Asian Pacific Journal of Allergy and Immunology (1987) 5 : 137-141

Evidence of Anti-Embryonation Immunity and Egg Destruction in Mice Sensitized with Immature Eggs of *Schistosoma japonicum*

Edito G. Garcia, Graham F. Mitchell*, Pilarita T. Rivera, Ruth R. Evardome, Rowena E. Almonte and Wilfred U. Tiu*

SUMMARY **BALB/c mice sensitized by repeated injections of immature eggs of the trematode worm, *Schistosoma japonicum*, were challenged with low numbers of cercariae and evidence was sought for inhibition of embryonation by examination of eggs in livers and intestines at days 40 – 42 of infection. In contrast to the situation in unsensitized control mice, a greater proportion of dead eggs was noted in tissues of many of egg-sensitized mice. There was also a decrease in the proportion of mature eggs relative to control mice. A substantial number of egg – sensitized mice contained no eggs in the liver though eggs were readily detected in their intestinal walls. The data support the concept that immune effector mechanisms act on eggs in a manner that prevents their full development into a miracidium and thus a rich source of immunopathologic antigens.**

In schistosomiasis mansoni and schistosomiasis japonica, one aspect of host resilience to chronic parasitism is the modulation of granuloma formation around eggs trapped in tissues.[1,2] This event, characterized by a reduction in granuloma size in chronically-infected individuals, is also seen in mice hyperimmunized with eggs.[3] During infection, reduced granulomatous hypersensitivity leads to reduced severity of hepatosplenic (and perhaps intestinal) disease. Clearly, when the cellular events of granuloma modulation in man are known, and the antigens involved in the process identified and produced, vaccination against severe diseases may become feasible. At the present time, the relative contributions of suppressor T cells, antibodies and other candidate immune mediators in this apparent "desensitization" remain unknown. In the *Schistosoma japonicum*-mouse model there is evidence that serum antibodies may mediate granuloma modulation though the specificities of these antibodies are unknown.[2,3]

In a recent hypothesis,[4] we proposed that one of the key events in granuloma modulation is inhibition of embryonation of the egg and its destruction at an immature (*i.e.*, pre-miracidial) stage of development. A consequence of this anti-embryonation immunity would be a failure of the egg to mature into a major producer of immunopathologic antigens leading to inhibition of T cell-dependent granuloma formation. This hypothesis therefore de-emphasizes immunoregulation as the central event in granuloma modulation and focuses on the neglected aspect of immune effects on the egg.[5] If immune effector cells and molecules were able to attack unembryonated or embryonating eggs, then their target antigens would become prime candidates for inclusion in a vaccine designed to prevent severe immunopathologic disease in schistosomiasis.

Whilst some support for the hypothesis has been forthcoming[3,6] definitive evidence will probably come only when a reliable *in vitro* system is developed that enables egg maturation to occur. It could then be used to assay for inhibition of embryonation and thus inhibition of immunopathologic antigen production. In the meantime, we have continued to pursue *in vivo* experiments involving measurement of granuloma size and assessment of the numbers of eggs and their state of development in egg-sensitized mice. The basic

From the Department of Parasitology, College of Public Health, University of the Philippines, Manila 2801, Philippines and The Walter and Eliza Hall Institute of Medical Research, Melbourne 3050, Australia*
Correspondence to: Dr. Edito G. Garcia, College of Public Health, 625 Pedro Gil St., Ermita, Manila 2801, Philippines.

system is an adaptation of that devised by von Lichtenberg[7] in which lung granulomas were measured. However, in natural infection it is granuloma formation in livers and intestinal walls that is the cause of most pathology and we have therefore altered the design of anti-embryonation experiments accordingly. We report here the results of studies using immature eggs for sensitization of mice, followed by challenge with low numbers of cercariae and examination of individual eggs or egg clusters in livers and intestines.

MATERIALS AND METHODS

Mice and rabbits

Male and female BALB/c mice were bred in the Manila laboratory from stocks originally supplied by The Walter and Eliza Hall Institute of Medical Research. Rabbits were purchased from a local Manila breeder. Animals were infected with cercariae of *S. japonicum* obtained from field-collected *Oncomelania hupensis quadrasi* snails or snails bred and infected in the Manila laboratory.[8]

Egg sensitization

Donors of viable immature eggs were rabbits killed from day 26 (D + 26) to day 29 (D + 29) of infection while mixtures of immature and mature eggs were obtained from rabbits killed between D + 50 and D + 65 of infection. Eggs were recovered by digestion of livers of infected rabbits as previously described.[9] Mice were injected on the same day eggs were obtained, subsutaneously (SC) and intraperitoneally (IP) with equal number (of eggs) per route of injection. In experiments 1 and 2, five weekly injections of 10,000 eggs were given to the mice. The mice used for experiments 1 and 2 were infected with 4 cercariae 1 and 4 days, respectively, after the second injection of eggs. In experiment 3, the mice received 23 injections of a mixture of mature and immature eggs at 1 to 3 week intervals followed by 3 weekly injections of whole immature eggs. These mice were also infected with 4 cercariae after the last injection.

Assessment of egg numbers and their development

Egg-sensitized mice and unsensitized controls were killed at day 40 to 42 of infection. The entire small intestine and 3 samples of liver were taken for assessment of egg development. A 5-mm portion from the inferior margin or border of each of the 3 lobes of the liver were obtained for assessment of the status of eggs in the liver. The number and state of development of single eggs in liver samples was determined microscopically after pressing the tissue between microscope slides. In the case of eggs in the intestinal wall, many were in clusters. Thus, determinations were made on single eggs or on clusters, the latter being recorded as mature, immature, mixed mature plus immature and dead. Immature eggs were in multicellular stages. Mature eggs had intact miracidia. Dead eggs had a black tinge and contained dark granules. In the analysis of data, all mice with no eggs in the liver and intestine (*i.e.*, uninfected mice) were excluded. So were mice with less than 50 eggs in either intestinal wall or in liver samples. This was done to limit the potential sampling error (in the case of the liver) and distortions brought about by assessments on too few eggs. The number of excluded mice was 9 out of a total of 53 mice in the 3 experiments.

RESULTS

In 2 of 3 experiments, BALB/c mice received 5 injections of viable immature eggs of *S. japonicum* by SC and IP routes over several weeks. On day 8 or day 12 of this injection regime, they were infected with 4 cercariae in anticipation of egg-laying commencing soon after the last of the egg injections. In

Table 1 Summary of data from three experiments on the numbers of eggs and their state of development in the liver and intestines of BALB/c mice immunized with immature eggs of *Schistosoma japonicum* and challenged with cercariae 40 to 42 days before sacrifice.

Parameter	Organ examined	Unsensitized mice	Egg–sensitized mice
No. of mice with > 5% dead eggs present*	Liver	0/15	7/29
	Intestines+	0/15	19/29
No. of mice with a ratio of % immature eggs to % mature eggs of > 5*	Liver	1/15	8/29#
	Intestines+	3/15	18/29
No. of mice with eggs in intestines but *not* in liver sample		0/16	15/37

* Only mice with > 50 eggs in the liver sample or intestines were included in the analysis.
\+ Eggs in intestines of unsensitized mice were invariably counted as clusters, mixed clusters of mature and immature eggs being included in the mature category; in sensitized mice, approximately 50% of determinations were on single eggs rather than clusters (see Fig. 1).
\# If mice with no eggs in liver sample are excluded, this figure is 8/19.

S. japonicum infection, egg-laying starts on days 24 to 27.[12] The eggs require 10 to 12 days to mature[10] and can survive in tissues for over 10 days. In the third experiment, mice were infected after a prolonged immunization regime. All mice were killed 40-42 days after cercarial challenge and liver samples and intestines were examined. Control mice were uninjected but were infected in parallel with the experimental group. Data on number and state of development of eggs in mice with worms for all 3 experiments are presented in Figure 1 and Table 1.

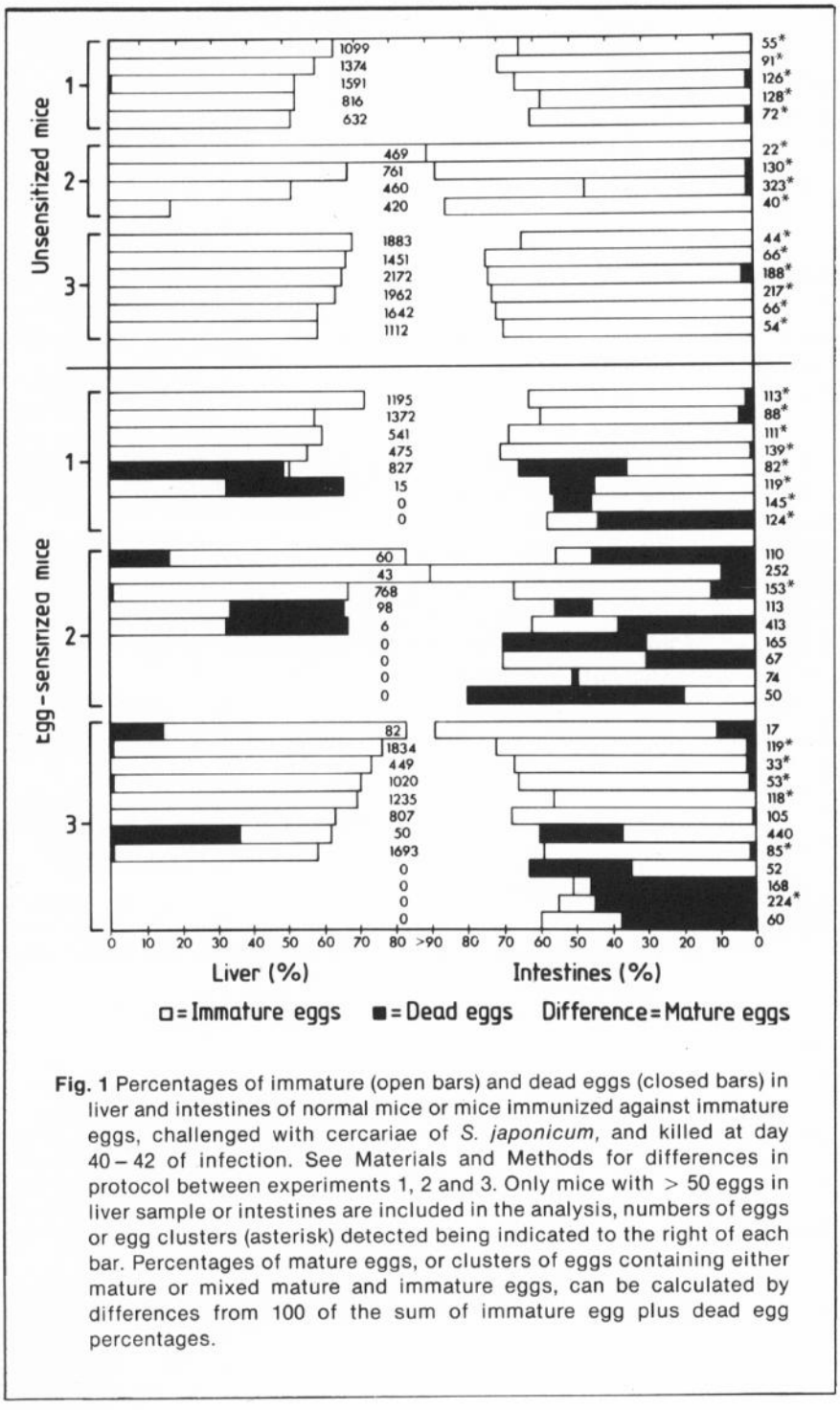

Fig. 1 Percentages of immature (open bars) and dead eggs (closed bars) in liver and intestines of normal mice or mice immunized against immature eggs, challenged with cercariae of *S. japonicum*, and killed at day 40 – 42 of infection. See Materials and Methods for differences in protocol between experiments 1, 2 and 3. Only mice with > 50 eggs in liver sample or intestines are included in the analysis, numbers of eggs or egg clusters (asterisk) detected being indicated to the right of each bar. Percentages of mature eggs, or clusters of eggs containing either mature or mixed mature and immature eggs, can be calculated by differences from 100 of the sum of immature egg plus dead egg percentages.

Several aspects of the data are notable. Of the immunized mice, 10 had no mature eggs in both the liver and intestines although they had dead and immature eggs. There is a very obvious increase in dead eggs, particularly in the intestines, in many (though not all) mice immunized with immature eggs. In contrast, dead eggs are rare in tissues of normal mice infected for 40 to 42 days. Sensitized mice with a high proportion of dead eggs in the intestines generally had high counts of dead eggs, or no eggs detectable, in the liver (Fig. 1). Conversely, mice in which the bulk of intestinal eggs were viable were also likely to have few dead eggs in the liver. Thus, events in the liver and intestine within one mouse appear to be positively correlated. Individual sensitized mice are either efficient or inefficient destroyers of eggs.

When expressed as a ratio (Table 1), the percentage of immature and mature eggs is clearly shifted towards a deficiency of mature eggs in tissues of egg-sensitized mice, an effect that is again more apparent in the intestine than in the liver. Such a decrease in mature eggs relative to immature eggs could result from either inhibition of embryonation or accelerated destruction of mature eggs or both.

Additional indications that egg production and/or fate is affected in egg-sensitized mice comes from several other peripheral observations. The number of mice with eggs in intestines (and therefore obviously infected) but with no eggs detectable in liver samples was approximately 40% in the egg-sensitized group but was zero in the unsensitized group (Table 1). Moreover, egg clusters were counted invariably in the intestines of unsensitized mice while only a few single eggs were found in 50% of similarly infected egg-sensitized mice.

DISCUSSION

Studies reported here demonstrated that a high proportion of mice sensitized repeatedly by injection with immature eggs and challenged with low numbers of cercariae were able to restrict the number of mature eggs present in the liver and intestinal wall. Whilst dead eggs were rare in unsensitized mice, they were common in mice immunized with immature eggs. The fact that 10 of the infected immunized mice had no mature eggs is highly suggestive that the eggs were killed before maturation. However, it is difficult to discount the killing of eggs at completion of maturation when they start producing immunopathologic antigens. If eggs were killed before completion of embryonation, then it would be most probable that secretions and excretions of the immature and maturing eggs were responsible for induction of anti-embryonation immunity. The injection regime used in 2 of the 3 studies reported in Fig. 1 (5 injections over a month or so) using viable immature eggs, could not be expected to result in efficient modulation of *lung* granuloma formation to intravenously injected mature eggs.[1]

One implication of the data reported here is that anti-embryonation immunity could reduce transmission by retarding access of mature eggs into the intestinal lumen. It is known that, in *chronic* schistosomiasis japonica, fecal egg determinations can be most unreliable. Further, it is conceivable that differences in fecal egg count in rabbits infected with comparable numbers of parasites but using different geographical isolates, may be related in part to differences in the efficiency of egg maturation.[13] Immune "expulsion" of eggs from the intestinal wall has been proposed in mouse infections with *S. mansoni*[14] but not *S. japonicum*.[15] Still to be assessed in the present system are any anti-fecundity effects of induced anti-egg immunity that perturb oviposition.[13,16]

The next stage in this project is to determine whether serum antibodies in mice sensitized with immature eggs will transfer anti-embryonation and/or inhibition of maturation and/or egg destruction in minimally-infected mice. Moreover, it will be important to link any immunological effect on eggs with granuloma modulation, *i.e.*, small granuloma size. This has not yet been demonstrated formally although we have reported previously that sera from chronically-infected humans will reduce lung granuloma size in egg-sensitized mice injected intravenously with eggs[6] and that egg-sensitized mice inhibit maturation of eggs harvested from the uterus of female *S. japonicum* worms and trapped in the lung after intravenous injection. Clearly, further progress in this project, and the pursuit of an anti-disease vaccine in schistosomiasis, is dependent upon identification of antigens of immature eggs that are the target of an aggressive immune attack. Batteries of monoclonal antibodies to eggs will be useful in this regard[17,18] as well as cDNA libraries, if the target antigens are proteins.

ACKNOWLEDGEMENTS

This work was supported by the U.S. Agency for International Development, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the Australian Development Assistance Bureau.

REFERENCES

1. Warren KS. The secret of the immunopathogenesis of schistosomiasis *in vivo* models. Immunol Rev 1982; 6:189-213.
2. Olds GR, Olveda R, Tracy JW, Mahmoud AAF. Adoptive transfer of modulation of granuloma formation and hepatosplenic disease in murine schistosomiasis japonica by serum from chronically infected animals. J Immunol 1982; 128:1391-3.
3. Garcia EG, Mitchell FG, Tapales FP, Tiu WU. Reduced embryonation of *Schistosoma japonicum* eggs as a contributory mechanism in modulation of granuloma in chronically-sensitized mice. Southeast Asian J Trop Med Pub Hlth 1983; 14:272-3.
4. Garcia EG, Mitchell GF. Vaccination against severe hepatosplenic disease in schistosomiasis japonica: an hypothesis. Acta Med Philippines 1982; 18:107-12.
5. James SL, Colley DG. Eosinophil-mediated destruction of *Schistosoma mansoni* eggs. J Reticuloendothelial Soc 1976; 20:359-74.
6. Garcia EG, Mitchell GF, Beall JA, Tiu WU. *Schistosoma japonicum:* the modulation of lung granuloma and inhibition of maturation of eggs in mice using human sera. Asian Pacific J Allergy Immunol 1985; 3:156-60.
7. Von Lichtenberg F. Host response to eggs of *S. mansoni*. I. Granuloma formation in the unsensitized laboratory mouse. Am J Pathol 1962; 11:267-76.
8. Mitchell GF, Garcia EG, Anders RF, Valdez CA, Tapales FP, Cruise KM. *Schistosoma japonicum:* Infection characteristics of mice of various strains and a difference in the response to eggs. Int J Parasitol 1981; 11:267-76.
9. Garcia EG, Tapales FP, Valdez CA, Mitchell FG, Tiu WU. Attempts to standardize the circumoval precipitin test (COPT) for schistosomiasis japonica. Southeast Asian J Trop Med Pub Hlth 1981; 12:384-94.
10. Vogel J. Uber entwicklung. Lebensdauer und Tod der Eier von *Bilharzia japonica* im Wirtsgewebe. Dtsche Trop Z 1942; 46: 57-69.
11. Kawanaka M, Hayashi S, Ohtomo H. Nutritional requirements of *Schistosoma japonicum* eggs. J Parasitol 1983; 69:857-61.
12. Pesigan TP, Farooq M, Hairston NG, *et al.* Studies on *Schistosoma japonicum* infections in the Philippines. I. General considerations and epidemiology. Bull WHO 1958; 18:345-455.
13. Cheever AW, Duvall RH, Minker RG. Quantitative parasitologic findings in rabbits infected with Japanese and Philippine strains of *Schistosoma japonicum*. Am J Trop Med Hyg 1980; 29:1307-15.
14. Doenhoff MJ, Musallam R, Bain J, McGregor A. *Schistosoma mansoni* infections in T-cell deprived mice, and the ameliorating effect of administering homologous chronic infection serum. I.

Pathogenesis. Am J Trop Med Hyg 1979; 28:260-73.

15. Moloney NA, Doenhoff MJ, Webbe G, Hinchcliffe P. Studies on the host-parasite relationship of *Schistosoma japonicum* in normal and immunosuppressed mice. Parasite Immunol 1982; 4:431-40.
16. Damian RT. Immunity in schistosomiasis. A holistic view. Cont Topics Immunobiol 1984; 12:359-420.
17. Cruise KM, Mitchell GF, Tapales FP, Garcia EG, Huang S-R. Murine hybridoma-derived antibodies producing circumoval precipitin (COP) reactions with eggs of *Schistosoma japonicum*. Aust J Exp Biol Med Sci 1981; 59:503-14.
18. Tiu WU, Garcia EG, Davern KM, Beall JM, Mitchell GF. Analysis of the specificities of circumoval precipitin (COP) positive monoclonal antibodies to *Schistosoma japonicum* eggs. (manuscript in preparation)