ANAEMIA IN SCHISTOSOMIASIS WITH PARTICULAR REFERENCE TO ITS CAUSATION

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ABSTRACT

The clinical picture of schistosomiasis and its symptomatology of anaemia represent an important health problem in parts of the tropics. The multiple factors responsible for production of anaemia are reviewed. In this work an attempt was made to answer the questions raised by these earlier investigations. Particular attention was given to the question whether haemolysis involved in pathogensis of anaemia is due to an immunological process.

Haematological changes were studied in mice infected with S. mansoni. It was found that animals infected with light doses of cercariae developed progressive anaemia, reticulocytosis and enlarged spleen. Radioactive studies showed considerable shortening of the erythrocyte life span, associated with destruction of these cells in the spleen. These findings confirmed that the anaemia was of a haemolytic nature.

The main mechanism responsible for the severe haemolysis during the subacute and chronic stages of infection (22 - 80 weeks) was found to be immunological. Evidence for this included the serological abnormalities, i.e. positive direct agglutination test, slight elevation of serum gamma globulins, and the presence of similar globulins in eluates from pooled red cells of infected mice, thus indicating that the red cell membrane was coated with immune complexes. In addition "holes" on the surface of the erythrocytes taken from a number of infected mice were also seen ultrastructurally. This finding suggests that complement fixation was involved in the immunological process.

The other possible mechanism for haemolysis, was that worm/egg toxins either lyse red cells directly or through the damaged tissues. It was postulated that this factor might play a role before the immune process took place.

Evidence was obtained that the following were contributory factors aggravating the anaemia. a) Non-continuous blood loss in animals heavily infected. b) Phagocytosis of damaged red cells inside the enlarged spleen.

In the present study it was also shown that at later stages of infection along with severe anaemia, progressive glomerulonephritis was produced in mice infected with light doses of cercariae. An immunological mechanism for induction of the renal damage is postulated.

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PART I

INTRODUCTORY CHAPTERS

CHAPTER I

INTRODUCTION

Infection with different species of schistosoma cercariae is widespread in tropical and subtropical countries; for instance 14 million out of Egypt's total population of 30 million are infected with either S.haematobium or S. mansoni, or by both species (Farooq, 1967).

Many patients infected with S. mansoni develop anaemia; this state of health could lead to suffering of the patients as well as disastrous results on the economy of the country, especially those countries which depend most on the people affected with schistosomiasis, that is the farmers who live in villages that are in endemic areas.

The importance of anaemia in schistosomiasis leads to the question being asked "What is, or are, the causes of anaemia in schistosomiasis?" Throughout recent years much research work has been concerned with the mechanism of anaemia in schistosomiasis; but there are still many gaps to be filled, and more questions have arisen from previous research work.

It is an anaemia of complex and different causes and in the following chapters an attempt is made to answer some of the causes.

The questions concerned with causation of anaemia and how they were investigated in mice experimentally infected with S. mansoni. are summarized in the following points:

I- Is anaemia due to blood loss?

Haemorrhage through the gastrointestinal tract was measured in stools of control and infected mice after injection with ^{51}Cr tagged red cells.

2- Is anaemia of a haemolytic nature?

The ^{51}Cr and osmotic fragility methods were used. Also by the first method the role of spleen in the development of anaemia was studied.

3- Does anaemia result from immunological reaction?. Or during infection are red cells coated with immunoglobulins and complement?

The agglutination and elution tests were used to prove presence of globulins on the surface of red cells and if so the haemagglutination was applied to study the nature of the globulins.

4- Do toxins from either adult schistosomal worms and their eggs, or damaged infected tissue (liver and spleen) have a direct haemolytic effect on the red cells?

In vivo studies were applied to see if anaemia develops in normal animals injected with these toxins separately. Also in vitro studies (blood agar plate method) were used to prove that these toxins have a haemolytic activity.

CHAPTER 2

REVIEW OF LITERATURE

Schsitosomiasis was reported as a common cause of anaemia by Day (1911); Hutchinson (1928); Greany (1952); and Saif (1959). It was of a microcytic-hypochromic type in patients infected with S. mansoni (Molina et al, 1936), sometimes normocytic (Kenawy, 1958). On the other hand Cutinho (1956) described anaemia as macrocytic. Anaemia was produced in mice experimentally infected with S. mansoni. by DeWitt (1959), and in monkeys infected with S. japonicum by Vogel and Minning (1953).

The most important factors that might contribute in the production of anaemia in schistosomiasis are reviewed below:

(1) Blood loss:

In a parasitological review; Foy & Nelson (1963) concluded that in the early stages of S. mansoni infection blood loss in combination with toxaemia results in a hypochromic anaemia. Sabour et al (1967) considered chronic faecal blood loss as one of multiple factors that could account for the anaemia in patients with Egyptian splenomegaly.

(2) Haemolysis and anaemia:

Haemolytic mechanism was proved to play a part in anaemia of patients infected with S. mansoni. by the reduction of their erythrocyte life span; which was associated with evidence of intrasplenic destruction of erythrocytes as revealed by increased surface radio-activity over the spleen (Woodruff et al, 1966). The role of the spleen in erythrocyte destruction and anaemia was explained by three theories:

- Sequestration of erythrocytes inside a hyperactive spleen (congestive splenomegaly) was suggested by Jamra et al (1964).

- Erythrostasis: Doan (1949) postulated that the sequence of events in hypersplenism might be: abnormal erythrocyte stasis within splenic sinuses with increased mechanical intercellular friction; loss of erythrocyte

potassium; leading to increased fragility; pathological concentration of lysolecithin, i.e. an ideal environment for the establishment of a vicious cycle of cell withholding and destruction. This theory of erythrocyte pooling accompanied with hypervolaemia was applied by Sabour et al (1967) to explain partly the cause of anaemia in Egyptian splenomegaly.

- An inhibitory splenic hormone may induce the bone marrow to produce short life spanned red cells or cells more susceptible to phagocytosis was suggested by Shafei et al (1972).

(3) Immunological mechanism:

The evidence of immunological events produced by antigen-antibody reactions in vivo was well documented in human schistosomiasis mansoni infection. Eggs, cercariae, worms, and worm secretion-excretion products stimulate antibody production (Kagan and Norman, 1963), and an increase in sera immunoglobulins (antibodies) of patients infected with S. mansoni was demonstrated by Antunes et al, 1971. The effect of these immunological reactions on the development of anaemia was not fully investigated. However, Woodruff (1973) drew attention to the importance of an immune process as the cause of anaemia in a wide variety of infective diseases i.e. Kala-azar; Trypanosomiasis; malaria and schistosomiasis. This process involved the development of an immune complex on the red cell surface which binds complement (C'_3, C'_7, and C'_8) and lead to red cell destruction by lysis or phagocytosis in the spleen or other reticuloendothelial systems.

Dacie (1962) advanced two main hypotheses for the development of auto-immune haemolytic anaemia, I) an alteration in the erythrocytes which has the effect of making them seem "foreign" or "not self" to the antibody-forming tissues thus antigenic. 2) development of anti-erythrocyte antibodies due to an unusual responsiveness or intrinsic activity of the antibody-forming tissue. Both hypotheses were used to elucidate in part the mechanism of anaemia in schistosomiasis. Kurata (1966) demonstrated

circulating auto-antibodies in sera of rabbits infected with S. japonicum against rabbit erythrocytes coated with liver or colon extracts "autoantigens". Also Mahmoud (1971) proved by a positive antiglobulin test that there was an "alteration" of the antigenicity of erythrocytes from anaemic mice infected with S. mansoni.

More work is needed to investigate the nature of antibodies (auto-antibodies?) if present/and whether the complement is fixed to the immuno-complexes on red cell surface in schistosoma infection.

(4) Toxins and anaemia:

The direct haemolysis of red cells due to toxic substances from worms and eggs of the schistosoma parasite were mentioned very cautiously and vaguely in literature. In other diseases e.g. malaria, the existence of a lytic factor from the parasite correlated with production of anaemia was acknowledged on a hypothetical basis (Maegraith, 1969).

Schistosome toxins might also induce the bone marrow to form fewer red cells in anaemic mice infected with S. mansoni as suggested by Nasser and Platt (1967).

From this review it appears that anaemia in schistosomiasis might be produced either by one or a combination of several causes, hence the following studies were undertaken to throw more light on these causes.

CHAPTER 3

PARASITOLOGICAL METHODS

In experimental infection with S. mansoni it is of importance to chose a suitable host that produces comparable if not similar lesions to those of human patients; accordingly mice were used for the following studies because:

- Mice infected with S. mansoni developed symptoms that closely resembled those in human patients, namely hepatomegaly; splenomegaly; esophageal varices; ascites, and anaemia (DeWitt and Warren, 1959).
- Mice are easy to handle and can be kept in the laboratory under constant observation.
- They survive for a considerable period of time, hence the opportunity to study anaemia at later stages of infection.

Tisilius original strain of male mice (weight 18 - 22gm.) were infected with the Egyptian-Wellcome strain of S. mansoni cercariae; kindly supplied by the Burroughs Wellcome Research Laboratories at Beckenham, Kent.

Shedding of cercariae (Australorbis glabratus)

The infected snails were kept in beakers with clear water under a bright light for one hour to shed the cercariae. Cercarial counts were done in triplicate, 1ml. of the cercarial suspension was transferred to petri dishes with few drops of Bouins fixative to immobilise the cercariae, they were counted under the dissecting microscope.

Method of infection

Mice were infected cutaneously according to the method of Ashry (1966). They were placed first in warm tap water to urinate and defaecate, then put in a clean suitable cage. The measured number of cercariae in a graduated cylinder were poured over the mice and diluted with water until it covered 1/3 of their bodies. They were left for ½ hour under a warm light during which the cage was gently agitated to ensure an even distribution of

cercariae. The water was examined for any remaining cercariae; if none were detected the water was drained and the mice were dried in a warm room. They were kept in separate cages fed on a pellet diet and water, and were checked every day for sick or dead mice.

Stool examination

To prove that mice were infected bisexually their stools were collected into tubes, emulsified with saline (NaCl 0.9%), a drop was delivered onto a slide and examined under the microscope for fertile eggs.

Worm collection

Infected livers have white areas on their surfaces and could thus be recognised as infected. They were taken from the killed mice, cut into pieces and put into petri dishes containing normal saline, the dishes were placed in an incubator at 37C° for two hours; then examined for live worms. Also each piece of the liver was pressed between two glass plates and held against a light to look for any trapped worms. The worms were collected in sterile bottles and stored in the deep freeze at -20C°, to be later used as antigen.

CHAPTER 4

HAEMATOLOGICAL METHODS

Collection of blood

The mouse was anaesthetized by both intraperitoneal injection of 0.3ml. veterinary Nembutal (Pentobarbitone Sodium, Abbott), and ether inhalation for few seconds. Fixed on a dissecting board the mouse thoracic cavity was opened, a cut was made through the heart, and its blood with a micro-pipette was drawn into tubes containing anticoagulant (EDTA-sequestrene) for further experiments.

Collection of serum and storage

The collected blood was centrifuged for a few minutes, without disturbing the red cell deposit the serum was drawn into plastic tubes and kept at -20C°.

Haemoglobin estimation

The cyanmethaemoglobin method was employed, by which blood is diluted in a solution containing potassium cyanide and potassium ferricyanide "Drabkin's Reagent" Drabkin and Austin, 1932. The haemoglobin and its derivatives are rapidly converted to cyanmethaemoglobin. The absorbance of the solution is then measured in a photoelectric colorimeter.

0.05ml. of blood was added to 10ml. of the reagent in tubes stoppered with a rubber bung and inverted several times, they were left at room temperature for one hour. The optical density of the solution was then measured and compared with the standard in a photoelectric colorimeter. The cyanmethaemoglobin standard solution was obtained from BDH chemicals Ltd. (B.S.3985). Calculation for haemoglobin values was done as follows:

$$\text{Haemoglobin (gm./100ml.)} = \frac{\text{O.D. blood sample}}{\text{O.D. standard}} \times \frac{\text{Conc. std. (mg./100ml.) X Dilution factor}}{1{,}000}$$

(Dacie, 1968)

Determination of packed cell volume (PCV)

Dacie's (1968) micro method was used. The blood was allowed to enter capillary tubes (75 mm. in length), the tubes were sealed by cristaseal and centrifuged in a microhaematocrit for five minutes. The packed cell volume was measured by a reading device. Each blood sample had duplicate tubes; the mean of their readings represent the packed cell volume.

Reticulocyte count

A drop of 0.5% solution of new methylene blue dye in absolute alcohol was dried on a slide, with a heat strerilized metal loop one drop of blood was mixed with the stain and left ten minutes in a wet chamber. A blood film was made with slide spreader and counterstained by Wright's stain.

The reticulocytes were counted under the oil-immersion objective. Successive fields of the slide were surveyed until a total of 500 cell were counted; the number of reticulocytes among them was then divided by 5 which gave the reticulocyte percentage in 100 red cells.

CHAPTER 5

ANAEMIA IN MICE INFECTED WITH S. MANSONI

Introduction

Prior to the investigations done on the causes of anaemia in mice experimentally infected with S. mansoni, two pilot experiments were planned to establish certain basic facts; namely the degree of anaemia; effect of different doses of cercariae and duration of infection on the anaemia.

Experiment I: To study the anaemia in infected mice

Thirty mice were used in two groups, the first was infected with 50 cercariae of S. mansoni, and the second group as their controls. Two mice from each group were killed at intervals of 6, 9, II, I5, I9 and 23 weeks after infection; and their haematological picture was studied.

Results

Infected mice started to show first signs of anaemia after six weeks of infection. The packed cell volume (PCV) and haemoglobin values were markedly decreased at I5 and I9 weeks of infection as illustrated in Fig. I(a, b); also reticulocyte count increased at I9 weeks. The almost identical values between the two groups observed at 23 weeks of infection was probably due to the fact that the two infected mice killed at this stage were not anaemic.

Comment

- anaemia develop in mice infected with S. mansoni after the prepatent period (6 weeks); it appeared to progress with the infection.
- the tendency for reticulocytosis indicated that the bone marrow was hyperactive.

Experiment 2: The effect of dose and duration of infection on anaemia

I) A group of 50 mice were infected with a heavy dose of 500 cercariae; another group of 10 mice was used as their controls. After I4 days from infection , mice were killed weekly until 7 weeks; when the experiment was terminated due to increased mortality among the infected group.

HAEMATOLOGICAL RESULTS OF INFECTED MICE WITH 50 CERCARIAE OF S. MANSONI AND THEIR CONTROLS

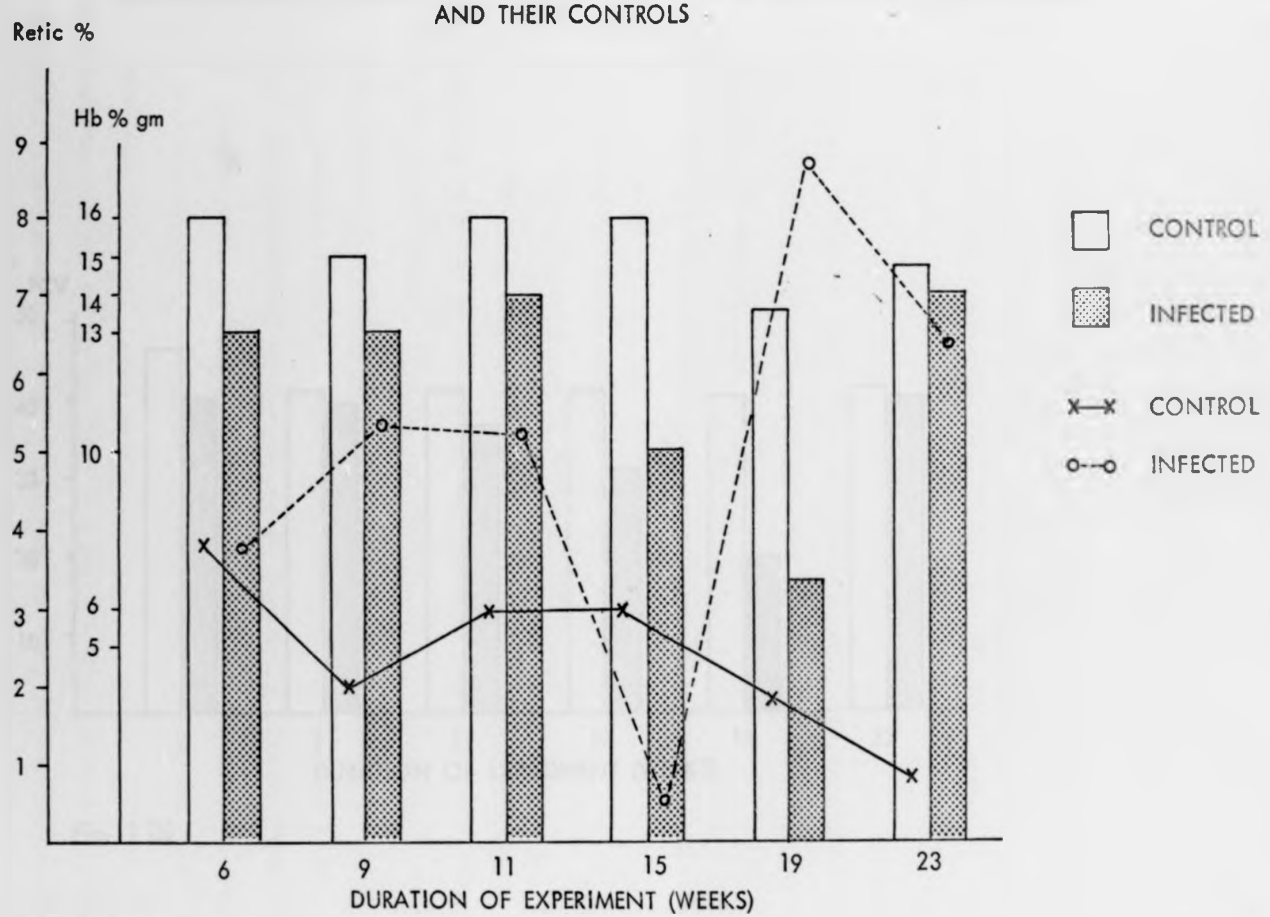


Fig. 1 (a)

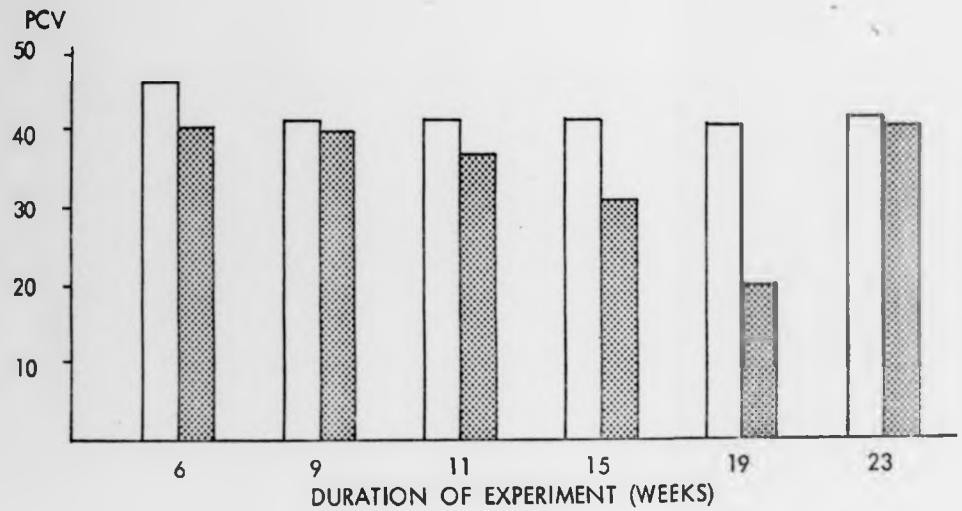


Fig. 1 (b)

2) Fifty mice were infected with 50 cercariae; with their controls. They were killed at 4 weekly intervals.

3) To study the development of anaemia during prolonged periods of infection a low dose of 25 cercariae was given to a third group of 30 mice, these were killed at intervals which commenced at 35, 38, 42, 45, 75, and 82 weeks after infection.

Results

To prove that there was statistical differences in the haematological values of infected group and their controls the following paired comparison t-test (analysis of variance) was used:

$$t = \frac{\bar{d}}{\sqrt{vas(\bar{d})}}$$ on f degrees of freedom (Armitage, 1971)

where $\bar{d}$ = mean difference

$vas(\bar{d})$ = variance of the mean difference

$\sqrt{vas(\bar{d})}$ = standard error

I) Mice infected with 500 cercariae did not pass eggs in their stools during the period of the experiment; 7 weeks. An average of 65 - 70 pairs of worms were counted from each infected liver by the end of seven weeks. The mean haemoglobin values declined at 6 and 7 weeks of infection (9.8 and 11.7 gm./100ml.), also packed cell volume decreased especially after 6 weeks 27.5%, as shown in table I. There was no increase in the reticulocyte counts. The mean cell haemoglobin concentration (MCHC) remained within the normal range, table I.

2) Mice infected with 50 cercariae had 7 - 8 pair of worms in their livers. They developed anaemia as evidenced by decrease in their haemoglobin values which fluctuated between 8.9, 10.3, 9.4, and 12.7 gm./100 ml. at respectively 14, 18, 22, and 37 weeks of infection. When taken individually one mouse infected for 14 weeks; had a very low Hb value of 3.8 gm./100 ml. As can be seen in table 2, the MCHC values of the infected mice remained slightly

Table I

Comparison between haematological values of nice infected with 500 cercariae and their controls.

Duration of infection	Mice	2	3	4	5	6	7	p
Hb	Cont.	I4.I (I)	I4.8 (I)	I4.5 (I)	I4.2 (I)	I4.I (I)	I5.I (I)	<0.3
	Inf.	I4.5 (I)	I4.5 (I)	I4.I (I)	I5.8 (I)	9.8 (6)	II.7 (7)	
PCV	Cont.	38	42	42	40	40	42	<0.2
	Inf.	39	39	4I	42	27.5	33.7	
Retic.	Cont.	3	I	2	I	2	3	<0.5
	Inf.	2	3	3.8	2	4.2	2.2	
MCHC	Cont	37.I	35.2	34.5	35.5	35.2	36.I	<0.7
	Inf.	37.I	37.I	34.3	37.6	35.8	34.6	

( ) no. of mice

p probability of t-test

Table 2

Comparison between haematological values of mice infected with 50 cercariae and their controls.

Duration of infection	Mice	I0	I4	I8	22	26	32	37	p
Hb	Cont.	I6.2 (3)	I4.2 (3)	II.8 (2)	I3.7 (2)	I6.4 (I)	I4.I (I)	I5.0 (I)	<0.0I
	Inf.	I4.4 (6)	8.9 (6)	I0.3 (3)	9.4 (3)	I3.0 (2)	I2.5 (2)	I2.7 (I)	
PCV	Cont.	40.3	39.3	34.5	40.0	44.0	40.0	40.0	<0.02
	Inf.	37.I	27.I	3I.3	29.0	35.5	36.5	37.0	
Retic. %	Cont.	I.2	I.7	0.5	2	2.6	2	3	<0.05
	Inf.	4.9	9.2	4.3	2I	4.2	4	4	
MCHC	Cont.	40.3	36.3	34.2	34.3	37.2	35.2	37.5	<0.00I
	Inf.	38.9	32.6	33.0	3I.7	36.7	34.2	34.3	

( ) number of mice

p probability of t-test

lower than those of the control group.

When haematological indices of the infected and control groups of mice were compared statistically; significant differences were obtained as shown in table 2; in which probability of the t-test for Hb, PCV and reticulocyte counts were respectively <0.01, <0.02 and <0.05.

3) Severe anaemia was observed in mice infected with 25 cercariae; when first killed at 35 weeks of infection. They maintained a very low packed cell volume and haemoglobin values until the end of the experiment (82 weeks). The reticulocyte counts were increased especially when there was a great drop in PCV and haemoglobin levels (Fig. 2, a and b).

From thirty infected mice used at beginning of the experiment; few survived the long durations of infection. Therefore, one mouse from each group was killed at intervals of 35 - 82 weeks, this lead into a different t-test being evaluated according to Armitage, 1971:

$$t = \frac{\bar{d}}{Se(d)} \quad \text{on } n - 1 \text{ degrees of freedom}$$

where Se(d) = standard error

The control mice had haemoglobin indices of between 12.1 - 14.5 gm./100 100 ml., whereas those of infected mice 2.5 - 9.0 gm./100 ml. with a significant difference $p < 0.01$. The lowest packed cell volume of 12% was at 35 weeks with a reticulocyte count of 44%, table 3. There was depression in MCHC values of 21.0, 28.0, and 18.5% after 35, 38, and 42 weeks of infection.

Discussion

Although mice infected with 500 cercariae developed anaemia at an early stage of infection (6 weeks), yet large number of infected animals did not survive past the sixth week after exposure. Similarly, Saud (1965) reported death of 100% of mice infected with 300 cercariae of S. mansoni after 54 days. Thus high doses of cercariae are not suitable for studying

HAEMATOLOGICAL RESULTS OF MICE INFECTED WITH 25 CERCARIAE OF S. MANSONI AND THEIR CONTROLS

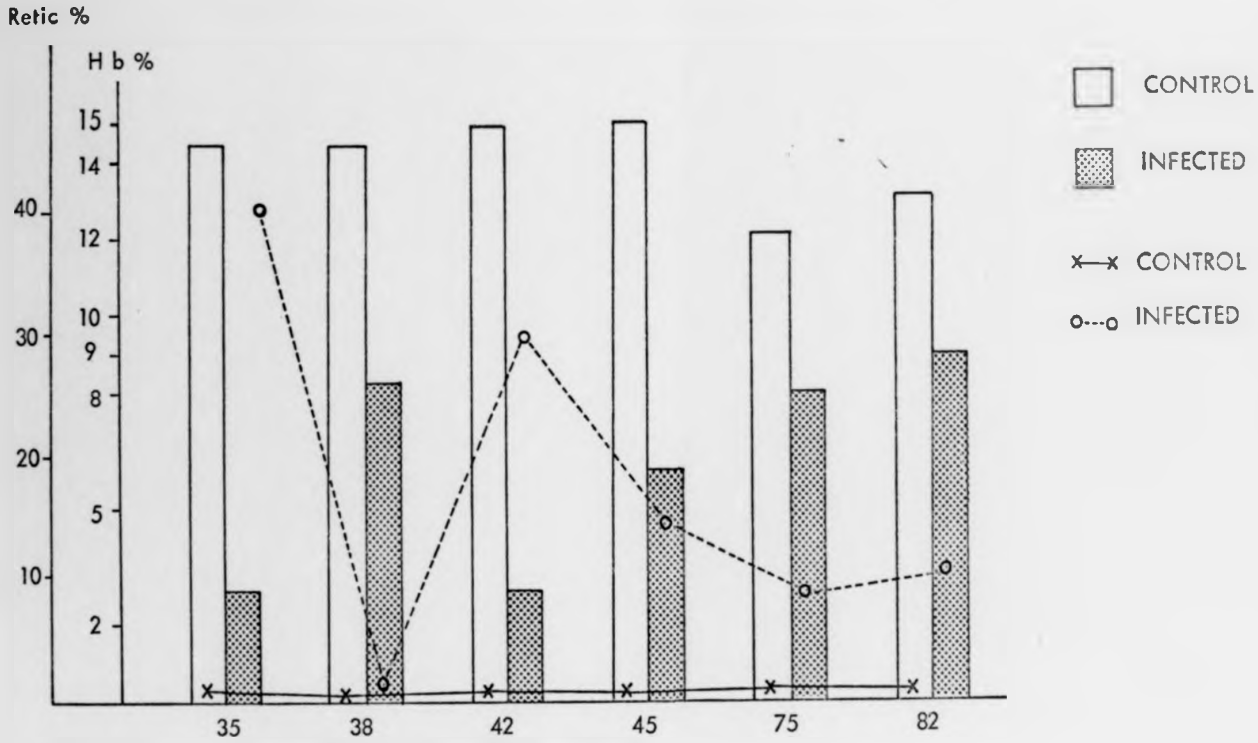


Fig. 2 (a)

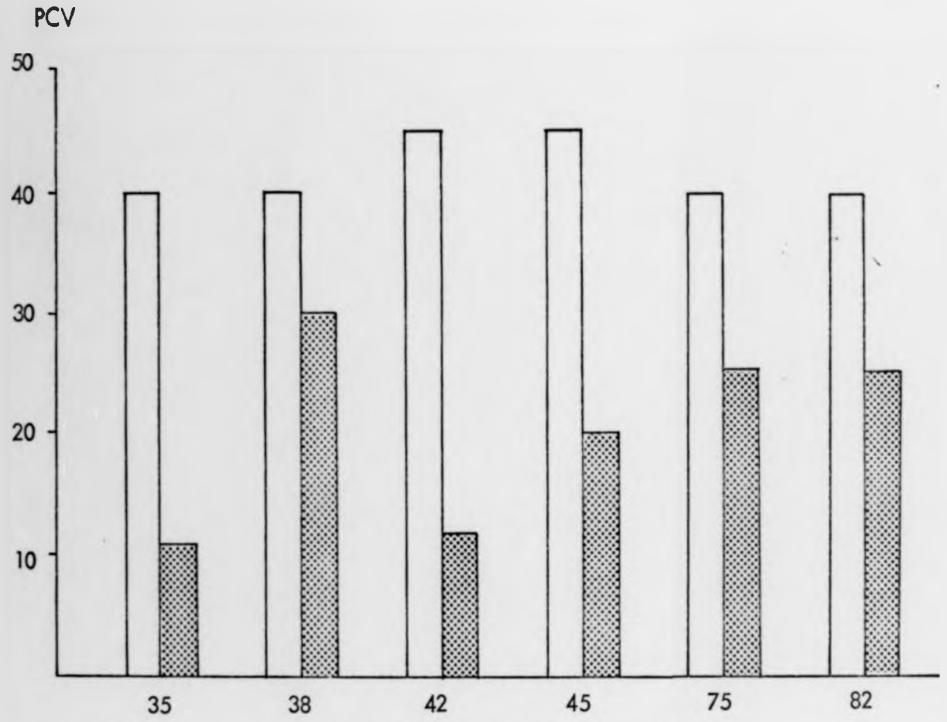


Fig. 2(b)

Table 3

Comparison between haematological values of mice infected with 25 cercariae and their controls.

Duration of infection	Mice	35	38	42	45	75	82	p
Hb	Cont. (I)	I4.5	I4.5	I4.8	I4.8	I2.I	I3.0	<0.0I
	Inf. (I)	2.5	8.4	2.6	6.3	8.2	9	
PCV	Cont.	40	40	45	45	40	40	<0.0I
	Inf.	I2	30	I4	20	25	24	
Retic. %	Cont.	2	I	2	2	3	2	<0.0I
	Inf.	44	4	30	I6	6	I2	
MCHC	Cont.	36.2	3I.6	32.8	32.8	30.2	30.2	<0.4
	Inf.	2I.0	28.0	I8.5	3I.5	32.8	37.5	

( ) number of mice

p probability of t-test

anaemia of mice at long terms of infection.

Within I4 weeks of infection with 50 cercariae mice developed a moderate anaemia. By contrast mice infected with 25 cercariae and harbouring I - 2 pair of worms, developed severe hypochromic anaemia, splenomegaly, reticulocytosis with hyperactive bone marrow. These symptoms were maintained almost the same through period of the experiment (35 - 82 weeks). These mice produced the typical pathophysiological state of schistosomal infection.

From these observations the following points could be concluded in regard to the relation between anaemia and infection with S. mansoni in mice:

- Anaemia develops with the minimal load of worms (I - 2 pairs).
- The intensity of infection does not determine the severity of anaemia.
- There is a probable relationship between the time of the onset of anaemia and the number of worms present, i.e. with heavy infections the time is usually reduced.
- The intensity and duration of infection interelate in their effect on the progress of anaemia, meaning that when mice are infected with a large dose of cercariae which resulted in shorter periods of infection, thence less is the chance that infected mice would produce the chronic type of anaemia.

Difficulties are encountered when human and mice schistosomal infections are compared; particularly in case of its intensity. For example Cheever (I969) found that the livers of infected mice with one pair of worms for one year contained 75 times more eggs per gram than the livers of the most heavily infected "asymptomatic" human subjects. However, the work done by Warren (I963), and the present data pointed that mice infected with low dose of cercariae (25 or 50), survived long periods of infection and produced the nearest syndromes (enlarged spleen and anaemia) that might be compared to those of diseased humans. Therefore, both doses were used to study the mechanisms of anaemia in the following chapters, but bearing in mind that the experimental infections were done on an animal that has major differences from man, especially in relation to size and age.

PART 2

HAEMOLYSIS AND ANAEMIA

CHAPTER I

OSMOTIC FRAGILITY STUDY ON RED CELLS OF MICE INFECTED WITH S. MANSONI

Introduction

Guest (1948) stated that normal human erythrocytes suspended in the series of hypotonic salt solutions employed for testing red cell fragility behave like nearly perfect osmometers up to a maximum volume of spheres within the limits of their surface area; the same principles govern the swelling and haemolysis of some abnormal types of erythrocytes with different characteristics of abnormal fragility or resistance to osmotic haemolysis.

The factors that control a cells osmotic fragility are complex, but of major importance is its shape which in turn depends on its volume and surface area, and the functional state of its surface membrane (Dacie, 1968). For example, the decreased resistance to osmotic lysis seen in hereditary spherocytosis was explained as due to the fact that many of the cells are in a spherical shape; which has less capacity to swell in hypotonic solution than the normal biconcave or flat red cells (Harris, 1963). Spheroidicity, in contrast to normal biconcavity, may predispose to sequestration and destruction of erythrocytes in the spleen (Emerson et al, 1956). Therefore, the *in vitro* study of osmotic fragility of red cells is an indirect method for the diagnosis of their increased haemolysis *in vivo*.

Abnormal changes in the osmotic fragility of erythrocytes during schistosomal infection were reported in a few number of references; e.g. Saeed (1970), reported decrease in osmotic fragility of red cells taken from anaemic mice infected with 100 cercariae of *S. mansoni*.

The objective of the following experiment was to study the osmotic fragility of red cells from normal mice, and whether it alters throughout their infection with *S. mansoni*.

Materials and methods

30 mice were infected with 50 cercariae of S.mansoni ; with I6 mice as their controls. Blood samples for haematological and osmotic fragility studies were collected from mice killed after 8 weeks post exposure, then at four weekly intervals until 28 weeks of infection.

The osmotic fragility test was done according to Dacie's method (I968): 0.05ml of heparinized blood was added to each of twelve tubes filled with 5ml volumes of progressively hypotonic concentrations of sodium chloride solution (buffered at pH 7.4), i.e. 0.90, 0.75, 0.65 .....0.I0% Nacl. The tubes were inverted several times to mix the blood with saline solution, and left for thirty minutes at room temperature, samples were remixed and centrifuged for five minutes. Then with a photoelectric colorimeter;the amount of haemolysis in each tube was compared with that in the I00% lysis tube (0.I% Nacl). A "fragility curve" were drawn on graph paper from the main percentage of haemolysis in each tube against the corresponding concentration of salt solution. And the median corpuscular fragility (i.e. the concentration of saline causing 50% haemolysis) was recorded.

Results

Infected mice developed mild anaemia after 8 weeks of infection (Hb= I2.2 gm/I00ml, PCV= 34.0%). Their haematological indices remained lower than the normal group until the end of the experiment (28 weeks). The lowest Hb and PCV values was observed after I2 weeks post infection, they were respectivly 9.7 gm/I00 ml and 26.7%. There was no significant rise in the reticulocyte count (Table I).

The spleens of infected mice were enlarged and their relative splenic weights were significantly different from those of the control group ($p < 0.00I$. Table I).

Generally, the control "fragility curve" were almost sigmoid in pattern as illustrated in Fig. I, which also shows that curves of the red cells

Table I

Comparison of haematological indices and relative splenic weight of mice infected with S.mansoni and their controls

Duration of infection	Mice	8	I2	I6	20	24	28	p
Hb	Cont.	I4.5 (4)	I3.2 (4)	I4.3 (2)	I4.I (3)	II.9 (2)	I4.8 (I)	<0.0I
	Inf.	I2.2 (4)	9.7 (4)	I2.I (2)	I0.2 (3)	II.0 (2)	I2.4 (2)	
PCV	Cont.	4I.0	4I.0	4I.5	4I.6	37.5	40.0	<0.00I
	Inf.	34.0	26.7	35.0	3I.3	34.0	35.5	
Retic.	Cont.	2.3	0.9	I.I	2.I	2.5	3.0	<0.0I
	Inf.	3	4	3.2	6.I	7.5	6	
Body weight	Cont.	40	30	39	39	40	46	<0.5
	Inf.	38	35	35	40	4I	48	
Spleen weight	Cont.	0.26	0.I8	0.24	0.24	0.39	0.22	<0.00I
	Inf.	0.45	0.5I	0.50	0.67	0.47	0.44	
S/W	Cont.	0.64	0.58	0.60	0.60	0.89	0.47	<0.00I
	Inf.	I.I6	I.48	I.54	I.63	I.I5	0.92	

( ) number of mice

$$S/W \quad \frac{\text{spleen weight}}{\text{body weight}} \times I00$$

p probability of t-test

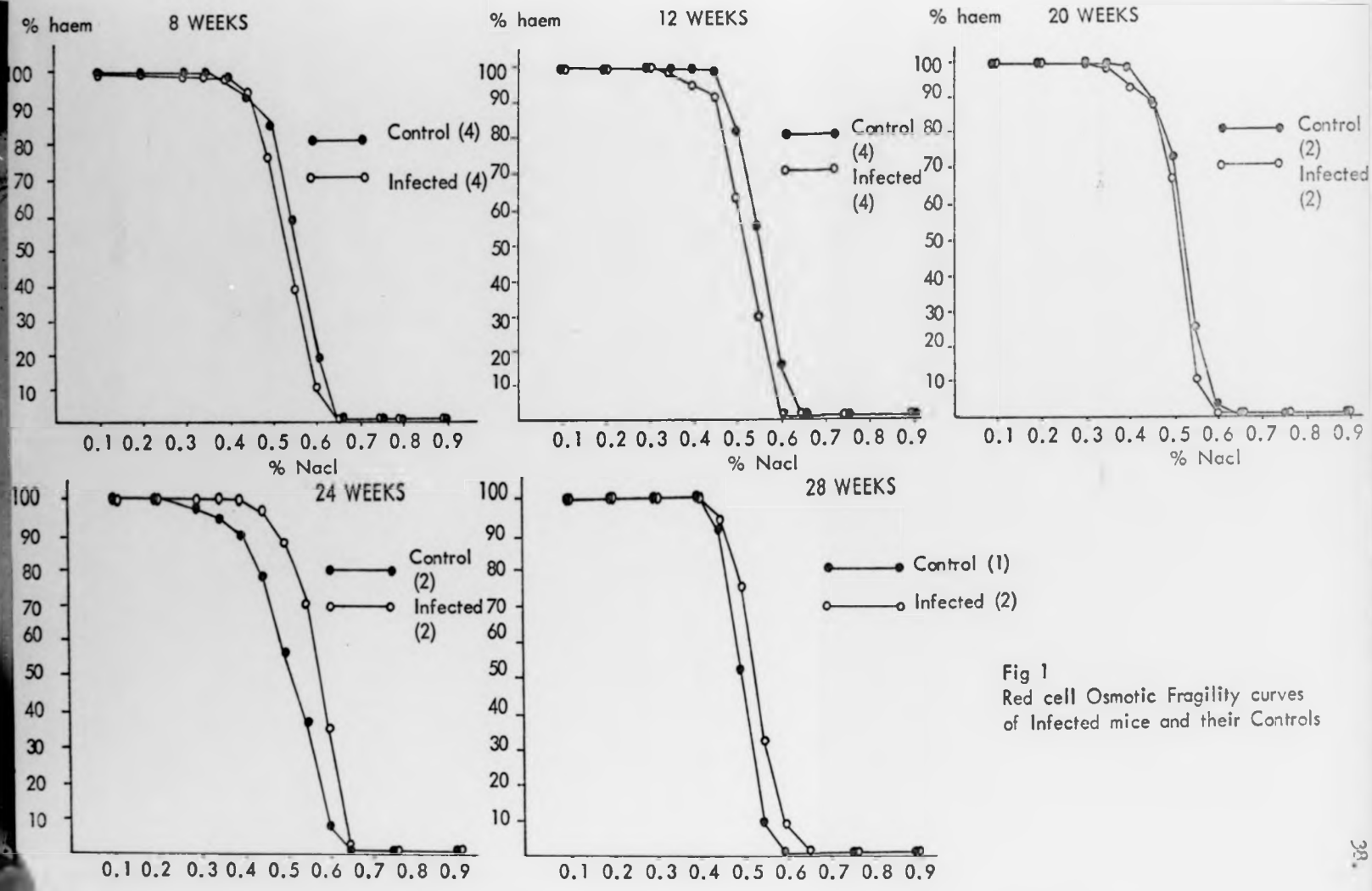


Fig 1
Red cell Osmotic Fragility curves of Infected mice and their Controls

from infected mice after 8 and I2 weeks of infection slightly shifted to the left, this may well be within the normal range. The only significant deviation from the normal type of curve was observed when red cells "fragility curve" of mice infected for 24 weeks shifted to the right (Fig. I). The median corpuscular fragility of this group was 0.58%, whereas those of the control mice 0.51% (Table 2).

Discussion

The resemblance observed in the red cell osmotic fragility curves of the control and infected mice, was probably due to the fact that infected mice in this experiment developed a very mild picture of anaemia; and their red cell osmotic fragility curves had no "tails" which denotes the absence of very fragile cells. However, in few cases of haemolytic anaemia, e.g. hereditary spherocytosis, the osmotic fragility test yields results in the accepted normal range (Harris, I963).

Nevertheless, the infected mice for 24 weeks showed increase in their red cells osmotic fragility, which indicates a change in their shape. In contrast, Saeed (I970) demonstrated a clear shift to the left in the erythrocytes osmotic fragility curves of mice infected with I00 cercariae of S. mansoni after 24 weeks; he suggested that with the development of anaemia the red cells became thinner than the normal, and were able to absorb more water before bursting; the result being an unusual resistance to haemolysis in hypotonic solutions.

In active cases of auto-immune haemolytic anaemia of the warm-antibody type, erythrocyte osmotic fragility is moderately or markedly increased which runs parallel with the degree of spherocytosis in the peripheral blood films (Dacie, I962). Although the exact mechanism which bring about the change in erythrocyte shape is still not exactly known, but one of the possibilities was that the spherocytosis develops as the result of metabolic damage suffered by the erythrocytes in consequence of adsorption

Table 2

Median corpuscular fragility (MCF)
of infected mice and their controls

Duration of infection	8	I2	20	24	28
Control	0.56	0.55	0.52	0.5I	0.50
Infected	0.54	0.52	0.5I	0.58	0.53

of antibody to its surface *in vivo* (Dacie, 1962).

In conclusion, in mildly anaemic mice infected with *S. mansoni* their erythrocyte population have a few fragile cells that renders them more prone to splenic sequestration. The osmotic behaviour to hypotonic saline solution might be different when mice develop severe anaemia.

CHAPTER 2

THE RADIOACTIVE STUDIES IN MICE INFECTED WITH S. MANSONI

Introduction

Gray and Sterling (1950) introduced a procedure for labelling red cells *in vitro* with radioactive sodium chromate (^{51}Cr) which made it possible to measure their rate and site of destruction *in vivo*. In clinical investigation departure from the normal rates would be taken as an index of haemolytic activity involved in producing the anaemia in question.

Woodruff et al (1966) and Shafei et al (1972) found by the use of the isotope method a reduced life span of erythrocytes taken from anaemic patients infected with *S. mansoni*. and the site of their destruction was the spleen as revealed by increased surface radioactivity over this organ. Similar results was obtained in experimental infection of mice (Mahmoud, 1971).

When mice infected with *S. mansoni* developed anaemia in the previous chapter it was necessary to study the erythrocyte life span in the following experiment in order to determine if their anaemia was of a haemolytic nature and whether the animals were passing blood in their stools.

Materials and methods

Survival of erythrocytes was estimated from both control (normal) mice, and mice infected with 50 cercariae killed after 15 weeks.

^{51}Cr labelling technique

Based on the technique used by Mollison and Veal (1955) the following steps were performed:

- two mice were used, each killed by neck dislocation. With the mouse thoracic cavity opened it was turned face downward; then the heart was held from its lower end by forceps and cutting through it, the blood was dripped straight into sterile bottles with 1 ml. ACD (acid-citrate-dextrose solution) as the anticoagulant.

- sterile isotonic solution of sodium chromate ($Na_2Cr_{51}O_4$) 0.50 uc/ml. blood, was added with continuous shaking to the blood and left at room temperature for 20 minutes.

- the blood suspension was washed three times with warm saline (37C°), and resuspended with sufficient amount of saline.

- 0.2 ml. of labelled erythrocytes were injected into the tail vein of control and infected mice, also a standard solution was prepared from the remaining erythrocyte suspension.

^{51}Cr counting of blood samples

Radioactivity of blood samples was measured first from mice killed after one hour from injection with labelled erythrocytes (day 0 sample), subsequently at 4, 8, and I2 day intervals a number of mice were killed and their Hb, PCV and reticulocyte count were estimated in part of each sample. 0.05 ml. of the remaining blood suspension was then haemolysed with I0 ml. distilled water in counting tubes, their radioactivity was measured in a well-type scintillation counter, for I00 seconds.

Measurment of erythrocyte life span

After subtraction of the background count from each reading and correction for radioactivity decay were made, the percentage survival of ^{51}Cr on day t was estimated by Dacie's & Lewis quotient (I975)

$$^{51}Cr \text{ survival on Day t (\%)} = \frac{\text{counts per min./ml. of blood on Day t}}{\text{counts per min./ml. of blood on Day 0}} \times 100$$

The values obtained were plotted against time on semi-logarithmic graph paper in order to find the half life span of ^{51}Cr labelled erythrocytes.

Residual organ activity

Spleen and liver from the killed mice were weighed and transferred to the counting tubes for radioactive reading, and the spleen:liver activity ratio was calculated.

Radioactivity in the stools

To prove whether infected mice had haemmorrhage into their gastrointestinal tracts; radioactivity was measured from their stools which had been collected either:

- daily for six days after injection with ^{51}Cr tagged red cells from infected (50 cercariae) and a control group of mice.
- two mice infected with 25 cercariae were injected with their own erythrocytes after being labelled with ^{51}Cr, later one gram of their stools were collected after 2, 6, 10, and 12 days from injection.

Results

The infected group of mice developed mild anaemia as proved by their decrease in Hb and PCV values, with a significant difference of respectively $P < 0.01$ and $P < 0.01$ when compared to the control group (Table I).

The percentage survival of ^{51}Cr labelled erythrocytes from anaemic infected mice on 4, 8, and 12 days after injection were less than the normal mice as shown in table 2, and it was found that the ^{51}Cr red cell half life ($^{51}CrT\frac{1}{2}$) of infected mice (5 days) were shorter than the control mice (10 days) as illustrated in Fig I.

Weight of the spleen and the relative splenic weights (R.S.W) of infected mice were elevated and differed significantly from control mice, $P < 0.001$ (table 3). Initially after 4 and 8 days ^{51}Cr activity was found in both spleen and liver organs, by the end of the experiment (12 days) excess splenic counts were more marked than excess hepatic counts in infected mice, the ratio of which (spleen/liver counts) was 1.93 whereas those of the control mice 0.92 (Table 3).

Blood radioactivity in stools taken from control mice were discarded because usually they were not above the background. Mice infected with both doses (25 and 50 cercariae) lost some blood in their stools which was not a continuous loss as shown in tables 4 and 5. When the infected mouse had severe anaemia (Hb = 7.9 gm/100 ml.) it passed more red cells in its stool

Table I

Mean haematological indices of mice infected with 50 cercariae and their controls

	Day after injection	4	8	I2	P*
PCV	Control	40.5 (2)	38.0 (2)	40.0 (2)	<0.0I
	Infected	25.I (6)	32.8 (6)	33.6 (6)	
Hb	Control	I4.4	I3.4	I4.5	<0.0I
	Infected	7.8	I0.8	I0.6	
Reticul. %	Control	2.8	I.5	I.8	<0.00I
	Infected	I6.6	3.2	8.5	

P* probability of t-test (Analysis of variance)
( ) number of mice

Fig. 1 Survival curve of ^{51}Cr tagged erythrocytes in mice infected with 50 cercariae of S. mansoni (15 weeks after infection) and their controls

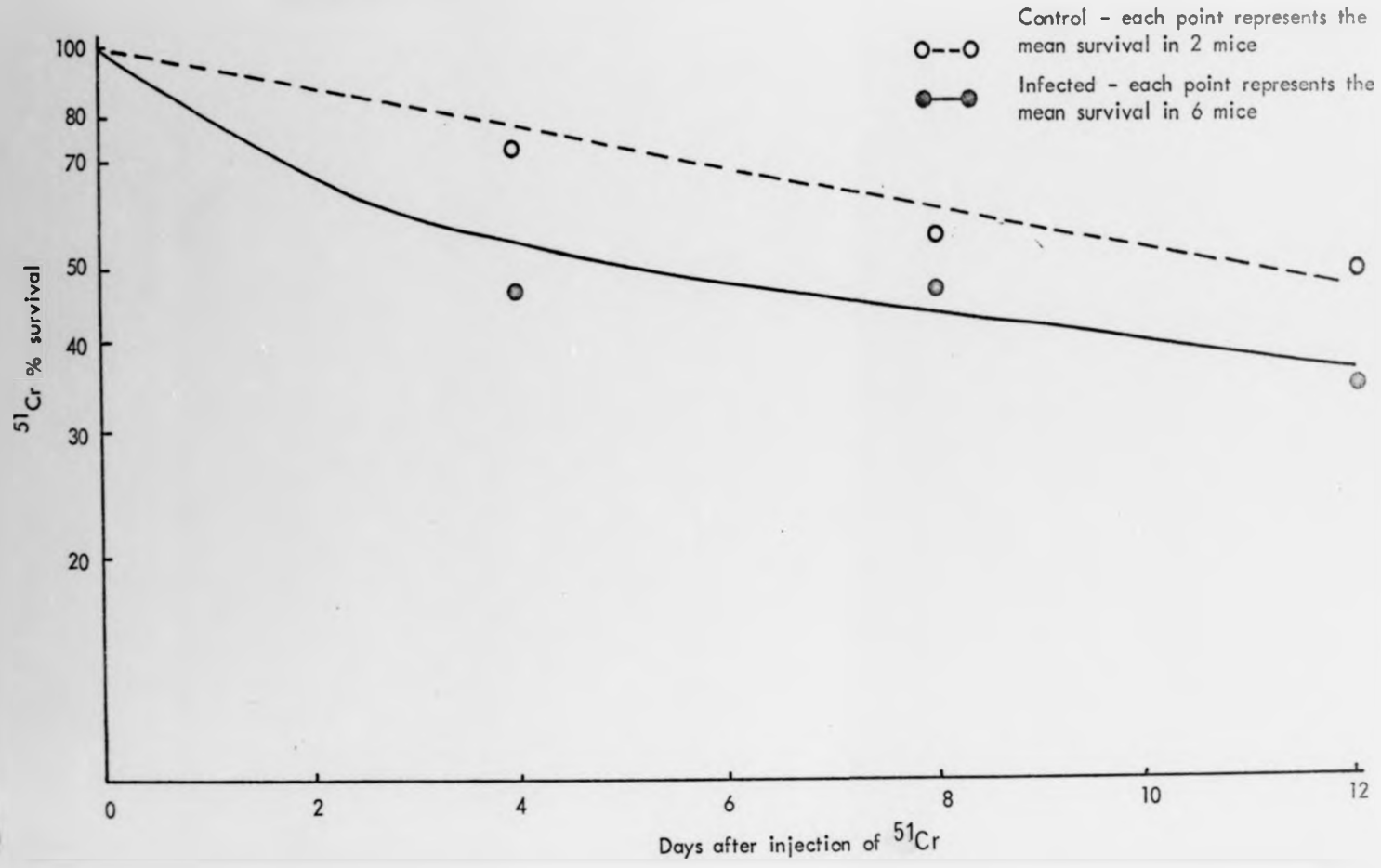


Table 2

% survival of ^{51}Cr labelled red cells in mice infected with 50 cercariae and their controls

Days after injection	Normal	Infected	P of paired comparison t-test
4	71 (2)	46.4 (6)	<0.05
8	52.6	44.9	
12	46.6	33.4	

( ) number of mice

Table 3

The mean relative splenic weight (R.S.W.) and spleen/liver residual radioacivity (after I2 days from injection of ^{5I}Cr tagged erythrocytes) of infected and control mice

	Day after injection	4	8	I2	P*
Spleen weight	Control	0.25 (2)	0.I5 (2)	0.I0 (2)	<0.00I
	Infected	0.6I (6)	0.50 (6)	0.60 (6)	
R.S.W.	Control	0.65	0.52	0.26	<0.00I
	Infected	I,75	I.33	I.57	
Spleen/liver ratio	Control			0.92 (2)	
	Infected			I.93 (6)	

P= probability of t-test (Analysis of variance)

( )= number of mice

Table 4

^{51}Cr count in stools of mice infected with 50 cercariae

Mice	Hb gm/100ml	^{51}Cr count					
		Days after injection					
		1	2	3	4	5	6
1	12.9	709	247	488	144	104	92
2	10.7	213	156	121	68	64	67
3	11.7	511	217	289	187	67	61
4	11.2	980	436	378	225	141	138

Table 5

^{51}Cr count in one gram of stool from mice infected with 25 cercariae

Mice	Hb gm/100ml	^{51}Cr count			
		Days after injection			
		2	6	10	12
1	7.9	11989	10903	2013	2700
2	13.2	2565	915	1090	123

as indicated by excess of ^{51}Cr activity than the one with milder anaemia (Hb = 13.2 gm./100ml.), table 5.

Discussion

It was shown from these results that anaemia produced in mice infected with S. mansoni was accompanied with reduction in their erythrocyte life span to approximately half the normal; which agrees with the work done previously on schistosomiasis in human patients (Woodruff, et al, 1966) and T.O. mice (Mahmoud, 1971) although he proved that the $^{51}CrT_{\frac{1}{2}}$ life span of red cells from normal mice was 13 days instead of 10 days (Fig. 1), this might be due to using different technical procedures.

These findings indicate that in schistosoma infection red cells were destroyed more rapidly than those in the normal. Several factors might be involved in the elimination of these cells:

1 - Immunological factor: during schistosoma infection the pathological alteration of red cells brought about by antibody-antigen complexes on their surface would initiate their destruction by phagocytosis in the reticulo-endothelial system (Woodruff, 1973).

2 - In infected mice the progressive accumulation of ^{51}Cr in their enlarged spleens with increase in the spleen/liver ratio activity signified that this organ was the main site for red cells destruction. The exact role by which the spleen in schistosoma infection pools and traps the red cells is not known; many theories have been suggested namely:

a) Hypervolaemia resulting from pooling of red cells themselves inside bilharizial spleens was one of the causes for production of chronic anaemia (Farid et al, 1966).

b) The sequestration-phagocytosis theory (Doan and Wright, 1946; and Jandl, 1955); the spleen as a reticuloendothelial organ captures and destroys the altered erythrocytes.

c) During infection the release of a splenic hormone may induce the bone

narrow to produce red cells with diminished survival time (Shafei et al, 1972).

These two factors responsible for the increase in red cell destruction will be discussed in more detail in part 3.

3- Blood loss: Although mice passed in their stools red cells as shown by ^{51}Cr study and its excess when anaemia were severe, bleeding cannot account entirely for the anaemia because:

- The ^{51}Cr activity measured in the spleen (espacially by the end of the experiment) and the liver exceeded the radioactivity of the stools, therefore most of the red cells were destroyed inside those organs.
- The blood loss was not a continuous one as indicated from the gradual decrease in ^{51}Cr activity of the stools collected (table 4 and 5) which proves that infected mice did not bleed every day, unlike human patients infected with S. mansoni who had chronic faecal blood loss (Sabour et al, 1967).

Hence the bleeding factor through the intestine of mice might be considered as a contributory more than the main factor in the genesis of their anaemia.

PART 3

IMMUNOLOGICAL MECHANISM AND DEVELOPMENT OF HAEMOLYTIC ANAEMIA

CHAPTER I

THE AGGLUTINATION TEST

Introduction

Haemolytic anaemia was studied in mice infected with S. mansoni, and it was found that there was considerable shortening of the life span of their erythrocytes (Woodruff, 1973, and this study). This anaemia cannot be attributed entirely to blood loss caused by chronic haemorrhage; for it also develop when the mice are known to be free from blood loss resulting from the presence of schistosoma ova as in infection with irradiated cercariae (Mahmoud, (Woodruff 1972). This phenomenon suggested that an immunological mechanism is involved in the haemolytic destruction of red cells.

One of the major serological tests used in the diagnosis of acquired haemolytic anaemia is the direct agglutination (antiglobulin) test, introduced by Coombs, Mourant and Race (1945), in which "incomplete" antibodies firmly attached to red cells which lack the property of causing direct agglutination in vitro are detected by the use of antiglobulin serum (Dacie, 1960). But a positive agglutination test does not always mean that the anaemia in question is of auto-immune type because other proteins (non-immune globulins) would be detected by the same test, for instance in case of various poisons, which alter the red cell surface leading to adsorption of serum proteins nonspecifically and these may well react with the antibody in the antiglobulin serum (Weiner, 1959).

Gamma globulins bound on the surface of red cells were demonstrated by the use of agglutination test in anaemic patients with infectious diseases, e.g. in P. vivax malaria (Parrett-Connor, 1967) and trypanosoma infections (Woodruff, 1973). A negative agglutination test has been described previously in humans with bilharizial hepatosplenomegaly (Farid et al, 1964). After obtaining a positive direct antiglobulin test

with erythrocytes of mice experimentally infected with 25 cercariae of S. mansoni. Mahmoud (I971) concluded that the presence of auto-antibodies acting against autoantigens "altered" their erythrocyte surface.

The purpose of the following experiment was to determine whether antibodies were bound to erythrocytes of mice infected with S. mansoni, and if so, what kind of antibodies they are.

Materials and methods

Blood samples for the direct and indirect agglutination test were taken from mice infected with 25 or 50 cercariae with their controls; killed after long periods of infection (30 - 82 weeks). These tests was performed as follows after the work of Long et al (I963):

Direct agglutination test

- Blood was immediately transferred into small bottles containing sequestrene, and was centrifuged in order to separate plasma for the indirect agglutination test.
- Red cells were washed three times with warmed (37C°) buffered saline pH 7.4, to prevent absorption of cold agglutinins on the surface of erythrocytes (Dacie, I968). A blood suspension of 2% was then prepared with PBS.
- On a translucent tile, two drops of red cell suspension were thoroughly mixed with two drops of anti-mouse globulins; two-fold diluted at I/4 to I/5I2.
- Control, normal saline replaced the antiglobulin.
- The tiles were left at room temperature (20C°) for 5 - 7 minutes, after which a drop of each dilution were examined microscopically for agglutination which was scored according to Lindsey et al scheme (I966):

4+ = virtually every cell agglutinated; agglutination easily seen without magnification.

3+ = many 3 - I0 cell clumps and at least one large agglutinate seen in each field; unagglutinated cells uncommon.

2+ = 5 - I0 small clumps of cells in each field; unagglutinated cells numerous.

I+ = 2 - 4 clumps in each field, unagglutinated cells predominant.

negative - = no agglutination.

Indirect agglutination test

- In small test tubes; two volumes of the serum under test were mixed with one volume of a 50% suspension in buffered saline of thrice washed normal mouse red cells.
- Control tubes containing saline instead of serum were set up similarly.
- The tubes were incubated at 37C° for one hour and a half.
- Cells were washed three times with PBS, and treated as for the direct agglutination test.

The anti-mouse globulins used in both tests were the "Broad spectrum" (anti-IgG, IgM, and IgA); anti-IgG; anti-IgM; anti-C'_3 (supplied by Nordic pharmaceuticals and Diagnostics), all the globulins were produced in rabbits.

Results

The direct agglutination test gave negative reaction with erythrocytes from mice infected with 50 cercariae. But when red cells from mice infected with 25 cercariae were mixed with the "Broad spectrum" positive agglutination was observed after 35, 38, and 42 weeks of infection; when their Hb values dropped respectively to 2.5, 8.4, and 2.6 gm./100ml. The end point of agglutination was respectively at a dilution of I/32, I/4, and I/16 of the antiglobulin sera (Table I). The test was negative after 45, 75, and 82 weeks post-exposure. Normal controls were consistently negative.

Negative reaction was obtained when the same blood samples were mixed with the specific anti-globulins (IgG, IgM, and C'_3).

The indirect agglutination test was negative with sera from mice infected with both doses. The absence of positive results was probably due to the fact that globulins in the tested sera were not strong enough to sensitize the red cell surface from normal mouse to bring their agglutination later.

Table I

Direct agglutination test in mice
infected with 25 cercariae

Duration of infection	Hb %	Dilution of "Broad spectrum" anti-IgG, IgM, IgA							
		4	8	I6	32	64	I28	256	5I2
35	2.5	+	+	+	+	0	0	0	0
38	8.4	+	0	0	0	0	0	0	0
42	2.6	+++	+ +	+	0	0	0	0	0
45	6.3	0	0	0	0	0	0	0	0
75	8.2	0	0	0	0	0	0	0	0
82	9	0	0	0	0	0	0	0	0

each infected mouse had a normal control which gave negative agglutination.

In the light of these results new groups of mice were infected with 25 and 50 cercariae of S.mansoni in order to repeat the direct agglutination test and studies of the blood picture on a larger number of mice and also at an earlier stage of their infection. The animals were killed as follows:

Experiment I: Mice infected with 25 cercariae

- Selected group: in which mice having a pale colouring of the eyes and tail were choosen to be killed at different durations of infection.
- Non pale infected mice with a similar duration of infection to these of the first group were killed so as to make a comparative pair. They were referred to in the tables and figures as the random group.
- Control group: normal healthy mice.

Experiment 2: Mice infected with 50 cercariae; also killed selectively at I4, 20, 23, 28, 35, 48, and 54 weeks of infection with their controls.

To facilitate adsorption of globulins (especially the complement) if any was present onto the surface of red cells to be tested, one drop of whole fresh blood was mixed with 2ml of a solution containing 6% sucrose and 0.I5% Sodium Chloride and incubated at 37C° for I5 minutes, after being washed twice, the cells were ready for the direct agglutination test (Mollison, et al /I964), with diluted "Broad spectrum", anti-IgG, IgM, and C_3.

Haematological Studies

Experiment I: Both selected and random group of mice infected with 25 cercariae developed severe anaemia, yet the severity was greater in the first group. There was marked decrease in haemoglobin and packed cell volume (PCV) values of both groups, from I4 weeks after infection when the mice were first killed, there were almost the same low values up to 66, 70, and 80 weeks when this decrease became milder as illustrated in Fig. I and 2, which also shows how mice from the selected group had lower values of Hb and PCV than those of the random group (except at 53 weeks of infection).

Mice from the random group had the most depressed Hb value of 5.5, 5.2,

Fig 1 Comparison of Hb values of mice infected with 25 cercariae (selected, random) and their controls

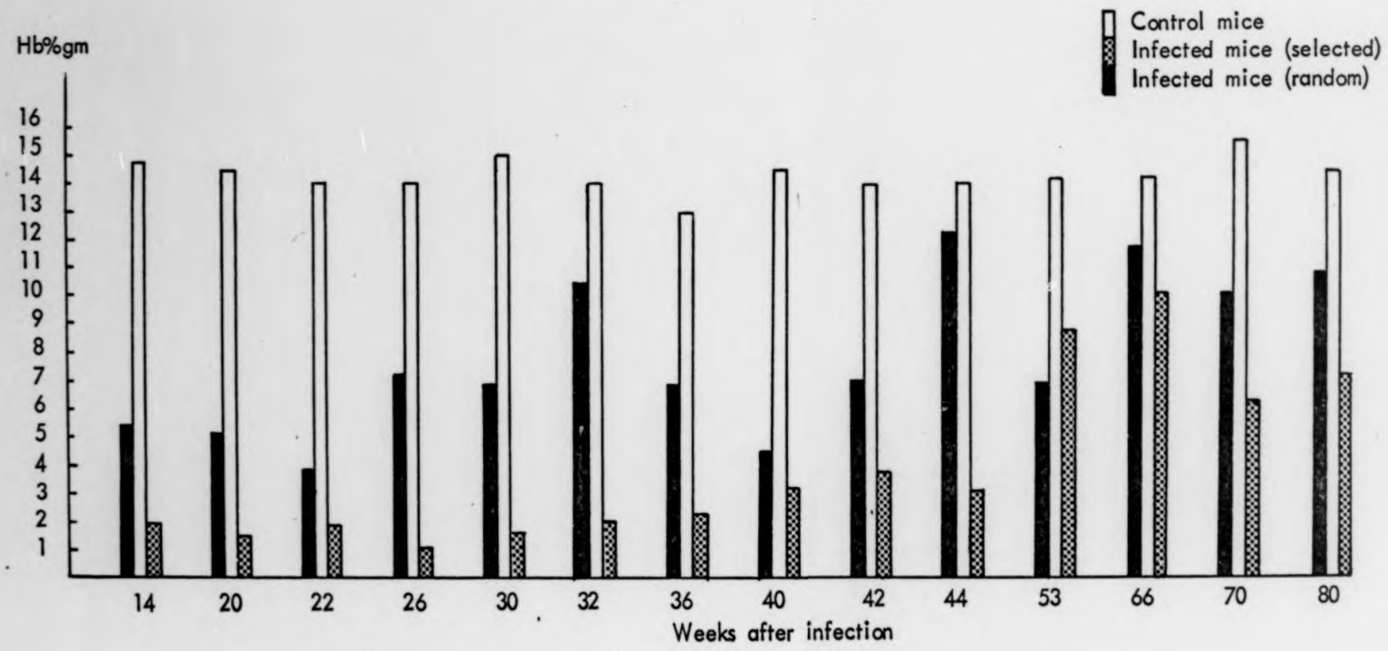
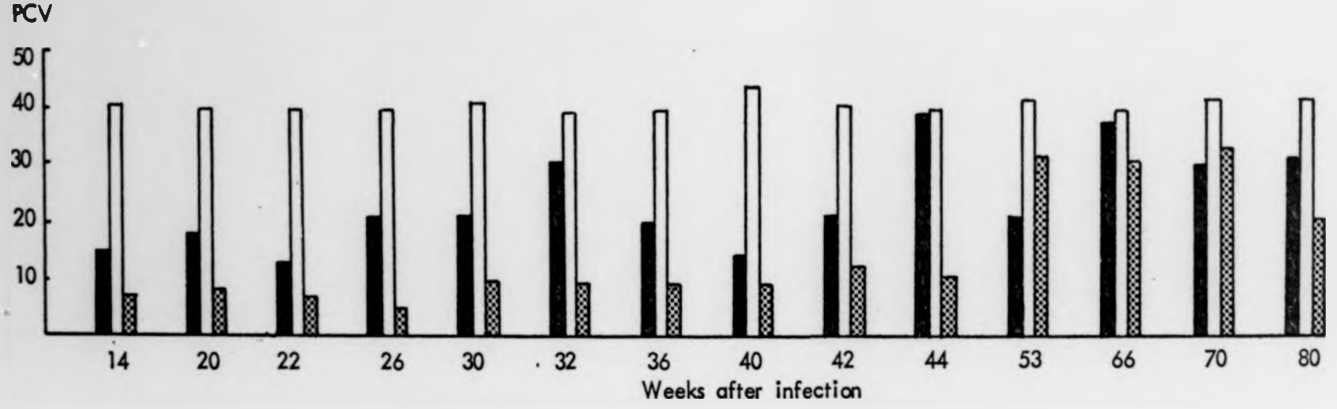


Fig 2 Comparison of PCV values of mice infected with 25 cercariae (selected, random) and their controls



3.9, 7.2 and 6.8 gm/100 ml at 14, 20, 22, 26, and 30 weeks of infection, it was less in the selected mice i.e. 1.9, 1.5, 1.9, 1.1 and 1.6 gm/100 ml at the same duration. Packed cell volume of the three groups, control, selected and random ranged respectively from 38-44, 5-33 and 14-39 as seen in Table 2. The mean cell haemoglobin concentration (MCHC) usually declined in both infected groups e.g. when haemoglobin value was 2.4 gm/100 ml (selected) and 5.2 gm/100 ml (random) it was respectively 23.6, and 28.4% (Table 2).

Parallel to the decrease in Hb and PCV values, the reticulocyte count increased in both infected groups; this was usually manifested when PCV was at its lowest value for example when it was 10% (selected) reticulocyte count was 67.5% (Table 2). The increase was less in the mice from random group as demonstrated in Fig. 3.

To verify the differences statistically between the three groups of mice in their haematological indices; the paired comparison t-test was used:

$$t = \frac{\bar{d}}{\sqrt{\text{vas}(\bar{d})}}$$ (Armitage, 1971)

Each haematological value had three probabilities for the t-test which was calculated in the following manner:

P for Control V Selected (infected)
Control V Random (infected)
Random V Selected

The probabilities obtained were highly significant (<0.001) in PCV, Hb, MCHC and reticulocyte count when each two groups were compared (Table 2).

Experiment 2: Mice infected with 50 cercariae when killed selectively had severe anaemia with diminished Hb, PCV values and increased reticulocyte count especially at 20, 35 and 48 weeks of infection (Fig. 4a and b). The decrease in PCV value fluctuated between 18, 7, 9.5, 15.5, 9, and 10% until the last mouse that survived (54 weeks of infection), it became 25%; the

Table 2 Comparison between haematological values of mice infected with 25 cercariae (selected and random) and their controls

Duration in weeks		14	20	22	26	30	32	36	40	42	44	53	66	70	80	P of t-test
Hb	Control	14.8 (3)	14.5 (3)	14.1 (3)	14.1 (2)	15.0 (2)	14.1 (3)	12.9 (2)	14.5 (2)	13.9 (2)	14 (2)	14.2 (2)	14.3 (2)	15.5 (2)	14.4 (2)	
	Selected	1.9 (1)	1.5 (2)	1.9 (3)	1.1 (1)	1.6 (2)	2.1 (2)	2.4 (3)	3.3 (2)	3.8 (2)	3.2 (1)	8.7 (2)	10 (1)	6.2 (1)	7.2 (1)	<.001* <.001
	Random	5.5 (4)	5.2 (3)	3.9 (3)	7.2 (3)	6.8 (2)	10.5 (2)	6.8 (1)	4.5 (1)	6.9 (3)	12.2 (1)	6.8 (2)	11.6 (1)	10.0 (1)	10.7 (2)	<.001
PCV	Control	42.3	40	40.3	40	41.5	39	39.5	44	40.5	40	41.5	38.5	41.5	41.5	<.001
	Selected	7	7.5	7	5	10	8.5	9.3	8.5	13	11	32	31	33	21	<.001
	Random	15.2	17.6	13.3	21.6	21.5	31	20	14	20.6	39	20.5	37	30	32	<.001
MCHC	Control	34.9	36.3	34.8	35.3	36.1	36.1	32.7	32.9	34.3	34.9	34.2	37.1	37.4	34.6	<.001
	Selected	27.1	21	27	22	16.5	25.1	23.6	38.7	28.9	29	27.5	32.2	18.7	34.2	<.001
	Random	36	28.4	28.2	33.4	31.6	34	34	32.1	33.5	31.2	33.1	31.3	33.3	33.4	<.001
Retic. %	Control	1.2	1.9	1.6	1.7	2.7	3	3.5	1.1	2.5	2.3	1.6	0.9	3.2	1.9	<.001
	Selected	34	32	36	35	67.5	41.5	35.3	29.5	26	19	9	6	5	1.4	<.001
	Random	16.6	24.1	9	8.6	13.5	7.5	15	1	8.1	5.2	23.5	1.2	3	9.2	<.001

( ) number of mice

* probability first control V selected
second control V random
third selected V random

Fig 3 Comparison of reticulocyte counts of mice infected with 25 cercariae and their controls

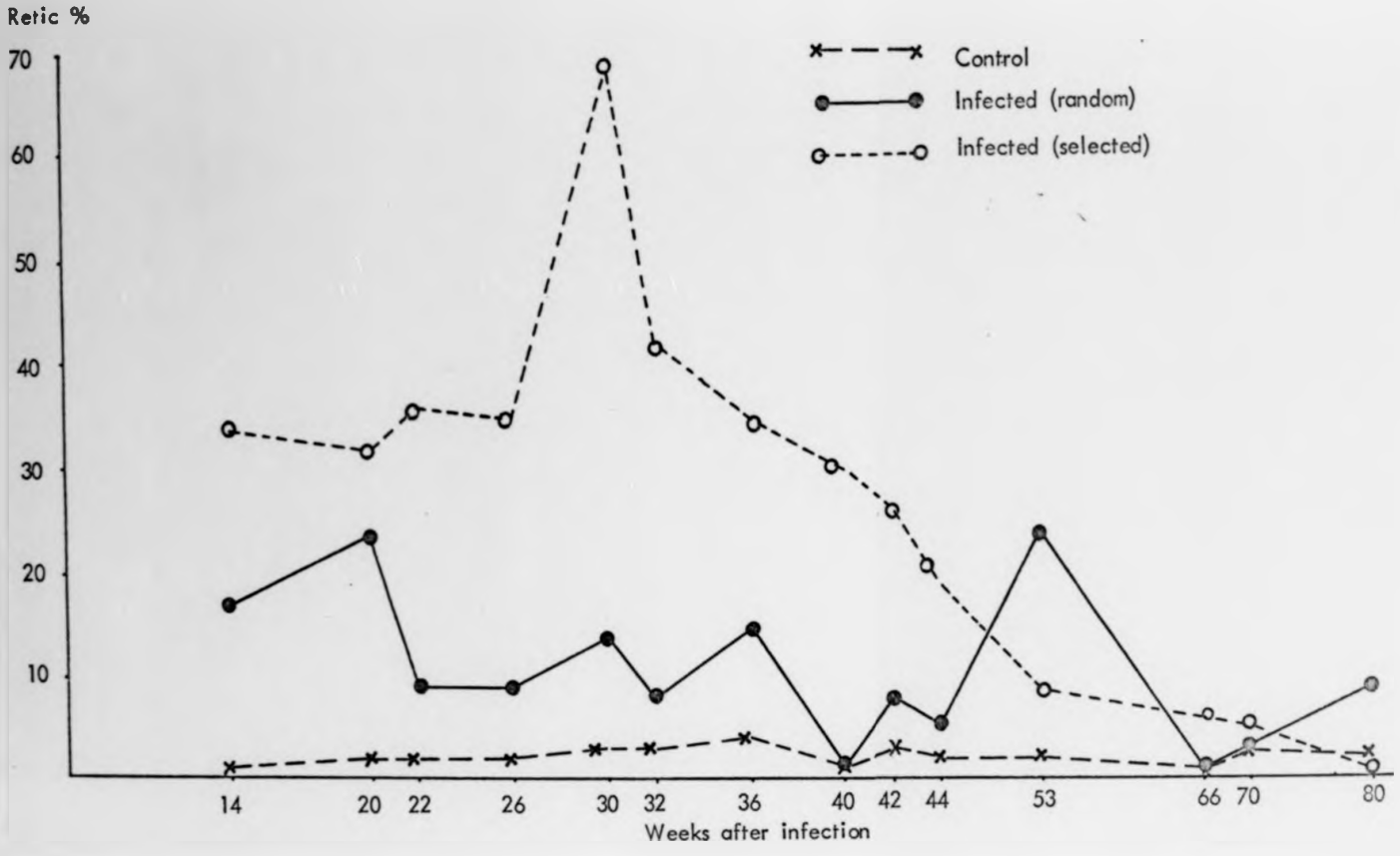


Fig 4 (a) Haematological results of mice infected with 50 cercariae of S. mansoni and their controls

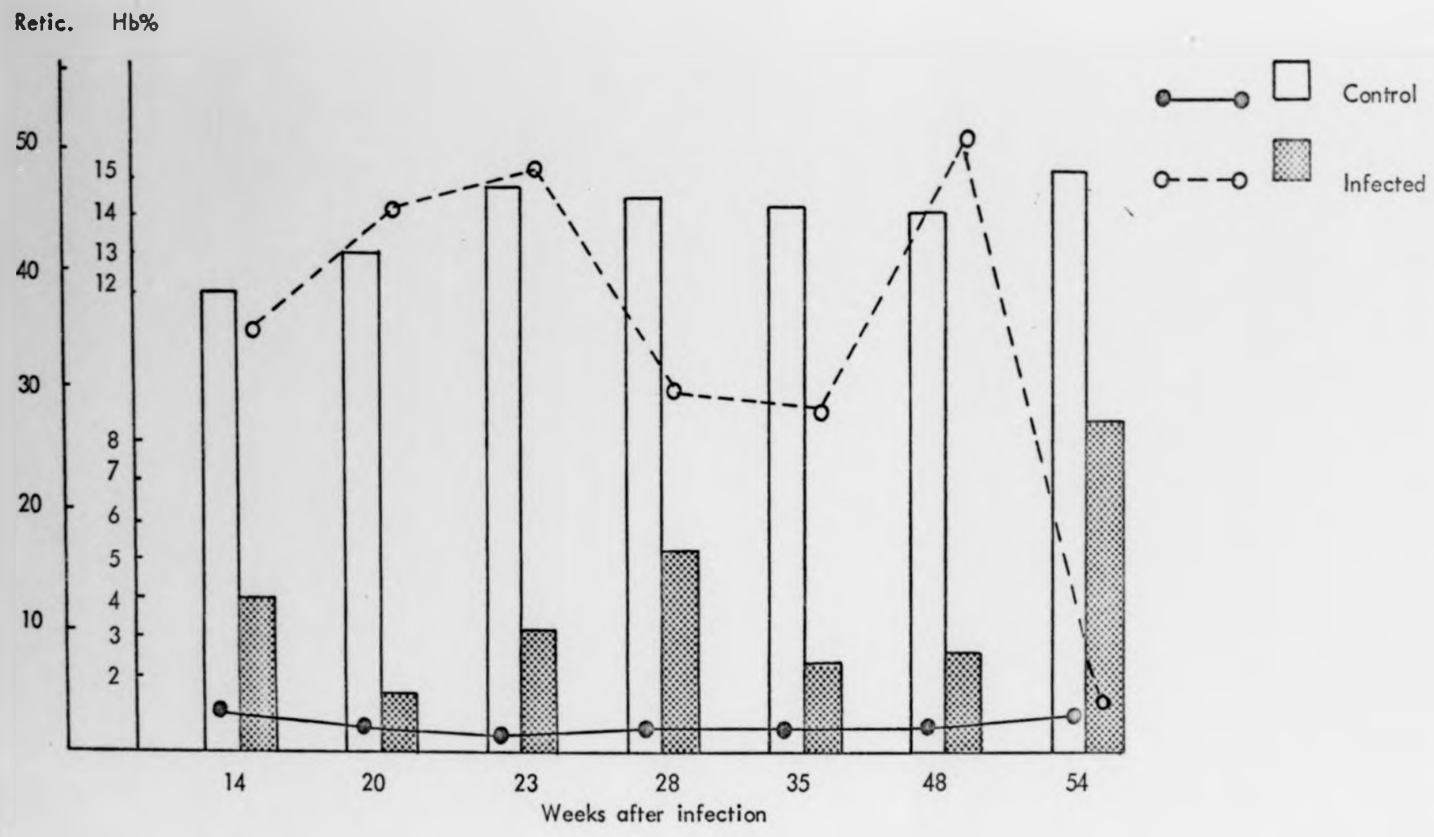
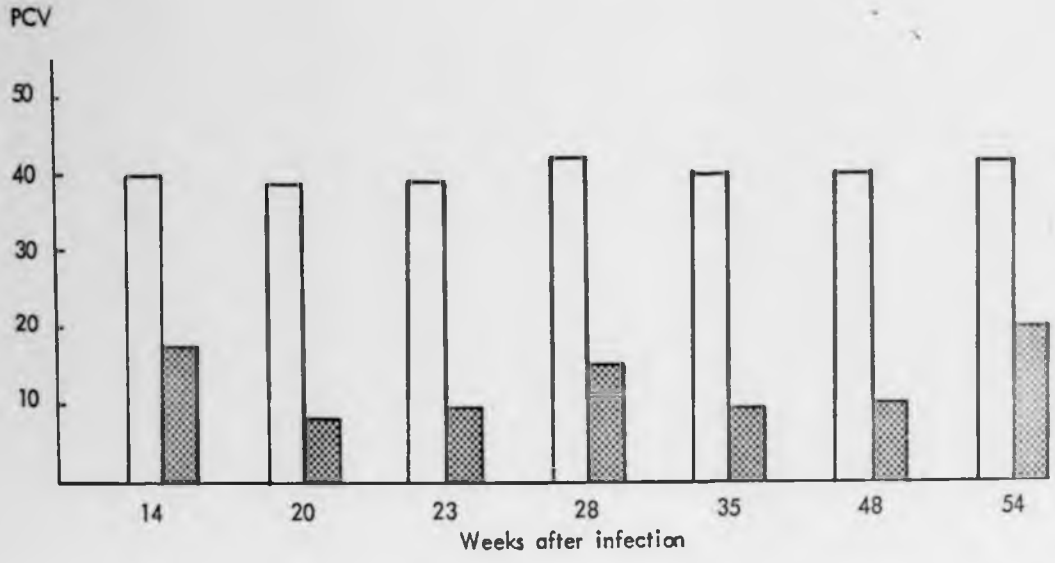


Fig 4(b)



haemoglobin values also declined in the infected mice ranging between I.7 to 8.6 gm/I00 ml, compared to those from control mice which ranged from I2.I to I5.2 gm/I00 ml (Table 3).

There were statistically significant differences between control and infected mice as indicated by the paired comparison t-test . In case of Hb and PCV, values were respectively <0.02 and $<0.0I$. The difference in the case of reticulocyte count was also highly significant being $<0.00I$ (Table 3). The reticulocyte count increased irregularly, usually increasing as PCV values decreased, for example it was 35, 45, and 50% at I4, 20, and 48 weeks of infection (Table 3).

The MCHC decreased through the whole period of the experiment in the infected mice e.g. at I4 weeks of infection it was 20.3 although the probability of the t-test between the two groups were not significant (<0.3) as shown in Table 3.

Splenic enlargement

With the production of severe anaemia in mice infected with 25 or 50 cercariae (experiment I and 2) the spleen increased in size which was a prominent feature whether the mice were obviously sick or were taken from the cage at random (Table 4 and 5).

Relative splenic weights (R.S.W.) were elevated in mice infected with 25 cercariae compared with their control group, with statistical significant difference of $p<0.00I$ (Table 4), this elevation in the two infected groups progressed during the period of the experiment almost identically except at 20, 26, 36, 53, and 80 weeks of infection when it became higher in mice from the random group than those of selected group as shown from Fig. 5.

Mice infected with 50 cercariae had also increased R.S.W. values which reached a maximum level of 4.5% at 23 weeks of infection (Fig. 6).

Direct Agglutination Test

A number of infected mice with 25 cercariae (selected group) gave positive

Table 3

Comparison between the haematological values of mice infected with 50 cercariae and their controls

Duration in weeks		I4	20	23	28	35	48	54	P of t-test
Hb	Control	I2.I	I3.I	I4.8	I4.5	I4.2	I4.I	I5.2	<0.02
	Infected	4 (2)	I.7 (I)	3.I (2)	5.2 (2)	2.4 (I)	2.6 (I)	8.6 (I)	
PCV	Control	40	39	39	42	40	40	42	<0.0I
	Infected	I8	7	9.5	I5.5	9	I0	25	
MCHC	Control	30.2	33.5	35.2	34.5	35.5	35.2	36.I	<0.3
	Infected	20.3	24.2	32.4	30.4	26.6	26.0	30.4	
reticu.	Control	3	I.8	I	2	2	2	3	<0.00I
%	Infected	35	45	48	29	28	50	3	

( ) number of mice killed; one control was killed each time

Table 4 Mean relative splenic weight of mice infected with 25 cercariae (selected and random) and their controls

Duration in weeks		14	20	22	26	30	32	36	40	42	44	53	66	70	80	P of t-test
Body weight	Control	34.6	36.3	33.3	36.5	38	38.6	42	38.5	37	42.5	43	37.5	33.5	43	<.02
	Selected	34	56	33	41	37	34	37	40	45	42	47	48	64	32	<.6
	Random	32	41.6	30.3	39	37	45	37	45	31.3	45	35	48	34	30	<.001
Spleen weight	Control	0.23	0.19	0.16	0.17	0.20	0.16	0.15	0.20	0.10	0.30	0.20	0.15	0.20	0.15	<.001
	Selected	0.20	0.50	0.70	0.40	0.72	0.66	0.60	0.70	0.60	0.50	0.75	0.50	0.50	0.10	<.001
	Random	0.42	0.71	0.66	1.00	0.75	0.45	1.20	0.40	0.50	0.60	0.70	0.40	0.50	0.30	<.6
R.S.W.	Control	0.68	0.52	0.47	0.47	0.51	0.42	0.35	0.59	0.26	0.69	0.46	0.39	0.65	0.34	<.001
	Selected	0.58	0.88	2.17	0.97	1.92	2.14	1.64	1.72	1.32	1.19	1.62	1.04	0.78	0.31	<.001
	Random	1.27	1.70	2.24	2.56	2.02	0.99	3.24	0.88	1.55	1.33	1.99	0.83	1.47	1.05	<.4

$$\text{R.S.W.} = \frac{\text{spleen weight}}{\text{body weight}} \times 100$$

probability first control V selected

second control V random

third selected V random

Table 5

Relative splenic weight of mice infected with 50 cercariae and their controls

Duration in weeks		I4	20	23	28	35	48	54	P of t-test
Body weight	Control	4I	40	35	35	35	35	39	<0.5
	Infected	35	4I	40.5	35.5	39	34	45	
Spleen weight	Control	0.37	0.I4	0.23	0.3I	0.32	0.32	0.22	$<0.I$
	Infected	0.90	0.74	I.82	0.95	0.90	0.70	0.50	
R.S.W.	Control	0.90	0.35	0.65	0.88	0.9I	0.87	0.56	$<0.I$
	Infected	2.64	I.80	4.59	2.65	2.30	2.05	I.II	

R.S.W. Fig 5 Comparison of R.S.W. of mice infected with 25 cercariae (selected, random) and their controls

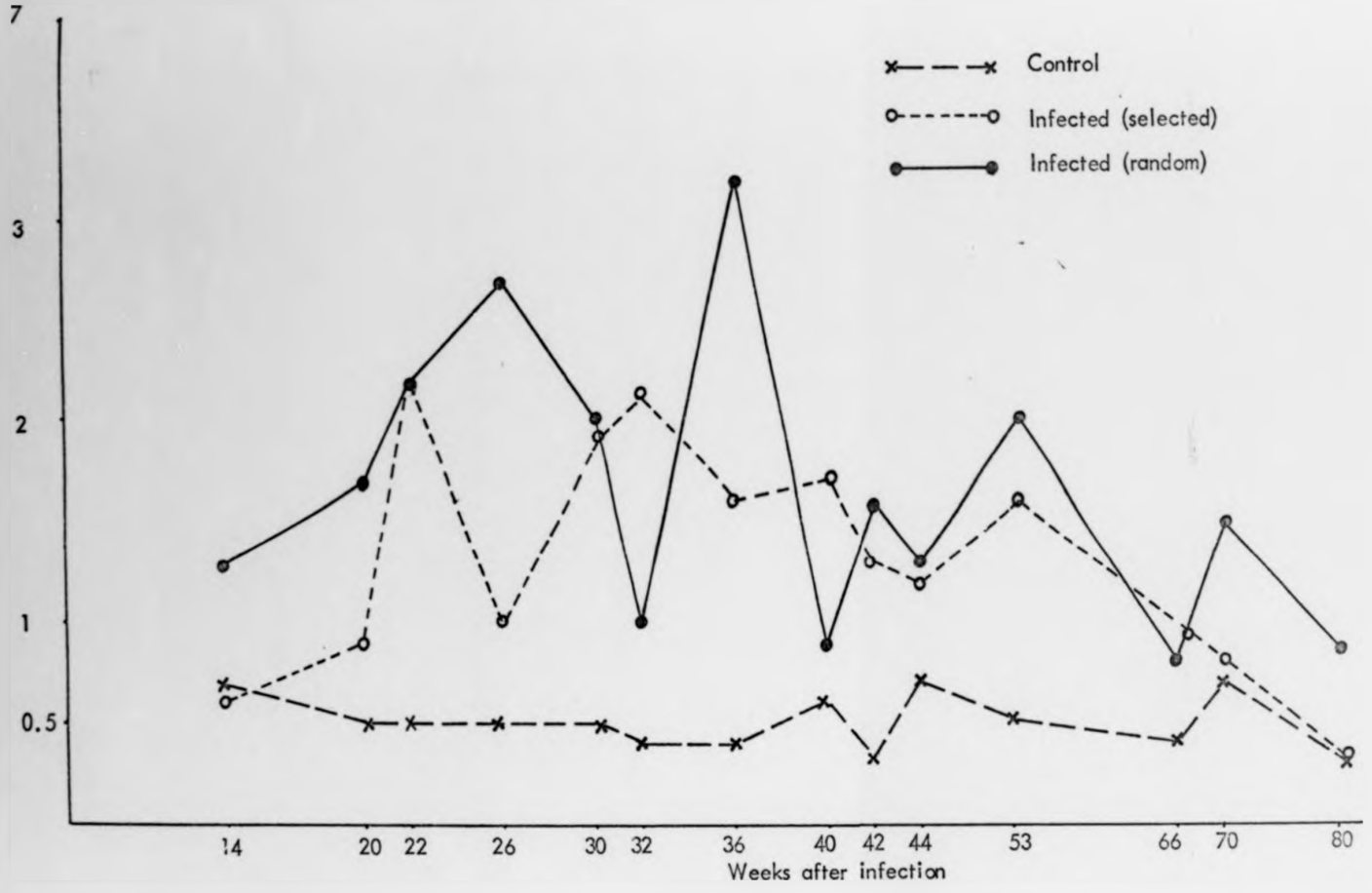
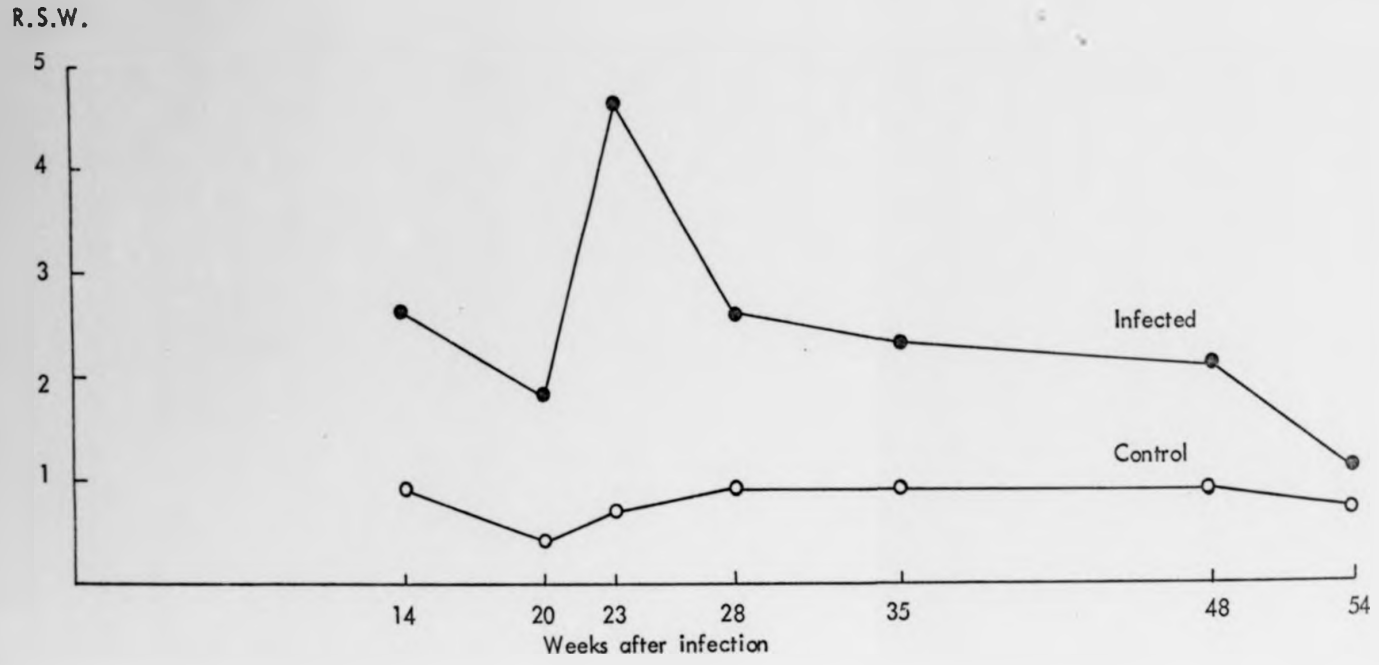


Fig 6 Relative splenic weight of mice infected with 50 cercariae (severe anaemia) and their controls



agglutination when their blood was mixed with the diluted "Broad spectrum" antiglobulin serum, which started after 22 weeks continued positive until 66 weeks after infection when it became negative. During this period strength of agglutination varied among the individual infected mice for example at a dilution of the "Broad spectrum" I/4 it was either 3+, 2+, or I+. It was noted that the agglutination was usually positive when there was marked decrease in PCV values (8-30). In the strongest reaction with antiglobulin the end point was at I/I28. The red cells in this case were from an infected mouse which had a PCV value of 7% (see Table 6).

Infected mice from the random group this test was mostly negative with "Broad spectrum"; except for two mice at 22 and 30 weeks of infection when they gave positive agglutination respectively at I/4 (PCV = 8%, Hb = I.8 gm/ I00ml) and at I/8 (PCV= 2I%, Hb = 7.2 gm/I00ml) of I+ strength. No agglutination was observed in the case of erythrocytes taken from the control group.

Mice infected with 50 cercariae had positive agglutination reaction after 28 and 35 weeks of infection in which respectively two mice (PCV= 9, 22%) had I+ agglutination at I/4 and one mouse (PCV = 9%) had I+ agglutination at I/8 dilution of "Broad spectrum".

When the specific antisera (anti-IgG, IgM, and C'_3) were used in the direct agglutination test, it was not possible to obtain positive results from infected mice of both experiments and at early or late infections.

Discussion

Haematological studies of infected mice

Following the results obtained from the previous two experiments few points would be summarized referable to the production of anaemia in mice infected with low dose of S.mansoni (25 or 50 cercariae):

I- Infected mice developed mild anaemia (Hb = I0-I2 gm./I00ml) which progressed into severe anaemia (Hb = 4-9 gm/I00ml); this could become manifested

Table 6

Direct agglutination test on red cells taken from mice infected with 25 cercariae (selected group)

Duration in weeks	No. of Inf. mice	PCV	Dilution of Br.sp. (anti-IgG. IgM and IgA)							
			4	8	I6	32	64	I28	256	5I2
I4	I	7	0	0	0	0	0	0	0	0
20	I	8	0	0	0	0	0	0	0	0
	2	7	0	0	0	0	0	0	0	0
22	I	6	0	0	0	0	0	0	0	0
	2	7	0	0	0	0	0	0	0	0
	3	8	+	+	+	0	0	0	0	0
26	I	5	+++	+	0	0	0	0	0	0
30	I	I0	+	+	0	0	0	0	0	0
	2	I0	0	0	0	0	0	0	0	0
32	I	8	+	0	0	0	0	0	0	0
	2	9	0	0	0	0	0	0	0	0
36	I	7	++	+	+	+	+	+	0	0
	2	8	+	+	0	0	0	0	0	0
	3	I3	0	0	0	0	0	0	0	0
40	I	9	+	0	0	0	0	0	0	0
	2	8	+	0	0	0	0	0	0	0
42	I	20	++	+	+	0	0	0	0	0
	2	6	+	+	0	0	0	0	0	0
44	I	II	+	+	0	0	0	0	0	0
53	I	30	+	0	0	0	0	0	0	0
	2	34	0	0	0	0	0	0	0	0
66	I	3I	0	0	0	0	0	0	0	0
70	I	33	0	0	0	0	0	0	0	0
80	I	2I	0	0	0	0	0	0	0	0

Control normal mice gave negative agglutination test.

furthermore when haemoglobin value deteriorated to I-3.8 gm/I00ml, the infected mouse usually died i.e. once the mice are severely anaemic they never recover their normal blood values although there was increase in their bone marrow output of reticulocytes (30-80%).

2- The onset and severity of this anaemia depends on each individual infected mouse, how it reacts to infection, and on the sex of worms they harbour. Thus infected mice that survived long period of one year and a half; when killed had either mild (Hb = I0.7 gm/I00ml) or moderetely severe (Hb= 7.2 gm/I00ml) anaemia, usually harboured one to five male or one male and one female worm, whereas those killed after 30 or 32 weeks of infection with very severe anaemia (Hb = I.4 and I.9 gm/I00ml) had two male and 3-4 female worms in their livers. Similarly, Mahmoud (I973) proved that mice infected with either bisexual sterile worms or male worms, developed at later stages of the infection milder anaemia than those infected with bisexual fertile worms, the difference in severity of anaemia due to the larger antigenic stimulus resulting from bisexual infections.

3- The low Hb, PCV and MCHC values obtained indicates that anaemia tended to be hypochromic for most of the period of the experiment.

Direct agglutination test

The positive reactions acquired after adding to the "Broad spectrum" antiglobulin serum red cells from mice lightly infected with S.mansoni; provides evidence for the passive sensitization of these cells with globulins, rendering them more susceptible to destruction. This in fact causes their severe anaemia. It was particularly noted that when these mice had a low PCV value (5-20%) their red cells agglutinated.

Differences in the strength and end point of agglutination among the infected mice indicates that their red cells are coated gradually with variable quantities of globulins. This process may depend on the following mechanisms:

I- The level of these globulins in the sera of infected mice varying according to the antigenic effect of worms and eggs in their tissues.
2- The extent of damage to erythocytes themselves, i.e. number of antigenic sites on the red cell membrane.

Thus it appears that some red cell surfaces could be completely free from globulins (negative results after 20 weeks of infection); others have two or more molecules attached to its surface (I+ at I/4), when red cells became coated with a considerable number of globulin molecules (positive at I/I6 or I/I28), then their destruction increases markedly resulting in the very severe anaemia seen in some of the infected mice.

A positive direct antiglobulin test is a pointer to the presence of auto-antibodies (Dacie, I968). They could be either IgG or too few molecules of IgM (Dacie, Lewis I975). Elevation in the gamma globulins of sera from infected mice with schistosomiasis have been found by Hillyer et al (I967), also later in this study when sera from infected mice were tested by the agar plate method (Chapter I-Part 5). It was not possible though to detect them on the surface of red cells from the infected mice when the specific anti-globulin sera; anti-IgG, and anti-IgM were used, on the other hand the negative reaction does not negate the possibilty that they were present, which could be explained by either of the following circumstances:
- If the antiglobulin serum is added rapidly to the washed cells a weakly positive reaction is obtained which becomes completely negative on standing for a few minutes (Rosenfield et al, I95I).
- The specific antiglobulin sera used were relatively impotent and only capable of detecting strongly sensitized red cells (Dacie, Lewis's I975).

Alternatively small amounts of both globulins (IgG and IgM) may coat the surface of red cells in mice infected with S. mansoni that can only be detected by the "Broad spectrum" antiglobulin sera.

Although red cells had no detectable C_3 on its surface after using the

anti-C'_3, other evidences suggested otherwise for example presence of the IgM molecule on the membrane of some red cells, which is the antibody most capable of fixing the complement (Woodruff, I973). Further evidence was the "holes" caused by C'_3 fixation (Dacie, & Worlledge I969) on the surface of few erythrocytes from the same infected mice, seen by the scanning electron microscope (Part 5).

The results thus obtained prove that the haemolytic anaemia in mice infected with S. mansoni was partly caused by an immunological mechanism brought about in two probable ways :

I- Interaction between antigen-antibody with or without complement fixation on the surface of red cells rendering them more susceptible for phagocytosis by the reticulo-endothelial cells in the spleen or liver.

2- Complement fixation by immuno-complexes on the membrane of red cells causing their lysis.

One of the major hypothesis for the etiology of auto-immune anaemia is the modification of red cell antigens leading to termination of immune tolerence.(Dacie, I970).

It is an established fact that schistosome parasites produce soluble antigens found in host tissues and blood stream outside the parasite structure itself (Weller, I976). The red cell membrane might be modified by these antigens either by direct adsorption of the antigen molecule (Bruninga, I97I, and Mahmoud, I97I); or cell membrane might be coated with damaged (altered) tissue components from antigenic toxic material released from eggs or worms, as suggested by Kurata (I966). He proved that red cells sensitized with liver or colon extracts (auto-antigens) agglutinated when incubated with sera (auto-antibodies) from rabbits infected with S. japonicum. More detailed studies on this is recorded in chapter 3.

Splenic enlargment

Infected mice developed the "Hypersplenism" syndrome when they had low

PCV and Hb values, with increase in their bone marrow output of reticulocytes and enlarged spleen (sometimes four times its normal size, Table 2 and 4).

The causes of splenic enlargment are diverse and involve more than one factor: In the case of schistosomiasis: a) Worm and egg toxins circulating in the blood were considered responsible in bringing about structural changes in tissues hence the spleen (Mohamed, 1936), which in the initial phase of infection involves intense proliferation of reticulo-endothelial cells, followed by plasma cell differentiation (Andrade, 1964). b) Congestive splenomegaly induced by overgrowth of fibrous tissue, dilatation of veins and sinuses; haemorrhage with thickening of the reticulin fibers (Sabour, et al 1967). c) Red cell pooling (Bowdler, 1975).

Although the enlarged spleen proved to be the principal site for the destruction of infected red cells (Woodruff, et al 1966 and this study), yet the specific role it plays in trapping these cells is still not fully comprehended; many theories have been suggested:

1- Erythrostasis: Blood passes slowly inside the bilharizial spleen due to its fibrotic and congested tissue (Farid, et al 1966), hence the red cells becomes closely packed and this leads to metabolic death from glucose deprivation (Jandl, 1967).

2- The spleen acts as a fine filter and tends to retain the antibody-coated cells, this retention probably facilitates phagocytosis (Dacie, 1970). Following the work done in this chapter it was known that a number of damaged red cells had on their surface small amounts of a non-complement-fixing antibody (IgG), these cells are destroyed predominantly by the spleen (Mollison, et al 1965, and Dacie, 1970). Moreover the number of lymphocytes and plasma cells was proved to increase in spleens of mice infected with S. mansoni (Saeed, 1970).

Although these facts suggest that the spleen contributes to the production of anaemia, nevertheless it is difficult to conclude that it

is one of the important causes because:

1- A number of infected mice with no increase in their spleen size had severe anaemia e.g. Hb = 1.9gm./100ml., its spleen weight was 0.20 (Table 2, 4).

2- Removal of the spleen from infected mice does not alter the production of anaemia (Saeed, 1970).

Presence of the enlarged spleen would aggravate the condition of anaemia probably the best description of this organ in cases of schistosoma infection was made by Woodruff (1973) in which he stated that "The spleen is but a graveyard of the damaged red cells".

CHAPTER 2

ELUTION OF ANTIBODIES FROM RED CELLS

Introduction

In human acquired haemolytic anaemia of the autoimmune type; several observers (Kidd, 1949, Vaughan, 1956, and Weiner, 1957) have been able to elute antibody from erythocytes, which usually gave a positive antiglobulin test. These workers have studied the specificity of these immunoglobulins by the indirect antiglobulin test.

The elution technique has not been much used to elucidate the pathogensis of anaemia in experimental schistosomiasis. On the other hand, Kobayashi et al (1976) proved that eluate from erythrocytes of calves infected with T. congolense (African trypanosomiasis) contained IgG and IgM, which reacted against antigen of the parasite; thus he postulated that trypanosome antigen-antibody-complement complexes, deposited on the surface of erythrocytes of infected calves, resulted in their immune elimination leading to clinical anaemia.

In the previous chapter it was mentioned that mice infected with a low dose of S. mansoni cercariae, had profound anaemia. The direct antiglobulin test showed that some of their erythrocytes were coated with small amounts of globulins. In the present experiment an attempt was made to elute and study the characteristics of globulins bound to the surface of erythrocytes.

Materials and methods

A group of 30 mice were infected with 50 cercariae of S. mansoni, with 15 normal mice as their controls. They were killed at 4 weekly intervals during the early stages of infection, and later at 31, 38, 42, 55, and 73 weeks.

Blood samples from both infected and normal mice were collected for haematological estimations. When more than one mouse was killed at a certain stage of the experiment, blood samples were pooled for elution

which was done after the technique of Landsteiner and Miller (1925):

- A volume of 0.9% NaCl solution equal to the volume of saline which will be used for the eluate was added to washed packed sensitized red cells, and mixed thoroughly.
- The suspensions were centrifuged at 1,200 - 1,500g for ten minutes. Then the supernatant was taken off as control; diluted on a second row with the eluate.
- The same volume of saline was added to the packed cells, and placed in a water-bath at 56C° for 5 - 10 minutes and shaken repeatedly.
- The mixture was then centrifuged rapidly while still warm, and the cherry-red supernatant fluid was removed, this is the eluate for use in the indirect antiglobulin test.

The eluate and the saline of the last wash (control) were examined to see if they contained antibody: One volume of a 50% suspension of normal mouse red cells was added to 10 volumes of the eluate and control, then incubated at 37C° for one hour, the mixture was examined macroscopically for agglutination. Cells were washed four times in a large volume of 0.9% NaCl (warmed to 37C°), and a 10 - 20% suspension was made in 0.9% NaCl. On a white translucent tile, two drops of this suspension was then mixed with an equal volume of anti-mouse globulin ("Broad spectrum", IgG, and C_3^1) diluted at 1/4 to 1/512.

Results

Reduction in mean haemoglobin and packed cell volume values of infected mice began after 12 weeks of infection (Hb = 10.8gm./ 100ml., PCV = 29.3%). later at 31, 55, and 73 weeks post-exposure, the mice had very low Hb values; 5.50, 5.7, and 7.5 gm./100ml. Anaemic mice had their reticulocyte count elevated (Table I), especially after 31 weeks of infection; 44%, when PCV dropped to 19.5%.

The paired comparison t-test was statistically significant ($p < 0.01$)

Table I Comparison between mice infected with 50 cercariae and their controls

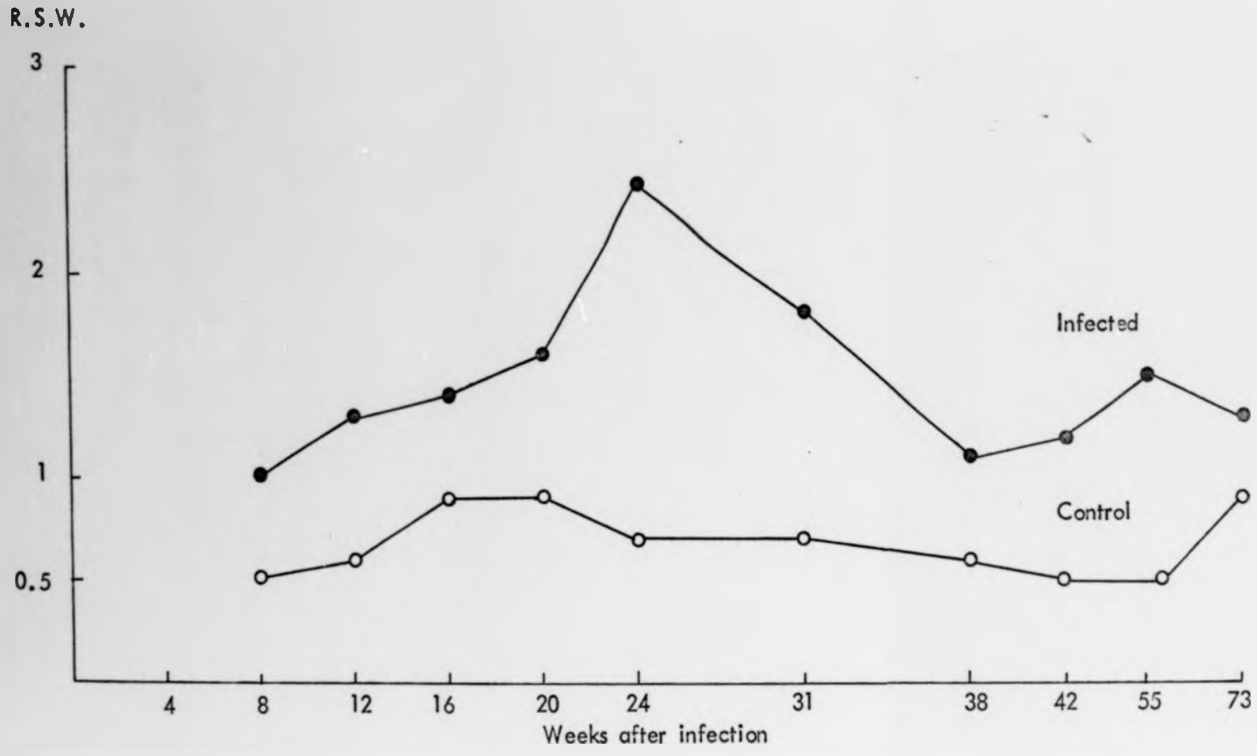
Duration in weeks	Mice	8	I2	I6	20	24	3I	38	42	55	73	P*
Hb	Control	I4.5 (2)	I4.5 (I)	I4.0 (I)	I0.6 (I)	I3.0 (I)	I5.0 (I)	I4.8 (I)	I5.4 (I)	I3.0 (I)	I2.I (I)	<0.0I
	Infected	I3.2 (3)	I0.8 (3)	I0.6 (3)	II.6 (2)	I0.8 (2)	5.5 (2)	I4.4 (I)	I2.4 (I)	5.7 (I)	7.5 (I)	
PCV	Control	40.5	40.0	4I.0	40.0	38.0	42.0	45.0	45.0	40.0	40.0	<0.0I
	Infected	34.3	29.3	3I.0	38.0	32.5	I9.5	38.0	39.0	I8.0	20.0	
reticul. %	Control	2.5	3	3	2	2	4	2	0.8	2	3	<0.0I
	Infected	4.3	5.6	5.6	5.5	5.5	44	I.6	3.8	I3	I.6	
R.S.W.	Control	0.5I	0.6I	0.87	0.90	0.73	0.72	0.58	0.50	0.45	0.90	<0.05
	Infected	0.99	I.32	I.42	I.56	2.36	I.8I	I.II	I.23	I.52	I.37	

( ) number of mice

P* probability of t-test

$$\text{R.S.W.} = \frac{\text{spleen weight}}{\text{body weight}} \times \text{I00}$$

Fig 1 Relative splenic weight of mice infected with 50 cercariae (mild anaemia) and their controls



when PCV, Hb values and reticulocyte counts were compared between the normal and infected mice (Table I).

The ratio of spleen to body weight was increased through the whole period of the experiment (Fig. I), with significant probability <0.05 between the infected and control group indicating hypersplenism.

The different antiglobulins used failed to agglutinate normal red cells coated with the eluate from erythrocytes of infected mice killed at several stages of infection (8 - 73 weeks). With these negative results, another attempt was made to concentrate the eluates by placing them in cellophane bags for seven days at 4C°. The results were also negative.

Discussion

The negative results encountered after the addition of eluates coated erythrocytes of infected mice with low dose of 50 cercariae to the antiglobulin sera ("Broad spectrum", IgG, and C_3); does not exclude the possiblity that auto-antibodies or globulins were adherent to the surface of red cells. Such results maybe due to one of four possible causes:

- Failure to dissociate the antigen-antibody complex because of unusual stability (Komninos, & Rosenthal/1953). Later, when a different method of elution was tried based on the principle of alcohol precipitation of proteins in the cold (Weiner, 1957), it was possible to detect by the agar plate method (Chapter I-Part 3) weak globulins in eluate of erythrocytes from a number of anaemic infected mice.
- By the direct agglutination test it was shown in the previous chapter, that globulins gradually coated some erythrocyte surfaces and this lead to the very severe anaemia seen in the infected mice (Hb = I - 5gm./I00ml.). Unlike them the present group of infected mice had a moderate to severe anaemia (Hb = 5 - I2gm./I00ml.), indicating that at the time the mice were killed; their erythrocytes were not coated with a sufficient amount of globulins to be eluted.

- The dissociation of low affinity antibody, while the red cells were being washed; especially in case of IgG (Gilliland et al, 1970).
- A different type of globulins (haemolytic antibody) probably was present in the eluate, as suggested by Dacie/(1951) in human acquired haemolytic anaemia; as these antibodies are hardly adsorbed at all by normal corpuscles under physiological conditions.

J. De Gruchy

CHAPTER 3

DETECTION OF AUTOANTIBODIES IN SERA OF MICE INFECTED WITH S. MANSONI BY HAEMAGGLUTINATION TEST

Introduction

There are several ways in which micro-organisms may cause an auto-immune response including I) Liberation of normal or damaged tissue components which then cause auto-antibody production; 2) Modification of normal tissue components by enzyme; 3) Combination of normal tissue components with products of the micro-organism. (Asherson, I968).

Conditions similar to those processes afore-mentioned that might excite production of auto-antibodies are present in schistosoma infection. Firstly there is tissue damage due to worm and egg antigens/toxins (e.g. enzymes, metabolic end products), as seen for example in the liver (Daugherty, I955 and Warren, I96I). Secondly the possibility of denaturing of colonic tissues by the infection (Salem et al, I972) and thirdly new antigens might be produced by combination of tissue haptens with parasitic antigens and vice versa, and antibodies against these new antigens might cross-react with the normal tissue constituents (Shamma et al, I965).

It is possible to hypothesise that in S. mansoni infection, red cells during circulation in vivo inside the damaged tissues (particularaly liver and spleen); their surface either adsorbs the tissue antigens or becomes modified by them so that red cells appear foreign or antigenic to which antibodies form in the serum. These might agglutinate the red cells resulting in less resistant cells towards the action of erythrophagocytosis. This process is outlined in Fig. I; following the work of Kurata (I966); in which he proved by the passive haemagglutination test presence of auto-antibodies in the sera of rabbits infected with S. japonicum, against liver or colon extracts and when the titre of these "auto-antibodies" was at its maximum; anaemia was most conspicuous. The following study was carried out

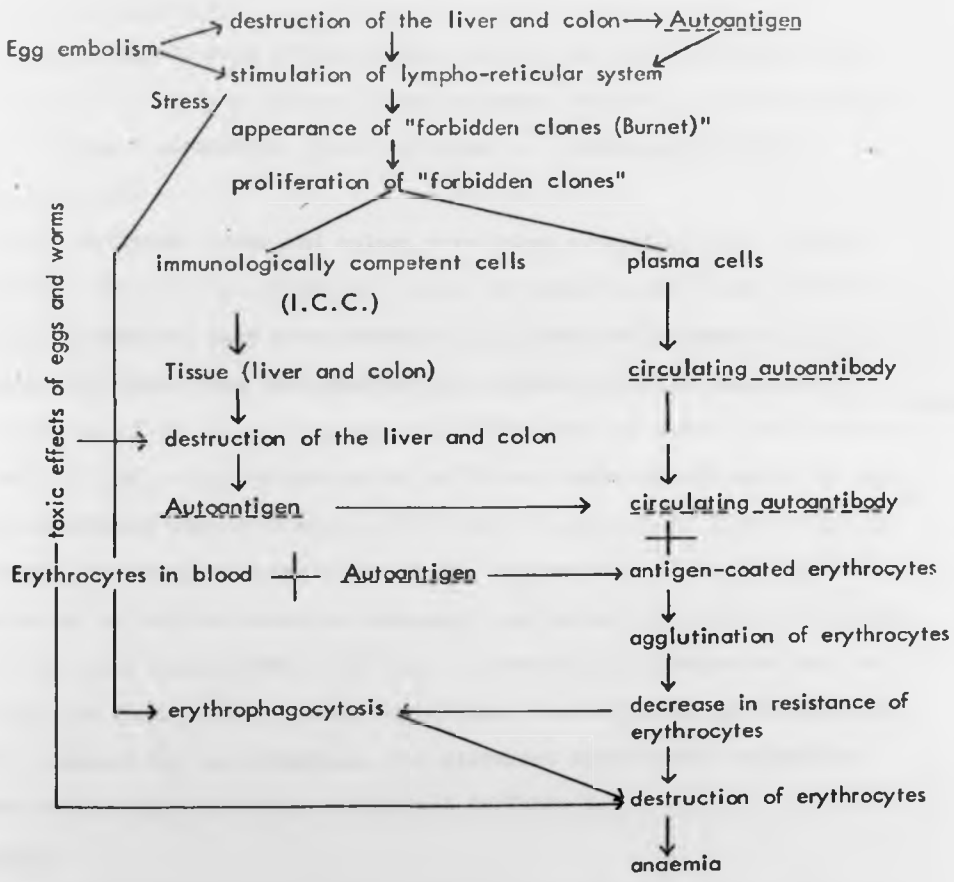


Fig 1 Pathogenesis of anaemia in schistosomiasis japonica from the immunological point of view.

From Kurata (1966)

to determine whether this hypothesis of auto-immunity is partly responsible for production of anaemia in mice infected with S. mansoni.

Materials and methods

The principal steps of the experiment were as follows: Normal mouse erythrocytes + antigen (either liver or spleen extract) ⟶ antigen-coated erythrocytes + antibodies (infected serum) ⟶ haemagglutination of erythrocytes.

- Tissue antigens: Liver and spleen were taken separately from control (normal) mice, also at different periods of infection from mice infected with 50 cercariae; they were extracted by alcohol as illustrated in Fig. 2.
- Sera were taken from both control and infected mice (50 cercariae).
- Procedure of the passive haemagglutination test was done after Middlebrook & Dubos (1948): 0.I ml. of a 40% suspension of thrice washed normal mouse red cells were sensitized for two hours at 37°C with 3 ml. of either liver or spleen extract; the final concentration of this suspension was 0.25%. Then 0.I ml. was added to twofold serial dilutions of the tested sera (0.25 ml.); after they had been inactivated at 56°C for 30 minutes. The mixture was left at 37°C for two hours, then at room temperature for I8 hours. The erythrocytes were observed for agglutination. The different erythrocyte suspensions used in this experiment are summarized in Table I.

Results

No agglutination of red cells could be observed after either two hours at 37°C or I8 hours at room temperature; subsequent to their sensitization with both liver or spleen extracts, even using extracts of early infection (I0 weeks) or older infections (23, 24, or 26 weeks) did not alter the result haemolysis occurred instead and after I8 hours. Which was not the case when erythrocytes were incubated with the sera (normal or infected) alone (Table I).

To find out if this haemolysis was caused by the action of the extract

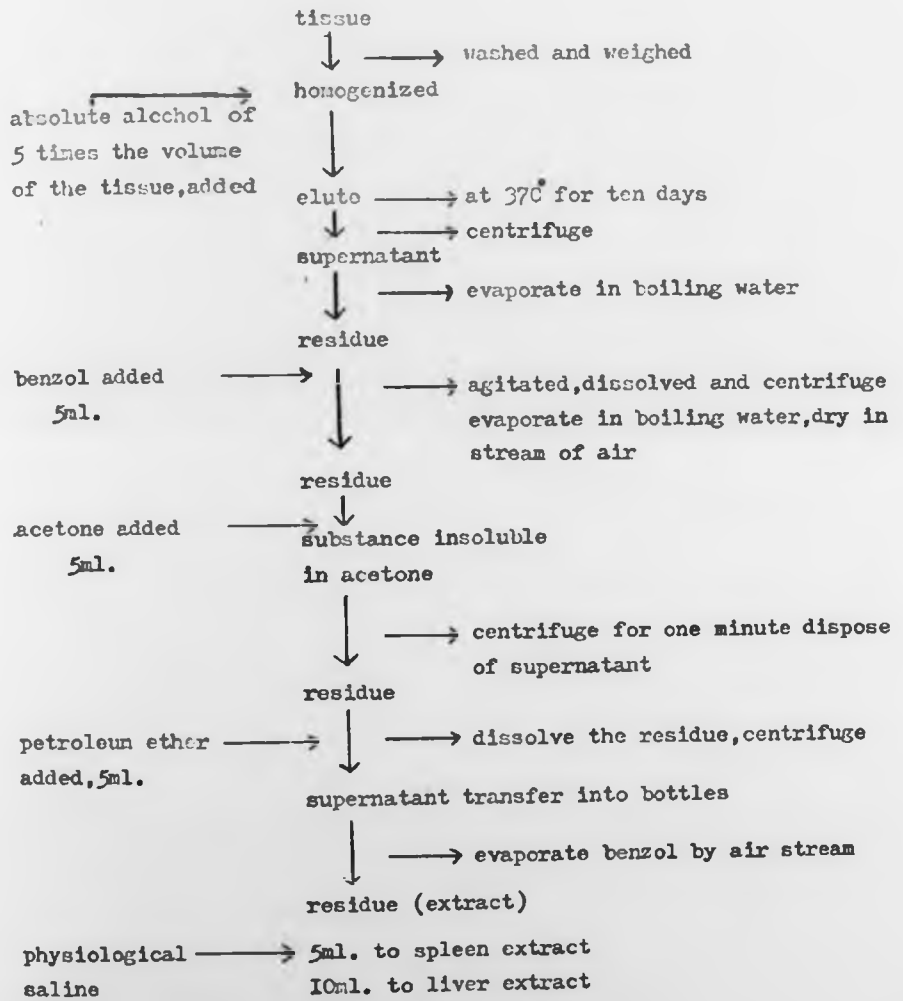


Fig. 2 Procedure to produce the liver or spleen extract, after Kurata (1966).

Table I Results of the haemagglutination test

SUSPENSIONS			HAEMAGGLUTINATION		HAEMOLYSIS	
Antigens (extract)	Red cells 40%	Serum diluted I/4 - I/128	2 hours 37C°	18 hours Room temp.	2 hours 37C°	18 hours R. temp.
Control saline	0.1 ml.	———	-ve	-ve	-ve	-ve
———	0.1 ml.	normal	-ve	-ve	-ve	-ve
———	0.1 ml.	infected	-ve	-ve	-ve	-ve
Normal liver	0.1 ml.	normal (2)	-ve	-ve	-ve	-ve
10 weeks infected liver	0.1 ml.	infected 10 weeks (4)	-ve	-ve	-ve	+ve
23 weeks infected liver	0.1 ml.	infected 23 weeks (3)	-ve	-ve	-ve	+ve
26 weeks infected liver	0.1 ml.	infected 26 weeks	-ve	-ve	-ve	+ve
Normal spleen	0.1 ml.	normal	-ve	-ve	-ve	-ve
23 weeks infected spleen	0.1 ml.	infected 23 weeks	-ve	-ve	-ve	+ve
26 weeks infected spleen	0.1 ml.	infected 26 weeks	-ve	-ve	-ve	+ve

( ) number of sera tested

along the following experiment was carried out:

An haemolytic assay of liver and spleen

A- 0.1 ml. of thrice washed normal mouse erythrocytes (40%) + 3 ml. alcoholic extract of liver or spleen separately; left for 24 hours at room temperature in bottles.

Red cells +	Normal liver	-ve haemolysis
	Infected liver	+ve haemolysis
	Normal spleen	-ve haemolysis
	Infected spleen	+ve haemolysis
Control (red cells + saline)		-ve haemolysis

Both extracts were diluted at I/4 to I/I28 to determine the end point of haemolysis, it took place in all dilutions when positive.

B- Saline extract of both spleen and liver were prepared by:

-The whole tissue was frozen at -20C° overnight (to destroy the trapped erythrocytes inside the tissue).

-The tissue was thawed and homogenized with isotonic saline, filtered and centrifuged several times.

-3 ml. of the supernatant was mixed with 0.I ml. of thrice washed normal mouse erythrocytes (40%), incubated at 37C° for one hour.

-Centrifuged and any haemolysis was noted.

Red cells +	Normal liver	-ve haemolysis
	Infected liver	+ve haemolysis
	Normal spleen	-ve haemolysis
	Infected spleen	+ve haemolysis

Control was the same as in A.

Discussion

In general terms, autoimmune disease may arise because the antibody-producing system is abnormal or because it is presented with antigen in unusual amounts or of altered form (Asherson, I968).

Kurata (I966) assumed that destruction of liver and colon in rabbits

infected with S. japonicum , was the result of either toxic effects of the eggs and worms, or egg embolism which also stimulate the lymphoreticular system and cause the mutation of the clones in haematopoietic organs, with sequential production of circulating auto-antibodies and infiltration of lymphoid cells inside liver and colon which adds to their damage (Fig. I). Subsequently erythrocytes in blood became coated with auto-antigens of the damaged tissues; to be agglutinated by the circulating auto-antibodies (Fig. I).

Further evidence of auto-immune process involved in the pathogenesis of schistosoma infection was proved by the detection of auto-antibodies in sera of patients with hepatic schistosomiasis against liver (Ekladios et al, 1971) and spleen (Ageeb et al, 1971) antigens.

Although the same procedure used by Kurata (1966) was followed in the present experiment, results of haemagglutination test was negative; probably due to either: That liver and spleen extracts are not easily adsorbed by the normal mouse red cells; or the short duration (2 hours) of the crude exposure *in vitro* of normal red cells; compared with the prolonged opportunity for contact by the blood cells *in vivo* (Wagiley, *et al* 1948), was not sufficient to coat the cells with tissue extracts.

Explanations for the haemolysis observed include 1) that agglutination was so strong that red cells were haemolysed 2) it is possible that liver and spleen extracts themselves are directly haemolytic; as was proved by using the extract alone without the serum; and that the extract lytic activity can also be shown without incubating the red cells at 37C; i.e. it was active at room temperature. This was done as a preliminary step to investigate the haemolytic role of these tissues, which will be discussed in details in the following chapter.

PART 4

SCHISTOSOMA TOXINS AND ANAEMIA

HAEMOLYTIC EFFECTS OF SCHISTOSOMA TOXINS ON THE DEVELOPMENT OF ANAEMIA

Introduction

Mohamed (1931) stated that helminths secrete or excrete toxins which are distributed equally in the blood and tissues resulting in a general eosinophilia; the eosinophil cell secrete antitoxic products to neutralize these toxins. Toxic products of worms, dead worms, and eggs produced by the worms are one of the factors involved in the pathogenesis of hepato-splenic schistosomiasis (Warren, 1961). The role of these toxins in the development of anaemia whether they are metabolites, enzymes or digestive ferments of worms or eggs needs more investigation.

Although a hypothetical haemolytic toxin secreted by the worms was suggested by Mahmoud & Woodruff (1972), the identification of a specific anti-erythrocytic toxin in S. mansoni infection has been rare. However, Huan Chi et al (1976) demonstrated by in vitro studies, that the incubation of purified trypanosomes with normal mouse red cells had a strong lytic effect upon these cells.

The other possible lytic factor involved in the destruction of red cells, is the tissue lysins normally present in balance with its inhibitor in tissues and sera, under pathological conditions such as blackwater fever this balance was suggested to be upset in the direction of increased erythrocytes lysis (Maegraith et al, 1943 a, b, and Zuckerman, 1966).

Thus it was plausible to ask two questions: Do schistosome toxins have a direct haemolytic effect on red cells?. What effect do the toxins have on the host tissues that might influence the destruction of the erythrocytes?. The following experiments were undertaken to answer those questions.

Materials and methods

Experiment I: To test for a possible direct haemolytic effect of adult worms on normal mouse red cells. A crude concentrated extract was prepared

from frozen worms (-20C), then added to a 4% suspension of thrice washed normal mouse red cells in small bottles. The suspension was left for two hours at 37C then successively for 18 hours at room temperature, and examined for haemolysis.

The second attempt was to inject worm extract into normal mice in order to show if they would produce anaemia. 0.20 mg. were weighed from pooled frozen worms; homogenized with 15 ml. normal saline. Five normal mice were used, two served as controls which received only sterile saline, three mice were injected with the worm extract; the injection was intraperitonealy as follows:

0.1 ml. daily for the first week

0.1 ml. three times for the second week

0.1 ml. twice for the third week

Then once weekly until 32 weeks

Experiment 2: The haemagglutination test done in the previous chapter resulted in haemolysis of the red cells when mixed with either liver or spleen alcoholic or saline extracts in small bottles. In this experiment alcoholic extracts were employed under more sterile conditions by using blood agar plates to eliminate the possibility of bacterial contamination.

2% of washed normal erythrocytes was added to 1.5% melted agar (Oxoid agar) at 45C, mixed well then distributed into plastic sterile small petri dishes (5cm. diameter). Two drops of liver or spleen alcoholic extracts taken from normal and mice infected with 50 cercariae of S. mansoni, were put separately in holes made in the agar. The plates were left at room temperature in a humid chamber for 72 hours, the control plates contained PBS, water and alcohol extracted alone by the same procedure of the tissues.

The strength of haemolysis was measured by the size of the clear zone around the hole containing the extract.

Defibrinated sheep red cells were used instead of mouse erythrocytes

because the mouse cells were haemolysed intensely leaving a clear area that covered almost the whole surface of the plate after two hours from the addition of the extract.

Results

Experiment I: Normal mouse erythrocytes were not haemolysed when they were incubated for two hours at 37C° or for I8 hours at room temperature with the worm extract.

Injection of worm toxins into normal mice did not alter their blood picture, e.g. after I2 and 32 weeks from the first injection the haemoglobin values were respectively I3.7 and I3.5 gm./I00 ml., and the reticulocyte count was between I - 4%. Also there was no change in the spleen weights (Table I).

Experiment 2: Out of three normal liver extracts, two produced a small clear area of +Ive haemolysis in the blood agar plates. Whereas infected liver extracts of 8, 24, 32, and 37 weeks post-exposure, produced positive haemolysis that increased during the period of infection, as shown in Table 2, and illustrated in Fig. I, i.e. the liver extract of the longest duration of infection (37 weeks) gave +4ve strength of haemolysis (Fig I).

No haemolysis was observed in the plates containing either normal or infected spleen extracts of 8, I2, 22, 24, and 37 weeks post-exposure, table I.

Discussion and conclusions

Ponder (I95I) stated that, lysins which produce haemolysis in vivo can be divided into a group of haemolytic substances which are essentially foreign to the individual, such as the bacterial lysins, and the many agglutinins and lysins of the immune type, and a group of naturally occurring haemolytic substances such as, the fatty acids, lysolecithin and other lytic materials obtainable from tissues and plasma (the "tissue lysins").

Table I

Blood indices of mice injected with schistosome worm extract

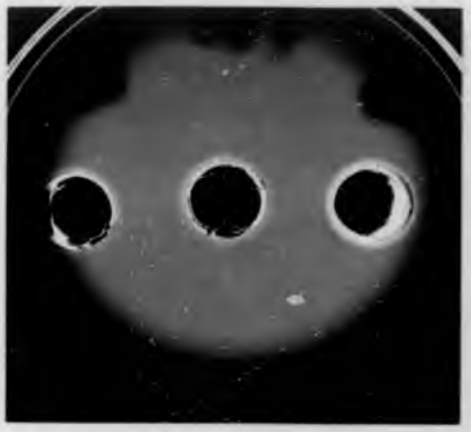
No. of weeks	Mice	Body weight	Spleen weight	Ratio*	PCV	Hb	Retic.%
I2	Control	44	0.2	0.45	40	I3	2
	Injected	44	0.2	0.45	4I	I3.7	4
32	Control	40	0.2	0.44	40	I3	I.2
	Injected	40	0.2	0.44	39	I3.5	I.6
	Injected	48	0.3	0.62	39	I3.5	I

$$\text{ratio } * = \frac{\text{splean weight}}{\text{liver weight}} \times \text{I00}$$

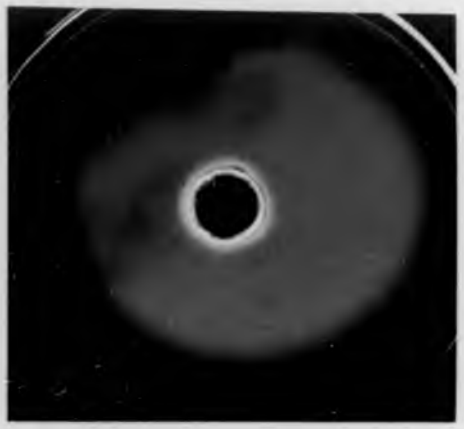
Table 2

Results of haemolysis in blood agar plates

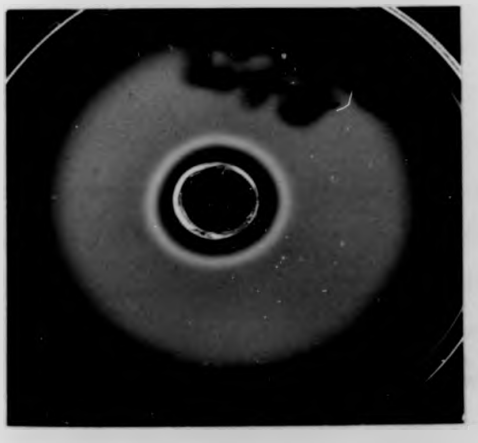
Extract	Duration in weeks	Haemolysis
Normal liver		+Ive
Normal liver		-ve
Normal liver		+Ive
Infected liver	8	+Ive
"	I2	-ve
"	24	+3ve
"	32	+3ve
"	37	+4ve
Normal spleen		-ve
Normal spleen		-ve
Infected spleen	8	-ve
"	I2	-ve
"	22	-ve
"	24	-ve
"	37	-ve
Controls - PBS		-ve
water		-ve
alcohol		-ve



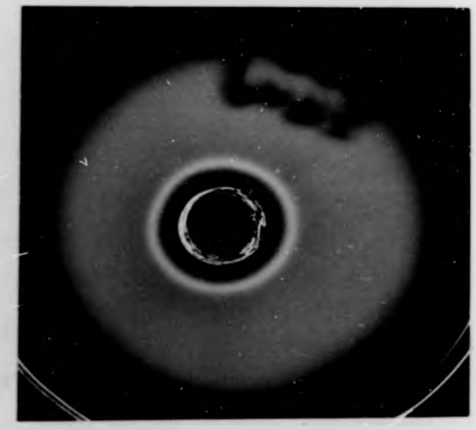
I- Holes with PBS, water and alcohol, -ve haemolysis.



2- Hole with normal liver extract -ve haemolysis.



3- hole with infected liver extract (24 weeks), +3ve haemolysis.



4- Hole with infected liver extract (37 weeks), +4ve haemolysis.

Fig. I: Photogaghs of agar blood plates, holes contain alcoholic extracts of normal liver and livers taken from mice infected with 50 cercariae of S. mansoni.

In S. mansoni infection, whether worms or eggs secrete haemolytic substances similar to the bacterial lysins, is a matter of conjecture; since its existence was not demonstrated in vivo. However, anaemia which developed at the acute stage of infection in experimental animals; i.e. before the immune phenomena took place (proved by the agglutination test to be usually after 22 weeks), was an indication that a toxic factor maybe involved.

Schistosome worms ingest and lyse red cells inside their gut to derive nutritional substrates (Zussman et al, 1970, Lawrence, 1973, and Senft, 1976). A proteolytic enzyme which hydrolyses the haemoglobin of the ingested erythrocytes was described by Senft & Maddison (1975), he suggested that when the worm regurgitates it expels the haemoglobin proteolytic enzyme which might escape the liver and eventually induce an immune response by the host. Thus it seem reasonable to assume that vomitus from the worms might contain injurious and toxic substances to the erythrocytes; like the proteolytic enzyme; or the factor that lysed red cells inside the worms and/or end products of the worm metabolism. Which explain the early death and anaemia (6 - 7 weeks) of mice infected with 500 cercariae (Part I, Chapter 5), due to presence of large number of worms releasing high rate of toxins.

In the present experiment, erythrocytes when incubated with emulsified worms did not haemolyse, nor injection of worm saline extract into normal mice produced anaemia, which was due to either: a) The worms were frozen before being used which might have inhibited their haemolytic activity if any were present. b) The concentration of worms was not effective to produce haemolysis or anaemia. Experiments are needed to study the toxins and their chemical nature in vivo. when the worms are alive and secreting these toxins.

In the previous chapter positive haemolysis of normal mouse red cells incubated with normal liver, and the increase of haemolysis with infected

liver or spleen extracted with alcohol or saline, was also shown in case of liver alcoholic extract; under more sterile conditions with the agar blood method in this chapter. It is premature to draw a reliable conclusion especially as these experiments were done in vitro: yet the following points might be put forward to explain the increase in haemolytic activity of tissue extracts taken from the infected mice:

I- In normal tissues there is equilibrium between the lytic agent and its inhibitory factors (possibly a single factor) present in the tissues and in the blood stream; any alteration in this equilibrium by extraneous factors (presumably here the schistosome toxins) will results in increase of erythrocyte destruction. This theory was advanced by Maegraith et al (1943,a). Later (1943,b) hesuggested that increased haemolysis might occur either as the result of a) excess of the lytic agent or b) reduction in quantity or effect of the inhibitory factor; the second condition has been proved in few cases of blackwater fever. Schistosoma toxins probably exert both factors.

2- Toxins of both worms and eggs (circulating with the blood or present in the tissues), are in continuous contact with tissue cells; especially when infection is of long durations, which lead into their necrosis; dissolution and degeneration;as in the case of liver damage (Mohamed, 1936 and Cameron, et al 1964). These might provoke some chemical changes in the structure of the cells releasing a haemolytic factor later. Evidence of this was the increase in haemolytic activity in agar blood plates with livers taken from mice infected with 50 cercariae after 37 weeks.

The absence of haemolysis with spleen extracts from infected mice in blood agar plates indicated that the lytic factor of spleen (proved in the previous chapter when normal mouse red cells were incubated with spleen extract from infected animals), are species specific because sheep red cells were used in preparing the blood agar.

There has been much speculation as to the nature of the tissue haemolytic agent since methods of its preparation differed widely. For instance, Mangraith et al (1943a), and Herberman (1964) considered the lytic agent of saline tissue extracts to be a protein; acting enzymatically. Whereas Ponder (1951) regard it as identical to lysolecithin (alcohol-soluble). In this study extraction with both saline or alcohol and further with petroleum and ether (refer to method in the previous chapter), produced a lytic factor which was more potent by the second extraction. Therefore, it appears that there might be more than one type of lytic agent, i.e. an enzyme and lipid.

The question remains as to the origin of the lytic agent extracted from tissues (particularly the liver) of infected mice, whether in addition to the tissue lysin, egg/worm toxic lytic factor was also extracted.

These observations argues in favor of the existence of a schistosoma toxin secreted by both worms or eggs, which either haemolyse red cells directly or indirectly through the affected tissues.

PART 5

DIAGNOSTIC METHODS

CHAPTER I

DOUBLE DIFFUSION STUDIES IN S. MANSONI

Introduction

Serum total proteins as well as their individual fractions show variable changes in S. mansoni infection, usually the gamma-globulin level rises which was taken as an index of the intensity of immune reaction and antibodies formation (El-Hawary et al, 1971). Fractions of the gamma-globulin (IgG, IgM) have been shown to be elevated in sera from individuals with S. mansoni infection (Hillyer, 1969, Antunes et al, 1971 and Bassily, et al 1972).

The qualitative analysis of globulins in normal and their changes in pathological sera was made possible by Ouchterlony's double-diffusion method (1949); in which contents of wells made in gel-agar are allowed to diffuse towards each other resulting in the formation of specific precipitin lines. This permits direct comparison of various antigens and anti-sera with one another and allows direct identification of antigens in unknown mixtures, further more it directly reveals the identity of cross reaction (Fife, 1971).

Using Ouchterlony's agar-plate precipitin test as the basis for the following experiments in which the aim was to study:

1- Identification of globulins in sera from control (normal) and their possible changes in mice infected with S. mansoni; and in eluates from antibody-coated pooled red cells.

2- Nature of these globulins of infected sera whether they are immunoglobulins (antibodies) against worm or tissue (liver) extracts.

Materials and methods

Sera to be tested in this experiment were collected from control and infected mice with S. mansoni (25 or 50 cercariae), killed in the previous experiments, they were stored at -20°.

Eluates were prepared according to the method of Weiner's ethanol

elution (1957) which was as follows:

- red cells were pooled from two mice; washed with PBS; then centrifuged; supernatant was removed.
- the tube was corked and exposed to -6C° to-35C° until the cells were laked.
- thawed; ten times their original volume of 50% (v/v) ethanol pre-cooled in the deep freeze, was added.
- the tube was rapidly inverted and the alcohol thoroughly mixed with the laked cells.
- re-corked and immediately returned to the deep freeze for 30-60 minutes to precipitate the antibody proteins.
- the tube was then centrifuged for five minutes; supernatant was removed as completely as possible, then refilled with distilled water.
- the tube was shaken by hand so that the sediment would mix thoroughly with distilled water, and re-centrifuged.
- supernatant was removed, and saline was added to the sediment which was stirred or broken up and thoroughly mixed with it.
- the tube was incubated at 37C° for 30-60 minutes, centrifuged and the supernatant which was the eluate transferred to a clean tube.

The anti-sera used included separately "Broad spectrum" (anti-IgG, IgM, and IgA), anti-IgG, anti-IgM, and anti-C_3 were all obtained from Nordic Pharmaceuticals and diagnostics. These anti-sera were all raised in rabbits.

Crude extracts of both frozen male and female adult worms (collected from the infected livers) were prepared by homogenizing them with saline.

Some of the alcoholic liver extracts used in the haemagglutination test were used in this experiment.

Diffusion method was carried out in small flat plastic plates containing 0.5% agarose made up in 0.85% NaCl with antibiotics. Agarose was preferred to agar because it give more sharply defined lines (Hillyer, & Frick 1967). With a cork borer five wells were cut in the agarose, one in the middle of the

plate and four around it at a measured distance (approximately 5 mm.).

After adding the reagents to these wells (2 drops); the plates were kept in a humid chamber at room temperature for three days subsequently at 4C. Results were read after 48 hours to seven days. At the end of one week the plates were photographed because some lines would develop over a long period usually not less than one week (Wilson & Pringle, 1954).

Procedures and results

I- The first set of plates contained the anti-sera (antibody) in the central well with undiluted tested sera (antigen) in peripheral wells.

With the "Broad spectrum" (anti-globulin sera), straight well defined precipitin lines were formed of almost identical intensity with control (normal) and infected sera of different ages and dose of infection (Fig. I, 2-A).

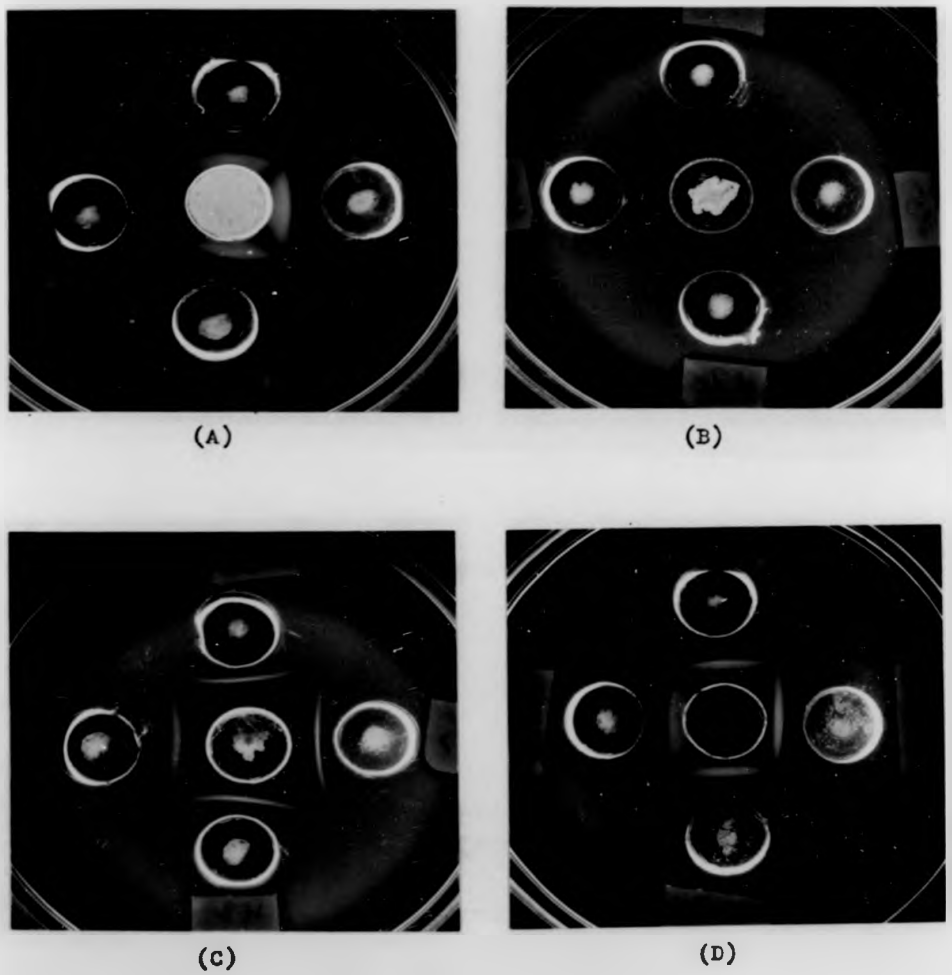
No precipitation was obtained using the specific anti-sera; the anti-IgG (Fig. I-B). Anti-IgM plates had thin curved precipitin lines (Fig. I-C), which indicates that the antigen in this case has a lower diffusion coefficient than that of the antibody (Crowle, 1975).

Plates containing anti-C'_3 gave similar straight lines to that of anti-globulin sera in both tested sera, with less intensity (Fig. I and 2-D).

Quantitative tests

The strongest reaction was obtained when tested sera were against the "Broad spectrum" (Table I), hence sera of controls and those of infected mice were diluted with saline to find the end point (concentration of reactant that fails to form a distinct precipitin line). Plates were charged individually with each sera (e.g. two-fold diluted control in the peripheral wells against the anti-globulins in centre).

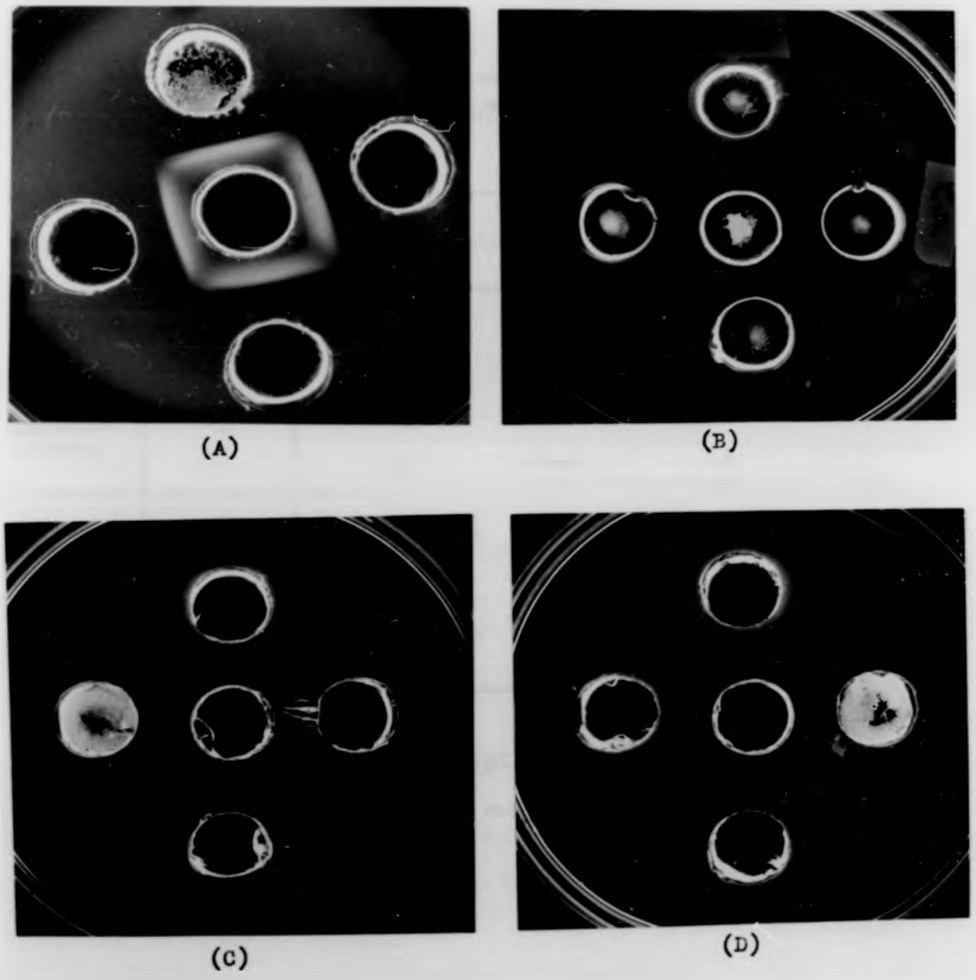
The end point of control sera were between I/128-I/256 whereas those of sera from mice infected with 50 cercariae was at higher dilutions that varied among the sera used of different durations of infection e.g.



(A) (B)

(C) (D)

Fig. I: Agarose diffusion. Antisera in central well, peripheral wells with tested sera from normal mice (top well), and mice infected with 25 cercariae for 35, 42, and 45 weeks. (clockwise). A) Broad spectrum with short thick precipitin lines. B) Anti-IgG with no reaction. C) Anti-IgM with smooth curved precipitin lines. D) Anti-C_3^1.



(A) (B)

(C) (D)

Fig. 2: Agarose diffusion. Antisera in central well, peripheral wells with tested sera from normal mice (top well), and mice infected with 50 cercariae for 10, 32, and 52 weeks of infection (clockwise). A) Broad spectrum (immunoglobulins). B) Anti-IgG. C) Anti-IgM. D) Anti-C'_3.

Table I

Results of diffusion in agarose plates, centre well with antisera against tested sera of control and infected mice.

Sera	Weeks of infection	Antisera			
		B.sp*	IgG	IgM	C'_3
Control		+3	-ve	+I	+3
	35	+4	-ve	+I	+3
Infected 25cerc.	42	+4	-ve	+2	+2
	45	+I	-ve	+2	+4
Control		+4	-ve	trace	+I
	I0	+4	-ve	trace	+I
Infected 50 cerc.	32	+4	-ve	trace	+I
	52	+4	-ve	trace	+I

*: Broad spectrum (anti-IgG, IgM, and IgA)

strength of the reaction was estimated according to thickness of precipitin lines.

successively at 14, 20, 42, 48, and 75 weeks the end point was at a dilution of I/2048, I/1024, I/4096, I/2048, and I/1024. The end point in the case of sera from mice infected with 25 cercariae was also at higher dilution e.g. at 45 weeks it was I/1024, the sera of 75 weeks gave distinct precipitin lines at I/4096 (Table 2). Number of the diluted sera formed two precipitin lines, which was probably due to the fact that the antigens were available in two distinctly different molecular weights (Kabat, & Mayer 1961).

The end point of precipitation with diluted sera from control mice against anti-IgM was at I/4, slight increase of this globulin was seen with plates charged with the infected sera; the end point at I/32 (25 cercariae), and at I/64 (50 cercariae) as seen in table 3.

In Fig. 3 (A,B) precipitin lines disappeared at a dilution of I/128 in plates with control sera against the anti-C'_3. Sera from infected mice (50 cercariae) had increase in their complement up to I/512 (Fig. 3, C and D). Sera from mice infected with 25 cercariae had the end point at I/128 (Table 3).

The second set of plates were charged with eluates from control and infected pooled red cells seperately against anti-IgG, anti-IgM, and anti-C'_3 no reaction was obtained. Precipitin lines were formed against the anti-globulin (short and straight) in four out of eleven eluates used; with end point at a low dilution, of I/4 from two mice infected with 25 cercariae and killed successively after 42 and 82 weeks, and of I/16 and I/4 from two mice infected with 50 cercariae and killed after 28 and 48 weeks. No globulins were present in eluates from normal red cells (Table 4).

2- Plates charged with sera from control and infected mice (peripheral wells) of 10, 14, 28, and 45 weeks post-exposure; against both antigens from worm and liver extracts (taken from mice infected with 50 or 500 cercariae) gave no reaction; although they were left for more than one week (ten days at room temperature).

Table 2

Results of precipitation in double diffusion plates with serial dilutions of tested sera against the antiglobulin "Broad spectrum"

Sera	Duration in weeks	1/4---64	128	256	512	1024	2048	4096	8192
Control		+ve	+ve	-ve					
		+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
		+ve	-ve	-ve					
Infected 50cerc.	14	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
	15				+ve	t*	-ve	-ve	
	20		+ve	+ve	+ve	-ve			
	28		+ve	+ve	+ve	+ve			
	35		+ve	+ve	-ve	-ve			
	42	+ve	+ve	+ve	+ve	+ve	t	-ve	-ve
	48		+ve	+ve	t	t			
	54		+ve	t	-ve	-ve			
	75			+ve	+ve	-ve	-ve		
Infected 25 cerc.	13	+ve	+ve	+ve	+ve	+ve	+ve	t	-ve
	42	+ve	+ve	t	-ve	-ve	-ve	-ve	
	45			+ve	+ve	-ve	-ve		
	75				+ve	+ve	+ve	+ve	

t* = trace

Table 3

Results of precipitation in double diffusion plates with serial dilutions of tested sera against anti-IgM and anti-C'_3.

Anti-sera	Sera	I/4	8	I6	32	64	I28	256	5I2
IgM	Control	t*	-ve	-ve	-ve				
	Infected 50 cerc. 42 weeks	t	t	t	t				
	Infected 25 cerc. 75 weeks	+ve	+ve	+ve	-ve				
C'_3	Control	+ve	+ve	+ve	+ve	t	-ve	-ve	-ve
	Infected 50 cerc. I4 weeks	+ve	+ve	+ve	+ve	+ve	+ve	t	-ve
	Infected 25 cerc. 75 weeks	+ve	+ve	+ve	+ve	t	-ve	-ve	-ve

t* = trace

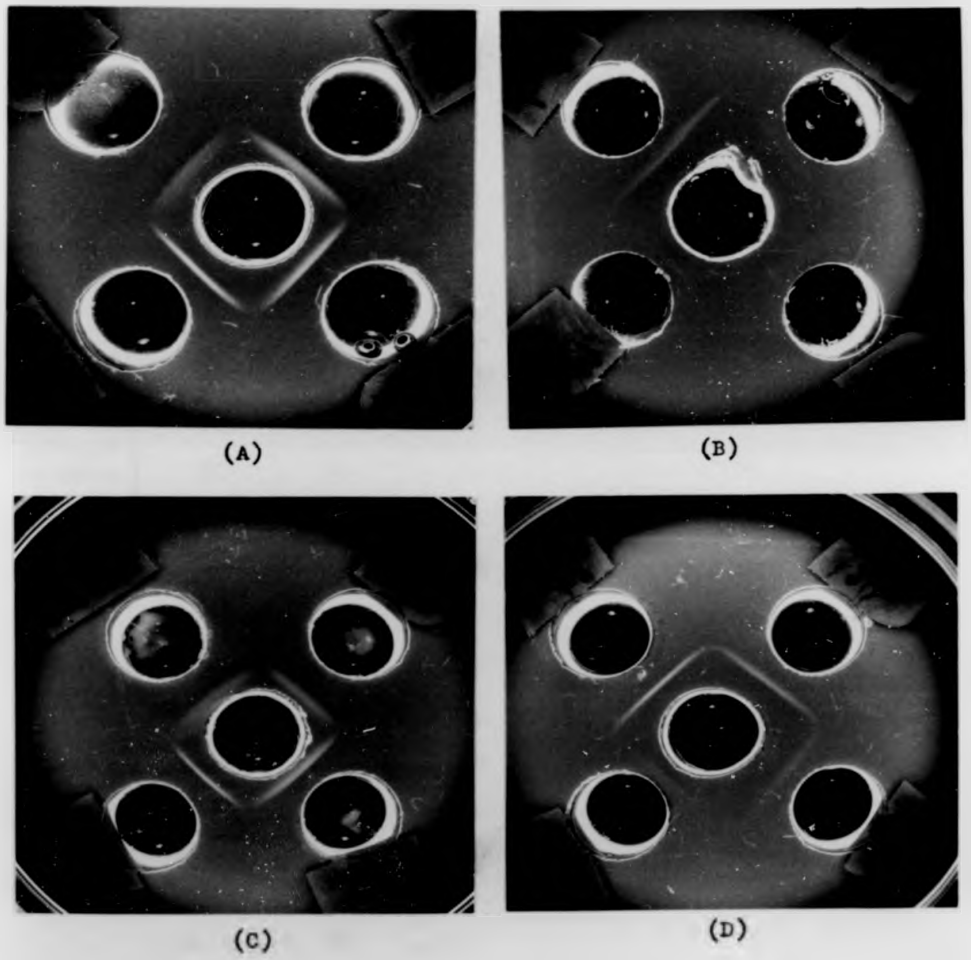


Fig. 3: Double diffusion in plates charged with anti-C_3^1 in central well peripheral wells with diluted tested sera (I/4 - I/512). A-B) Control sera with end point at I/128. C-D) Sera from mouse infected with 50 cercariae of 14 weeks of infection, end point at I/512.

Table 4

Results of precipitation in double diffusion plates with serial dilution of eluates from control and infected red cells (each is from pooled cells of two mice) against the anti-globulin.

Eluate	Duration in weeks	UN*	I/4	8	I6
Control		-ve	-ve	-ve	-ve
	23	-ve	-ve	-ve	-ve
	28	+ve	+ve	+ve	+ve
Infected 50 cerc.	35	-ve	-ve	-ve	-ve
	48	trace	trace	-ve	-ve
	I4	-ve	-ve	-ve	-ve
	26	-ve	-ve	-ve	-ve
	30	-ve	-ve	-ve	-ve
Infected 25 cerc.	33	-ve	-ve	-ve	-ve
	40	-ve	-ve	-ve	-ve
	42	trace	trace	-ve	-ve
	82	+ve	+ve	-ve	-ve

UN* = undiluted eluate

Discussion

I- Double-diffusion test showed that globulins are present in sera of both normal and infected mice; but they increased during the period of infection with either 25 or 50 cercariae. The most elevated globulins was at a much older infection, 75 weeks (Table 2). Evans and Stirewalt (1957) found that with progress of the disease in mice infected with S. mansoni there was an absolute increase in their gamma and beta globulins.

Serum immunoglobulins IgG and IgM are formed specifically as a result of exposure to S. mansoni antigens in humans and mice (Hillyer, 1969), also at the acute stage of human schistosomiasis those globulins have a higher level than the control group (Antunes et al, 1971). The strongest immunoprecipitins were found to be against the egg antigenic material (Hillyer and Frick, 1967). Therefore, toxins from live eggs deposited in the tissues of mice used in this chapter might be the reason for the persistence of IgM.

The negative results with anti-IgG are not comparable to those of Fahey et al, (1964) who described in normal mouse serum two classes of 7Sγ- globulins, this result could be explained either by:

- the quantity of IgG is minimal that it was not traced by double-diffusion.
- the anti-IgG used did not have the appropriate antibody to be precipitated bearing in mind that this globulin is composed of more than one subclass.

The complement was just detectable in normal and there was a slight increase in sera from infected mice. The increased amount might represent the C'_3 that had not been fixed by immunocomplexes on the surface of erythrocytes.

Four out of eleven eluates from pooled red cells of anaemic (refer to blood picture in chapter I-Part 3) mice infected with both doses of 25 or 50 cercariae; had immunoglobulins in them as shown by forming

precipitin lines identical to those obtained with sera against the anti-globulins ("Broad spectrum" anti-IgG, IgM, and IgA). This step was a further proof that some red cells are covered with small amounts of globulins from their sera usually at the later stages of infection; rendering them more susceptible to be destroyed by the reticuloendothelial system.

2- The attempt to prove that sera globulins have antibodies against the worm extract failed which might be due to:

- defect in the worm extract used.
- antibodies in tested sera were already combined with the circulating worm antigen.
- diminished reactivity to adult worms (Hillyer and Frick, 1967).

Further studies are needed on serum of infected mice against the egg antigen which appears to be the most antigenic of the extracts used by Smithers (1960), forming four strong precipitin lines.

Although Ekladios et al (1971) detected auto-antibodies in sera of patients with hepatic schistosomiasis against liver antigens, no antibodies were found in the present study engaging the same procedure which coincides with the results of haemagglutination test done in chapter 3 - Part 3.

To summarize the results, the following points are mentioned in regard to mouse globulins:

- they have more than one fraction, that differ in their molecular weight.
- they increase in mice infected with S. mansoni, especially at later stages of the infection.
- they are weak in their nature.
- they coat some of the infected red cells, hence responsible for their destruction.
- immunoglobulin increase may not be due to specific antibody activity against the worm antigen.

CHAPTER 2

SCANNING ELECTRON MICROSCOPY STUDIES OF RED CELLS FROM MICE INFECTED WITH S. MANSONI

Introduction

The surface appearance of red cells and their possible changes during infection with S. mansoni in mice was investigated in this chapter by the scanning electron microscope (S.E.M.), which provides a three dimensional view of surface structure without involving preparation of haemoglobin "ghosts" cells (Lewis et al, 1968).

Pathological changes during infection have been observed on the membrane of murine red cells by the S.E.M., for example in malarial infection (P. berghei), apertures were found by Arnold et al (1971). Also knobs which represented budding virus were present on murine Friend erythroleukemia cells (De Harven et al, 1973).

Materials and methods

Red cells examined were taken from mice killed for the agglutination test (Chapter I - Part 3), they were infected with either 25 or 50 cercariae of S. mansoni.

The following steps were taken to prepare the erythrocytes for examination with S.E.M.:

- immediately after the mouse was killed a small drop of blood was placed onto round cover slips (13 mm. diameter, no.1) spread to make a thin film.
- the drop was left till almost dry, few drops of the fixative (3% gluteraldehyde in 0.066M Cacodylate buffer, pH 7.2) were added and left for 45 - 60 minutes.
- to remove the fixative, cells were washed with 0.066M Cacodylate buffer twice for 15 minutes.
- The cells were then dehydrated by series of alcohol i.e. 10, 20, 40, 60, 70, 80, 90, 100, 100% for five minutes each.

- the cover slips were left at 4C in absolute alcohol until examined.

Cover slips were mounted on metal stubs, and coated with a layer of gold then examined by the scanning electron microscope (Bedford college stereoscan unit). The instrument was operated at an accelerating voltage of 20 or 30kV, covering all the cover slip with low and high power magnifications.

Results

Some cells taken from mice infected with low doses of cercariae (25 or 50) had "holes" on their surface characterized by discontinuation of membrane. The "holes" varied in their size and number, for example Figs. 3 and 10 shows red cells with a large deep "hole" on their surface. Other cells from different samples showed several smaller "holes" scattered over the surface as illustrated in Figs. 5, and 6. The majority of these cells lost their normal biconcave appearance, they became either swollen (Figs. 5 and 8) or irregular with depressions of the membrane (Fig. 6).

Also a number of cells had on their surface pits where the membrane appears not to be broken, Fig. 4, these cells might be reticulocytes as was concluded by Hattori (1972).

Other defects of the membrane noted were small processes (Figs. 7 and 8), or protrusions with rounded ends (Fig. 5).

Most cells taken from the control normal mice had a smooth uniform surface (Fig. 1), except for few sporadic cells on which one very small "hole" was found (Fig. 2), which differed from those observed on the surface of erythrocytes taken from infected mice by their much smaller size and lesser numbers, also shape of the normal cells was not altered (i.e. concave disc).

Out of ten samples examined from the control mice only two had one cell with the occasional "hole" on its surface, whereas those taken from twenty infected mice, twelve samples had cells with "holes" on their membrane,

Fig. I- Erythrocyte from control (normal) mouse with smooth surface. X I5,200.

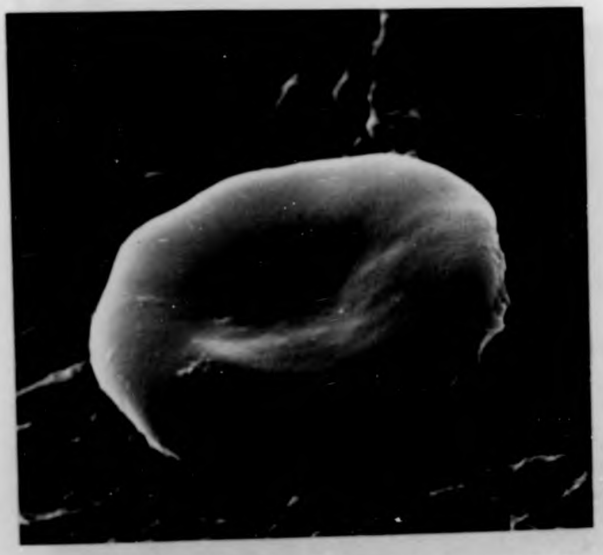


Fig. 2- Erythrocyte from control (normal) mouse with occasional odd "hole" on its surface. X I4,000.

Fig. 3- Erythrocyte from infected mouse (50 cercariae for 20 weeks) with large deep "hole",and its possible fragment still near by on its surface X 30,000.

Fig. 4- Erythrocyte from infected mouse (50 cercariae for 20 weeks) with pits on its surface. X 15,000.

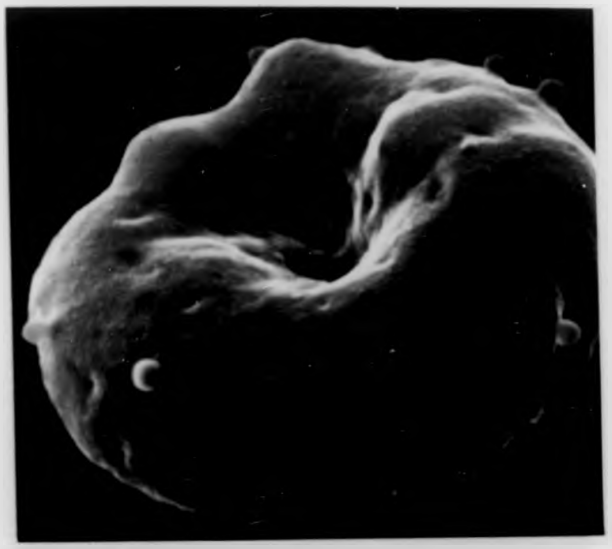


Fig. 5- Erythrocyte from infected mouse (25 cerc. for 36 weeks) with small "holes" and protrusions on the surface. X 28,000.

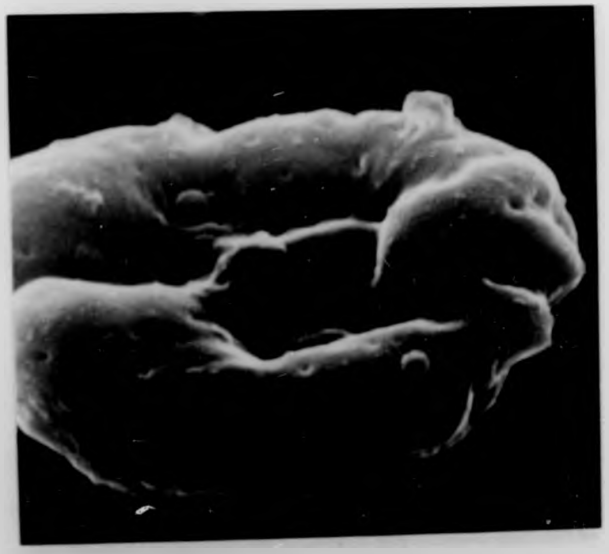


Fig. 6- Erythrocyte from infected mouse (25 cerc. for 40 weeks) showing irregularity in shape, depression of cell membrane, and small "holes" scattered over the surface. X 28,000.

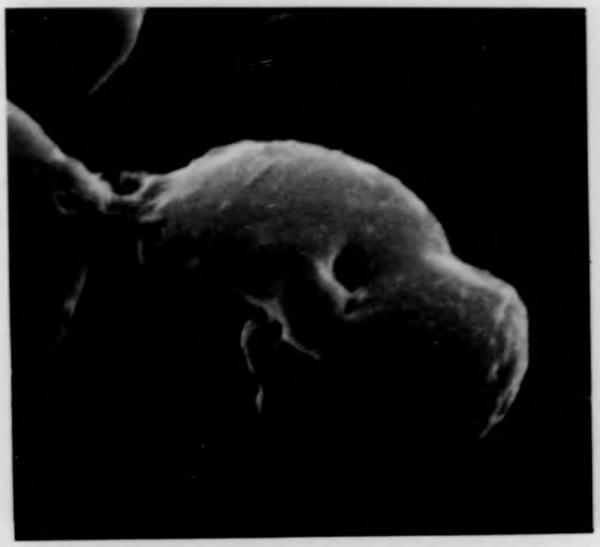


Fig. 7- Erythrocyte from infected mouse (25 cerc. for 36 weeks) with one small pointed process on its surface. X 26,000.

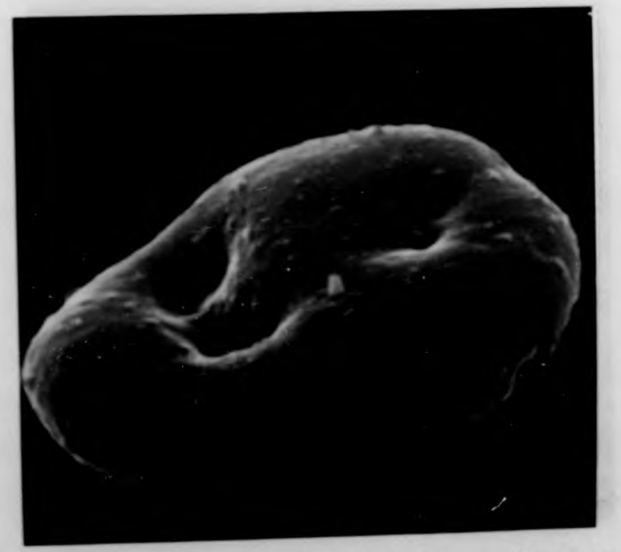


Fig. 8- Erythrocyte from infected mouse (50 cerc. for 48 weeks) with two processes and small "holes" on its surface. X 28,000.

Fig. 9- Defect in erythrocyte from patient infected with P. falciparum. X I0,000 (from Balcerzak et al I972).

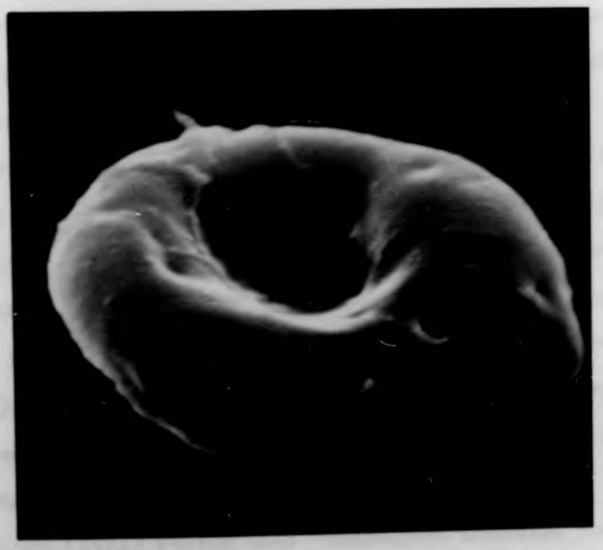


Fig. I0- Defect in erythrocyte of infected mouse (25 cerc. for 48 weeks) with one large "hole" and few smaller "holes" on cell membrane.
X 22,000.

usually one to four damaged cells in the whole drop from the positive samples.

The two-sample (unpaired) t-test was applied to find a statistical difference between two groups of 8 controls and 8 mice infected with 25 cercariae. In a low power field the number of cells with "holes" was counted per 50 cells. The difference was highly significant $P < 0.01$. From Table I one could see that infected mice were severely anaemic as in the case of mouse infected for 30 weeks with haemoglobin value of I.9gm./I00ml., had four cells with "holes" on their surface.

The samples taken from mice killed at earlier durations of infection (8 to I3 weeks), failed to have these damaged cells.

Discussion

Many authors described defects on the membrane of the red cell after using the S.E.M., which are broadly in agreement with the ones mentioned in this study. Clarke (Salsbury) (I967) found in certain cases of haemolytic and dyshaemopoietic anaemia, large pores on the membrane of some red cells. Pits or small processes were occasionally seen on erythrocytes from patients with severe iron deficiency anaemia (Hattori, I972).

Marked deformities on erythrocytes from patients with P. falciparum infection was discovered by Balcerzak et al (I972), in parasitized cells the organism produced a highly irregular red cell surface defect also the cells without parasites had a surface cavity defect (Fig. 9), which differed from those found on erythrocytes of mice infected with S. mansoni (Fig. I0). They concluded the possibilty that these morphological observations would explain the premature destruction of red cells in malarial infections.

Bruninga (I97I) stated that "Complement reaction sequence begins with the attachment of antibody (single IgM molecule or IgG "doublet") to a specific antigen on or attached to the cell surface. The components, i.e.

Table I

Number of cells with "holes" on their surface in mice infected with 25 cercariae and their controls

Mice	Duration in weeks	Number of cells with "holes"	Hb
CONTROL		I*	I5.3
		0	I3.0
		0	I4.3
		0	I3.7
		0	I4.0
		0	I4.0
		0	I3.8
		0	I4.0
INFECTED	30	4	I.9
	36	I	I.3
	36	2	4.5
	38	0	8.4
	40	I	3.0
	40	2	3.6
	42	I	5.9
	42	I	3.2

* Fifty cells were counted from each sample
The t-student test (unpaired) had a P $<0.0I$

C'_4, C'_2, C'_3, ... C'_8 ,C'_9, are then sequentially activated and bound, the end result being irreversible cell damage, with the formation of characteristic ultrastructural "holes"". During infection with S. mansoni immune complex would develop on the red cell surface leading to complement fixation (Woodruff, 1973 and this study), therefore the "holes" observed here and their infrequency in the controls suggests that antigen-antibody-complement fixation is responsible for the haemolysis due to schistosomiasis; especially when the examined samples were taken from anaemic infected mice (Table I).

The variations in number and size of the "holes" could be attributed to the number of antibody molecules bound on the membrane of red cell.

Destruction of these cells might possibly be explained by one of two basic mechanisms, bearing in mind that a single site of damage caused by the complement is sufficient for cell lysis (Kabat & Mayer, 1961):

- The damaged cells with "holes" would be destroyed by erythrophagocytosis in the reticuloendothelial system.
- The "holes" permit rapid exchange of small molecules, which leads to an increase in the osmotic pressure of the cell, resulting in an influx of water and swelling of the cell. The original small "hole" defects then enlarge enough to allow macromolecules such as haemoglobin to escape, the end result being "colloid osmotic lysis" (Green et al, 1959 and Bruninga, 1971).

The complement "holes" have been reported to have a diameter of 80 - 100A, observed by the electron microscope (Rosse et al, 1966, and Polley et al, 1971). Since the scanning electron microscope resolution differs from that of the electron microscope, therefore; the "holes" described in the present study might represent the enlarged "holes" due to swelling of red cells as mentioned above.

PART 6

IMMUNOPATHOLOGICAL STUDIES ON KIDNEYS OF MICE INFECTED WITH S. MANSONI

IMMUNOPATHOLOGICAL STUDIES ON KIDNEYS OF MICE INFECTED WITH S. MANSONI

Introduction

Patients infected with S. mansoni and without clinical manifestation of renal disease, bear in their kidneys histological glomerular changes characterized by moderate proliferation of axial cells and focal thickening of the basement membrane (Andrade and Queiroz, 1968); these changes progress and worsen toward a picture of chronic glomerulonephritis in advanced cases of hepatosplenic schistosomiasis (Andrade et al, 1971).

The frequency of these glomerular lesions in schistosomaisis, especially in patients with chronic hepatosplenic form of the disease, suggested that the parasitic infection may play a role in the development of their nephrotic syndrome (De Lima et al, 1969, and Queiroz et al, 1973).

Further evidence supporting the relationship between schistosomal infection and development of kidney injury is that similar renal lesions to those of humans can be reproduced experimentally by infection with S. mansoni in hamesters (Hillyer et al, 1974) and mice (De Rousse et al, 1974, and Mahmoud and Woodruff, 1975).

Da Silva et al (1970), used electron microscopy to study early glomerular lesions of patients infected with S. mansoni; they found electro-dense deposits located in the basement membrane near to mesangial cells, these deposits appeared to contain immunoglobulins and complement as seen by immunofluorescence, which suggests that the kidney lesions were of immunological origin.

Combined immunofluorescent, light microscopy and ultrastructural techniques were employed in the following experiments in order to study the nature and pathogenesis of early and late kidney lesions, particularly in the glomeruli, in mice infected with low doses of S. mansoni cercariae.

Materials and methods

Kidneys examined were taken from groups of mice infected with either 25 or 50 cercariae of S. mansoni and healthy control mice, killed for the agglutination test mentioned in chapter I-Part 3.

Light microscopy

Appropriate areas of kidney tissue were cut into small pieces, fixed in buffered formalin, and embedded in paraffin wax. From these blocks 4-6u thick sections were cut and stained by the conventional haematoxylin and eosin method. Specimens examined were from two mice killed after 48 and 92 weeks of infection with 25 cercariae, and from two control mice.

Electron microscopy

Kidneys from three controls and five infected mice were examined; two mice infected with 25 cercariae (killed after 48 and 92 weeks of infection) and three mice infected with 50 cercariae (killed after 20, 28, and 75 weeks of infection). Renal specimens were processed as follows:

I- As soon as the mouse was killed, its kidneys were sliced open and their surfaces bathed in 4% phosphate buffered glutaraldehyde fixative, thin slices from the exposed surface areas were transferred to wax and cut into I mm. cubes in a drop of fresh glutaraldehyde fixative using a razor blade washed in toluene.

2- The cubes were then fixed in 4% phosphate buffered glutaraldehyde, pH 7.4 for a further one hour at cool room temperature.

3- Washed in buffered solution (Cacodylate with Sucrose, pH 7.4) the specimens were post-fixed in cold (0 - 4C) 2% OsO_4 in cacodylate buffer for one hour.

4- Washed in cacodylate buffer solution; three changes for I5 minutes each.

5- After dehydration in 30% alcohol (methanol), renal specimens were stained in 0.5% uranyl acetate in 30% alcohol for 30 minutes.

6- Then dehydration for 5 minutes each through graded dilutions of alcohol (i.e. 60 - 70 - 90 and absolute). Later specimens were washed in toluene

(two changes 10 minutes each) and left overnight in fresh Araldite.

7- The specimens were embedded in Araldite in plastic capsules and polymerized over 48 hours at 60C.

8- The blocks were removed from the plastic capsules and sectioned on a microtome using a glass knife. Sections of I - 2u thickness were placed on slides, stained with toluidine blue and examined by light microscopy to identify areas containing glomeruli before thin sections were cut and mounted on Smetharsi New 200 copper grids for viewing in the electron microscope.

The buffer; fixative solutions and embbeding media were prepared as the follows:

1) Buffer solution 0.1M

Sodium cacodylate $(CH_3)_2AsO_2Na.3H_2O$	21.4 g.
Distilled water	I litre

2) Buffer for washing

(0.1M Cacodylate + 0.2M Sucrose)

Buffer solution	250 ml.
Distilled water	250 ml.
Sucrose	34.23 g.

Adjust pH to 7.2 with N.Hcl or N.NaOH

3) 2% OsO_4 fixative

Buffer solution	6.6 ml.
Distilled water	13.4 ml.
OsO_4	0.4 g.
Sucrose	0.016 g.
$Cacl_2$	0.02 g.

Allow osmium tetroxide to dissolve in water overnight then add buffer solution and adjust pH to 7.2. If the solution is slightly hypotonic then sucrose must be added. Ca ions are for good fixation.

4) 4% Glutaraldehyde fixative

Buffer solution	50 ml.
25% glutaraldehyde solution	I6 ml.
Distilled water	34 ml.

Adjust pH to 7.2 with N.HCL

5) ARALDITE – FLUKA (embedding media)

Resin	80 ml.
Hardener	I00 ml.
Plasticiser	2 ml.
Accelerater	4 ml.

Mix resin, hardner, plasticiser and stir for 20 minutes. Add accelerater, stir for I5 minutes. Degas.

Immunofluorescence method

Kidneys from 24 mice infected with 25 cercariae killed at I4, 20, 22, 26, 30, 32, 36, 38, 40, 42, 44, 48, 54, 75, 82, and 92 weeks of infection; and from II mice infected with 50 cercariae killed at I4, 20, 22, 28, 36, 48, 54, and 75 weeks of infection. The method of fluorescence staining was done following Ehrich and Voller (I972):

Kidneys were removed immediately after the mice were killed; and with a scalpel incision was made in their middle then snap-frozen in liquid nitrogen and kept at -70C°. Sections of 4 - 6u were cut in a cryostat, they were kept on the slides at -25C° until processed. Later the sections were washed with PBS pH 7.2 for I5 minutes, subsequently stained in a humid chamber for 40 minutes with conjugate anti-globulin labelled with fluorescein isothiocyanate (supplied by Nordic Pharmaceuticals and Diagnostics), two conjugates were used, fluorescein Swine anti-mouse IgG (for the detection of different immunoglobulins) diluted with PBS I/5 and I/40, also

I/5 of fluorescein rabbit anti-mouse BIC (C_3^1). After treatment with the conjugate sections were counter-stained with a few drops of Evans blue at I/10,000 in distilled water for one minute this stain was used to eliminate unclear and difficult images (Coudert et al, 1968). Sections were washed in PBS for ten minutes, then mounted with buffered glycerine, examination was done on a Leitz fluorescence microscope.

Results

Infected mice were anaemic, and mostly had their spleens enlarged as shown in chapter I-Part 3.

Light microscopy

Progressive pathological changes were seen in the kidneys of infected mice; at 48 weeks of infection the appearance was of a subacute glomerulonephritis. Few of the glomeruli were normal, the majority varied in their lesions, mostly they displayed larger tufts with increased mesangial matrix and hypercellularity due to proliferation of mesangial cells and occasional polymorph leucocytes, thus a large number of tuftal capillaries were closed (Fig. I, A). Epithelial cells of Bowman's capsule were usually swollen or proliferated at one side to form crescentic structures composed of flattened cells/or hyaline material (Fig. I, B), other glomeruli had foci of hyaline necrosis. In addition some of the convoluted tubules showed cloudy swelling, while others contained hyaline casts. There was also some degree of interstitial cellular infiltration, mostly plasma cells and mainly around the glomeruli.

Chronic glomerulonephritic changes which involved most of the renal tissue were seen in kidney sections from the mouse with the longest (92 weeks) infection. Except for a small number of glomeruli with mesangial thickening and hypercellularity, the majority of glomeruli either had only a few remaining cells (mostly fibroblasts and polymorphs) or had lost their cells completly. The cells had been replaced by hyaline

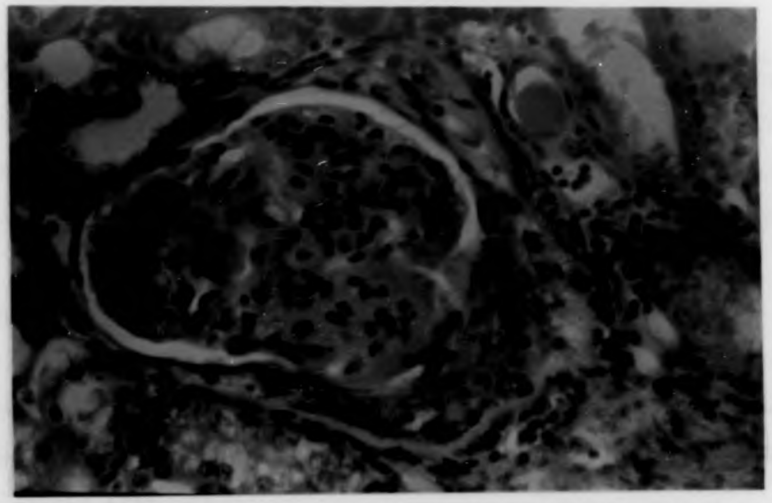


Fig.I, A- Mouse infected for 48 weeks. Glomerulus showing marked proliferation of cells. A tuftal adhesion is evident. X250.

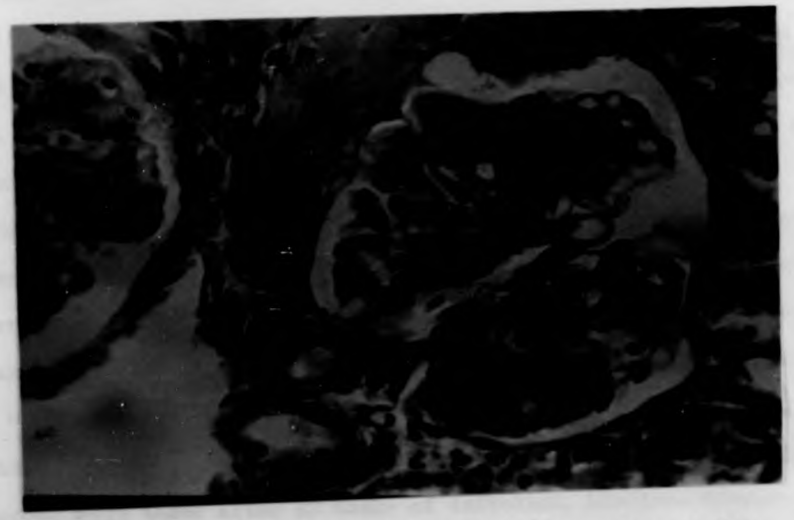
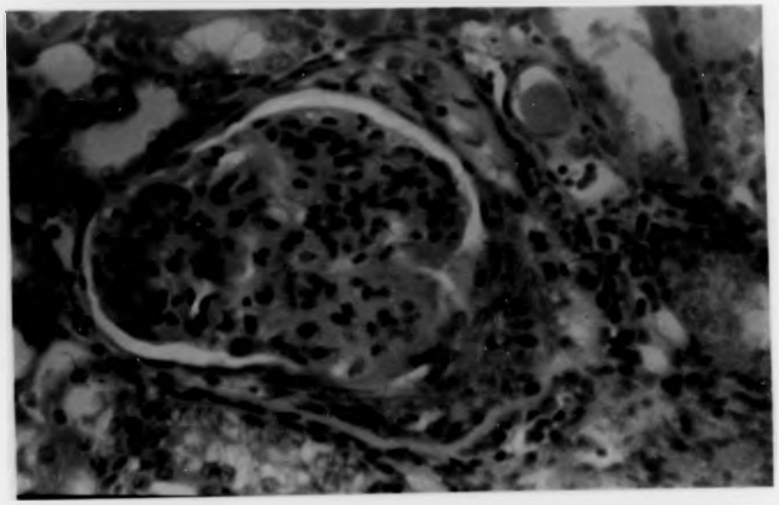


Fig. I, B- Mouse infected for 48 weeks. Glomerulus showing crescentic structure composed of flattened cells and hyaline material.



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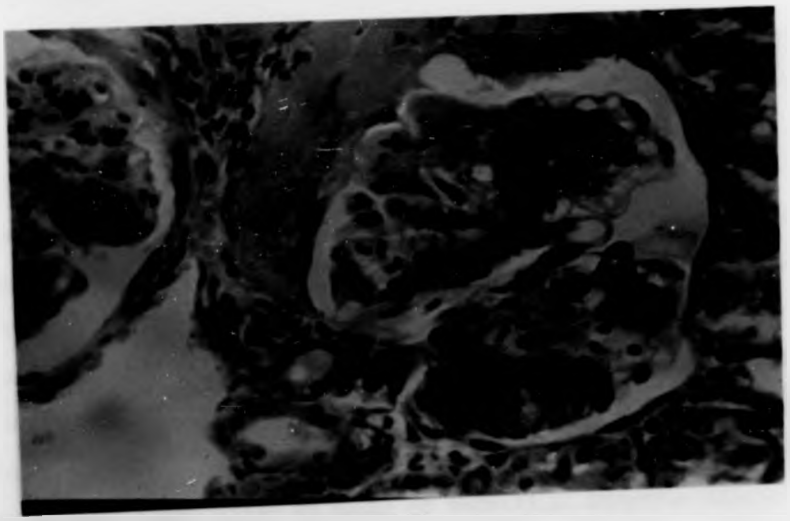

Fig. I, B- Mouse infected for 48 weeks. Glomerulus showing crescentic structure composed of flattened cells and hyaline material.

discs (Fig. 2, A). Some of the tubules were atrophied with flattened cells, several containing hyaline casts (Fig. 2, B). Tubules were seperated by fibrous tissue which was infiltrated with plasma cells and lymphocytes.

None of these changes were seen in the control mice, even in the older ones.

Electron microscopy

No ultrastructural changes were seen in the earlier stages of infection i.e. 20 and 28 weeks; however in some glomeruli of the later clusters of electron-dense deposits were present in well defined areas of the mesangial matrix (Fig. 3).

Electron microscopy of glomeruli from the mouse infected for 75 weeks revealed similar but more widely scattered electron-dense deposits which consisted of a homogenuous substance, localized on the endothelial side of the glomerular basement membrane usually near to or beneath mesangial cells and within the expanded mesangial matrix (Fig. 4, A and B). These deposits also showed varying degrees of electron density (Fig. 4, A and Fig. 5). Endothelial cells were swollen. Glomerular basement membrane showed irregular thickening and wrinkling particularly at the site of deposition of the electron-dense material (Fig. 4,,A and B).

There were no subepithelial deposit in any of the examined cases. However in glomeruli of the kidney from the mouse infected for 48 weeks the irregularly thickened basement membrane had in several areas, marked nodular swellings on the epithelial side. These are illustrated and compared with normal control tissue in figures 6, A, B, and C.

Kidney sections which showed the most damaged appearance by light microscopy were those after 92 weeks of infection. Electron micrographs of their glomeruli revealed few electron-dense deposits in the mesangium where most of the cells had been replaced by necrotic material. Foot processes of the epithelial cells were markedly fused in many areas

Fig. 2, A- Mouse infected for 92 weeks. Glomerulus with few cells and abundent hyaline material. X250.

Fig.2, B- Mouse infected for 92 weeks. Many tubules with hyaline casts.

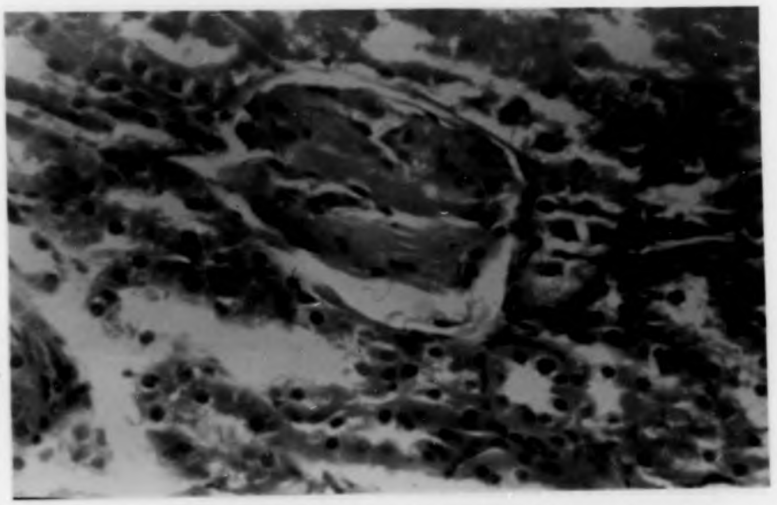

Fig. 2, A- Mouse infected for 92 weeks. Glomerulus with few cells and abundent hyaline material. X250.

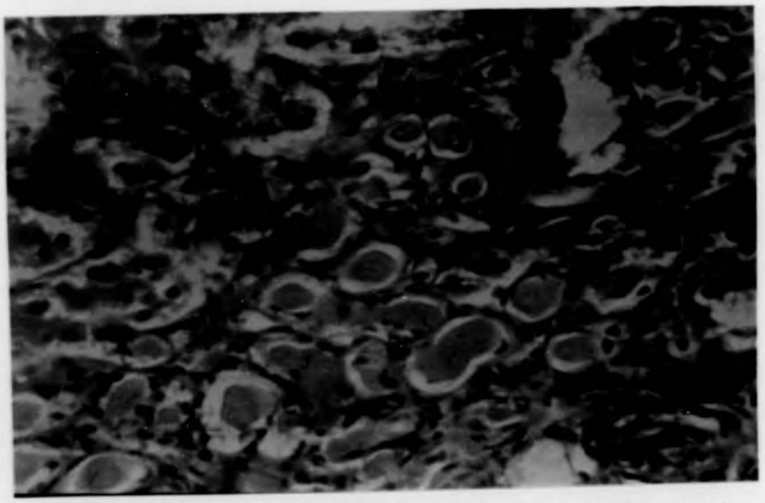

Fig.2, B- Mouse infected for 92 weeks. Many tubules with hyaline casts.

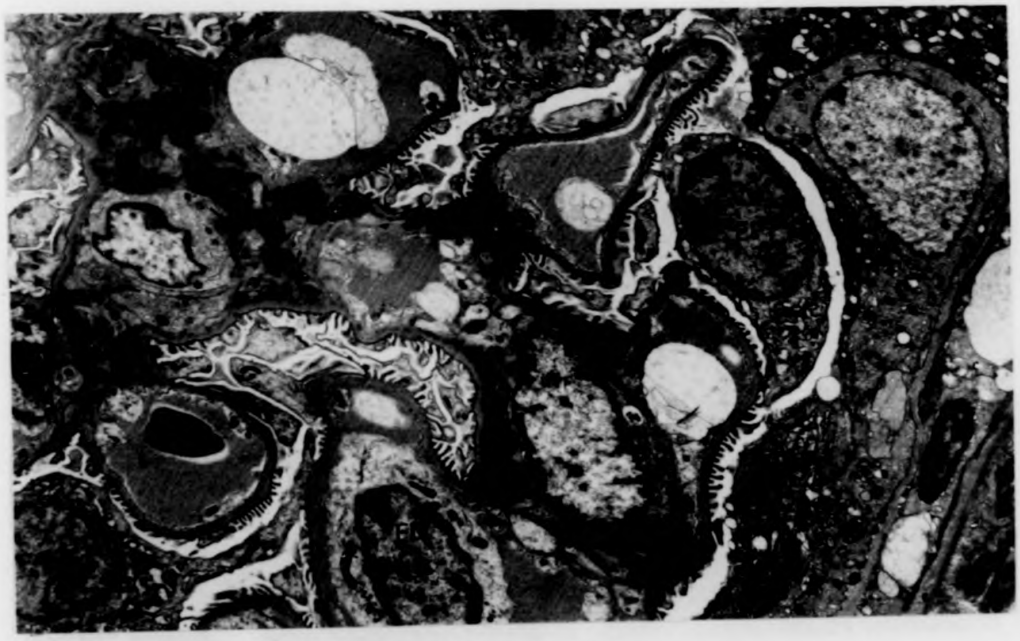

Fig. 3 - Electronmicrograph of mouse glomerulus (infected for 28 weeks), showing the electron-dense deposits within the mesangium (arrows) with no apparent damage to the basement membrane. EN, endothelial cell, EP, epithelial cell. X5,000.

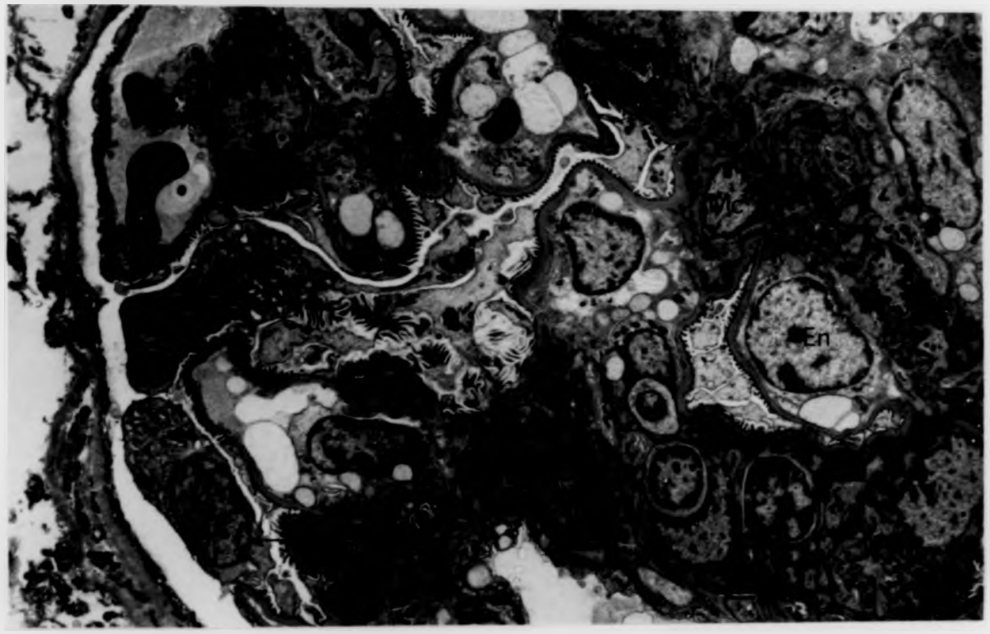

Fig. 4,A – Mouse infected for 75 weeks. Electronmicrograph of glomerulus showing electron-dense deposits (D) on endothelial side of the basement membrane near to mesangial cells (Mc), and glomerular basement thickening and wrinkling beneath the deposits (arrows). En, endothelial cell, EP, epithelial cell, MM, increased mesangial matrix. X 3,200.

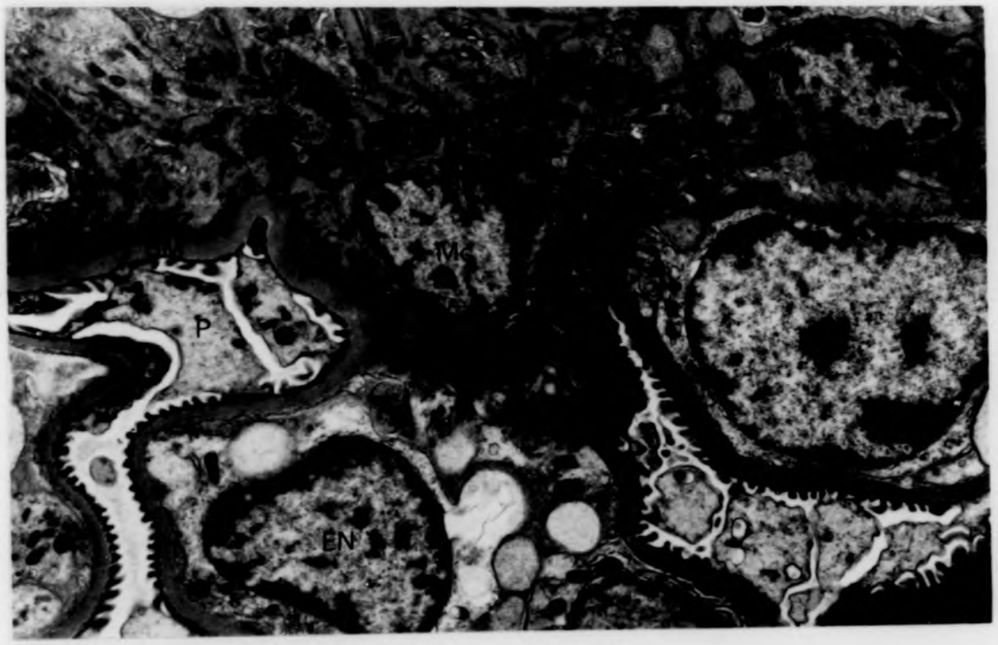

Fig. 4,B - Detail of Fig. 4,A. Large electron-dense deposits (D) on the endothelial side of irregularly thickened glomerular basal membrane (BM), near to a mesangial cell (Mc). EN, endothelial cell, MM, mesangial matrix, P, podocyte. X 8,000.

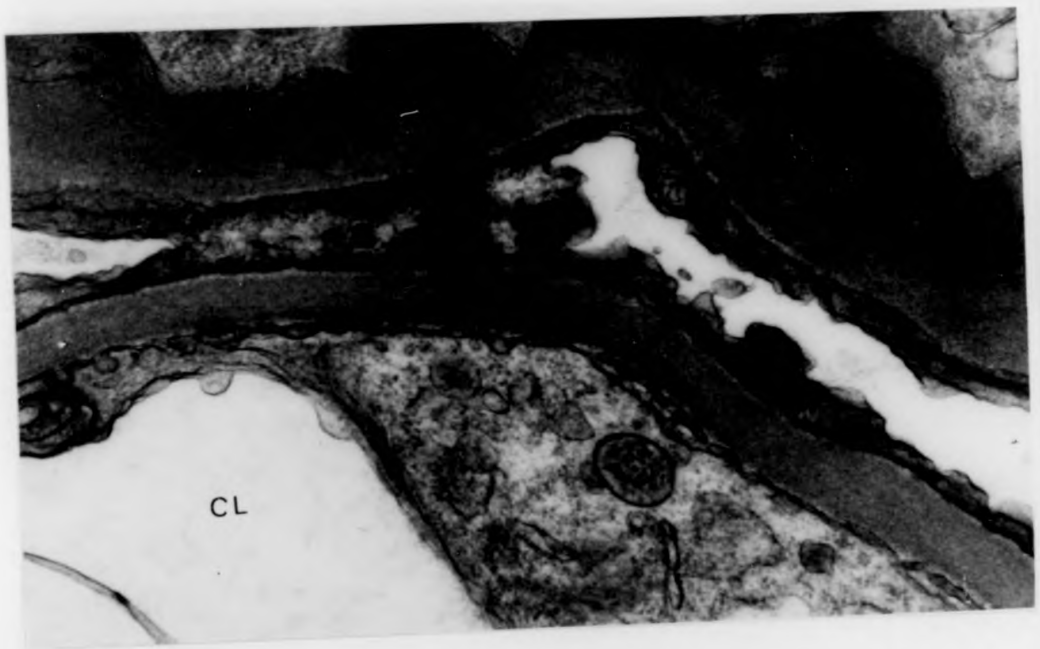

Fig. 5 - High power electronmicrograph of glomerular basement membrane (mouse infected for 75 weeks), showing the difference in density of the deposits (D). BM, basement membrane; CL, capillary lumen. X 42,000.

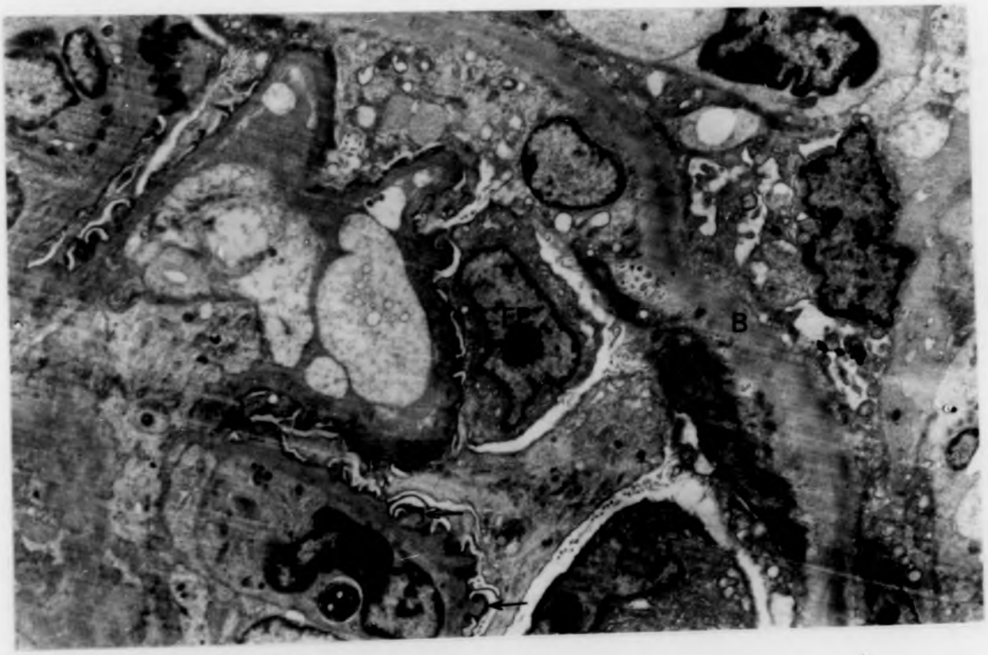

Fig. 6,A - Mouse infected for 48 weeks. Electronmicrograph of glomerulus showing nodular swelling of the basement membrane (arrows) and loss of the fenestrated shape of the endothelium. EP, epithelial cell; BM, glomerular basement membrane; B, Bowman's capsule. X 5,000.

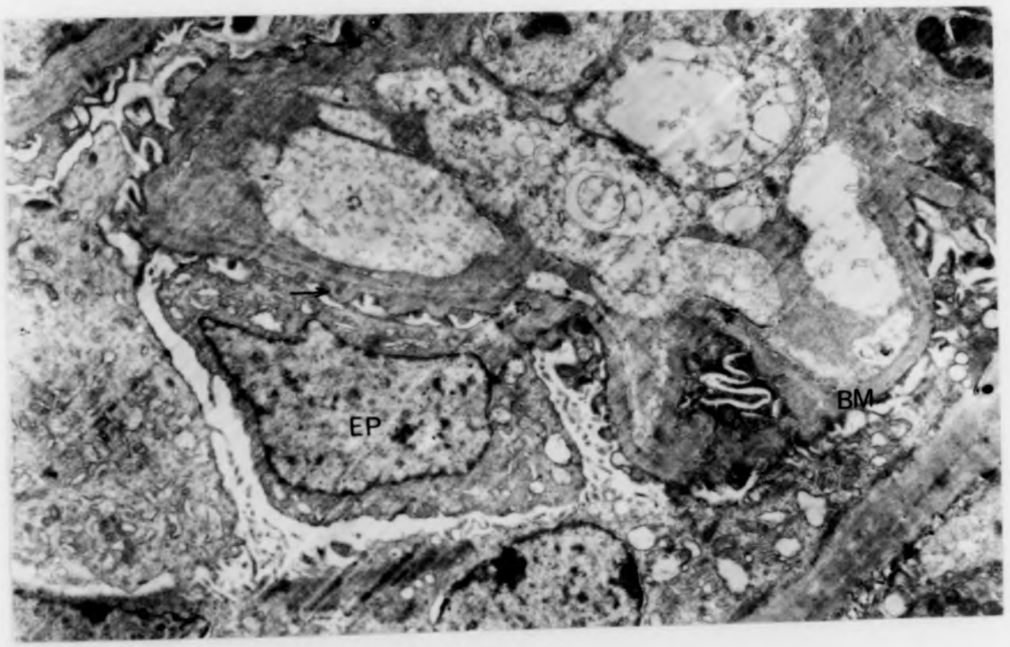

Fig. 6,B - Mouse infected for 48 weeks. Electronmicrograph with high magnification of glomerulus showing the nodular swelling of glomerular basement membrane (arrows). BM, glomerular basement membrane; EP, epithelial cell. X 8,000.

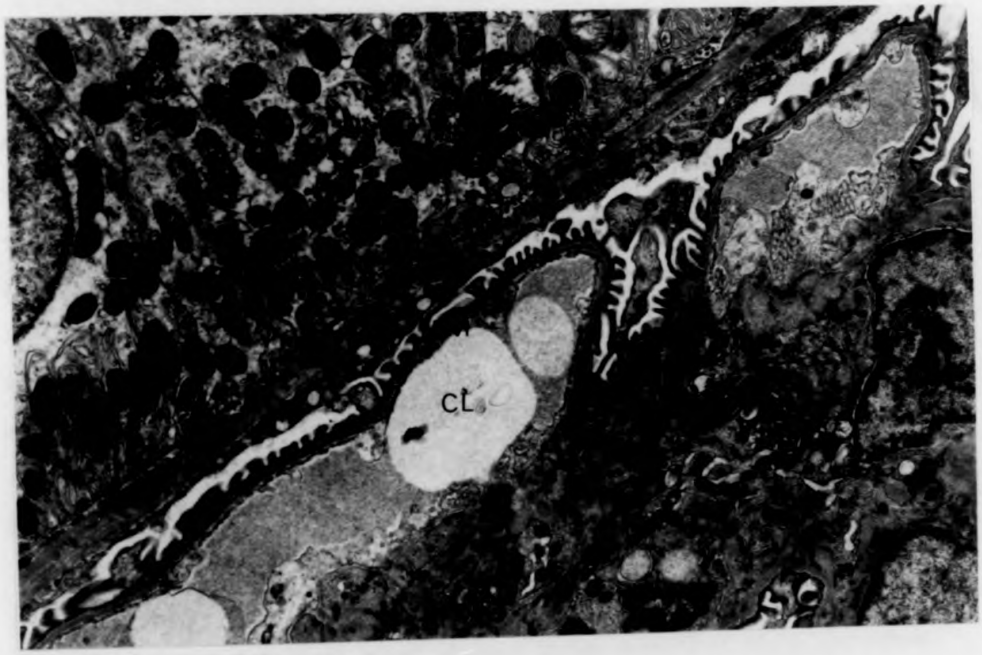

Fig 6,C - Control mouse glomerulus to compare with Fig. 6,A. BM, glomerular basement membrane; P, podocyte; CL, capillary lumen. X 8,000.

(Fig. 7); there was also irregular thickening of the basement membrane (Fig. 8, A).

Immunofluorescence microscopy

Frozen kidney sections from mice infected with 25 and 50 cercariae stained with the labelled anti-mouse gamma globulin (IgG) gave no fluorescence, but with the labelled anti-mouse C_3^1, specific apple green fluorescence were seen as lumpy granular deposits mainly in mesangial area of the glomeruli. Both conjugates did not react with the control kidney sections.

In brief mice infected with 25 or 50 cercariae gave positive fluorescence after the 30 weeks of infection had elapsed, except for two mice infected with 25 cercariae which had moderate to weak fluorescence after respectively 22 and 26 weeks of infection (Table I). Deposition of the complement in glomeruli of animals infected with both doses was progressive with the infection (Table I).

Discussion

The present studies showed that mice lightly infected with S. mansoni (25 or 50 cercariae) developed progressive diffuse glomerulonephritis, manifested by histological renal changes which resembled those described in advanced cases of human hepatosplenic schistosomiasis (Andrade et al, 1971). The absence of glomerular lesions in control mice indicates the significence of schistosomal infection as an etiological factor in nephropathy.

Histologically renal lesions were seen at later stages of the infection (48 weeks), they showed in many glomeruli mesangial expansion, mesangial cell proliferation, focal hyalinization and capsular crescent formation. These changes became more severe after 92 weeks of infection with several glomeruli damaged and replaced by hyaline casts. Renal lesions were produced in mice that had one to four worm pairs, similarly hamsters

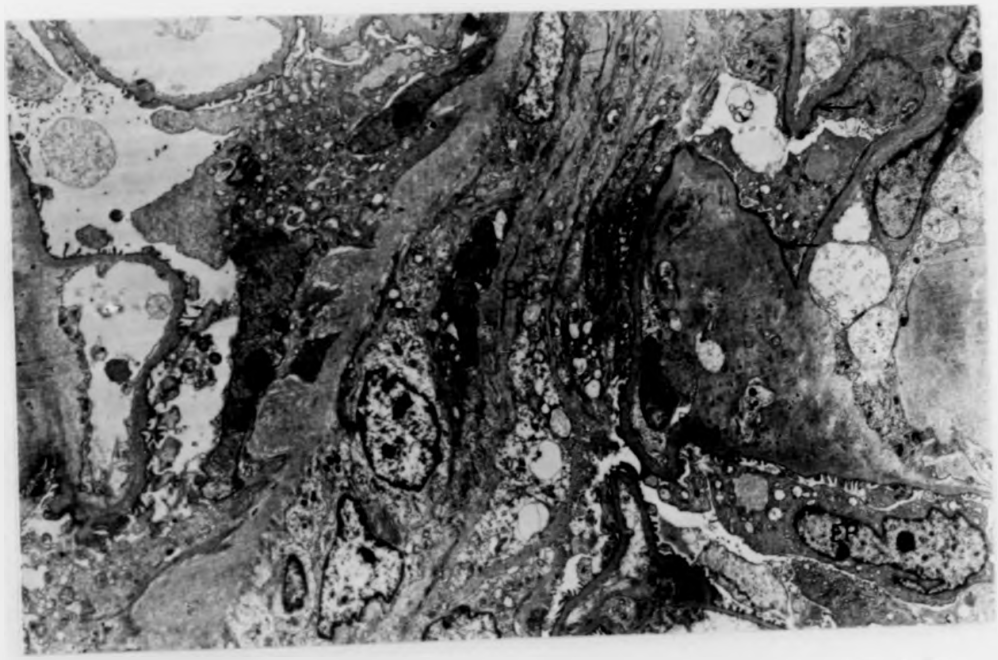

Fig. 7 - Electronmicrograph of mouse glomerulus (infected for 92 weeks) showing part of Bowman's capsule (BC) with swollen epithelial cells and fibrin; several foot processes of glomerular epithelial cells were fused (arrows). BM, basement membrane; EP, epithelial cell; D, deposit. X 3,200.

Fig. 8,A - Mouse infected for 92 weeks. High power magnification of thickened basement membrane (BM); FP, foot process. X 50,000.

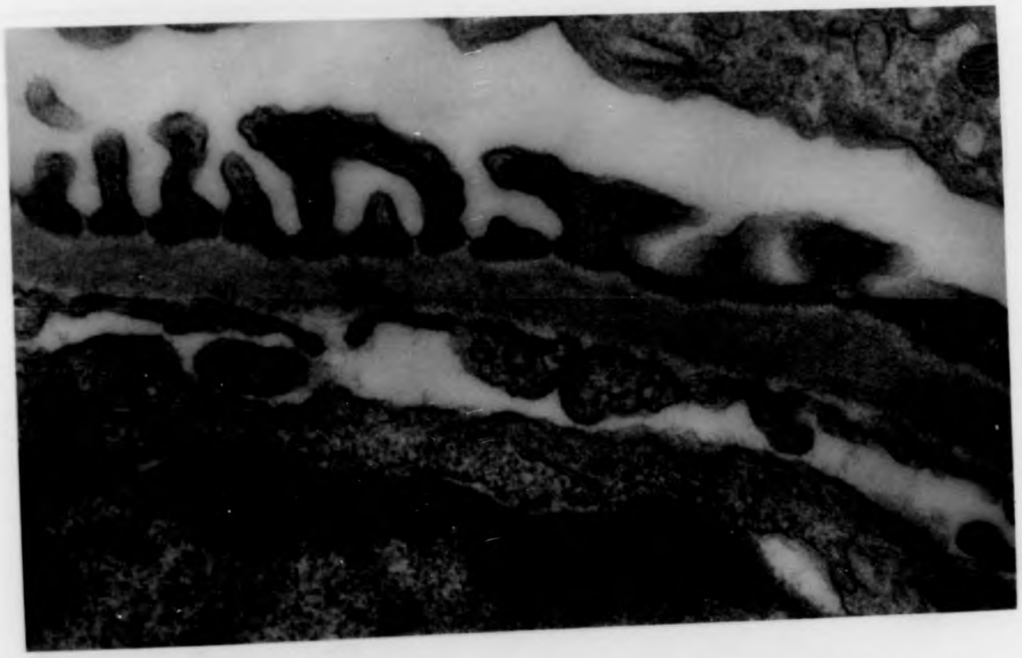

Fig. 8,B - To compare with Fig. 8,A the thickness of glomerular basement membrane from control mouse glomerulus. EN, endothelial cell; FP, foot process. X 50,000.

Table I

Fluorescence intensity observed in glomeruli of renal sections from infected mice and their controls, using I/5 labelled anti-mouse complement.

Duration of infection (weeks)	Mice	Control	Infected 25 cerc.		Infected 50 cerc.	
	No.	Intensity	No.	Intensity	No.	Intensity
14	1	-ve	1	-ve	1	-ve
					2	-ve
20	2	-ve	2	-ve	3	-ve
			3	-ve		
22	3	-ve	4	-ve	4	-ve
			5	-ve	5	-ve
			6	+ve		
26	4	-ve	7	w		
28	5	-ve			6	-ve
					7	-ve
30	6	-ve	8	-ve		
			9	-ve		
32	7	-ve	10	w		
			11	+ve		
36	8	-ve	12	+ve	8	+++ve
			13	+ve		
			14	+ve		
38	9	-ve	15	+ve		
40	10	-ve	16	w		
			17	w		
42	11	-ve	18	+++ve		
44	12	-ve	19	+++ve		
48	13	-ve			9	+++ve
54	14	-ve	20	-ve	10	+ve
			21	-ve		
75	15	-ve	22	+++ve	11	+++ve
82	16	-ve	23	+++ve		
92	17	-ve	24	w		

w = pale green, +ve = specific fluorescence.

infected with S. mansoni with the same worm load had focal glomerulonephritis at 37 - 44 weeks of infection in contrast hamsters with heavier worm load (40 -100 adult pairs) after 7 - 11 weeks post-exposure exhibited a marked tubular dilatation; atrophy and focal glomerulonephritis, on the other hand hamsters without renal pathology had as many as 50 to 200 worm pairs (Hillyer, & Lewert (1974), therefore in experimental infection with S. mansoni it appears that production of glomerular lesions does not depend on the worm load of the host; but probably the importance of a large number of worms lies in the fact that mild glomerular lesions would be produced earlier than with the lower load of worms.

Electron microscopy showed that the glomeruli had both mesangial and endothelial cells swollen, with electron-dense deposits seen either in the mesangium or underneath mesangial cells on the endothelial side of the glomerular basement membrane. The electron-dense deposits observed in the infected mice were reminiscent of those described in human kidney specimens from cases of hepatosplenic schistosomiais (De Brito et al, 1969, and Rocha et al, 1976).

By the immunofluorescence studies it was found that the electron deposits corresponded to the complement (C_3'). Other globulins were probably present in smaller quantities impossible to detect with fluorescin tagged anti-IgG. The complement appears to be continously fixed by antigen-antibody complexes and not trapped nonselectively within the glomeruli due to two factors which support this theory:

- The inflammatory reactions observed in the renal sections namely proliferation of mesangial cells and migration of polymorphs both would act as phagocytes against the immuno-complexes.
- The persistence and progression of the damage in the glomeruli which was accompanied by increase of the complement deposition as seen by the immunofluorescence method (Table I).

Previous work done by Andrade and Susin (1974) showed in mice infected with 150 cercariae of S. mansoni, glomerular electron deposits which contained IgG. Tada et al (1975) proved the presence of IgG, IgM, IgA, IgE, complement, and schistosoma antigens in the glomeruli of monkeys infected with S. japonicum. Thus it appears that an immunological mechanism might participate in the development of glomerulonephritis.

Although the exact mechanism of glomerular injury is still obscure, yet from the current studies and previous literature it is possible to suggest the following:

I- Circulating schistosomal antigens in the host were demonstrated by Berggren et al (1967); and Gold et al (1969), these antigens form complexes with antibodies; which deposit in the glomeruli depending on size of the complexes; their concentration (i.e. excess antigen or antibody); and whether they escaped their elimination by the reticuloendothelial system.

Soluble immuno-complexes usually deposit on the epithelial side of glomerular basement membrane of the kidney (Dixon et al, 1961), however in the present work they were located on the endothelial side of the aforementioned membrane; this might be due to the deposition of large complexes which appear to penetrate the basement membrane with difficulty, so subepithelial deposits were infrequent (De Brito et al, 1975). Once the immuno-complexes were inside the glomeruli they would intiate the inflammatory lesions observed.

2- The S. mansoni worms and their ova produce a range of somatic and metabolic antigens (Andrade, 1961). During long periods of infection with low doses of cercariae repeated and prolonged action of these antigens/toxins accompanied by the damage following deposition of antigen-antibody-complement complexes would alter the structure of host glomerular tissue and stimulate an auto-immune reaction akin to that produced experimentally in animals. Experimental glomerular changes were produced after repeated

antigenic stimulation (Taufer et al, 1959). Mellors (1965), concluded that the glomerular membranous lesions in NZB/BI mice, were induced by an auto-immune mechanism; these lesions showed homogenous swelling of the capillary basement membrane. Therefore, in the present study irregular thickening of the basement membrane in kidneys of infected mice were probably due to such mechanism.

Other antigens might also be present in the glomeruli, such as DNA which was found to be retained in the glomeruli of hamsters infected with high dose of S. mansoni cercariae (Hillyer and Lewert, 1974).

In conclusion mice infected with a low dose of S. mansoni produce along with anaemia; enlargement of spleen; glomerulonephritis induced by auto-immune phenomena and/or host damaging antigen-antibody-complement complexes.

PART 7

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Anaemia is one of the clinical manifistations of S. mansoni infection (Day, 1911, and Shousha, 1949). It was described to be either hypochromic-microcytic (Molina et al, 1936), macrocytic (Suárez, 1934, and Pessa et al,. 1955), or normocytic (Kenawy, & El Mawla 1958).

The causes of this anaemia were explained by multiple factors: i.e. blood loss, haemolysis, splenic sequestration, haemodilution, and iron deficiency.

The aim of this study was to investigate development of anaemia in mice experimentally infected with S. mansoni. and the factors involved in its causation. The following topics of anaemia of schistosomiasis have been viewed:

I. The relation between infection and the development of anaemia

Anaemia commences after the prepatent period (6 - 8 weeks). It developed with the minimal load of worms I - 2 pairs, and its severity was not determined by the intensity of infection.

The dose of infection appears to effect the progress of anaemia; in that mice infected with heavy dose of 500 cercariae died after 5 - 6 weeks probably due to toxaemia, thus the animals did not survive long to produce the chronic type of anaemia.

Three points were concluded in case of anaemia of mice infected with light doses of 25 or 50 cercariae:

- during the course of infection, anaemia tended to be hypochromic in type as evidenced by the marked decrease in haemoglobin and MCHC values.
- when anaemia develops it progress from mild to severe and the infected mouse does not regain its normal blood values, which usually deteriorated until the animal died, although its bone marrow output of reticulocytes was increased by 30 - 80% compared to the control.
- the time at which anaemia occurs and progress into a severe one depends on each individual animal, how it respond to the infection and on the sex

of worms inside its tissues. Usually the infected mice which had severe anaemia after 30-32 weeks harboured two male and 3-4 female worms, whereas those that survived longer durations of infection (50 - 80 weeks), had in their livers either I-7 male or one male and one female worms.

2. The mechanism(s) involved in the aetiology of anaemia of schistosomiasis

A- Blood loss

It has been shown by injection of ^{51}Cr tagged erythrocytes in infected mice, that they pass some blood in their stools yet this loss was not a continuous one. Simlilarly, Farid et al (I969) stated that blood loss in human schistosomiasis is intermittent and not constant and though it maybe severe for a few days it usually ceases for prolonged periods. Also mice infected with irradiated cercariae; i.e. eliminating presence of the eggs responsible for haemorrhage; produced anaemia (Mahmoud & Woodruff I972). Thus it seems that blood loss in infected mice is only a subsidiary contributory factor to their anaemia, and it possibly becomes effective only when the animals are infected with heavy doses of cercariae.

B- Haemolysis and anaemia

Labelling with chromium 5I showed diminution in erythrocyte life span of patients infected with S. mansoni, associated with increased take up of radioactivity by the spleen, from which a clear lead was obtained indicating that haemolysis was taking a part in the production of anaemia (Woodruff et al, I966).

In this study the $^{51}Cr\frac{1}{2}$ life span of red cells from mildly anaemic infected mice was proved to be half (5 days) the controls (I0 days), also red cells destruction took place largely inside the spleen as demonstrated by increased accumulation of radioactivity in this organ. In addition to the presance of few fragile (deformed) erythrocytes in a number of blood samples taken from infected mice, tested by the osmotic fragility test, which suggested an increased rate of haemolysis during infection. Several factors were found

to be responsible for the production of haemolytic anaemia including:

i) Immunological factor

Evidence of the passive sensitization of red cells with immunocomplexes which renders them more susceptible for destruction, was provided by the positive direct agglutination (antiglobulin) test obtained after the addition of erythrocytes from severly anaemic mice (PCV = 5 - 20%, Hb = I - 9gm./I00ml.) infected with light dose of cercariae; to the diluted antiglobulin sera "Broad spectrum" (anti-IgG, IgM, and IgA). The antiglobulin reaction commenced after 22 weeks and remained inconstantly positive until later stages of infection (53 weeks). Due to the variations in strength and end point of agglutination, it was possible to assume that red cells during infection become gradually coated with variable amounts of globulin depending on their concentration in the plasma, and extent of damage on the surface of erythrocytes.

Erythrocytes reacting positively in the agglutination test, was then used for antibodies elution by the alcohol method (Weiner, I957). A number of eluates proved to consist of gamma globulins by formation of weak precipitin lines in agar plates; against the "Broad spectrum" antiglobulin serum. These lines were identical to those obtained from sera of infected mice which had a higher titre of immunoglobulins than the control group as shown by the immunodiffusion method. Therefore, red cells are covered with small amounts of antibodies from their sera usually at later stages of infection.

Although an attempt was made to study the characteristics of the immunoglobulins bound to red cells by using the specific anti-IgG and anti-IgM sera in the direct agglutination test, proved to be negative. However, it was reasonable to conclude that red cell surface was coated with small amounts of both IgG and IgM, that can only be detected by the "Broad spectrum".

Druninga (1971) stated that, the complement reaction sequence begins with the attachment of antibody to a specific antigen on or attached to the cell surface. The nine components of complement (i.e. C_4', C_2', C_3', ... C_8', C_9') are then sequentially activated and bound, the end result being irreversible damage to the cell membrane with formation of "holes" causing osmotic lysis of the red cells. A similar process might also take place in the severe anaemia of infected mice, as indicated by the presence of "holes" that varied in their size and number on the red cells surface seen by the scanning electron microscope. Woodruff (1972) denoted the importance of complement fixation on erythrocyte surface leading to its destruction, and hence haemolytic anaemia due to infectious diseases, i.e. Kala-azar, trypanosomiasis and schistosomiasis.

Dacie (1962), proposed two leading hypotheses for the aetiology of auto-immune haemolytic anaemia: I) an alteration in the erythrocytes which has the effect of making them seem "foreign" to the antibody-forming tissues thus antigenic. 2) development of anti-erythrocyte antibodies due to an unusual responsiveness or intrinsic activity of the antibody-forming tissue. The serological abnormalities summarized above, namely positive direct antiglobulin test, slight elevation of serum gamma globulins and presence of "holes" on the damaged infected erythrocytes, accompanied by severe anaemia, indicates an "alteration" of the antigenicity of the red cells, with formation of immuno-complexes of a possible auto-immune nature on their membrane, thus responsible for their prompt removal from the circulation by:

- antigen-antibody reaction with or without complement fixation, which results in phagocytosis of erythrocytes by reticuloendothelial cells of spleen or liver.
- complement fixation by immunocomplexes, leading to the osmotic lysis of red cells.

The red cell membrane might be "altered" by two ways due to the presence of schistosome parasite:

- Adsorption of schistosome antigen molecules directly (Bruninga, 1971, and Mahmoud, 1971).
- Cell membrane might be coated gradually with damaged "altered" tissue components as a result from the continuous release of egg or worm toxins over prolonged periods of infection. This process was proved by Kurata (1966), when red cells coated with liver or colon extracts (auto-antigen) were agglutinated with sera (auto-antibodies) from rabbits infected with S. japonicum.

Additional evidence for an auto-immune mechanism responsible for the pathogenesis of schistosomiasis is the detection of auto-antibodies in sera of patients with hepatic schistosomiasis against liver (Ekladios et al, 1971) and spleen (Ageeb et al, 1971) antigens.

ii) Destruction of red cells inside the spleen

By means of radioisotopic studies, it was found that many of the erythrocytes are destroyed inside the enlarged spleen of infected mice by the increase of radioactivity over this organ relative to the liver. The exact role played by the spleen in trapping erythrocytes is far from known, yet two major theories have been introduced:

- The sequestration-phagocytosis theory; suggested by Doan and Wright (1946). The present study showed that few red cells might be coated by few molecules of a noncomplement fixing antibody (IgG); these cells are destroyed by phagocytosis predominantly by the spleen (Mollison, et al 1965, and Dacie, 1970), especially the number of lymphocytes and plasma cells was described to be increased in the spleens of mice infected with S. mansoni (Saeed, 1970).
- Erythrostasis which results in metabolic death from glucose deprivation (Jandl, 1967).

Since splenectomy in mice (before exposure to infection) did not improve the blood picture (Saeed, 1970), it appears thus in murine schistosomiasis the spleen is not primarily responsible for development of haemolytic anaemia, and presence of the enlarged spleen would aggravate its picture.

iii) Schistosome haemolytic toxins

Although incubation of normal erythrocytes with emulsified worms, and injection of saline worm extract in normal mice did not prove that worms had a haemolytic activity. Nevertheless, the studies done by incubating normal erythrocytes with tissue (particularly the liver) alcoholic or saline extracts, showed the presence of a lytic factor in small number of normal livers; and it increased markedly in case of infected livers, similar results was seen under more sterile conditions with the agar-blood method using the alcoholic extract.

It is difficult to conclude a reliable fact from these observations, since the experiments were done <u>in vitro</u> which is no mimic to the <u>in vivo</u> haemolysis of red cells. However, two explanations for the increase in the lytic activity of tissue (liver) are put forward:

- In normal tissues there is equilibrium between the lytic agent and its inhibitory factors, present in the tissues and in the blood stream, any alteration in this equilibrium by extraneous factor (presumably here the schistosome toxins) will result in increase of erythrocyte destruction (Macgraith, *et al* 1943 a).
- Worm or egg secreting or excreting toxins (enzymes, or metabolite end products) for long periods of infection would probably exert a toxic chemical change in the structure of tissue components, thus releasing a haemolytic factor.

The question remains as to the origin of the extracted lytic factor, whether it was from eggs/or worms present inside the liver or/and in addition to the tissue lysins.

Therefore, the schistosoma toxins secreted by both worms or eggs would haemolyse red cells either directly (indicated by the acute anaemia seen before the immunological process commences, proved by agglutination test to be after 22 weeks, and by severe anaemia of a number of mice infected with heavy dose of 500 cercariae), or indirectly through the affected tissues.

Immunopathological renal changes in kidneys of mice infected with light dose of cercariae

By means of light and electron microscopy and immunofluorescent microscopy, renal lesions were studied in mice infected with light dose of cercariae for periods ranging from 14 - 92 weeks. At later stages of infection (48 - 75 weeks), infected mice developed progressive diffuse renal lesions, characterized generally by mesangial expansion, mesangial cell proliferation, focal hyalinization, and crescent formation. In advanced lesions (particularly in glomeruli of mouse killed after 92 weeks), several glomeruli were replaced by hyaline casts. In addition, there were electron-dense homogenuous deposits in the mesangium or underneath mesangial cells on the endothelial side of the glomerular basement membrane, which showed irregular thickening.

Immunofluorescence studies proved that the glomerular electron deposits corresponded to the complement C_3^1 (the test was positive essentially after 30 - 92 weeks). The complement was not trapped non selectively since the persistence and progression of glomerular damage was accompanied by increase of the complement deposition, in addition to signs of inflammatory reactions was indicated by the migration ofa few polymorphs and proliferation of mesangial cells, both act as phagocytes against immunocomplexes. These immunofluorescence findings are in keeping with previous reports on murine schistosomiasis by other investigators (Andrade et al, 1974, and Mahmoud and Woodruff, 1975) demonstrating the deposition of immunoglobulins and complement in the affected glomeruli. Also Tada (1975) proved by the

same immunohistochemical method, localisation of IgG, IgM, IgA, and IgE immunoglobulins mostly in the mesangial area of the glomeruli of monkeys infected with S. japonicum. accompanied by the deposition of schistosoma antigens.

Thus these observations strongly suggests that renal injury in mice infected with light dose of cercariae is of an immunological origin brought about by two possible mechanisms:

- The occurance of circulating schistosomal antigens (Berggren et al, 1967), form immunocomplexes with the host antibodies, these might deposit in the glomeruli if they were in excess, and have escaped their elimination by the reticuloendothelial system. Such immunocomplexes deposit on the epithelial side of the glomerular basement membrane (Dixon, 1961). The infrequency of subepithelial deposits in this study might be due to deposition of large complexes on the endothelial side of glomerular basement membrane that find difficulty to pentrate the membrane as suggested by De Brito et al (1975).
- During long periods of infection, repeated action of schistosoma worms or egg antigens/toxins, accompanied by the damage following deposition of antigen-antibody-complement complexes would alter the mouse glomerular tissue components, i.e. become auto-antigenic, thus possibly stimulates an auto-immune reaction. Such mechanism was shown by the irregular thickening of the glomerular basement membrane.

In conclusion, mice infected with light dose of S. mansoni cercariae, develop in conjunction with an enlarged spleen, severe anaemia, progressive glomerulonephritis. These three features are probably induced by one common immunological mechanism, i.e. auto-immune phenomena and/or host damaging antigen-antibody-complement complexes.

PART 8

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