STUDIES ON NEMATODE SECRETORY ANTIGENS IN IMMUNODIAGNOSIS WITH SPECIAL REFERENCE TO TOXOCARIASIS

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ABSTRACT

Immunodiagnosis of nematode infections in man and animals is often inaccurate and unreliable. A rationale is presented for a novel serologic approach which uses *in vitro* cultivation of the relevant parasitic stages to isolate *in vitro* released secretory antigens for use in immunoenzymatic assays. The rationale was tested in the toxocariasis model in man and experimental animals and found to be valid.

An *in vitro* cultivation system is described for the maintenance, in synthetic medium, of actively metabolizing *Toxocara canis* infective larvae producing 200 pg of antigen per larva per day. Toxocaral secretory antigen was shown to be a simple mixture of at least three genus specific, protein antigens with a major component molecular weight of 42 k daltons and isoelectric point of pI = 9.5.

An enzyme-linked immunosorbent assay designed to quantitate anti-toxocaral responses was validated in assays of over 1000 sera from human subjects and experimental animals. Results indicated an advance from existing methodologies. The assay was further evaluated in practice for the serodiagnosis of visceral and ocular toxocariasis. In man, these forms exhibited distinct serological patterns. A realistic model of experimental ocular toxocariasis was developed in the rhesus monkey (*Macaca mulatta*), and results indicated that intraocular toxocaral larvae are capable of inducing retinal pathology in animals sensitized by a previous toxocaral infection, but not in animals which are *Toxocara* naive.

Seroepidemiologic studies demonstrated that Iceland and Sudan are non-endemic, England is endemic and Czechoslovakia and Ghana are hyperendemic for toxocariasis. Within these areas subpopulations were identified with higher prevalence of infection. Seroepidemiology in

naturally infected dogs revealed that tolerance to toxocaral secretions may occur in pups less than six months of age.

The studies indicate that *in vitro* derived, nematode secretory antigens, used in enzyme-linked immunosorbent assays can provide highly sensitive, genus specific, and reliable immunodiagnostic and seroepidemiologic data on a basis that is both economical and practical. It is proposed that this approach might be applied to the diagnosis of other tissue-invasive nematode infections.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

GENERAL INTRODUCTION

The serology of helminthic disease in man is perhaps the least reliable discipline in laboratory medicine today. This is particularly so for nematode infections, in which immunodiagnostic procedures are often inadequate because poorly defined and non-specific antigens are used indiscriminately in serological techniques of inappropriate design.

The inadequacy of helminth immunodiagnosis has been a persistent and major problem. Clinicians, throughout their careers, regularly encounter patients who suffer a medley of signs and symptoms matching no known syndrome or disease. In helminthology especially, the protean nature of clinical presentation sets diagnostic pitfalls, while clinical signs may mimic a wide variety of infectious processes. In situations conducive to helminth transmission, multiple parasitic (and non-parasitic) infections often prevail. For added complication, the distinction between 'infection' and disease' is a concept easily misinterpreted when considered within the phenomenon of parasitism. Thus laboratory methods, both parasitological and serological, hold pre-eminent roles in the diagnosis of helminthic disease.

In theory, indirect serological diagnosis poses a more attractive technical alternative than direct parasitological diagnosis, however this promise has not been fully realized in practice, partly due to historical - but still justified - lack of confidence in helminthic serology. Nevertheless, there are circumstances in which serology affords the only practical means for establishing diagnosis. These include: early diagnosis during long prepatent periods (eg. onchocerciasis); low intensity or single-sex nematode infections in which ova or larvae may not be liberated in clinical samples (eg. anisakiasis); infections with nematodes which multiply within the

host (eg. trichiniasis); infections of low intensity which may become life-threatening during immunosuppressive therapy (eg. strongyloidiasis); infections in which man is a paratenic host to tissue-invasive larval stages (eg. toxocariasis).

Additional to these special cases, need is growing for improved serological tools, both in the developed and developing worlds, for use as diagnostic aids for all helminthic infections. In temperate regions there is concern with increased importation of disease resulting from the frequency and ease of commercial and recreational travel to the tropics. Contemporary political phenomena which displace large refugee populations from tropical to temperate regions have highlighted current deficiencies. Within the tropics, the requirement for high levels of specificity in immunodiagnosis is compounded by multi-parasitism of individual hosts. The implementation of large-scale eradication or control measures has created the need for seroepidemiologic monitoring procedures sensitive to both light and early infections as well as being practical and economical in field conditions.

During the past two decades, significant progress has been won in the development of immunologic procedures with greatly enhanced sensitivity. However, the application of these to the unique challenges of parasitology has only magnified the problem: lack of specificity. It is noteworthy that historical advances in immunodiagnosis for helminthic disease have occurred principally as a result of improvements in antigen quality, not test methodology. Development of helminth antigens has failed to keep pace with development of the assays in which they are used. Workers have been preoccupied with the use of whole worm, somatic antigens which, in their crude state, are ill-defined, heterogeneous mixtures from which the fractionation of specific antigen has proved formidably difficult. Cross-reaction has been the rule rather

than the exception. Throughout these years, a few workers have suggested that nematode secretions might provide a more satisfactory source of diagnostic antigen, but the lack of immunoassays suitably efficient in antigen consumption has impeded progress in this direction. Recent advances in both these fields now allow further assessment of the potential role of secretory products as a practical source of antigen.

In the present work, toxocariasis was selected as a model nematode infection because it exemplifies the problem. Aside from being a readily available, cosmopolitan infection, distributed widely from the tropics to the subarctic, toxocariasis has been difficult to diagnose either clinically, parasitologically or serologically. Clinically, no pathognostic features occur. The spectrum of disease ranges from occult, subclinical infection to severe morbidity with possible mortality. Parasitologically, patent infections never occur and definitive diagnosis is obtained only through biopsy, a procedure rarely successful because parasites may be widely dispersed in tissues and impracticable to locate. Serologically, the aetiologic agents (Toxocara spp.) are known to share antigens with, among others, Ascaris lumbricoides, the most commonly occuring nematode in man.

Using the toxocariasis model, this thesis describes the development of an immunoassay using nematode secretory antigens and assesses its ability to provide sensitive, and reproducible data on a practical basis for use in serodiagnosis and seroepidemiology.

CHAPTER 1

REVIEW OF LITERATURE

A. Toxocariasis: Historical Perspective

Toxocara canis (Nematoda) was first described by Werner in 1782 and Toxocara cati by Shrank in 1788. Yet almost 200 years passed before their aetiology in human disease was proved (Beaver, Snyder, Carrera, Dent, and Lafferty, 1952) and their life cycles elucidated (Sprent, 1956, 1958). The history of events leading to the recognition of toxocariasis as a disease entity in man focuses attention on the problems responsible for the delay in its discovery; problems which still exist for the contemporary diagnostician.

As early as the 1920's, the medical literature contains numerous case reports of persistent eosinophilia, hepatomegaly, pulmonary infiltration and poor health with unknown aetiology. Many of these cases were in children and some cases showed more than one member of a family affected. Numerous eponyms were applied including familial eosinophilia, Frimodt-Möllers syndrome, Loeffler's syndrome, Weingarten's disease and eosinophilic pseudoleukemia. The syndrome appeared to involve a hypersensitivity reaction and various allergic aetiologies were proposed involving pollen, bacteria and drugs.

In 1921, Fülleborn discussed the possibility of larval infestation and associated hypersensitivity in man and Chandler (1925) and Schwartz (1932) recorded the suspicion that early development of Toxocara canis including invasion of the lung probably occured in man.

Thomson, Wilson and McDonald in 1937 reported for the first time the observation of numerous white pin-head sized lesions resembling tularemic foci on the surface of the liver of an adult patient with

illness characterized by prolonged eosinophilia.

Atmar (1940) described similar lesions on the liver surface of an adult patient with "familial eosinophilia". Familial eosinophilia was defined (Atmar, 1940) as "the presence of an increase in mature eosinophilic leucocytes in the blood of several members of a family in which none of the known causes of eosinophilia such as allergic states, dermatoses and parasitic infestations was found".

In 1947 Perlingiero and György reported the presence of eosinophilic granulomata in the liver of a child with a syndrome characterized by chronic eosinophilia, fever, pulmonary distress, hyperglobulinaemia and leukocytosis.

In 1949 Zeulzer and Apt described seven cases of disseminated visceral lesions in children 18 months to three years of age presenting with the same clinical syndrome. Liver lesions were demonstrated at biopsy or autopsy in each case. These authors interpreted the lesions as representing an expression of a "common pathologic process of variable localization and severity, the nature of which is regarded as an allergic-hyperergic tissue response to undertermined antigens".

In 1950 Loeffler's syndrome (transient pulmonary infiltration with blood eosinophilia) was reviewed by Nemir, Heyman, Gorvoy and Ervin (1950) with a report of eight cases, all occurring in children.

In the same year Mercer, Lund, Bloomfield and Caldwell (1950) described the case of a 2-year-old child suffering from headache, fever, abdominal distension and pain. The child had extreme eosinophilia and at laparotomy showed numerous lesions typical of those previously described. In biopsy material from these lesions, nematode larvae were detected. Ascariasis was suspected and on treatment the child passed one Ascaris lumbricoides specimen but little improvement in clinical condition resulted. The patient's history revealed pica and that he was

frequently left to play on a dirt basement floor. A check of this soil revealed embryonated parasite ova identified as Ascaris sp. However the photomicrograph of these ova in this paper shows clearly the ova of Toxocara sp. rather than Ascaris sp.

In 1950 Wilder reported a now classic work in which she re-cut serial sections of 46 eyes enucleated from patients three to 13 years of age with suspected retinoblastoma. The histologic examination of these lesions showed eosinophilic abscesses surrounded by epithelioid and giant cells with no evidence of neoplasm. Since these lesions were similar to those caused by nematodes elsewhere in the tissues, the sections were examined with a view to detecting the responsible organism. Nematode larvae or their residual hyalin sheaths were found in 52% of the eyes examined. The larvae were identified by B.G. Chitwood as being similar to third stage hookworm larvae. These same sections were later examined by Nichols (1956) who identified the larvae not as hookworm but as Toxocara sp.

In 1951 Beautyman and Woolf discovered nematode larvae in the brain of a child who had died from poliomyelitis. The larvae were identified as those of Ascaris lumbricoides however the authors recorded the larval dimensions as 300 to 350 microns in length and 15 to 20 microns in width. Sprent (1955) reported that the measurements in this paper are more suggestive of Toxocara canis than of Ascaris lumbricoides.

The following year Beaver et al. (1952) of Tulane University, published their classic paper describing three cases of eosinophilia with liver lesions and hyperergic syndrome in children in which Toxocara sp. larvae in liver biopsy were identified for the first time. In two cases the children played with dogs and in one case Toxocara canis ova were recovered from soil in the back yard of the patient's home.

These authors introduced the term "visceral larva migrans" (VLM) to describe the prolonged migration of nematode larvae through the internal organs of man. The invading larvae are generally of species naturally adapted to hosts other than man, remain immature and eventually perish in the tissues. The syndrome as they defined it is "usually a relatively benign disease, characterized chiefly by sustained eosinophilia, pneumonitis and hepatomegaly and probably is due both to direct tissue damage by migrating larvae and to allergic responses to their products. Its severity varies with the number of larvae in the tissues and the immune or allergic state of the infected individual".

In 1953 Brill, Churg and Beaver confirmed Beaver's (1952) observation by reporting the VLM syndrome in the autopsy of a 2-year-old child who died after a course of anorexia, lethargy, fever and abdominal pain. Typical lesions were observed in the liver, lungs, kidney, and myocardium. A toxocaral larva was identified in a lung section. The child was a known dirt-eater who played with several cats. There was no known contact with dogs.

Also in 1953 Smith and Beaver, in an experiment of questionable ethics, infected two mentally defective children aged two and three years, with 200 embryonated eggs of Toxocara canis. Both developed significant peripheral eosinophilia (52% and 45% respectively) within three months and some degree of hepatomegaly within one month. Eosinophilia persisted for at least 13 months after infection.

Karpinski, Everts-Suarez and Sawitz (1956) on reviewing two cases of VLM in children approximately two years old, who were both dirt-eaters and who associated with pet cats with proven Toxocara cati, suggested T. cati as a possibly significant cause. They emphasized that cats are more prone to defecate in children's sandboxes, under porches, and inside homes than are dogs and hence T. cati have a greater likelihood

of being ingested by crawling children.

Dent, Nichols, Beaver, Carrera and Staggers (1956) described a case of a 19-month-old child who died of what was at first recorded as overwhelming homologous serum hepatitis due to blood transfusion. At autopsy numerous granulomata were found in the liver, lung, brain, kidney, spinal cord, and myocardium. Live Toxocara canis larvae were recovered from many of these lesions. Sample digest estimates indicated 60 larvae per gram of liver and five larvae per gram of muscle. In press preparations there were three to five larvae per gram of brain. There was an estimated minimum of 50,000 larvae present in the body.

In the periodfrom 1952 to 1962 approximately 150 cases of visceral larva migrans syndrome were reported in the literature (Beaver, 1966) with new cases beingreported less frequently unless accompanied by unusual features. It was realized that other nematodes could be implicated in the syndrome (eg. Capillaria hepatica from rodents; Dirofiliaria immitis from dogs; Ancylostoma caninum, Ancylostoma braziliensis, Gnathostoma spinegerum, Uncinaria stenocephala from dogs and cats; Anisakis marina from fish; Toxocara vitullorum from calves; Ascaris suum from pigs). Therefore it was proposed that VLM be redefined to describe the migration of all nematode species in host tissues (Beaver, 1956). Sprent (1954, 1962) more than any other worker, led the way towards a re-examination of the concept of larva migrans and in 1969 redescribed it as a biological phenomenon rather than a clinical syndrome (Sprent, 1969). In response, Beaver (1969) withdrew his original definition, which he described in retrospect as placing erroneous emphasis on a picture of "the abnormal, aimless wandering of a hapless and frustrated, ill-fated worm having no happier prospect than eventual death after an indefinite period of inflicting damage to a physiologically inadequate, over responsive host". Beaver's new

definition was narrowed to include as causative agents only those species of nematode larvae undergoing prolonged migration and long persistance in paratenic hosts. By introducing the biological concept of paratenesis, he proposed Toxocara canis as the prototype of visceral larva migrans just as Ancylostoma caninum is prototypic of cutaneous larva migrans. Since this time the term 'VLM' has been gradually replaced in the medical literature by the specific aetiology when known (eg. toxocariasis), a convention which will be followed in this thesis.

A historical review of the relevant toxocariasis literature subsequent to 1956 is cumbersome unless some attempt at categorization is made. The remaining literature will be reviewed in terms of the parasites involved, the syndrome they cause, and the diagnosis of the syndrome, both clinical and serological.

B. Toxocariasis: Natural History

It is significant to note that as late as 1956, the life cycle and biology of *Toxocara canis* were still incompletely understood despite the incrimination of *Toxocara spp.* in human disease. The growing realization since 1952 of the importance of these parasites in pediatric medicine initiated renewed interest in various aspects of their life history on the part of several noted parasitologists.

In 1957 Schacher published an exhaustive study of *Toxocara canis* and *T. cati* larvae. He concluded as did Sprent and English (1958) that human infection with *Toxocara* is limited to the second-stage larva in the tissues.

In 1958 Sprent published a now classic work which for the first time effectively elucidated the complex life cycle of *T. canis* in the dog and its inherent epidemiologic implications.

1. Toxocara canis in Canidae

A summary of the work by Sprent (1958) shows that adult *T. canis* worms live in the small intestine of the dog were they produce large numbers of non-infective ova which are liberated to the environment via faeces. Each adult female worm may shed at least 200,000 ova per day (Schantz and Glickman, 1978). Under suitable conditions of warmth and moisture the ova become infective in two to six weeks to dogs and other animals which may ingest them. On ingestion, second-stage larvae are released in the small intestine and penetrate the mucosa. Newly hatched larvae exhibit thigmotropic behaviour and enter the portal circulation to the liver, passing through sinusoids to the hepatic vein, through the right side of the heart to the pulmonary artery and into the lung capillaries. When this occurs in pups less than five weeks of age the larvae pursue the classical cycle of tracheal

migration (ie. they moult to third-stage larvae, break out of the lung capillary bed into alveoli, ascend the respiratory tree, and are swallowed to the stomach and gut, moulting to fourth-and fifth-stage larvae en route, and develop to sexually mature adults in the upper small intestine). If this occurs in pups older than five weeks of age, or in any other mammal of any age, the larvae undergo somatic migration (ie. no ecdyses occur and second-stage larvae pass through the lung capillary bed, back to the left side of the heart via the pulmonary vein, and are disseminated via the arterial blood to all parts of the body where they persist in somatic tissues as second-stage larvae for long periods of time).

Pups under five weeks of age have little opportunity to ingest large numbers of T. canis ova. In an effort to explain the high rates of infection (approaching 100%) in young pups Sprent (1958) discovered that the most important means of infection is via the prenatal route. Previously dormant larvae in somatic tissues (particularly retro-peritoneal tissues) of the pregnant bitch mobilize after the 42nd day of gestation (Douglas and Baker, 1959) presumably due to hormonal stimulus related to pregnancy in the bitch. The reanimated larvae migrate through the placenta to the foetus ultimately to give rise to patent infection at three weeks of age (Yutuc, 1949). This interesting phenomenon has received relatively little attention (Scothorn, Koutz, and Groves, 1965) and awaits elucidation. The possibility that immunosupression during pregnancy might be implicated has not been adequately investigated.

Patent infection of pups commences four to five weeks after birth (Webster, 1958) and persists for approximately six months before adult worms are expelled from the gut (Sprent,1958). That this expulsion is immunologically mediated has not been tested. Soulby (1965) quoted

from various observations that bitches, after parturition, re-acquire patent T. canis infection and attributed this occurrence to a weakening of immunity. Sprent and English (1958) proved it was due to the bitch's habit of ingesting their pups' faeces which may contain advanced-stage larvae. Patent infection in the bitch is expelled more rapidly than in pups thus suggesting an immunologic mechanism. Predation on animals infected with paratenic second-stage T. canis larvae presents another source of non-patent infection in older dogs and other carnivores. Trans-mammary neonatal infection of pups with T. canis is less common (Sprent, 1961; Stone and Smith, 1973; Stage, 1976).

Thus it appears that T. canis has five possible life cycles in canids: 1) in utero infection resulting in tracheal migration and patent infection; 2) ingestion of trans-mammary larvae or ova from the environment resulting in tracheal migration and patent infection in young pups; 3) ingestion of ova resulting in somatic migration and non-patent infection in older pups and mature dogs; 4) post-parturient direct migration resulting in patent infection in the lactating bitch; 5) predation on paratenic hosts resulting in somatic migration and non-patent infection in older pups and mature dogs.

Age resistance to tracheal migration and patent toxocariasis in pups older than five weeks has been the subject of much research (Fülleborn, 1929; Hinman and Baker, 1936; Pullar, 1946; Noda, 1956; Boch, 1956; Ehrenford, 1957; Noda, 1958; Griesemer et al, 1963; Henson, 1971) but has not been adequately explained. The hypothesis that somatic migration and age resistance are manifestations of specific acquired immunity is not supported by experimental evidence from ascarid naive pups which indicates that somatic migration does not depend on previous exposure (Greve, 1971; Oshima, 1976; Dubey, 1978).

2. Toxocara cati in Felidae

Sprent (1956) reviewed and contributed to the elucidation of the life cycle and biology of Toxocara cati by demonstrating that cats of any age may acquire patent infection by ingestion of infective T. cati ova which hatch in the upper alimentary tract releasing larvae that undergo tracheal migration and return to the gut as advanced-stage larvae which mature to adults. Cats may also be infected by ingesting rodents carrying paratenic infection with T. cati second-stage larvae. Prenatal infection has been recorded but is of minor importance. Neonatal infection has not been observed.

Since the work of Schacher (1957) it has been accepted that the infective egg of the genus Toxocara contains a second-stage larva, however newer evidence (Paulo, 1972) suggests that two ecdyses occur in the egg of Toxocara and Ascaris and that the infective stage of these parasites is the third-stage larva. This is questionable since it implies five ecdyses in the life cycle.

3. Toxocara spp. in Paratenic Hosts Including Man

Paratenesis is a biological phenomenon in which infective-stage, parasitic larvae pass without essential development through a series of transport hosts to the final host; the transport hosts serving at the same time to maintain infective-stage larvae from one season of transmission to the next (Beaver, 1969). The spectrum of host specificity of the infective-stage is necessarily very broad whereas the host specificity of the adult stage is restricted to the definitive, carnivorous host. Man and all mammalian species can serve as paratenic hosts for Toxocara spp. When infective T. canis or T. cati ova are ingested, they hatch in the small intestine and second-stage larvae penetrate the mucosa, enter the portal or lymphatic circulation and are

carried to the heart, hence to be distributed to all tissues of the body via the arterial circulation (Woodruff, 1970). Studies of predeliction for a particular tissue in man have not been done, however, autopsy cases, in particular those reported by Dent (1960), Beaver (1956), and Mikhael et al. (1970) would suggest that most of the ingested larvae remain in the liver, or that the host response is most vigorous in that organ. No significant larval growth or development ensues in paratenic hosts and second-stage larvae persist indefinitely with dimensions of approximately 400 x 20 μ for T. canis (cf. 400 x 16 μ for T. cati), (Nichols, 1956).

Adult T. cati have been reported in man on 21 occasions (Ehrhard and Kernbaum, 1979). There have been few indisputable records of adult T. canis infection in man (Sprent, 1956; Bisseru, Woodruff and Hutchison, 1966; Wiseman and Lovel, 1969).

In monkeys Toxocara canis larvae are known to persist for at least nine years in the liver and probably reside in that organ (Beaver, 1962; Beaver, 1966). Aljeboori, Stout and Ivey, (1970) found, in baboons, that the largest number of T. canis larvae per gram of organ occurred in the liver although the lung sometimes contained lower but comparable numbers per gram. These authors maintain that the distribution of larvae is an important factor in determining the character of the disease that will result from toxocaral infection and suggest that the liver "protects" the lung from "verminous pneumonia" (intra-alveolar hemorrhage and oedema). Their observations indicate that there is some impediment, possibly of immunologic nature, to the progress of larvae through the liver and that pulmonary symptoms are directly related to the number of parasites reaching the lung per unit of time.

Sprent (1955b) reported striking differences in the migratory behaviour of T. canis and T. cati larvae in mice. A relatively large

proportion of T. canis larvae migrating in the tissues of mice were found to be distributed to the brain while the larvae of T. cati were rarely recovered from the brain. Sprent (1958) confirmed Nichols' (1956) observation that the diameter of second-stage T. canis was slightly greater than that of T. cati and deduced that the difference in migratory behaviour might be related to the difference in larval diameter in relation to blood vessel calibre of the host. Sprent (1955a) proposed that the larvae proceed along vessels and leave them at a point where larval diameter approaches vessel diameter. This has been offered in explanation of the "filtering" of T. canis larvae from the blood in the lungs of pups less than five weeks of age but seems an unsatisfactory interpretation.

Many authors (Lee, 1960; Oshima, 1961; Olson, 1962; Dubey, 1968; Burren, 1971; Olson and Petteway, 1972; Lee, Min, and Soh, 1974; Kayes and Oaks, 1976, 1978) have studied the distribution of toxocaral larvae in mice. Burren (1971) supported Sprent's (1955a,b,) observation in mice by reporting that 100% of an inoculum of T. canis eventually resided in the brain while only 8.3% of an equivalent number of T. cati were found in the brain. The predeliction tissue in this case was the cerebellum. Olson, Izzat, Petteway and Theinhard (1970) investigated ocular T. canis infection in mice and found that larvae entered the eye within three days of infection and that haemorrhage coincided with invasion. The posterior eye harboured more larvae. Olson and Petteway (1971) showed that T. cati did not enter the eye of mice. These same authors in 1972 demonstrated that T. canis larvae invade the spinal cord of mice and persist there for at least four months.

Toxocara larval distribution in the rat and guinea pig has been discussed by Beaver (1962) and Burren (1972). Burren found that the cerebellum of Toxocara infected rats and gerbils contained the greatest

number of larvae per unit tissue weight whereas no significant single tissue predeliction was determined for the hamster. The ability of rats to solve complex maze problems after being infected with 20,000 eggs of T. canis was studied by Olson and Rose (1966) and was found to be significantly reduced within one week of infection. This learning deficit persisted for at least four weeks. The cerebral cortex and the cerebellum harboured the most larvae.

Lautenslager (1970) observed differences in the migratory behaviour of T. canis and T. cati larvae in the rabbit. T. canis larvae that reached the brain tended to remain while T. cati larvae did not. T. cati larvae that reached the eye tended to remain while those of T. canis had less tendency to remain. Occasional tracheal migration was observed with T. cati in the rabbit.

Toxocaral infection has been studied in higher domestic animals including swine (Done and Gibson, 1958; Stevenson, 1979), sheep (Schaeffler, 1960) and cattle (Greenway and McGraw, 1970). Infection in chickens and pigeons has been reported by Galvin (1964), in turtles by Merdivenci and Zezen (1965).

In invertebrates, second-stage T. cati larvae have been recovered from the tissues of experimentally infected earth worms and cockroaches, but not from beetle larvae or wood lice (Sprent, 1956). Pegg (1970, 1971) in a series of experiments suggested that the adult house-fly, Musca domestica, could become infected with T. canis larvae on ingesting infective ova and thus act as a source of infection to other animals including the dog. Edwards (1971) disputed this work pointing out that it is physically impossible for adult Musca domestica to ingest Toxocara ova and suggested that they merely act as vectors for the ova. It is possible to infect fly maggots with Toxocara canis larvae (Edwards, 1971).

C. Visceral Toxocariasis in Man

1. Epidemiology

Toxocariasis appears to be a world-wide zoonosis occurring wherever man co-exists with canidae and felidae populations (Sprent, 1969). There have been many prevalence studies of patent T. canis infection in dogs (reviewed by Cypess, 1978), which uniformly record this infection as amongst the most common parasitoses of canidae, infecting on average, 80% of pups and 20% of older dogs. Distribution ranges from equatorial regions, (Dada, Adegboye, Mohammed, 1979) to the sub-arctic (Viens, 1975). In dogs, Toxocara is one of the few helminths whose distribution is not influenced by climatic factors (Ng and Kelly, 1975; Becker, Selby, Hutcheson and Hacker, 1977) a feature which testifies to the parasitological advantage of prenatal infection. Intensity of infection in dogs may be seasonally affected (Woodruff, Thacker and Shah, 1964; Wiseman, 1969) as may egg output (Chieffi and Müller, 1978). Prevalence studies (by 17 authors) of Toxocara infection in definitive hosts reviewed by Mok (1969) indicate that prevalence rates for Toxocara canis range from 6.4% to 100% with a world average of 35.5% however, such figures are influenced profoundly by the age (often unknown) of the dogs studied. Prevalence of Toxocara cati ranged from 8% to 61% with an average of 40.4%. Prevalence has been found high in well-cared-for as well as free-ranging and feral animals (Jacobs, Pegg and Stevenson, 1976; Selby et al, 1977; Beck, 1973).

Of more importance to the epidemiology of toxocariasis in man is the distribution of dog and cat faeces and the dispersal, survival, and infectivity of Toxocara spp. ova in the human environment. Borg and Woodruff (1973) were first to survey systematically,soil samples in public areas and found ova of Toxocara spp. in 24.4% of 800 samples,

many of which were collected in urban areas in the UK. This led to intensive studies of urban dogs (Beck, 1973, 1975, 1976; Fox, 1976) and urban soil samples (Dubin, Segall and Martindale, 1975; Read and Thompson, 1976; Ghadirian et al, 1976; Chieffi and Muller, 1976; Dada, 1979; Quinn, Smith, Bruce and Girdwood, 1980). It was estimated that New York city's dog population of 500,000 deposited 170,000 kg of faeces per day on city streets (Beck, 1973) (0.34 kg per day per dog). Houston estimated 63 million kg of dog faeces per year (Arambulo, and Steele, 1976). Toxocara spp. ova are the most commonly occurring parasite ova in sewage sludge in Canadian cities and probably enter the system through storm sewer run-off from city streets (Graham, 1976).

Owing to their impervious "shell", Toxocara ova are extremely resistant to adverse conditions and to disinfectants. They may remain viable for several years in moist soil and are resistant to long exposures (three days) to cresol disinfectants (eg. 2.5% chlorxylenol) (Lautenslager, 1973). Ova survive at least four years in 1% formaldehyde (Headlee, 1936), withstand composting for at least one year (Pegg and Donald, 1978) but do not survive freezing at -15^{o}C. Ghadirian et al. (1976) found that ova can endure a Canadian winter in frozen soil presumably due to the insulative effects of soil and snow cover. To kill toxocaral eggs in children's sandpits required the application of steam at 160^{o}C under 10 atmospheres of pressure for five hours (Knappen, Frachimont and Otter, 1979). Ova are susceptible to direct sunlight and desiccation (Lautenslager, 1972). An intriguing possibility for control of ova in soil is the use of ovicidal fungi (Lysek, 1978).

Bourke and Yeates (1961) suggested common ways in which children may become infected;

1. By handling pups of age three weeks to six months.
2. By contact with objects contaminated with infective ova

either inside or outside the home.

3. By ingestion of soil containing ova.

4. By direct contamination of hands due to contact with a nursing bitch or her immediate environment.

Pegg (1970) suggested that the common house fly may carry Toxocara ova and may contaminate food with them; a potential source of infection for adults. As predicted by Woodruff (1970), Toxocara ova have been found on soil-contaminated foods (eg. lettuce leaves) (Anuar and Ramachandran, 1977) which thus constitute a potential source of infection for adults as well as children. Local cultural habits may also predispose adults to toxocariasis. For example, beetle-eating in Malaysian folk medical practice (Chu, Palmmieri and Sullivan, 1977) and the Korean custom of eating raw beef liver, a source of Toxocara vitullorum larvae (Lee, Min, Chung, and Chang, 1976). Congenital transmission of toxocariasis in man has been suggested by de Savigny and Tizard (1977) and induced in experimental paratenic hosts (Lee, Min, and Soh, 1976).

Early investigators recognized toxocariasis mainly in children less than four years of age (reviewed by Mok, 1968). These cases usually recorded a history of pica or geophagia and ownership of a pet dog or cat from which Toxocara ova were being shed (Snyder, 1961; Beaver, 1962; Shrand, 1964; and Huntley, Costas and Lyerly, 1965). Boys appeared to be infected twice as commonly as did girls (Snyder, 1961; Beaver, 1962). Later workers realized that infection was not uncommon in adults (Woodruff, 1970; Rook and Staughton, 1972). Ehrhard and Kernbaum (1979) reviewed 1,920 case reports of which 17.7% were adult, 44% were less than three years of age and the average age was 9½ years. As more cases in both adults and children were seen it also became evident that histories of pica and of pet ownership were not essential

features (Woodruff, 1970) since the presence of ova in the environment can be ubiquitous. In North America it was estimated that one household in three owns a dog (Beck, 1975). It has been reiterated that toxocariasis has much greater incidence in man than is realized (Beaver, 1962; Sprent, 1963; Mok, 1968; Woodruff, 1970, Borg and Woodruff, 1973, Schantz and Glickman, 1978). Woodruff (1970) has shown by skin tests that at least 2% of healthy persons in Britain have been infected with Toxocara. "It is evident that either subclinical Toxocara infections are common or that when patients are examined during the symptomatic phase, the diagnosis is overlooked unless one of the more dramatic manifestations of the disease forces it on the physician's attention" (Rook and Staughton, 1972).

Much of the epidemiology of toxocariasis has been revealed from seroepidemiologic studies and these are reviewed separately in Section E.2.

2. Clinical Manifestations

The frequency of various manifestations noted in toxocariasis has been surveyed from the world literature describing 350 cases reported between 1952 and 1978 (Table 1 and 2). Such surveys may be misrepresentative since the motive to publish case reports in recent years is biased toward those with more unusual features. Common features (ie. those occurring in more than 50% of patients) are reviewed in more detail below.

Eosinophilia

Few parasitic diseases of man provoke a more severe or chronic eosinophilia than toxocariasis. Eosinophilia is the most consistent clinical feature: Snyder (1961) found that 100% of 20 cases and Shrand (1964) found that 100% of 40 cases had leucocytosis with an eosinophilia greater than 30%; Beaver (1962) reported that in approximately 80% of recorded cases, eosinophils represented more than 50% of circulating blood leucocytes. Two of these cases exceeded 80% of 90,000/mm^3 of leucocytes. Approximately 13% of cases have eosinophilia greater than 80%. (Ehrhard and Kernbaum, 1979).

In experimental human infection (Smith and Beaver, 1953; Chaudhuri and Saha, 1959) eosinophilia was a striking sequel as early as 13 days post infection with 100 to 200 embryonated eggs.

Archer (1963) feels that the most important factor on which eosinophilia depends is local histamine release in tissue chronically damaged by parasites. The raised blood histamine level may stimulate eosinophils in the bone marrow (Snyder, 1961). Archer (1963) also states that the parasite may release histamine as a result of antigen-antibody reaction. Follow-up studies (Zeulzer and Apt, 1949; Bourke and Yeates, 1961; Ashton, 1960; Irvine and Irvine, 1959; Huntley et al. 1965) have shown that eosinophilia declines to normal, usually in the space of a few

TABLE 1

NATURE AND FREQUENCY OF CLINICAL FEATURES IN 350 CASES* OF TOXOCARIASIS IN ADULTS AND CHILDREN

Feature	Total n=350**	Adults n=57	Children n=256
Hepatomegaly	74.6%	47.0%	79.4%
Fever	69.3%	71.4%	68.9%
Pulmonary Involvement	66.7%	41.7%	71.6%
(Radiologic Anomalies 70.5%=94/133)	47.6%	59.6%	43.7%
Gastro-intestinal Involvement	44.8%	63.4%	37.5%
Malnutrition	44.2%	36.4%	45.9%
Central Nervous System Involvement	35.6%	33.0%	36.1%
Splenomegaly (always associated with hepatomegaly)	32.9%	17.8%	34.8%
Anorexia	31.1%	30.0%	30.9%
Pallor	26.2%	12.0%	29.2%
Cutaneous Involvement	24.1%	29.3%	22.9%
Lymphadenopathy	21.2%	19.0%	21.3%
Cardiac Involvement	11.1%	9.7%	12.5%
Oedema	11.0%	4.9%	13.5%

* Surveyed from published cases in the literature according to Ehrhard and Kernbaum (1979).

** Age unknown in 28 cases.

TABLE 2

NATURE AND FREQUENCY OF
LABORATORY FINDINGS IN TOXOCARIASIS (350 CASES)*

FEATURE	FREQUENCY	
Hypereosinophilia (blood)		
> 400 X 10^6/l	100 %	
> 5,000 X 10^6/l	75.8%	
> 10,000 X 10^6/l	57.0%	
> 30,000 X 10^6/l	20.0%	
> 5%	98.7%	
> 30%	85.1%	
> 50%	52.5%	
> 80%	12.6%	
Hypereosinophilia (marrow)	96.3%	
Erythrocyte Sedimentation Rate Elevated	89.0%	
Hyperleucocytosis	83.8%	
Hypergammaglobulinaemia (> 12 g/l)	81.0%	
Heterophile Antibodies	74.4%	
Hyperglobulinaemia (>30 g/l)	73.8%	
Anti-Toxocara Antibodies	67.6%	
Isohaemagglutinins (anti-A, anti-B)	66.6%	
Anemia	62.7%	
Hypoalbuminaemia (<40 g/l)	62.2%	
Rheumatoid Factor (IgM anti-IgG)	50.0%	
Larvae detected histopathologically	42.0%	
(in liver biopsy)		(37.0%)
Non-specific Serological Reactions	42.0%	
(to Ascaris spp.)		(31.8%)
Coprology Positive for Parasites	31.0%	
(for Trichocephalus trichiura)		(19.7%)
(for Ascaris lumbricoides)		(13.8%)

* Surveyed from published cases in the literature according to Ehrhard and Kenrbaum (1979).

years, however, possible subsequent features of visceral toxocariasis such as ophthalmitis and encephalitis may be accompanied by a normal eosinophil count. Olson and Izzat (1972) also suggest the link between eosinophilia and allergic injury in toxocariasis and state that these occurrences reflect IgE-targetcell-Toxocaral antigen reactions leading to release of vasoactive amines and activation of kinins. They also suggest that the role of complement mediated release and activation of mediators should be considered.

Kayes (1978) and Kayes and Oaks (1980) reported that T. canis specifically sensitizes a sub-population of T-lymphocytes (in murine toxocariasis) that triggers the full display of peripheral eosinophilia and the participation of these eosinophils in the inflammatory response to migrating larvae. In T-cell deprived mice, no granulomas occur and eosinophilia is reduced.

Jones and Kay (1976) Butterworth (1977) and Austen (1978) have shown that eosinophils have a repertoire of catabolic activities appropriate for handling products of mast cells, and can modulate Type I hypersensitivity. This prompted Roitt (1979) to speculate that parasites may deliberately attract eosinophils to defend against attack by mast cells, thus casting new light on Kayes' work (vide supra). Toxocariasis would seem an excellent model in which to examine Roitt's hypothesis since high IgE levels are common (Hogarth-Scott, Johansson and Bennich, 1969) and larvae are often found alive within eosinophilic granulomas (Mok, 1968).

Hepatic Involvement

Among 40 children with toxocariasis reported by various authors, Shrand (1964) found hepatomegaly described in 87%. Liver biopsy had been performed on 25 of these children and characteristic lesions were found in 20. Larvae were found in only six cases. Huntley et al.

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(1965) in a review of 51 patients with toxocariasis noted hepatomegaly in 65% and elevated serum glutamic-oxaloacetic transaminase (SGOT) levels in 20%. Elevated SGOT has also been reported by Lewis and his associates (1962). Hepatomegaly is more common in children with toxocariasis than it is in adults (Ehrhard and Kernbaum, 1979).

Pulmonary Involvement

Pulmonary manifestations in toxocariasis have been well documented (Zeulzer and Apt, 1949; Dent, Nichols, Beaver, Carrerra and Stagger, 1956; Heiner and Kevy, 1956; Beaver and Danaraj, 1958; Chaudhuri and Saha, 1959; Dent, 1960; Williams and Henning, 1961; Lorentz, 1962; Haddow and Grant, 1970). Beaver (1962) estimated the incidence of pulmonary involvement to be about 50%; Ehrhard and Kernbaum (1979) reported 42% and 72% in adults and children respectively. Mok (1968) points out that it is not certain whether dyspnoea, cough, wheezes and infiltration seen in chest x-rays are the result of direct invasion of Toxocara larvae or a hypersensitivity reaction. Pneumonitis resulting from toxocariasis can lead to life-threatening respiratory distress or death (Jung and Pacheco, 1960; Beshear and Hendley, 1973). Moreau, Mary and Junod (1971) on examining two patients with protracted and severe bronchopulmonary disease and conclusive evidence of Toxocara infection concluded that toxocariasis apparently belongs to the family of immune-complex diseases and that certain asthmas and bronchopulmonary conditions presenting with high eosinophil counts could be attributable to toxocaral infestation. Brown in 1972 found evidence of toxocariasis in nine of 40 consecutive unselected asthmatic patients in a prospective study. Woodruff (1973) found anti-toxocaral antibody in 19.5% of asthmatics compared to 5.8% of controls.

Central Nervous System Involvement

Toxocaral granulomata and larvae have been demonstrated in brain tissue in man (Beautyman and Woolf, 1951; Dent et al. 1956; Dickson and Woodcock, 1959; Moore, 1962; Brain and Allan, 1964; Wood, Ellison, Kelly and Kaufman, 1965, Mikhael et al, 1974). Huntley, Costas and Lyerly (1965) in their review of 51 cases found 28% had convulsions. The convulsions were recurrent in nine of these patients. Ehrhard and Kernbaum (1979) recorded CNS involvement in 36% of 350 cases with approximately equal incidence in adults and children. Woodruff, Bisseru and Bowe (1965) suggested that epilepsy may be caused by granulomatous tissue formed around disintegrating larvae in the brain since toxocariasis had been demonstrated by skin test evidence in 7.5% of 349 epileptics while prevalence in the normal population was estimated by skin tests to be 2.1%.

Encephalitis, encephalopathies and meningitis have been reported in fifteen cases of toxocariasis and merit comment because this manifestation is often fatal. Mortality, usually in children, has been recorded by Beautyman and Woolf, 1951; Dent et al, 1956; Van Thiel, 1960; Moore, 1962: Schoenfeld, et al, 1964; Sumner and Tinsley, 1967; Schoehet, 1967; Mikhael et al, 1974. Toxocaral eosinophilic meningoencephalitis with reversable flaccid paraplegia in an adult has been reported (Müller-Jensen et al, 1973). The finding of larvae or eosinophilic granulomas within the central nervous system at autopsy suggests that neurologic disturbance is caused by actual larval invasion of the nervous tissues. Woodruff, Bisseru and Bowe, (1966) found that 13.6% of patients with poliomyelitis had evidence of toxocariasis (cf. 2.1% in controls). Khalil, Khattab, El-Fattah, Khalid, Awaad, and Rifaat (1971) reported a 5.8% incidence of toxocariasis in 102 infants from one to three years of age suffering with poliomyelitis in the

United Arab Republic.

Woodruff (1968) introduced the important concept that toxocaral larvae, on leaving the lumen of the bowel to migrate in the tissues, may act as vectors for viruses or other microorganisms or result in lesions which could afford foci for the growth of potential pathogens circulating at the time. Experimental support was provided by Pavri et al. (1975) using Japanese encephalitis virus and Toxocara canis in mice. The public health implications of this possible association of toxocariasis with the transmission of, or predisposition to, other microbial infections are insidious and far-reaching to the degree that ultimately such association may be the most important feature of toxocariasis.

Cutaneous Involvement

Skin lesions in toxocariasis have been described carefully by Dent and Carrera (1953), Smith and Beaver (1956), Heiner and Kevy (1956), Mok (1958), Friedman and Hervada (1960), Brain and Allen (1964), Shrand (1964), and Rook and Staughton (1972). The types of lesion are varied and include tender nodules in palms and soles, atypical erythema nodosum, purpura, fine papular rash, urticaria, usually on abdomen and extremities, and recurrent circular painless subcutaneous lesions in the loin and extremities.

It is difficult to determine the frequency of skin lesions in childhood infections from the literature. Arean (1964) reports this manifestation "in severe cases". Dent and Carrera (1953) describe it in "most of our seven cases". These authors record cutaneous lesions most commonly in children, however Rook and Staughton (1970) have described a severe case of extensive pruritus and erythema with nodules due to Toxocara in a 72 year old gardener. Ehrhard and Kernbaum (1979) record the frequency to be approximately 24% of published cases and

slightly higher in adults than in children.

Cardiac Involvement

Myocarditis has been reported in 7 cases (Dent et al. 1956; Friedman and Hervada, 1960; Becroft, 1964; Woodruff, 1965; Vargo et al, 1977) two patients recovered while five died. It is believed that myocarditis may be the result of the direct larval invasion of the myocardium (Mok, 1968) or a hypersensitivity reaction in response to the parasite (Sprent, 1963) or a potentiation of viral infection (Becroft, 1964).

Hyperglobulinemia

Heiner and Kevy (1956) reported elevated serum gammaglobulin levels ranging from 35 to 56 g/l. Ehrhard and Kernbaum (1979) found hypergammaglobulinemia (> 12 g/l) in 81% of 116 published cases. Shrand (1964) calculated that 30% of toxocariasis cases were accompanied by reports of elevated serum globulins. Snyder (1961) found five of eight patients had elevated globulin levels from 12 to 82 g/l but that hyperglobulinemia was a feature restricted to the more severe cases. Hyperglobulinemia defined as >30 g/l was noted in 73.8% of 153 published cases.

Huntley, Costas, Williams, Lyerly and Watson (1968) performed quantitative serum protein determinations on 50 patients with toxocariasis and 30 controls and showed that the albumin concentrations were significantly reduced in the patient group and associated with gammaglobulin levels greater than 12 g/l. Hypoalbuminemia less than 40 g/l occurred in 62.2% of 135 cases (Ehrhard and Kernbaum, 1979).

Mok (1968) suggests that the hypergammaglobulinemia may be due to production of specific antibody against Toxocara larvae or their products, or be partially or wholly nonspecific globulin, or perhaps reflect altered liver function.

Anti-A, anti-B and Anti-IgG Factors

In 1956 Heiner and Kevy found anti-human blood group antigen A and B titers greater than 1,000 in their three patients. Shrand (1964) reported an anti-A titer of 4,000 and an anti-B titer of 500 in his patient. In the 51 patients reviewed by Huntley et al. (1965) two-thirds had isohemagglutinin titers above 250 while all controls were less than 250. Ninety-four percent of the 51 patients had elevated anti-B titers. Huntley, Lyerly and Patterson (1969) reported that the isohaemagglutinins in 12 of 13 patients were IgG class, suggesting that human A and B blood group antigens might be present in toxocaral larvae. In a summary of 78 cases, elevated isohaemagglutinins were present in 66.6% and heterophile antibodies were present in 74.4% (Ehrhard and Kernbaum, 1979).

IgM anti-IgG antibody (rheumatoid factor) was elevated in 26 of 59 children with toxocariasis during the acute phase but returned to normal with clinical recovery (Huntley et al, 1966). Ehrhard and Kernbaum (1979) reported elevated rheumatoid factor in 50% of 34 cases.

Anaemia

Snyder (1961) reports that erythrocyte counts revealed anaemia "in most cases" and haemoglobin concentrations varying from 5.8 to 14 g/100ml with less than 11 g/100ml in 80% of cases. Huntley et al. (1965) described anaemia in 50.9% of 51 patients and Ehrhard and Kernbaum (1979) noted it 62.7% of 117 cases. The possibility that anaemia (and malnutrition occurring in 45.9% of 172 cases, loc.cit.) might be predisposing factors in pica rather than sequelae of toxocariasis has not received critical analysis in the literature.

D. Ocular Toxocariasis

Wilder, (1950) while surveying histologically 46 eyes enucleated for suspected retinoblastoma and subsequently diagnosed (because no tumour had been found) as Coats' disease, pseudoglioma, or endophthalmitis, found nematode larvae in 24 and tissue reactions characteristic of nematode endophthalmitis in the remaining 22. At that time the larvae found in Wilder's series were thought to be hookworm and it was not until six years later that Nichols (1956) when reviewing this series, ascertained that the larvae present in five eyes were those of Toxocara canis, the remainder being too incomplete to assess. All 46 cases were retrospectively diagnosed as ocular toxocariasis. Since then, over 330 cases of intraocular Toxocara have been noted in the world literature (Table 3) and it has become evident that, in comparison with visceral toxocariasis, ocular toxocariasis is the expression of a clinically and epidemiologically distinct form.

1. Epidemiology

Shrand (1964) and Ferguson and Olson (1967) noted that ocular toxocariasis is found usually in children older than the one- to four-year-old age group common in visceral toxocariasis. In a review of 245 cases, Henderson (1970) reported a mean age of 7.5 years with a range of 2 to 31 years. Schantz, Meyer, and Glickman (1979) observed a mean age of 8.6 years for their study group but noted that the date of onset preceded diagnosis by an average of 12.6 months, thus resulting in an average age of 7.5 years. Ehrhard and Kernbaum (1979) found 50% of 430 cases were less than 16 years of age and that mean ages varied according to the different ocular manifestations. The older age of patients with ocular toxocariasis might be explained in part by the difficulty in recognizing unilateral loss of vision in the very young child (Schantz et al, 1979), however ocular lesions are rarely seen in

visceral toxocariasis. The possibility that ocular toxocariasis might be a late-onset manifestation of visceral toxocariasis receives support from two case reports (Snyder, 1961; Bourke and Yeates, 1961) in which the visceral form was followed four years later by the ocular form. The relative frequency of ocular invasion as a proportion of total infections is unknown.

Pica does not appear in the histories of 100 cases reviewed by Zinkham (1978), however Schantz et al. (1979) found geophagia in 41% of 17 cases and attributed its detection to careful inverviewing technique. In the latter study, all cases and controls reported exposure to pets, and only exposure to puppies was significantly associated with the study group. Contact with dogs or cats was recorded in 33 of 245 cases (Henderson, 1970) but it was claimed that most investigators had not sought this information from patients.

Toxocara canis (cf. T. cati) appears to be the primary cause of human ocular toxocariasis. Of 86 enucleated eyes containing nematode fragments, Toxocara was identified to genus in 66 of which 63 could be speciated to T. canis (Henderson, 1974). T. cati has never been conclusively identified in the human eye although it has been suspected in at least one case (Harris, 1961). Olson and Petteway (1971) found that T. cati did not invade the eye in experimentally infected mice but that T. canis could readily do so and attributed this to the predeliction for brain tissue exhibited by T. canis.

Although epidemiologic studies of visceral toxocariasis commonly reveal a patient male:female ratio of approximately 2:1 (Snyder, 1961; Huntley et al. 1965; Mok, 1968; de Savigny and Tizard, 1977; de Savigny, Voller, and Woodruff, 1979; Ehrhard and Kernbaum, 1979), this relationship is less evident in ocular toxocariasis where ratios of 1.3:1 prevail (Henderson, 1970; Ehrhard and Kernbaum, 1979). The higher prevalence in males of both forms of toxocariasis is usually attributed

to culturally acquired behaviour patterns which result in a slightly higher level of hygiene in female children (Snyder, 1961). Some authors (Kernbaum, Tazi and Champagne, 1978; Ehrhard and Kernbaum, 1979) invoke as explanation, the superior humoral immune response of females afforded by the presence of the second X-chromosome.

In animals, experimental infections with varying numbers of larvae administered orally in a single dose have shown that the probability of ocular invasion increases with the number of ingested larvae (Olson, 1976). If this finding can be extended to the pathogenesis of ocular toxocariasis in man, ocular involvement should be more common in the most severe cases (ie. visceral toxocariasis). Paradoxically, the converse is true (see Section D.2.). If immune processes operate to protect the chronically infected host, then ocular invasion is more likely to occur in the individual not previously exposed to Toxocara and in whom relatively small numbers of larvae migrate unrestrained by the host's immune defences (Schantz et al, 1979). This is supported indirectly by the observation that ocular toxocariasis is extremely rare in dogs (Rubin and Saunders, 1965) however the specific immune status of Toxocara-infected dogs has not been examined. Factors governing the incidence of ocular toxocariasis in man also remain unknown. It is perhaps significant that anti-Toxocara antibody assays are negative in some cases of ocular toxocariasis (Duguid, 1961b, 1963; Woodruff, 1970; Kagan, 1979).

2. Clinical Manifestations

Ocular toxocariasis is encountered most commonly in the older child who presents with strabismus, uniocular visual disturbance, or blindness in one eye. Bilateral involvement is rare and has been suspected in only 12 of 430 cases (Hudomel, 1965; Ehrhard and Birnbaum, 1979). With rare exceptions (Unsworth et al, 1965; Danis, Parmentier, Maurus and Otten, 1966; Byers and Kimura, 1974; Negrel, 1977) ocular toxocariasis

does not co-exist with visceral toxocariasis and only occasionally (Bourke and Yeates, 1961; Snyder, 1961) can a prior history of visceral toxocariasis be documented. Thus the great majority of cases exhibit no hypereosinophilia, hepatomegaly, fever, pulmonary involvement, hyperglobulinaemia, immune anti-A or anti-B antibody, or elevated IgE levels (Woodruff, 1970; Henderson, 1970; Zinkham, 1978; Schantz et al, 1979). In contrast to eosinophilic granulomas which feature in visceral toxocariasis, the majority of inflammatory cells in some ocular lesions are monocytes and macrophages (Zinkham, 1978) although this is contrary to the reports of Wilder (1950) Rey (1962), Arean and Crandall (1963) and many others.

Schlaegel (1972) catalogued 14 clinically distinct forms of intraocular toxocaral infection to which Reese (1971) and Karel, Peleska, Uhlikova, and Hubner (1977) have contributed an additional two. The most frequently recorded manifestations in a survey of world literature (Table 3) are chronic endophthalmitis (classically described by Wilder, 1950; Duguid, 1961a) posterior-pole granuloma (Ashton, 1960; Duguid, 1961b; Ashton, 1969) and peripheral granuloma (Greer, 1963; Hogan, Welch and Spencer, 1965; Wilkinson and Kimura, 1971). All other aspects are of relatively infrequent expression.

Chronic Endophthalmitis

Endophthalmitis is the most common form of ocular toxocariasis (Perkins, 1966) and may in some cases be a sequel to or contemporaneous with larval granulomatosis, either posterior or peripheral (Duguid, 1963). Most reports describe posterior segment lesions although involvement of the anterior segment has been observed (Baldone, Clark and Jung, 1964). Endophthalmitis may progress to vitreous haze, subretinal exudation, and retinal detachment (Woodruff, Ashton and Stott, 1961; Duguid, 1961a; Rey, 1962). Vitreous bands associated

with retinal folds may also occur (Wilkinson and Welch, 1971). Of crucial diagnostic importance is the fact that the inflammatory mass seen in toxocaral endophthalmitis may masquerade clinically as retinoblastoma which also occurs most commonly in children (Wilder, 1950). Of 158 enucleations for retinoblastoma, 147 occurred in cases of endophthalmitis (Ehrhard and Birnbaum, 1979). The mean age of cases is 6 years 4 months (loc. cit.).

Posterior Pole Granuloma

Typically, the posterior pole granuloma is white or greyish, solitary, well defined, and most often located in the peri-macular region, often between the disc and the macula and sometimes in the macula. It is on average 3 to 4 disc-diameters in size (occasionally as large as 5 to 6 diameters) and may be slightly raised in the vitreous (Ashton, 1960; Duguid, 1961b; Ashton, 1969).

Among the merits of Ashton's (1960) publication is the proposal that if the larval granuloma can be clinically differentiated from retinoblastoma, surgical removal should not be necessary as the "larva in the granuloma may sooner or later die and the lesion heal by fibrosis". Woodruff (1970) confirmed this and described eight cases of ocular toxocariasis, diagnosed on the basis of clinical history supported by skin test results, who did not undergo enucleation. A five year follow-up revealed nothing suggestive of retinoblostoma. As a result,enucleation of this form is now relatively rare. A survey of 500 eyes of children enucleated for suspected retinoblostoma revealed non-malignant conditions in 265 of which only 18 (3.6%) were a result of toxocaral larval granulomatosis, this being the sixth ranked cause of mis-diagnosis (Howard and Ellsworth, 1965). Retinal granulomas due to Toxocara have often been found in adults (Raistrick and Hart, 1975; Raistrick and Hart, 1977) and it is remarkable that the mean age of

TABLE 3

REPORTED CASES OF OCULAR TOXOCARIASIS

Reference	Year	Symptomatology	No. of Patients
Wilder	1950	Endophthalmitis	46
Benedict	1958	Endophthalmitis	1
Irvine	1959	Retinitis-peripheral	1
Ashton	1960	Granuloma-posterior pole	4
Jung and Pacheco	1960	Nodule on Iris	1
Bourke and Yeates	1961	Endophthalmitis	1
Duguid	1961a	Endophthalmitis	4
Duguid	1961b	Granuloma-posterior pole	18
Harris	1961	Granuloma-posterior pole	1
Snyder	1961	Retinal haemorrhage	1
Woodruff et al.	1961	Endophthalmitis	1
Rey	1962	Endpohthalmitis	1
Braun-Vallon et al.	1963	Endophthalmitis	1
Brett	1963	Endophthalmitis	1
Greer	1963	Endophthalmitis	4
Baldone et al.	1964	Iridocyclitis	1
Braun-Vallon et al.	1964	Endophthalmitis	1
De Haan	1964	Endophthalmitis	1
Appelman et al.	1965	Iritis	1
Hogan et al.	1965	Granuloma-peripheral	1
Hudomel	1965	Granuloma-posterior pole	1
Huntley et al.	1965	Endophthalmitis	1
Remky and Kraft	1965	Granuloma-posterior pole	2
Unsworth et al.	1965	Granuloma-posterior pole	1
Bar-Ishak and Stein	1966	Granuloma-posterior pole	1
Danis et al.	1966	Granuloma-posterior pole	1
De Buen et al.	1966	Endophthalmitis	1
Maumenee	1966	Keratitis, larva in cornea	2
Otten et al.	1966	Choroidoretinitis	1
Perkins	1966	Uveitis	15
Wanieski et al.	1966	Endophthalmitis	1
Yoshioka	1966	Endophthalmitis	1
Divisova et al.	1967	Uveitis-posterior	1
Ferguson and Olson	1967	Endophthalmitis	1
Kagan	1968	Endophthalmitis	1
Nolan	1968	Endophthalmitis	1
Rubin et al.	1968	Retinal haemorrhage	2
Van Metre	1968	Endophthalmitis	4
Wiseman and Woodruff	1968	Iridocyclitis	14
ibid.		Granuloma-posterior pole	2
ibid.		Uveitis	1
Zinkham	1968	Endophthalmitis	6
Ashton	1969	Endophthalmitis	41
ibid.		Granuloma-posterior pole	46
Hogarth-Scott et al.	1969	Endophthalmitis	1
Makley et al.	1969	Endophthalmitis	2

Cont. .....

TABLE 3

(Cont.)

Reference	Year	Symptomatology	No. of Patients
Bird et al.	1970	Optic neuritis	1
Hardy-Smith et al.	1971	Anterior uveitis	1
Mullaney and Horan	1971	Endophthalmitis	1
Reese	1971	Orbital lesion	3
Wilkinson and Welch	1971	Endophthalmitis	13
ibid.		Granuloma-posterior pole	10
ibid.		Peripheral retinitis	18
Karel et al.	1972	Granuloma-posterior pole	1
O'Connor	1972	Sub-retinal tube	1
Kouba et al.	1973	Granuloma	3
Phillips and McKenzie	1973	Papillitis	1
Siam	1973	Granuloma-posterior pole	3
Byers and Kimura	1974	Larval embolization	1
Swartz et al.	1974	Endophthalmitis	3
Gutow et al.	1975	Granuloma (with worm tracks)	1
Raistric and Hart	1975	Granuloma-posterior pole	1
Valnickova et al.	1975	Granuloma-posterior pole	1
Raistric and Hart	1976	Retinal haemorrhage	1
ibid.	1976	Choroidoretinitis	2
Wolter et al.	1976	Granuloma (with worm tracks)	3
Hart and Raistric	1977	Granuloma-posterior pole	8
Huismans	1977a	Granuloma-posterior pole	2
Huismans	1977b	Choroidoretinitis	1
Karel et al.	1977	Larva in lens capsule	1
Leisegang	1977	Nodule on Iris	1
Shields et al.	1977	Endophthalmitis	1
Zinkham	1978	Endophthalmitis	6
Schantz	1979	Endophthalmitis	5
ibid.		Granuloma	12
Glickman et al.	1979	Lesion on Iris	1
Biglan et al.	1979	Endophthalmitis	2
		Granuloma-peripheral	2
		Vitritis	1
		Total	353

posterior pole granuloma due to Toxocara is 17 years 8 months (cf. supra) (Ehrhard and Kernbaum, 1979).

Peripheral Granuloma

It is necessary to distinguish between the peripheral granuloma discovered at enucleation in cases of chronic endophthalmitis (noted in four of ten cases by Wilkinson and Welch in 1971), and the peripheral granuloma visible by ophthalmoscopy in a relatively quiet eye. The latter is usually on the peripheral retina at the equator or associated with peripheral structures. Thus toxocariasis has become a newly recognized aetiology of chronic cyclitis, pars planitis, and peripheral choroidoretinitis (Ehrhard and Kernbaum, 1979). The existence of such lesions suggests the possibility of a different route of larval invasion, possibly via ciliary vessels rather than retinal arteries. Compared to endophthalmitis and posterior granuloma, peripheral granuloma is recognized most commonly in yet an older age range (Schlaegel, 1972).

Other Forms

These include; larval embolism in retinal arterioles (Byers and Kimura, 1974); isolated retinal haemorrhage (Rubin et al, 1968; Byers and Kimura, 1974); papillitis (Bird, Smith and Curtin, 1970); iritis (Jung et al, 1960, Appelmans et al, 1965); iridocyclitis (Baldone et al, 1964; Wiseman and Woodruff, 1968; Woodruff, 1970; Smith and Greer, 1971); keratitis (Baldone et al, 1964; Maumenee, 1965); cataract (Karel et al, 1977); orbital lesions (Reese, 1971); pars planitis (Ehrhard and Kernbaum,1979); larva in lens (Karel et al,1977).

Hypotheses

Contrasts between the clinical features of ocular and visceral toxocariasis have posed many questions for which various explanations have been offered.

Woodruff (1970) suggests that ocular toxocariasis is predominantly

unilateral since the possibility of larvae entering both eyes must be small even in heavy infestations. Woodruff (1964) also points out that the predeliction of the parasite for the eye is more apparent than real. If a Toxocara larva should enter the eye by chance, it is likely to cause damage which will result in symptoms leading to a precise histologic diagnosis whereas if the larva is held elsewhere it is less likely to give rise to any specific symptoms leading to recognition.

Ashton (1967) postulates that the larvae, with a diameter of 18-21 μ, are readily able to pass through the central retinal artery (diameter 100 μ near the disc) and are trapped near the posterior pole within small arterioles of 20 μ in calibre (especially those temporal to the disc in the macular region) or they are swept on to be impacted at the retinal periphery.

Mok (1968) speculates that after leaving the retinal vessel "the larva commonly burrows from the retina into the vitreous inducing the formation of granulation tissue on the retinal surface. The larva attracts eosinophils, epitheleoid cells, giant cells, lymphocytes and plasma cells in different proportions.

To explain why ocular toxocariasis is usually manifested either by chronic endophthalmitis or by retinal granuloma (or a process in evolution between the two) Duguid (1963) proposed that the former occurs in the non-immune host with high "tissue sensitivity" in which the larva passes rapidly into the eye and causes damage leading to chronic inflammation, exudation, retinal detachment, etc. In contrast the retinal granuloma is the response of an immune host with low tissue sensitivity and high resistance to the larva. The granuloma is seen as a foreign body reaction healing by fibrosis in analogy with tuberculosis. Elegant work by Kayes and Adams (1978) chronicling the development of persistent toxocaral granulomas suggests strongly that these are Type IV

hypersensitivity reactions (Gell, Coombs, and Lachmann,1975) to living worms and evidence of cell mediated immunity. Wilkinson and Welch (1971) suggest that the site of the lesion is determined by chance and that the observed differences in clinical form are secondary to variations in nematode activity and host response. Ehrhard and Kernbaum (1979) provide circumstantial evidence and reasoned argument to indicate that chronic endophthalmitis occurs in response to a dead larva while retinal granuloma is the response to a living larva or its products.

It now appears that further clinical studies of natural ocular infections in man are unlikely to offer new insights regarding pathogenesis. To examine these speculations critically, an experimental animal model for ocular toxocariasis must be developed.

In summary, the body of literature describing the natural history of toxocaral infection in definitive and paratenic hosts is extensive. Within this, particular attention has been focused on the visceral and ocular manifestations of toxocaral disease in man. But commensurate with knowledge gained from past studies is a lengthening list of new questions: does age resistance to patent toxocaral infection in young canidae have an immunological basis? Is the epidemiology of human toxocariasis related to the size of dog populations or to the persistance and survival of larvated ova in soil? Are cats a significant source of human toxocariasis? How long do toxocaral larvae persist in human tissues? What is the local and world-wide period prevalence of human toxocariasis? Are particular sub-populations at higher risk? Is toxocariasis an unrecognized cause of eosinophilia and hepatomegaly in the tropics? What is the nature and

extent of association between toxocariasis and other disease entities such as asthma, epilepsy, poliomyelitis, multiple sclerosis etc.? Why do visceral and ocular toxocariasis appear to be clinically distinct entities? Is ocular toxocariasis (granuloma vs. endophthalmitis) an expression of altered immunoreactivity to Toxocara? Does congenital toxocariasis occur in man?

It is conceivable that the answers to these and similar problems could be efficiently sought through the careful use of serodiagnostic and seroepidemiologic information. The following section reviews the current 'state of the art' of toxocariasis serology.

E. Immunoassays for Toxocariasis

The serology of toxocariasis has been a field of constant activity since 1960. The large number of publications reflects both the need for such procedures and perhaps the unsatisfactory outcome of much of this research. As yet there is no standard procedure accepted internationally as the test of choice or reference. An analysis of the complete literature is provided in Table 4 indicating the assay used, the source and nature of the antigen employed, and the final application of the method. It is readily apparent that few procedures have evolved beyond the research stage to application in either immunodiagnosis or seroepidemiology. Historically, this lack of success has lead to a proliferation of reports describing essentially the same type of antigen used in a panopoly of methods. Unfortunately, relatively little effort was devoted to the investigation of alternative sources of antigen.

1. Immunodiagnosis

Immunoreactivity to Toxocara has been measured by a variety of assays exemplified by the following works: Agglutination (Fellers, 1953); immunodiffusion (Kagan, 1957); bentonite flocculation (Sadun, Norman and Allain, 1957); haemagglutination (Jung and Pacheco, 1958; Kagan, Norman and Allain, 1959; Krupp, 1974; de Savigny and Tizard, 1977); in vitro larval precipitate test (Olson, 1960; Lamina, 1970); Schultz-Dale test (Sharp and Olson, 1961); direct cutaneous anaphylaxsis for delayed hypersensitivity (Duguid, 1961b); direct cutaneous anaphylaxis for immediate hypersensitivity (Woodruff, Thacker and Shah, 1961b); passive cutaneous anaphylaxis (Ivey, 1965); direct immunofluorescence (Hogarth-Scott, 1966); indirect immunofluorescence Bisseru and Woodruff, 1968); Prausnitz-Küstner reaction (Dobson, Campbell and Webb, 1967); immunoelectrophoresis (Jeska, 1967a,b); complement fixation (Fernando, 1968a,c,) conglutinating complement absorption test

(Fernando and Soulsby, 1974); Soluble antigen fluorescent antibody assay (de Savigny and Tizard, 1977); paper radioimmunosorbent assay (Girdwood, Smith, Bruce, and Quinn, 1978); enzyme-linked immunosorbent assay (de Savigny and Voller, 1978; Glickman, Schantz, Dombroske and Cypess, 1978; de Savigny, Voller and Woodruff, 1979); immunoradiometric assay (de Savigny and Voller, 1979).

A more detailed analysis of some key papers is provided in Table 5; contributions being selected not exclusively for their scientific merit (deficient in some) but also for their historical impact on the direction of subsequent work. Each of the assays described in Table 5 has been used in national reference diagnosis for toxocariasis. Some of these and other works are discussed below with a view to highlighting the problems encountered and the changes in approach which have evolved.

Applications Using Somatic Antigen

Historically, Fellers (1953) was first to report the use of a serologic technique to detect circulating antibody to Toxocara canis. At a time when relatively sophisticated serological techniques for other parasitic diseases were in use (eg. Sabin and Feldman, 1948), this initial approach was perhaps optimistically simple. "Pulverized" fragments of adult T. canis were tested for agglutination in the serum of a patient with toxocariasis. Although the result was positive, no specificity was demonstrated and normal sera were not investigated.

Heiner and Kevy (1956) used a saline extract of emulsified adult toxocaral worms as antigen in immunodiffusion tests by which precipitin lines were demonstrated with the serum of three children with toxocariasis but not three other family members or two other patients tested.

Sadun, Norman and Allain in 1957 described the use of bentonite flocculation, complement fixation, and precipitin tests using an acid-soluble protein fraction (according to Melcher, 1943) of adult

Toxocara worms as a source of antigen. Flocculation was the most reproducible of the three tests and detected antibody two to three weeks after experimental high-dose infections in rabbits. No cross-reaction could be demonstrated in humans and animals infected or immunized with various viruses, rickettsiae,bacteria, protozoa, and helminths with the exception of Ascaris, Clonorchis and Echinococcus. Sensitivity was low, flocculation being reactive in only six of 24 children having high eosinophilia and symptoms comparable to those of toxocariasis, and in only one of three monkeys infected with 900,000 T. canis embryonated eggs.

Kagan, Norman, and Allain (1959) proceded to study the use of the tannic acid haemagglutination and bentonite flocculation tests using as antigen Toxocara and Ascaris; a) whole worm and isolated tissue saline extracts; b) polysaccharide whole worm and tissue antigens prepared by ethanol fractionation; and c) a protein antigen prepared by the method of Melcher (1943). All but two of the 14 antigens isolated had serologic activity; however no specificity could be demonstrated. Despite their deficiencies, these haemagglutination and bentonite flocculation tests using adult somatic antigens have formed the basis of the national reference test for toxocariasis at the Communicable Disease Center of the US Public Health Service, Atlanta, until as recently as 1978.

Jung and Pacheco (1960) used an ethanol extract of adult T. canis and A. lumbricoides worms in a tannic acid haemagglutination test and were able to demonstrate titers for either Toxocara and/or Ascaris antibody above 1:80 in 79.3% of 29 cases of toxocariasis. However 10% of healthy controls also had titers greater than 1:80.

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Vinke (1962) evaluated Jung and Pacheco's Toxocara and Ascaris haemagglutination antigens by testing 197 persons in Curaçao. It was concluded that "these antigens were not reliable in the diagnosis of larva migrans infections".

In 1963 Huntley and Mooreland described gel diffusion studies with extracts of adult Toxocara and Ascaris worms. Nine antigenic components were demonstrated in Toxocara of which five were common with those of Ascaris. In human toxocariasis, two of three cases showed precipitins to Toxocara antigen while one of one case of Ascaris infection also showed precipitins to this antigen.

Woodruff et al. (1964) reported the use of an intradermal skin test using an aqueous extract of dried adult T. canis to measure Type I (immediate type) hypersensitivity. At an antigen dilution of 1:1000 no cross-reactions were observed.

Mitchell (1964) described an indirect fluorescent antibody test using formalin fixed second-stage T. canis larvae as antigen. In T. canis infected animals specific antibody appeared within two weeks. A weak reaction with anti-Ascaris sera appeared after repeated infection. Sera from a group of rabbits and monkeys immunized or infected with various bacterial, viral and parasitic antigens gave no reaction. Suspected human cases of toxocariasis were not tested, however 35% of normal adult blood donors gave a significant reaction in the test.

Jeska (1967) analysed the antigens of Toxocara canis and detected, by immuno-diffusion, 40 components in the adult worm, some of which were shared with larval stages.

Fernando (1968a) reported a singificant rise in heterophile antibody levels in rabbits after challenge infection of 100,000 T. canis preceded by an initial infection of 1200 T. canis two weeks previously. Forssman antigen was present in eggs and in adult intestine but not in the cuticle.

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In these animals (Fernando, 1968b) serum precipitins detected by agar gel diffusion were reactive with two antigens. It was shown that saline extracts of adult T. canis were deficient in at least three antigenic components present in extracts of infective ova.

In a review of toxocaral serodiagnosis, Kagan (1968) discussed the failures and suggested that "first, there may be no antibody in the serum during the chronic stages of visceral larva migrans; second, the antigens employed for diagnosis may not be sufficiently sensitive". It is significant that Kagan failed to identify antigen specifity as contributory.

In another review of the work to 1968 Soulsby (1968) restated the fact that only second-stage larvae of T. canis were responsible for toxocariasis in man but that antigens from the adult stage had been used in diagnosis. This author warned that such antigens were "relatively unpurified and liberal interpretation of reactions obtained with them should be guarded against".

Bisseru and Woodruff (1968) developed an indirect fluorescent antibody test using second-stage T. canis larvae as antigen. Reactive results showed fluorescence of the cuticle as well as fluorescent precipitates at the larval orifices. Cross-reactions were noted with Ascaris lumbricoides and subsequent absorption of reactive sera with Ascaris antigen was necessary. No cross-reactivity with other helminths was observed. Of normal, skin test negative, adults, 18.5% reacted positively while 34.5% of 29 patients with toxocariasis (clinical diagnosis with positive skin test) reacted positively.

Investigations using adult stage antigens and even heterologous species continued. Oliver-Gonzalez et al. (1969) analysed the serologic activity of adult Ascaris suum perienteric fluid fractionated with Sephadex G-200 and purified by cellulose acetate electrophoresis. Using

this antigen in tannic acid-indirect haemagglutination tests, 95% of children with presumed toxocaral visceral larva migrans were reactive. However 65% of adults from an ascaris endemic area also were reactive.

Hogarth-Scott, Johansson, and Bennich (1969) on examining the sera from 26 patients with clinical histories suggestive of toxocariasis demonstrated Toxocara reactive homocytotropic antibody as measured by passive cutaneous anaphylaxis in baboons. There was complete correlation between the presence of homocytotropic antibody and a 10- to 15-fold increase in serum levels of IgE.

Fernando,Vasudevan, Hamza, Pandithagunawardene and Samarasinghe (1970) studied precipitin reactions in monkeys (Macaca sinica) experimentally infected with T. canis and in children with suspected toxocariasis. Using saline extracts of adult and embryonated ova of T. canis and A. lumbricoides as antigen, these authors found four to five genus-specific antigens predominantly in the embryonated ova extract, and one antigen common to both genera. Of 53 children with the visceral larva migrans syndrome, ten had precipitin reactions specific for T. canis, the same ten showed precipitin reactions for the common group specific antigen, and seven reacted only with Ascaris antigen.

Wiseman and Woodruff in 1970 released an evaluation of their skin test for immediate hypersensitivity to Toxocara adult worm antigen. All of nine patients with proved toxocaral infection were reactive; of 122 with proved non-toxocaral helminthiases, 2.5% were reactive.

Aljeboori and Ivey (1970) described an improved indirect micro-haemagglutination test for detecting anti-Toxocara antibody in which they demonstrated crude larval or egg antigens to be more sensitive than adult antigens as indicators of antibody. Antibody was detected in experimentally infected baboons within one week, reached peak titers in two to three weeks and remained detectable for six to seven months.

Cross-reactions occurred with Ascaris suum infected baboons; however, encouraged by the sensitivity of the larval antigen these workers suggested that "attempts should be make to isolate specific antigens from the larval stage". Sera from cases of human toxocariasis were not tested by this method.

Lamina (1970a) used saline extracts of adult T. canis and various ascarids and could demonstrate no specific results by immunodiffusion.

Ball, Voller, and Taffs (1971) took advantage of accurately known histories of three patients to evaluate immediate and delayed skin sensitivity to extracts of T. canis, A. suum, and Necator americanus. Crude adult Toxocara antigen cross-reacted with Ascaris and Necator antigens and these authors recommended caution in the interpretation of both reactive and non-reactive results in the T. canis skin test. "A positive response may not be specific, and a negative response may indicate only that the infecting dose was below the threshold for the test or that infection occurred too recently or too long ago".

Beaufine-Ducrocq, Couzineau, Beauvais and Lariviere (1971) described an imaginative technique whereby embryonated eggs of T. canis were placed in a small section of washed hamster intestine, tied at each end, wrapped in hamster muscle, frozen at -20°C, sectioned by cryostat, and stored at -20°C. Immunofluorescence studies using Evans Blue as a counterstain showed titers of 160, 320, and 640 in three suspected cases of toxocariasis. Titers of A. lumbricoides carriers were not greater than 40.

Krupp (1974) examined sera collected from 237 patients with possible toxocariasis from areas within the U.S. using an indirect haemagglutination test with adult and larval Toxocara antigens. Of these, 35% were reactive while 5% (one of 19) were reactive in the uninfected control group.

Viens, Strykowski, Richards and Sonea (1975) described an indirect fluorescent antibody test using T. canis larvae sectioned in hamster muscle according to Beaufine-Ducrocq et al. (1971) but found absorption of test sera was necessary to avoid cross-reaction with ascariasis.

Relatively little effort has been devoted to the investigation of cell mediated immunity (CMI) in toxocariasis even though it is thought that such responses are more specific than humoral manifestations (Potaro, Kowalski, Howell and Ash, 1977). Wong, Embil and Ozere (1976) examined macrophage migration inhibition and lymphocyte transfromation using a crude extract of embryonated ova as antigen. Cross-reaction occurred between Toxocara and Ascaris. Regarding CMI, it is noteworthy that the intradermal skin tests used by Duguid (1961b) and Khalil et al. (1971, 1976a, 1976b) measured Type IV (delayed) hypersensitivity to toxocaral antigens, however neither of these authors discussed the significance of this aspect.

Cypess and Glickman (1976) working in New York were first to report use of the enzyme-linked immunosorbent assay (ELISA) for toxocariasis. These authors used a crude extract of embryonated T. canis ova but found that absorption of all test sera with Ascaris antigens was necessary to avoid non-specific results. Nevertheless 18% of healthy adults were positive. Cypess et al. (1977) later showed that the presence of C-reactive protein (CRP) in patients' sera could lead to false positives in this and other assays for toxocariasis using antigen prepared from ova. Despite these deficiencies, ELISA using somatic egg antigen was introduced as the reference diagnostic test at the U.S. Public Health Service, Communicable Disease Center (CDC) Atlanta in 1977.

Speiser and Weiss (1979) evaluated ELISA using toxocaral egg antigen obtained from the CDC and found extensive cross-reactivity with

filariasis and minor cross-reactivity with ascariasis, ancylostomiasis, strongyloidiasis, and echinococc o sis.

At the present time, although genus specific antigens have been identified in somatic tissues of Toxocara spp.(Jeska, 1969) the technical problems associated with their isolation and purification from the mixture of predominantly shared antigens have not been solved. Thus after two decades, no genus-specific immunoassays using somatic antigens are available for toxocariasis.

Following the development of in vitro cultivation for Toxocara canis larvae (de Savigny, 1975) the further introduction of techniques using toxocaral somatic antigens declined dramatically in favour of assays using in vitro derived secretory antigens (Table 4).

Applications using Secretory Antigen

In 1960, Olson, aside from being the first to use larval stages of T. canis as antigen, was also credited with the first use of "metabolic" antigen. The system employed was the in vitro larval precipitate test in which living, second-stage toxocaral larvae, obtained from the liver and lungs of experimentally infected mice, were incubated at 37°C in a wax-sealed, hanging drop preparation of patient's serum. The preparations were observed after 24 hours for the presence of immune precipitates of metabolite (excretions and secretions) adherent to the pores of living larvae. Sera of seven of 17 children with clinical histories suggestive of toxocariasis were reactive by this test. One of three patients with Ascaris infection was reactive.

Hogarth-Scott (1966) described a direct immunofluorescent technique for the demonstration of antibody to the excretory and secretory antigens of second-stage T. canis, T. cati, and Toxascaris leonina larvae. Living, artificially hatched, second-stage larvae of these parasites were incubated in fluorescein-conjugated human sera and sera from

experimentally infected animals. The preparations were observed for the presence of fluorescent precipitates at the oral, anal and excretory pores. Intra-genus cross-reactions were detected between T. canis and T. cati larvae, however no reaction was observed with Toxascaris leonina in either the homologous or heterologous systems. No reaction occurred in sera from a clinically normal human group. Approximately 29% of sera from patients suspected of previous clinical visceral larva migrans were found to react positively by this test.

Hosoi (1969) studied the antigenic activity of somatic antigen from saline extracts of freeze-dried adult T. canis worms and the excretory and secretory antigen prepared from adult maintenance medium. Haemagglutination titres with homologous rabbit antisera were 5,120 for somatic antigen and 20,480 for secretory antigen. Immunoelectrophoresis of secretory antigen versus homologous antibody revealed only two components.

Lamina (1970b) reported the third application of an in vitro larval precipitate test in which living T. canis larvae were placed in serum from guinea pigs infected with T. canis, T. cati, Ascaris suum, and Parascaris equorum. This test system showed genus-specific results and detected specific anti-Toxocara excretion-secretion antibody six days post-infection and continued to detect antibody for at least 199 days.

Diconza (1972) characterized rat precipitins to T. canis larval secretory and somatic antigens. Larval precipitating antibodies were present in rats between 21 and 183 days post-infection with 1000 T. canis ova. The precipitins were stable at heating to 60°C for one hour. G-200 and DEAE-cellulose fractionated precipitins were stable at 70°C for 15 minutes and resistant to 2-mercaptoethanol. Immunoelectrophoretic analysis of serum fractions indicated that the fraction containing fast 7S globulin was primarily responsible for

precipitin activity.

In the fifteen years following the discovery of toxocaral secretory antigen, despite promising specificity and sensitivity, no routine diagnostic or epidemiologic applications of these antigens emerged because of the impracticality of demonstrating labelled or unlabelled immunoprecipitates at the orifices of live parasites. However, in 1975 de Savigny reported the development of an *in vitro* culture method whereby *Toxocara* larvae could be maintained as infective second-stage larvae in a synthetic, chemically defined medium. Using this system, a simple procedure was described for extracting genus-specific larval secretory antigens in pure form.

de Savigny and Tizard (1975, 1977) described the first use of *in vitro* derived nematode secretory antigens in conventional serologic techniques; haemagglutination and soluble antigen fluorescent antibody (SAFA). Both assays were highly sensitive, detecting antibody in experimentally infected animals within nine days of infection, in animals infected with as few as ten larvae, and in 85% of cases of suspected toxocariasis in man. Specificity at the genus level was established and reproducibility was satisfactory. The haemagglutination test using secretory antigen was subsequently introduced by the Ontario Ministry of Health as the reference diagnostic test for toxocariasis in Canada.

Stevenson and Jacobs (1977) compared the performance of toxocaral somatic antigens (in indirect fluorescent antibody assays) with that of secretory antigens (in *in vitro* larval precipitate tests) in experimental infections of pigs with *Ascaris suum*, *Toxocara canis*, *T. cati*, *Toxascaris leonina* and *Parascaris equorum*. Results confirmed the genus-specific characteristics of secretory antigen and permitted use of the *in vitro* larval precipitate test in the investigation of toxocariasis

in a pig population in which ascariasis was endemic (Stevenson, 1979).

de Savigny and Voller (1978) and de Savigny, Voller and Woodruff (1979) described the application of secretory antigens in the indirect enzyme-linked immunosorbent assay (ELISA) and reported high degrees of sensitivity, specificity, reproducibility, and antigen efficiency (further described in this thesis) and recommended its use in central reference immunodiagnosis and in seroepidemiology.

In Scotland, Girdwood et al. (1978) reported the application of in vitro culture derived, toxocaral secretory antigen in the paper radio-immunosorbent test (PRIST) and found no cross-recations in any of 16 helminthic infections tested.

In the USSR, Ignashenkova, Yampolskaya, Ermolin, and Lysenko (1978) and Yampolskaya, Ermolin and Lysenko (1978) independently evaluated the Toxocara ELISA of de Savigny and Voller (1978), confirmed the absence of cross-reactions with ascariasis, echinococcosis and other parasitic diseases, and recommended it for clinical use. Yampolskaya and Ignashenkova (1979) further demonstrated the usefullness of this assay in seroepidemiologic studies (see E.2.).

In Canada, Yang and Kennedy (1979) compared the secretory antigen Toxocara ELISA to the haemagglutination reference test using the same antigen and found superior sensitivity by ELISA with no loss of specificity. There were no cross-reactions with ascariasis, trichinosis, or echinococcosis.

de Savigny and Voller (1979) devised an immunoradiometric assay (IRMA) for toxocariasis using secretory antigen but did not achieve any further increase of sensitivity compared to ELISA.

The practical aspects and rationale of the use of nematode secretory antigens in immunodiagnosis have been reviewed recently by de Savigny (1979).

The foregoing illustrates the spectrum of escalating technological sophistication which has been applied to the immunodiagnosis of toxocariasis. The analysis in Table 5 of several applications represents a cross-section of this spectrum and highlights many of the difficulties encountered by serologists when attempting to evaluate assay performance. Sensitivity, defined as the proportion of specifically diseased (or infected) individuals found positive by the assay (Martin, 1977) can rarely be determined with accuracy because the criteria which constitute clinically diagnosed toxocariasis have not been standardized and definitive parasitological diagnoses are generally not available. Relatively few authors have estimated the sensitivity of their applications or stated the criteria used for clinical diagnosis, thus, meaningful comparisons cannot be made. Specificity, defined as the proportion of healthy individuals found negative by the assay, can also be misleading because the prevalence of subclinical toxocariasis may vary between and among populations. Again, many of the proposed methods in the literature have neglected the examination of specificity in this way or have tested only very small samples. Similar criticisms of much of the literature can be raised concerning the search for cross-reactivity with other helminthiasis. However, cross-reactions when encountered are, to a degree, presumptive, since any patient who has acquired toxocariasis is perhaps more likely than "normal" controls to have acquired other geohelminthic infections concurrently. A further difficult feature in the evaluation of these assays is the general lack of any critical assessment of reproducibility.

TABLE 4

IMMUNODIAGNOSTIC APPLICATIONS IN TOXOCARIASIS

Author	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
TERTIARY ASSAYS						
Direct Cutaneous Anaphylaxis						
Jung & Pacheco	1958	ITH	Adult,L_2	●		*
Sprent & English	1958	ITH	Adult	●		*
Duguid	1961b	DTH	Adult,L_1	●		**
Sharp & Olson	1962	ITH	L_2	●		*
Duguid	1963	DTH	Adult,L_1	●		**
Woodruff *et al.*	1964	ITH	Adult	●		**
Woodruff *et al.*	1966	ITH	Adult	●		***
Dobson *et al.*	1967	ITH	Adult	●		*
Wiseman & Woodruff	1967	ITH	Adult	●		*
Bisseru	1968	ITH	Adult	●		***
Wiseman *et al.*	1968a	ITH	Adult	●		*
Wiseman *et al.*	1968b	ITH	Adult	●		***
Woodruff & Bradstreet	1968	ITH	Adult	●		**
Wiseman *et al.*	1969	ITH	Adult	●		*
Bradford	1970	ITH	Adult	●		***
Wiseman & Woodruff	1970	ITH	Adult	●		**
Ball *et al.*	1971	ITH,DTH	Adult	●		*
Khalil *et al.*	1971	DTH	Adult,L_1	●		***
Wiseman & Woodruff	1971	ITH	Adult	●		***
Collins & Ivey	1975a	ITH	Adult,L_2	●		*
Hogarth-Scott & Feerey	1976	ITH	Adult,	●	○	*
Khalil *et al.*	1976a,b	DTH	Adult,L_1	●		***
Khalil *et al.*	1979	DTH	Adult,L_1	●		***
Passive Cutaneous Anaphylaxis						
Ivey	1965	PCA	Adult	●		*
Ivey & Slanga	1965	PCA	Adult	●		*
Dobson *et al.*	1967	PCA	Adult	●		*
Hogarth-Scott	1967	PCA	Adult	●		*
Ivey	1967	PCA	Adult	●		*
Takeuchi	1970	PCA,ART	Adult	●		*

TABLE 4

(Cont.)

Author	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Collins & Ivey	1975b	PCA	Adult,L_2	●	○	*
Hogarth-Scott & Feerey	1976	PCA	Adult	●		*
Daffala	1978	PCA	Adult	●		*
Schultz-Dale Test						
Sharp & Olson	1961	S-D	L_2	●		*
Sharp & Olson	1962	S-D	L_2	●		*
Olson & Sharp	1963	S-D	Ova	●		*
Olson & Sharp	1963	S-D	Ova	●		*
SECONDARY ASSAYS						
Precipitin Reactions						
Heiner & Kevy	1956	ID	Adult	●		*
Kagan	1957	ID	Adult,Ova	●		*
Sadun et al.	1957	ID	Adult, Ova	●		*
Jung & Pacheco	1958	ID	Adult,L_2	●		*
Huntley & Moreland	1963	ID	Adult	●		*
Ivey & Slanga	1965	ID	Adult	●		*
Ivey	1965	ID	Adult	●		*
Jeska	1967a	ID,IEP	Adult	●		*
Jeska	1967b	ID,IEP	Adult,Ova	●		*
Capron	1968	ID	Adult	●		*
Fernando	1968b,c	ID	Adult,Ova	●		*
Perlmutter et al.	1968	ID	Adult,L_2	●		**
Jalayer	1969	ID	Adult	●		*
Jeska	1969	ID	Adult	○		*
Takeuchi	1970	RPT	Adult	●		*
Fernando et al.	1970	ID	Adult,Ova	●		*
Lamina	1970	ID	Adult	●		*
Khalil et al.	1971	ID	Adult	●		***
Fernando & Soulsby	1974	ID	Adult	●		*
Lamina	1974	ID	Adult	●		**
Dafalla	1975	CPT	Adult	●		*

TABLE 4

(Cont.)

Author	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Zheleva	1975	ID,IEP	Adult	●		*
Khalil et al.	1976a,b	PAT	Adult	●		***
Triboulez-Duret et al.	1976	ID	Adult	●		*
Zyngier	1976	ID	Adult	●		*
Cypess et al.	1977	ID	Adult,Ova	●		*
Enayat & Pezeshki	1977	CIEP	Adult	●		*
Girdwood et al.	1978	CIEP	L_2		▲	*
Glickman et al.	1978	ID	Adult	●		*
Khalil et al.	1978	PAT	Adult	●		**
In Vitro Larval Precipitate Test						
Olson	1960	IVLP	L_2		▲	*
Richards et al.	1962	IVLP	L_2		▲	**
Sharp & Olson	1963	IVLP	L_2		▲	*
Lamina	1968	IVLP	L_2		▲	*
Jalayer	1969	IVLP	L_2		▲	*
Lamina	1970	IVLP	L_2		▲	**
Diconza	1972	IVLP	L_2		▲	*
Fernando & Soulsby	1974	IVLP	L_2		▲	*
Lamina	1974	IVLP	L_2		▲	**
Stevenson & Jacobs	1976	IVLP	L_2		▲	***
Stevenson & Jacobs	1977	IVLP	L_2		▲	**
Stevenson	1979	IVLP	L_2		▲	***
Agglutination						
Fellers	1953	AGG	Adult	● ○		*
Sadun et al.	1957	BF	Adult,Ova	●		*
Jung & Pacheco	1958	IHA	Adult,L_2	●		*
Kagan et al.	1959	BF,IHA	Adult	●		**
Jung & Guillermo	1960	IHA	Adult	●		*
Vinke et al.	1962	IHA	Adult	●		***
Vinke et al.	1964	IHA	Adult	●		***
Ivey & Slanga	1965	BF,IHA	Adult	●		*
Ivey	1965	IHA	Adult	●		*

TABLE 4

(Cont.)

Author	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Zheleva	1975	ID,IEP	Adult	●		*
Khalil et al.	1976a,b	PAT	Adult	●		***
Triboulez-Duret et al.	1976	ID	Adult	●		*
Zyngier	1976	ID	Adult	●		*
Cypess et al.	1977	ID	Adult,Ova	●		*
Enayat & Pezeshki	1977	CIEP	Adult	●		*
Girdwood et al.	1978	CIEP	L_2		▲	*
Glickman et al.	1978	ID	Adult	●		*
Khalil et al.	1978	PAT	Adult	●		**
In Vitro Larval Precipitate Test						
Olson	1960	IVLP	L_2		▲	*
Richards et al.	1962	IVLP	L_2		▲	**
Sharp & Olson	1963	IVLP	L_2		▲	*
Lamina	1968	IVLP	L_2		▲	*
Jalayer	1969	IVLP	L_2		▲	*
Lamina	1970	IVLP	L_2		▲	**
Diconza	1972	IVLP	L_2		▲	*
Fernando & Soulsby	1974	IVLP	L_2		▲	*
Lamina	1974	IVLP	L_2		▲	**
Stevenson & Jacobs	1976	IVLP	L_2		▲	***
Stevenson & Jacobs	1977	IVLP	L_2		▲	**
Stevenson	1979	IVLP	L_2		▲	***
Agglutination						
Fellers	1953	AGG	Adult	● ○		*
Sadun et al.	1957	BF	Adult,Ova	●		*
Jung & Pacheco	1958	IHA	Adult,L_2	●		*
Kagan et al.	1959	BF,IHA	Adult	●		**
Jung & Guillermo	1960	IHA	Adult	●		*
Vinke et al.	1962	IHA	Adult	●		***
Vinke et al.	1964	IHA	Adult	●		***
Ivey & Slanga	1965	BF,IHA	Adult	●		*
Ivey	1965	IHA	Adult	●		*

TABLE 4

(Cont.)

Author	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Wood et al.	1965	IHA	Adult	●		***
Jeska	1967a	IHA	Adult	●		*
Hosoi	1969a,b	IHA	Adult	●	▲	*
Aljeboori & Ivey	1970	IHA	Adult,L_2	●		*
Krupp	1974	IHA	L_2	●		**
de Savigny & Tizard	1975	IHA	L_2		▲	*
de Savigny & Yang	1976	IHA	L_2		▲	***
Hogarth-Scott & Feerey	1976	IHA	Adult	●		*
Triboulez-Duret et al.	1976	IHA	Adult	●		*
Zheleva	1976	IHA	Adult	●		*
Zyngier	1976	IHA	Adult	●		***
de Savigny & Tizard	1977	IHA	L_2	●	▲	**
Enayat & Pezeshki	1977	IHA	Adult	●		*
Glickman et al.	1978	IHA	Adult	●		*
Complement Fixation						
Sadun et al.	1957	CF	Adult,Ova	●		*
Fernando	1968a,c	CF	Adult,Ova	●		*
Jalayer	1969	CF	Adult	●		*
Fernando & Soulsby	1974	CCAT	Adult	●		*
Triboulez-Duret et al.	1976	CF	Adult	●		*
Zyngier	1976	CF	Adult	●		*
Ruitenberg et al.	1976	CF	Adult	○		*
PRIMARY ASSAYS						
Immunofluorescence Assay						
Mitchell	1964	IFA	L_2	●		*
Hogarth-Scott	1966	FA	L_2		▲	*
Bisseru & Woodruff	1968	IFA	L_2,Ova	● ○		**
Hogarth-Scott et al.	1969	FA	L_2		▲	*
Baufine-Ducroq et al.	1971	IFA	Ova	●		*
Brown	1972	IFA	L_2	● ○		***
Tettamanti et al.	1972	IFA	L_2	● ○		*
Baufine-Ducroq et al.	1973	IFA	Ova	●		**

TABLE 4

(Cont.)

Authors	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Garrow & Kane	1973	IFA	L_2	● ○		***
Kane	1973	IFA	Ova	●		*
Annen et al.	1975	IFA	L_2	● ○		*
de Savigny & Tizard	1975	SAFA	L_2		▲	*
Viens et al.	1975	IFA	L_2	● ○		**
de Savigny & Yang	1976	SAFA	L_2		▲	***
Ruitenberg & Buys	1976	IFA	Adult	○		*
Beggs	1977	IFA	L_2	○		**
de Savigny & Tizard	1977	SAFA	L_2		▲	**
Jacobs et al.	1977	IFA	L_2	● ○		***
Viens	1977	IFA	L_2	● ○		***
Girdwood et al.	1978	IFA	L_2		▲	*
Weiland et al.	1978	IFA	Adult,L_2	● ○		*
Welch & Dobson	1978	IFA	Adult	●		*
Speiser & Weiss	1979	IFA	L_2	● ○		*
Welch et al.	1979	IFA	L_2	●		***
Immunoenzymoassay						
Cypess & Glickman	1976	ELISA	Ova	●		*
Cypess et al.	1977	ELISA	Ova	●		*
Glickman & Cypess	1977	ELISA	Ova	●		***
de Savigny & Voller	1978	ELISA	L_2		▲	*
Woodruff et al.	1978	ELISA	L_2		▲	***
Weiland et al.	1978	ELISA	Adult,L_2	●		*
Glickman et al.	1978	ELISA	Ova	●		*
Ignashenkova et al.	1978	ELISA	L_2		▲	*
Yampolskaya et al.	1978	ELISA	L_2		▲	***
Schantz et al.	1978	ELISA	Ova	●		***
de Savigny et al.	1979	ELISA	L_2		▲	**
Glickman et al.	1979a,b	ELISA	Ova	●		***
Yang & Kennedy	1979	ELISA	L_2		▲	**
Speiser & Weiss	1979	ELISA	Ova	●		**

TABLE 4

(Cont.)

Authors	Year	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Garrow & Kane	1973	IFA	L_2	● ○		***
Kane	1973	IFA	Ova	●		*
Annen et al.	1975	IFA	L_2	● ○		*
de Savigny & Tizard	1975	SAFA	L_2		▲	*
Viens et al.	1975	IFA	L_2	● ○		**
de Savigny & Yang	1976	SAFA	L_2		▲	***
Ruitenberg & Buys	1976	IFA	Adult	○		*
Beggs	1977	IFA	L_2	○		**
de Savigny & Tizard	1977	SAFA	L_2		▲	**
Jacobs et al.	1977	IFA	L_2	● ○		***
Viens	1977	IFA	L_2	● ○		***
Girdwood et al.	1978	IFA	L_2		▲	*
Weiland et al.	1978	IFA	Adult,L_2	● ○		*
Welch & Dobson	1978	IFA	Adult	●		*
Speiser & Weiss	1979	IFA	L_2	● ○		*
Welch et al.	1979	IFA	L_2	●		***
Immunoenzymoassay						
Cypess & Glickman	1976	ELISA	Ova	●		*
Cypess et al.	1977	ELISA	Ova	●		*
Glickman & Cypess	1977	ELISA	Ova	●		***
de Savigny & Voller	1978	ELISA	L_2		▲	*
Woodruff et al.	1978	ELISA	L_2		▲	***
Weiland et al.	1978	ELISA	Adult,L_2	●		*
Glickman et al.	1978	ELISA	Ova	●		*
Ignashenkova et al.	1978	ELISA	L_2		▲	*
Yampolskaya et al.	1978	ELISA	L_2		▲	***
Schantz et al.	1978	ELISA	Ova	●		***
de Savigny et al.	1979	ELISA	L_2		▲	**
Glickman et al.	1979a,b	ELISA	Ova	●		***
Yang & Kennedy	1979	ELISA	L_2		▲	**
Speiser & Weiss	1979	ELISA	Ova	●		**

TABLE 4
(Cont.)

Authors	Years	Assay	Antigen			Use
			Stage(s)	Somatic	Secretory	
Other Assays						
Dobson et al.	1967	PK	Adult	●		*
Hogarth-Scott	1968	MAGR	L_2	○		*
Wong et al.	1976	MMI,LT	Ova	●		*
Girdwood et al.	1978	PRIST	L_2		▲	***
de Savigny & Voller	1979	IRMA	L_2		▲	*
Welch & Dobson	1979	LT	L_2	●		*
Kayes & Oaks	1980	MMI	L_2	●		*

Legend

*	Research (no diagnostic or epidemiologic applications)
**	Serodiagnosis
***	Seroepidemiology
●	Whole Worm Antigen
○	Cuticular Antigen
▲	Secretory Antigen
AGG	Agglutination
ART	Arthus Reaction
BF	Bentonite Flocculation
CCAT	Conglutinating Complement Absorption Test
CF	Complement Fixation
CIEP	Counter-immunoelectrophoresis
CPT	Capillary Tube Precipitin Test
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme-linked Immunosorbent Assay
FA	Direct Fluorescent Antibody Assay
ID	Immunodiffusion
IEP	Immunoelectrophoresis
IFA	Indirect Fluorescent Antibody Assay
IHA	Indirect Haemagglutination
IRMA	Immunoradiometric Assay
ITH	Immediate Type Hypersensitivity
IVLP	In vitro Larval Precipitate Test
LT	Lymphocyte Transformation
MAGR	Mixed Anti-globulin Reaction
MMI	Macrophage Migration Inhibition
PAT	Precipitin Adsorption Test
PCA	Passive Cutaneous Anaphylaxsis
PK	Prausnitz-Kustner
PRIST	Paper Radioimmunosorbent Test
RPT	Ring Precipitin Test
SAFA	Soluble Antigen Fluorescent Antibody Assay
S-D	Schultz-Dale Test

TABLE 5

COMPARATIVE ANALYSIS OF REFERENCE IMMUNODIAGNOSTIC PROCEDURES FOR TOXOCARIASIS*

Assay	Antigen	Criteria for Clinical Diagnosis	Sensitivity % (n_1)	Specificity % (n_2)	Predictive Value +	Predictive Value −	Cross-Reactivity % (n_3)	Precision	Subjects Tested	Reference Diagnosis	Source
BF	Adult Somatic	"Eosinophilia and symptoms comparable with VLM"	25% (24)	99.6% (282)	97.9%	56.9%	Ascaris 31/45 Clonorchis 3/6 Echinococcus 1/7 Trichinella 1/79 Total 26.3% (137)	No Data	443	USA 1959-1978	Sadun, Norman and Allain (1957)
IHA	Adult Somatic	Not Stated	50% (14)	No Data	No Data		Ascaris No Data	No Data	14	USA 1959-1978	Sadun et al. (1959)
ITH	Adult Somatic	One of : -unexplained eosinophilia; -urticaria; -choroidoretinitis; -asthma	31.4% (35)	96.4% (55)	89.6%	58.4%	Trichuris 1/37 Hookworm 1/20 4 other spp. 0/16 Total 2.7% (73)	No Data	175	UK 1964-1970	Woodruff, Thacker and Shah (1964)
		Parasitological Diagnosis	100% (9)	98.7% (156)	98.7%	100%	Not Stated Total 2.4% (85)	No Data	284		Wiseman and Woodruff (1970)
IFA	Larval Somatic	Clinical criteria (not stated) plus positive ITH reaction	34.5% (29)	81.5% (27)	74.0%	55.6%	Ascaris 3/3 Wuchereria 1/2 7 other spp. 0/9 Total 14.2% (14)	No Data	128	UK 1968-	Bisseru and Woodruff (1970)
IHA	Larval Secretory	Eosinophilia > 20% plus any 2 of: -hepatomegaly;fever; -pneumonitis; -hyperglobulinaemia; -geophagia	85.2% (27)	98.7% (150)	98.5%	86.9	Ascaris 0/4	No Data	325	Canada 1975-1979	de Savigny and Tizard (1977)
ELISA	Ova Somatic	Any 5 of: -leucocytosis; -eosinophilia; -hepatomegaly; ↑IgG; -↑IgM;↑anti A; ↑anti B	78.3% (23)	92.3% (39)	85.7%	87.8%	No Data (Sera absorbed with Ascaris Antigen)	No Data	62	USA 1978-	Glickman et al. (1978)
ELISA	Larval Secretory	Eosinophilia > 20% plus any 2 of: -hepatomegaly;fever; -pneumonitis; -hyperglobulinaemia; -geophagia	100% (20)	97.4% (922)	97.5%	100%	Trichuris 1/5 Hookworm 1/11 6 other spp. 0/51 Total 2.9% (67)	CV = 7.7%	1009	UK Canada USSR Switzerland 1979-	de Savigny Voller and Woodruff (1979)

* Data in Table 5 were calculated from information given in the citations. Calculations were done according to formulae in Table 12.

n_1 Number of toxocariasis patients tested.

n_2 Number of healthy subjects tested.

n_3 Number of non-toxocaral helminth infected patients tested.

2. Seroepidemiology

Although the seroepidemiology of toxocariasis has been the subject of relatively few studies, much has been learnt from these regarding the nature and extent of toxocariasis in man. Skin hypersensitivity data, especially those compiled by Woodruff and his colleagues, have been particularly productive in this regard. Since the inception of the work reported herein, ELISA has rapidly supplanted the skin test as the prime source of seroepidemiologic information (Table 6).

The period prevalence of toxocariasis (subclinical infection or past infection) in man as surveyed from the literature indicates that asymptomatic infections are wide-spread. Prevalence generally ranges from 2 to 3% in temperate regions and arid tropical regions, and up to 30% in humid tropical regions. In practice, skin test results using crude antigen appear to be relatively more specific than serological assays using the same antigens. This has been noted in other helminthiases (Grove, 1979) but is unexplained. There have been some anomolous reports in the literature: eg. Kane (1973) reported 56.3 percent and Wood et al. (1965) reported 45.3 percent of healthy adults in the UK and USA respectively with positive Toxocara IFA and IHA results. Such works were not considered in the survey for Table 6 unless the authors had shown prevalidation of the assays in terms of non-specific reactivity.

The most comprehensive survey of period prevalence was reported by Yampolskaya et al. (1978) who, using larval secretory antigens and methods supplied by de Savigny, examined sera from approximately 1000 children living in diverse biomes within the USSR. The prevalence of ELISA positivity was proportional to the prevalence of larvated toxocaral ova found in sympatric soil samples. Furthermore, there was an absence of toxocaral infection in dogs (and man) in the arctic (confirmed by

TABLE 6

PREVALENCE OF ANTI-*TOXOCARA* IMMUNOREACTIVITY IN MAN*

Location	Percent Positive	No. Tested	Assay	Reference	
Canada					
Toronto	2.7	150	IHA,SAFA	de Savigny et al.	(1977)
Montreal	4.5	940	IFA	Viens et al.	(1977)
Toronto	3.5	310	ELISA	Yang et al.	(1979)
Cyprus	2.0	200	ITH	Bradford	(1970)
Egypt					
Siwa	2.2	446	DTH,PAT	Khalil et al.	(1976b)
Luxor	3.7	135	DTH,PAT	ibid.	
Aswan	2.9	300	DTH,PAT	ibid.	
Mansourah	2.0	400	DTH,PAT	ibid.	
Kenya					
Nairobi	0	160	ITH	Wiseman et al.	(1971)
Masai	10.7	75	ITH	ibid.	
Malaysia	5.5	199	ITH	Bisseru	(1968)
Malta	0.95	105	ITH	Wiseman et al.	(1971)
Nigeria					
Ibadan	4.1	193	ITH	ibid.	
Tanzania					
Dar es Salaam	8.2	134	ITH	ibid.	
Uganda					
Kampala	30.8	123	ITH	ibid.	
UK					
England	1.35	156	ITH	Wiseman et al.	(1970)
England	1.86	485	ITH	Woodruff	(1970)
England	2.1	329	ITH	Woodruff et al.	(1966)
Scotland	2.0	200	PRIST	Girdwood et al.	(1978)
England	2.6	922	ELISA	de Savigny et al.	(1979)
USA					
Georgia	0.4	282	BF	Sadun et al.	(1957)
Louisiana	1.1	190	IHA	Jung et al.	(1960)
Texas	2.8	70	IVLP	Richards et al.	(1962)
New York	18.0	104	ELISA	Glickman et al.	(1977)
USSR					
Arctic circle	0	290	ELISA	Yampolskaya et al.	(1978b)
Kamchatka	0	84	ELISA	ibid.	
West Siberia	1.0	100	ELISA	ibid.	
Samarkand	1.1	90	ELISA	ibid.	
Belev	5.5	254	ELISA	ibid.	
Moscow	5.4	93	ELISA	ibid.	
Georgia	8.2	61	ELISA	ibid.	

Surveyed from published seroepidemiologic studies on sample populations of at least 50 healthy subjects.

observations in Canada by Eaton and Secord,(1979).

Groups having closer contact with dogs than that of the general population have been studied. Jacobs, Woodruff, Shah and Prole (1978) tested by IFA, 34 professional dog handlers employed at a greyhound training establishment known to be contaminated with Toxocara ova (Jacobs and Prole, 1976). Positives were found 1.5 times more frequently in the exposed group compared to controls but the difference was not statistically significant. Glickman and Cypess (1977) using ELISA found no statistically significant association between toxocariasis and the occupational exposure of animal hospital employees, nor between toxocariasis and dog ownership. The design and interpretation of this study has been criticized (Hamble, 1977) and the results invalidated. Woodruff, de Savigny and Jacobs (1978), using ELISA, found that dog breeders and exhibitors had a significantly higher prevalence of seropositivity (15.7%) compared to the general population (2.6%) and that this difference was proportional to the number of years spent working with breeding animals.

Knowledge of period prevalence in healthy adults has allowed several authors to examine the suspected interrelationship between toxocariasis and other disease states (see Section C.2.). In asthmatics, immunoreactivity to Toxocara, compared to that in the general population, was more prevalent by factors of 8-fold (Wiseman and Woodruff, 1968); 3.4-fold (Woodruff, 1973) 4.9-fold (Viens, 1973) and 7-fold (Girdwood, et al, 1978). In paralytic poliomyelitis, positives were more frequent by factors of 6.5-fold (Woodruff, Bisseru and Bowe, 1966) and 4.1-fold (Khalil et al. 1971). In epilepsy, positives were more prevalent by factors of 3.6-fold (Woodruff, Bisseru and Bowe, 1966); 4.4-fold (Viens, 1977) and 3.7-fold (Glickman, Cypess, Crumrine and Gitlin, 1979). No association between toxocariasis and toxoplasmosis was observed

(Wiseman, Fleck and Woodruff, 1970). Historically, this was an important finding because the life cycle of Toxoplasma gondii was incompletely understood at the time and it was believed that Toxocara cati ova acted as protective vehicles for transmission of Toxoplasma (Hutchison, 1965). The subsequent discovery of the T. gondii sexual reproductive cycle and oocyst stage in cat faeces (Frenkel, Dubey and Miller, 1969) was supported by these negative findings in toxocariasis seroepidemiology.

Seroepidemiologic studies implicating toxocariasis in the transmission and pathogenesis of potentially severe microbial infections led Woodruff (1968) to conclude that the "eradication of ascariasis, including toxocariasis, from a region would be certain to bring with it much benefit to the health of the community concerned and probably not least among these benefits would be the prevention of many bacterial and viral illnesses".

CHAPTER II

TOXOCARAL SECRETORY ANTIGEN: PRODUCTION AND CHARACTERIZATION

INTRODUCTION

In a review of immunodiagnosis for parasitic diseases, Fife (1971) wrote "I believe that we may have tended to overlook what ultimately may prove to be the best source of antigen, namely, the secretions and excretions of living parasites".

This source has been neglected because the prerequisite is *in vitro* cultivation of helminths, a field which has provided many challenges but yielded few successes. In 1963, Silverman optimistically announced "Though it is clear that the task is not an easy one, there is little doubt that all parasitic organisms will be induced to survive, develop, and reproduce *in vitro* when sufficient intelligent effort is applied". Such achievement has proved elusive; no parasitic helminth species has been yet cultured through a single life-cycle *in vitro*. Although the problem of *in vitro* maintenance (ie. without development) is less difficult, the maximum life-span of maintenance cultures ranges from several days to only a few weeks. The exception is *Toxocara canis* which can be maintained *in vitro*, in synthetic medium, for over 1.5 years (de Savigny, 1975), thus providing a useful model for the study of nematode secretory antigens.

Historical Perspective

It was Chandler in 1932 who introduced the concept of the helminth metabolite / host anti-metabolite relationship. Sarles (1938) was credited with the first observation of this phenomenon, on describing the development of precipitates at oral, excretory, and anal pores of

nematode larvae (Nippostrongylus) incubated in immune serum, but not when incubated in normal serum. Taliaferro and Sarles (1939) confirmed this occurrence in vivo. Direct evidence supporting the view that excretions and secretions of helminths elicit an immune response in the host was provided by Thorson (1951, 1953) who demonstrated that immunization of rats with secretory antigen collected from Nippostrongylus muris provided partial protection against infection with the same parasite. The majority of literature concerning nematode secretory antigens describes their use as protective antigens (reviewed by Clegg and Smith, 1978).

Concerning diagnosis, the specific precipitation of nematode secretions by immune serum has been observed for a variety of nematodes both in vivo and in vitro (reviewed by Thorson, 1970) but the immunological nature of this was not elucidated until Jackson (1959, 1960) and Taffs and Voller (1962) used fluorescein-labelling to demonstrate incorporation of antibody in precipitiates. Hogarth-Scott (1965) presented evidence for antigenic specificity of secretory antigens at the genus level. Sadun and Norman (1957) were first to use secretory antigen (Trichinella) in serodiagnosis. Subsequent applications of nematode secretory antigens in immunodiagnosis are summarized in Table 7. Early diagnostic applications of toxocaral secretory antigens utilized the original phenomenon, ie. direct demonstration of precipitates at the orifices of living larvae following incubation for periods (up to 48 hours) in patient's serum (Olson, 1960; Lamina, 1970; Stevenson, 1977). A high degree of specificity and adequate sensitivity was reported but the in vitro larval precipitates (IVLP) test was slow laborious, and required a ready supply of live parasites. Use of IVLP was largely restricted to research applications and detection of antibody in experimentally infected animals. De Savigny and Tizard

(1975, 1977) initiated the use of nematode secretory antigens (Toxocara) in conventional serological tests (HA, SAFA) intended for routine application. These were shown to be genus specific, highly sensitive, and were found suitable as national reference tests in a public health laboratory service (Canada).

Despite encouraging results from diagnostic work using nematode secretory antigens, little is known of the nature of such antigens (Clegg and Smith, 1978). This chapter describes studies of (a) the long-term in vitro maintenance of Toxocara canis larvae including the dynamics of antigen production, and (b) the physico-chemical and immunological characteristics of toxocaral secretions elaborated in vitro.

TABLE 7

APPLICATION OF NEMATODE SECRETORY ANTIGENS IN SERODIAGNOSIS

Species	Stage	Antigen Source*	Test System	Reference	
Nippostrongylus muris	L_3	A	IVLP	Sarles	(1938)
Ascaris lumbricoides	L_3	A	IVLP*	Lejkina	(1948)
Trichinella spiralis	L_1	B	BF	Sadun and Norman	(1957)
Trichinella spiralis	L_1	A	FA	Jackson	(1959)
Nippostrongylus muris	L_3; L_4; L_5	A	FA	Jackson	(1960)
Toxocara canis	L_2	A	IVLP	Olson	(1960)
Ascaris suum	L_3	A	FA; IFA	Taffs and Voller	(1962)
Toxocara canis	L_2	A	FA	Hogarth-Scott	(1966)
Toxocara cati	L_2	A	FA	Hogarth-Scott	(1966)
Toxascaris leonina	L_2	A	FA	Hogarth-Scott	(1966)
Toxocara canis	L_2	A	IVLP*	Lamina	(1968)
Heterakis gallinae	L_3	A	IVLP	Tareneko and Krivutenko	(1974)
Toxocara canis	L_2	C	HA; SAFA*	de Savigny and Tizard	(1975)
Litomoisoides carinii	L_5	B	IEP; CF; HA	Takoaka et al.	(1975)

TABLE 7
(Cont.)

Species	Stage	Antigen Source*	Test System	Reference	
Necator americanus	L_5	B	ELISA	Ogilvie et al.	(1976)
Toxocara canis	L_2	A	IVLP	Stevenson and Jacobs	(1977)
Toxocara canis	L_2	C	ELISA*+	de Savigny and Voller	(1978)
Anisakis marina	$L_?$	C	CIEP	Watt et al.	(1979)
Ascaris suum	L_2; L_3; L_4	C	ELISA	de Savigny et al. (Unpublished)	
Ascaris lumbricoides	L_3; L_4	C	ELISA	de Savigny (Unpublished)	
Brugia pahangi	L_3; L_5; Mf	C	ELISA	de Savigny et al. (Unpublished)	
Necator americanus	L_5	C	ELISA	de Savigny et al. (Unpublished)	
Onchocerca gutturosa	L_5; Mf	C	ELISA	de Savigny et al. (Unpublished)	

A = In vitro secretion in testserum
B = In vitro secretion in complex culture medium
C = In vitro secretion in synthetic culture medium

\+ See Table 4 for subsequent applications of Secretory Antigen in Toxocara ELISA
* Routine diagnostic application, all others used in research only

MATERIALS AND METHODS

Acquisition of Parasite Ova

Toxocara canis adults were obtained from pups four weeks to six months of age at a local animal shelter. Pups were dosed with piperazine phosphate* (500 mg/kg) in the afternoon and faeces were collected the following morning. Adult ascarids were removed from faeces and washed in several changes of cold tap water to remove adherent faecal material. Female adults were segregated in individual petri dishes containing 1% formalin. The anterior and posterior fifths of each worm were severed and discarded. The bifurcate uterus was extruded from the remaining portion of the worm by applying pressure with a siliconized glass rod. Each uterus was placed in a separate petri dish containing 1% formalin and stripped free of ova. Ova in each petri dish were examined microscopically before pooling to confirm their identity as *T. canis* (cf. *Toxascaris leonina*). Ova so obtained were embryonated by incubation in a monolayer under 1% formalin at a depth of 3 mm in petri dishes. Embryonation proceeded for 30 days at room temperature with daily resuspension. Thereafter ova were pooled and stored at $4^{o}C$ in 1% formalin. For estimations of larvated ovum numbers, samples were suspended in distilled water and counted microscopically by serial dilution in gridded petri dishes.

In vitro Hatching of *Toxocara canis* Ova

Embryonated ova were washed three times in distilled water by centrifugation at 85 g for three minutes and resuspended in 1.5 M sodium hypochlorite (NaOCl) for 30 minutes at $37^{o}C$ to remove the albuminous coat. The suspension was then washed in distilled water until no chlorine odour was detectable (at least ten washings).

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* Candizine, Norden Laboratories, Lincoln, Nebraska.

In vitro hatching was achieved by modification of Fairbairn's (1961) technique for *Ascaris* ova. A 1 ml distilled water slurry of decoated *Toxocara* ova was mixed with 2 ml of a 0.1M sodium bisulphite, 0.25M sodium chloride, 0,0025% tween 80 solution, and 2 ml of a 0.1M gassed sodium bicarbonate solution. This suspension was gassed for 15 minutes with a mixture of N_2-CO_2 (95%:5%) at 37°C. The suspension was sealed immediately and incubation continued for a further 1.75 hours. Following incubation the larval suspension was washed twice in Hanks' Balanced Salts Solution* (HBSS) with 100 units/ml penicillin and 200 µg/ml streptomycin and held overnight in HBSS at 37°C in a sterile Baermann apparatus consisting of five layers of lens paper in a sedimentation flask. Most viable hatched larvae migrated through the lens paper while unhatched eggs, hatching debris, and dead larvae retained on or in the paper. Larvae collected in this manner did not require further washing and were dispensed into 10 ml of maintenance medium for counting as described above.

In vitro Cultivation of Toxocaral Larvae

The method of de Savigny (1975) was used. Briefly, *T. canis* second-stage larvae recovered by Baermann filtration were dispensed into silicone stoppered roller drum tubes (18 x 150 mm) containing HMEM (Eagle's Minimal Essential Medium with Hanks' Salts)* with 100 units/ml penicilin and 200 µg/ml streptomycin. A typical culture consisted of 10^6 larvae at a concentration of 10^4 /ml. Culture tubes were incubated vertically, without rotation at 37°C. Every seventh day tubes were examined and any showing contamination were discarded. Any in which mortality exceeded 5% were not used for secretory antigen production. Dead larvae could be removed by Baermann filtration. In satisfactory

* All culture media from GIBCO-Europe, Dundee, Scotland.

cultures, larvae were allowed to sediment and spent medium was aspirated aseptically. Fresh volumes of sterile medium were dispensed by a 5 ml Cornwall Syringe and the tubes reincubated. Spent medium (approximately 100 ml per week) was pooled, centrifuged to remove any larvae aspirated inadvertantly, and stored at -70°C.

Preparation of Toxocaral Secretory Antigen

Each month, the pooled harvest (approximately 500 ml) of HMEM containing larval metabolites, excretions, and secretions, was dialysed and simulataneously concentrated 20-fold in distilled water to 25 ml by hollow-fibre ultrafiltration (Amicon CH3 with H1C10 cartridge) retaining all components of molecular wight greater than 10,000 daltons. The retentate was designated as Toxocaral Secretory Antigen. Protein concentration was estimated by a micro-modification of the methods of Lowry, Rosebrough, Farr, and Randall (1951) and Garver, Cremer, and Sussdorf (1977) using bovine serum albumin as a standard. Concentrated antigens were stored at -70°C or lyophilized.

Dead larvae (1×10^4) were incubated in HMEM at 37°C for two years and the culture supernatant was examined periodically for the release of somatic antigen. Larvae remained morphologically intact during this period.

Preparation of Toxocara canis Adult Secretory Antigen

Adult T. canis obtained as described above were axenized by incubation for 1 hour in 0.0015 M NaOH to purge intestinal contents, followed by incubation for one hour in saline containing 1000 units/ml of penicillin and 1 mg/ml of streptomycin. Ten adults were cultured in HMEM containing glucose supplement (22.5 g/l), 600 units/ml of penicillin, and 600 µg/ml of streptomycin at 37°C for 18 hours under 5% CO_2 and 95% N_2. Secretory antigen was extracted from culture fluids by centrifugation through Amicon "Centriflo Cones"(Type CF25) at 500 X g.

Preparation of Toxocara canis Larval Somatic Antigen

A suspension of 10^5 freshly hatched, second-stage Toxocara canis larvae in 2 ml of phosphated buffered saline was sonicated. The supernatant resulting from centrifugation at 10,000 X g at 4^oC was harvested as larval somatic antigen.

Preparation of Ascaris suum Secretory Antigen

Adult Ascaris suum were collected at a local abbatoir from pig intestines. Ova were obtained, embryonated and hatched as described for T. canis. In vitro hatched Ascaris second-stage larvae were cultured at 37^oC in Medium-199 containing 25 mM HEPES buffer, 100 units/ml of penicillin, 200 µg/ml of streptomycin and gassed with N_2-CO_2-O_2 (90:5:5). Media were supplemented with glucose (1 mg/ml) and the synthetic tripeptide, glycyl-L-histidyl-L-lysine acetate (M.W. 400) (Sigma, London) at 200 ng/ml. (Stromberg, Khoury and Soulsby, 1977).

Ascaris suum third stage larvae were obtained from rabbit lungs seven days following experimental infection with 100,000 larvated ova administered by stomach intubation. These advanced-stage larvae were cultured by the same method as Ascaris second-stage larvae but at density of 200/ml during which time moulting to fourth-stage occurred.

Ascaris L_2 and L_3-L_4 secretory antigens were extracted from culture fluids by diafiltration through an Amicon PM10 membrane in a stirred cell (Amicon Model 52).

Preparation of Necator americanus Secretory Antigen

Infective third-stage Necator americanus larvae were obtained from faecal culture by standard methods and used to infect one- to two-day old golden hamsters percutaneously (approximately 200 per hampster) by the method of Burt and Ogilvie (1975). Adult Necator were retrieved at necropsy 42 days after infection, axenized for 2 hours at 37^oC, and washed 20 times in HBSS containing 500 units/ml penicillin and 500 µg/ml

streptomycin. Ninety nine Necator adults were maintained in vitro for 20 days at a concentration of 5 worms/ml in tripeptide supplemented medium as described for Ascaris. Antigens were extracted by diafiltration through an Amicon PM10 membrane.

Preparation of Brugia pahangi Secretory Antigen

Adult Brugia pahangi were obtained from peritoneal cavities of experimentally infected girds (Courtesy of Dr. D. Denham). Ten adults were maintained for 10 days in medium NCTC-135 containing 200 units/ml penicillin and 200 µg/ml streptomycin at 37°C. Antigens were extracted as described for Toxocara.

Preparation of Antiserum to Secretory Antigens

Antiserum reactive to toxocaral secretory antigen was raised in a New Zealand White rabbit by intrasmuscular inoculation of 50 µg of secretory antigen in Freund's complete adjuvant, followed 28 days later by subcutaneous administration of 50 µg of the same antigen in saline. Antiserum was obtained by venesection seven days later. An immunoglobulin fraction retaining 96% of the antibody response was obtained by ammonium sulphate precipitation (Hudson and Hay, 1976) followed by sodium acetate precipitation to facilitate the removal of lipoproteins (Harboe and Ingold, 1973). Antibody to Necator americanus adult secretory antigen, Ascaris suum third-stage larval secretory antigen, Brugia pahangi adult secretory antigen and Toxocara canis larval somatic antigen (sonicate) was raised in C57BL/6 female mice by intraperitoneal inoculation of 25 µg of antigen in Freund's complete adjuvant according to the schedule of March and Cooney (1976). Peritoneal fluid (approximately 5 ml/mouse) containing antibody was obtained by paracentesis weekly for four weeks commencing 30 days after the first dose. Clot formation in immune ascites fluid was minimized

by acid precipitation of fibrinogen (Chiewslip and McCown, 1972).

Enzyme Immunoassay of Toxocaral Secretory Antigen

An enzyme immunoassay was developed to monitor *in vitro* production of larval secretory antigen during cultivation. The double antibody sandwich method (Voller, Bidwell and Bartlett, 1976) was employed using the rabbit immunoglobulin prepared above for both the first antibody (solid phase) and the second antibody (labelled with alkaline phosphatase by the one-step glutaraldehyde method (Voller, *et al*, 1976). The primary immunoglobulin was adsorbed to polyvinyl chloride micro-ELISA plates at an optimal dilution of 1:100 in coating buffer (see appendix for details of buffers) for 18 hours at $4^{o}C$. Wells were washed three times in phosphate buffered saline containing 0.5% tween 20 (PBS-Tween) and were then incubated for two hours at room temperature with samples (thought to contain secretory antigen) diluted 1:5 in PBS-Tween, and with known antigen standards in PBS-Tween. Anti-*Toxocara* immunoglobulin-alkaline phosphatase conjugate at a dilution of 10^{-3} was added for 18 hours at $4^{o}C$. Following further washes, the enzyme substrate, 4-nitrophenyl phosphate, at 1 mg/ml in diethanolamine buffer, was added. Hydrolysis was proportional to the quantity of enzyme-conjugate bound. The reaction was terminated with the addition of 3M NaOH after 30 minutes at room temperature and the amount of chromogenic hydrolysate (4-nitrophenyl) produced, was determined spectrophotometically at 405 nm. Antigen concentrations of unknowns were determined by interpolation from a standard curve.

Characterization of Toxocaral Secretory Antigen

Polyacrylamide Gel Electrophoresis. Heterogeneity of secretory antigens was studied by polyacrylamide gel electrophoresis (PAGE) in horizontal, thin-layer, 3.5% and 10% gels (Fernstrom and Moberg, 1977)

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using tris-glycine buffer, pH 8.9, and a field strength of 15 V cm^{-1} at 10°C.

Analytical Isoelectric Focusing. Antigen heteroegeneity and isoelectric point (pI) were investigated using horizontal, thin-layer (1 mm) polyacrylamide gels (5% gel, 3% cross-linking, 3.0% ampholyte) (Winter, Ek, and Andersson, 1977) with pH gradients ranging from 3.5 to 9.5 and from 9 to 11 maintained by constant power of 25W at 10°C. Samples were concentrated (Amicon CS 15) and eluted in 1% glycine buffer before isoelectric focusing. Triton-X 100 (0.5%) was added to gels and samples to enhance solubility of antigens at their respective pI's (Angellis, Inglis and Fishman, 1976). Gels were stained for proteins using Coomassie Brilliant Blue, (Winter, et al. 1977), glycoproteins using periodic-acid Schiff's stain (Zacharius, Zell and Morrison, 1969) and lipoproteins using Sudan Black (Uriel, 1971).

Molecular Weight Determinations. Molecular weights of secretory antigen protein moieties (2-mercaptoethanol reduced) were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Weber and Osborn, 1969) calibrated using oligomeric markers (BDH, Poole, England) in 5% and 10% gels with a field strength of 5 V cm^{-1} at 10°C. Proteins were stained by Coomasie Blue (Fernstrom and Moberg, 1977) and glycoproteins by periodic-acid Schiff's stain (Segrest and Jackson, 1972).

Bi-Dimensional Immunoelectrophoresis. Antigen heterogeneity was further examined using a micro-modification of two-dimensional (Laurell-crossed) immunoelectrophoresis (Weeke, 1973). Antigen (containing 16 µg protein) was electrophoresed in 1.3 mm thick gels of 1% agarose in barbital buffer (ionic strength = 0.1 , pH 8.6) on glass plates (50 X 50 X 1 mm). First dimension electrophoresis was done at 5 V cm^{-1}

for 30 minutes at 15°C. An agarose strip 15 mm wide containing electrophoresed antigen was retained on the plate and the agarose on the remainder of the plate was replaced with another gel, 1.1 mm thick, containing rabbit anti-*Toxocara* immunoglobulin at a concentration of 250 μg cm^{-2}. Second dimension immunoelectrophoresis was run at 2 V cm^{-1} for 12 hours. The use of intermediate gels containing antisera to heterologous helminths was attempted to further assess antigen specificity. Antigens electrophoresed or isoelectrically focused in polyacrylamide gels were also examined in two-dimensional immunoelectrophoresis by use of laying-on techniques (Smith, Soderholm, and Wadstrom, 1977). Gels were pressed, washed, dried and stained for protein with Coomassie Brilliant Blue as described by Deans *et al*. (1978).

RESULTS

Acquisition of Parasite Ova

All pups readily took the flavoured piperazine anthelmintic and, of those treated as described, approximately 40% yielded ascarids, which when washed free of faeces and residual piperazine, were alive. On embryonation of ova obtained from adult female T. canis, approximately 50% of ova showed microscopic evidence of embryonation to the state containing motile larvae (Fig. 1). Microbial contamination of ova suspensions was never observed. Embryonated ova, stored at $4^{o}C$, remained infective to laboratory hosts for at least three years and could be hatched in vitro yielding viable larvae for in vitro cultivation for at least two years.

In Vitro Cultivation of Toxocaral Larvae

In vitro hatching of T. canis by Fairbairn's (1961) method for Ascaris resulted in a high yield (>98%) of larvae from embryonated ova. In contrast, recovery of larvae from pepsin digests of brain, liver and lung of experimentally infected mice and of liver and lung of experimentally infected rabbits was attempted, but yields never exceeded 40% of inoculum. Background hatching, as observed (Fairbairn, 1961) for Ascaris lumbricoides ova, did not occur with T. canis.

Various Baermann filter systems in use for Ascaris were investigated, including Whatman No.12, ED-512, and Kim-Wipe Tissues, but were found unsatisfactory for Toxocara. The relatively large size and rapid penetrating ability of Toxocara compared to Ascaris was such that five layers of lens paper optimally allowed the majority of active larvae to penetrate in staggered fashion, but retained hatching debris and dead larvae. Cuticles of the first ecdysis were not seen.

The enhanced survival of T. canis larvae when maintained in HMEM is contrasted in Fig. 2 which demonstrates a culture half-life of 16 months.

Table 8 compares the mean lengths of T. canis larvae, sampled from various media between one and 500 days after in vivo. Only HMEM maintained larvae showed a dimensional relationship approximating available data from in vivo systems during early phases of culture. There is evidence of growth of larvae in vitro during later phases.

The appearance of T. canis larvae maintained in vitro alters after approximately four months (Figs. 3,4). An area of diffuse, dark, pigmentation appears in the procorpus and oesophageal region of an increasing proportion of larvae. These larvae show no gross differences in motility, behaviour, or infectivity compared to unpigmented forms. Pigmentation does not appear to be related to mortality.

Quantitative production of toxocaral secretory antigen was monitored using the double antibody sandwich ELISA (Fig. 5a). Results (Fig. 5b) revealed little variation in antigen output (approximately 200 pg/larva/day) between the first week and 18 months of culture, however production during the first day post-hatching was significantly higher.

Characterization of Toxocara Larval Secretory Antigen

Immunoelectrophoresis of secretory antigen (16 µg) against serum from a rabbit experimentally infected with 10^4 T. canis detected a single component with cathodal mobility. Immunoelectrophoresis against hyperimmune serum from a rabbit immunized with Toxocara secretory antigen revealed a minor component with anodal mobility, in addition to the same cathodal antigen (Fig. 6).

Polyacrylamide gel electrophoresis resolved one protein component with cathodal mobility. No glycoproteins or lipoproteins were detected.

SDS-Polyacrylamide gel electrophoresis indicated one major protein component with a molecular weight of 42,000 and two minor components

with molecular weights of 120,000 to 160,000 (Fig. 7).

Isoelectric focusing revealed a major component with pI 9.5 and a two minor components with pI 5.2 and 5.5 (Fig. 8).

Because of the high pI of the major component, it was necessary to modify the two-dimensional electrophoresis to detect antigens migrating in both directions (Fig. 9) and this revealed three components, one with cathodal mobility, two with anodal mobility. Neither these, nor other components were detected in intermediate gels containing antibody to Necator adult secretory antigen, Ascaris larval (L_3-L_4) secretory antigen, or Toxocara larval somatic antigens.

TABLE 8

Mean length in Microns (± 2 S.E.) of samples of 100 *Toxocara canis* second-stage larvae following *in vitro* hatching and maintenance in various synthetic media compared with data from *in vivo* sources.

Culture Period (Days)	*in vivo**	*in vitro*					
		HMEM	EMEM	M199	HBSS	PBS	SAL
0	411			419 (5.8)			
14	429	427 (5.0)	410 (7.2)	412 (6.8)	392 (9.8)	353 (3.2)	400 (4.6)
40	430	438 (5.0)	388 (9.8)	403 (4.7)	Dead	Dead	Dead
100	ND	450 (5.9)	ND	ND	†	†	†
300	ND	453 (3.1)	ND	ND	†	†	†
500	ND	485 (4.9)	ND	ND	†	†	†

* Means of 500 larvae recovered from rabbit tissue (Lautenslager, 1970)

ND Not Done

Fig. 1 Toxocara canis

(a) Non-infective, unembryonated ova 24 hours after dissection from the uterus. Both fertile and infertile ova are present. Fertilized ova are in the morula stage.

X 300

(b) Infective, larvated ova following embryonation in 1% formalin at room temperature for 28 days. Fertile ova contain motile second-stage larvae while infertile ova are unchanged.

X 300

d ova 24 hours after
Both fertile and infertile
d ova are in the morula stage.
X 300

lowing embryonation in 1%
e for 28 days. Fertile ova
larvae while infertile ova

X 300

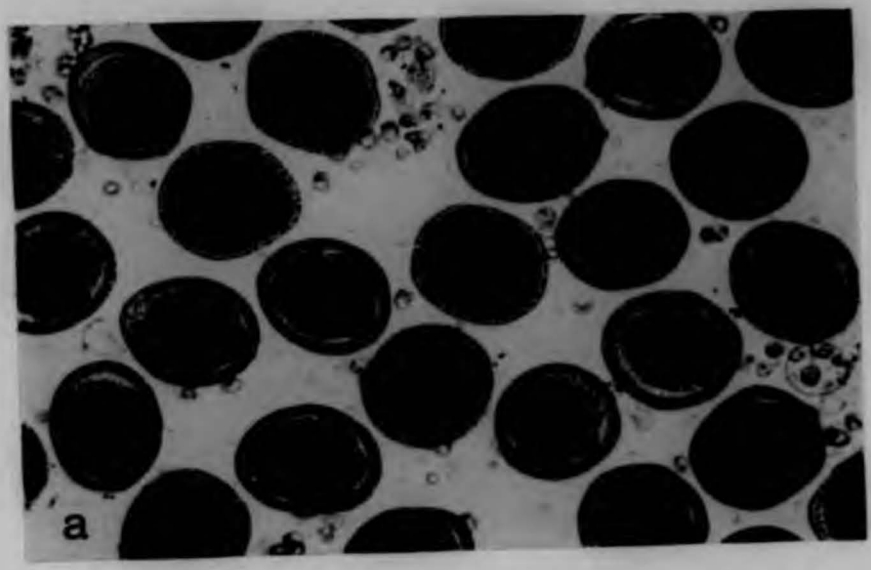

FIGURE 1

d ova 24 hours after
Both fertile and infertile
d ova are in the morula stage.
X 300

owing embryonation in 1%
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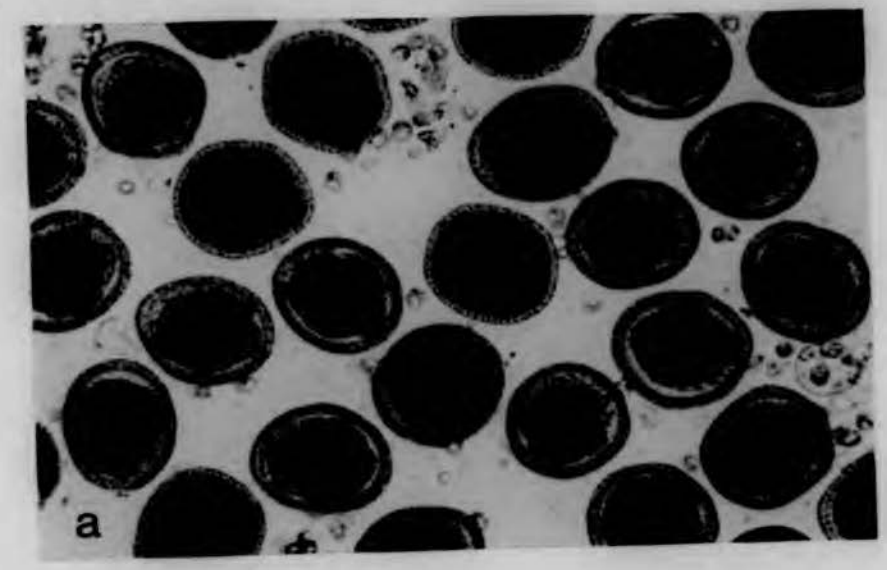
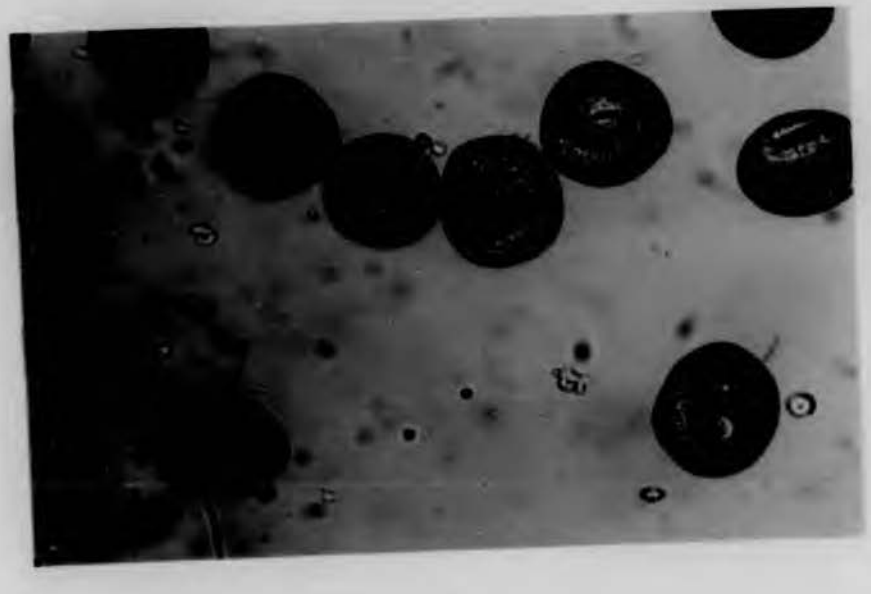
X 300





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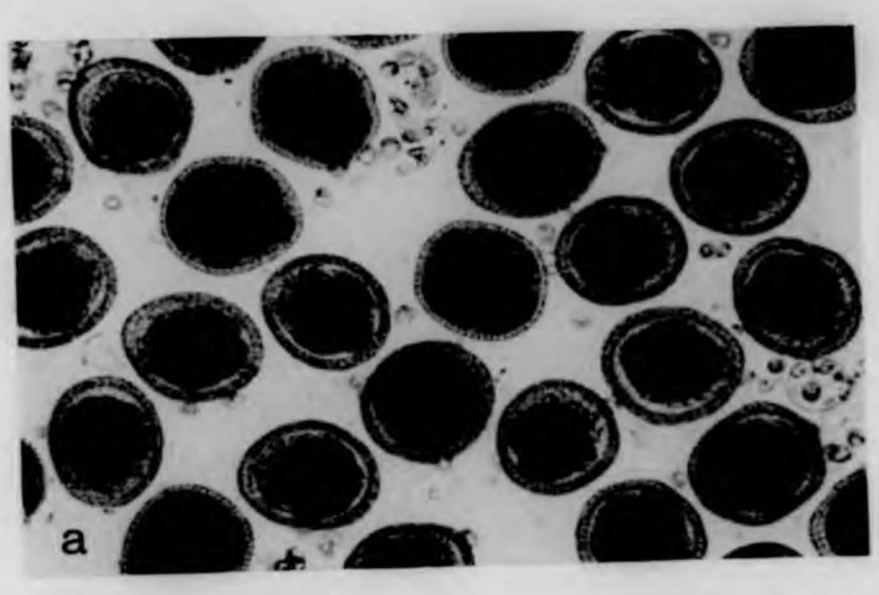

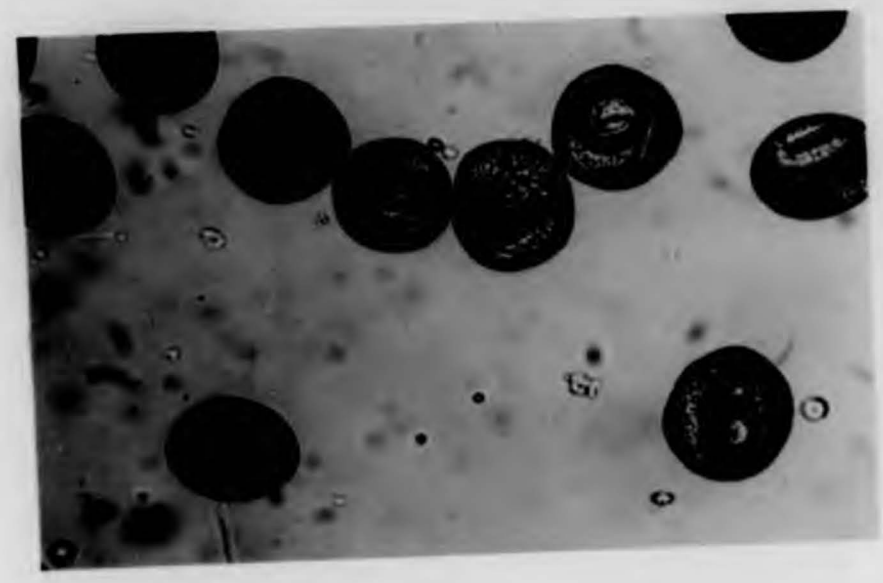

FIGURE 1

Fig. 2 *In vitro* survival of *Toxocara canis* second-stage larvae when cultured in various synthetic media.
Culture half-life in HMEM is 490 days (16 months).

- ● HMEM - Eagle's Minimal Essential Medium with Hanks' Salts
- ■ EMEM - Eagle's Minimal Essential Medium with Earls' Salts
- ▲ M199 - Medium 199
- △ HBSS - Hanks' Balanced Salts Solution
- □ PBS - Phosphate Buffered Saline
- ○ SAL - Saline

anis second-stage larvae

tic media.

0 days (16 months).

ial Medium with Hanks' Salts

ial Medium with Earls' Salts

Solution

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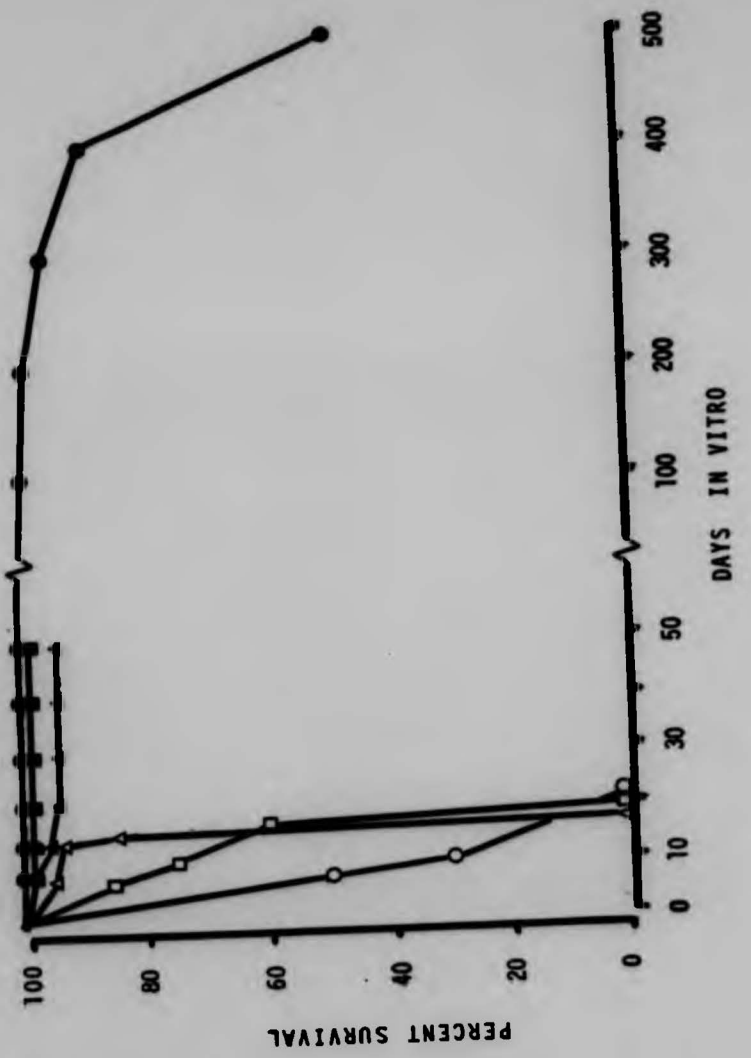

FIGURE 2

Fig. 3 Toxocara canis second-stage larva (from Nichols, 1956).

A	Anus
BA	Buccal Apparatus
DES	Dorsal Eosophageal Gland
DESN	Nucleus of DES
EB	Eosophageal Bulb
EC	Excretory Column
EN	Nucleus of Excretory Cell
EP	Excretory Pore
ET	Excretory Tubule
GN	Ganglionic Nucleus
GP	Genital Primordium
IS	Isthmus
INT	Intestine
MC	Metacorpus
NR	Nerve Ring
PC	Procorpus

(from Nichols, 1956).

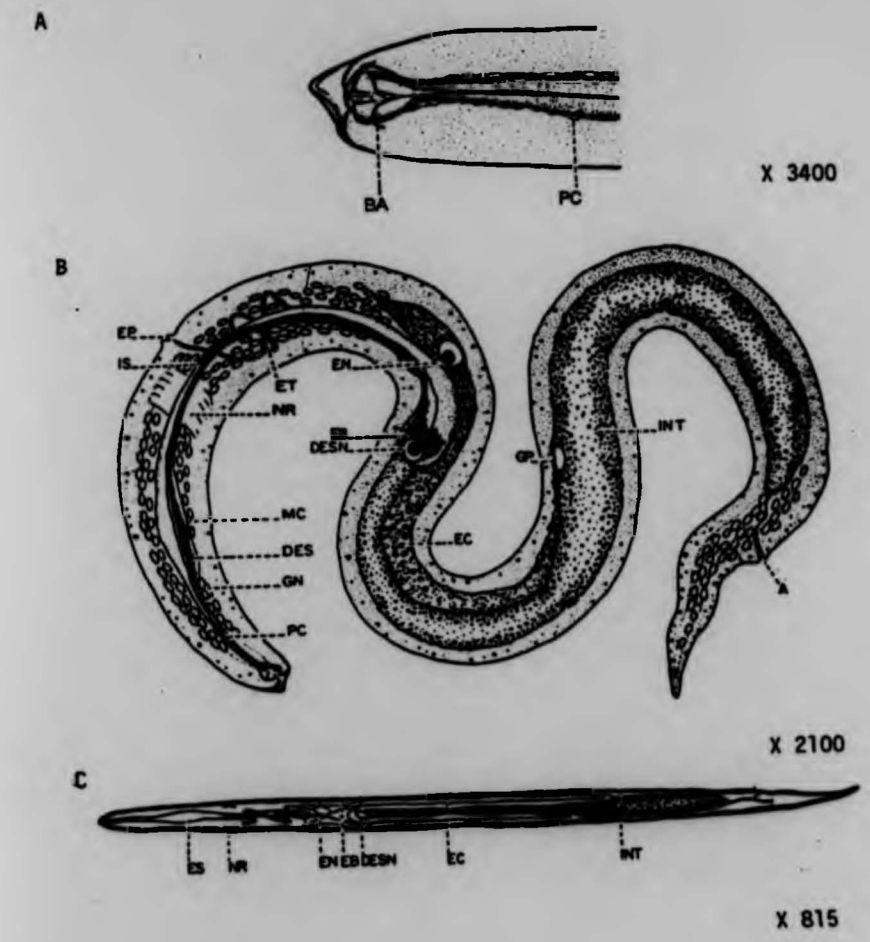

FIGURE 3

Fig. 4 *Toxocara canis* second-stage larvae *in vitro*.
(Heat shocked for photomicrography)

a) Cultured in synthetic medium for 48 hours. X 300

b) Cultured in synthetic medium for 18 months X 300
showing pigmentation of the procorpus.

c) Cultured in immune serum for two hours X 625
showing oral and excretory pore precipitates.

vae *in vitro*.

phy)

m for 48 hours. X 300

m for 18 months X 300

procorpus.

r two hours X 625

pore precipitates.

FIGURE 4

rvae in vitro.

aphy)

um for 48 hours. X 300

um for 18 months X 300

e procorpus.

or two hours X 625

pore precipitates.

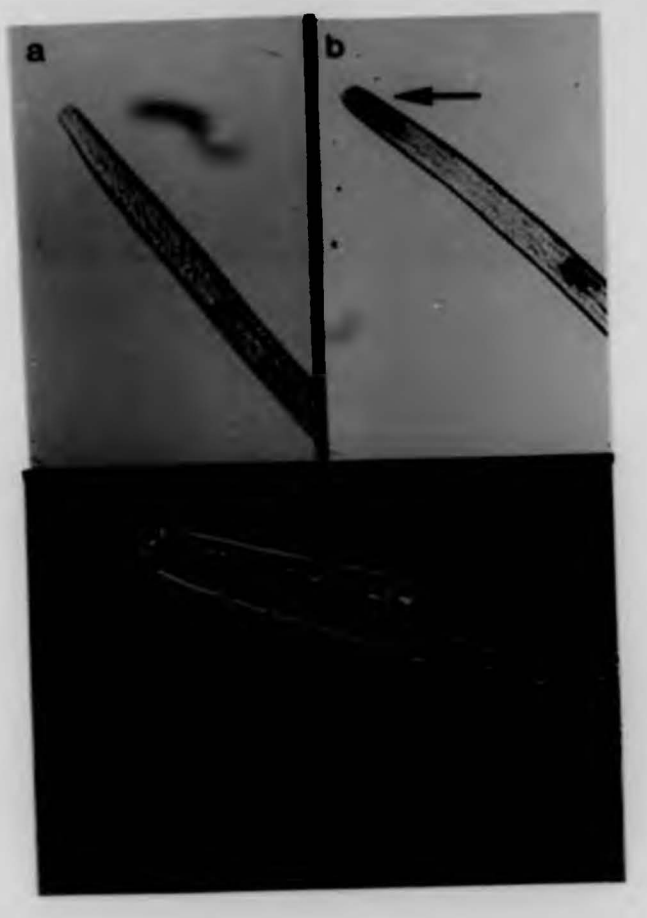

FIGURE 4

rvae in vitro.

aphy)

um for 48 hours. X 300

um for 18 months X 300

e procorpus.

or two hours X 625

pore precipitates.

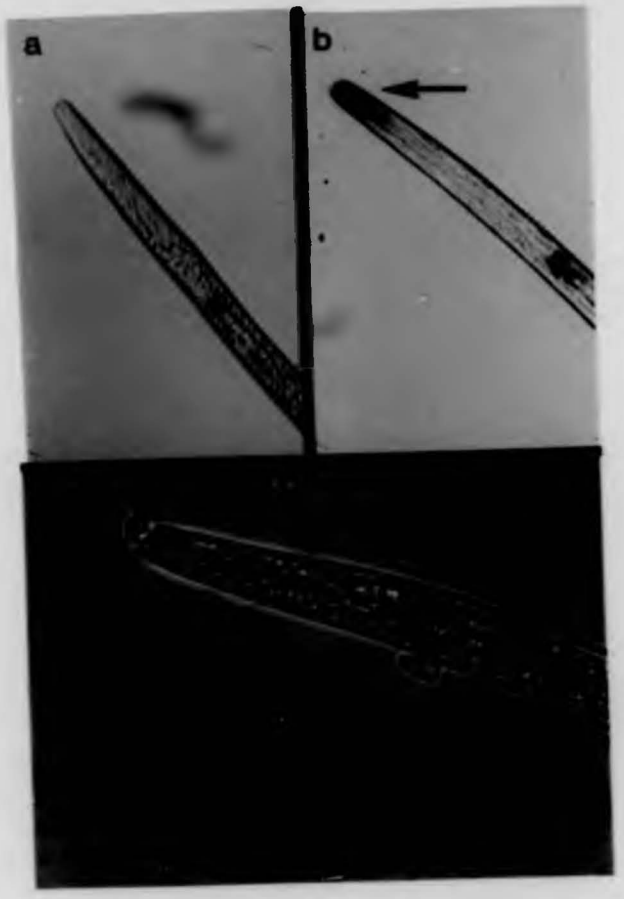

FIGURE 4

Fig. 5a Enzyme immunoassay standard curve for quantitation of Toxocara secretory antigen. Vertical lines indicate 95% confidence limits.

Fig. 5b Production of Toxocara larval secretory antigen, released in vitro during 18 months of culture, quantitated by enzyme immunoassay.

curve for quantitation
en. Vertical lines indicate

l secretory antigen,
months of culture,
assay.

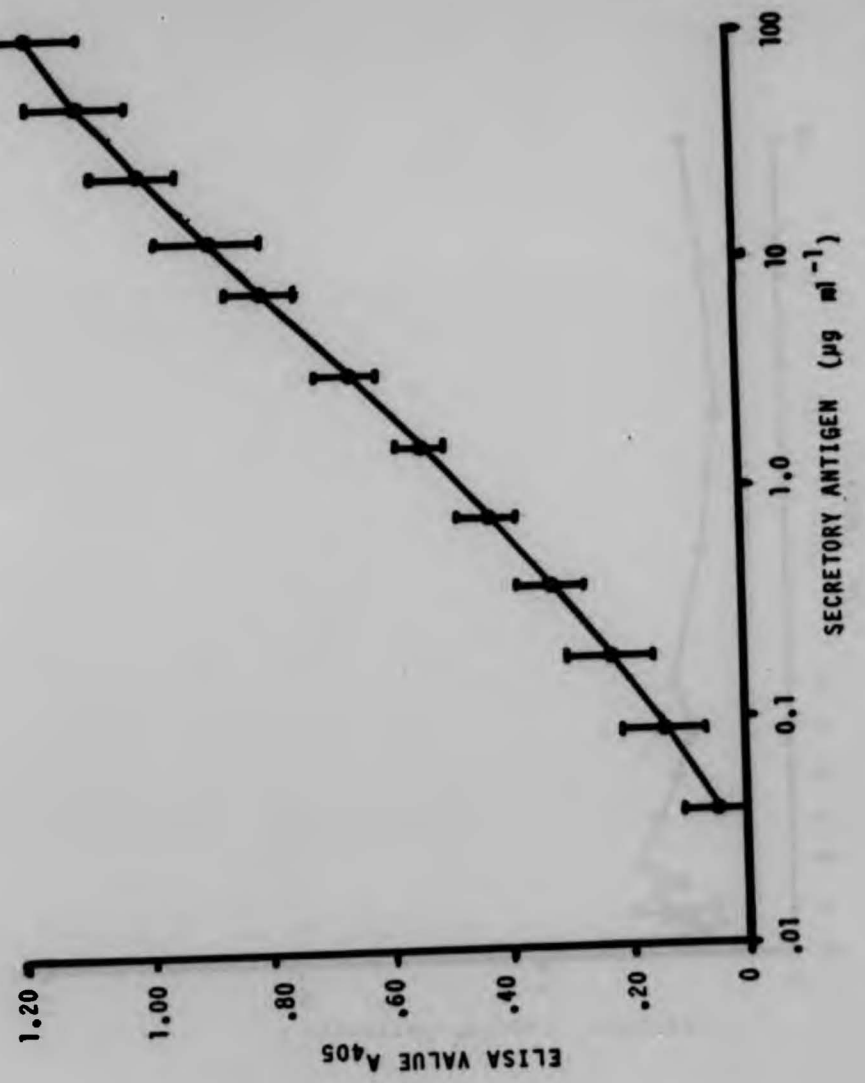

FIGURE 5a

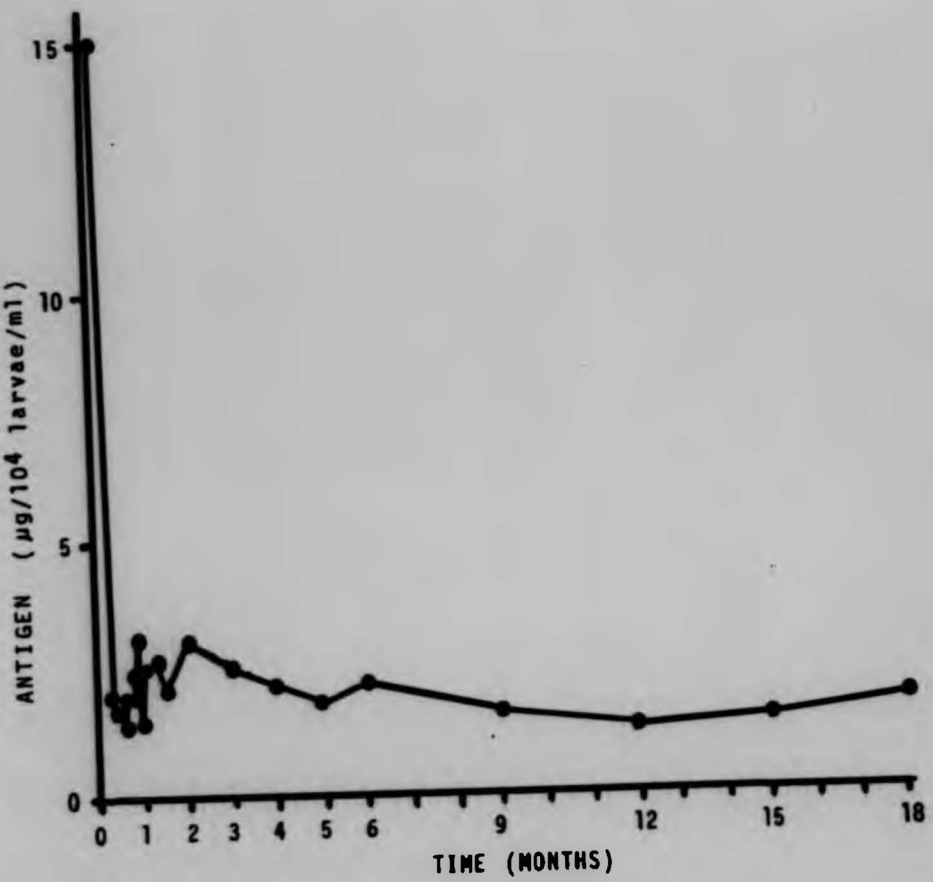
15
10
5
0
ANTIGEN (µg/10⁴ larvae/ml)
0 1 2 3 4 5 6 9 12 15 18
TIME (MONTHS)

FIGURE 5b

Fig. 6 Immunodiffusion and Immunoelectrophoresis of *Toxocara* larval secretory antigen

(a) Immunodiffusion

Centre well: Rabbit anti-*Toxocara* secretory antigen hyperimmune serum

Periphery: *Toxocara* secretory antigen (0.5 µg to 16 µg)

(b) Immunodiffusion

Centre well: *Toxocara* secretory antigen (8 µg)

Periphery: Immune sera

1. Rabbit anti-*Toxocara canis* secretory antigen
2. Mouse anti-*Toxocara canis* somatic antigen
3. Swine anti-*Ascaris suum*
4. Cat anti-*Brugia pahangi*
5. Mouse anti-*Necator americanus* secretory antigen
6. Rabbit anti-*Toxascaris leonina*

(c) Immunodiffusion

Centre row: Rabbit anti-*Toxocara* secretory antigen hyperimmune serum

1. *Necator americanus* adult secretory antigen
2. *Toxocara canis* larval secretory antigen
3. *Toxocara canis* larval somatic antigen
4. *Toxocara canis* adult secretory antigen
5. *Ascaris suum* larval (L_3-L_4) secretory antigen
6. *Toxocara canis* larval secretory antigen

(d, e) Immunoelectrophoresis

All wells: *Toxocara canis* larval secretory antigen (16 µg) electrophoresed at 10 V cm^{-1} for 30 minutes

(d) Trough: Antiserum from Immunized Rabbits

Rabbit anti-*Toxocara* secretory antigen hyperimmune serum.

(e) Trough: Antisera from Infected and Control Rabbits

1. Rabbit anti-*Toxascaris leonina*
2. Rabbit anti-*Ascaris suum*
3. Rabbit anti-*Toxocara canis*
4. Normal rabbit serum

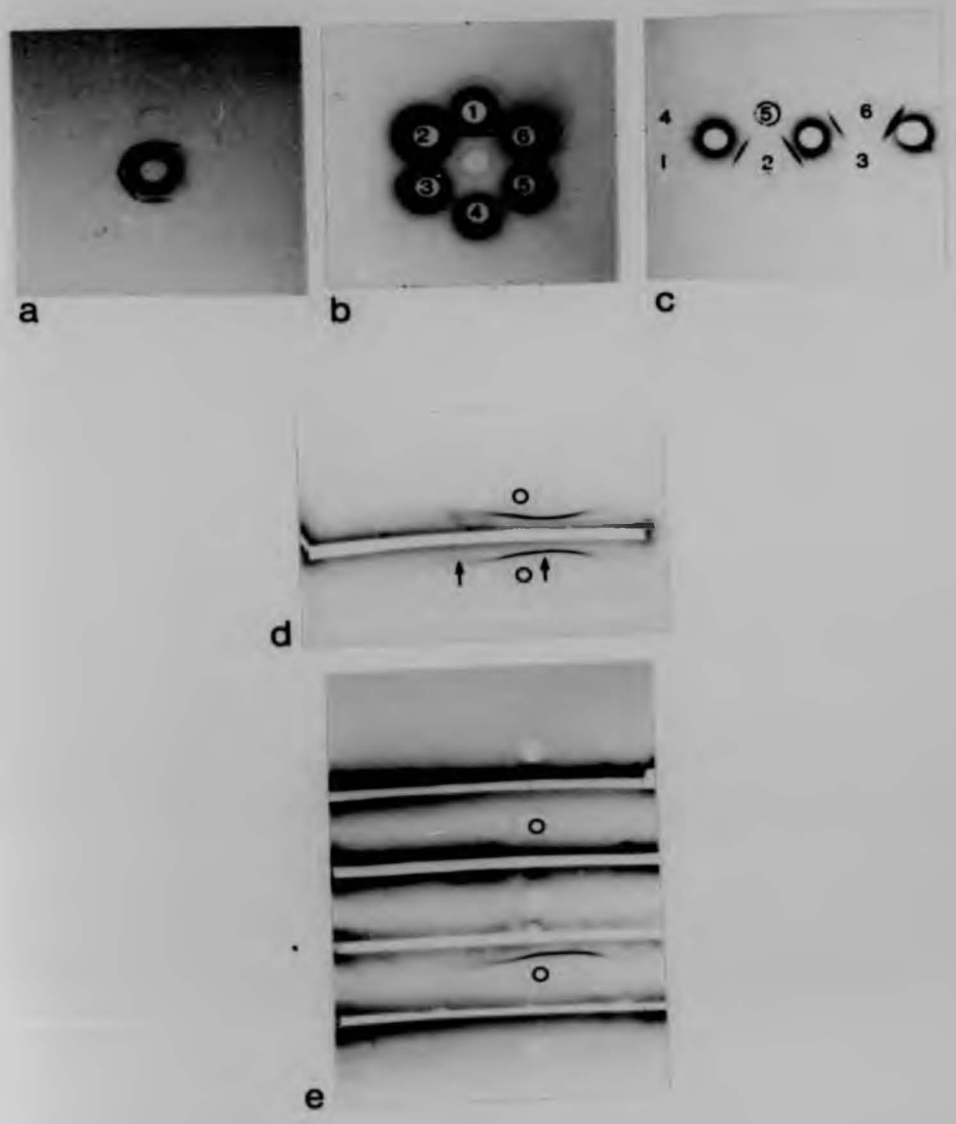

FIGURE 6

trophoresis of Toxocara larval

cara secretory antigen
m
ry antigen (0.5 µg to 16 µg)

ry antigen (8 µg)

cara canis secretory antigen
ara canis somatic antigen
is suum
pahangi
or americanus secretory antigen
scaris leonina

cara secretory antigen
m
us adult secretory antigen
arval secretory antigen
arval somatic antigen
dult secretory antigen
val (L_3-L_4) secretory antigen
arval secretory antigen

arval secretory antigen (16 µg)
at 10 V cm^{-1} for 30 minutes

mmunized Rabbits
cara secretory antigen hyperimmune

fected and Control Rabbits
scaris leonina
ris suum
cara canis
rum

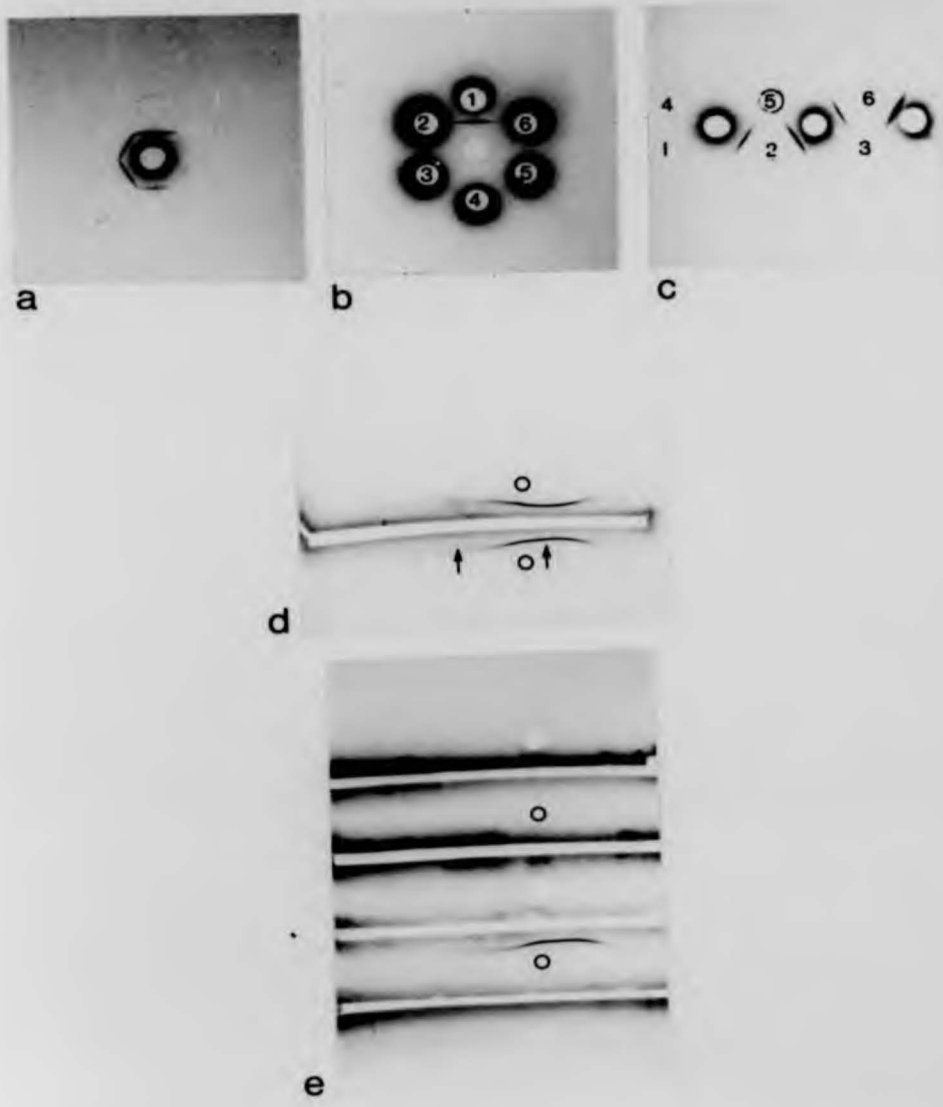

FIGURE 6

Fig.7 Molecular weight estimation, by SDS polyacrylamide gel electrophoresis, of Toxocara larval secretory antigens.

		MW K Daltons
(a) High molecular weight markers	(●)	56.0
		112
		168
(b) Low molecular weight markers	(■)	14.3
		28.6
		42.9
		57.2
		71.5
		85.8
(c) Toxocara canis secretory antigen	(X)	42.0
(d) Albumin (human)	(□)	68.0
(e) Ovalbumin	(○)	43.0

SDS polyacrylamide gel
rval secretory antigens.

		MW K Daltons
rs	(●)	56.0
		112
		168
s	(■)	14.3
		28.6
		42.9
		57.2
		71.5
		85.8
tigen	(X)	42.0
	(□)	68.0
	(○)	43.0

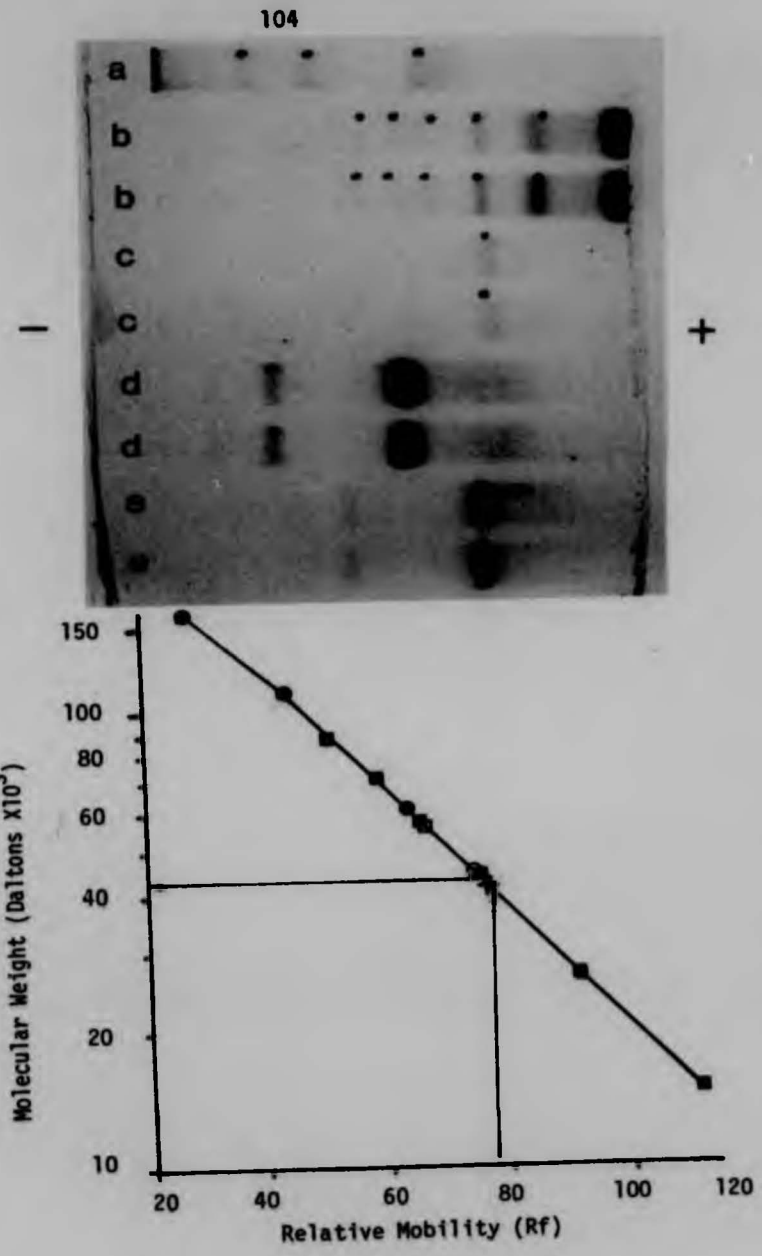

FIGURE 7

SDS polyacrylamide gel
rval secretory antigens.

		MW K Daltons
rs	(●)	56.0
		112
		168
s	(■)	14.3
		28.6
		42.9
		57.2
		71.5
		85.8
tigen	(X)	42.0
	(□)	68.0
	(○)	43.0

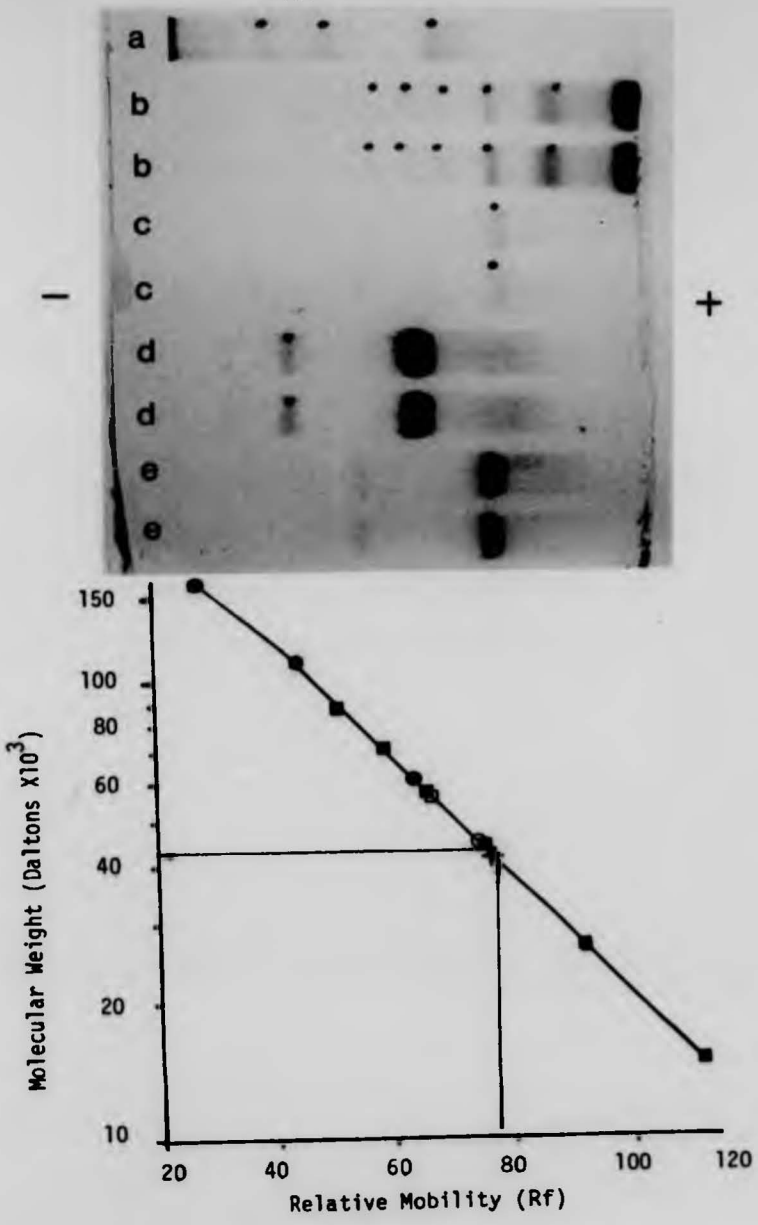

FIGURE 7

Fig. 8 Analytical isoelectric focusing of Toxocara larval secretory antigen.

(a) Toxocara canis Secretory Antigen Batch $B_1$78 (15 µg)
(b) " Batch $B_5$78 (15 µg)*
(c) Ascaris suum Somatic Antigen (70 µg)

The figure illustrates the high pI value (9.5) and the relative purity of toxocaral secretory products in contrast to Ascaris somatic antigens (perienteric fluid) in which at least 40 components are resolved in the original gel. Plot shows the pH gradient across the gel

* See Table 10 for description of antigen batches.

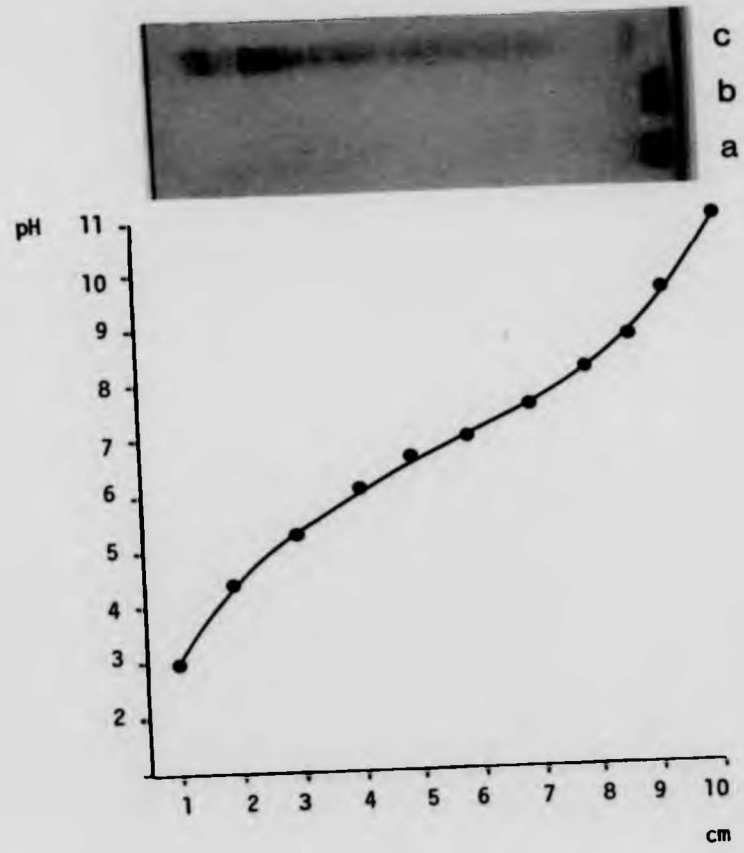

FIGURE 8

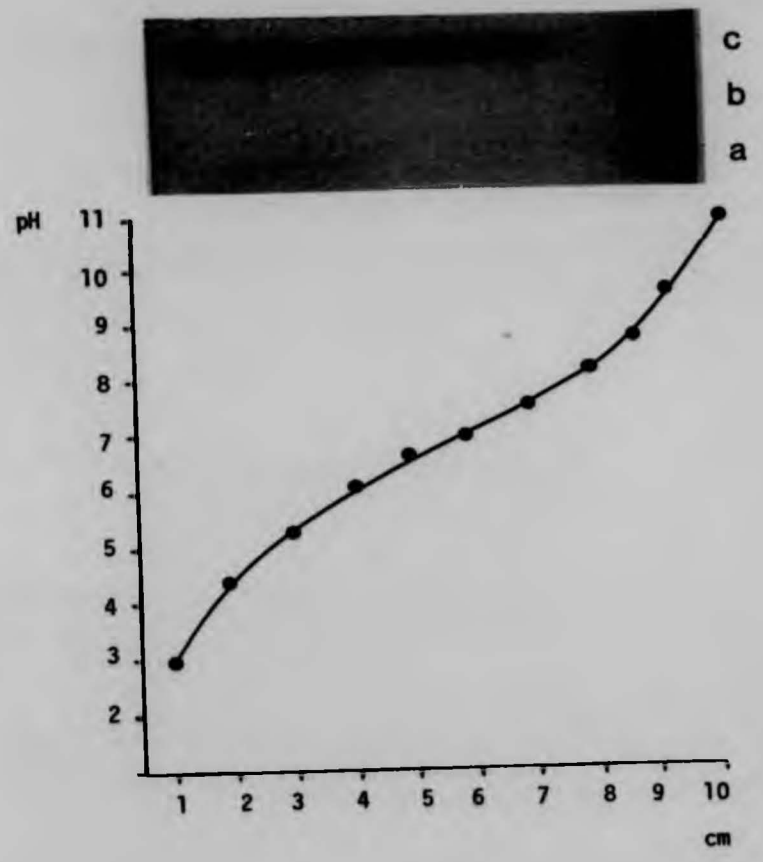

FIGURE 8

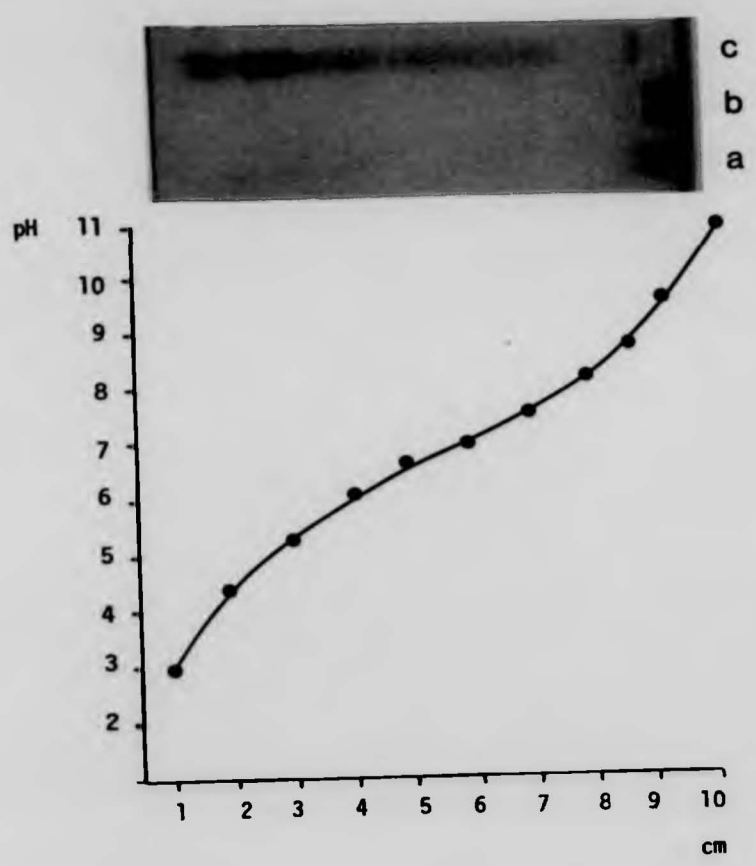

FIGURE 8

Fig. 9 Bi-dimensional immunoelectrophoresis of Toxocara secretory antigen (16 µg).

(a,b) First dimension: Electrophoresis of antigen in 1% agarose at 5 V cm^{-1} for 30 minutes.
Second dimension: Immunoelectrophoresis in 1% agarose containing rabbit hyperimmune antiserum (globulin fraction at 250 µg cm^{-1}) to Toxocara secretory antigen, at 2 V cm^{-1} for 12 hours.

(a) Anodal Antigens
(b) Cathodal Antigen

(c) First dimension: Analytical isoelectricfocusing of antigen in polyacrylamide gel followed by "laying on" an antibody containing gel.
Second dimension: Immunoelectrophoresis as in (a) and (b). Cathode at top.

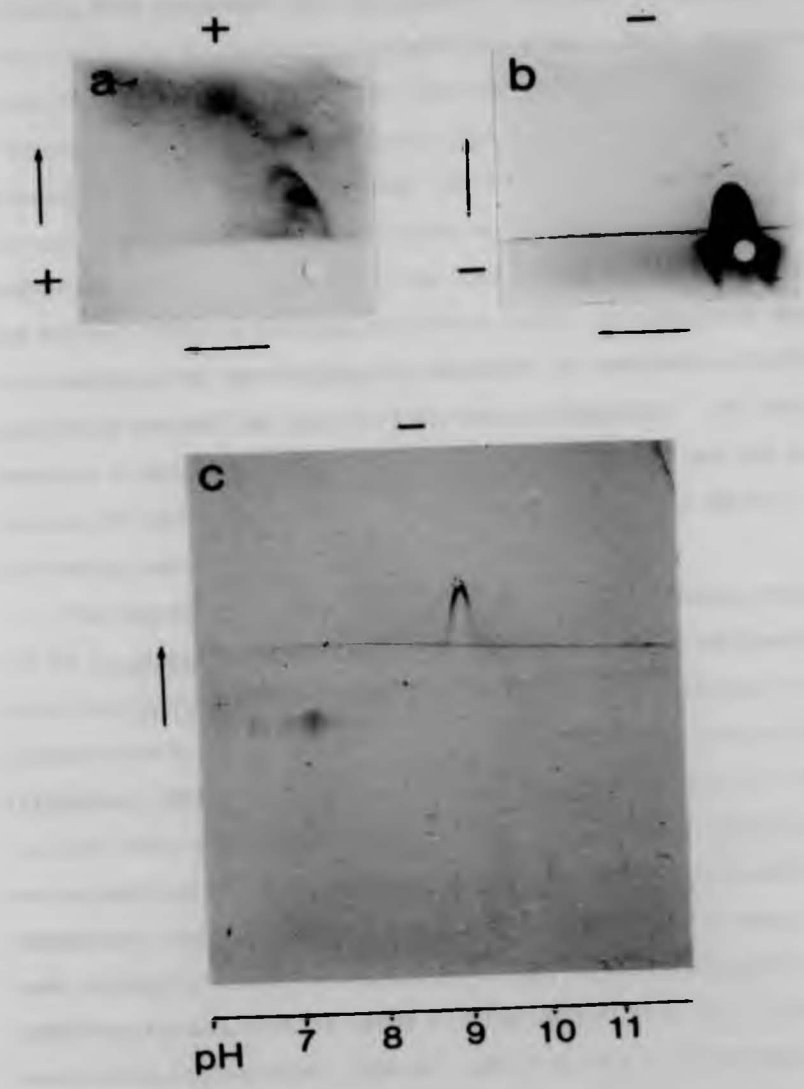

FIGURE 9

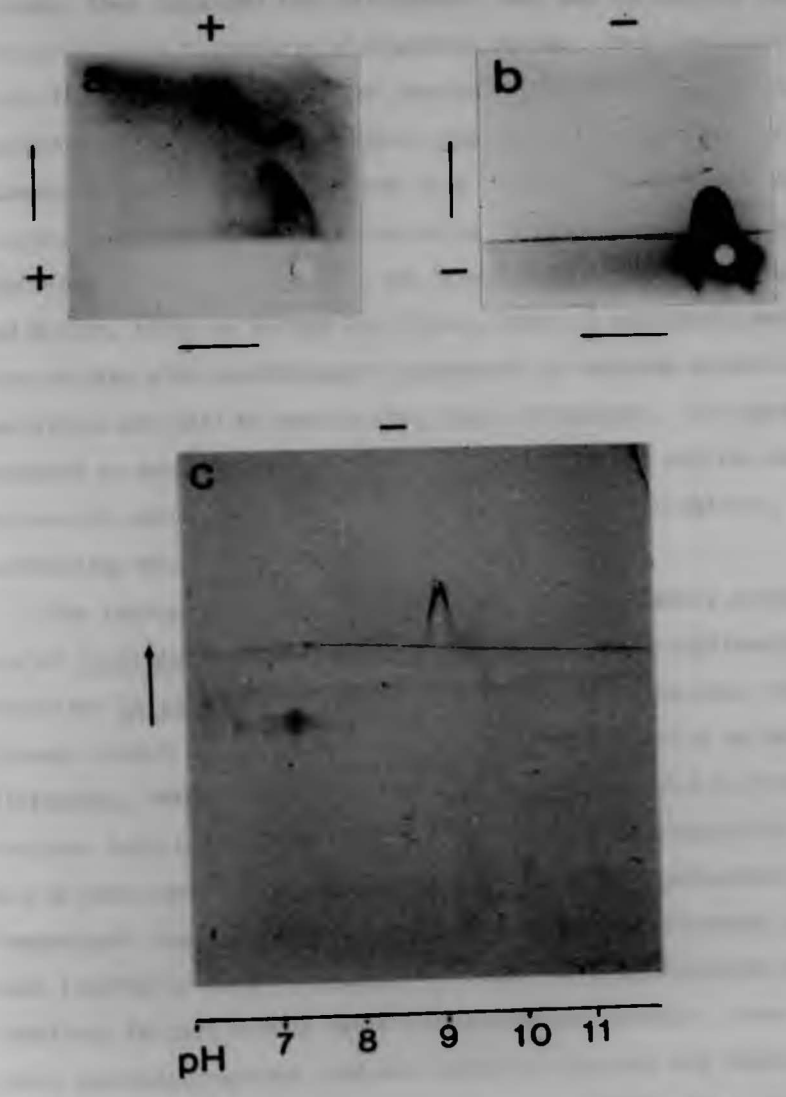

FIGURE 9

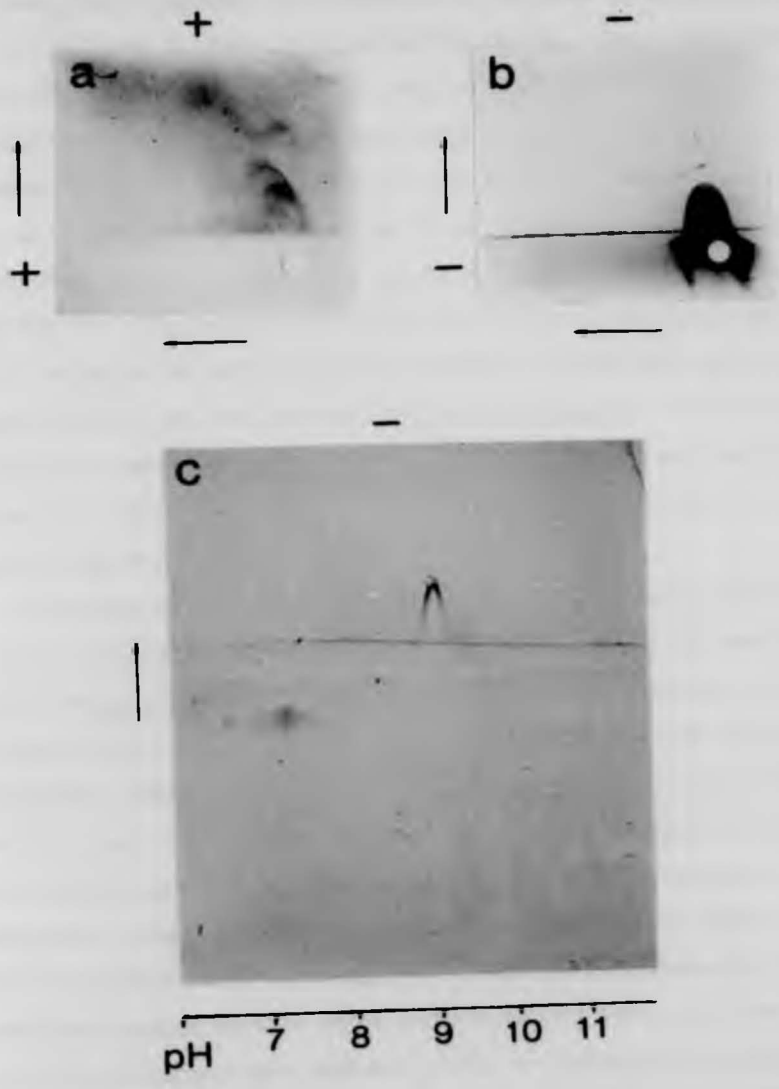

FIGURE 9

resis of Toxocara secretory

ophoresis of antigen in 1%
30 minutes.
oelectrophoresis in 1% agarose
mmune antiserum (globulin fraction
ara secretory antigen, at 2 V cm^{-1}

ical isoelectricfocusing of
e gel followed by "laying on"
el.
oelectrophoresis as in (a) and

DISCUSSION

When Beaver et al. (1952) first defined toxocaral visceral larva migrans, they suggested that pathogenesis was due, in part,to the host's immune response to products of migrating larvae. Such immunogenic products,once ambiguously called 'metabolic' antigens, have been named 'excretory' and 'secretory' antigens (Thorson, 1953) or 'ES' antigens (Campbell, 1955). It is now known that nematode 'exoantigens' contain enzymes arising from glandular secretion at oral and excretory pores (Ogilvie and Jones, 1973), thus the term 'secretory' antigen (Despommier and Muller, 1976; de Savigny and Tizard, 1977) is adequately descriptive when dealing with non-dialysable components of nematode excretions and secretions and will be used in this thesis throughout. In light of on-going research on metabolic activities of nematode cuticles and the possible release of cuticular antigens (Maizels, de Savigny and Ogilvie, 1980) this terminology may require future modification.

The logical requisite for study of nematode secretory antigens is the use of in vitro cultivation. Historically, long-term cultivation of parasites in vitro has had the motive of culturing parasites from ova, through larval stages to adults, and completing the cycle to fertile ova (Silverman, 1965). Achievement of this goal is expected to further insights into essential physiologic mechanisms of host-parasite relationships and to make available models for study of genetic, developmental and immunologic aspects. To this end, workers have not hesitated, and indeed, have invariably found it necessary, to use media supplemented with undefined factors such as serum proteins,embryo extracts, liver extracts, yeast extracts, peptones, and cell cultures (Douvres and Tromba, 1970; Silverman and Hansen, 1971; Hansen and Hansen, 1978). In studies of protective immunity elicted by secretory anitgens, such supplements have not posed a serious disadvantage since the effect of extraneous proteins is

irrelevant or otherwise controlled (Stromberg and Soulsby,1976, 1977; Clegg and Smith, 1978). However, use of supplements when producing secretory antigens for immunodiagnostic purposes has created serious problems since secretory products, usually present in low concentration (µg/ml), must be isolated in pure form, uncontaminated by supplementary protein, usually present at high concentration (>10 mg/ml) (Guerrero, Green, Silverman, Meradante, 1974.)

It has been known that second-stage larvae of T. canis liberate secretory antigen when maintained in vitro in serum (Olson, 1960; Hogarth-Scott, 1966). However, aside from this thesis and previous work by the author (de Savigny, 1975) there have been no reports of in vitro cultivation of toxocaral larvae nor of the production of secretory antigen for the purpose of comprehensive characterization and serological evaluation.

In contrast Ascaris suum, perhaps owing to the relative ease with which the parasite is obtained, has received considerable attention in terms of in vitro cultivation (Fairbairn, 1961; Arean and Crandall, 1961, 1962; Crandall and Arean, 1964; Cleeland, 1963; Levine and Silverman, 1969; Justus and Ivey, 1969; Guerrero and Silverman, 1972; Stromberg, Khoury and Soulsby, 1977; Stromberg, 1979).

In vitro studies by Levine and Silverman (1968) showed that HMEM supplemented with porcine serum was a superior medium for the development of A. suum from the second to fourth larval stages. However HMEM, without protein supplementation, failed to support Ascaris larvae longer than 19 days. Other workers (Douvres and Tromba, 1970) have experienced similar difficulty in maintaining Ascaris larvae longer than three weeks in non-supplemented medium. The longest maintenance records are: 110 days using protein supplementation (Cleeland, 1963); 65 days using tri-peptide supplementation (this thesis); and 21 days using no

supplements (de Savigny, 1975).

It is significant therefore, that in the present study T. canis larvae were maintained for over 500 days, in synthetic, unsupplemented medium. The results indicate that subtle differences in the composition of media such as exist between HMEM, EMEM, and M199 can have a demonstrable effect on survival (Fig. 1) and dimensions (Table 8) of larvae. Evidently, HMEM contains all the essentials in satisfactory proportion for the maintenance of actively metabolizing second-stage T. canis larvae, and as such, should prove a valuable basal medium for parasitologists investigating larval development, ecdysis, migratory stimuli, chemotherapy, and physiology. These results also suggest a fundamental difference in the nutritive requirements of the second-stage larvae of Toxocara canis and Ascaris suum. More importantly, they re-affirm the contention that long-term cultivation of all nematode parasites is a practicable goal.

The relative ease by which long-term cultivation of T. canis has been achieved may be due to the biological phenomenon of paratenesis (characteristic of this parasite), which allows the biological "clock" (Chuw and Pasternak, 1969,Zuckerman, 1974) of the second-stage larva to stop for long periods for larval maintenance (without development) in paratenic hosts. It has been noticed in some nematode species (de Savigny, unpublished observations) that mortality in vitro commences at the approximate time that ecdysis in vivo should occur. Perhaps efforts in the in vitro cultivation field should be redirected towards the stopping of the parasite's "clock" rather than improvement of culture systems and media which may now be adequate.

The design of the Toxocara in vitro culture system excluded in vivo steps. Although this resulted in higher yields of larvae compared with in vivo hatching, of greater importance or antigen production,

was the elimination of additional host antigen contaminants and mediators of the host's immune system. The assumption was initially made that *in vitro* hatched larvae would secrete the same products as would *in vivo* derived larvae. Immune precipitates observed at the oral and excretory pores of *in vitro* hatched larvae (Fig. 2) incubated in serum from rabbits with active infection, and electrophoretograms (Fig. 5,8) of secretory antigen from *in vitro* hatched larvae versus the same immune rabbit serum, support this assumption. However, it is possible that the host may induce production of additional secretory products which are not evoked under *in vitro* circumstances. Quantitative production of secretory antigen assayed by ELISA remained relatively constant at approximately 200 pg/larva/day throughout 18 months of culture. This production represents a daily turnover of at least 0.1% of the larval biomass (estimated for a single larva as 125 ng). Antigen production remained constant despite the appearance at five months, of opacities in the procorpal region. The nature of this development was not investigated but it might reflect decline of the culture, accumulation of waste metabolites, redistribution of internal components, pathological change in secretory glandular function, or perhaps crystalization and incorporation of phenol red (HMEM pH indicator). Stained crystals have been noted in the intestinal lumen of *Haemonchus contortus* and *Ostertagia ostertagi* *in vitro* and were thought to be by-products of degenerative processes (Bird, Waller, Dash and Major, 1978). Nematode "age pigment granules" (Epstein, Himmelhoch and Gershon, 1972) and dark pigmentation associated with ageing (Kisiel, Himmelhoch and Zuckerman, 1973) are known. It is thought that pigment results from the peroxidation of lipids (Chio, Reiss, Fletcher and Tappel, 1969). Procorpal opacity was not correlated with mortality, however the possible loss of

staining after death could not be excluded. It is noteworthy that larval mortality increased as the mean length of second-stage larvae approached the minimum length of third-stage larvae (slow,free-running of the "clock"?). A theory that genetic programming of protein synthesis may encompass age related phases, in which genes produce useless enzymes, received support from studies which demonstrate that inactive enzyme molecules accumulate with age in nematodes (Gershon and Gershon, 1970; Zeelon, Gershon and Gershon, 1973). The foregoing considerations raised the question of possible qualitative and quantitative changes in secretory antigen production in ageing cultures. However no physico-chemical differences were identified in antigen harvested at 15 months compared to that produced at one month. Immunological qualitative differences are investigated in Chapter III.

The observed thermo-phototropic behaviour exhibited by toxocaral larvae might provide a useful means of purifying live larvae from dead or moribund larvae and other contaminants.

Little is known concerning the chemical nature of nematode secretions. Ammonia, urea, peptides, amino acids, amines, uric acid and other organic acids have been found in the excretions and secretions of a variety of nematodes *in vitro* (Haskins and Weinstein, 1956; Weinstein, 1959; Stirewalt, 1963). Such low molecular weight components, if present, were removed during the diafiltration step of toxocaral secretory antigen preparation. Enzymic activity in nematode secretions has been demonstrated (Thorsen, 1963) including lipases and proteases. Olson and Izzat (1972) suggested that the preciptates observed at the orifices of *T. canis*. larvae incubated in homologous immune serum represent an enzyme-anti-enzyme reaction such as the anti-dehydrogenase activity described by Dusanic (1966) in experiments with *Trichinella spiralis*. It has been logical to speculate that nematode secretions should contain enzymes

since these are known to be necessary for hatching and ecdysis (Fairbairn, 1961) and penetration of host tissues (Lewart and Lee, 1954; Stirewalt, 1966). Protease activity has been demonstrated in Ascaris secretions (Hinck and Ivey, 1976) but it is not known if this arises from oesophageal (oral) or excretory pore secretions. It is generally accepted that the nematode excretory system is in fact a glandular secretory system (Weinstein, 1959; Romanowski, Rhoades, and Malakatis, 1973), and in ascarids, is probably associated with extracorporeal digestion of host tissue (Gibson, 1975). The excretory system of several nematode species contains alkaline phosphatase (Parshad and Guraya, 1977), a feature which may compromise the use of secretory antigens in enzyme immunoassays using alkaline-phosphatase as a label if this phosphatase is released into culture supernatants.

The anterior secretory gland of Nippostrongylus brasiliensis, Trichonstrongylus spp., Oesophagostomum spp., Necator americanus, Ostertagia spp., and Cooperia spp. secrete acetylcholinesterase (Sanderson and Ogilvie, 1971, Ogilvie and Yeates, 1974; Rothwell, Anderson, Bremer, Dash, Le Jambre, Merritt and Ng, 1976; Masaba and Herbert, 1978; Beaver and Dobson, 1978). Acetylcholinesterase (AChE) was found to be a useful marker for in vitro cultivation (Burt and Ogilvie, 1971) and to be antigenically genus specific (Rothwell et al. 1976). AChE production increased proportionately to host immunity (Sanderson and Ogilvie, 1971), showed changes in iso-enzyme pattern during response (Jones and Ogilvie, 1972), and stimulated a vigorous humoral responses (Yeates and Ogilvie, 1975). Since all nematodes which secrete AChE are gut-dwelling, Rothwell (1974) postulated that, because host intestinal structures are innervated by cholinergic fibres, nematode secretions containing AChE could influence intestinal function at the site of infection and act as a "biochemical holdfast" for nematodes. Although the adult stage of

Toxocara spp. is gut-dwelling, AChE was not demonstrated in secretions of adults (Rothwell, et al. 1976) nor larvae (this study) when assayed by the colormetric method of Ellman, Courtney, Valentino, Featherstone (1961).

Using techniques capable of high resolution, this work demonstrated that secretions of toxocaral larvae contain a single major protein component and at least two minor components. Compared with other helminth crude somatic antigens, which usually consist of dozens to hundreds of components, crude secretory antigen is a relatively simple mixture. This finding is in concordance with studies of secretory antigen heterogeneity in other species, which show the presence of between one and seven components (Crandall and Zam, 1968; Neilson, 1969; Jenkins and Wakelin, 1977) although Day, Howard, Prowse, Chapman and Mitchell (1979) reported over 20 components in Nematospiroides dubius secretions. The Coomassie Blue protein staining technique, with a sensitivity of 39 ng/mm^2 (Switzer, Merril and Shifrin, 1979), suggests that components which were barely detected have an estimated content of 200 ng. This would represent in the order of 2% of the protein applied to the gel. The amount of protein in the major band is not known, however components present at concentrations less than 20 µg/ml are probably not detected. The high pI value (9.5) of the major component imparted cathodal mobility at pH 8.6 and suggests that this is the major antigen seen in immunoelectrophoretic studies. The unusually high pI value of the major fraction should facilitate its concentration, isolation and purification by preparative isoelectric focusing.

SUMMARY

Studies of long-term *in vitro* maintenance for *Toxocara canis* second-stage larvae confirmed the value of the HMEM culture system as a reliable quantitative source of larval secretory antigen during at least 18 months of culture. An enzyme immunoassay, developed to quantitate larval secretions released during culture, indicated a daily production of approximately 200 pg per larva. Morphological changes in the procorpal region of cultured larvae after five months *in vitro* are described. The life span of the *Toxocara canis* larval culture (half-life 16 months) is the longest on record for any metazoan parasite and supports the contention that *in vitro* cultivation of nematodes, in synthetic media, is a practicable goal.

Studies, of antigen heterogeneity revealed toxocaral secretory products as a simple mixture containing one major protein component and two minor components, the former having a molecular weight of 42×10^3 daltons and pI of 9.5, while the minor components were of molecular weight 120×10^3 to 160×10^3 daltons and pI of 5.2 and 5.5. Glycoprotein, lipoprotein and acetylcholinesterase were not detected. Single and bi-dimensional immunoelectrophoretic studies indirectly identified the 42,000 molecular weight component as the major secretory antigen evoking a humoral response in the host during natural infection.

CHAPTER III

THE ENZYME-LINKED IMMUNOSORBENT ASSAY IN HELMINTHOLOGY - DEVELOPMENT AND VALIDATION FOR TOXOCARIASIS -

INTRODUCTION

Enzyme Immunoassay

The serological diagnosis of infectious diseases has relied for many years on assays which use secondary gross immunologic phenomena to demonstrate the primary event (ie. the antigen-antibody interaction). Such secondary serologic techniques suffer from various deficiencies including: insensitivity (precipitation); favouring of certain antibody classes (agglutination, haemagglutination, complement fixation); sensitivity to interfering agents (complement fixation); use of live pathogens (virus neutralization); and use of *in vivo* systems (passive cutaneous anaphylaxis). These shortcomings prompted development of immunoassays designed to measure primary reaction of antigen with antibody rather than secondary immunologic effects. In such assays, either of the reaction partners may be labelled with a marker whose purpose is to allow (1) perception and amplification of the primary antigen-antibody reaction, and (2) quantitation of antigen or antibody. Various labels have been used: bacteriophages (viroimmunoassay: Hamovitch, Hurwitz, Novik and Seal, 1970); free radicals (spin immunoassay: Leute, Ullman, and Goldstein, 1972); organometallic complexes (metalloimmunoassay: Cais, Dani, Eden, *et al.*, 1977); radioisotopes (radioimmunoassay for antigens: Yalow and Berson, 1960, and Ekins, 1960; immunoradiometric assay for antibody: Miles and Hayes, 1968) and fluorophores (fluoroimmunoassay for antigen: Adler and Lau, 1971, immunofluorescence assay for antibody: Coons, Creech

and Jones, 1941. These assays each have high sensitivity but also various limitations for practical application such as: unstable and/or hazardous reagents; costly equipment: slow test performance; low potential for automation; low potential for field use; narrow applicability.

The introduction of antibody-enzyme conjugates prepared without significant loss of either anitbody or enzymic function (Avrameas and Uriel, 1966; Nakane and Pierce, 1966) permitted the introduction of enzyme immunoassays, developed independently in Sweden (Engvall and Perlmann, 1971) and Holland (Van Weemen and Schuurs, 1971) for the purpose of antigen (hapten) quantitation. Later, Engvall and Perlmann, (1972) developed the immunoenzymatic assay for antibody quantitation which they named enzyme-linked immunosorbent assay (ELISA). Both types of assay elegantly combined biological specificity provided by antibodies, with biological amplification, afforded by enzymes, yet avoided many problems inherent in other isotopic and non-isotopic immunoassays. Practical application of ELISA in medicine was not significant until Voller, Bidwell, Huldt, and Engvall (1974) developed the microplate modification, following which, the introduction of enzyme immunoassays in many fields for quantitation of both antigens and antibodies, followed an exponential course. Applications have been comprehensively reviewed by: Engvall and Carlsson (1976); Scharpe, Cooreman, Blomme and Laekeman (1976); Voller, Bartlett and Bidwell (1976a); Wisdom (1976); Engvall (1977); Schuurs and Van Weemen (1977); Voller, Bartlett and Bidwell (1978); Engvall and Pesce (1978); O'Beirne and Cooper (1979); Voller, Bidwell and Bartlett (1979).

Enzyme immunoassays are either heterogeneous (ie. requiring the separation of bound from unbound enzyme-labelled reactant to permit the measurements of either "bound" or "free" enzyme activity (Voller,

Bartlett and Bidwell, 1978; O'Beirne and Cooper, 1979) or homogeneous (ie. no separation of bound or unbound reactants required) (Rubinstein, Schneider and Ullman, 1972). It may soon be possible to quantitate antibody by homogeneous immunoassay (Zuk, Rowley and Ullman, 1979; Rubinstein, 1980) although no applications have yet been perfected. Only heterogeneous assays have been used for immunodiagnosis of infectious diseases, and of these, ELISA has found widest applicability (Voller, Bidwell and Bartlett, 1979). In ELISA (Fig. 10) antigen is passively adsorbed to a solid phase which is then incubated with test antiserum. The presence of specific antibody reacting with (and bound to) the solid phase is detected using enzyme-labelled anti-immunoglobulin conjugate, followed by the appropriate enzyme-substrate. Each of these stages (ie. antigen, antibody, enzyme labelled-anti-immunoglobulin and substrate) is punctuated by a "bound-free" separation step. In the final stage, it is assumed that the rate of enzyme hydrolysis of substrate is directly proportional to the amount of enzyme-labelled antibody bound to the solid phase, which in turn is proportional to the amount of primary antibody bound; thus a single test produces a quantitative result expressed on a continuous scale. ELISA for detection of antibody is now widely used in: studies of autoimmune disorders (reviewed by Pesce, Kant, Ooi and Pollak, 1978); virology (Bidwell, Bartlett and Voller, 1977); bacteriology (Carlsson and Lindberg, 1978); mycology (Carlsson and Bernandes, 1976); protozoology (Voller, Bartlett and Bidwell, 1979); and helminthology (reviewed below.)

Use of ELISA in helminthology

Parasitology is often late to benefit from advances in other fields, however parasitologists have been quick to appreciate that the relative merits of ELISA (ie. sensitivity, reliability, economy and practicality) are of particular value to their field, and furthermore, that ELISA might play a special role in the developing world. Many early

applications of ELISA were devoted to parasitological, and especially protozoological problems (reviewed by: Voller, Bartlett and Bidwell, 1976b; Bout, Dugimont, Farag and Capron, 1976: Anon, 1976; Ruitenberg and Van Knappen, 1977; Hillyer and Kagan, 1979; Voller, Bidwell and Bartlett, 1979).

Trematodes. ELISA was introduced by Huldt, Lagerquist, Phillips, Draper, and Voller (1975) to detect antibody in patients with schistosomiasis, initially by use of the macro (tube), and later the micro (plate) formats (Voller, Bartlett and Bidwell, 1976). Schinski, Clutter and Murrell (1976) evaluated various tests and found ELISA and immunoradiometric assay to have the highest sensitivity. Deelder, Ruitenberg, Kornelius and Steerenberg (1977) demonstrated that useful data could be obtained when results were read by eye, but in field application using adult worm antigens, ELISA lacked sensitivity and specificity (Polderman and Deelder, 1977). McLaren, Draper, Roberts, et al. (1978) described the first use of ELISA in epidemiological studies of schistosomiasis and encountered cross-reactivity with other helminths but found specificity superior to that of IFA or CF. Other applications in schistosomiasis include those of Bout, Dugimont, Farag and Capron (1976); Voller, Bartlett, Bidwell and Edwards,(1977); Kelsoe and Weller (1978); Farag and Barakat (1978); Farag, Baraket, Awadalla and El-Gohany (1978); Salih, Bartlett, and Voller (1978); McLaren, Long, Goodgame and Lilleywhite (1979); Hillyer, Ruiz-Tiben, Knight, Gomes De Rios and Pelley (1979); Maddison, Kagan, Chandler, Gold et al. (1979); Speiser and Weiss (1979). Madwar and Voller (1977) and Stek (1978) used ELISA to detect circulating schistosomal antigens, the latter reporting 100% specificity. Although most authors reported enhanced sensitivity when using ELISA compared to conventional tests,

few provided data concerning specificity. Of those detecting cross-reactivity, none was able to improve specificity without resorting to antigen purification (Bout et al., 1976).

ELISA has been used in the diagnosis of fascioliasis (Bout, et al. 1976; Grelch and Horchver, 1977; Burden and Hammet, 1978; Hillyer, 1978; Hillyer and de Weil, 1979; Speiser and Weiss, 1979; Carlier, Bout and Capron, 1979); however cross-reactions were variable. There have been no applications of ELISA for paragonimiasis or opisthorchiasis.

Cestodes. Echinococcosis (hydatid disease) has been the subject of several applications; Bout et al. 1975; Capron, Bout and Dugimond, 1975; Sorice, Delia and Castagnari (1977); Ruitenberg, (1977); Felgner (1978 a,b,); Ambroise-Thomas, Des Georges and Monget (1978); Matossian, McLaren, Draper, et al.(1979) with acceptable results. Other workers (Farag et al. 1975; Ballard, Gavilova and Zorikhina, 1979; Speiser and Weiss, 1979) emphasized that purified antigens were more effective than crude antigens in ELISA for hydatidosis. Even so, Speiser (1980) reported cross-reactions with fascioliasis and filariasis.

Arambulo, Walls, Bullock and Kagan (1978) described a reproducible ELISA for cysticerciasis in man using cysticercal antigens however cross-reactions were observed with Echinococcus and Schistosoma. Cross-reactivity could be avoided by using Taenia antigens however sensitivity was compromised. In veterinary applications, ELISA, using somatic cysticercal antigen, performed well in the diagnosis of experimentally infected animals, but failed in field application because of high cross-reactivity with other helminths, including nematodes (Craig and Rikard, 1980).

Nematodes . The application of ELISA for nematode infections has received comparatively little attention, most of which has been directed to toxocariasis (reviewed in Chapter I, Section E) and trichinosis.

The first application of ELISA in parasitology was for trichinosis in pigs (Ljungstrom, Engvall and Ruitenberg, 1974). Considerable effort was devoted to the technical development of large-scale, automated, serologic screening procedures needed in veterinary applications for trichinosis detection (Ruitenberg, Steerenberg, Brosi and Buys, 1974; Ruitenberg, Steerenberg and Brossi, 1975; Ruitenberg, Ljungstrom, Sterenberg and Buys, 1975; Ruitenberg, Steerenberg, Brosi and Buys, 1976; Ruitenberg, Van Amstel, Brosi and Steerenberg, 1977; Ruitenberg and Van Knappen, 1977 a,b; Saunders, Clinard, Bartlett, Peterson, Sanders, Payne and Martinez, 1977; Saunders, Clinard, Bartlett and Sanders, 1977). Arising from these studies was an automated ELISA system, operated by two workers, and capable of processing 4000 sera per day. However during this period, virtually no critical study examined the validity of using crude Trichinella spiralis antigen. Recently the problem of extensive false positivity was recognized and this now represents the limiting factor in the implementation of such programmes (Clinard, 1979). Trichinosis in man has been diagnosed using ELISA by Engvall and Ljungstrom (1975) Ambroise-Thomas, Des Georges and Monget (1978) and Manweiller, Stumpf, Felgner, and Lederer (1978).

Bartlett, Bidwell and Voller (1975) designed ELISA procedures using crude whole worm extracts of Onchocerca volvulus, Onchocerca gutturosa Necator americanus, Dipetalonema viteae, Litomosoides carinii, Brugia pahangi and Ascaris suum. O. volvulus antigens obtained from human tissue gave unacceptably high non-specific ("background") values

in ELISA, possibly due to contamination of antigens with host immunoglobulin. Extensive cross-reactivity was encountered among the other species and these authors did not recommend ELISA using crude antigen as an alternative to conventional serodiagnostic methods for nematode infections.

Marcoullis, Salonen and Grasbeck (1978) used sequential affinity chromatography to purify O. volvulus whole worm extracts for use in ELISA but high backgrounds and cross-reactivity persisted.

Speiser and Weiss (1979) evaluated ELISA using antigen of seven helminth species, including Ascaris suum and Dipetalonema viteae, against panels of sera from nine parasitologically diagnosed helminthic diseases and concluded that when using crude antigens and testing at a single serum dilution, ELISA was inadequate for specific immunodiagnosis.

Ogilvie, Bartlett, Godfrey, Turton and Yeates (1976) described the successful application of ELISA using the secretory antigens of Necator americanus. Secretions of one adult worm provided sufficient antigen for 300 quantitative assays of high sensitivity. Cross-reactions were noted with Ancylostoma but not Onchocerca.

The salient features of the literature describing helminthological applications of ELISA are: (1) ELISA is particularly suited to large-scale diagnostic and seroepidemiological studies; (2) ELISA has equal or superior sensitivity in the detection of helminthic infection compared to conventional serologic methods; (3) ELISA, using crude helminth antigen has inferior and often unacceptable specificity compared to conventional methods; (4) few studies have provided critical assessments of specificity and cross-reactivity; (5) few studies investigated the use of purified helminth antigens in ELISA; (6) there have been no comprehensive studies of the use of helminth

secretory antigens in ELISA; (7) at the inception of this work, there had been no application of ELISA for toxocariasis.

This chapter describes the development and validation of ELISA for toxocariasis using larval secretion as antigen.

MATERIALS AND METHODS

Toxocara Enzyme-linked Immunosorbent Assay

The immunoenzymatic assay used was the indirect microplate enzyme-linked immunosorbent assay (ELISA) described by Voller, Bidwell and Bartlett (1976). ELISA was adapted for toxocariasis by using as antigen, *Toxocara canis* second-stage larval secretory products, obtained from *in vitro* culture in chemically defined, low molecular weight medium, and extracted as described in Chapter II. Optimal test parameters including solid-phase, dose and incubation times for antigen, test serum, and conjugate were determined experimentally.

In the optimized system, antigen was diluted to 100 ng/ml of protein in carbonate buffer, pH 9.6 and by employing passive adsorption at $4^{o}C$ for 18 hours, 200 µl quantities of antigen (20 ng) were coated onto the wells of disposable polyvinyl ELISA plates (Cooke, Microtiter 1-220-29). Plates were washed by three, three minute rinses in phosphate buffered saline containing 0.05% Tween 20 (PBS-Tween, pH 7.4) to remove unadsorbed antigen. Plates were given a quick rinse in distilled water to remove buffer salts before drying at $37^{o}C$ for 30 minutes and packaging individually in heat-sealed polyester-aluminum foil sleeves containing silica gel desiccant. Sealed *Toxocara* ELISA plates were stored at $4^{o}C$. To test sera, wells were incubated for 2 hours at room temperature with duplicate, 200 µl volumes of patients' or reference sera diluted 10^{-3} in PBS-Tween. Further washing, as above, removed unbound serum components. To assay specifically bound antibody, wells were incubated for 18 hours at $4^{o}C$ with 200 µl of alkaline phosphatase-labelled anti-human IgG (γ chain specific) prepared by the one-step glutaraldehyde method

(Avrameas, 1969; ad modum Voller, Bidwell and Bartlett, 1976)*. Following washing, 200 µl of enzyme substrate, 4-nitrophenyl phosphate (1 mg/ml), diluted in diethanolamine buffer, pH 9.8, were added at 20°C. Enzyme hydrolysis of substrate was monitored photometrically. After the appropriate interval, as determined by the reaction rates of reference standards, hydrolysis was terminated by addition of 50 µl of 3M sodium hyroxide. Results (ELISA values) were determined photometrically (Dynatech Vitatron ELISA Reader or Flow Multiskan) at 405 nm and expressed in absorption units (A_{405} cm^{-1}). Unless otherwise indicated, all ELISA values reported are mean results of duplicate assays.

Controls

Replicates of positive and negative standard sera were tested in each ELISA plate within all assays. After ten minutes of substrate reaction, two of the replicate standards were terminated on each plate and the A_{405} determined photometrically (A_1). Termination time for the remainder of the plate was then calculated using the known "target" A_{405} (A_2) of each standard according to the formula:

$$\text{Expected Substrate Reaction Time (min)} = \frac{(A_2) \times (10 \text{ min})}{(A_1)}$$

Following termination of the remainder of the wells according to the calculated reaction time, mean ELISA values of the remaining replicates were compared to target ELISA values. If these were disparate by a factor greater than the coefficient of variation (CV %) of the assay, all ELISA values would then be corrected by multiplication by the factor:

(A_{405} Expected / A_{405} Observed).

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* Anti-human IgG alkaline phosphatase conjugate was prepared by Dr. Ann Bartlett, Nuffield Laboratories of Comparative Medicine, London.

The same positive and negative standard sera were used as references throughout this study and the positive negative ratio was monitored to detect drifts in assay sensitivity.

In addition, a quality control serum was included in each row of each ELISA plate to assess intra-assay and inter-assay precision, calculated as the coefficient of variation (CV % = $\frac{S\ D}{\bar{x}}$ X 100). Results of an assay were rejected if the intra-assay CV was greater than 10%. Results of a study were rejected if the inter-assay CV was greater than 15%.

The ELISA protocol described above was modified to measure anti-Toxocara, class specific immunoglobulin responses in experimentally infected mice, rabbits, pigs, and rhesus monkeys, by use of species specific conjugates (one-step glutaraldehyde method). For some species, peroxidase conjugates were used in conjunction with o-phenylenediamine (34 mg/100 ml) and $H_2\ O_2$ (40 µl 100/ml) substrate in citrate-phosphate buffer, pH 6.0. Reaction was terminated with 50 µl of 5 N $H_2\ SO_4$ and results read photometrically at 492 nm.

Toxocara IgM-ELISA

To measure IgM anti-Toxocara responses by ELISA, 50 µl volumes of test sera were fractionated by micro-column chromatography on Bio-Gel A-5m (Pyndia, Krech, Price and Wilhelm, 1979) and the IgM peak tested at an estimated dilution of 1:100 using the IgG ELISA protocol with anti-IgM (µ chain specific) enzyme-conjugate. Approximately 30 sera per day could be fractionated by this method.

Toxocara Immunoradiometric Assay

For performance comparisons with ELISA, an immunoradiometric assay (IRMA) was devised using the ELISA protocol but substituting radioisotope labelled anti-human IgG in place of the enzyme conjugate. Care was taken that all parameters were held constant except the enzymic or

isotopic labels and their detection methods. Isotope labelling (^{125}I) of the same antibody preparation used in enzyme conjugates was done by the chloroglycouril method of Markwell and Fox (1978)*. Results were read by excising individual wells into a rack gamma-counter (Nuclear Enterprises, NE 1600). Conjugates (phosphatase-, peroxidase-, and ^{125}I- labelled anti-IgG) were adjusted to the same molar concentration of antibody. Equivalence of antibody molar concentration in enzyme conjugates was confirmed by polyethyleneglycol (PEG) precipitation of ^{125}I-labelled IgG.

Toxocara Somatic Antigen ELISA

For comparative purposes, an ELISA using Toxocara larval somatic antigen was developed. Antigen, prepared as described in Chapter II, was used at an experimentally determined optimum concentration of 2.5 μg ml^{-1}. All other parameters were identical to those of Toxocara ELISA using secretory antigen. The positive threshold was taken as the mean, plus two standard deviations, of the ELISA values of 50 healthy adults.

Antisera

To initiate validation of Toxocara ELISA, sera from three groups of subjects were tested: (1) healthy adults; (2) patients with non-toxocaral helminthiasis; and (3) patients with clinical toxocariasis.

Sera from 922 healthy adults were obtained from blood donors native to the UK, attending blood donor clinics in south-east England. Donors, had a mean age of 34.5 years (range 19-65) and a male:female ratio of 1.01:1.

Sera from 96 subjects with non-toxocaral helminthiasis were obtained from European patients examined at the Hospital for Tropical Diseases, London (Dr. D. Ridley) and the Swiss Tropical Institute, Basle,

* ^{125}Iodine labelling was done by Dr. Ray Edwards, St. Bartholomew's Hospital, London.

(Dr. F. Speiser) and diagnosed as having a single parasitologically proved, helminthic infections, and whose sera contained demonstrable antibodies to their respective parasites.

Sera from 49 subjects with presumed clinical visceral toxocariasis were obtained from Canada (Courtesy of Dr. J. Yang), Czechoslovakia (Dr. M. Uhlikova), Sweden (Dr. H. Carlsson), Switzerland (Dr. F. Speiser), UK (Prof. A.W. Woodruff), USA (Dr. K.Walls). Using information obtained from clinical findings and laboratory data available for each patient, cases were assigned a score from 0 to 8, based on the number of criteria for toxocariasis (Tables 1 and 2) which they displayed. The scoring method of Cypess et al. (1979) was used with the modification that criteria were weighted according to their known frequency of occurrence in clinical visceral toxocariasis (eg. hypereosinophilia >20% was given extra weighting, while features whose frequency in Tables 2 and 3 was less than 50%, were given less weighting). Criteria and their respective scores were as follows: Hypereosinophilia >20%, geophagia, pica, were each scored a value of 2. Hypereosinophilia of 10% to 19%, hyperglobulinaemia (>12 g/l) leucocytosis ($>10^4/mm^3$), elevated anti-A or anti-B isohaemagglutinins, and rheumatoid factor were each scored a value of 1. Central nervous system involvement, cutaneous involvement, lymphadenopathy, pallor, anaemia, and oedema were each scored a value of 0.5. Patients with a total score of 0 to 2 were designated as "doubtful" cases of clinical visceral toxocariasis and were excluded from the evaluation. Patients with a score of 3 to 4 and patients with a score 5 or higher were designated, respectively, as "possible" and "probable" cases. Only those with the latter scores (ie. equal or higher than 3) were included in the category of clinical visceral toxocariasis for use in evaluation of Toxocara ELISA sensitivity.

Sera were obtained from eight rhesus monkeys (Macaca mulatta) experimentally infected by stomach intubation with 0, 10, 100, 1000, or 5000 Toxocara canis ova/kg, administered in a single dose under anaesthesia (Ketamine hydrochloride, 1 mg/kg intramuscularly). Two additional animals received trickle doses of 5 ova/kg weekly, for six months, administered via inoculated portions of food.

Sera were obtained from four rabbits (New Zealand White) experimentally infected (by stomach intubation) with Ascaris suum (10,000 ova/kg) Toxascaris leonina (5,000 ova/kg), Toxocara cati (5,000 ova/kg) or Toxocara canis (5,000 ova/kg).

Sera were obtained, (courtesy of Dr. P. Stevenson, Royal Veterinary College) from 13 ascarid naive pigs (Landrace and Large White) experimentally infected by stomach intubation with 1,000 to 160,000 Toxocara canis and 5,000 to 160,000 Ascaris suum infective ova, in single or trickle doses, at 7-9 weeks of age (14.5 to 23.5 kg) and bled up to 14 weeks post-infection. Additional sera were obtained from eight pigs (courtesy of Dr. D. Hart, Bristol Eye Hospital) infected intravenously with 1,000 in vitro hatched, T. canis larvae.

All sera were stored at -70^{o}C without preservative.

Processing of Toxocara ELISA Data.

To evaluate the most effective means of expressing Toxocara ELISA test results, all currently available methods were examined using data from tests on five sera representing a spectrum of Toxocara ELISA reactivity. Methods included: qualitative and semi-quantitative methods; the titration method; the absorbance method; the positive:negative ratio method; the percent positive method; the reference value/percentile method; the effective dose method (Leinikki and Passila, 1977); the multiple of normal activity (MONA) method (Felgner, 1978); and the

standard curve method (de Savigny and Voller, 1980).

To prepare the Toxocara ELISA standard curve, sera from 41 subjects, selected as having ELISA values in a spectrum from 0.05 to 4.0 (A_{405}), were titrated to their respective titre end-points in $\log_2$ or in half $\log_{10}$ dilutions. The dose-response curve for each patient was plotted and the titre end-point interpolated from the point at which the titration curve intersected the positive threshold ELISA value (A_{405} = 0.50). Toxocara ELISA values for all sera, at a serum dose of 10^{-3} were then plotted as a function of their respective titre end-points to generate a standard curve. The standard curve was then used to transform Toxocara ELISA values to estimates of serum titre expressed on a continuous scale.

RESULTS

Development and Optimization of Toxocara ELISA

Optimal Antigen Parameters. Initial experiments indicated that polyvinyl chloride was more satisfactory than the commonly used polystyrene carriers as a solid-phase for toxocaral secretory antigen. Polyvinyl adsorbed 81.7% of 20 ng/well (16 ng) after 18 hours at $4^{o}C$, approximately five-fold more than that adsorbed by polystyrene (Fig. 11). By increasing the antigen concentration to 200 ng/well, the percent adsorbed decreased to 19.2%, although the absolute amount adsorbed was higher (38 ng). For all solid phases, adsorption occurred asymtopically, approaching a plateau level after approximately six hours at $4^{o}C$.

A two-dimensional titration was used to estimate the optimal working concentration of toxocaral antigen for ELISA. Antigen was titrated in one dimension by adsorption for 18 hours at $4^{o}C$ while negative, low, medium and high-titred standard sera were titrated in the second dimension. Positive:negative ratios of ELISA values were calculated for each antigen dilution to identify the dose allowing optimal distinction (ie. the highest ratio) between positive and negative responses. A discrete optimum of 100 ng ml^{-1} was determined which was satisfactory for all levels of seroreactivity. Above or below 100 ng/ml, sensitivity was reduced.

Antigen incubation time was examined using antigen protein at 100 ng ml^{-1}. Results (Table 9) indicated that adsorption for as few as five minutes was sufficient to distinguish positive and negative responses, and that positive:negative ratios were not substantially improved by increased adsorption time. Because more reproducible results were obtained with longer incubation times, and because it suited work flow patterns for large scale seroepidemiological studies,

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TOXOCARA ELISA

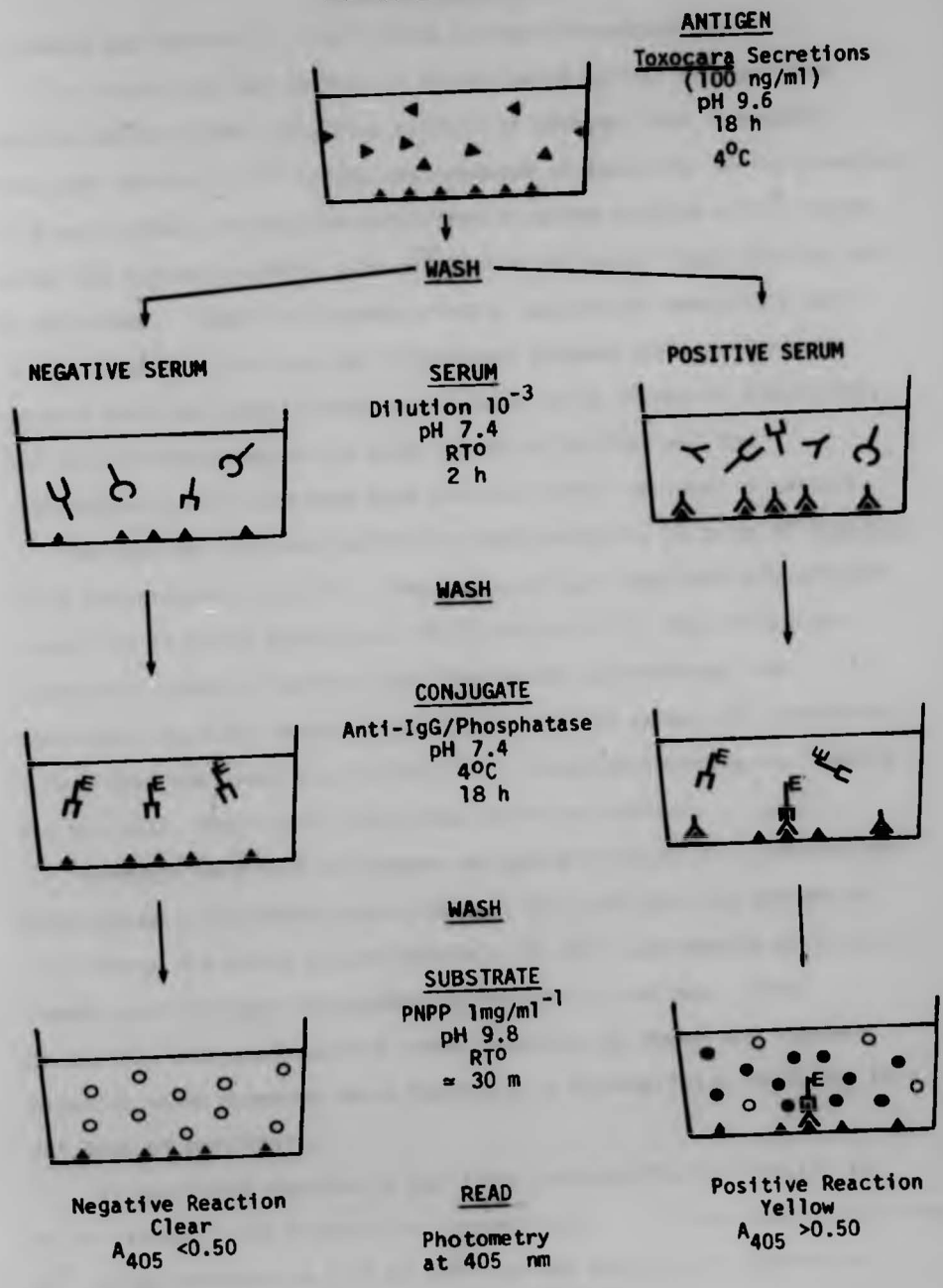

FIGURE 10

18 hours was selected as the routine antigen adsorption period.

To assess the reliability of the estimated optimal antigen dose and incubation conditions, five batches of antigen, four harvested from one culture of 10^5 larvae and produced at intervals during a period of eight months, and one harvested from a second culture of 10^6 larvae after two months *in vitro*, were diluted to 100 ng ml^{-1} and used to coat ELISA plates. Eight sera representing a spectrum of reactivity were tested. Results indicated no differences between batch performance greater than that anticipated due to inter-assay variation (Table 10), and that antigen prepared at eight months of culture was not qualitatively different from that prepared after one week of culture.

Antigen stability and shelf-life were evaluated in terms of *Toxocara* ELISA performance (Table 11). Best results were obtained with antigen stored dry on ELISA plates under desiccant at 4°C. The antigen was remarkably stable to various heat treatments and although some treatments resulted in statistically lower ELISA values, all variations were within the usual inter-assay CV %. Repeated freezing and thawing had the most significant deleterious effect on antigen.

Attempts were made to improve antigen binding by (1) pre-coating ELISA plates with bovine serum albumin (BSA) and coupling antigen to solid-phase BSA using glutaraldehyde or by (2) pre-coating wells with rabbit anti-*Toxocara* immunoglobulin followed by antigen. Both procedures increased positive serum responses by about 50%, however negative serum responses were increased up to nine-fold, resulting in a net loss of sensitivity.

It was found possible to use larval culture fluids directly in ELISA without prior dialysis or concentration. Culture supernatant from 10^4 larvae cultured in 2 ml of HMEM for one day could be used at an optimal dilution of 1:100 by adsorption to polyvinyl ELISA plates for

18 hours at 4°C (ie. sufficient for 480 duplicate assays). Again, excess of culture antigen reduced sensitivity. The apparent simplicity of this approach was abandoned in favour of dialysed antigen because of the difficulties of protein estimation (and thus standardization) in HMEM, the possibility of non-specific reactivity to penicillin, and the potential for microbial contamination of HMEM during antigen incubation in non-sterile ELISA plates.

ELISA dose-response curves for positive and negative standard sera were compared in polyvinyl and polystyrene ELISA plates (Fig. 12) and found to be both parallel and equally sensitive despite the lower content of antigen on the polystyrene. To achieve comparable results, it was necessary to increase the substrate reaction time in polystyrene by at least 4-fold compared to that in polyvinyl, but precision was sacrificed (see: Reproducibility, pg 144).

Optimal Serum Dilution. Results of two-dimensional titrations of antigen and antisera indicated that the optimal serum dilution for use in the IgG Toxocara-ELISA was 10^{-3}. This is a function of the combined effects of (a) the nature of the negative serum dose-response curve, and (b) the prozone phenomena commonly seen in positive sera. The serum working dilution (demonstrated in Fig. 13) is constant for all levels of seroreactivity. The optimal time for serum incubation at a dilution of 10^{-3} was between 1.5 and 2.5 hours at room remperature (Fig. 14). Two hours was selected for the routine protocol.

Optimal Conjugate Dilution. For reasons of reagent economy and logistics, the conjugate incubation step was overnight at 4°C. Optimal dilution of enzyme-conjugate was estimated initially by titration against a known quantity of IgG (100 ng ml^{-1}) adsorbed to polyvinyl ELISA plates. Conjugate dose was selected as that dilution producing an ELISA

TABLE 9

EFFECT OF ANTIGEN ADSORPTION TIME ON *TOXOCARA*-ELISA REACTIVITY

Antigen Adsorption Time (20 ng/well)	ELISA Value (A_{405})*		Positive:Negative Ratio
	Negative	Positive	
5 m	0.085	2.52	29.6
15 m	0.11	3.88	35.2
30 m	0.12	4.10	34.1
60 m	0.13	4.28	32.9
2 h	0.15	4.64	30.9
3 h	0.16	4.87	30.4
6 h	0.17	5.07	29.8
18 h	0.20	6.06	30.3

* ELISA values are means of duplicate assays in one experiment.

TABLE 10

PERFORMANCE OF *TOXOCARA* ELISA USING DIFFERENT SECRETORY ANTIGEN BATCHES. (AT 20 ng/well)

Serum Code	ELISA Value (A_{405})						
	Antigen Batch No.						
	$B_1$78	$B_2$78	$B_3$78	$B_5$78	$B_2$79	Mean	Coefficient of Variation (S.D.)
	Months in Culture						
	0.25	2	4	8	2		
1	.11	.10	.15	.12	.10	.116	18.1% (.021)
2	.49	.35	.41	.45	.50	.44	14.1% (.062)
3	.95	.85	.83	.88	.80	.86	6.6% (.057)
4	1.35	1.65	1.40	1.37	1.10	1.37	14.2% (.195)
5*	1.75	1.75	1.75	1.75	1.75	---	----
6	2.40	2.45	2.43	2.25	2.25	2.36	4.2% (.098)
7	2.95	3.24	3.05	3.15	2.95	3.07	4.1% (.127)
8	3.90	3.99	4.30	4.18	3.90	4.05	4.4% (.179)

B_{78} Culture of 10^5 *T. canis* larvae.

B_{79} Culture of 10^6 *T. canis* larvae.

5* Positive standard serum - results adjusted to 1.75

TABLE 11

THERMOSTABILITY OF *TOXOCARA* LARVAL SECRETORY ANTIGEN ASSESSED BY PERFORMANCE IN *TOXOCARA* ELISA

Antigen Treatment		*Toxocara* ELISA Values A_{405} (± SD)		
Temperature	Time	negative	weakly positive	strongly positive
+4°C	6 mon	.133 (.007)	.70 (.022)	2.06 (.038)
-70°C	6 mon	.135 (.006)NS	.70 (.095)NS	2.05 (.057)NS
+4°C	6 mon	.138 (.013)NS	.65 (.015)///	2.01 (.043)NS
20°C	7 day	.133 (.009)NS	.67 (.022)NS	1.97 (.022)///
37°C	2 day	.135 (.013)NS	.74 (.039)NS	2.01 (.089)NS
45°C	1 day	.120 (.008)///	.73 (.034)NS	1.99 (.037)NS
56°C	30 min	.128 (.010)NS	.71 (.024)NS	1.94 (.049)///
80°C	15 min	.125 (.010)NS	.65 (.049)NS	1.97 (.015)///
100°C	5 min	.118 (.009)///	.65 (.028)//	1.89 (.031)///
-70°C/+20°C	10 X	.115 (.013)/	.68 (.025)NS	1.68 (.048)///
Control (No Antigen)		.045 (.006)	.052(.007)	.045 (.013)

* Standard *Toxocara* secretory antigen coated on polyvinyl ELISA plates at 20 ng per well and stored at 4°C with desiccant. All other antigens stored in PBS in 20 ul volumes at 0.3 mg ml^{-1} in sealed glass capillary tubes.

NS Difference not statistically significant at P = 0.05

/ Significant Difference P < .05

// " P < .01

/// " P < .001

ELISA values are means of quadruplicate assays in one experiment.

gen to various solid

e-response curves for
and polystyrene antigen

2 min
2 min

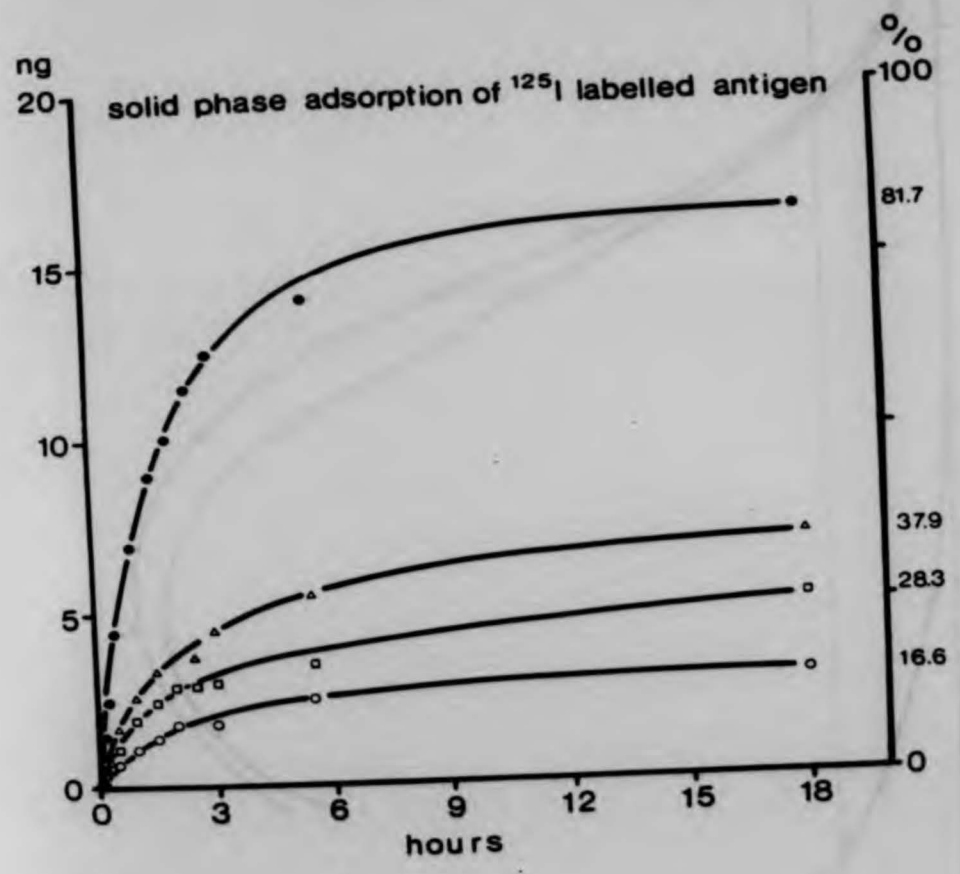

FIGURE 11

Fig. 11 Adsorption of ^{125}I-labelled antigen to various solid phases at pH 9.4.

- ● Polyvinyl chloride
- △ Polystyrene "Removawell"
- □ Polystyrene Tube
- ○ Polystyrene Plate

Fig. 12 Comparison of Toxocara ELISA dose-response curves for assays performed with polyvinyl and polystyrene antigen solid phase.

- ● Polyvinyl A_{405} / 22 min
- ○ Polystyrene A_{405} / 72 min

igen to various solid

se-response curves for
and polystyrene antigen

22 min
72 min

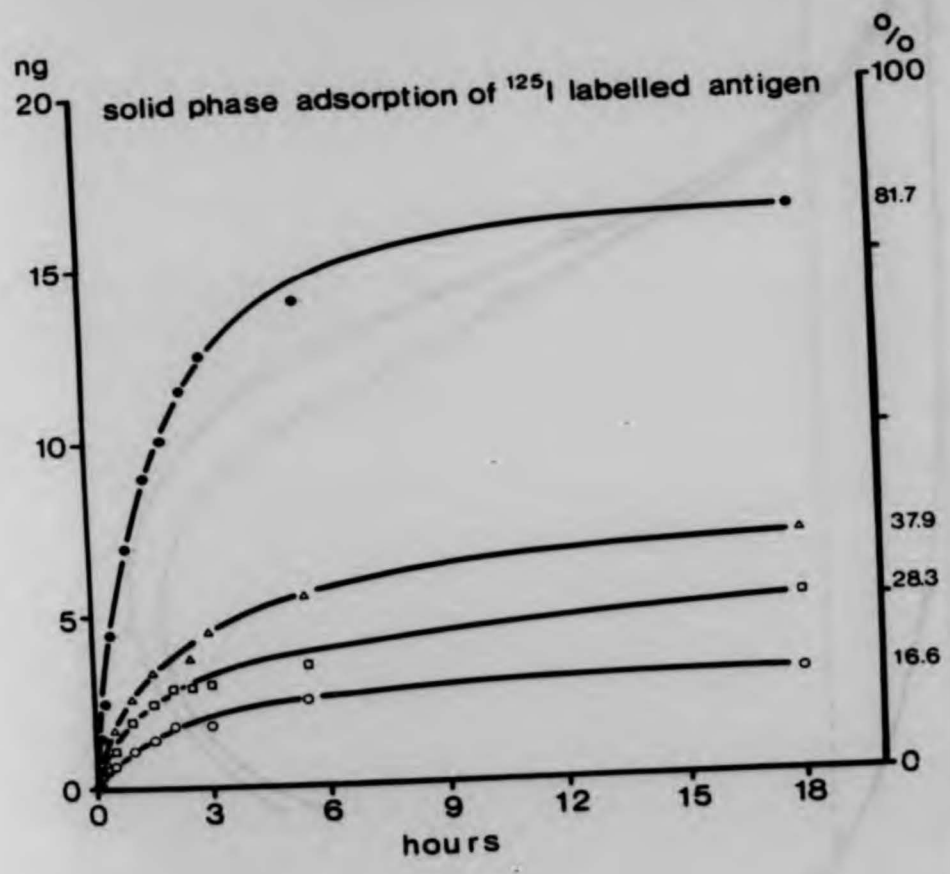

FIGURE 11

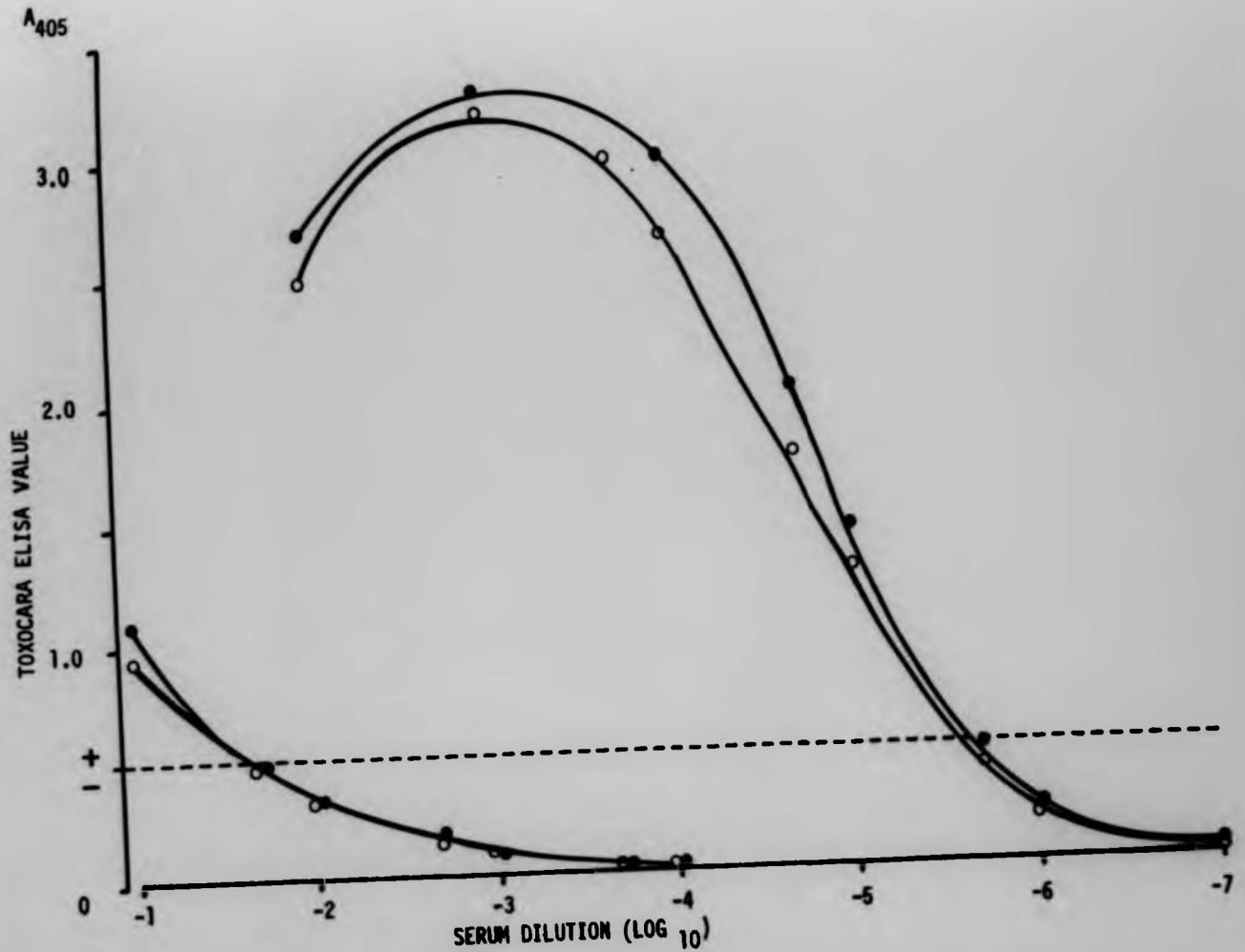
A405
3.0
2.0
1.0
+
−
0
TOXOCARA ELISA VALUE
-1
-2
-3
-4
-5
-6
-7
SERUM DILUTION (LOG 10)

FIGURE 12

Fig. 13 Determination of the optimal serum dilution for use in Toxocara ELISA. The dilution resulting in the maximum positive:negative ratio is arrowed.

Fig. 14 Determination of optimal serum incubation time for Toxocara ELISA. Time of maximal positive:negative ratio is arrowed

- ● Positive serum
- ○ Negative serum
- ▲ Positive:Negative Ratio

serum dilution for use in
resulting in the maximum
rowed.

m incubation time for
mal positive:negative

o

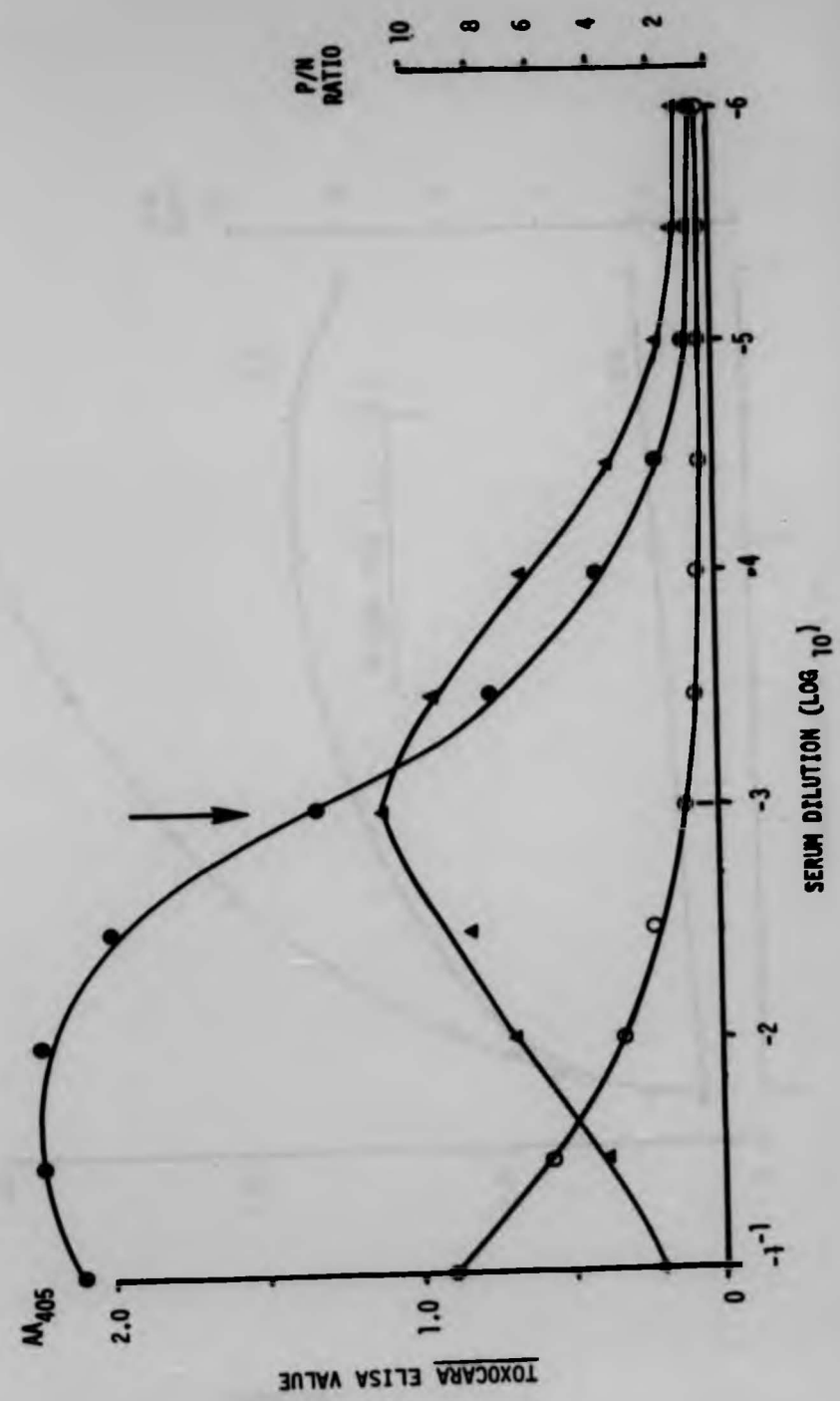

FIGURE 13

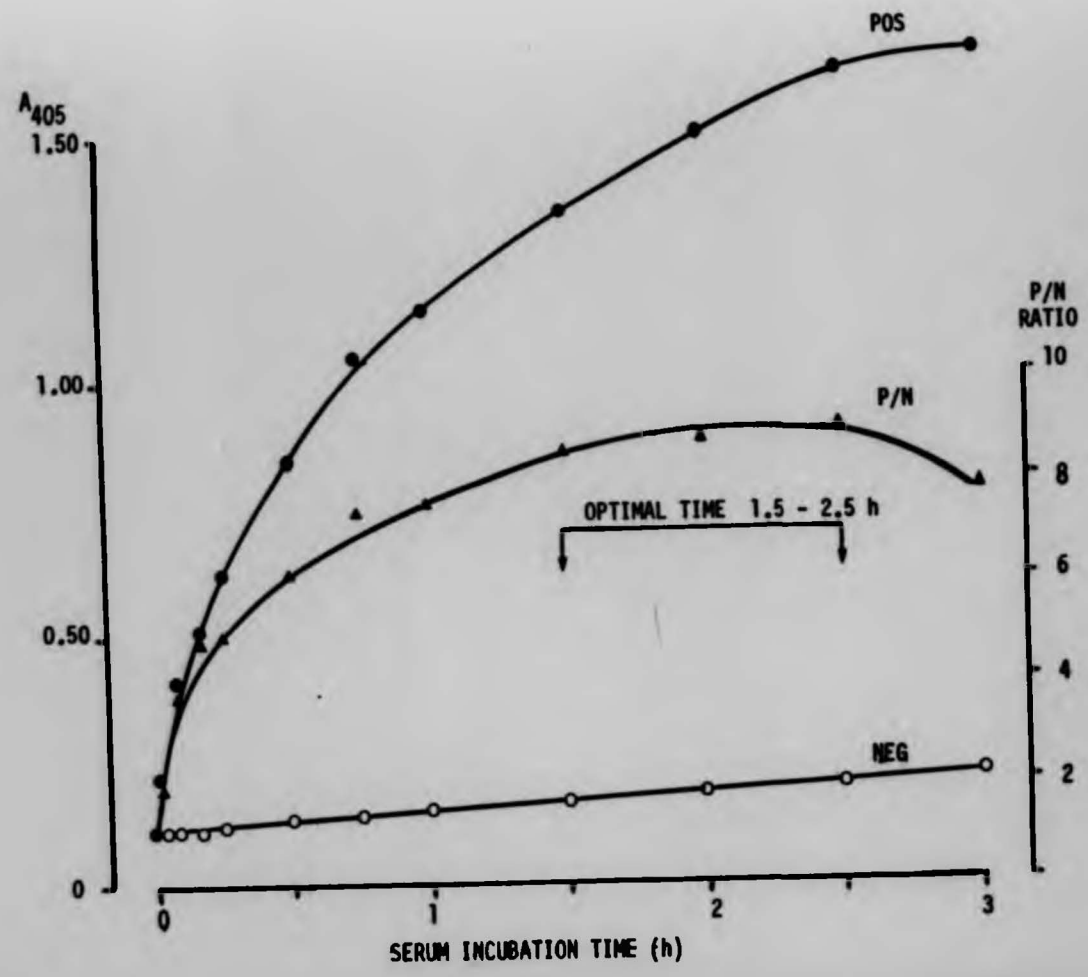
POS
A_{405}
1.50
1.00
0.50
0
P/N
RATIO
10
8
6
4
2
P/N
OPTIMAL TIME 1.5 - 2.5 h
NEG
0
1
2
3
SERUM INCUBATION TIME (h)

FIGURE 14

Fig. 15 Anti-human IgG (γ)-alkaline phosphatase conjugate titrations in the Toxocara-ELISA system.

a) Titrations

b) Positive:Negative Ratios indicating optima.

Human Anti-Toxocara sera

● High reactivity

▲ Moderate reactivity

■ Low reactivity

○ Non-reactive

In this experiment 20 ng of conjugated antibody per ml (conjugate dilution of 1:2000) was selected for routine use.

TOXOCARA ELISA

phosphatase conjugate titrations

indicating optima.

onjugated antibody per ml

) was selected for routine use.

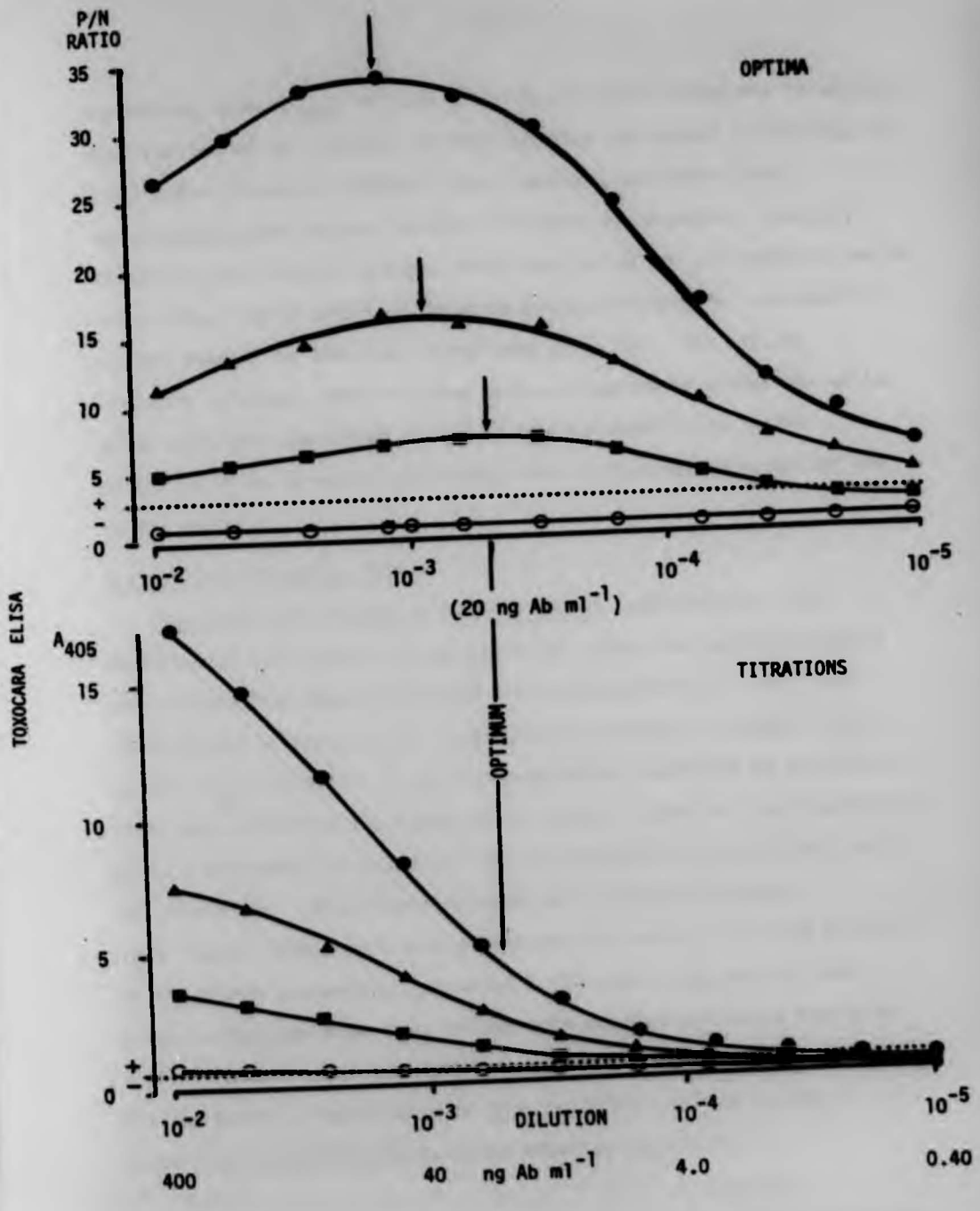

FIGURE 15

absorbance value (A_{405}) of 1.00 after 30 minutes of substrate incubation. Confirmation of the validity of this approach was sought by testing, in the routine protocol, negative, low-, medium-, and high-titred anti-*Toxocara* sera against various dilutions of conjugate. Contrary to antigen and antisera optima, which were valid for all levels of serum reactivity, high-titred sera required progressively more conjugate for optimal reactivity than low-titred sera (Fig. 15). The optimum conjugate dilution predicted from tests on IgG coated plates was valid principally for low-titred sera. To enhance sensitivity to the detection of low levels of antibody, this optimum was selected for use in the protocol.

Validation of Toxocara ELISA

Toxocara ELISA Values in Healthy Adults (Specificity). The distribution and limits of *Toxocara* ELISA values in healthy UK adults were established from a survey of 922 blood donors (Fig. 16). The distribution is approximately log-normal with median and modal ELISA values (A_{405}) of 0.17. A small subpopulation (confirmed by probability plot) was evident at the higher ELISA values. Based on the probability plot, a minimum ELISA value of 0.50 was selected to discriminate this sub-population. This "positive-negative" threshold value was approximately three multiples of the median "normal" value and occurred at the 97.4th percentile. Elevated ELISA values ($A_{405}>0.50$) were clearly disparate from those of the main population, ranged from 0.50 to 1.35 (3 to 8 times the median negative) and included 2.6% of healthy adults. Specificity of *Toxocara* ELISA, defined as the proportion of healthy adults found negative, was 97.4%

Toxocara ELISA Values in Clinical Visceral Toxocariasis (Sensitivity). Sera from 32 cases of visceral toxocariasis, diagnosed

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Toxocara ELISA Values in Clinical Visceral Toxocariasis (Sensitivity). Sera from 32 cases of visceral toxocariasis, diagnosed

according to clinical criteria, were tested, of which 87.5% had elevated ELISA values (A_{405}>0.50) and 8.4.% had values above the upper limit observed in healthy controls (A_{405}>1.35). ELISA values ranged as high as A_{405} = 6.0 (40 times the median negative value) with a mean of 2.96 (geometric mean = 2.08), significantly higher then that of both healthy and helminth-infected controls (P<0.001) (Students t test). Using A_{405} = 0.50 as a titre end-point, Fig. 17 illustrates the titre equivalents of ELISA values for representative negative and positive sera. Serum titres range from 1:30 to 1:1,000,000.

Sensitivity of Toxocara ELISA, defined as the proportion of patients with clinical toxocariasis found positive, was 87.5%.

Toxocara ELISA Values in Non-Toxocaral Helminthiasis (Cross-Reactivity). Sera from 96 Europeans with single, parasitologically proved, helminthic infections were tested (Fig. 18). ELISA values of all patients were within the range observed for healthy controls. Although ELISA values in this group were raised slightly, there was no statistically significant difference in mean ELISA value (log transformed data, Student's t test, P>0.50) nor in the proportion (6.3%) with elevated (positive) values (Chi-Square, P>0.50)*. Correcting for the toxocariasis seropositivity rate of 2.6% in healthy adults, prevalence adjusted cross-reactivity of Toxocara ELISA was thus 3.7%.

Toxocara ELISA Predictive Values. Predictive values for both positive and negative Toxocara ELISA results, calculated as shown in Table 12, were 97.1% and 88.6% respectively. The influence of various settings of ELISA positive threshold value on sensitivity, specificity and predictive value is given in Table 13.

---

* Yates's Correction for Continuity

Toxocara ELISA Reproducibility. Quantitative reproducibility was governed from day-to-day by use of a predictive equation (see methods) to estimate the termination time of substrate incubation in each assay. This equation is valid only if enzyme kinetics remain linear throughout the substrate incubation. Because of the broad spectrum of sero-reactivity encountered in clinical toxocariasis, kinetics of enzyme-substrate hydrolysis (regression of ELISA value on time) for both phosphatase and peroxidase conjugates were compared in Toxocara ELISA using negative, low, and high titred sera. Results, analysed by linear regression (Fig. 19), indicated that phosphatase/PNPP kinetics were more suitable in this regard, remaining linear throughout 100 minutes. Peroxidase/OPD kinetics tended to plateau after 40 minutes, and at no time, were linear. (Correlation coefficients, $r = 0.999$ and $r = 0.940$ respectively). The convergence of the three peroxidase regression lines on the abscissa at a common point, and the fact that each curve had a discrete maximum response value suggests that the fault is not substrate limitation, but that it is a time-dependent inhibition of enzyme activity, possibly dictated by the initial rate of enzyme activity.

Intra-assay precision was assessed both experimentally and in practice. Experimentally, six sera selected as representing a spectrum of Toxocara-ELISA reactivity were tested repeatedly (16 times each) within one assay. The regression of standard deviation on mean ELISA value for each serum was computed (Fig. 20) and the total coefficient of variation (CV = 11.1%) was then estimated from the slope of the calculated linear regression line. In practice, intra-assay precision of Toxocara-ELISA results was monitored during assays of over 5,000 human sera (10% of which constituted quality controls or standards) tested during period of three years. Intra-assay precision ranged

TABLE 12

CALCULATION OF SOME ASSAY PARAMETERS FOR VALIDATION OF *TOXOCARA* ELISA

Qualitative *Toxocara* ELISA Results	Health Status: Clinical Toxocariasis (D+)	Health Status: Healthy Subjects (D-)	Total
Positive (T+) $A_{405} < 0.50$	a	b	a + b
Negative (T-) $A_{405} > 0.50$	c	d	c + d
Total	a + c	b + d	a+b+c+d = N

Data from validation experiments: a = 28, b = 24, c = 4, d = 898, N = 954

Sensitivity = p (T+/D+) = a (a+c) X 100 = 87.5%

Specifivity = p (T-/D-) = d (b+d) X 100 = 97.4%

Predictive Value*

Positive Result = p (D+/T+) = a'/(a'+b') X 100 = 97.1%

Negative Result = p (D-/T-) + d'/(c'+d') X 100 = 88.6%

* To account for differences in sample size of groups (D+) and (D-), data were transformed to percentage(') of (D+) and (D-) respectively, before calculating predictive values.

TABLE 13

SENSITIVITY, SPECIFICITY, AND PREDICTIVE VALUES*
OF *TOXOCARA* ELISA AT DIFFERENT POSITIVE THRESHOLD LEVELS

ELISA Positive Threshold Value A_{405}	Sensitivity (%)	Specificity (%)	Predictive Value	
			Positive Result (%)	Negative Result (%)
0.25	87.5	87.7	87.7	87.5
0.30	87.5	92.1	91.7	88.0
0.40	87.5	96.0	95.6	88.5
0.50	87.5	97.4	97.1	88.6
0.75	87.5	99.0	98.9	88.8
1.00	87.5	99.8	99.8	88.9
1.25	84.4	99.9	99.9	86.5
1.50	78.1	100	100	82.0

* Parameters refer to detection of clinical visceral toxocariasis. For subclinical toxocariasis and ocular toxocariasis, see Chapter IV.

TABLE 14

SUMMARY OF *TOXOCARA* ELISA EVALUATIVE PARAMETERS

Parameter	Value	n
Sensitivity	87.5%	32
Specificity	97.4%	992
Cross-Reactivity	3.7%	96
Predictive Value		
Positive	97.1%	954
Negative	88.6%	
Precision		
Intra-assay ($\bar{x}$)	7.3%	5000
Inter-assay ($\bar{x}$)	12.7%	

n = size of relevant populations studied.

Fig. 16 Distribution of Toxocara ELISA values in the healthy adult population (n = 922; M:F = 1.01:1). 2.6% have elevated ELISA values.

LISA values in the healthy

M:F = 1.01:1). 2.6% have

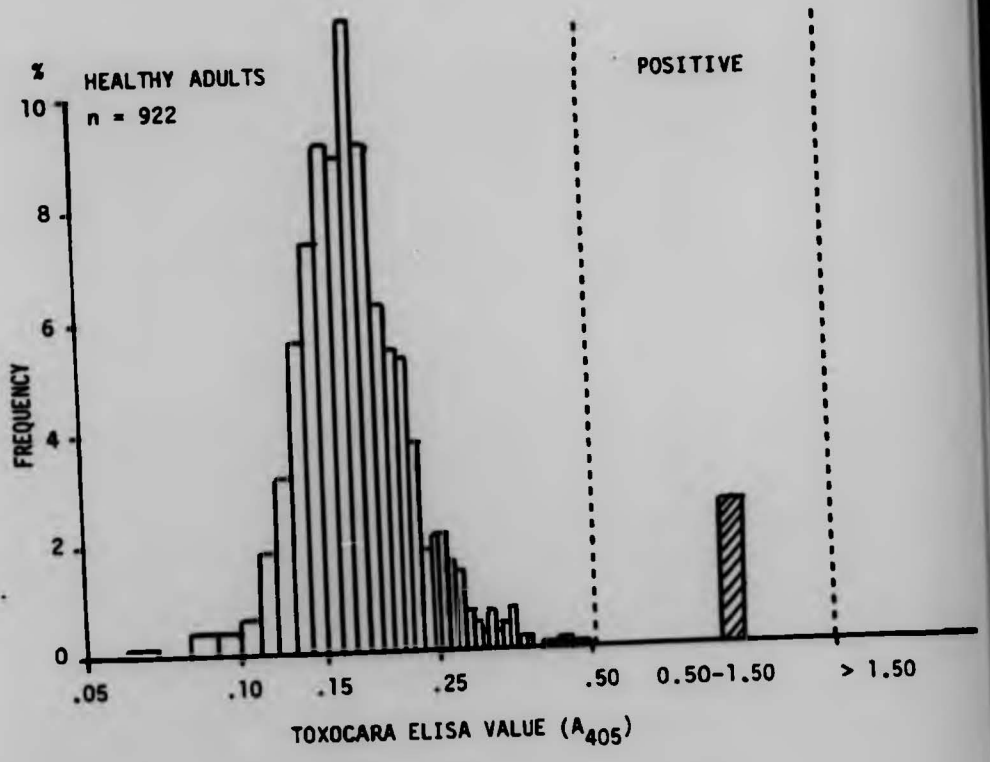

FIGURE 16

Fig. 17 Dose-response curves in Toxocara ELISA.

Serum	A_{405}	Titre 1:
Strong Positive	3.80	550,000
Moderate Positive	1.75	5600
Weak Positive	0.55	1100
Negative Standard	0.15	70

ocara ELISA.

A_{405}	Titre 1:
3.80	550,000
1.75	5600
0.55	1100
0.15	70

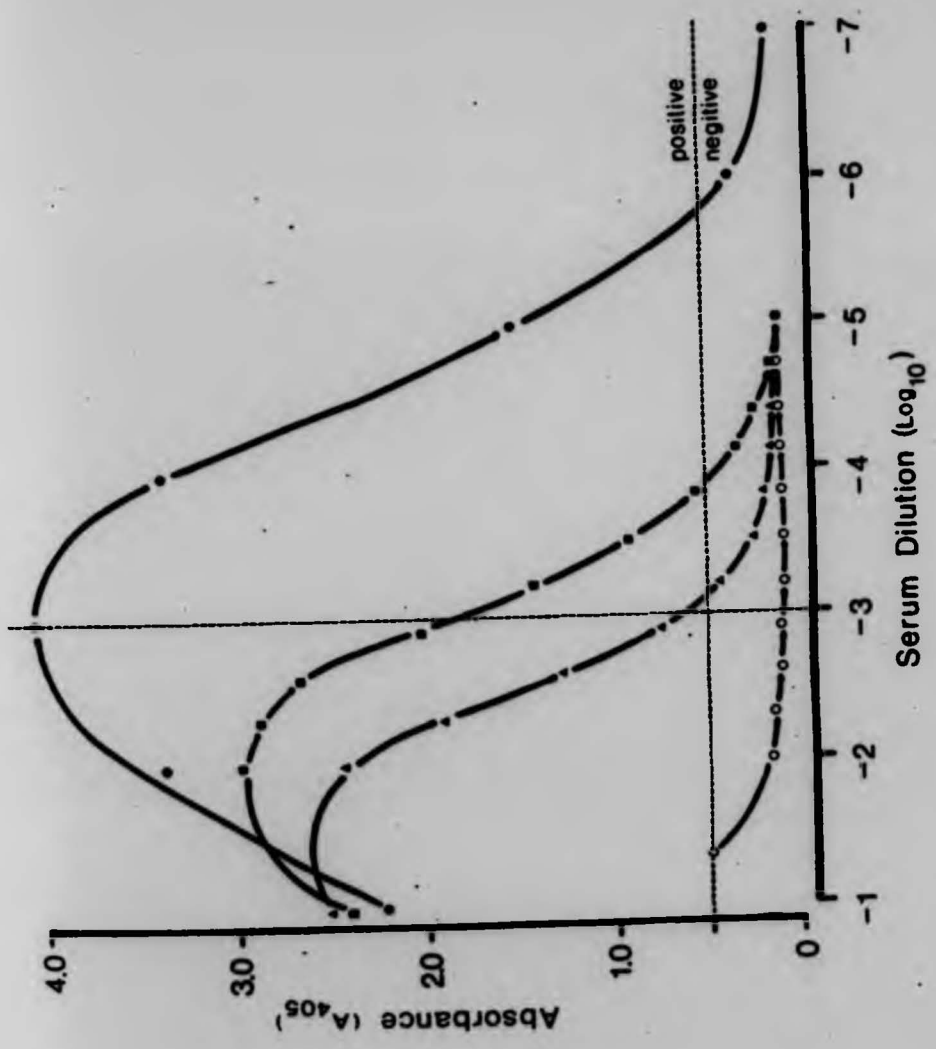

FIGURE 17

Fig. 18 Anti-*Toxocara* ELISA values in patients with suspected clinical visceral toxocariasis (n = 32); healthy adult subjects (n = 992); and Europeans with parasitologically diagnosed, serologically reactive, non-toxocaral helminthiasis (n = 96).

FIGURE 18

n patients with suspected clinical
2); healthy adult subjects (n = 992);
gically diagnosed, serologically
nthiasis (n = 96).

from coefficients of variation (CV %) of 4.2% to 11.7% with a mean of 7.3%. Inter-assay precision was 12.7 CV %. Although numerical ELISA values of highly reactive sera appeared to display more arithmetic variation than those of negative sera, there was no statistically significant correlation between percent coefficient of variation and reactivity of sera (Kendall's rank correlation, $P>0.10$). Sera sampling (pipetting) errors and intra-plate variation of antigen adsorption were considered the most likely sources of within-assay variability. Intra-plate variability of Toxocara ELISA results was therefore compared in various polyvinyl and polystyrene ELISA formats. Cooke 1-220-29 polyvinyl chloride plates had the highest reactivity, lowest range of variation between highest and lowest readings ($\pm$ 11%) and best CV % (5.6%). Other brands of polyvinyl and polystyrene showed severe, non-random, intraplate variability (Fig. 21) and inferior antigen adsorption.

Since it was planned to use comparisons of frequency distributions of healthy populations as a means to detect subtle differences between populations with respect to period prevalence of infection (Chapter V) it was necessary to establish whether individual ELISA values observed in negative subjects (ie. A_{405} distributed log normally from 0.05 to 0.50) are spurious, or characteristic of the subject. Serum specimens from 35 subjects were collected serially over a period of four months and assayed in a blind protocol. Results (Fig. 22) show that, within negative range, Toxocara ELISA values are reproducibly characteristic of the individual, at least within a four months time-frame. Rate of change of positive ELISA values in patients with toxocariasis is discussed in Chapter IV.

Antigen Inhibition of Toxocara ELISA. As a further assessment of specificity of Toxocara ELISA, negative, low, medium, and high-titred

anti-Toxocara sera were diluted to 10^{-3} and pre-incubated with various doses of Toxocara secretory antigen for two hours at room temperature (ie. homologous inhibition). Absorbed sera were then tested in Toxocara ELISA. Results (Fig. 23) indicated that less than 2 ng (< 50 picomoles) of secretory antigen was sufficient to cause 50% inhibition (ID_{50}) of the ELISA response of all but the highest titred sera.

The response of even the highest titred sera could be inhibited to the level of the negative serum.

Non-specific (heterologous) inhibition of Toxocara ELISA was attemped using bovine serum albumin, Ascaris suum somatic antigen, and Necator americanus adult secretory at doses ranging from 5 picograms of antigen. All failed to induce significant (ID_{50}) inhibition. Toxocara somatic antigen inhibition approached ID_{50} at a level of 5 µg, suggesting the possible presence of 0.2% secretory antigen in somatic preparations (calculated from log distance between inhibition curves (Fig. 24).

Comparison of Toxocara ELISA and IRMA

Conjugate Performance. The dose-response curves of phosphatase, peroxidase, and isotope-labelled anti-human IgG conjugates in the standard immunoassay test system using a positive serum are shown in Fig. 25. The 125-iodine conjugate clearly displays the most linear dose-response relationship, while both enzyme-labelled conjugates appear to approach a maximum limiting response value despite increasing dose. However to consider the relative influences of "background" non-specific binding of labelled ligand,and the relative ability of the assays to distinguish positive and negative sera in practice, the positive:negative ratios (P/N) were calculated at each dose of conjugate and plotted in Fig. 26. Enzyme conjugates show an optimal

Fig. 19 Comparison of enzyme-substrate reaction kinetics of phosphatase and peroxidase conjugates in Toxocara ELISA.

(a) Alkaline phosphatase / PNPP

(b) Peroxidase / OPD

● High titred serum

▲ Low titred serum

○ Negative serum

ate reaction kinetics of

conjugates in Toxocara ELISA.

/ PNPP

FIGURE 19

Fig. 20 Intra-assay precision of Toxocara ELISA values at different levels of serological reactivity. Each of six sera were tested 16 times in one assay.

The calculated linear regression line is y = 0.11 X -0.005
Correlation coefficient r = 0.990 (P <0.001)
Coefficient of variation = (slope of regression line X 100) = 11.1%.

54

Toxocara ELISA values at
cal reactivity. Each
times in one assay.

sion line is y = 0.11 X -0.005
0.990 (P <0.001)
slope of regression

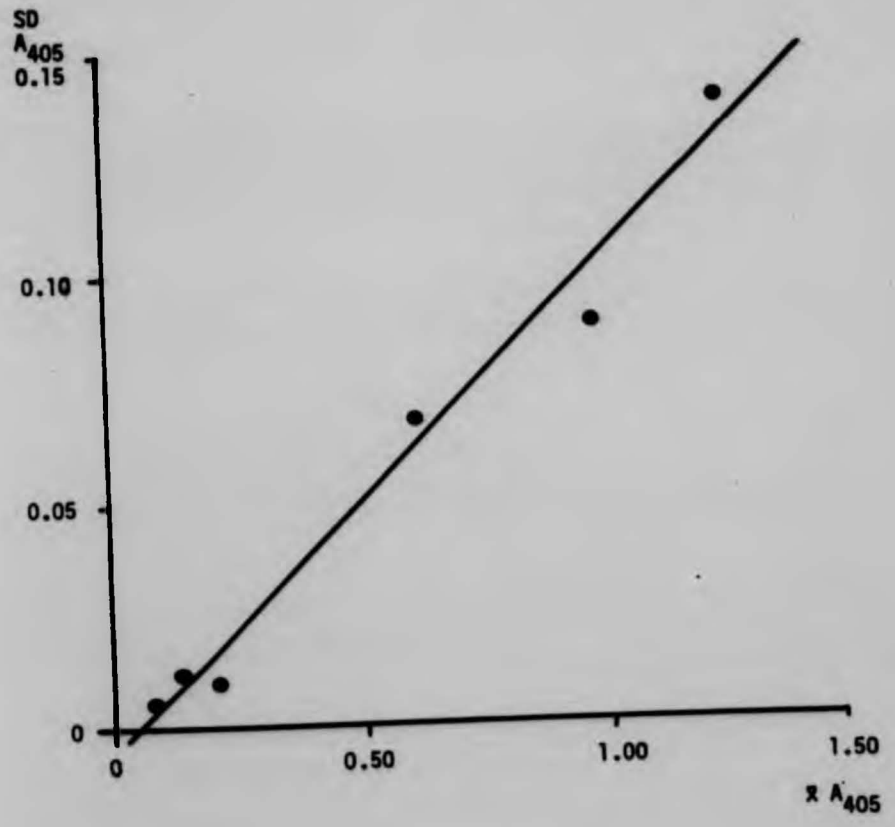

FIGURE 20

ra ELISA values at
reactivity. Each
s in one assay.

n line is y = 0.11 X -0.005
990 (P <0.001)
pe of regression

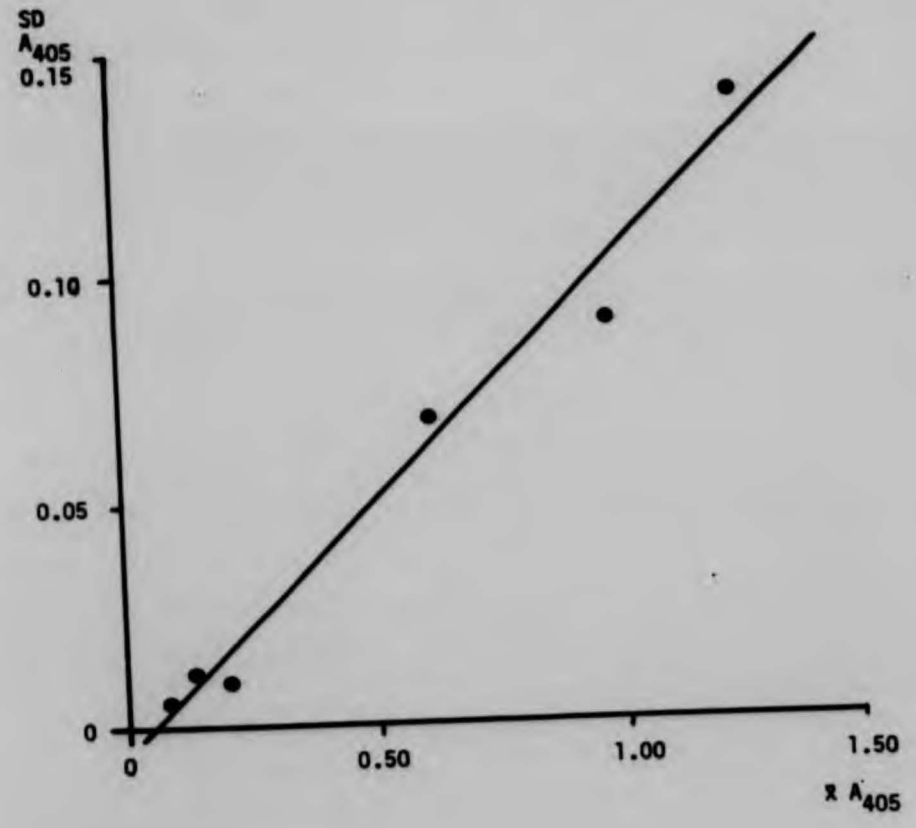

FIGURE 20

Fig. 21 Comparison of Toxocara ELISA variability using different solid phase carriers for antigen. Each horizontal line indicates the mean ELISA value (± 95% confidence interval) of 96 replicate assays of a weakly positive serum tested within one plate. Plotted data indicate the mean ELISA values (+ SD) of individual rows (n = 12 wells) and columns (n = 8 wells) within each plate. Oscillation of plotted data around the horizontal line indicates the presence or absence of non-random variability (tested by Kendall's Rank Correlation).

● Polyvinyl (Cooke 1-220-29)

Variability:	Non-Random	$\tau = 0.76$ ($P < 0.001$)
	Range	± 11% of $\bar{x}$
	Coefficient	CV = 5.6%

▲ Polyvinyl (Greiner Irradiated Prototype Plate)

Variability:	Random	$\tau = 0.24$ ($P > 0.10$)
	Range	± 16% of $\bar{x}$
	Coefficient	CV = 6.2%

■ Polyvinyl (Greiner Non-Irradiated Prototype Plate)

Variability:	Non-Range	$\tau = -0.91$ ($P < 0.001$)
	Range	± 30% of $\bar{x}$
	Coefficient	CV = 21.9%

○ Polystyrene (Dynatech Removawells)

Variability:	Random	$\tau = -0.15$ ($P > 0.50$)
	Range	± 30% of $\bar{x}$
	Coefficient	CV = 12.4%

△ Polystyrene (Dynatech 129-A)

Variability:	Non-Random	$\tau = +0.64$ ($P < 0.05$)
	Range	± 27% of $\bar{x}$
	Coefficient	CV = 22.8%

riability using different
n. Each horizontal line
(± 95% confidence interval)
kly positive serum tested
indicate the mean ELISA
s (n = 12 wells) and columns
. Oscillation of plotted
indicates the presence
ility (tested by Kendall's

m — τ = 0.76 (P <0.001)
± 11% of $\bar{x}$
nt — CV = 5.6%

ted Prototype Plate)
τ = 0.24 (P >0.10)
± 16% of $\bar{x}$
nt — CV = 6.2%

adiated Prototype Plate)
τ = -0.91 (P <0.001)
± 30% of $\bar{x}$
nt — CV = 21.9%

ovawells)
τ = -0.15 (P >0.50)
± 30% of $\bar{x}$
nt — CV = 12.4%

-A)
om — τ = +0.64 (P <0.05)
± 27% of $\bar{x}$
nt — CV = 22.8%

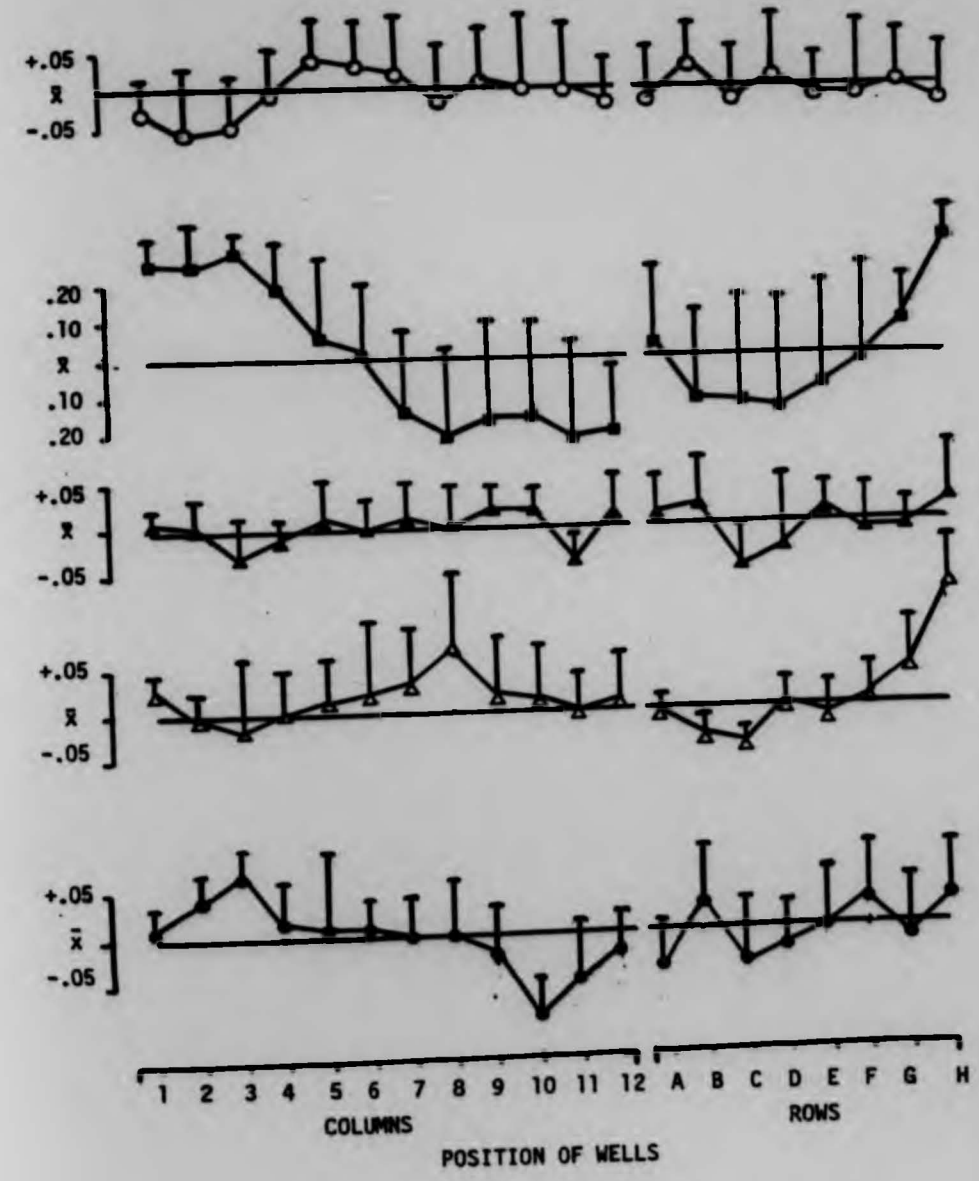

FIGURE 21

Fig. 22 Reproducibility of Toxocara ELISA quantitative results in 35 serologically negative subjects sampled twice with a four month interval.

ELISA quantitative results in
ubjects sampled twice with a four

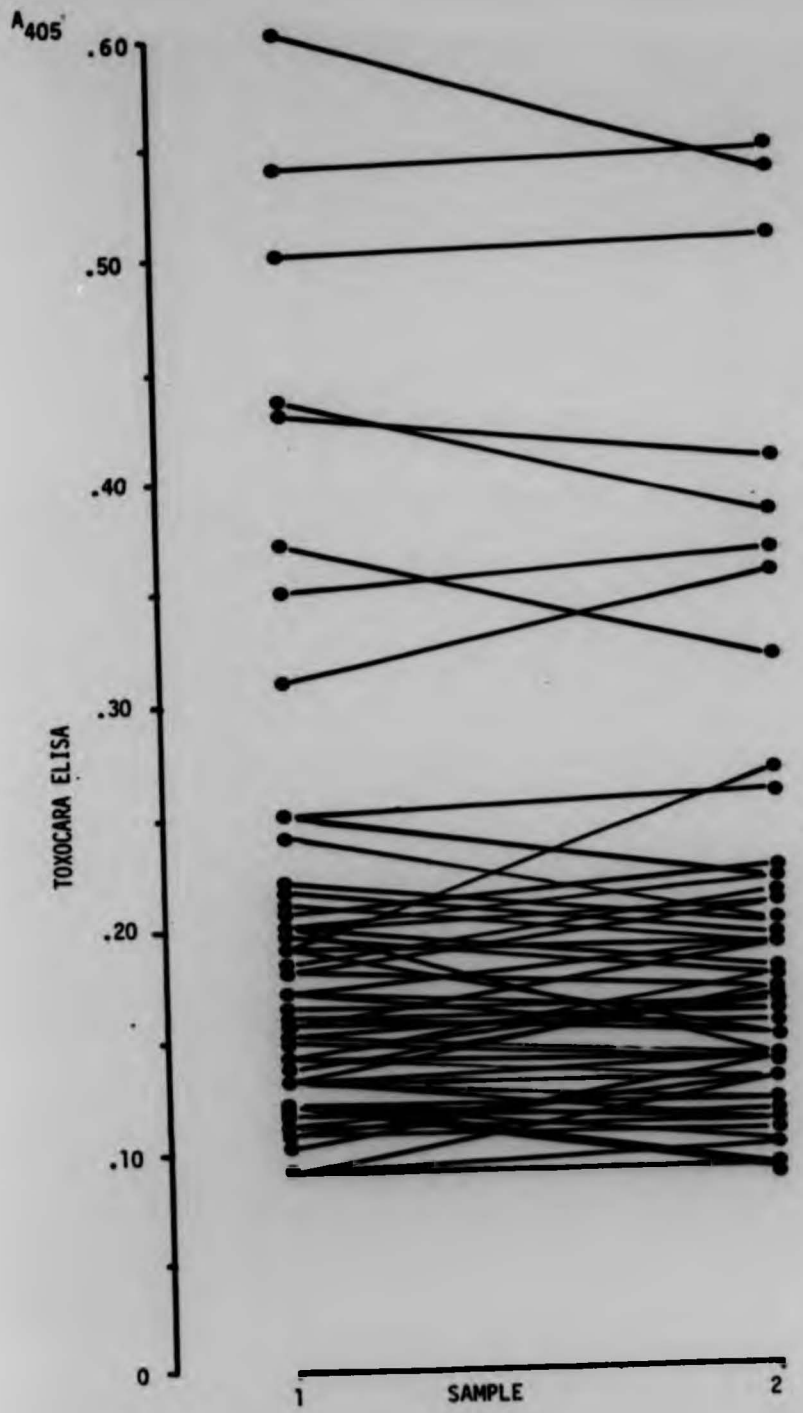

FIGURE 22

Fig. 23 Homologous antigen inhibition of Toxocara ELISA. Six positive sera and one negative serum were preincubated with varying doses of homologous antigen before assay in Toxocara ELISA.

— ID_{50}

● Toxocara canis secretory antigen

Fig. 24 Heterologous antigen inhibition of Toxocara ELISA. Aliquots of one positive serum were preincubated with varying doses of heterologous and homologous antigen.

— ID_{50}

● Toxocara canis secretory antigen

▲ Toxocara canis somatic antigen

✕ Necator americanus secretory antigen

■ Ascaris suum somatic antigen

○ Bovine serum albumin

n of Toxocara ELISA.
gative serum were preincubated
gous antigen before assay in

tory antigen

ion of Toxocara ELISA.
rum were preincubated with
us and homologous antigen.

tory antigen
ic antigen
ecretory antigen
antigen

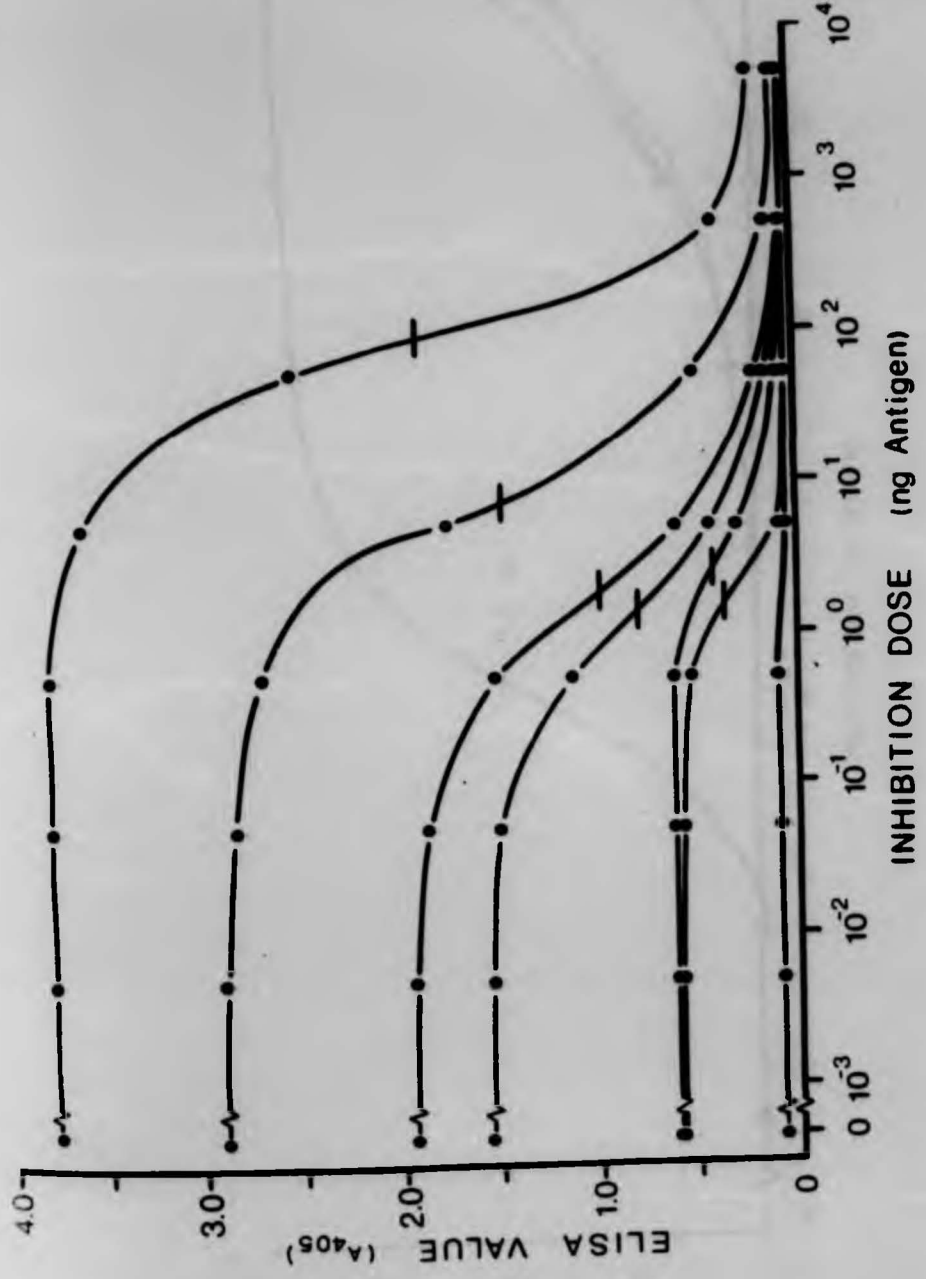

FIGURE 23

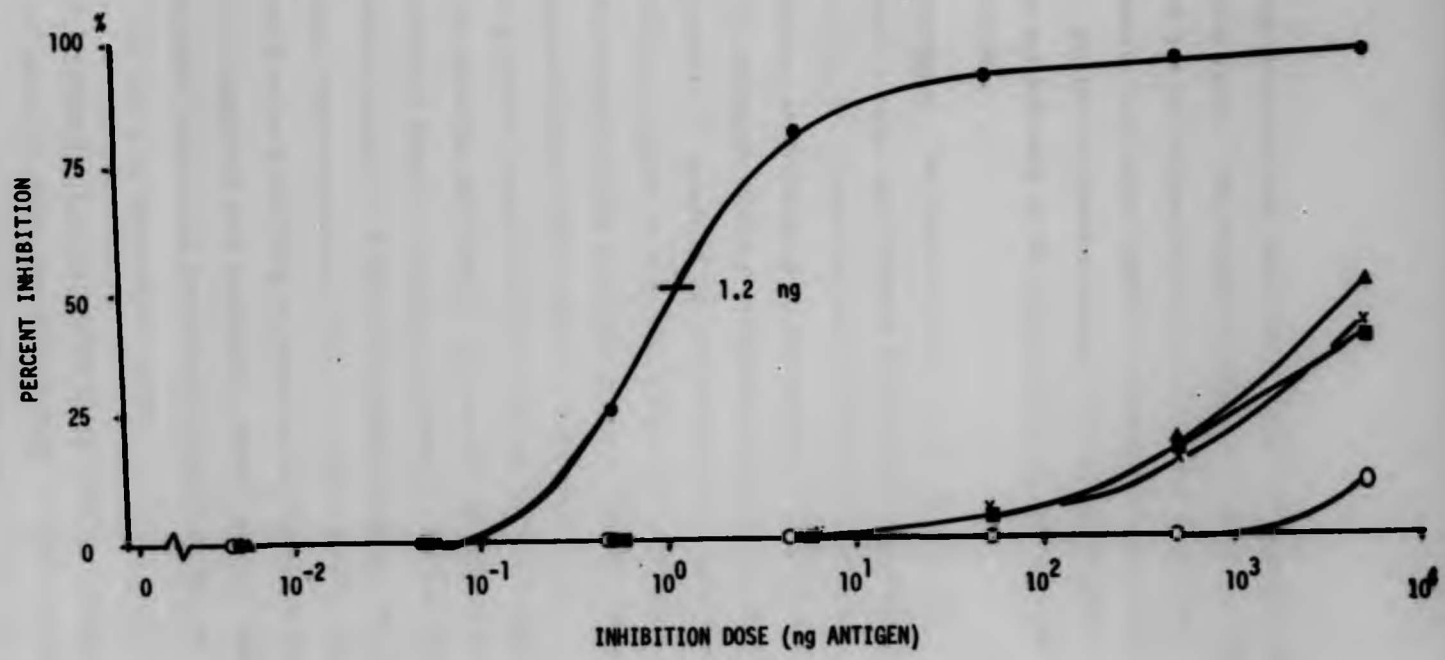
100 %
75
50
25
0
PERCENT INHIBITION
1.2 ng
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
INHIBITION DOSE (ng ANTIGEN)

FIGURE 24

labelled antibody concentration, above which there is a decrease in positive:negative ratio. The isotope conjugate, in contrast, shows no optimal value and can be used across a broad range of working dilutions. However, both enzyme-labelled conjugates produced higher P/N ratios (ie. greater distinction between positive and negative reactivity than was achieved by the isotope-labelled reagent under identical conditions.

Assay Performance. The dose-response of high, medium, and low-titred patient's sera, when titrated in the ELISA and IRMA systems, are compared in Fig. 27. Concerning both the kinetics and titre end-points there are no differences in dose-response although the isotope-labelled conjugate displays considerably higher non-specific binding. The results of assays of 90 sera tested at a single serum dilution of 10^{-3} are compared in Fig. 28. Since it is difficult to compare data expressed as ELISA absorbance values with data expressed as IRMA disintegrations per minute, data for both assays were transformed to positive : negative ratios in order to assess more objectively, the relative abilities of these assays to quantitatively distinguish different levels of antibody reactivity. The correlation coefficient between assays ($r = 0.93$) is reasonable, however the slope of the calculated linear regression line ($y = .501x + 2.439$) indicates that ELISA should be more sensitive in measuring strong reactivity but less sensitive in measuring weak reactivity. Both ELISA and IRMA exhibited comparable intra-assay precision with coefficients of variation of 4.4% and 5.8% respectively (n=10).

Comparison of Toxocara ELISA using Secretory and Somatic Antigens

Results of parallel assays for anti-Toxocara antibody to secretory antigens and somatic antigens in 137 sera are shown in Fig. 29. In

Fig. 25 Comparison of phosphatase, peroxidase and ^{125}-Iodine labelled anti-human IgG conjugates.

Fig. 26 Determination of optimum concentration of conjugate antibody when labelled with phosphatase, peroxidase, or ^{125}-Iodine.

Fig. 27 Serum dose-response curves for high, medium, and low titred sera when titrated in ELISA and IRMA.

Fig. 28 Comparison of ELISA and IRMA results from assays for anti-Toxocara antibody in the sera of 90 subjects and patients.

$n = 90$

$r = 0.93$

$y = 0.50 x + 2.35$

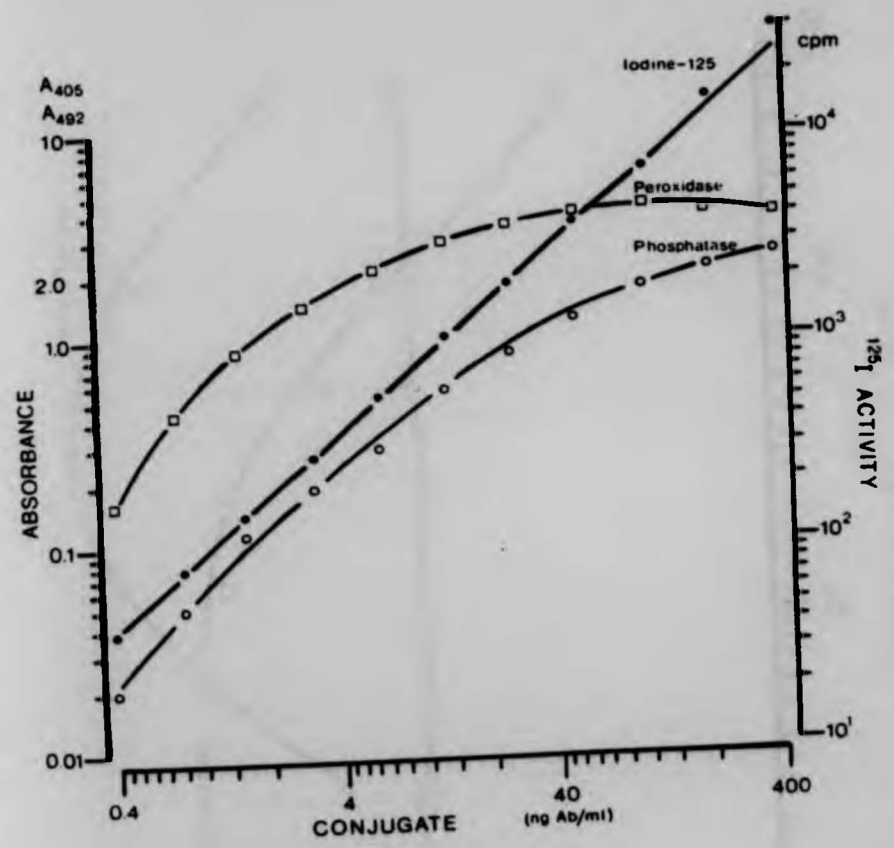
A405
A492
ABSORBANCE
10
2.0
1.0
0.1
0.01
cpm
Iodine-125
Peroxidase
Phosphatase
10^4
10^3
10^2
10^1
125I ACTIVITY
0.4
4
40
400
CONJUGATE
(ng Ab/ml)

FIGURE 25

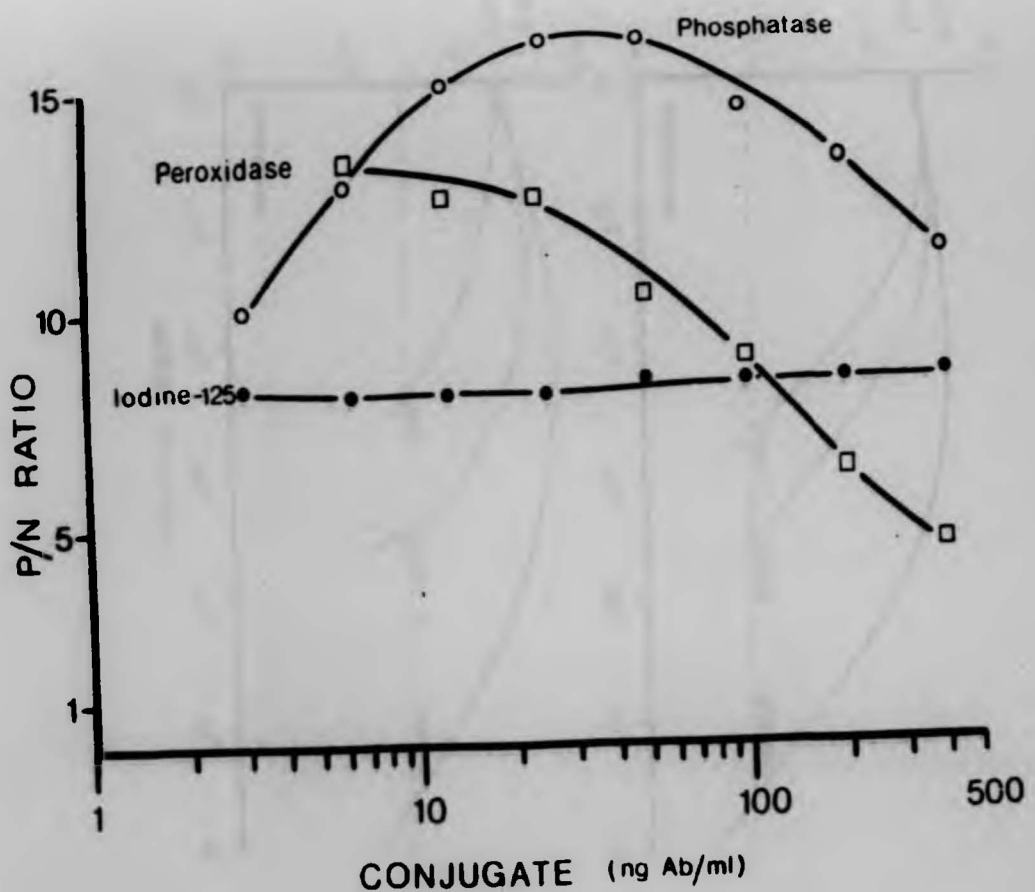
Phosphatase
Peroxidase
Iodine-125
15
10
5
1
P/N RATIO
1
10
100
500
CONJUGATE (ng Ab/ml)

FIGURE 26

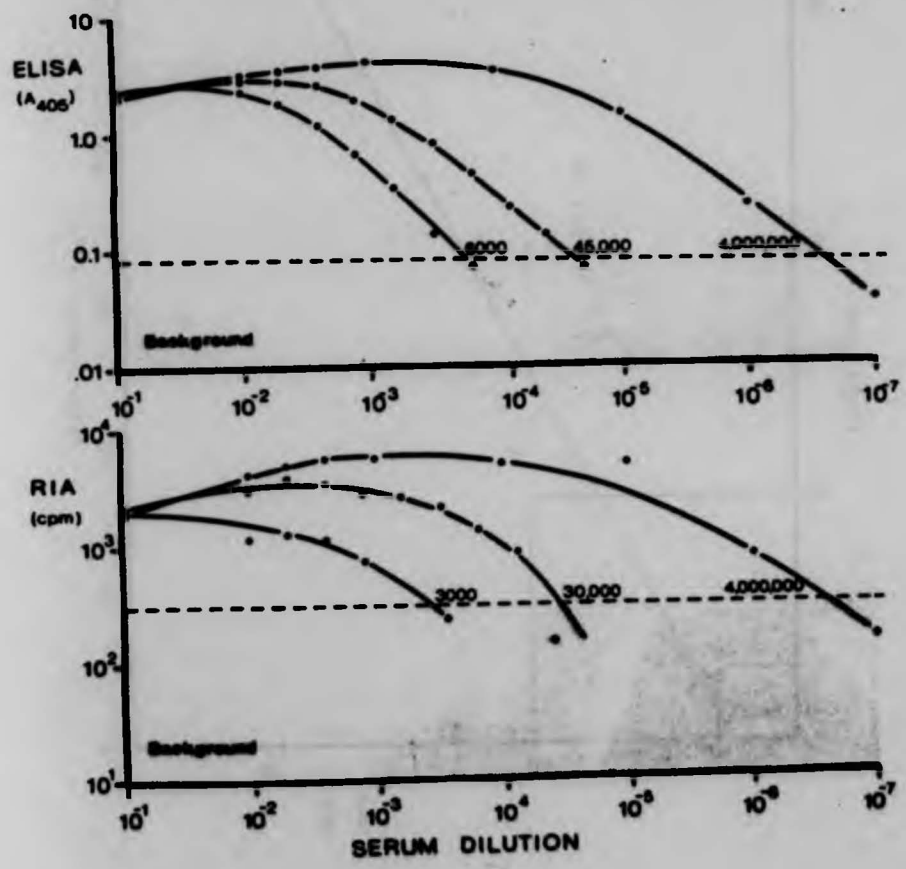
ELISA
(A_{405})
10
1.0
0.1
.01
6000
45,000
4,000,000
Background
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
RIA
(cpm)
10^4
10^3
10^2
10^1
3000
30,000
4,000,000
Background
SERUM DILUTION

FIGURE 27

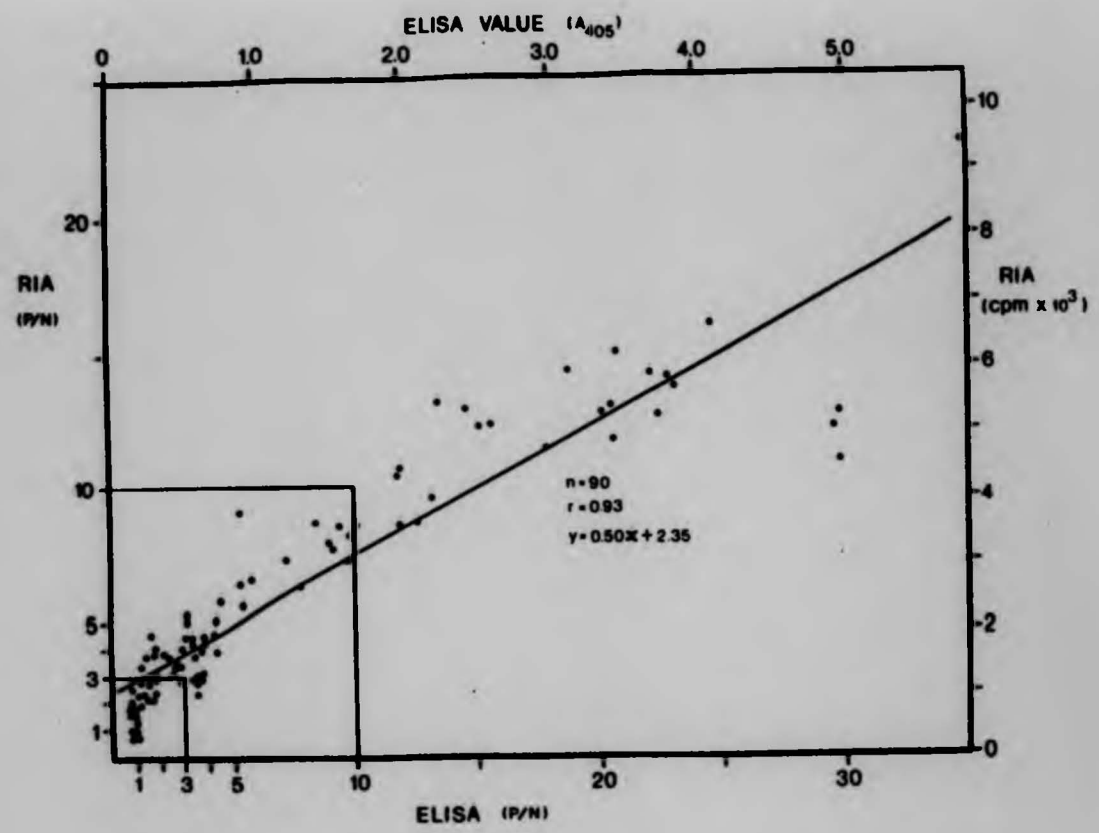
ELISA VALUE (A_{405})
0
1.0
2.0
3.0
4.0
5.0
RIA
(P/N)
20
10
5
3
1
RIA
(cpm x 10^3)
10
8
6
4
2
0
n=90
r=0.93
y=0.50x+2.35
1
3
5
10
20
30
ELISA (P/N)

FIGURE 28

Fig. 29 Comparison of Toxocara ELISA results using secretory antigen and somatic antigen.

	Legend	Secretory	Somatic
●	Healthy Subjects n = 66 (Specificity)	98.5% (65/66)	95.5% (63/66)
O	Toxocariasis Patients n = 16 (Sensitivity)	100% (16/16)	93.8% (15/16).
X	Non-toxocaral Helminthiasis Patients n = 55 (Cross-reactivity)	7.3% (4/55)	30.9% (17/55).
CV %	Coefficient of Variation (Precision)	9.4%	5.6%

The calculated linear regression line for all data (n = 137) is plotted ($y = 0.491x + 0.306$).
Correlation coefficient $r = 0.30$ ($P < 0.001$).

Fig. 29 Comparison of Toxocara ELISA results using secretory antigen and somatic antigen.

	Legend	Secretory	Somatic
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esults using secretory

	Secretory	Somatic
	98.5% (65/66)	95.5% (63/66)
	100% (16/16)	93.8% (15/16).
	7.3% (4/55)	30.9% (17/55).
	9.4%	5.6%

on line for all data (n = 137)
).
30 (P< 0.001).

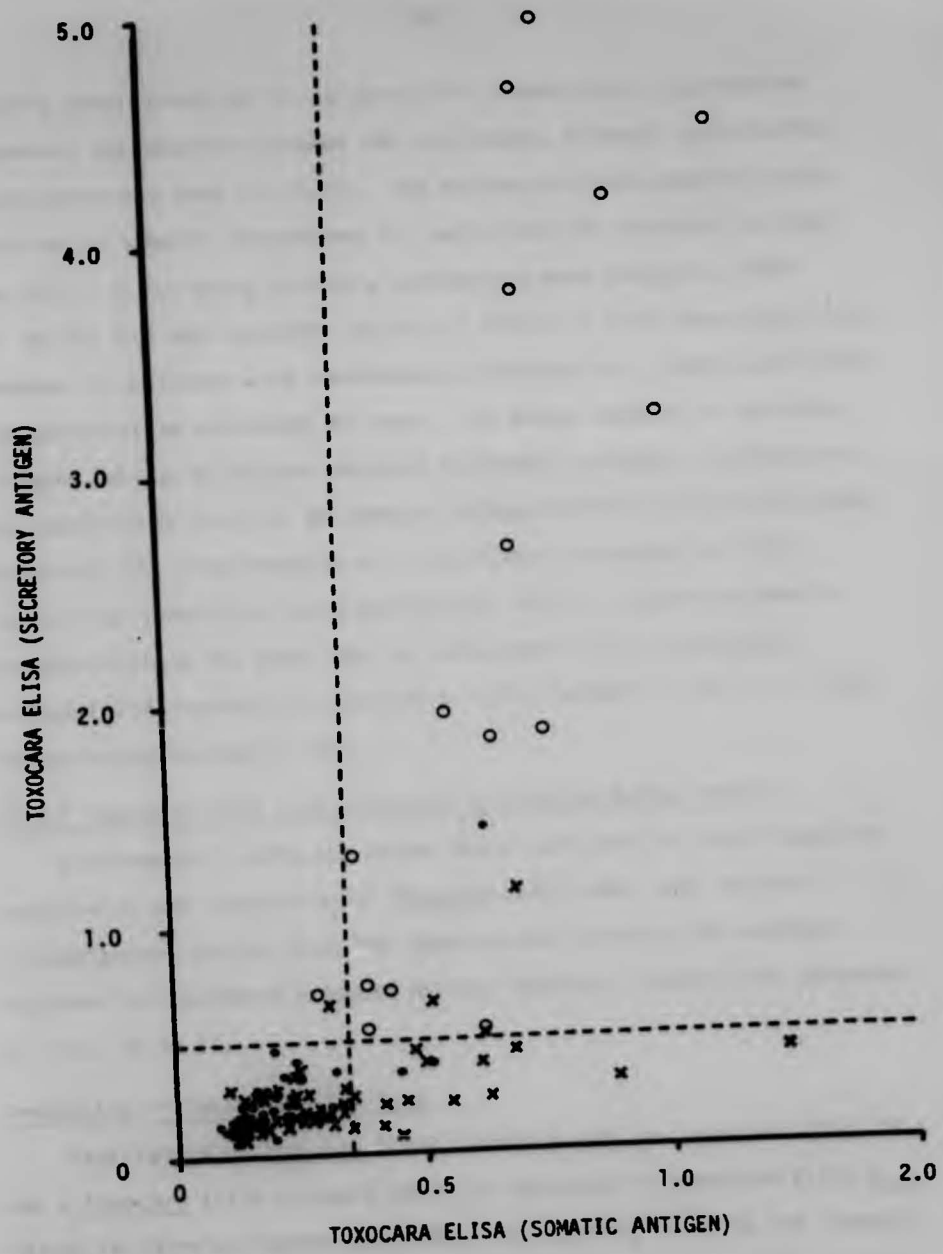

FIGURE 29

healthy populations and in patients with toxocariasis, qualitative agreement was observed between the two assays, although quantitative correlation was poor (r= 0.30). The maximum positive:negative ratio achieved by somatic antigen was 9:1 while that for secretory antigen was 33:1. ELISA using secretory antigen was more sensitive (100% cf. 93.8%) and more specific (98.3% cf. 95.0%) in both these populations. However, in patients with non-toxocaral helminthiasis, both qualitative and quantitative agreement was poor, 7.3% being reactive to secretory antigens, while 30.9% were reactive to somatic antigens. By adjusting the specificity level of the Somatic Antigen-ELISA to that of Secretory Antigen-ELISA, cross-reactivity of the former decreased to 16.4%, however its sensitivity also declined (to 68.8%). Adjusting Somatic Antigen-ELISA to the same level of cross-reactivity as Secretory Antigen-ELISA lowered its sensitivity still further (to 56.3% cf. 100% for Secretory Antigen-ELISA).

Use of Toxocara ELISA in Experimentally Infected Animal Models

Experimentally infected animal hosts were used to investigate the sensitivity and specificity of Toxocara ELISA under more defined circumstances, and to study the dynamics and character of serologic responses to secretory products during infection. Results are presented in Figs. 30 to 34.

Processing of Toxocara ELISA Data

Parallelism of Toxocara ELISA titration data was assessed (Fig. 35) and a Toxocara ELISA standard curve was prepared to transform ELISA A_{405} values to titre estimates (Fig. 36). The validity of using the standard curve method for sera which may contain IgM anti-Toxocara antibodies was assessed using an IgM anti-Toxocara positive human serum (Fig. 37). The ability of the standard curve method to generate results linearly

proportional to titration results was examined in practice by comparisons with five other methods of handling quantitative data obtained from a panel of toxocariasis sera (Fig. 38). Examples of Toxocara ELISA results in their final form, obtained by the use of the standard curve method and eight other methods, are illustrated in Table 19.

Fig. 30 Anti-*Toxocara* ELISA values in experimentally infected swine.

Legend:

Code	Infection				Weeks Post Primary Infection of ELISA Result
	Mode	Route	Dose	Schedule	
A	Ova	p.o.	5250	150 ova daily for 5 days each week for 7 weeks	7
B	Ova	p.o.	160,000	10,000 ova weekly for 6 weeks, then 100,000 at week 10 post-infection	14
C	Ova	p.o.	1000	single dose	10
D	Ova	p.o.	10,000	single dose	10
E	Ova	p.o.	2000	primary dose of 1000 followed by secondary dose of 1000 at 8 weeks post-infection	11
F	Larvae	i.v.	1000	single dose	2-4

p.o. per oral

i.v. intravenous

experimentally infected swine.

Schedule	Weeks Post Primary Infection of ELISA Result
0 ova daily r 5 days each ek for 7 weeks	7
,000 ova weekly r 6 weeks, then 0,000 at week post-infection	14
ngle dose	10
ngle dose	10
rimary dose of 00 followed by econdary dose of 00 at 8 weeks st-infection	11
ingle dose	2-4

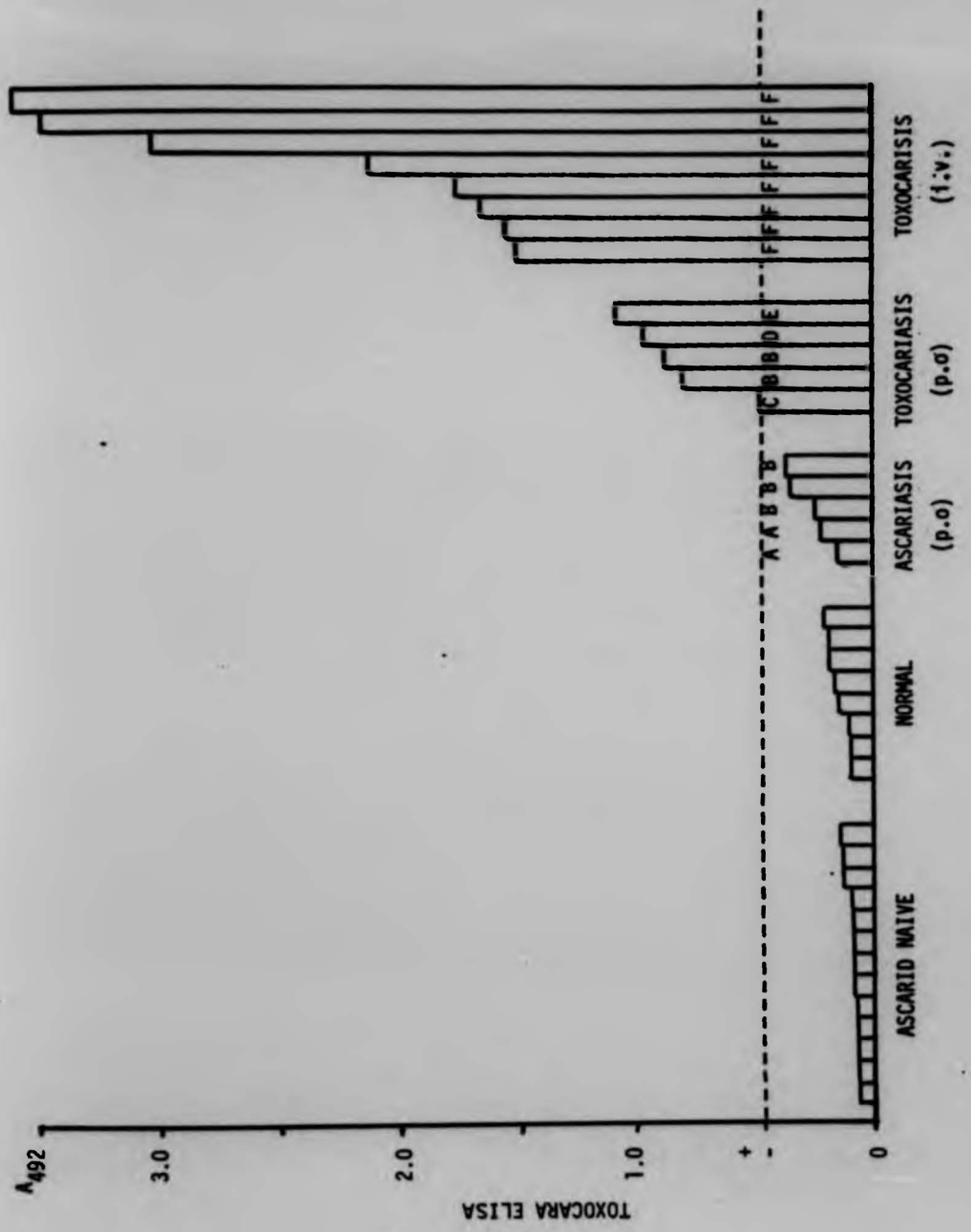

FIGURE 30

Fig. 31 Anti-*Toxocara* ELISA values in experimentally infected rabbits.

- ● *Toxocara canis*. Primary dose/kg of 10,000 ova followed by 1,000 and 10,000 at three week intervals. (↓)
- ▲ *Toxocara canis*. Trickle dose of 10 ova/kg/week for 26 weeks.
- ■ *Toxocara cati*. Single dose of 5,000 ova/kg.
- △ *Toxascaris leonina*. Single dose of 5,000 ova/kg.

experimentally infected

dose/kg of 10,000 ova followed
hree week intervals. (↓)

dose of 10 ova/kg/week

se of 5,000 ova/kg.

gle dose of 5,000 ova/kg.

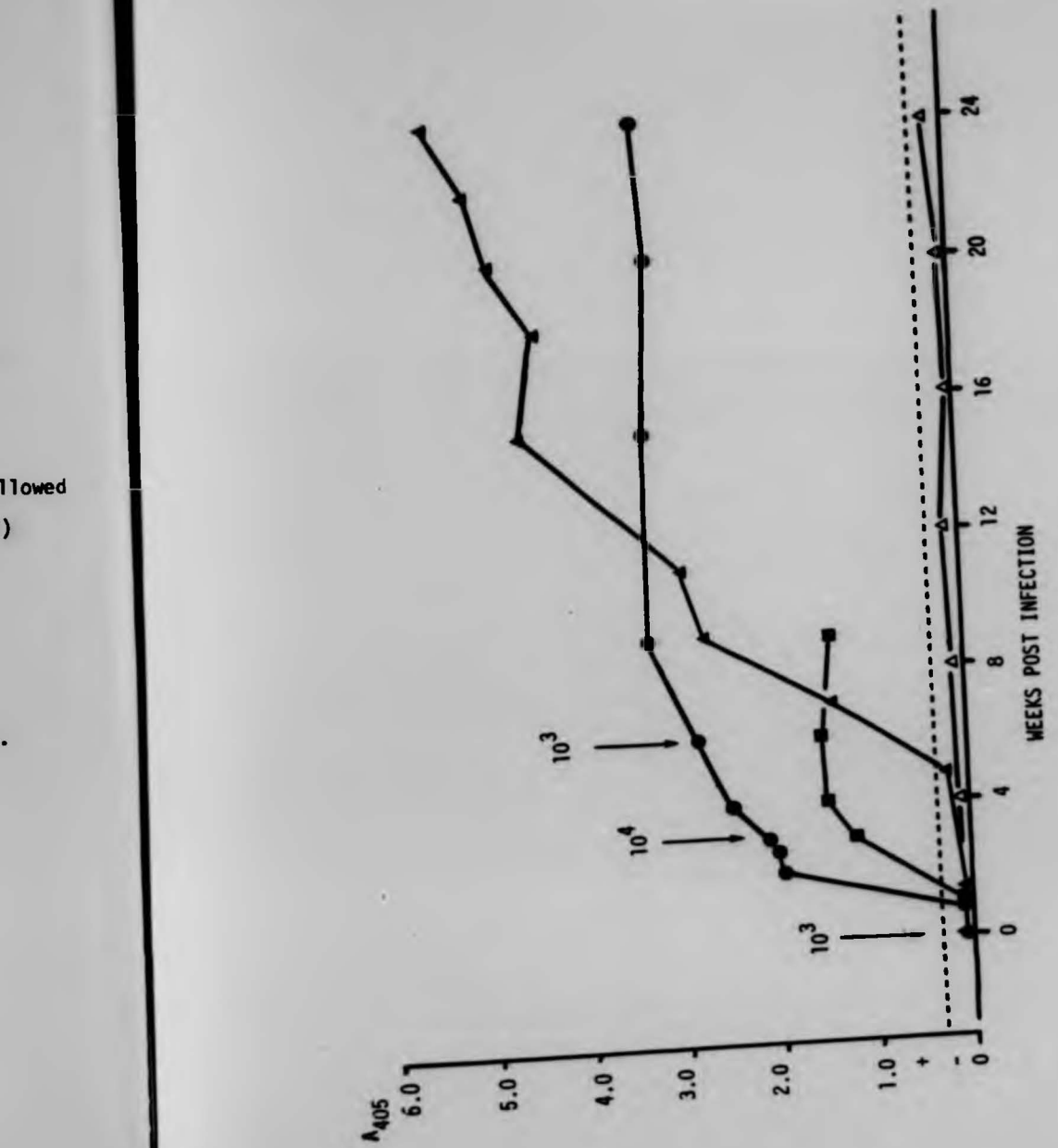

FIGURE 31

In experimentally infected

y dose/kg of 10,000 ova followed
three week intervals. (↓)

le dose of 10 ova/kg/week

dose of 5,000 ova/kg.

ingle dose of 5,000 ova/kg.

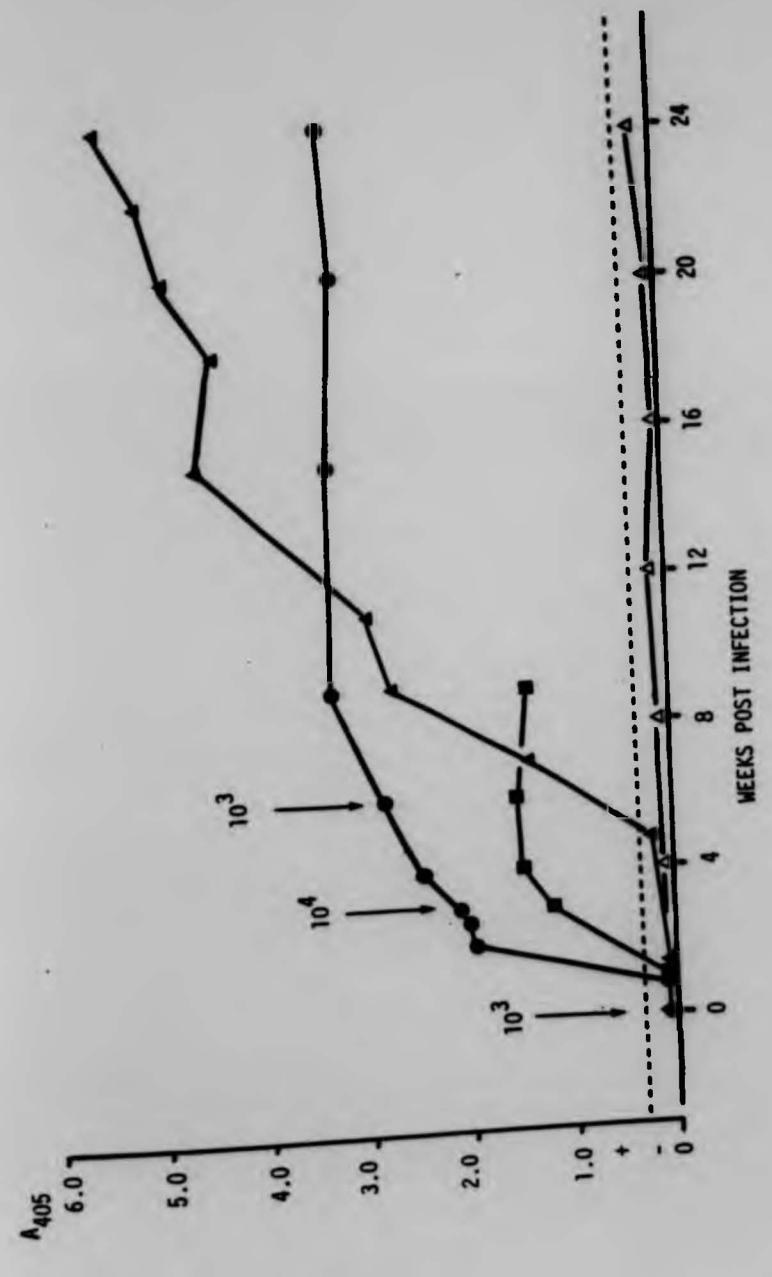

FIGURE 31

Fig. 32 Anti-*Toxocara* antibody in rhesus monkeys experimentally infected with *Toxocara canis*.

● 5,000 ova/kg

▲ 1,000 ova/kg

■ 100 ova/kg

△ 10 ova/kg

○ 0 ova (control)

hesus monkeys experimentally

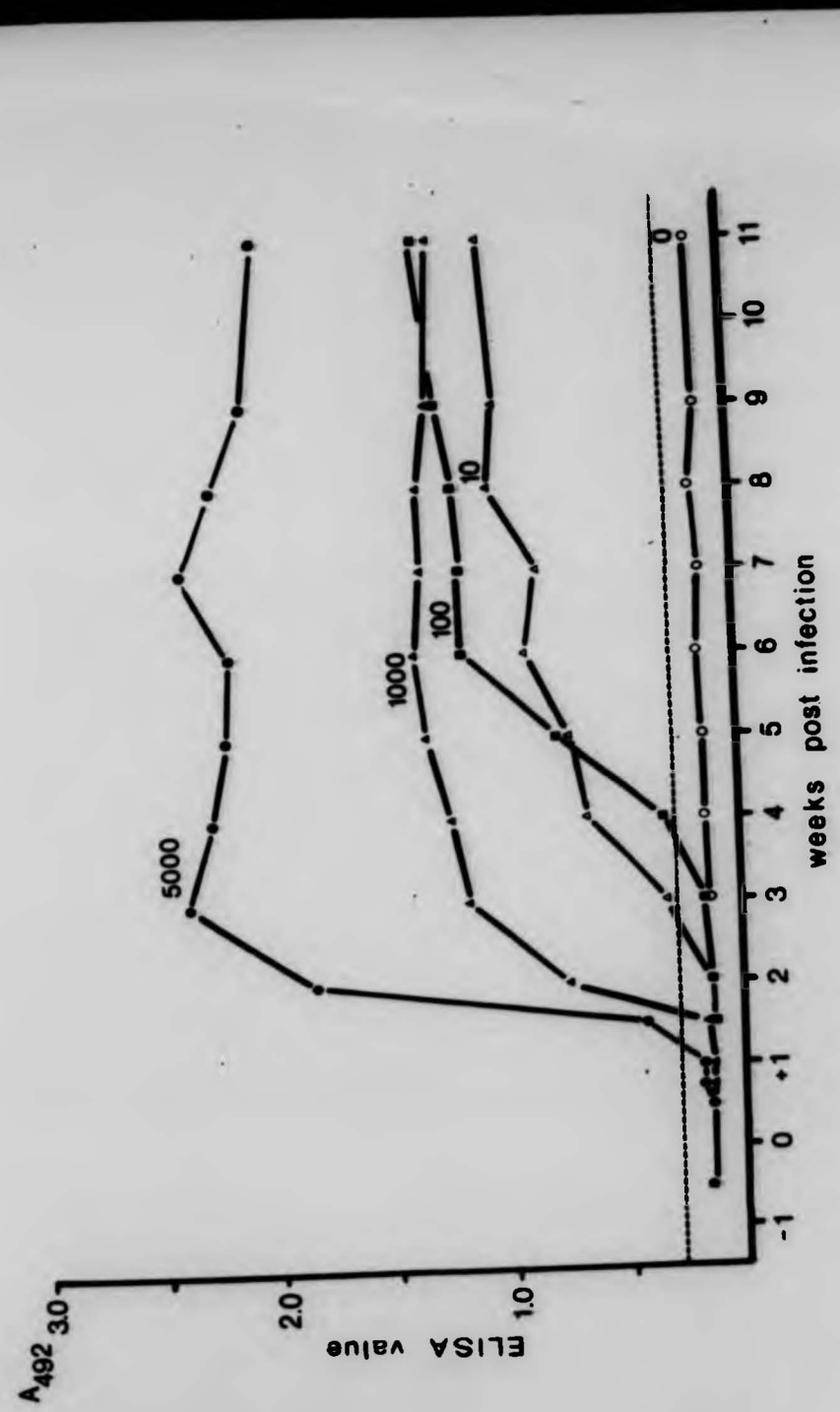

FIGURE 32

Fig. 33 Anti-*Toxocara* IgG responses in rhesus monkeys experimentally infected with *Toxocara canis*.

● 5,000 ova/kg in single dose

▲ 1,000 ova/kg in single dose

○ 5 ova/kg in trickle dose weekly for six months accumulating to 135 ova/kg.

rhesus monkeys experimentally

ose

ose

dose weekly for six months

5 ova/kg.

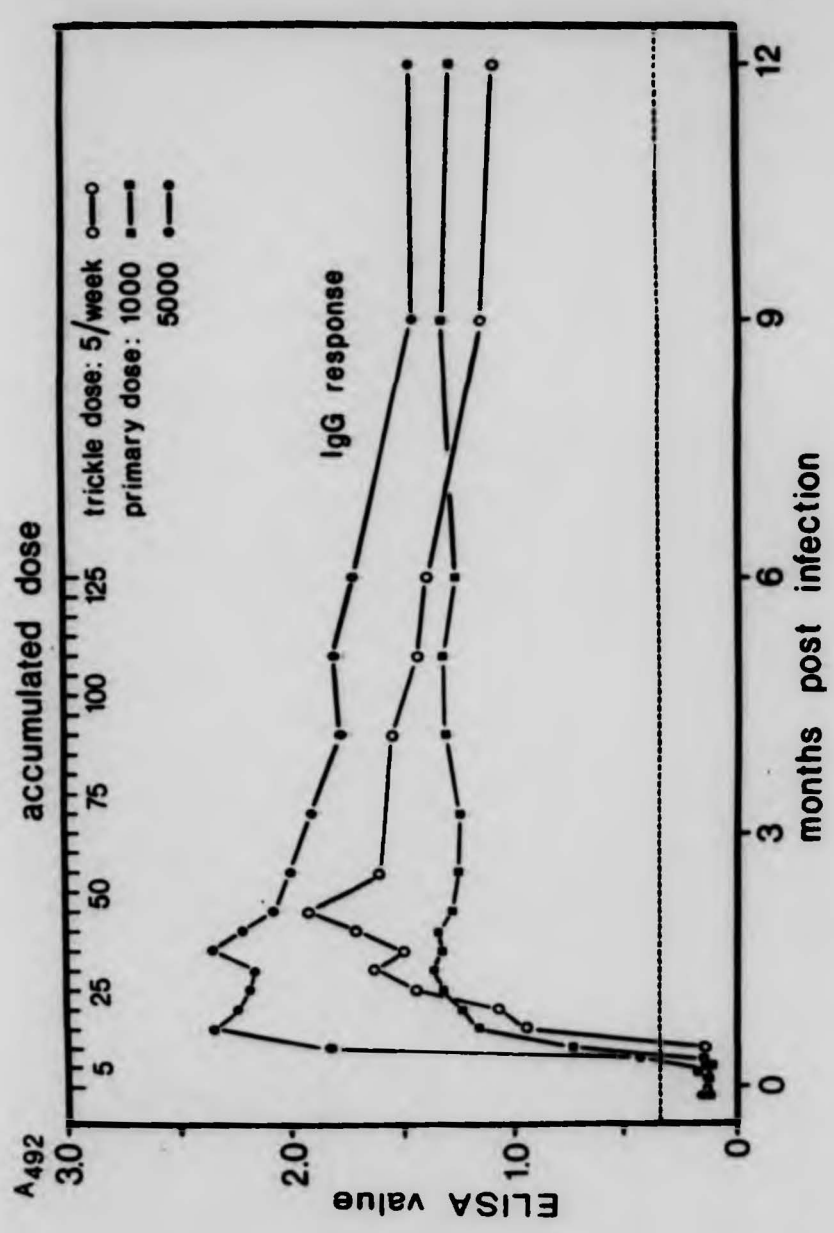

FIGURE 33

Fig. 34 Anti-*Toxocara* IgM responses in rhesus monkeys experimentally infected with *Toxocara canis*.

● 5,000 ova/kg in single dose

▲ 1,000 ova/kg in single dose

○ 5 ova/kg weekly in trickle dose for six months accumulating to 135 ova/kg.

rhesus monkeys experimentally

dose

dose

trickle dose for six months

5 ova/kg.

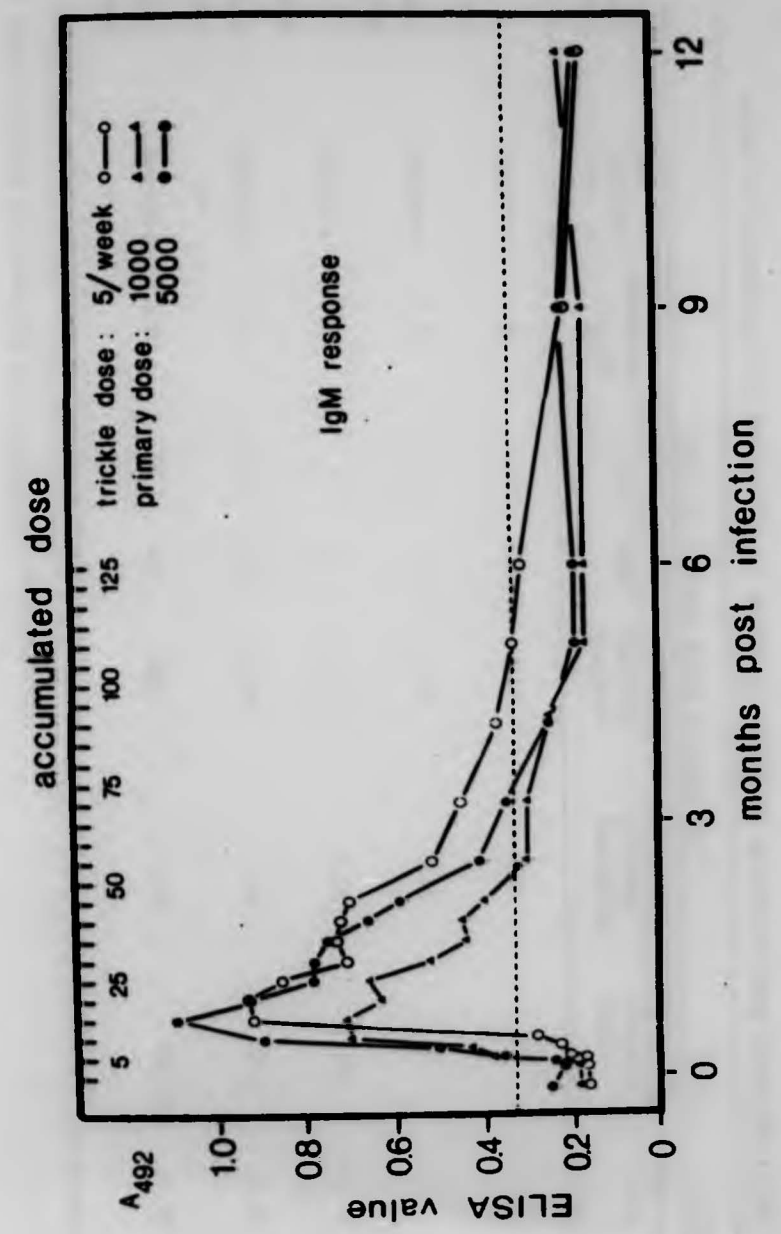

FIGURE 34

TABLE 15

MEANS OF COMMUNICATING ESTIMATES OF RELATIVE ANTIBODY ACTIVITY FROM TOXOCARA ELISA DATA

Relative Specific Antibody Activity	Assay at a Single Serum Dilution (10^{-3})							Titration	
	Semi Quantitative	Absorbance (A_{405}^{nm})	P/N	MONA	Percent of Positive Standard	Percentile of Normal Population	Units from Standard Curve	ED (Log)	Titre 1:
Weak Negative	Negative	0.09	0.6	0.4	4.5%	0.02	35	-0.37	30
Standard Negative	Negative	0.15	1.0	1.0	7.5%	0.46	90	0	70
Weak Positive	Positive +	0.55	3.7	12	27.5%	0.98	1100	+1.19	1100
Moderate Positive	Positive ++	1.75	11.7	107	87.5%	1.00	7010	+1.90	5600
Strong Positive	Positive +++	3.80	25.3	463	190%	1.00	800,000	+3.89	550,000

Taken from ELISA data for human IgG anti-Toxocara antibody activity.

P/N = Positive:Negative Ratio MONA = Multiple of Normal Activity (n = 1.9). ED = Effective Dose

Fig. 35 Parallelism of Toxocara ELISA serum dose-response curves.

Fig. 36 Toxocara ELISA standard curve relating ELISA absorbance values to titre estimates.

Fig. 37 IgG anti-Toxocara antibody dose-response curves for positive (●) and negative (▲) sera and for the same sera (○, △) following the removal of IgM by fractionation on Bio-Gel A-5m micro-chromatography.

ISA serum dose-response

rve relating ELISA absorbance

dose-response curves for
(▲) sera and for the same
removal of IgM by fractionation
natography.

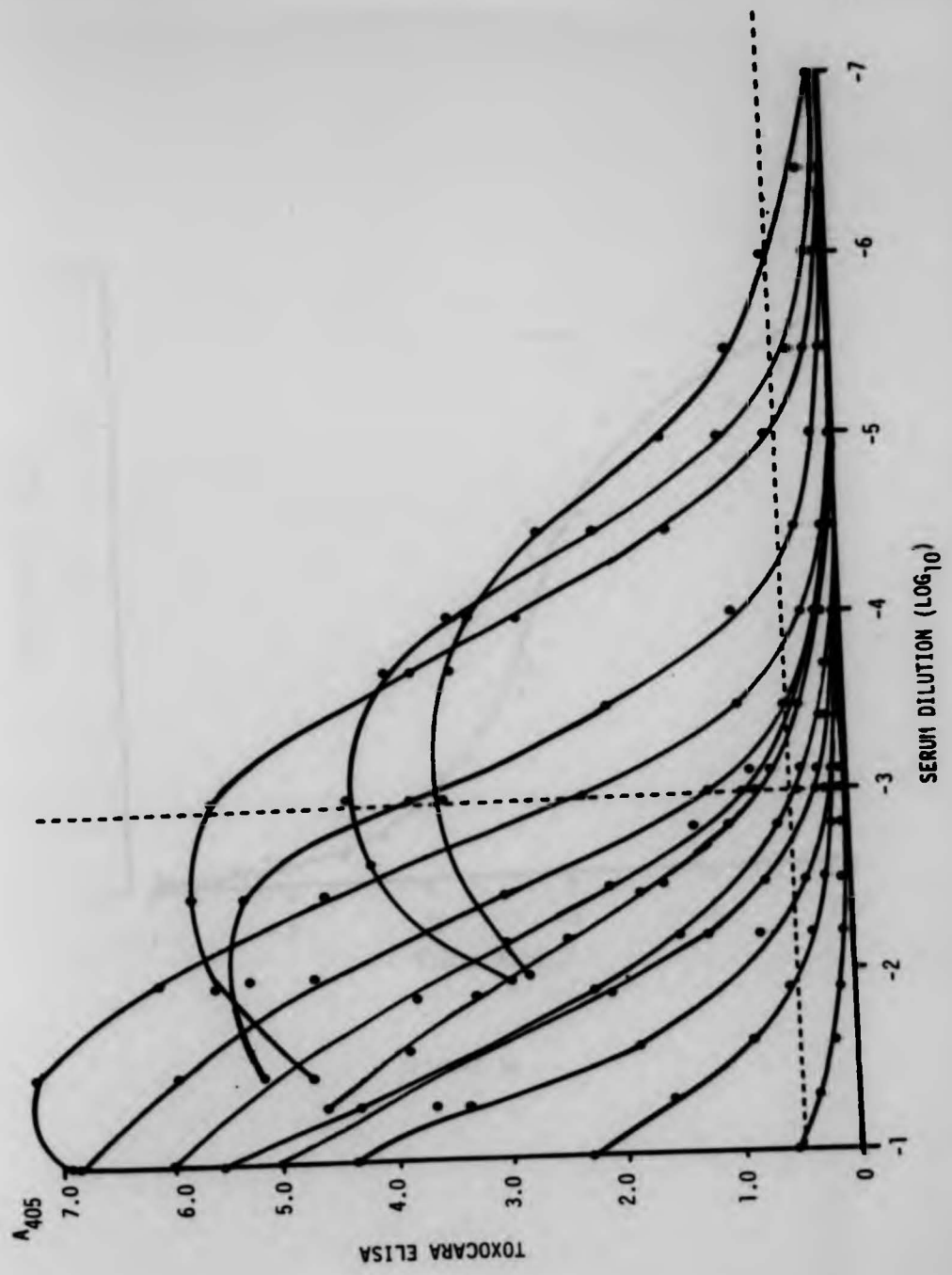

FIGURE 35

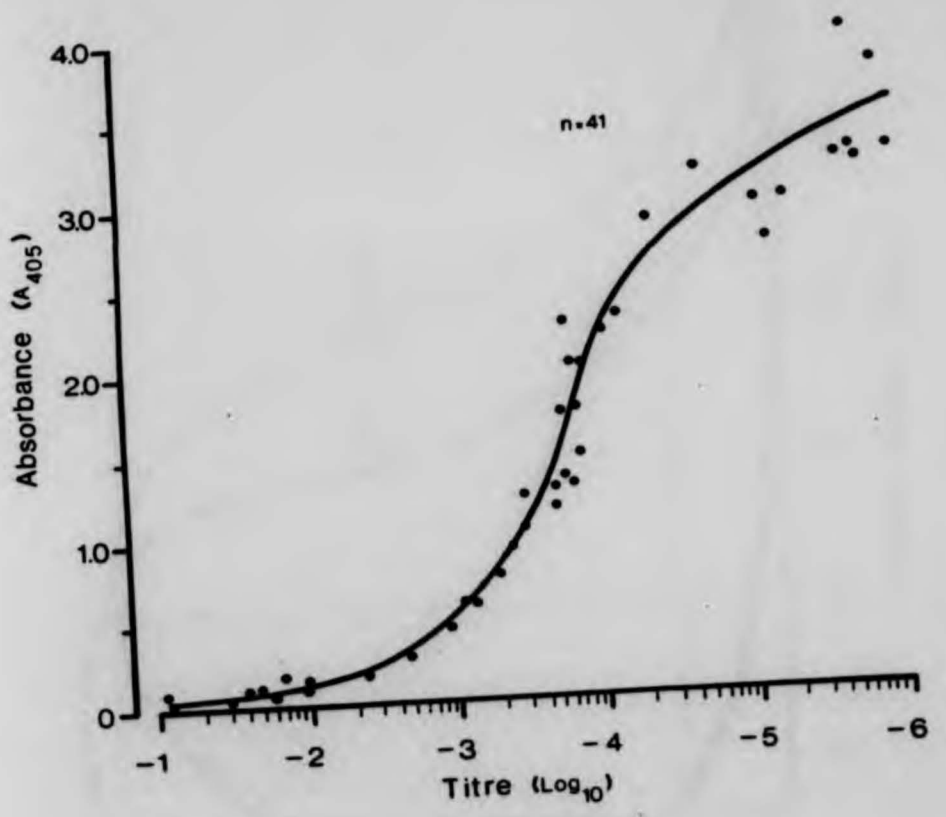

FIGURE 36

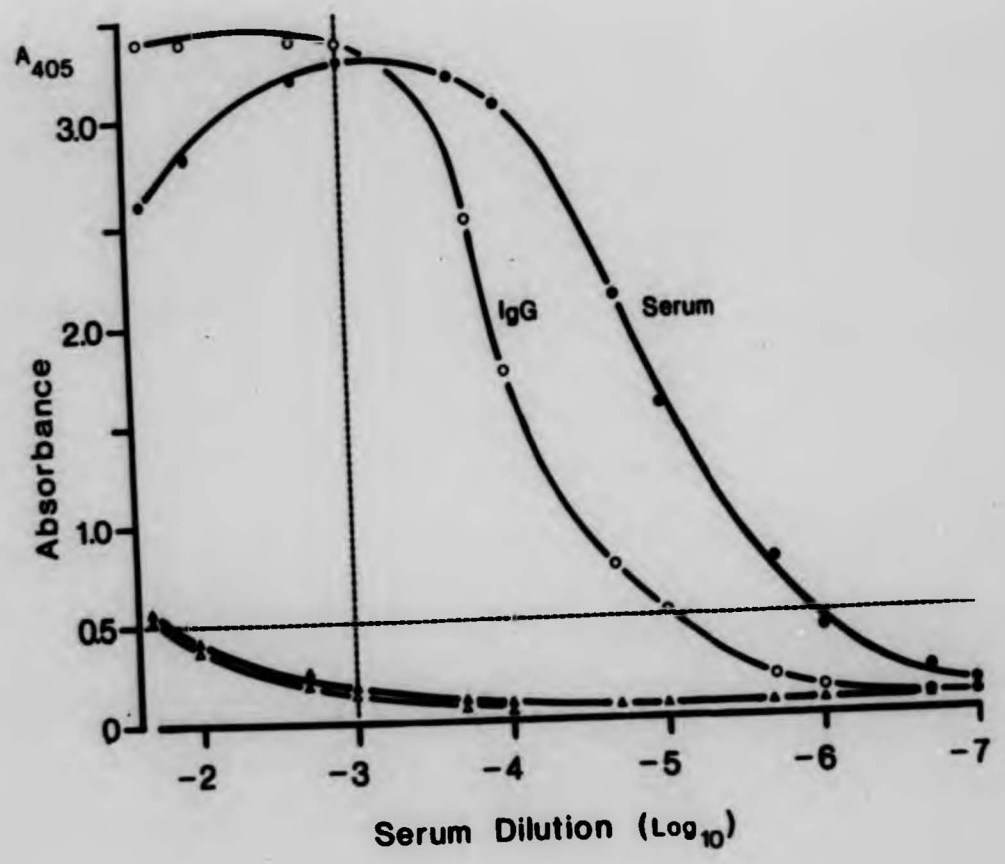
A405
3.0
2.0
1.0
0.5
0
Absorbance
IgG
Serum
-2
-3
-4
-5
-6
-7
Serum Dilution (Log10)

FIGURE 37

Fig. 38 ELISA data obtained from tests on five patients with anti-Toxocara titres ranging from weak negative (1:30) to strong positive (1:300,000) and treated by various data handling methods. The data products of each method have been plotted versus serum titre to illustrate the degree of linear relationship of the results with titre. Correlation (r) and slope (a) of the calculated linear regression lines are as follows:
(note r = 1.0 and a = 1.0 indicates a perfect linearly proportional relationship between the data product and titre.

Method	Correlation (r)	Slope (a)
Absorbance	0.97	0.39
P/N	0.97	0.39
% Pos.	0.97	0.39
MONA	0.97	0.74
Percentile	0.66	0.29
E.D.	0.99	0.99
Standard Curve	0.99	1.02

Note: Only E.D. and Standard Curve units exhibit a satisfactory relation to titre, and that MONA improves the linear relationship of the P/N ratio.

ests on five patients with
ng from weak negative (1:30) to
) and treated by various data
a products of each method have
titre to illustrate the degree
the results with titre. Correlation
alculated linear regression lines

indicates a perfect linearly
between the data product and

rrelation (r)	Slope (a)
0.97	0.39
0.97	0.39
0.97	0.39
0.97	0.74
0.66	0.29
0.99	0.99
0.99	1.02

ard Curve units exhibit a
titre, and that MONA improves
f the P/N ratio.

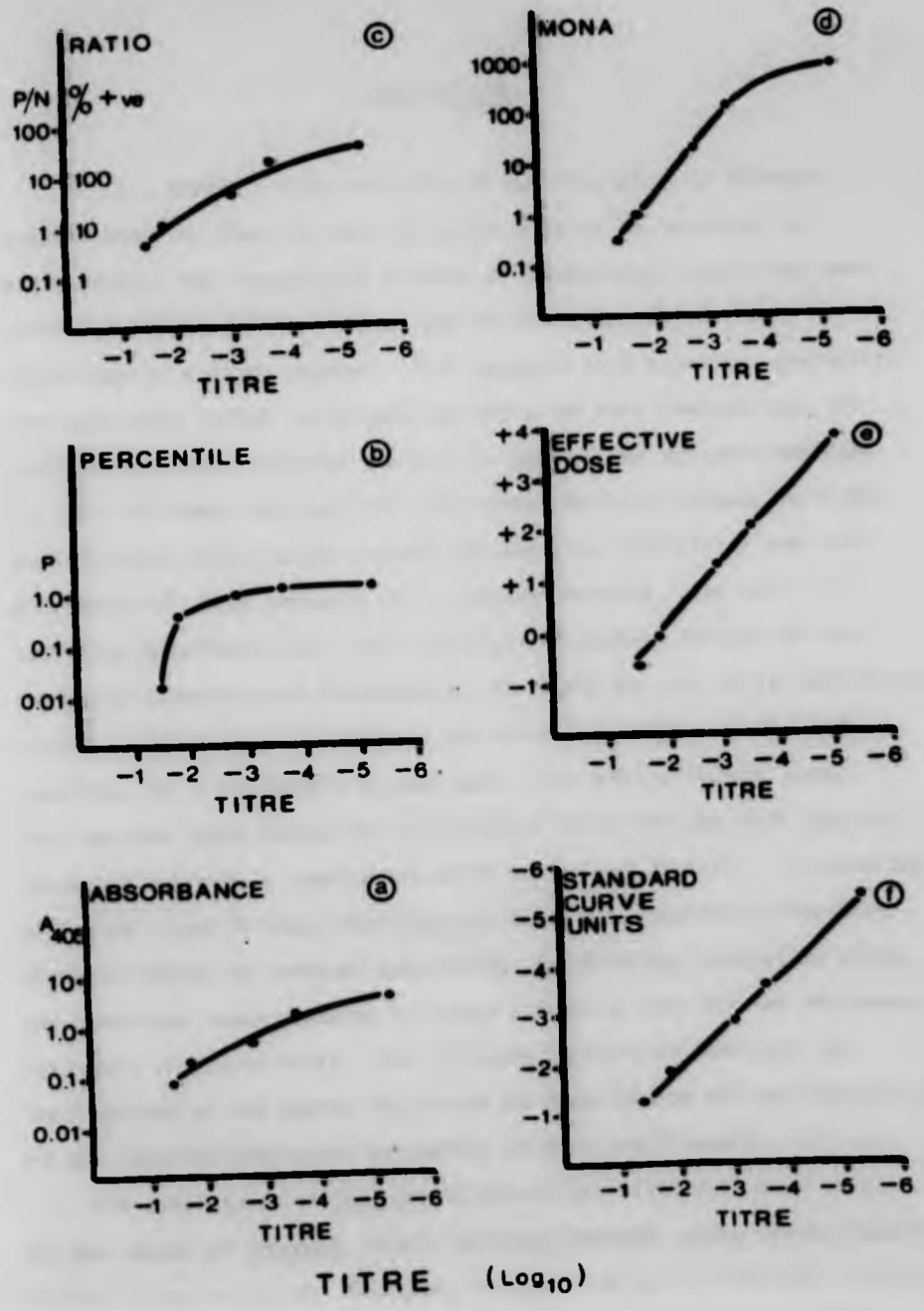

FIGURE 38

DISCUSSION

It is a peculiar characteristic of the literature of helminth immunodiagnosis, that for each parasitic disease in question, an extraordinary and imaginative variety of immunoassays assays has been recruited for use with a limited and relatively unimaginative assemblage of antigen sources. This suggests that helminth serologists have generally failed to acknowledge the axiom that immunoassays for specific antibody responses are only as good as the antigens employed. The use of crude, non-specific and uncharacterized antigens in highly sophisticated immunoassays capable of exquisite sensitivity and high precision will most probably give valueless results. The use of a specific, relatively pure, and biologically relevant antigen in even the least sophisticated immunoassays, is likely to give fully satisfactory results. Since the best antigens are usually available in the least quantity, it is desireable to use them in the most efficient assay. This was the logic behind the selection of ELISA for use with Toxocara secretory antigen, a combination which has proved fruitful. A comparison of Tables 5 and 14 shows that Toxocara ELISA has superior performance characteristics in terms of sensitivity, specificity, predictive value, and precision, when compared to assays currently used for the reference diagnosis of toxocariasis. The following discussion considers the implications of the choice of antigen on assay design and the implications of the observed evaluative parameters on assay performance.

The development of Toxocara ELISA was facilitated, in many ways, by the nature of Toxocara larval secretory antigen which proved ideally suited to use in ELISA. Secretory antigen binding to polyvinyl chloride was rapid, reproducible, irreversible, and efficient. Solid phase - adsorbed secretory antigen retained its antigenic reactivity and

exhibited enhanced stability and shelf-life compared to soluble storage at -70°C. The high immunogenicity of secretory antigen in infected hosts evoked strong serological responses, allowing the assay of serum at high dilution (10^{-3}) thus minimizing the possibility of non-specific reactions (Field, Shanker, Murphy, 1980). The predominantly single-component nature of the antigen helps explain not only the low optimal antigen dose (20 ng per test, ie. $\leq$500 pmol of major antigen per test), but also contributes to the economy of this application, since protein concentration and percent adsorption are inversely related (Hermann and Collins, 1975; confirmed in the isotope-labelled antigen adsorption experiment). The pI of the secretory antigen's major component (pI = 9.5) is such that this component remains uncharged during antigen coating (pH = 9.4), a feature which may assist hydrophobic interaction between antigen and the solid phase, and contribute to the observed reproducibility and efficiency of antigen adsorption. It is estimated that, at 20 ng of secretory product per test, it is possible to perform 30,000 assays with the antigen extracted from one month's culture of 10^6 larvae.

These antigen related considerations made the development and optimization of the assay a relatively uncomplicated undertaking, and are consistent with the contention that highly sensitive immunoassay procedures are best suited by the use of purified antigen.

The discrimination between positive and negative is a crucial element in the design of all assays for specific antibody activity, both quantitative and qualitative (de Savigny and Voller, 1980). Although the development and optimization of Toxocara-ELISA gave few problems, the setting of the "positive : negative" threshold level, as with all clinical laboratory tests, required careful consideration. This setting can profoundly influence the sensitivity, specificity, predictive value

and the degrees of false positivity and false negativity of the assay result (Vecchio, 1966; Nissen-Meyer, 1974). Various methods for determining the positive / negative threshold ELISA value have been reported in the literature, the most common being to set the minimum positive response value (Absorbance) at two (or three) multiples of the mean response value of the negative reference group. This method is useful only when small numbers of reference negative sera (<10) are available, but is unreliable due to insufficiency of data. When more reference sera (between 10 and 100) are available, authors tend to set the positive threshold value at the mean negative value plus two (or three) standard deviations of the mean. This method is rarely valid because it assumes ELISA values in the "normal" reference population are distributed normally (ie. gaussian), when they, as are most other measures of serum constituents in normal populations, are distributed with a positive skew (Mainland, 1971, de Savigny et al.,1979) which is usually log-normal (Roberts, 1969). Therefore false positives occur at +2 and +3 standard deviations. When assay results of healthy populations have non-gaussian distribution and cannot be transformed to a normal form (Harris and De Mets, 1972), non-parametric methods must be used, ie. the median value 50^{th} percentile in place of the mean, and the 97.5^{th} or 99.5^{th} percentiles in place of +2 and +3 standard deviations respectively (Herrera, 1958; Elveback and Taylor, 1969; Mainland, 1969; Werner and March, 1975; Harris, 1975). The upper limit of "normal" can be determined using these methods with a minimum of 120 sera from the reference negative group (Reed, Henry and Mason, 1971).

In this study, the frequency distribution of ELISA values in a reference "negative" group of 922 healthy adult blood donors was bimodal, the first mode having a log-normal distribution and the second

mode being too thinly populated to categorize, but clearly representing a sup-population with high ELISA values. A probability plot (Caputo, 1972) of log-transformed ELISA values indicated that these distributions overlap at an ELISA value of 0.50. This value corresponded to the 97.4^{th} percentile estimate of the population and was approximately three multiples of the median value (3 X 0.17 = 0.49). Setting the positive threshold value at 0.50 would imply that 2.6% of the reference negative population would be classed as serologically positive for toxocariasis. Epidemiologic studies (Woodruff et al. 1966) have indicated that the period prevalence of toxocariasis in healthy UK adults is 2.1% (intradermal Type I hypersensitivity). Considering that the level of 0.50 closely approximated the threshold indicated by three different approaches (ie. 97.5^{th} percentile; three multiples of median value; and discrimination of current estimates of period prevalence) this value was selected as the minimum positive response for both qualitative and quantitative Toxocara ELISA data throughout this study.

Although standard methods for evaluation of diagnostic assays have been published (Thorner and Remein, 1961; Cochrane and Holland, 1971; Grab and Pull, 1974; Martin, 1977) they appear to have been conspicuously avoided in much of the literature of helminth immunodiagnosis. Since every assay has potential errors, these must be identified and measured if results of a test are to be interpreted intelligently (Draper, Voller and Carpenter, 1972). In the pre-validation of Toxocara ELISA, the parameters of sensitivity, specificity, predictive value, cross-reactivity, and precision, were examined, each by a variety of approaches as discussed below.

Sensitivity of an immunoassay for antibody activity can be defined most objectively as the ability of a test to correctly identify those individuals with the specified disease (Martin, 1977). This definition

must be differentiated from the common usage of sensitivity (Sonksen, 1974) to describe the minimal detection limit of a test to small quantities of antibody. To determine the sensitivity of a serological assay, clearly defined "diseased" and "healthy" groups are needed (Thorner and Remein, 1961) and the methods used to define this health status should be non-serological and approximately 100% effective (Martin, 1977). This requirement is a major obstacle to objective assessment of immunoassays for toxocariasis. Presently, the only alternative to serological diagnosis of toxocariasis is clinical diagnosis. The criteria which constitute clinically diagnosed visceral toxocariasis have not been standardized or agreed and definitive parasitological diagnoses are rarely available. In this work, for purposes of serological evaluation only, criteria were established to select from cases of clinically suspected visceral toxocariasis, those which would have a high probability of being true cases of toxocariasis. Using information obtained from the clinical findings and laboratory data available for each patient, cases were assigned a score based on the number of criteria for toxocariasis (Tables 1 and 2) which they displayed. Criteria were weighted (see methods) according to their known frequency of occurrence in toxocariasis. Only those patients with a score of three or higher were included in the group designated as clinically diagnosed visceral toxocariasis, ie. those patients with persistent hypereosinophilia in association with at least two, three, or four (dependent on their weighted value) features of toxocariasis. Of 32 cases of clinical visceral toxocariasis diagnosed accordingly, 87.5% were serologically positive. This clinical group also showed a bimodal distribution (Fig. 18) with the minimum positive result being 1.38 while the maximum negative result was 0.18. Although the objective measure of sensitivity for Toxocara ELISA is 87.5%, it should be

appreciated that this parameter refers to clinical, visceral toxocariasis as defined above and does not consider subclinical and ocular toxocariasis (see Chapter IV). If the criterion for clinical toxocariasis is set at a score of 3.5 rather than 3, the sensitivity of the assay becomes 100%.

Non-human primates have been recommended as ideal experimental models of human toxocariasis (Kuntz, 1973) and were used extensively in this work to evaluate the efficacy of Toxocara ELISA. From these experiments on Macaca mulatta, less objective, but clinically useful, measures of sensitivity were obtained. Seroconversion of Toxocara infected animals occurred between five and seven days following high-dose infection (5,000 ova) and within four weeks following low dose infection (10 ova). All infected animals seroconverted while all controls remained negative. During single dose infections, anti-Toxocara IgG (and IgM) seroconversion intervals, peak antibody titres, and peak antibody intervals post infections, were all dose dependent, as was hypereosinophilia. Dose dependency persisted for approximately three months. There have been few studies of toxocariasis in rhesus monkeys, the most comprehensive being those of Wiseman (1969) who observed similar haematological responses following infection with comparable doses of T. canis. Eosinophilia in both studies peaked at 3-4 weeks post-infection and persisted for a maximum of three months. There was minimal hypereosinophilia in response to single low dose infections. Wiseman (1969) reported positive intradermal tests for Type I hypersensitivity commencing four to six weeks post infection. Serologic responses were not reported.

The use of high-dose and single-dose experimental infections, although traditionally popular in helminth (and especially toxocariasis) serological studies (Sadun et al.1957; Mitchell, 1964; Fernando, 1968b;

Wiseman, 1969; Fernando et al.1970; Aljeboori, and Ivey, 1970; Tomimura, 1976), serve to indicate minimum thresholds of sensitivity to detection of infection (cf. disease) but have little relevence to natural infection (eg. dose size and mode of infection are unnatural; hypereosinophilia is transient). The results of low intensity, trickle-dose infections (5 ova per week) as used in this study of toxocariasis in rhesus monkeys have more realistic implications regarding ELISA sensitivity in naturally acquired infections. In such infections, hypereosinophilia was prolonged and peak antibody responses (both IgG and IgM) rapidly overwhelmed the dose-dependency phenomenon seen in single-dose infections. Animals harbouring only 25 larvae, acquired during a five week period, had a relative antibody activity exceeding that of animals infected with 100 or 1,000 larvae in single doses, and by nine weeks (45 larvae), antibody activity approached the response level evoked by 5,000 larvae in a single dose. Six months post-infection, similar positive plateau levels of IgG response were reached by all doses and modes of infection. Plateaus persisted in all infected animals for at least 18 months (experiment terminated). Hence the level of IgM anti-Toxocara ELISA values are a more promising indicator of recency of infection since all dose and mode regimens resulted in serological reversion to negative within five months post-infection. IgM-ELISA failed to confer an advantage to the early detection of infection when compared to IgG-ELISA, both assays sero-converting synchronously. Although this may reflect an insensitivity inherent in the design of the IgM-ELISA, these transiently positive IgM responses are nevertheless historically significant. Other studies (Crandall and Crandall, 1972; Diconza, 1972; Fernando and Soulsby, 1974; de Savigny and Tizard, 1977) in rats, monkeys (Macaca sinica), and man, have failed to detect any convincing evidence of an IgM response to toxocaral secretory antigen.

IgM responses in our monkeys (Macaca mulatta) were observed in sera fractionated by gel chromatography (ie. in the absence of IgG). It is possible that transient IgM anti-secretion responses have remained undetected in past studies because of insensitivities in previous methods and / or competitive inhibition by the rapid and pronounced IgG response which in turn may suppress full expression of the IgM response. Attempts were made to measure anti-Toxocara IgE specific responses in these monkeys, using various anti-human IgE preparations for assay of protein A (100 mg / 500 µl) absorbed and unadsorbed rhesus sera at a dilution of 1:5 but without success.

Often, in attempts to evaluate immunoassays, helminth serologists compare results of newly developed assays to those of existing assays (Bout et al. 1976; Deelder et al. 1977) rather than comparing both, independently, to clinical criteria (Speiser and Weiss, 1979). The former comparisons establish relative sensitivity only, and were not included in this study. However, two alternative assays were developed, the Toxocara immunoradiometric assay (IRMA) using isotopic label and the Toxocara ELISA using soluble somatic larval antigens, in order to compare independently, the effects of isolated parameters (ie. isotopic vs. non-isotopic conjugate labels; and secretory vs. somatic antigens) within comparable immunoassay systems.

Isotopic immunoassay is generally regarded as the immunoassay of highest sensitivity, and the one to which all other tests are generically compared. In Toxocara-IRMA, the dose-response curves of isotopic and non-isotopic conjugates suggested that use of isotopic labels should confer higher sensitivity. In practice, the results of actual tests indicated the converse. This was probably due to the higher non-specific binding of isotope-labelled antiglobulin compared to enzyme conjugates. Possible explanations include the higher molecular weight of the latter,

which could also be responsible for the lower maximal values obtained in tests with enzyme conjugates. Their relatively large size could result in stearic hindrance at the solid phase surface and could influence the apparent affinity of antiglobulin conjugate for bound antibody. In practice ELISA was slightly more sensitive to high levels of antibody while IRMA was slightly more sensitive to low levels of antibody. Qualitatively, as found by others (Schinski, Clutter and Murrell, 1976; Voller, Bartlett, Bidwell and Edwards, 1977) ELISA and IRMA were essentially equivalent, although ELISA was the more attractive alternative from a practical viewpoint.

High ELISA values, titres, or positive:negative ratios are often given as measures of assay sensitivity (Kagan, 1978). In Toxocara-ELISA, remarkably high ELISA values ($A_{405}>8.0$) representing end-point serum titres greater than 10^{-6} and positive:negative ratios greater than 50:1, have been observed in some cases of clinical toxocariasis in man. Although this reflects, to some extent, the immunogenicity of toxocaral larval secretions during natural infections, such data are merely numerically interesting, are not reliable indices of assay sensitivity, and should not be used as evaluative parameters for immunoassays (de Savigny and Voller, 1980).

No advantage in assay sensitivity was gained using somatic Toxocara-ELISA, either independently, or in parallel.

As a final feature of the sensitivity of Toxocara-ELISA, the optimal working dilution of serum at 10^{-3} requires a minimum of 1 µl of serum per duplicate assay. In practice, 10 µl are used, to ensure higher precision at the serum sampling stage.

Specificity, an equally important parameter to be quantified in the evaluation of immunoassays, is usually inversely related to sensitivity (Lobel and Kagan, 1978). Thus the high sensitivity of Toxocara-ELISA

necessitated careful examination of specificity from several viewpoints.

Specificity of an assay for detection of disease (cf. detection of infection) is most objectively defined as the ability of a test to correctly identify those individuals without the specific disease (Martin, 1977). Again, clearly defined "diseased" and "healthy" groups are required. In toxocariasis, this definition can mislead because the prevalence of subclinical toxocariasis infection can exist and vary between and among populations. A reference group of blood donor sera representing 922 subjects resident in southern England was used to assess assay specificity. Ideally each of these subjects should have been screened for the absence of eosinophilia and other criteria of clinical toxocariasis, but for practical reasons, acceptance as a blood donor was used as the only criterion for inclusion in this group. Of these 97.4% were negative in Toxocara-ELISA and thus, the objective measure of specificity was 97.4%. This parameter is not stable from one population to another since it is influenced by the sensitivity of the assay to the prevalence of subclinical toxocariasis, past toxocariasis, and non-toxocaral helminthisases with which the assay may cross-react. Like sensitivity, specificity,as described here, refers only to visceral toxocariasis (see Chapter IV for ocular toxocariasis).

A second aspect of specificity is that of assay design, as opposed to disease detection. This was assessed by the use of homologous antigen inhibition, which indicated 50% inhibition doses in the order of one to two nanograms. All levels of seroreactivity including negative sera, could be inhibited by homologous antigen down to a level of approximately $A_{405} = 0.05$. These results confirm that the assay has high specificity in the sense that the A_{405} ELISA result is a true measure of specific antibody response to homologous antigen, and not to non-specific serum

factors.

Specificity can be examined by a third means: at the antigen level. Antigen specificity of Toxocara-ELISA was investigated by heterologous antigen, competitive inhibition. Heterologous proteins and helminth antigens failed to induce 50% inhibition at levels as high as five micrograms, ie. 5,000 times the homologous antigen ID_{50}. At 5 µg of heterologous antigen, inhibition in the range of 10 to 40% became evident. It is possible, but unlikely, that this implies minor antigenic cross-reactivity since the relative concentration of heterologous protein is extremely high (5 µg / 200 µl of test serum diluted 10^{-3}) and exceeds the total IgG concentration of the serum dilution. As a result, non-specific influences due to altered protein concentration may account for the observed inhibition.

Having established the sensitivity and specificity of Toxocara ELISA, primarily by the use of selected populations, it is important to know what its predictive value will be when used in large, unselected populations (Vecchio, 1966) (ie. what is the probability that a subject yielding a positive result actually has the disease?). Conversely, what is the likelihood that a subject with a negative result does not have the disease? This probability cannot be estimated directly from sensitivity and specificity parameters obtained in the preliminary test evaluations since it is related to disease prevalence in the population under study (Nissen-Meyer, 1974; Stewart, 1974; Martin, 1977; Archer, 1978). In reference serodiagnosis, the population tested is clinically pre-selected and will have a relatively high prevalence while in seroepidemiologic applications, prevalence may be low. Assuming the period prevalence of toxocariasis in UK is 2.6%, the predictive values (for detection of toxocaral infection) of positive and negative Toxocara ELISA results are 97.1% and 88.6% respectively. Predictive values have

been published for only one of the 167 published immunodiagnostic methods for toxocariasis (ELISA: Glickman et al. 1978; positive = 85.7%, negative = 87.8%). Predictive values are rarely used in the evaluation of helminth immunodiagnostic tests, however they can provide valuable insights to the performance of assays in various populations, especially where prevalence rates are expected to differ. For instance, in a hypothetical population, if disease prevalence is 10% and assay sensitivity and specificity are both as high as 90%, there will be, in the first 1,000 subjects tested, a larger number of false positives (100) than of true positives (90). As the prevalence falls, false positivity rates increase. Thus at 1% prevalence, there will be 9 true positives (out of ten) compared with 100 false positives in the first 1,000 persons tested.

According to the calculated predictive values (Table 13) for disease detection, the use of Toxocara-ELISA in a population with a prevalence of clinical visceral toxocariasis of 1%, will detect eight true positives (out of ten) and no false positives in the first 1,000 persons tested. In the same population, the US CDC Toxocara-ELISA of Glickman et al. (1978) would detect eight true positives (out of ten) but 52 false positives in the first 1,000 persons tested.

It was shown in this study that the setting of the positive: negative threshold exerts a moderate influence on predictive value (Table 13). The use of such information becomes important when deciding a particular application of the assay. For example, in seroepidemiologic studies, where it is desired to have a test of high sensitivity, the consequences of false positive detection are not as serious as in immunodiagnostic applications where false positive patients may undergo unnecessary treatment. Conversely, in immunodiagnostic applications,

there are circumstances, such as may occur in ocular toxocariasis, where a false negative serological result may lead to unnecessary enucleation of an eye. The use of predictive values allows one to estimate the probability of a false positive or false negative result for any particular ELISA reading, and thus increases the usefulness of the assay for the diagnostician.

Cross-reactivity is the fourth evaluative parameter of importance to helminth immunodiagnosis. Although cross-reactivity is often discussed synonomously with specificity (Fife, 1971, Kagan, 1974), and is influenced by sensitivity (Kagan and Lobel, 1978), these parameters are discrete, can vary independently, and should be measured separately. Cross-reactivity of an assay, is a function of antigen specificity (as apposed to assay specificity) and may be defined as the proportion of diseased individuals (suffering from a specified disease other than the one under test) who react positively in the assay.

By definition, the cross-reactivity of Toxocara-ELISA, determined from assays of 96 sera from Europeans with defined, non-toxocaral, helminthic infections, was 6.3%. However the nature of toxocariasis further confounds the interpretation of this result, since the prevalence of toxocaral infection in a population with non-toxocaral helminthic infection, especially geohelminthic infection, can be expected to be higher than the prevalence of toxocariasis in a healthy population. For example 19.7% and 13.8% of published toxocariasis case histories record co-existing Trichocephalus trichiurus and Ascaris lumbricoides infections respectively (Ehrhard and Kernbaum, 1979). It would be difficult to exclude the possibility of toxocariasis occurring in a helminth infected population. Therefore it became necessary to examine cross-reactivity in experimentally infected animals. These data failed to demonstrate qualitative cross-reactivity in Toxocara-ELISA beyond

the species level despite, as in the case of Ascaris suum, trickle-dose and repeated high dose heterologous infections of a type known to induce high levels of anti-Ascaris antibody. Competitive inhibition of Toxocara-ELISA, by heterologous helminth antigens also indicated the absence of significant cross-reactivity. Thus Toxocara-ELISA, according to animal experiments, appears to be a genus specific immunoassay, a finding consistent with current understanding of the genus-specific nature of toxocaral secretory antigens (Hogarth-Scott, 1966; Lamina, 1970; de Savigny and Tizard, 1977; Stevenson, 1979; Chapter II).

Combining the data describing sensitivity and specificity allows the following interpretation of the cross-reactivity parameter. Considering (a) the absence of significant cross-reactivity in animal experiments and heterologous inhibition studies, and (b) high assay specificity; the sub-population representing 2.6% of healthy adults with elevated ELISA values ($0.50 < A_{405} < 1.35$) can be identified as valid positives, ie. with significant anti-toxocaral antibody levels. Since the group is clinically healthy, this range of ELISA values should therefore reflect current, sub-clinical toxocariasis, or past history of clinical or subclinical toxocariasis. If so, this would indicate that the period pervalence of toxocariasis in the UK is at least 2.6%. Although the cross-reactivity level (prevalence in the non-toxocaral helminthic infection group) of 6.3% is not significantly different from the prevalence in the healthy population, the true measure of cross-reactivity should be adjusted downward to 3.7% to compensate for the "background" prevalence in healthy subjects.

The final parameter used to evaluate Toxocara-ELISA was precision (reproducibility). Precision may be defined as the ability of an assay to give consistent results in tests on the same sample and is measured in quantitative immunoassays by the coefficient of variation (CV %).

Intra-assay variation ranged from 4.2 to 11.7 CV % while inter-assay variation was less than 15 CV %; both parameters indicating suitability of the assay for use.

It was recognized early in this study that inter-assay precision of Toxocara-ELISA was critically influenced by the termination of the enzyme-substrate reaction and that this could be effectively controlled for alkaline-phophatase conjugates using the methods described. It was initially assumed that the major source of intra-assay variation would be sampling error, however these studies incriminate the antigen solid-phase as contributing greatly to variability. The reproducible quality of antigen production, the high stability of secretory antigen and the long shelf-life of pre-coated Toxocara-ELISA plates contribute to the observed precision.

Because Toxocara-ELISA was designed primarily as a seroepidemiological tool, the enzyme-substrate reaction step was allowed to run longer than would be necessary for mere discrimination of positive and negative responses. This was done to allow scope for negative values to emerge into a frequency distribution which could be used to detect subtle differences between populations, in addition to the proportion positive (Chapter V). This being so, it was important to establish, not just the quantitative reproducibility of positive results but also of negative results. The high reproducibility of the assay proved that distribution of negative values was not just a function of accumulated errors, ie. sample treatment, instrument bias, intra-individual variation of serum constituents, etc. (Bakelund, Winkel, Stalland, 1974) but was a reproducible function of the individual patient and remained so, serially, within at least a four month period. This indicates that, within the negative result range, ELISA is responding to specific or non-specific serum related factor(s) characteristic of individual patients. This

phenomenon has been noticed in other ELISA systems (Voller, Bartlett and Bidwell, 1979, personal communication; Speiser, 1980, personal communication) and remains unexplained. Hypotheses include serum globulin levels, rheumatoid factor, C-reactive protein and other acute phase proteins, and are worth investigation as a possible cause of increased ELISA "backgrounds" in healthy tropical populations.

The reproducible nature of quantitative Toxocara ELISA data allows the following general conclusions: Toxocara ELISA values less than A_{405} = 0.50 suggest the absence of significant toxocaral infection or disease and are found in 97.4% of the healthy adult population (UK). Values between 0.50 and 1.50 may be expected in 2.6% of the healthy population and probably indicate a relatively low level of circulating anti-Toxocara antibody such may be expected in light, current infections and following past infections. Toxocara ELISA values greater than 1.50 indicate significant levels of specific antibody, and are associated with active or recent clinical toxocariasis disease. Such values do not occur in the healthy population.

Although ELISA results expressed as absorbance values are useful in research and seroepidemiologic applications, they are a source of confusion when used in routine serodiagnostic applications. Development of Toxocara ELISA commenced in 1977 at a time when ELISA methodologies were primarily tools of research and none had yet been introduced into routine serodiagnostic service. ELISA, in many disciplines, is now emerging from the realm of the research laboratory to that of routine diagnostic laboratory. There is therefore a need for reconsideration of the means by which laboratory results of ELISA are

communicated to the clinician. No matter how sensitive and accurate an assay is, its result has little value unless it is in a form intelligible to the user, the clinician. Toxocara ELISA results presented in this thesis are in absorbance units (A_{405}). Such values, although useful in research, should be considered as raw laboratory intermediate data, analogous to disintegrations per minute in radioimmunoassay, and should be further refined before they are reported from the laboratory.

The main deficiency of the absorbance value result is that it is not linearly proportional to relative antibody activity (ie. an ELISA value of 3.0 does not indicate three times the antibody activity of an ELISA value of 1.0, but rather 35 times). Values linearly proportional to relative antibody activity should be useful in comparing one patient to another, or comparing one form of toxocariasis to another, or in following the response of a single patient to treatment.

It was considered that in order to convey an impression of relative antibody activity, some form of mathematical transformation was required. Of all methods available, only the effective dose method and the standard curve method were shown to be capable of linear transformation of ELISA data. Of these, only the standard curve method produced results in easily understood units.

The main argument against the use of the standard curve is that it presupposes parallelism in the dose response curves of patients at all times during the course of their infections. In theory, parallelism of dose response curves should not be presumed. The concentration range of specific antibody in pathological sera from patients with toxocariasis can span up to six $\log_{10}$ dilutions. The antibody affinities of such sera cannot always be assumed to be of the same distribution (Werblin and Siskind, 1973; Macario and de Macario, 1975; Hirschfeld,

(1980). In tests for specific antibody responses of a particular immunoglobulin class, the dose response curve can be altered by the presence or absence of responses in other immunoglobulin classes (Carlsson and Lindberg, 1977; Keren, 1979). The current work demonstrated that removal of IgM from a highly reactive serum would diminish the prozone phenomenon and increase the slope of the dose-response curve. Finally, ELISA measures the combined effects of antibody concentration and antibody affinity since secondary antibody-enzyme complexes may exert an interfering effect on the attachment of primary antibodies (Butler, Feldbush, McGivern and Stewart, 1978). Thus the expression of results in absolute units (ie. "Specific anti-Toxocara Antibody Concentration = 70 ng IgG/ml of Serum") would seem to be precluded.

In practice, Toxocara ELISA dose-response curves of 40 out of 42 sera, representing a spectrum of serological reactivity levels, were remarkably parallel. This may be due to the relative rarity of IgM responses to toxocaral secretory antigens; however, the usual explanation that IgM competition is responsible for the prozone phenomenon does not seem valid since plateaux were occasionally observed in IgG fractions. This has also been noted by Lehtonen and Viljanen (1980) who found that, at high antibody concentrations, there was superior antibody binding to solid phase antigens dispersed at low density compared to high density, and explained this as due to avidity effects. An alternative explanation could consider the diffusion of substrate towards, and substrate product away from, surface-bound enzyme conjugate. In either case, the low density of antigen used in Toxocara ELISA should minimize prozone phenomena. It was considered therefore, that a standard curve could be used to predict, with reasonable accuracy, titre end-points on a continuous scale, given ELISA absorbance values obtained from tests at a single serum dilution. This decreases the tedium, expense, and

inefficiency of preparing serial dilutions to quantitate positive results, while avoiding the major errors and loss of precision introduced by such titrations. Errors due to prozones occur only in high titred sera and should have no influence on the qualitative interpretation of the result. Since the standard curve is prepared from data pooled retrospectively from several assays, its validity is enhanced. The standard curve is sigmoidal and exhibits heteroscedasticity (non-uniform variance) along its length, such that values in the central part of the curve (ie. at the positive-negative threshold) have the highest precision. The curve remains valid for any given series of Toxocara ELISA assays but should be recalibrated when a new test reagent (eg. new batch of conjugate) is introduced. The curve is not valid for high Toxocara ELISA values. It should be possible by using curve-fitting techniques and curvilinear regression, (Ashton, 1972; Marschner, Erhart and Scriba, 1974) with weighting to correct for variance, (Robard and Hutt, 1974) to transform the sigmoidal standard curve to linear form and derive its equation. The equation can then be used by a programmable calculator linked to the ELISA photometer, to transform ELISA values directly to titres or other units (de Savigny and Voller, 1980 a,b). However it is not always necessary to straighten standard curves and the advantages of non-linear estimations of immunoassay standard curve data have been discussed by Kamp (1980). Once it has been established that ELISA dose-response curves are predominantly parallel, the ELISA standard curve method can be exploited further, as shown by Leinikki, Shekarchi, Dorsett and Sever (1979), by calibrating it with known concentrations of the affinity purified, antigen specific, IgG fraction of a standard serum. Thus, results can be expressed in absolute terms of ng of IgG per millilitre of serum. Since the recognition that ELISA dose-response curves can be parallel in

practice, the standard curve method has become increasingly used in other ELISA systems (Tracy, Alport, Yunginger, and Hamburger, 1980; Karch, Halbert, Klima and Steinberg, 1980; Van Loom and Van der Veen, 1980).

SUMMARY

A novel enzyme-linked immunosorbent assay (ELISA) was developed to quantitate immunoglobulin class-specific antibody responses to *Toxocara canis* larval secretory antigens in both man and animals. *Toxocara*-ELISA was evaluated objectively, according to the parameters of sensitivity, specificity, predictive value, cross-reactivity, and precision with the following salient points arising:

Sensitivity

- detects 87.5% of clinically diagnosed cases of visceral toxocariasis (n = 32).
- detects minimal infection levels of at least 10 larvae per kg host tissue.
- detects antibody between one and four weeks of infection.
- detects antibody for at least 18 months post-infection.
- requires 20 ng (<500 pMol) of antigen per test.
- requires a minimum of 1 µl of serum per test.
- sensitivity is comparable to a *Toxocara* immunoradiometric assay.
- detects antibody in pathologic sera to dilutions of 10^{-6}.

Specificity

- detects 97.4% of healthy adults as negative (n = 922).
- homologous antigen 50% inhibition dose is<2 ng.

Predictive Value

- of positive result = 97.1%
- of negative result = 88.6%

Cross-Reactivity

- no cross-reactivity detected in non-toxocaral, helminth infected, experimental animals.

- 3.7% (prevalence adjusted) of non-toxocaral, helminth infected patients are positive (n = 96)
- heterologous antigen 50% inhibition dose is greater than 5,000 ng.
- strong intra-genus cross-reactivity to Toxocara cati confirmed.

Precision

- Intra-assay CV = 7.3%
- Inter-assay CV = 12.7%
- Antigen quality is reproducible between batches
- Antigen is thermostable and exhibits long shelf-life.

Experience gained from Toxocara-ELISA during the evaluation suggests the following interpretation: Toxocara ELISA values less than $A_{405} = 0.50$ indicate no evidence of clinical toxocariasis and occur in 97.4% of the healthy adult population (UK). ELISA values $0.50 < A_{405} < 1.50$ indicate relatively low-levels of circulating anti-Toxocara antibody such as might be expected in light, current, subclinical infections or following past infections. These values occur in 2.6% of the healthy adult population and reflect the minimum period prevalence of toxocaral infection. ELISA values greater than $A_{405} = 1.50$ are associated with active or recent clinical visceral toxocariasis (disease). Such values did not occur in the healthy adult population (sample size, 992).

A standard curve method was designed to transform Toxocara ELISA data into more comprehensible measures of relative antibody activity for use in routine serodiagnostic applications.

This chapter reports the first application of nematode secretory antigen in an enzyme immunoassay system. The use of highly specific antigen in such a sensitive serological system has facilitated the

design of a quantitative assay with high sensitivity, specificity and precision in addition to inherent practical advantages which allow processing of large numbers of sera with efficiency and economy. Toxocara ELISA, as designed, constitutes a potential advance on currently available methods used in central reference immunodiagnosis and seroepidemiology.

CHAPTER IV

SERODIAGNOSTIC APPLICATIONS OF *TOXOCARA* ELISA

INTRODUCTION

The unquantified zoonotic potential of toxocariasis, whose reservoir was first recognized in 1952 as residing in the companion animals of man (Beaver *et al*. 1952) has stimulated a rapid demand for reliable diagnostic procedures. In the absence of any practical means of obtaining a parasitological diagnosis of toxocariasis in man, this demand has been answered by an increasing frequency of published immunodiagnostic methods (Table 4 describes 166 methods) of which only a few have been used routinely (Table 5). The large number of publications and their low application rate illustrate both the need for such methods and the challenge involved.

Toxocariasis in man occurs in two clinically distinct forms - visceral and ocular - each presenting different facets of the diagnostic problem.

Clinical visceral toxocariasis is seen most commonly in young children,one to four years of age, who have poorly developed habits of personal hygiene and are excellent candidates for oral-faecal transmission of geohelminths. Since manifestation and severity of symptoms is proportional to the number of infective ova consumed (and to host immune response factors), the most severe cases are often found in children with a history of eating soil from premises contaminated by dog and cat faeces. It is also possible that significant numbers of parasite ova could be consumed by individuals of any age, who fail to wash their hands adequately after playing or working in soil and before

eating.

The diagnosis of toxocariasis has been as much a clinical art, as it is a diagnostic science. The visceral form is characterized by chronic and extreme eosinophilia, associated variously with hepatomegaly, transient pulmonary involvement, fever and hyperglobulinaemia (Tables 1 and 2). These features, considered singly or in combination are not pathognostic and occur commonly in many areas, (especially tropical regions) as a result of non-toxocaral infection. Definitive diagnosis is established only by the biopsy demonstration of larvae in tissue; a procedure performed often in the early years following the recognition of toxocariasis as a disease entity, but rarely done now. Immunodiagnostic methods currently provide the only option to parasitological diagnosis. Requirements of a suitable immunodiagnostic assay for visceral toxocariasis include: (1) a high degree of sensitivity in order to identify, as possible candidates for treatment and for surveillance, all those with significant disease, including most of those with light or recent infection; (2) a high degree of specificity, to avoid unnecessary or inappropriate treatment of healthy individuals or those with other infections; (3) a low degree of cross-reactivity, to allow its use, with confidence, in individuals from areas of helminth endemicity; (4) a capacity for accurate and reproducible quantitation of the antibody response to allow assessment of the severity of infection and of response to treatment; and (5) operable using small volumes of serum available from paediatric populations.

Ocular toxocariasis is a clinically distinct expression of the disease and occurs more commonly in older children, often in the absence of significant eosinophilia and other features associated with visceral toxocariasis. Since ocular toxocariasis may result in uniocular

blindness, it commands clinical attention. Of critical importance is the differential diagnosis of ocular toxocariasis and retinoblastoma. The clinical similarities between these conditions dictate a necessity for high levels of both sensitivity and specificity in toxocariasis immunoassays, since a false negative may lead to unnecessary enucleation of an eye, while a false positive may unnecessarily delay treatment of a neoplasm with possible disastrous results for the patient. Additional to the requirements set out for visceral toxocariasis, immunoassays for ocular toxocariasis must be suitable for use with small volumes of sample such as are available from ocular anterior chamber fluid.

The Toxocara ELISA developed and validated in Chapter III is a candidate assay with potential to meet the diagnostic needs of both visceral and ocular toxocariasis. In this chapter, Toxocara ELISA is evaluated in practice, in immunodiagnostic applications in both man and canidae, with consideration given to the dynamics of the humoral response in natural infections, its relevance to clinical expression, the persistance of antibody following infection, and the effect of chemotherapy on assay results. The development of the first laboratory model of ocular toxocariasis is also described and used to further evaluate Toxocara ELISA applications.

MATERIALS AND METHODS

Toxocara ELISA. All assays used *Toxocara canis* larval secretory antigen, prepared as described in Chapter II, in the *Toxocara* ELISA protocol, designed and validated in Chapter III. *Toxocara* ELISA was modified to assay canine IgG anti-*Toxocara* antibody by use of appropriate dilutions of rabbit anti-dog IgG (heavy and light chains) antisera (Miles-Yeda Ltd., Rehovot) fractionated as described by Voller, Bidwell and Bartlett (1976) and conjugated by the one-step glutaraldehyde method (Avrameas, 1969) with alkaline phosphatase (Miles Laboratories, Slough, UK). Results were expressed as absorbance values.

Sera. Sera from 49 patients with clinically presumed visceral toxocariasis were obtained from Canada, Czechoslovakia, Sweden Switzerland, UK and USA, as acknowledged in Chapter III.

Lyophilized serum samples (136) collected serially from ten patients, during a minimum of 10 months and as long as eight years following the onset of clinical visceral toxocariasis (clinical score≥ 3.0), were obtained from Dr. M. Uhlikova and J. Hübner (Prague), as randomized, coded samples for assay in a blind protocol.

Sera from 11 patients with clinically diagnosed ocular toxocariasis were obtained from the Toxocaral Reference Laboratory, UK, courtesy of Professor A.W. Woodruff.

Sera from ascarid naive beagle dogs were obtained from White Eagle Laboratories, Kansas City.

Sera, from beagle dogs, collected before and after various anthelminthic therapies, were obtained courtesy of Dr. V. Stejskal, Astra Pharmaceuticals, Södertalje, Sweden.

Experimental Ocular Toxocariasis. Five experiments were conducted in four animals (Macaca mulatta) whose previous experience with Toxocara canis infection was as follows: one animal with chronic visceral toxocariasis arising from high-level, single-dose infection; one with chronic visceral toxocariasis arising from low-level, trickle-dose infection; one with recent toxocariasis arising from high-level, single-dose infection; and two controls, without primary exposure to Toxocara. In each ocular infection experiment, 1000 in vitro hatched Toxocara canis larvae were administered to halothane anaesthetized animals by catheterization of the ophthalmic branch of the right internal carotid artery via the right femoral artery. Confirmation of accurate catheterization was obtained by intercurrent administration of radio-opaque contrast medium (Urografin 370) and X-ray image intensification*. Confirmation of successful infection was obtained by either primary or secondary serological responses to secretory antigens. Ocular infection was monitored by retinal fluorescense angiography at both five minutes pre- and five minutes post-inoculation, and at 48 hours post-inoculation. Direct ophthalmoscopy and fundus photography (Kowa RC-2 Retinal Camera) were done regularly commencing three days post-inoculation. Serum, and blood samples were collected for anti-Toxocara antibody assays and heamatology. Aqueous humor (150 µl per eye) was obtained by paracentesis of the anterior chamber using a 27 gauge needle introduced through the lateral cornea, entering at the corneal-limbal junction.

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* Catheterizations were done by Dr. B. Kendall, Hospital for Nervous Diseases, Queen's Square, London, and Prof. G. DuBoulay, Nuffield Laboratories of Comparative Medicine, Regent's Park, London.

RESULTS

Visceral Toxocariasis in Man. Clinical features of 49 patients with eosinophilia and presumed visceral toxocariasis are shown in Table 16. Within this group, the correlation between clinical scores (see Chapter III) and Toxocara ELISA values is shown in Fig. 39. Sera from ten patients with clinical score 3.0 were followed serially, for periods of ten months to 8 years by a single assay in a randomized blind protocol. Results are shown in Fig. 40. The salient points are as follows: All patients were serologically positive and remained so throughout the study period; rising ELISA values were not commonly seen; peak responses generally occurred during the first month following onset of symptoms, although in three patients, maximum responses were delayed until two, three and six months respectively; all patients ultimately had decreasing antibody responses during the study period. Dependent on the peak response, maximal ELISA results may decline to the past exposure range postulated in Chapter III (0.50 A_{405} 1.50) during a period of two to five years, following onset of symptoms, at a mean rate of -1.22 (0.44 SD) absorbance units (A_{405}) per year (Table 17). Since all patients had received a course of treatment with Thiabendazole, it could not be determined if this decline was a normal feature or had been influenced by chemotherapy.

Ocular Toxocariasis in Man. Toxocara ELISA values were positive in ten of 13 cases of clinically presumed ocular toxocariasis (Table 18). The mean positive value (1.30 0.77 SD) was significantly lower than that observed in visceral toxocariasis ($P<0.001$) and seven were in the 'past-exposure' range of A_{405} = 0.50 to 1.50. Serial samples were available from four patients (Fig. 40). Each showed a long persistance

TABLE 16

CLINICAL FEATURES IN PATIENTS WITH PRESUMED VISCERAL TOXOCARIASIS

Patient	Age (y)	Sex	Eosinophilia	Geophagia/Pica	Hepatomegaly	Pulmonary involvement	Fever	Globulinaemia	Splenomegaly	Cutaneous involvement	Pallor	Leucocytosis	CNS involvement	Lymphadenopathy	Clinical Score	ELISA Value (A 405)	
JA*	50	F	70%		•				•			•			8	5.16	+
JA	1	F	85%	•	•	•				•		•			7.5	2.75	+
BM	2	M	63%	•	•		•					•			7	4.80	+
RT	12	M	77%			•	•		•			•	•		7	4.50	+
EH	2.5	M	40%	•	•	•	•	•							7	3.24	+
NB	1	F	30%	•	•				•	•	•	•			7	3.02	+
ZI	1	M	50%		•	•	•						•	•	6.5	4.22	+
SJ	7.5	F	75%	•	•			•			•				6.5	3.56	+
KT	2	F	72%	•							•	•			6	4.35	+
HI	2	M	49%	•			•					•			6	2.91	+
SL	2.5	F	49%	•	•			•							6	1.35	+
SK	4	F	62%		•				•		•	•	•		5.5	5.10	+
KA	49	F	52%		•			•		•		•			5.5	3.96	+
BP	4	M	70%		•	•					•	•			5.5	3.81	+
AS	2.5	M	50%		•	•									5	6.00	+
KI	2	F	31%	•	•		•					•			5	4.11	+
BM	47	F	55%		•				•					•	4	3.81	+
RF	60	F	10%		•		•								3.5	.05	-
KA	40	F	79%		•							•			3	4.50	+
BM	1	M	27%									•			3	3.92	+
PV	2	M	47%									•			3	3.18	+
HH	2	F	64%			•									3	3.09	+
TM	4	F	74%			•									3	2.35	+
JP	3	M	10%		•	•									3	1.99	+
FV	15	F	32%				•								3	1.15	+
JD	15	M	23%			•									3	.18	-
CB	14	M	20%			•									3	.18	-
SF	14	F	45%												3	.15	-
BD	3	M	50%												2.5	1.97	+
PG	4	M	25%							•					2.5	.37	-
CG	6	M	12%							•	•				2.5	.17	-
EH	50	M	31%	•											2.5	.16	-
BT	47	F	10%				•								2.5	.14	-
SB	40	F	23%				•								2	3.52	+
SH	30	F	28%												2	3.33	+
TS	60	M	66%												2	.35	-
BB	50	M	20%			•									2	.23	-
CR	7	M	21%												2	.18	-
PN	14	M	12%								•				2	.11	-
AF	47	F	16%				•			•					2	.09	-
AT	51	F	40%							•					1.5	.42	-
CB	37	M	19%												1.5	.50	-
RS	30	M	16%												1	.17	-

* Biopsy confirmed.

TABLE 17

RATE OF CHANGE PER YEAR OF POSITIVE TOXOCARA ELISA REACTIVITY IN CLINICAL TOXOCARIASIS*

Patient	Clinical Score	ELISA		Toxocariasis
		$\bar{A}_{405}$ Maximum	ΔA_{405}/Year	
HH	3.0	4.80	-1.84	Visceral
BM	4.0	4.00	-1.10	Visceral
KZ	5.5	4.05	-0.26	Visceral
BP	5.5	3.81	-1.17	Visceral
SK	5.5	5.10	-1.31	Visceral
KI	6.0	4.37	-1.70	Visceral
ZI	6.5	4.20	-1.08	Visceral
BN	7.0	4.79	-1.55	Visceral
NB	7.0	3.01	-1.05	Visceral
RF	7.0	4.55	-1.11	Visceral
BA	N/A	2.95	+0.02	Ocular
HM	N/A	0.75	+0.18	Ocular
RD	N/A	2.25	-0.10	Ocular
DP	N/A	1.33	-0.06	Ocular
		Mean (± SD)	-1.22(0.44)	Visceral n=10
		Mean (± SD)	+0.01(0.12)	Ocular n= 4

* Data, from 14 patients sampled serially during a period of at least ten months, expressed as the rate of change of the peak antibody response to the minimum or plateau response. Sign indicates whether the response is declining (-) or increasing (+) with time. Difference of means is statistically significant ($P < 0.001$).

N/A Not Applicable

TABLE 18

TOXOCARA SEROLOGY IN PATIENTS WITH PRESUMED OCULAR TOXOCARIASIS

Patient	Country of Origin	Toxocara FAT	Toxocara ELISA Quant.	Toxocara ELISA Qual.
K.G.	U.K.	+	0.75	+
R.H.	U.K.	+	0.34	-
K.M.	U.K.	+	1.38	+
M.L.	Jamaica	+	0.16	-
J.S.	Nigeria	+	0.18	-
D.P.	U.K.	+	1.45	+
M.R.	U.K.	+	0.65	+
R.R.	U.K.	+	0.87	+
R.D.	U.K.	+	2.25	+
C.M.	U.K.	+	0.70	+
G.N.	St. Vincent	+	1.00	+
B.A.	CSSR	ND	2.95	+
H.M.	CSSR	ND	0.75	+

ND Not Done

Mean Positive ELISA Value A_{405} = 1.30 (± .77 SD)

Percent Positive FAT = 100% (n = 11)

ELISA = 76.9% (n = 13)

Fig. 39 Relationship between Toxocara ELISA value and toxocariasis clinical score in 49 patients with eosinophilia and presumed visceral toxocariasis.
Calculated linear regression line is $y = 0.575x + 0.039$.
Correlation coefficient $r = 0.64$ ($P < 0.001$).

ISA value and toxocariasis
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ne is y = 0.575 χ + 0.039.
(P <0.001).

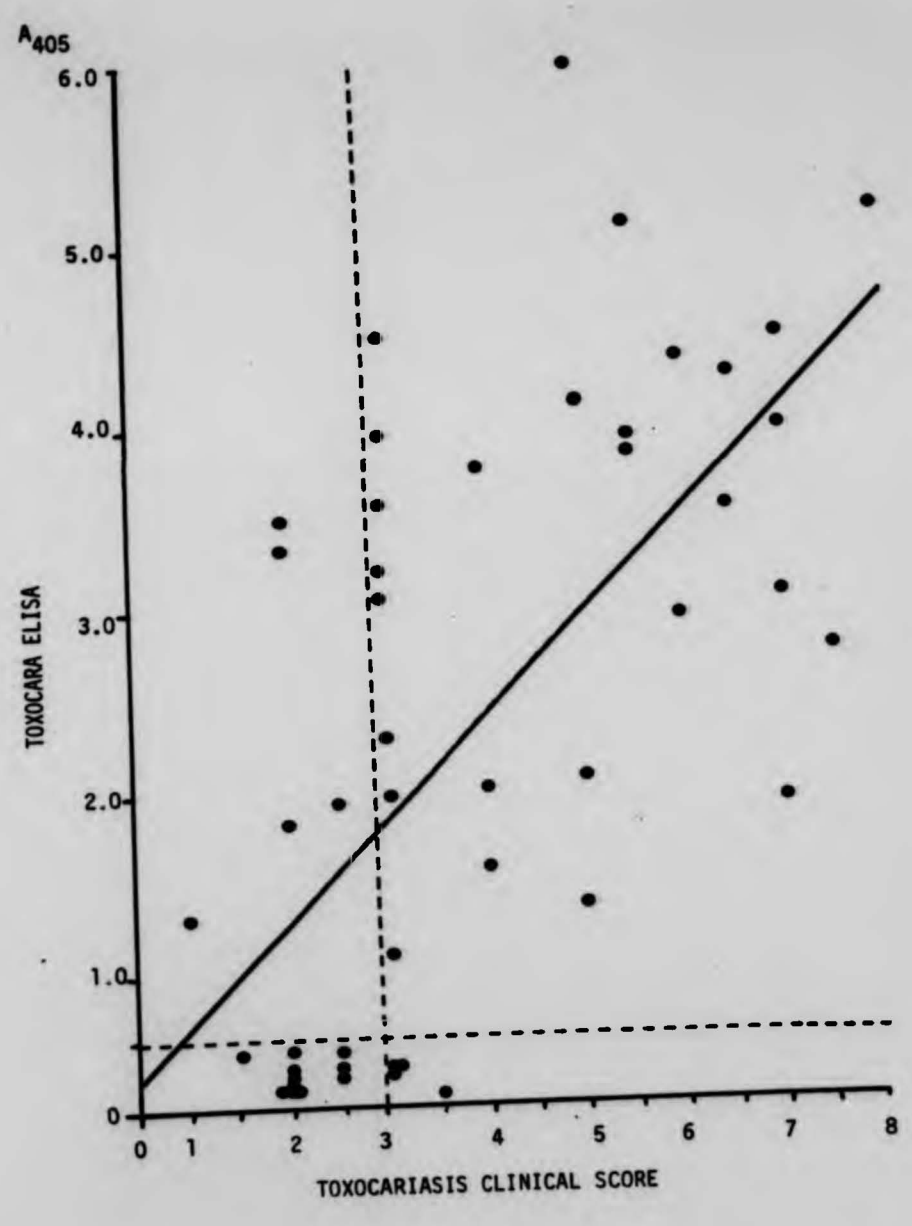

FIGURE 39

Fig. 40 Dynamics of positive Toxocara ELISA values in visceral (———) and ocular (-----) toxocariasis.

ISA values in

---) toxocariasis.

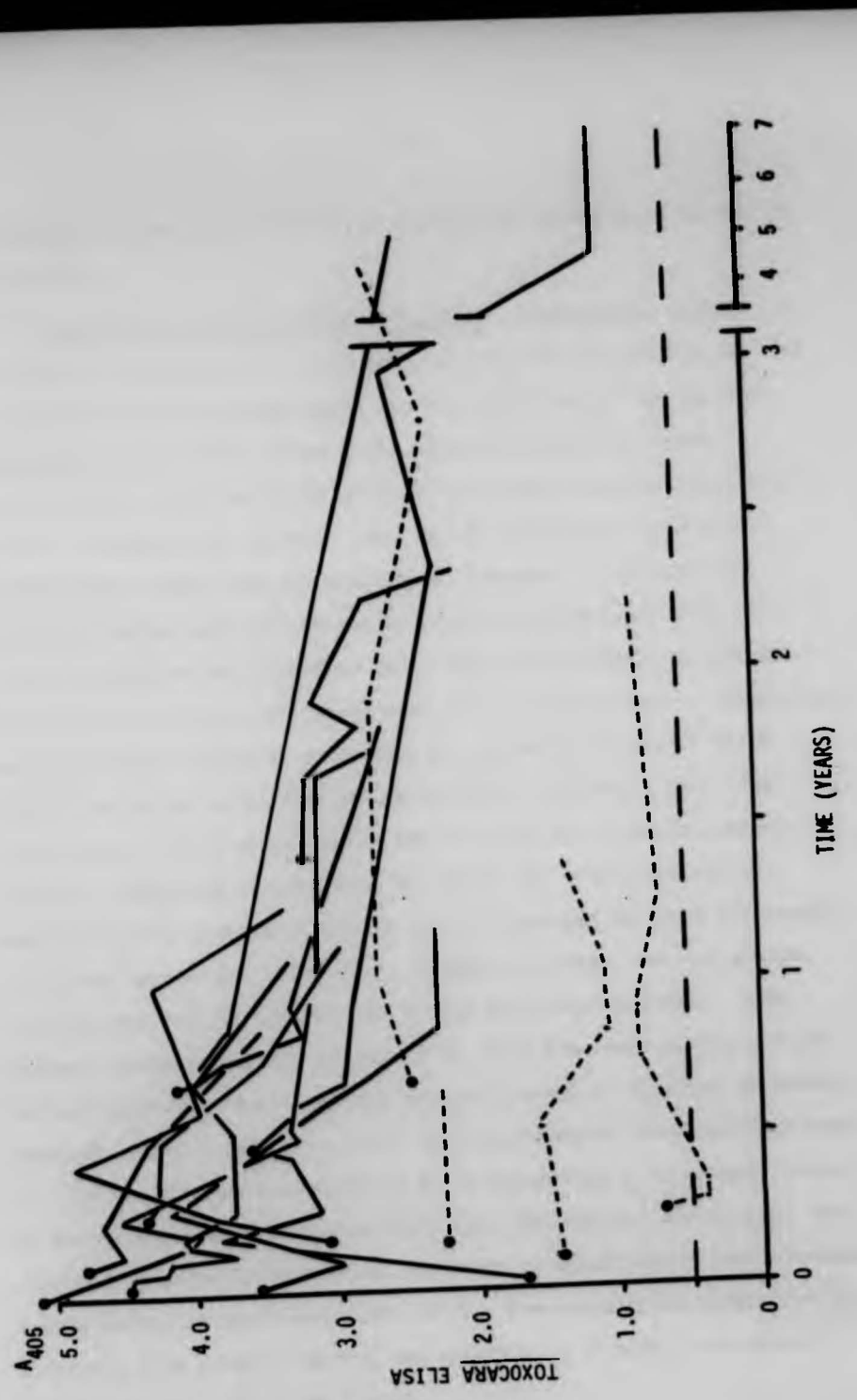

FIGURE 40

of positive values (for up to four years) with no evidence of decline (Table 17).

Ocular Toxocariasis in Rhesus Monkeys. Experimental ocular toxocaral lesions could be induced by intraocular inoculation of live larvae into rhesus monkeys which had been previously infected with Toxocara canis at least seven weeks previously and which were serologically positive at the time of intra-ocular administration of larvae. Experimental lesions could not be induced by intra-ocular infection in normal (non-presensitized) monkeys. In susceptible animals, lesions were manifested by retinal haemorrhages (Fig. 41a) first observed on the second or third days post-inoculation and which were often, but not always associated with retinal vessels. Haemorrhages resolved without residual pathology or evolved to a central white lesion surrounded by an area of haemorrhage or necrosis (Fig. 41b). Fluorescence angiography, during the first 48 hours showed nothing unusual. Beginning between five to eight days post-inoculation, additional small, white, discrete lesions appeared in areas previously free from haemorrhage (Fig. 41c). These were often, but not always, located near retinal vessels and slowly increased in number. Some lesions increased rapidly in size (Fig. 41d) and later healed without retinal scarring. Small lesions tended to occur in clusters or become confluent along a path (Fig. 41e) and occassionally healed without trace.

In Mmu 121, presensitized by a six-month-long trickle-dose infection 16 months prior to intraocular challenge, the initial haemorrhage was followed by white lesions which continued to appear and became innumerable over a period of seven weeks (Fi. 41f). Endophthalmitis progressed to a vitreal haze at eight weeks, and cataract at 18 weeks, preventing further observation of the fundus.

Fig. 41 Retinal lesions induced during experimental ocular toxocariasis in rhesus monkeys.

(a) Mmu 121 - Trickle-dose presensitized animal at 4 days post-ocular inoculation of T. canis larvae.

(b) Mmu 130 at 9 days post ocular inoculation

(c) Mmu 121 at 11 days "

(d) Mmu 121 at 15 days "

(e) Mmu 121 at 21 days "

(f) Mmu 121 at 28 days "

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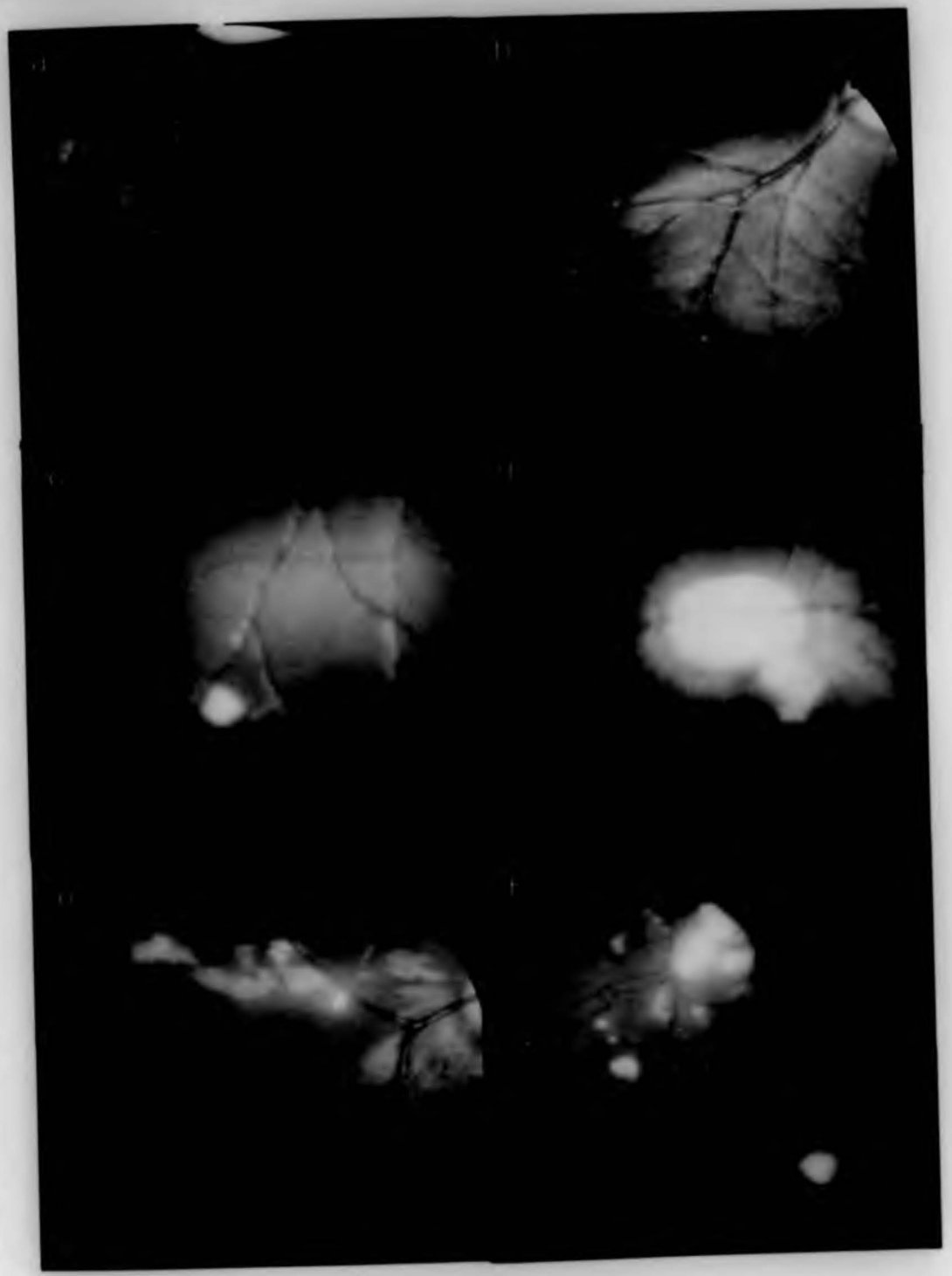

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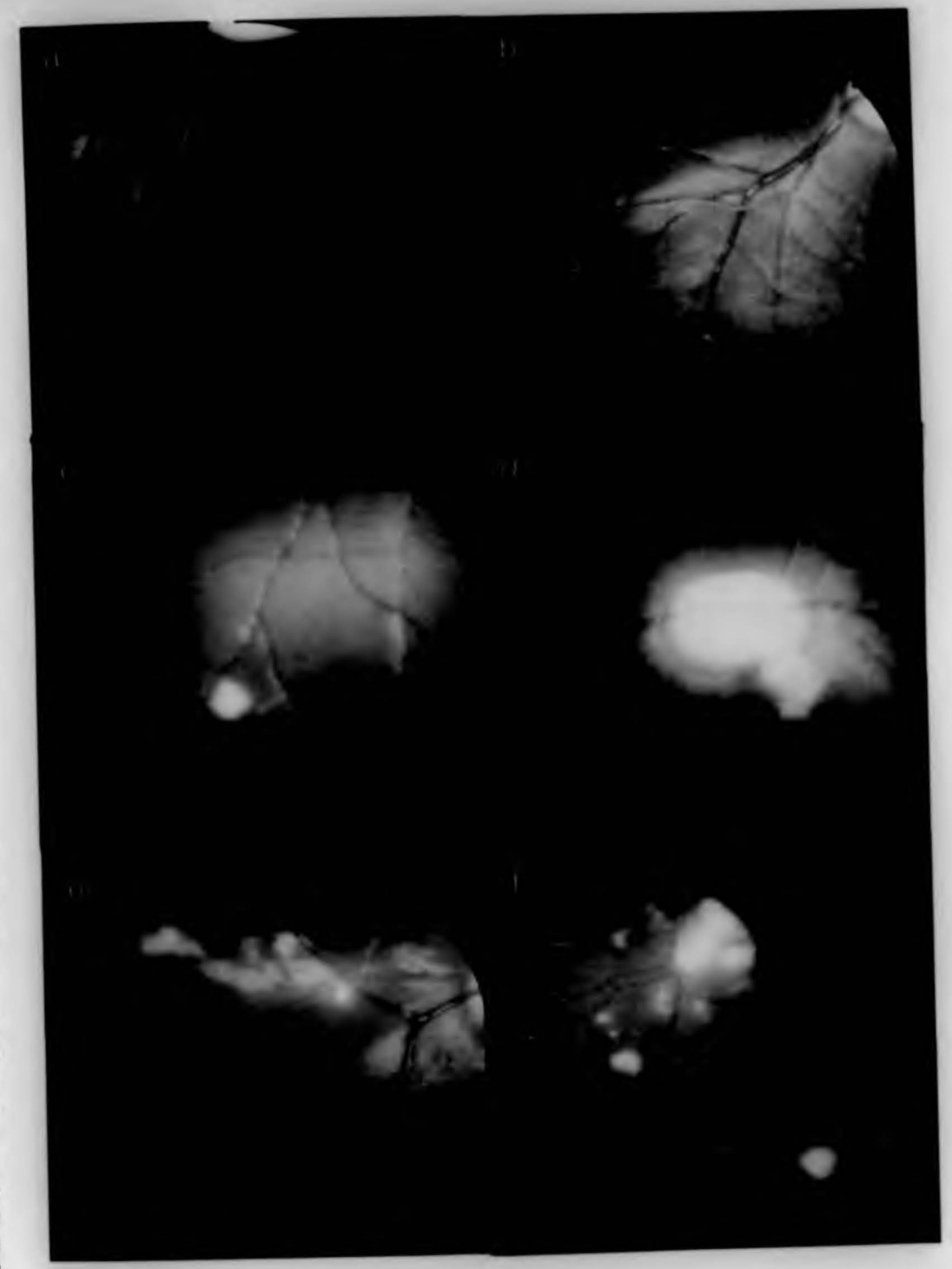


In Mmu 130, presensitized by a single-dose infection 18 months prior to intraocular challenge, a similar pattern, but milder course, was followed initially. Haemorrhages secondary to central white areas were noted in the superior nasal and temporal quadrants within the first seven days, followed by the progressive appearance of many small, discrete, white lesions in the inferior and superior temporal quandrants by 18 days. Vitreal haze obscured the superior temporal quadrant at 4 weeks, and large white lesions appeared in the inferior temporal quadrant. Both lesions and vitreal haze spontaneously regressed at 9 weeks coincident with the 11^{th} week of pregnancy in this animal.

In Mmu X53, which was unsensitized at the time of primary intraocular inoculation, no haemorrhages or lesions were detected throughout 6 weeks despite seroconversion in Toxocara ELISA by the first week post-infection. Secondary intraocular challenge at six weeks post-primary infection resulted in a white lesion and retinal haemorrhage within 48 hours. Haemorrhages persisted until 9 days and were followed by increasing numbers of white lesions until seven weeks post secondary infection. At this time lesions were at the maximum size attained. The largest lesion, in the superior temporal quadrant, measured several disc diameters. By 66 days lesions had decreased in number and size resulting in a few peripheral clusters of small lesions, or mottled depigmentation which showed no variation in location throughout the remainder of the observation period (134 days). This animal had the mildest reaction of three presensitized animals.

In Mmu 12, which was unsensitized at the time of primary intraocular inoculation of larvae, no haemorrhages or lesions were detected throughout the study period (eight weeks).

Toxocara ELISA IgG values in presensitized animals showed a weak

Fig. 42 IgG anti-*Toxocara* antibody responses during experimental visceral and ocular toxocariasis in rhesus monkeys.

▲ Mmu 212
■ Mmu 130
● Mmu X53
○ Mmu 12

ponses during experimental

is in rhesus monkeys.

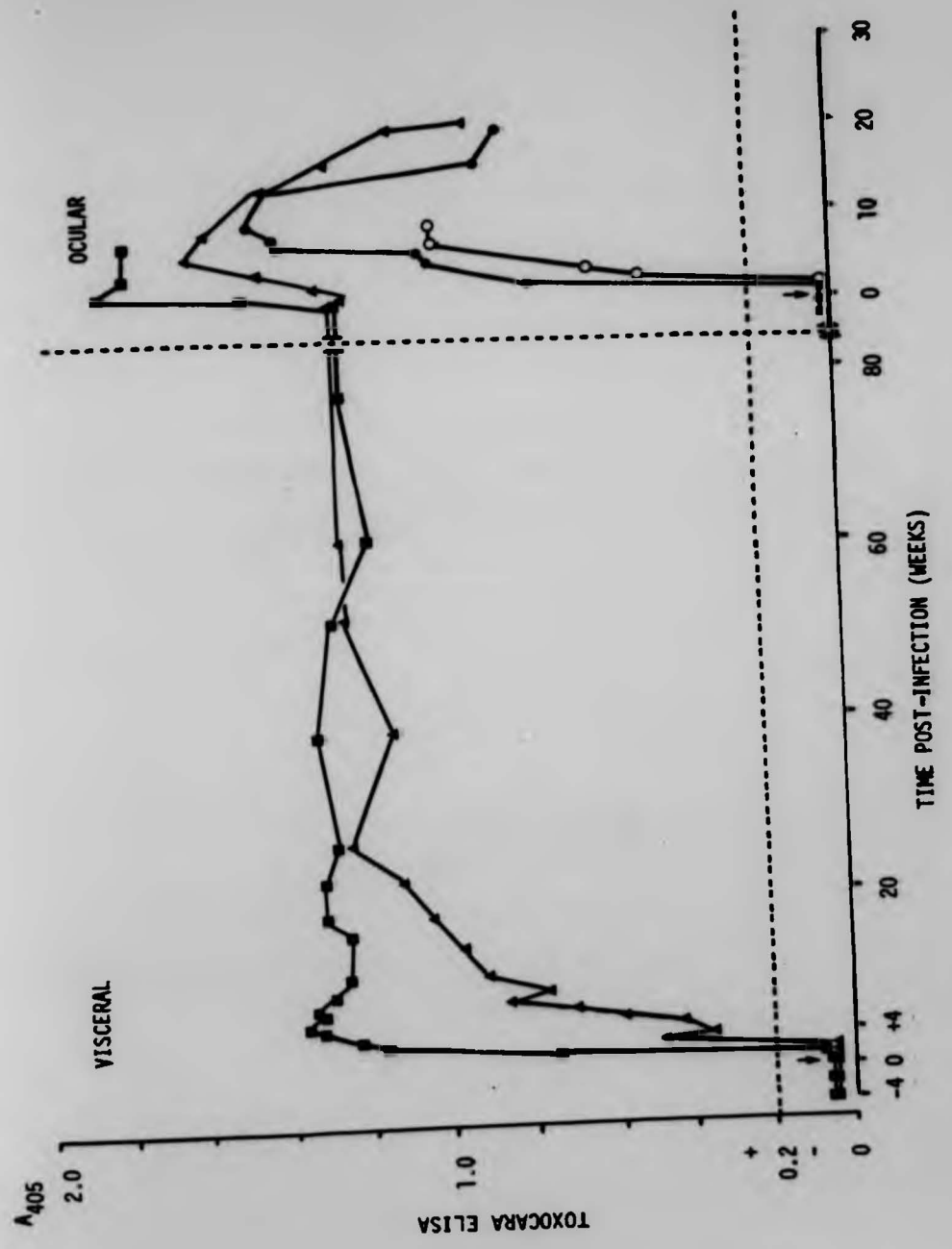
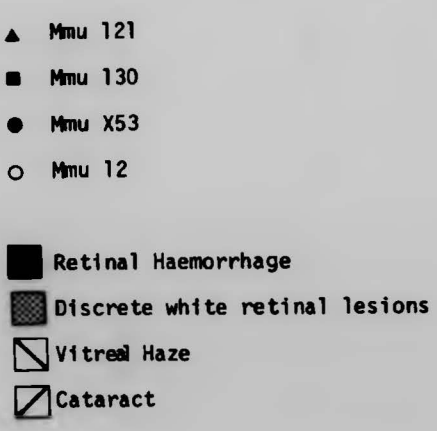


FIGURE 42

Fig. 43 IgG anti-Toxocara antibody responses in relation to ocular symtomatology during experimental ocular toxocariasis in rhesus monkeys.



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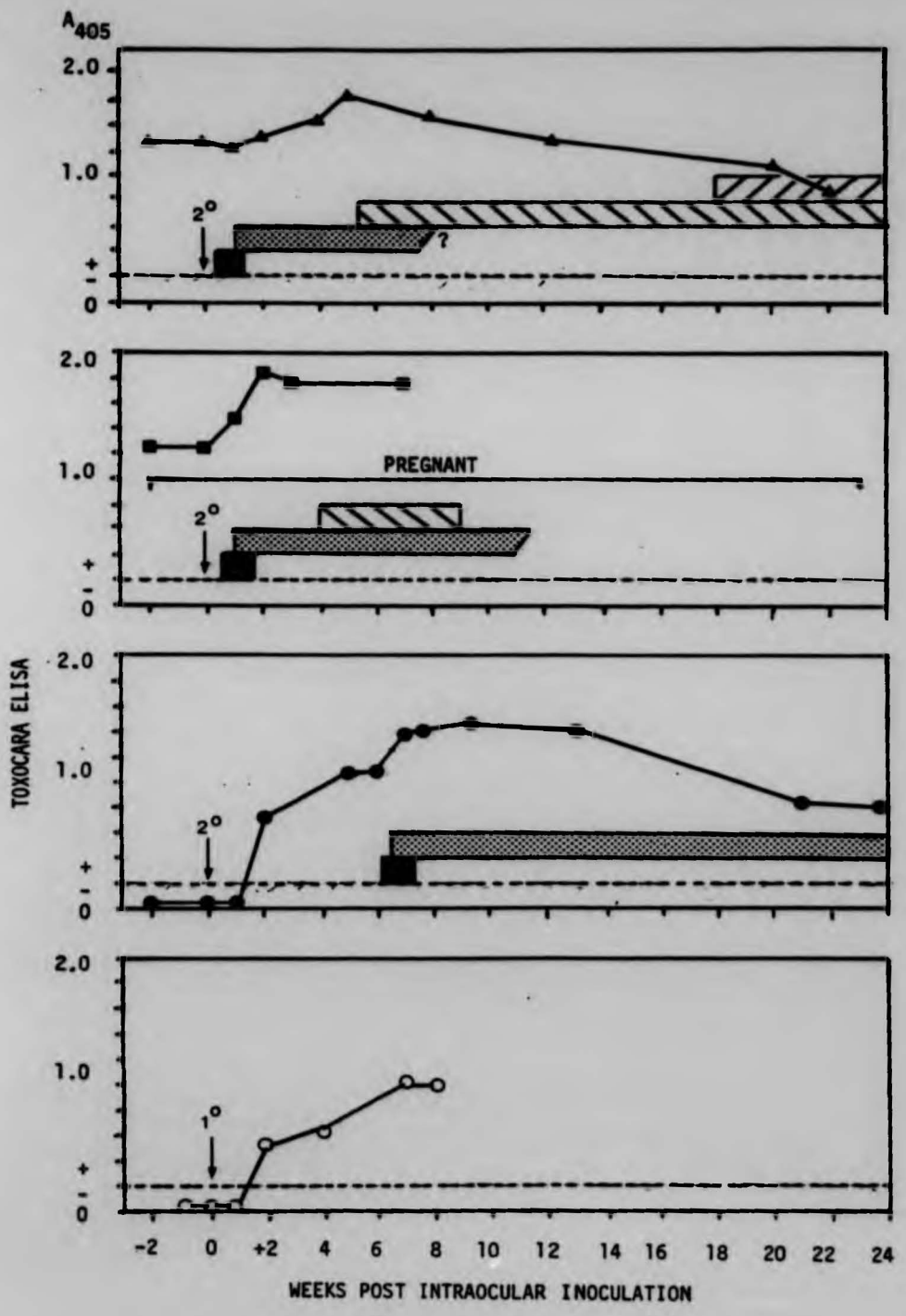

FIGURE 43

Fig. 44 Peripheral eosinophilia during experimental visceral and ocular toxocariasis in rhesus monkeys.

▲ Mmu 121

■ Mmu 130

● Mmu X53

○ Mmu 12

g experimental visceral

esus monkeys.

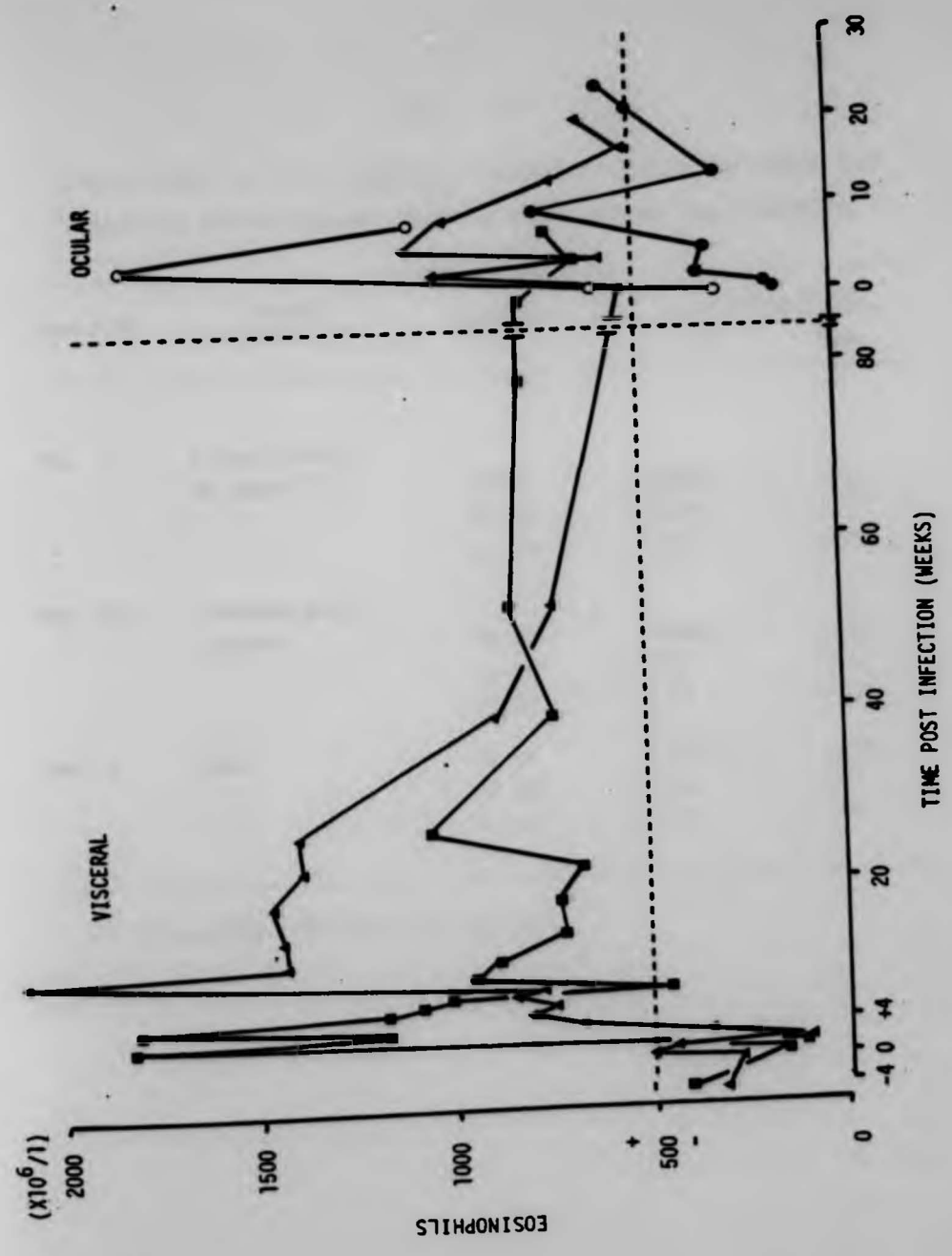

FIGURE 44

TABLE 19

COMPARISONS OF ANTI-*TOXOCARA* ANTIBODY LEVELS IN SERUM AND AQUEOUS HUMOR DURING EXPERIMENTAL OCULAR TOXOCARIASIS

Rhesus No.	Ocular Symptomatology	Specimen*	Toxocara ELISA	
			Titre	A_{405}
Mmu 121	Endophthalmitis 22 weeks P.I.	Serum	1:8000	0.90
		Rt eye	1:1100	1.10
		Lt eye	< 1:20	0.02
Mmu X53	Discrete white Lesions	Serum	1:8000	0.91
		Rt eye	< 1:20	0.01
		Lt eye	< 1:20	0.01
Mmu 12	None	Serum	1:8500	0.98
		Rt eye	< 1:20	.02
		Lt eye	< 1:20	.02

* All intraocular infections in right eye.

secondary humoral response following intraocular challenge, peaking at two to four weeks post-secondary infection with peak values higher than those observed during the preceding systemic infection. Serological responses declined to or below pre-ocular infection levels by 16 weeks (Fig. 42, 43). Eosinophilia was a mild and transient response to intraocular challenge (Fig. 44). Toxocara IgM ELISA was negative throughout all intraocular infections, including those with primary exposure to Toxocara by the ocular route.

Toxocara ELISA was also used to assay local IgG anti-Toxocara antibody production in aqueous humor obtained by anterior chamber paracentesis of infected and contra-lateral eyes. The low protein content of aqueous humor necessitated the experimental determination of the optimal aqueous sample dilution (10^{-2}) for use in ELISA. Results of assays of aqueous humor at 10^{-2}, and of matched serum samples at 10^{-3}, are provided in Table 19. In toxocaral endophthalmitis the ratio of Toxocara IgG ELISA values measured between the infected eye and its fellow (non-infected) eye was 55:1, and between the values in the aqueous of the infected eye, and in matched serum was 1.22:1. Both results indicate significant local antibody production in the infected eye and no antibody, either produced or passively acquired, in the fellow eye. In those monkeys with discrete toxocaral retinal lesions, no antibody was detected in infected eyes.

Chemotherapy as Monitored by Toxocara ELISA. The influence of chemotherapy on anti-Toxocara responses in 6 to 8 months old, naturally infected, beagle dogs who had received a course of 5% Diethylcarbamazine/ 2% Morantel (Banminth®) administered in four treatments at six week intervals commencing at three weeks of age (Fig. 45), revealed 15.4% positive, compared to 96.6% positive in age-matched, untreated dogs, and 0% positive in age-matched ascarid naive dogs. The influence of

Fig. 45 Influence of regular treatment with Diethylcarbamazine/Pyrantel compared with non-treatment on anti-Toxocara ELISA responses in naturally infected dogs.

Fig. 46 Influence of a single treatment with Dichlorophen/Toluene or Diethylcarbamazine/Pyrantel (Morantel) on anti-Toxocara ELISA responses in naturally infected dogs.

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nses in naturally infected

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(Morantel) on anti-Toxocara
infected dogs.

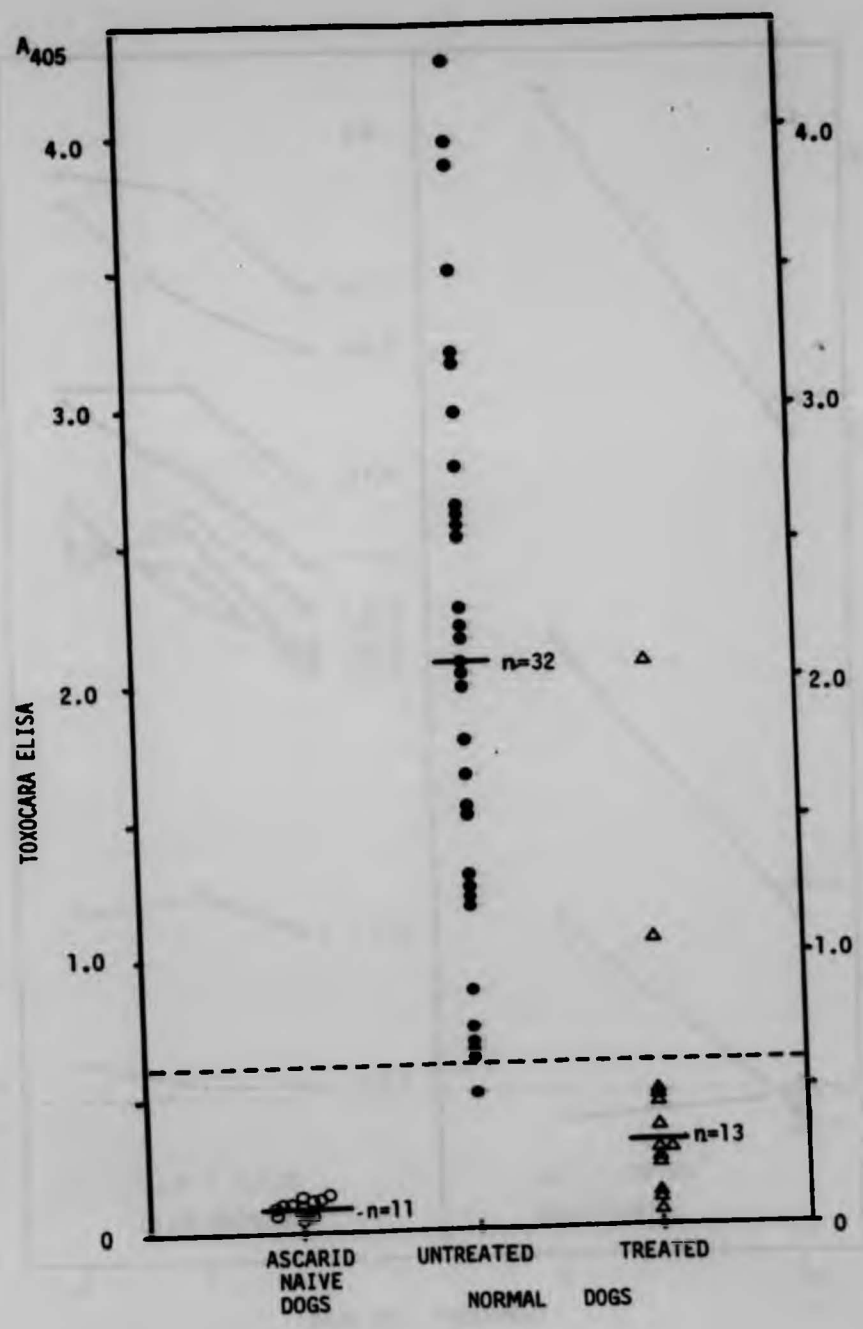

FIGURE 45

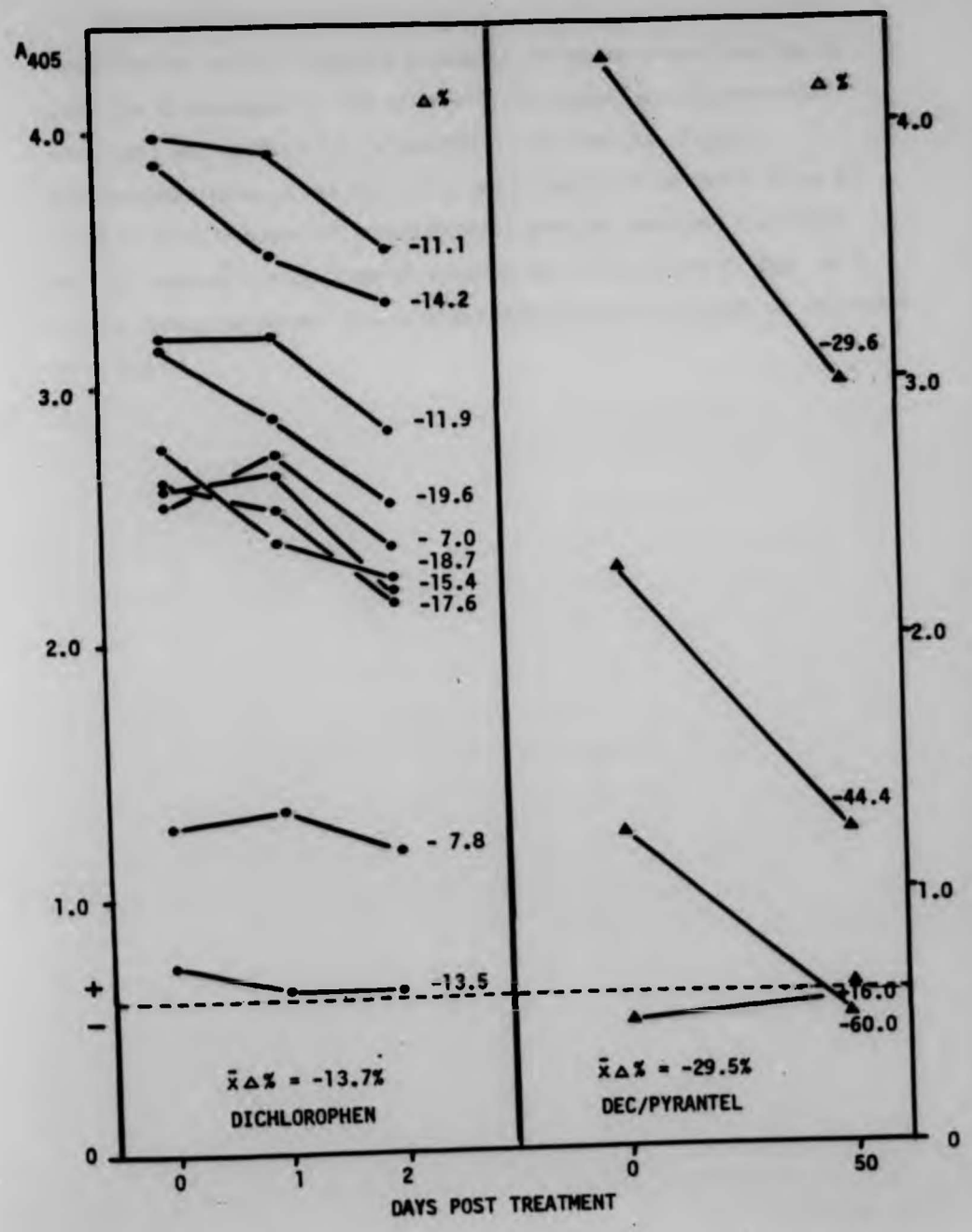
A405
Δ%
4.0
3.0
2.0
1.0
+
−
0
-11.1
-14.2
-11.9
-19.6
- 7.0
-18.7
-15.4
-17.6
- 7.8
-13.5
x̄Δ% = -13.7%
DICHLOROPHEN
Δ%
-29.6
-44.4
-16.0
-60.0
x̄Δ% = -29.5%
DEC/PYRANTEL
4.0
3.0
2.0
1.0
0
0
1
2
0
50
DAYS POST TREATMENT

FIGURE 46

chemotherapy on anti-*Toxocara* responses following single treatments with 50% dichlorophen / 50% toluene (Vermithana®) or DEC/Morantel (Fig. 46) was manifested by a decline of all positive titres. Dichlorophen/toluene resulted in a mean dimunition of ELISA value by 13.7% within 48 hours of administration while DEC/Morantel treatment was followed by a reduction of response by 29.5% within 50 days of a single dose. Responses in untreated dogs remained constant or increased with age.

DISCUSSION

"An immunoassay result is valueless until it, together with its interpretation, has reached the clinician" (Challand, Goldier and Landon, 1974). The correct interpretation of results requires qualitative and quantitative information concerning (a) technical aspects related to the antigen and test method (Chapters II and III) (b) performance characteristics of the assay, ie. sensitivity specificity, etc. (Chapter III), and (c) natural biological phenomena related to the disease in question (Grab and Pull, 1974). The results of this chapter describe some of the diagnostic applications to which Toxocara ELISA may be put and, at the same time, provide insights into how the assay results can be related to some of the biological phenomena associated with toxocariasis. To complete the evaluation of its serodiagnostic applicability, consideration was also given to how best the results may be communicated to the clinician.

Regarding the immunodiagnosis of the visceral form of toxocariasis, it was anticipated from studies in Chapter III using experimental trickle-dose infections in rhesus monkeys, that the magnitude of the Toxocara ELISA A_{405} value would not be related to worm burden, but rather to the mode of infection. In practice, ELISA values in man were found to be approximately proportional to the toxocariasis clinical score, (ie. the weighted multiplicity of symptoms and signs) which in turn should be correlated with the numbers of invading larvae (Beaver, et al,1952). Although this latter claim may be so, it was noted that peak titres in some cases occurred later in the course of infection than would be expected during a single dose infection, suggesting that some patients were still acquiring a trickle-dose mode of infection, even

after initial diagnosis of the condition. Therefore, ELISA values which continue to rise in patients with clinical toxocariasis indicate the need for a careful reassessment of the epidemiological setting of the patient. It also follows that low intensities of infection, acquired over a period of time, may contribute significantly to the morbidity pattern of toxocariasis.

The relationship of ELISA values to low Toxocara clinical scores illustrates the inadequacy of any scoring system for toxocariasis. Patients with eosinophilia plus only one symptom compatible with toxocariasis, were deleted from the group used to calculate the sensitivity parameter in Chapter III. Yet these patients have either strongly positive or unequivocably negative ELISA values with no intermediate spectrum. This indicates that the scoring method used, although necessarily objective, has little relation to reality when dealing with those cases of clinical toxocariasis with few symptoms. In the absence of alternatives, the sensitivity parameter of Toxocara ELISA (78.5%), estimated in Chapter III by this method, probably represents an underestimate of assay sensitivity. This was expected since, if any scoring method worked well, there would be no need for serological confirmation of the diagnosis. This therefore, highlights the value of Toxocara ELISA as an adjunct to the clinical diagnosis of visceral toxocariasis and the differential diagnosis of unexplained eosinophilia and hepatomegaly syndromes.

Because Toxocara ELISA measures the host response to products of living and migrating larvae, it may be considered that ELISA values should be better indicators of active infection and of efficacy of treatment than would be responses to somatic antigens, which persist after death of the parasite. It is difficult to evaluate this theme in

man, no record of decline of ELISA value into the negative range was recorded. Considering chemotherapy, ELISA values did not decrease appreciably in infected but, untreated rhesus monkeys, but did decline consistently in man following chemotherapy with thiabendazole. Whether this decline was due to an anthelminthic action of thiabendazole on tissue larvae, or due to its anti-inflammatory effects (Van Arman and Campbell, 1975) or simply a function of the natural history of infection in man cannot be determined from this study. The efficacy of thiabendazole in treatment of toxocariasis is contentious. It was found effective by Nelson, McCornell and Moore (1966), Aur, Pratt and Johnson (1971), and Congdon and Ames (1973) but ineffective, in favour of diethylcarbamazine (DEC), by MacDougall (1969), Wiseman, Woodruff and Pettitt (1971) and Dafalla (1972). Recently, Nicholas and Stewart (1979) have shown that the benzimidazoles (fenbendazole and oxfendazole) kill Toxocara larvae in mouse tissues and may kill larvae in dog tissues (Dubey, 1979). Since the tissue of female dogs is the prime reservoir of toxocariasis, chemotherapy of toxocariasis in canidae is of interest to many. Knowledge of the dynamics of the humoral response in such studies is potentially useful and Toxocara ELISA was selected for use by one such group (Astra Pharmaceuticals, Sweden). The two drugs used in their study were known to be effective against intestinal phases only. Dichlorophen kills adults in the intestine and causes release of antigens which may be processed by gut lymphoid tissue to raise an anti-larval response systemically. DEC/Morantel immobilizes adult worms which are expelled intact from the gut.

Neither preparation had any hitherto recognized effect on toxocaral larvae in tissues nor any immunosuppressive effects. It remains questionable whether the observed anthelminthic effect of DEC

against other tissue invasive nematodes (eg. onchocercal microfilariae) results from direct action by the drug on parasites or from modulation of the host's immune response to the parasite (Rew, 1978). It is difficult therefore to explain the rapid drop in ELISA value within 48 hours of treatment with dichlorophen and the inhibition of seroconversion in DEC/Morantel treated animals. Others have recorded an even more dramatic drop of serologic titre, also in the canine response to nematode secretory antigen (Dirofilaria immitis) within two hours following DEC treatment (Desowitz, Barnwell, Palumbo, Una and Perri, 1979) while levels of antibody to somatic antigens remained unaltered. It was postulated that treatment stimulated larvae to a high rate of secretory antigen production which effected a rapid change in antibody level through the production of immune complexes of antibody and secretory antigen and their subsequent clearance. DEC has recently been shown to be lethal to Onchocerca volvulus microfilariae in vitro (Langham and Kramer, 1980) an action long suspected in vivo, but unproven.

The results of this study also indicate that the regular use of DEC/Morantel during the first six months following birth in pups, substantially alters the serologic profile of treated populations compared to untreated. No serological studies of toxocariasis in canidae are available in the literature so it is not known whether this profile indicates that DEC/Morantel has immobilized (or killed) larvae in tissues, preventing release of secretory antigens, potentiated the immune elimination of larvae,or merely suppressed seroconversion in otherwise infected animals. The key finding is that Toxocara ELISA is sensitive to rapid changes in antibody level which may accompany chemotherapy, and may therefore be a tool useful to such studies.

Seroepidemiological studies are undertaken in Chapter V to further elucidate the normal humoral responses to toxocaral secretions in naturally infected dog populations.

Ocular toxocariasis in man is a realm of many unanswered questions: principally, why does ocular toxocariasis occur more frequently in older children and in the absence of the usual manifestations of visceral toxocariasis; and what are the pathophysiologic and/or immunologic mechanisms which decide the type of ocular lesion which ensues?

The literature contains many reports of cases of ocular toxocariasis, some histologically proved, in whom circulating anti-Toxocara antibody could not be demonstrated (Duguid, 1961b, 1963; Woodruff, 1970; Kagan, 1979; Walls, 1979; Schantz, Meyer and Glickman, 1979). In this study, the apparent sensitivity of Toxocara ELISA to the detection of ocular toxocariasis was 76.9%, a relatively low figure considering the demonstrated sensitivity of the assay and the fact that most of these cases had positive indirect fluorescent antibody titres against somatic toxocaral antigens. Like visceral toxocariasis, it is possible that not all cases of clinically diagnosed ocular toxocariasis are true cases, although this is less likely since most had responded to specific treatment for toxocariasis. In addition to the apparently low qualitative sensitivity, those patients with positive ELISA values had, as a group, significantly lower mean ELISA values compared to mean positive values in the visceral toxocariasis group. Combining the experience gained thus far with Toxocara ELISA, this suggests that primary systemic toxocaral infections were probably acquired by these patients years previously, or that they had very light infections acquired principally by a single dose. Since this assay has been shown

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to detect an infection dose of at least 10 larvae, it would suggest that some cases of ocular toxocariasis are due to very low total worm loads. The possibility that patients with ocular toxocariasis might show congenital differences in response to toxocariasis compared with patients with visceral toxocariasis must also be entertained in light of the significant difference between these two groups in persistance of positive ELISA values. The small number of patients for whom serial samples were available had persistant positive ELISA values for as long as five years without the usual decline rate seen in visceral toxocariasis. There have been no HLA tissue typing studies made of patients with ocular and visceral toxocariasis but considering the many different manifestations of these two clinical courses of infection, host factors must be examined more closely.

The uncertainties described which attend studies on human ocular toxocariasis, and especially the difficulty of determing the history of experience of the patient with prior systemic toxocaral infection, prompted the development of the animal model described in this Chapter. Since the work of Suyemor (1925) describing intravascular Ascaris lumbricoides larvae circulatory through guinea pig eyes 30 hours after peroral infection, rodents, and in particular, mice, rats, and guinea pigs, have been used as models of intraocular nematode infection. Recent studies in these models have taught: (1) that endophthalmitis can be induced in the guinea pig eye by intraocular Type III and Type IV hypersensitivity reactions (Silverstein and Zimmerman, 1959); (2) that intraocular Type I (IgE mediated) hypersensitivity reactions can occur in guinea pigs passively sensitized with Ascaris summ serum (Soulsby, Rockey and Laties, 1974); (3) that primary intravitreal injection of live Ascaris suum larvae evokes a local (intraocular), but not systemic (serum) IgE response (six day P-K test), while killed

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larvae do not, and that following secondary intravitreal challenge with either live or killed larvae, both evoke local and systemic IgE production (Donnelly, Rockey and Soulsby, 1979); (4) that ascarid antigens can be detected in aqueous humor by a reversed Prausnitz-Kustner (P-K) test up to 189 days post intravitreal inoculation (Donnelly, Rockey, Stromberg and Soulsby, 1979).

The most recent intravitreal infections in guinea pigs (Rockey, Donnelly, Stromberg and Soulsby, 1979) indicated that Toxocara second-stage larvae attracted an eosinophil rich infiltrate within 24 hours which persisted for at least 51 days. By day 12, larvae were surrounded by granulomas consisting almost entirely of eosinophils, many of which were firmly adherent to the larval cuticles. Degranulation of adherent eosinophils and deposition of granules was observed, as was the ingestion of granules by larvae. Similar responses were observed to live Ascaris larvae and to Ascaris secretory antigens, but not to dead Toxocara or Ascaris larvae, which evoked only a minimal inflammatory reaction with a few eosinophils and a diffuse mononuclear cell infiltrate. Dense eosinophil infiltration of the choroid, not immediately adjacent to a parasite larva, was accompanied by destruction of the overlying retina. Intraocular inflammatory responses were most severe in animals which had been systemically pre-sensitized to ascarid antigens.

Such studies, although useful as models of isolated immune functions, have little relevance to the natural history of ocular toxocariasis. Firstly, the immunologic responses of guinea pigs, especially those of the Type I category,cannot easily be extrapolated to man. Secondly, the intravitreal infections in guinea pigs required the inoculation of 5000 larvae directly into the vitreous, whereas ocular toxocariasis in man results from the invasion by one or a few

larvae, entering via the retinal or choroidal blood vessels. Thus it would seem logical that the appropriate model should be to inoculate live larvae into the eye via the ophthalmic artery of a host whose orbital and vascular anatomy is more akin to that of man than is the guinea pig. Initial experiments by Woodruff and Hart (1978) attempted this approach in normal pigs, but failed to induce ocular lesions, despite a rapid and vigorous seroconversion in the infected animals. Similar results were obtained in this study when larvae were introduced via the ophthalmic arteries of two normal rhesus monkeys. However, larvae inoculated by this route, into three rhesus monkeys previously exposed to either systemic, or intraocular toxocaral infection, rapidly evoked, in sequence, many of the characteristics of ocular toxocariasis seen in man as isolated events, including: retinal haemorrhage; discrete posterior-pole and peripheral granulomatous lesions; and endophthalmitis leading to vitreal haze. Thus this model in rhesus monkeys is proposed as a more valid alternative to the guinea pig model for studies designed to elucidate the mechanisms of ocular toxocariasis in man.

The significant finding of these ongoing studies is that prior experience with toxocaral infection is required before the presence of an intraocular larva can result in a pathological response, and that a trickle-dose primary infection is a more potent sensitizing infection in this regard. The implication is that toxocaral ocular lesions are immunologically mediated. The reasons for this may be associated with the unique immunobiology of the eye. Except for the conjuctiva, the eye has no lymphatic drainage and is consequently unassociated with any local lymph nodes (Rahi and Garner, 1976). Processing of antigen has, therefore, to take place at a distant site before sensitized lymphocytes migrate towards the antigenic source. Furthermore, there is a tendency for intraocular antigen to remain sequestered within certain components of the eye such as the vitreous (Hall and O'Connor), and to be

separated from the systemic circulation by the blood - aqueous barrier (Allansmith, Whitney, McLelland and Newman, 1973). Most antibody present in the eye in response to intraocular antigen is produced locally by immunologically competent cells attracted from distant lymphoid tissue to the uvea and limbus, which behave as accessory lymph nodes. Thus primary intraocular infection would be expected to elicit a weak and much delayed intraocular immunologic response, possibly in the absence of a systemic immunologic response. In contrast, secondary ocular challenge in an animal, already highly sensitized systemically, should result in an earlier and much potentiated intraocular response.

These considerations may be relevant to the observations in the rhesus model, of rapid reaction to intraocular larvae in systemically presensitized animals and no reaction in naive animals. In immune animals, the intravascular progress of a larva through the eye may be retarded to a degree that a cellular response or Type IV reaction can be mounted to the immobilized larva, thus explaining the progressive increase of numbers and intensity of lesions. Alternatively, a retarded larva, slowly secreting antigen into an immune animal, may stimulate a Type III reaction in the eye since complement is present in the aqueous humor (Chandler, Leder, Kaufman and Caldwell, 1974). The increasing number of lesions, and their disappearence and reappearance at alternate sites on the retina - an unexpected observation - might also be explained by the effects of several larvae living and dying in the retina or choroid, or moving between the two, or by one or a few larvae, residing at one nidus until the host's reaction becomes too hostile, forced to move to a new location.

Since, at the time of secondary intraocular challenge, all animals had reached equivalent levels of serologic reactivity (ELISA values), one might argue this as evidence against an immunological mechanism,

considering the more severe ocular pathology in trickle-dose sensitized animals compared to high-dose sensitized animals. However trickle-dose infections may induce stronger functional immunity to tissue-invasive stages of ascarids and humoral antibody levels are not an index of cell-mediated responsiveness (Ogilvie and Jones, 1973). Furthermore it must be appreciated that some larvae, which survive the immune response evoked by trickle-dose infection, may be "adapted" and thus capable of long survival in the face of the response (Ogilvie and Love, 1974). It could not be determined whether the larvae entered the fundus via the choroidal vessels or the retinal vessels since both originate from the ophthalmic artery. Larvae which enter via the central retinal artery would be expected to gain access to the fundus through retinal arterioles or capillaries. However retinal arterioles are narrow, 8 to 15 μ in luminal calibre (cf. 20 μ diameter of T. canis L_2), and are without anastomoses. Retinal capillaries are anatomically unique and,being designed for gaseous rather than nutritive exchanges, are thick walled, with high internal pressures and flow rates (Hogan and Feeney, 1963). A larva entering by this route might become impacted in an arteriole or capillary, resulting in local, retinal ischaemia, or if the larvae escaped, resulting in haemorrhage. Lesions observed in the fundus were not always located near visible retinal arterioles or other retinal vessles, suggesting that larvae may have gained entrance from the choroid. Larvae entering via the choriocapillaris might have an easier time since these vessels anastomose extensively, being designed to provide the major portion of fluid and nutrients moving towards the retina, and are anatomically polarized such that the thinest aspect of the vessel wall is nearest to the retina (Bernstein and Hollenberg, 1965). Thus larvae entering by this route might easily invade the choroid and move periodically

between it and the retina, evoking the transient lesions observed.

The hypothesis that ocular toxocaral lesions are the expression of cell mediated immunity receives fortuitous support from an experiment in nature whereby rapid resolution of endophthalmitis was observed in an animal which had become pregnant and was thus experiencing suppressed cell mediated immune functions (Ogilvie and Jones, 1973; Tizard, 1977).

The use of aqueous humor as a diagnostic sample in the differential diagnosis of ocular toxocariasis and retinoblastoma is gaining acceptance, since anterior chamber paracentesis is a reasonable procedure if retino-blastoma cannot be ruled out (Shields, Lerner, and Feldberg, 1977). In such samples the aqueous: serum ratios of lacticdehydrogenese (LDH) are reliably increased above 1.5 in cases of retinoblastoma (Feldberg, McFall and Schields, 1977; Swartz,Herbst and Goldberg, 1974) but are less than 0.50 in other conditions including nematode endophthalmitis. If specimens are collected for LDH ratios, the small volumes of sample required by Toxocara ELISA, would allow simultaneous estimation of intraocular IgG responses to Toxocara. Local antibody production occurs only in response to intraocular infection or immunization, is specific to the infected eye, and can occur in the absence of a systemic response (Thompson and Olson, 1950; Witmer, 1964; Allansmith et al. 1973; Rahi and Garner, 1977). Biglan, Glickman and Lobes (1979), using ELISA have reported aqueous:serum antibody ratios greater that unity in four of six cases of ocular toxocariasis, endophthalmitis showing the highest ratios (up to 8-fold higher than serum antibody titres). In such studies it is necessary to allow for the aqueous:serum protein ratio which in the rhesus is 0.005:1 (Gaasterland, Pederson, MacLelland and Roddy, 1979). In Toxocara ELISA specimens of aqueous are tested at a dilution of 10^{-2} (cf. 10^{-3} for

serum).

In experimental ocular toxocariasis, intraocular antibody was detectable during endophthalmitis but not during the discrete lesion (granuloma?) phase. Serum antibody levels were high in both forms. It follows that a negative intraocular antibody result cannot rule out a toxocaral aetiology, but a positive result, especially one with a high aqueous:serum quantitative ratio, is useful evidence in favour of a diagnosis of ocular toxocariasis.

These serological studies of ocular toxocariasis in man and rhesus monkeys support the hypothesis that ocular toxocariasis is a late manifestation of previously acquired systemic toxocariasis possibly acquired as low-dose and trickle-dose infections. The lesions seen are probably immunologic in nature, and possibly due to cell mediated responses. These findings help explain why patients with ocular toxocariasis are older and why their serological responsiveness to toxocaral antigens is relatively weak.

SUMMARY

Toxocara ELISA was evaluated in practice, in a variety of serodiagnostic applications for visceral and ocular toxocariasis in man.

In visceral toxocariasis: positive ELISA values were found to persist indefinitely; rising values were encountered infrequently; peak values were high and occurred within one month of onset of symptoms; all peak values tended to decline with time, with a mean rate of -1.22 A_{405} units per year, before reaching the past exposure range (A_{405} = 0.50 to 1.50). It is suggested that delayed peak values may reflect current acquisition of toxocaral infection by the patient.

In ocular toxocariasis in man, 76.9% of clinically presumed cases had detectable antibody to *Toxocara* larval secretions. Of these, the mean ELISA responses were significantly lower than those seen in visceral toxocariasis, and the majority corresponded to the level associated with current light infection or past infection. Of those patients followed serially, ELISA values were not seen to decline with time. These data are consistent with the hypotheses that ocular toxocariasis occurs in low dose infections, or following past visceral infections.

An experimental model of ocular toxocariasis was developed in the rhesus monkey and is proposed as a more realistic model than those in current use. Experience from the model suggests that prior visceral toxocariasis is required for the manifestation of pathological responses to toxocaral larvae entering the eye via the ophthalmic artery. Following intraocular challenge in *Toxocara* presensitized animals, retinal heamorrhage was observed during the first week,

followed by the appearance between two and seven weeks post-challenge, of increasing numbers of small, white, discrete lesions, usually in the peripheral retina. In a trickle-dose presensitized animal, endophthalmitis with vitreal haze evolved by eight weeks. In less sensitized animals, lesions gradually resolved, leaving a few areas of mottled depigmentation in the peripheral retina by 20 weeks. Intraocular inocualtion of larvae in normal, unsensitized animals resulted in no detectable pathology. In sensitized animals, intraocular challenge resulted, in a weak secondary IgG anti-Toxocara serological response which returned to or below prechallenge levels within 20 weeks, Eosinophilia was also weak and transient. During toxocaral endophthalmitis, local production of IgG anti-Toxocara antibody could be demonstrated in aqueous humor by ELISA.

The use of Toxocara ELISA to monitor the serological response to experimental anthelmintic chemotherapy was demonstrated in canid experimental models.

CHAPTER V

SEROEPIDEMIOLOGIC APPLICATIONS OF *TOXOCARA* ELISA

INTRODUCTION

The true extent of morbidity in the world due to parasitic agents is not precisely known (Lobel and Kagan, 1978). Because parasitic, and especially nematode infections, are ubiquitous in man (Stoll, 1947; Bloom, 1979) and because they are often chronic in nature, the health and energy of populations or sub-populations may be subtly undermined. Data describing the prevalence and incidence of parasitism are necessary to permit fuller understanding of host-parasite relationships and to permit optimal use of available resources in public health programmes. It is in this context that seroepidemiology can play an important role, since large population groups can be studied efficiently, to provide much of the essential information (Lucas, 1976).

The seroepidemiology of toxocariasis has been the subject of relatively few studies (reviewed in Chapter I). Nevertheless, the greater part of our knowledge regarding the nature and extent of toxocariasis in man has arisen from these, and is mainly provided by skin test data for Type I hypersensitivity to toxocaral adult antigens (pioneered by Woodruff and his co-workers; Woodruff, 1970). The period prevalence of toxocariasis, as determined by skin tests, indicated the existence of wide-spread asymptomatic infection in man. Prevalence rates were found to vary greatly between populations and ranged from approximately 2% in temperate and sub-tropical regions to 30% in tropical climates. This finding would, at first, seem inconsistent with the uniformly high prevalence of toxocariasis in canidae and felidae

populations, and the co-existance of these companion animals with man throughout virtually all of his habitats. The important epidemiologic associations between toxocariasis and other forms of ill-health such as poliomyelitis, epilepsy and asthma have also been founded largely on skin test data (Woodruff, Bisseru and Bowe, 1966; Wiseman and Woodruff, 1968). It has been suggested that the validity of skin test results should be confirmed by other means (Ball, Voller and Taffs, 1971) however reference serodiagnostic tests for toxocariasis, such as the indirect fluorescent antibody assay (Bisseru and Woodruff, 1968) and the bentonite flocculation and haemagglutination assays (Kagan et al. 1959), were found to be either impractical for large scale surveys (IFA) or too insensitive (BF, HA) for use in seroepidemiology.

The development of Toxocara ELISA offers a practical means to reassess these earlier epidemiologic findings, and to study in greater depth the nature and distribution of toxocariasis. Toxocara ELISA has been shown to detect antibody to biologically relevant antigens of the infective larval stage (Chapter II); to have both high and defined levels of sensitivity and specificity (Chapter III); and to be capable of detecting antibody to past infections (Chapter IV). The assay is inherently more economical, efficient, and reproducible when testing large numbers of sera. The most important advantage of Toxocara ELISA, for seroepidemiologic applications, is its potential ability to describe the distribution of serological reactivities of individuals within a population in objective, numerical terms, on a continuous scale, with high precision and resolution.

This chapter describes several seroepidemiologic applications of Toxocara ELISA including: an assessment of toxocariasis endemicity in man in relation to certain geographic and political parameters, in

relation to age and sex within populations, in relation to specific sub-populations, and in relation to other forms of morbidity. The first seroepidemiologic survey of toxocariasis in dogs is also described.

MATERIALS AND METHODS

Toxocara ELISA. The Toxocara ELISA method using larval secretory antigen, was adapted to measure either human IgG responses (Chapter III) or canine IgG responses (Chapter IV) as required.

Filter Paper Blood Samples. A modification of Toxocara ELISA was designed for use with finger-prick capillary blood samples collected and stored dry on absorbant paper (Whatman No.2 filter paper). Standardization of ELISA for capillary blood was as follows: From a patient with clinically diagnosed, serologically positive toxocariasis, simultaneous specimens were obtained as capillary whole blood on filter paper and as venous blood serum. A 6 mm diameter disc of filter paper, fully impregnated with dried blood,was punched-out using a single-hole stationary punch (Ryman, Vangaurd) into a tube containing 1.5 ml of PBS-T. Blood was eluted overnight at $4^{o}C$ before simultaneous two-fold titrations of eluate and serum were tested in the standard Toxocara ELISA. ELISA dose-response curves of eluate and serum were plotted and the log-distance between the linear components of the curves was taken as the dilution factor relating eluate to serum. From this, it was determined that a 6 mm disc of dried capillary blood, eluted by 2.5 ml of PBS-T at $4^{o}C$ for 18 hours was equivalent to a 10^{-3} dilution of venous serum. Eluates prepared in this way could be assayed directly in Toxocara ELISA without further dilution.

Sera. Over 4,000 human and canine sera were tested to evaluate the adaptability of Toxocara ELISA to seroepidemiologic applications. Sera were obtained from various sources as acknowledged in Table 20.

TABLE 20

SOURCES OF SERA USED TO ASSESS THE SEROEPIDEMIOLOGIC APPLICABILITY OF TOXOCARA ELISA

Group	Country of Origin	Number	Source
Healthy Adults	Iceland	307	Dr. O. Jensson, Reykjavik Blood Bank, Reykjavik.
Healthy Adults	UK	922	Blood Donor Clinics (616) Brentwood (Essex) (306) Milton Keynes (Bucks)
Healthy Adults	CSSR	400	Dr. M. Uhlikova Postgraduate Medical Institute, Prague.
Healthy Adults	Sudan	270*	Dr. S. Salih, U. of Khartoum Khartoum.
Healthy Adults	Ghana	259	Dr. I. Quakyi Dept. of Med. Sci. U. of Accra, Accra.
Healthy Children	CSSR	936	Dr. J. Hubner Postgraduate Medical Institute, Prague.
Seizure Patients (Adult)	UK	323	Dr. A. Richens, Institute of Neurology, London.
		53	Prof. A.W. Woodruff, Hospital for Tropical Diseases, London.
Seizure Patients (Children)	USA	102	"
Multiple Sclerosis	UK	117	Dr. L.F. Taffs, National Institute, for Medical Research, Mill Hill.

Cont.

TABLE 20
(Cont.)

Group	Country of Origin	Number	Source
Others			
Dog Breeders	UK	102	Dr. D.E. Jacobs, Royal Veterinary College, London.
Cat Breeders	UK	67	Prof. A.W. Woodruff HTD, London.
Dogs	UK	299	Dr. D.E. Jacobs R.V.C. London.
	Total	4,157	

* 225 Subjects had parasitologically proved schistosomiasis.

RESULTS

In Iceland, the study population consisted of 307 healthy adult residents of Reykjavik, aged 19 to 63 with a mean of 31.1 ± 8.4 years. All Icelandic sera were negative by Toxocara ELISA. The frequency of ELISA values revealed a log-normal distribution (Fig. 47) with median and 100th percentile values of 0.15 and 0.48 A_{405}, respectively.

In the UK, serum specimens were obtained from 922 healthy adults residing in the south of England whose ages ranged from 19 to 65 years with a mean of 34.5 ± 12.1 years. Sexes were represented by a male:female ratio of 1:01 to 1. Toxocara ELISA was positive in 24 (2.6%) with a male:female ratio in the positive group of 2:1 (ie. 1.7% of females and 3.4% of males were positive). The significance of sex-relatedness of qualitatively positive Toxocara ELISA values was tested and found statistically significant (X^2 = 3.385, P <0.05; Wilcoxon Rank Sum Test for Unpaired Samples, P <0.01). Within the positive population, males, as well as occurring more frequently, also tended to have significantly higher mean positive ELISA values (0.82) compared with females (0.60). The significance of this sex-related difference in quantitative ELISA values was confirmed by the method of Cherian and Hill (1978) which corrects for imprecision in the assay. Regression analysis of ELISA values with age (19 to 65 years) revealed no significant association (P >0.10).

Having established the prevalence of positive Toxocara ELISA reactivity in healthy UK adults, various UK sub-populations were surveyed for comparisons based on their frequency distributions. Results are given in Table 21 and Figure 48.

In a sample of 376 adult patients receiving treatment for epilepsy, Toxocara ELISA positives were observed three times more frequently than

was expected in the healthy population (X^2 = 18.0, P <0.001).

Sera from 53 children with epilepsy (mean age of 11.9 ± 4.6 years) and 49 age matched controls (mean age 11.5 ± 4.3 years) were surveyed for anti-Toxocara antibody levels. There were 4.7 times as many positive reactors (9.4%) among the seizure patients compared with controls (2.0%), however this difference was not significant (X = 1.35, P >0.10). Although the male:female ratios in the seizure and control groups were 0.93 and 0.82 respectively, the male:female ratios of positive reactors was 6:1.

Serum specimens from 102 dog breeders and exhibitors (80 female and 22 male) were surveyed. Ages ranged from 18 to 69 with a mean of 42.3 ± 13.3 years. Positive ELISA values were found in 15.7% (16 of 102), approximately six times more frequently than expected in the control population (X^2 = 38.4, P <0.001). Significance was greater in females of whom 18.8% were positive, compared to 1.7% in the conrol population (X^2 = 44.4, P <0.001). There were 23.1% positive reactors among the 52 dog show exhibitors who were 40 years of age or over, compared with 8% positive reactors under that age. This difference was statistically sig nificant (P <0.02) and consistent with continued exposure to infection from long contact with breeding animals. Country dwellers, who had, on average, been breeding from 7.4 bitches for 19.4 years, gave more positive ELISA readings (28.1%) than inhabitants of villages, or cities (10%, P <0.05) who had owned on average 4.7 animals for 12.5 years. The distribution of Toxocara ELISA values obtained from tests on cat breeders and exhibitors was not significantly different from that of the control population (Fig. 48).

Sera from 400 healthy adults of age 19 to 60 years and 936 healthy children of age 1 to 10 years were obtained from various urban and rural settings in the Czechoslovak Soviet Socialist Republic (CSSR). Findings

are described in Table 22 and Figure 49. Results indicate a rising prevalence of positive reactors with age, from 1.3% in children one year of age, to 37% in adults 50 years of age (Fig. 50). A predominance of positive findings was observed in rural areas (Table 22), in male children (Fig. 51) and in female adults (Fig. 52). Mean negative values were low in young children and rose slowly throughout life (Fig. 53). Mean negative values of rural inhabitants were consistently higher than those of urban inhabitants (Fig. 53).

Serum samples from adults native to an arid tropical region (Khartoum, Sudan, n = 273) and a humid tropical region (Accra, Ghana, n = 259) were surveyed and revealed 0.7% and 30.0% positive Toxocara ELISA reactors respectively. Data describing the sex and age-structure of these groups were not available.

Sera were obtained from 299 dogs of mixed breed, with ages ranging from one month to 12 years (means 5.1 $\pm$ 3.5 years) and a male:female ratio of 1.26.1. All of 18 dogs less than six months of age were serologically negative by Toxocara ELISA, despite a high prevalence of patent infection in this age group. Seroconversion and increasing prevalence of positive reactions were observed between 6 months and 18 months, coincident with declining patency rates (Fig. 54). Prevalence and intensity of positive reactions were at a maximum at two years of age. Positive reactors were significantly more prevalent in intact female dogs (70% of 60) but not spayed females, compared with male dogs (42.9% of 98), (X^2 = 5.51, P <0.02) (Table 23).

TABLE 21

SUMMARY OF TOXOCARA ELISA SEROEPIDEMIOLOGIC SURVEYS OF 3,857 SERA FROM VARIOUS POPULATIONS

Source	Group	n	Toxocara ELISA				
			Mean Negative	Median	Total % Positive	% High Level Positive	Mean Positive
Iceland	Healthy Adults	307	0.16	0.15	0	0	N/A
UK	Healthy Adults	922	0.17	0.17	2.6	0	0.75
	Cat Breeders	67	0.19	0.18	1.5	0	1.45
	Dog Breeders	102	0.18	0.16	15.7 †	0	0.73
	Multiple Sclerosis	117	0.18	0.17	2.6	0	0.59
	Epilepsy	376	0.18	0.17	7.7 †	1.0	0.95
USA	Healthy Children	49	0.21	0.18	2.0	0	0.51
	Epileptic "	53	0.22	0.19	9.4	0	0.61
Sudan	Healthy Adults	270	0.18	0.17	0.7	0	0.56
Ghana	Healthy Adults	259	0.29	0.36	30.0 †	3.2	0.89
CSSR	Healthy Adults	400	0.25	0.30	25.7 †	5.3	0.95
	Healthy Children	936	0.12	0.15	15.8 †	4.5	0.97

† Difference between UK healthy adults statistically significant ($P < 0.001$).

TABLE 22

SUMMARY OF TOXOCARA ELISA SEROEPIDEMIOLOGIC SURVEY OF 1336 HEALTHY ADULTS AND CHILDREN IN THE CSSR.

Source	Toxocara ELISA percent Positive*						Total % Positive
	Urban			Rural			
	Neg	Low	High	Neg	Low	High	
Children 1-10 y.							
Prague (n = 248)	230	14	4	N/A			7.3%
	7.3%						
Prostejov: 2 towns 42 villages (n = 258)	94	7	2	111	34	10	20.5%
	8.7%			28.4%			
Tachov: 4 towns 41 villages (n = 430)	274	34	15	77	18	12	18.3%
	15.2%			28.0%			
Total (n = 936)	598	55	21	188	52	22	
	674			262			
	12.7%			28.2%			15.8%
	Low 11.3% High 4.5%						
Adults 18-60 y.							
Prague (n = 200)	169	27	4				15.5%
	15.5%						
Kolin: town (n=88)	68	15	5				22.7%
	22.7%						
Kolin: villages (n=112)				60	40	12	46.4%
				46.4%			
Total (n = 400)	237	42	9	60	40	12	
	288			112			
	23%			46%			25.7%
	Low 20.5% High 5.2%						

Fig.47 Frequency distributions of Toxocara ELISA values in samples of healthy adult populations in Iceland, England and Sudan.

Fig.48 Frequency distributions of Toxocara ELISA values in dog breeders and cat breeders sampled in the UK.

Fig.49 Frequency distributions of Toxocara ELISA values in healthy adults and children in both urban and rural environments in the CSSR.

↑ Indicates position of the mode.

cara ELISA values in samples
Iceland, England and Sudan.

cara ELISA values in dog
ed in the UK.

ocara ELISA values in
both urban and rural

mode.

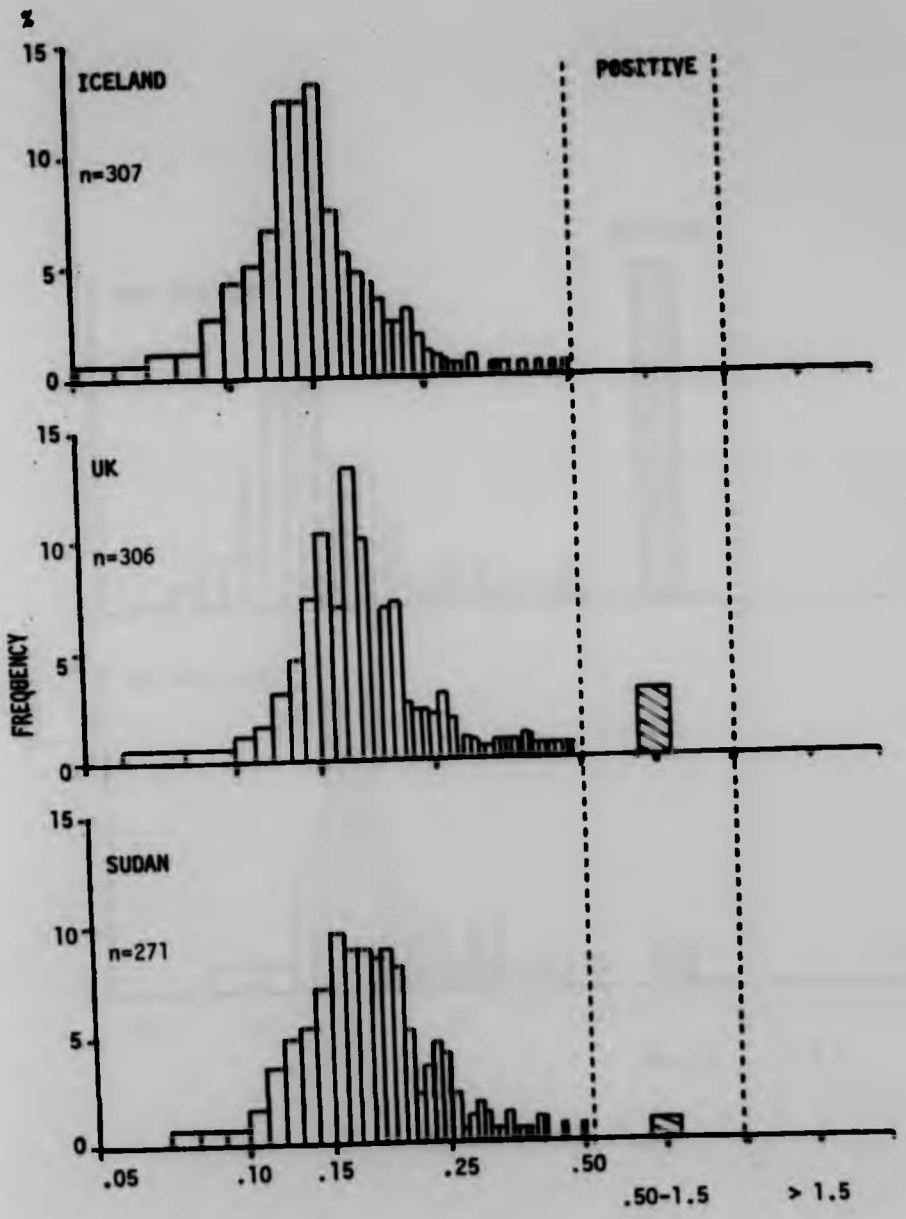

FIGURE 47

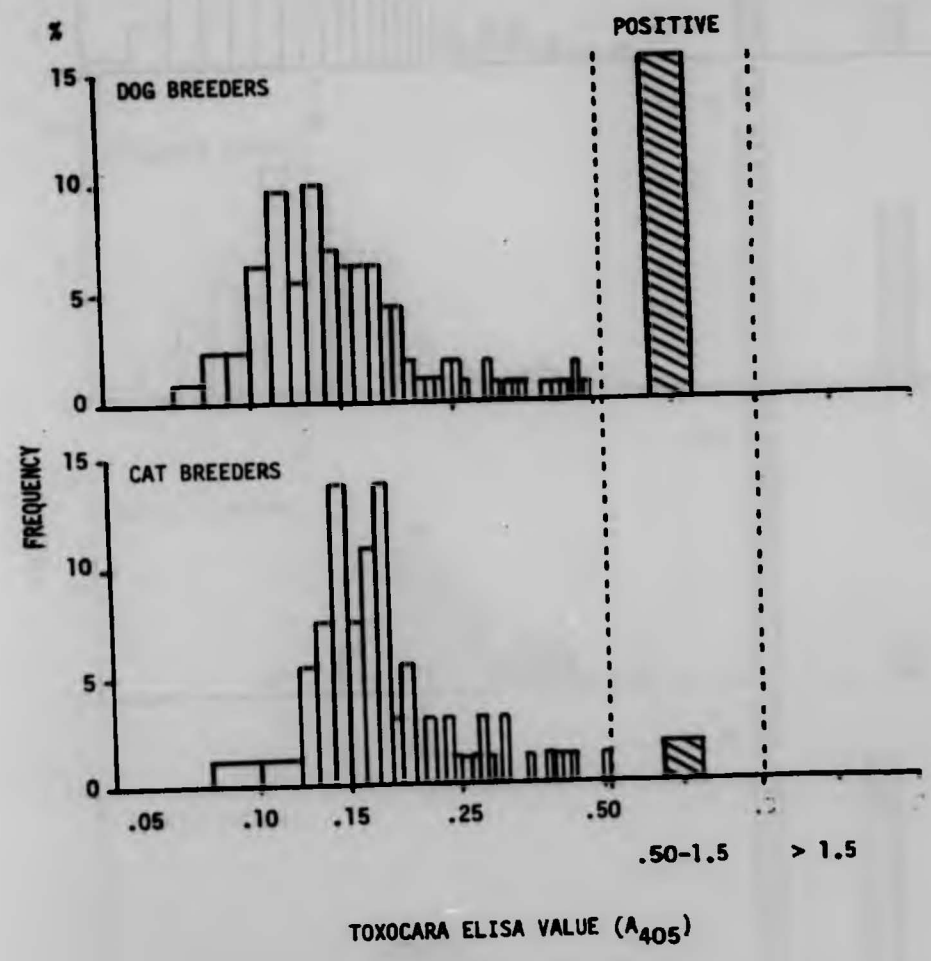
%
POSITIVE
15
DOG BREEDERS
10
5
0
FREQUENCY
15
CAT BREEDERS
10
5
0
.05
.10
.15
.25
.50
.50-1.5
> 1.5
TOXOCARA ELISA VALUE (A_{405})

FIGURE 48

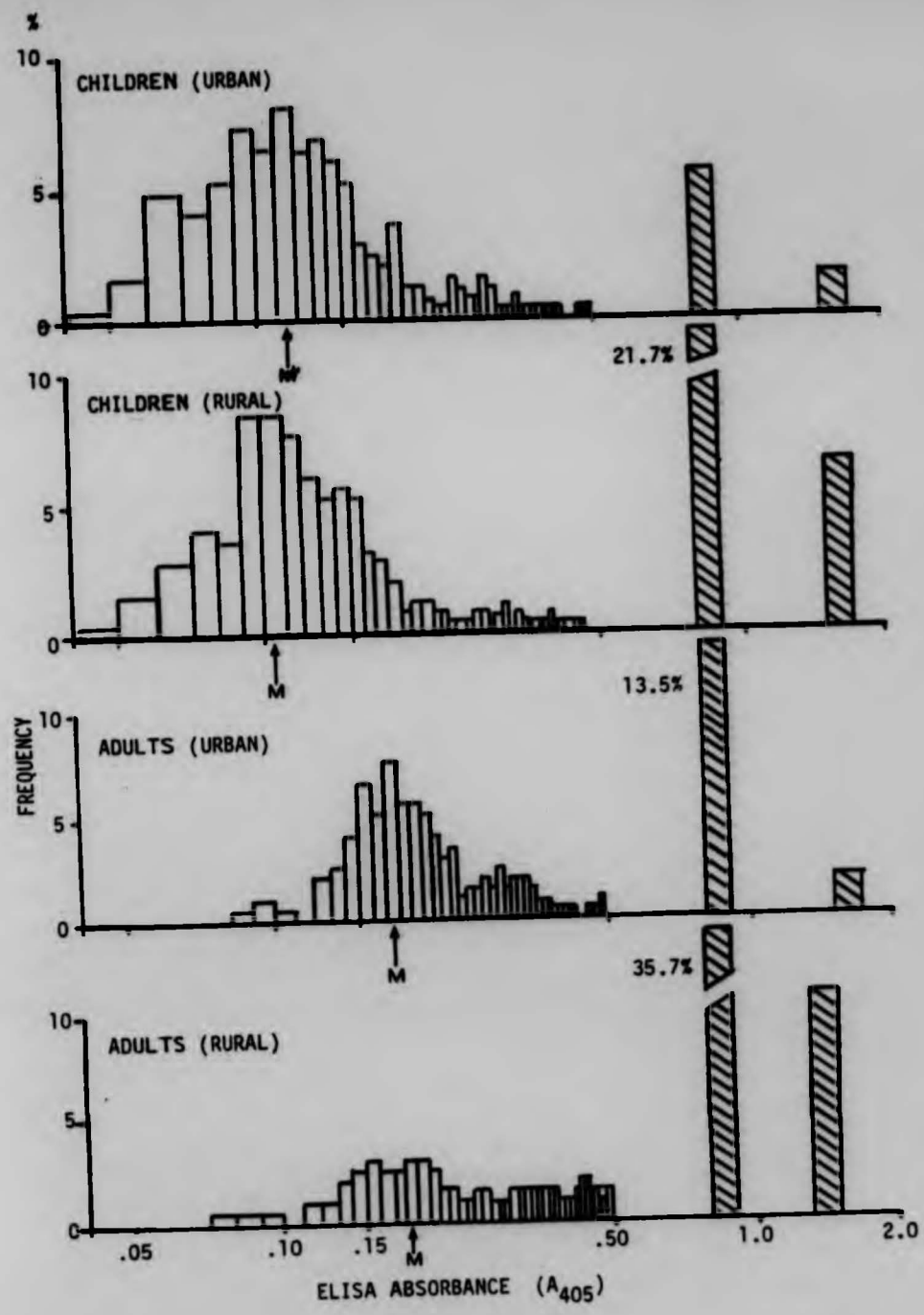
%
10
CHILDREN (URBAN)
5
0
M
21.7%
10
CHILDREN (RURAL)
5
0
M
13.5%
FREQUENCY
10
ADULTS (URBAN)
5
0
M
35.7%
10
ADULTS (RURAL)
5
0
.05
.10
.15
M
.50
1.0
2.0
ELISA ABSORBANCE (A_{405})

FIGURE 49

Fig.50 Prevalence of positive Toxocara ELISA values in the CSSR as a function of age.

Fig.51 Prevalence of positive Toxocara ELISA values in children in the CSSR as a function of sex and age.

Fig.52 Prevalence of positive Toxocara ELISA values in adults in the CSSR as a function of sex and age.

ELISA values in the

a ELISA values in children
ex and age.

ra ELISA values in adults
ex and age.

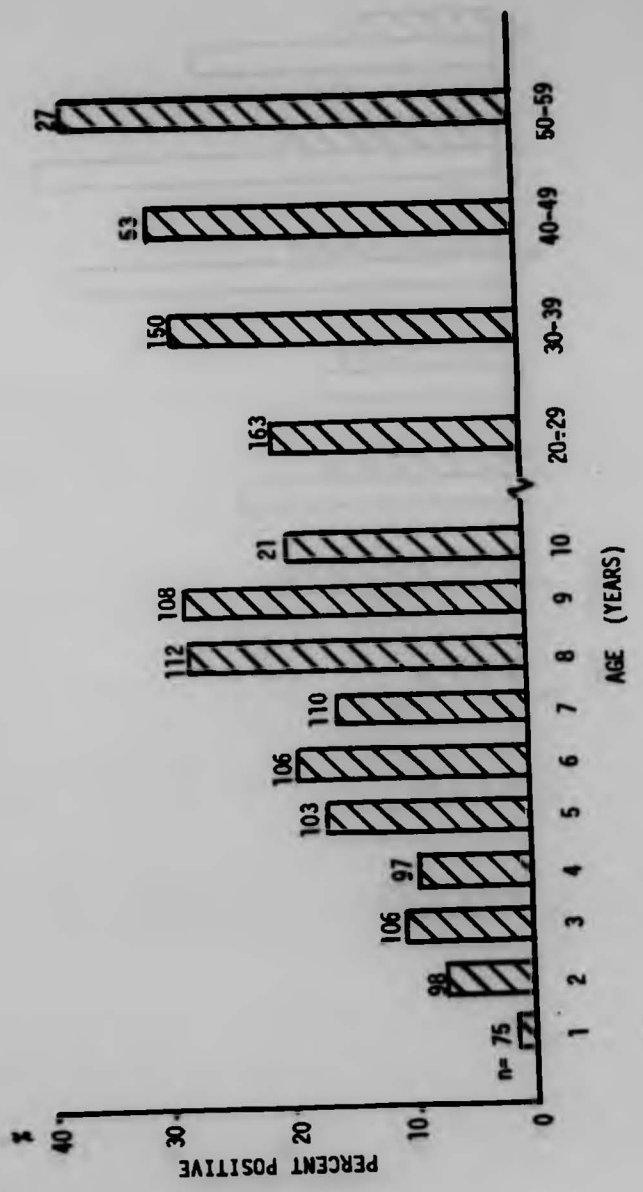

FIGURE 50

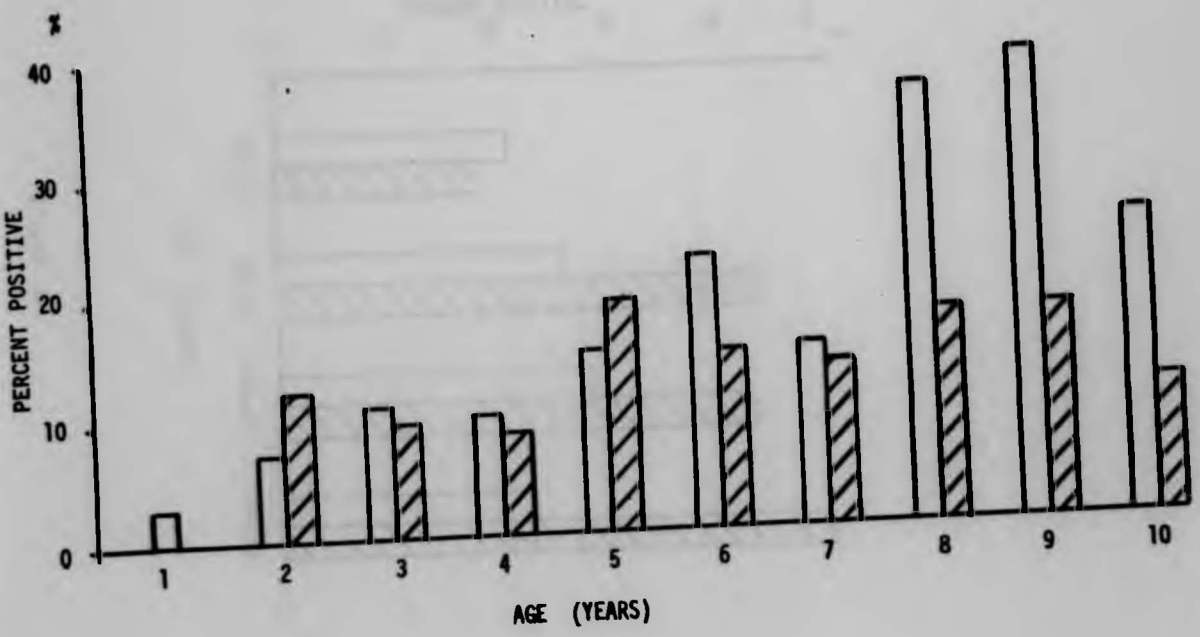

FIGURE 51

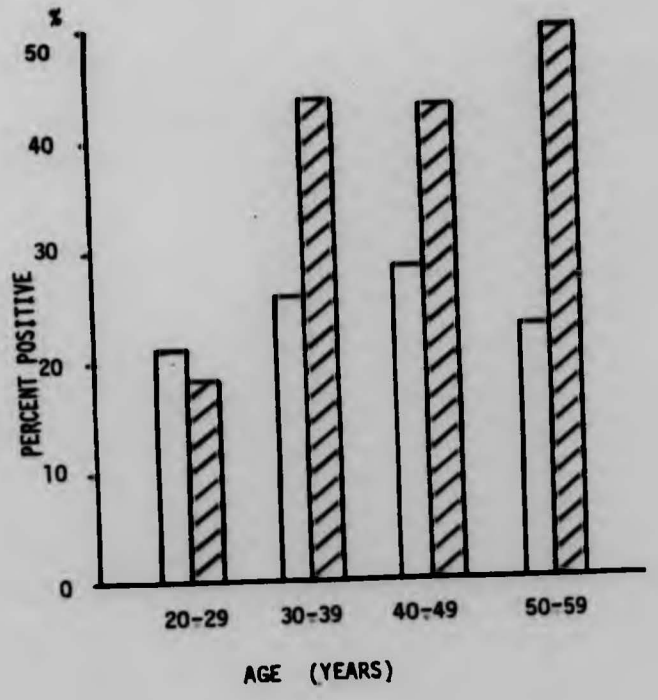
%
50
40
30
20
10
0
PERCENT POSITIVE
20-29
30-39
40-49
50-59
AGE (YEARS)

FIGURE 52

Fig. 53 Mean negative Toxocara ELISA values in inhabitants of urban, suburban and rural areas of the CSSR as a function of age.

- ● Urban (City)
- △ Suburban (Town)
- ○ Rural (Country-side and Villages with populations less than 200)

alues in inhabitants of

s of the CSSR as a

Villages with populations

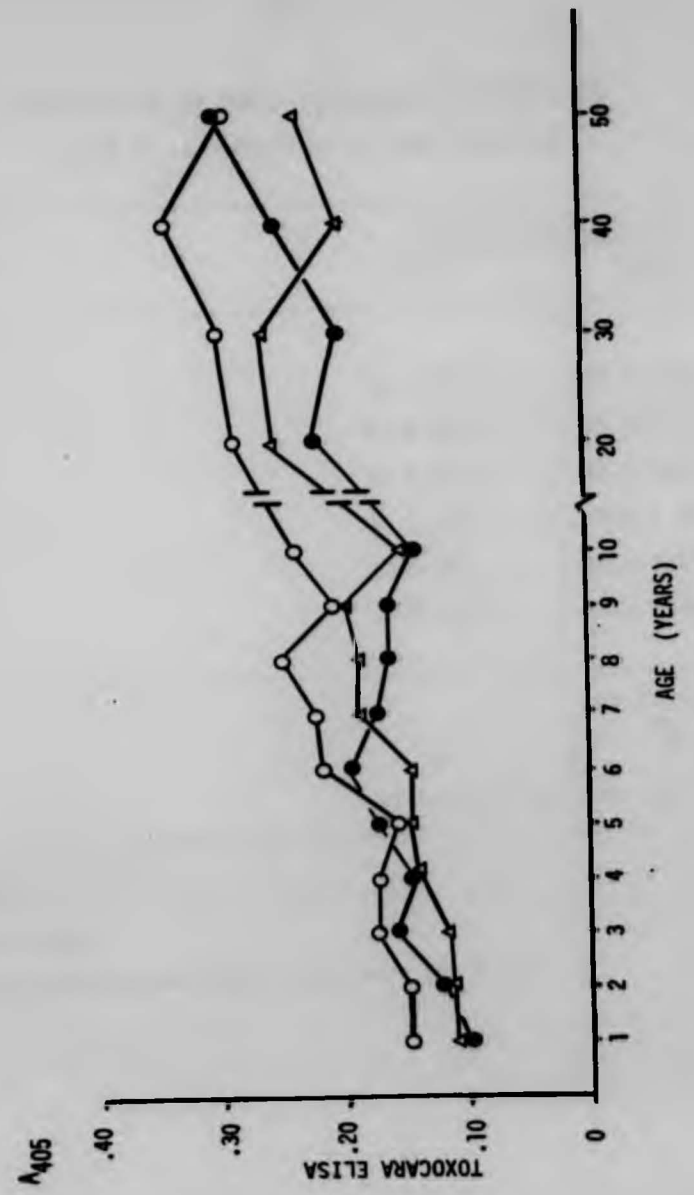

FIGURE 53

TABLE 23

PREVALENCE OF ANTI-*TOXOCARA* ANTIBODY IN DOGS AS A FUNCTION OF AGE AND SEX

Age (years)	Percent Positive (n)	
	Male	Female
< 2	18.4 (23)	28.7 (7)
2-3	63.6 (22)	75.0 (13)
4-5	36.4 (11)	64.3 (14)
6-7	41.7 (12)	66.6 (8)
8-9	33.3 (15)	66.6 (15)
> 9	66.6 (15)	54.5 (14)
Totals	♂ 42.9 (98)	63.3 (79) ♀ 42.1 (19) ♀ 70.0 (60)†

♀ Female

♀ Spayed Female

† Statistically Significant Difference ($P < 0.02$).

Fig. 54 Mean and percent positive *Toxocara* ELISA values in normal dogs as a function of age.

Mean ELISA value (± 2 SE)

● Mean Positive ELISA value

○ Percent Positive

PERCENT POSITIVE
% (○)
100
75
50
25
0
≥10
8-9
6-7
4-5
3
2
1
0.5-1
<0.5
AGE (YEARS)
A405 (●)
1.5
1.0
0.5
0
+
-
TOXOCARA ELISA

FIGURE 54

Fig. 55 Adaptation of Toxocara ELISA for use with capillary. whole blood samples collected and dried on absorbent paper.

- ● Titration of positive serum
- o Titration of eluate collected in 1.5 ml of PBS-T from a 6 mm disc of dried capillary blood on filter paper, collected simultaneously from the same patient.

Log distance between Toxocara ELISA dose-response curves is 2.77 (= 1:600). Thus, the eluate is equivalent to a 1:600 dilution of venous serum.

Hence:

Elution Volume (ml)	Equivalent serum Dilution
0.5	1:200
1.0	1:400
1.5	1:600
2.0	1:800
2.5	1:1000

for use with capillary.
and dried on absorbent

erum
lected in 1.5 ml of PBS-T
ed capillary blood on
simultaneously from the

ELISA dose-response
us, the eluate is equivalent
serum.

)	Equivalent serum Dilution
	1:200
	1:400
	1:600
	1:800
	1:1000

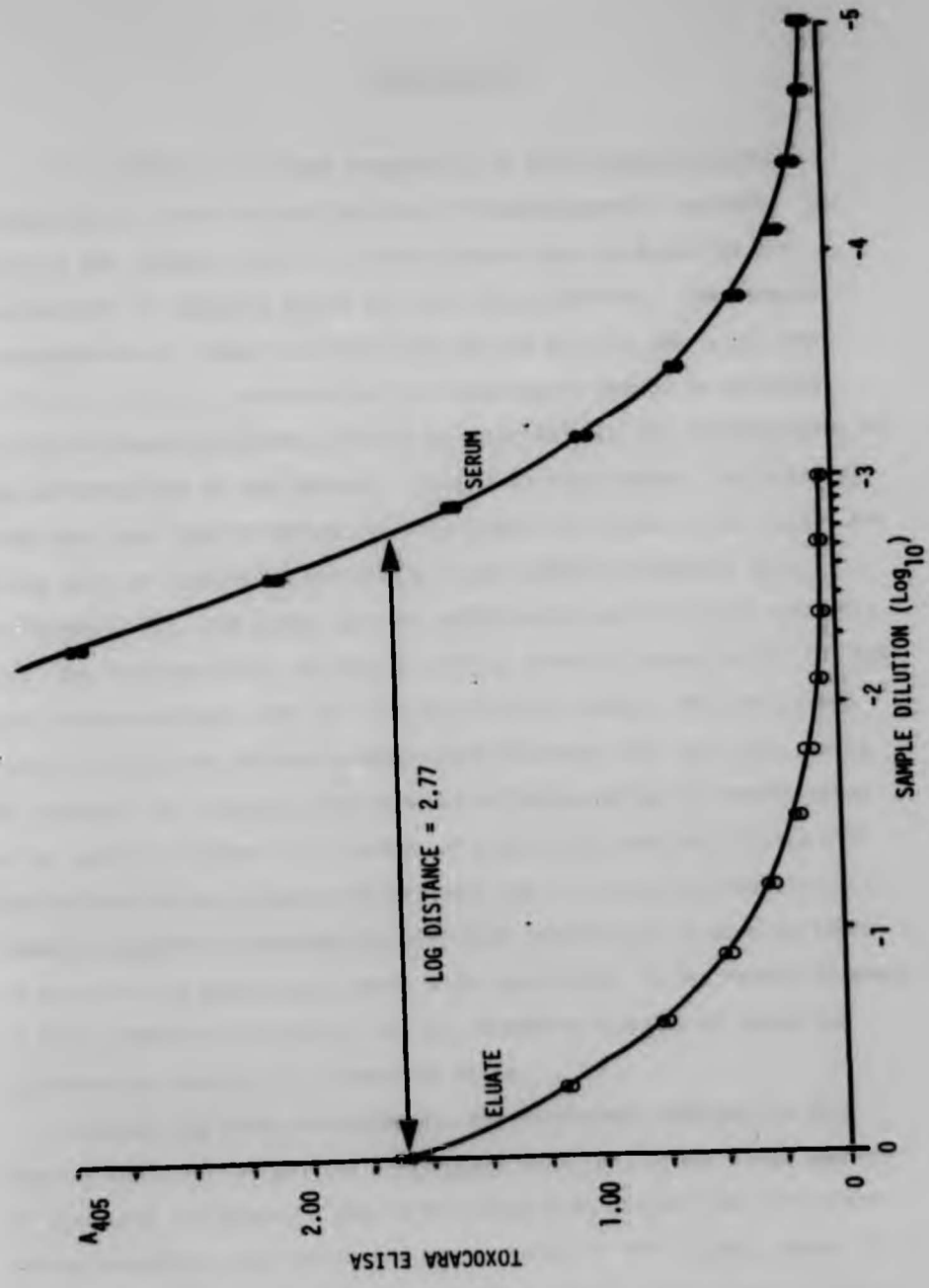

FIGURE 55

DISCUSSION

It is difficult, if not impossible, to study the epidemiology of toxocariasis in man without the use of immunodiagnostic methods. The goal of the studies reported in this Chapter was to determine the usefullness of Toxocara ELISA for such investigations. The purpose of seroepidemiologic investigations is to obtain data on the levels and pattern of specific seroreactivity in population groups in relation to the relevant variables and thus to contribute to the understanding of the epidemiology of the disease. To achieve these goals, the assay(s) used must meet the following criteria (Lobel and Kagan, 1978): (a) the assay must be capable of processing large numbers of samples with efficiency; (b) the assay must be sufficiently sensitive and specific; (c) the interpretation of results must be free of subjectivity; (d) the cost mustbe minimal; and (e) the test must be rapid. One can extend this list with the following desireable features; (f) the test should be suitable for use with test samples collected either as venous serum or as capillary blood; (g) levels of sensitivity and specificity, with defined predictive values, must be known and be flexible enough to suit changing patterns of endemicity (ie. high sensitivity is more suitable in areas of low endemicity, while high specificity is desireable in areas of high endemicity); and (h) the test should be capable of producing quantitative results on a numerical scale.

Considering these requirements, the experience reported in this chapter confirms the ability of Toxocara ELISA to process large numbers of sera with efficiency. Over 4000 assays were carried out during the seroepidemiologic evaluation and these represent the largest number of healthy subjects surveyed for toxocariasis by any single method to date.

The requirement set by Lobel and Kagan (1978) that the test must be rapid, needs qualification. Toxocara ELISA cannot be considered a rapid test, results being available only after an overnight incubation. However this feature of the assay was deliberately designed for seroepidemiologic applications, for which a rapid through-put of samples was considered more desireable than a rapid result in hand. (The converse is true for serodiagnostic applications.) Using Toxocara ELISA, one experienced worker can independently process 300 to 400 serum samples per day. The use of capillary blood samples collected on absorbant paper obviates the serum dilution step and allows a higher through-put. Using a "through-the-plate", automated ELISA reader (eg. Flow Multiskan) in conjunction with capillary blood samples, a single worker could process in the order of 1000 samples daily. Concerning the requirements of sensitivity, specificity, predictive value and objectivity, these have been established previously for Toxocara ELISA. The material cost of Toxocara ELISA was estimated to be £ 0.03 per assay (1979 costs). An important feature of Toxocara ELISA for seroepidemiologic applications is the quantitative nature of the result. Using the high precision of the assay, it was possible to compare subtle differences in distribution of Toxocara ELISA values obtained in various populations and subpopulations.

The performance evaluation of Toxocara ELISA in seroepidemiologic applications focused attention on three human environments which were considered, for various reasons, to represent toxocariasis non-endemic, endemic, and hyperendemic regions. Selected subpopulations of subjects with potentially higher risk of acquiring toxocaral infection were also investigated. Serologic data were summarized by three methods: proportion of persons reactive (Lobel and Kagan, 1978); mean positive titres (Draper et al.1972); and frequency distribution of the sample

population (Kagan, 1973). It is emphasized that these investigations were intended as validation studies of Toxocara ELISA applicability to seroepidemiologic investigations, and were not intended to represent definitive epidemiologic studies in themselves. Nevertheless, several important findings have emerged and are discussed below.

Iceland was selected as a potentially non-endemic region for toxocariasis. Iceland currently occupies a unique epidemiological setting in this respect since it is the only country in the western world where dog ownership and dog importation is prohibited by law and free-ranging dogs have been eliminated. This legislation has been strictly enforced, especially in urban areas, since 1924 (Jorundarson, 1979). Some dogs have been kept, illegally, in Reykjavik during the last five to six years (Palsson, 1979) and there is an estimated population of no more than 200 dogs per 100,000 human inhabitants. The implications of the Toxocara ELISA survey of the Reykjavik sample group (representing 0.3% of the total population) are several. Although the median and modal ELISA values are the same as those recorded in healthy adults in the UK, there were no positives detected in Reykjavik, and the distribution of values was unimodal, suggesting that Reykjavik is a non-endemic area for toxocariasis according to the classification of Healy and Gleason (1972). This is a useful finding and supports the qualitative interpretation of results in other populations when using 0.50 A_{405} as the positive threshold (assuming modal values are similar to those of the Icelandic population). Epidemiologically, the results indicate that restrictive legislation can be effective in minimizing the epizootic cycle of toxocariasis. Contrary to popular impression (Anon, 1979) the climate of Iceland is not severe and should not be a major factor in inhibiting transmission of Toxocara spp. ova. Cats in Reykjavik are unrestricted

and are popular as housepets. Toxocara cati is a common finding by Icelandic veterinarians (Palsson, 1979) confirming the potential for development and transmission of infective toxocaral ova in Icelandic soils. These epidemiological features considered together with the demonstrated ability of Toxocara ELISA to detect paratenic T. cati infection, support the hypothesis that T. cati is not a major source of toxocariasis in man. The wild fox in Iceland has a large population in remote areas and is a considerable nuisance to agriculture. Eradication measures destroy 4000 foxes per year but the population is uncontrolled and represents a possible source of T. canis ova in rural areas.

These studies of toxocariasis were the first in Iceland. Further work should exploit its unique epidemiological situation to investigate: the prevalence of toxocaral antibodies in native Icelandic subjects older than 60 years of age (ie. pre-legislation); the prevalence of toxocaral ova in rural and urban soils; the prevalence of toxocaral antibodies in rural inhabitants; the possible role of foxes in the transmission of toxocariasis in rural areas.

The results of Toxocara ELISA surveys of healthy adult subjects in the UK suggest that the UK is an endemic area with regard to toxocariasis infection and has a period prevalence of at least 2.6%. It may be questioned whether this 2.6%, found to have low levels of circulating anti-Toxocara antibody, is a reflection of the incidence of recent light infections occurring in adults or is evidence of the prevalence of long-past toxocariasis acquired in childhood. Antibody responses to nematode secretory antigens are expected to be better indicators of active or recent infection than are responses to somatic antigen (de Savigny and Tizard, 1977), however, the antibody response to secretory antigen even during active, but light infections tends to be

very high. Therefore these low-level responses suggest past infection. It is not known for how long Toxocara larvae persist and continue to migrate in tissues during natural infections in man. That the 2.6% prevalence of antibody is a result of childhood exposure gains indirect support from the lack of increasing ELISA values during adult life and the adult male:female ratio of 2:1 in positive reactors which is similar to that observed in clinical visceral toxocariasis occurring in childhood (Snyder, 1961; Huntley et al. 1965).

To evaluate the performance of Toxocara ELISA in an area of high toxocariasis endemicity, Czechoslovakia (CSSR) was selected. Clinicians in the CSSR have been concerned by the apparent high incidence of cases of unexplained chronic hypereosinophilia (Uhlikova and Hübner, 1968, 1971, 1979) many of which have been diagnosed retrospectively as toxocariasis using Toxocara ELISA (Chapter IV). A seroepidemiological survey of 1136 healthy adults and children was organized to provide a fundamental base of information regarding the prevalence and intensity of positive reactions in the healthy population as a guide by which to assess the significance of positive reactions observed in the clinically diseased population. Several facts and many questions emerged regarding the epidemiology of toxocariasis in the CSSR. In children, modal values were found to be significantly lower than those in adults and were similar to the pattern seen in young, serologically negative animals when compared to adult serologically negative animals. The shift of an entire distribution of ELISA values, to higher values with age has been noted with other ELISA systems (Seawright, Bartlett, Sanders, Clinard, Mills, Martinez, Payne, Bishop and Zimmerman, 1979) and probably reflects the accumulated response to total antigenic exposure throughout life. Rural subjects also had higher negative modal values than did urban

inhabitants. This feature of ELISA assays emphasizes the need for careful selection of relevant reference control groups in the evaluation of ELISA data. The studies in healthy children also suggest a changing pattern of infection with age which might have an epidemiologic basis. In young children, males and females had equivalent prevalences of positive reactions, while in older children, males predominated. Teenaged children were not surveyed, however in adults, the pattern was reversed such that females predominated in positive serological reactivity. Highly positive Toxocara ELISA values which are assumed to be associated with recent toxocariasis, occur with equal prevalence in children (4.5%) and adults (5.3%), while low positive ELISA values predominate in adults (20.5%) over children (11.3%). This observation is consistent with the suggestion that low positive ELISA values in adults may be residual from previous infection in childhood. The remaining point regarding ELISA reactivity is the high prevalence of positives in rural adults (36.0%) and rural children (18.1%) compared to urban adults (15.5%) and urban children (7.3%).

These findings indicate that the CSSR is a hyperendemic region for toxocariasis. The significantly higher prevalence of infection observed compared with the UK raises the question as to why this should be so. Climate and soil types are comparable in these two countries as is the prevalence of toxocariasis in dogs (20%) and cats (50%) (Vokoun and Slezakova, 1975: Uhlikova and Hübner, 1979). A survey of the Czechoslovak literature revealed the following: the role of soil and vegetables in the epidemiology of geohelminthiasis has been recognized in Czechoslovakia (Lysek, 1959), however the incidence of geohelminth ova in gardening soils was low (Lysek, 1968). Palicka and Pazdiora (1971) surveyed soil, and sand from children's sand pits, in towns, housing settlements, and "children's collectives" at monthly intervals

throughout the course of one year, and found no Ascaris lumbricoides or Trichocephalus trichiurus ova but did detect Toxocara spp.ova in an undisclosed proportion of samples. Giboda and Kratochiv (1968) recorded a high incidence of geohelminth infection in certain children's collectives. Rheka and Rochunova (1973) reported a 15 year longitudinal study of several thousand children and found a low incidence of human geohelminths including Trichocephalus trichiurus (3.5%) and Ascaris lumbricoides (4.1%). Of epidemiological significance may be the practice of using sewage sludge as fertilizer. Sewage sludge may contain viable Toxocara spp. ova (Graham, 1976) which survive sewage treatment. Sedlacek and Stoklosova (1977) have called for safety regulations in the CSSR, governing the use of sludge in agriculture and have proposed certain hygienic precautions for the pretreatment of sludge for use as manure.

As yet no satisfactory reason has emerged for the high prevalence of positive Toxocara ELISA values in the CSSR.

Two tropical populations, in which multiple parasitic infections were expected, were surveyed. In the relatively arid region of Khartoum, the proportion positive (0.7%) was indicative of a non-endemic area and was lower than that seen in the UK despite concurrent schistosomiasis and other helminth infection in many of these subjects. In the relatively humid tropical environment of Accra, the proportion positive was similar to the CSSR and corresponded to an area hyperendemic for toxocariasis. These findings are supported by those of Yampolskaya et al.(1978) who, using Toxocara ELISA with secretory antigen, found both seropositivity and prevalence of viable ova in soil, to be highest in humid regions and lowest in arid regions. Since both Khartoum and Accra have substantial free-ranging dog populations, it follows that environmental

conditions, especially aridity, can exert a significant effect on the transmissibility of toxocariasis to man and that this effect may be detected in Toxocara ELISA serological profiles.

The high modal ELISA value of the healthy Ghanaian population merits comment because it illustrates the irrelevence of European standard sera when assays are transposed for use with tropical populations. It is important that assays be standardized gainst relevant local reference groups whose health status has been determined by other means. In many areas of Africa, high "background" values are commonly seen in indirect assays which use anti-globulin reagents (Voller, 1979, personal communication) and are possibly due to the relatively high levels of circulating serum globulins, rheumatoid factors, and immune complexes. In these assays, the distribution of negative values may overlap the (European) positive threshold value leading to a high prevalence of false positives. By standardizing the assay with appropriate controls, it should be possible to re-establish a satisfactory threshold value at a higher discriminating level. Since it is unlikely that positive responses are additive to the high negative modal value, there will be some loss of sensitivity, especially in the detection of the "past-exposure" range of values. Therefore seroepidemiologic prevalence estimates may be overestimated, using European reference standards, and under-estimated, using African reference standards. However, it may be possible to obtain a more accurate estimate of prevalence by making use of the known sensitivity and predictive value parameters of Toxocara ELISA (Chapter III) as follows. In Ghana, 9 of 259 (3.5%) of the sample population had positive ELISA values of A_{405} = 1.25 or greater. At this value, there should be few or no false positives, and at this value, it is known that the assay sensitivity is 84.4% (Table 3). Thus the adjusted estimate of toxocariasis prevalence in Ghana would be 4.1%. The validity

of this logic needs further investigation.

Having established the Toxocara ELISA serological profile of the UK population, it was then possible to compare subpopulations in an epidemiological context.

The possibility of an association between house pets and multiple sclerosis (MS) was raised by Cook and Dowling (1977) in a careful study of MS patients and controls matched for age, sex, neighbourhood and socio-economic status. No difference in ownership of cats or dogs was found. However, exposure to small indoor pets (cats or dogs) was significantly higher in the MS group ($P < 0.001$). Exposure to small pets was particularly striking during the ten years before onset of initial symptoms of MS, when compared to the control group. Of 49 MS patients, 33 had a young pet dog or cat in their homes, within one year before onset of the first neurological symptom. Familial MS was also found to be associated with young pets. These authors proposed the transmission of an infectious agent from dogs or cats to man, in attempt to explain the temporal relationship with MS. Serological studies by Krakova and Koestner (1978) and Nathanson, Palsson and Gudmundsson (1978) failed to incriminate canine distemper virus in the aetiology of MS although measles antibody titres were significantly elevated. Since it has been shown that viral infection can become more severe with simultaneous Toxocara infection (Pavri, Chalsasi, Dastur, Goverdhan and Lalitha, 1975), the possibility of dual viral and toxocaral infection is worth consideration. The absence of reports of T. canis larvae in tissues of MS patients need not disqualify the suggestion, considering the difficulty with which these are detected (Woodruff, 1970). In the current study no significant difference was observed in the prevalence of toxocaral antibody in 117 MS patients compared to healthy controls. Thus, there is no support for the

hypothesis implicating toxocaral infection in the aetiology of multiple sclerosis.

Toxocaral antibodies have been shown to occur in epilepsy with higher prevalence than expected in the normal population in several studies (Woodruff, Bisseru and Bowe, 1966; Viens, 1977; Glickman, Cypess, Crumrine and Gitlin, 1979). In the current study, toxocaral antibody was found approximately three times more commonly in adult epileptics and five times more commonly in epilepsy in children although the significance of these results in uncertain. It has been suggested that the migration of toxocaral larvae in the brain may be the cause of some forms of epilepsy (Woodruff, Bisseru and Bowe, 1966). Glickman et al. (1979a) found a higher incidence of geophagia in epileptics, and no significant difference in seropositivity between idiopathic epilepsy and epilepsy of known aetiology. These authors concluded that toxocariasis is not causally linked to epilepsy but that epilepsy may predispose patients to acquiring toxocariasis. The use of Toxocara ELISA in a prospective study of children with recently diagnosed epilepsy should resolve this issue.

Toxocara ELISA was used to examine selected subpopulations of the healthy population, who may stand a higher risk of acquiring toxocariasis. Results demonstrated that dog show exhibitors and breeders were at particular risk of acquiring sub-clinical toxocariasis and that this risk was proportional to the number of bitches whelped per annum and the number of years engaged in dog breeding. In contrast, cat show exhibitors and breeders appeared to be at no significantly increased risk of toxocariasis. Again this supports the contention that T. cati is not a major source of human toxocariasis. Others have reported an absence of increased seropositivity in employees of dog kennels of the type which do not engage in breeding, but are nevertheless, heavily

contaminated with toxocaral ova (Jacobs, Woodruff, Shah and Prole, 1977; Glickman and Cypess, 1977). The results of the current work would thus illustrate the particular risk of young pups, and post-parturient bitches in the epidemiology of toxocariasis.

The high intensity of patent infection in newborn and young pups, which decreases with age and, reappears in post-parturient females, strongly suggests the slow development of effective immunity in the neonate against adult T. canis, and the supression of this immunity in pregnant and in lactating bitches. The availability of Toxocara ELISA prompted the first serodiagnostic/seroepidemiologic survey of anti-Toxocara humoral responses in naturally infected dogs of all ages. Such studies have not previously been contemplated because of the adequacy of parasitological diagnosis and the general lack of interest expressed by parasite immunologists in the canine-toxocaral host-parasite relationship. The important finding of this survey was the absence of serological responsiveness to Toxocara larval secretory antigens during the first five months of life. This result was unexpected in view of the high prevalence of patent infection in these animals, the concurrent migration of larvae through tissues and the high sensitivity of Toxocara ELISA. These serological observations are consistent with the following hypothesis:

In utero infection induces in the foetus a specific tolerance to Toxocara canis antigens, including the secretory antigens of the second-stage larvae, and that this tolerance is broken after approximately 5 months following birth. The breaking of tolerance is accompanied by the development of specific immunity which is manifested by the elimination of adult worms from the gut, the suppression of further development and migration of larvae and their subsequent relegation to dormancy in the tissues. This survey in dogs also encountered a higher

prevalence of positive reactions in intact adult female animals compared to males or spayed females, which might be explained by the mobilization of dormant second-stage larvae known to occur during each pregnancy.

There are few parasites which have exploited the prenatal route of infection as successfully as have Toxocara canis but the mechanisms whereby this is accomplished have not received adequate study. The suggestion that specific tolerance is operating in utero and in neonatal life, as part of the host-parasite relationship in canine toxocariasis, should be investigated further. Not only could this represent an interesting model of immunological tolerance in nature, but it might provide an effective approach for the control of toxocariasis. Interruption of prenatal transmission of toxocariasis in canidae could have significant public health implications for man.

SUMMARY

The performance of Toxocara ELISA was evaluated in various seroepidemiologic applications. In addition to previously described parameters of sensitivity, specificity and precision, the assay was found particularly suitable for use with a high through-put of samples at minimal cost, and was adaptable for use with capillary blood samples collected on absorbent paper. The objective and quantitative nature of the assay result was of particular value for seroepidemiologic applications and allowed analysis of the proportion positive, the intensity of the positive response and the numerical description of a population by the frequency distribution of its ELISA values. Comparison of frequency distributions was used to identify differences between populations with regard to relevant variables.

Over 4000 sera were assayed from presumed, toxocariasis non-endemic (Iceland, Sudan), endemic (UK, USA) and hyperendemic (Czechoslovakia, Ghana) areas.

Within an endemic area (UK) subclinical toxocariasis was detected in the healthy population 2-fold more frequently in males than in females, 3-to 5-fold more frequently in epileptics than in healthy subjects, and 6-fold more frequently in dog breeders (but not cat breeders) compared with healthy subjects. Patients with multiple sclerosis showed no significant serological association with toxocariasis. In the endemic area, the positivity rate did not increase with the age of the population. In the hyperendemic area, positivity rates increased progressively with the age of the population. Rural areas had higher negative modal values and higher positivity rates compared with urban areas. Male children and female adults had predominantly higher positivity rates for their age groups.

In non-endemic areas, it was shown that political factors (legislation prohibiting dogs) and environmental factors (aridity) can effectively inhibit transmission of toxocariasis to man and that this is reflected in the Toxocara ELISA serological profile. No evidence was seen to implicate Toxocara cati as a significant source of human toxocariasis.

A seroepidemiologic survey of naturally (prenatally) infected dogs revealed an absence of specific humoral response to toxocaral antigens in 100% of pups less than 6 months of age and a high prevalence of antibodies in dogs older than six months. It is postulated that tolerance to toxocaral antigens is induced in utero and that manipulation of immunoreactivity in neonatal pups may provide a means to control patent toxocariasis in the dog. Interruption of the prenatal transmission cycle of toxocariasis in canidae could have significant puplic health implications for man.

CHAPTER VI

GENERAL DISCUSSION

The success or failure of any immunodiagnostic test is a function of the antigen used. It is significant that the history of helminth immunodiagnosis reveals a general disregard for this axiom. The past dogma has implied that success would be won from the search for new assays of improved design rather than from a search for alternative antigens of improved quality. As a result, the literature for any one parasite (eg. Toxocara canis) describes the application, in virtually every immunoassay system yet devised, of a limited variety of unpurified and uncharacterized antigens. Now that immunoassays have achieved relatively high levels of sensitivity and technical sophistication without yielding the promised advances in helminth diagnosis, attention is being redirected to antigens and their sources (Pelley, Warren and Jordan, 1977; Capron and Wakelin, 1978). Because of the neglected importance of the role antigens in nematode serology, this discussion will focus on antigen related aspects, and in so doing, will describe the rationale of the current study. Although this study was based on the toxocariasis model, the arguments put forth apply to all tissue-invasive nematodes.

Nematodes are metazoan endoparasites and as such are highly complex. In toxocariasis, nematode larvae are able to reside in intimate contact with host tissues and are effectively able to elude the host's immune mechanisms. These two features: antigenic complexity and successful parasitism, should alert the serologist to possible pitfalls in the development of satisfactory serodiagnostic methods. In toxocariasis

the literature of the past 25 years is replete with proposed methods, most of which have resulted in assays of unsatisfactory sensitivity and specificity, or of limited practical value. Most efforts were based on the classical approach of parasite antigen production, ie. (1) obtain parasites from naturally or experimentally infected animals and (2) grind and solubilize parasites to extract antigens by chemical or physical means.

With the wisdom of hindsight, it can be said that such an approach is seldom worth-while when applied to metazoans which enjoy successful host-parasite relationships. To develop immunoassays for such infections, an approach philosophy is even more essential than it is for other microbial diseases. From this philosophy, a strategy must be formulated that considers both (a) the source of parasite material (ie. *in vivo* or *in vitro*) and (b) the source of antigen from within the parasite (ie. whole worm fractions, surface components, or secreted products). This discussion will deal with each of these aspects.

Considering the source of parasite material, it must soon be accepted that the development of suitable *in vitro* cultivation techniques for helminths is essential for the practical preparation of acceptably pure nematode antigens. It is difficult to imagine the relative excellence of serology that now exists for the various bacterial and viral diseases without *in vitro* cultivation of the organisms in question. Certainly as *in vitro* cultivation techniques improved for each organism, improvement, or indeed inception, of serologic techniques followed closely (eg. gonorrhoea). In the serology of parasitic diseases, *in vitro* cultivation for antigen production is the exception rather than the rule (eg. *Entamoeba histolytica*). Helminth serology is currently at the same level of development as that achieved by bacterial serologists of the 1930's. Hence, it is reasonable to expect major

advances in helminth serology when *in vitro* culture techniques are developed and applied. The advantage of *in vitro* cultivation is basically the same as posed for bacteria and viruses: technical simplicity in obtaining organisms clearly divorced from acquired host antigens including mediators of the host's immune system.

It follows that the use of synthetic culture media, unsupplemented by host factors, or other undefined components, is mandatory in this scheme. This is necessary, not only to obtain antigens of high purity, but also to allow their adsorption to immunoassay solid-phases without the competitive effects of other macromolecules. The recent use of synthetic tripeptides (Pickart and Thaler, 1973; Pickart, Thayer and Thaler, 1973; Stromberg, Khoury and Soulsby, 1977) as replacements for serum supplements, is a major advance in this context.

In the present work, antigens apparently specific for *Toxocara spp.* were derived from parasites hatched and maintained *in vitro* in synthetic medium, without exposure to an *in vivo* milieu. The observed specificity is unlikely to be attributed solely to the absence of acquired host antigens or immune mediators.

Having obtained "clean" parasites from *in vitro* sources, target antigens can then be considered within three categories: whole worm extracts; surface determinants; and secretory products. It must be appreciated that any helminth antigen may exhibit a variety of characteristics including: cross-reactivity with host antigens; cross-reactivity with heterologous helminth antigens; weak immunogenicity; protective (specific) immunogenicity; or immunosupressive functions. Furthermore, these characteristics do not occur with equal probability in each of the three potential sources of antigen.

Considering whole worm extracts, which include both surface (cuticular) and internal (somatic) antigens , the tradition of grinding whole adult or larval worms for the production of antigen is analogous to the emulisfaction of a whole cow for the production of insulin (Thorson, 1963). Arbitary extractions of antigen from helminth emulsions using alcohol, ether, acids, buffers etc., are based on little scientific logic but remain popular and are used in most recently published serodiagnostic procedures for toxocariasis (Kagan and Norman, 1979; Walls, 1979). The whole worm antigen approach fails to consider important aspects of parasite biology. Firstly, as a metazoan, nematode antigenic structure is not simple and contains several hundred potential antigens arising from functionally distinct organs and tissues (Crofton, 1966). Many of these do not evoke a serological response until the death and disintegration in vivo of the parasite and are thus poor serological antigens. Furthermore, their presence in diagnostic antigen preparations can compromise serological tests by inhibiting the performance of those few specific antigens which are present in diminished proportions. (eg. competitive inhibition of solid-phase antigen adsorption; proteolytic degradation by exposure to parasite intracellular products). Secondly, the whole worm antigen approach fails to consider those aspects of the host-parasite relationship which so profoundly influence serodiagnostic strategies. The long persistance of metazoan parasites in host tissues indicates that they have evolved a variety of successful mechanisms for survival in the face of natural and acquired immune responses of their hosts (Bloom, 1979). Assuming that all parasites had free living ancestors in the remote past, the evolution of host-parasite associations has necessarily involved a progressive series of modifications and adaptations leading toward mutual tolerance.

The concept of adaptive tolerance implies that those genetic changes which tended to decrease the harm or increase the benefit to either parasite or host were favoured by the process of natural selection (Linicome, 1978). According to this view, the degree of mutual tolerance achieved in any host-parasite relationship is a function of its evolutionary age and rate. It is therefore conceivable that ultimately (or presently), there may exist near-perfect host-parasite relationships in which, in a portion of the host population, immunologic interactions do not occur. Sprent (1962) has suggested that this state could be achieved by "selective obliteration of counter patterns in the host" and a "selective convergence of antigen structure between host and parasite". Evidence for convergent molecular evolution is found in the ubiquitous sharing of antigens between parasites and their hosts, both in plants and animals (reviewed by Vay and Adler, 1976). Antigen sharing has been termed "molecular mimicry" (Damian, 1964) and is essential to host-parasite compatibility possibly through the regulatory effects of concomitant immunity (Terry, 1977). In nematodes, shared antigens are usually authochtonous (Biguet, Capron, Tran Van Ky, and Rose, 1965; Capron, Dessaint, Camus and Capron, 1976) but may also be acquired, as described for some trematodes (Clegg, 1974; Smithers and Terry, 1976). That common antigens occur outside the phenomenon of parasitism in phylogenetically unrelated organisms (eg. Salmonella typhi and Trichinella spiralis share antigens), suggests that antigen sharing can arise by coincidence and that its occurrence within a host-parasite relationship would be enhanced by mutative and selective pressures. On theoretical grounds, this concept is supported by the view that the universe of antigens is not infinite, but limited, and perhaps rather small (Jerne, 1960).

The logical extension of antigen sharing between parasite and host is antigen sharing among parasites which parasitize the same host. Ascarid nematodes have been favourite subjects of studies on cross-reactivities. The presence of heterophile antigens, Forsmann antigens, blood group cross-reacting antigens, C protein, etc. are well documented (Soulsby, 1962; Biguet, Capron, Tran Van Ky and Rose, 1965; Harrison and Ridley, 1975). In addition, the relatively close phylogenetic relationship of many nematode genera has resulted in naturally occurring cross-reactivity between antigens not shared with their hosts (Petithory, Brumpt and Bahno, 1973; Cohen and Sadun, 1976).

Functional immunity in helminth infections appears to be specific, at least at the generic level (Soulsby, 1962). Somatic and whole worm antigens used in vaccination experiments produce only a low degree of protective immunity (Weinstein, 1959; Clegg and Smith, 1978). Therefore it may be considered that helminth whole worm antigens have a low content of functional antigens and hence, are potentially poor sources of specific antigens.

The second possible source of serological antigens to be considered are the nematode surface components. Since the development of fluorescent antibody techniques, it has been possible to study serological responses to nematode cuticles (Taffs and Voller, 1962) which in theory should be valuable because functional host immunity to the parasite probably operates primarily at the host-parasite interface. Unfortunately host selective pressures exert a major influence on the antigenic structure of the cuticle. Although it is known that the surface of certain nematodes contains genus, species, and stage specific markers (Mackenzie, Preston and Ogilvie, 1978) it is logical that the cuticle of the most

successful parasites should contain a mosaic of weak or nonspecific antigens. Experimental evidence of the non-specificity of nematode cuticles is abundant(Ambrois-Thomas, 1976). The apparent absence of an effective functional response to nematode cuticles prompted Damian (1962) to coin the term "eclipsed antigen" to describe parasite antigenic determinants which resemble those of the host. Implicit in the definition is the idea that the host will not recognize an eclipsed antigen as foreign and thus will not produce antibodies against it. The survival value to the parasite is obvious and the concept has been further expanded by Dineen (1963 a, 1963 b) who suggested that the immune responses of the host exert selective pressures which favour the survival of variants of the parasite surface which display reduced antigenic disparity with the host. It is also suggested that during evolution of the host-parasite relationship, a reduction of disparity would only be necessary with those antigenic characters of the parasite which stimulate responses which adversely affect survival of the parasite (ie. functional antigens). Dineen has termed these antigens "fitness" antigens and stated that the natural host will fail to recognize fitness antigens, or will show relatively little immunological responsiveness to them while reacting with normal vigour to those antigens of the parasite which do not influence fitness of the organism. Conversely, it would be expected that in the unnatural host, there will be serological responses to a greater range of antigens because there has not been the same reduction in disparity. Toxocara canis in its natural host, the Canidae is an excellent example of parasitism well tolerated by the host. Toxocara infection in a less natural host such as man, demonstrates a less perfect parasitism resulting occasionally in death of the host and inevitably in cessation of the life cycle of the parasite. That the human immune

response to functional (fitness) antigens of the infective stage of T. canis is a factor preventing further development in the life cycle is a possibility although natural immunity, nutritional and physiological factors must be considered. Ogievetskaya (1979) has examined experimentally the phylogenetic disparity of antigens and found that the capacity of a protein to induce synthesis of specific immunoglobulins is directly correlated with protein evolution rate. The evolution rate of a protein serves as a measure of its foreigness which can be evaluated as the number of amino acid differences between the immunogen and the host's homologous protein. Structural components such as actin and collagen have low evolution rates (less than 2 amino acid changes in 10^8 years) and are poor immunogens while enzymes in general have high evolution rates and are good immunogens. This would suggest that nematode cuticular collagen, which comprises the greater part of the cuticle (Chappell, 1979), should be a weaker source of antigen than nematode enzymes.

This leads to the final general source of nematode antigens: the secreted products. The concepts discussed above, when applied to the tissue invasive nematode (eg. Toxocara spp.) suggests a scenario in which a large, complex metazoan, has evolved a cuticular and somatic camouflage of non-specific or host-similar antigens and effectively evades the host's immune response, while migrating through host tissues. Since the second-stage toxocaral larvae lacks biting mouth parts, it progresses through tissue by secreting digestive enzymes from its oral (and possible excretory) pore into host tissue. Tissue digested by these "exo-enzymes" is then ingested as the larvae proceeds. Although cuticular antigens may be host-similar or non-functional, and although many somatic whole worm antigens may be hidden from the immune system, secreted

digestive enzymes are more likely to be host dissimilar, since they (1) digest host tissue; (2) have high molecular evolution rates, and (3) are functional (ie. their inactivation would halt the worm's migratory progress, inhibit its up-take of nutrients, and possibly result in its encystment and destruction by other immune effector mechanisms). Therefore, as functional antigens, secreted products are more likely to be protective. The superior protective value of nematode secretory antigens compared to whole worm extracts are well known (Rothwell and Love 1974; Poulain, Petit, Pery and Luffan, 1976; Despommier and Müller, 1976; Wakelin and Selby, 1973; Depommier, Campbell and Blair, 1977). It follows,that as protective antigens, secretory products are more likely to be specific antigens (Soulsby, 1962). In addition to high levels of potential specificity, nematode secretory products have other characteristics which make them ideal serological antigens.

Regarding isolation and purification, nematode secreted products, even in the crude state, are relatively simple mixtures of only a few antigens, compared to the complexity of crude cuticular and whole worm extracted antigens.

Regarding sensitivity, the small biomass of worm material present during light infections will eventually present a substantial quantity of secretory antigen to the hosts immune system. Moreover, this presentation will have a natural, adjuvant-like basis resulting from its slow and continued release.

Regarding biological relevance, studies have shown that secretory antigens are the first helminth antigens to evoke an immune response (Catty, 1969). It is also considered that anti-secretion antibody is temporally more closely related to active infection, then anti-whole worm antibody which may persist (or even increase) following death of the

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parasites (Fife, 1971). It is tempting to speculate that the presence or intensity of anti-secretion antibody correlates with protection. Because secretory antigens usually contain enzymes (Sanderson and Ogilvie, 1971; Ogilvie, Rothwell, Bremner, Schizerling, Noland and Keith, 1973; Mitchell, 1979) it has been suggested that anti-enzyme antibody might interfere with parasite feeding, or with essential metabolic functions, thus leading to worm damage and increased susceptability to other effector mechanisms of the host's immune system (Mills and Kent, 1965). In experimental Dipetalonema infections, secretory products can enhance microfilaraemia (Hague, Chassoux, Ogilvie and Capron, 1978). Immunological inteference with this process might effect worm fecundity as has been reported in secretory antigen vaccine studies with Trichinella (Despommier and Muller, 1970). Aside from the successful protective responses in nematode infections evoked by immunization with secretory products, there is little direct experimental evidence that the presence of anti-secretions antibody indicates protection (Rothwell and Merritt, 1975; Mitchell, 1979).

In contrast to their protective effects it has been suggested that some secretory antigens might also be immunosuppressive (Terry, 1977). The chronicity of toxocariasis in apparently immunocompetent hosts has not been adequately explained. Nematode released antigens might, in some circumstances, be polyclonal B-cell mitogens thus causing immunosuppression which would be manifested by an increased susceptibility to (or association with) certain infectious diseases. Goose (1977) working with secretory antigens from the non-nematode, Fasciola hepatica, found them cytotoxic to lymphoytes in vitro. Faubert and Tanner (1970) reported that serum from Trichinella spiralis infected mice would agglutinate and kill lymph node cells in vitro and prolong allografts in vivo. However there is, as yet,

no direct evidence that the soluble factors responsible for these immunosuppressive actions are contained in nematode secretory antigen preparations. Further elucidation of the role of nematode secretions in the host-parasite relationship is necessary before a more complete understanding of the biological relevance of anti-secretion serological responses is possible.

Thus, in balance, there appear to be compelling arguments in favour of the use of secretory antigens in nematode serology, as opposed to whole worm extracts, or surface antigens. In the present work, results support this view. Given a satisfactory in vitro cultivation system using synthetic medium to maintain the appropriate parasite stage, it was shown that secretory antigens can be produced in usuable amounts. Since the best antigens are usually available in the least quantity and are in the highest demand, it is necessary to use them in the most efficient assays. ELISA proved to be essential for this purpose. Without ELISA or an assay of similar characteristics, this work involving many thousands of assays, could not have been contemplated. It was the deliberate marriage of antigen, selected following a rational examination of the host-parasite relationship, with assay, selected for its qualities of economy, sensitivity and practicality, which resulted in the described applications. The approach evolved an immunoassay capable of providing specific and sensitive quantitation of circulating anti-Toxocara antibody. Low level infections were detectable rapidly after onset, and cross-reactions could not be demonstrated in animal models nor in a clinical survey. It is proposed that a similar advance may be achieved by application of the above strategy to other tissue invasive nematode infections of man including, onchocerciasis, anisakiasis, strongyloidiasis and trichinosis.

Although some of the concepts discussed in this chapter have been in existence for over a decade, parasitologists and immunologists have been slow to exploit them. The study emphasizes the value of *in vitro* cultivation to nematode serology and the importance of a strategy directed toward the selection of biologically relevant antigens for use in appropriate assays. The role of the immune response to secretory antigens merits further study, particularly as regards protection, hypersensitivity, and immunosuppression. From such studies should come a better understanding of the host-parasite relationship and its relevance to immunopathology and immunodiagnosis.

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APPENDIX 1

Published Portions of this Work

De Savigny, D., Voller A., and Woodruff, A.W. (1979).
Toxocariasis: serological diagnosis by enzyme immunoassay.
Journal of Clinical Pathology 32, 284-288.

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Brit. med. J. II 1747-1748.

APPENDIX 2

Formulation of Reagents for Toxocara ELISA

Coating Buffer

-0.05 M Carbonate-bicarbonate (for antigen dilution)

$NaCO_3$	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.20 g
$D.H_2O$	→ 1000 ml (pH 9.6)

PBS-T

- phosphate buffered saline with 0.05% Tween 20. (for serum dilution, conjugate dilution, and washing steps).

NaCl	8.0 g
KH_2PO_4	0.2 g
$Na_2HPO_4 . 12H_2O$	2.9 g
KCl	0.2 g
NaN_3	0.2 g
Tween 20	0.5 ml
$D.H_2O$	→ 1000 ml (pH 7.2)

Diethanolamine Buffer

- 10% Diethanolamine (for substrate dilution)

Diethanolamine	97 ml
$MgCl_2.6H_2O$	0.1 g
NaN_3	0.2 g
$D.H_2O$	≈ 800 ml

HCl (1M) Titrate to pH 9.8

$D.H_2O$	→ 1000 ml (pH 9.8)

Substrate

4 - Nitrophenyl phosphate	20 mg
Diethanolamine Buffer	20 ml

Terminating Reagent

3M NaOH

NaOH	12 g
$D.H_2O$	→ 100 ml