Schistosoma japonicum : The Modulation of Lung Granuloma and Inhibition of Egg Maturation in Mice by Human Sera*

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SUMMARY Human sera taken from patients with chronic schistosomiasis japonica have been demonstrated to have two effects on mice. Sera from those patients reduced the size of granuloma in mice sensitised for accelerated granuloma formation to eggs entrapped in the lungs of mice injected with the sera shortly before and at day 2 after intravenous egg challenge. The sera with this effect on the mouse lung granuloma models caused large segmented precipitates in the optimised circumoval precipitin test (COPT). Such sera also reduced the rate at which eggs matured in the liver and intestines of mice infected with *S. japonicum*. The results strongly support our postulate that a major cause of granuloma modulation in cases of chronic schistosomiasis japonica is antiembryonation immunity and that mice provide useful models for the analysis of our postulate. Identification of egg antigens responsible for the anti-embryonation effect should facilitate progress towards the development of a vaccine against granulomatous disease.

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Many manifestations of disease in cases of schistosomiasis mansoni and japonica result from granulomatous and fibrotic responses to antigens released from eggs entrapped in the liver, intestines, lungs and other organs. These immunopathological responses have been studied in some detail in mouse models. In *Schistosoma japonicum* infection, as in *S. mansoni* infection, it is generally accepted that T cells of the delayed hypersensitivity type (i.e. T_D cells) are the principal initiators of these reactions.[1] Whilst it has been demonstrated clearly that the mature egg containing a miracidium is the richest source of "immunopathologic antigens",[2-4] the range and nature of such antigens and their epitopes still remain virtually unknown.[5]

In cases of chronic infection and in mice hyperimmunised or chronically sensitised with eggs, granuloma formation is reduced. This phenomenon of granuloma modulation, also termed "endogenous desensitisation",[6] opens up the prospects for vaccination against disease. The principal mechanisms are believed to involve suppressor T cells (T_S cells) perhaps with anti-idiotype reactivity, antibody-dependent accelerated destruction of eggs, and reduced embryonation of eggs (discussed in references 7 and 8). At the laboratories using *S. japonicum* (Philippines) in mice, attention has been focused on the destructive or inhibitory effects of immune response on the egg, particularly in view of the evidence for antibody-mediated effects in *S. japonicum* granuloma modulation.[9] If particular antibody specificities and types inhibit egg embryonation after oviposition by the female worm[10] or mediate accelerated destruction of the mature[11] or immature egg, then the potential for vaccination is improved greatly.[7] This is especially so if the antigens or epitopes responsible for these processes are quite different from those that stimulate T_D cells involved in granulomatous hypersensitivity.

In order to facilitate the analysis of *S. japonicum* granuloma formation and its modulation, we have

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developed a radioisotopic variant[12] of the mouse lung granuloma model[13] and numerous anti-egg monoclonal antibodies[14] (Tiu and Mitchell, unpublished observations). The monoclonals produce prominent circumoval precipitates in the circumoval precipitin test (COPT) and most have been found to be directed towards carbohydrate determinants. At least two of them may be used to absorb antigen(s) that sensitise mice for accelerated granuloma formation as determined by radioisotopic assay[8] (Tiu, unpublished observation). However, the epitopes recognised by these monoclonals seem not to be major immunogenic determinants in man; infected individuals with or without hepatosplenic disease are not high responders to them, at least in terms of antibody production detected in sensitive competitive radioimmunoassays.[15] We have, therefore, turned to a more systematic analysis of anti-egg antibody specificities in sera from clinically and parasitologically defined patient groups. To assist in this, we have attempted to develop an assay for the modulating effects of human sera on lung granuloma formation in egg-sensitised mice. Using a histological read-out for granuloma formation, in the first instance, we describe here a modulating effect of some but not all schistosomiasis japonica sera on the mouse lung granuloma model. In a previous paper,[10] we demonstrated reduced embryonation of freshly recovered uterine eggs of *S. japonicum* injected intravenously into chronically egg-sensitised mice which exhibited modulated granuloma response. Also reported in this paper are the effects of human sera on the maturation of eggs in tissues of infected mice.

MATERIALS AND METHODS

Mice and granulomas.

Young, inbred BALB/c mice, maintained under conventional conditions in the Manila laboratory, were used. They were infected using the coverslip method of application of cercariae to the shaved abdomen of restrained mice, the cercariae having been obtained from field-collected or laboratory infected *Oncomelania hupensis quadrasi* snails.[16] Eggs for sensitisation and challenge were obtained from the livers of infected rabbits or BALB/c mice and purified using methods described previously[17] except that fresh rather than lyophilised eggs were generally used. Donors were killed 50-60 days after infection and the eggs were obtained by digestion of macerated livers. Recipient mice were injected intraperitoneally five times with 3,000-5,000 eggs each time from infected rabbits at 10- to 27-day intervals. A subcutaneous injection of 3,000 eggs was also given at the same time as the first intraperitoneal injection of eggs. One week after the last sensitising injection, the mice were challenged intravenously with 2,000 freshly digested eggs from infected mice to initiate granulomas in the lungs. On the same day and also two days later, they were injected intraperitoneally with a total of 2.0 ml of human sera. Each mouse was injected with serum from one donor, that was either COPT-positive or -negative (Fig. 1). The mice were killed after five days, the lungs inflated and fixed with 10 per cent formalin. Sections were then cut from the tissue embedded in paraffin. Granulomas or eggs (with no surrounding reaction) were measured using a graduated ocular lens and the volume calculated from the formula $V = 4/3\ \pi\ a^2 b$ (where a is the shorter radius, and b the longer). Serial sections of one granuloma were examined to identify the section with maximum dimensions. "Granulomas" were classified as having no reaction (0), minimal reaction (±) (i.e. size 1 to 3 x 10^{-4} mm^3 and usually involving a single layer of inflammatory cells), and positive reaction (+) (i.e. size greater than 3 x 10^{-4} mm^3). The only error in such a classification is likely to be the overestimation and underestimation of granulomas around unusually large or small eggs. For some of the mice (two mice per group), granuloma development was assessed according to the number of cell layers around eggs (i.e. one or greater than or equal to 3 layers as minimal and positive reaction categories, respectively) to take into account any differences in the size of the *S. japo-*

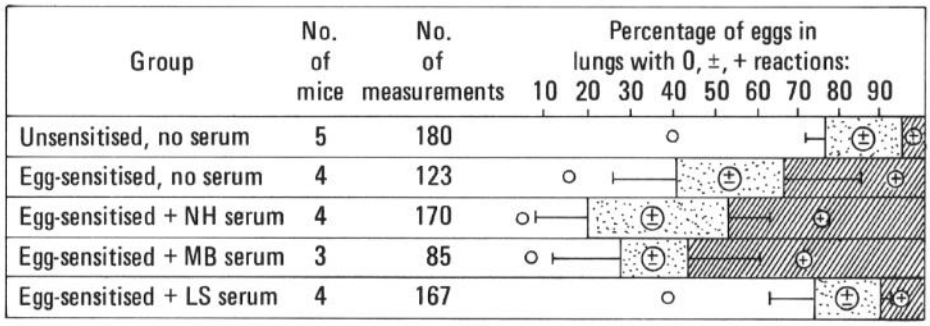

Fig. 1 Certain human sera have a modulating effect on granuloma formation in the lungs of egg-sensitised mice. Reactions around eggs in the lungs of mice are expressed as the percentage of those examined (from 85 to 180 measurements in groups of three to five mice, each mouse being injected with serum from one individual; combined data from two independent experiments show that there were no reaction around the egg (0) (open sections), a minimal granulomatous reaction of 1 to 3x10^{-4} mm^3 volume (±) (dotted sections) and a positive reaction (+) (cross-hatched sections) (size of greater than 3 x 10^{-4} mm^3). NH = negative human sera as determined by COPT; MB = medium sized bleb reactors in COPT; LS = large segmented precipitate in COPT. Arithmetic mean values and standard errors of the mean are indicated.

nicum eggs. Data obtained using this alternative assessment method did not alter the ranking of the groups shown in Figure 1.

In the experiment in which live and lyophilised egg challenges were compared, mice were initially injected with 3,000 eggs intraperitoneally and 2,000 eggs subcutaneously. They received two more weekly intraperitoneal injections of 5,000 eggs. They were challenged 13 days after the last sensitisation with 1,500 live or lyophilised eggs with 1.5 ml of either large segment COPT positive (see below) or normal human sera injected shortly before challenge and 0.5 ml at day 2 (after egg challenge). Mice were killed for assessment of lung granulomas at day 5.

To determine the effects of the sera on egg maturation in infected mice, BALB/c mice were infected with 10 cercariae percutaneously; serum injections commenced on day 24, the approximate time of commencement of egg deposition by *S. japonicum* female worms. Injection volumes and days of injection were as follows: day 24, 1.5 ml; day 27, 0.75 ml; day 29, 0.5 ml; day 31, 0.75 ml; day 34, 0.75 ml; day 36, 0.5 ml; and day 38, 0.75 ml. The human sera used were either COPT-positive, giving segmented precipitates, or they were COPT-negative. Mice were killed at either day 38 or day 41. They were perfused to flush out worms,[12,16] then the intestines and liver were removed. The intestines were cut along their length, cut further into segments and each segment pressed between two slides. Egg clusters were counted and the state of development (of eggs in each cluster) noted microscopically. Similarly, three sections taken from corresponding regions of the liver of each mouse were examined by the same method.

Human sera and the circumoval precipitin test (COPT).

Sera were collected from individuals attending the Institute of Public Health for schistosomiasis serology. The performance of an optimised COPT has been described in detail,[17] reactions being classified into small blebs, medium-sized blebs (MB) and large segmented precipitates (LS). MB and LS sera, together with sera from individuals negative in the sensitive COPT, were not pooled but rather injected into individual mice. Thus the number of mice used in the experiments reflects the number of individual human sera assessed, large numbers of mice per group being impractical because of the amounts of each human serum available. Sera were also obtained from four rabbits exposed for up to 215 days previously to approximately 300 cercariae of *S. japonicum* (Philippines).

RESULTS

Effect of human sera on granuloma formation in egg-sensitised mice.

Figure 1 shows that the formation of granuloma in egg-sensitised mouse recipients of LS sera was reduced in comparison to that seen in sensitised mice injected with normal (NH) or MB serum. Sera from apparently uninfected donors and MB sera were ineffective for reducing granuloma formation in egg-sensitised mice. We believe the latter to be a particularly good control for the modulating effect of COPT-positive LS sera. The geometric mean size of granulomas greater than 1×10^{-4} mm^3 in recipients of COPT-positive LS sera was $2.38\ (1.35) \times 10^{-4}$ mm^3 with fewer than 10 per cent being greater than 5×10^{-4} mm^3; in recipients of MB sera, it was $5.90\ (1.27) \times 10^{-4}$ mm^3 with more than 60 per cent being of a size greater than 5×10^{-4} mm^3. These measurements were performed on three mice per group in the one experiment.

Using a similar protocol but with sera from four infected rabbits (two being infected for more than 200 days and with the sera showing LS COPT reactions), no inhibition of granuloma formation could be demonstrated in egg-sensitised mice. In this experiment, the proportion of negative reactions around eggs in egg-sensitised mice was approximately 50 per cent (compared with greater than 80 per cent in unsensitised mice). This figure of 50 per cent was unchanged by injection of sera from infected rabbits.

In the experiment in which groups of 3-5 mice sensitised with live eggs were challenged with live or lyophilised eggs and injected with LS human or normal human sera, significant granuloma modulation was not observed in the mice challenged with lyophilised eggs. The percentages of eggs with granuloma reaction in mice injected with LS sera + live eggs, LS sera + lyophilised eggs, NH sera + live eggs and NS sera + lyophilised eggs were 14 ± 2, 54 ± 15, 29 ± 4 and 64 ± 8, respectively. It should be stressed that the same sera injected in sensitised mice challenged with live eggs were also injected in mice challenged with lyophilised eggs.

Effect of human sera on maturation of eggs in infected mice.

Data in Tables 1 and 2 establish that human COPT-positive sera, these being LS reactors, alter the ratio of immature to mature eggs in the intestines and livers of infected mice. BALB/c mice infected with low numbers (i.e. two to eight) of adult worms were injected with a total of 5.5 ml of human sera in seven dose between days 24 and 38 after infection. Five LS COPT-positive and five COPT-negative sera were injected into two groups of five mice that were killed 39 (2 x 3 mice) or 41 days (2 x 2 mice) after infection. In the livers (Table 1), a significant ($P < 0.05$) increase in the ratio of immature to mature eggs was recorded in the recipients of LS sera. Since the total number of eggs present in the liver sections was comparable in the two groups of mice, the data suggest that the

Table 1 Maturity of eggs in livers of BALB/c mice injected with *Schistosoma japonicum** and injected with human sera between days 24 and 38 after infection

Mouse No.	No. of immature eggs	No. of mature eggs	Ratio of immature to mature
	Mice injected with COPT–positive sera		
1	536	216	2.5
2	718	543	1.3
3	1,209	1,046	1.2
4	603	148	4.1
5	179	90	2.0
			2.2 ± 0.5**
	Mice injected with COPT – negative sera		
6	969	1,238	0.8
7	969	968	1.0
8	816	986	0.8
9	90	395	0.2
10	268	218	1.2
			0.8 ± 0.2**

*Numbers of adult worms in mice Nos. 1 to 5 were 4,4,4,4 and 2 respectively (mean ± SEM = 3.6 ± 0.4) and in mice Nos. 6 to 10 were 8,6,4,3 and 4 respectively (mean ± SEM = 5.0 ± 0.9).
**$P < 0.05$, Mann-Whitney U-test.

Table 2 Maturity of egg clusters in intestines of BALB/c mice infected with *Schistosoma japonicum* and injected with human sera between days 24 and 38 of infection (same mice as in Table 1).

Mouse No.	No. of clusters of immature eggs	No of clusters of mixed eggs	No. of clusters of mature eggs	Ratio of immature to other egg clusters
	Mice injected with COPT–positive sera			
1	276	47	38	3.2
2	212	54	36	2.4
3	292	73	48	2.4
4	433	28	15	10.1
5	54	4	1	10.8
				5.8 ± 1.9*
	Mice injected with COPT – negative sera			
6	92	22	270	0.3
7	109	63	179	0.5
8	270	35	382	0.7
9	71	8	210	0.3
10	120	11	279	0.4
				0.4 ± 0.1*

*$P < 0.01$, Mann-Whitney U-test.

serum delayed the maturation of the eggs rather than mediating the destruction of mature eggs. More striking data were obtained from the sections of intestines in which egg clusters were examined (Table 2). These clusters were classified according to whether the eggs they contained were mature, immature, or consisted of mixed mature and immature eggs. As evidenced by the ratios of immature egg clusters to others (mature plus mixed), a highly significant difference ($P < 0.01$) was recorded in recipients of LS COPT-positive sera compared with normal sera. In fact, 69 to 91 per cent of the egg clusters contained only immature eggs in recipients of infected LS sera while this percentage was 24 to 39 per cent in recipients of normal sera. Again, total tissue egg numbers were comparable, and since infection levels were also not significantly different, the data strongly suggest that sera from humans with chronic schistosomiasis japonica inhibit embryonation of eggs.

DISCUSSION

An assay has been developed that is capable of detecting the modulating effect of human sera on *S. japonicum* eggs in the mouse lung model of granuloma formation. Egg-sensitised mice were injected with sera on the day of, and for some days after, intravenous egg challenge. Because the assay is time-consuming and difficult to quantitate, it is not suitable for the mass screening of human sera, determination of minimal effective amounts, antibody isotype analyses, etc. However, the radioisotopic assay for lung granulomatous hypersensitivity to *S. japonicum* eggs should be suitable for these purposes.[12]

The present data indicate that granuloma modulation can be demonstrated using certain human sera in mice, and that the same human sera can inhibit or reduce efficiency of embryonation. The sera which showed a modulating effect and inhibition of embryonation were those that produced large segmented precipitates in the optimised COPT. In this test, *S. japonicum* eggs were incubated for 1-2 days with neat human sera and precipitates examined microscopically. Segmented precipitates reflect multiple antibody-antigen interactions. In contrast, bleb reactions are

observed with monoclonal antibodies[14] and are seen in patients likely to be recently infected.[18]

That mouse sera can induce modulating effects on the *S. japonicum* egg granuloma/mouse system has been clearly demonstrated by Olds *et al.*[9] In our laboratories, we previously failed to show any modulating effect of human and hyperimmune mouse antisera, as well as mouse monoclonal antibodies, using lyophilised eggs for sensitisation and challenge.[4,8] A repeat experiment reported here confirms our past observation. The effects of hyperimmune sera, reported by Olds *et al*,[9] involved a more physiological reagent: viable eggs. The use of lyophilised eggs would negate opportunities for detecting accelerated destruction of reduced embryonation of eggs. Conceivably, direct effects on the viable egg underlie the modulating effect of certain human sera in mice rather than effects mediated through released antigen or T_D cells responding to such antigen.

Evidence concerning the effect of human sera on *S. japonicum* eggs comes from the second line of experimentation reported here, i.e. inhibition of egg maturation in infected mice. The data presented in Tables 1 and 2 provide very definite encouragement to pursue the objective of vaccination against severe schistosomiasis japonica using antigens from immature eggs and induction of anti-embryonation-immune responses.[7]

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REFERENCES

1. Warren KS. The secret of the immunopathogenesis of schistosomiasis: *in vivo* models. Immunol Rev 1982; 61:189-213.
2. Hang LM, Warren KS, Boros DL. *Schistosoma mansoni:* antigenic secretions and the etiology of egg granulomas in mice. Exp Parasitol 1974; 35:288-98.
3. Hamburger J, Pelley RP, Warren KS. *Schistosoma mansoni* soluble egg antigens: II. Determination of the stage and species specificity of their serological reactivity by radioimmunoassay. J Immunol 1976; 117:1561-6.
4. Mitchell GF, Garcia EG, Cruise KM, Tiu WU, Hocking RE. Lung granulomatous hypersensitivity to eggs of *Schistosoma japonicum* in mice analyzed by a radioisotopic assay and effects of hybridoma (idiotype) sensitization. Aust J Exp Biol Med Sci 1982; 60:401-16.
5. Mitchell GF, Cruise KM. Schistosoma antigens: immunochemical identification of antigens of *Schistosoma mansoni* and *Schistosoma japonicum.* In: Pearson TW, ed. Parasite Antigens and their Interactions with the Immune System. New York: Marcell Dekker, 1985.
6. Domingo EO, Warren KS. Endogenous desensitization: changing host granulomatous response to schistosoma eggs at different stages of infection with *Schistosoma mansoni.* Am J Pathol 1968; 52:369-77.
7. Garcia EG, Mitchell GF. Vaccination against severe hepatosplenic disease in schistosomiasis japonica: an hypothesis. Acta Med Philipp 1982; 18:107-12.
8. Mitchell GF, Cruise KM, Garcia EG, Valdas MA, Munoz JJ. Attempts to modify lung granulomatous responses to eggs of *Schistosoma japonicum* eggs in low and high responder mouse strains. Aust J Exp Biol Med Sci 1983; 61:411-24.
9. Olds GR, Olveda R, Tracy JW, Mahmoud AAF. Adoptive transfer of modulation of granuloma formation and hepatosplenic disease in murine schistosomiasis japo ε' by serum from chronically infected animals. J Immunol 1982; 128:1391-3.
10. Garcia EG, Mitchell GF, 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 Publ Hlth 1983; 14: 272-3.
11. James SL, Colley DG. Eosinophil-mediated destruction of *Schistosoma mansoni* eggs. J Reticuloendothelial Soc 1976; 20: 359-74.
12. 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.
13. 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.
14. Cruise KM, Mitchell GF, Tapales FP, Garcia EG, Huang SR. Murine hybridoma-derived antibodies producing circumoval precipitation (COP) reactions with eggs of *Schistosoma japonicum.* Aust J Exp Biol Med Sci 1981; 59:503-14.
15. Mitchell GF, Garcia EG, Cruise KM. Competitive radioimmunoassays using hybridoma and anti-idiotype antibodies in identification of antibody responses to, and antigens of, *Schistosoma japonicum.* Aust J Exp Biol Med Sci 1983; 61:27-36.
16. Garcia EG, Mitchell GF, Espinas FJM, Tapales FP, Quicho LP, Tiu WU. Further studies on resistance to reinfection with *Schistosoma japonicum* in mice. Asian Pacific J Allerg Immun 1984; 2:27-31.
17. 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 Publ Hlth 1981; 12: 384-94.
18. Lewert RM, Yogore MG Jr, Martin WR, Blas BL. Schistosomiasis japonica in Barrio San Antonio, Basey, Samar in the Philippines. IV. "Atypical" precipitates in the circumoval precipitin test – a reaction of recent infection. Am J Trop Med Hyg 1980; 29:431-4.