed through the lung will be missed. In addition, measurement of resistance is confined to the early stage of parasite development, and the long term effects on parasite growth and development are not recorded. In order to enable full realization of the immunization effect, these two techniques were therefore chosen for this study.

For unknown reasons, the lung recovery rate of 8.1-9.0% in control mice on day 5 after challenge was much lower than that reported by Janecharut *et al*,[6] in which a recovery rate of 20% was obtained. Variations in recovery rates could be attributable to parasite factors, including parasite strain and the host immune status.[11] Furthermore, different species of schistosomes showed variations of peak lung recovery rate on different days in different hosts, being 24% on day 5 or 6 in *S. mansoni* infection in mice and 28% in rats,[12,14,20] 15% on day 8 in *S. haematobium* infections in hamsters,[21] and up to 12% and 10% on day 4 in *S. japonicum* infections in mice and rats, respectively.[24]

Nevertheless, protective efficacy of antigens from different stages of the parasites showed good correlation between these two techniques (Tables 1, 2). Whilst percent worm reduction in mice immunized with schistosomulae and adult worm antigens was not different, they were significantly higher than those immunized with cercarial antigens. Our findings thus lend support to the report by Sadun *et al*,[19] on the poor immunogenicity of cercarial antigens compared with adult worm homogenates or their secretory-excretory products, and to work of Katz and Colley[9] on *S. mansoni* infection in

mice. Cercarial extract is not entirely devoid of protective efficacy, since injections of freeze-thawed cercariae of *S. mansoni* mixed with BCG conferred 63% protection against challenge with cercariae of the same species.[7] The molecules conferring resistance in *S. mekongi* have not been identified, whereas in *S. mansoni* several candidate antigens have been characterized. A minimally glycosylated protein of 97 kDa (Sm-97) found in both schistosomulae and adult worms was claimed to be protective on the grounds that mice immunized with nonliving, freeze-thawed lysate of schistosomulae and adult worms were up to 55% protected and their sera of immunized mice recognized the Sm-97 antigen.[8,16] Other candidate molecules include a 38 kDa glycoprotein, a 28 kDa antigen (P28) on the surface membrane, and a cluster of 22-26 kDa in schistosomula-released products (SRP).[1] The gene encoding P28 has been cloned, and the recombinant protein (Sm28 GST) gave a mean protection close to 60% in rats and 45% in mice.[2] In an attempt to find an *in vitro* correlate of protective immunity in *S. mekongi* infection, we observed significantly higher mean ln ELISA titers in mice immunized with schistosomulae or adult worm antigens than those immunized with cercarial antigens. Yet this apparent association is inadequate to suggest that the increase in ELISA titer be used as an indicator for resistance in mekongi schistosmiasis. Obviously, more work has to be carried out to clarify such association.

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