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"DEVELOPMENT OF A VACCINE TO PREVENT HEPATO-SPLENIC DISEASE IN SCHISTOSOMA JAPONICUM INFECTIONS"

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FINAL REPORT

"DEVELOPMENT OF A VACCINE TO PREVENT HEPATOPLENIC DISEASES IN *SCHISTOSOMA JAPONICUM* INFECTIONS"

EXECUTIVE SUMMARY

INTRODUCTION AND OBJECTIVE

This project is a continuation of AID Grant no. 5.002 (1985-1988) wherein we proved the validity of our hypothesis "antibodies of certain isotypes and specificities can inhibit embryonation and cause early destruction of *S. japonicum* eggs at pre-miracidial stage (anti-embyonation immunity) to cause modulation of granuloma formation and disease abatement". The destruction of eggs at pre-miracidial stage precludes the production of immunopathologic antigens responsible for T-cell dependent granuloma formation since mature eggs with miracidia are the principal source of these antigens. The important findings at that time were: (a) anti-embryonation immunity was induced by injections of viable intact immature eggs, (b) anti-embryonation immunity was demonstrated in chronic human infections, and (c) BALB/c mice sensitized with immature egg injections had at D+75 of infection no manifestations of hepato-splenic disease in contrast to infected naive controls which had splenomegaly and portal hypertension.

The objectives are further dissection and analysis of anti-embryonation immunity coupled with isolation, identification and possible production of the molecules responsible for its induction and vaccination trials (of the molecules).

Further dissection of anti-embryonation immunity was given priority to be sure that the vaccine based on it had more desirable effects and no objectionable ones before starting efforts for its production. In brief it came out that our anti-disease vaccine based on anti-embryonation had no objectionable features. Vaccination based on anti-embryonation immunity does not increase susceptibility to infection but on the contrary, it may augment resistance by less formation of blocking antibodies of egg origin, it does not need reboosting after its induction but it become more intense with time; and it is also anti-transmission as export of mature eggs with miracidia with the feces is curtailed. In other words our anti-disease vaccine based on anti-embryonation can be used alone to diminish incidence of transmission and prevent severe diseasse.

In viewing the project accomplishments in the context of international development, efforts of other organizations should be taken into consideration. The UNDP/World Bank/WHO (WHO-TDR) since its organization has started a program of research (of which we are participants since 1980) to develop vaccine(s) against schistosomiasis and a several other globally prevalent tropical diseases. Up to date, results indicate a vaccine to prevent infection with *S. japonicum* maybe long in coming. Thus although a vaccine against infection is more desirable, an anti-disease vaccine which is also anti-transmission has a place in schistosomiasis control.

The present project has improved our research and research training capabilities through procurement and repair of laboratory equipments, provide apportunities for young faculty to work in actual research, contribute teaching materials particularly for our courses in immuno-parasitology and molecular biology of parasite infections.

We have not accomplished all of our objectives dependent on further dissection and particularly "to isolate, identify and produce the molecules responsible for anti-embryonations immunity for vaccination trials". However significant work has been accomplished and this would be priorities for a succeeding grant.

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DEVELOPMENT OF A VACCINE TO PREVENT HEPATO-SPLENIC DISEASE IN *SCHISTOSOMA JAPONICUM* INFECTIONS

1. RESEARCH OBJECTIVES

The objectives or work plan includes the further dissection and characterization of anti-embryonation immunity particularly in relation to immunopathology, resistance to infectious and epidemiologic observations on morbidity (Domingo, et. al., 1980) and immunity coupled with isolation, identification and possible production of the molecules or antigens responsible for its induction and vaccination trails (of these molecules).

This project is essentially a continuation of US-AID Project no. 5.002 wherein we proved the validity of our hypothesis "that antibodies of certain isotypes and specificities can inhibit maturation or embryonation or cause early destruction of *S. japonicum* eggs (anti-embryonation immunity) deposited in the tissues at the pre-miracidial stage, to cause modulation of granuloma formation and disease abatement" (Garcia, et. al. 1989, 1992). The destruction of *S. japonicum* eggs at the immature or pre-miracidial stage precludes the production of immunopathologic antigens responsible for T-cell dependent granuloma formation since mature eggs with miracidium are the principal source of such antigens. Anti-embryonation immunity focuses on the immune effects on the eggs (Garcia, et. al., 1992) rather than immunoregulation (Olds et. al., 1985; Stavitsky, 1987) as the central factor in granuloma modulation.

It is generally accepted that manifestations of disease in *Schistosoma japonicum* and *S. mansoni* (Hang, L.M., 1974; Hamburger, et. al., 1976 and Warren, K.S., 1982) infections result from granulomatous and fibrotic responses to antigens from mature eggs entrapped in the tissue of the liver in the intestines, lungs and other organs. The more serious clinical manifestations of hepato-splenomegaly, increase portal vein pressure, ascites and collateral veins are readily ascribable to obstructive granuloma formation and fibrosis with resulting impedence to blood flow through the intra-hepatic portal tributaries (Garcia, E.G., 1975).

Schistosomiasis, a snail transmitted disease, is endemic in tropical and subtropical rural areas of China, Philippines, Indonesia, Malaysia and Laos along the Mekong River. In the endemic areas infections of farmers and their kins, are relatively frequent. Programs to control or eliminate this disease are not sufficient due to technical and financial constraints. Thus, there is a need for a vaccine to prevent disease. Prevention of disease with reduction of morbidity by vaccination will reduce cost of treatment, diminish man-days lost, due to illness, and improve the working efficiency of farmers to contribute to agricultural productivity.

We have had WHO-TDR and AIDAB support to study the immunology of *Schistosoma japonicum* infections with the ultimate objective of developing a vaccine to prevent establishment of infection. The conception and demonstration of anti-embryonation immunity was an off-shoot of our studies on immunology of schistosomiasis. Anti-embryonation

immunity has had partial support from our other grants. Please note, starting with our first AID project no. 5.002 of 1985, all of our scientific publications on anti-embyronation immunity acknowledged support from both USAID, AIDAB and WHO-TDR.

2. METHODS AND RESULTS:

The presentation of the different studies or experiments performed to attain the project objectives does not follow the chronological order the experiments were undertaken. The order of presentationn is designed to make understanding, appreciation and correlation of what was done easier.

Priority was given to further dissection of anti-embryonation immunity to have basis for the rational use of an anti-embryonation vaccine. For example there is no wisdom is producing our anti-disease vaccine if it increases susceptibility to infection.

2.1 Secretions and/or excretions and not somatic antigens of the immature egg are responsible for anti-embryonation immunity.

We have pressumed that excretions and/or secretions oozing through micropores of the egg shell and not somatic antigens of the immature egg as responsible for the induction of anti-embryonation immunity at the start of our studies on this subject. The immature eggs in an infection, without anti-embryonation immunity, develop in 10-12 days to intact mature eggs thus providing no chance for the liberation of somatic antigens into the tissues. In all of our past experiments (Garcia, E.G., et. al., 1987, 1989) and those reported here, intact freshly recovered immature eggs were injected (on the same day they were recovered) to induce anti- embryonation immunity.

To document the above presumption and to provide basis for our later attempts to isolate secretions and excretions for use as vaccines, two sets of BALB/c mice were sensitized with 6 and 13 injections, respectively, with immature egg- homogenates given weekly or bi-weekly, depending on availability. The homogenate injected was equivalent to ± 20,000 eggs, the dose of intact immature eggs we used for sensitizing in previous experiments. The mice with naive controls were infected with 6-8 cercariae and killed on D+42 of infection to count and determine state of development of eggs in equal and identical portions of the liver. The results of the egg-counts of the mice that had 13 injections rather than those that receive 6 injections are shown below, do not indicate that the mice developed anti-embryonation immunity.

Table 1. Number and percent of dead eggs in livers of mice sensitized with 13 injections of immature eggs homogenate and naive controls at D+42 of infection.

Sensitization	No. of Mice*	Total no. of eggs counted	Percent of dead eggs
Immature egg homogenate	5	6,589	0.31%
Unsensitized controls	9	10,222	0.28%

* Mice from both groups with less than 500 eggs were not included in the analysis to prevent any diluting or concentrating effect on the number of dead eggs.

We interpreted the results as an unequivocal proof that somatic antigens are not responsible for induction of anti-embryonation immunity. The mice that received 13 injections which would have been hypersensitized if somatic molecules were responsible for anti-embryonation immunity. The group that recieved 6 injections also had very few dead eggs.

Evidence of anti-embryonation immunity we look for, is the presence of a significant number of dead eggs from 3% to 35% as noted in our past studies (Garcia, E.G., et. al., 1987), in the liver and/or intestines at D+42 of infection. Please note if the sensitized mice are killed at D+75 of infection (Garcia et. al., 1989) dead eggs would range from 60 to 80% of eggs in the tissues.

S. japonicum starts laying immature eggs at D+24-26 of infection (Pesigan et. al., 1958). These immature eggs require 10-12 days to embryonate with the development of a miracidium and survive for another 10-12 days in the tissues. Thus at D+42 of infection, there should be very few or no dead egg (in the tissue) in the absence of anti-embryonation immunity. It should be mentioned that the required 10 to 12 days for immature eggs to embryonate provides ample time for anti-embryonation immunity to act on the immature eggs.

Donor rabbits or mice for immature eggs were killed at D+26-27 of infection to get the liver. At this duration of the infection, the eggs in the liver are 1 to 3 days old. Intact tissue free immature eggs were recovered from the liver by trypsin digestion of the homogenized liver, followed by sieving to separate the eggs from undigested tissues, and several washings with centrifugation with physiologic saline to make the eggs tissue free. Centrifugation was at low speed to ensure that the eggs are not subjected to pressure which may affect their viability.

2.2 Effect of anti-embryonation immunity on susceptibility to infection.

It was important to know the effect of the induction of anti-embryonation immunity on susceptibility to infection since an anti-pathology vaccine should augment resistance rather than increase susceptibility (to infection). BALB/c mice were sensitized with 5 weekly injections of 20,000 freshly recovered intact immature *S. japonicum* eggs. The sensitized group with an equivalent number of naive controls of the same age were infected after the 2nd injection of eggs, with 25 cercariae applied with a wire loop to the moistened shaved abdominal skin. (Since *S. japonicum* starts egg-depositionat D+24 to 25, infection after the second dose of immature eggs are given allows 24 days to inject the 3 other weekly dose of injections before immature eggs are deposited in the tissues. This shorthens the duration of the experiment by at least 3 weeks). Both groups of mice were killed at D+42 of infection and the number of flukes recovered by perfusion of the portal system compared to determine the effect of anti-embryonation on susceptibility or resistance to infection.

The table below shows no increase in susceptibility to infection due to anti-embryonation immunity, as indicated by the number of flukes recovered from the two mice groups (see section 2.4.2). Please note, comparison of the number of flukes was made after ascertaining that the sensitized mice had anti-embyonation immunity.

Table 2. Number of flukes recovered from infected mice sensitized with immature eggs and unsensitized controls at D+42 of infections.

Group	No. of Mice	Total No. of Flukes	Average No. of Flukes per mice	Range of flukes recovered
Sensitized with immature eggs	9	134	14.9	8-22
Unsensitized	12	221	15.7	10-20

Usually for assaying resistance to infection that may have been induced experimentally, mice or rabbits are killed at D+30 of infection at which time the flukes are already mature. In this particular experiment the mice were killed at D+42 to demonstrate evidence of anti-embryonation immunity before comparing the number of flukes recovered from each groups.

2.3 Effect of anti-embryonation immunity on the fecundity of S. japonicum.

It is possible that in addition to early destruction of immature eggs, anti-embryonation immunity may also reduce the fecundity of S. japonicum as an additional mechanism in causing disease abatement. Reduction of eggs laid by the schistosome female will limit the number of eggs available for serving as nidi for granuloma formation. In view of the large egg laying capacity of S. japonicum in comparison to S. mansoni, anti-fecundity effect of several percent, although statistically significant, will not have any measureable effect on the amount of morbidity. S. japonicum female empties its uterus to deposit 200-300 eggs in the tissues 12 times in 24 hours (Pesigan, T.P. et. al., 1958). This evidenced by the appearance of egg clusters in the intestines and liver. Thus we calculated that a reduction of fecundity should be at least 30% to have any effect on morbidity.

Groups of mice and rabbits sensitized with immature egg injections and corresponding naive controls were infected with cercariae and killed on D+40-45 to recover the flukes. Each female fluke was mounted on a slide and the number of eggs in the uterus counted. The average number of eggs per female of the sensitized and unsensitized groups were compared for determining anti-fecundity effect. In brief, the eggs counts did not show readily detectable reduction of eggs in the uterus from the experimental mice and rabbits.

2.4 Effect of anti-embryonation immunity on the production of blocking antibodies and on resistance to reinfection.

These experiments were undertaken relevant to the effect of anti-embryonation immunity on the production of blocking antibodies in view of its very important implications to the development of vaccines to prevent estasblishment of infection based on immune effects on the tegumen or membrane of the re-infecting schistosomules.

The failure to reject re-infecting schistosomules of S. mansoni has been attributed to "blocking antibodies" elicited in response to epitopes of egg antigens expressed in membranes of young schistosomules (Butterworth, A.C. et. al.Parasitology, 1987). This was based on a study which showed high levels of anti-egg antibodies associated with susceptibility to re-infection after treatment. The anti-egg antibodies block potentially protective IgM antibodies by either direct competition for a single epitope or by steric inhibition of the binding of antibodies with specificity for other surface epitopes of the membrane of the schistosomule.

2.4.1 Difference in number of membrane antigens recognized by blocking antibodies of immature egg and mature egg origin.

It is logical to state that mature eggs with miracidia will have more antigenic molecules since it is a more complex developmental stage than the immature egg without a miracidium. Consequently the probability is greater that lesser number of immature egg antigens will express epitopes of the schistosomular membrane. Thus an animal with anti-embryonation immunity will produces less blocking antibodies of egg origin. To test this assumption, we determined, by Western Blots, the difference in number of molecules with epitopes shared by immature and mature egg with schistosomular membrane antigens. It came out that hyperimmune sera of rabbits sensitized with repeated injections of mature eggs recognized 94 KD, 70 KD, 50 KD, 30 KD and 20 KD membrane molecules obtained by freeze-thawing, of D+15 early hepatic schistosomules. Only two molecules of the membrane antigens, 70 KD and 50 KD, were recognized by sera of rabbits sensitized with repeated injections of intact immature eggs.

The findings strongly suggest that an 1infected animal with anti-embryonation immunity will be able to reject re-infecting schistosomules if the inference of Butterworth are correct, i.e. blocking antibodies prejudice resistance to reinfection by either competing for a single epitope or by steric inhibition of the binding of antibodies with specificity for other surface epitopes of the membrane of the schistosomules.

2.4.2 Resistance to re-infection of animals with anti-embryonation immunity.

Based on the preceeding observations a primary infection with 400 cercariae was given to a group of rabbits that received 6 weekly injections of immature eggs. These rabbits were challenged with 100 cercariae at D+70 of the primary infection with a group of naive rabbits to serve as challenge controls. At D+21 of the challenge infection, all rabbits were killed and the count of worm recovered by perfusion of the portal systems from each of the 2 groups compared. The rabbits were killed at D+21 to be able to differentiate the larger schistosome from the earlier primary infection from the smaller flukes of the challenge infection.

Table 3. Number of flukes recovered from egg sensitized rabbits challenged at D+70 primary infection.

Particulars	Sensitized with 6 injections of immature egg	Controls
No. of rabbits	7	7
No. of flukes recovered	104	319
Mean no. of flukes	14.9	45.6
Maun-Whitney U-test	p = 0.005	

The results indicate that the sensitized rabbits with a previous primary infection of 400 cercariae had been able to more efficiently reject the challenge of 100 cerariae with a level of protection of 67.3%. We have not observed as high level of protection in the various vaccination trials, to prevent establishment of infection, using cloned molecules (Sj26, Sj28, Sj23 and paramyosin) from our cDNA expression library of S. japonicum proteins prepared from adult parasite materials.

2.4.3 Effect of sensitizing with D+15 membrane antigen on resistance to infection.

One implication of the preceeding observations is that the membrane antigens protected by blocking antibodies may actually be vital antigens for developing a vaccine based on immune attack (on the membrane). Consequently we immunized a set of BABL/c mice with membrane antigens obtained by freeze-thawing of D+15 schistosomules. However no significant level of protection was observed as shown in table 4.

Table 4. Number of flukes recovered from mice sensitized with D+15 schistosomule membrane antigen and naive controls at D+29 of infection.

Group	No. of Mice	Total no. of flukes recovered	Range no. of flukes recovered	Mean no. of flukes recovered
Sensitized	7	72	5-17	10.2
Unsensitized	9	91	5-17	10.1

From this results we inferred blocking anti-bodies, also of membrane origin, have prevented aggressive immune attack on the vital antigen so that no level of protection was observed. It is easy to make this inference since there must be scores of membrane molecules. Extrapolation of this inference, although not yet supported by experimental evidence, suggests that although cloned molecules of the membrane or tegument of the schistosomules may be developed as effective vaccines to prevent establishment of infection, their effectivity will only probably be for 2 or 3 re-exposures to cercariae. In the rejection of the schistosomules, their ensuing destruction will liberate large number of molecules which will stimulate blocking anti-bodies of membrane origin. The preceeding inference may also explain why crude extracts or homogenate of schistosomules and adults worms do not induce measurable resistance when tried as vaccines.

2.5 Further Studies on the sex ratio of flukes in *Schistosoma japonicum* infections.

The impression has been gained over years of working with *Schistosoma japonicum* Philippines that eggs harvested from animals late in infections give rise to cercariae that are very biased towards males although the male to female ratio is closer to one when the eggs are harvested early in infections. Also it has been observed that usually there are more male than female worms in the experimental animals, infected usually from a pool of cercariae from several snails. We entertained the possibility that the female miracidium or egg is more prone to anti-embryonation immunity and/or some other immune response.

To examine the influence of duration of infection on the ratio of male to female miracidium *O.h. quadrasi* snails were infected individually with miracidium hatched from eggs from rabbits killed at different days

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of infection. Between D+65 to D+70 each snail was crushed and cercariae from one snail were infected to mice (each mouse was infected with cercariae from one snail only). The mice were killed on D+30 to determine sex of the cercariae with which they were infected, as indicated by the sex of the mature flukes. The results are shown in the table below.

Table 5. Sex of infection of cercariae in snails as determined by the sex of flukes in the mice.

Duration of infection	No.of snails		% of female
	Male	Female	
D+48	14	7	33.3%
D+49	18	7	35.7%
D+77	20	0	0%
D+77	19	1	4%
D+100	24	0	0%

With the above findings of more male miracidia from chronic infections it was decided to determine sex of infection in field snails and naturally infected field rats which maybe extrapolated to indicate sex of infection in humans. Shown below are the relevant observations.

Table 6. Sex of cercariae in fields snails as determined in mice.

Snail Infections	Sorsogon	%	Mindoro	%
Male worms only	46	85.18%	47	90.38%
Female worms only	5	9.25%	5	9.61%
Mix sex	3	5.55%	0	0%
Total	54		52	

Note: Data from both endemic areas present observations from two separate collection from different snail areas.

Table 7. Sex of S. japonicum field rat infection in Irosin, Sorsogon and Victoria, Oriental Mindoro.

Infection in Rats	Sorsogon		Mindoro	
	No.	%	No.	%
Mixed Male & Female	80	91.9%	25	71.4%
Male worms only	6	6.8%	9	25.7%
Female worms only	1	1.1%	1	0.2%
TOTAL	87		35	

Table 8. Male to female ratio of *S. japonicum* worms recovered from infected field rats.

Area	No. of rats	No. of worms		Male-female ratio
		Male	Female	
Sorsogon	87	3,548	1,764	2.03
Mindoro	35	699	499	1.40

The data presented confirmed our expectations that the sex ratio of male to female cercariae in field snails and of adults recovered from naturally infected field rats would be heavily biased for males. This can be easily explain by the fact that most human and animal *S. japonicum* infections in endemic areas would be chronic.

One hypothesis to account for the apparent bias towards male miracidia in eggs from chronically infected animals is that anti-embryonation immunity and other unidentified immune response over time act preferentially against heterogametic female miracidium. If valid this hypothesis has implication on granuloma modulation in experimental and human schistosomiasis. If female miracidia are a richer source of immunopathologic antigens, their selective loss would account for reduced granuloma size in chronic infections and contribute to reduction of transmission.

The heavy bias for males in both snails and animal host explain or account for the difference between seropositive and apparently parasite positive individuals in endemic areas, the observation that the

greater proportion of human infections in endemic areas are asymptomatic (Domingo et. al., 1980) and with significant portion of the asymptomatic group apparently resistant to nfection. It should be mentioned that Vogel and Minning in 1953 were able to induce high level of resistance in rhesus monkeys by continous trickling infection with male cercariae only. Many of residents in endemic areas are probably similarly exposed to male cercariae only and become immune or will have few female schistosomes so that they are asymptomatic.

2.6 Production of Hybridoma secreting monoclonal antibodies which have anti-embryonation activity.

The intent in producing hybridoma secreting monoclonal antibody (McAb) with anti-embryonation activity was to have an antibody for screening a future cDNA expression library, we planned to construct from immature eggs, for molecules which can be cloned for testing as anti-embryonation vaccine.

BALB/c mice were given 10 sensitizing injection of 20,000 freshly recovered immature eggs at 7-10 day intervals. A month after the 10th injection, a booster injection of immature eggs was given. On the 3rd day after the booster the mice were killed and the spleen cells recovered anf fused with mice myeloma cells (SP2) to produce hybridomas which were cloned. Each clone culture was expanded to get enough culture supernate from which the monoclonal antibody was concentrated by $(NH_4)_2SO$ precipitation. Determination of anti- embryonation activity was by injection of the concentrated McAB starting at D+24 of infection of BALB/c mice. The BALB/c mice were killed at D+42 for counting eggs and determining of their development.

The clone HI had apparent anti-embryonation activity, as 5.06% of eggs in McAb injected mice were dead in contrast to only 1.11% in the naive controls. We have not been able to use this hybridoma in view of a number of developments.

2.7 Evaluation of Sj 26 (glutathione-S-transferase) as antigen for inducing anti-embryonation immunity.

One of the molecules cloned from our cDNA expression library, Sj 26, Mr 26,000 is an enzyme found in adults and eggs of *S. japonicum*. BALB/c mice which received injections of Sj26 and shown to have titers (against Sj 26) before challenge did not show any evidence of the induction of anti-embryonation immunity.

2.8 Production, isolation and identification of secretion and/or excretion responsible for the induction of anti-embryonation immunity.

Although aseptically digested tissue free viable immature eggs can be used as vaccine for humans, their production is quite expensive and have only a limited

shelf-life. Thus attempts were started towards making a molecular or defined vaccine which can be produced by recombinant cDNA technics or synthesized if the desired molecules are proteins. The succeeding paragraphs present what we are able to do.

2.8.1 We have a cDNA library in XL1 blue strain of E. Coli made from S. japonicum worms. It is made in a lambda phage vector called "lambda Zap II". This library was screened with hyperimmune sera of rabbits that received eight injections of immature eggs at intervals of 7 to 10 days. A number of clones have been isolated. Partial DNA sequence had been determined for 4 lambda gt 11 clones. These are SjIrV, major egg antigens (40KD) and heatshock proteins 70 and 86.

2.8.2 In view of the availability of DME (Dulbecco's Modified Eagle) we incubated immature eggs, about 100,000 for 5 days and concentrated the culture supernate. We made immunoblots of the culture supernate with SDS-Page but no bands were seen on staining with protein stain.

2.8.2 On the presumption, that immature eggs have their own reserve of nutrients to support their embryonation, we incubated immature eggs obtained at D+26 to 27 of infection in phosphate buffered saline with 0.06% glucose. Incubation was for 5 days with collection of the culture fluid daily. This culture fluid was concentrated and immunobloted with SDS-Page. Two molecules a 70 KD and a 50 KD were detected with Coomasie stain.

The mixture was used to sensitize ELIZA plates and positive reactions were seen with sera of mice and rabbits immunized with immature eggs. However mice that received 6 injections given every week did not show evidences of anti-embryonation immunity. A replicate could not be done in view of time constraint and we did not have enough of the molecules to complete the vaccination schedule.

2.8.3 We have accumulated about 4 ml (packed volume) of tissue-free immature eggs in anticipation of constructing a cDNA expression library (from immature eggs).

3. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER.

The real impact and relevance of this project has to be evaluated with reference to the accomplishments of the first project also supported by AID. Findings in the present project make the case for an anti- pathology vaccine based on anti-embryonation immunity stronger.

3.1 Schistosomiasis japonica is endemic in rural areas of China, Philippines, Malaysia, Laos and Indonesia. Present programs of control are not adequately effective due to financial and technology constraints. An anti-infection or anti-disease vaccine which is also anti-transmission at affordable cost will improve control programs.

3.2 Anti-embryonation immunity, first described by us, is a fairly recently recognized immune response at least in S. japonicum infections and not yet appreciated by many researchers. In view of biological differences (Mitchel, et. al. 1991) between S. japonicum and S. mansoni it is not known whether the findings can be extrapolated to S. mansoni.

3.3 Previous findings demonstrated the effectiveness of anti-embryonation immunity to prevent serious disease in S. japonicum infection (Garcia et. al., 1989). Evidences indicate that humans also develop anti-embryonation immunity (Garcia, et. al., 1985) but relatively late in natural infections. From the observations reported an anti-embryonation vaccine is relatively safe, requires no boosting, and also is anti-transmission. It does not increase susceptibility to infection, but may increase resistance to infection.

3.4 As is, the crude vaccine of viable intact immature eggs can be used but is very costly to produce and has a limited shelf-life. The ideal would be a molecularly defined vaccine which can be produced at affordable cost. A start has been made towards this by our laboratory which would be the object of futher studies.

3.5 This project has complemented or supplemented our other researches in improving our research and research training capabilities, particular in immunoparasitology. This was made through purchase of some equipment, use of project materials and researches as study materials for our graduate students.

4. PROJECT OUTPUTS

4.1 The paper "Anti-embryonation immunity as a granuloma modulating mechanism in schistosomiasis caused by Schistosomiasis japonicum" was presented in the meeting "Aquaculture and Schistosomiasis" sponsored by BOSTID, National Research Council and IKLARM, held in Manila August 6-10, 1991.

4.2 "Anti-embryonation as a basis for an Anti-Pathology Vaccine in S. Japonicum infections was presented. Synposium on Schistosomiasis, sponsored by Rockefeller Foundations, held in Manila and Leyte, Philippines, November, 1992.

4.3 "Development of anti-pathology vaccine against S. japonicum based on anti-embryonation immunity" Joint US-Philippines Meeting on use of "Biotechnology in the Study of Parasitic Disease", Manila, Philippines, Oct. 23-26, 1995.

4.4 Publications

The attached list of references includes most of our publications on anti-embyonation immunity.

Garcia, E.G.; Mitchell, G.F.; Tiu, W.U. and Rivera, P.T. 1992. Anti-embryonation as a granuloma modulating mechanisms in Schistosomiasis caused by Schistosoma japonicum. In National Research Council 1992, "Aqua Culture and Schistosomiasis". Proceeding of a network meeting, Manila, Phillipines, pp. 167-177. National Academy Press. Washington, D.C.

Mitchell, G.F.; Garcia, E.G.; Rivera, P.T.; Tiu, W.U. and Davern, K.M., 1994. Evidence for and Implications of Anti-embryonation Immunity in Schistosomiasis. Experimental Parasitology 79: 546-549.

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5. PROJECT PRODUCTIVITY

The project did not accomplish all of the proposed goals more particularly to isolate, identify and possibly produce the antigens or molecules responsible for the induction of anti-embryonation immunity for vaccination trials. We have however made some significant progress on this goal.

The further dissection and charactherization of anti-embryonation immunity has provided observations which can justify a very strong recommendation for human trials of a molecularly defined anti-embryonation vaccine when produced or in combination with anti-infection vaccines when produced.

Reasons for the above are:

1. The frequency of electric cutages or brown-out in 1991 and 1992 which prevented us from working full time.

2. Financial constraints due to the delay in implementing the study. The proposal was submitted in April, 1988 and the contract for its implementation was signed only in August, 1991. The three years delay forced us to absorb increase in prices of commodities due to inflation. This shrunk our operating budget. The shrinking budget particularly affected our supply of animals/feeds.

3. We were adviced on April 6, 1994 that we had exceeded the budgetary allotment for animals/feeds in October, 1993. All purchases of animals and feeds from October were disallowed. AID-Manila stated it would approve a budget re-alignment provided there is prior AID-Washington approval. Due to some communication snag, Washington approval came only in July, 1994 and the budget modification signed in September, 1994. We practically curtailed purchase of animals from April to July, 1994.

4. A four-day review or audit of our financial management and accounting system was conducted in October, 1994 by SGV Consulting in behalf of AID-Manila. In brief, SGV found our accounting systems not up to their standards and recommended AID-Manila furnish the project (which AID had not done at the start) a copy of the AID financial manual and for the project to set-up an accounting system in accordance with the AID manual and recommendations of SGV.

   We were caught in discussion and writing of lengthy comments on SGV findings and recommendations as the contract between SGV and AID provided that draft of the financial review to be discussed with the grantee before SGV makes a final report. Finally, in January, 1995 SGV submitted its final report. AID-Manila decided that not to push us to implementing a new accounting systems. Starting February, 1995 the principal investigator advance expenses to be re-imbursed monthly.

5. We did, on request, got a one-year extension up to August 31, 1995 but without additional funds. To meet expenses for commodities, the principal investigator and co-investigator voluntarily reduce their stipends or honoraria. Also only 1 technical help was supported by AID from April to September, 1995

6. FUTURE WORK:

It is imperative that the work be continued to isolate, identify and produce the molecules responsible for anti-embryonation immunity for vaccination trials. It should be stressed that vaccination to prevent serious disease in schistosomiasis, based on anti-embryonation immunity is the most effective anti-disease intevention and not saddled with risks unlike vaccination to promote immunoregulation (Garcia, et. al., 1985; Mitchell, G. F., et. al., 1994).

Further at the present stage of development, it appears that a vaccine to prevent infection is long in causing. All the cloned molecules that have been tried in different laboratories have not consistenty given protection of sufficient level.

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ANTI-EMBRYONATION IMMUNITY AS A GRANULOMA MODULATING MECHANISM IN SCHISTOSOMIASIS CAUSED BY *SCHISTOSOMA JAPONICUM*

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ABSTRACT

In a recent hypothesis we proposed that one of the key events in granuloma modulation is the inhibition of maturation or embryonation of the egg and its destruction at an immature (i.e., pre-miracidial) stage of development. One consequence of this anti-embryonation immunity is the failure of the egg to mature and produce immunopathologic antigens responsible for T-cell dependent granuloma formation. This hypothesis focuses on immune effects on the egg rather than on immunoregulation as the central factor in granuloma modulation.

The more important observations that support anti-embryonation immunity are: (1) maturation of fresh uterine eggs injected into the pulmonary vasculature of chronically egg-sensitized mice, with modulated granuloma response, was inhibited; (2) sera taken from humans with chronic schistosomiasis injected into BALB/c mice reduced the rate of maturation of eggs deposited in the liver and intestines and reduced the size of granuloma in mice sensitized for accelerated granuloma formation; (3) BALB/c mice sensitized by repeated injection of viable immature eggs and challenged with few cercariae had at D+42 of infection significant increase of dead eggs and a decrease in the proportion of mature eggs to immature eggs in the liver and intestines; and (4) infected mice sensitized with immature viable eggs prior to the onset of egg-laying had at D+75 of infection lesser and smaller granuloma relative to controls with portal pressure and relative spleen weight comparable to uninfected controls. We consider these findings as sufficient basis for the development of a vaccine to prevent hepato-splenic disease. An anti-embryonation vaccine will also be anti-transmission.

INTRODUCTION

Manifestations of disease from *Schistosoma japonicum* and *S. mansoni* result from granulomatous and fibrotic responses to antigens released from eggs entrapped in the liver, intestines, lungs, and other organs (Warren 1982). The more common and more serious clinical manifestations of hepatosplenomegaly, increased portal pressure, ascites, and collateral circulation are readily ascribed to obstructive granuloma formation around eggs with subsequent fibrosis and impedance to blood flow

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through the liver. In *S. japonicum* infection, as in *S. mansoni* infection, it is generally accepted that CD4 T-cells of the delayed hypersensitivity type are the principal initiators of these reactions. It has also been demonstrated that the mature or embryonated egg containing a miracidium is the source of the immunopathologic antigen (Hang et al. 1974, Hamburger et al. 1976, Mitchell et al. 1982). The speed and size of the granulomatous response is dependent on the duration and intensity of sensitization with egg antigens. After initial sensitization, there is an accelerated formation of larger and more destructive granulomas. However, as the infection becomes chronic, or with prolonged sensitization, the size of granuloma formed is reduced or modulated (Domingo and Warren 1968, Olds et al. 1982, Garcia et al. 1983).

This phenomenon of granuloma modulation, previously called endogenous desensitization (Domingo and Warren 1968), opened the prospects for vaccination against disease. Immunization to promote modulated granuloma response could lead to a reduced likelihood of severe hepatosplenic disease in infections with *S. japonicum* or *S. mansoni*. The principal mechanisms involved in granuloma modulation, probably operating singly or in combination, include: (1) reduced efficiency of maturation or embryonation of eggs in tissues, thus preventing them from secreting immunopathologic antigens, i.e., anti-embryonation immunity (Garcia et al. 1983, Mitchell et al. 1991); (2) suppressor T cell mediated inhibition of CD4 T-cells or their products responsible for granuloma formation (Stavitsky 1987, Olds 1989, Boros 1986); and (3) antibody mediated inhibition of anti-egg-response, probably by diversion of antigens from T cells or inhibition of antigen recognition by anti-idiotype antibodies (Pelley and Warren 1978, Olds et al. 1982, Garcia et al. 1985).

It is not the intent of this brief review to discuss in detail the different postulated mechanisms of granuloma modulation, but to recount the series of observations made in our laboratory on anti-embryonation immunity that indicate that it may be the principal mechanism involved in granuloma modulation, at least in *S. japonicum*. Anti-embryonation immunity focuses on the effects of the immune response on the egg rather than on immunoregulation as the central factor in granuloma modulation.

TABLE 1 Evidence for Maturation Arrest of Eggs of *Schistosoma japonicum* in BALB/c Mice* Sensitized by Weekly Intraperitoneal Injections of Eggs for 28-33 Weeks

Mice	No. of Mice	No. of Mice with < 25 Mature Eggs	Percentage of Mature Eggs in Lungs
Egg-sensitized	11	8	25.8 ± 8.6
			$p < 0.025$**
Age-matched controls	11	3	59.8 ± 11.3

* Mice were injected intravenously with 50-100 uterine eggs from *S. japonicum* adult worms and lungs examined for mature eggs (as distinct from immature and egg shells) at days 12-16. Mean numbers of eggs examined in each mouse of the egg-sensitized and control groups were 46.4 ± 18.9 and 58.7 ± 20.0, respectively. Examination was made by compressing the lungs between glass slides and microscopy.

** P values determined by the Mann-Wnitney U statistics (Sokal and Rohlf 1969).

EVIDENCE OF ANTI-EMBRYONATION IMMUNITY

Of the postulated mechanisms of granuloma modulation, reduced embryonation of eggs in the tissues or anti-embryonation has more attractive features in terms of disease prevention. If egg maturation can be halted, the release of immunopathologic egg products is curtailed and both the sensitization and expression for immunopathology should be reduced (Garcia and Mitchell 1982, 1985; Garcia et al. 1985, 1987, 1989). The continuous deposition of immature eggs in the tissues and their destruction results in continuous reboosting of anti-embryonation immunity.

The initial suspicion that *S. japonicum* eggs in the liver are inhibited from maturing in chronic infections was aroused in connection with our attempts to standardize intact eggs as antigen for the circumoval precipitin test (COPT) (Oliver-Gonzales 1954, Garcia et al. 1981). We wanted to determine at what point, during infection, donor rabbits should be killed for eggs (recovered by digestion of livers) so as to have an optimal proportion of mature eggs that are the more suitable antigen for the COPT. (In the course of this study it was also demonstrated that immature and dead eggs are less suitable as antigens for the test.) It was observed that egg batches recovered from livers of infected rabbits at time points earlier or later than 55th to 60th day of infection were less suitable for the performance of COPT. Differences presumably reflected immaturity of eggs at early time points and loss of viability at later time points (after D+60). Thus, the possibility was entertained that embryonation of maturation of eggs was retarded or inhibited in addition to the previously reported observation of accelerated egg destruction in long-term infected animals (James and Colley 1976, Olds and Mahmoud 1980).

INHIBITION OF EMBRYONATION IN MODULATED MICE

Chronically egg-sensitized mice (28 weekly injections) with modulated granuloma response inhibited the maturation of intravenously injected freshly recovered immature uterine eggs (Table 1). On D+12 after immature egg challenge (based on the fact that the *S. japonicum* ovum needs 10-12 days to mature), in the chronically egg-sensitized mice, only 25.8% of the eggs were mature compared to 59.8% in the lungs of the unsensitized controls. This led us to infer that reduced embryonation of *S. japonicum* eggs results in chronically egg-sensitized mice of infected hosts and anti-embryonation may be a granuloma modulating mechanism (Garcia et al. 1983).

INHIBITION OF MATURATION OR EMBRYONATION OF *S. JAPONICUM* EGGS IN INFECTED MICE BY INJECTION OF SERA FROM HUMANS WITH CHRONIC INFECTION

The preceding observations prompted us to determine if anti-embryonation immunity is induced in humans with chronic infections (Garcia et al. 1985).

Sera from humans with clinically defined chronic *S. japonicum* infections and classified by the nature of precipitates formed when reacted with eggs in the standardized COPT were injected from D+24 to D+38 of infection into BALB/c mice exposed to four cercariae. The start of injection at day 24 coincides with the earliest period that *S. japonicum* oviposits in mice. Control mice were injected with COPT-negative sera following the same dosage and schedule. The mice were killed at D+41 of infection. At this time point there should be none or very few dead eggs in the control mice

injected with COPT negative sera. *Schistosoma japonicum* starts ovipositing from day 24 to day 27 after cercarial penetration and the eggs need 10 days to mature or embryonate and survive another 10-14 days in the tissues.

TABLE 2 Maturity of Eggs in Livers of BALB/c Mice Infected 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
Injected with COPT-positive sera			
1	536	216	2.[illegible]
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**
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. 1-5 were 4,4,4,4, and 2, respectively (mean ± SEM = 3.6 ± 0.4) and in mice nos. 6-10 were 8,6,4,3, and 4, respectively (mean ± SEM = 5.0 ± 0.9).
** $p < 0.05$, Mann-Whitney U-test.

Tables 2 and 3 show the number and maturity of eggs in the livers and intestines of the mice injected with human sera. As these tables demonstrate, there is significant inhibition of maturation of eggs in the infected mice injected with sera from humans with chronic *S. japonicum*, which form large segmented (LS) precipitates in the standard COPT. This is evidenced by the much higher proportion of immature eggs to mature eggs in the livers and intestines of the mice that received COPT-positive sera. This indicates that some serum factor, probably antibody, inhibited maturation (of the eggs).

EVIDENCE OF ANTI-EMBRYONATION IMMUNITY AND EGG DESTRUCTION IN MICE SENSITIZED WITH IMMATURE EGGS OF *S. JAPONICUM*

The next experiment undertaken demonstrated that anti-embryonation immunity is induced by injections or sensitization with viable immature *S. japonicum* eggs (Garcia et al. 1987). Mice were

sensitized by injections of viable intact immature eggs recovered by the digestion of livers of infected rabbits killed from D+25 to D+29 of infection. In experiments 1 and 2, five weekly injections of 10,000 eggs were given to mice and infected with four cercariae 1 and 4 days, respectively, after a second injection of eggs. In experiment 3, the mice had 23 injections of a mixture of mature and immature eggs at 1 to 3-week intervals followed by three weekly injections of immature eggs. They were infected with four cercariae after the last injection.

TABLE 3 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

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
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*
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.

The results of the assessment of egg development in the intestines and livers of the egg-sensitized mice and unsensitized controls killed at day 40-42 of infection are shown in Figure 1. In the analysis of the data, mice that had no eggs in the liver and intestines (uninfected mice) as well as those with less than 50 eggs in either intestinal wall or in liver samples were excluded. This was done to limit the potential sampling error (in the case of the liver) and distortion brought about by too few eggs. This excluded 9 of 53 mice in the three experiments. The notable aspects of these data follow.

- There is a very obvious increase in dead eggs, particularly in the intestines in most of the immunized mice.
- Ten of the 29 immunized or egg-sensitized mice had no mature eggs either in the liver or intestines, although they had both dead and immature eggs. In contrast, all of the 15 unsensitized mice had mature eggs.

- When expressed as a ratio, the percentage of immature to mature eggs is clearly shifted to a deficiency of mature eggs in tissues of egg-sensitized mice.

The fact that 10 of the infected egg-sensitized mice had no mature eggs strongly suggests that the eggs were killed before maturation, in which case it would be most probable that secretion and excretion of the immature and maturing eggs were responsible for the induction of anti-embryonation immunity. One implication of the data is that anti-embryonation could reduce export of mature eggs with the feces and consequently contribute to reduction of transmission as well as amelioration of intestinal pathology.

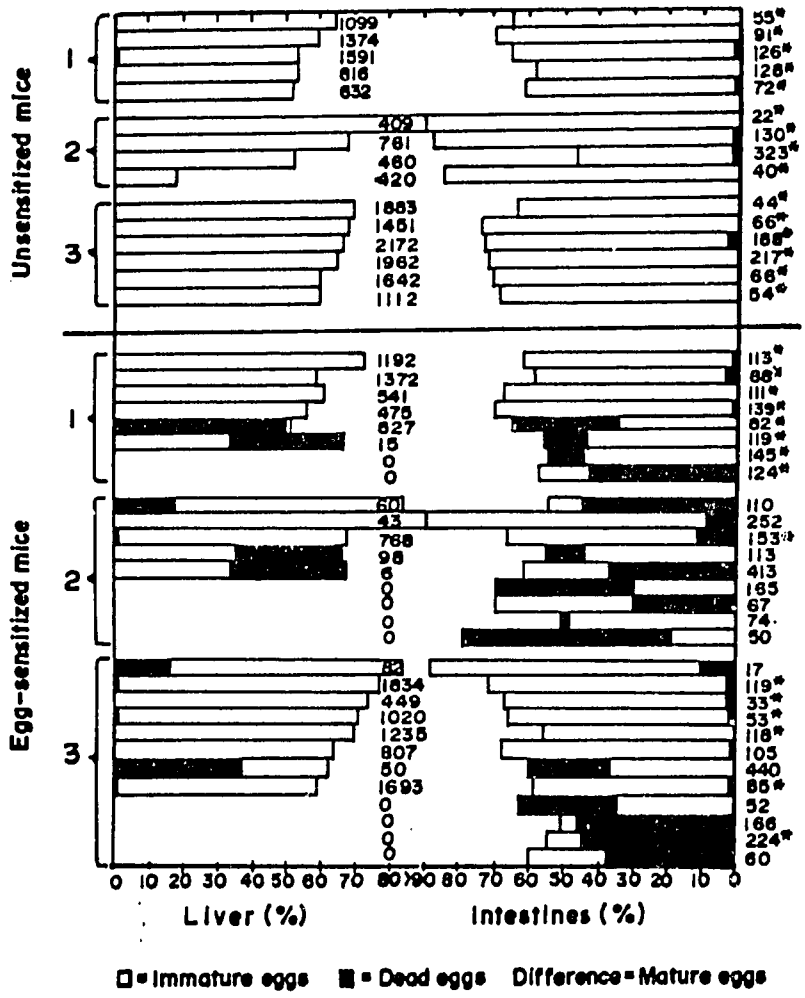

FIGURE 1 Percentages of immature eggs (open bars) and dead eggs (closed bars) in liver and intestines of normal mice or mice immunized against immature eggs, challenged with cercariae of *S. japonicum*, and killed at day 40-42 of infection. Only mice with > 50 eggs in liver sample or intestines are included 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.

EFFECTS OF INDUCTION OF ANTI-EMBRYONATION IMMUNITY ON LIVER GRANULOMAS, RELATIVE SPLEEN WEIGHT, AND PORTAL PRESSURE OF INFECTED MICE

This experiment was undertaken to determine if mice sensitized for the induction of anti-embryonation immunity prior to egg deposition will develop hepatosplenic disease (Garcia et al. 1989).

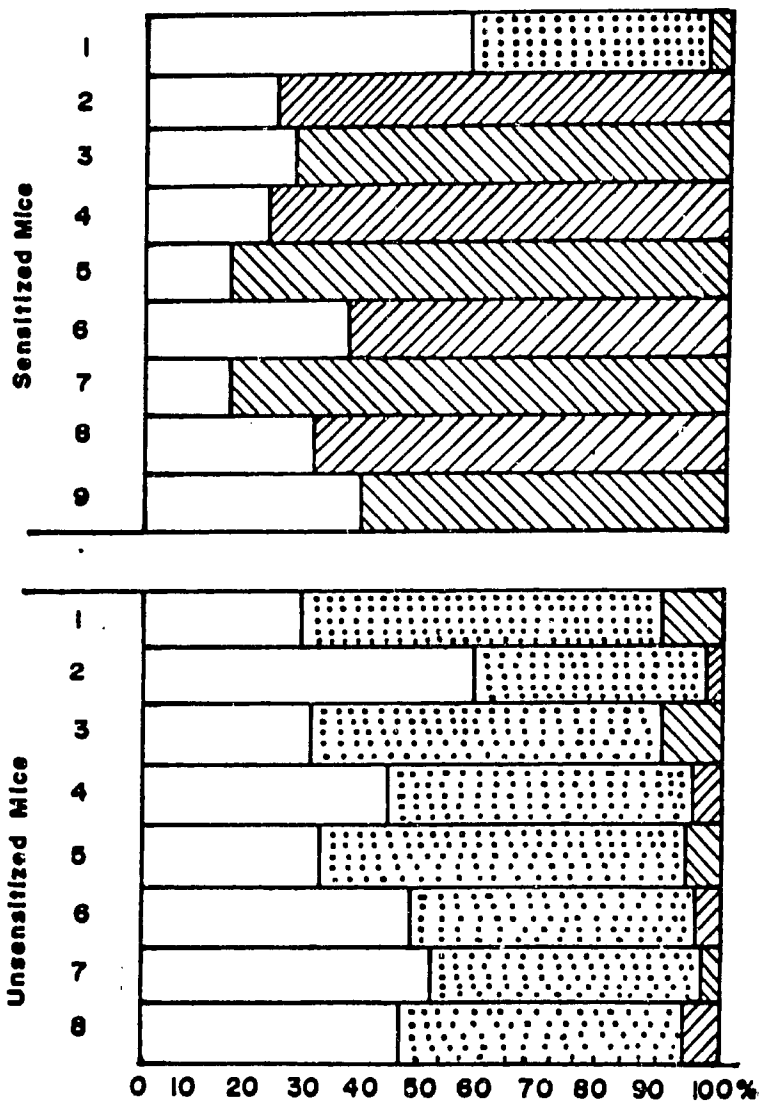

FIGURE 2 Percentage of immature (open sections), mature (dotted sections), and dead eggs (cross-hatched sections) in intestines of egg-sensitized and unsensitized mice at D+75 of infection. Only mice with > 50 eggs or egg clusters in the intestines are included in the analysis, most clusters being confined to the unsensitized group.

In this study, BALB/c mice received five weekly injections of 10,000 viable immature eggs by subcutaneous and intraperitoneal routes. On the tenth day of the sensitization regimen, the mice were infected with four cercariae in anticipation of egg-laying commencing after the last of the egg injections. The mice were sacrificed on day 75 of infection for determination of number and size of granulomas in the liver, portal vein pressure, and relative spleen weight as well as evidence of anti-embryonation immunity. Controls included an equal number of unsensitized infected mice and unsensitized uninfected mice of corresponding ages for determining normal values of portal vein pressure and relative spleen weight. No count of eggs in the liver was made. However, serial sections were made for assessing granuloma formation. Each egg or granuloma was followed up serially to identify the section with maximum dimensions that was used for calculating granuloma volume and amount of reaction. "Granuloma" were classified as having no reaction (0), minimal reaction (±) (i.e., size 1-3 × 10^{-4} mm^3 and usually involving 1-2 layers of inflammatory cells) and positive (+) (i.e., size greater than 3 × 10^{-4} mm^3). The only error in such classification is likely to be either overestimation or underestimation of granulomas around unusually large or small eggs. The observations on the state of development of eggs in the intestines, assessment of granulomas in the liver, portal vein pressure, and relative spleen weight are presented in Figures 2, 3, and 4, respectively. The notable aspects of these observations follow.

- At D+75 of infection only one of the egg-sensitized mice had mature eggs, with the remaining eight mice having only dead or immature eggs in the intestines. In contrast, there were fewer dead eggs but a greater proportion (40-64%) of mature eggs in the unsensitized group (Figure 2).
- The egg-sensitized mice had a much higher (61%) number of eggs without granulomatous reaction in contrast to the unsensitized controls where only 8% had no reaction ($p < 0.001$) (Figure 3).

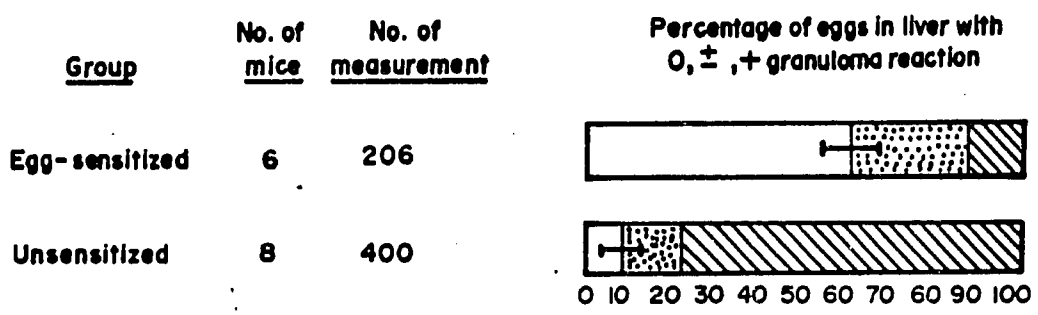

FIGURE 3 Percentage of eggs in liver with no granulomatous reactions (open section, with SEM), minimal granulomatous reactions (dotted section), and obvious granulomatous reactions (cross-hatched section). A minimal reaction is a granuloma of 1-2 layers of cells, whereas an obvious reaction consists 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.

- The mean portal venous pressure (207 mm of $H_2O \pm 76.31$) of the unsensitized group was significantly higher ($p < 0.005$) than that (122.3 ± 54.19 mm of H_2O) of the egg-sensitized group (Figure 4).
- The mean relative spleen weight in the unsensitized group is much greater ($p < 0.001$) than that of the sensitized group.
- The portal vein pressure and spleen weight of the egg-sensitized group were comparable to normal uninfected mice of similar age (Figure 4).

The observations above demonstrated that the induction of anti-embryonation immunity prior to infection, through sensitization by injection of viable immature eggs, has resulted in reduced number and size of granuloma in the liver of the infected mice with prevention of portal hypertension and its consequences. The number of adult schistosomes from four cercariae, extrapolated for a 60-kg human would mean a worm burden of 3,000 flukes, which is much higher than the usual worm burden in infected humans.

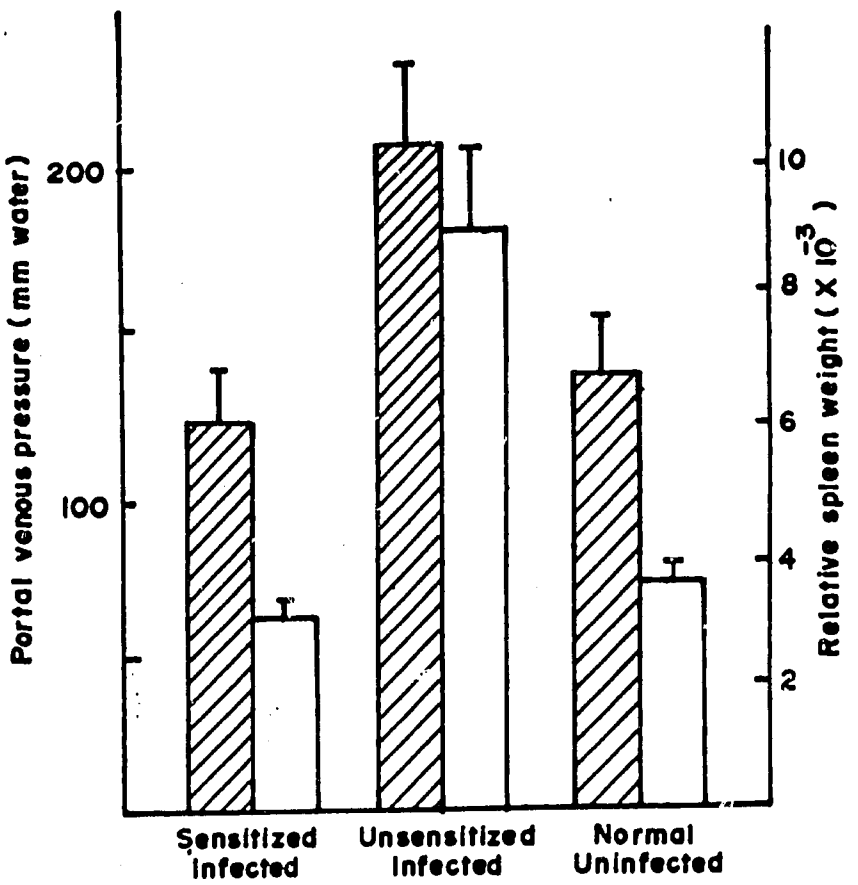

FIGURE 4 Portal venous pressure (cross-hatched bar) and relative spleen weight (open bar) of infected egg-sensitized mice, unsensitized infected mice, and uninfected controls at D+ 75 of infection. SEM are indicated.

DISCUSSION AND CONCLUSIONS

The observations reported here provide unequivocal evidence that an anti-embryonation vaccine can prevent the development of hepato-splenic disease, the more serious consequence of schistosomiasis caused by *S. japonicum*. The fact that *S. japonicum*, which is deposited in the multicellular stage, requires 10-12 days to embryonate and develop a miracidium, provides ample time for anti-embryonation immunity to act. The observation of no mature eggs in many of the sensitized mice suggests that anti-embryonation immunity may be the principal granuloma modulating mechanism in chronic infections.

An alternative strategy of altering disease susceptibility in cases of schistosomiasis by promoting suppressor T-cell mediated immunoregulation may not only be difficult to achieve but may also be associated with some changes. Defective anti-egg antibody responses or granulomatous encapsulation of eggs and sequestering of egg products may lead to a hepato-toxic effect, at least in schistosomiasis caused by *S. mansoni*.

We have yet to investigate the molecular basis of anti-embryonation immunity to determine how to: (1) identify the antigen responsible for its induction; (2) isolate and produce said antigen(s); and (3) determine how the immune response inhibits embryonation.

Inhibition of the 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 (and this seems certain to be the case with regard to schistosomiasis), then vaccination against severe disease may have a place in disease control. This is especially so if the strategy also leads to reduced transmission of infection in locations where nonhuman reservoirs of adult worms are not of epidemiological significance. Anti-embryonation responses directed towards immature eggs in the intestinal wall should lead to a reduction in the transmission of infection. Accelerated destruction of eggs should also reduce the export of eggs to the environment via the feces.

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MINIREVIEW

Evidence for and Implications of Anti-embryonation Immunity in Schistosomiasis

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The hypothesis that granuloma modulation and the disease abatement in chronic schistosomiasis japonica could be ascribed to relatively simple antibody-mediated effects on egg maturation and egg viability arose from studies performed in the Philippines on *Schistosoma japonicum* infections in mice. This novel hypothesis that was formulated more than a decade ago (Garcia and Mitchell 1982) has not yet been integrated into the schisto literature. One reason for this is that events proposed to account for this type of granuloma modulation may be confined to *S. japonicum*, even *S. japonicum* (Philippines).

Infections with *S. japonicum* (Philippines) differ in many respects from those of related trematode parasites, including *S. mansoni*, even though broad biological events in infected hosts show obvious similarities (Cheever 1987). From detailed studies of *S. japonicum* (Philippines) infections in mice, it is clear that this organism is a highly infective, highly pathogenic, and rapidly maturing parasite (that, as a schistosomule, does not linger in the lungs) (reviewed in Mitchell *et al.* 1991). Yet the prevalence of overt clinical disease in endemic areas of the Philippines is not obviously different from that in schistosomiasis mansoni-endemic areas of Africa and South America (Domingo *et al.* 1980). As has been demonstrated in naturally infected rats (Mitchell *et al.* 1990), *S. japonicum* (Philippines) worm burdens in humans presumably show an overdispersed distribution with the bulk of infected individuals being lightly parasitized. Moreover, the phenomenon of granuloma modulation is very obvious in *S. japonicum* (Philippines)-infected mice (Olds *et al.* 1982). All evidence points to this decrease in T cell-dependent granulomatous hypersensitivity to egg antigens being mediated by anti-egg antibodies (of unknown specificity) (Mitchell *et al.* 1991; Olds *et al.* 1982; Stavitsky 1987). Low worm burdens and reduced immunopathology will limit the extent of clinical disease in mammalian hosts.

The phenomenon of granuloma modulation in chronic schistosomiasis, first described by Andrade and Warren (1964), is clearly an immune-mediated event, like granuloma formation itself. From recent studies of the *S. mansoni*/mouse system, especially analysis of granuloma macrophages and Th1 or Th2 T-cell lines and clones (Flores-Villanueva *et al.* 1993), it is

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most likely that granuloma modulation, *in that system*, results from an interplay between cytokines.

In granuloma modulation in murine schistosomiasis japonica, we have focused on antibody effects on the source of immunopathologic antigens—the maturing eggs (literature cited in Garcia *et al.* 1992). The female worm deposits eggs, generally as clusters in *S. japonicum*, that mature (embryonate) over a period of 12 to 14 days in the tissues of the host. [As indicated above, *S. japonicum* (Philippines) is a rapidly maturing parasite (at least in rabbits and rodents) that commences egg laying at around 24 days of infection, with eggs appearing in the feces after Day 35.]

The underlying rationale for our anti-embryonation studies in the Philippines stems from the fact that schistosomiasis is a classical immunopathologic disease (Warren 1982), and the existence of immunopathology that is induced by eggs is a major deterrent to schisto vaccine development (cf. chemotherapy and chemoprophylaxis) (Mitchell 1994). The possibility of vaccine-induced sensitization in a small proportion of (genetically susceptible) vaccines with a vaccine that will probably be <100% effective is enough to deter vaccine developers, even those that include altruistic objectives in their corporate strategy. Any anti-disease component of a multicomponent schisto vaccine that is (and should be) aimed primarily at reducing or preventing (eliminating?) infection must be directed toward preventing immunopathologic antigens being produced. This can be achieved by induction of anti-fecundity immunity and/or through prevention of egg maturation (i.e., anti-embryonation immunity involving destruction of the egg long before it matures to an egg that contains a miracidium).

The first hint that immune-mediated maturation arrest or accelerated destruction of eggs might occur in *S. japonicum* (Philippines) infections came from our attempts to optimize the principal serological test used in the Philippines for diagnosis—namely, the circumoval precipitin/precipitation test (COPT), in which lyophilized eggs are incubated with sera for 24–48 h. The antigen–antibody reaction—in effect, an "aqueous Ouchterlony" reaction—develops over this time period and has the extraordinary appearance of a segmented exudate emanating from the surface of the egg. Notably, *S. japonicum* (Philippines) eggs have one or a very limited number of formed precipitates, perhaps indicative of a limited number of pores in the egg shell from which antigens emanate. Sera from recently infected individuals, some batches of eggs, and monoclonal antibodies reacting with eggs that otherwise give segmented precipitates all produce vacuolated precipitates (blebs) indicative of a restricted number of antigen–antibody interactions. Unembryonated eggs as found in the uterus of female worms do not produce COP reactions. Moreover, when eggs are harvested at various stages from infected rabbits for performance of the COPT, the quality of eggs decreases rapidly after 55–60 days of infection with an increased frequency of blebs rather than segmented precipitates. One of several possible explanations for these results could be arrested development (and early destruction) of eggs.

A Von Lichtenberg (1962) assay was used to demonstrate that fewer uterine eggs matured when injected into previously egg-sensitized mice compared with uterine egg maturation in unsensitized recipients. Moreover, granuloma formation in the lungs of egg-sensitized mice could be inhibited by *human* sera that produce large segmented precipitates, but not blebs, in the optimized COPT. Such human sera were also effective at inhibiting egg maturation in livers of infected mice. Antibodies in human sera might not be expected to engage efficiently in many immune effector mech-

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anisms in mice. Thus, through simply binding to antigen and occluding pores in embryonating eggs *in vivo*, antibodies may reduce metabolite export or nutrient uptake through pores in the egg shell, with subsequent maturation arrest and death of the maturing miracidium.

In an additional assay for anti-embryonation immunity, mice were infected with a low number of cercariae during a 5-week course of injections of live eggs enriched for immature eggs by harvesting from rabbits in the early stages of infection. When killed at about 6 weeks of infection, the egg-sensitized infected mice contained a lower proportion of mature eggs and a higher proportion of dead eggs in livers and intestines relative to mice not immunized with live immature eggs. Moreover, the mice had fewer granulomas around eggs in the liver, no splenomegaly, and reduced portal pressures.

In terms of the implications of anti-embryonation immunity for transmission dynamics in endemic areas, it is of interest that sex ratios of worms in experimental schistosome infections are often markedly skewed toward males (Liberatos 1987). *Oncomelania hupensis quadrasi* snails were collected from an endemic area in the Philippines (Sorsogon) and crushed to harvest cercariae and the parasites from single snails injected into single mouse recipients. The ratio of male infections to female infections in these mice can be as high as 6:1. In the *S. mansoni*/mouse system we have demonstrated that the population of miracidia obtained from liver eggs in infected mice becomes progressively enriched for male miracidia during the course of infection. Thus, miracidia harvested at 75 days of infection result in sex ratios of worms in secondary recipients of approximately 2, whereas this ratio with miracidia harvested at >100 days of infection is closer to 5 (Mitchell *et al.* 1990, 1991). These results require confirmation using simpler assays—e.g., *in situ* hybridization with sex-specific probes and eggs in short-term and long-term infected mice. It is possible that immune responses to eggs in a situation of modulated granulomas may be directed preferentially to female miracidia (W chromosome-encoded antigens?). If these are a richer source of immunopathologic antigens than eggs containing male miracidia, then reduced net granuloma size will be a consequence of their preferential destruction.

A further consequence of a decreased proportion of female cercariae in endemic areas will be that many vertebrate hosts might be exposed to male-only trickle infection. Although not reproducible in mice, such infections in a rhesus monkey/*S. japonicum* (Chinese) system have been shown by Vogel and Minning (1953) to lead to impressive resistance to subsequent mixed cercarial challenge. Low worm burdens in stable endemic situations, and a low frequency of obvious clinical disease, may thus be a reflection of anti-egg events in infected individuals.

Finally, antibody-mediated anti-embryonation immunity, if it exists in human schistosomiasis, provides encouragement to vaccine development in which an anti-disease *adjunct* to an anti-infection vaccine is sought. Promoting antibody production, or memory for antibody production, by way of vaccination in any genetic low responders (in terms of inducible granuloma-modulating responses), should be less demanding than promoting immunoregulatory T-cell responses.

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abled this productive Melbourne–Manila, "North–South" collaboration to address diverse aspects of the host–parasite relationship in schistosomiasis japonica since 1980.

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REVIEW ARTICLE

On vaccination against Schistosomiasis japonica (Philippines)

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For over a decade, two major research collaborations existed in the Philippines involving the UP Manila College of Public Health (CPH), the Research Institute for Tropical Medicine (RITM) of the Department of Health, and Australian and US investigators. The focus was on the development of vaccines against Schistosoma japonicum (Philippines). No schistosomiasis vaccine exists anywhere in the world and it is therefore appropriate to summarize progress to date and speculate on likely future developments.

It has been proven that good research that combines laboratory investigations and field work, and that has vaccination as an ultimate objective, can lead to numerous spinoffs such as knowledge relevant to existing forms of schistosomiasis control and even commercial products. This has certainly been the case for these research collaborations. For example, a key reagent useful in molecular biology and the production of vaccines in general, has emerged from research on an enzyme, glutathione S-transferase, of S. japonicum. This reagent, known as the "pGEX expression system" and marketed by Pharmacia is finding wide application among researchers and vaccine producers.

Chronic schistosomiasis japonica is an immunopathologic disease in which major manifestations are the consequence of immune responses to eggs of the parasite. These eggs are trapped in organs and tissues, particularly liver and intestines, but also in the lungs and the brain. Antigens released by trapped eggs stimulate inflammatory immune responses that lead to granuloma formation and fibrosis. Such reactions in the liver lead to portal hypertension with resultant hepatosplenomegaly, esophageal varices, etc. Because schistosomiasis is an immunopathologic disease, great care is required to ensure that the eventual vaccine does not increase the severity of immunopathologic responses to eggs in some vaccinees. Safety is a major consideration in schistosomiasis vaccine development.

A vaccine to prevent schistosomiasis could operate to prevent infection or prevent disease. Much has been written about both of these approaches to vaccination against human schistosomiasis based on data from experimental animals and clinical observations. It can be difficult for the non-specialist to distill out of the literature that which is likely to be relevant to the vaccine objective in humans as distinct from an interesting (or uninteresting!) laboratory phenomenon unlikely to apply to, or be applied in, human vaccination.

One successful means of vaccinating against infection with certain veterinary schistosomes and Schistosoma mansoni in animal models is to expose hosts to irradiated cercariae. Cercariae can be obtained from infected snail intermediate hosts. These attenuated, yet living, cercariae do not develop into adult worms but induce high level protection against subsequent challenge infection. Notwithstanding these successes, there has been no demonstration that the approach works with S. japonicum (Philippines). In the S. mansoni/mouse model it has been shown that inflammatory responses mediated by T-cells in the lungs are important for expression of resistance after exposure to irradiated cercariae. It may be relevant that schistosomules (that develop from skin-penetrating cercariae) of S. japonicum (Philippines) seem to pass very quickly through the lungs thereby allowing the parasites to escape an immune attack in vaccinated mice.

Mice infected with S. japonicum can be highly resistant to re-infection. However, this resistance has more to do with severe liver pathology and portal

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system changes in the infected mice such that incoming challenge parasites encounter a physiologically-hostile environment rather than an immunologically-hostile environment (a more relevant situation to the vaccine researcher). Analysis of this type of resistance in mice has therefore not resulted in any important leads in vaccine research.

Strains of inbred mice differ in susceptibility to first infection. An analysis of the immune responses differentially induced in a resistant mouse strain has lead to the identification of particular parasite enzymes: the glutathione S-transferases (GSTs) as candidate vaccine molecules. However, the mouse strain that showed impressive resistance to first (or repeated) infection and high anti-GST responsiveness, was again shown to have portal system peculiarities that militated against parasites homing to and maturing in their preferred location - the portal system and liver. The contribution of anti-GST responses to resistance in this mouse model remains unproven and, indeed, vaccination of suspectible strains of mice with purified worm GSTs and recombinant GSTs produced from bacteria have been largely unsucessful. Interestingly, the GSTs have been proposed as strong candidates for an S. mansoni vaccine.

High and low apparent resistance to reinfection in human after praziquantel treatment and analysis of immune responses in relatively resistant versus susceptible individuals should identify antigens (and thus vaccine candidates) better recognized by the resistants. Results to date have been uninformative and no particular antigen has been identified as a vaccine candidate. It is possible that particular broad classes of immune response - e.g. antibody isotypes or T-cell subpopulations responding to multiple antigens, rather than particular antibody or T-cell specificities to a single antigen, are the basis of resistance versus susceptibility to reinfection after drug cure of existing infection in individuals living in endemic areas with repeated exposure to infection. This would not be an encouraging result for the development a molecular vaccine but by no means a fatal blow either.

Various antigens isolated from schistosomes and/or produced by recombinant DNA methods in bacteria have some promise as prototype vaccines in animal systems. These include surface and internal proteins of schistosomules and adults like the GSTs, paramyosin, molecules well-recognized by mice given irradiated cercariae, and many others. Although many show only low levels of protective immunity, it is disappointing to record that no one has shown better protection using a mixture of antigens all of which when individually tested have some protective effect. A limited number of the molecules show definite promise (e.g. 70% protection in animals) although the ultimate test of efficacy in humans is some time off and some restraints are imposed by the fact that many are related to proteins present in humans. Indeed, it is not a straightforward decision to make on just what group of individuals will be best suited to clinical trials of a prophylactic schistosomiasis vaccine. Vaccination of informed older individuals cleared of infection by praziquantel treatment and at high risk of reinfection may be worthy of careful consideration. It is important to emphasize that, in schistosomiasis, a vaccine need not be 100% effective to be useful in a public health sense a reduced worm burden will lead to reduced disease. Additionally, schistosomiasis japonica vaccines, unlike schistosomiasis mansoni vaccines, can be evaluated and used in relevant animal hosts such as carabaos, pigs, dogs etc.

Consideration of the issues that were raised above and the regulatory and technical complexities of vaccine development and production leads to the valid conclusion that adequately effective schistosomiasis vaccines designed to reduce or prevent infection will not be available in the immediate future. This also applies to vaccines aimed to preventing or reducing severity of disease. A disease-preventing vaccine may be designed to inhibit egg production by the worms or to accelerate egg death particularly early after oviposition by the female worm and prior to production, by the developing (or embryonating) miracidium inside the egg, of those antigens that stimulate immunopathology. This latter concept of "anti-embryonation immunity" has been pioneered in the Philippines and is worthy of increased research effort particularly if safe, effective and approved prophylactic vaccines are slow to come on stream. An anti-embryonation vaccine would reduce disease, reduce transmission in any area where animal reservoirs are unimportant, and might be useable in both infected and uninfected individuals in an endemic area. Anti-embryonation immunity is an attractive hypothesis to account for the granuloma size decrease and disease abatement seen in chronic schistosomiasis. And, as an hypothesis, it has been developed in the S. japonicum/mouse model. However, no mechanism has been identified other that it is likely to be mediated by antibodies. Identification, characterization and recombinant DNA work on target antigens in immature embryonating eggs are currently in progress. Again, this approach to a schistosomiasis vaccine is still a long way from human application.

Because of the commercial realities of the Western biopharmaceutical world, the countries that stand to benefit most from human parasite vaccines - the less industrially-developed nations of the tropics - will need to become lead players in the development and manufacturing of such vaccines. This is a big challenge, bearing in mind the infrastructural deficiencies in many developing countries. A type of "cottage industry approach" may be worth considering with technical assistance from vaccine manufacturers

of the industrialized world and financial assistance from aid agencies. Because of the costs and time frames involved, an established commercial organization will rigorously scrutinize any R&D project and assess it according to technical feasibility (risk), commercial attractiveness, and strategic fit. Human parasite vaccines do not usually fare well in such an assessment. Thus alternative strategies are required if parasite vaccines are to become a reality in the next decade and there is scope for "assisted initiative" on the part of endemic countries. An upgrading of local skills in vaccine manufacture, regulatory affairs, clinical trials and intellectual property matters is also required urgently.

It can be rightly inferred from this discussion that schistosomiasis control in the Philippines will be dependent on chemotherapy, health education, snail control and environmental sanitation for quite some time to come. Vaccines are still at the experimental stage though the need for them will become urgent with the first evidence of praziquantel resistance in S. japonicum (Philippines). Continued research that combines "the bench, biotechnology, the bedside, and the bush" is thus fully justified in order to increase future options for schistosomiasis control. Philippine investigators must ensure that they are in the driver's seat on this long journey.

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