# SCHISTOSOMA JAPONICUM: EFFECTS OF INDUCTION OF ANTI-EMBRYONATION IMMUNITY ON LIVER **GRANULOMAS, SPLEEN WEIGHT AND** PORTAL PRESSURE IN INFECTED MICE

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#### Summary

BALB/c mice sensitized with injections of viable immature S. japonicum eggs had significantly fewer and smaller granulomas in the liver, lower portal pressure and smaller spleens at D+75 of infection compared to similarly infected unsensitized controls. The portal pressure and spleen weights of the mice sensitized with immature eggs were not different from *uninfected* unsensitized mice of similar ages at D+75 of infection. The results strongly support our hypothesis that it should be possible to prevent serious hepatosplenic disease in schistosomiasis japonica by vaccination to induce anti-embryonation immunity.

# Introduction

Manifestations of disease in cases of schistosomiasis japonica and mansoni result from granulomatous and fibrotic responses to antigens released from eggs entrapped in the liver, intestine, lungs and other organs (Warren, 1982). In S. japonicum infection, as in S. mansoni infection, it is generally accepted that CD4<sup>+</sup> T cells of the delayed hypersensitivity type are principal initiators of these reactions. While it has been demonstrated that the mature egg containing a miracidium is the source of immunopathologic antigens (Hang et al., 1974; Hamburger et al., 1976; Mitchell et al., 1982), the nature of such antigens and their epitopes still remain virtually unknown particularly for S. japonicum (Mitchell and Cruise, 1986).

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After initial sensitization with crude egg antigens or isolated fractions, there is accelerated formation of large destructive granulomas. However, in chronic infections and in mice hyperimmunized with eggs, granuloma formation is reduced or modulated (Warren, 1982; Domingo and Warren, 1968; Olds *et al.*, 1982).

The phenomenon of granuloma modulation, also termed "endogenous desensitization" (Domingo and Warren, 1968), opened up the prospects of vaccination against disease particularly the more frequent serious sequelae seen in the hepatosplenic syndrome. The large granulomas formed after the initial sensitization, not only block blood flow but also destory large surrounding areas which are later replaced by fibrotic tissue. Thus, immune intervention to induce modulated responses should cause disease abatement including reduced severity of hepatosplenic disease.

In a recent hypothesis (Garcia and Mitchell, 1982, 1985; Garcia et al., 1983), we proposed that one of the key events in granuloma modulation is inhibition of egg maturation (i.e. anti-embryonation immunity) and its destruction at an early (i.e. pre-miracidial) stage of development. If our hypothesis is true, then we have a basis for developing a vaccine against granulomatous disease in schistosomiasis japonica. A consequence of this anti-embryonation immunity will be failure of the eggs to mature into producers of antigens which stimulate T cell-dependent granuloma formation and fibrosis. Initial evidence for our hypothesis was the demonstration of reduced embryonation of viable immature S. japonicum eggs injected intravenously and filtered in the pulmonary microvasculature of chronically egg-sensitized mice (Garcia et al., 1983). That anti-embryonation immunity may also be induced in human infections was indicated by the inhibition of maturation of eggs in BALB/c mice infected with few cercariae following injection of sera from humans chronic infection (Garcia et al., 1985). Further, we have recently demonstrated a high proportion of dead eggs in livers and intestines of mice injected with viable immature S. japonicum eggs which were subsequently infected with small numbers of cercariae (Garcia et al., 1987). These series of observations support the concept that immune effector mechanisms act on eggs in a manner that prevents their full embryonation into miracidia and thus prevent the development of the egg into a rich source of immunopathologic antigens for granuloma formation.

This paper presents observations on liver granulomas, portal venous pressure and relative spleen weight at day 75 of inection in mice sensitized with injections of viable immature eggs. The intent is to test whether severe hepatosplenic disease will not occur after infection in mice sensitized for anti-embryonation immunity prior to egg deposition by the adult schistosome.

# **Materials and Methods**

# Mice, rabbits and snails

Male and female BALB/c mice were bred in the Manila laboratory from stocks orginally supplied by the Walter and Eliza Hall Institute of Medical Research

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in Australia. 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 those bred and infected in the laboratory (Mitchell *et al.*, 1981).

## Egg sensitization

Donors of viable immature eggs were rabbits killed from day 26 (D+26) to day 28 (D+28) of infection since S. *japonicum* worms start laying eggs from D+24 to D+27 of infection (Pesigan *et al.*, 1958). The immature eggs require 10-12 days to embryonate or mature. Eggs were recovered by digestion of livers of infected rabbits as previously described (Garcia *et al.*, 1981). They were injected (on the same day they were obtained) subcutaneously (SC) and intraperitoneally (IP) with equal numbers of eggs into each site.

## Assessment of portal venous pressure and spleen weight

The infected egg-sensitized mice and naive controls were sacrified at D+75 of infection to determine their portal pressure and relative spleen weight. The mice were injected with heparin and anesthesized with Nembutal. The abdomen was opened, the portal vein isolated and the pressure, expressed in millimeters of water, determined by inserting a winged infusion set to which a three-way valve, serological pipette, and millimeter scale had been attached. The solution used was heparinized physiologic salt solution. After determination of the portal pressure the mice were perfused, and the spleen isolated and weighed. Spleen weight was expressed relative to the total live body weight of the particular mouse.

#### Assessment of egg numbers and development

After determination of the portal pressure, the entire intestines were isolated and divided into segments pressed between two microscope slides. The number and the state of development of eggs (either singly or in clusters) were determined according to previously described criteria (Garcia *et al.*, 1983, 1985).

## Measurement of granulomas

Serial sections of livers were cut from tissue embedded in paraffin. Each egg or granuloma was followed serially to identify the section with maximum dimensions (of the egg or ganuloma). "Granulomas" were classified as having no reaction, minimal and obvious reaction or according to their volume (Garcia *et al.*, 1985). All data are expressed as arithmetic mean ( $\pm$  SEM) and groups compared using a Mann-Whitney U test.

## Results

In this study, BALB/c mice received five weekly injections of 10,000 viable immature S. *japonicum* eggs by SC and IP routes. On the 10th day of the sensiti-

zation regimen, the mice were infected with four cercariae in anticipation of egglaying commencing soon after the last of the egg injections. The mice were sacrified on D+75 of infection for determination of liver granuloma number and size, portal vein pressure, spleen weight as well as evidence of induction of anti-embryonation immunity. Controls included an equal number of unsensitized infected mice as well as unsensitized uninfected mice of corresponding ages for determining normal values for portal pressure and spleen weight. The observations are presented in Figures 1, 2 and 3.

As seen in Figure 1, anti-embryonation immunity was induced in the eggsensitized group. This is evidenced by the significantly higher proportion of dead eggs in the intestines of the egg-sensitized mice compared with the unsensitized infected mice where there was a higher proportion of mature eggs. Enumeration and determination of the state of development of eggs in the liver was not possible, since this organ was sectioned for determining granuloma formation.

Other aspects of the data are notable. These are: (1) the egg-sensitized mice had a much lower number of granulomas in the liver (p < 0.001) since 61% of the eggs had no reaction in contrast to the unsensitized controls where only 8% of the eggs had no reaction (Figure 2); (2) the mean portal venous pressure of the unsensitized infected mice was significantly higher (p < 0.005) than that of the sensitized infected mice, the latter being comparable to normal uninfected mice (Figure 3); and (3) the mean spleen weight in the unsensitized infected mice is much greater (p < 0.001) than that of the sensitized infected and uninfected groups whose spleen weights were comparable (Figure 3).

#### Discussion

The study herein reported has demonstrated the induction of anti-embryonation immunity through sensitization of mice with viable immature eggs, resulting in reduced number and size of granuloma in the liver of (of these mice) after infection with four cercariae thus preventing haemodynamic changes leading to portal hypertension and splenomegaly. The latter are the most prominent manifestations of severe hepatosplenic disease in schistosomiasis. The number of schistosomes arising from four cercariae, extrapolated to a human being of 60 kilograms would mean a worm burden of 3,000 adult flukes. This is very much higher than calculated worm burdens in human infections. This may indicate that for the usual worm burden in infected humans it should be possible to prevent hepatosplenic disease by vaccination to induce anti-embryonation immunity prior to the start of egg deposition by resident *S. japonicum* worms. Granuloma modulation should also cause abatement of pathology in the intestines (Domingo and Warren, 1969).

It is important to emphasize that a component of anti-embryonation immunity may be reduced production of eggs by the female worm (i.e. antifecundity immunity) through chronic effects of induced immune responses on worm pairs.



Figure 1. Percentage of immature (open sections), mature (dotted sections) and dead eggs (crosshatched sections) in intestines of egg-sensitized and unsensitized mice at D+75 of infection. Only mice with > 50 eggs or egg clusters in the intestines are included in the analysis, most clusters being confined to the unsensitized group.



Figure 2. Percentage of eggs in liver with no (open section, with SEM), minimal (dotted section) and obvious granulomatous reactions (cross-hatched section). A minimal reaction is a granuloma of 1-2 layers of cells while an obvious reaction consists of 3 or more layers of cells around the egg. Only mice with > 50 eggs or egg clusters in the intestines are included in the analysis.



Figure 3. Portal venous pressure (cross-hatched bar) and relative spleen weight (open bar) of infected egg-sensitized, unsensitized infected mice and uninfected controls at D+75 of infection. SEM are indicated.

The late readout we have used (75 days of infection) compared with earlier studies (Garcia *et al.*, 1987) may uncover such effects. Thus, the term we have coined - namely anti-embryonation - may embrace reduced egg production and even accelerated destruction of eggs up to the miracidial stage in addition to postulated inhibitory effects on the newly laid egg.

Inhibition of establishment or persistence of infection is clearly a more desirable consequence of vaccination than inhibition of disease (but consider tetanus and diphtheria immunoprophylaxis). However, if the former is difficult to achieve through conventional vaccination, then immunization to prevent severe pathology has a place in disease control. This is especially so if the vaccination strategy also leads to reduced transmission. Anti-embryonation immunity directed towards immature eggs in the intestinal wall should lead to reduce export of eggs to the environment.

An alternative strategy of altering disease in schistosomiasis by promoting suppressor T cell or anti-idiotype-mediated immunoregulation (Stavitsky, 1987) may not only be difficult to achieve but could be associated with some danger. Thus, defective anti-egg antibody responses or defective granulomatous encapsulation of eggs and sequestering of egg products may well lead to hepatotoxic effects or diffuse collagen deposition in heavy infection as shown in schistosomiasis mansoni (Dunne and Doenhoff, 1983).

The next step in our pursuit of an anti-disease vaccine in schistosomiasis japonica is the identification of immature egg antigens that are targets of any aggressive immune effector mechanism responsible for anti-embryonation. Batteries of monoclonal antibodies to immature egg antigens will be useful in this regard as well as cDNA libraries, if the target antigens are proteins (W.U. Tiu *et al.*, in preparation). Moreover, establishment of an *in-vitro* system of egg embryonation (Kawanaka *et al.*, 1983) would greatly facilitate the analysis of antibody ( $\pm$  cell) effects on this process.

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