Further Studies on Resistance to Reinfection with *Schistosoma japonicum* in Mice*

Edito G. Garcia, M.D., M.P.H.
Graham F. Mitchell, B.V.Sc., Ph.D.
Fems Julia M. Espinas, B.S.P.H., M.S.
Fe P. Tapales, B.S.P.H.
Lucivic P. Quicho, B.S.M.T., M.Sc. P.H.
Wilfred U. Tiu, B.S. Bio.

SUMMARY **Resistance to second infection in *Schistosoma japonicum*-infected BALB/c mice was determined by counting the number of immature worms arising from challenge infection 20 days after cercarial challenge, and the number of petechiae on the lungs several days after cercarial challenge. Only a proportion of the high resistance to reinfection observed at about 50 days of a primary infection could be ascribed to a loss of schistosomules as evidenced by lung petechiae numbers 4 to 6 days after challenge. Expression of high level resistance to reinfection depended on the presence of substantial numbers of both male and female worms in the primary infection, as single-sex primary infections were not associated with any resistance to reinfection. In a primary infection, an apparent loss of worms at times longer than 50 days after infection is most readily explained by a selective loss of heavily infected and thus severely diseased mice. Long-term survivors that contain few worms were not resistant to reinfection when challenged many months after first exposure to cercariae. The data are in line with the possibility that liver pathology, and in particular the consequences of portal hypertension in mice with heavy mixed-sex infections, contributes significantly to expression of "concomitant immunity" in this model parasite system.**

ASIAN PACIFIC J ALLERG IMMUN 1984; 2 : 27-31.

Analyses of the mechanisms underlying expression of concomitant immunity in schistosomiasis[1] are fundamental to the strategy of developing vaccines effective against first infection. Recently it has become clear that the mouse models of schistosomiasis have some limitations in the study of concomitant immunity (i.e. resistance to reinfection in already infected hosts). Much controversy exists regarding the relative contributions of "pathology-associated resistance" as distinct from "classical anti-parasite immune effector mechanisms" in expression of reinfection resistance in mouse models.[2,3] There is no doubt that liver pathology is severe in mice with mixed-sex *Schistosoma japonicum* infections at $\geq$ 50 days, and mortality can be high in mice exposed to doses of 20 to 25 cercariae. Since even one worm pair in a mouse can be considered a high worm burden relative to that in man,[4] the real possibility exists that the mouse models of schistosomiasis japonica and schistosomiasis mansoni are useful for analyses of disease processes but are less useful for analysis of concomitant immunity.

In a previous publication, it was reported that mice infected for 50 days or longer with Philippine *S. japonicum* expressed high level resistance to challenge infection.[5] Thus, the number of immature worms recovered 20 days after challenge infection was approximately 80 per cent lower in already-infected mice than in age-matched challenge controls. In the experiments

*From the Department of Parasitology, Institute of Public Health, University of the Philippines, Ermita 2801, Manila, Philippines and Immunoparasitology Unit, The Walter and Eliza Hall Institute of Medical Research, Melbourne 3050, Australia.

Supported by the Schistosomiasis and Research Strengthening Components of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the Rockefeller Foundation Great Neglected Diseases Programme.

reported in this paper, answers have been sought to several questions raised in a previous report.[5] Are worms of a primary infection lost progressively with time, particularly during the period of expression of high-level resistance to reinfection? Does re-exposure to cercariae result in some loss of the existing worm burden in already-infected mice? Do mice with male-only or female-only infection (and thus no egg production and hepatic disease) show any resistance to reinfection? Since mice that display resistance to reinfection contain eggs in the lungs as a result of shunting of eggs past the liver via the collateral circulation, do lung granulomas alone inhibit establishment of a challenge infection? The answer to these four questions appears to be no. Experiments were designed to determine whether lung petechiae numbers,[5,6-8] like immature worm numbers, were also reduced in mice with primary infection. If this was so, then it would suggest that resistance to reinfection is being expressed at lung or pre-lung stages in the early migration pathway of the challenge worms.

MATERIALS AND METHODS

Mice

Male and female BALB/c mice, obtained originally from the Walter and Eliza Hall Institute, were bred and maintained under conventional conditions and first used in experiments when 6 to 20 weeks (usually 9 to 12 weeks) of age. In one experiment, local outbred white mice were used. In each experiment, infected mice for challenge, and challenge control mice, were matched by age and sex.

Parasites, infections and injections

Techniques for infection of mice using the percutaneous coverslip method and cercariae derived from laboratory-bred or field-collected (from Mindoro) *Oncomelania hupensis quadrasi* snails have been described.[5] Also described are the methods used for enumeration of worms by portal perfusion and liver dissection as well as enumeration of lung petechiae at 4 to 6 days after cercarial challenge. Eggs from rabbits or mice infected for 40 to 65 days[9] were digested at 37°C from chopped, homogenised livers using trypsin in phosphate buffer, pH 8.5, followed by sieving, centrifugation and further treatment with pepsin in acid saline buffer pH 1.8 to further reduce tissue contamination. Eggs were used fresh without lyophilization. Eggs to be injected intraperitoneally in Freund's complete adjuvant (FCA) (Difco Labs., Detroit, Michigan, USA) were suspended in phosphate buffer, pH 7.3, and emulsified in a 1:1 mixture with FCA.

Unless stated otherwise, mice were given 20 cercariae percutaneously in the primary infection and 25 cercariae percutaneously at the same abdominal site in the challenge infection. The time interval between primary and secondary (challenge) infection was usually 50 days. Mice were killed 4 to 6 days later for lung petechiae or 20 days or so later for determination of worm numbers (mature males and females and/or immatures). In most experiments reported in the paper, cercariae used were obtained from field-collected snails rather than a laboratory isolate. In some experiments (Table 3), mice were infected with cercariae derived from single individual snails, in order to achieve single-sex infections.

Statistical analysis

Data were expressed as the arithmetic mean ± standard error of the mean. Statistical analysis was performed using a Student's t-test with a criterion of rejection at $p > 0.05$.

RESULTS

Stability of the primary worm burden and effects of challenge on primary worms

One of the questions raised in the previous report[5] was whether the number of adult worms was reduced at the time of maximal expression of resistance to reinfection in *S. japonicum*-infected mice. Another question was whether reinfection caused a loss of worms from primary infection. In Table 1,

Table 1. Primary adult worm burdens at different times of infection and effects of challenge on existing worm burdens

Experiment No.	No. of cercariae administered		No. of mice	Day of assay	No. of mature worms
	Day 0	Days 40-51			
1*	20	0	11	40	12.0 ± 1.4
		0	12	50	9.1 ± 1.0
		0	13	59	6.2 ± 0.7
	20	25 (day 40)	6	59	7.0 ± 0.8
2+	20	0	10	33	13.4 ± 1.2
		0	10	40	12.9 ± 1.5
		0	7	53	12.0 ± 1.0++
	20	3 x 20 (days 40, 45, 51)	6	53	9.0 ± 1.2++

*BALB/c males and females, 8-11 weeks old at day 0. Of 100 initially infected mice, only 42 were available for assay over the 59-day experimental period. Average percentage of female worms in primary worm burden = 31%.

+BALB/c females, 15-19 weeks old at day 0. Of 48 initially infected mice, 33 were availabe for assay over the 53-day experimental period. Average percentage of female worms in primary worm burden = 28%.

++Not significant.

young BALB/c mice were infected with 20 cercariae per mouse and worm burdens were determined at days 40, 50 and 59 by portal perfusion. Additional infected mice were re-exposed to 25 cercariae per mouse at day 40 of primary infection. The results demonstrate an apparent progressive loss of primary infection worms. However, the total number of initially-infected mice surviving for the duration of the experiment was < 50 per cent. Thus it is likely that the apparent progressive loss of primary-infection worms reflects selective mortality in heavily infected and thus severely diseased mice. This conclusion is supported by data in another experiment (experiment 2). When older BALB/c mice were infected with 20 cercariae per mouse and killed at days 33, 40 and 53, no loss of worms was noted with increasing duration of primary infection, and large numbers of initially infected mice survived the entire time course of the experiment. In both experiments reported in Table 1 no accelerated loss of primary-infection worms was induced by challenge of infected mice with additional cercariae.

Examination of lung petechiae numbers as an index of resistance to reinfection

Data reported previously[5] indicated that number of lung petechiae in mice exposed to cercariae 4 days previously were reduced in previously-infected mice as compared with control mice. In the present study, involving 6 to 12-week old outbred or BALB/c mice, lung petechiae at day 4, and mature plus immature worm numbers at 15, 20 or 22 days after challenge, were determined in previously infected and control mice. Fifty days separated first (20 or 25 cercariae per mouse) and second (25 or 50 cercariae per mouse) percutaneous infection. As shown in Table 2, resistance to reinfection was 72 per cent as assessed by immature worm numbers and 44 per cent by lung petechiae in challenged infected mice compared with challenge control mice. The data can be interpreted as supporting the notion of two phases of expression of resistance to reinfection. That is, attrition of challenge parasites occurs either early (lung or pre-lung stage) or late (post-lung stage) in the establishment of parasites.[10]

Failure to demonstrate resistance to reinfection in mice infected with male-only or female-only worms

Mice with single-sex infections of *S. japonicum* do not develop hepato-splenic disease and worms in either a male-only or female-only infection remain immature and stunted. For unknown reasons, male-only infections are far easier

Table 2 Comparison of two indices of resistance to reinfection in mice, lung petechiae and immature worm numbers

Exposure to cercariae		Infection status		
First (day – 50)	Second (day 0)	No. of lung petechiae (day 4)	No. of worms (day 15-22)	
			Mature	Immature
+	+	9.5 ± 1.1 (22)	8.1 ± 2.3[+]	3.9 ± 1.6 (10)
–	+	16.9 ± 1.9 (20)	0	13.8 ± 2.9 (18)
		44% resistance		72% resistance
		$P < 0.01$		$P < 0.05$

Number of mice indicated in brackets; combined data from 3 experiments
+Approximately 30% female worms.

Table 3 Lack of resistance to reinfection in BALB/c mice infected with male-only or female-only worms

Experiment No.	Exposure to cercariae		Number of Mice	Number of worms (days 20 to 28)
	First (day 50)	Second (day 0)		
1	+ (males only)	+	18	29.1 ± 2.1*
	–	+	17	15.0 ± 1.6*
	+ (males only)	–	15	12.9 ± 1.3
2[++]	+ (males only)	+	8	21.6 ± 2.4*
	–	+	8	12.5 ± 1.5*
	+ (males only)	–	8	6.9 ± 1.3
3	+ (females only)	+	12	22.2 ± 5.6[+]
	+ (males only)	+	12	23.9 ± 3.6*
	–	+	10	10.6 ± 1.8

*27 to 39% of worms present were females in these 5 determinations, this ratio of males to females being in line with previous data (Mitchell *et al*, 1981; see also Tables 1 and 2).
[+]64% of worms present were females; this higher proportion of females was expected as primary infection was with female worms only (see text).
[++]In this experiment, additional mice infected for 70 rather than 50 days with male-only worms were also not resistant to reinfection.

to generate regularly than female-only infections, and males invariably predominate in mixed-sex infections.[11] To test for resistance to reinfection in single-sex infections, group of 6 to 7-week old male and female BALB/c mice were exposed to cercariae collected from a series of single snails, and representative mice were killed (prior to challenge of remaining mice) to ascertain that male-only or female-only infections had been established (experiments 1 and 3, Table 3). In experiment 2 (Table 3), mice exposed to pooled cercariae were fortuitously found to be infected with male worms only. The infected animals were given a challenge infection of 25 cercariae either 50 or 70 days after the first infection (12 or 20 cercariae per mouse). Total worm numbers were determined 20 to 28 days after challenge. Results shown in Table 3 indicate that no resistance is demonstrable in single-sex infections; this result is entirely different from that obtained in mice with mixed-sex primary infections.[5] It was demonstrated further that BALB/c mice with one female worm each and numerous males, were also not resistant to reinfection.

Failure to demonstrate resistance to infection in uninfected, egg-sensitised mice with induced lung granulomas

To determine whether egg granuloma formation in the lungs was responsible for expression of resistance to reinfection in infected mice, egg-sensitised mice with induced lung granulomas were challenged with *S. japonicum* cercariae and the numbers of lung petechiae and worms were determined. Male and female BALB/c mice aged 6 to 8 weeks were injected by the intraperitoneal route with 2,000 freshly collected eggs at weekly intervals (the first injection was either with or without FCA). They were then injected intravenously with 1,000-2,000 freshly collected eggs to induce granulomas,[12] challenged percutaneously 3-6 days later with 25 cercariae and killed at either 4 to 6 or 25 to 29 days for determination of lung petechiae or worms in the portal system and liver, respectively. Mean worm burden in 17 egg-sensitised mice with lung granulomas was 13.8 ± 0.9, and in 16 control mice not injected with eggs, 12.8 ± 1.5. Number of lung petechiae in sensitized and control mice also did not differ. Thus, the presence of lung granulomas, without liver pathology, is insufficient to inhibit establishment of *S. japonicum* injection.

DISCUSSION

The results presented in this paper complement those reported recently,[5] although they tend to negate various discussion points raised in the previous paper. As assessed by the number of challenge worms which become established,[13] high-level resistance to reinfection has again been demonstrated in mice with mixed-sex *S. japonicum* infections involving several female worms (Table 2). This resistance has been demonstrated using cercariae from field collected snails from Mindoro, the previous study having used an "isolate" from Mindoro maintained in snails in the laboratory.[5] Experiments are in progress to determine whether primary and challenge infection of mice with cercariae from different areas endemic for schistosomiasis japonica in the Philippines result in different levels of resistance to reinfection.

In a primary infection involving 5-15 mixed-sex worms, but in which males invariably predominate, an apparent loss of worms which is observed in some experiments late in the infection is most readily explained by deaths of heavily infected mice. Loss of such mice then leads to an artificially low assessment of worm burdens in mice at later times (Table 1). A similar consequence of infection is likely to operate in the *S. mansoni*/mouse system.[14] No evidence could be obtained for the notion that reinfection has any consequence for the established worm burden (Table 1). This is in keeping with the tenets of concomitant immunity, i.e., that expression of resistance to reinfection has no effect on the established worms.[15] Mice with male-only or female-only worm infections show no resistance to reinfection (Table 3) and such resistance seen in mice with mixed-sex infections cannot be ascribed readily to the presence of lung granulomas and thus impedence of worm passage through the lungs. However, it is not known whether lung pathology in egg-sensitised mice injected intravenously with eggs differs in intensity or character from lung disease induced by eggs in infected mice.

Lung petechiae numbers, if they are a true reflection of schistosomule numbers reaching and penetrating the lungs *en route* to the liver,[5] only account for partial resistance to reinfection seen in infected mice. The data together support the results of extensive experiments by Smithers and colleages which strongly suggest that resistance to reinfection is expressed in two distinct phases in the *S. mansoni*/mouse system: early (at 1 to 2 days) and late (at 1 to 2 weeks).[10,16,17] Additional data indicate or suggest that challenge worm attrition in reinfection experiments may occur at the post-lung stage.[18,19] The available evidence in the *S. japonicum*/mouse system, which can only be considered to be indirect, indicates that challenge worm attrition in reinfected mice may occur early (pre-lung) and late (pre-adult). Although the first phase is unlikely to be due to "concomitant pathology" in infected mice, it is an open question whether the second phase has anything to do with direct effects of anti-worm immune responses.[2,3] Portal hypertension is pronounced in mice infected with *S. japonicum*. The mean portal hydrostatic pres-

sure was significantly elevated from 28 mm of water in uninfected mice to 61 mm of water in mice exposed 50 days previously to 20 cercariae (7 or 8 mice per group) (E.G. Garcia, unpublished observations). Thus, pre-adults of the challenge infection may experience some difficulty in reaching their desired definitive locations in the liver and portal system.[1-3]

Mice with male-only or female-only infections are not resistant to reinfection nor are mice with one female worm plus numerous males. These results are compatible with data obtained in the *S. mansoni*/mouse system.[14,20,21] However, it must be cautioned that the results with single-sex infections are not a reliable indication that the liver pathology of mixed-sex infections is necessarily responsible for resistance to reinfection against post-lung pre-adults in mouse models. Both males and females in single-sex infections of mice are stunted. Thus, induced anti-schistosome immune responses may not resemble those induced by fully mature worms.[22] Whilst it remains extremely difficult to prove or disprove the possibility that liver and lung disease is partly or wholly responsible for resistance to reinfection, the parabiosis experiments of Dean *et al*[2] cast serious doubts on the utility of at least the *S. mansoni*/mouse model in analysis of the immunology of resistance to reinfection. Results presented here suggest that the *S. japonicum*/mouse model may also suffer similar deficiencies and that a vigorous search for *immunological* correlates of reinfection resistance, particularly that expressed against post-lung stages, may not be warranted.

ACKNOWLEDGEMENTS

We thank Nori Castrodes, Evelyn Bilanes, Elizabeth G.C. Tadina and Roberto Gabion for their technical help and Conchita Conlu for typing the manuscript.

REFERENCES:

1. Smithers SR, Doenhoff M. Schistosomiasis. In: Cohen S, Warren KS, eds, Immunology of Parasitic Infections, 2nd ed. Oxford: Blackwell Scientific 1982:527-607.
2. Dean DA, Bukowski MA, Clark SS. Attempts to transfer the resistance of *S. mansoni*-infected and irradiated cercariae-immunized mice by means of parabiosis. Am J Trop Med Hyg 1981; 30:113-20.
3. Wilson RA. Is immunity to *S. mansoni* in the chronically infected mouse an artefact of pathology? In: Proceedings of the Third European Multicolloquium in Parasitology, Cambridge, England, 1980:37.
4. Damian RT. Immunity in schistosomiasis: a holistic view. Con Top Immunobiol 1984; (in press).
5. Garcia EG, Mitchell GF, Tiu WU, Tapales FP, Valdez CA. Resistance to reinfection with *Schistosoma japonicum* in the mouse. Southeast Asian J Trop Med Pub Hlth 1983; 14:133-9.
6. Oliver L. A comparison of infections in mice with three species of schistosomes, *Schistosoma mansoni, Schistosoma japonicum* and *Schistosoma douthiti*. Am J Hyg 1952; 55:22-35.
7. Giron-Garcia R. A study of the migratory pathway of *S. japonicum* from the pulmonary circulation to the portal vein of white mice. Masteral thesis for MSPH, Institute of Public Health, University of the Philippines, 1980.
8. Garcia EG, Tiu WU, Mitchell GF. Innate resistance to infection with *Schistosoma japonicum* in a proportion of 129/J mice. J Parasitol 1983; 69:613-5.
9. Garcia EG, Tapales FP, Valdez CA, Mitchell GF, Tiu WU. Attempts to standardize the circumoval precipitin test (COPT) for schistosomiasis japonica. Southeast Asian J Trop Med Pub Hlth 1981; 12:384-95.
10. Smithers SR, Miller KL. Protective immunity in murine schistosomiasis mansoni: evidence for two distinct mechanisms. Am J Trop Med Hyg 1980; 29:832-41.
11. Mitchell GF, Garcia EG, Anders RF, Valdez CA, Tapales FP, Cruise KM. *Schistosoma japonicum:* infection characteristics in mice of various strains and a difference in the response to eggs. Int J Parasitol 1981; 11:267-76.
12. von Lichtenberg F. Host response to eggs of *S. mansoni*. I. Granuloma formation in the unsensitized laboratory mouse. Am J Pathol 1962; 41:711-31.
13. Doenhoff M, Bickle Q, Long E, Bain J, McGregor A. Factors affecting the acquisition of resistance against *Schistosoma mansoni* in the mouse. I. Demonstration of resistance to reinfection using a model system that involves perfusion of mice within three weeks of challenge. J Helminthol 1978; 52:173-86.
14. Colley DG, Freeman GL Jr. Differences in adult *Schistosoma mansoni* worm burden requirements for the establishment of resistance to reinfection in inbred mice. I. CBA/J and C57BL/6 mice. Am J Trop Med Hyg 1981; 29:1279-85.
15. Smithers SR, Terry RJ. Immunity in schistosomiasis. Ann N Y Acad Sci 1969; 160:826-40.
16. Smithers SR, Gammage K. Recovery of *Schistosoma mansoni* from the skin, lungs and hepatic portal system of naive mice and mice previously exposed to *S. mansoni:* evidence for two phases of parasite attrition in immune mice. Parasitology 1980; 80:289-300.
17. Miller KL, Smithers SR, Sher A. The response of mice immune to *Schistosoma mansoni* to a challenge infection which bipasses the skin: evidence for two mechanisms of immunity. Parasite Immunol 1981; 3:25-31.
18. Dean DA, Minard P, Murrel KD, Vannier WE. Resistance of mice to secondary infection with *Schistosoma mansoni*. II. Evidence for a correlation between egg deposition and worm elimination. Am J Trop Med Hyg 1978; 27:957-65.
19. Blum K, Cioli D. *Schistosoma mansoni:* age-dependent susceptibility to immune elimination of schistosomula artificially introduced into preinfected mice. Parasite Immunol 1981; 3:13-24.
20. Dean DA, Minard P, Stirewalt MA, Vannier WE, Murrell KD. Resistance of mice to secondary infection with *Schistosoma mansoni*. I. Comparison of bisexual and unisexual initial infections. Am J Trop Med Hyg 1978; 27:951-6.
21. Bickle Q, Bain J, McGregor A, Doenhoff M. Factors affecting the acquisition of resistance against *Schistosoma mansoni* in the mouse. III. The failure of primary infections with cercariae of one sex to induce resistance to reinfection. Trans R Soc Trop Med Hyg 1979; 73:37-41.
22. Mota-Santos TA, Toledo MI, Correa MC, Correa-Oliviera R, Gazzinelli G. Schistosomiasis from *S. mansoni* in mice: the relationship between acquired immunity and serum levels of lethal antibody. Parasite Immunol 1981; 3:319-27.