ABSTRACT

This thesis is concerned with observations on the following serological tests: countercurrent immunoelectrophoresis (CIE), Enzyme-Linked Immunosorbent assay (ELISA) and the Thin Layer Immunoassay (TIA) in Schistosoma haematobium infections in man and experimental animals.

CIE was found to be less sensitive and needing a higher concentration of antigens than the two other tests.

ELISA proved to be very sensitive and specific for detecting antibodies in sera from patients with S.haematobium infections. There was strong cross reaction between S.mansoni antigens and sera from S.haematobium infections. Soluble egg antigen was more reactive than either adult worm antigen or cercarial antigen, and cercarial antigen gave a high percentage of false positives with sera from other helminth infections.

The ELISA proved to be a promising test for serological monitoring of chemotherapy in hamsters, baboons and humans.

A comparison was made of a new serological method, TIA and ELISA, in the detection and quantification of antibodies in schistosomiasis using adult worm antigen and sera from known S.haematobium and S.mansoni cases. TIA produced a small number of false positives with sera from other helminth infections whereas ELISA gave none. TIA has the advantage of being extremely simple to perform but has the disadvantage of requiring large amounts of antigen.

It was possible to detect circulating schistosome antigen in the sera of infected baboons, and to some extent in humans, but not in hamsters using the ELISA test.

Demonstration of the antigen-antibody complexes in the kidneys of infected hamsters, using the electron microscope and immunofluorescence was of limited success.

Additional attempts were made to study the pathological changes in relation to serology at different times after treatment of groups of hamsters infected with S.haematobium. The antibody levels declined in response to treatment. The inflammatory reactions progressively diminished but were still locally present at 20 weeks post treatment.

CONTENTS

ACKNOWLEDGEMENTS

I am very grateful to my supervisors Dr. C.C. Draper and Professor G.S. Nelson, without whose encouragement, advice and help this work could not have been carried out. I am greatly indebted to Professor R. Terry, Brunel University, for his great help and assistance. I would also like to thank Professor Ö. Ouchterlony and all at his department for giving me the opportunity to work in their laboratory in Göteborg, Sweden.

Special thanks are also due for the help I have received from Dr. C. James, M. Taylor, M. Doenhoff and all at Winches Farm, and from my own colleagues here at the Ross Institute, especially Dr. M. Mclaren, Miss J. Lillywhite and Mr. A. Au.

Thanks go to Mr. M. Guy, Ross Institute; Mr. H. Furze, Helminthology Department; Mrs. S. Smith and Mr. A. Radalowicz, Statistical Department; all the staff of the School library, for their kind help on many occasions.

I would also like to thank Mrs. Patricia Foley for the effort and time she has spent on typing this work.

I gratefully acknowledge the photographic skills and patients of Mr. C. Webb and his colleagues in the Visual Aid Department here at the School.

I should like to thank the Egyptian Government for its financial support.

LIST OF TABLES

LIST OF FIGURES

Chapter I

INTRODUCTION

Schistosomiasis has been called "the greatest unconquered parasitic disease now afflicting men and animals" (Weir, 1969). It affects more than 350 million people in tropical and sub-tropical areas of the world. Schistosoma haematobium (Bilharz, 1852; Weinland, 1858), Schistosoma japonicum (Katsurada, 1904) and Schistosoma mansoni (Sambon, 1907), the three major species of schistosomes which infect man, are widely distributed. Figure 1 shows the world distribution of human schistosomiasis.

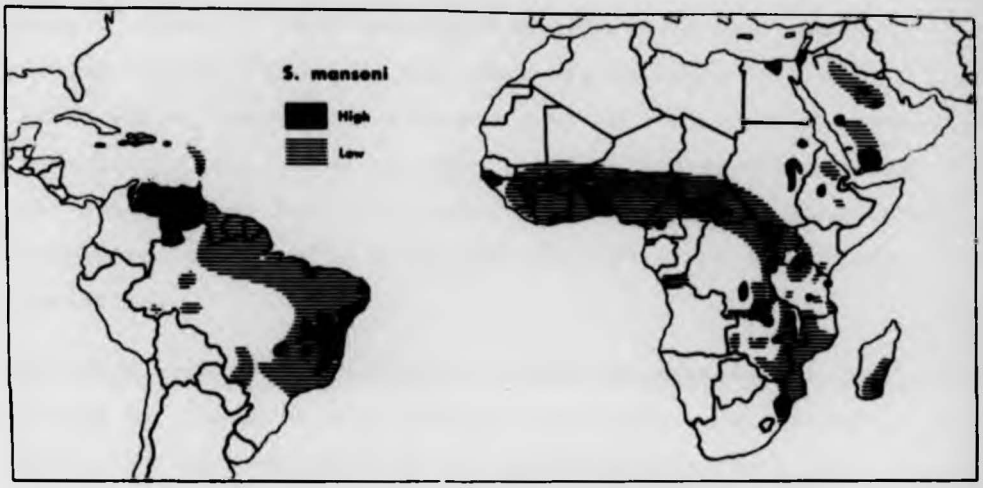
It was considered by the World Health Organization in 1965 and more recently by the 1976 Special Programme for research and training in tropical diseases, a joint project of the World Health Organization and the United Nations Development Programme, as second in importance only to malaria among tropical diseases.

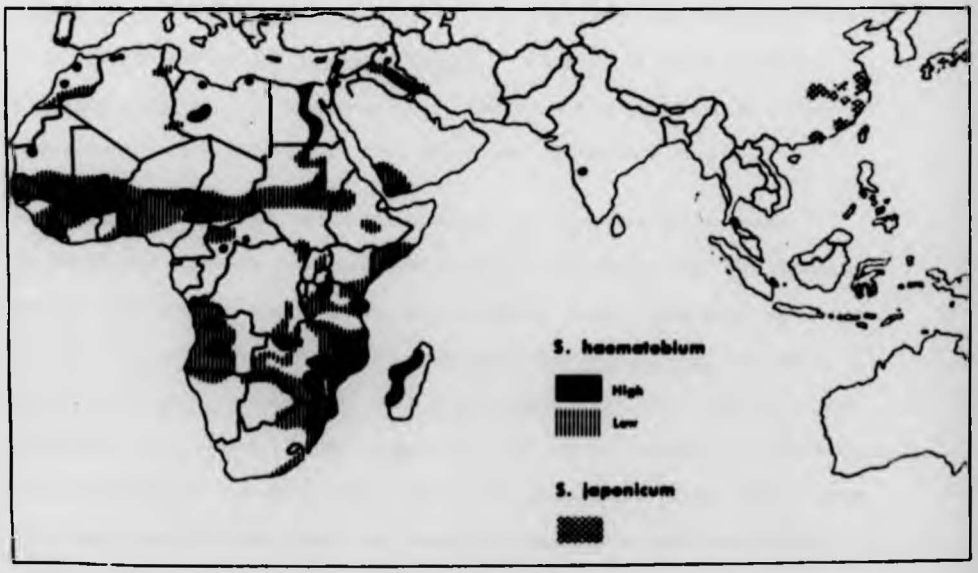
The schistosome worms are not typical flukes in all respects, but do, however, possess some characteristics of digenetic trematodes. Firstly, they have a complicated life cycle through a succession of stages: egg; miracidium; schistosomule; and adult worm. Secondly, an alternation of generations occurs in which there is a sexual generation, parasitic in definitive vertebrate hosts including man; a short-lived free swimming stage infective for molluscs; an asexual generation parasitic in molluscs; and a

Figure 1

World distribution of the three major species of schistosome found in man, S.mansoni, S.haematobium and S.japonicum

Map redrawn from Tropical Health : A Report on a Study of Needs and Resources, National Academy of Sciences - National Research Council, Washington, D.C., 1962.


S. mansoni
High
Low


S. haematobium
High
Low
S. japonicum

short-lived free swimming stage infective for vertebrate hosts. However, they show two features which are somewhat unusual in the digenea: the sexes are separate in the adult worms, and the adult worms live in the blood of their definitive host. Although they have evolved separate sexes, the paired worms remain together forming a kind of functional hermaphrodite. Nearly all other trematodes are true hermaphrodites.

The life cycle of the parasite is shown diagramatically in Figure 2. The male worm carries the female in a ventral groove of the body known as the gynaecophoric canal and migrates with her into the veins draining the intestine (S.mansoni and S.japonicum) or to the vesical plexus draining the bladder (S.haematobium). Figure 3 shows adult S.haematobium: the larger, slimmer female lies in the gynaecophoric canal of the shorter, stouter male.

Although the adult schistosomes, in contrast to many infectious agents, do not multiply directly in the human host, the adult paired worms produce vast numbers of eggs. These are produced and laid singly by S.mansoni or in groups by S.haematobium and S.japonicum. The adult worm attempts to deposit the eggs in the fine venules surrounding the intestine or bladder, so that they can find their way through the intestinal or bladder wall to the external environment in faeces or urine. But some eggs always

Figure 2

Schematic life cycle of the Schistosoma species which infect man

Reproduced from Jordan, P. and Webbe, G. (1969) 'Human Schistosomiasis', published by Heinemann, London.

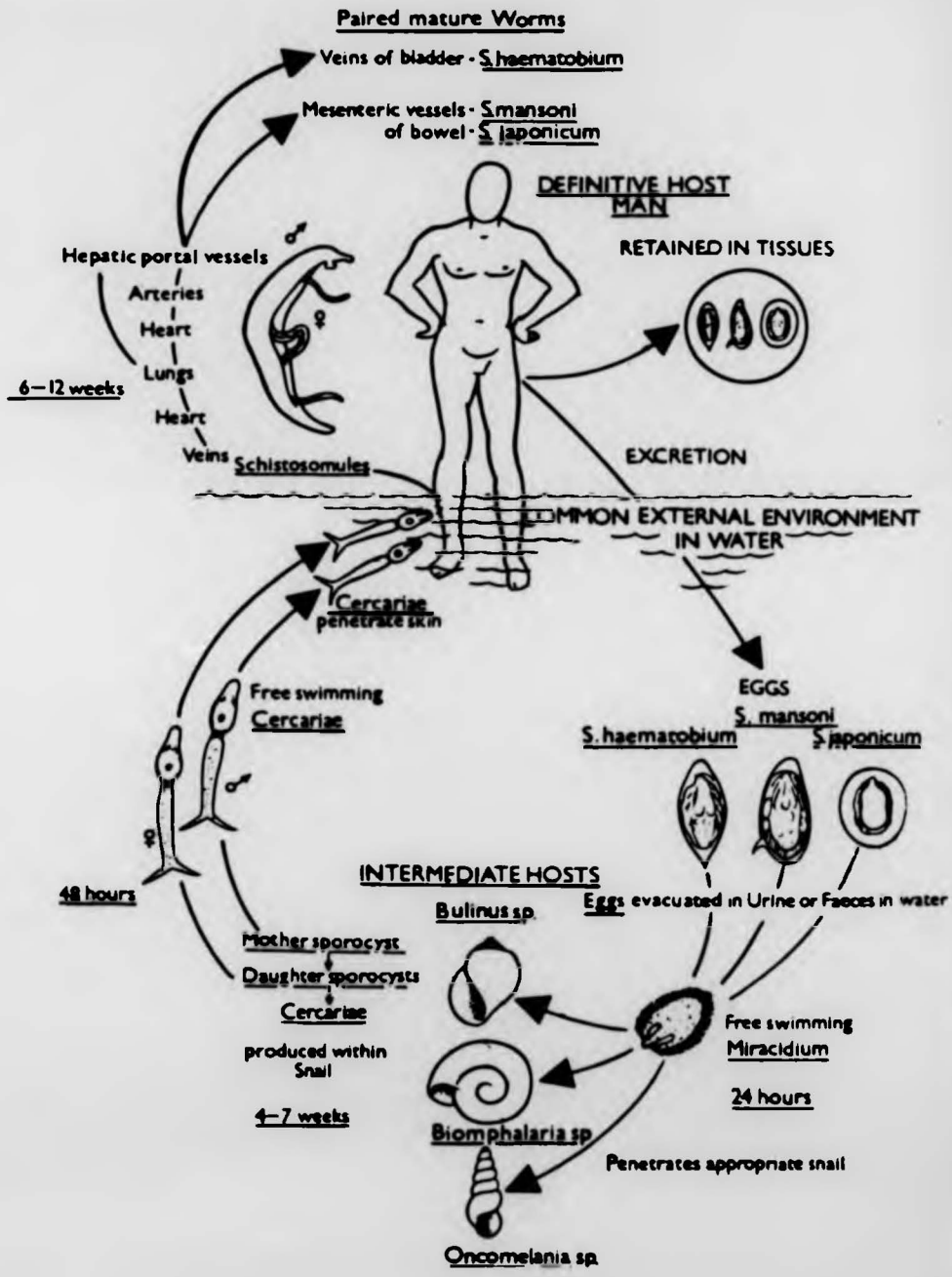

Paired mature Worms
Veins of bladder - S.haematobium
Mesenteric vessels - S.mansoni
of bowel - S. japonicum
DEFINITIVE HOST
MAN
RETAINED IN TISSUES
Hepatic portal vessels
Arteries
Heart
Lungs
Heart
Veins
6–12 weeks
Schistosomules
EXCRETION
COMMON EXTERNAL ENVIRONMENT
IN WATER
Cercariae
penetrate skin
Free swimming
Cercariae
EGGS
S. mansoni
S. haematobium
S. japonicum
48 hours
INTERMEDIATE HOSTS
Eggs evacuated in Urine or Faeces in water
Bulinus sp
Mother sporocyst
Daughter sporocysts
Cercariae
produced within
Snail
4–7 weeks
Free swimming
Miracidium
24 hours
Biomphalaria sp
Penetrates appropriate snail
Oncomelania sp.

Figure 3

S.haematobium adults : male and female in copula

Figure 3

S.haematobium adults : male and female in copula

remain within the body; they may be swept back by the flow of venous blood to the liver or other organs or become lodged in the intestinal or bladder wall. These eggs become a focus of immunological responses, in which the host mounts a cell-mediated immune reaction against them (Andrade, 1965; Boros and Warren, 1970), and they are eventually surrounded by characteristic granulomata (Warren, 1974). These pathological changes can obstruct the normal portal blood flow giving rise to portal hypertension (Warren, 1972), and in severe cases where large number of eggs are involved, an intense inflammatory reaction occurs which is followed by fibrosis. In S.haematobium infections this can lead to fibrosis of the ureters and bladder and possibly even to cancer of the bladder (Ferguson, 1911; Gelfand, 1964; Forsyth, 1967) and in S.mansoni infections to hepatic portal fibrosis (Andrade et al., 1962).

Those eggs which do escape and are voided in urine or faeces contain a fully developed embryo. If the voided eggs fall into fresh water they hatch to give rise to a free-swimming ciliated larva or miracidium. Miracidia are equipped specifically to find and infect snail hosts. They are chemo-sensitive, attracted only to certain snails, and are able to penetrate their soft tissues quickly by means of powerful enzymes (MacInnis, 1963). In a suitable snail host, the larva changes into a first generation or

mother sporocyst, a sac-like structure with germinal cells arranged in layers along the wall of the sporocyst. The second generation or daughter sporocysts develop from the germ balls within the central cavity of the mother sporocyst and migrate usually to the snail liver or ovotestis, where further growth occurs, and bud off more germ balls which develop into cercaria, the final larval stage. Some 4 - 5 weeks after miracidial penetration, cercariae escape from the snail in large numbers; it has been estimated that one miracidium can give rise to as many as 100,000 cercariae. They swim actively with the aid of their large-forked tails and, should they find a suitable host, the head section rapidly penetrates the skin with the aid of enzymes contained in its penetration glands, while the tail section is left behind at the skin surface (Clegg and Smithers, 1968; Ghandour and Webbe, 1973). After penetration of the skin the larva called a schistosomulum, undergoes a number of important structural and physiological changes. These include loss of the cercarial glycocalyx and replacement of the trilaminate tegumental outer membrane with a multilaminate (5 - 7 layers) membrane, identical to that of the adult worm (Hockley and McLaren, 1973). The organism also changes physiologically from one that can survive in fresh water, into one that is rapidly killed by fresh water. It has become adapted to saline and serum and unable to survive in water even for a brief time (Clegg and Smithers, 1968).

It may be that the cercarial fibrillous coat has some water-proofing properties which are no longer required once the parasite enters the host.

The route of migration of the flukes to their final destination is not exactly known, but after a short sojourn in the skin, they reach the lungs by way of the right heart. Here they stay for a day or so before migrating to the liver where they mature and mate and from thence to the mesenteric or vesical veins where the female begins to lay eggs some 4 - 10 weeks after cercarial penetration.

IMMUNOLOGY OF SCHISTOSOMIASIS

Innate immunity

The skin is the first defensive barrier of the host to schistosome infection and it has been related mainly to innate resistance. It appears that there is no selectivity in the penetration of the epidermis of mammals by the cercariae, although some rodents show ranging susceptibilities to infection (Warren and Peters, 1967). Clegg and Smithers (1968) were able to show differences in cercarial death within the skin of various rodent species. Furthermore there is direct evidence that increased host resistance in older mice is correlated with cercarial death in the skin (Ghandour and Webbe, 1973). They also demonstrated that the number of cercariae which die during penetration of mouse abdominal skin steadily increases with age following emergence from the snail. A review of innate immunity is given by Smithers and Terry (1969a).

Concomitant immunity

This is a situation in which the invading schistosomula are destroyed by the host's immune response while the adult worms, from a primary infection which stimulated this response, are unaffected. This concept of concommitant immunity was first introduced by Smithers and Terry (1969b). It was based on the observation that established adult S.mansoni. derived from an initial infection, persisted

long after resistance had developed to a challenge infection (Smithers and Terry 1965a). Thus the adult worms that provoked an immunity were themselves unaffected by it and immunity and infection were concomitant. Support for this concept has been observed from studies of S.mansoni in the baboon (Taylor et al., 1973; Damian et al., 1974, 1976), S.mansoni in the mouse (Sher et al., 1974b), and S.haematobium in the baboon (Webbe et al., 1976). It was concluded from studies which had been carried out by Webbe et al. (1979), that baboons infected with S.haematobium could develop not only a strong immunity to the homologous infections but also a marked degree of acquired immunity to S.mansoni. It was also shown by Smithers and Terry (1967) who transferred adult worms directly into the portal system of rhesus monkeys, that adults were largely responsible for stimulating this resistance. It was supported recently by the transfer of adult S.haematobium in the baboon (Webbe et al., 1976) and adult S.mansoni in the mouse (Boyer and Kalfayan, 1978), although resistance to reinfection was not as strong as in rhesus monkeys. Concomitant immunity has the important biological advantage of preventing overcrowding of the parasites in the host. Schistosome worms derived from early infections, in conjunction with host immunity, may create a barrier to the continual reinfection which would otherwise threaten the long-term survival of both host and

parasite (Smithers, 1972). There has been great interest as to whether concomitant immunity operates in human schistosomiasis. Studies by McCullough and Bradley (1973), Bradley and McCullough (1973), showing the relative stability of egg count in school children with S.haematobium infections, seemed to support the concept.

The destruction of the invading schistosomula by the immune response of the host, whilst the adult worms of a previous infection are unaffected, has focused on an interesting hypothesis to explain this phenomenon. Smithers et al. (1969) have suggested that antigens synthesized by the host become firmly bound to or incorporated in the tegument of the schistosome. It has also been shown that these host antigens are not merely gross contaminants as they resisted prolonged washing of the worms (Clegg et al., 1970). These host antigens act as an immunological disguise preventing the attack of the immune response on the worms. The very young schistosomula migrating through the lungs in the first few days after infection do not have these host antigenic materials and may therefore be vulnerable to attack by the immune response provoked by the adult worms. However these adult worms which have host antigens escape the effect of immune response (Clegg et al., 1971).

The existence of host-parasite cross-reacting antigens has been demonstrated by Damian (1964, 1967) and Capron et al. (1965). This idea was first suggested by Sprent (1962),

that some of these antigens are in fact synthesized by schistosomes and mimic host molecules. Damian et al. (1973) have described an antigen which cross-reacts with mouse alpha 2 - macroglobulin (α_2-M) and is found on the surface of adult worms grown in rhesus monkeys as well as on those grown in mice. They also suggested that this antigen is of parasitic origin, as there is no cross reactivity between primate and murine α_2-M. On the other hand, there is evidence that other host antigens are indeed synthesized by the host and are acquired by the parasite. Goldring et al. (1976) have shown that schistosomula cultured in human blood of various ABO groups, have molecules of exactly the same specificity as the blood used in the culture. There is some evidence, although not yet fully convincing, that the acquired antigens are most likely to be glycolipids (Clegg, 1972, 1974; Dean, 1974).

Diagnosis of Schistosomiasis

Although demonstration of schistosome eggs in urine or faeces remains the only certain evidence of active infection, negative stool or urine examinations do not exclude infection. Eggs are not easily found in early or in light infections as well as in cases which have been partially cured by chemotherapy. Also the extensive inflammatory reaction causes many eggs to be trapped in the tissues and never to reach the lumen of the intestine or bladder. Clinicians have

found great difficulties in diagnosing the disease from the clinical picture only, as it is frequently not well defined especially in children (Pellegrino et al., 1959). Most of the available quantitative parasitological techniques are insensitive, laborious, and need well-trained personnel. Accordingly, there have been many attempts to develop serodiagnostic tests which could be relied upon for individual patients as regards the activity and the intensity of the infection, for assessment of chemotherapeutic cure and more importantly, for epidemiological surveys and for the evaluation of control measures. It has been recommended by the WHO Scientific Working Group on Schistosomiasis (WHO, 1978) and more recently by an independent group (Hoffman et al., 1979), that the use of such serologic tests should be advanced, as alternatives or in addition to the current available parasitological techniques. The serologic tests should ideally be sensitive, specific, quantitative and characterised by technical simplicity and low costs, in order to suit developing countries, where schistosomiasis, as well as other infections, are frequent.

The sensitivity of a serodiagnostic test is defined as the percentage of individuals with positive serologic results among those infected with schistosomiasis as proved by parasitological methods. The specificity is the ability of the test to distinguish schistosome infections from other parasitic diseases. Moreover, species specificity means that the test should be able to differentiate between various species of

schistosome. The reliability of these tests for schistosomiasis may vary considerably depending on whether the tests are applied to individual cases or conducted under field conditions in developing areas where most people are affected by nutritional deficiencies and other concomitant parasitic infections (Sadun, 1976). The most important of the factors which determine the reliability of such techniques are the nature and degree of the purification of the antigens used.

In the following, the relative values of different immunological methods currently in use for the diagnosis of schistosomiasis are discussed. Table 1 gives a summary of these tests, together with others described later in the thesis.

The Complement Fixation Test (CFT)

The complement fixation test (CFT) was the first immunological technique to be used in the diagnosis of schistosomiasis by Fujinami and Nakamura (1909). Yoshimoto (1910) was among a group of Japanese workers who were able to demonstrate the presence of complement fixing antibody in patients with S. japonicum infection using an alcoholic extract of adult S. japonicum as antigen. Subsequently, Fairley (1919) obtained encouraging results in the diagnosis of S. haematobium and S. mansoni infections using alcoholic as well as saline extracts of infected snail hepatopancreas in a CFT. The disadvantage of many antigens was their tendency to react with syphilitic sera, particularly the alcoholic extracts prepared from infected snail livers. Chaffee et al. (1954) prepared an improved antigen by removing the lipids from

Table 1

The value of different immunological methods for the diagnosis of schistosomiasis

Test	Sensitivity	Specificity	Reproducibility	Correlation with degree of infection	Ease of Operation	Suitability for field use	Ability to detect circulating antigen in blood or urine	Assessment of chemotherapeutic cure
(1) Complement Fixation Test (CFT)	+	+	++	-	+	+	+	±
(2) Intradermal Test (ID)	+	+	+	-	++	+	-	-
(3) Indirect Fluorescent Antibody Test (IFAT)	++	+	++	-	+	-	-	-
(4) Soluble Antigen Fluorescent Antibody Test (SAFAT)	++	+	++	-	++	-	-	-
(5) Defined Antigen Substrate Sphere System (DASS)	++	++	++	-	+	-	+	N.K.
(6) Indirect Haemogglutination Test (IHA)	++	++	++	-	+	+	-	-
(7) Slide Indirect Haemogglutination Test (SIHA)	++	++	++	-	+	+	-	-
(8) Cercarien-Hüllen Reaktion (CHR)	++	++	+	-	+	-	-	N.K.
(9) CircumOval Precipitin Test (COPT)	++	++	+	-	+	+	-	±
(10) Miracidial Immobilization Test (MIT)	+	+	+	-	+	-	-	N.K.
(11) Slide Flocculation Test (SFT)	+	+	+	-	++	-	-	-
(12) Schistosomiasis Plasma Card Test (SPCT)	+	+	+	-	++	+	-	-
(13) Urine Precipitation Test (UPT)	++	++	+	N.K.	++	+	+	±
(14) Counter Current Immuno Electrophoresis (CIE)	+	+	+	±	++	-	+	-
(15) Radio Immuno Assay (RIA)	+++	+++	++	+	++	+	N.K.	±
(16) Enzyme Linked Immunosorbant Assay (ELISA)	+++	+++	++	+	++	++	+	±
(17) Thin Layer Immuno Assay (TIA)	++	++	++	N.K.	+++	++	N.K.	±

- Of no use
+ Moderate
+++ Very good
± Possible use
++ Good
N.K. Not known

desiccated adult schistosomes with anhydrous ether in the cold prior to final extraction with buffered saline. They reported that with this method cross-reactions with sera from syphilitic patients could be eliminated. They also achieved the use of the CFT on a quantitative basis. It has been reported by many workers (Davies and Eliakim, 1953, 1954; Horstman et al., 1954; Pellegrino et al., 1959; Sleeman, 1960; Rieber et al., 1961; Rifaat and Khalil, 1965), that better results were obtained by using extracts of adult worms as antigen. Also, partial purification of this antigen (Schneider et al., 1956, Sadun et al., 1959; Sleeman, 1960) and precise standardization of reagents (Kent and Fife, 1963) have greatly improved the specificity and the performance of the test. The sensitivity of the test has been further improved by using more purified antigen preparations (Sato et al., 1969b; Sawada et al., 1969b), and it has been considered as one of the most reliable precedures when conducted under optimal conditions (Kim, 1975). However, this reaction requires more technical expertise than that needed to perform many other immunodiagnostic tests (Fife, 1971; Sadun, 1976). Field studies carried out with this test (Schofield, 1959; Buck and Anderson, 1972), showed that the CFT lacks sensitivity in children and in persons with chronic infections. Evaluation studies of different serological tests (Umaly et al., 1974a) using different schistosome antigen preparations, indicated that the CFT was the least sensitive among the tests evaluated.

The assessment of chemotherapeutic cure by a reliable serological test is very desirable. The CFT has been tried for this purpose (Chaffee et al., 1954; Meillon and Hollingham, 1958; Schofield, 1959; Sadun et al., 1963b; Dennis et al., 1972; Soulsby, 1975), but all regarded the test as unsuitable because CF antibodies remained at a high level for a long time, as long as 5 years, after treatment.

The anti-complementary activity which occurs in large numbers of sera from tropical areas (Buck et al., 1970) represents another great problem that seriously limits the usefulness of the CFT as a diagnostic tool for epidemiological surveys.

Thus, the CFT has certain inherent advantages and limitations, and judgement should be exercised in selecting it for a given purpose (Fife, 1971).

The Intradermal test (ID)

The second immunological method used for the diagnosis of schistosomiasis is the intradermal test (ID), and since the first preliminary report by Fairley and Williams (1927), trials have been undertaken using this method.

The antigens for the test have been made from different stages of various human schistosomes. Antigens of non-human schistosomes as well as of other trematodes have also been used as diagnostic antigens, but in some instances have proved to be quite insensitive (Khalil and Hussan, 1932; Hassan and Betashe, 1934; Culbertson and Rose, 1942; Kagan et al., 1966).

In performing the test, small quantities of antigen are injected intradermally together with a saline control. In positive reactions, wheals often with pseudopodia develop, which reach their maximal size within 10 - 15 minutes (immediate reaction). There are difficulties in interpretation of the results, some (Pellegrino and Macedo, 1956; Pellegrino et al., 1959), considered a wheal area up to 0.9 cm^2 as negative, 1-1.1 cm^2 as doubtful, and 1.2 cm^2 or greater as positive, using 0.05 ml of antigen containing 20 µg N/ml. Ishizaki (1964) recommended that a wheal area of 0.7 cm^2 caused by 0.02 - 0.1 ml of antigen containing 30 µg N/ml should be considered positive. The test is usually performed on the flexor surface of the forearm and, less frequently, on the outer surface of the arm. It has been shown, however, that skin tests carried out on the back are more intense (Pellegrino et al., 1959; Kagan et al, 1961).

The ID test is not particularly specific. Sadun et al., (1959) observed moderate cross-reactions with some other trematode infections, even after purification of the antigen. Sawada et al. (1968) found 3.3% of false positive reactions on examination of 30 clonorchiasis patients. More recently, it has been reported by Yogore and Lewert (1974), that a false-positive ID test response of 37.5% was found in Filipinos infected with capillaria philippinensis infections. It is also well established that individuals exposed to cercariae of non-human schistosomes develop hypersensitivity to antigens of human schistosomes (Sadun and Biocca, 1962; Moore et al. 1968).

The ID test is, therefore, a measure of recent and past experience with any schistosome cercariae (Kagan, 1968) and so it should not be used for epidemiological studies unless concomitant presence of non-human schistosomes can be excluded (Fife, 1971).

Reported data on the sensitivity of the ID test cannot be properly evaluated, because of the diversity of the antigens used and of the criteria for interpreting results, together with the lack of uniformity in individuals in the groups in which the test has been applied (Kagan et al., 1961). However, it has been recognised that the intensity of reactions to the test is greater in adults than children, the reaction is more pronounced in men than in women and it is more intense in coloured persons than in white (Kagan et al., 1961). Warren et al., (1973), confirmed that the ID test is less sensitive in children and that the delayed reaction is less sensitive but more specific. The sensitivity of the test has been slightly improved by using more purified antigens (Williams et al., 1965, Sawada et al., 1968, 1969a; Sato et al., 1969a; Enders et al., 1974), but in spite of this a positive reaction is not always sufficient evidence to confirm a diagnosis of bilharziasis.

The ID test has been used as an epidemiological tool. A recent study by Yogor and Lewert (1974) showed that the ID test could be useful and acceptable for assessing schistosomiasis in the Philippines for those older than 12 years, but they found

that prevalence will be underestimated by about a third in younger age groups.

Warren et al. (1973) reported that the prevalence of delayed skin reactions was 66% for St. Lucia, while Moriearty and Lewert (1974) found it to be 29% for Uganda. However, Kagan (1978) stated that one could not generalize about the sensitivity and specificity of a particular serologic test.

More recently it has been concluded by Hiatt et al., (1978), that the many sources of variability involved and the unsatisfactory sensitivity and specificity of the test limit its epidemiologic usefulness.

It has been agreed (Kagan and Pellegrino, 1961; Kagan, 1968), that the ID test should not be used as a criterion for the assessment of chemotherapeutic cure. Yokogawa, (1969), stated that the ID test has value only as an auxiliary method of diagnosis and he also considered that treatment should not be given to individuals with a positive ID reaction until the diagnosis was confirmed by parasitological examination.

The indirect fluorescent antibody (IFA) test.

The indirect fluorescent antibody (IFA) test is another senological technique introduced by Sadun et al. (1960) for diagnosis of schistosomiasis, using cercarie of S. mansoni as antigen. Other varieties of antigens have been employed in this technique, including experimental liver granulomas (Magalhães-Filho et al., 1965), hepatopancreas of infected

snails (Gigase et al., 1967), and frozen sections of adult worms (Coudert et al., 1968; Pothier and Lourdes-Sampaio-Xavier, 1969a and b). Much of this experimentation has been carried out in attempts to make the test less expensive and elaborate and to facilitate storage and expedition of antigenic materials. The technique has also been improved and rendered more practical by using counterstaining (Sadun et al., 1962; Cookson, 1963; Coudert et al., 1968; Niel et al., 1970). The use of eluates from dried blood smears on absorbent paper in place of serum has made the test a practical diagnostic and epidemiological tool (Anderson et al., 1961; Sadun et al., 1961). Additional modifications have been introduced by Camargo et al., (1965), using worm particles as antigen fixed on microscope slides. Coudert and his coworkers (1968) have developed a similar method using sections of adult worms instead of worm particles fixed on slides. These modifications permit economy in the use of antigen and satisfactory results have been obtained.

However, Kagan (1968) stated that the performance of the test is highly technical and careful standardization is necessary. Conflicting reports on the sensitivity of the test have been given. Pellegrino (1963) reported 86% sensitivity in 307 patients with active schistosomiasis, compared to 96% in the CFT and 92% in a flocculation test. The IFA test was found to be 81% sensitive in known positive cases, but gave up to 40% false positives in a group of Puerto Ricans who were negative for S.mansoni by other

parasitological and serological tests (Kagan et al., 1965).

In comparative studies between IFA and ID tests Foster (1965) found the former test to be more sensitive but still 9% of known positives were negative. Between 95-98% sensitivity among known positive cases have been obtained using the IFA test (Coudert et al., 1968; Ambroise-Thomas et al., 1972; Umaly et al., 1974a; Wilson et al., 1977). Although Hoshino et al., (1970b), reported 100% sensitivity for the IFA test in 105 patients with Manson's schistosomiasis, compared to 96% in the hemagglutination test, still 2.6% false positive occured among a non-parasitized group. In general, it was found that the IFA test provided more reliable results than the CF or the immunoelectrophoresis tests (Vernes et al., 1969).

Toussaint and Anderson (1965) and Toussaint (1966) introduced the soluble antigen fluorescent antibody technique (SAFA), which involves coating a soluble antigen onto cellulose acetate paper discs before processing it in the normal manner. The results are read on a fluorometer, thus elminating the subjective readings of the IFA test. Another advantage is that a relatively large number of tests can be read with great economy of scarce antigenic materials. Compensation can also be made for non-specific fluorescence.

Sadun and Gore (1967) compared the IFA test employing fixed whole cercariae with the SAFA test using soluble antigens. In

the latter, egg, cercarial and adult somatic antigens, as well as cercarial and adult metabolic antigens were used. They found that the highest degree of sensitivity was obtained when fixed whole cercariae were used as the source of antigen and when somatic products of delipidized cercariae were used in the SAFA test. The highest degree of specificity was obtained when fixed intact cercariae were used as the source of antigen or when cercarial somatic and adult metabolic products were used in the SAFA test. On the other hand, adult somatic and metabolic antigens conferred to the test the lowest degree of sensitivity, while cercarial metabolic antigen resulted in the lowest degree of specificity.

However, the IFA test has the disadvantage of cross-reacting with antibodies to Trichinella spirals and bovine, avian and rodent schistosomes (Sadun et al., 1960; Sadun and Biocca, 1962; Cookson, 1963; Pellegrino and Biocca, 1963; Moore et al., 1968; Amin et al., 1969). The test, therefore, has to be used with care, since exposure to avian schistosomes will incite production of detectable antibodies (Kagan, 1968). Neither is the test reliable for assessment of chemotherapeutic cure (Sadun et al., 1963b, Rifaat et al., 1969; Silva et al., 1971 and 1975).

The defined antigen substrate spheres (DASS) system

Van-Dalen et al. (1973) introduced a new IFA technique model by using soluble antigens covalently bound to agarose beads for microfluorometry. This model, the defined antigen

substrate spheres (DASS) system, has been used by Deelder and Ploem (1974) for the immunodiagnosis of S. mansoni infections in animals and men. It proved to be specific, reproducible and more sensitive than the SAFA and IFA techniques (Deelder and Streefkerk, 1975; Deelder et al., 1975b).

Furthermore, storage of the antigen-bound beads in a freeze-dried state proved to be possible and the results of the test could readily be observed with the naked eye (Deelder et al., 1977). Although the DASS system allows the use of different stages of the worm for antigen fractions, and Deelder et al. (1975c) have tried to simplify and optimize the system, it is still technically complex and expensive equipment is needed.

The indirect hemagglutination (IHA) test

The indirect hemagglutination (IHA) test was patterned after the method developed by Boyden (1951) and Stavitsky (1954), which is based on the adsorption of protein antigens on sheep erythrocytes treated with tannic acid and their subsequent hemagglutination by specific antiserum. This technique was initially adapted and evaluated to the antigen-antibody system in schistosomiasis by Kagan (1955a) and Kagan and Oliver-Gonzalez (1955 and 1958). They found the test to be valuable for the diagnosis of acute infection in children and to be more sensitive than the cercarien Hüllen reaktion (CHR). It has been used for sero-epidemiological studies and has been claimed to be a valuable technique to determine the prevalence and distribution of parasitic diseases (Cuadrado and Kagan, 1967).

However, the IHA test gives no index of chemotherapeutic cure (Kagan and Oliver-Gonzalez, 1958; Hoshino et al., 1970b; Silva et al., 1971 and 1975).

Tien-Wei et al. (1966) developed a slide indirect hemagglutination test (SIHT) with formalin-preserved sheep erythrocytes sensitized with an extract of eggs of S. Japonicum. They concluded that the test was of value in the diagnosis of clinical schistosomiasis with relatively high sensitivity and specificity. Its sensitivity was also found higher than that of the CHR.

Using more purified antigens, prepared from adult schistosome worms by chemical procedures, gel filtration, carboxy methyl (CM) cellulose and DEAE sephadex columns chromatography, the sensitivity and specificity of the IHA test have been improved (Sato et al., 1969b; Sawada et al., 1969b). Camargo (1970) and Hoshino et al. (1970a) introduced a modification for the IHA test by employing chromium chloride rather than tannic acid as the sensitizing agent for binding the antigen to the erythrocytes, and found this technique to be more reproducible. Moreover, the sensitized chromium chloride-treated erythrocytes were stable for several months in the refrigerator. Further studies have been carried out by Hoshino et al. (1970c), to standardize the test and the results show good reproducibility and relatively high sensitivity. Umaly et al. (1974a) reported a sensitivity of 85-90% among parasitologically proven cases of S. mansoni and S. haematobium, and none of the antigens

used in the IHA test reacted with the control sera.

The cercarien Hüllen reaktion (CHR)

The cercarien Hüllen reaktion (CHR) of Vogel and Minning (1949a) is a test which uses live schistosome material as antigen. It is a serological phenomenon resulting in the formation of a membranous sheath around living cercariae when these are placed in the sera of infected patients. No such reaction develops in normal serum. This phenomenon was first described and reported by Papirmeister and Bang (1948). The nature of the reaction and the active factors in the serum have been studied by Stirewalt and Evans (1955), Evans et al. (1955), Evans and Stirewalt (1959) and Kemp (1972). The reaction is not species or stage-specific, as cross-reactions between different species of schistosomes have been reported (Vogel and Minning, 1949b; Meleny and Moore, 1954; Hendricks and Cort, 1956; Dönges, 1966; Leflore and Martin, 1968; Umaly et al., 1974a). However, the test is specific and does not cross-react with other helminth infections (Standen, 1952; Abdallah and Raggal, 1962; Fraga de Azevedo and Rombert, 1964; Dönges, 1966; T'ien-Wei et al., 1966). The sensitivity was found to be higher in the younger infected individuals (Jordan and Goatly, 1963; Fraga de Azevedo and Rombert, 1964). However, the advantage of the test seems to derive from the fact that it is positive early in infections (Kagan, 1955b; Mao, 1958; Fraga de Azevedo and Rombert, 1964). Furthermore it was found to be positive in serum of animals infected with

worms of one sex (Mayer and Pifano, 1951).

Since the CHR must be made with living, infectious cercariae, the test is not too useful as a routine procedure (Kagan, 1968).

The circumoval precipitin test (COPT)

The circumoval precipitin test (COPT) of Oliver-Gonzalez (1954) uses live schistosome eggs as antigen. He observed that when S. mansoni eggs were incubated at 37°C for 24 hours with serum from patients infected with S. mansoni, a precipitate formed around the eggs in the form of globules of varying sizes and shapes. It has been confirmed that the test is both sensitive (Rodriguez-Molina et al., 1956 and 1958; Liu et al., 1958; Kloetzel, 1959; Anderson and Niamark, 1960; Rodriguez-Molina et al., 1962 and 1966; Abdallah and Raggal, 1962; Fraga de Azevedo and Rombert, 1964; Yogore et al., 1968; Rifaat et al., 1969, Ruiz-Tiben et al., 1979), and specific for each species of the genus schistosoma (Newsome, 1958; Oliver-González et al., 1955a; Yogore et al., 1968), there being only a slight reaction when eggs of S. mansoni are incubated in the serum of patients infected with S.haematobium and S. japonicum. However, a low sensitivity of the test has been reported elsewhere as low as 71% among 164 patients with active schistosmiasis in Brazil (Pellegrino and Oliveira-Andrade, 1962). Jachowski and Anderson (1961) found the test not species specific, and this was confirmed later by Shoeb et al. (1967a), who found that living S. haematobium ova can be used as antigen for performing the test in all types of

bilharzial infection. The test is very sensitive in detecting antibodies in animals infected with one pair of worms (Bruijning, 1964) and becomes positive early after infection, as soon as 2 weeks in some instances (Yokogawa et al., 1967). The test has been used in the field (Lewert and Yogore, 1969) and for quantitative purposes (Cancio et al., 1965) and the results were encouraging. While some workers (Oliver-González et al., 1955b; Kloetzel, 1959; Bruijning, 1961; Cancio et al., 1967; Shoeb et al., 1967b; Rifaat et al., 1969) found the test a valuable means of evaluating chemotherapeutic cure, others reported that the test remains positive for long periods after treatment (Sadun et al., 1963b; Rodriguez-Molina et al., 1959 and 1966; Yokogawa, 1969). The use of lyophilized eggs has made the test of practical use in diagnosis where a source of fresh eggs is not readily available (Sala et al., 1962; Yogore et al., 1968; Lewert and Yogore, 1969). The test has a drawback since it will not detect antibodies in unisexual infections (Oliver-González et al., 1955b; Bruijning, 1964).

Rodriguez-Molina (1970) reported that the COP test is no longer used except as an experimental tool. Recent studies, however, show that the test can be used as additional proof of cure after experimental chemotherapy of infected monkeys with promising compounds (Mello et al., 1979). Ruiz-Tiben et al., (1979), concluded from their comparative studies of immunodiagnostic tests in Puerto Rico that the COP test is

sufficiently sensitive and specific to be a useful diagnostic tool for screening populations for infection with S. mansoni. They also found that the test would be valuable in estimating prevalence and incidence of infection in epidemiological work and in making clinical diagnosis. But limitations due mainly to the laborious preparation of antigen, as well as the long time consumed for reading the reactions, discourages routine work.

The miracidial immobilization test (MIT)

A third test using live schistosome material as antigen is the miracidial immobilization test (MIT). The reaction as described by Senterfit (1953) involves the incubation of live miracidia in serum and observing whether or not they become immobilized.

Although this test detects early infection (Senterfit, 1953 and 1958a) and gives a positive result with sera of animals experimentally infected with worms of one sex (Senterfit, 1958b), it does cross-react with trichinella antiserum (Senterfit, 1953 and 1958b), and is considered to be more of academic interest than of diagnostic value (Kagan and Pellegrino, 1961; Yokogawa, 1969).

The cercarial slide flocculation test (SFT)

The cercarial slide flocculation test (SFT) employing cholesterol-lecithin crystals coated with lipid-free cercarial antigen was first described and applied for schistosmoasis by Brandt and

Finch (1946). This test was, as is frequently the case, developed from a test applied to another disease, in this instance trichinosis (Suessenguth and Kline, 1944). The principle of the test is the addition of a drop of antigen-cholesterol-lecithin suspension to the previously inactivated serum and the mixture is then shaken. The degree of flocculation is indicative of the reactivity of the serum. Evaluation of this technique has increased its sensitivity and extended its usefulness (Anderson, 1960; Anderson and Naimark, 1960; Jachowski and Anderson, 1961; Pellegrino, 1964; Sadun et al., 1965). Moreover, the test is relatively simple to perform, requires no specialized equipment or facilities and the antigen-sensitized particles can be stored for a long time without deterioration.

However, there appears to be a significantly high degree of flocculation with sera from patients with other parasitic diseases, especially Trichinella infections (Kagan, 1968). It is well established that there is a curious one-way cross between schistosome antigens and Trichinella antibodies (Anderson et al., 1963; Sadun, 1963). Thus, reactions in serodiagnostic tests for schistosomiasis must be interpreted with care in cases in which trichinosis cannot be excluded (Fife, 1971). The substitution of bentonite for cholesterol in performing the flocculation test did not significantly increase its specificity (Wright et al., 1947; Allain et al., 1972).

Schistosomiasis plasma card (SPC) test

A schistosomiasis plasma card (SPC) test has been described by Sadun et al., (1963a). Using a capillary tube, 0.03 ml of unheated plasma was placed on a card especially designed for ten separate samples. One drop of antigen suspension (approximately $\frac{1}{60}$ ml) was distributed to each sample. Using a separate toothpick for each test sample, the plasma and antigen suspensions were mixed and spread until they filled the entire test surface. The card was then rocked by tilting to and fro for 4 minutes and read immediately by inspection with the naked eye. Specimens showing characteristic clumping were reported as positive and those showing no clumping at the end of the 4 minutes were reported as negative. For a permanent record, the cards should be allowed to air dry, care being taken not to shake them until they are completely dried. Because of the economy, rapidity and simplicity of this test, and the fact that it provides a permanent record for future reference, the procedure made it possible to employ the flocculation reaction under field conditions. However, one of the main drawbacks of the test is that several samples can be read only with dificulty, since an atypical pattern of clumping occurs in the cards after 4 minutes. The test also showed a high percentage of positive reactions among individuals without a definite diagnosis of schistosomiasis.

Because of the extreme dryness and the presence of dust in many of the African areas where the field tests were performed,

it was necessary to shake the cards for 4 minutes inside a flat plastic box, which made the test more cumbersome.

Urine precipitin reaction (UPR)

Another approach to serodiagnosis has been attempted in measuring worm material in the host's system, as the measurement of antibody level alone gives no true indication of infection levels nor of active infection. Okabe and Tanaka (1958 and 1961) and Sherif (1962) were the first to demonstrate the presence of antigenically reactive materials in the urines of infected animals and men. Using the urine precipitin reaction of Okabe and Tanaka (1958), Shoeb et al., (1968), found that the test was highly sensitive for early diagnosis of active infection in patients infected with S. haematobium and/or S. mansoni and completely negative four months after treatment.

This review indicates that none of the methods mentioned fulfils all the requirements for good immunological diagnosis of schistosomiasis (cf. table1). In general the sensitivity of the described methods is poor, false negative cases among the parasitological positive patients have been reported. The specificity of these techniques is not of a high enough standard, cross-reactions with sera of non-human schistosome infections and failures to differentiate human species of schistosomes are common. None of the tests correlates accurately with active infection or load of infection and

can, therefore, not be used for assessment of chemotherapeutic trials.

The main aim of this thesis is to explore the sensitivity and specificity of some serological methods (CIE, ELISA and TIA) in the detection of anti-schistosome antibodies in a variety of animal and human sera. ELISA, being one of the most sensitive and established techniques, has been used for serological monitoring of chemotherapeutic cure and for detection of circulating antigen in humans and animals infected with S. haematobium and/or S. mansoni.

A substantial part of this thesis is concerned with comparative studies between ELISA and TIA, in the detection of antibodies in schistosomiasis. A preliminary report of this comparison has been published (Ismail et al., 1979).

Chapter II

MATERIALS AND METHODS

This chapter deals with the basic, general methods and techniques which were used throughout the study. The more specific techniques are described in the appropriate sections of the thesis.

Snail maintenance

The Ghanian strain of *S.haematobium* was maintained in *Bulinus (Bulinus) truncatus rohlfsi* and the Puerto Rican strain of *S.mansoni* in *Biomphalaria glabrata*. The snails were kept in all glass aquaria of 5.5 litres capacity at a density of about 20 snails per tank so as to avoid the adverse effects of overcrowding (Wright, 1960). The tanks were filled with tap-water stored for a minimum of one week before use in a reservoir stocked with fish (*Lesbistes reticulatus*) and plants. The water was kept between 25-28°C using individual heaters and thermostats. Gentle aeration was provided through porous air-stones from a compressor (Hy-Flo pump), and crustaceans (Daphnia species) were allowed to flourish in order to keep the water free from bacteria and debris. Lighting was by fluorescent tubing, the natural diurnal rhythmn of 12 hours of daylight followed by 12 hours of darkness proved to be very satisfactory both for breeding purposes and for maintaining infected

snails. All the snails were fed regularly with dried scalded lettuce leaves, additionally very small amounts of Bemax (wheat germ) and Tetramin (fish food) were given once a week.

Snail infections

The liver of mice infected with S.mansoni and of hamsters harbouring S.haematobium infection were the source commonly used to obtain eggs for hatching and subsequent infection of snails. Eggs were extracted from rodent livers according to the method of Webbe and James (1971). This was carried out by macerating the tissue, sieving it (No.60 mesh, 250 microns), and washing in physiological saline, followed by sedimentation for 20 minutes. The sediment was washed three times, with a final wash in distilled water at 4-5°C. Hatching was then carried out in petri dishes, containing distilled water, in a lighted incubator at 29-30°C.

Snails were infected individually in haemagglutination plates with one ml. of aquarium water per snail at a temperature of 27-29°C. The required number of miracidia were picked up with a pasteur pipette under a dissecting microscope and introduced in each well. The plate was then covered with a sheet of glass and maintained at 27-29°C, and the snails were left undisturbed for a minimum of 5 hours to ensure maximum penetration by the

miracidia before being transferred to the tanks. The length of the prepatent period of infected snails kept between 25-28°C was approximately 28 days for S.mansoni and 36 days for S.haematobium. However, snails infected with S.haematobium were also imported directly from Ghana. The methods and techniques describing the importation and maintenance of schistosomes have been well reviewed by Webbe and James (1971).

Final hosts

Syrian golden hamster (Mesocricetus auratus)

Male golden hamsters, WO strain at 6-8 weeks old and weighing 60-80 grammes were supplied by Wrights Limited, Latchingdon, Essex.

Mice (Mus musculus)

Male 20 gram white mice TO strain were supplied by Tucks Limited, Rayleigh, Essex.

Adult male mice and hamsters were used throughout since they are known to be more susceptible to schistosomes than adult females (Purnell, 1966).

Baboons

All the sera from the baboons (Papio anubis) used in the experiments were obtained from Dr. C. James at Winches Farm. Details of these animals are given in the appropriate sections of the thesis.

Rabbits

Adult 2.5-3.0 kilogram New Zealand white rabbits were used for immunization against worm and egg materials. They were supplied by Hop Rabbits of Kent.

Methods of infection

Mice

Infection was carried out by the subcutaneous infection method as described by Preston and James (1972). The mice were held by the skin over the back of the neck and the appropriate volume of cercarial suspension infected through this loose skin using a 21 G, 1½ needle.

Hamsters

They were infected under sedation using veterinary Nembutal (Abbots Laboratories Limited., 60 mg/ml), one part of Nembutal was diluted with nine parts of physiological saline (0.85% Nacl) and the dosage rate was 6.5 mg/100 g body weight. The percutaneous method of exposure to cercariae (ring method), as described by Smithers and Terry (1965b) was used. The hamsters were anaesthetised by an intraperitoneal injection of the diluted Nembutal. The fur was then clipped from the lower abdomen and the area was swabbed with aquarium water. Each animal was laid on its back with a heavy metal ring of nickle-plated brass with a capacity of 5 ml

over the swabbed area. The skin was wetted before locating the ring in order to form a seal and so prevent spillage under the ring. The cercarial suspension was gently stirred and a volume containing the required number was pipetted into the ring; 15-30 minutes were allowed for cercarial penetration of the skin.

An estimation of the total number of cercariae present in a suspension was made by counting the number of cercariae (killed and stained by a drop of Lugol's iodine) present in three measured aliquots of the suspension. The mean of these counts was used for evaluation of the total number present.

Baboons

Infection was carried out by the percutaneous pouch method of Webbe and James (1971), using "Sernylan" (phencyclidine hydrochloride 20 mg/ml) intramuscularly as an anaesthetic, at a dose of one mg/kg. Briefly, the anaesthetized animal was placed on its back with the legs and arms tied in a vertical position. A pouch was made by lifting up the loose skin of the groin with spring-clip clothes pegs (Smithers and Terry, 1965b). The cercarial suspension was placed in this pouch, which usually holds 10-30 ml of water. Penetration was allowed for 30-40 minutes, after which the fluid was recovered and examined for any remaining cercariae.

Perfusions of animals for recovering of adult schistosomes

Mice and hamsters

Perfusion was carried out by the technique of Smithers and Terry (1965b). Animals were killed with undiluted Nembutal injected intraperitoneally (dosage rate 20-30 mg per animal) containing 10 units of hepatin per ml. Nembutal anaestheized the worms slightly causing them to lose their hold on the walls of the blood vessels and they were easily washed out. A 20 litre plastic carboy complete with outlet of rubber tubing and 20 gauge needle, containing citrated saline (0.85% sodium chloride, 1.5% sodium citrate) was positioned above the perfusion apparatus. The pressure for the perfusion was provided by a rotars peristaltic pump. The abdominal and thoracic cavities of the dead animal were opened and the rib cage was removed. The animal was then well stretched by means of spring clips by one front leg and one back leg, onto a vertical sheet of perspex. The portal vein was cut and the needle from the citrate saline outlet was placed into the left ventricle of the heart. The perfusate flowing from the opened hepatic portal vein passed through a 42 gauge stainless steel mesh into a beaker, while the worms were retained by the mesh. Perfusion continued until the fluid leaving the animal was free of blood. The needle was then removed and with the pump still operating, the

coils of the intestine were lifted and washed down in order to dislodge any worms adhering to them. The mesh was then removed and inverted in a dish of clean physiological saline and the worms collected. The viscera, with their surrounding fat deposits, were thoroughly searched for worms and each liver was squashed between two heavy glass plates and examined under a dissecting microscope.

Baboons

They were first anaesthetised by an intra-muscular injection of "Sernylan". After bleeding from the heart, heparin (25,000 units) was injected direct into the heart and allowed to pass around the body for a few minutes. The animal was then killed by an overdose of Euthatal (pentobarbitone sodium 200 mg/ml) intracardially, and opened. The animal was perfused with citrated saline through a glass canula tied into the aorta. The perfusate was collected from an incision in the hepatic portal vein, into a conical flask by a suction pump. Perfusion continued until all the blood vessels were clear of blood, aided by gentle massaging of the intestine. The worms were collected by sedimentation in urine flasks and then counted.

After perfusion of mice, hamsters and baboons, the adult worms were washed with phosphate buffered saline (PBS) pH 7.6 (its composition is given in the appendix),

counted, and distributed in small tubes and kept at -60°C until used.

Recovery of eggs from tissues

For the purpose of making pure egg antigen, free of host materials, very clean samples of schistosome eggs were necessary. The digestion method of Smithers (1960b) was used with modifications.

The method was as follows:

(1) Mice infected with 150 S.mansoni cercariae and hamsters infected with 300 S.haematobium cercariae were killed and perfused 8 weeks and 20 weeks after infection respectively.

(2) The livers and intestines, including the caecae were removed and placed in separate dishes.

(3) The livers were slightly crushed between glass plates and incubated in normal saline (0.85% sodium chloride) at 37°C for half an hour. They were then removed and washed in fresh normal saline; most of the adult worms possibly present were removed in this way.

(4) The mesentry was removed from each intestine which was then opened and washed thoroughly. The cleaned gut tissue was then minced using scissors.

(5) Ten ml. of 1.7% saline was added for each liver or gut and the tissue was homogenized at low speed for 2 minutes in an MSE homogenizer.

(6) One mg of trypsin (crystallized trypsin, Grade II, Sigma Labs.) per liver or gut was added to the homogenate, which was then incubated at 37°C for 2 hours with occasional shaking.

(7) After incubation, the digest with ten times its volume of 1.7% saline, was poured through two sieves, 180 and 300 mesh, and the filtrate collected and transferred to centrifuge tubes.

(8) The deposit was washed six times with 1.7% saline by centrifugation at 1000 rpm for 2 minutes and the resulting sediment contained clean live eggs, identified by their flame cell movement when viewed under the high power of the microscope. The concentration of saline used was sufficient to prevent premature hatching of the eggs without affecting them adversely (Oliver-González, 1954; Coker and Lichtenberg, 1956). This clean suspension of live eggs was used immediately for the preparation of soluble egg antigen.

Collection of cercariae for preparation of antigen

The infected snails were removed from the aquaria, placed in a small volume of aquarium water and exposed to the light of a fluorescent lamp to stimulate the cercarial shedding process. The cercarial suspension was removed hourly and shedding continued in an additional small volume of aquarium water. After removal of the snails, the

cercarial suspensions were combined and allowed to stand undisturbed for about 5-10 minutes to permit sedimentation of snail faeces and other debris. The supernatant solution containing the living cercariae was then placed in the refrigerator (4°C) to facilitate spontaneous sedimentation of cercariae. Effective sedimentation was usually achieved within 4-6 hours, and this treatment had little, if any, deleterious effect on the organisms. On the other hand, incubation in the cold for periods longer than 6 hours significantly reduced viability and resulted in massive clumping of the cercariae (Fife et al., 1967). After sedimentation of the cercariae, the supernatant fluid that contained some snail products was removed by aspiration. The cercarial concentrate was immediately adjusted to the original volume with dechlorinated water and the organisms were resuspended by gentle stirring. Harvests prepared in this manner usually contained thousands of living cercariae. These cercariae were used immediately for preparation of cercarial antigen.

Antigens

Three antigenic extracts were prepared each for S.haematobium and S.mansoni:

Adult worms of S.haematobium and S.mansoni were collected from infected hamsters and mice respectively and washed thoroughly in phosphate buffered saline (PBS) pH 7.6,

before being stored at -60°C. Eggs and cercariae were used immediately after their preparation for extraction of antigen.

Preparation of antigens

Adult worm antigen (A/W)

A freeze-thaw extract of adult worms was prepared according to the method of Murrell et al. (1974) with some modifications. The previously washed frozen adult worms were allowed to thaw at 37°C. The supernatant was discarded in order to minimize contamination with hamster or mouse antigens. The adult worms (5-6 ml packed worms in 10 ml PBS) were then frozen in liquid nitrogen (-160 to -170°C) and rapid thawing was obtained by placing the frozen worms in a 37°C water bath. Between each cycle of freezing and thawing, the worms were centrifuged at 800 G. for 10 minutes, the supernatant removed and held at 4°C, and the worms resuspended in the initial volume of PBS. The cycle of freezing and thawing was repeated six times and the pooled supernatants were centrifuged firstly at 800 G. for twenty minutes and then at 100,000 G. for two hours at 4°C. The ultracentrifugation (100,000 G.) was carried out in a superspeed 65 MK2 ultracentrifuge (MSE). The supernatant of this was used as antigen.

Capron's method of preparation of antigen for TIA work

Crude soluble antigen was prepared from adult S.haematobium and S.mansoni worms according to the technique of Capron et al. (1968). Briefly, washed adult worms were homogenized in hypotonic saline (0.017 M Nacl), and the homogenate centrifuged as described above. The supernatant of this was used as antigen after dialysis against distilled water for 48 hours.

Soluble Egg Antigen (SEA)

A soluble egg antigen was prepared according to the method of Boros and Warren (1970). A clean suspension of live eggs extracted from the intestines and livers of infected hamsters or mice as described above, were homogenized at a concentration of 50,000 eggs per ml of phosphate buffered saline (PBS) pH 7.6. This was carried out in a glass tissue homogenizer (Gallenkamp) maintained in an ice bath until no intact eggs were seen by examining a drop of the suspension mixed with a drop of Lugol's iodine under the microscope; (the intact eggs stained brown). This material was ultracentrifuged (100,000 G.) for two hours at 4°C. The supernatant of this was used as antigen.

Cercarial antigen

This was prepared according to the original method of Fife et al. (1967) as modified by Murrell et al. (1975),

with further modifications. The cercariae were distributed in flat-bottomed dishes at a concentration of 1,000 to 5,000 per ml and incubated at 4°C for 5 hours. Cercarial suspensions more dilute than this were concentrated before refrigeration, using a suction Millipore filter (Stirewalt, 1971). After incubation, the cercarial suspensions containing exo-antigen were further subjected to sonication in a MSE 150 watt ultrasonic disintegrator MK2, in which the amplitude meter is calibrated in microns peak to peak and indicates the actual movement that is taking place at the transducer/probe interface. This was carried out twice, firstly at 12 microns and secondly at 18 microns, one minute each with five minutes interval. To avoid the effect of heating during sonication, the whole process was conducted in an ice bath. Following sonication, the cercarial suspensions containing exo- and somatic antigens were ultracentrifuged (100,000 G.) at 4°C for two hours. The supernatant of this was used as antigen.

After preparation of all antigens, the protein concentration for each was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Afterwards, the antigen was freeze-dried in 0.5 ml aliquots using (Edwards Freeze Dryer, Model EF03) and was then stored at -60°C until

used. The antigen used for TIA work was dispensed as one ml aliquots and stored at -60°C until used.

Antisera

Antisera were prepared in separate adult New Zealand white rabbits (2.5-3.0 kg) against adult S.haematobium and egg materials. The adult frozen worms were washed twice with phosphate buffered saline (PBS) (pH 7.6) by centrifugation at 100 G. for 10 minutes.

A 2 ml packed volume of adult worms (approximately 500 worms) was homogenized with 5 ml of pH 7.6 PBS in a tissue grinder (Gallen Kamp). The homogenate in a volume of 0.5 ml was emulsified with an equal volume of Freund's complete adjuvant (with Mycobacterium butyrium, Difco Laboratories, Detroit, Michigan) and injected at 2 intramuscular sites on the rabbit's back near each extremity. The immunization procedure consisted of 7 biweekly injections of antigen emulsified in Freund's complete adjuvant. The rabbits were bled once a week after each injection and the serum of the final bleeding which showed the greatest numbers of antigen-antibody precipitin bands on preliminary testing in agar gel with schistosome antigens was used in these studies.

A clean suspension of live S.haematobium eggs (100,000 eggs) was used for antigenic extraction by the method already described and emulsified undiluted in equal volume

of Freund's complete adjuvant. The immunization procedure was the same as described above for adult worm antigen.

Collection of antisera

The antisera were collected and used for a trial of detection of circulating antigen. The rabbits were bled from the marginal veins of the ears during the immunization procedure. The ears were first bathed with xylene to dilate the blood vessels and then thoroughly washed with warm water. A 21 gauge needle was inserted into the vein and the blood was collected in centrifuge tubes and then placed in an incubator at 37°C for one hour. The clot was separated from the wall of the tube and incubated further for one hour at 4°C to retract the clot. The sample was then centrifuged at 1000 G. for 10 minutes and the serum was drawn off with the aid of a pasteur pipette, dispensed into 0.2 ml aliquots and stored at -20°C until required.

At the end of the immunization procedure, the rabbits were deeply anaesthetized with ether and bled directly from the heart, after which they were killed. Each rabbit was opened, the rib cage removed and the blood was collected in centrifuge tubes. This was treated as described above to obtain serum, which was then distributed in 2 ml aliquots and kept at -20°C until used.

Collection of serum from animals and humans

Hamsters

To obtain serum, individual hamsters were bled from

the orbital sinus according to the technique of Riley (1960). The blood was collected in micro-capped centrifuge tubes and then placed at 37°C for one hour. A further one hour's incubation at 4°C to retract the clot preceded centrifugation of the samples at 1000 G. for 10 minutes, after which the serum was drawn off and kept at -20°C in small tubes until examined.

Baboons

The animals were bled under sedation. The blood was withdrawn from the saphenus vein on the back of the hind legs using 10 ml syringe and 20 gauge needle. Having collected the blood in centrifuge tubes, it was dealt with as above for separation of serum which was kept at -20°C until used.

Humans

The human sera examined in these studies were from: (a) Known cases of S.haematobium from El-Minya, Upper Egypt. Figure 4, a map of Egypt, shows the area where these sera were collected. North of Cairo in the Nile Delta both species of schistosomes, S.haematobium and S.mansoni, occur while south of Cairo only S.haematobium is found except for sporadic cases in immigrants (Scott, 1937; Malek, 1975). Before bleeding, the patients were examined clinically and a case history compiled including age, sex, exposure to water, blood in urine,

Figure 4

Map of Egypt, showing the area (arrow) where sera were collected. North of Cairo in the Nile Delta both species of schistosomes : S.haematobium and S.mansoni occur, while south of Cairo only S.haematobium is found - except for sporadic cases in immigrants.


N
Haifa
Israel
TEL AVIV
Gaza
Mansoura
Mahalla al-Kubra
Zagazig
Mersa Matruh
Alexandria
Port Said
Suez Canal
Damanhur
Tanta
Ismailiya
Suez
Giza
CAIRO
Eilat
Fayoum
Beni Suaif
Al-Minya
Sharm al-Shaikh
Asyut
Suhaj
Egypt
Naga Hamadi
Al-Kharga
R Nile
Aswan
500 Km
Wadi Halfa

previous schistosome treatment and social status. The urine from each patient was then examined for live S.haematobium eggs, The blood was collected from positives as well as from patients negative for S.haematobium ova in the urine. Patients negative for S.haematobium infection were carefully selected as controls for the positive cases. They had a negative history of S.haematobium and no S.haematobium ova in urine. Other helminth infections or exposure to schistosome cercariae cannot, however, be entirely excluded. The sera were separated as described above and kept frozen at -20°C until transferred to London.

(b) Known cases of S.mansoni from St. Lucia (kindly provided by Dr. P. Jordan and Dr. J. Cook in 1974 and 1975).

(c) Patients from Gabon (provided by Dr. G. Languillat in 1975), some were from an area where both S.intercalatum and/or S.haematobium occur and the others were from an area where people were exposed to infections but no parasitological data are available.

(d) Cases from an outbreak of human cercarial dermatitis in the U.K. due to the cercariae of a bird schistosome (Knight and Worms, 1972). These sera were provided by Dr. R. Knight.

(e) U.K. hospital patients with parasitic infections other than schistosomes (provided by Dr. D.S. Ridley).

These included cases of W.bancrofti, Loa Loa, Onchocerca volvulus and T.saginata infections. However, it cannot be excluded that some of these patients may also have been exposed to schistosomiasis.

(f) Patients from Kapsabet, a highland area of Kenya (surveyed in 1969). No schistosome infections were found although there was a high prevalence of infections with Ascaris, Necator, Trichuris, Taenia species and Fasciola hepatica. When tested on two occasions by the WHO standard skin test for schistosomiasis (Olivier and Uemura, 1973), many of these children gave false positive reactions, on both or on one occasion. Sera were obtained at the time of each test.

(g) New Guinea children and adults infected with W.bancrofti or Taenia species.

(h) U.K. hospital patients with clinically proven hydatid infections.

(i) Known cases of S.haematobium and S.mansoni from Sudan, (kindly provided by Dr. S.Y. Salih) and these sera were selected (no active malarial infection, no malarial parasites in the peripheral film and no other intestinal parasites in the stools). All patients were treated with hycanthone intramuscularly at a dose of 3 mg per kilogramme body weight. Parasitological

cure, as judged by the absence of schistosome ova in the stool and/or urine, was achieved in all patients who reported for fullow-up 6 weeks after the treatment.

Chapter III

TRIALS OF IMMUNOELECTROPHORETIC METHODS

Introduction

The use of precipitation in gel methods in the serology of parasitic diseases has been reviewed by Kagan (1974), who noted that though these may have the advantage of relative simplicity, they also have the disadvantage of needing concentrated sera and antigens. Of recent workers using Ouchterlony double diffusion tests as were described by Crowle (1973), Madwar and Voller (1975 and 1977) were able to detect antibodies and possibly circulating antigens in sera from patients with S.haematobium and/or S.mansoni infections.

Other workers (Berggren and Weller, 1967; Teesdale, 1973; Bawden and Weller, 1974) found the test sensitive in the detection of schistosome circulating antigen, but only in animals very heavily infected with S.mansoni.

It was also reported by Hillyer (1976) that the test was relatively insensitive in the detection of anti-DNA antibodies in the sera from chimpanzees infected with S.japonicum.

As already mentioned, one of the drawbacks of agar gel test is the high concentration of antigen and antiserum required; another is the time required to perform the

test. Accordingly, the high infection levels required to produce a positive result suggest that double diffusion test would be of little value for the diagnosis of human schistosomiasis.

The principle of the method hereafter termed immuno-electrophoresis (IE) is the electrophoretic separation of an antigen in a gel medium followed by diffusion of precipitating antibodies in the same gel at right angles to the direction of electrophoresis (Fig.5). This increases both the speed and sensitivity of the immunoprecipitation system as well as giving analysis of the antigen. Capron et al., (1966) were able to characterise the antigens of the three species of human schistosomiasis, using IE, and though differences in the precipitin bands could be discerned, many of these are common to one or other species. It was reported by Biguet et al. (1965) that precise diagnosis could be established by studying a single specific arc, but they pointed out that this test was not sensitive in distinction of schistosome species (S.mansoni and S.haematobium). Using IE, Hillyer and Frick (1967) showed evidence indicating the presence of stage specific antibodies to egg antigens in mice infected with S.mansoni. Furthermore, Hillyer (1969) reported that sera from humans with recent S.mansoni infection gave stronger precipitin lines to cercarial, adult and egg extracts than did sera

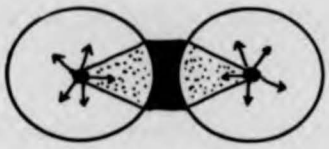
of the chronic cases. Deelder et al. (1975b) found that IE is far less sensitive than the IFA test or DASS system in the detection of antibodies in sera of patients with S.mansoni infections.

Because of its speed, simplicity and greater sensitivity over other gel diffusion methods, countercurrent immunoelectrophoresis (CIE) has become a most attractive technique. The principle of this test is that under certain electrophoretic conditions the antigen and the antibody move in opposite directions simultaneously. The negatively charged antigen moves towards the anode, while the antibody moves towards the cathode by endosmosis set by in this gel, so that they will meet, react and precipitate somewhere between their respective origins. Figure 5 is a diagram illustrating the advantages and principle of CIE. It also shows a double CIE: the specimen for test, which may contain antibody or antigen or both, is placed in a central well with the antigen on the cathodal side and specific antiserum on the anodal side.

Countercurrent immunoelectrophoresis has been used in tropical and other infections for the detection of antibodies and also for circulating antigen. A review of the application of this test in these infections is given by Draper (1976). In comparative studies, Deelder

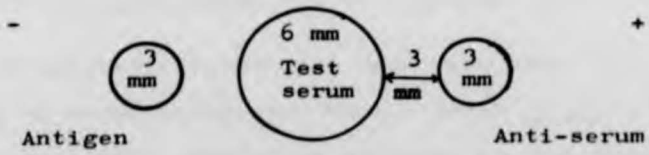
Figure 5

Diagrams illustrating the principle of different gel diffusion methods and the advantages of CIE.

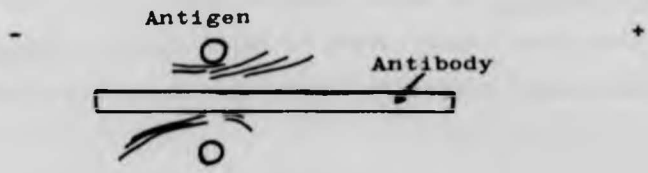


Double diffusion, only the portions moving along the shaded path precipitate.

CIE, the greater portions of the reactants move towards each other intensifying the reaction



Double countercurrent immunoelectrophoresis



Immunoelectrophoresis

et al. (1975c) found that CIE is superior to the other immunoprecipitation techniques in the detection of antibodies in sera of individuals infected with S.mansoni. One disadvantage of CIE is that the class of antibody cannot be ascertained as in IFA or ELISA tests (Draper, 1976).

Double CIE was developed by Phillips and Draper (1975) for the detection of both antibody and circulating antigen in sera of patients with S.mansoni infections. The test was found to be sensitive in detecting antibodies, and possibly for circulating antigen, but in a subsequent use of this test, most of the precipitin bands at the anodal side were found to be non-specific due to acidification of the sera before testing (Draper, 1976). Using the same test, but with the sera not acidified, Madwar and Voller (1977) also reported the possible detection of both antigens and antibodies in sera from patients with S.haematobium and/or S.mansoni infections.

Immunoelectrophoretic techniques have been used for monitoring of chemotherapeutic cure. Dodin et al. (1966), Capron et al. (1969), showed an increase in the number of precipitin bands during the progression of chemotherapy using a crude whole worm antigen, both in S.haematobium and S.mansoni; though some of these bands completely disappeared as a result of cure, the other bands persisted

up to one year after treatment (Capron *et al.*, 1969).

This chapter is concerned with a trial of immunoelectrophoretic methods for the detection of antibodies in sera from patients with *S.haematobium* infections.

Materials and Methods

Antigens

S.haematobium crude adult worm and soluble egg antigens were prepared according to the methods described in Chapter II.

Sera

The sera chosen in this study were from known cases of *S.haematobium* from El-Minya, Upper Egypt, (there is no *S.mansoni* in this area), as described in Chapter II. Also, sera from Egypt and East Africa from people infected with various helminths other than schistosomes were used as controls.

Anti-sera

Anti-sera prepared in rabbits by the method described in Chapter II against *S.haematobium* adult worms were used, together with normal rabbit serum as control.

Choice of gel medium

In the previous work with schistosomiasis Scapin and Tendler (1975), Phillips and Draper (1975), used agar gel, while Dodin *et al.*, (1966), Capron *et al.*, (1969),

used agarose gel. Work with other systems such as bacterial and fungal infections here also used agarose gel (e.g. Bänffer, 1972; Coonrod and Rytel, 1972; Remington et al., 1972).

A mixture of equal quantities of agar and agarose as suggested by Gordon et al. (1971) and used by Combridge and Shaw (1971), Kleger and Kaufman (1973), MacKenzie and Philpot (1975), resulted in increased migration of reagents and improved resolution of lines of precipitation with virus and mycotic infections. Kelkar and Niphadkar (1974) found that pure agarose has disadvantages; movement of serum proteins in agarose is anodal and the y-globulin sweeps out of the wells, probably by diffusion. There is hardly any electroendosmotic movement. They also stated that the ideal gel composition would be that in which the electroendosmotic movement of the globulins equals the anodal migration of the antigen.

It has been reported that sharpening and better resolution of antigen-antibody precipitin lines were observed when dextran was included into agar medium (Ceska and Grossmüller, 1968; Ceska, 1969). Presumably the effect of dextran is to exclude antigen-antibody complexes more efficiently in the agar medium.

Trials were carried out with different mixtures of agar,

agarose and dextran and observations showed that good resolution, separation and definition of precipitin lines were obtained when CIE was performed with gels made of a mixture of oxoid ion agar, agarose (type III, Sigma Lab.) and dextran (grade C, M.W. 60,000-90,000, BDH Lab.), in a proportion of 0.75%, 0.25% and 2% respectively in barbitone/acetate buffer with 0.1% sodium azide added as preservative. Its composition is given in the appendix.

For IE 1% agar in the above buffer was used.

Choice of buffer

The choice of the buffer was investigated in the light of results of other workers using this technique in a variety of different systems. Jameson (1968), used a buffer for bridging the agar slide that had a lower ionic strength than the buffer in the agar. Pesendorfer et al. (1970) and Vergani (1971) used the same buffer in both agar and tank, while Zuckerman and Taylor (1970), Wallis and Melnick (1971), and Combridge and Shaw (1971) used discontinuous buffer system in which the ionic strength of the buffer in the slides was less than that used for bridging them, for detecting serum hepatitis (Australia) antigen and/or antibody. Wallis and Melnick (1971) reported that under the conditions of discontinuous buffer systems, y-globulin and antigen in serum hepatitis patients could be made to

move rapidly towards each other giving increased speed and sensitivity. This system is known as discontinuous countercurrent immunoelectrophoresis (DCIE). In addition, because of the increased electroendosmosis in DCIE, the precipitin lines between antigen and antibody form at some distance from the wells and thus are not confused with non-specific lines which may occur close to wells in the CIE test. The above different systems were applied for testing the human sera mentioned earlier in this chapter, using barbital buffer ranging between 0.015 M and 0.13 M. Apart from some improvement in resolution of precipitin lines, no increase in sensitivity was noted when discontinuous buffer systems were substituted for the system where the ionic strength was the same in both the agar and tank. The buffer chosen for use in the tank and the preparation of the slides was that recommended by Pesendorfer et al. (1970) - (0.13 M, pH 8.4). Its composition is given in the appendix.

Immunoelectrophoresis (IE)

Immunoelectrophoresis (IE) was initially used for comparing adult worm and egg antigens using sera from known S.haematobium cases. It was carried out at room temperature in a Shandon electrophoresis tank (Model U77) using a Shandon V500/150 stabilised power supply (fig. 6).

Microscopic slides (24 x 77 mm and 39 x 78 mm) were each

Figure 6

Shandon Electrophoresis Apparatus with a Shandon V500/150 power supply unit used in immunoelectrophoresis tank

Figure 6

Shandon Electrophoresis Apparatus with a Shandon V500/150 power supply unit used in immunoelectrophoresis tank

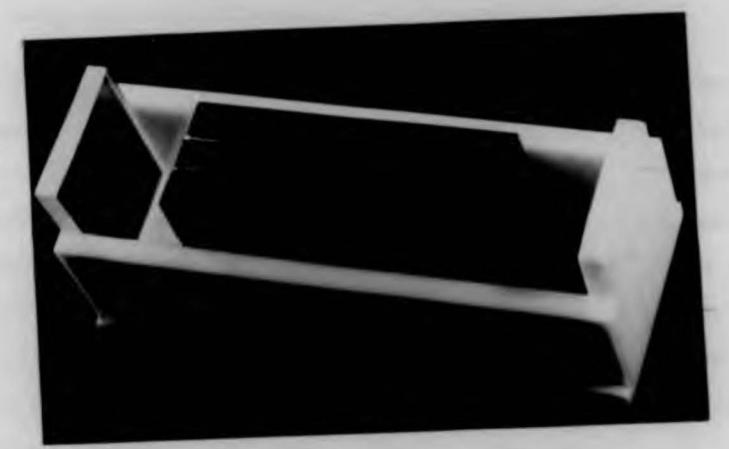
coated with 2-4 ml of 1% molten oxoid ion agar in barbital buffer pH 8.4 (0.13 M), with addition of 0.1% sodium azide as preservative. Wells and troughs were cut using special cutter shown in Fig.7. The wells were each filled with 10-20 µl of antigen containing 10 mg protein/ml. Electrophoresis was performed for 1½ hours using constant voltage at 100 volts/slide.

After the run had been completed, the troughs were filled with undiluted sera (100 µl each) and the slides allowed to incubate for 24-48 hours in a humidity chamber. After this period the slides were read in a diffuse light and the precipitin lines were recorded. For permanent preparation the slides were washed in normal saline for 24 hours and stained with Coomassie blue (Ouchterlony and Nilsson, 1973). The composition of the stain and method is given in the appendix.

Countercurrent immunoelectrophoresis (CIE)

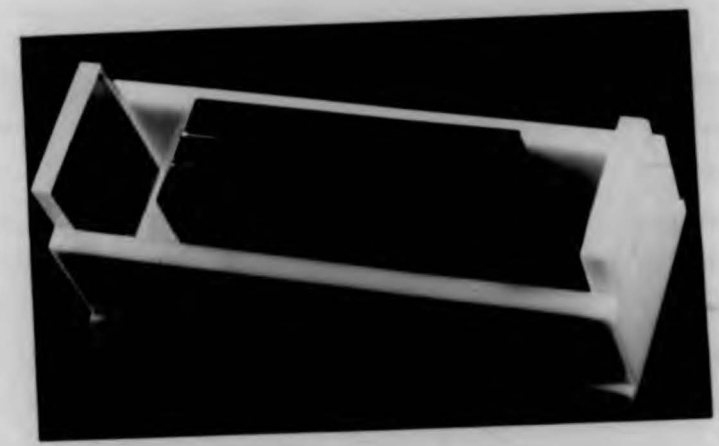
Microscopic slides (24 x 76 mm) were cleaned and precoated with molten buffered agar:agarose mixture before use. Two ml of molten buffered gel (as described earlier in this chapter) were added to each slide. A set of 3 paired wells were cut, each 3 mm in diameter and 5 mm from its partner. The wells nearer to the anode were each filled with 10 µl undiluted serum to be tested. The slides were transferred to the electrophoresis tank (the same

Figure 7



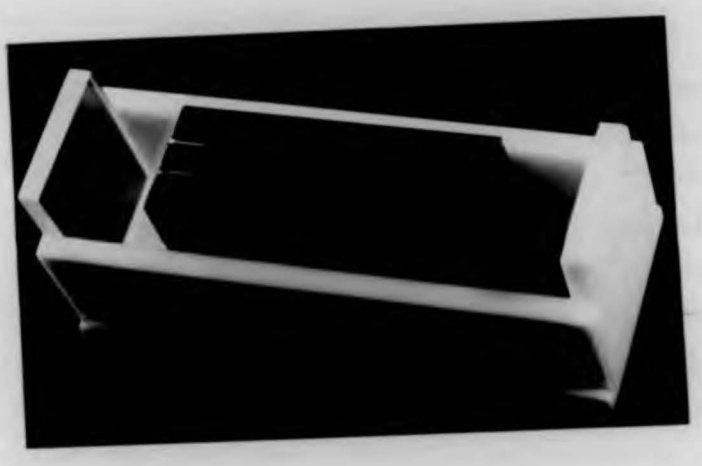
The immunoelectrophoresis gel cutter

Figure 7



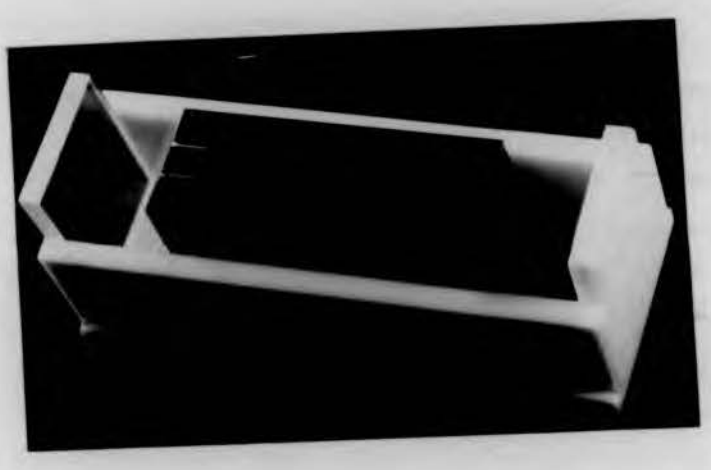
The immunoelectrophoresis gel cutter

Figure 7



The immunoelectrophoresis gel cutter

Figure 7



The immunoelectrophoresis gel cutter

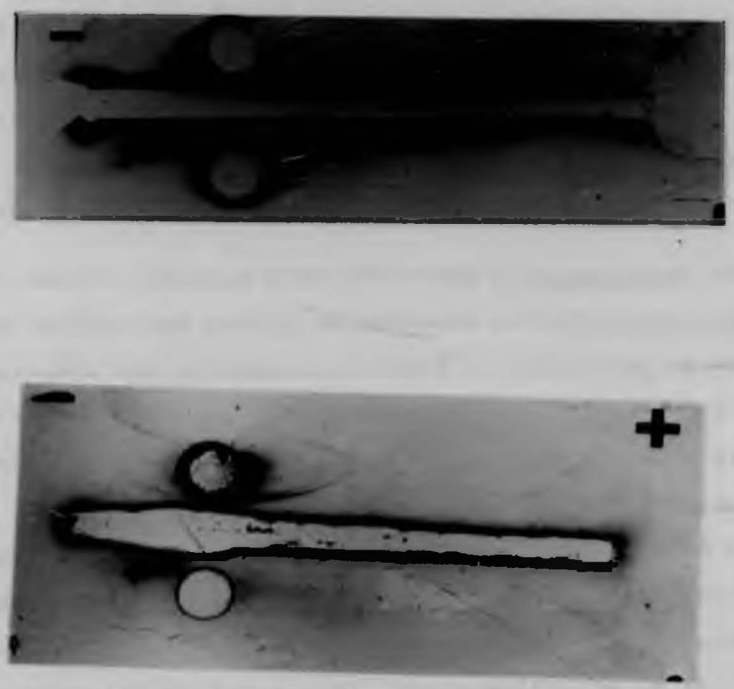
apparatus as above). Constant current was used at 12 mA/slide for 45 minutes after which the slides were removed from the tank and examined for the presence of precipitin lines. Permanent stained slides were prepared as in immunoelectrophoresis.

Different sizes and shapes of wells other than the ones used above were tried with different distances between, with no improvement. Some attempts were made using double CIE for the detection of antibodies as well as antigen in sera from patients with S.haematobium infection. The sera were tested with and without acidification. The slides were prepared as mentioned above and wells were cut in a pattern as shown in Fig.5. The central well was filled with 20 µl of the test sera. Then 5 µl of anti-sera (rabbit anti-S.haematobium adult worm) was placed into the well nearest to the positive pole and 5 µl of worm antigen was placed in the peripheral well nearest to the negative pole. The slides were placed into the tank and the run was carried out as described above. After the run, the slides were removed from the tank and read under diffuse light.

Results

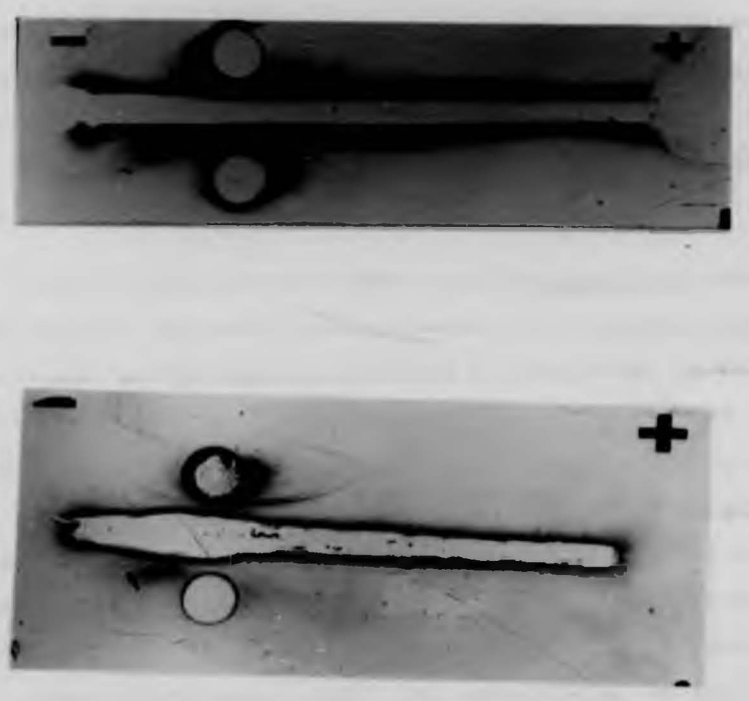
Fig.8 shows two of the immunoelectropherograms comparing S.haematobium adult worm and soluble egg antigens against sera from patients with S.haematobium infections. It

Figure 8



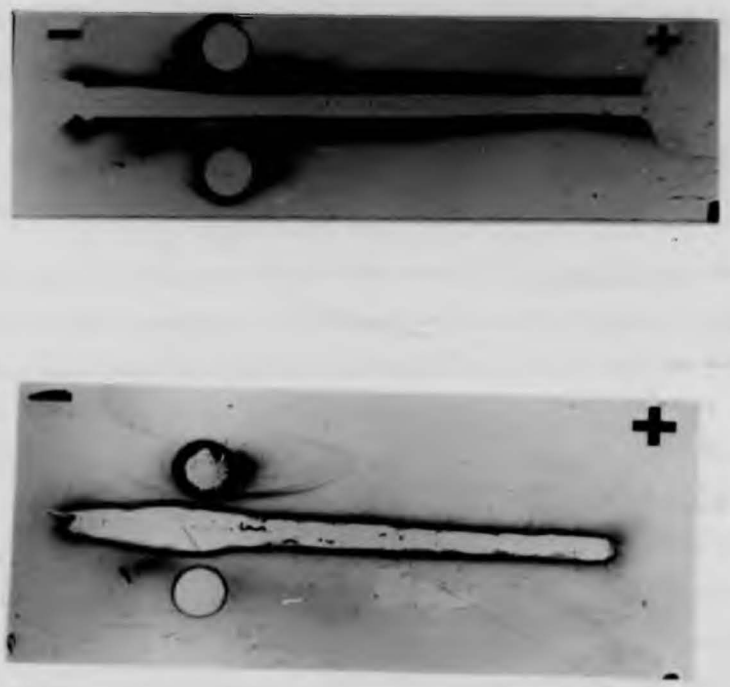
Immunoelectropherograms of two sera from S.haematobium infected individuals (sera No. 91,226) against S.haematobium adult worm antigen (top wells) and egg antigen (bottom wells)

Figure 8



Immunoelectropherograms of two sera from S.haematobium infected individuals (sera No. 91,226) against S.haematobium adult worm antigen (top wells) and egg antigen (bottom wells)

Figure 8



Immunoelectropherograms of two sera from S.haematobium infected individuals (sera No. 91,226) against S.haematobium adult worm antigen (top wells) and egg antigen (bottom wells)

appears that the main antigenic fractions of egg antigen move cathodally, while those of the adult worm antigen seem to move anodally, a finding already described by other authors (Deelder et al. 1975c). Accordingly, the use of egg antigen in CIE might prove to be less satisfactory.

The results obtained with CIE using S.haematobium adult worm antigen and sera from patients with S.haematobium infections are presented in Table 2. As shown in the table, only two out of ten sera examined (4-9 years old) were positives, each giving one precipitin line. Also, the sensitivity of the test in the old age group ($\geq$40 years old) was low; two out of five sera examined were positives with one precipitin line each. On the other hand, in the teen-age group (10-19 years old) and middle age group (20-39 years old), the sensitivity of the test was moderate; five out of ten sera examined (10-19 years old) were positives with one or two precipitin lines each and 15 out of 25 sera examined (20-39 years old) were positives with two or three precipitin lines each. These results were in agreement with those reported by Hwu et al. (1978).

Also, Figs 9.1, 9.2 and 9.3 illustrate the results for S.haematobium adult worm antigen when reacted against sera from some patients with S.haematobium infections as well as

Table 2

Results obtained with CIE on sera from patients with *S.haematobium* infection using *S.haematobium* adult worm antigen

Age group (years)	Number examined	Number of positives	Number of precipitin lines
4 - 9	10	2	1
10 - 19	10	5	1 or 2
20 - 39	25	15	2 or 3
≥40	5	2	1

Figure 9

Fig. 9.1 Samples 1 and 2 from patients aged 20-39 years old
Samples 3 and 4 from patients aged 10-19 years old
Sample 5 control serum
Sample 6 from patient aged >40 years old

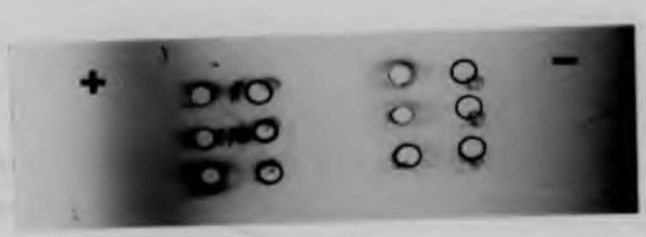
Fig. 9.2 Samples 1 and 2 from patient aged 20-39 years old
Samples 3 and 4 from patients aged 10-19 years old
Samples 5 and 6 control sera

Fig. 9.3 Samples 1, 2 and 3 from patients aged 4-9 years old
Samples 4 and 5 control sera
Sample 6 from patient aged >40 years old

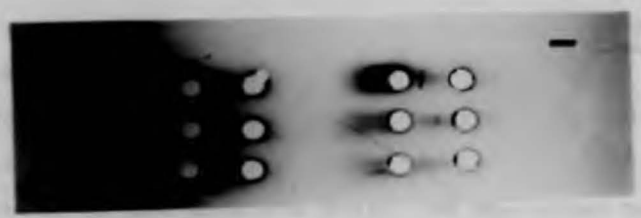
Precipitin lines formed between S.haematobium adult worm antigen and sera from patients with S.haematobium infection as well as control sera when tested by the CIE

Figure 9

9.1



9.2



9.3

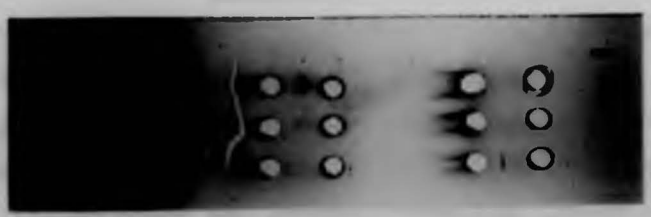
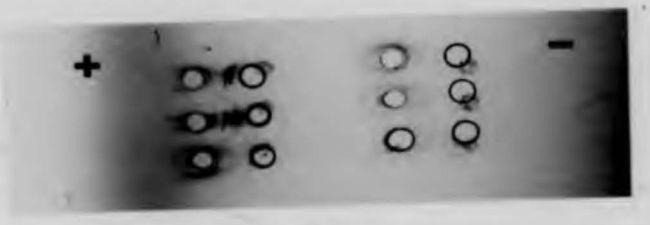
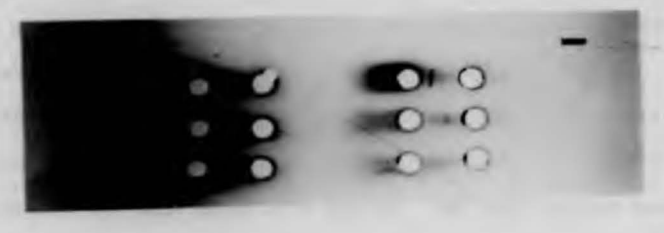


Figure 9

9.1



9.2



9.3

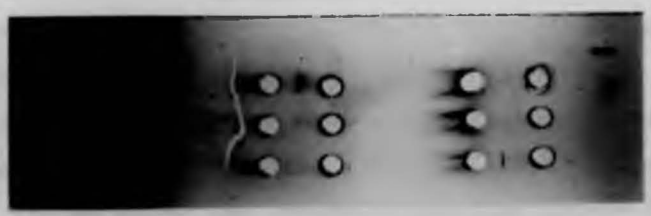
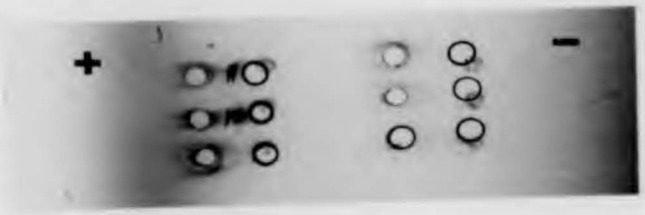
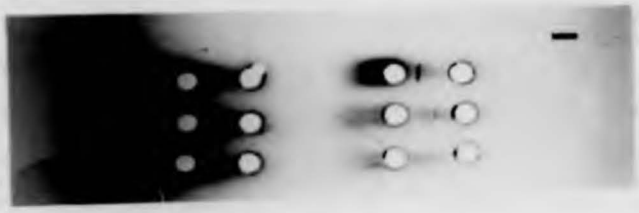


Figure 9

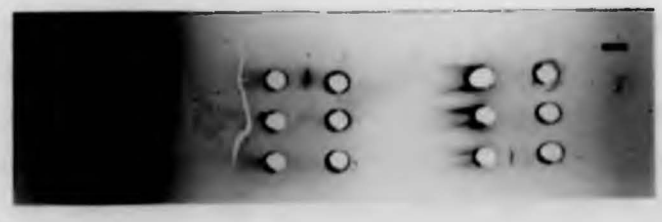
9.1



9.2



9.3



control sera.

None of the control sera (from people with various helminths other than schistosomes) gave precipitin lines except one case out of ten which showed faint, vague precipitin line (Fig. 9.2), which could easily be differentiated from sharp and pronounced precipitates formed against positive sera.

Attempts at detection of circulating schistosome antigen using double CIE and the sera mentioned above gave inconsistent results. When the test sera were acidified, non-specific precipitin lines were found adjacent to the wells nearest to the positive pole. On the other hand, when the sera were tested without acidification, there were no reactions in some while others gave very faint precipitin lines which could not be defined.

Discussion

In this study, CIE proved to be less sensitive in detecting precipitating antibodies in sera from patients with S.haematobium infection in certain age groups, particularly the young (4-9 years old) and old (≥40 years old) age groups. In the other age groups, (teen-age (10-19 years old) and middle age (20-39 years old), the test gave moderate sensitivity. Not only were a higher number of precipitin lines found in the middle age groups than in

those in the young and old age groups, but also the precipitates were more pronounced (Fig. 9.1, 9.2 and 9.3).

Explanations for negative immunoprecipitin reactions may be that the antibody level was too low to precipitate, or the antibody, if present, may not be of precipitin type. It was shown by Hillyer (1969) that sera from acute cases with S.mansoni infection gave stronger precipitin lines to cercarial and egg as well as adult worm extracts than did sera of the chronic cases. It was also confirmed later by Reis et al. (1970) that the gel diffusion test was sensitive with sera from patients with acute stages of schistosomiasis but was relatively insensitive with sera from patients with chronic cases.

Scapin and Tendler (1975) reported excellent sensitivity in a CIE test with eight sera from patients with schistosomiasis that were also positive in double diffusion tests. However, besides the small number of patients they examined, they gave no details regarding the age and/or the load of worms each patient might be expected to be carrying.

Because of the relatively large quantities of antigen and sera required, only small number of sera were tested using CIE. However, the test as so far tried indicated a low sensitivity in detecting antibodies in sera from patients with S.haematobium infections. It agrees with conclusion

those in the young and old age groups, but also the precipitates were more pronounced (Fig. 9.1, 9.2 and 9.3).

Explanations for negative immunoprecipitin reactions may be that the antibody level was too low to precipitate, or the antibody, if present, may not be of precipitin type. It was shown by Hillyer (1969) that sera from acute cases with S.mansoni infection gave stronger precipitin lines to cercarial and egg as well as adult worm extracts than did sera of the chronic cases. It was also confirmed later by Reis et al. (1970) that the gel diffusion test was sensitive with sera from patients with acute stages of schistosomiasis but was relatively insensitive with sera from patients with chronic cases.

Scapin and Tendler (1975) reported excellent sensitivity in a CIE test with eight sera from patients with schistosomiasis that were also positive in double diffusion tests. However, besides the small number of patients they examined, they gave no details regarding the age and/or the load of worms each patient might be expected to be carrying.

Because of the relatively large quantities of antigen and sera required, only small number of sera were tested using CIE. However, the test as so far tried indicated a low sensitivity in detecting antibodies in sera from patients with S.haematobium infections. It agrees with conclusion

of Deelder *et al.* (1975a) that although CIE was found to be superior to other immunoprecipitation techniques in the detection of antibodies in sera from individuals with *S.mansoni* infections, the test is far less sensitive and uses more valuable antigenic materials than other methods (Deelder and Streefkerk, 1975). No further trials, therefore, were carried out with these techniques.

Chapter IV

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES IN S.HAEMATOBIUM INFECTIONS

Introduction

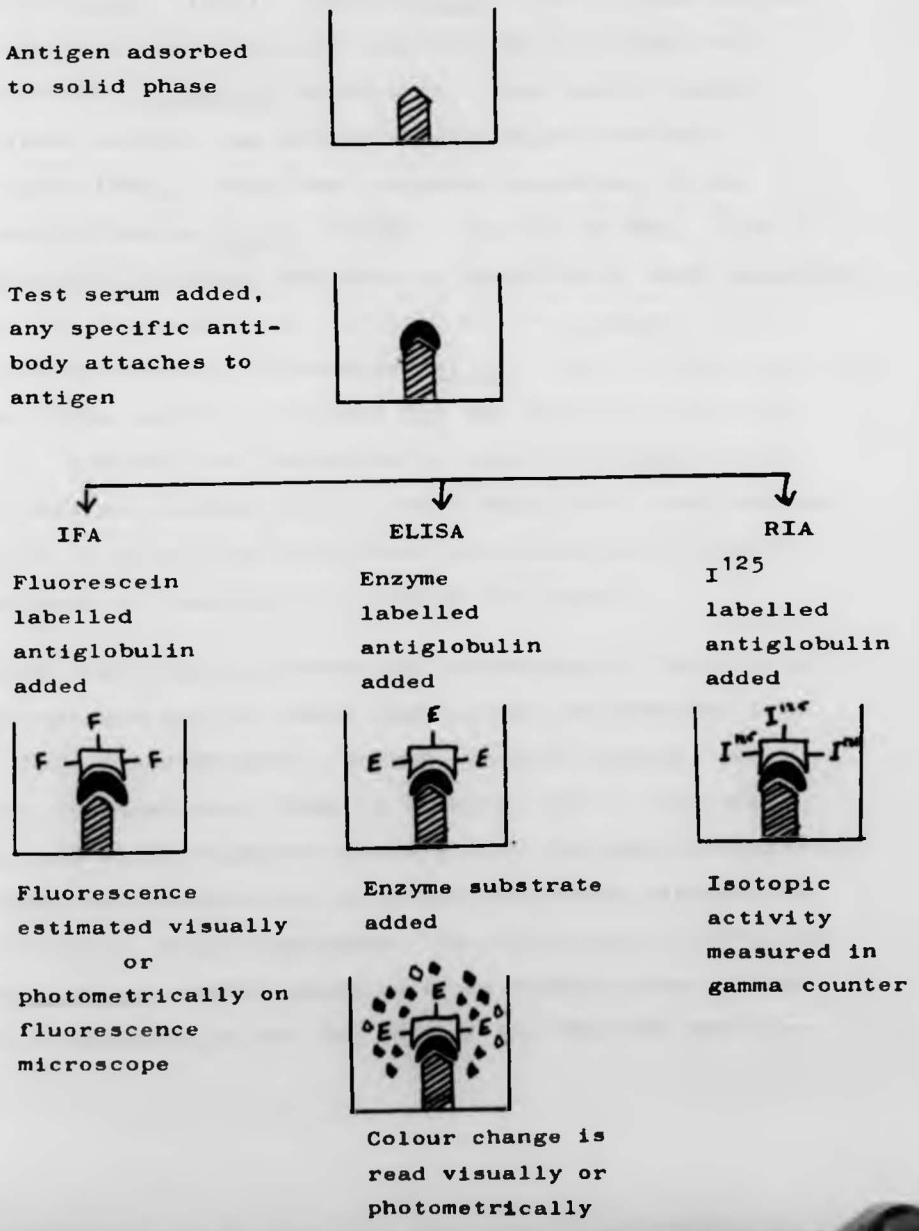
The use of labelled antigens or antibodies with various markers has been found to be particularly useful for assays of biological substances.

Fluorescent dyes have been used for this purpose for many years and, as reviewed in the first chapters of this thesis, the IFA test is one of the best established diagnostic methods in the serological diagnosis of schistosomiasis. However, the IFA test is time-consuming, not easy to perform in an automated manner and the reading of the results is subjective.

Other labels used for antigens or antibodies are isotopes utilized in the radio immunoassay method (RIA). The principle of RIA for measuring antibody is to coat the solid phase with an antigen and then to incubate the test sample thought to contain antibody. If the antibody is present it will combine with the antigen fixed on the solid phase. Subsequently the specific antiglobulin labelled with an isotope (I^{125}) is added and the isotopic activity measured with a gamma counter. Fig.10 illustrates the principle of RIA.

Figure 10

Schematic illustration of the principle of ELISA, compared to the two other methods, RIA and IFA tests.



RIA was found to be sensitive and reliable in detecting antibodies to schistosomiasis (Schinski et al., 1976; Voller et al., 1977). Pelley et al. (1977) reported that RIA was very sensitive and specific in detecting antibodies with S.mansoni infections. They used a highly purified soluble egg antigen, named major serologic antigens (MSA_1), which was prepared according to the method of Pelley et al. (1976). The use of MSA_1, with a high degree of stage and species specificity which parallel that of the sensitizing activity of the S.mansoni eggs in granuloma formation (Hamburger et al., 1976; Warren and Domingo, 1970a and b), accounted for the sensitivity of the test. However, the isotopically labelled reagents used with RIA are costly, have a short shelf life, some health hazard is associated with their production and expensive equipment is required for reading the results.

Enzyme immunoassays combine the advantages of the immunofluoresecnce and the radio immunoassay and overcome some of their disadvantages. Enzyme labelled reagents are safe, comparatively cheap to prepare, have a long shelf life and yield objective results with the same sensitivity as RIA, yet results can be determined either visually or with fairly simple equipment. Ferreira et al. (1974) and Capron et al. (1975) found that the immuno enzyme method was as accurate as the IFA test or the IHA test and its

simplicity made it suitable for immunodiagnosis of human parasitic diseases.

These studies deal with the enzyme-linked immunosorbent assay (ELISA), pioneered by Engvall and Perlmann (1971, 1972). The basic ELISA test depends on two assumptions: (i) that the antigen or the antibody can be attached to a solid-phase support, yet retain its immunological activity and (ii) that either antigen or antibody can be linked to an enzyme, the complex retaining both its immunological and enzymatic activity. Antibodies or antigens have been linked to a variety of enzymes including peroxidase and alkaline phosphatase yielding stable, highly active reagents. The use of a specific enzyme substrate results in the production of a colour which can be assayed photometrically at an appropriate wave length. Fig.10 shows the principle of ELISA, compared to the two other methods, RIA and IFA. ELISA has been used in infectious and non-infectious diseases for the detection of antibodies and antigens. It has also been used for agricultural and veterinary purposes; to detect plant viruses and trichinella infections in pigs. The use of ELISA in these diseases has been well reviewed by Voller et al. (1979). Huldt et al. (1975) were the first to have used the ELISA to detect antibodies to schistosomes and they concluded that the test had potential for epidemiological studies.

Voller et al. (1976a) introduced a micro-ELISA system for detecting both antibody and antigen in schistosomiasis and other parasitic diseases, and Bout et al. (1976) reported a satisfactory sensitivity and specificity of the ELISA in diagnosis of parasitic diseases. A comparative study of the applicability of immunoperoxidase techniques in immunodiagnosis of S.mansoni infections in humans was carried out by Deelder et al. (1977). They found that the DASS system and the ELISA technique (macro and micro systems) were at least as specific and sensitive as the IFA test and also noted the convenience of the micro-ELISA system for field use. The ELISA was found as sensitive and reliable as the RIA in detecting antibodies with sera from patients with S.haematobium or S.mansoni infections (Schinski et al., 1976; Voller et al., 1977).

Recent studies on ELISA in S.mansoni infection indicated that the test has great sensitivity especially when soluble egg antigens were used (McLaren et al., 1978). Kelso and Weller (1978) also reported high sensitivity and specificity for the test in detecting antibodies with sera from patients with S.mansoni infections using adult worm anodic antigen which was non-covalently bound to the surface of poly (L-Lysine) coated wells in polysterine trays.

Studies carried out by Farag et al. (1978a and b) indicated that the extinction values of ELISA for S.haematobium and S.mansoni cases were higher with the homologous antigen. They used S.haematobium and S.mansoni adult worm antigens. They also concluded that the use of specific antigenic fraction(s) would play an important role in increasing the sensitivity and specificity of the ELISA. More recently, Lunde et al. (1979) were able to distinguish serologically between cases of acute and chronic schistosomiasis using the ELISA with both cercarial and worm antigens. Hassan et al. (1979) found that ELISA is slightly superior to IFA and IHA tests in detecting antibodies in schistosomiasis. Hillyer and De Rios (1979) reported high sensitivity of the test in detecting antibodies in sera from mice and humans with S.mansoni infections, but they found that the test gave extensive cross-reactions with sera from humans with fascioliasis, trichinosis, cysticercosis and echinococcosis. They also stated that further purification of the soluble egg antigen would be needed for the ELISA to be an effective seroepidemiological tool in areas where these parasites are endemic. However, they used a different method for conducting the test; the plates after being coated with antigen, sealed and stored

at 4°C for at least three days, the incubation of the serum was then carried out at 25°C for 18 hours. Probably these conditions are not optimal for the test.

It appears that ELISA has proved to be at least as sensitive as the RIA or the IFA test, having fewer disadvantages than the two other tests.

This chapter describes the application of ELISA on a larger scale than previously described to sera from human populations infected with S.haematobium, with other human and non-human schistosomes and/or with helminth infections other than schistosomiasis.

Materials and Methods

Antigens

Adult worm, soluble egg and cercarial antigens, each prepared from S.haematobium and S.mansoni, were used in these studies. They were prepared according to the methods described in Chapter II.

Sera

The human sera examined were mentioned in detail in Chapter II. Briefly, these were from: (a) known cases of S.haematobium from El-Minya, Upper Egypt, (b) known cases of S.mansoni from St. Lucia, (c) people from Gabon where both S.intercalatum and/or S.haematobium occur, (d) cases

from an outbreak of human cercarial dermatitis due to the cercariae of a bird schistosome, (e) U.K. hospital patients with parasitic infection other than schistosomiasis, (f) people from Egypt and East Africa infected with various helminths other than schistosomiasis, (g) New Guinea children and adults infected with W.bancrofti.

ELISA

The basic method of Engvall and Perlmann (1971, 1972) was used, but with the microtitre modification of Voller et al. (1976a). Optimum dilutions of antigen, test sera and conjugate were determined by chequer board titration as described by Voller et al. (1976b). This was carried out against positive and negative sera. In this investigation, three reference sera were used: strongly and weakly positive sera from humans with S.haematobium infections and a negative serum, pooled from several European nurses. These sera were diluted in PBS-Tween and different dilutions ranging between 1:100 and 1:1000 were used. Antigens were diluted in carbonate/bicarbonate buffer at different protein concentrations, ranging between 1 µg protein/ml and 20 µg protein/ml. The antigen concentration and serum dilution that gave the best discrimination between positive and negative sera were used in subsequent tests. It was found that the optimum antigen concentration was 5 µg protein/ml for all the three S.haematobium antigens and the optimum

dilution for the test serum was $\frac{1}{300}$. Similarly, chequer-board titration was carried out for S.mansoni antigens against positive and negative sera. It was found that the optimum concentration for soluble egg antigen was 2.5 µg protein/ml while for worm and cercarial antigens it was 5 µg protein/ml, and the optimum dilution for test serum was $\frac{1}{300}$.

The conjugate, alkaline phosphatase labelled anti-human immunoglobulin, was kindly supplied by Dr. A.Voller, Nuffield Institute, London and the optimum dilution of the conjugate as tested by ELISA against plates coated with varying amounts of immunoglobulin was found to be $\frac{1}{900}$ - $\frac{1}{1200}$, depending on the batch.

The test was carried out in a microtitre plate (Cooke Microtitre, Greiner M29 AR, Dynatech Laboratories). Two hundred µl of diluted soluble antigen, diluted in carbonate/bicarbonate buffer, was added to each well of the plate which was then covered and left overnight at room temperature for coating to occur. The next day the plate was washed three times, three minutes each, with a saline-Tween solution, to remove non-adsorbed antigen. After the last wash, the plate was shaken dry and 200 µl of diluted test serum was added to each well and the plate was incubated at room temperature for 2 hours.

After repeating the above washing procedure to remove unreacted components, 200 µl of the diluted conjugate, alkaline phosphatase labelled anti-human immunoglobulin diluted in PBS-Tween, was added to each well and the plate again incubated at room temperature for a further two hours. After further washing as before to remove unreacted conjugate, 200 µl of the enzyme substrate (para-nitrophenyl phosphate 1 mg/ml in 10% diethanolamine buffer) was added. The reaction was stopped in all wells by the addition of 50 µl of 3 M sodium hydroxide for each well, using a reference positive serum as described later. The contents of each well were removed and the absorbance at 400 nM was read in a spectrophotometer (Vitatron, UPM) (Fig.11). The ELISA values were given as Extinction (E) ≡ absorbance, at 400 nM with a 1 cm light path.

The composition of the reagents used in ELISA are given in the appendix.

Endpoint determination

Because of the high sensitivity of the test and the enzymatic reaction dependance, considerable day-to-day variability occurs, probably due to differences in the ambient temperature; a reference serum should therefore be used as a standard (Voller et al. 1976b).

McLaren et al. (1978) have described a method for

Figure 11

A spectrophotometer (Vitatron, UPM) and printer (Peak Height Reader-Printer Unit, MSE), used for reading and printing ELISA results

Figure 11

A spectrophotometer (Vitatron, UPM) and printer (Peak Height Reader-Printer Unit, MSE), used for reading and printing ELISA results

determination of the optimum value for the reference serum endpoint. Briefly, random samples of sera were taken from 3 groups, 10 sera each. These were from groups a, e and f, among sera examined in this chapter. Tests on a positive reference serum, consisting of a pool of sera from a group of individuals with a high titre of S.haematobium infection, were used, together with replicate tests on the sera mentioned above, using worm antigen. The reaction was stopped when the E_{400} for the reference serum had reached 0.25, 0.50, 0.75, 1.00 and 1.25 respectively. Means and 95% confidence intervals for the E_{400} for each group were calculated at the different E_{400} levels of the reference serum. A value of 0.75 for the E_{400} of the reference serum was found to be the point at which there were the greatest differences between the groups with small variation within the groups. In all subsequent tests several replicates of the reference serum were included. These were read at intervals and the test was stopped when one had reached an E_{400} value of 0.75; this occured after 15-30 minutes. The test sera were considered as positive if their E_{400} value was more than 0.25. This point was selected as none of the negative sera of the other two groups gave values above 0.25. A negative reference serum, from a pool of sera from European hurses, was also included in all subsequent tests.

Figure 12 is a photographic recording of an ELISA plate

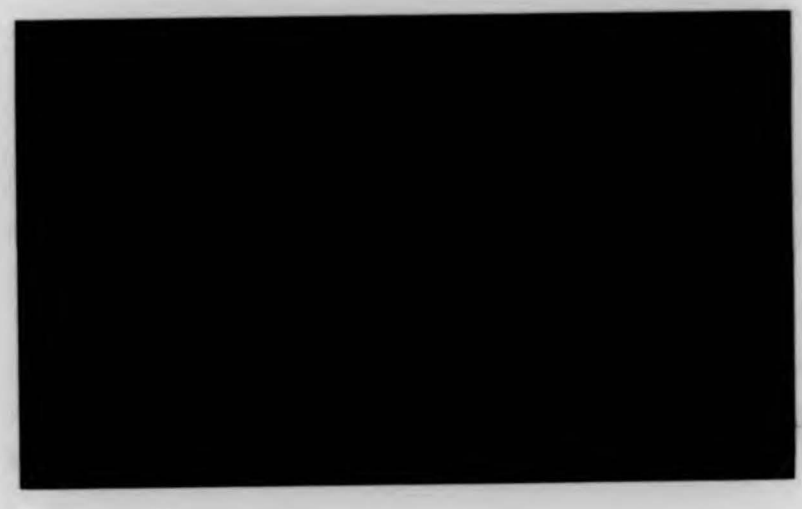
Figure 12

Photograph of an ELISA plate (alkaline phosphatase enzyme system) with S.haematobium adult worm antigen and sera from known Egyptian S.haematobium cases, with different levels of antibody (Rows C and D). Row E, wells Nos.1-4 were used for replicates of positive reference serum, wells Nos.5-8 were used for replicates of negative reference serum, while wells Nos.9-12 contained PBS, required for zeroing the spectrophotometer.

Figure 12

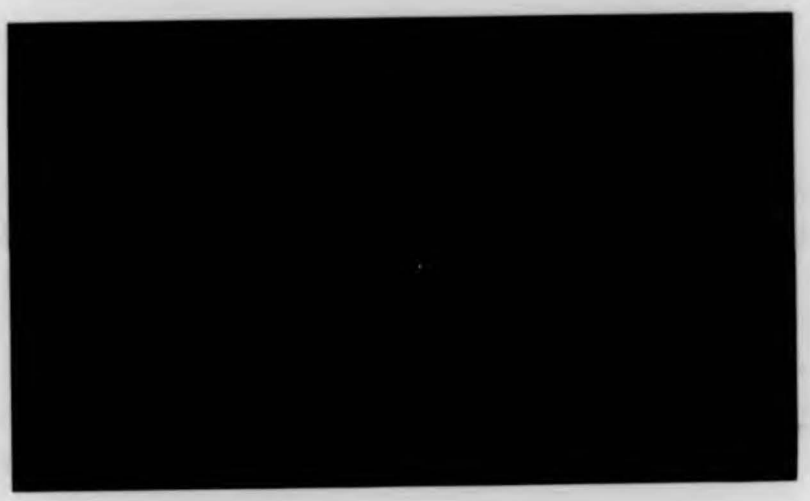
Photograph of an ELISA plate (alkaline phosphatase enzyme system) with S.haematobium adult worm antigen and sera from known Egyptian S.haematobium cases, with different levels of antibody (Rows C and D). Row E, wells Nos.1-4 were used for replicates of positive reference serum, wells Nos.5-8 were used for replicates of negative reference serum, while wells Nos.9-12 contained PBS, required for zeroing the spectrophotometer.

Figure 12



Photograph of an ELISA plate (alkaline phosphatase enzyme system) with S.haematobium adult worm antigen and sera from known Egyptian S.haematobium cases, with different levels of antibody (Rows C and D). Row E, wells Nos.1-4 were used for replicates of positive reference serum, wells Nos.5-8 were used for replicates of negative reference serum, while wells Nos.9-12 contained PBS, required for zeroing the spectrophotometer.

Figure 12



Photograph of an ELISA plate (alkaline phosphatase enzyme system) with S.haematobium adult worm antigen and sera from known Egyptian S.haematobium cases, with different levels of antibody (Rows C and D). Row E, wells Nos.1-4 were used for replicates of positive reference serum, wells Nos.5-8 were used for replicates of negative reference serum, while wells Nos.9-12 contained PBS, required for zeroing the spectrophotometer.

with positive sera from known Egyptian S.haematobium cases, with various levels of antibody, and positive and negative reference sera. The colour changes and the difference between positive and negative could be easily read visually.

Results

Detection of antibodies to S.haematobium infection in Egyptian adults and children using ELISA with different schistosome antigens

Tables 3.1, 3.2 and 3.3 show the results of ELISA with S.haematobium egg, worm and cercarial antigens, on sera collected from El-Minya, Upper Egypt, where only S.haematobium infection is present.

The egg antigen was slightly more reactive than the worm antigen, but both egg and worm antigens were more reactive than cercarial antigen. The cercarial antigen gave a high percentage of positive results with sera from patients without S.haematobium ova in the urine, but other helminth infections or exposure to schistosome cercariae could not, however, be entirely excluded from these patients; while egg and worm antigens gave no false positives. The correlation between the mean E_{400} value of the positives and the age of the patients appears from the tables. The mean E_{400} value obtained with the egg antigen increased and then

Table 3.1

Results of ELISA tests with a S.haematobium egg antigen on sera from Egyptian adults and children.

Age groups (years)	S.haematobium ova in urine	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
5 - 9	Present	27	27	100	0.70 ± 0.12
	Absent	2	0	0	0
	Total	29	27	93	0.70 ± 0.12
10 - 19	Present	114	114	100	0.76 ± 0.06
	Absent	4	0	0	0
	Total	118	114	97	0.76 ± 0.06
20 - 39	Present	245	243	99	0.67 ± 0.04
	Absent	17	0	0	0
	Total	262	243	93	0.67 ± 0.04
40 and over	Present	9	9	100	0.66 ± 0.14
	Absent	3	0	0	0
	Total	12	9	75	0.66 ± 0.14

* Ninety-five per cent confidence interval.

The increase and the subsequent decrease of the mean E_{400} value in the older age groups was significant (correlation coefficient $r = -0.11$ $p < 0.05$).

Table 3.2

Results of ELISA tests with a S.haematobium worm antigen on sera from Egyptian adults and children.

Age groups (years)	S.haematobium ova in urine	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
5 - 9	Present	27	25	93	0.72 ± 0.03
	Absent	2	0	0	0
	Total	29	25	86	0.72 ± 0.03
10 - 19	Present	114	105	92	0.72 ± 0.07
	Absent	4	0	0	0
	Total	118	105	90	0.72 ± 0.07
20 - 39	Present	245	234	96	0.85 ± 0.05
	Absent	17	0	0	0
	Total	262	234	89	0.85 ± 0.05
40 and over	Present	9	9	100	0.92 ± 0.43
	Absent	3	0	0	0
	Total	12	9	75	0.92 ± 0.43

* Ninety-five per cent confidence level.

The increase of the mean E_{400} value in the older age groups was significant (correlation coefficient $r = 0.12$ $p < 0.025$).

Table 3.3

Results of ELISA tests with a S.haematobium cercarial antigen on sera from Egyptian adults and children

Age groups (years)	S.haematobium ova in urine	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
5 - 9	Present	27	20	74	0.46 ± 0.07
	Absent	2	0	0	0
	Total	29	20	70	0.46 ± 0.07
10 - 19	Present	114	81	71	0.47 ± 0.03
	Absent	4	2	50	0.34 ± 0.05
	Total	118	83	70	0.46 ± 0.03
20 - 39	Present	248	189	77	0.46 ± 0.02
	Absent	17	7	41	0.39 ± 0.04
	Total	262	196	75	0.46 ± 0.02
40 and over	Present	9	5	56	0.52 ± 0.05
	Absent	3	1	33	0
	Total	12	6	50	0.53 ± 0.04

* Ninety-five per cent confidence interval.

The increase of the mean E_{400} value in the older age groups was not significant.

decreased in the older age groups, while with both the adult worm and the cercarial antigens the mean E_{400} continued to increase with age. The S.mansoni antigens - egg, worm and cercarial - proved to be as reactive as the S.haematobium antigens, against the same set of sera. This is shown in tables 4.1, 4.2 and 4.3. Figure 13 shows the relations between the three S.haematobium antigens used and the percentage of ELISA positives in the different age groups infected with S.haematobium. The relations were almost identical using the three S.mansoni antigens, although S.mansoni cercarial antigen was slightly more reactive than the S.mansoni worm antigen, particularly in the young and middle age groups (Fig.14).

Figure 15 shows the distribution of ELISA values (E_{400}) obtained with different S.haematobium antigens from a group of uninfected Egyptians and from a group with proven S.haematobium infection (all age groups). Cercarial antigen produced more low ELISA values than either worm or egg antigen. The distribution was similar using different S.mansoni antigens (Fig.16.).

Tables 5.1, 5.2 and 5.3 give the results for further ELISA tests using different S.haematobium antigens on sera from individuals with other schistosome infections or parasitic infections other than schistosomiasis. A strong cross-reaction occured between sera from individuals with a

Table 4.1

Results of ELISA tests with a S.mansoni egg antigen on sera from Egyptian adults and children.

Age groups (years)	S.haematobium ova in urine	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
5 - 9	Present	27	26	96	0.77 ± 0.10
5 - 9	Absent	2	0	0	0
	Total	29	26	90	0.77 ± 0.10
10 - 19	Present	114	110	97	0.67 ± 0.05
	Absent	4	0	0	0
	Total	118	110	93	0.67 ± 0.05
20 - 39	Present	245	237	97	0.71 ± 0.03
	Absent	17	0	0	0
	Total	262	237	91	0.71 ± 0.03
40 and over	Present	9	9	100	0.74 ± 0.19
	Absent	3	0	0	0
	Total	12	9	75	$0.74 + 0.19$

* Ninety-five per cent confidence interval.

The decrease of the mean E_{400} value in the older age groups was not significant.

Table 4.2

Results of ELISA tests with a S.mansoni worm antigen on sera from Egyptian adults and children.

Age groups (years)	S.haematobium ova in urine	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
5 - 9	Present	27	18	67	0.59 ± 0.12
	Absent	2	0	0	0
	Total	29	18	62	0.59 ± 0.12
10 - 19	Present	114	76	67	0.67 ± 0.08
	Absent	4	0	0	0
	Total	118	76	64	0.67 ± 0.08
20 - 39	Present	245	194	79	0.62 ± 0.04
	Absent	17	0	0	0
	Total	262	194	70	0.62 ± 0.04
40 and over	Present	9	7	78	0.88 ± 0.52
	Absent	3	0	0	0
	Total	12	7	58	0.88 ± 0.52

* Ninety-five per cent confidence interval.

The increase of the mean E_{400} value in the older age groups was not significant.

Table 4.3

Results of ELISA tests with a S.mansoni cercarial antigen on sera from Egyptian adults and children

Age groups (groups)	S.haematobium ova in urine	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
5 - 9	Present	27	21	78	0.52 ± 0.09
	Absent	2	1	50	0
	Total	29	22	75	0.54 ± 0.09
10 - 19	Present	114	89	78	0.56 ± 0.04
	Absent	4	1	25	0
	Total	118	90	76	0.56 ± 0.04
20 - 39	Present	245	204	83	0.52 ± 0.02
	Absent	17	5	29	0.45 ± 0.02
	Total	262	209	80	0.52 ± 0.02
40 and over	Present	9	5	56	0.53 ± 0.12
	Absent	3	1	33	0
	Total	12	6	50	0.53 ± 0.12

* Ninety-five per cent confidence interval.

The increase and the subsequent decrease of the mean E_{400} value in the older age groups was not significant.

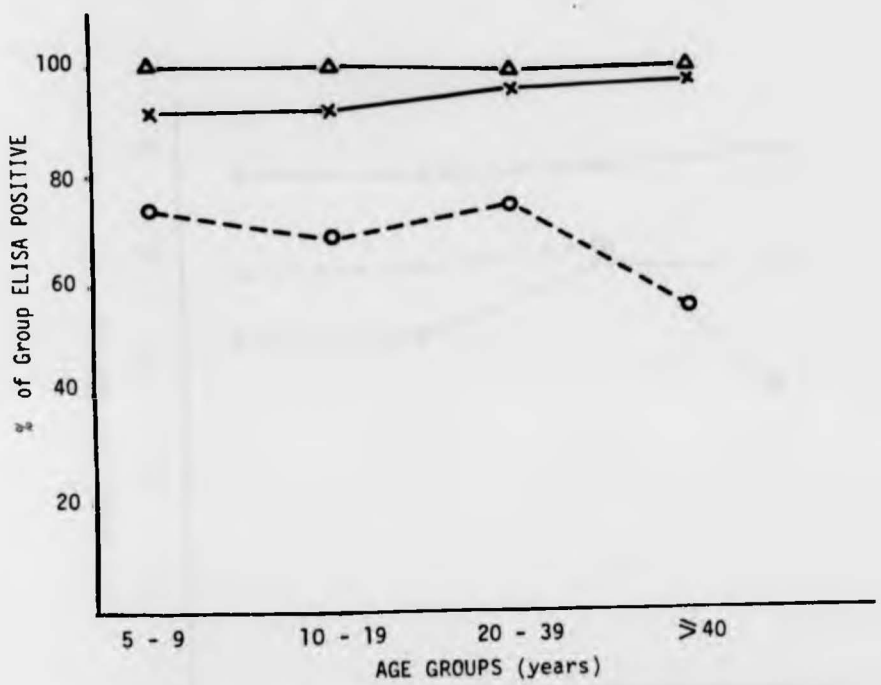


Fig.13 Showing the relation between different S.haematobium antigens and the percentage of group ELISA positive among Egyptian adults and children with S.haematobium infection.

△——△——△ Egg antigen

×——×——× Worm antigen

o----o----o Cercarial antigen

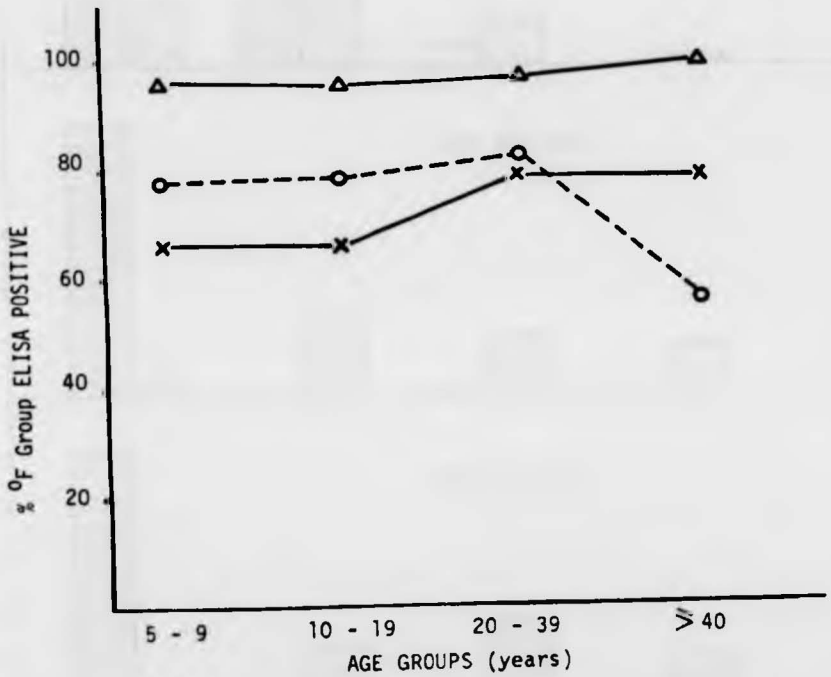


Fig.14 Showing the relation between different S.mansoni antigens and the percentage of group ELISA positive among Egyptian adults and children with S.haemotobium infection.

△——△——△ Egg antigen

×——×——× Worm antigen

o---o---o Cercarial antigen

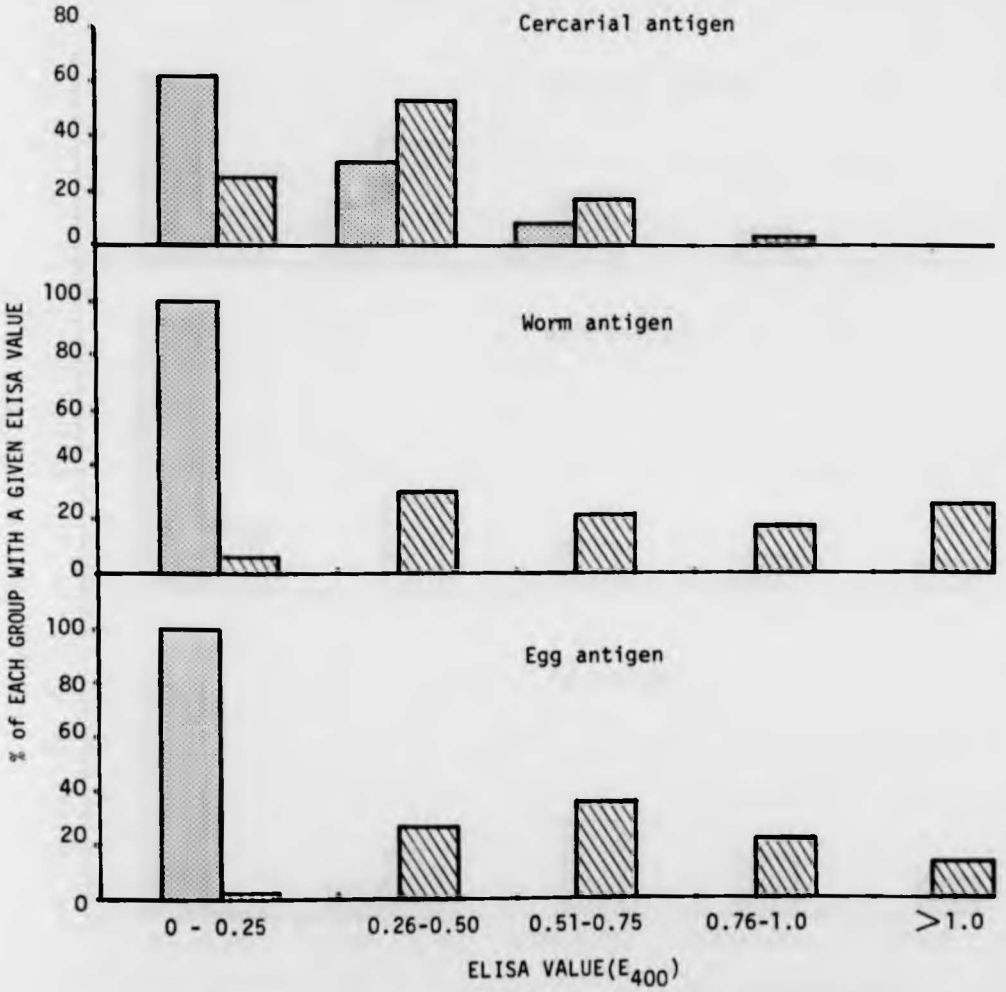


Fig.15 The distribution of ELISA values with different S.haematobium antigens of a group of uninfected Egyptians and a group with proven S.haematobium infection

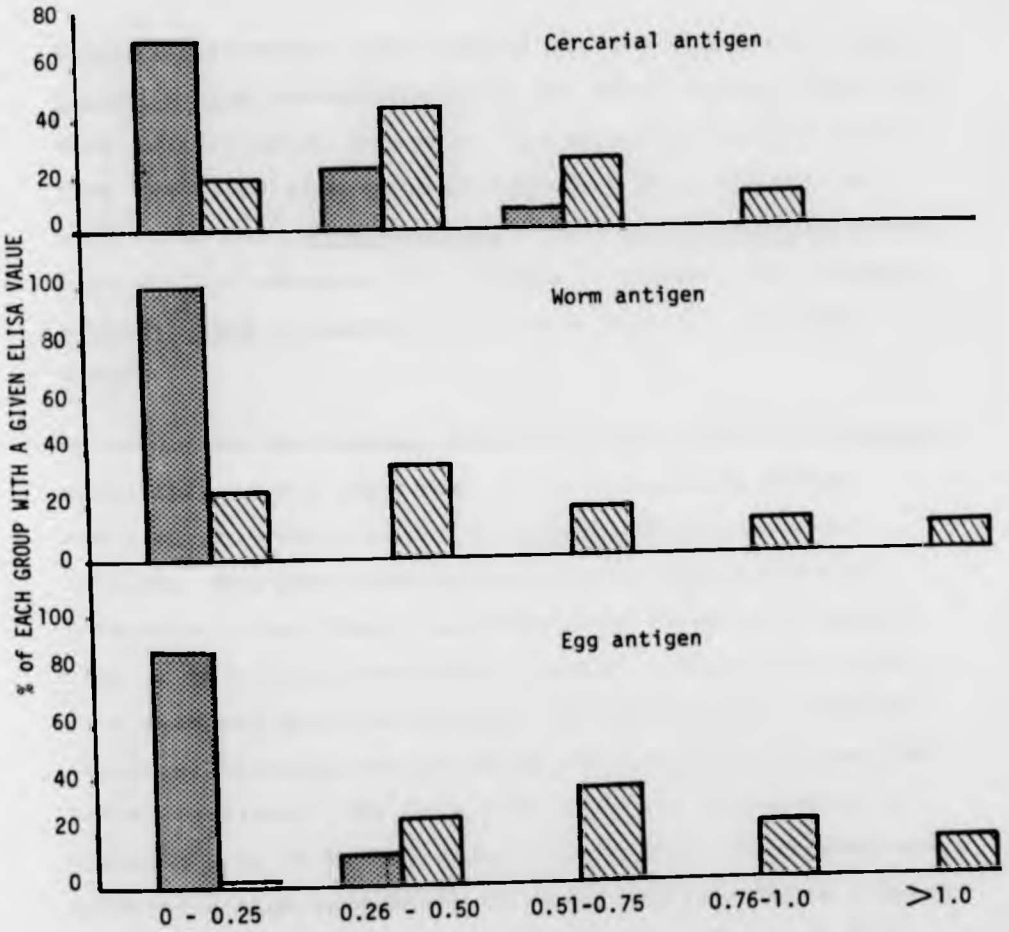


Fig.16 The distribution of ELISA values with different S.mansoni antigens of a group of uninfected Egyptians ▒ and a group with proven S.haematobium infection ▨

S.mansoni infection (St. Lucia) and all the fairly crude S.haematobium antigens used in the test, particularly the worm and cercarial antigens. A high proportion of positive reactions also occured with sera from Gabon; an area where both S.haematobium and/or S.intercalatum occur. Figure 17 illustrates the results of ELISA with different S.haematobium antigens on the sera from St. Lucia and Gabon.

As seen from the tables, sera from U.K. cases of cercarial dermatitis gave a high rate of false positive ELISA results, especially with the S.haematobium cercarial antigen. The sera from patients with various helminth infections other than schistosomiasis produced a proportion of false positive ELISA results. Twenty out of 28 sera examined gave false positive ELISA results with the cercarial antigen, but the worm and egg antigens gave 10% false positives. The sera from children in Kapsabet, a highland area of Kenya, where there is no schistosomiasis, produced a high proportion of false positive ELISA results, particularly with the cercarial antigen. The sera from New Guinea, where there is no schistosomiasis (Ewers and Jeffery, 1971), were all infected with Wachereria bancrofti and nearly all subjects were also infected with necator and strongyloides. These sera produced a low false positive ELISA rate when tested against egg and worm antigens, but gave a high rate with cercarial antigen.

Table 5.1

Results of ELISA tests with a S.haematobium egg antigen in different Schistosoma and other helminth infections

	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
St. Lucians infected with S.mansoni:				
Children	14	4	29	0.34 ± 0.08
Adults	36	12	33	0.37 ± 0.12
Gabon adults infected with S.intercalatum and/or S.haematobium	74	47	64	0.39 ± 0.04
U.K. cases of cercarial dermatitis	24	2	8	0.28 ± 0.04
U.K. hospital patients with parasitic infections other than Schistosomiasis	30	3	10	0.37 ± 0.12
Kenya children without Schistosomiasis:				
Skin test positive	93	4	4	0.45 ± 0.20
Skin test negative	78	2	3	0.32 ± 0.10
New Guinea children and adults infected with W.bancrofti	50	2	4	0.35 ± 0.14

* Ninety-five per cent confidence interval.

Table 5.2

Results of ELISA tests with a S.haematobium worm antigen in different Schistosoma and other helminth infections

	Number tested	Number positive	Percentage positive	Mean E_{400} of positives *
St. Lucians infected with S.mansoni:				
Children	14	11	79	0.55 ± 0.16
Adults	36	22	61	0.61 ± 0.16
Gabon adults infected with S.intercalatum and/or S.haematobium	74	55	74	0.52 ± 0.10
U.K. cases of cercarial dermatitis	24	0	0	0
U.K. hospital patients with parasitic infections other than Schistosomiasis	30	3	10	0.48 ± 0.27
Kenya children without Schistosomiasis:				
Skin test positive	93	2	2	0.35 ± 0.10
Skin test negative	78	2	3	0.39 ± 0.10
New Guinea children and adults infected with W.bancrofti	50	8	16	0.39 ± 0.08

* Ninety-five per cent confidence interval.

Table 5.3

Results of ELISA tests with a S.haematobium cercarial antigen in different Schistosoma and other helminth infections

	Number tested	Number positive	Percentage positive	Mean E_{400} of positivies *
St. Lucians infected with S.mansoni:				
Children	14	8	57	0.36 ± 0.08
Adults	36	24	67	0.48 ± 0.08
Gabon adults infected with S.intercalatum and/or S.haematobium	74	63	85	0.55 ± 0.04
U.K. cases of cercarial dermatitis	24	6	25	0.42 ± 0.15
U.K. hospital patients with parasitic infections other than Schistosomiasis	28	20	71	0.50 ± 0.20
Kenya children without Schistosomiasis:				
Skin test positive	93	36	39	0.38 ± 0.04
Skin test negative	78	19	24	0.32 ± 0.10
New Guinea children and adults infected with W.bancrofti	50	12	24	0.31 ± 0.02

* Ninety-five per cent confidence interval.

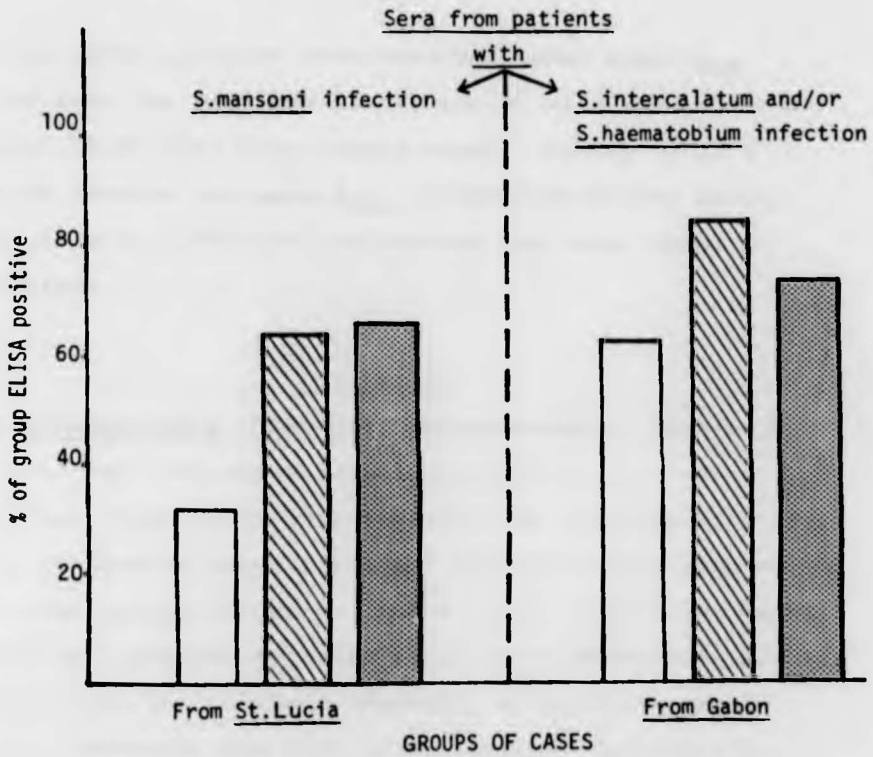


Fig.17 Diagram showing the percentage of group ELISA positive among patients with some other schistosome infection using different s.haematobium antigen preparations.

Soluble egg antigen

Adult worm antigen

Cercarial antigen

All the false positive reactions had lower mean E_{400} values than the true positives, except with the cercarial antigen where they were nearly equal. Figure 18 is a diagram showing the mean E_{400} of ELISA positives among patients with different schistosome and other helminth infections.

Discussion

In a S.haematobium infection, anti-cercarial, anti-worm and anti-egg (anti-miracidium) antibodies are produced. ELISA was found to be very sensitive in the detection of these antibodies using different homologous and heterologous schistosome antigens. ELISA also proved to be highly sensitive, compared to other serological methods (e.g. CIE, mentioned in the previous chapter), in detecting antibodies. However, the test lacks complete specificity. The false positive ELISA rates with sera from individuals with non-human schistosomes and with parasitic infections other than schistosomiasis were higher using cercarial antigens than when using worm or egg antigens. These results were in agreement with those reported by McLaren et al. (1978) using ELISA with egg antigen. On the other hand, Hillyer and De Rios (1979) reported low specificity of the ELISA with soluble egg antigen (SEA) of S.mansoni. They found extensive cross-reactivity when using serum from humans with fascioliasis, trichinosis, cysticercosis

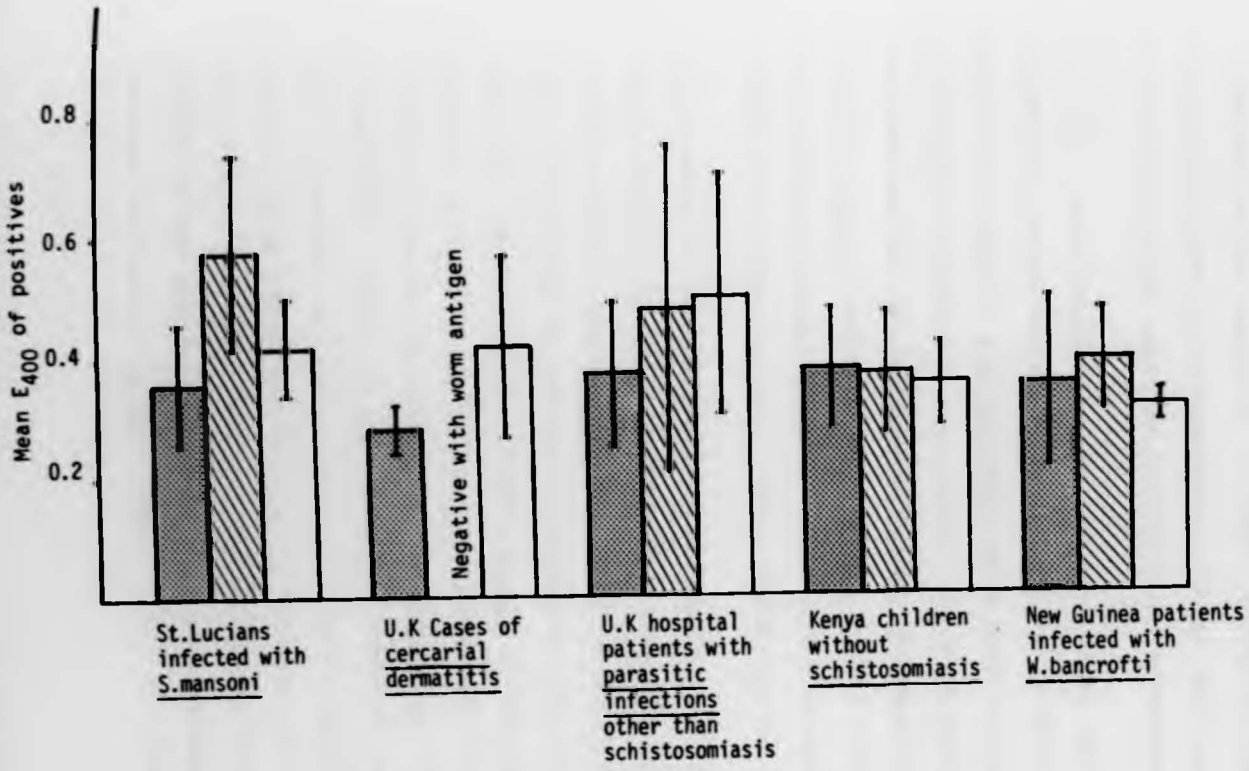


Fig.18 Diagrame showing the mean E_{400} of ELISA positives among patients with different schistasome and other helminth infections,using S.haematobium antigens

soluble egg antigen
Adult worm antigen
cercarial antigen

and echinococcosis. The false positive reaction could be explained by the presence of common antigens between schistosomes and other parasites. Their different method of conducting the test has already been mentioned.

Moreover, with crude antigens the test lacks species specificity which was demonstrated by the relatively high positivity rate of S.haematobium sera with different S.mansoni antigens and vice versa. This is expected as not only do the two species have many antigens in common (Capron et al., 1966, 1968; Warren, (1976), but the different stages of each species have shared antigenic components (Capron et al., 1965; Ramalho-pento et al., 1976).

Improvement of the sensitivity and specificity of the ELISA could, however, be achieved by purification of the antigens used. Egg antigen was found to be more reactive than either worm or cercarial antigens. This was in agreement with observations of Huldt et al. (1975) and McLaren et al. (1978) that egg antigen was more reactive than worm antigen in detecting antibodies to S.mansoni infection using ELISA. Umaly et al., (1974a), however, reported that cercarial antigen gave better results than worm antigen using other serological techniques (e.g. IHA and CF tests). The importance of the antigenic stimulus linked to the egg and its presence in the tissues has been recorded by Capron et al. (1966). This confirmed a previous

observation by Smithers (1960a) that schistosome egg extract, compared to worm and cercarial extracts, appeared to be the most antigenic in gel diffusion studies on S.mansoni.

The decrease of the mean E_{400} of ELISA positives, using egg antigen and the increase using worm or cercarial antigens with the age of patients might be related to the duration of active infection with egg production, rather than to the age of the patient. Hassan et al. (1979), using ELISA and other serological tests (IFA and IHA tests) with worm antigen found that antibodies detected in the sera of infected patients showed increasing values with the progress of the disease.

Using other serological tests, Jachowski and Anderson (1961) reported that the proportion of sera reacting in the COP test tended to decrease as the age of the patients increased while in the cercarial agglutination test, a high proportion of sera reacted regardless of the patient's age.

Although with some disadvantages mentioned above, ELISA has many advantages; minute quantities of serum and reagents are needed, large numbers of samples can be easily examined and fairly simple equipment is required. These advantages suggest that ELISA could be a convenient test for field use. However, for the ELISA to be more effective as a sero-epidemiological tool, further refinements of the antigens, using simple techniques, are needed.

Chapter V

THE USE OF THE ELISA FOR SEROLOGICAL MONITORING OF CHEMOTHERAPY AND ACQUIRED RESISTANCE IN BABOONS, HAMSTERS AND HUMANS

Introduction

Immunity (resistance to reinfection) to schistosome infections in experimental animals has been well reviewed by Stirewalt (1963), Smithers and Terry (1969a), Warren (1973), Kagan and Maddison (1975). Smith et al. (1976) reported that hamsters with a primary infection of S.haematobium or S.mansoni rapidly developed a high level of immunity to reinfection with either species of schistosome. Although some workers (Taylor et al., 1973) reported that the baboon develops a partial immunity to S.mansoni slowly, others (Webbe and James, 1973; Webbe et al., 1976) showed a high degree of acquired resistance to S.haematobium could develop in the baboon in a relatively short period.

Most attempts to transfer immunity passively from resistant to normal animals have been unsuccessful (Smithers and Terry, 1969a; Warren et al., 1972). There are conflicting opinions as regards the role of antibodies in the immune mechanisms. Although antibodies have been detected in the sera of immune monkeys and rodents which, in the presence of complement, destroy (Clegg and Smithers, 1972; Perez et al., 1974) and promote the opsonization by neutrophils

(Dean et al., 1974) of schistosomula cultured in vitro, some workers (Sher et al., 1974a; Perez et al., 1974) showed that these in vitro mechanisms may not be effective against a challenge infection in vivo. However, the passive transfer studies of Sher et al. (1975) have demonstrated that humoral factors play a major role in the effector mechanism of schistosome immunity. Therefore, there is a great need for highly sensitive assays not only to accurately measure antibodies, but also to be used for assessment of chemotherapeutic cure and, more importantly, for epidemiological surveys and for the evaluation of control measures.

According to the criteria adopted by a WHO Group (1966), a schistosomiasis patient is considered parasitologically cured when there is a complete disappearance of all eggs for at least six months after treatment in the absence of re-exposure. As reviewed in the first chapter of this thesis, none of the tests currently available, with the possible exception of the circumoval precipitin test (COPT) (Rifaat et al., 1969; Ruiz-Tiben et al., 1979), correlate with active infection and can thus not be used for the assessment of chemotherapeutic cure.

It was shown in Chapter IV that the ELISA is a sensitive and specific test, not only for the diagnosis of schistosome infections but also for the quantification of antibodies. Recently, Salih et al. (1978), reported the possible use of

ELISA for the assessment of chemotherapeutic cure in human S.mansoni infections. There are, however, no further studies on the application of the ELISA test for serological monitoring of resistance and the evaluation of chemotherapeutic cure in experimental animals and humans with schistosome infections.

The main objective of the experiments described in this chapter is to report on the ELISA results obtained from baboons and hamsters with different levels of infection, after treatment and reinfection with S.haematobium and/or S.mansoni. This chapter also deals with the application of ELISA for serological monitoring of chemotherapy in humans with S.haematobium or S.mansoni infections.

Methods, Experiments and Results

All the test sera mentioned in this chapter were examined using the ELISA technique against different S.haematobium antigens. The basic ELISA using the alkaline phosphatase system as described in the previous chapter was used for the detection of antibodies in sera from baboons mentioned in section B. Baboon sera were treated as human sera. All other test sera were examined using the ELISA-peroxidase system. Peroxidase enzyme was found to have high activity and is cheaper than alkaline phosphatase, and yields a visible (brown) reaction product (Voller et al., 1976 b). All test sera were used at a dilution of 1:300 and the

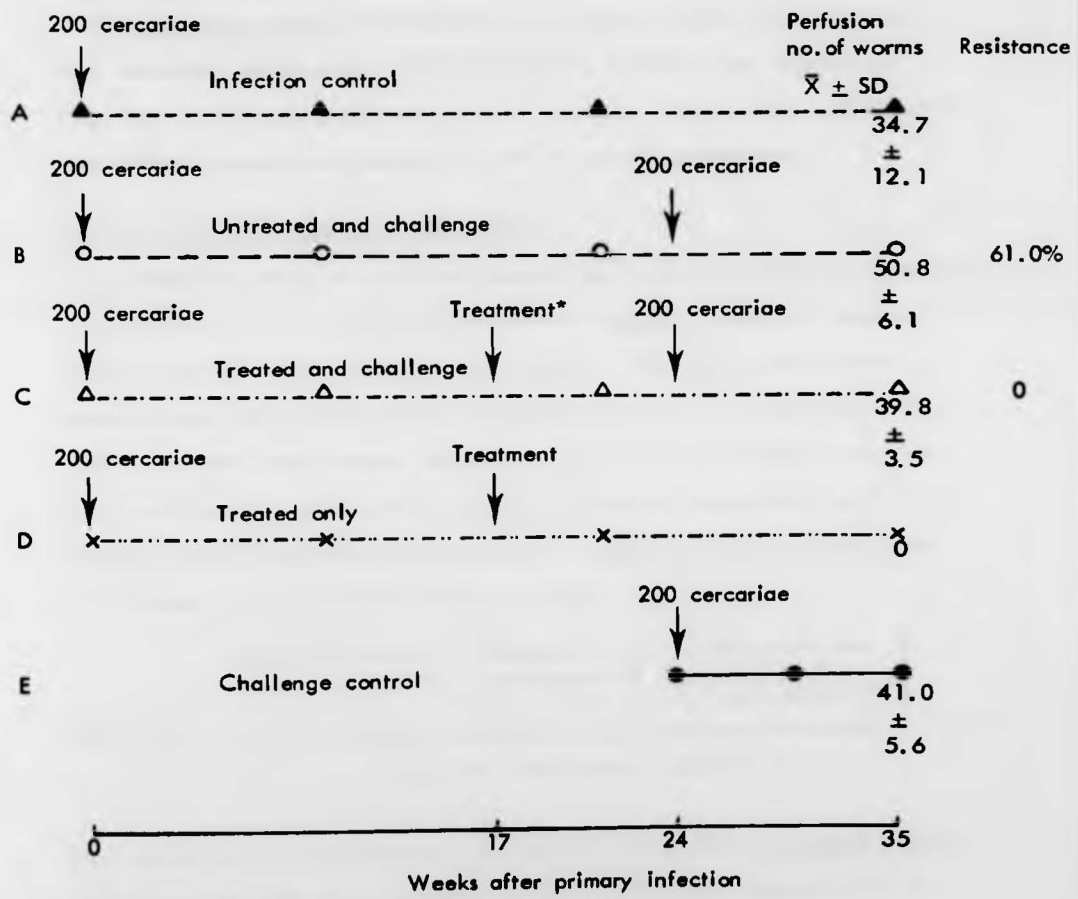
peroxidase-labelled anti-human IgG and anti-hamster conjugates (Miles) were each used at a dilution of 1:1000. A mixture of ortho phenylene diamine (O-PD) and hydrogen peroxide (H_2O_2))0.01% O-PD and 0.003% H_2O_2 in distilled water) was used as substrate, and 8N sulphuric acid as inhibitor. The amount of bound antibody was assayed photometrically at 492 nM. Sera were judged positive if E_{492} was more than 0.25 for human and baboon sera and 0.1 for hamster sera. Positive and negative hamster reference sera were from pooled sera collected from S.haematobium infected or clean (non-infected) hamsters respectively.

Section A

Two experiments were designed to study the effect of treatment on resistance to reinfection with S.haematobium in hamsters and the measurement of antibody levels by the ELISA technique. The hamster was used as an experimental model because it is readily available as a host for human schistosomes (Erickson et al., 1974). In experiment one, groups of 5 hamsters were infected with S.haematobium, treated with Niridazole (Ambilhar, Ciba) at 17 weeks, when the worms were mature, reinfected at 24 weeks as shown in Fig.19, and then perfused at 35 weeks. Sera were collected (cf. Chapter II) every two weeks and kept at -20°C until examined. The second experiment was designed to find out whether hamsters in which a S.haematobium infection was

Figure 19

Schedule of infection, treatment and reinfection of groups of 5 hamsters



* Niridazole 200 mg/Kg body weight, daily for 5 days, given orally by syringe with a curved blunt needle

terminated by Niridazole would be resistant to an immediate challenge infection. Groups of 5 hamsters were infected with S.haematobium, treated at 6½ weeks before the worms had reached maturity, reinfected at 7 weeks as shown in Fig.20, and then perfused at 21 weeks. Sera were collected every two weeks and kept at -20°C until examined.

Calculation of Immunity Results

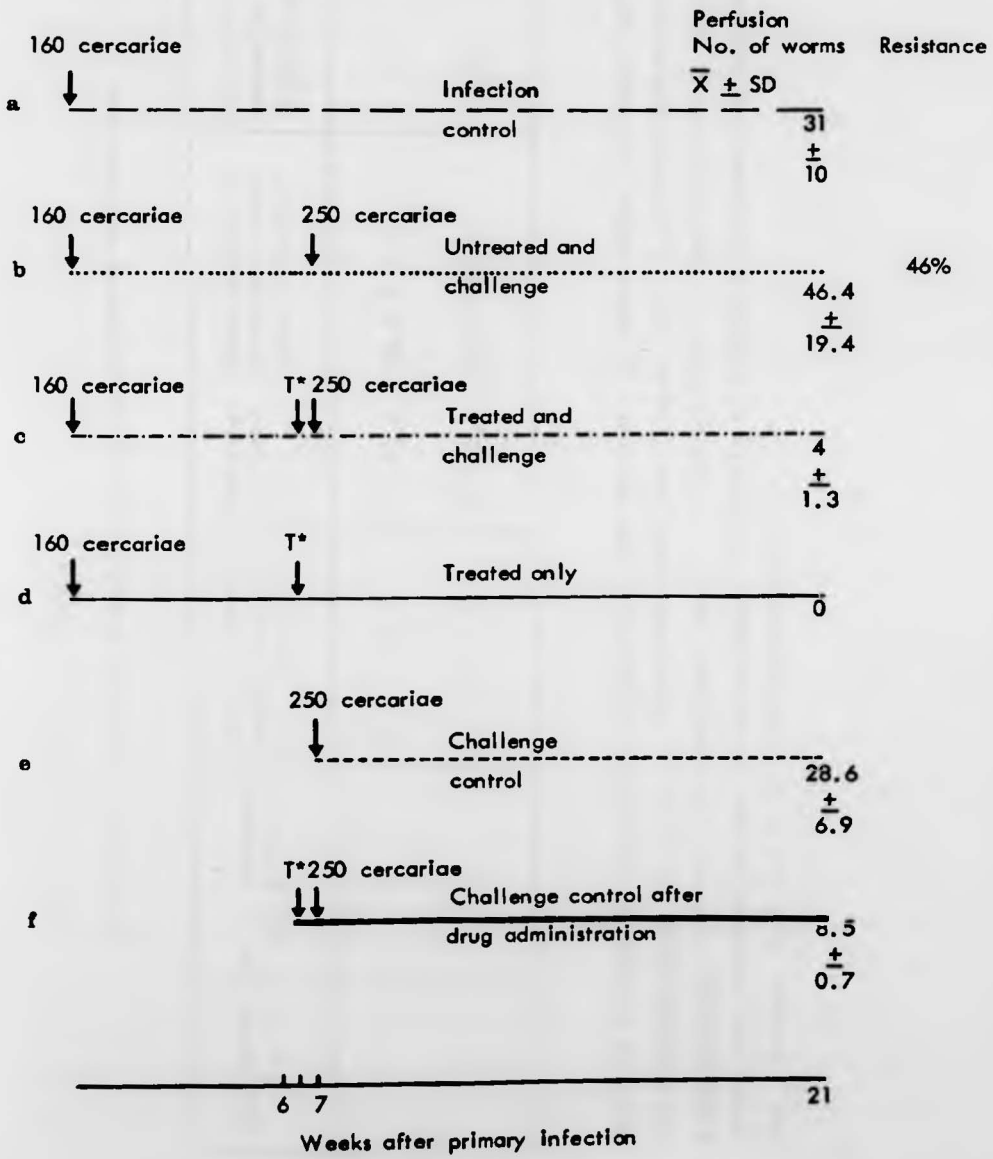
The immunity was calculated based on results from the portal perfusion assay described by Smith et al. (1976). Briefly, three groups of hamsters were used: one with the primary immunizing infection alone (primary infection control, P); one with the challenge infection alone (challenge control, C); and an experimental group (E) which received both the primary and challenge infection. Immunity was calculated according to the following equation:

$$\text{Immunity}_{\%} = \frac{\text{Mean of challenge control C} - \left[\text{Mean of experimental E} - \text{Mean of primary infection control P}\right]}{\text{Mean of challenge control C}} \times 100$$

The results of resistance to reinfection with S.haematobium obtained in the two hamster experiments are summarized in Table 6 and also illustrated in Figs.19 and 20. The hamsters reinfected 24 weeks after the primary infection showed a high degree of resistance (61%), while only 46% of resistance was obtained in hamsters reinfected 7 weeks after the primary

Figure 20

Schedule of infection, treatment and reinfection of groups of 5 hamsters



* Treatment, Niridazole 200 mg/kg body weight, daily for 5 days, given orally by syringe with a curved blunt needle

Table 6

Immunity by perfusion assay in the two experiments of hamsters

Experiment No.	No.of hamsters per group	Primary immunization control P	Challenge control C	Experimental E, primary & challenge infection	Per cent immunity $\frac{C-(E-P)}{C} \times 100$	Value of P
1	5	34.7 ± 12.1	41.0±5.6	50.8 ± 6.1	61	< 0.05
2	5	31.0 ± 10.0	28.6±6.9	46.4 ±19.4	46	< 0.05

Immunity assayed as described in Materials and Methods, 24 weeks after a primary infection with 200 S.haematobium cercariae in experiment No.1 and 7 weeks after a primary infection with 160 S.haematobium cercariae in experiment No.2. P values refer to the statistical significance of resistance obtained in the two experiments (analysis of variance has been carried out between the 3 groups, P, C and E in each experiment).

infection. In experiment No.1, chemotherapeutic eradication of the adult worms 17 weeks after the primary infection completely abolished all resistance 7 weeks later (group C). In experiment No.2, reinfection of hamsters was carried out immediately after the treatment as shown in Fig.20. Comparing groups c and f, it was found that Niridazole had a residual effect in the circulation and affected the challenge infection of the control group f. It was therefore not possible to assess the effect of treatment on resistance to reinfection of this group of hamsters. However, to confirm this observation, groups e and f of experiment No.2, were repeated on additional hamsters. The results are shown in Table 7. Niridazole greatly reduced the recovery of worms from an infection given to hamsters immediately after the administration of the drug.

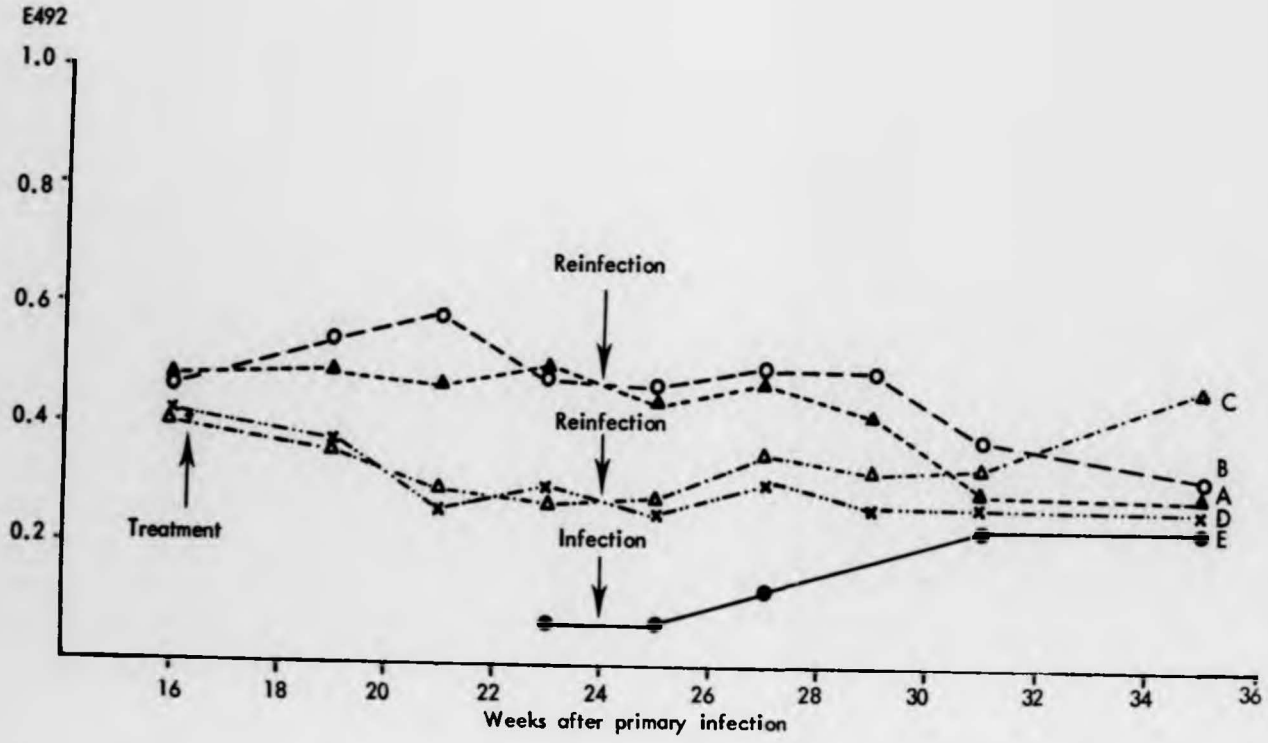
Figures 21.1, 21.2 and 21.3 show the antibody levels of hamsters in experiment No.1, as measured by the ELISA test, using three different S.haematobium antigens. The antibody levels declined in response to treatment in groups C and D and this was highly significant by the fifth week with all the three antigens ($p < 0.05$ with worm antigen, < 0.02 with cercarial antigen and < 0.001 with egg antigen), in contrast to the high antibody levels maintained in the untreated groups A and B. Two weeks later, the hamsters in groups B and C were reinfected together with a control group E. The

Table 7

The worm burden of a group of hamsters infected with S.haematobium, immediately after the administration of Niridazole, and an infected control group (group 2)

Group No.	No. of hamsters per group	Drug before infection	Infection no. of cercariae	Perfusion no. of worms $\bar{x} \pm S D$
1	5	Niridazole *	200	2.6 ± 1.5
2	5	-	200	28.8 ± 16.5

* 200 mg/kg body weight, orally for 5 days


E492
1.0
0.8
0.6
0.4
0.2
Reinfection
Reinfection
Infection
Treatment
C
B
A
D
E
16
18
20
22
24
26
28
30
32
34
36
Weeks after primary infection

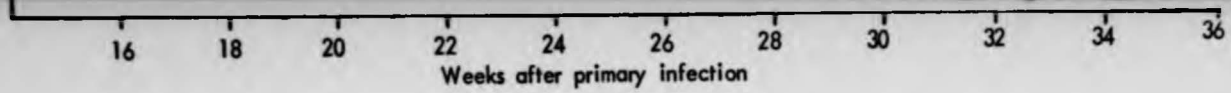


Figure 21.1

Diagram showing the antibody levels, using S.haematobium cercarial antigen in different groups of hamsters

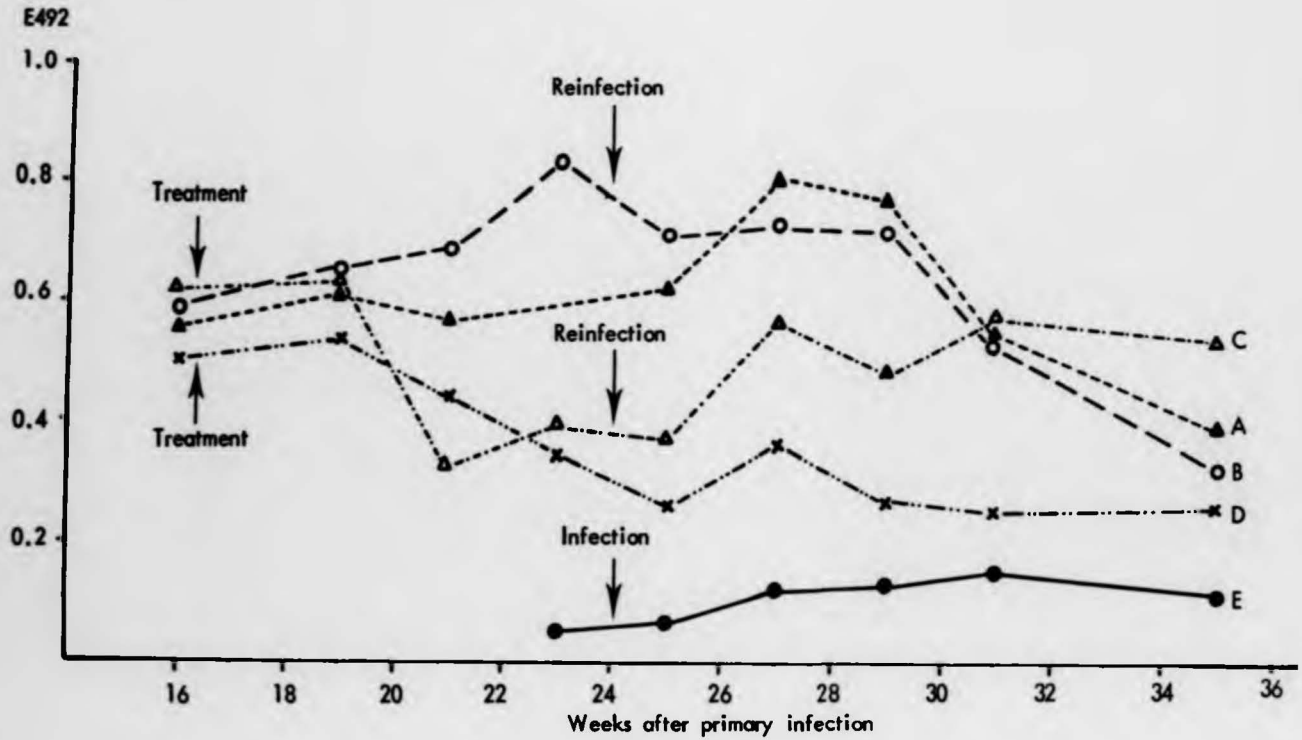
A, infection control group

B, untreated and challenge group

C, treated and challenge group

D, treated only group

E, challenge control group


E492
1.0
0.8
0.6
0.4
0.2
Reinfection
Treatment
Reinfection
Treatment
Infection
A
B
C
D
E
16
18
20
22
24
26
28
30
32
34
36
Weeks after primary infection

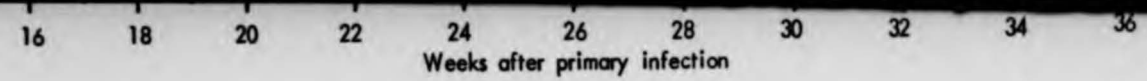


Figure 21.2

Diagram showing the antibody levels, using S.haematobium adult worm antigen in different groups of hamsters

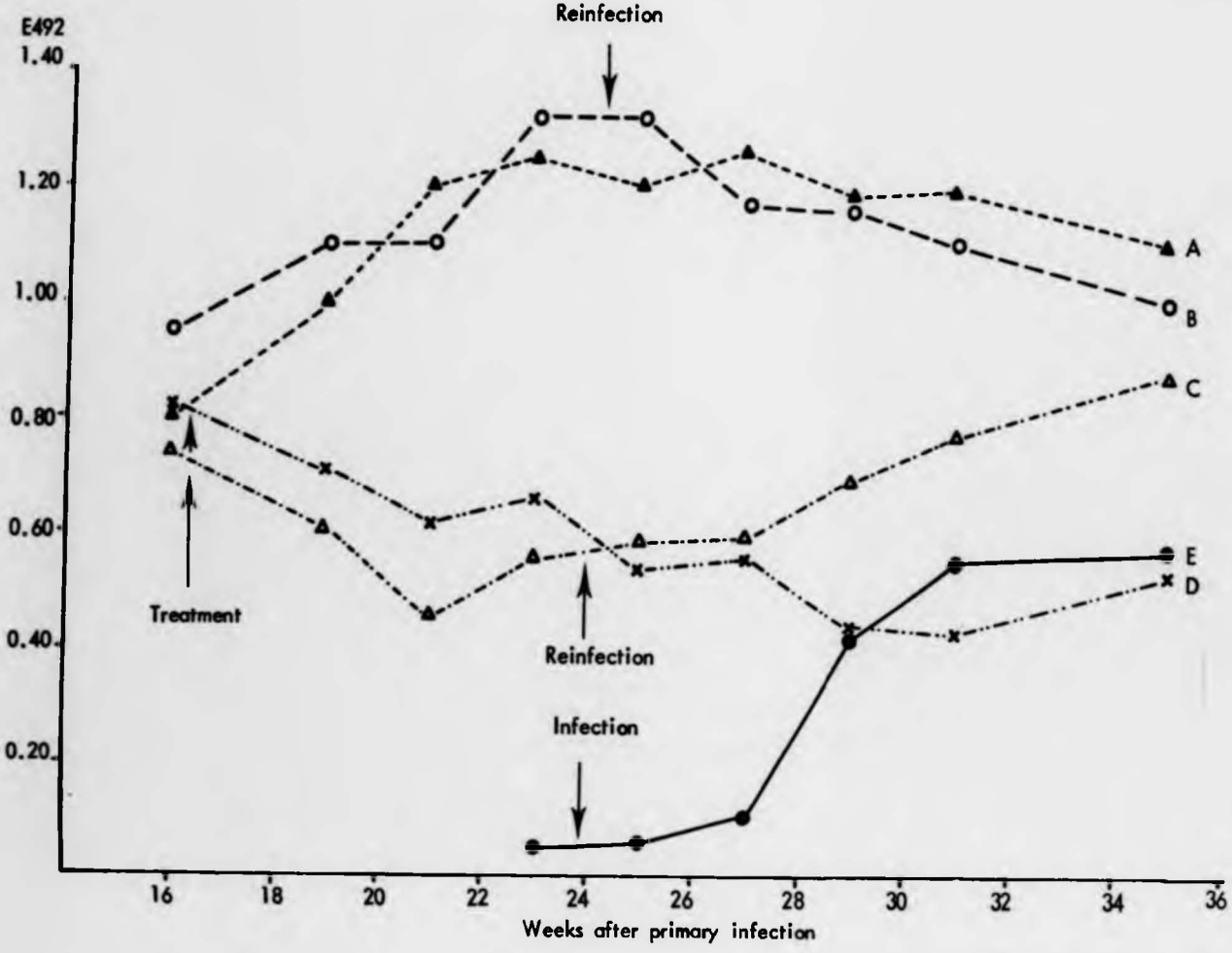
A, infection control group

B, untreated and challenge group

C, treated and challenge group

D, treated only group

E, challenge control group


E492
1.40
1.20
1.00
0.80
0.60
0.40
0.20
Reinfection
Treatment
Reinfection
Infection
A
B
C
D
E
16
18
20
22
24
26
28
30
32
34
36
Weeks after primary infection

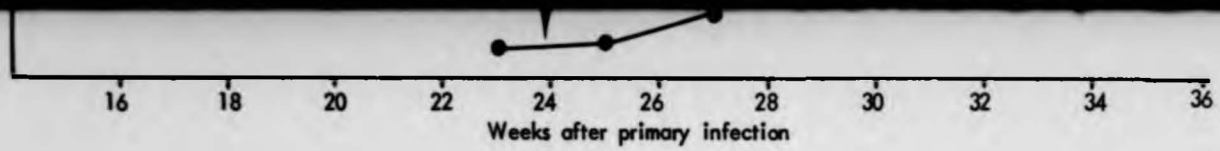


Figure 21.3

Diagram showing the antibody levels, using S.haematobium egg antigen in different groups of hamsters

A, infection control group

B, untreated and challenge group

C, treated and challenge group

D, treated only group

E, challenge control group

antibody level rose in response to reinfection in the treated group C, but continued to decline in the untreated group B. This was more apparent with worm and cercarial antigens than with egg antigen.

The measurements of antibody levels, using the ELISA test with the three different S.haematobium antigens in experiment No.2, are shown in Figs. 22.1, 22.2 and 22.3. The results confirm the observations shown in experiment No.1. The antibody levels showed a significant drop in the treated group d with all antigens, in contrast to the high antibody levels maintained in the untreated group a ($p < 0.01$ with worm antigen and < 0.001 with both egg and cercarial antigens). The antibody level rose after reinfection in the treated group c and also in the untreated group b; whereas it showed a decline in group d which was not reinfected.

In both experiments, egg antigen proved to be more reactive than either worm or cercarial antigens, confirming previous observations on human sera (cf. Chapter IV).

Section B

ELISA for serological monitoring of resistance to reinfection of baboons with S.haematobium or S.mansoni.

The results in this section have been published in a paper entitled "Cross resistance between Schistosoma haematobium and S.mansoni in the baboon" (Webbe et al., 1979).

Figure 22.1

Diagram showing the antibody levels, using S.haematobium cercarial antigen in different groups of hamsters

a, infection control group

b, untreated and challenge group

c, treated and challenge group

d, treated only group

e, challenge control group

f, challenge control group after administration of the drug.

I_1, infection with 200 S.haematobium cercariae

I_2, reinfection with 200 S.haematobium cercariae

T, treatment with Niridazole 200 mg/kg body weight orally, daily for 5 days.

matobium

sters

of the

ht orally,

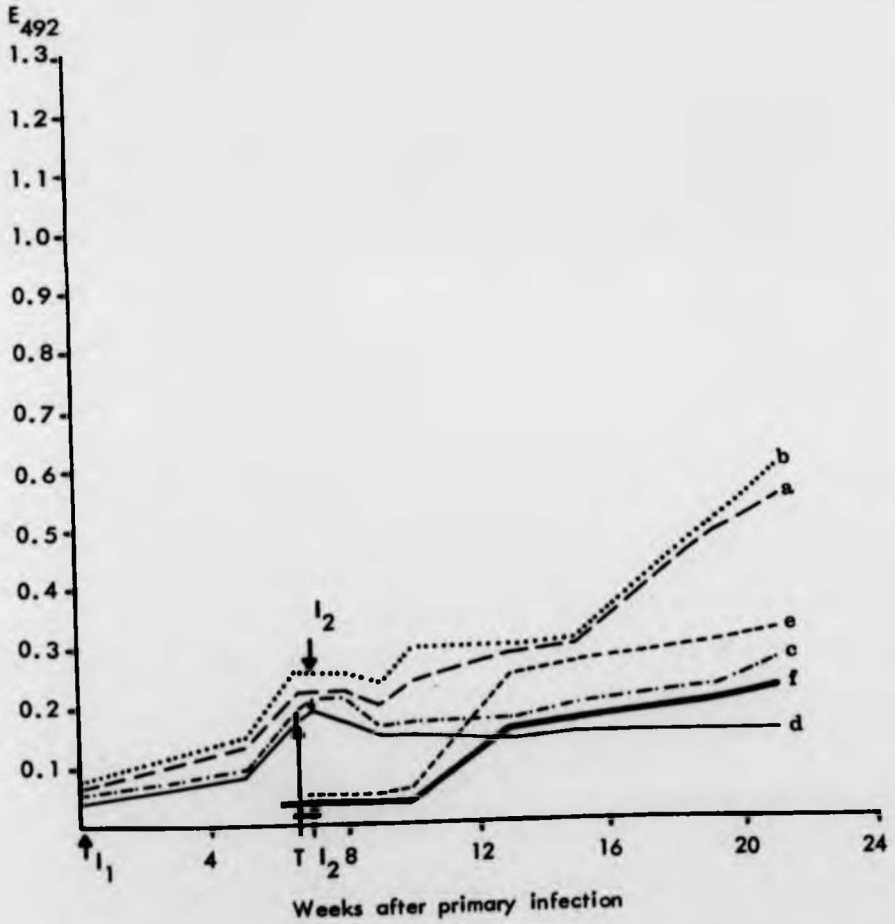


Figure 22.2

Diagram showing the antibody levels, using S.haematobium adult worm antigen in different groups of hamsters

a, infection control group

b, untreated and challenge group

c, treated and challenge group

d, treated only group

e, challenge control group

f, challenge control group after administration of the drug.

I_1, infection with 200 S.haematobium cercariae

I_2, reinfection with 200 S.haematobium cercariae

T, treatment with Niridazole 200 mg/kg body weight orally, daily for 5 days.

tobium

ters

the

t orally,

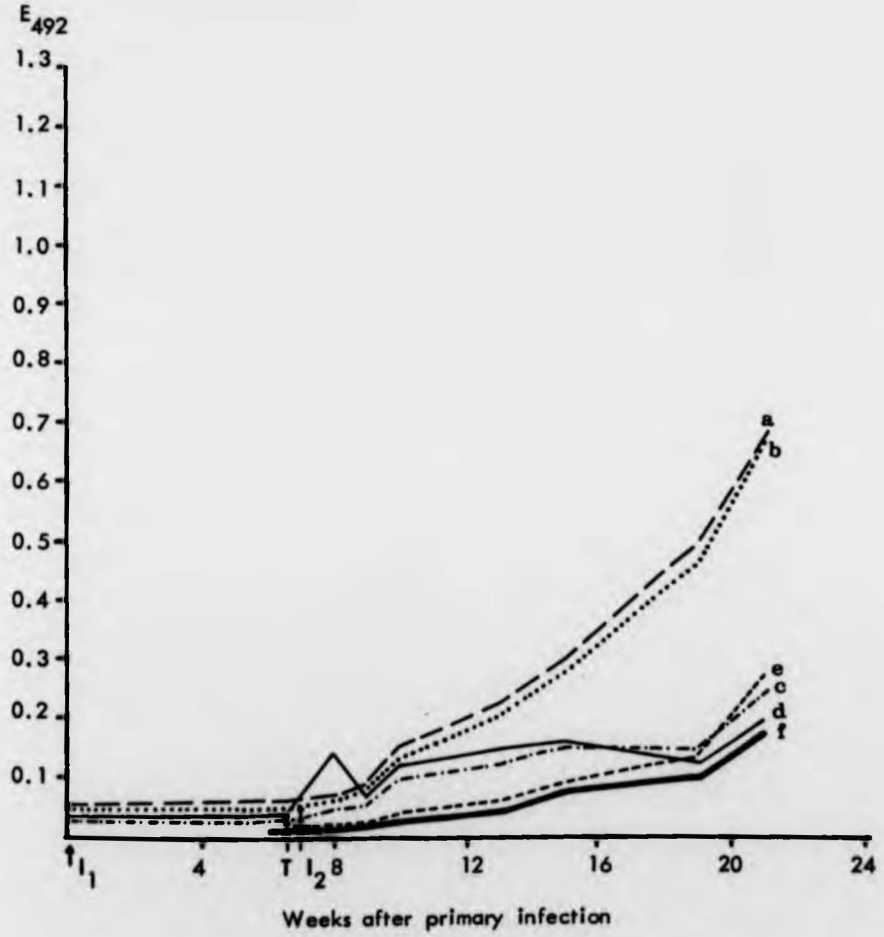


Figure 22.3

Diagram showing the antibody levels, using S.haematobium egg antigen in different groups of hamsters

a, infection control group

b, untreated and challenge group

c, treated and challenge group

d, treated only group

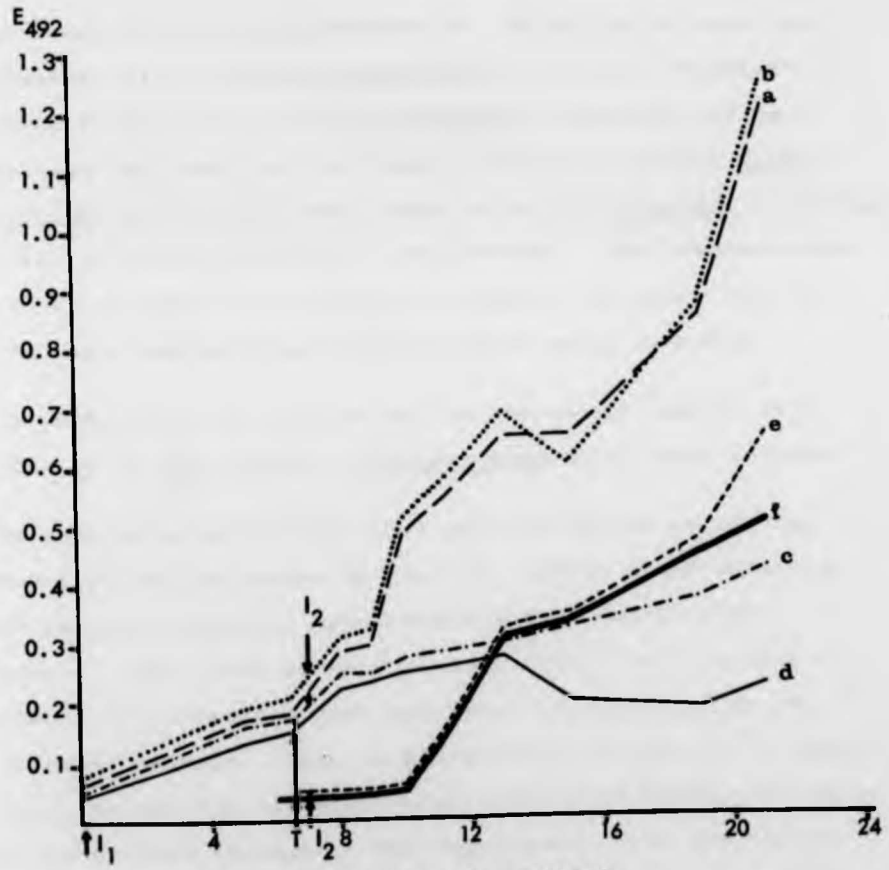
e, challenge control group

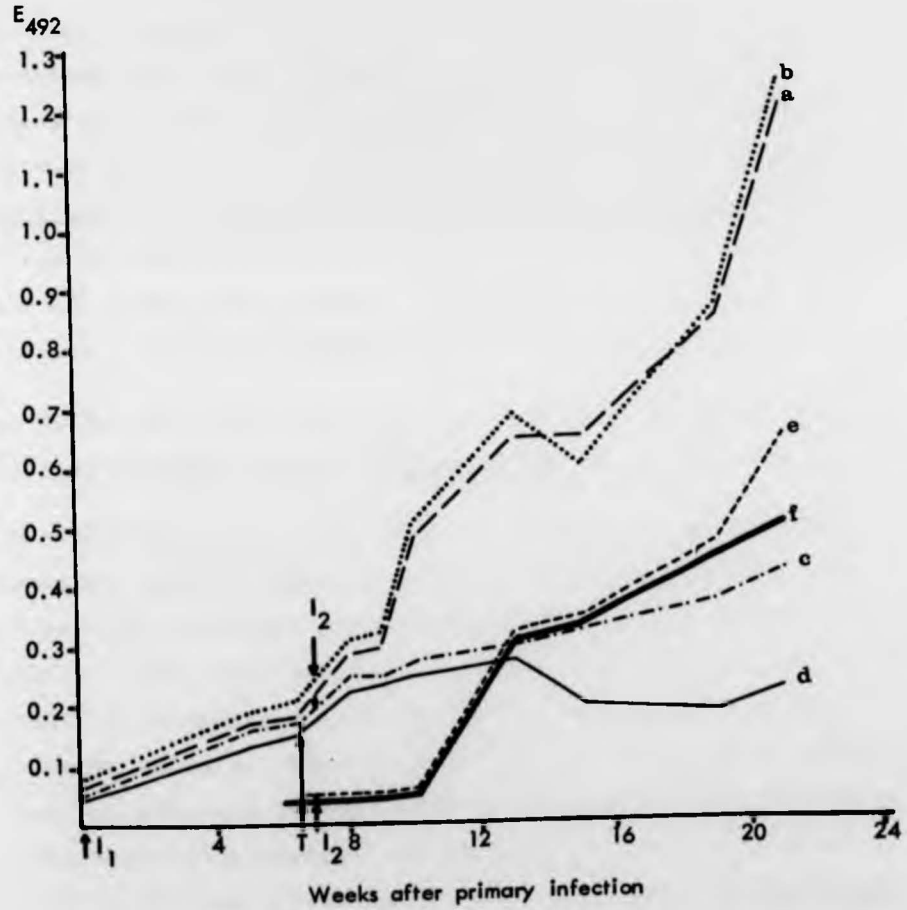
f, challenge control group after administration of the drug.

I_1, infection with 200 S.haematobium cercariae

I_2, reinfection with 200 S.haematobium cercariae

T, treatment with Niridazole 200 mg/kg body weight, orally, daily for 5 days.


E492
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
b
a
e
f
c
d
I2
I1
4
T
I2
8
12
16
20
24
Weeks after primary infection

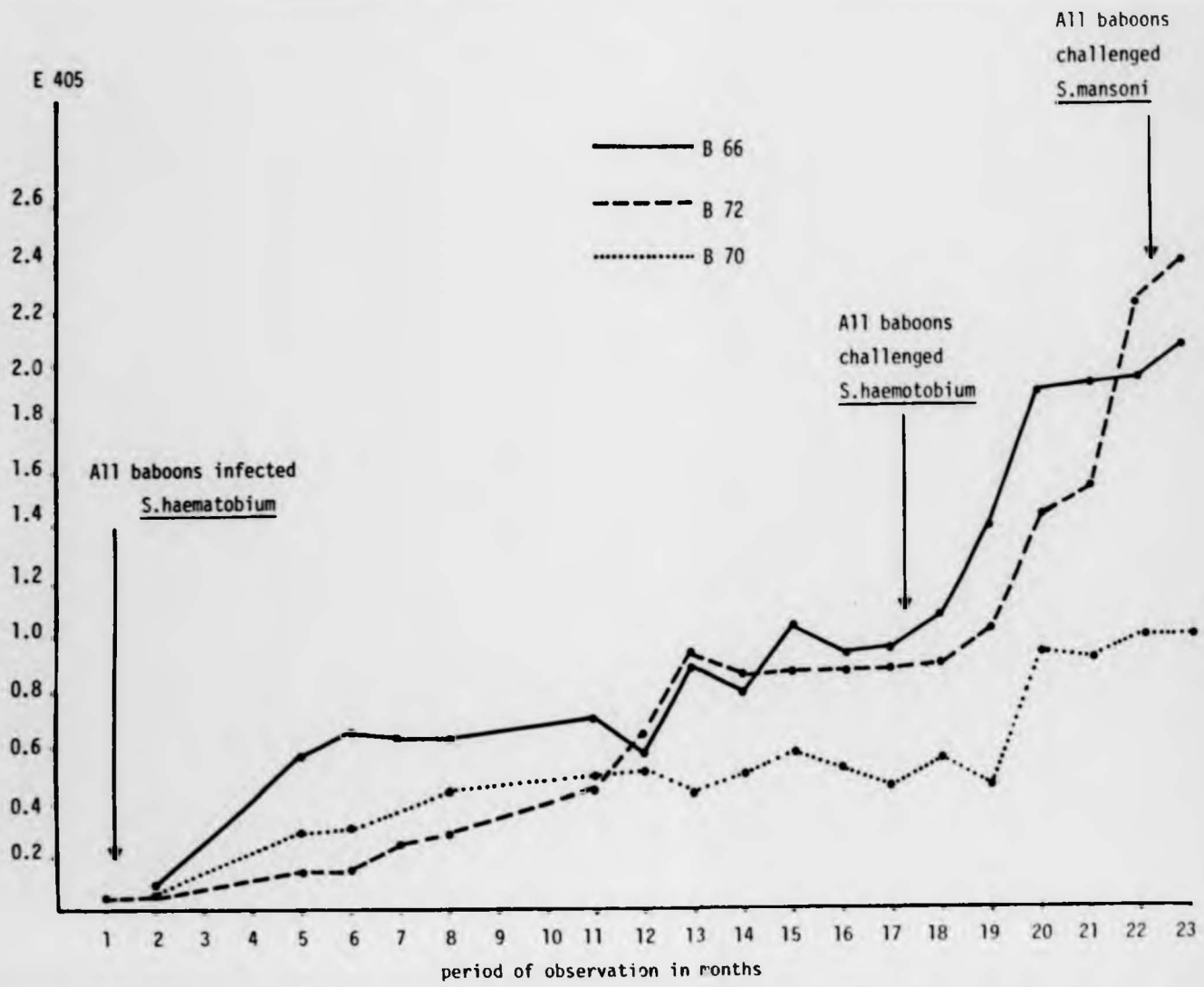

E492
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
b
a
e
f
c
d
I2
I1
4
T
I2
8
12
16
20
24
Weeks after primary infection

The experiments were carried out to determine whether infection of the baboon with S.haematobium could stimulate significant acquired immunity to S.mansoni.

The study included six baboons (4 - 6 kg) which were each immunized with 1,200 S.haematobium cercariae, "boosted" 15 months later with 1,000 S.haematobium cercariae per kg., (to test the level of resistance developed against S.haematobium) and finally challenged with 250 S.mansoni cercariae per kg 20 months after the immunization. The baboons were bled on average once monthly throughout the study and the sera were separated and kept at -20°C until examined.

The ELISA test was carried out on the serum samples at a dilution of $\frac{1}{300}$, using a S.haematobium adult worm antigen.

The results obtained show that the antibodies in all the immunized baboons began to rise two months after infection and reached a plateau approximately five months after patency. The level of this plateau could, to a certain extent, be correlated with the level of infection in the different animals. This is illustrated in Fig. 23.1, which gives the antibody levels, as measured with ELISA, for three of the baboons throughout the experiment. For one baboon (No.70) which had a low egg output and a final S.haematobium infection rate of only 5.1% of cercariae inoculated, the ELISA values remained below 0.6 and did not increase more


E 405
B 66
B 72
B 70
All baboons challenged S.mansoni
All baboons challenged S.haemotobium
All baboons infected S.haematobium
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
period of observation in months

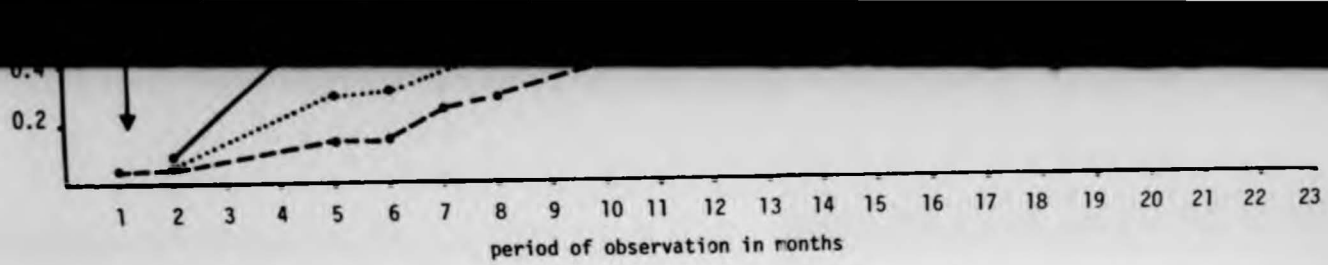


Figure 23.1

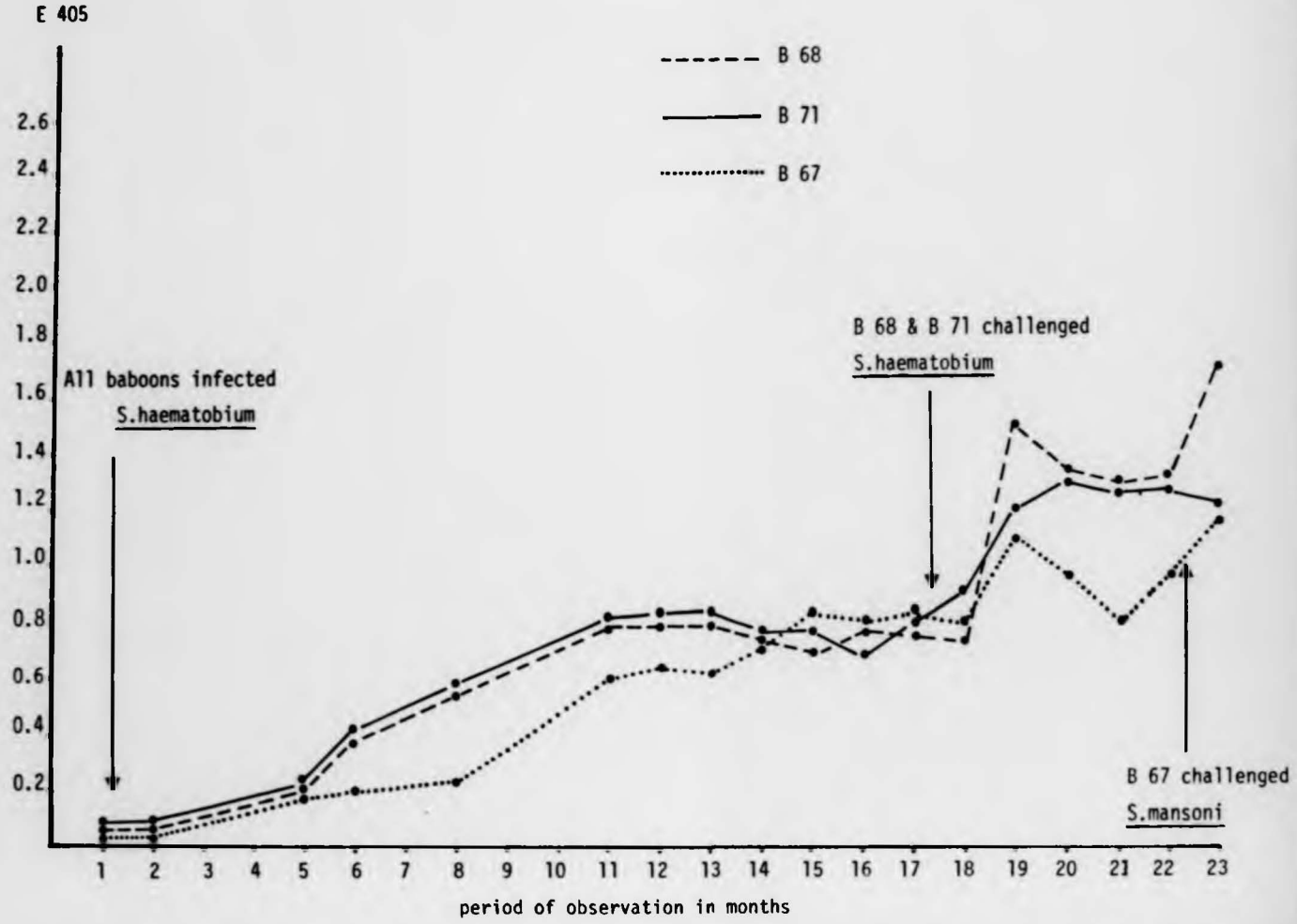
Diagram showing the antibody levels, using S.haematobium adult worm antigen in different baboons

than 1.0 even after the booster infection. For the two other baboons (Nos.66 and 72), the initial ELISA values were between 0.8 and 1.0, correlating well with the high worm burden and the initially high egg output. Figure 23.2 shows the ELISA values for the three other baboons of which one (No.67) did not receive the S.haematobium booster. The rise in the ELISA values occuring two months after the "booster" infection was largest in baboons Nos. 66 and 72 and smallest in baboons Nos.70 and 67.

Section C

ELISA for serological monitoring of chemotherapy in hamsters, baboons and humans.

Three groups of ten hamsters each were infected with 200 S.haematobium cercariae. The schedule of infection and treatment of these hamsters appears in Fig.24. The hamsters were bled on average once every two weeks and the sera were kept at -20°C until examined. At the end of the experiment, the hamsters were killed, the abdomen was opened and the whole left kidney was removed for electron microscopic and immunofluorescence examinations. The hepatic portal system was perfused and the adult worms thus recovered were counted. After perfusion, the whole of the right kidney, as well as samples of other tissues - liver, lung, intestine and urinary bladder - were removed for light microscopic examinations. The techniques dealing with these tissues and the


E 405
B 68
B 71
B 67
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
All baboons infected
S.haematobium
B 68 & B 71 challenged
S.haematobium
B 67 challenged
S.mansoni
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
period of observation in months

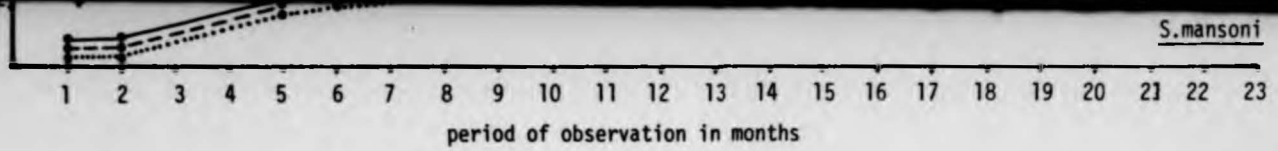
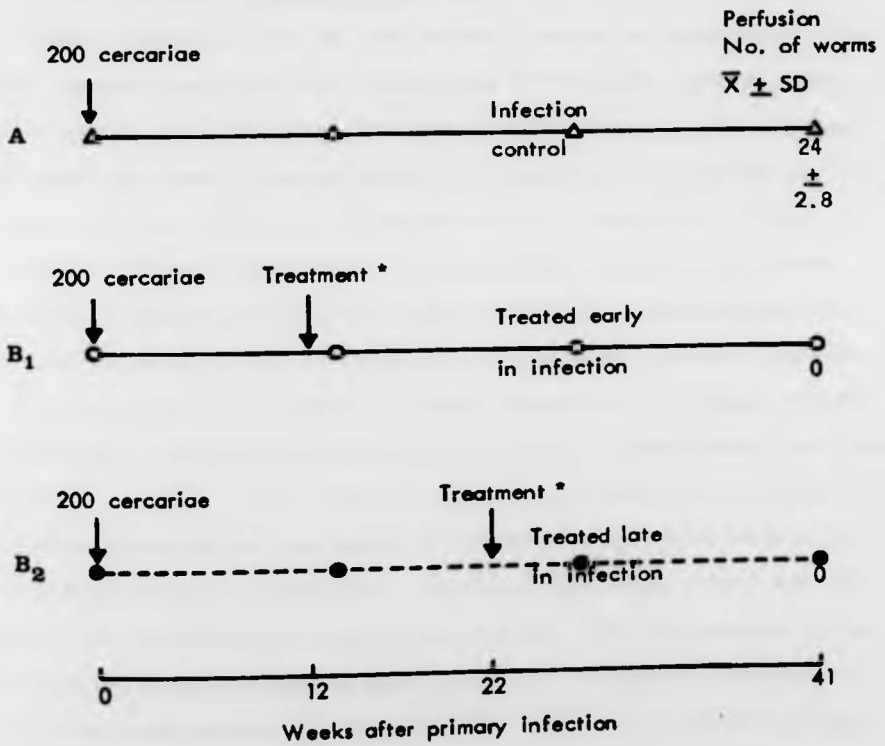


Figure 23.2

Diagram showing the antibody levels, using S.haematobium adult worm antigen in different baboons

Figure 24

Schedule of infection and treatment of groups of 10 hamsters

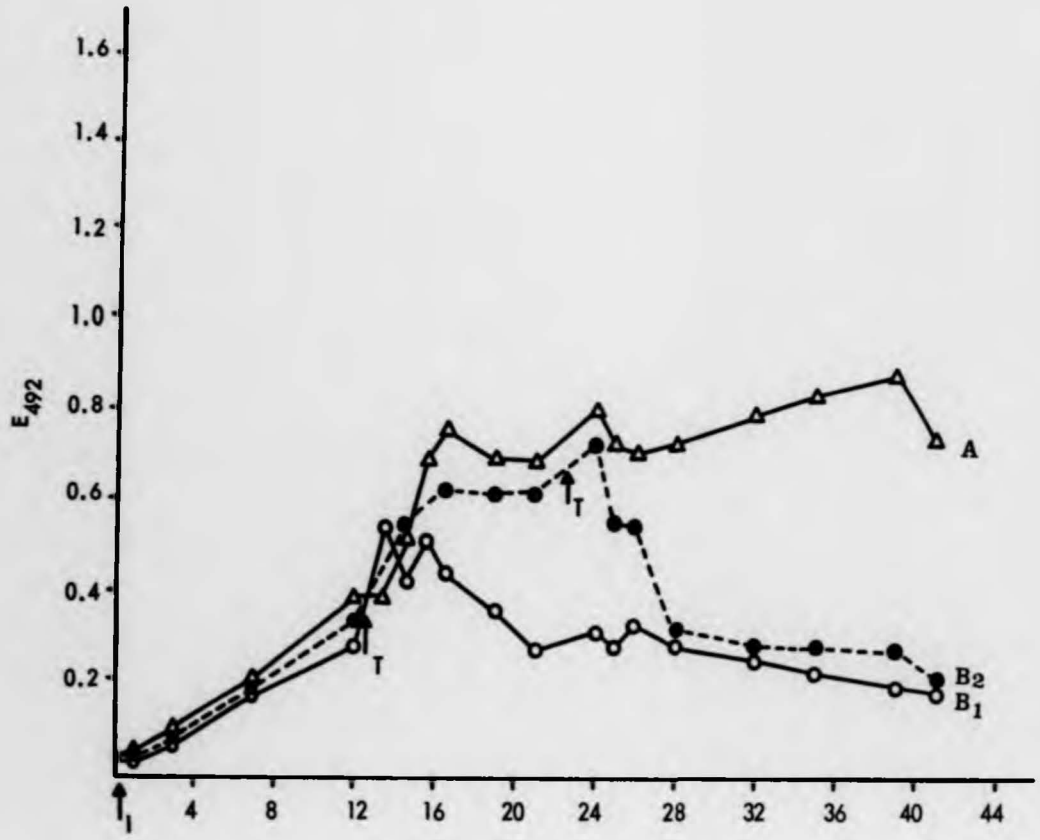


* Niridazole 200 mg/kg body weight, daily for 5 days, given orally by syringe with a curved blunt needle

results of their examinations are described in detail in Chapter VIII.

Figures 25.1 and 25.2 show the antibody levels, measured by ELISA, using S.haematobium adult worm and soluble egg antigens respectively in the three groups of hamsters. The sera became positive 4-8 weeks and 7-12 weeks after infection using egg and worm antigens respectively. Two groups of hamsters were treated with Niridazole; one group at 12 weeks and the other at 22 weeks after infection. After an initial insignificant rise, the antibody levels declined in both treated groups and this was highly significant by the sixth week after treatment, confirming previous observations mentioned earlier in this chapter, ($p < 0.02$ and < 0.001, using worm and egg antigens respectively in both treated groups). The figures also show that the decline of antibody levels in the group of hamsters treated later in infection occured abruptly, reaching the same level as the other treated group in only six weeks. The untreated group of hamsters maintained a high level of antibody throughout the forty-one weeks of observation, ($p < 0.001$ using both antigens, compared to the treated groups).

The serum from four baboons infected with 7,000 S.haematobium cercariae was used. Three of the baboons were treated and the fourth served as an untreated control.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
E_{492}
A
B_2
B_1
T
T
T_1
4
8
12
16
20
24
28
32
36
40
44
Weeks after primary infection

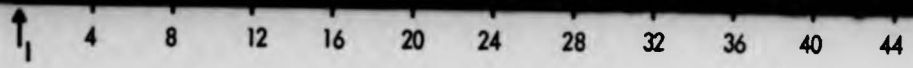


Figure 25.1

Diagram showing the antibody levels, using S.haematobium adult worm antigen in different groups of hamsters

A, infection control group

B_1, treated group, treatment started 12 weeks after infection

B_2, treated group, treatment started 22 weeks after infection

I, infection with 200 S.haematobium cercariae

T, treatment with Niridazole 200 mg/kg body weight orally, daily for 5 days

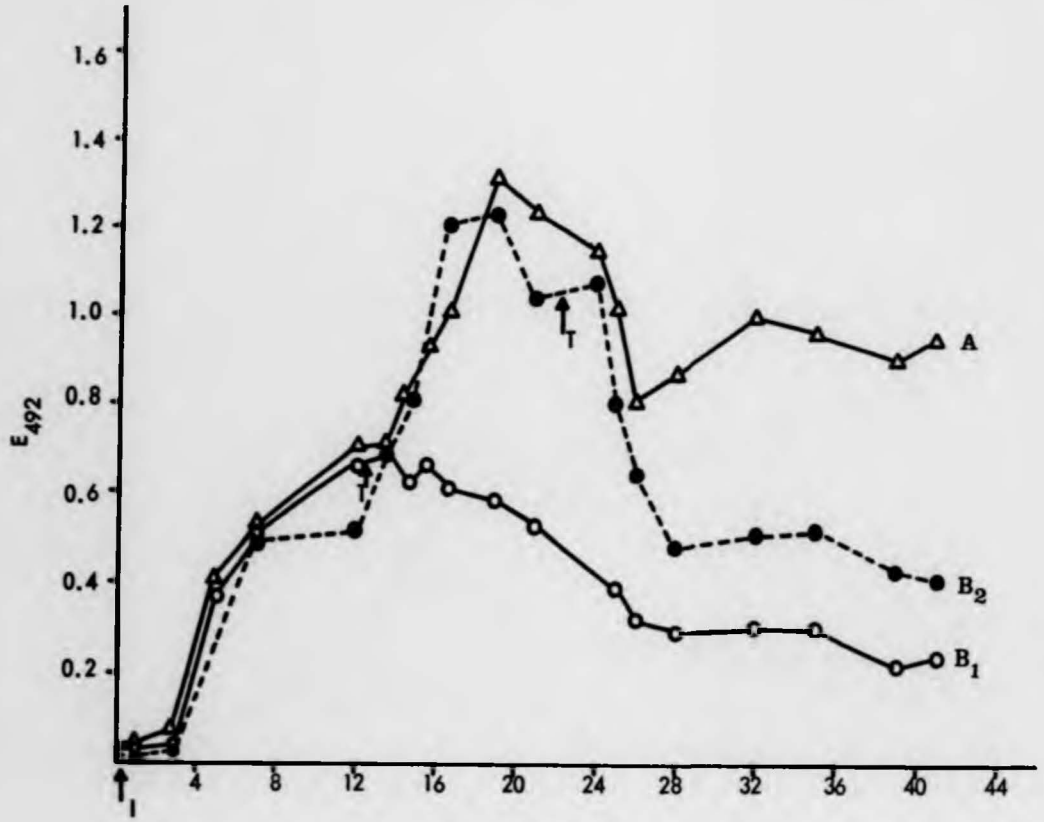

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
E_{492}
4
8
12
16
20
24
28
32
36
40
44
I
T
T
A
B_2
B_1
Weeks after primary infection

Figure 25.2

Diagram showing the antibody levels, using S.haematobium egg antigen in different groups of hamsters

A, infection control group

B_1, treated group, treatment started 12 weeks after infection

B_2, treated group, treatment started 22 weeks after infection

I, infection with 200 S.haematobium cercariae

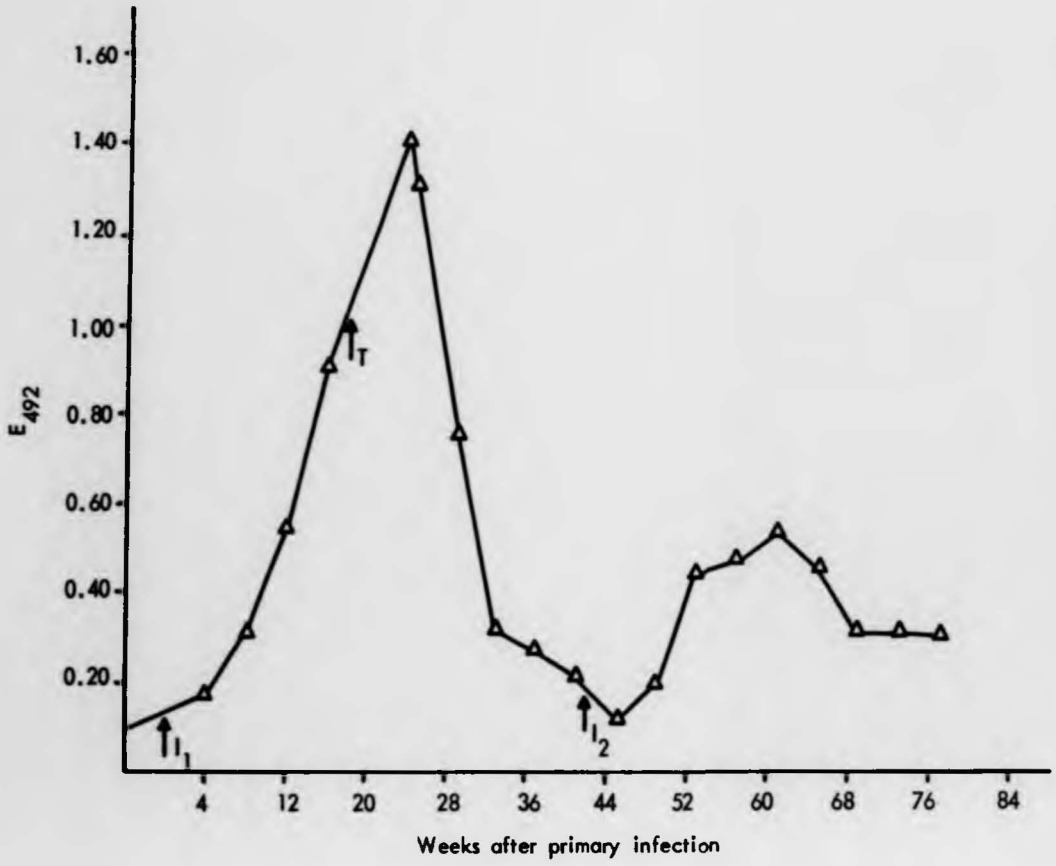
T, treatment with Niridazole 200 mg/kg body weight, orally, daily for 5 days.

The sera became positive 4-7 weeks after infection, confirming previous observations.

The baboon shown in Fig. 26.1 was treated with a dinitrothiophane derivative, (Hoffman-La-Roche Limited, Basel), 18 weeks after infection when the antibody levels were high. During the first two weeks after treatment, antibody levels rose to an unusually high peak, but thereafter declined, reaching almost background levels 22 weeks after treatment. At this time the baboon was reinfected and the antibody levels rose again, 4-6 weeks after reinfection, but failed to reach those of the primary infection. In contrast, the untreated baboon (Fig. 26.2) maintained high antibody levels throughout the 80 weeks of observation. The changes in the antibody levels, after treatment of the other two baboons, are shown in Figs. 26.3 and 26.4.

The baboon in Fig. 26.3 had only a low level of infection and was completely cured of infection by Niridazole. The antibody levels therefore - after the usual post-treatment rise - declined abruptly to almost pre-infection levels.

The baboon in Fig. 26.4 was treated with another of Hoffman-La-Roche's compounds (RO11-3128) but the first dosage did not have much effect on the infection as judged by egg output and the baboon was therefore retreated. The antibody level only declined temporarily after the first treatment and only the second more curative treatment had


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
E_{492}
I_1
I_T
I_2
4
12
20
28
36
44
52
60
68
76
84
Weeks after primary infection

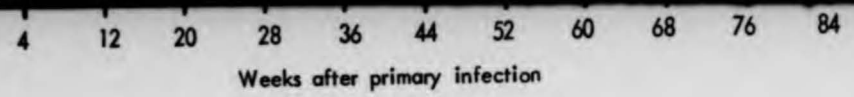


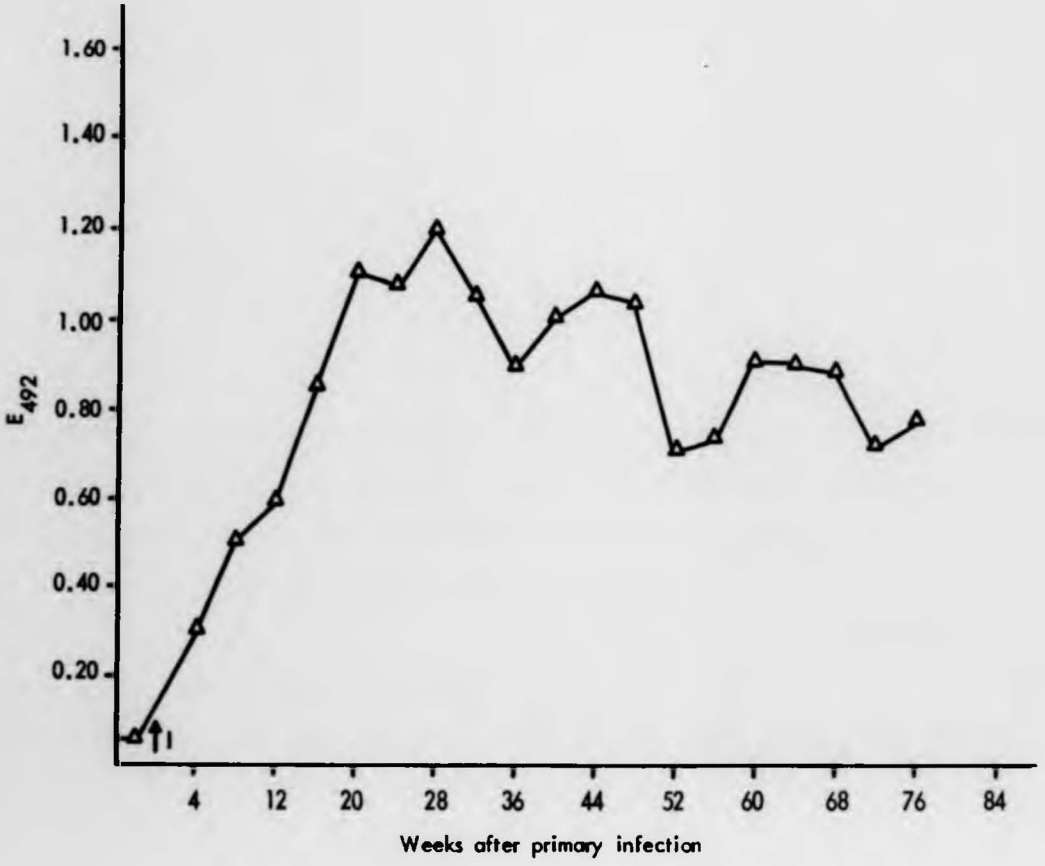
Figure 26.1

Diagram showing the antibody levels, using S.haematobium adult worm antigen in a treated and reinfected baboon

I_1, infection with 7,000 S.haematobium cercariae

I_2, reinfection with 7,500 S.haematobium cercariae

T, treatment with a dinitro-thiophane derivative (RO11-0761) Hoffman La Roche) 5 x 125 mg/kg body weight, orally by stomach tube.


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
E_{492}
I
4
12
20
28
36
44
52
60
68
76
84
Weeks after primary infection

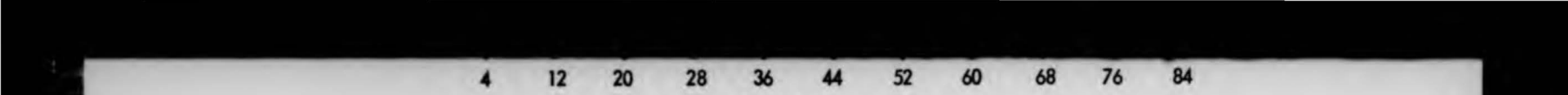


Figure 26.2

Diagram showing the antibody levels, using S.haematobium adult worm antigen in an untreated baboon

I, infection with 7,000 S.haematobium cercariae.

Figure 26.3

Diagram showing the antibody levels, using S.haematobium adult worm antigen in a treated baboon

I, infection with 7,000 S.haematobium cercariae

T, treatment with Niridazole 5 x 50 mg/kg body weight, orally by stomach tube.

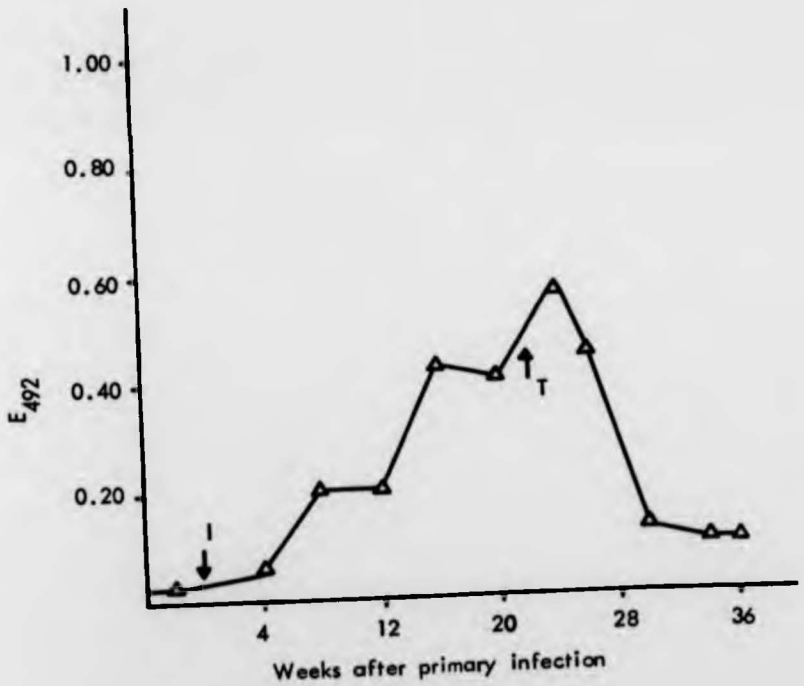

1.00
0.80
0.60
0.40
0.20
E_{492}
I
T
4
12
20
28
36
Weeks after primary infection

Figure 26.4

Diagram showing the antibody levels, using S.haematobium adult worm antigen in a treated baboon

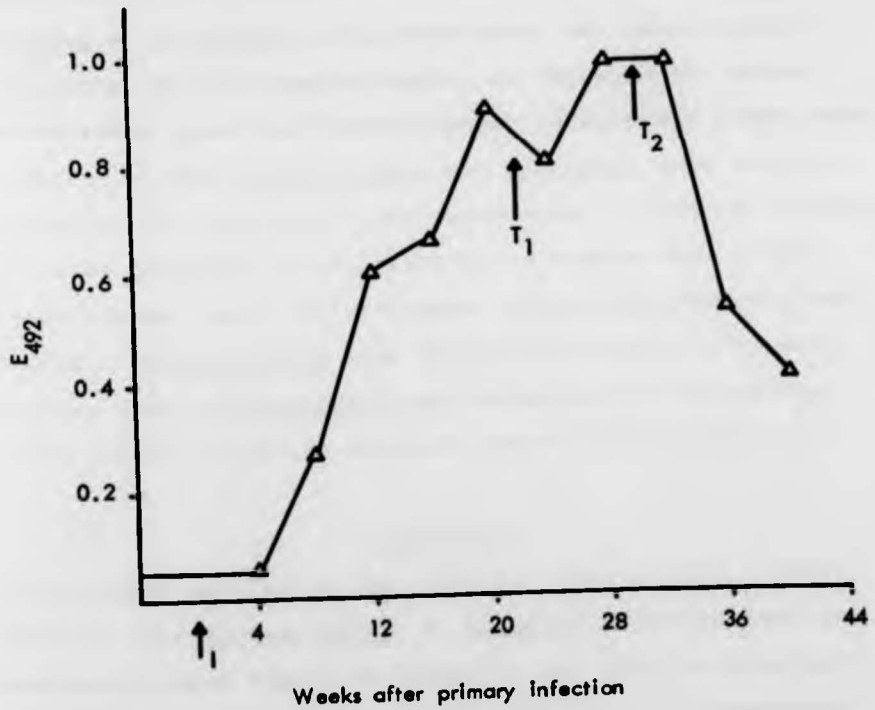
I, infection with 7,000 S.haematobium cercariae

T_1, treatment with RO11-3128, Hoffman La Roche's compound at a dose of 1 x 10 mg/kg body weight, orally by stomach tube

T_2, retreatment with 1 x 25 mg/kg body weight.

ematobium

e
s
ght,



any lasting effect on the antibody level of this baboon.

The human sera used in this study were from the Sudan (cf. Chapter II).

Table 8 shows the possible use of ELISA for serological monitoring of chemotherapy in humans infected with S.haematobium or S.mansoni using homologous and heterologous schistosome worm or egg antigens. As shown in the Table, the antibody level was significantly increased 6 weeks after treatment, using S.haematobium and S.mansoni worm antigens. The Table also shows that the percentage of patients showing a rise of antibody after treatment is larger when a homologous rather than a heterologous schistosome worm antigen is used. S.haematobium worm antigen was found to be more reactive than S.haematobium egg antigen in the detection of the early changes in antibody level after treatment.

Discussion

As mentioned earlier in this Chapter, Smith et al. (1976) reported that S.haematobium or S.mansoni infected hamsters developed a high degree of immunity, not only to homologous but also to heterologous reinfections. In this investigation, hamsters infected with S.haematobium demonstrated different degrees of immunity to homologous reinfection before and after oviposition. Immunity was higher (61%)

Table 8

Serum antibody levels (IgG) before and 6 weeks after a successful treatment with hycanthone in 8 patients with S.haematobium infections and 11 patients with S.mansoni infections

Type of schistosome infections	Number of patients	ELISA value E492								
		S.haematobium worm antigen			S.mansoni worm antigen			S.haematobium egg antigen		
		Mean E_{400} ± SE		% showing rise	Mean E_{400} ± SE		% showing rise	Mean E_{400} ± SE		% showing rise
		Before treatment	After treatment		Before treatment	After treatment		Before treatment	After treatment	
S.haematobium (1)	8	0.49 ± 0.1	0.81 ± 0.2	50.0	0.37 ± 0.08	0.56 ± 0.14	50.0	1.06 ± 0.16	1.10 ± 0.25	25.0
S.mansoni (2)	11	0.50 ± 0.12	0.80 ± 0.15	45.5	0.64 ± 0.10	0.98 ± 0.13	63.6	0.58 ± 0.10	0.68 ± 0.15	27.0

(1) Paired "t" test, significant at $p < 0.05$ with both worm antigens, but not significant with egg antigen.

(2) Paired "t" test, significant at $p < 0.01$ and < 0.001 with S.haematobium and S.mansoni worm antigens respectively, but not significant with egg antigen.

in hamsters challenged after oviposition than the group challenged before oviposition (46%). This could be explained by the fact that maturity and establishment of adult worms and the presence of eggs play an important part in the production of immunity to reinfection.

Chemotherapeutic eradication of adult worms 17 weeks after primary infection of hamsters with S.haematobium completely abolished all resistance to reinfection 7 weeks later. However, there are conflicting opinions as regards the effect of treatment on immunity to reinfection after cure. Although Warren et al. (1977) reported that immunity is residual or persistent after curative treatment (using Niridazole) in mice infected with S.mansoni, others (Cheever et al., 1965; Gold and Lengy, 1975) found no immunity after reinfection of mice or hamsters previously infected with S.mansoni and treated using Stibophen or Niridazole. This discrepancy may be due to the fact that the mechanism of immunity differs according to the host-parasite system, the time at which treatments and challenge infections are given and/or the type of drug used.

The antibodies as measured by the ELISA test in the baboons suggested no correlation with the degree of immunity, but a definite correlation with the level of infection.

ELISA also showed great sensitivity in monitoring chemotherapy

in humans and animals infected with S.haematobium or S.mansoni. The increase in antibody level after treatment is significant in human cases and baboons but not in hamsters.

The increase in antibody level in human cases after treatment is more apparent and significant using S.haematobium or S.mansoni worm antigens than using S.haematobium egg antigen. This is probably due to a massive lysis of worms as a result of treatment releasing an antigen which in a paradoxical way stimulates a sudden production of antibodies. Salih et al. (1978), using the ELISA test, Silva et al. (1971, 1975 and 1976), using other serological tests (IFA, IHA and immunodiffusion tests), were able to demonstrate a significant increase in antibodies reacting with worm antigens, during the first 2-6 weeks following treatment, in patients with S.mansoni infection successfully treated with Niridazole, Hycanthone or Oxamniquine.

These studies indicate that the ELISA test, besides its high sensitivity, economy and adaptability for field use, has a potentially useful application in the evaluation of the efficacy of drugs in the treatment of schistosomiasis.

Capron et al., (1974), observed that an unexpected rise in an otherwise declining antibody level often points to a recurrent infection. Therefore the ability of ELISA to monitor changes in antibody levels after reinfection is

particularly useful in long-term follow-up studies. Moreover, long-term follow-up studies of antibody levels in larger numbers of treated patients are recommended which would determine the usefulness of ELISA in assessing cure at varying intervals following chemotherapy.

Chapter VI

A comparison between a new serological method, thin layer immunoassay, TIA, and the enzyme-linked immunosorbent assay, ELISA, for the detection of antibodies in schistosomiasis

Introduction

As mentioned in the first chapter of this thesis, a simple, cheap, sensitive and specific serologic test is urgently needed for the diagnosis of schistosomiasis.

A test which shows particular promise is the enzyme-linked immunosorbent assay (ELISA), developed by Engvall and Perlmann (1971 and 1972). As shown in the previous two chapters of this thesis and from initial investigations by Huldt et al., (1975); McLaren et al., (1978); the ELISA was found to have great sensitivity in the detection of anti-schistosome antibodies.

More recently, the thin layer immunoassay (TIA) has been developed for the detection and quantification of specific antibodies (Elwing et al., 1976 and 1977). This test is characterized by its simplicity and does not require sophisticated technical apparatus for its performance. It is, therefore, of considerable potential value for studies carried out in developing countries, but has yet

to be fully evaluated. Accordingly, a comparative investigation has been carried out of TIA and ELISA for the detection and quantification of anti-schistosome antibodies in a variety of human and animal sera. A briefer version of this study has already been published (Ismail et al., 1979).

Materials and Methods

Antigens

Crude soluble antigens were prepared from S.haematobium and S.mansoni adult worms obtained from infected hamsters and mice respectively, according to the technique of Capron et al., (1968), as described in Chapter II.

Sera

The human and animal sera examined were described in detail in Chapters II and V respectively of this thesis.

ELISA

The basic method of Engvall and Perlmann (1971 and 1972) was employed, as described in Chapters IV and V, using the ELISA-peroxidase system.

TIA

This assay was carried out according to the methods of Elwing et al., (1976, 1977). Initial observations showed that the TIA performed best when the antigen was diluted to a final concentration of 100 µg protein/ml in 0.005%

ovalbumin in normal saline, e.g. 0.1 ml of 1% ovalbumin was added to 1.9 ml of antigen (100 μg protein/ml) and this mixture was diluted to 20 ml with normal saline. This was poured into the bottom of a polystyrene petri dish (8.5 cm diameter, Nunc A/S, Roskilde, Denmark) and allowed to remain for 30 min. at room temperature. During this time the material in solution coated the bottom of the dish. The solution was then poured away and kept for recoating and the dish washed three times, one minute per wash, with saline. A solution of 1% molten agar in saline was poured into the dish in quantity sufficient to give a layer 2.5 mm thick. After setting on a level surface, twelve 3 mm diameter wells were punched in the agar, and each was filled with 15 ml of undiluted test serum. Following incubation in a moist atmosphere, at room temperature, for 48 hours, the agar was rinsed off with saline. Reinforcement was carried out by adding 15 ml of rabbit anti-human IgG serum (Miles) for testing human and baboon sera, or rabbit anti-hamster IgG serum (Miles) for testing hamster sera. Each was diluted 1:500 in saline. Incubation was for one hour at room temperature, after which the solution was poured away and the plate dried. Visualization of specifically absorbed antibody was then carried out by inverting the dish over water at 60°C for one minute. Areas with absorbed antibody are characterized by a hydrophilic condensation pattern, with large

condensation drops, in contrast to the surrounding areas where the pattern is of tiny droplets, giving a "misting" effect. The mechanism underlying this phenomenon is unknown and is being investigated. A record of the condensation patterns was then made by contact copying the plate onto photographic paper (Ilfoprint YR 31P). The surface area of the positive hydrophilic spots was calculated from the print, using the mean of two diameters measured at right angles. The surface area is proportional to the logarithm of the antibody concentration, provided that the antibody level is not very low. Fig.27 shows a schematic illustration of the principle of diffusion into gel-thin-layer immunoassay, (Dig.TIA).

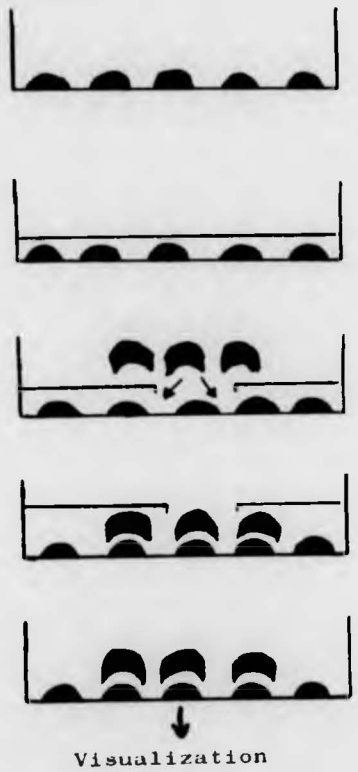
Figure 28 is a photographic recording of a typical plate with ten positive sera from known S.haematobium cases, with various levels of antibody, and two negative sera. The hydrophilic and hydrophobic areas are easily discerned.

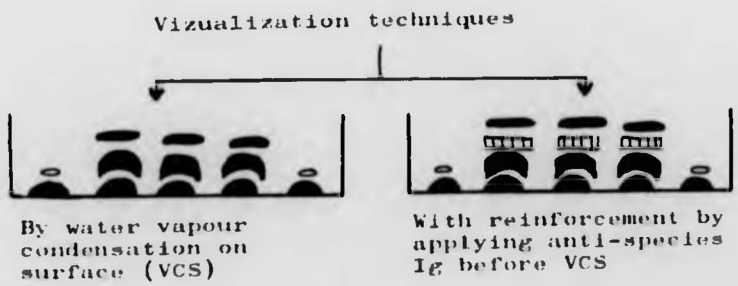
Results

The results obtained with the various human sera, using both S.haematobium and S.mansoni crude soluble adult worm antigens, are summarized in Table 9. Using sera from known schistosomiasis cases, and homologous antigens, the performance of TIA and ELISA were almost identical; 95.6% of the sera were positive by TIA and 96.4% by ELISA. Both tests gave high percentages of positive results when

Figure 27

Schematic illustration of the principle of diffusion into gel Thin Layer Immunoassay (DIG-TIA)

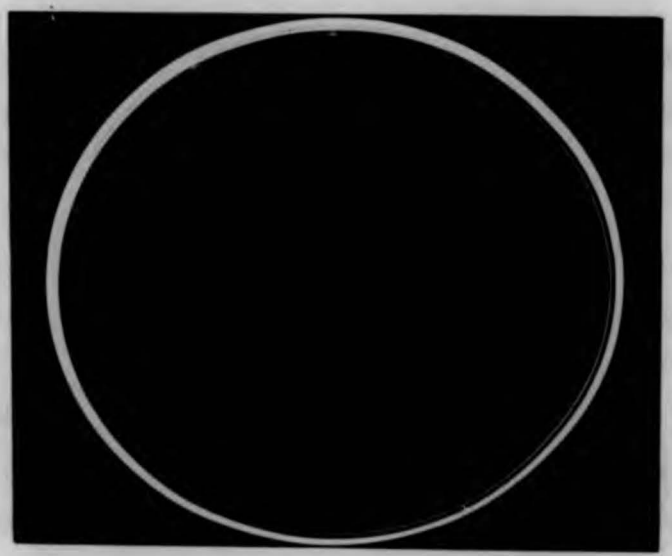




= Ag = Ab = Anti-Ig

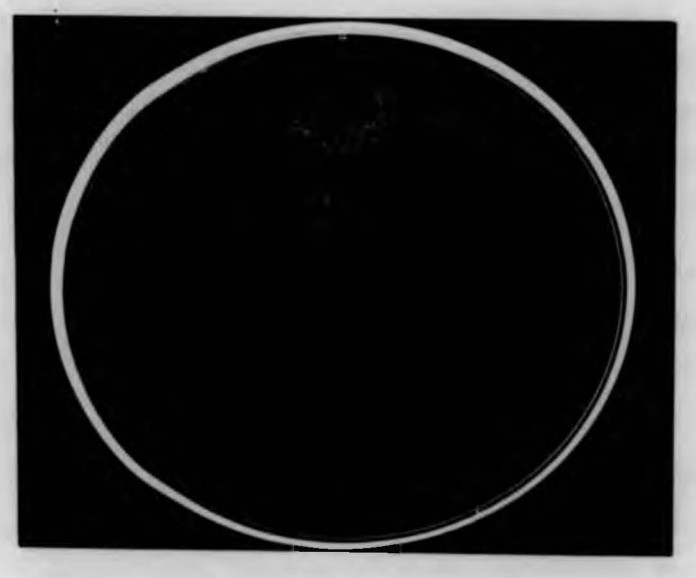
= Condensation drops

Figure 28



Photograph of TIA plate with S.haematobium adult worm antigen and ten sera from subjects with S.haematobium with different levels of antibody, Two normal sera are at bottom right and only the wells are visible.

Figure 28



Photograph of TIA plate with S.haematobium adult worm antigen and ten sera from subjects with S.haematobium with different levels of antibody, Two normal sera are at bottom right and only the wells are visible.

S.haematobium sera were tested against S.mansoni antigen and vice-versa; this is expected as the two species have many antigens in common (Capron et al., 1968). With sera from patients infected with helminths other than schistosomes, ELISA produce no false positive results whereas TIA produced 7% or 12% depending upon the antigen used. Interestingly, the majority of the false positives with TIA were from the U.K. cercarial dermatitis patients.

Figure 29 shows the correlation between ELISA and TIA results for S.haematobium human sera against S.haematobium worm antigen. The correlation was almost as good using S.mansoni worm antigen (Fig.30).

The Gabon sera used were either from an area where both S.intercalatum and/or S.haematobium occur, or from an area where people were exposed to these infections but no parasitological data are available; the two tests, ELISA and TIA, show a great concordance between each other.

The antibody levels measured by TIA correlate well with those found using the ELISA test on sera from the four baboons described in the previous chapter. In the untreated baboon (Fig.31.) the antibody level remained high with both tests throughout the 80 weeks of observation, but in the treated and reinfected baboon (Fig.32) the levels varied with the treatment and reinfection. Figures 33, 34 and 35 also show the good correlation obtained in the antibody levels,

S.haematobium sera were tested against S.mansoni antigen and vice-versa; this is expected as the two species have many antigens in common (Capron et al., 1968). With sera from patients infected with helminths other than schistosomes, ELISA produce no false positive results whereas TIA produced 7% or 12% depending upon the antigen used. Interestingly, the majority of the false positives with TIA were from the U.K. cercarial dermatitis patients.

Figure 29 shows the correlation between ELISA and TIA results for S.haematobium human sera against S.haematobium worm antigen. The correlation was almost as good using S.mansoni worm antigen (Fig.30).

The Gabon sera used were either from an area where both S.intercalatum and/or S.haematobium occur, or from an area where people were exposed to these infections but no parasitological data are available; the two tests, ELISA and TIA, show a great concordance between each other.

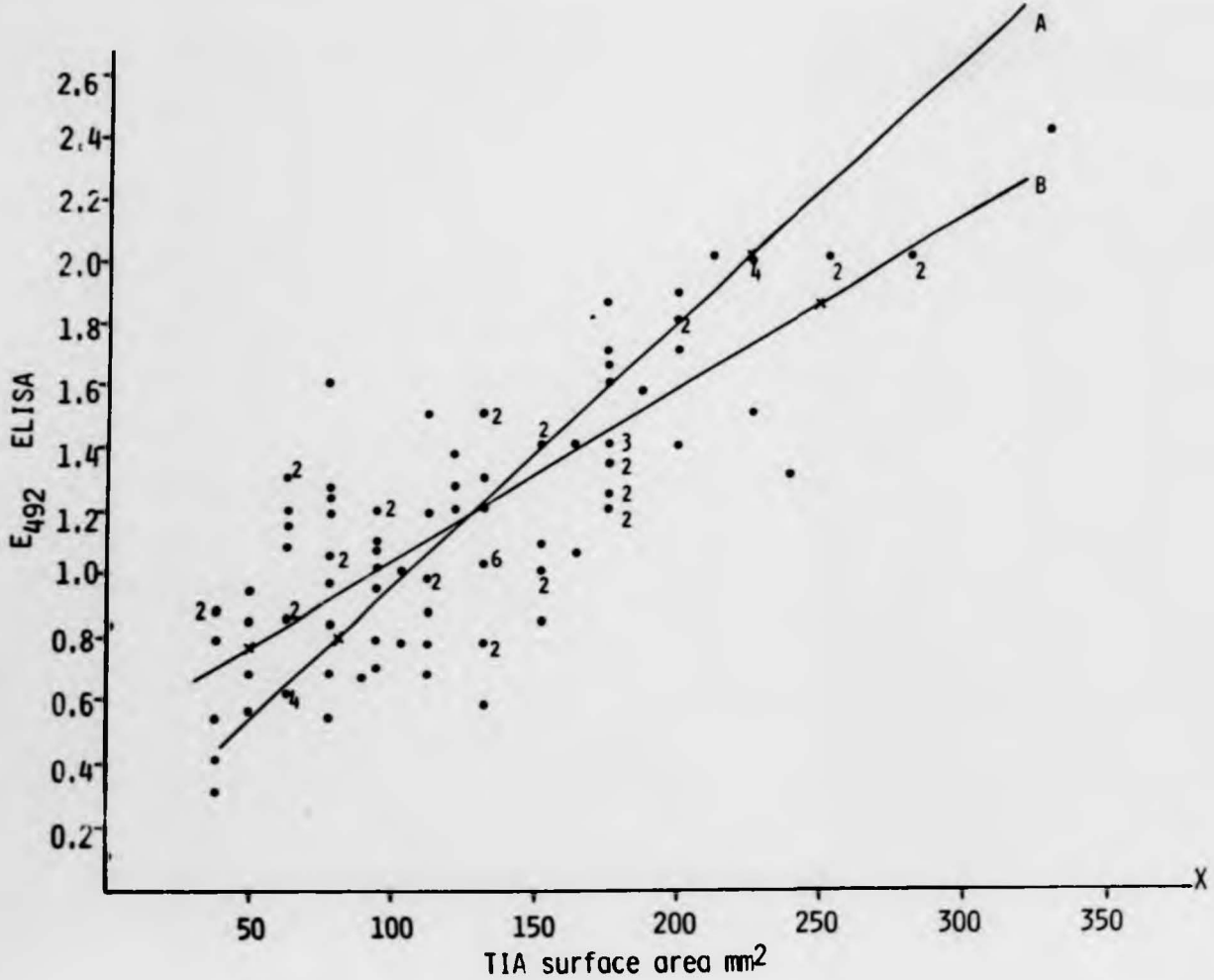
The antibody levels measured by TIA correlate well with those found using the ELISA test on sera from the four baboons described in the previous chapter. In the untreated baboon (Fig.31.) the antibody level remained high with both tests throughout the 80 weeks of observation, but in the treated and reinfected baboon (Fig.32) the levels varied with the treatment and reinfection. Figures 33, 34 and 35 also show the good correlation obtained in the antibody levels,

Table 9

Results obtained with ELISA and TIA on sera from patients with various helminth infections, using S.haematobium and S.mansoni antigens

Infection (No. of cases)	S.haematobium antigen				S.mansoni antigen			
	ELISA		TIA		ELISA		TIA	
	+	-	+	-	+	-	+	-
S.haematobium (107)	106	1	105	2	100	7	105	2
S.mansoni (30)	25	5	25	5	26	4	26	4
Cercarial dermatitis (10)	0	10	2	8	0	10	4	6
Hydatid (10)	0	10	0	10	0	10	0	10
Cysticercosis (10)	0	10	1	9	0	10	1	9
Other helminths (26)	0	26	1	25	0	26	2	24
S.haematobium &/or S.intercalatum (15)	13	2	13	2	13	2	14	1
Gabon sera, no parasitological data (35)	7	28	7	28	10	25	8	27

Sera were judged positive by ELISA if E_{492} level was more than 0.25; and positive by TIA if a clear cut hydrophilic condensation pattern was seen.


A
B
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
E_{492} ELISA
X
50
100
150
200
250
300
350
TIA surface area mm2

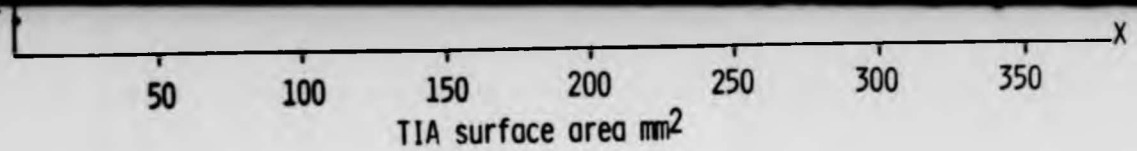


Figure 29

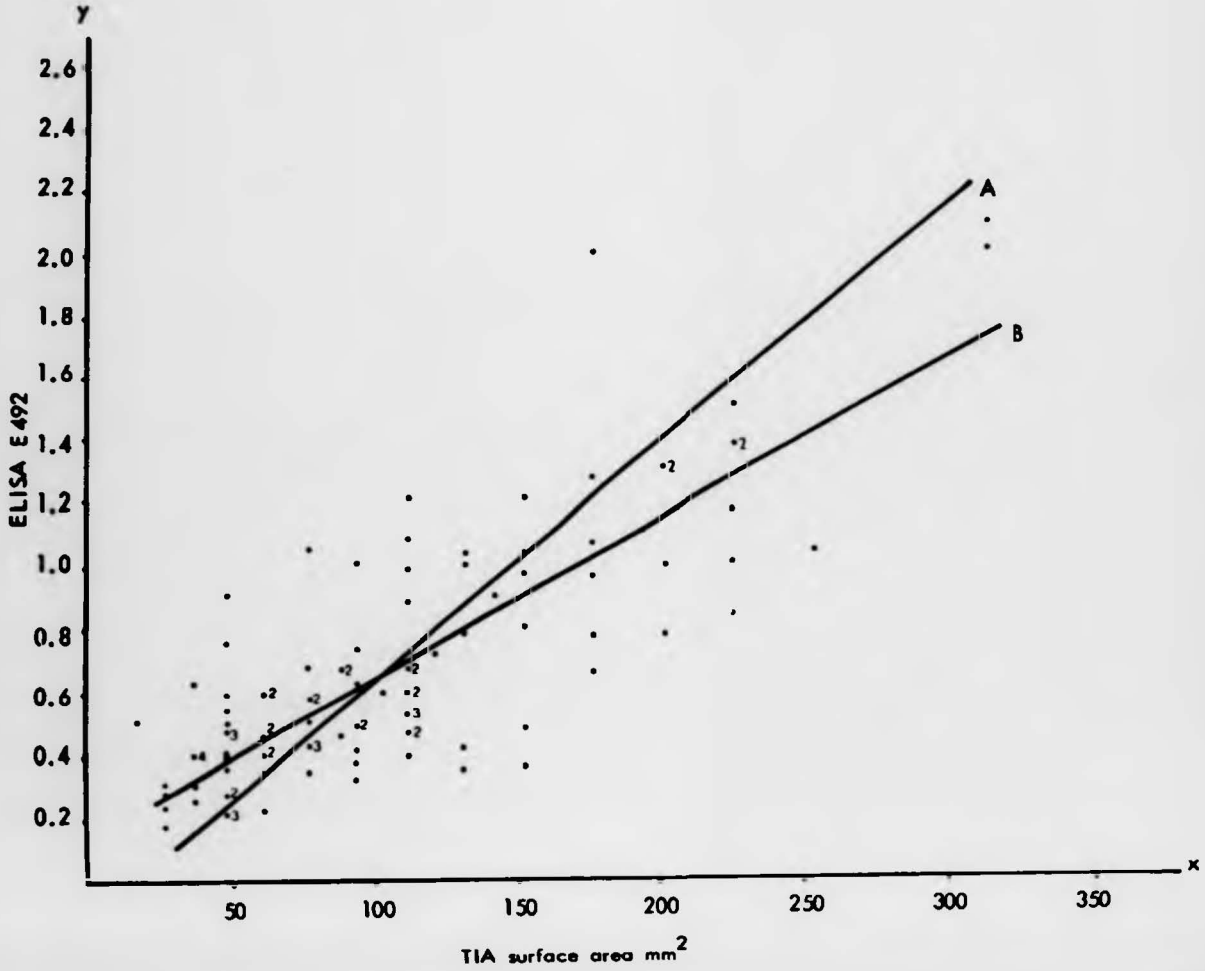
Scatter diagram showing the relation between the extinction of ELISA (E_{492}) and the surface area of TIA using human sera from S.haematobium cases and S.haematobium adult worm antigen

Correlation coefficient, r = 0.81 and P < 0.001

A = regression line of x on y.

B = regression line of y on x.

Numbers refer to numbers of sera represented by each dot when these are more than one.


y
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ELISA E 492
A
B
x
50
100
150
200
250
300
350
TIA surface area mm2

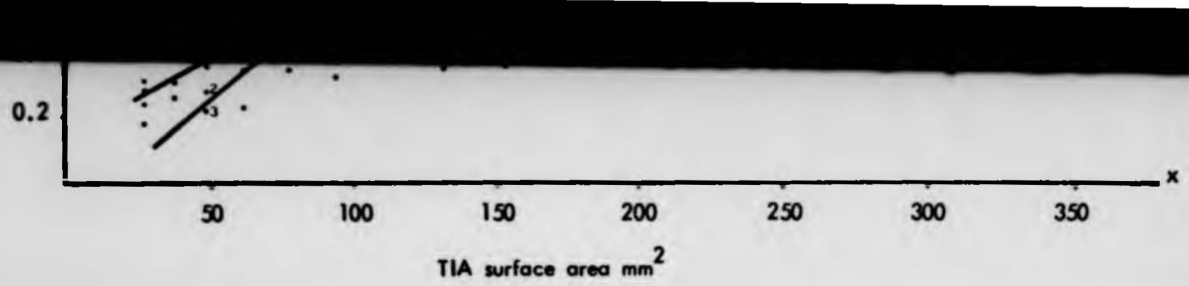


Figure 30

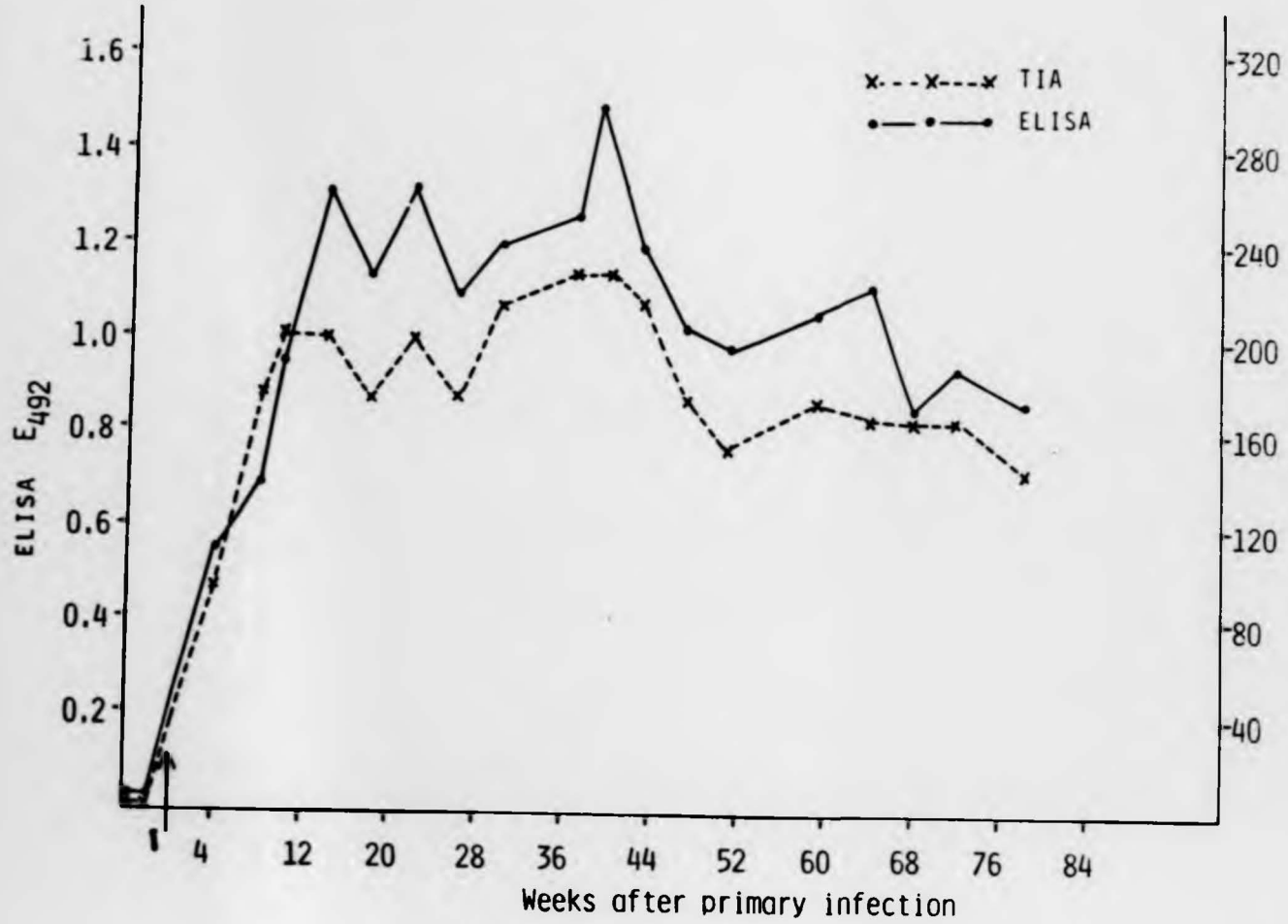
Scatter diagram showing the relation between the extinction of ELISA (E_{492}) and the surface area of TIA using human sera from S.mansoni cases and S.mansoni adult worm antigen

Correlation coefficient, r = 0.88 and P < 0.001

A = regression line of x on y.

B = regression line of y on x.

Numbers refer to numbers of sera represented by each dot when these are more than one.


TIA
ELISA
ELISA E_{492}
TIA surface area nm^2
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
320
280
240
200
160
120
80
40
1
4
12
20
28
36
44
52
60
68
76
84
Weeks after primary infection

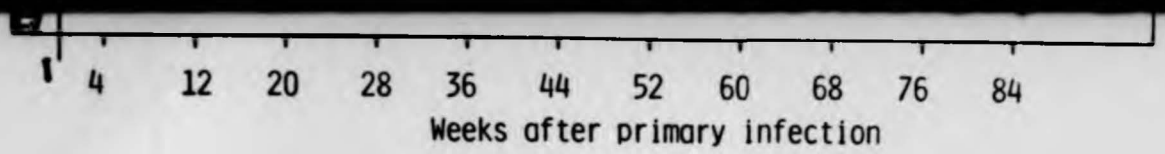
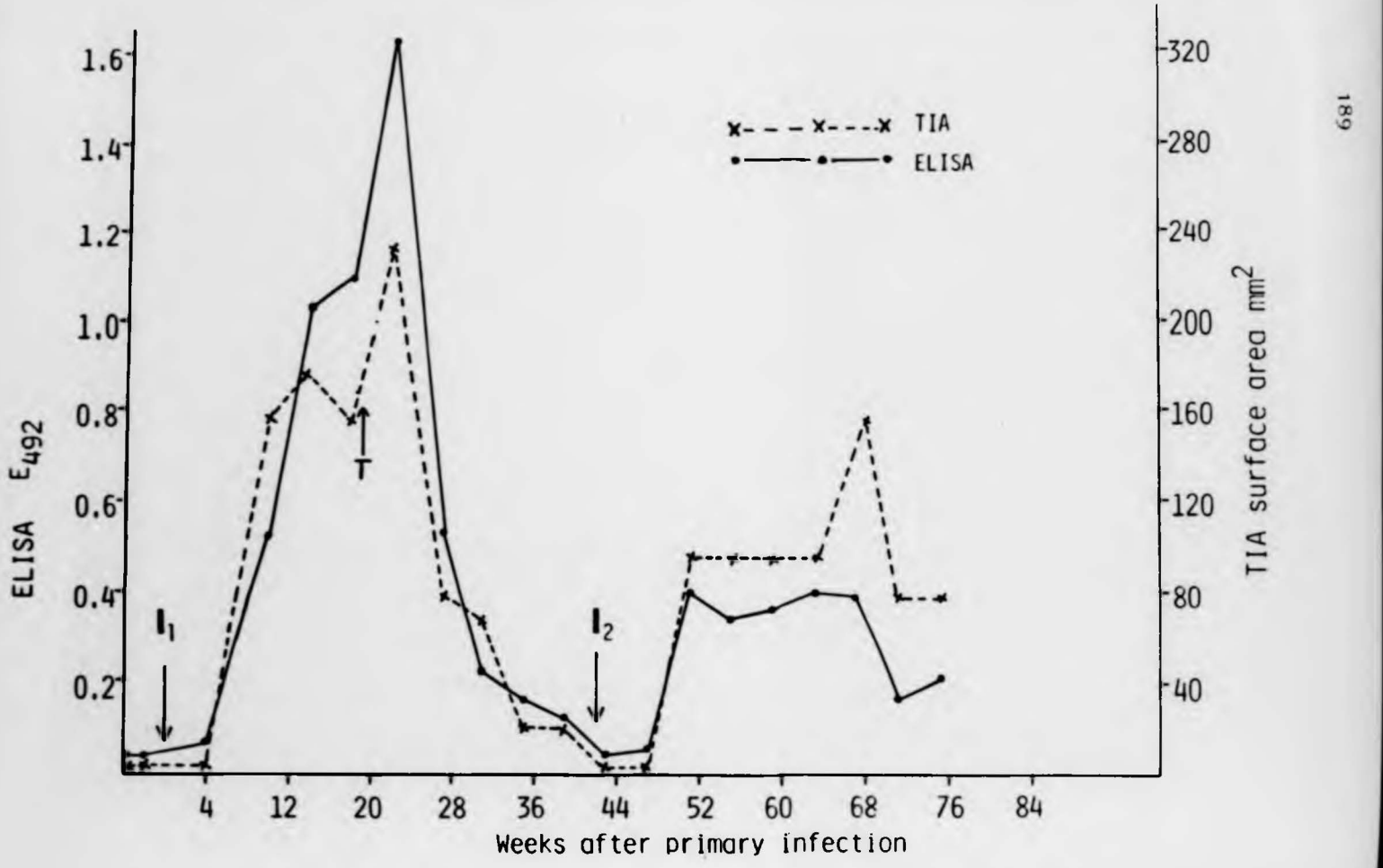


Figure 31

Diagram showing the relation between the antibodies measured by the ELISA (.) and the TIA (x), using S.haematobium adult worm antigen, in an untreated baboon

I, infection with 7,000 S.haematobium cercariae.


ELISA E_{492}
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
TIA surface area mm^2
320
280
240
200
160
120
80
40
TIA
ELISA
I_1
I_2
4
12
20
28
36
44
52
60
68
76
84
Weeks after primary infection

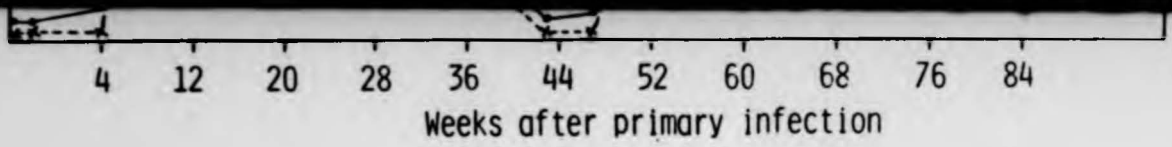


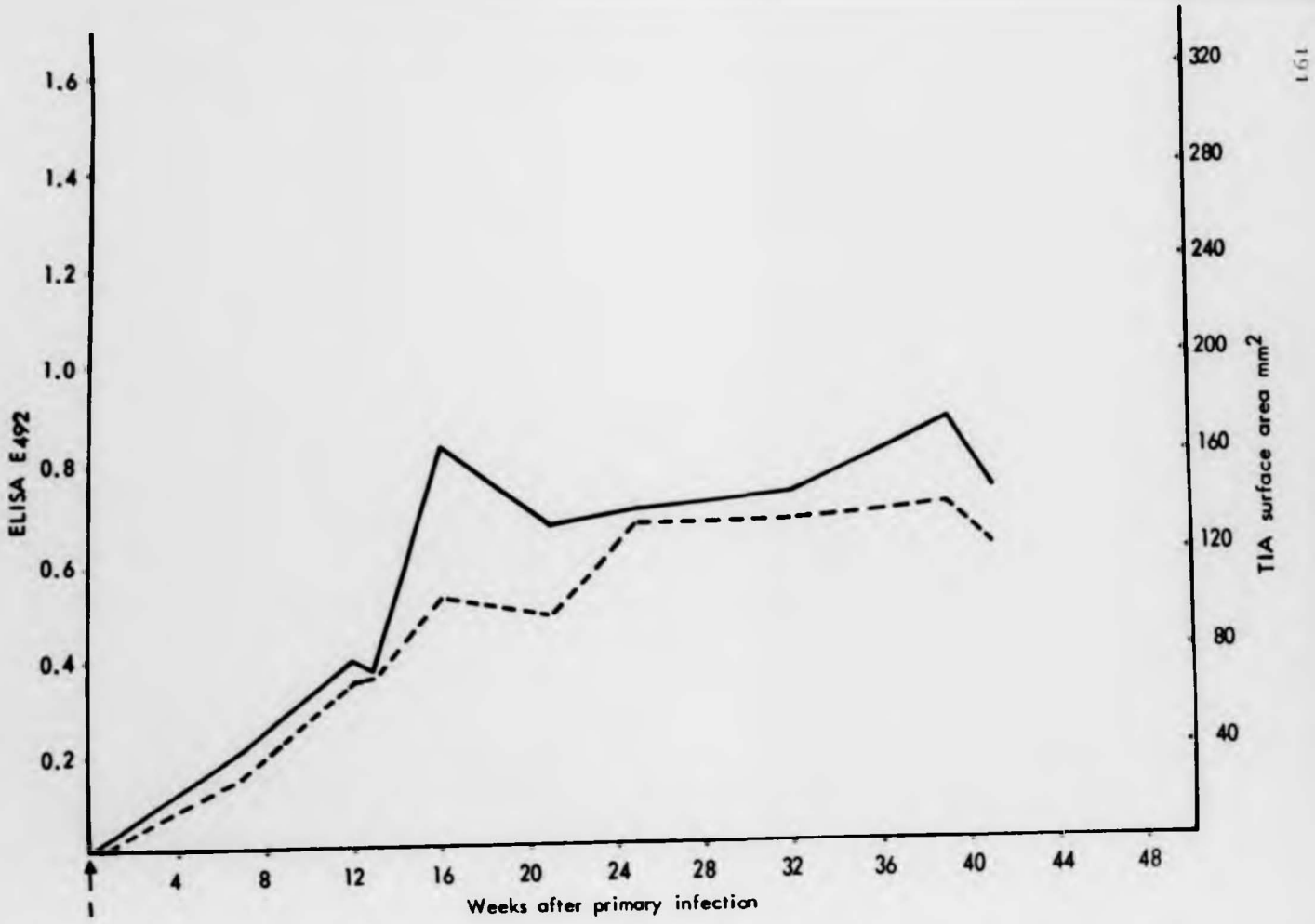
Figure 32

Diagram showing the relation between the antibodies measured by the ELISA (.) and the TIA (x), using S.haematobium adult worm antigen, in a treated and reinfected baboon

I_1, infection with 7,000 S.haematobium cercariae.

I_2, reinfection with 7,500 S.haematobium cercariae.

T, treatment with a dinitro-thiophane derivative.


ELISA E492
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
TIA surface area mm²
320
280
240
200
160
120
80
40
1
4
8
12
16
20
24
28
32
36
40
44
48
Weeks after primary infection

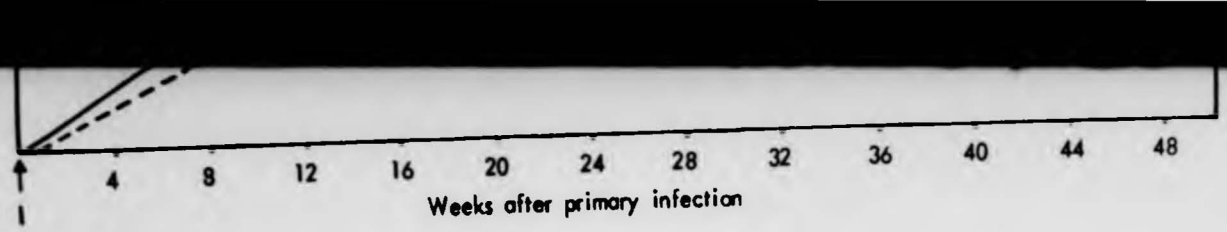
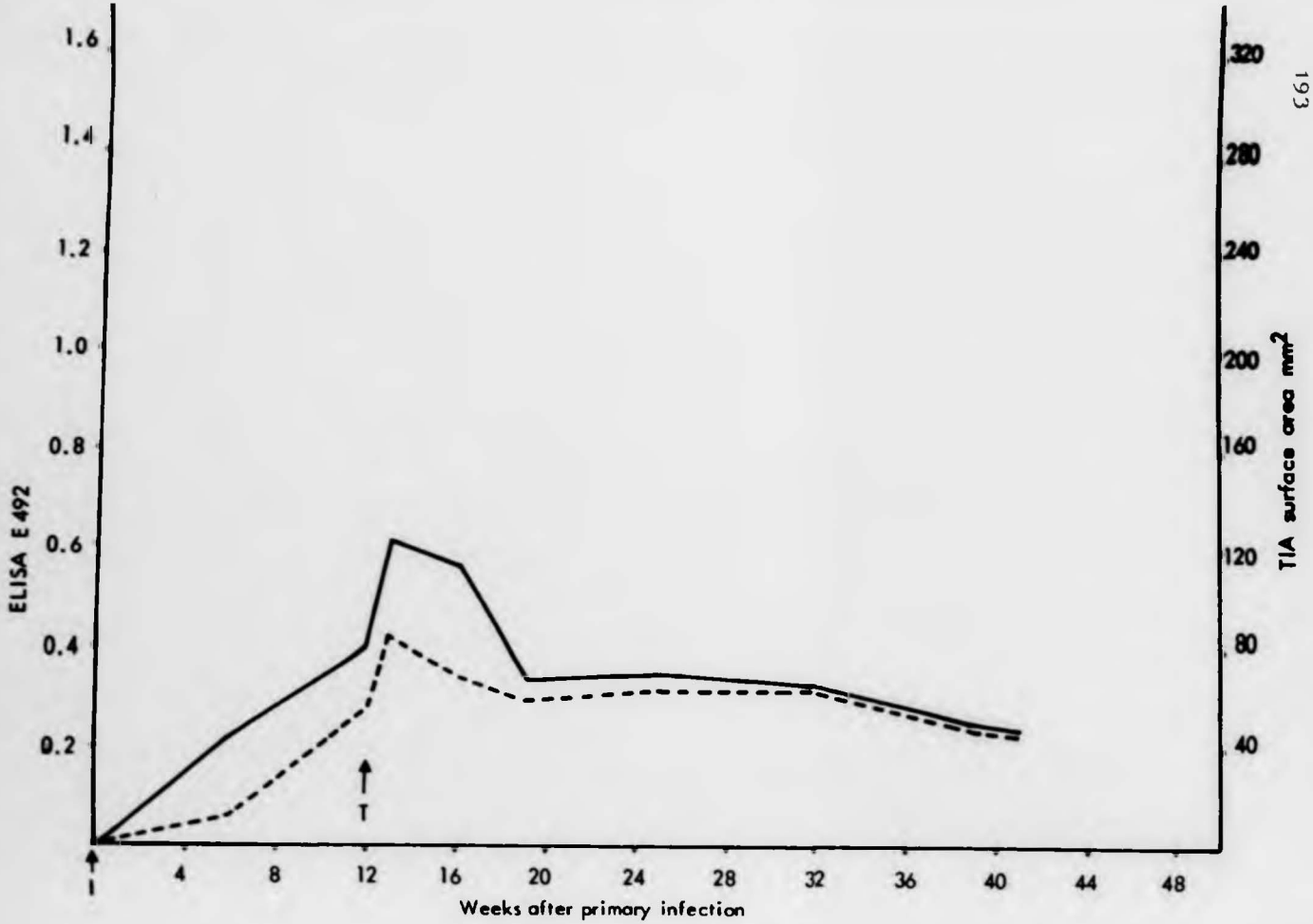


Figure 33

Diagram showing the relation between the antibodies measured by the ELISA (–) and the TIA (--), using S.haematobium adult worm antigen, in an untreated group of hamsters

I, infection with 200 S.haematobium cercariae.

Each point represents the mean of 5 hamsters, standard error ranges between 0.03 - 0.19.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ELISA E 492
320
280
240
200
160
120
80
40
TIA surface area mm²
I
T
4
8
12
16
20
24
28
32
36
40
44
48
Weeks after primary infection

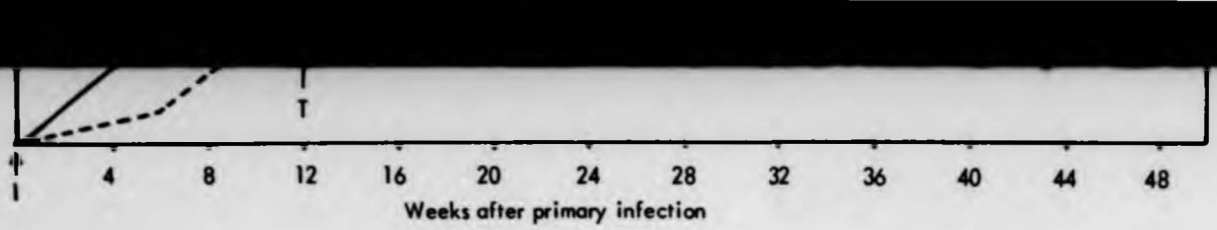


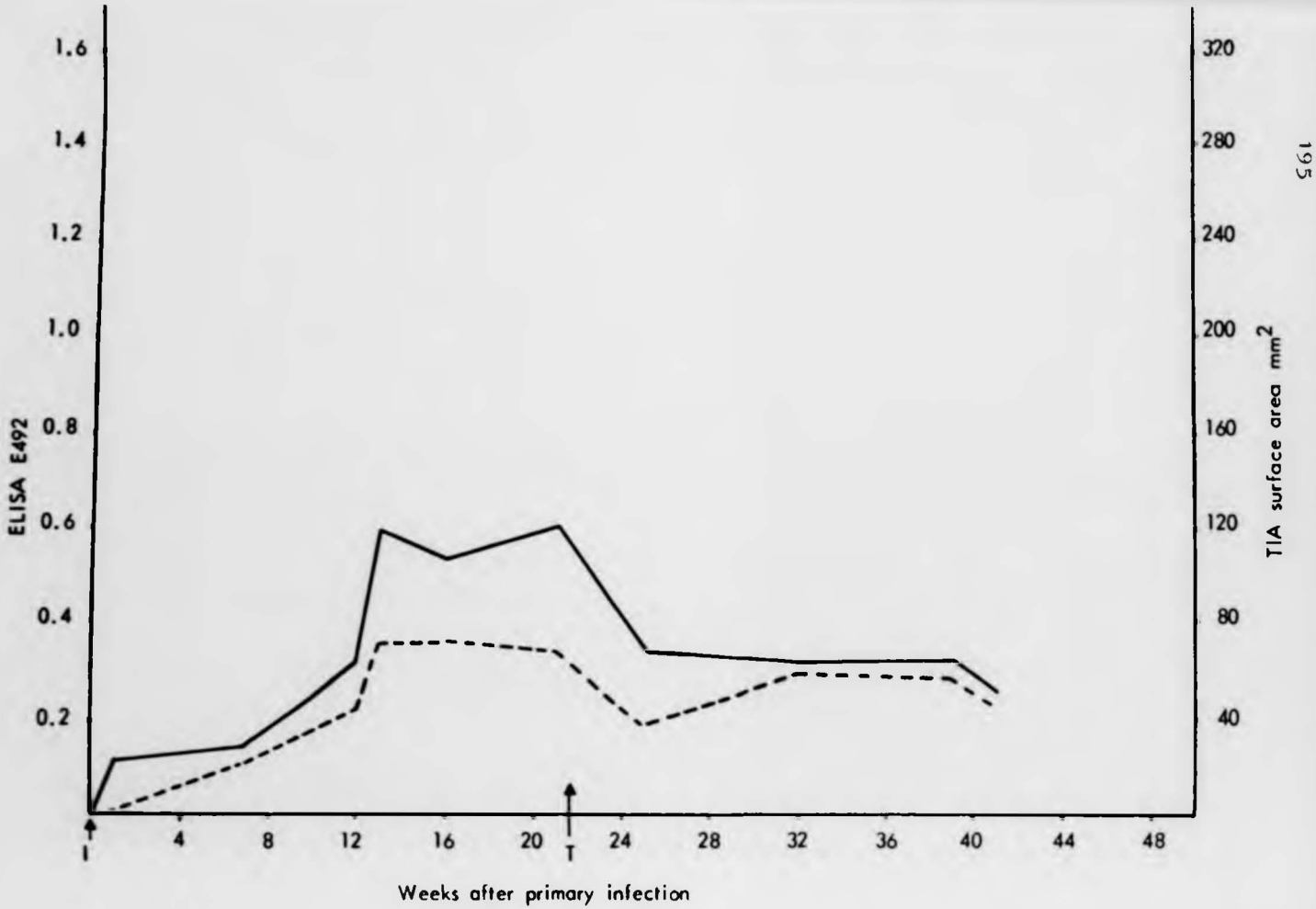
Figure 34

Diagram showing the relation between the antibodies measured by the ELISA (—) and the TIA (--) using S.haematobium adult worm antigen, in a treated group of hamsters

I, infection with 200 S.haematobium cercariae.

T, treatment with Niridazole (Ambilhar) 200 mg/kg body weight orally, daily / 5 days at 12 weeks after infection.

Each point represents the mean of 5 hamsters, standard error ranges between 0.003 - 0.1.


ELISA E492
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
TIA surface area mm2
320
280
240
200
160
120
80
40
I
T
4
8
12
16
20
24
28
32
36
40
44
48
Weeks after primary infection

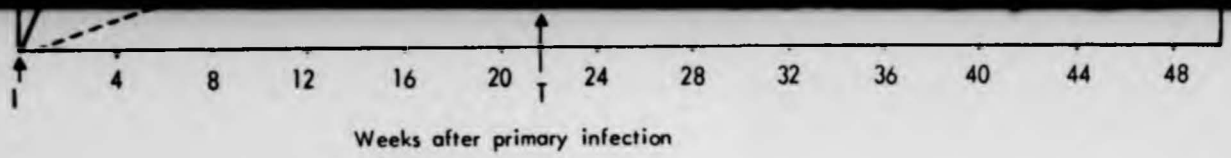


Figure 35

Diagram showing the relation between the antibodies measured by the ELISA (—) and the TIA (--) using S.haematobium adult worm antigen in a treated group of hamsters

I, infection with 200 S.haematobium cercariae.

T, treatment with Niridazole (Ambilhar) 200 mg/kg body weight orally, daily / 5 days at 22 weeks after infection.

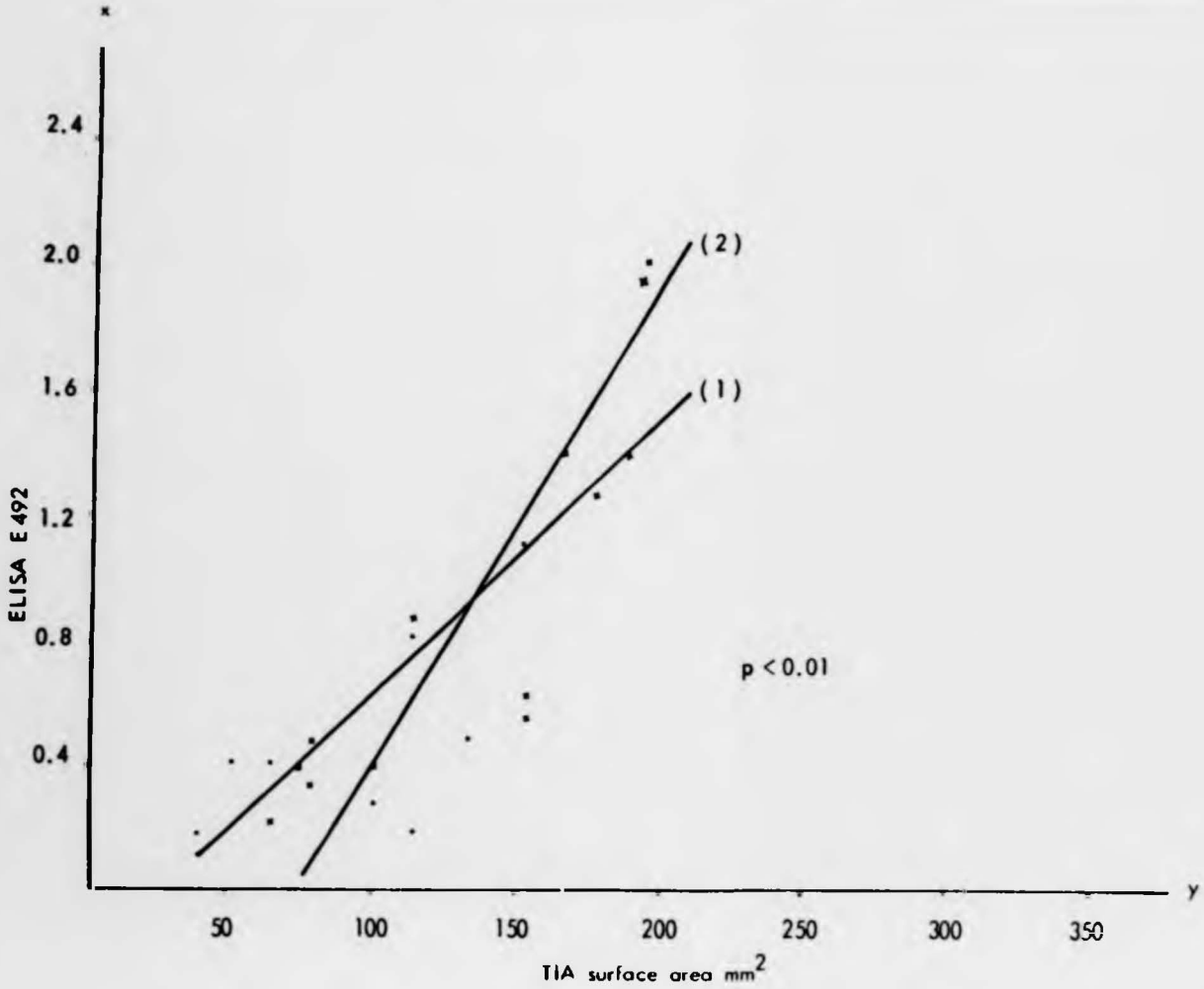
Each point represents the mean of 5 hamsters, standard error ranges between 0.003 - 0.1.

measured by the two tests, in three groups of the experimentally infected hamsters. The sera became positive 7-12 weeks after infection by both tests, and remained at a high level in the untreated hamsters but declined significantly in the two groups of treated hamsters reaching a low level 6 weeks after treatment (P being <0.001 and <0.01 in ELISA and <0.002 and <0.02 in TIA).

Finally, the two tests showed a very close correlation in monitoring chemotherapy in patients infected with S.haematobium or S.mansoni, using homologous and heterologous schistosome worm antigens (Figs. 36-37 and 38-39). The percentage of patients showing a rise of antibody after treatment is larger when a homologous rather than a heterologous schistosome worm antigen is used (Table 10 and 11).

Discussion

The primary aim of this investigation was to explore the sensitivity and specificity of TIA in detecting anti-schistosome antibodies, using ELISA as a standard for comparison. The results, using human, baboon and hamster sera, demonstrate that the sensitivity of TIA is fully comparable to ELISA, but that the specificity of TIA may be somewhat less. This may be due to shared antigens between different helminths which are present in crude


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E492
(2)
(1)
p < 0.01
y
50
100
150
200
250
300
350
TIA surface area mm2

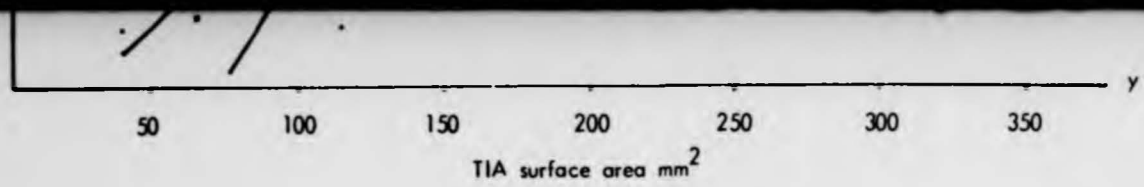


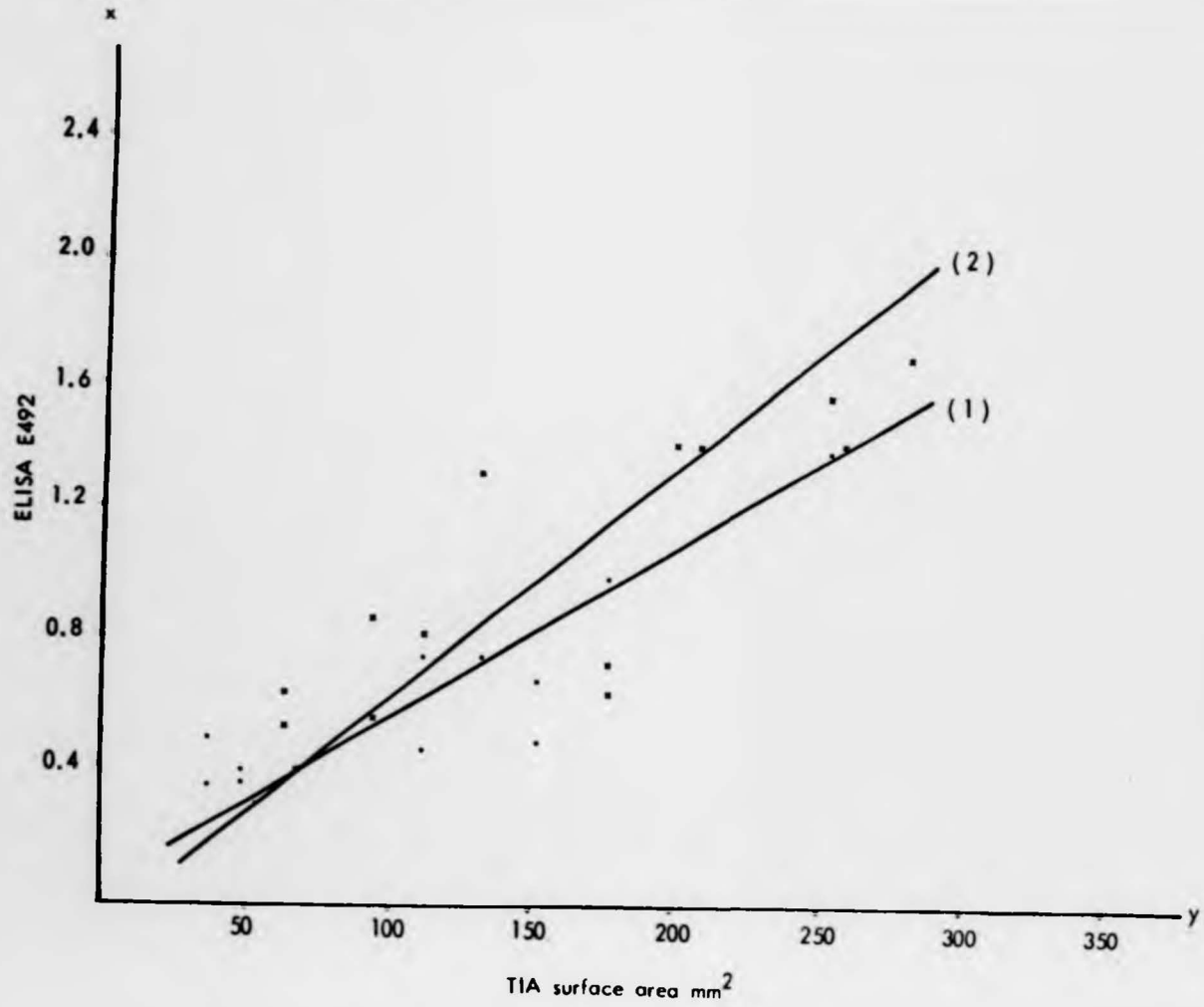
Figure 36

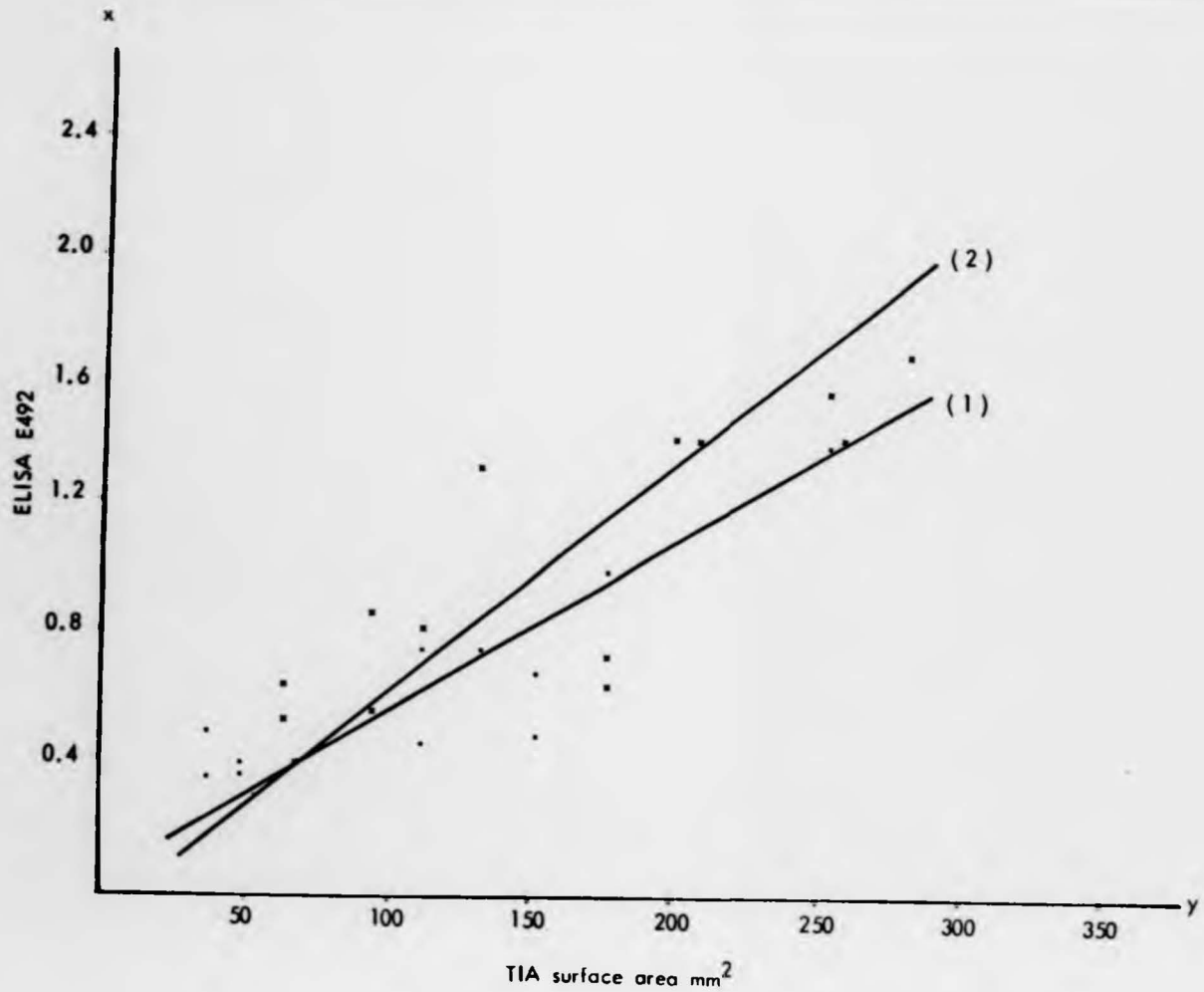
Scatter diagram showing the relation between the extinction of ELISA (E_{492}) and the surface area of TIA using human sera from S.haematobium cases, before (.) and after (x) treatment and S.haematobium adult worm antigen

Correlation coefficient, r is significant for both:

(1) Before treatment $r = 0.85$ $p < 0.01$

(2) After treatment $r = 0.81$ $p < 0.01$


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E492
(2)
(1)
50
100
150
200
250
300
350
y
TIA surface area mm2


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E492
(2)
(1)
50
100
150
200
250
300
350
y
TIA surface area mm2

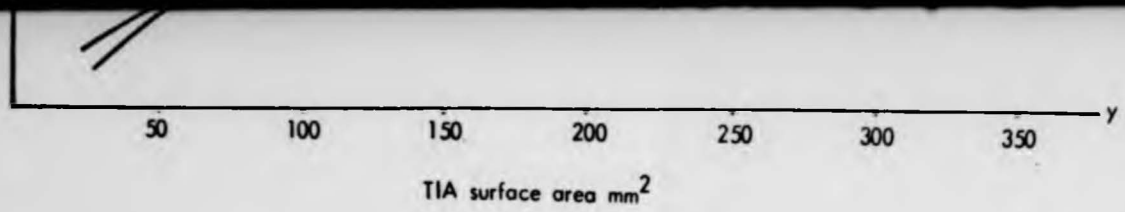


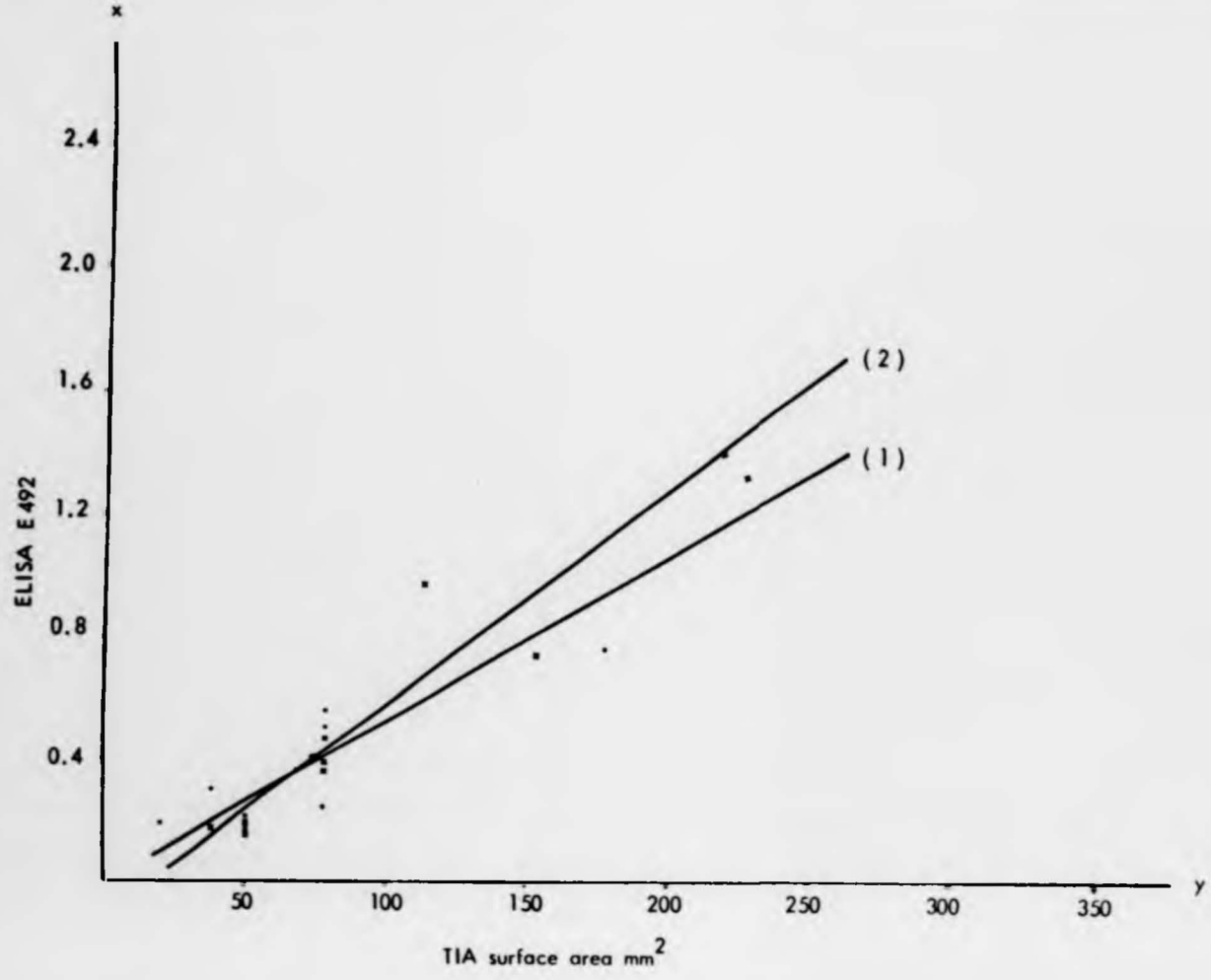
Figure 37

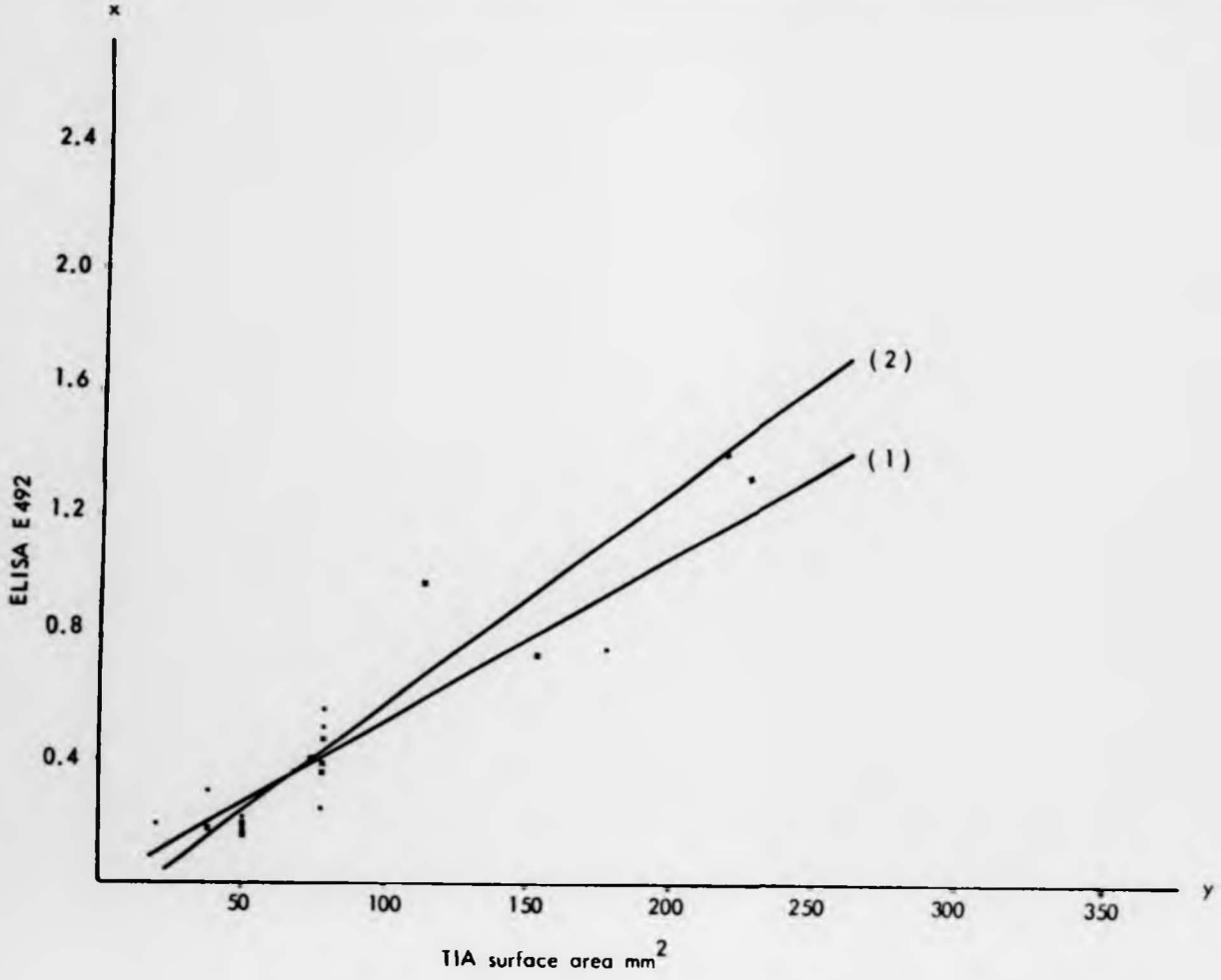
Scatter diagram showing the relation between the extinction of ELISA (E_{492}) and the surface area of TIA using human sera from S.mansoni cases before (.) and after (x) treatment and S.mansoni adult worm antigen

Corrélation coefficient, r is significant for both:

(1) Before treatment $r = 0.88$ $p < 0.001$

(2) After treatment $r = 0.81$ $p < 0.001$


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E 492
(2)
(1)
50
100
150
200
250
300
350
y
TIA surface area mm2


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E492
(2)
(1)
50
100
150
200
250
300
350
y
TIA surface area mm2

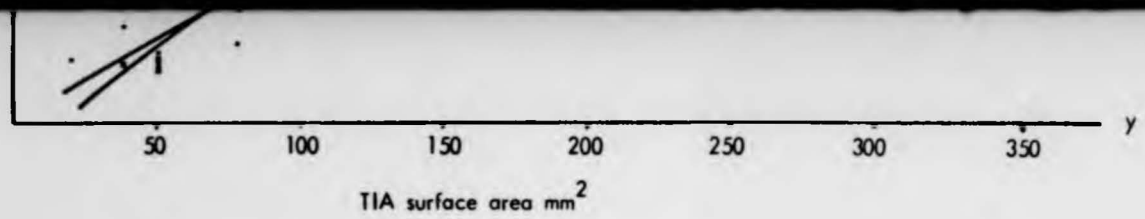


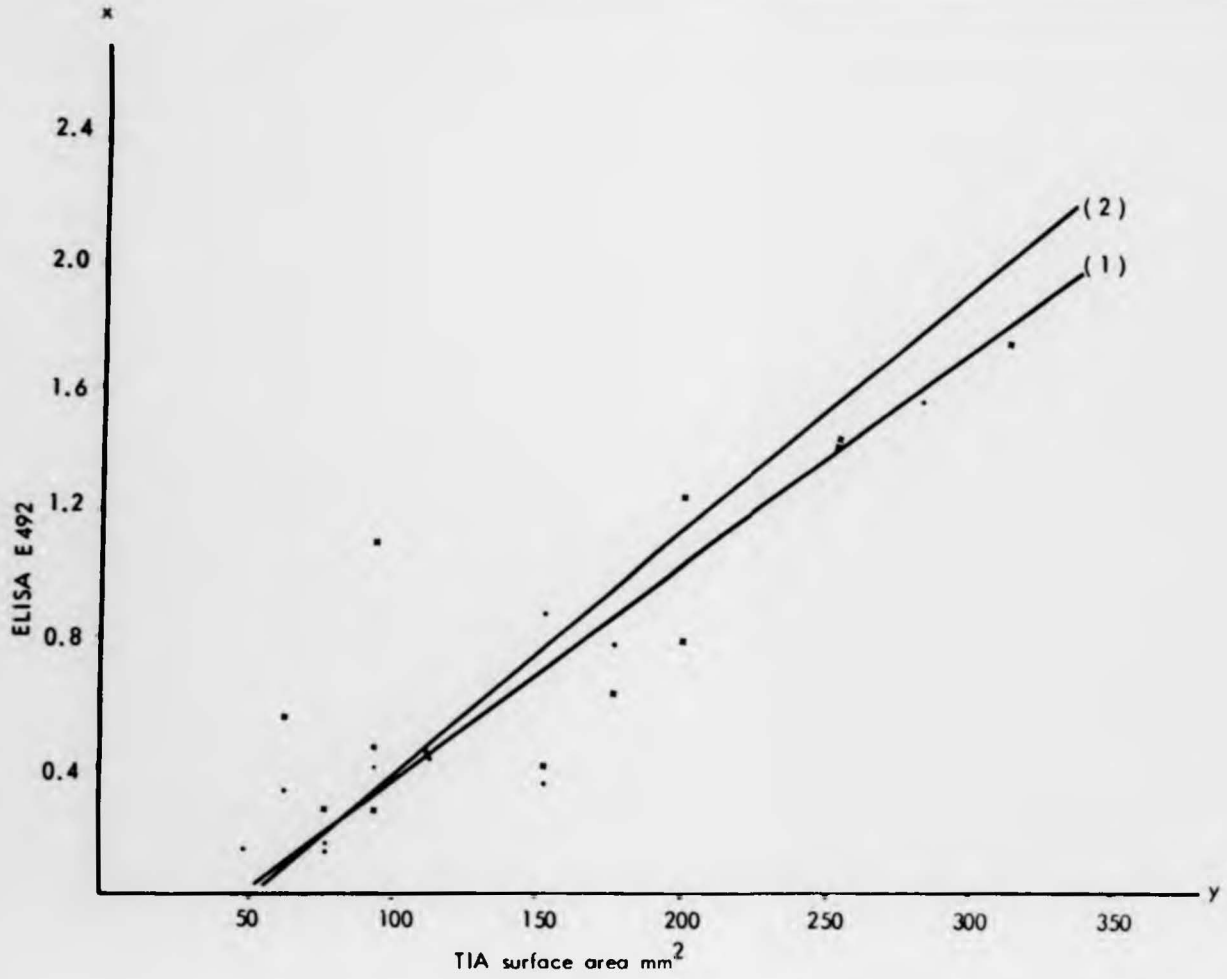
Figure 38

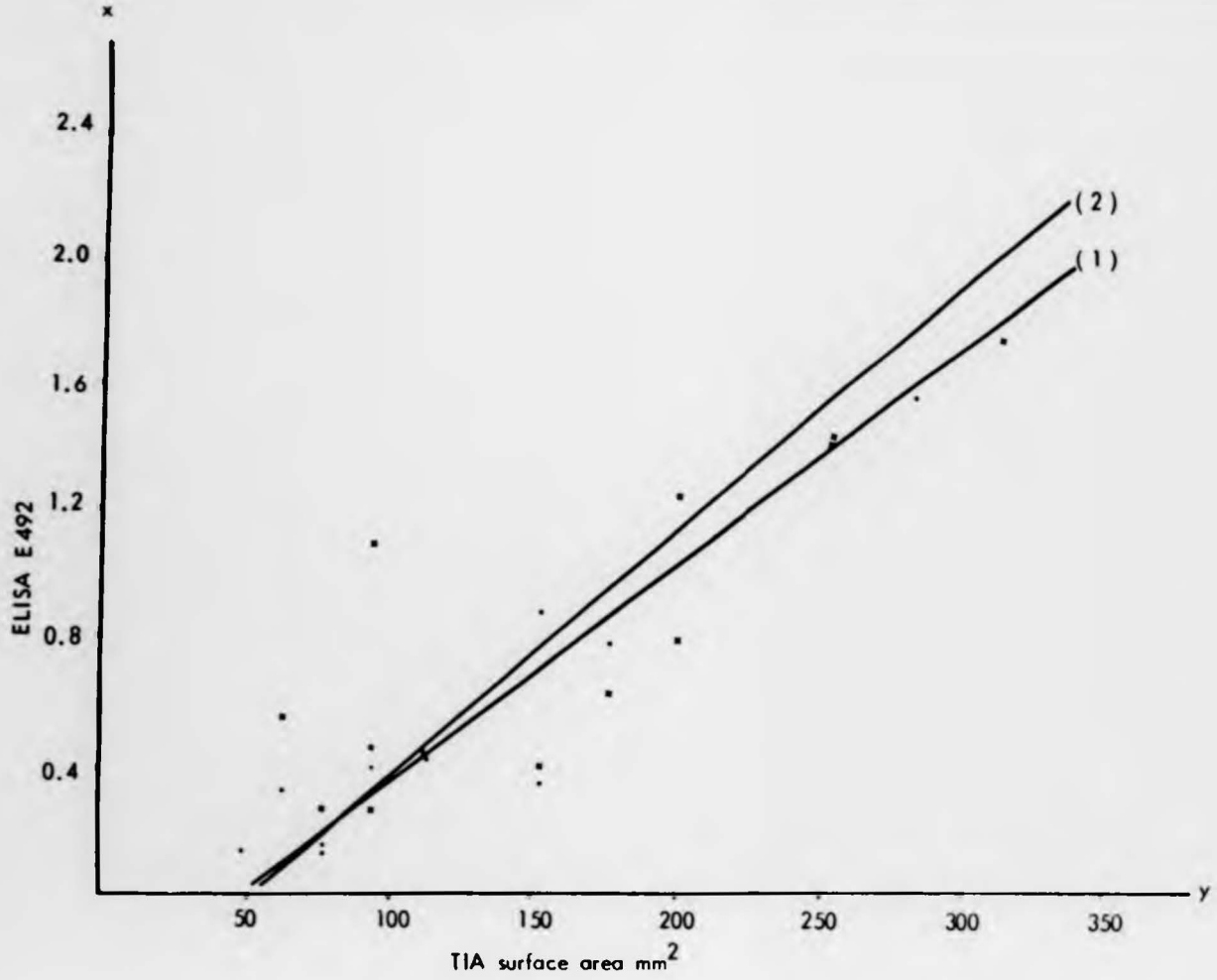
Scatter diagram showing the relation between the extinction of ELISA (E_{492}) and the surface area of TIA using human sera from S.haematobium cases before (.) and after (x) treatment and S.mansoni adult worm antigen

Correlation coefficient, r is significant for both:

(1) Before treatment $r = 0.85$ $p < 0.01$

(2) After treatment $r = 0.93$ $p < 0.001$


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E492
(2)
(1)
y
50
100
150
200
250
300
350
TIA surface area mm2


x
2.4
2.0
1.6
1.2
0.8
0.4
ELISA E492
(2)
(1)
y
50
100
150
200
250
300
350
TIA surface area mm2

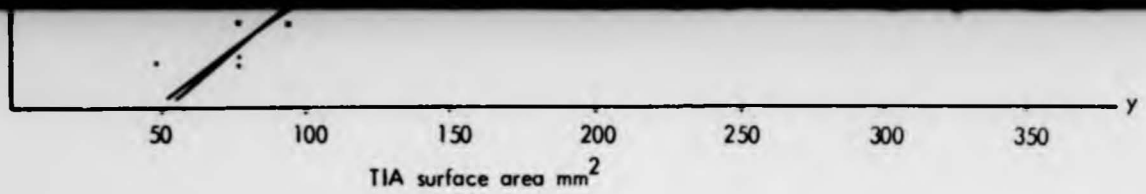


Figure 39

Scatter diagram showing the relation between the extinction of ELISA (E_{492}) and the surface area of TIA using human sera from S.mansoni cases before (.) and after (x) treatment and S.haematobium adult worm antigen

Correlation coefficient, r is significant for both:

(1) Before treatment $r = 0.94$ $p < 0.001$

(2) After treatment $r = 0.81$ $p < 0.001$

Table 10

Serum antibody levels (IgG) before and 6 weeks after successful treatment with Hycanthone in 8 patients with S.haematobium infections and 11 patients with S.mansoni infections against S.haematobium worm antigen using both tests, ELISA and TIA

Type of schistosome infection	Number of patients	ELISA value E_{492} Mean ± SE		% showing rise	TIA surface area mm^2 Mean ± SE		% showing rise
		Before treatment	After treatment		Before treatment	After treatment	
S.haematobium (1)	8	0.49 ± 0.10	0.81 ± 0.20	50.0	83 ± 15	126 ± 18	50.0
S.mansoni (2)	11	0.50 ± 0.12	0.80 ± 0.15	45.5	123 ± 20	157 ± 24	36.0

(1) Paired t test, $p < 0.05$ in ELISA and < 0.002 in TIA

(2) Paired t test, $p < 0.01$ in ELISA and < 0.05 in TIA

Table 11

Serum antibody levels (IgG) before and 6 weeks after successful treatment with Hycanthone in 8 patients with S.haematobium infections and 11 patients with S.mansoni infections against S.mansoni worm antigen using both tests, ELISA and TIA

Type of schistosome infection	Number of patients	ELISA value E_{492} Mean ± SE			TIA surface area mm^2 Mean ± SE		
		Before treatment	After treatment	% showing rise	Before treatment	After treatment	% showing rise
S.haematobium(1)	8	0.37 ± 0.08	0.56 ± 0.14	50.0	71 ± 17	98 ± 23	50.0
S.mansoni(2)	11	0.64 ± 0.1	0.98 ± 0.13	63.6	116 ± 21	151 ± 22	54.6

(1) Paired t test, $p < 0.05$ in ELISA and TIA

(2) Paired t test, $p < 0.001$ in ELISA and < 0.05 in TIA

preparations. Huldt et al., (1975) and McLaren et al., (1978) have shown that both sensitivity and specificity of ELISA were much improved by using antigens derived from schistosome eggs, rather than adult worms. Unfortunately, insufficient egg antigen was available for TIA at this time, but a comparison between the egg and the worm antigens in the TIA should be carried out.

TIA enjoys the advantage of being extremely simple to perform and requiring little in the way of sophisticated equipment. At present it suffers from the disadvantage of requiring higher concentrations of antigen than the ELISA, for example in the TIA an antigen solution containing 100 μg/ml can be used to coat a plate to test 12 sera and can be reused at least 4 times giving a total of 60 sera, whereas in the ELISA only 5 μg/ml of antigen is used to test a similar number of sera. Further investigations will hopefully lead to modifications which will in part at least overcome this disadvantage. TIA certainly has a great potential, particularly in respect of work carried out in the field.

Chapter VII

A trial of the ELISA test for the detection of circulating schistosome antigen in sera from humans and animals infected with S.haematobium

Introduction

Circulating schistosome antigens have been detected in plasma or serum of experimental animals and humans infected with schistosomes, using different serological techniques. Berggren and Weller (1967), Gold et al. (1969), Hillyer (1971), Nash et al. (1974), Deelder et al. (1976), Hirata (1976), using immunodiffusion (ID) and/or immunoelectrophoresis (IE), were able to detect circulating antigen in mice and hamsters heavily infected with S.mansoni or S.japonicum. Antigen has also been demonstrated in rabbits infected with S.japonicum (Hirata and Akusawa, 1975), in chimpanzees infected with S.japonicum (Hillyer, 1976) and in baboons infected with S.mansoni (Houba et al., 1976), using ID, IE or CIE respectively. Dodin et al., (1966), using ID and IE, detected circulating schistosome antigen in sera of patients on the seventh day of treatment. Using double counterimmunoelectrophoresis (DCIE), Phillips and Draper (1975) were able to demonstrate a combination of antigens and antibodies, as immune complexes, in sera of some patients suffering from S.mansoni. Hernández-Almenas and Hillyer,

(1973); Madwar and Voller, (1975); Carlier et al.. (1975); Hillyer et al., (1975 and 1976); using ID and/or IE, reported the presence of circulating antigen in sera from patients infected with S.mansoni or S.haematobium.

However, ID and IE are relatively insensitive procedures for the detection of antigenic reactants, therefore, Bawden and Weller (1974) have used CFT for the detection of circulating antigens. They were able to demonstrate antigen in sera of animals as early as the 18th day after infection, indicating that the antigen had a worm rather than an egg origin. Additionally, the antigen was not detectable in egg extracts. Furthermore, Nash (1974), Lichtenberg et al., (1974), using IF techniques, showed that the gut, and not the integument of the adult worm, is the site of production of circulating schistosome antigens. Nash (1974) also confirmed the previous suggestion of the polysacchoride nature of circulating antigen (Gold et al., 1969; Nash et al., 1974; Bawden and Weller, 1974). Recently IgG and IgM antibodies to a polysaccharide present in the epithelial cells of the gut of adult schistosomes, were detected by IF techniques, in high titres in infected patients and animals (Nash, 1978; Nash et al., 1978).

In comparative studies, using different serological methods (e.g. ID, DCIE, CFT and ELISA) for the detection of

circulating antigen and antibody in patients with S.haematobium or S.mansoni. Madwar and Voller (1977) reported that DCIE was more sensitive than the ELISA. They also stated that ELISA was still in the development phase. More recently, Ferreira et al., (1979), using the ELISA, were able to detect circulating antigen in mice with light bisexual and unisexual male worm S.mansoni infection.

The present investigation describes attempts to use the ELISA to detect circulating antigen in animals and humans infected with S.haematobium.

Materials and Methods

Antigen

Crude soluble antigen, prepared from S.haematobium adult worms as described in Chapter II, was used in this study as a reference.

Sera

The human sera examined in this study were chosen from Egyptian patients infected with S.haematobium as described in Chapter II. Ten sera from Egypt from people with helminth infections other than schistosomes were used as controls.

The animal sera were from:

i. Four baboons, infected with S.haematobium, variously

treated and reinfected as described in Chapter V.

ii. Two groups of four hamsters each, group one was infected with 250 S.haematobium cercariae, while the second group was infected with 500 S.haematobium cercariae each. The hamsters were each bled weekly, for ten weeks, after which they were then perfused as described in Chapter II.

Anti-worm serum

Anti-S.haematobium adult worm serum was prepared in rabbits as described in Chapter II.

The immunoglobulin fraction of this anti-worm serum was separated according to the technique of Voller et al. (1976c). Half of it was conjugated with alkaline phosphatase and kept at 4°C to be used as indicator for ELISA, while the other half was kept at -20°C in aliquots of 200 µl at a concentration of 1 mg protein per ml, till used.

The conjugation of the immunoglobulin with alkaline phosphatase was kindly carried out by Dr. D.E. Bidwell, Nuffield Institute, London Zoo, according to the method of Voller et al. (1976c).

ELISA for the detection of antigen

The double antibody sandwich method of ELISA for the detection of antigen was carried out according to the

original method of Voller et al., (1976a), but with some modifications. Each well of the microtitre plates was coated with 200 µl of the immunoglobulin fraction of the anti-worm serum made up to 10 µg protein/ml in carbonate-bicarbonate coating buffer. Coating was carried out at 37°C for three hours and plates were then washed as described in Chapter IV, using saline-Tween solution. The plates were then shaken dry and 200 µl of the test sera diluted $\frac{1}{10}$ in PBS-Tween was added for each well and incubated at 37°C for two hours afterwards. Plates were then washed as before. Two hundred µl of conjugated anti-worm immunoglobulin diluted $\frac{1}{1000}$ in PBS-Tween was added to each well and incubated overnight at 4°C. The plates were washed and 200 µl of the substrate (P-nitrophenyl phosphate 1 mg/ml in 10% diethanolamine buffer) was added to each well and incubated at 37°C for one hour and the reaction in all wells was then stopped by the addition of 50 µl of 3M NaOH. The adult worm S.haematobium antigen at a concentration of 5-10 µg protein/ml was used as a positive reference. The contents of each well were removed and the absorbance at 400 nM was read in a spectrophotometer. Samples with an extinction $E_{400} > 0.20$ were presumed to be positive. Fig. 40 shows a schematic illustration of the double antibody sandwich method of ELISA.

The specificity of the circulating antigen

This was determined according to the blocking assay of

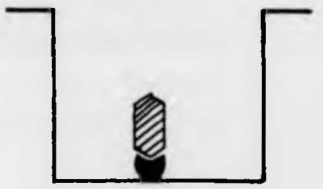
Figure 40

Schematic illustration of the double antibody sandwich method for microplate ELISA for the detection and measurement of antigen

(1) Antibody adsorbed to plate

Wash

(2) Add test solution, any specific antigen attaches to antibody



Wash

(3) Add enzyme labelled specific antibody

Wash

(4) Add enzyme substrate

Amount hydrolysed = antigen present

Reproduced from: Voller et al., 1976
Bull. W.H.O. 53, 55-65

Duermeyer et al.. (1978), using positive and negative sera, and shown in Fig.41. The immunoglobulin fractions of normal rabbit serum and of rabbit anti-worm serum were used as negative and positive sera respectively. Different concentrations of these sera (40-120 µg protein/ml) were tried and the best results in blocking the reaction were obtained when these sera were used at a concentration of 120 µg protein/ml. They were added after incubation of the test sera. Samples were regarded as positive if the ratio of the extinctions of the samples after incubation with the negative serum (no blocking) or the positive serum (blocking) was $\geq$ 2.0.

Results

The results show that 36 out of 50 known human Egyptian S.haematobium cases examined were positive for adult worm circulating antigen (Table 12). These sera positive for circulating antigen became negative after blocking the reaction as described above. None of the control sera examined (human Egyptian sera without S.haematobium ova in urine) gave positive ELISA values for circulating worm antigens.

Schistosomal worm circulating antigen and relevant antibody levels, in the experimentally infected baboons are shown in Figs. 42, 43, 44 and 45. The changes in antibody level with treatment and reinfection have been described in Chapter V.

Figure 41

Schematic illustration of the principle of blocking reaction

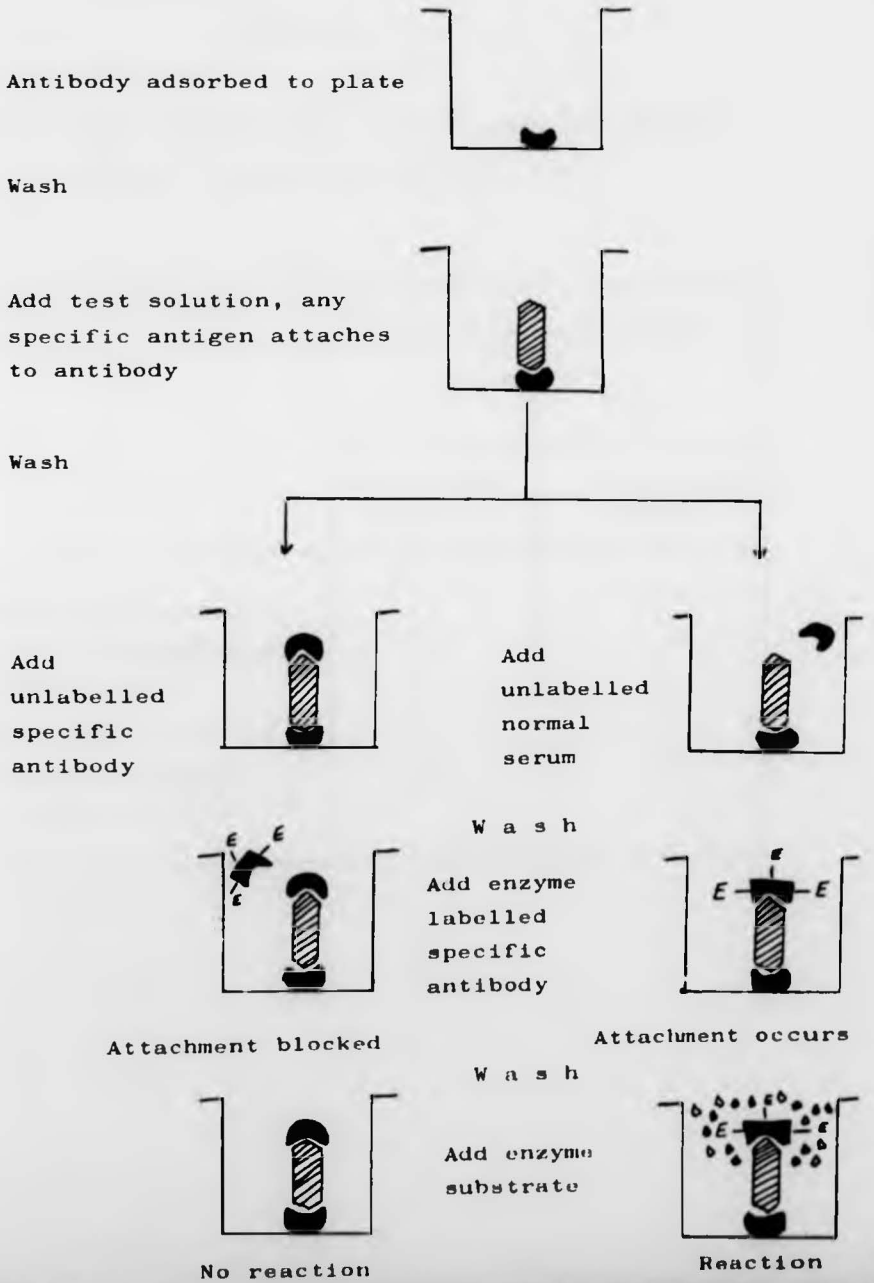


Figure 41

Schematic illustration of the principle of blocking reaction

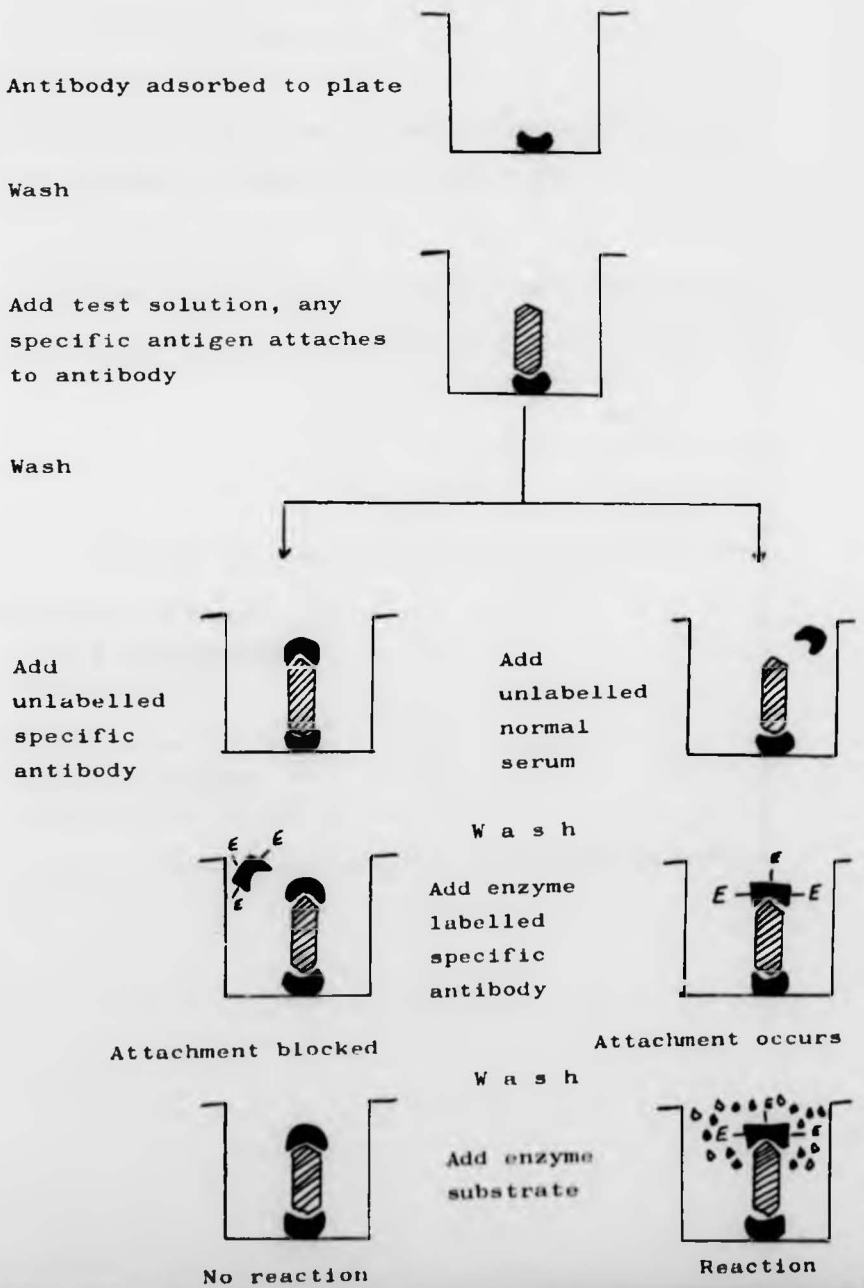
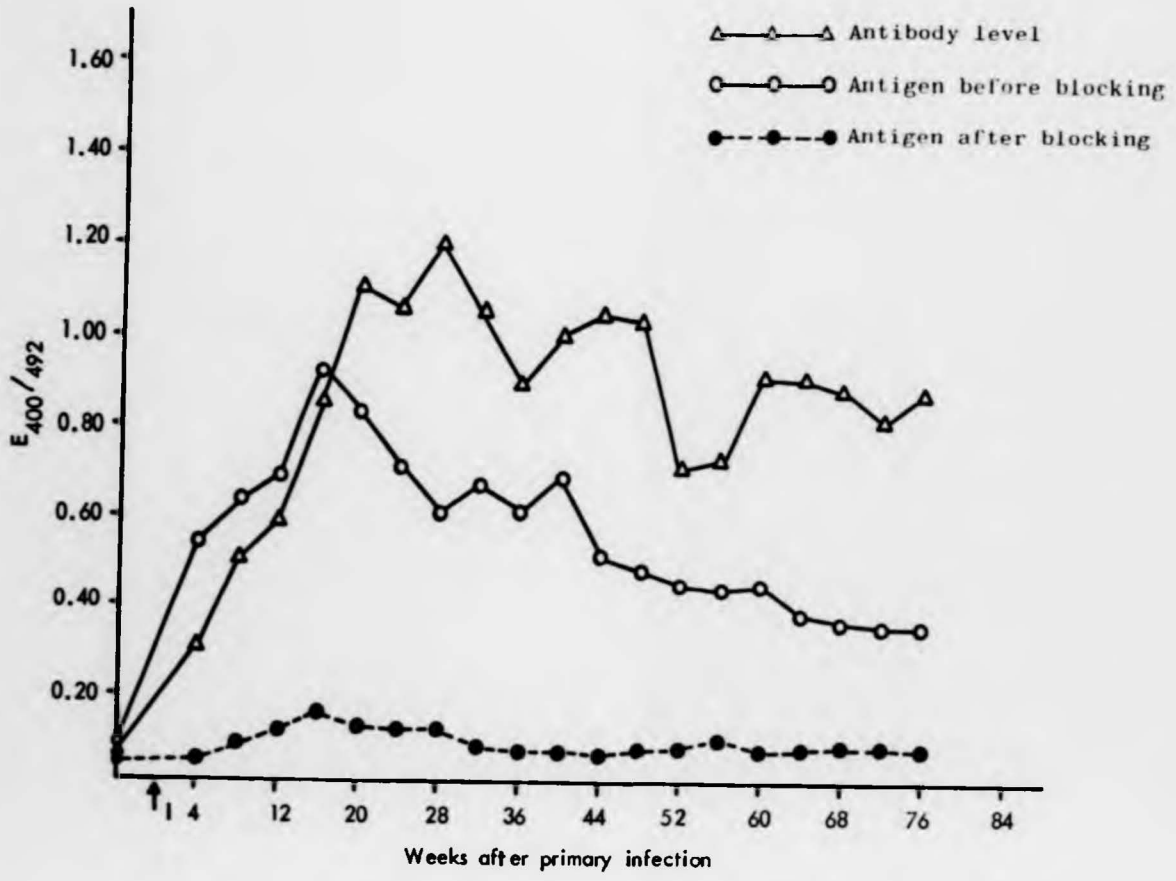


Table 12

The results of ELISA for a schistosome circulating antigen in Egyptian patients' sera

	No. of patients with	
	positive	negative
	E_{400} value > 0.2	E_{400} value ≤ 0.2
50 Egyptian patients with S.haematobium ova in urine	36	14
10 Egyptian patients without S.haematobium ova in urine	0	10


Antibody level
Antigen before blocking
Antigen after blocking
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
$E_{400/492}$
1
4
12
20
28
36
44
52
60
68
76
84
Weeks after primary infection

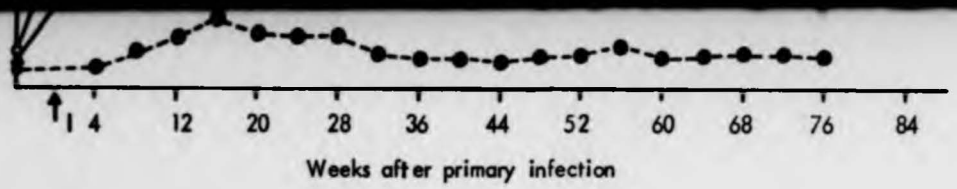
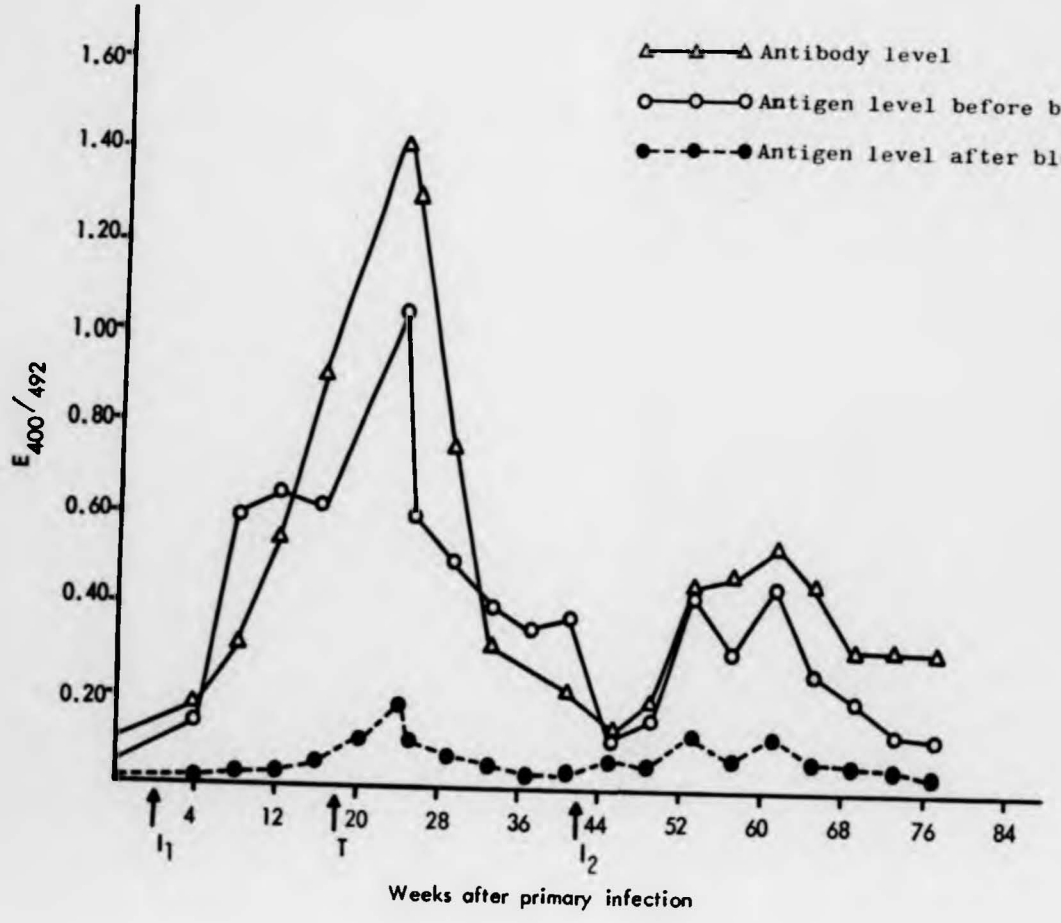


Figure 42

Diagram showing the antibody and antigen levels, in an untreated baboon

I, infection with 7,000 S.haematobium cercariae


Antibody level
Antigen level before blocking
Antigen level after blocking
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
$E_{400/492}$
4
12
20
28
36
44
52
60
68
76
84
I_1
T
I_2
Weeks after primary infection

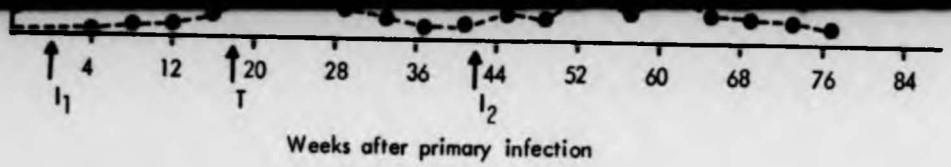


Figure 43

Diagram showing the antibody and antigen levels, in a treated and reinfected baboon

I_1, infection with 7,000 S.haematobium cercariae

T, treatment with a dinitro-thiophane derivative

I_2, reinfection with 7,500 S.haematobium cercariae

Figure 44

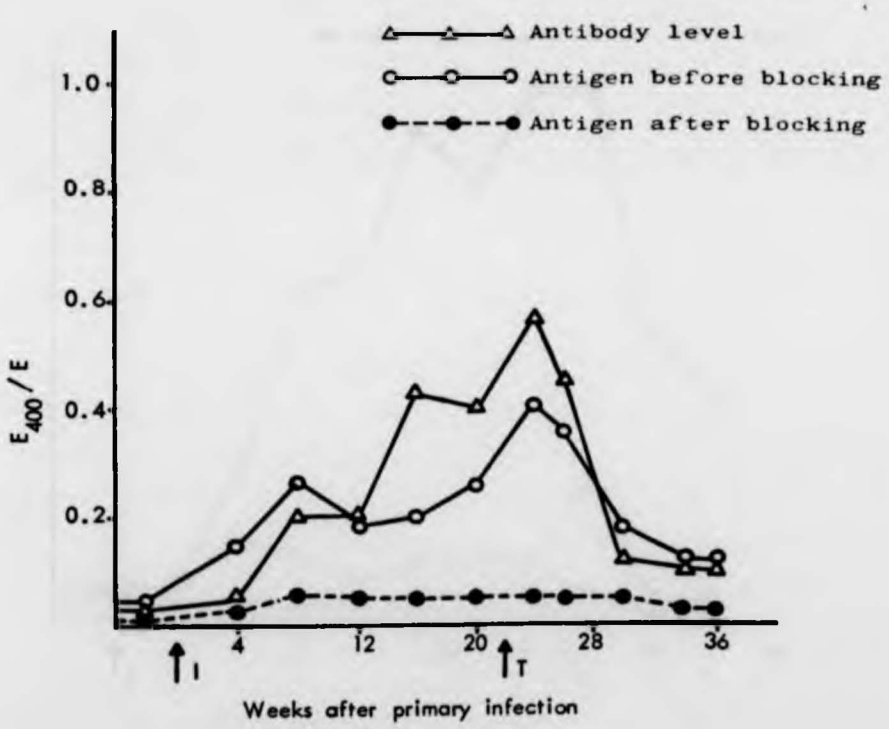


Diagram showing the antibody and antigen levels in a treated baboon

I, infection with 7,000 S.haematobium cercariae

T, treatment with Niridazole

Figure 45

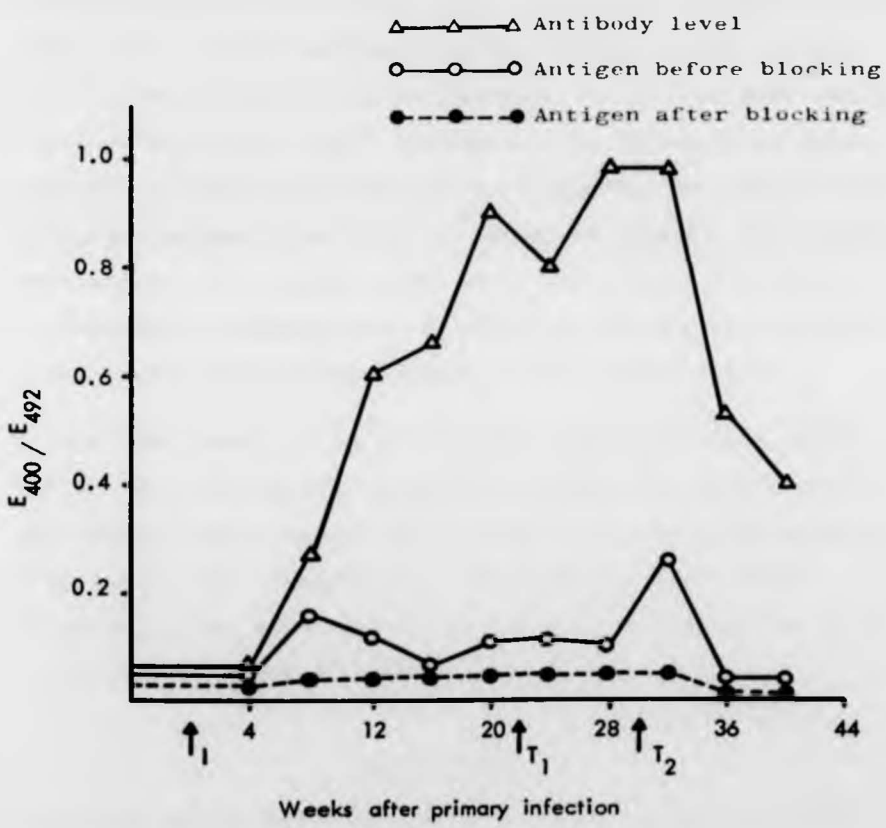


Diagram showing the antibody and antigen levels in a treated baboon

I, infection with 7,000 S.haematobium cercariae

T_1, treatment with RO11-3128 (Hoffman La Roche)

T_2, retreatment with the same drug as above

The circulating antigen began to rise 4-7 weeks after the primary infection, its appearance preceding that of detectable antibody. The circulating antigen varied similarly to the antibody before and after treatment and reinfection (Fig. 43). In the untreated baboon (Fig.42) the antigen level gradually declined in contrast to the antibody which remained at a high level throughout the 80 weeks of observation. In the baboon depicted in Fig.45, the circulating antigen remained at a very low level throughout the experiment except for a small peak after the second treatment. The positive reaction for circulating antigen was blocked using specific anti-worm serum in all the baboons.

None of the hamsters in either group gave positive ELISA values for circulating antigen, although the antibody was detectable from 7 weeks after infection confirming previous observation (cf. Chapter V). The average adult worm recovery in the first group of hamsters was 35 worms, and in the second group 75 worms.

Discussion

The ELISA has already proved to be very sensitive in the detection of antibody; it appears also to be a promising test for the detection of circulating schistosome antigen. The presence of both components, antigen and antibody, in the same sample of serum strongly suggests that they are

bound into immune complexes which split during processing as a result of low antibody affinity.

The probable explanation for the 14 Egyptian patients with negative tests for circulating antigen may be that the antigen if present in low amounts occured in the form of immune complexes with a high affinity-type antibody.

The presence of circulating antigen appears to be directly related to the worm burden in S.mansoni infections of mice and hamsters (Berggren and Weller, 1967; Gold et al. 1969; Bawden and Weller, 1974). It may have a possible role in immune complex nephropathy associated with S.mansoni (Hillyer et al., 1973; Hillyer, 1976; Madwar and Voller, 1977).

The failure to detect circulating antiben in hamsters infected with 250-500 S.haematobium cercariae was perhaps surprising. Bawden and Weller (1974) and Deelder et al. (1976) using the CF test and CIE technique were able to detect schistosome circulating antigens only in sera from hamsters and mice heavily infected with S.mansoni, with not less than 100 worms, but had to concentrate their sera and antisera before electrophoresis. The worm counts here were a little lower but a more sensitive technique was used. More recently, Ferreira et al. (1979) using the ELISA reported the detection of circulating schistosome antigens

in mice with light S.mansoni infections, but they also stated that antigens could not be detected in the serum from every infected mouse, which agrees with the results obtained from the S.haematobium human and baboon sera in the present study. There seems, therefore, to be quite a wide variation in the antigen response of different species and of individual animals.

Although small numbers of human and animal sera were used, this investigation showed that ELISA besides its high sensitivity in an antibody detecting system, also has a potential use in the detection of circulating schistosome antigen. It could be used for showing efficacy of chemotherapy.

The inconsistent results reported for the detection of circulating antigen using different methods including the ELISA suggest more investigation should be carried out, such as the characterization of these antigens and the preparation of more specific antisera.

Chapter VIII

Observations on pathology in relation to serological findings in hamsters infected with S.haematobium

Introduction

As shown in the previous chapters of this thesis, anti-schistosome antibodies significantly decrease after treatment of hamsters infected with S.haematobium. Warren et al. (1977) reported that modulation of immunopathology in chronic murine S.mansoni infections remains after curative treatment. There is also direct evidence for amelioration of pathology in chronic S.haematobium infections (Lichtenberg, 1971). Schiller and Haese (1973) found that termination of S.mansoni infection in mice by treatment with a nitro vinylfuran derivative, resulted in almost complete resolution of hepatic injury caused by eggs of these parasites. In endemic areas where patients with schistosomiasis may undergo curative therapy, questions have arisen as to whether the eradication of schistosomes is followed by pathological and serological changes and whether there is any correlation between these and the time at which chemotherapy was given in relation to the infection.

In an attempt to elucidate some of these problems, hamsters infected with S.haematobium were treated with Niridazole, and the pathological changes and antibody

levels were recorded for hamsters at different times after treatment.

Materials and Methods

The sera and tissue samples examined were from two hamster experiments. In the first experiment, 50 hamsters were infected with 200 S.haematobium cercariae each, as described in Chapter II, and divided into 3 groups. The first and second groups of 15 each were treated at 13 weeks (early) and 21 weeks (late) after primary infection respectively, the hamsters of the third group (20 hamsters) were not treated and were used as controls. Treatment consisted of 200 mg/kg body weight of Niridazole daily for 5 days given orally by syringe with a curved blunt needle. At different times after treatment, as described in the Result section, one or two hamsters from each group were killed by injection of undiluted Nembutal as described in Chapter II. After a blood sample was taken from the heart wedge biopsy samples from the livers and lung were removed for light micropsy. The hepatic portal system was then perfused, the adult worms recovered, washed and counted. The second experiment was described in detail in Chapter V. These hamsters were treated at 12 weeks (early) and 22 weeks (late) after infection and were all killed at 41 weeks after infection.

Serological examinations

Serum samples from the different hamsters were examined using the ELISA test for measuring antibody levels as described in Chapter V.

Histological examinations

Pieces of different tissue organs mentioned above were fixed for at least 24 hours in buffered ten per cent formalin (pH 7.0); its composition is given in the appendix.

After fixation, the tissues were embedded in paraffin wax. Sections were cut at 7 μ and routinely stained in Ehrlich's haematoxylin and Eosin (H and E). For specific staining of amyloid and of collagen, congo red and Ven Gieson's stains were used respectively. These were kindly done by Dr. S.P. Lucas at the Department of Morbid Anatomy, St. Thomas Hospital, London.

Immunofluorescent studies

Fresh kidney tissue samples were embedded in O.C.T. Tissue-Tek compound (Ames Company, USA), snap frozen in liquid nitrogen and then stored at -60°C until examined. Cryostat sections, 6 μ in thickness, were cut at -20°C, dried at room temperature and processed for the detection of immunoglobulin (IgG) and schistosome antigen deposits as described by Falcão and Gould (1975), using direct and

indirect immunofluorescent methods respectively.

Direct immunofluorescent method for the detection of IgG. The dried cryostat sections were washed three times with PBS and were then exposed to fluorescein isothiocyanate (FITC) - conjugated swine anti-hamster IgG (Nordic Innumological Labs.), at a dilution of 1:20, for 30 minutes at room temperature in a moist chamber. Three 10 minute washings with PBS were done after staining. They were then mounted in 0.01 M Tris-buffered glycerol, pH 9.6; its composition is given in the appendix.

Indirect immunofluorescent method for the detection of schistosome antigen

Preparations were made by first incubating the dried cryostat sections with 0.02 M sodium citrate, pH 3.2, at 37°C for two hours in order to elute any bound antibody. Some sections were examined without this elution. The sections were then washed three times with PBS, three minutes each, and fixed with a mixture of ether and 95% ethanol (50:50) for ten minutes and with 95% ethanol for a further 20 minutes. They were then incubated with unfluoresceinated rabbit anti-S.haematobium worm serum, diluted 1:2, for 30 minutes at room temperature. The wash procedure was repeated as above and the sections were incubated with FITC-goat anti-rabbit serum (Nordic Immunological Labs.), at a dilution of 1:40 for 30 minutes at room temperature. They were then washed

in PBS and mounted in buffered glycerol as described above.

Sections of normal uninfected hamster kidneys were used as controls. Sections stained with non-conjugated rabbit anti-hamster IgG (Miles) and with normal rabbit serum were included in both the direct and the indirect immunofluorescent test respectively.

The sections were examined with a Leitz fluorescent microscope with a Halogen lamp as the transmitted light source, using excitation filters KP 450 and 500 and interference filter K 530.

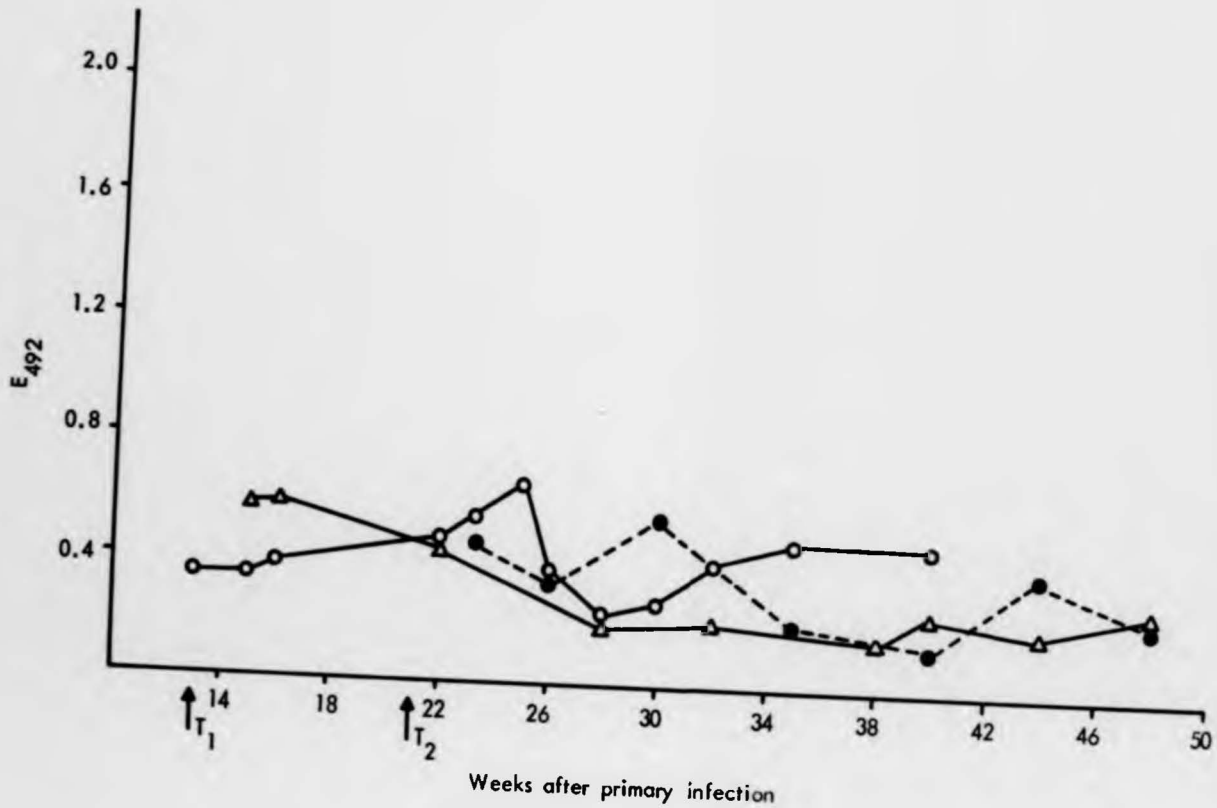
Electron microscopic examination (EM)

The electron microscopy of kidney tissues were kindly carried out by Dr. R.G. Bird at the London School of Hygiene and Tropical Medicine. One millimeter cubes of kidney tissues were fixed in 3% Glutaraldehyde in 0.066 M cacodylate buffer for 12 hours, transferred to 0.066 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated and embedded in epoxy resin (Araldite). Sections were examined with A.E.I. EM 801 electron microscope (A.E.I., U.K.).

Results

Serological findings

Figures 46.1, 46.2 and 46.3 show the antibody levels as measured by the ELISA, using S.haematobium cercarial, worm


2.0
1.6
1.2
0.8
0.4
E_{492}
T_1
14
18
T_2
22
26
30
34
38
42
46
50
Weeks after primary infection

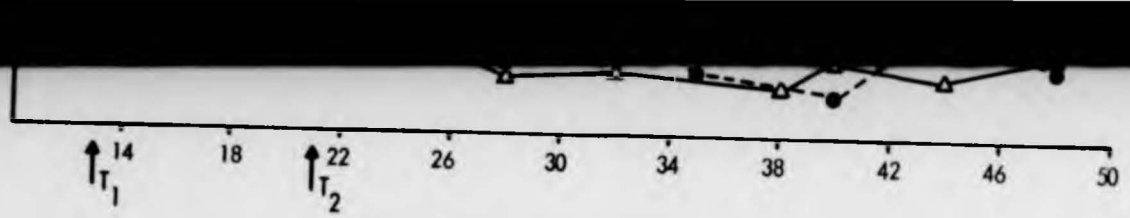
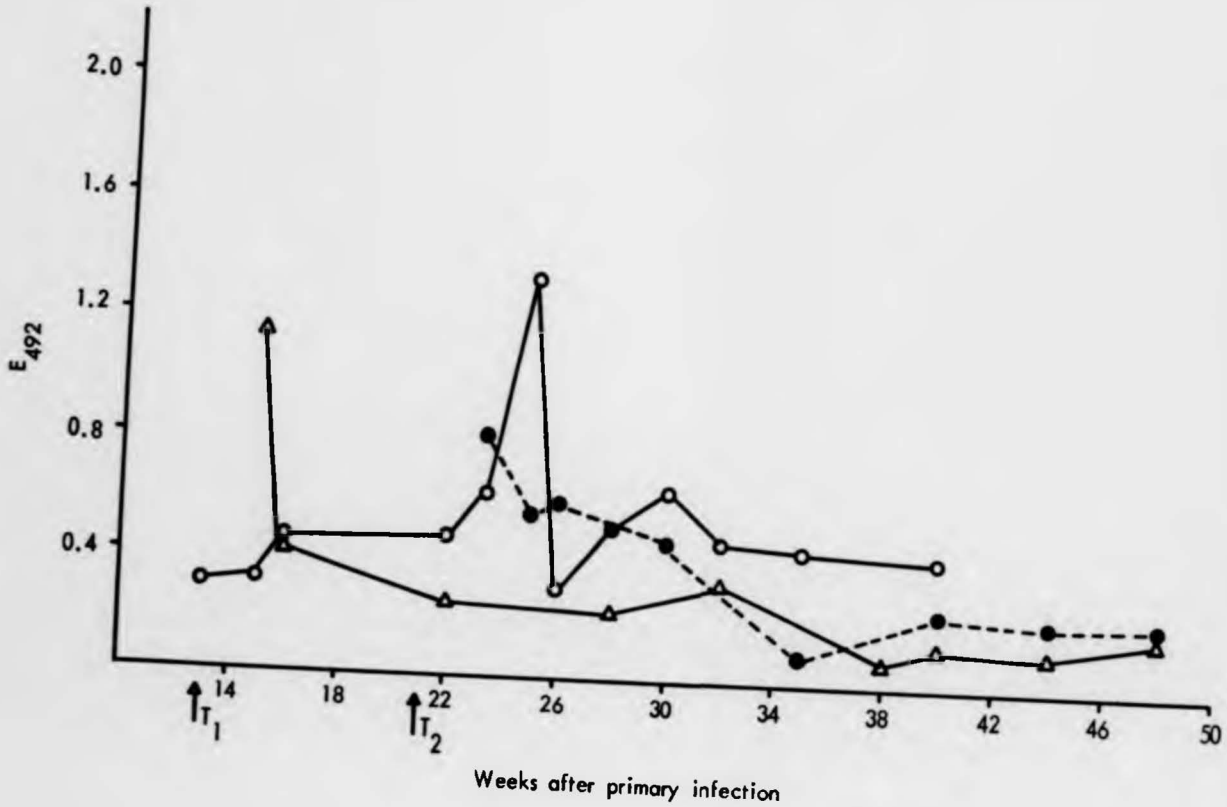


Figure 46.1

Diagram showing the antibody levels of different groups of S.haematobium infected hamsters, treated early in infection (-Δ-Δ-), treated late in infection (-●-●-) and untreated (-O-O-), using S.haematobium cercarial antigen

T_1 and T_2, treatment of the first and second groups respectively with Niridazole 200 mg/kg body weight orally daily for 5 days.


2.0
1.6
1.2
0.8
0.4
E_{492}
T_1
T_2
14
18
22
26
30
34
38
42
46
50
Weeks after primary infection

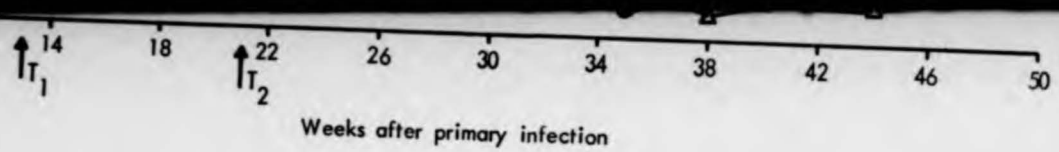
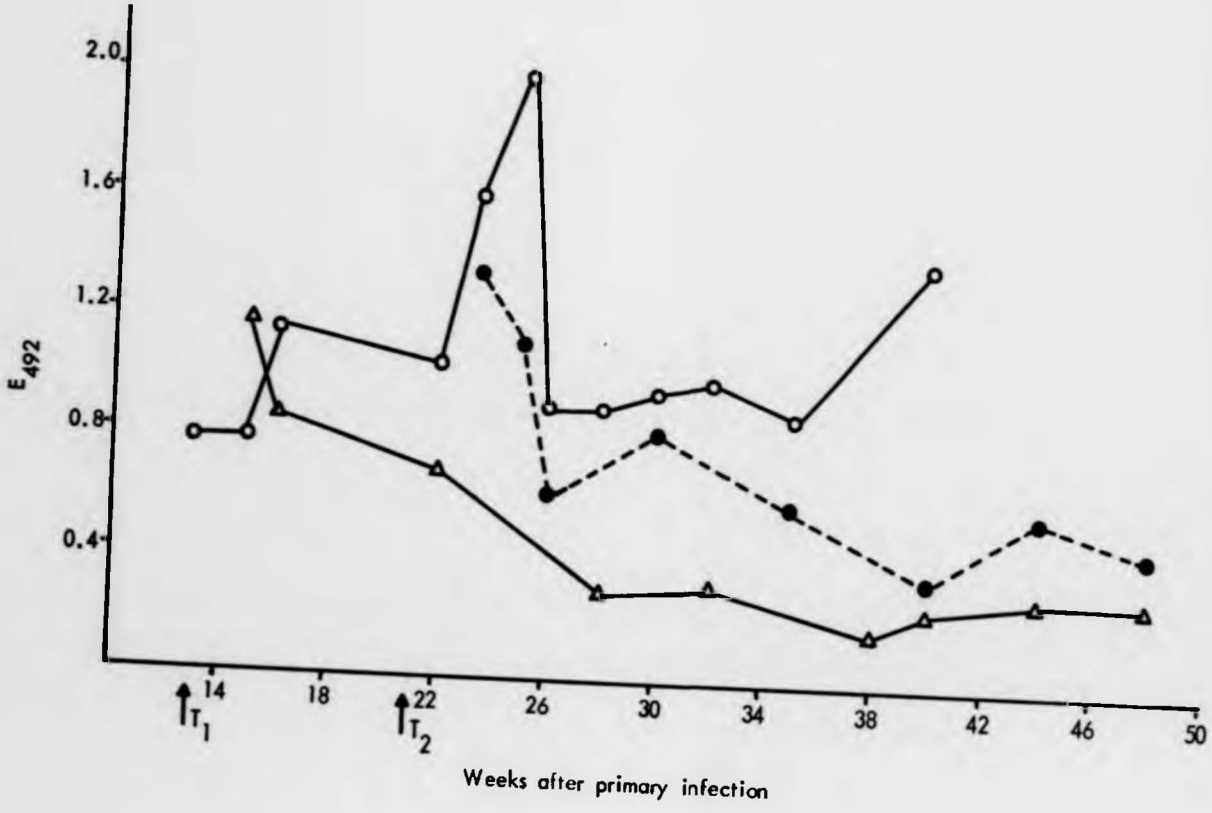


Figure 46.2

Diagram showing the antibody levels of different groups of S.haematobium infected hamsters, treated early in infection (-Δ-Δ-), treated late in infection (-●-●-) and untreated (-o-o-), using S.haematobium worm antigen

T_1 and T_2, treatment of the first and second groups respectively with Niridazole 200 mg/kg body weight orally daily for 5 days.


2.0
1.6
1.2
0.8
0.4
E_{492}
14
18
22
26
30
34
38
42
46
50
T_1
T_2
Weeks after primary infection

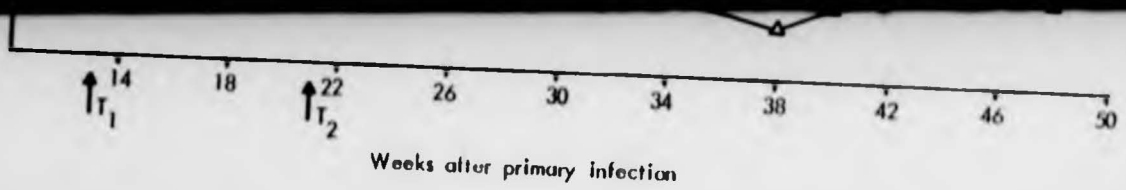


Figure 46.3

Diagram showing the antibody levels of different groups of S.haematobium infected hamsters, treated early in infection (-Δ-Δ-), treated late in infection (-●-●-) and untreated (-o-o-), using S.haematobium egg antigen

T_1 and T_2, treatment of the first and second groups respectively with Niridazole 200 mg/kg body weight orally daily for 5 days.

and egg antigens respectively. Each point represents the reading of one or the mean of two hamsters sacrificed at each time. The antibody level decreased after treatment in contrast to the high antibody level maintained in the untreated hamsters. As shown before, egg antigen proved to be more reactive than both cercarial and worm antigens.

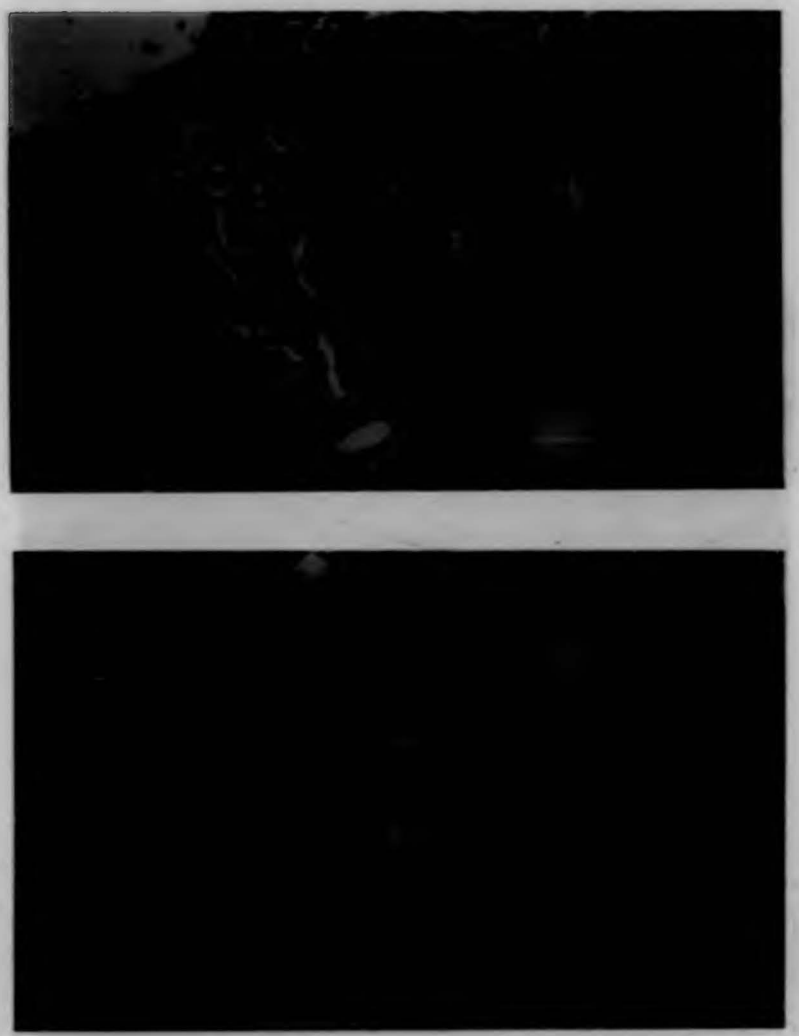
Parasitological findings

The adult worms recovered from the untreated hamsters were active, well developed and both sexes were present in comparable numbers. The total number of worms recovered ranged between 25-40 pairs per hamster. No worms were recovered from any of the treated hamsters.

Histologic observations

In the first experiment, which compares the histopathology of the groups treated early and late in infection, a similar rate of resolution of the perioval reaction was observed in both groups. By two weeks after either treatment, the granulomas were still mature around the degenerate eggs with central necrosis and pigmentation (Fig. 47). Portal tract inflammation was decreasing as was pigment load. By eight weeks after either treatment, portal inflammation was more localised, consisting mainly of foreign body giant cells containing pigments and slight chronic inflammatory cell infiltration in relation to dead eggs, but very scanty granulomas (Fig.48). The pigment was localised in clumps mainly in the portal tract

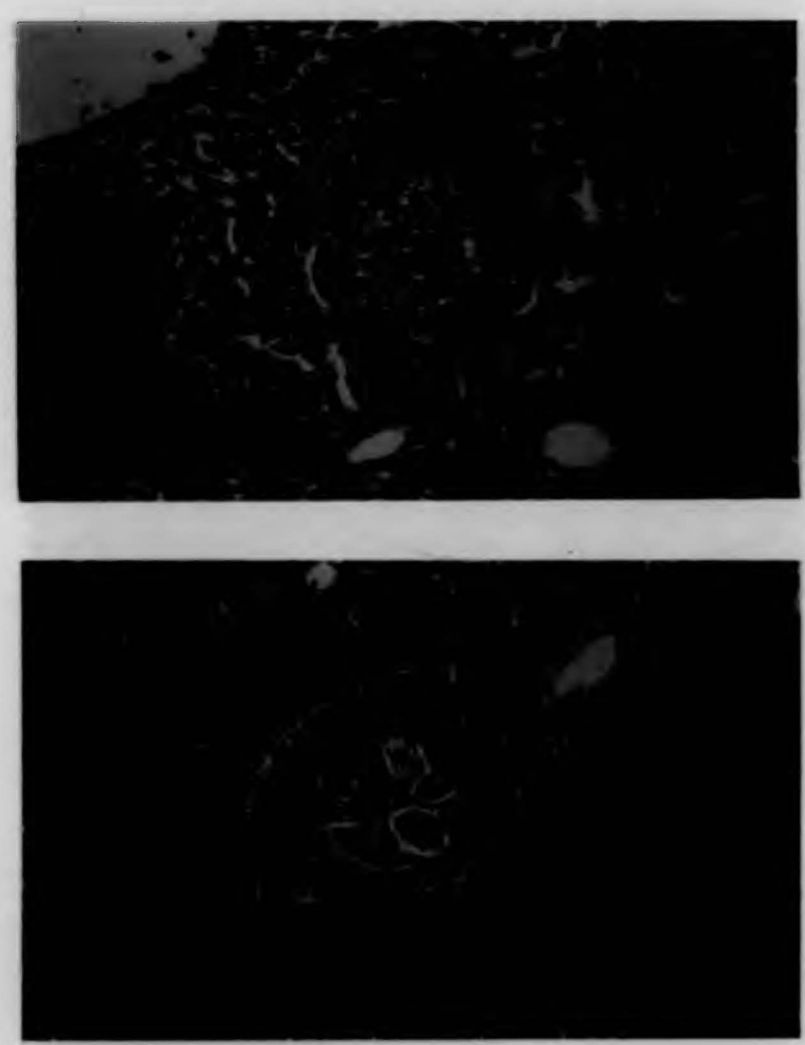
Figure 47



Liver sections from a hamster, two weeks after treatment at 13 weeks after infection, showing mature granulomas around degenerate eggs and pigment in macrophages

(H & E x 200)

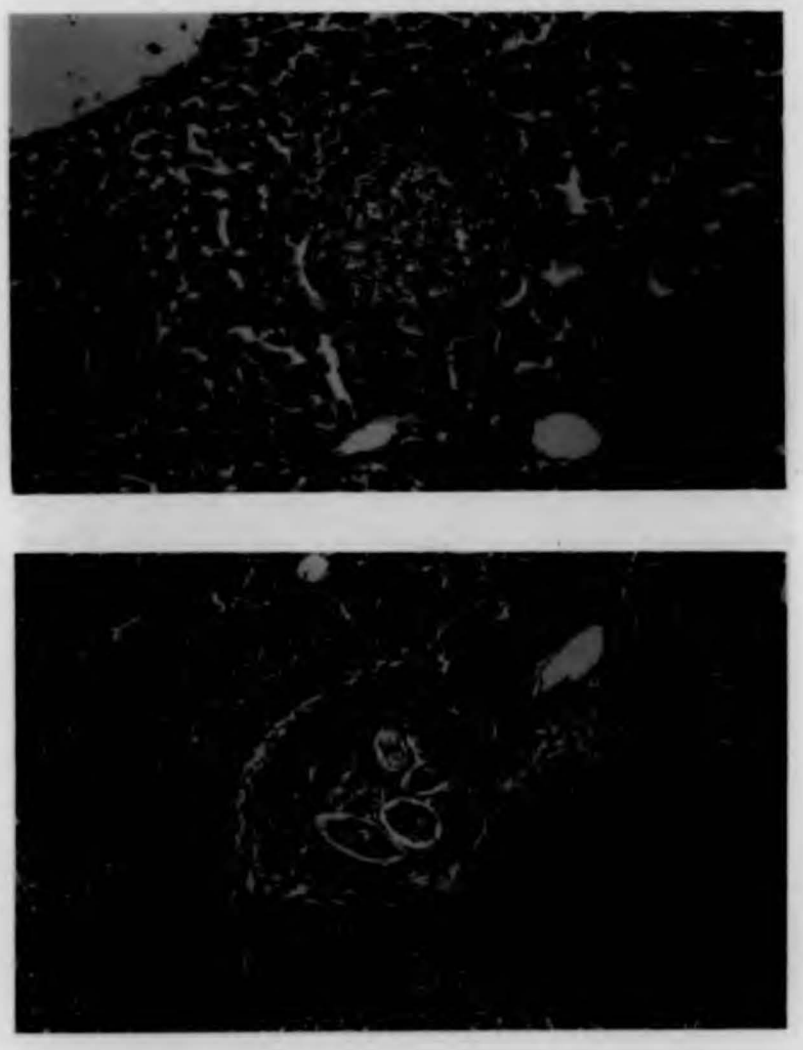
Figure 47



Liver sections from a hamster, two weeks after treatment at 13 weeks after infection, showing mature granulomas around degenerate eggs and pigment in macrophages

(H & E x 200)

Figure 47



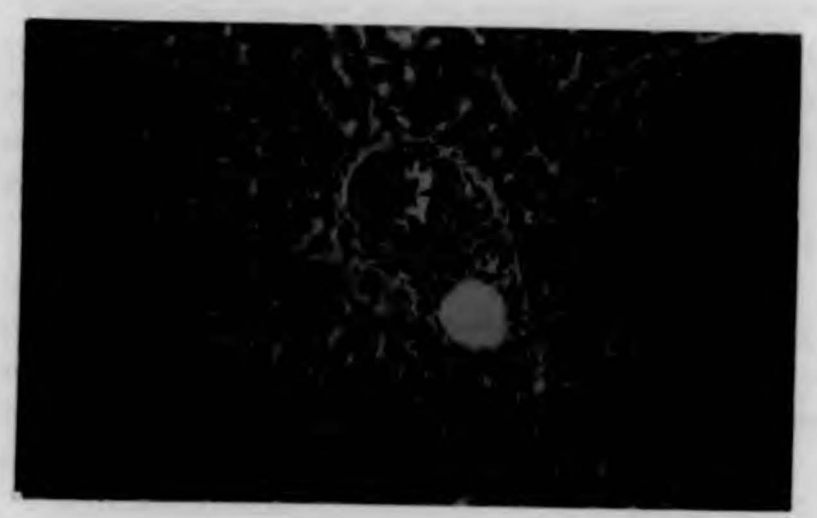
Liver sections from a hamster, two weeks after treatment at 13 weeks after infection, showing mature granulomas around degenerate eggs and pigment in macrophages

(H & E x 200)

Figure 48

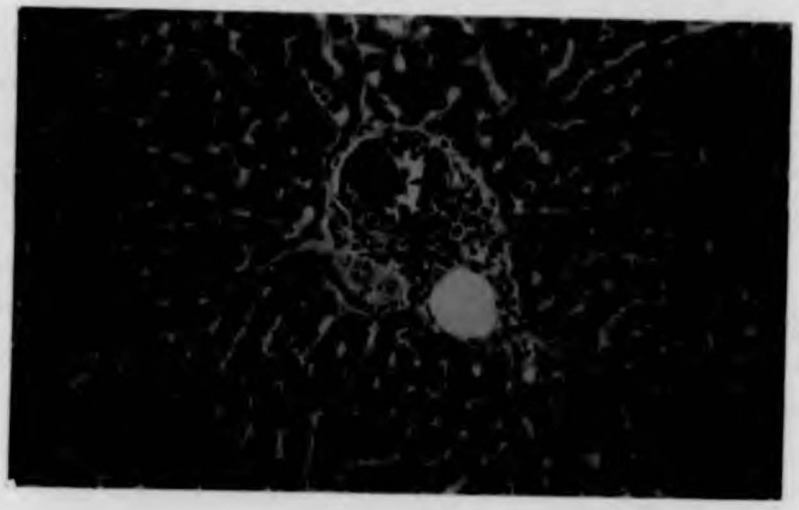
Liver section from a hamster eight weeks after treatment at 21 weeks after infection, showing calcified egg (arrow), foreign body giant cells, pigmentation and inflammatory cells (H & E x 200)

Figure 48



Liver section from a hamster eight weeks after treatment at 21 weeks after infection, showing calcified egg (arrow), foreign body giant cells, pigmentation and inflammatory cells (H & E x 200)

Figure 48

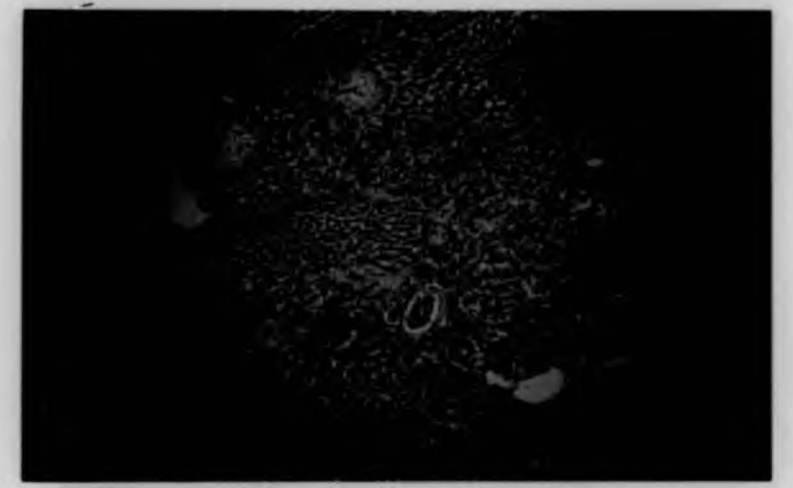


Liver section from a hamster eight weeks after treatment at 21 weeks after infection, showing calcified egg (arrow), foreign body giant cells, pigmentation and inflammatory cells (H & E x 200)

but also in large Kupffer cells within the lobules. The inflammatory reaction progressively diminished, but was still locally present at twenty weeks after treatment. The major difference between the two groups is in the development of fibrosis in the animals treated at 21 weeks after infection compared with animals treated at thirteen weeks. Portal tract fibrosis and bridging were seen in animals sacrificed at three weeks after late treatment with subsequent reduction at weeks four, sixteen and twenty after treatment. No fibrosis was seen in animals sacrificed at twenty weeks after late treatment which suggested a resolution of fibrosis with time (Figs. 49 and 50). The lungs from both early and late treated and untreated hamsters appeared normal except for occasional granulomas seen in some of the untreated animals.

Similar results were seen in the second experiment in which the period of observation was prolonged to 29 weeks. There was minimal schistosomal demage and any hepatic fibrosis that had been present had resolved. An intestinal section from a hamster, nineteen weeks after late treatment, with residual dead eggs and small associated giant cell infiltration, is shown in Fig.51. In untreated hamsters, at the same time after infection, there was a range of pathology in the liver and the bladder ranging from simple granulomas without fibrosis to severe portal

Figure 49

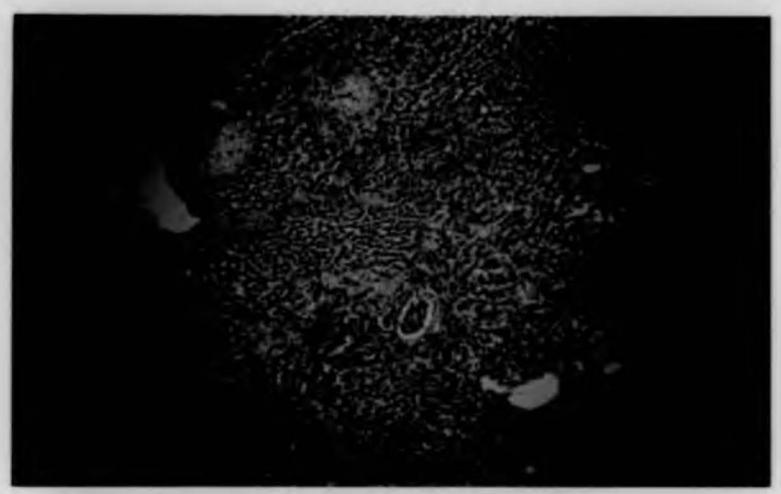


Liver section from one of the hamsters, three weeks after treatment at 21 weeks after infection, showing portal fibrosis and bridging, and pigmentation (V.G. stain x 50)

Figure 50

Liver section from one of the hamsters, twenty weeks after treatment at 21 weeks after infection, showing resolving focal portal inflammation and pigmentation but no fibrosis (H & E x 125)

Figure 49



Liver section from one of the hamsters, three weeks after treatment at 21 weeks after infection, showing portal fibrosis and bridging, and pigmentation (V.G. stain x 50)

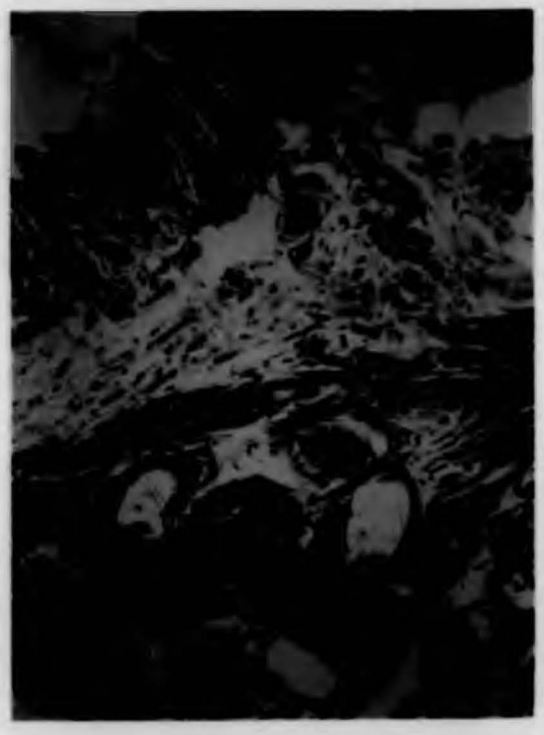
Figure 50

Liver section from one of the hamsters, twenty weeks after treatment at 21 weeks after infection, showing resolving focal portal inflammation and pigmentation but no fibrosis (H & E x 125)

Figure 51

Large intestine of a hamster, nineteen weeks after late treatment showing little reaction around dead eggs in the muscular layer (H & E x 125)

Figure 51



Large intestine of a hamster, nineteen weeks after late treatment showing little reaction around dead eggs in the muscular layer (H & E x 125)

tract fibrosis with little inflammation around dead eggs (Figs. 52 and 53). The intestinal pathology in untreated animals tended to be patchy, some areas having large numbers of eggs and granulomas and others appearing normal or with little inflammation (Fig. 54). Light microscopic examination of the kidneys of both early and late treated hamsters revealed no pathological changes. In the untreated hamsters, only three out of ten examined showed diffuse amyloid deposits in the glomeruli (Fig.55). No other remarkable changes were found in other parts of the kidney.

Direct immunofluorescence

Ninety per cent of more than 70 glomeruli sampled in the kidney sections of untreated hamsters, showed diffuse granular staining of the mesangium and basement membrane for IgG (Fig. 56.A). A few (6%) of the glomeruli examined showed extensive, lumpy deposits of IgG in the glomerular tufts (Fig. 56.B). Negative results were obtained with kidneys from uninfected control hamsters as well as from hamsters treated early in infection (Fig. 56.C). However, some hamsters treated late in infection (four out of ten examined) gave positive results similar to the untreated hamsters.

Indirect immunofluorescence

Negative results were obtained with all kidney sections

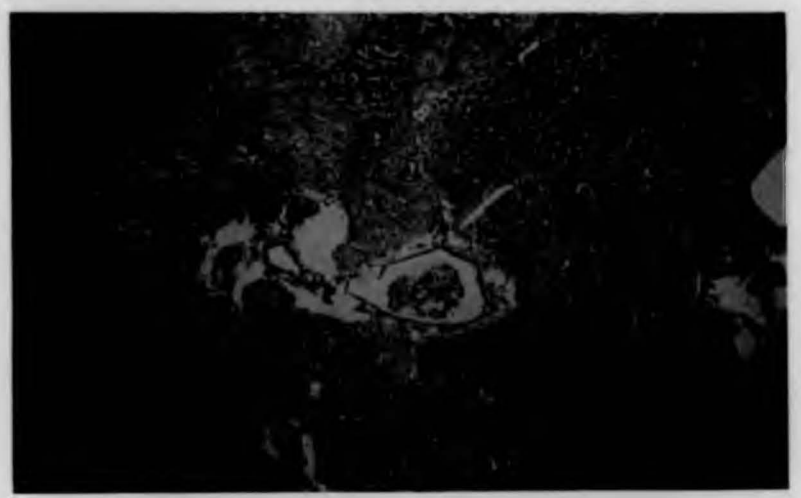
Figure 52

Liver of an untreated hamster, forty-one weeks after infection showing severe portal fibrosis with dead eggs (H & E x 50)

Figure 53

Bladder of an untreated hamster, forty-one weeks after infection showing numerous perioval granulomas and fibrosis in the submucosa and muscle layer. (Eggs were also present in serosa.) (H & E x 50)

Figure 52



Liver of an untreated hamster, forty-one weeks after infection showing severe portal fibrosis with dead eggs (H & E x 50)

Figure 53

Bladder of an untreated hamster, forty-one weeks after infection showing numerous perioval granulomas and fibrosis in the submucosa and muscle layer. (Eggs were also present in serosa.) (H & E x 50)

Figure 54

Large intestine of untreated hamster, forty-one weeks after infection showing numerous submucosal granulomas and eggs in the muscle layer and serosa. (H & E x 50)

Figure 54

Large intestine of untreated hamster, forty-one weeks after infection showing numerous submucosal granulomas and eggs in the muscle layer and serosa. (H & E x 50)

Figure 54

Large intestine of untreated hamster, forty-one weeks after infection showing numerous submucosal granulomas and eggs in the muscle layer and serosa. (H & E x 50)

Figure 55

Micrograph of a kidney section from an untreated hamster, forty-one weeks after infection; no pathological changes visible except diffuse amyloid deposits in the glomeruli which gave strong positive birefringence under polarised light. (Conge red x 200)

Figure 55

Micrograph of a kidney section from an untreated hamster, forty-one weeks after infection; no pathological changes visible except diffuse amyloid deposits in the glomeruli which gave strong positive birefringence under polarised light. (Conge red x 200)

Figure 55

Micrograph of a kidney section from an untreated hamster, forty-one weeks after infection; no pathological changes visible except diffuse amyloid deposits in the glomeruli which gave strong positive birefringence under polarised light. (Conge red x 200)

Figure 56

Direct immunofluorescence in kidney sections:

A. Diffuse granular staining of the mesangium and basement membrane of the glomerulus of an untreated hamster, forty-one weeks after infection.

B. Extensive, lumpy deposits of IgG in the glomerular tufts of an untreated hamster, forty-one weeks after infection.

C. No IgG deposits in the glomerulus of an early treated hamster.

(x 300)

tions:

sangium and
of an untreated
ction.

the glomerular
y-one weeks after

f an early treated

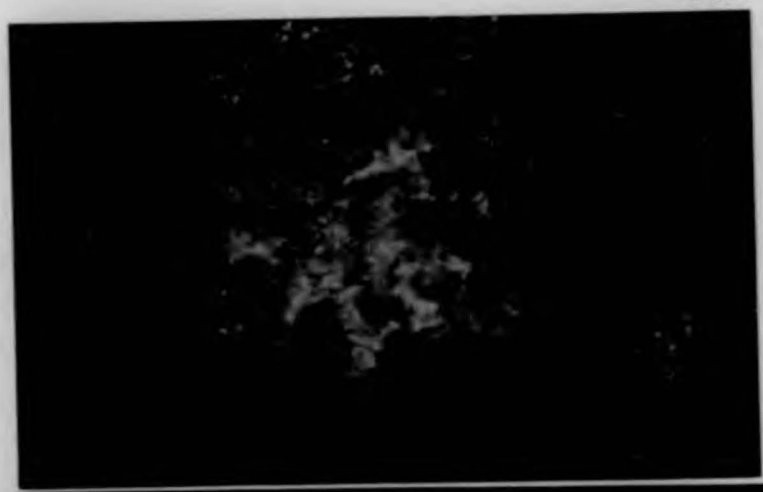


Fig. 56.A

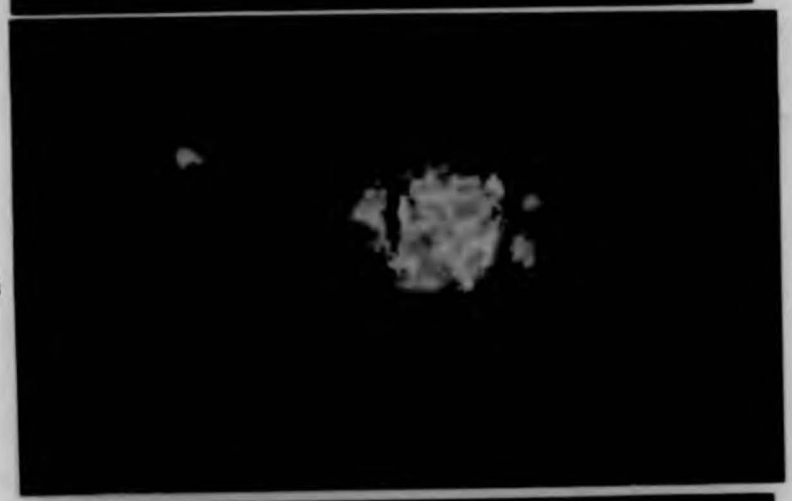


Fig. 56.B

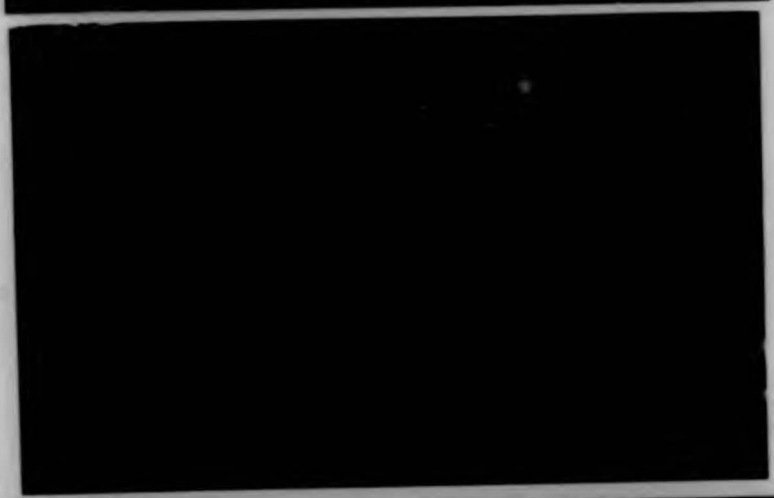


Fig. 56.C

tions:

sangium and
of an untreated
ction.

the glomerular
y-one weeks after

f an early treated

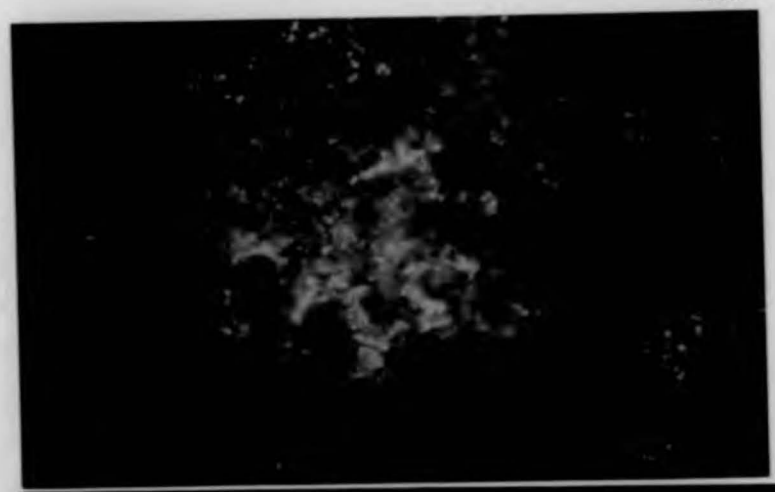


Fig. 56.A

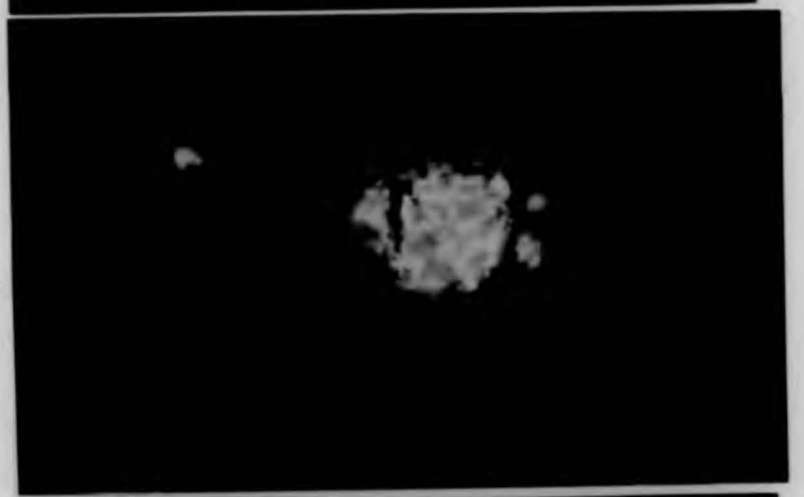


Fig. 56.B

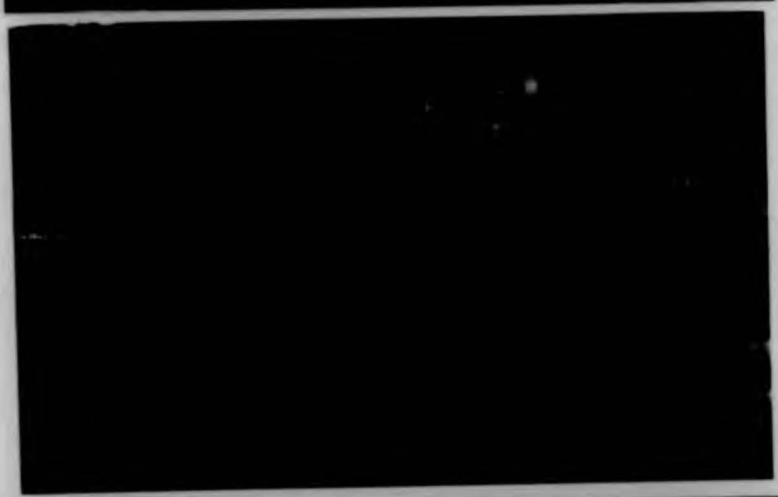


Fig. 56.C

tions:

sangium and
of an untreated
ction.

the glomerular
y-one weeks after

f an early treated

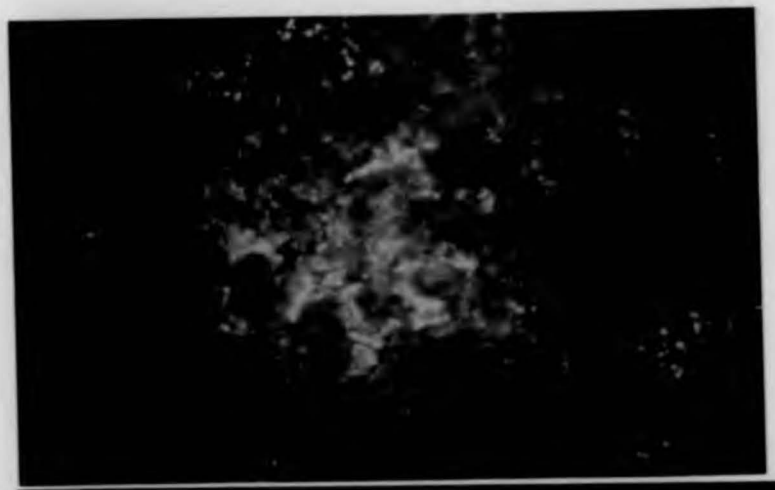


Fig. 56.A

Fig. 56.B

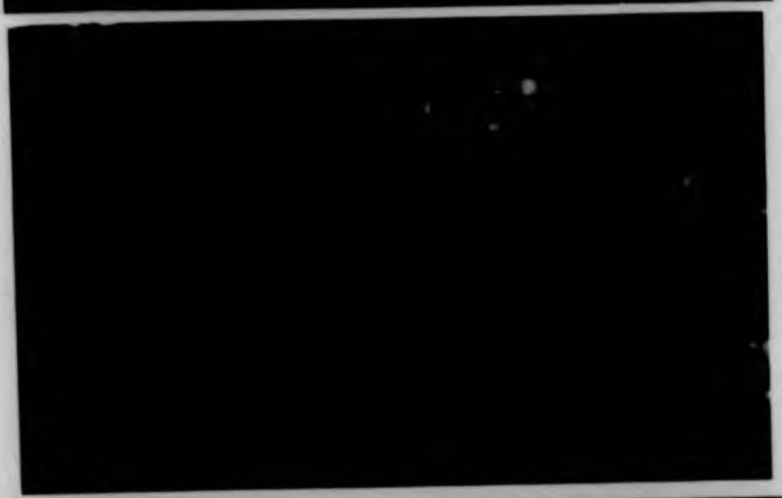


Fig. 56.C

from treated as well as untreated and uninfected control hamsters.

Electron microscopy

Among the kidney tissue specimens of the ten untreated hamsters examined, only two showed deposits within a slightly thickened basement membrane of the glomeruli (Fig.57). None of the early or late treated hamsters showed abnormalities on electron microscope examination. An electron micrograph of the kidney tissue specimen of a normal control (non-infected) hamster is shown in Figure 58.

Discussion

Although the antibody level was measured in different hamsters each time instead of serial observations on the same animals, these results agree with those given earlier in this thesis, showing that antibody level decreases after treatment of schistosome-infected hamsters. This was more evident in the hamsters treated early in infection than in the late treated ones.

The pathological findings caused by S.haematobium in hamsters confirm the conclusion reached by Schiller and Haese (1973), Sadun et al. (1974), from S.mansoni in mice and S.japonicum in chimpanzees respectively, that schistosomal hepatic fibrosis in these animals is eventually

Figure 57

Electron micrograph of a kidney section from an untreated hamster forty-one weeks after infection, showing deposits along the slightly thickened basement membrane (arrow). (x 12,600)

B, basement membrane
E, endothelial cell
M, pseudopodes of mesangial cells
RBC, red blood cell

Figure 58

Electron micrograph of a kidney section from a non-infected hamster, showing normal variation in thickness and no evidence of deposits or pathological thickening. (x 12,800)

tion from an
fter infection,
thickened
(x 12,600)

ls

tion from a
l variation
osits or
(x 12,800)

Figure 57

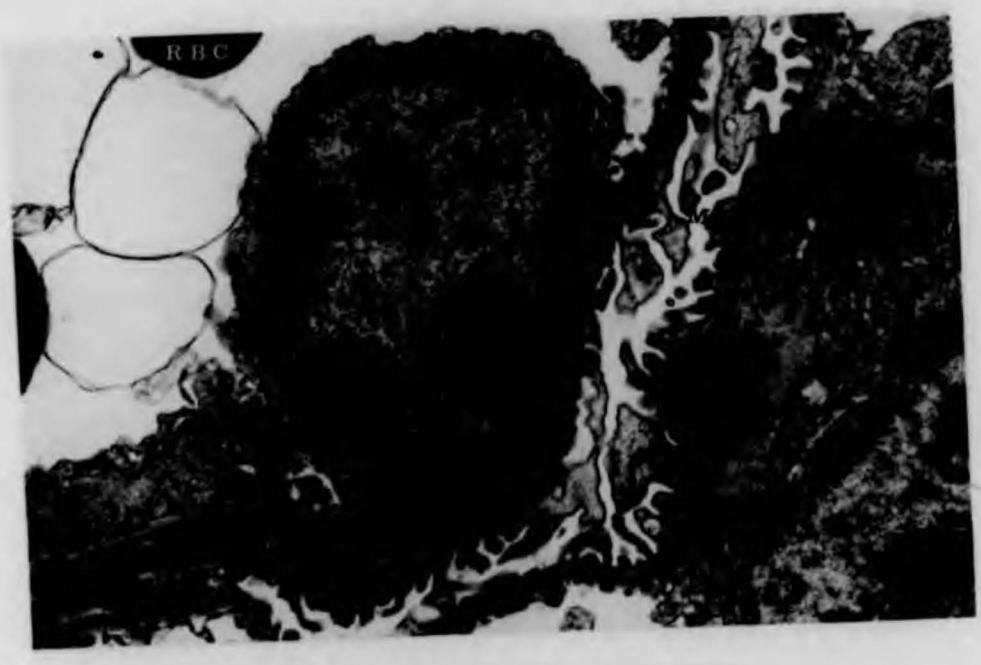
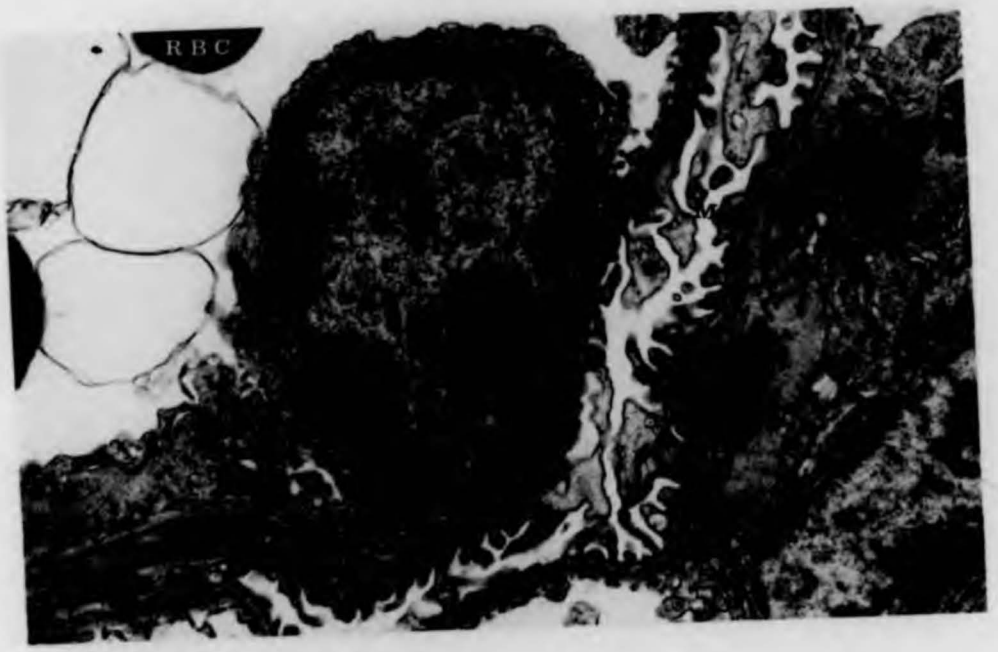


Figure 58

Figure 57



tion from an
fter infection,
thickened
(x 12,600)

ls

Figure 58

tion from a
l variation
osits or
(x 12,800)

almost completely reversible when infection is terminated by effective drug treatment, even when given at 21-22 weeks. This supports the WHO, Scientific Working Group on Research in Bilharziasis (chemotherapy), (1966) which stated that chemotherapy can prevent the establishment of hepatic fibrosis if given early enough in infection. Although dead eggs were found to persist within minute inert scars more than twenty weeks after treatment, most of the associated fibrocellular reactive tissue had disappeared. It appears from these observations that the timing of the initial course of treatment is an important factor as regards the histopathological response to treatment. The earlier the treatment is given the more rapidly and completely the lesions will resolve.

The pathological and serological changes after treatment of schistosome-infected hamsters are, to some extent, correlated with each other in that both decrease. However, other workers (Tada et al., 1975) reported that no correlation was found between some other serological techniques (COP and CF tests) and the pathological findings in Macaca monkeys infected with S.japonicum followed for twelve weeks without treatment.

The granular and lumpy deposits of IgG seen in the direct immunofluorescent studies support the concept of immune complexes being involved in the pathogenesis of schistosomal

renal disease. This may include the amyloid and EM deposits seen in the glomeruli in some of the untreated infected hamsters.

The absence of IgG deposits in kidney sections from hamsters treated early and their presence in some treated late suggested that treatment of schistosome-infected hamsters early enough in infection can ameliorate renal pathology.

The failure to detect parasite antigen in these deposits suggests that perhaps an inadequate antiserum was used or other antigens might be involved.

Brito et al. (1970) and Silva et al. (1970) were among the first to show that immunoglobulin and complement deposits, without parasite antigen, could be found in the glomeruli of human hepatosplenic S.mansoni schistosomiasis. Hillyer and Lewert (1974) reported that parasite antigens were found in the circulation of hamsters and men infected with S.japonicum and S.mansoni and in the case of antigen excess as immune complexes. They also suggested DNA might be the antigen (hapten) involved in the immune complexes. This was further supported by their findings regarding retention of DNA in the kidneys of hamsters infected with S.japonicum as compared to normal controls by autoradiographic and liquid scintillation counting

techniques.

There are conflicting opinions as regards the association between S.haematobium infection in man and the glomerular lesions. Although some workers (Sabbour et al., 1972; Ezzat et al., 1974), on the basis of clinical, laboratory and/or pathological findings, reported a frequent association between glomerulonephritis and S.haematobium infections in Egypt, others (Smith et al., 1974; Sadigursky et al., 1976), on the basis of Egyptian autopsy data, did not find such association.

Beaufils et al., (1978), found glomerular lesions in nine out of thirteen African immigrants in France with S.haematobium infections, but failed to domonstrate schistosomal antigen in the glomeruli. They concluded that renal symptoms such as proteinuria or the nephrotic syndrome could be due to such lesions. They also stated that in man, early diagnosis and adequate treatment of urinary schistosomiasis will perhaps decrease the incidence of glomerular disease in endemic regions.

CHAPTER IX

General Discussion and Summary

Schistosomiasis is a parasitic disease with a large impact on public health and economic development in the countries where this parasite occurs. There is currently a great emphasis on the implementation of control measures to combat the disease in the endemic areas. The need for a sensitive, specific and quantitative immunodiagnostic technique for schistosomiasis is desirable, not only for the diagnosis, but also for application to mass surveys and for the assessment of control measures.

Most of the generally available techniques (as reviewed in the first chapter of this thesis) have given results which have failed to inspire clinicians and epidemiologists with confidence in their sensitivity and specificity. It was, therefore, the main aim of the present study to investigate the sensitivity and specificity of newer serological techniques (CIE, ELISA and TIA) for the detection of anti-schistosome antibodies in a variety of animal and human sera.

The ELISA has been shown to be one of the most sensitive of the newer techniques for the detection of antibodies. Egg antigen was proved to be more reactive than both worm and cercarial antigens. CIE was found to be less sensitive

and required higher concentrations of antigen than the two other tests.

A comparative study has been made between ELISA and TIA and the results indicate excellent correlation between the tests in the quantification of anti-schistosome antibodies, both in humans and animals. TIA has the advantage of simplicity and low cost, but it has the disadvantage of requiring higher concentrations of antigen than ELISA. However, TIA is still in the development stage and after further evaluation it might be used as a supplement or alternative to the other serologic tests for work to be done in the field.

ELISA was found to be a promising test for the detection of circulating schistosome antigen in animals and humans infected with S.haematobium. However, this was a preliminary trial and further studies, using more specific anti-serum, should be carried out.

Chemotherapy is an important method for the control of schistosomiasis, especially if it is used together with other measures in well-designed programmes. Besides its main role in reducing the clinical severity, it has also the advantage of decreasing the rate of transmission. A method for the evaluation of treatment in schistosomiasis is urgently needed. Observations were, therefore, made on antibody levels in baboons and hamsters infected with

S.haematobium and in humans infected with S.haematobium or S.mansoni before and after treatment. The results suggest that the ELISA can be used to monitor the effect of chemotherapy.

The effect of treatment on immunity to reinfection has been studied using hamsters as a model. Groups of hamsters initially infected with S.haematobium cercariae showed different degrees of immunity to homologous reinfection before and after oviposition. Immunity, measured by worm assay, was higher in hamsters challenged after oviposition than in the group challenged before oviposition. However, curative treatment of these hamsters completely abolished all resistance to reinfection. The antibodies measured by the ELISA, significantly declined by the fifth week in response to treatment.

In a group of baboons infected with S.haematobium, there was no correlation between the antibody levels, as measured by the ELISA, and the degree of immunity to reinfection, but a definite correlation with the level of infection.

The pathological changes in relation to serology at different times after treatment of groups of hamsters infected with S.haematobium have been studied. The inflammatory reactions progressively diminshed and the antibody levels declined after treatment. The results also suggested the possible role of immune complexes in renal nephropathy, although schistosome antigen could not be detected in the glomeruli.

REFERENCES

ABDALLAH, A. and RAGGAL, M.H. (1962). The circumoval precipitin test as performed by the use of S.haematobium ova. A preliminary report. Journal of the Egyptian Medical Association, 45, 304-309.

ALLAIN, D.S., CHISHOLM, E.S. and KAGAN, I.G. (1972). Use of the bentonite flocculation test for the diagnosis of Schistosomiasis. Health Services Reports, 87, 550-554.

AMBROISE-THOMAS, O., AMIN, M., OMER, A.H. and FENWICK, A. (1972). Les bilharzioses en République Démocratique du Soudan. Résultats preliminarires d'une enquête séro-épidémiologique dans la région de Gezirah. Bulletin de la Societe de Pathologie Exotique, 65, 675-685.

AMIN, M.A., SAOUD, M.F. and NELSON, G.S. (1969). Cross serological reactions between human and animal schistosomes by the fluorescent antibody technique in some laboratory animals. Annals of Tropical Medicine and Parasitology, 63, 373-375.

ANDERSON, R.I. (1960). Serologic diagnosis of Schistosoma mansoni infections. I. Development of a cercarial antigen slide flocculation test. American Journal of Tropical Medicine and Hygiene, 9, 299-303.

ANDERSON, R.I. and NIAMARK, D.H. (1960). Serologic diagnosis of Schistosoma mansoni infection. II. Sensitivity of intradermal and serologic tests on individuals with an unequivocal diagnosis of schistosomiasis. American Journal of Tropical Medicine and Hygiene, 9, 600-603.

ANDERSON, R.I., SADUN, E.H. and SCHOENBECHLER, M.J. (1963). Cross absorption studies performed with Schistosoma mansoni and Trichinella spiralis antigens on sera from patients with trichinosis. Experimental Parasitology, 14, 323-329.

ANDERSON, R.I., SADUN, E.H. and WILLIAMS, J.S. (1961). A technique for the use of minute amounts of dried blood in the fluorescent antibody test for schistosomiasis. Experimental Parasitology, 11, 111-116.

ANDRADE, Z.A. (1965). Hepatic schistosomiasis, morphological aspects. In, Progress in Liver Disease. Volume II. Eds. H. Popper and F. Schaffner, 228-248. New York.

ANDRADE, Z.A., SANTANA, S. and RUBIN, E. (1962). Hepatic changes in advanced schistosomiasis. Gastroenterology, 42, 393-400.

BÄNFFER, J.R.J. (1972). Demonstration of precipitins against a treponemal antigen by counter-immunoelectrophoresis. Lancet, 1, 996-997.

BAWDEN, M.P. and WELLER, T.H. (1974). Schistosoma mansoni circulating antigen : detection by complement fixation in sera from infected hamsters and mice. American Journal of Tropical Medicine and Hygiene, 23, 1077-1084

BEAUFILS, H., LEBON, P., AURIOL, M. and DANIS, M. (1978). Glomerular lesions in patients with Schistosoma haematobium infection. Tropical and Geographical Medicine, 30, 183-191.

BERGGREN, W.L. and WELLER, T.H. (1967). Immunoelectrophoretic demonstration of specific circulating antigen in animals infected with Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene, 16, 606-612.

BIGUET, J., ROSÉ, F., CAPRON, A. et TRAN VAN KY, P. (1965). Contribution de l'analyse immunoelectrophoretique a la connaissance des antigèns vermineux. Incidences pratiques sur leur standardisation, leur purification et le diagnostic des helminthiasis par immunoélectrophorése. Revue d'Immunologie, Paris, 29, 5-30.

BILHARZ, T. (1852). Ein beitrag zur helminthographia humana, aus brieflichen mittheilungen des Dr. Bilharz in Cairo, nebst bemerkungen von Prof. Th. V. Siebold in Breslau. Zeitschr. Wissensch. Zool., 4, 53-71.

BOROS, D.L. and WARREN, K.S. (1970). Delayed hypersensitivity-type granuloma formation and dermal reaction induced and elicited by a soluble factor isolated from Schistosoma mansoni eggs. Journal of Experimental Medicine, 132, 488-507.

BOUT, D., DUGIMONT, J.C., FARAG, H. and CAPRON, A. (1976). Immunodiganosis of human parasitic diseases by ELISA. In, Immunoenzymatic techniques, pp. 175-182. Eds. C. Feldman et al., Amsterdam, North Holland.

BOYDEN, S.V. (1951). The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. Journal of Experimental Medicine, 93, 107-120.

BOYER, M.H. and KALFAYAN, L.J. (1978). Effect of transferred adult Schistosoma mansoni on resistance of mice to cercarial challenge. American Journal of Tropical Medicine and Hygiene, 27, 542-547.

BRADLEY, D.J. and McCULLOUGH, F.S. (1973). Egg output and the epidemiology of Schistosoma haematobium. Part II. An analysis of the epidemiology of endemic S.haematobium. Transactions of the Royal Society of Tropical Medicine and Hygiene, 67, 491-500.

BRANDT, J.L. and FINCH, E.P. (1946). A simple flocculation slide test for the diagnosis of schistosomiasis. American Journal of Clinical Pathology, 16, Supplement, 141-152.

BRITO, T., GUNJI, J., CAMARGO, M.E., PENNA, D.O. and SILVA, L.C. (1970). Advanced kidney disease in patients with hepatosplenic Manson's schistosomiasis. Revista do Instituto de Medicina Tropical de Sao Paulo, 12, 225-235.

BRUIJNING, C.F.A. (1961). The circumoval precipitin test in experimental Schistosoma mansoni infections. Tropical and Geographical Medicine, 13, 378-387.

BRUIJNING, C.F.A. (1964). The circumoval precipitin reaction in experimental schistosomiasis II. Tropical and Geographical Medicine, 16, 256-262.

BUCK, A.A. and ANDERSON, R.I. (1972). Validation of the complement fixation and slide flocculation tests for schistosomiasis. American Journal of Epidemiology, 96, 205-214.

BUCK, A.A. ANDERSON, R.I. and SASAKI, T.T. (1970). Chapter 5, in, Health and disease in Chad. (eds.) Buck, A.A., Anderson, R.I., Sasaki, T.T. and Kawata, K. Monographs in International Health. Baltimore: Johns Hopkins Press.

CAMARGO, M.E. (1970). Immunodiagnosis of some parasitic diseases at the Instituto de Medicina Tropical de São Paulo. Journal of Parasitology, 56,(No.4, Section II), 45.

CAMARGO, M.E., HOSHINO, S. and SILVA, L.C. da. (1965). A slide fluorescent antibody technique with adult worm antigen for the serological diagnosis of Schistosomiasis mansoni. Revista Instituto de Medicina Tropical de Sao Paulo, 7, 327-331.

CANCIO, M., RIVERA DE SALA, A., RAMIREZ de ARELLANO, G., RODRIGUEZ-MOLINA, R. (1967). Circumoval antibodies. Measurement during treatment of experimental schistosomiasis. American Journal of Tropical Medicine and Hygiene, 16, 729-734.

CANCIO, M., RIVERA DE SALA, A., RODRIGUEZ-MOLINA, R. and TORO-GOYCO, E. (1965). In vitro quantitation of the circumoval precipitin test in infections with Schistosoma mansoni. Experimental Parasitology, 16, 64-73.

CAPRON, A., BIGUET, J., ROSE, F. and VERNES, A. (1965). Les antigenes de Schistosoma mansoni II. Etude immunoélectrophorétique comparée de divers stade larvaires et des adults de deux sexes. Aspects immunologiques des relations hote-parasite de la cercaire et de l'adulte de S.mansoni. Annals de Instituto Pasteur, Paris, 109,798-810.

CAPRON, A., BIGUET, J., TRAN VAN KY, P. and MOSCHETTO, Y. (1969). Immunological studies in various types of schistosomiasis. Annals of New York Academy of Science, 160, 863-879.

CAPRON, A., BIGUET, J., VERNES, A. and AFCHAIN, D. (1968). Structure antigenique des helminthes. Aspects immunologiques des relations hote-parasite. Pathologie et Biologie, 16, 121-138.

CAPRON, A., DUGIMONT, J.C., FRUIT, J. and BOUT, D. (1975). Application of immunoenzyme methods in diagnosis of human parasitic diseases. Annals of New York Academy of Science, 254, 331.

CAPRON, A., PARIS, J., CAPRON, M. et DARWICHE, H. (1974). Valeur des methodes immunologiques dans la surveillance post therapeutique des parasitoses hepato-biliaires. Tijdschr. Gastroenterol., 17, 17-23.

CAPRON, A., VERNES, A., BIGUET, J., ROSE, F., CLAY. A.,and ADENIS, L. (1966). Les précipitines sériques dans les bilharzioses humaines et expérimentales á Schistosoma mansoni, S. haematobium et S.japonicum. Annals de Parasitologie Humane et Comparée, 41, 123-187.

CARLIER, Y., BOUT, D., BINA, J.C., CAMUS, D., FIGUEIREDO, J.F. and CAPRON, A. (1975). Immunological studies in human schistosomiasis. I. Parasitic antigen in urine. American Journal of Tropical Medicine and Hygiene, 24, 949-954.

CESKA, M. (1969). Immuno-precipitates of some purified and unpurified human plasma proteins with monospecific and polyvalent human plasma protein antisera. The Journal of Immunology, 103, 100-106.

CESKA, M. and GROSSMULLER, F. (1968). Immunoprecipitates in agar-Dextran media. Experintia, 24, 391-392.

CHAFFEE, E.F., BAUMAN, P.H. and SHAPILO, J.J. (1954). Diagnosis of schistosomiasis by complement-fixation. American Journal of Tropical Medicine and Hygiene, 3, 905-913.

CHEEVER, A.W., DE WITT, W.B. and WARREN, K.S. (1965). Repeated infection and treatment of mice with Schistosoma mansoni : functional, anatomic and immunologic observations. American Journal of Tropical Medicine and Hygiene, 14, 239-253.

CLEGG, J.A. (1972). The schistosome surface in relation to parasitism. In, Functional Aspects of Parasite Surfaces, p.23-40 (10th Symposium of the British Society for Parasitology). ed. A.E.R. Taylor. Blackwell Scientific Publications, London.

CLEGG, J.A. (1974). Host antigens and the immune response in schistosomiasis. In, Parasites in the immunized host : mechanisms of survival. Ciba Foundation Symposium, 25 (new series), 161-183.

CLEGG, J.A. and SMITHERS, S.R. (1968). Death of schistosome cercariae during penetration of the skin. II. Penetration of mammalian skin by Schistosoma mansoni. Parasitology, 58, 111-128.

CLEGG, J.A. and SMITHERS, S.R. (1972). The effects of immune rhesus monkey serum on schistosomula of Schistosoma mansoni during cultivation in vitro. International Journal for Parasitology, 2, 79-98.

CLEGG, J.A., SMITHERS, S.R. and TERRY, R.J. (1970). 'Host' antigens associated with schistosomes : observations on their attachment and their nature. Parasitology, 61, 87-94.

CLEGG, J.A., SMITHERS, S.R. and TERRY, R.S. (1971). Concomitant immunity and host antigens associated with schistosomiasis. International Journal for Parasitology, 1, 43-49.

COKER, C.H. and LICHTENBERG, F. (1956). A revised method for isolation of Schistosoma mansoni eggs for biological examination. Proceedings of the Society for Experimental Biology and Medicine, 92, 780-782.

COMBRIDGE, B.S. and SHAW, C. (1971). Immunoelectrophoresis using discontinuous buffer system to detect Australia antigen in pooled human plasma. Lancet, 2, 1184-1185.

COOKSON, L.O.C. (1963). Some investigations with the fluorescent antibody technique. II. Experiments with advanced fluorescent equipment. Central African Journal of Medicine, 9, 469-478.

COONROD, J.D. and RYTEL, M.W. (1972). Determination of aetiology of bacterial meningitis by counter-immuno-electrophoresis. Lancet, 1, 1154-1157.

COUDERT, J., GARIN, J.P., AMBROISE-THOMAS, P., POTHIER, M.A. and THAI, K.T. (1968). Diagnostic sérologique par immuno-fluorescence sur soupes á la congélation d'infections á Schistosma mansoni, S.haematobium et S.intercalatum. Acta Tropica (Basel), 25, 109-132.

CROWLE, A.J. (1973). Immunodiffusion. Academic Press, New York and London.

CUADRADO, R.R. and KAGAN, I.G. (1967). The prevalence of antibodies to parasitic diseases in sera of young army recruits from the United States and Brazil. American Journal of Epidemiology, 86, 330-340.

CULBERTSON, J.T. and ROSE, H.M. (1942). Skin tests on schistosomiasis with antigen from pneumoneces medioplexus. American Journal of Hygiene, 36, 311-315.

DAMIAN, R.T. (1964). Molecular mimicry : antigen sharing by parasite and host and its consequences. American Naturalist, 98, 129-149.

DAMIAN, R.T. (1967). Common antigens between adult Schistosoma mansoni and the laboratory mouse. Journal of Parasitology, 53, 60-64.

DAMIAN, R.T., GREENE, N.D. and FITZGERALD, K. (1974). Schistosomiasis mansoni in baboons. II. Acquisition of immunity to challenge infection after repeated small exposures to cercariae of Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene, 23, 78-80.

DAMIAN, R.T., GREENE, N.D. and HUBBARD, W.J. (1973). Occurrence of mouse α2-macroglobulin antigenic determinants on Schistosoma mansoni adults, with evidence on their nature. Journal of Parasitology, 59, 64-73.

DAMIAN, R.T., GREENE, N.D., MEYER, K.F., CHEEVER, A.W., HUBBARD, W.J., HAWES, M.E. and CLARK, J.D. (1976). Schistosoma mansoni in baboons. III. The course and characteristics of infection, with additional observation on immunity. American Journal of Tropical Medicine and Hygiene, 25, 299-306.

DAVIES, A.M. and ELIAKIM, M. (1953). Skin test and complement fixation test in schistosomiasis. Internationaux de Medicine Tropicale et du Paludisme, Instanbul, 3, 309-310.

DAVIES, A.M. and ELIAKIN, M. (1954).The value of different antigens in the diagnosis of chronic bilharziases by skin and complement fixation test. American Journal of Tropical Medicine and Hygiene, 3, 728-741.

DEAN, D.A. (1974). Schistosoma mansoni : adsorption of human blood group A and B antigens by schistosomula. Journal of Parasitology, 60, 260-263.

DEAN, D.A., WISTAR, R. and MURRELL, K.D. (1974). Combined in vitro effects of rat antibody and neutrophilic leuocytes on schistosomula of Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene, 23, 420-428.

DEELDER, A.M., KLAPPE, H.T., VAN DEN AARDWEG, G.J. and VAN MEERVEKE, E.H. (1976). Schistosoma mansoni : demonstration of two circulating antigens in infected hamsters. Experimental Parasitology, 40, 189-197.

DEELDER, A.M., KORNELIS, D., RIER, W.B. and TEN-JET-FOEI, C.L. (1975a). Immunodiagnosis of human schistosomiasis using different immunoprecipitation techniques. Zeitschrift Für Parasitenkunde, 47, 111-118.

DEELDER, A.M. and PLOEM, J.S. (1974). An immunofluorescence reaction for Schistosoma mansoni using the defined antigen substrate spheres (DASS) system. Journal of Immunological Methods, 4, 239-251.

DEELDER, A.M., RUITENBERG, E.J., KORNELIS, D. and STEERENBERG, P.A. (1977). Schistosoma mansoni : comparison of the immunoperoxidase techniques, DASS and ELISA, for human diagnosis. Experimental Parasitology, 41, 133-140.

DEELDER, A.M., SNOIJINK, J.J. and PLOEM, J.S. (1975b). Immunoprecipitation and class-specific immunofluorescence titration of human serum antibodies to Schistosoma mansoni antigens. Zeitschrift Für Parasitenkunde, 46, 195-201.

DEELDER, A.M., SNOIJINK, J.J. and PLOEM, J.S. (1975c). Experimental optimization of the DASS system for immunodiagnosis of some helminth infections. Annals of New York Academy of Sciences, 254, 119-136.

DEELDER, A.M. and STREEFKERK, J.G. (1975). Schistosoma mansoni : immunohistoperoxidase procedure in defined antigen substrate spheres (DASS) system as serologic field test. Experimental Parasitology, 37, 405-410.

DENNIS, D.T., TANAKA, H. and DE RAMOS, S.W. (1972). Complement-fixation reactions in schistosomiasis : quantitative studies. Japanese Journal of Experimental Medicine, 42, 445-450.

DODIN, A., RATO VON DRAHERTY, MOREAU, J.P. and RICHAUD, J. (1966). Étude immunologique de bilharziens traités par le CIBA 32644-Ba. ActaTropica (Basel), supplement, 9, 35-44.

DÖNGES, J. (1966). Der serologische nachweis von schistosoma-infektionen mittels cercarien von posthodiplostomum brevicaudatum. Eine modifikation der cercarienhüllen-reaktion von Vogel und Minning. Zeitschrift für Tropenmedizin und Parasitologie, 17, 413-426.

DRAPER, C.C. (1976). The use of counterimmunoelectrophoresis in immunodiagnosis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70, 93-97.

DUERMEYER, W., VEEN, J.V.D. and KOSTER, B. (1978). ELISA in hepatitis A. Lancet, 1, 823-824.

ELWING, H., NILSSON, L.-Å. and OUCHTERLONY, Ö. (1976). Visualization principles in thin-layer immunoassays (TIA) on plastic surfaces. International Archives of Allergy and Applied Immunology, 51, 757-762.

ELWING, H., NILSSON, L.-Å. and OUCHTERLONY, Ö. (1977). A simple spot technique for thin-layer immunoassays (TIA) on plastic surfaces. Journal of Immunological Methods, 17, 131-145.

ENDERS, B., ECONOMY, D., HUNGERER, K.D. and ZWISLER, O. (1974). Evaluation of a purified schistosoma-skin test-antigen for the diagnosis of human bilharzia infection. Report of field trials in Ethiopia, Rhodesia, Sierra Leone and Brazil. Revista do Instituto de Medicina Tropical de Sao Paulo, 16, 305-316.

ENGVALL, E. and PERLMANN, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry, 8, 871-874.

ENGVALL, E. and PERLMANN, P. (1972). Enzyme-linked immunosorbent assay, ELISA, III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. Journal of Immunology, 109, 129-135.

ERICKSON, D.G. JONES, C.E. and TANG, D.B. (1974). Schistosomiasis mansoni, haematobia and japonica in hamsters : liver granuloma measurements. Experimental Parasitology, 35, 425-433.

EVANS, A.S. and STIREWALT, M.A. (1959). Serologic reactions in Schistosoma mansoni infections. V. Localization of CHR and cercarial agglutinating factors in electrochromatographically fractionated human sera. Experimental Parasitology, 8, 1-9.

EVANS, A.S., STIREWALT, M.A. and MACKENZIE, M. (1955). Serologic reactions in Schistosoma mansoni infections. II. Cercarial behavior in electrophoretically separated fractions of sera of infected and uninfected mice. Experimental Parasitology, 4, 419-426.

EWERS, W.H. and JEFFREY, W.Y. (1971). Parasites of man in Niugini. Milton, Queensland : Jacaranda Press.

EZZAT, E., OSMAN, R.A., AHMED, K.Y. and SOOTHILL, J.F. (1974). The association between Schistosoma haematobium infection and heavy proteinuria. Transactions of the Royal Society of Tropical Medicine and Hygiene, 68, 315-318.

FAIRLEY, N.H. (1919). The discovery of a specific complement fixation test for bilharziasis and its practical application to clinical medicine. Journal of the Royal Army Medical Corps., 32, 449-460.

FAIRLEY, N.H. and WILLIAMS, F.E. (1927). A preliminary report on an intradermal reaction in schistosomiasis. Medical Journal of Australia, 2, 811-818.

FALCÃO, H.A. and GOULD, D.B. (1975). Immune complex nephropathy in schistosomiasis. Annals of Internal Medicine, 83, 148-154.

FARAG, H.F. and BARAKAT, R.M.R. (1978a). The enzyme-linked immunosorbent assay in the diagnosis of human bilharziasis. Tropenmedizin und Parasitologie, 29, 12-14.

FARAG, H.F., BARAKAT, R.M.R., AWADALLA, H.N. and EL-GOHARY, Y. (1978b). Diagnosis of bilharziasis (S.haematobium and S.mansoni) by the ELISA using the homologous antigen. Tropenmedizin und Parasitologie, 29, 413-416.

FERGUSON, A.R. (1911). Associated bilharziasis and primary malignant disease of the urinary bladder, with observation on a series of forty cases. Journal of Pathology and Bacteriology, 16, 76-94.

FERREIRA, A.W., CALDINI, A.L.M., HOSHINO-SHIMIZU, S. and CAMARGO, M.E. (1979). Immuno-enzymatic assay for the detection of Schistosoma mansoni antigens in serum of mice harbouring bisexual or unisexual light worm infections. Journal of Helminthology, 53, 189-194.

FERREIRA, A.W., CAMARGO, M.E. and NAKAHARA, O.S. (1974). Pesquisa de anticorpos anti-Schistosoma mansoni pela tecnica anti-globulinica de immunoperoxidase. Comparação com as reações de immunofluorescencia e de hemaglutinacao. Revista Instituto de Medicina Tropical de Sao Paulo, 16, 341-345.

FIFE, E.H. Jr. (1971). Advances in methodology for immunodiagnosis of parasitic diseases. Experimental Parasitology, 30, 132-163.

FIFE, E.H. Jr., SLEEMAN, H.K. and BRUCE, J.I. Jr. (1967). Isolation and characterization of a serologically active exoantigen of Schistosoma mansoni cercariae. Experimental Parasitology, 20, 138-146.

FORSYTH, D.M. (1967). Clinical patterns in urinary schistosomiasis. In, Bilharziasis, Ed. F.K. Mostafi, pp. 86-91. Springer-Verlag, Berlin and New York.

FOSTER, R. (1965). Skin antigen tests and fluorescent antibody technique in the diagnosis of schistosomiasis. East African Medical Journal, 42, 117-121.

FRAGA DE AZEVEDO, J. and ROMBERT, P.C. (1964). On the value of the cercariae membrane reaction (CHR) and the circumoval precipitin reaction (COP) in the diagnosis of bilharziases. Annales de la Societe Belgie de Medicine Tropicale, 44, 239-254.

FUJINAMI, I. and NAKAMURA, H. (1909). cited by Kagan, I.G. and Pellegrino, J. (1961). Bulletin of the World Health Organization, 25, 611-674.

GELFAND, M. (1964). Chronic urinary schistosomiasis and its relationship to hypertension. Central African Journal of Medicine, 10, 1-8.

GHANDOUR, A.M. and WEBBE, G. (1973). A study of the death of Schistosoma mansoni cercariae during penetration of mammalian host skin : the influence of the ages of the cercariae and of the host. International Journal for Parasitology, 3, 789-794.

GIGASE, P.L., VAN MEIRVENNE, N. and JANSSENS, P.G. (1967). Quantitative fluorescent antibody technique in human and experimental schistosomiasis. Annales de la Societe Belge de Medicine Tropicale, 47, 143-155.

GOLD, D. and LENGY, J. (1975). Failure to immunize mice against Schistosoma mansoni by therapeutic eradication of the adult worm burden. Annals of Tropical Medicine and Parasitology, 69, 265-266.

GOLD, R., ROSEN, F.S. and WELLER, T.H. (1969). A specific circulating antigen in hamsters infected with Schistosoma mansoni, detection of antigen in serum and urine, and correlation between antigenic concentration and worm burden. American Journal of Tropical Medicine and Hygiene, 18, 545-552.

GOLDRING, O.L., CLEGG, J.A., SMITHERS, S.R. and TERRY, R.J. (1976). Acquisition of human blood group antigens by Schistosoma mansoni. Clinical Experimental Immunology, 26, 181-187.

GORDON, M.A., ALMY, R.E., GREENE, C.J. and FENTON, J.W. (1971). Diagnostic mycoserology by immunoelectro-osomophoresis. American Journal of Clinical Pathology, 56, 471-474.

HAMBURGER, J., PELLEY, R.P. and WARREN, K.S. (1976). Schistosoma mansoni soluble egg antigens : determination of the stage and species specificity of their serological reactivity by radioimmuoassay. Journal of Immunology, 117, 1561-1566.

HASSAN, A. and BETASHE, M. (1934). Fasciola gigantica, an antigen for the skin reaction in human schistosomiasis. Journal of Egyptian Medical Association, 17, 991-993.

HASSAN, F., ABDEL-WAHAB, M.F., NOSSEUR, A., SEDEEK, S., SHEHATA, A. and MASOOD, M.A. (1979). Evaluation of enzyme-linked immunosorbent assay in the immunodiagnosis of schistosomiasis. Journal of Tropical Medicine and Hygiene, 82, 3-7.

HENDRICKS, J.R. and CORT, W.W. (1956). A study of the in vitro actions of antiserum on the cercariae of certain bird schistosomes. Journal of Parasitology, 42, 557-564.

HERNÁNDEZ-ALMENAS, N. and HILLYER, G.C. (1973). Immunologic studies in humans with schistosomiasis mansoni. Boletin de la Asociacion Medica Puerto Rico, 65, 231-232.

HIATT, R.A., CLINE, B.L. and KNIGHT, W.B. (1978). Limitations of the intradermal test for schistosomiasis mansoni : experiences from epidemiologic studies in a Puerto Rican community. American Journal of Tropical Medicine and Hygiene, 27, 535-541.

HILLYER, G.V. (1969). Immunoprecipitins in Schistosoma mansoni infections. IV. Human infections. Experimental Parasitology, 25, 376-381.

HILLYER, G.V. (1971). Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of hamsters and man infected with schistosomes. Proceedings of the Society for Experimental Biology and Medicine, 136, 880-883.

HILLYER, G.V. (1976). Schistosome antigens in the circulation of chimpanzees infected with Schistosoma japonicum. American Journal of Tropical Medicine and Hygiene, 25, 432-436.

HILLYER, G.V., CAMPOS, J.A., LLUBERES, R. and CANGLIANO, J.L. (1975). Schistosomal nephropathy, 1. Preliminary studies of a patient with schistosomiasis mansoni and glomerulonephritis in Puerto Rico. Boletin de la Asociacion Medica Puerto Rico, 67, 339-344.

HILLYER, G.V. and FRICK, L.F. (1967). Immunoprecipitins in Schistosoma mansoni infections. I. Mouse infections. Experimental Parasitology, 20, 321-325.

HILLYER, G.V. and GOMEZ DE RIOS, I. (1979). The enzyme-linked immunosorbent assay (ELISA) for the immunodiagnosis of schistosomiasis. American Journal of Tropical Medicine and Hygiene, 28, 237-241.

HILLYER, G.V., HERNÁNDEZ-ALMENAS, N., KNIGHT, W.B. and CLINE, B. (1976). Circulating antigens, antibodies and immunoglobulins in the serum of humans with schistosomiasis mansoni. Boletin de la Asociacion Medica Puerto Rico, 68, 128-132.

HILLYER, G.V. and LEWERT, R.M. (1974). Studies on renal pathology in hamsters infected with Schistosoma mansoni and S.japonicum. American Journal of Tropical Medicine and Hygiene, 23, 404-411.

HILLYER, G.V., SADUN, E.H. and BRUCE, J. (1973). Schistosomiasis japonica : a possible association between complexes and renal pathology. Ninth International Congress on Tropical Medicine and Malaria, 1, 83-84.

HIRATA, M. (1976). Circulating antigen in animals infected with Schistosoma japonicum. 2. Appearance of circulating antigen in infected mice. Japanese Journal of Parasitology, 25, 396-401.

HIRATA, M. and AKUSAWA, M. (1975). Circulating antigen in animals infected with S.japonicum. Detection and characteristics of the antigen in rabbits. Japanese Journal of Parasitology, 24, 250-254.

HOCKLEY, D.J. and McLAREN, D.J. (1973). Schistosoma mansoni : changes in the outer membrane of the tegument during development from cercaria to adult worm. International Journal for Parasitology, 3, 13-25.

HOFFMAN, D.B. Jr., LEHMAN, J.S. Jr., SCOTT, V.C., WARREN, K.S. and WEBBE, G. (1979). Control of schistosomiasis. Report of a workshop. American Journal of Tropical Medicine and Hygiene, 28, 249-259.

HORSTMAN, H.A., CHAFFEE, E.F. and BAUMAN, P.H. (1954). Schistosomiasis mansoni in Puerto Rican soldiers. American Journal of Tropical Medicine and Hygiene, 3, 914-917.

HOSHINO, S., CAMARGO, M.E. and CORREA, N.S. (1970a). A hemagglutination test for Manson's schistosomiasis using chromium chloride, formalin treated human erythrocytes, sensitized with worm extracts. Revista Medicina Tropical de São Paulo, 12, 192-194.

HOSHINO, S., CAMARGO, M.E. and SILVA, L.C. DA (1970b). A comparison between the hemaglutination test with formalin treated erythrocytes and the immunofluorescence test with worm particles, for schistosomiasis. Revista Instituto de Medicina Tropical de São Paulo, 12, 136-143.

HOSHINO, S., CAMARGO, M.E. and SILVA, L.C. DA (1970c). Standardization of a hemagglutination test for schistosomiasis with formalin-treated human erythrocytes. American Journal of Tropical Medicine and Hygiene, 19, 463-470.

HOUBA, V., KOECH, D.K., STURROCK, R.F., BUTTERWORTH, A.E., KUSEL, J.R. and MAHMOUD, A.A. F. (1976). Soluble antigens and antibodies in sera from baboons infected with Schistosoma mansoni. Journal of Immunology, 117, 705-707.

HULDT, G., LAGERQUIST, B., PHILLIPS, T., DRAPER, C.C. and VOLLER, A. (1975). Detection of antibodies in schistosomiasis by enzyme-linked immunosorbent assay (ELISA). Annals of Tropical Medicine and Parasitology, 69, 483-488.

HWU, H., BANZON, T.C. and CROSS, J.H. (1978). Counter-immunoelectrophoresis in the detection of antibodies to Schistosoma japonicum. American Journal of Tropical Medicine and Hygiene, 27, 276-280.

ISHIZAKI, T. (1964). A new criterion for the positive intradermal reaction to the antigen of Schistosoma japonicum. American Journal of Tropical Medicine and Hygiene, 13, 674-680.

ISMAIL, M., DRAPER, C., OUCHTERLONY, Ö., NILSSON, L.-Å. and TERRY, R. (1979). A comparison between a new serological method, thin layer immunoassay (TIA), and the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies in schistosomiasis. Parasite Immunology, 1, 251-258.

JACHOWSKI, L.A. and ANDERSON, R.I. (1961). Evaluation of some laboratory procedures in diagnosing infections with Schistosoma mansoni. Bulletin of the World Health Organization, 25, 675-693.

JAMESON, J.E. (1968). Rapid and sensitive precipitin test for the diagnosis of Farmer's lung using immuno-osmophoresis. Journal of Clinical Pathology, 21, 376-382.

JORDAN, P. and GOATLY, K.D. (1963). Observations on pericercarial reactions of sera in schistosomiasis in Tanganyika. Transactions of the Royal Society of Tropical Medicine and Hygiene, 57, 184-190.

KAGAN, I.G. (1955a). Hemagglutination after immunization with schistosome antigens. Science, 122, 376-377.

KAGAN, I.G. (1955b). Studies on the serology of schistosomiasis. 1. The in vitro activity of cercariae and miracidia in serum of experimental, natural and immunized hosts. Experimental Parasitology, 4, 361-376.

KAGAN, I.G. (1968). Serologic diagnosis of schistosomiasis. Bulletin of New York Academy of Medicine, 44, 262-277.

KAGAN, I.G. (1974). Advances in the immunodiagnosis of parasitic infections. Zeitschrift Fur Parasitenkunde, 45 163-195.

KAGAN, I.G. (1978). Recent advances in the diagnosis of schistosomiasis. In, Proceedings of the International Conference on Schistosomiasis (Cairo-Egypt, 18-25 October 1975). Ed. Abdallah, A. The Ministry of Public Health, Arab Republic of Egypt.

KAGAN, I.G., KAISER, R.L. and KENT, N. (1966). Comparison of Schistosoma mansoni, S.japonicum and Trichinella spiralis antigens in skin tests on persons with schistosomiasis mansoni and haematobium. American Journal of Tropical Medicine and Hygiene, 15, 719-724.

KAGAN, I.G. and MADDISON, S.E. (1975). Research studies on the immunology of schistosomiasis. In, Dynamic Aspects of Host Parasite Relationship, II. Ed. A Zuckerman, pp.163-176. New York, John Wiley.

KAGAN, I.G. and OLIVER-GONZALEZ,J.(1955). Preliminary studies on hemagglutination in schistosomiasis. Journal of Parasitology, 41 (suppl.), 26.

KAGAN, I.G. and OLIVER-GONZALEZ, J. (1958). Hemagglutination studies with schistosome antigens. Journal of Parasitology, 44, 457-460.

KAGAN, I.G. and PELLEGRINO, J. (1961). A critical review of immunological methods for the diagnosis of bilharziasis. Bulletin World Health Organization, 25, 611-674.

KAGAN, I.G., PELLEGRINO, J. and MEMORIAL, J.M.P. (1961). Studies on the standardization of the intradermal test for the diagnosis of bilharziasis. American Journal of Tropical Medicine and Hygiene, 10, 200-207.

KAGAN, I.G., SULZER, A.J. and CARVER, K. (1965). An evaluation of the fluorescent antibody test for the diagnosis of schistosomiasis. American Journal of Epidemiology, 81, 63-70.

KATASURADA, F. (1904). Schistosomum japonicum, ein neuer menschlicher parasit, durch welchen eine endemische krankheit in verschiedenen gegenden japans verursacht wird. Annotationes Zoologicae Japaneses, 5, 146-160.

KELKAR, S.S. and NIPHADKAR, K.B. (1974). Agarose and counterimmunoelectrophoresis. Lancet, 11, 1394-1395.

KELSOE, G.H. and WELLER, T.H. (1978). Immunodiagnosis of infection with Schistosoma mansoni : enzyme-linked immunosorbent assay for detection of antibody to circulating antigen. Proceeding of National Academy of Science USA, 75, 5715-5717.

KEMP, W.M. (1972). Serology of the cercarien-hüllen reaktion of Schistosoma mansoni. Journal of Parasitology, 58, 688-692.

KENT, J.F. and FIFE, E.H. Jr. (1963). Precise standardization of reagents for complement fixation. American Journal of Tropical Medicine and Hygiene, 12, 103-117.

KHALIL, M. and HASSAN, A. (1932). A preliminary note on a new skin reaction in human schistosomiasis. Journal of Egyptian Medical Association, 15, 129-130.

KIM, C.W. (1975). The diagnosis of parasitic diseases. Progress of Clinical Pathology, 6, 267-288.

KLEGER, B. and KAUFMAN, L. (1973). Detection and identification of diagnostic histoplasma capsulatum precipitates by counterelectrophoresis. Applied Microbiology, 26, 231-238.

KLOETZEL, K. (1959). A reação de precipitação periovular na esquistossomose. II. Correlação aos dados clinicos. Revista do Instituto de Medicina Tropical de São Paulo, 1, 129-137.

KNIGHT, R. and WORMS, J.J. (1972). An outbreak of cercarial dermatitis in Britain. Transactions of the Royal Society of Tropical Medicine and Hygiene, 66, 21.

LEFLORE, W.B. and MARTIN, W.E. (1968). Serology of marine termatodes. II. The action of antisera against the cercariae of certain heterophyids. Journal of Parasitology, 54, 305-309.

LEWERT, R.M. and YOGORE, M.G. Jr. (1969). A field circumoval precipitin (FCOP) test for schistosomiasis japonica. Transactions of the Royal Society of Tropical Medicine and Hygiene, 63, 343-348.

LICHTENBERG, F. Von, BAWDEN, M.P. and SHEALEY, S.H. (1974). Origin of circulating antigen from the schistosome gut. An immunofluorescent study. American Journal of Tropical Medicine and Hygiene, 23, 1088-1091.

LICHTENBERG, F. Von, EDINGTON, G.M., NWABUEBO, I., TAYLOR, J.R. and SMITH, J.H. (1971). Pathologic effects of schistosomiasis in Ibadan, Western State of Nigeria. II. Pathogenesis of lesions of the bladder and ureters. American Journal of Tropical Medicine and Hygiene, 20, 244-254.

LIU, H., WU, Y. and LU, S.T. (1958). The value of the circumoval precipitin test in the diagnosis of schistosomiasis japnonica - 1,626 clinical observations. Chung Hua I Hsueh Tsa Chih (National Medical Journal of China), 44, 640-648.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 139, 265-275.

LUNDE, M.N., OTTESEN, E.A. and CHEEVER, A.W. (1979). Serological differences between acute and chronic schistosomiasis mansoni detected by enzyme-linked immunosorbent assay (ELISA). American Journal of Tropical Medicine and Hygiene, 28, 87-91.

MACINNIS, A.J. (1965). Responses of Schistosoma mansoni miracidia to chemical attractants. Journal of Parasitology, 51, 731-746.

MACKENZIE, D.W.R. and PHILPOT, C.M. (1975). Counterimmunoelectrophoresis as a routine mycoserological procedure. Mycopathologica, 57, 1-7.

MADWAR, M.A. and VOLLER, A. (1975). Circulating soluble antigens and antibody in schistosomiasis. British Medical Journal, 1, 435-436.

MADWAR, M.A. and VOLLER, A. (1977). Serological investigations on patients with schistosomiasis with special reference to circulating antigen. Tropenmedizin und Parasitologie, 28, 57-62.

MAGALHÃES-FILHO, A., KRUPP, I.M. and MALEK, E.A. (1965). Localisation of antigen and the presence of antibody in tissues of mice infected with Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene, 14, 84-99.

MALEK, E.A. (1975). Effect of the Aswan High Dam on prevalence of schistosomiasis in Egypt. Tropical and Geographical Medicine, 27, 359-364.

MAO, C.P. (1958). Research on schistosomiasis japonica in China. American Journal of Tropical Medicine and Hygiene, 7, 58-62.

MAYER, M. and PIFANO, F. (1951). La cercaria-reaccion de Vogel-Mining en la schistosomiasis mansoni. Revista de sanidad y Asistencia Social (Caracas), 16, 461-474.

McCULLOGH, F.S. and BRADLEY, D.J. (1973). Egg output stability and the epidemiology of Schistosoma haematobium. I. Variation and stability in Schistosoma haematobium egg counts. Transactions of the Royal Society of Tropical Medicine and Hygiene, 67, 475-490.

McLAREN, M., DRAPER, C.C., ROBERTS, J.M., MINTER-GOEDBLOED, E., LIGHTHART, T., TEESDALE, C.H., OMER, A.H.S., VOLLER, A. and BARTLETT, A. (1978). Studies on the enzyme-linked immunosorbent assay (ELISA) test for Schistosoma mansoni infections. Annals of Tropical Medicine and Parasitology, 72, 243-253.

MEILLON, B. de, and HOLLINGHAM, E.A. (1958). Observations on the bilharzia complement-fixation test in school-children after successful treatment. Bulletin of the World Health Organization, 18, 1108-1109.

MELENY, H.E. and MOORE, D.V. (1954). Observations on immunity to superinfection with Schistosoma mansoni and Schistosoma haematobium. Experimental Parasitology, 3, 128-139.

MELLO, R.T. de, PEREIRA, L.H. and PELLEGRINO, J. (1979). The circumoval precipitin test as a control of cure in the experimental schistosomiasis mansoni. Revista Instituto de Medicina Tropical de São Paulo, 21, 1-4.

MOORE, G.T., KAISER, R.L., LAWRENCE, R.S., PUTNAM, S.M. and KAGAN, I.G. (1968). Intradermal and serologic reactions to antigens from Schistosoma mansoni in schistosome dermatitis. American Journal of Tropical Medicine and Hygiene, 17, 86-91.

MORIEARTY, P.L. and LEWERT, R.M. (1974). Delayed hypersensitivity in Ugandan schistosomiasis mansoni. 3. Examination of serological responses and clinical states. American Journal of Tropical Medicine and Hygiene, 23, 190-196.

MURRELL, K.D., DEAN, D.A. and STAFFORD, E.E. (1975). Resistance to infection with Schistosoma mansoni after immunization with worm extracts or live cercariae : role of cytotoxic antibody in mice and guinea pig. American Journal of Tropical Medicine and Hygiene, 24, 955-962.

MURRELL, K.D., VANNIER, W.E. and AHMED, A. (1974). Schistosoma mansoni : antigenic heterogeneity of excretions and secretions. Experimental Parasitology, 36, 316-330.

NASH, T.E. (1974). Localization of the circulating antigen within the gut of Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene, 23, 1085-1087.

NASH, T.E. (1978). Antibody response to a polysaccharide antigen present in the schistosome gut. I. Sensitivity and specificity. American Journal of Tropical Medicine and Hygiene, 27, 938-943.

NASH, T.E., OTTESEN, E.A. and CHEEVER, A.W. (1978). Antibody response to a polysaccharide antigen present in the schistosome gut. II. Modulation of antibody response. American Journal of Tropical Medicine and Hygiene, 27, 944-950.

NASH, T.E. PRESCOTT, B. and NEVA, F.A. (1974). The characteristics of a circulating antigen in schistosomiasis. Journal of Immunology, 112, 1500-1507.

NEIL, G., PINON, I.M. and GENTILINI, M. (1970). Immunofluorescence appliquée au diagnostic serologique de la bilharziose. (A propos d'une technique simple de préparation des coupes à la congélation). Bulletin de la Societe de Pathologie Exotique, 63, 356-362.

NEWSOME, N.M. (1958). Species-specific serological tests for bilharzia. Annals of Tropical Medicine and Parasitology, 52, 82-86.

OKABE, K. and TANAKA, T. (1958). A new urine precipitin reaction for schistosomiasis japonica. A preliminary report. Kurume Medical Journal, 5, 45-52.

OKABE, K. and TANAKA, T. (1961). Urine precipitin reaction for schistosomiasis japonica. Kurume Medical Journal, 8, 24-37.

OLIVER-GONZALAZ, J. (1954). Anti-egg precipitins in the serum of humans infected with Schistosoma mansoni. Journal of Infectious Disease, 95, 86-91.

OLIVER-GONZALEZ, J., BAUMAN, P.M. and BENENSON, A.S. (1955a). Species specificity of the anti-egg precipitin in schistosome serums. Journal of Infectious Disease, 96, 95-100.

OLIVER-GONZALEZ, J., BAUMAN, P.M. and BENENSON, A.S. (1955b). Immunological aspects of infections with Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene, 4, 443-452.

OLIVIER, L.J. and UEMURA, K. (1973). Techniques, statistical methods and record forms. In, Epidemiology and Control of Schistosomiasis, Ch.14, ed. Ansari, N. Basel, S. Karger.

OUCHETERLONY, O. and NILSSON, L.-A. (1973). Immunodiffusion and immunoelectrophoresis. In, Handbook of Experimental Immunology, Vol.2, Ch.19. Ed. D.M. Weir, Blackwell, Oxford.

PAPIRMEISTER, B. and BANG, F.B. (1948). "The in vitro action of immune sera on cercariae of Schistosoma mansoni". American Journal of Hygiene, 48, 74-79.

PELLEGRINO, J. (1963). Diagnostico serologico da esquistossomose mansonica. I. Estude comparativo entre as reacoes de floculacao, de imunofluorescencia e de fixacao do complemento. Revista Instituto de Medicino Tropical de Sao Paulo, 5, 147-153.

PELLEGRINO, J. (1964). Diagnostico sorologico da esquistossomose mansonica. IV. Valor de reacao de floculacao em levantamentos epidemiologicos. Revista do Instituto de Medicina Tropical de Sao Paulo, 6, 111-113.

PELLEGRINO, J. and BIOCCA, E. (1963). Diagnostico sorologico da esquistossomose mansonica. II. Reacao de imunofluorescencia com cercarias de Schistosoma bovis e cercaria caratinguensis. Revista Instituto de Medicino Tropical de Sao Paulo, 5, 257-260.

PELLEGRINO, J. and MACEDEO, D.G. (1956). Novo criterio de leitura de reacao intradermica na esquistossomosse. Revista Brasileira Malariologia, 8, 499-509.

PELLEGRINO, J. and OLIVEIRA-ANDRADE, F. (1962). A reacao peri-ovular no diagnostico da esquitossomose mansonica. Revista do Instituto de Medicina Tropical de Sao Paulo, 4, 163-169.

PELLEGRINO, J., REZENDE, C.L. DE, MEMORIA, J.M.P., MOURAO, O.G. and BRENER, Z. (1959). Diagnostico de laboratoria de esquistossomose mansoni na crianca. Journal de Pediatria (Rio de Janeiro), 24, 211-230.

PELLEY, R.P., PELLEY, R.J., HAMBURGER, J., PETERS, P.A. and WARREN, K.S. (1976). Schistosoma mansoni soluble egg antigens. I. Identification and purification of three major antigens, and the employment of radioimmunoassay for their further characterization. Journal of Immunology, 117, 1553-1560.

PELLEY, R.P., WARREN, K.S. and JORDAN, P. (1977). Purified antigen radioimmunoassay in serological diagnosis of schistosomiasis mansoni. Lancet, 2, 781-785.

PEREZ, H., CLEGG, J.A. and SMITHERS, S.R. (1974). Acquired immunity to Schistosoma mansoni in the rat : measurement of immunity by the lung recovery technique. Parasitology, 69, 349-359.

PESENDORFER, F., KRASSNITZKY, O. and WEWALKA, F. (1970). Immunelektrophoretischer nachweis von "Hepatitis-associated-antigen" (AU/SH-Antigen). Klinishe Wochenschrift, 48, 58-59.

PHILLIPS, T.M. and DRAPER, C.C. (1975). Circulating immune complexes in schistosomiasis due to Schistosoma mansoni. British Medical Journal, 2, 476-477.

POTHIER, M.A. and LOURDES-SAMPAIO-XAVIER, M. de. (1969a). Premiers resultats a propos du diagnostic serologique de la bilharziose par immuno-fluorescence sur coupes a la congelation de Schistosoma haematobium. Annals de Parasitologie Humaine et Comparee, 44, 387-390.

POTHIER, M.A. and LOURDES-SAMPAIO-XAVIER, M. de. (1969b). Le diagnostic de la bilharziose par immuno-fluorescence sur coupes as la congelation de Schistosoma mansoni et de Schistosoma haematobium. Bulletin de la Societe de Pathologie Exotique, 62, 711-716.

PRESTON, J.H. and JAMES, C. (1972). Infection of hamsters with terminal-spined schistosomes. Journal of Helminthology, 46, 291-296.

PURNELL, R.E. (1966). Host-parasite relationships in schistosomiasis. II. The effects of age and sex on the infection of mice and of hamsters with cercariae of Schistosoma haematobium. Annals of Tropical Medicine and Parasitology, 60, 94-99.

RAMALHO-PINTO, F.J., GOLDRING, O.L., SMITHERS, S.R. and PLAYFAIR, J.H.I. (1976). T-cell helper response to antigens of Schistosoma mansoni in CBA mice. Clinical Experimental Immunology, 26, 327-333.

REIS, A.P., KATZ, N. and PELLEGRINO, J. (1970). Immuno-diffusion tests in patients with Schistosoma mansoni infection. Revista Instituto de Medicina Tropical de São Paulo, 12, 245-248.

REMINGTON, J.S., GAINES, J.O. and GILMER, M.A. (1972). Demonstration of candida precipitins in human sera by counter-immunoelectrophoresis. Lancet, 1, 413.

RIEDER, S., ANDERSON, R.I. and RADKE, M.G. (1961). Serologic diagnosis of Schistosoma mansoni infections. III. Isolation and purification of antigen from adult S.mansoni for complement fixation test. American Journal of Tropical Medicine and Hygiene, 10, 351-355.

RIFAAT, M.A., ISMAIL, I. and EL-MAHALLAWAY, M.N., AWWAAD, S. and ESSAWY, M. (1969). Comparative study of some immunological tests for schistosomiasis before and after treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene, 63, 338-342.

RIFAAT, M.A. and KHALIL, H.M. (1965). The use of antigens prepared from adult Fasciola gigantica, Schistosoma mansoni and Schistosoma bovis in the diagnosis of urinary schistosomiasis by the complement fixation test (CFT). Journal of the Egyptian Public Health Association, 40, 197-205.

RILEY, V. (1960). Adaptation of orbital bleeding technique to rapid serial blood studies. Proceeding of the Society for Experimental Biology and Medicine, 104, 751-754.

RODRIGUEZ-MOLINA, R. (1970). Circumoval precipitin test for schistosomiasis. New England Journal of Medicine, 22, 104-105.

RODRIGUEZ-MOLINA, R., LICHTENBERG, F., OLIVER-GONZALEZ, J. and SALA, A.R. DE. (1959). Studies on immunity to Schistosoma mansoni. II. The circumoval precipitin reaction to S.mansoni in mice treated with stibophen American Journal of Tropical Medicine and Hygiene, 8, 565-569.

RODRIGUEZ-MOLINA, R., OLIVER-GONZALEZ, J., SALA, A.R. DE. (1962). The circumoval precipitin test in Schistosoma mansoni. Journal of the American Medical Association, 182, 1001-1004.

RODRIGUEZ-MOLINA, R., OLIVER-GONZALEZ, J. and SALA A.R. DE. (1966). Circumoval precipitin test in Schistisomiasis mansoni. A study of 400 patients. Boletin de la Asociacion Medica de Puerto Rico, 58, 159-165.

RODRIGUEZ-MOLINA, R., OLIVER-GONZALEZ, J. and SERRANO, D.G. (1956). Studies on immunity to schistosomiasis mansoni : evaluation of the circumoval precipitin tests as a diagnostic procedure in clinical schistosomiasis mansoni. Report of 46 cases. Boletin de la Asociacion Medica de Puerto Rico, 48, 389-392.

RODRIGUEZ-MOLINA, R., OLIVER-GONZALEZ, J. and SERRANO, D.G. (1958). Studies on immunity to bilharziasis mansoni : evaluation of the circumoval precipitin test. Bulletin of the World Health Organization, 18, 1105-1107.

RUIZ-TIBEN, E., HILLYER, G.V., KNIGHT, W.B., RIOS, G.I. DE, and WOODALL, J.P. (1979). Intensity of infection with Schistosoma mansoni : its relationship to the sensitivity and specificity of serologic tests. American Journal of Tropical Medicine and Hygiene, 28, 230-236.

SABBOUR, M.S., EL-SAID, W. and ABOU-GABAL, I. (1972). A clinical and pathological study of schistosomal nephritis. Bulletin of World Health Organization, 47, 549-557.

SADIGURSKY, M., ANDRADE, Z.A., DANNER, R., CHEEVER, A.W., KAMEL, I.A. and ELWI, A.M. (1976). Absence of schistosomal glomerulopathy in Schistosoma haematobium infection in man. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70, 322-327.

SADUN, E.H. (1963). Seminar on immunity to parasite helminths. Experimental Parasitology, 13, 72-82.

SADUN, E.H.(1976). Serodiagnosis of schistosomiasis. In, Immunology of Parasitic Infections. Eds. S. Cohen and E. Sadun. Oxford, Blackwell.

SADUN, E.H., ANDERSON, R.I. and SCHOENBECHLER, M.J. (1963a). A plasma card test for rapid serodiagnosis of schistosomiasis (SPC). Proceeding of the Society for Experimental Biology and Medicine, 112, 280-283.

SADUN, E.H., ANDERSON, R.I. and WILLIAMS, J.S. (1961). Fluorescent antibody test for the laboratory diagnosis of schistosomiasis in humans by using dried blood smears on filter paper. Experimental Parasitology, 11, 117-120.

SADUN, E.H., ANDERSON, R.I. and WILLIAMS, J.S. (1962). The nature of fluorescent antibody reactions in infections and artificial immunizations with Schistosoma mansoni. Bulletin of the World Health Organization, 27, 151-159.

SADUN, E.H., ANDERSON, R.I., WILLIAM, B. DE WITT, and JACHOWSKI, L.A. Jr. (1963b). Serologic reactions to Schistosoma mansoni. II. Quantitative studies in human patients treated with stibophen. American Journal of Hygiene, 77, 146-149.

SADUN, E.H. and BIOCCA, E. (1962). Intradermal and fluorescent antibody tests on humans exposed to Schistosoma bovis cercariae from Sardinia. Bulletin of World Health Organization, 27, 810-814.

SADUN, E.H. and GORE, R.W. (1967). Relative sensitivity and specificity of soluble antigens (metabolic and somatic) and whole cercariae in fluorescent antibody tests for schistosomiasis in humans and rabbits. Experimental Parasitology, 20, 131-137.

SADUN, E.H., LICHTENBERG, F.V., ERICKSON, D.G., CHEEVER, A.W., BUEDING, E.E. and ANDERSON, J.S. (1974). Effects on chemotherapy on the evolution of schistosomiasis japonica in chimpanzees. American Journal of Tropical Medicine and Hygiene, 23, 638-661.

SADUN, E.H., LIN, S.S. and WALTON, B.C. (1959). Studies on the host-parasite relationship to Schistosoma japonicum. III. The use of purified antigens in the diagnosis of infections in human and experimental animals. Military Medicine, 124, 428-436.

SADUN, E.H., SCHOENBECHLER, M.J. and BENTZ, M. (1965). Multiple antibody response in Schistosoma mansoni infections : antigenic constituents in eggs, cercariae and adults (excretions and secretions) determined by flocculation reactions, cross absorption and double diffusion studies. American Journal of Tropical Medicine and Hygiene, 14, 977-995.

SADUN, E.H., WILLIAMS, J.S. and ANDERSON, R.I. (1960). A fluorescent antibody technique for the serodiagnosis of schistosomiasis in humans. Proceeding of the Society for Experimental Biology and Medicine, 112, 280-283.

SALA, A.R. DE, CANCIO, M. and RODRIQUEZ-MOLINA, R. (1962). Preservation of eggs of Schistosoma mansoni for the circumoval precipitin test. American Journal of Tropical Medicine and Hygiene, 11, 199-200.

SALIH, S.Y., BARTLETT, A. and VOLLER, A. (1978). Detection of antibodies by enzyme-immunoassay in human Schistosoma mansoni infections : a clinical and chemotherapeutic study. Tropenmedizin und Parasitologie, 29, 409-412.

SAMBON, L.W. (1907). Remarks on Schistosomum mansoni. Journal of Tropical Medicine and Hygiene, 10, 303-304.

SATO, K., SAWADA, T. and SATO, S. (1969a). Studies on the purification of skin test antigen SST for the diagnosis of schistosomiasis japonica. II. The purification of antigen SST by disc-sephadex G_{25} column electrophoresis and disc electrophoresis. Japanese Journal of Experimental Medicine, 39, 347-353.

SATO, S., SAWADA, T. and TAKEI, K. (1969b). Studies on complement fixation and hemagglutination tests with purified antigens SPA and SSCD2 in infections with Schistosoma japonicum. Japanese journal of Experimental Medicine, 39, 355-358.

SAWADA, T., SATO, K. and SATO, S. (1969a). Studies on the purification of skin test antigen SST for the diagnosis of schistosomiasis japonica. I. The characterization and purification of antigen SST by zone electrophoresis and gel-filtration technique. Japanese Journal of Experimental Medicine, 39, 339-345.

SAWADA, T., SATO, S., TAKEI, K., MOOSE, J.W. and WILLIAMS, J.E. (1968). Immunodiagnosis of schistosomiasis. 3. Further purification of antigen SSC1 by DEAE sephadex A50 column cromatography. Experimental Parasitology, 23, 238-243.

SAWADA, T., TAKEI, K., SATO, S. and MATSUYAMA, S. (1969b). Immunodiagnosis of schistosomiasis. IV. Studies on complement fixation and hemagglutination tests with antigens prepared from adult schistosome worms. Japanese Journal of Experimental Medicine, 39, 541-547.

SCAPIN, M. and TENDLER, M. (1975). Immunoelectro-osmophoresis as a rapid method for soluble antigenic fraction of Schistosoma mansoni. The Journal of Parasitology, 03, 501-502.

SCHILLER, E.L. and HAESE, W.H. (1973). Histologic processes of healing in hepatic injury due to eggs of Schistosoma mansoni in mice following curative chemotherapy. American Journal of Tropical Medicine and Hygiene, 22, 211-214.

SCHINSKI, V.D., CLUTTER, W.C. and MURRELL, K.D. (1976). Enzyme- and 1251-labelled anti-immunoglobulin assays in the immunodiagnosis of schistosomiasis. American Journal of Tropical Medicine and Hygiene, 25, 824-831.

SCHNEIDER, M.D., RADKE, M.G. and COLEMAN, M.T. (1956). Immunologically reactive substance from Schistosoma mansoni. Experimental Parasitology, V, 391-397.

SCHOFIELD, F.D. (1959). The schistosomal complement fixation test. Transactions of the Royal Society of Tropical Medicine and Hygiene, 53, 64-74.

SCOTT, J.A. (1937). The incidence and distribution of the human schistosomes in Egypt. American Journal of Hygiene, 25, 566-614.

SENTERFIT, L.B. (1953). Immobilization of Schistosoma mansoni miracidia by immune serum. Proceedings of the Society for Experimental Biology and Medicine, 84, 5-7.

SENTERFIT, L.B. (1958a). Immobilization of the miracidia of Schistosoma mansoni by immune sera. I. The nature of the reaction as studied in hamster sera. American Journal of Hygiene, 68, 140-147.

SENTERFIT, L.B. (1958b). Immobilization of the miracidia of Schistosoma mansoni by immune sera. II. The occurrence of the antibody during the course of schistosomiasis. American Journal of Hygiene, 68, 148-155.

SHER, F.A., KUSEL, J.R., PEREZ, H. and CLEGG, J.A. (1974a). Partial isolation of a membrane antigen which induces the formation of antibodies lethal to schistosomes cultured in vitro. Clinical Experimental Immunology, 18, 357-369.

SHER, F.A., MACKENZIE, P. and SMITHERS, S.R. (1974b). Decreased recovery of invading parasites from the lungs as a parameter of acquired immunity to schistosomiasis in the laboratory mouse. Journal of Infectious Disease, 130, 626-633.

SHER, F.A., SMITHERS, S.R. and MACKENZIE, P. (1975). Passive transfer of acquired resistance to Schistosoma mansoni in laboratory mice. Parasitology, 70, 347-357.

SHERIF, A.F. (1962). Schistosomal metabolic products in the diagnosis of bilharziasis. Bilharziasis : Ciba Foundation Symposium. Little, Brown and Co., Boston, U.S.A., 226-238.

SHOEB, S.M., BASMY, K., HASEEB, N.M. and EL-GHONEMY, M.T. (1967a). The value of the circumoval precipitin test using S.haematobium ova as antigen in the diagnosis of the different types of bilharziasis. Journal of Egyptian Medical Association, 50, 564-570.

SHOEB, S.M., BASMY, K., HASEEB, N.M. RIFAAT, M.A. and KHALIL, H.M. (1967b). The value of the circumoval precipitin test (C.O.P.T.) in the assessment of cure from bilharziasis and in the evaluation of antibilharzial chemotherapy. Journal of Egyptian Medical Association, 50, 29-38.

SHOEB, S.M., BASMY, K., HASEEB, N.M., SAIF EL-DIN, S. and RIFAAT, M.A. (1968). Evaluation of the "Urine precipitin test" in the diagnosis and assessment of cure of bilharziasis. Journal of Egyptian Medical Association, 51, 367-376.

SILVA, L.C. DA, BRITO, T., CAMARGO, M.E., BENI, D.R. DE, LOPES, J.D. and GUNJI, J. (1970). Kidney biopsy in the hepatosplenic form of infection with Schistosoma mansoni in man. Bulletin of World Health Organization, 42, 907-910.

SILVA, L.C. DA, CAMARGO, M.E., HOSHINO, S., GUNJI, J., LOPES, J.D., CHAMONE, D.F., MARTINEZ, J.O. FERRI, R.G., ROTHSTEIN, W., SILVA, G.R.DA, CENEVIVA, A.C. and CARDOSO, E.J. (1971). Serum antibody and eosinophil changes after treatment of human manson's schistosomiasis with Niridazole or Hycanthone. Revista do Instituto de Medicina Tropical de Sao Paulo, 13, 121-130.

SILVA, L.C. DA, HOSHINO-SHIMIZU, S., KANAMURA, H., STRASSMANN, P.G., CAMARGO, M.E., SETTE, H. Jr., LOPES, J.D., CHAMONE, D.A.F., RAIA, S. and SILVA, G.R. DA. (1975). Serum antibody changes after chemotherapy of patients with schistosomiasis mansoni. A statistical analysis. Revista Instituto de Medicina Tropical de Sao Paulo, 17, 327-331.

SILVA, L.C. DA, HOSHINO-SHIMIZU, S., KANAMURA, H., STRASSMANN, P.G., CAMARGO, M.E., SETTE, H. Jr., LOPES, J.D., CHAMONE, D.A., RAIA, S. and SILVA, G.R. DA. (1976). Serum antibody changes after repeated chemotherapeutic series in "Parasitologically cured" patients with schistosomiasis mansoni. Revista do Instituto de Medicina Tropical de Sao Paulo, 18, 206-210.

SLEEMAN, H.K. (1960). Isolation and study of specific complement fixing antigen from adult Schistosoma mansoni. American Journal of Tropical Medicine, 9, 11-17.

SMITH, J.H., KAMEL, I.A., ELWI, A. and LICHTENBERG, F. Von. (1974). A quantitative post mortem analysis of urinary schistosomiasis in Egypt. I. Pathology and pathogenesis. American Journal of Tropical Medicine and Hygiene, 23, 1054-1071.

SMITH, M.A., CLEGG, J.A. and WEBBE, G. (1976). Cross-immunity to Schistosoma mansoni and S.haematobium in the hamster. Parasitology, 73, 53-64.

SMITHERS, S.R. (1960a). Gel-diffusion studies on Schistosoma mansoni. Transactions of the Royal Society of Tropical Medicine and Hygiene, 54, 8.

SMITHERS, S.R. (1960b). The isolation of viable schistosome eggs by a digestion technique. Transactions of the Royal Society of Tropical Medicine and Hygiene, 54, 68-70.

SMITHERS, S.R. (1972). Recent advances in the immunology of schistosomiasis. British Medical Bulletin, 28, 49-54.

SMITHERS, S.R. and TERRY, R.J. (1965a). Naturally acquired resistance to experimental infections of Schistosoma mansoni in the rhesus monkey (Macaca mulatta). Parasitology, 55, 701-710.

SMITHERS, S.R. and TERRY, R.J. (1965b). The infection of laboratory hosts with cercariae of Schistosoma mansoni and the recovery of the adult worms. Parasitology, 55, 695-700.

SMITHERS, S.R. and TERRY, R.J. (1967). Resistance to experimental infection with Schistosoma mansoni in rhesus monkeys induced by the transfer of adult worms. Transactions of the Royal Society of Tropical Medicine and Hygiene, 61, 517-533.

SMITHERS, S.R. and TERRY, R.J. (1969a). The immunology of schistosomiasis. In, Advances in Parasitology, Ed. B. Dawes, 7, 41-93. Academic Press, London and New York.

SMITHERS, S.R. and TERRY, R.J. (1969b). Immunity in schistosomiasis. Annals of the New York Academy of Science, 160, 826-840.

SMITHERS, S.R., TERRY, R.J. and HOCKLEY, D.J. (1969). Host antigens in schistosomiasis. Proceedings of the Royal Society (Series B), 171, 483-494.

SOULSBY, E.J.L. (1975). Diagnosis of helminth infections. In, Clinical Aspects of Immunology. Eds. Gell, P.G.H., Coombs, R.R.A. and Lachman, P.J. Oxford, Blackwell.

SPRENT, J.F.A. (1962). Parasitism, immunity and evolution, pp.149-165 in, The Evolution of Living Organisms, Ed. Leeper, G.W. Symposium of the Royal Society of Melbourne, Dec. 1959. Melbourne University Press, Melbourne, Australia.

STANDEN, O.D. (1952). The in vitro effect of normal and immune serum upon the cercariae of Schistosoma mansoni. Journal of Helminthology, 26, 25-42.

STAVITSKY, A.B. (1954). Micromethods for the study of proteins and antibodies. 1. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. Journal of Immunology, 72, 360-367.

STIREWALT, M.A. (1963). Seminar on immunity to parasite helminths. IV. Schistosome infections. Experimental Parasitology, 13, 18-44.

STIREWALT, M.A. (1971). Penetration stimuli for schistosome cercariae. In, Aspect of biology of symbiosis. Ed. Cheng, T.C. pp.1-23. Park Press, Baltimore, Maryland.

STIREWALT, M.A. and EVANS, A.S. (1955). Serologic reactions in Schistosoma mansoni infections. 1. Cercaricidal, precipitation, agglutination and CHR phenomena. Experimental Parasitology, 4, 123-142.

SUESSENGUTH, H. and KLINE, B.S. (1944). Simple rapid flocculation slide test for trichinosis in man and in swine. American Journal of Clinical Pathology, 14, 471-484.

TADA, T., KONODO, Y., OKUKURA, K.O., SANO, M. and YOKOGAWA, M. (1975). Schistosoma japonicum : immunopathology of nephritis in Macaca fascicularis. Experimental Parasitology, 38, 291-302.

TAYLOR, M.G., NELSON, G.S., SMITH, M. and ANDREWS, B.J. (1973). Studies on heterologous immunity in schistosomiasis. 7. Observations on the development of acquired homologous and heterlogous immunity to Schistosoma mansoni in baboons. Bulletin of the World Health Organization, 49, 57-65.

TEESDALE, C.H.M. (1973). Immunological studies in schistosomiasis. (thesis). London University Library, Senate House, London.

T'IEN-WEI, H., TEH-FU, T., CHIEN-LIANG, C. and CH'ENG-CHOU, H. (1966). Indirect hemaggluination test (slide method) in the diagnosis of schistosomiasis japonica. Chinese Medical Journal, 85, 96-106.

TOUSSAINT, A.J. (1966). Improvement of the soluble antigen fluorescent antibody procedure. Experimental Parasitology, 19, 71-76.

TOUSSAINT, A.J. and ANDERSON, R.I. (1965). Soluble antigen fluorescent-antibody technique. Applied Microbiology, 13, 552-558.

UMALY, R.C., OELERICH, S. and HAAS, J. (1974a). Immunodiagnostic test for bilharziasis. (II) Reactivity of various schistosome antigens in different serological tests. Tropenmedizin und Parasitologie, 25, 413-421.

UMALY, R.C., OELERICH, S. and HAAS, J. (1974b). Immunodiagnostic test for bilharziasis. III. Serodiagnosis of Schistosoma haematobium. Infection and influence of chemotherapy on immunodiagnostic tests. Tropenmedizin und Parasitologie, 25, 422-432.

VANDALEN, J.P.R., KNAPP, W. and PLOEM, J.S. (1973). Microfluorometry on antigen-antibody interaction in immunofluorescence using antigens covalently bound to agarose beads. Journal of Immunological Methods, 2, 383-392.

VERGANI, C. (1971). Cross-over electrophoresis for the rapid detection of serum hepatitis (Australia) antigen and antibody. Journal of Clinical Pathology, 24, 86-87.

VERNES, A., FRUIT, J., BOUTHEMY et CAPRON, A. (1969) L'immunofluorescence indirecte appliquee au diagnostic des bilharzioses. Etude de la specificite de la reaction sur coupes a congelation et comparasion avec les techniques d'immuno-electrophorese et de fixation du complement. Bulletin de la Societe de Pathologie Exotique, 62, 548-556.

VOGEL, H. und MINNING, W. (1949a). Hullenbildung bei bilharzia-cercarien im serum bilharzia infizierter tiere und menschen. Zentralblatt fur Bakteriologie, 153, 91-105.

VOGEL, H. und MINNING, W. (1949b). Weitere beobachtungen uber die cercarien-hullenreaktion, eine seroprazipitation mit lebenden bilharzia-cercarien. Zeitschrift fur Tropenmedizin und Parasitologie, 1, 378-386.

VOLLER, A., BARTLETT, A. and BIDWELL, D.E. (1976a). Enzyme immunoassay for parasitic diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70, 98-106.

VOLLER, A., BIDWELL, D.E. and BARTLETT, A. (1976b). Enzyme immunoassays in diagnostic medicine. Bulletin of the World Health Organization, 53, 55-65.

VOLLER, A., BIDWELL, D. and BARTLETT, A. (1976c). Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In, Manual of Clinical Immunology, Eds. N.R. Rose and H. Friedman, Chapter 69, pp.506-512. American Society for Microbiology.

VOLLER, A., BIDWELL, D.E. and BARTLETT, A. (1979). Eds. The enzyme-linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications. Dynatech Europe, Borough House, Rue du Pre, Guernsey, G.B.

VOLLER, A., BIDWELL, D.E., BARTLETT, A. and EDWARDS, R. (1977). A comparison of isotopic and enzyme-immunoassays for tropical parasitic diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene, 71, 431-437.

WALLIS, C. and MELNICK, J.L. (1971). Enhanced detection of Australia antigen in serum hepatitis patients by discontinuous counter-immunoelectrophoresis. Applied Microbiology, 21, 867-869.

WARREN, K.S. (1972). The immunopathogenesis of schistosomiasis : a multidisciplinary approach. Transactions of the Royal Society of Tropical Medicine and Hygiene, 66, 417-434.

WARREN, K.S. (1973). Regulation of the prevalence and intensity of schistosomiasis in man : immunology or ecology? The Journal of Infectious Diseases, 127, 595-609.

WARREN, K.S. (1974). Schistosomiasis : new concepts of immunity, immunodiagnosis and immunopathology. In, Advances in the Biosciences, 12, (Ed. G. Raspe), 637-647. Pergamon Press.

WARREN, K.S. (1976). Schistosomiasis : a multiplicity of immunopathology. Journal of Investigative Dermatology, 67, 464-469.

WARREN, K.S., COOK, J.A. and JORDAN, P. (1972). Passive transfer of immunity in human schistosomiasis mansoni : effect of hyperimmune anti-schistosome gamma globulin on early established infections. Transactions of the Royal Society of Tropical Medicine and Hygiene, 66, 65-74.

WARREN, K.S. and DOMINGO, E.O. (1970a). Schistosoma mansoni : stage specificity of granuloma formation around eggs after exposure to irradiated cercariae, unisexual infections, or dead worms. Experimental Parasitology, 27, 60-66.

WARREN, K.S. and DOMINGO, E.O. (1970b). Granuloma formation around Schistosoma mansoni, S.haematobium and S.japonicum eggs. Size and rate, cellular composition, cross-sensitivity and rate of egg destruction. American Journal of Tropical Medicine and Hygiene, 19, 292-304.

WARREN, K.S., KELLERMEYER, R.W., JORDAN, P., LITTELL, A.S., COOK, J.A. and KAGAN, I.G. (1973). Immunological diagnosis of schistosomiasis. I. A controlled study of intradermal (immediate and delayed) and serologic tests in St. Lucians infected with Schistosoma mansoni and in uninfected St. Vincentians. American Journal of Tropical Medicine and Hygiene, 22, 189-198.

WARREN, K.S., PELLEY, R.P. and MAHMOUD, A.A.F. (1977). Immunity and immunopathology following reinfection of mice cured of chronic schistosomiasis mansoni. American Journal of Tropical Medicine and Hygiene, 26, 957-962.

WARREN, K.S. and PETERS, P.A. (1967). Comparison of penetration and maturation of Schistosoma mansoni in the hamster, mouse, guinea pig, rabbit and rat. American Journal of Tropical Medicine and Hygiene, 16, 718-722.

WEBBE, G. and JAMES, C. (1971). The importation and maintenance of schistosomes of human and veterinary importance. In, Ninth Symposium of the British Society for Parasitology. Eds. A.E.R. Taylor and R. Muller. Oxford :Blackwell, pp.77-107.

WEBBE, G. and JAMES, C. (1973). Acquired resistance to Schistosoma haematobium in the baboon. Transactions of the Royal Society of Tropical Medicine and Hygiene, 67, 28-29.

WEBBE, G., JAMES, C., NELSON, G.S., ISMAIL, M. and SHAW, J.R. (1979). Cross resistance between Schistosoma haematobium and S.mansoni in the baboon. Transactions of the Royal Society of Tropical Medicine and Hygiene, 73, 42-54.

WEBBE, G., JAMES, C., NELSON, G.S., SMITHERS, S.R. and TERRY, R.J. (1976). Acquired resistance to Schistosoma haematobium in the baboon (Papio anubis) after cercarial exposure and adult worm transplantation. Annals of Tropical Medicine and Parasitology, 70, 411-424.

WEINLAND, G. (1858). See HEMMING, F. ed. Official list of generic names in zoology. London, International Trust for Zoological Nomenclature, 1958, p.24.

WEIR, J.M. (1969). The unconquered plague. Rockefeller Foundation Quarterly Report, 2, 4-23.

WILLIAMS, J.E., MOOSE, J.W., SAWADA, T., TAKEI, K. and SATO, S. (1965). Studies on the immunodiagnosis of schistosomiasis. I. Intradermal skin tests with fractionated antigens. Journal of Infectious Diseases, 115, 382-386.

WILSON, M., FRIED, J., McQUAY, R.M. and SULZER, A.J. (1977). Evaluation of the indirect immunofluorescence and complement fixation tests for the serodiagnosis of schistosomiasis. American Journal of Tropical Medicine and Hygiene, 26, 1159-1163.

WORLD HEALTH ORGANISATION, (1965). Snail control in the prevention of bilharziasis. WHO Monograph Series, No. 50, Geneva.

WORLD HEALTH ORGANISATION, (1966). Chemotherapy of bilharziasis. Report of a WHO Scientific Group. WHO Technical Report Series, 317, 3-70.

WORLD HEALTH ORGANISATION, (1976). Special programme for research and training in tropical diseases. TDR/WP/76.5, p.2.

WORLD HEALTH ORGANISATION, (1978). Scientific working group on schistosomiasis. Epidemiology and control of schistosomiasis : present situation and priorities for further research. Bulletin of the World Health Organisation, 56, 361-369.

WRIGHT, C.A. (1960). The crowding phenomenon in laboratory colonies of fresh water snails. Annals of Tropical Medicine and Parasitology, 54, 224-232.

WRIGHT, W.H., BOZICEVICH, J., BRADY, F.J. and BAUMAN, P.M. (1947). The diagnosis of schistosomiasis japonica. V. The diagnosis of schistosomiasis japonica by means of intradermal and serological tests. American Journal of Hygiene, 45, 150-163.

YOGORE, M.G. Jr. and LEWERT, R.M. (1974). Schistosomiasis japonica in the Philippines : results of standardized immediate intradermal (ID) tests. American Journal of Tropical Medicine and Hygiene, 23, 393-399.

YOGORE, M.G Jr., LEWERT, R.M. and SILAN, R.B. (1968). The circumoval precipitin (COP) test in schistosomiasis japonica. American Journal of Tropical Medicine and Hygiene, 17, 65-71.

YOKOGAWA, M. (1969). Immunological aspects of schistosomiasis. Proceedings of the 4th South East Asian Seminar in Parasitology and Tropical Medicine and Other Snail-transmitted Helminthiasis, Bangkok. Ed. C. Harinasuta, pp. 121-130.

YOKOGAWA, M., SANO, M. and ARAKI, K. (1967). Immunodiagnosis of schistosomiasis japonica. III. Circumoval precipitin test. Japanese Journal of Parasitology, 16, 77-84.

YOSHIMOTO, S. (1910). Ueber die komplement-bindongreaktion bie der schistosomumkrankhiet in Japan. Zeitschrift fur Immunitatsforschung, 5, 438-445. Cited by I.G. Kagan and Pellegrino, J. (1961). Bulletin of the World Health Organisation, 25, 611-674.

ZUCKERMAN, A.J. and TAYLOR, P.E. (1970). Immunoelectro-osmosis in the rapid detection of the Australia hepatitis-associated antigen and antibody. Shandon Instruments Applications, Number 32.

APPENDICES

1

Buffers and Reagents for Immunoelectrophoresis

Phosphate Buffered Saline (PBS) pH 7.6

Sodium chloride	170.00 g
Disodium hydrogen phosphate	25.60 g
Sodium dihydrogen phosphate $2H_2O$	3.12 g
Distilled water	20,000.00 ml

Barbitone/Acetate buffer 0.13 M pH 8.4

Sodium diethyl barbiturate	8.142 g
Sodium acetate	6.476 g
0.1 N Hcl	90.000 ml
Distilled water to	1000.000 ml

Agar/Agarose mixture (1%) with Dextran in Barbitone/Acetate Buffer

Oxoid Ion Agar	0.75 g
Agarose	0.25 g
Dextran	2.00 g
Barbitone/Acetate buffer	100.00 ml

Coomassie blue staining

(A) Coomassie brilliant blue	1 g
Acetic acid	100 ml
Ethanol	450 ml
Distilled water	450 ml
(b) Acetic acid	100 ml
Ethanol	250 ml
Distilled water	650 ml

Stain with (A) for 5-15 minutes
Destain with (B) for 5-10 minutes

2

Buffers for ELISA

Coating carbonate/bicarbonate buffer 0.05 M pH 9.6

Sodium carbonate	1.59 g
Sodium hydrogen carbonate	2.93 g
Sodium azide	0.20 g
Distilled water	1000.00 ml

Incubation buffer

PBS	1000.00 ml
Tween 20	0.50 ml
Sodium azide (NaN_3)	0.20 g

(Omit NaN_3 when using peroxidase)

Washing buffer

Sodium chloride	45.0 g
Tween 20	2.5 ml
Distilled water	5000.0 ml

Diethanolamine buffer pH 9.8

Diethanolamine	97.0 ml
Distilled water	800.0 ml
Magnesium chloride $6H_2O$	100.0 mg
Sodium azide	0.2 g

Adjust to pH 9.8 with 1 M Hcl

Substrates

(1) For alkaline phosphatase conjugates

Add 5 mg tablet p-nitrophenyl phosphate (Sigma) to 5.0 ml diethanolamine buffer

(2) For peroxidase conjugates

Stock solution

Ortho-phenylene diamine (O-PD)	100.0 mg
Absolute methanol	10.0 ml

Mix thoroughly and store in the dark at $4^{\circ}C$, stable for one week

Working solution

Distilled water	99.0 ml
Stock O-PD	1.0 ml
Hydrogen peroxide 3%	0.1 ml

Substrate Inhibitors

3 M Sodium hydroxide for alkaline phosphatase conjugates

8 N Sulphuric acid for peroxidase conjugates

3

Reagents for Histological and Immunofluorescent Studies

Buffered 10% formalin pH 7.0

Formalin	100.0 ml
Tap water	900.0 ml
Sodium dihydrogen phosphate H_2O	4.0 g
Disodium hydrogen phosphate	6.5 g

Tris-buffered glycerol 0.01M pH 9.6

Tris "hydroxymethyl" methan	One part
Glycerol	Nine parts