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"GROUNDWORK FOR DEVELOPMENT OF VACCINE TO PREVENT HEPATO-SPLENIC DISEASE IN SCHISTOSOMIASIS JAPONICA"

5.002

TERMINAL REPORT
(April, 1988)

Date of Start of Project: September, 1985

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OBJECTIVES

The primary objective of this project is to prove the validity of ur hypothesis that "antibodies of certain specificities or isotypes can nhibit embryonation (anti-embryonation immunity) or cause early destruc-ion of S. japonicum eggs deposited in tissues to cause modulation of ranuloma formed (around the eggs) and prevent severe hepatosplenic isease". This disease modulating effect of anti-embryonation immunity s due to the failure of the egg to mature and become producer of immuno-athologic antigens responsible for T-cell dependent granuloma formation.

This is in accordance with the provisos of the approval:

"Approval only for two years (if necessary, three) only in order to test basic hypothesis and determine what role is played by anti-granuloma or anti-embryonation antibodies. Once the groundwork has been completed and received, a follow-up proposal would be appropriate".

"Please submit revised workplan and budget. Recommend deletion of hybridoma work and recombinant DNA work at this time".

Proof of our hypothesis will provide an immunologic mechanisms which will serve as basis for developing a vaccine to modulate granuloma formation and disease in schistosomiasis japonica.

II. BACKGROUND AND RATIONALE

In many, if not most of the countries where schistosomiasis is endemic, programs to eliminate or control schistosomiasis or reduce its transmission are ineffective in view of various constraints including financial. This dictates a need for immunologically-based intervention measures like vaccination to prevent establishment of infection or if this is long in coming, vaccination to reduce or modulate morbidity or disease and/or anti-transmission.

Clearly, inhibition or prevention of establishment of infection is a more desirable consequence of vaccination than inhibition of disease (but consider tetanus and diphtheria immunoprophylaxis). If the former is difficult to achieve (the immune evasion mechanism of human schistosomes are not well understood) through conventional vaccination, and such is still long in coming, then immunoprophylaxis against severe pathology has a place in schistosomiasis control (Garcia EG & Mitchell GF, 1982; 1985). This is particularly so if the vaccination strategy like one based on anti-embryonation immunity (our hypothesis) also leads to transmission control. Anti-embryonation immunity should lead to reduced export of mature eggs into the environment and thus contribute to transmission control.

Chronic schistosomiasis japonica is an immunopathologic disease in which many of the clinical manifestations result from production or formation of granuloma and fibrosis due to T cell dependent immune responses to

antigens emanating from eggs entrapped in the liver (Warren KS, 1973; Pelley RM & Warren KS, 1978). Increased portal pressure, hepatosplenomegaly, collateral circulation and ascites are readily ascribed to obstructive granuloma formation around eggs and subsequent fibrosis with impedence to blood flow through the liver.

A major development in schistosomiasis research was the recognition that granuloma formation in chronically infected or sensitized hosts or animals was modulated, i.e. the granulomatous hypersensitivity was reduced (Domingo EO & Warren KS, 1968; Warren KS, 1977; Garcia EG et al., 1983). From the very first observation of this phenomenon, it was appreciated that immunization to promote modulated granuloma response could lead to reduced likelihood of severe hepatosplenic disease. Modulation of granuloma formation could result from several mechanisms operating singly or in combination (Garcia EG & Mitchell GF, 1982, 1985). These are: 1) reduced embryonation or maturation of eggs entrapped in tissues due to anti-embryonation immunity (Garcia EG et al, 1983; Garcia EG & Mitchell GF, 1985; Garcia EG et al, 1985); 2) suppressor T-cell mediated inhibition of anti-egg responses presumably by inhibitory effects on T_d cells or their products responsible for immunopathologic reactions (Green WF & Colley DG, 1978; Philipps SM et al, 1980; Chensue SW et al, 1981); 3) accelerated destruction of mature eggs subsequent to the formation of a miracidium, the maximum immunopathologic antigen producing stage (James SL & Colley DG, 1976; Olds GR & Mahmoud AAF, 1980); and 4) antibody mediated inhibition of anti-egg responses either by diversion of antigens away from T_d cells (opsonizing antibodies) or inhibition of antigen recognition by T_d cells (e.g. anti-idiotype antibodies).

1. Demonstration that mature embryonated eggs of S. japonicum, not immature eggs, are the more important source of immunopathologic antigens for granuloma formation.

Although a review of the literature show that mature and not immature eggs are the major source of antigens for granuloma formation (Hamburger J et al, 1976; Hang LM et al, 1974) it was important that this was also true for our S. japonicum-mouse models. Our hypothesis for granuloma modulation is dependent on prevention or inhibition of maturation or embryonation of immature eggs deposited in the tissues which prevent their becoming rich exporters of immunopathologic antigens.

Mature eggs recovered at day 50 of infection and immature eggs recovered at day 27 of infection by digestion of the livers rabbits with pepsin and trypsin (Smithers SR, 1960; Garcia EG et al, 1981) were injected intravenously into separate sets of BALB/c mice previously sensitized for accelerated granuloma formation (four weekly injections of mixture of mature and immature eggs). The intravenously injected eggs are filtered in the pulmonary micro-vasculature so that by examination of the lung sections, the granuloma response to each egg can be visualized. On D+4 after egg-challenge the mice were killed, the lungs isolated, serially sectioned and stained. Granuloma in the lung sectioned are then measured using a system based on or an adoptation of that devised by von Lichtenberg (1962).

In the 4 mice challenged with immature D+27 eggs; 44 to 53% of the eggs had no reaction or granuloma while in the mice (5) challenged with mixture of mature and immature eggs only 23 to 35% of the eggs had no granulomatous reactions. This indicates maturing immature eggs are poorer

exporter of antigens for inducing granuloma formation.

It should be mentioned that in mice S. japonicum starts laying eggs between D+24 and D+27 of cercarial penetration (Pesigan TP et al, 1958). The immature egg require 10 days to embryonate with formation of a miracidium. Thus it is possible that some of the immature eggs were already 3 days old at the time of injection into the mice and consequently 7 days old at the time the lungs were sectioned for granuloma assay. This may also indicate that the 7-day old eggs already export some immunopathologic antigens.

2. Inhibition of maturation or embryonation of S. japonicum eggs in infected mice by injection of sera from humans with chronic infection.

This study was undertaken to determine if anti-embryonation immunity is induced in humans with chronic infections.

Sera obtained from humans with clinically defined chronic S. japonicum infections and classified by the nature of the precipitates formed when reacted with S. japonicum eggs in the standardized circumoval precipitin test (COPT) were injected to BALB/c mice infected from cercariae. Injection volumes and days of injections were as follows: day 24, 1.5 ml; day 27, 0.75 ml; day 29, o.5 ml; day 31; 0.75 ml; day 34, 0.75 ml; day 36, 0.5 ml and day 38, 0.75 ml. The start of injection at day 24 coincides with the earliest period that S. japonicum oviposits in mice. Control mice were injected 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 serums. As stated earlier S. japonicum starts ovipositing from day 24 to day 27 after cercarial infection. The eggs need 10 days to mature or embryonate and will survive after embryonation for another 10 days.

The mice were perfused with citrated saline to flush out worms from the portal system and then the intestine and liver removed. The intestines were cut along their length and further cut into segments which were pressed between two slides for counting the egg cluster and determination of their state of development. Three sections from each lobe of the liver of each mice were also examined. Immature eggs were in multicellular stage; mature eggs had intact miracidia while dead eggs had a black tinge and contained dark granules.

Tables 1 and 2 show the number and maturity of eggs in the livers and intestines of mice injected with human sera. As seen in these tables there is definitely significant inhibition of maturation of eggs in the infected mice injected with sera from humans with chronic schistosomiasis japonica. This is evidenced by the much higher ratio of immature eggs to mature eggs in the livers and intestines of the mice that received COPT positive sera. This indicate that some serum factor probably antibody, inhibited maturation (of the eggs). It is worthwhile mentioning that the same sera caused modulation of granuloma in mice previously egg-sensitized for accelerated granuloma formation.

The data presented provided very definite encouragement for pursuing vaccination against severe disease using antigens from immature eggs to induce anti-embryonation immunity.

Table 1: Maturity of Eggs in Livers of BALB/c Mice Injected with *Schistosoma japonicum** and Injected with Human Sera Between Days 24 and 38 After Infection.

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

* Numbers of adult worms in mice Nos. 1 to 5 were 4,4,4,4 and 2 respectively (mean ± SEM = 3.6 ± 0.4) and in mice Nos. 6 to 10 were 8,6,4,3 and 4 respectively (mean ± SEM = 5.0 ± 0.9).

** $p < 0.05$, Mann-Whitney U-test.

Table 2. Maturity of Egg Clusters in Intestines of BALB/c Mice Infected with *Schistosoma japonicum* and Injected with Human Sera Between Days 24 and 38 of Infection (same as in Table 2).

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

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

3. Evidence of anti-embryonation immunity and egg destruction in mice sensitized with immature eggs of S. japonicum.

These experiments were undertaken to determine if anti-embryonation immunity can be induced by vaccination or sensitization with viable immature S. japonicum eggs.

Mice were sensitized by injection of viable intact immature eggs recovered by digestion of livers of infected rabbits killed from D+25 to D+29 of infection. The eggs were injected into mice on the same day they were obtained subcutaneously and with an equal number (of eggs) intraperitoneally. In experiments 1 and 2 five weekly injections of 10,000 eggs were given to mice and infected with 4 cercariae 1 and 4 days respectively after the second injection of eggs. In experiment 3, the mice had 23 injections of a mixture of mature and immature eggs at 1 to 3 weeks intervals followed by 3 weekly injection of whole immature eggs. They were infected with 4 cercariae after the last injection.

Egg-sensitized mice and unsensitized controls were killed at day 40 to 42 of infection. The entire small intestine and 3 samples of the liver were taken for assessment of egg development. The liver samples were 5 mm portion from the inferior or lower border of each of the three lobes of the liver. The tissue were pressed between microscope slides. In case of eggs in the intestinal wall, many were in clusters. Thus, determination were made on single eggs or on clusters. Immature eggs were in multicellular stage with yellowish tinge, dead eggs had dark granules and a blackish tinge, mature eggs had intact miracidia.

In the analysis of data, all mice that had no eggs in the liver and intestines (i.e. 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 out of 53 mice in the 3 experiments.

The results of the three trials are presented in Table 3 and Figure 1. Several aspects of the data are very notable.

TABLE 3. Summary of data from three experiments on the numbers of eggs and their state of development in the liver and intestines of BALB/c immunized with immature eggs of Schistosoma japonicum and challenged with cercariae 40 to 42 days before sacrifice.

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

* Only mice with > 50 eggs in the liver sample or intestines were included in the analysis.

\+ Eggs in intestines of unsensitized mice were invariably counted as cluster; mixed clusters of mature and immature eggs being included in the mature category; in sensitized mice, approximately 50% of determinations were on single eggs rather than clusters.

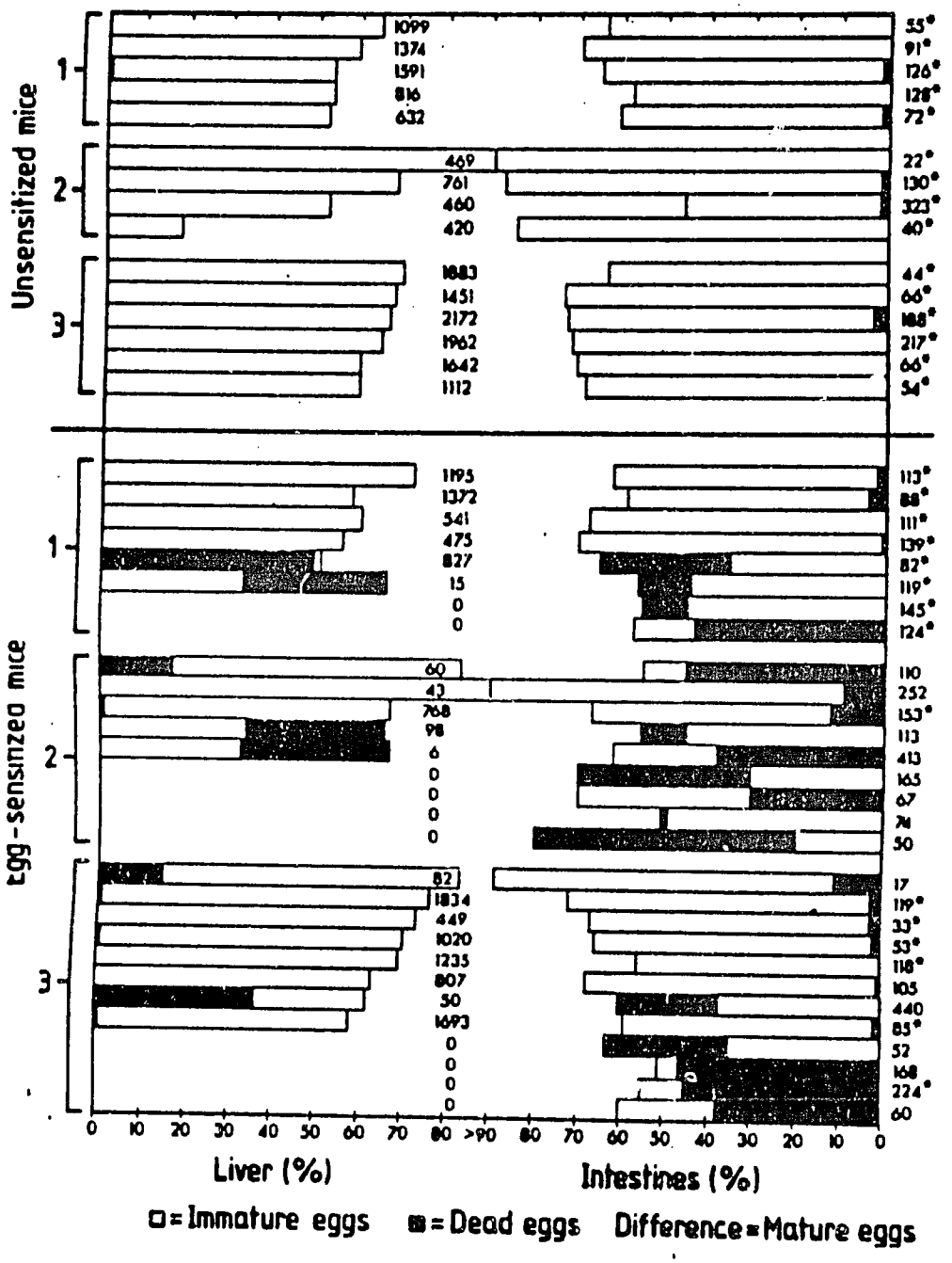

Fig. 1 Percentages of immature (open bars) and dead eggs (closed bars) in liver and intestines of normal mice or mice immunized against immature eggs, challenged with cercariae of *S. japonicum*, and killed at day 40 – 42 of infection. See Materials and Methods for differences in protocol between experiments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

a) Ten of the 29 immunized or egg-sensitized mice (Figure 1) had no mature eggs both in the liver and intestines although they had both dead and immature eggs. In contrast all of the 15 unsensitized mice had mature eggs.

b) There is a very obvious increase in dead eggs (Figure 1) particularly in the intestines in most of the immunized mice.

c) When expressed as a ratio (Table 3), the percentage of immature and mature eggs is clearly shifted to a deficiency of mature eggs in tissues of egg-sensitized mice.

d) Additional indications that the fate of eggs in egg-sensitized is affected come from peripheral observation. The number of mice with eggs in the intestines (and therefore infected) but with no eggs in the liver was approximately 40% of the egg-sensitized group but was zero in unsensitized group.

The observations demonstrated that a high proportion of mice sensitized by repeated injections of viable immature eggs and challenged with low numbers of cercariae were able to restrict the number of mature eggs in the liver and intestinal wall.

The fact that 10 of the infected egg-sensitized mice had no mature eggs is highly suggestive that the eggs were killed before maturation. If eggs were killed before maturation, then it would be most probable that secretion and excretion of the immature and maturing eggs were responsible for the induction of anti-embryonation immunity. However, it is difficult to exclude killing of eggs at completion of maturation.

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 also of intestinal pathology.

4. Effects of induction of anti-embryonation immunity on liver granulomas, spleen weight and portal pressure in infected mice.

This experiment was undertaken to determine if mice sensitized for the induction of anti-embryonation immunity (prior to infection) will develop hepatosplenic disease after infection.

In this study BALB/c mice received five weekly injections of 10,000 viable immature eggs by subcutaneous and intraperitoneal routes. On the 10th day of the sensitization regimen, the mice were infected with 4 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 spleen weight.

The infective dose of 4 cercariae ensures that each mice will have at least a female worm. A single pair of S. japonicum is enough to induce hepatosplenic disease (Warren KS and Berry EG, 1972).

The mice were injected with heparin and anesthesized with nembutal. The abdomen was opened, the portal vein isolated, the venous pressure,

expressed in millimeters of water was determined by inserting a winged infusion set connected to a 3-way valve attached to a serologic pipette with a millimeter scale. The solution used for determining portal pressure was heparinized physiologic salt solution. After determination of the portal pressure the mice were perfused (to recover worms) and the spleen isolated and weighed. Spleen weight was expressed relative to the total body weight of the particular mouse.

The entire intestine were isolated and divided into segments which were pressed between two slides for counting eggs and determination of their state of development.

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 which was used for calculating granuloma value and amount of reaction. "Granuloma" were classified as having no reaction (0), minimal reaction ($\pm$) (i.e. size 1 to 3 x 10^{-4} mm^3) and usually involving a single to two layers of inflammatory cells) and positive (+)(i.e. size greater than x 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 and portal vein pressure and relative spleen weight is presented in Figures 2, 3 and 4 respectively.

The very notable aspects of the observations are:

a) There were more (61 to 87%) dead eggs in the intestines of the immature egg-sensitized mice group. In contrast there were fewer dead eggs but greater proportion (40-64%) of mature eggs in the unsensitized group (Figure 2).

b) The egg-sensitized mice much higher (61%) number of eggs without granulomatous reaction in contrast with the unsensitized controls where only 8% had no reaction ($p < 0.001$)(Figure 3).

c) 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).

d) The mean relative spleen weight in the unsensitized group is much greater ($p < 0.001$) than that of the sensitized group.

e) The portal vein pressure and spleen weight of the egg-sensitized were comparable to normal uninfected mice of similar age (Figure 4).

The above observations have demonstrated that the induction of anti-embryonation immunity prior to infection, through sensitization by injection of viable immature eggs, have resulted in reduced number and size of granuloma in the liver of the infected mice with prevention of portal hypertension and its consequences like splenomegaly. The number of adult schistosomes from 4 cercariae, extrapolated for a human being of 60 kilograms would mean a worm burden of 3,000 flukes which is much higner than the usual worm burden in infected human.

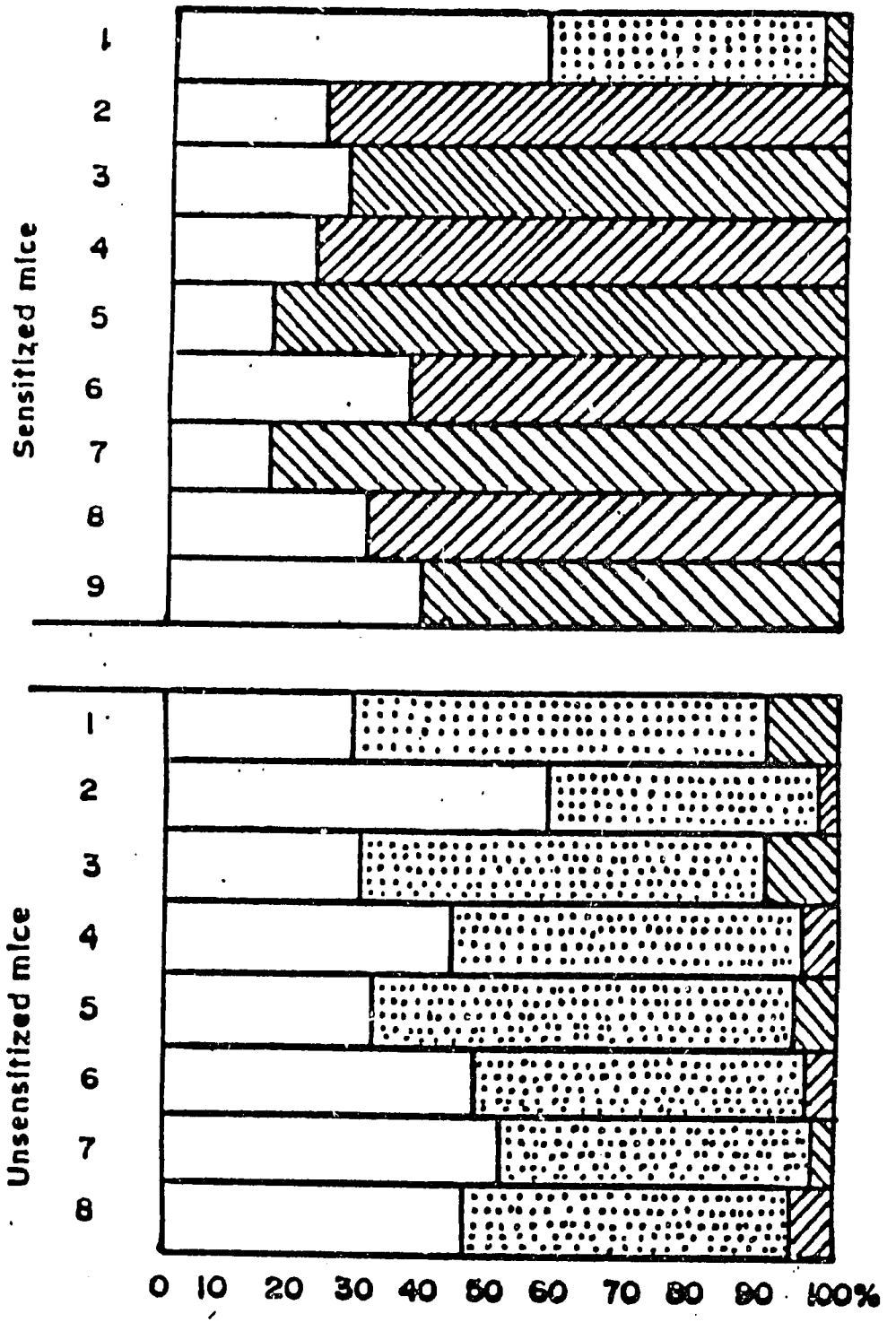

Fig. 2. Percentage of immature (open sections), mature (dotted sections) and dead eggs (cross-hatched sections) in intestines of egg-sensitized and unsensitized mice at D+75 of infection. Only mice with > 50 eggs or egg clusters in the intestines are included in the analysis, most clusters being confined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

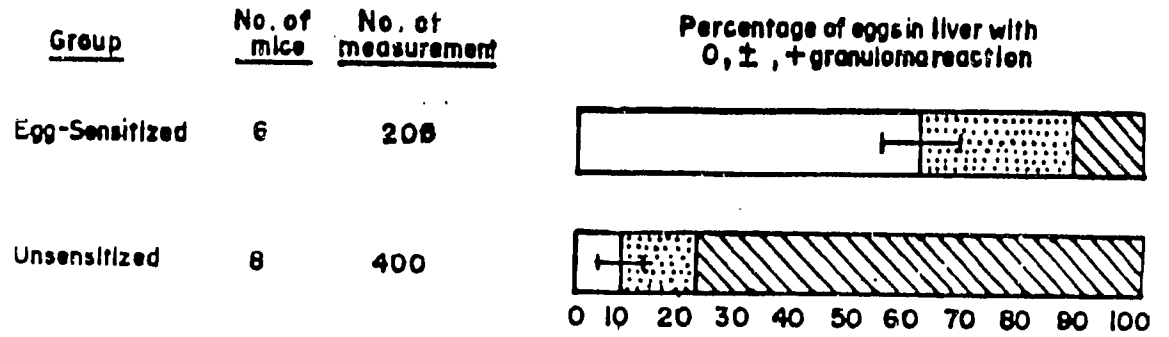

Fig. 3. Percentage of eggs in liver with no (open section, with SEM), minimal (dotted section) and obvious granulomatous reactions (cross-hatched section). A minimal reaction is a granuloma of 1-2 layers of cells while an obvious reaction consists of 3 or more layers of cells around the egg. Only mice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

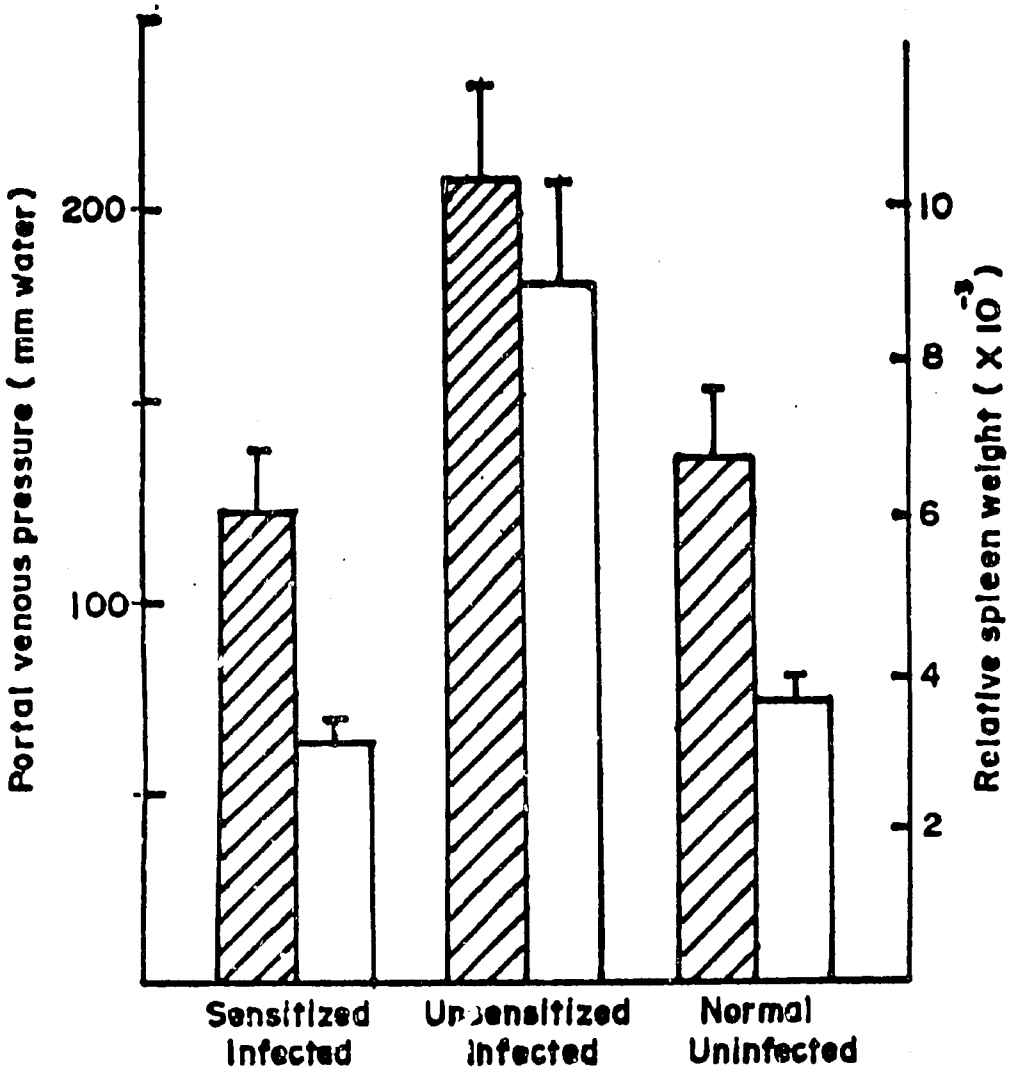

Fig. 4. Portal venous pressure (cross-hatched bar) and relative spleen weight (open bar) of infected egg-sensitized, unsensitized infected mice and uninfected controls at D+75 of infection. SEM are indicated.

5. Effect of induction of anti-embryonation immunity on susceptibility to infection.

This experiment was done to show that induction of anti-embryonation immunity does not increase susceptibility to infection.

Mice were sensitized for anti-embryonation immunity following the regimen of injection with viable immature eggs described earlier. The sensitized mice were infected with 25 cercariae at the same time a similar number of unsensitized mice were also infected. At day 32 of infection the mice were killed and the portal vein perfused to recovere adult fluke followed by detailed dissection of the mesentery and intestines for the recovery of remaining flukes.

In the 10 egg-sensitized mice the flukes recovered ranged from 8 to 25 with an average of 17.8 fluke per mice. In the unsensitized group the flukes recovered ranged from 14 to 21 with an average of 17.4 worms. We inferred that this indicate that induction of anti-embryonation immunity does not increase susceptibility to infection. Consequently, in so far as infection is concerned, vaccination to induce anti-embryonation immunity would be safe.

III. DISCUSSIONS

The series of observations herein reported provide unequivocal evidence that anti-embryonation immunity is induced by sensitization with immature viable eggs and the restriction or inhibition of maturation or embryonation of eggs results in lesser number and smaller granuloma in the liver which in turn prevented increase of portal venous pressure and

splenomegaly. It should be stressed that the worm burden resulting from infection with 4 cercariae is equal to 3,000 adult worms when extrapolated for a human being of 60 kilograms. Warren equates in mice, to 10,000 flukes in humans. Both calculations are way above usual estimated worm burden of infections in endemic areas indicating that an adequately developed anti-embryonation vaccine will protect most human infections from developing hepatosplenic disease.

Inhibition of maturation of eggs also cause amelioration of intestinal pathology as previously reported by Warren.

A very important implication of vaccination to induce anti-embryonation immunity, is reduced export of mature eggs with the feces. This will decidedly contribute to transmission control.

The next step in the development of an anti-disease vaccine in schistosomiasis based on anti-embryonation immunity is the isolation and identification of antigens of immature eggs that are targets of aggressive immune attack. Batteries of monoclonal antibodies to immature eggs will be useful as well as cDNA libraries if target antigens are proteins. The indications that eggs are killed before maturation point to excretions and secretions of the maturing immature egg as the antigens responsible for the induction of anti-embryonation immunity.

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