69-10,609

ALJEBOORI, Tarik I., 1937-

CELLULAR AND HUMORAL RESPONSES IN BABOONS INFECTED WITH TOXOCARA CANIS.

The University of Oklahoma, Ph.D., 1968
Biology

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CELLULAR AND HUMORAL RESPONSES IN BABOONS INFECTED WITH TOXOCARA CANIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1968

CELLULAR AND HUMORAL RESPONSES IN BABOONS

INFECTED WITH TOXOCARA CANIS

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ACKNOWLEDGMENT

The author wishes to express his indebtedness to Dr. M. H. Ivey, his major advisor and the chairman of his advisory committee. Dr. Ivey gave generously of his time, his friendly advice, guidance, and assistance in writing of this manuscript. Appreciation is also extended to other faculty members of the Department of Laboratory Practice, School of Health, for their encouragement and assistance during the graduate program.

He wishes also to acknowledge a considerable debt of gratitude to Dr. C. Stout of the Department of Pathology for his help in the preparation and interpretation of the histological sections. Special consideration is extended to Dr. Y. Gutierrez, Department of Laboratory Practice for technical drawings and Mr. A. F. Hutton, Chief Technologist for the use of electrophoresis equipment. Acknowledgment is due to Mrs. Tommy Hewett for typing the reading and final draft of this manuscript. The Iraqi Government is also due thanks for the graduate study scholarship.

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CELLULAR AND HUMORAL RESPONSES IN BABOONS INFECTED WITH *TOXOCARA CANIS*

CHAPTER 1

INTRODUCTION

Beaver *et al.* (1952) added a previously unrecognized human pathogen to the list of animal parasites which cause disease in man. The disease was characterized by chronic, extreme eosinophilia with hepatomegally and some degree of pulmonary infiltration. The highest incidence occurs in young children having tissues infected with larvae of dog ascarids of the genus *Toxocara* and possibly others. The above workers suggested the term "visceral larva migrans" for this disease.

Since 1952, two factors have led to an increasing interest in visceral larva migrans. These factors are: (1) The realization that the disease is more common than previously thought. (2) The high degree of damage which the disease causes to such organs as the brain (Dent, *et al.*, 1956), heart, (Friedman and Hervada, 1960), and retina (Irvine and Irvine, 1959; Ashton, 1960; Woodruff *et al.*, 1961; Duguid, 1961.)

Infections with *Toxocara canis* in various species of experimental animals have been reported by many workers in recent years. The animal species selected was determined by the objective which each worker hoped to attain. Interest focused especially on studying the onset of both cellular and humoral responses of infected animals, as well as any changes

in serum proteins during infection with T. canis, the major causative agent of visceral larva migrans. Most studies with small laboratory animals and some larger domestic animals have revealed that these animal species demonstrate an acute eosinophilic response as contrasted with the chronic response of humans.

It is difficult to diagnose human infections of T. canis clinically, due to the fact that the symptoms involved mimic those of other disease conditions. Brill, et al. (1953) reported that a diagnosis may be obtained by demonstrating larvae in granulomatous nodules of the liver. Smith and Beaver (1955) suggested that microscopic examination both of stained and serial sections and of digested tissues may be necessary for such a diagnosis. Furthermore, Heiner and Kevy (1956) reported that a leukocytosis with a marked increase in eosinophils accompanied by a hypergammaglobulinemia may be useful in making a diagnosis. Excluding autopsy, the demonstration of the larvae can only be accomplished by liver biopsy. This procedure is associated with risk and may also give inconclusive results in mild cases. This lack of a convenient diagnostic tool has generated much interest and study of the value of various serological tests for the presumptive diagnosis of T. canis infections.

Serological studies on experimental animals and suspected human cases of visceral larva migrans have been numerous. Most of the common serological techniques have been used, including agar-diffusion precipitin test, complement-fixation tests, flocculation tests, skin tests, hemagglutination tests, Schultz-Dale tests, passive cutaneous anaphylaxis, and indirect and direct fluorescent tests. Although the detection of Toxocara antibodies is possible with all of the above serological

tests, interpretation is complicated by cross reactions with other helminths, especially Ascaris.

Two aspects of experimental visceral larva migrans research demand further attention: (1) Further attempts to find a suitable experimental animal that responds to infection with T. canis in a manner similar to humans, and (2) intensified studies of the various stage specific antigens of Toxocara and Ascaris in an attempt to find more specific antigens for serological tests.

The objectives of this study were as follows:

1. To investigate the suitability of a primate, the baboon, (Papio anubis) as a potential experimental model for studying the cellular and the humoral responses and the associated serum protein changes in this host after infection with T. canis.
2. To investigate and evaluate four different types of T. canis antigens (adult, larval, infective eggs, and hatching fluid) by using the microhemagglutination test.
3. To investigate and compare the specificity of the T. canis antigens with their counterpart Ascaris suum antigens in order to evaluate the usefulness of the microhemagglutination method as a diagnostic technique for T. canis.
4. To investigate and study the histopathological effects as well as larval recovery from different organs of baboons infected with T. canis. This objective origi-

nally was not planned, but it was included when some of the animals died shortly after the second infection.

CHAPTER II

LITERATURE REVIEW

Visceral larva migrans, an important zoonotic human disease, was first defined by Beaver et al. (1952) as a prolonged migration of larval nematodes in tissues other than skin. Typically, the syndrome manifests itself as a chronic extreme eosinophilia accompanied by eosinophilic granulomatous lesions in various organs, an enlarged liver, and some degree of pulmonary infiltration. The larvae of dog ascarids of the genus Toxocara are incriminated most frequently. The syndrome occurs primarily in children with a history of geophagia.

Toxocara canis is a common intestinal roundworm of dogs and it is especially common among younger dogs. The infective egg hatches in the small intestine of dog after being ingested. The larva penetrates the wall of the intestine and is carried to the lung. The larva then migrates via the trachea and esophagus to the intestine where it matures. The success of this parasite in maintaining a high prevalence and wide distribution doubtless is due to the fact that it is well tolerated by the dog and to several special adaptations for transmission. Douglas and Baker (1959) reported that infection in the dog may be acquired by ingestion of eggs or there may be prenatal transfer of larvae from the tissues of the mother to the fetus, where they may reside for a year or more.

Schacher (1957) and Sprent (1958) reported that dogs can become infected when the larvae in the mother's tissues pass to the fetus (in utero), where they remain in the lungs until birth. Furthermore, it has been shown that the dog acquires patent infection by two other methods. Sprent (1958) showed that the dog may become infected by eating a paratenic host which harbors the infective larvae. Moreover, Sprent (1961) stated that sexually mature female dogs do not acquire patent infections by ingestion of eggs, as reported by several workers, but may acquire patent infection due to the ingestion of larvae passed in the feces of their newborn.

Toxocara infection in humans occurs predominantly in very young children, one to four years old, with a history of close contact with infected dogs and, especially, geophagia. An alternative method of infection is via consumption of food contaminated with infective eggs of Toxocara, particularly uncooked, leafy vegetables. In humans, the infective eggs hatch in the stomach or the intestine after being swallowed. The larvae migrate through the wall of the intestine, probably enter the blood vessels or lymphatics, and are carried to various organs of the body. Toxocara canis larvae apparently do not reach maturity in humans. Beaver (1962) demonstrated that larvae survive for long periods of time in paratenic hosts and can be recovered from the liver, lungs, and brain after several months.

Numerous workers have considered various aspects of host-parasite relationships in experimental animals and humans infected with T. canis. This literature review of T. canis will be confined to the objectives of the present study as outlined in the introduction: histopatho-

logy, antibody, cellular, and serum protein responses in baboons infected with T. canis, and the evaluation of the sensitivity and specificity of four different Toxocara antigens with their counterpart Ascaris antigens in serological tests.

A clinical syndrome of children characterized by fever, hepatomegally, leukocytosis with marked eosinophilia, and hyperglobulinemia was reported by Perlingiero and Gyorgy (1947). They also found small, gray-white lesions scattered over the surface of all lobes of the liver and numerous eosinophilic granulomas in biopsy tissue from a selected area. Mercer et al. (1950) and Behrer (1951) recovered larvae from human liver lesions. The latter worker suggested that the cause of eosinophilia was the keratin which the human ascarid larvae sheds during its life cycle moults. During the same period, Wilder (1950) reported finding nematode larvae in eosinophilic granulomas in enucleated eyes of children from different parts of the United States. Beautyman and Woolf (1951), found a similar larva in the left thalamus of a child whose death was due to poliomyelitis. Most of the cases with chronic eosinophilia and ill-defined etiology were given different names, such as familial eosinophilia, eosinophilic pseudoleukemia, and Weingarten's disease. Beaver et al. (1952) described three human cases and identified the larvae in one of these as T. canis, a dog ascarid. They suggested that this syndrome develops in patients with an allergic diathesis and suggested that the disease be called "visceral larva migrans". Furthermore, Smith and Beaver (1953) were able to establish the syndrome in two mentally defective young children by experimentally infecting them with T. canis.

Necropsy findings, histopathological observations, and larval

recovery from different organs, both in humans and experimental animals, have been reported by many workers. Brill et al. (1953) reported autopsy findings in a 2-year-old child with eosinophilia and disseminated visceral lesions. Many necrotic and granulomatous foci with eosinophilic infiltration were found in the liver, kidneys, heart, and lungs. A Toxocara larva was demonstrated in one of the pulmonary nodules. Dent et al. (1956) reported that necropsy of a 19-month-old child who died of serum hepatitis revealed granulomatous lesions, with and without the larvae of T. canis, widely scattered throughout the viscera and the central nervous system.

Although the larvae have been recovered most abundantly from the liver, brain, and lungs, they have also been found in other organs. Nichols (1956) showed that the larvae which Wilder (1950) recovered from human eyes were those of T. canis. Eye involvement due to Toxocara larvae has been shown by several other workers (Irvine and Irvine, 1959, Ashton, 1960; Woodruff et al., 1961; Duguid, 1961). Friedman and Hervada (1960) reported a case of visceral larva migrans with severe myocardial involvement. Becroft (1964) reported autopsy findings of a 15-month-old child who died suddenly from an acute myocarditis and pneumonia. Multiple granulomatous lesions were seen in the liver and in one lesion a T. canis larva was identified. Furthermore, the myocardium showed patchy interstitial infiltration, largely lymphocytic, and no granulomas were found in any organs other than the liver. Correa et al. (1966) described an incidental lesion due to T. canis larva in the myocardium of a 14-year-old girl who died suddenly, probably from food intoxication. The lesion consisted of a thick fibrous capsule surrounded by loose connective tis-

sue and some eosinophilic infiltration. Moore (1962) demonstrated granulomata in the brain of a child whose death was attributed to lead poisoning and toxocariosis. The granulomata were spherical and were composed of a necrotic area around each larva surrounded by disintegrated leukocytes, followed by an area of epithelioid cells. Surrounding the epithelial cells there was a band of fibrocytes and collagen and within the interstices of this zone there were lymphocytes, plasma cells, and eosinophils. Brain and Allan (1964) reported a case of central nervous system involvement due to T. canis larvae.

Studies on the behavior of T. canis and related ascarid larvae in experimental animals, as well as larval recovery from different organs, have been reported by several workers. Sprent (1952, 1955) and Smith and Beaver (1953) reported that T. canis larvae remained in the liver of mice for a few days, then migrated to the muscles, brain, and in lesser numbers, to other organs. After six months to a year a large proportion of the larvae still persisted in the brain and muscles and were infective when fed to other animals. Sprent (1952 and 1953) was able to recover 16.0 to 26.8 per cent of the T. canis larvae from the tissues of infected mice. Oshima (1961) found that, 44 to 48 hours after inoculation, 98 per cent of the larvae recovered from mice were in the liver and lungs. Lee (1960) reported that whereas 60 per cent of the larvae could be recovered from mice given a single infection, recovery was 20 per cent less in mice given an additional infection. Furthermore, he stated that the super infection had no apparent effect on the extent of encapsulation, but it did increase the relative number of encapsulated larvae in the liver.

Done et al. (1960) using the pig as an experimental animal, found that T. canis larvae migrated to all parts of the body. Lesions characteristic of visceral larva migrans were found in the liver, lung, heart, brain, spinal cord, gastro-splenic lymph nodes, pancreas, tongue, adrenal diaphragm, and voluntary muscles. Marked local eosinophilia and granuloma formation were observed, even in the early stages of infection. Higashikawa (1961) reported that larvae of T. canis migrated to the liver, brain, muscle, kidney, and heart of mice. After 240 days, fibrinoid degeneration of collagen was seen and the larvae were surrounded by hard fibrous capsules. In the brain, however, the larvae were usually free and cellular reactions were not clear, except in certain stages. Beaver (1962) reported that the distribution of the larvae of T. canis in tissues of various experimental animals (mice, rats, hamsters, guinea pigs, rabbits, and monkeys) varies considerably. In general, the organs most affected were liver, muscle, brain, and lungs. After 12 to 15 months, a significant number of larvae were recovered from the brain of the mouse, rat, hamster, and guinea pig, but in the rabbit and the monkey there were many in the liver and few in the brain. In the brain, the larvae usually remained free, while in all other organs they become enclosed in fibrous capsules. Sheep, chickens, and goats have been infected with T. canis (Schaeffler, 1960; Galvin, 1964; and Sinha, 1966). Fernando (1968) recovered larvae from the liver and lungs of rabbits infected with T. canis and also observed white foci in the liver, kidney, and lungs. Moreover, the animals which resisted the effects of reinfection with a lethal dose of larvae showed an enhanced antibody response as shown by complement-fixation and agar-diffusion precipitin tests. The immunity

was directed primarily against the migration of larvae to the lungs.

Much has been written on the cellular and humoral responses of a host to parasites. It has been suggested that both responses play an important role in the functional response of the host to many parasitic nematodes. Sarles (1938) demonstrated acquired resistance in rats infected with Nippostrongylus muris and suggested that the resistance was aided by humoral antibody, since a precipitate was formed on the cuticle and the oral and excretory orifices of the larvae and in the intestine of mature and immature adults exposed to immune sera. The importance of the hosts' cellular reaction to parasitic nematode infection has been studied by several workers. Taliaferro and Sarles (1939), working with N. muris in rats, indicated that immunity against this nematode was largely dependent upon humoral factors with secondary cellular cooperation. Moreover, they stated that eosinophils in large numbers were associated with the immune reaction; Larsh and Race (1954), working with Trichinella spiralis, made similar observations. Wells (1962) reported that resistance to N. muris was due to the production of antibodies and that the cellular reactions played a significant, but subsidiary role.

In spite of the extensive work done on the eosinophil, there is still no agreement or conclusive evidence available on its functions or on the factors that induce its increase in both tissues and blood. Eosinophilia usually accompanies conditions such as parasitic infections, certain tumors, allergic disorders, polyarteritis nodosa, and fungal infections. Generally, tissue parasites and parasites that spend at least part of their life cycle outside the digestive tract are more eosinophilogenic than are those that reside only in the digestive tract. Mild

or severe cases of eosinophilia have been observed in patients infected with Ascaris, Toxocara, Strongyloides, Trichinella, and many other helminths. Several workers have shown that both tissue and blood eosinophils increased in animals experimentally infected with various parasitic nematodes, for example, T. canis in rabbits, (Sadun et al., 1957); Litmosoides carinii in cotton rats, (Olson, 1959); Haemonchus contortus in sheep, (Soulsby, 1960); N. muris in rats, (Wells, 1962); Trichinella spiralis in mice, (Scardino and Zaiman, 1962); T. canis in guinea pigs, (Sharp and Olson, 1962; and Olson and Schulz, 1963).

It has been reported that injections of helminth extracts, foreign protein, or histamine will also induce eosinophilia in blood, lungs, and other organs. Campbell et al. (1935) pointed out that the protein must be foreign, it must be injected repeatedly, and the time interval following the first injection must correspond with that usually found necessary for sensitization to develop. They concluded that the allergic reaction, rather than the foreign protein itself, that stimulated blood eosinophilia. Campbell (1943) reported eosinophilia in guinea pigs injected with A. suum keratin following prior sensitization with the material. Speirs (1955) also demonstrated an increase in the number of eosinophils in the peritoneal cavity of mice after multiple intra-peritoneal injection of Ascaris extract. Soulsby (1962) demonstrated that the cuticles of Ascaris and Nippostrongylus larvae were antigenic and observed that larvae with antibody absorbed to the cuticle attracted eosinophils and transitional macrophages in vitro. Other cells, including lymphocytes, mast cells, and polymorphs, were not attracted.

Vaughn (1952) demonstrated eosinophilia in guinea pigs injected with a high molecular weight, protein-free polysaccharide extracted from A. suum. The eosinophilia disappeared after an antihistamine was given. Vaughn (1953) and Archer (1959) stimulated tissue eosinophilia by local injection of histamine. Litt (1961, 1962, 1963, 1964) claimed that such an eosinophilia is an incipient type compared to that caused by the presence of immune complexes. Furthermore, he showed that antigen-antibody complexes produced eosinophilia by their mere presence, in both unsensitized and sensitized animals. He also showed that histamine was not a major mediator of eosinophilia. However, Arean (1958) reported that the accumulation of eosinophils at the site of the injection of Ascaris eggs was too rapid to be explained on the basis of an antigen-antibody reaction. In addition, Huntly and Costas (1965) reported on a child which exhibited massive eosinophilia in spite of an almost complete absence of immune globulins. They suggested that the eosinophilia of parasitic disease may be unrelated to an antigen-antibody reaction. Uvnas (1960, 1963) reported that Ascaris extracts are potent histamine releasers by virtue of their ability to degranulate mast cells. He also stated that the extracts initiated the release of an enzyme related to phospholipase which causes a slow contraction of smooth muscles. Stewart (1953) showed that the self-cure reaction which occurs in sheep infected with H. contortus is associated with a rise in blood histamine. He also demonstrated that antihistamine eliminates the self-cure phenomenon but does not have any effect on the antibody response to the parasite.

Contradictory views prevail concerning the histamine content of eosinophils and the eosinophilogenic properties of histamine. Vaughn

(1953) reported that eosinophils contain an antihistamine substance and he believed it to be the chief component of their granules. He advocated the view that histamine is cytotactic for eosinophils and that one of the functions of the eosinophil is the detoxification of histamine. Vaughn (1961) injected polysaccharides and protein extracts of Ascaris into subcutaneous tissues of immune and non-immune guinea pigs and found evidence suggesting that eosinophils function as agents which absorb neutralize, remove, and otherwise deal with substances lethal to other cells. Litt (1964) stated that eosinophils contain histamine and he maintained that histamine was not eosinophilogenic.

Phagocytosis is the other major role suggested for eosinophils. They have been shown to phagocytize disrupted mast cell granules (Welsh and Geer, 1959), heterologous blood cells (Archer and Hirsch, 1963), and antigen-antibody complexes (Archer and Hirsch, 1963; Sabesin, 1963; Litt, 1964). The latter two workers suggested that phagocytosis and disposal of antigen-antibody complexes were specific functions of eosinophils. Such complexes may also be removed by neutrophils and macrophages, as noted by Patterson et al. (1962). Speirs (1958) proposed that neutrophils and eosinophils undertake the phagocytosis of antigenic material in inflammatory foci. He postulated that eosinophils play an early and important role in antibody formation.

Smith and Beaver (1953) established visceral larva migrans infections in two young children by giving each child 200 infective T. canis eggs. They reported, that although they remained asymptomatic, the children developed eosinophilia which persisted for more than 13 months. One child developed an eosinophilia of 52% while the other child reached a

peak eosinophilia of 20% within one month after oral infection. Chaudhuri and Saha (1959) demonstrated a marked eosinophilia (26%) thirteen days after infecting a human volunteer orally with 100 T. canis eggs. On the 30th day after infection the eosinophilia peaked at 62% and it remained between 40 and 50% for five months thereafter.

Several workers have shown that maximum eosinophilic responses in various animal species develop within two weeks after infection with nematodes: T. canis in rabbits, (Sadun et al., 1957); Dictyocaulus viviparus in cattle, (Weber and Rubin, 1958); Trichinella in rats, (Scardino and Zaiman, 1962). Wells (1962) demonstrated a marked eosinophilia in rats 12 days after infection with N. muris which was accompanied by a marked decrease in the number of mast cells. He suggested that this decrease in mast cells was due to their disruption and the release of histamine which apparently attracted eosinophils whose function was to remove the excess histamine.

Olson and Schulz (1963) showed that the onset and the extent of eosinophilia were dose dependent in guinea pigs infected with T. canis. A maximum eosinophilia of 39% was observed approximately two weeks after infection. They suggested that the decline in the eosinophil count after the second week of infection may be related to the negative larval recoveries with animals necropsied on day 14. However, Beaver (1962) reported that 26.4% of the larvae were recovered from guinea pigs one year after they were infected with T. canis. Ivey (1965) accomplished even higher larval recoveries from the livers of guinea pigs 10 weeks after infection with T. canis. Sinha (1966), using the goat as an experimental animal, found a marked increase (24%) in the eosinophilic response between the

5th and 7th week after infection with T. canis. He concluded that this increase in the eosinophil counts was not related to the number of infective eggs fed.

A substantial amount of work has been done on the serological diagnosis of T. canis infection in both experimental animals and in human cases suspected of being infected with visceral larva migrans. By using the agar-diffusion precipitin technique Heiner and Kevy (1956) demonstrated a reaction between Toxocara larval antigen and human serum from a suspected case of visceral larval migrans. Sadun et al. (1957) reported that specific antibodies were produced in rabbits infected with T. canis. Using adult Toxocara antigen in the bentonite flocculation test, the antibodies were detectable in a reproducible manner two to three weeks after infection. Complement fixation and precipitin tests gave less reproducible results. These workers also detected antibodies in Rhesus monkeys that had received 900,000 infective T. canis eggs over a period of one year. The antibodies were detected at a titer of 1:5 to 1:40 by flocculation tests one year after the last infection. The authors concluded that sufficient information was not yet available regarding the sensitivity and specificity of these tests to justify their use for the laboratory diagnosis of visceral larva migrans.

Jung and Pacheco (1958) showed that the skin test did not appear to be of value in distinguishing Ascaris from Toxocara infections with the use of either adult or larval antigens of both species. However, Duguid (1961), using adult and infective egg Toxocara antigens, and Woodruff et al. (1964), using adult Toxocara antigen, reported promising

positive skin test results in cases of suspected visceral larva migrans. Kagan (1958) found that antigens of Toxocara and Ascaris worms cross react in the hemagglutination test with antisera prepared against adult worm extracts of either species. Absorption with heterologous antigen removed cross-reacting antibodies and produced genus-specific antisera. Olson and Schulz (1963) reported a positive in vitro larval precipitin test using guinea pig sera collected 28 days after infection with T. canis.

Antigenic cross-reactions between nematode parasites are a major problem in understanding and interpretating positive serological tests. The cross-reactions between Ascaris and Toxocara systems have been confirmed using bentonite flocculation and hemagglutination tests (Kagan, et al., 1959); also hemagglutination test (Jung and Pacheco, 1960); precipitin tests (Olson, 1960); Schultz-Dale tests (Sharp and Olson, 1962); and agar-diffusion precipitin techniques (Huntly and Moreland, 1963). Ivey (1965), using Schultz-Dale desensitization techniques, presented evidence that T. canis larval antigens are of more diagnostic importance than are adult antigens. Furthermore, Ivey (1967) reported that antibodies in rabbits and guinea pigs could be detected by passive cutaneous anaphylaxis tests the second week after infection. Mitchell (1964) reported a cross reaction between Ascaris and Toxocara using the indirect fluorescent antibody test. However, Hogarth-Scott (1966), using the direct immunofluorescence technique, found no cross reactions when sera from individuals infected with other nematodes were tested with Toxocara larvae.

In recent years, electrophoretic techniques have been widely

used both in human and veterinary medicine for analysis of protein fractions for diagnostic purposes. Kagan and Goodchild (1961) stated that parasitologists were also finding increasing use for electrophoretic techniques in the analysis of helminth infections. Changes in host serum protein patterns after nematode infections have been shown by several workers. Leland et al. (1955) noted that in rats infected with N. muris the total serum protein was slightly increased, the beta globulin was significantly increased, and the increase in the beta globulin was dose dependent. Sadun et al. (1957) reported that rabbits infected with T. canis develop a hypergammaglobulinemia and an increase in the alpha-1 globulin fraction of the serum. Weber (1957) studied the serum protein changes in cattle infected and reinfected with Dictyocaulus viviparus in an attempt to correlate the increase in gamma globulin with antibody formation and eosinophilia. He found an increase in the gamma globulin and stated that the increase in the gamma globulin and the eosinophils appeared to be associated with increased antibody titers in the serum of the infected animals.

Leland et al. (1959) demonstrated an increase in the alpha-2 globulin level, a hypoproteinemia, and a decrease in the albumin in calves infected with Trichostrongylus axei. Turner (1959), studying Strongyloides papillosus infections in lambs, found an increase in both beta and gamma globulins. The beta globulin reached its highest concentration the third week after infection. He attributed these increases to "antibody" formation. Andersen et al. (1960) noted an increase in one or more of the serum globulins and a marked decrease in albumin in calves infected with Ostertagia ostertagi.

Nash (1965) found an increase in the gamma globulin in mice infected with T. canis. The results of antigen-antibody absorption studies suggested that the increase was not due to the production of antibodies but was probably due to bacterial invasion of the extra-intestinal tissues following penetration of larvae. Beaver et al. (1952, 1956) and Huntly and Costas (1965) noted a hypergammaglobulinemia in diagnosed and suspected cases of visceral larva migrans. Snyder (1961) also reported that hyperglobulinemia was a feature in most of the severe cases of visceral larva migrans. Furthermore, Beaver (1962) reported that there was a direct relationship between the degree of eosinophilia and hyperglobulinemia in most, but not all, cases diagnosed as visceral larva migrans.

Shedlousky and Scudder (1942) found that inflammation and tissue damage were associated with an increase in the alpha globulin fraction. Stauber (1954) reported that hyperglobulinemia and albuminemia are characteristic of many conditions, including parasitic infections. He also pointed out that most of the previous reports did not take into consideration the effect of the pathological process on the total circulating plasma or serum protein. Leland (1961) stated that changes in the serum protein fractions may be the result of changes in the rate of synthesis or loss of certain fractions by internal destruction or external leakage. Furthermore, he suggested that the changes in serum protein of a hosts infected with parasitic helminths are associated with inflammation of tissue damage, hence the changes cannot be considered as specific for the parasite. From the use of isotopic techniques and other evidence, it has been concluded that the liver is the chief, if not the sole site of forma-

tion of albumin, fibrinogen, and the beta globulins (Frankel and Reitman, 1963). Leland (1961) stated that several attempts have been made to explain the changes in serum protein fractions of animals infected with helminth parasites as being related to immunity, especially when the gamma globulin was involved. Frankel and Reitman (1963) stated that antibodies are formed primarily in the cells of the reticuloendothelial system and that their chemical and physical properties are those of normal serum globulins (especially gamma globulin). Kabat and Mayer (1964) reported that both beta-2 and gamma globulins are known to contain antibodies.

CHAPTER III

MATERIALS AND METHODS

Source of Adult Toxocara and Ascaris

Adult Toxocara canis were obtained from naturally infected dogs and from puppies experimentally infected at three weeks of age with 120 to 140 infective Toxocara canis eggs. The worms were recovered from the experimentally infected puppies 6 to 8 weeks after infection. Mature Ascaris suum were obtained from a local slaughter house. After washing the worms thoroughly with running tap water, male and female adults were separated and either used fresh or stored at -20°C for subsequent antigen preparations.

Embryonation of Toxocara and Ascaris Eggs

Eggs were collected from the distal one-third of the uteri of fresh mature female worms. One volume of eggs was treated with five volumes of two per cent sodium hydroxide for one hour to remove the uterine tissues and mucous. After several washings with distilled water, the eggs were suspended in 0.1N sulfuric acid containing an aqueous solution of merthiolate (1:10,000 concentration). The method of Fairbairn (1961), with minor modifications, was used for embryonation. Approximately 0.5 ml of packed eggs were suspended in 30 ml of the 0.1N sulfuric acid-merthiolate (1:10,000 concentration) solution in a 125 ml Erlen-

meyer flask. The eggs were incubated at room temperature for one month with periodic gentle shaking by hand. After embryonation, the eggs were stored in the same solution at 4°C until used as described below.

Hatching of Toxocara and Ascaris Eggs

The method of Fairbairn (1961), with slight modifications, was used to hatch the infective eggs. The infective eggs were washed three times with distilled water. Approximately 5 to 6 ml of eggs were deshelled by placing them in fresh 5.25 per cent sodium hypochlorite (2 ml of sodium hypochlorite/1 ml of eggs), followed by agitation in a water bath shaker kept at a temperature of 35°C. Complete removal of the egg shell was determined by microscopic examination. Deshelling required 45 to 90 minutes for Toxocara eggs and 2.5 to 3 hours for Ascaris eggs. After 10 washings with distilled water to remove the sodium hypochlorite, the deshelled eggs were transferred to a siliconized 125 ml Erlenmeyer flask containing 15 ml of a 0.25 M sodium chloride-0.1 M sodium bisulfite solution and incubated at 39°C for 2 to 3 minutes. An equal volume of pregassed (95% nitrogen and 5% carbon dioxide) 0.1 M sodium bicarbonate was then added. The sodium bicarbonate was gassed by bubbling it until a pH of 8 was obtained. The flask containing the hatching medium and the deshelled eggs was then stoppered, kept at 39°C in the water bath, and gassed (95% nitrogen, 5% carbon dioxide) for 15 minutes. Next, the flask was placed in a water bath shaker at 39°C and agitated until hatching was complete. The eggs of T. canis hatched in about 90 minutes or less, while Ascaris suum eggs required three to four hours.

Preparation of Antigens

Adult Toxocara Antigen

Adult Toxocara antigen was prepared as follows: 20 fresh or frozen mature Toxocara males or females were suspended in 30 ml of cold (1°C), phosphate buffered saline (PBS/0.15 M pH 7.2-7.3) and disrupted for 2 minutes in a chilled Waring Blender. The mixture was disintegrated further by sonic vibration using a 10 kilocycle Raytheon Sonic Oscillator (Model DF-101) for about 60 minutes. The power was set at an output of 1.25 amp and ice water (1°C) was circulated through the oscillator jacket during the sonication process to minimize the heat effect. Complete disruption was determined by microscopic examination of the suspension. The suspension was kept overnight at 5°C and then centrifuged at 1,000 X G for five minutes to remove the larger particles. The supernatant fluid was centrifuged at 10,000 X G for 30 minutes at 5° and the supernatant fluid was collected. After dialysis against 4 one liter changes of distilled water over a period of 36 hours at 5°C, the dialysate was then lyophilized and kept at 4°C until used.

Infective Egg Antigen

Fully infective Toxocara eggs were washed several times in distilled water at low speed centrifugation in a calibrated centrifuge tube and the volume of eggs was measured. One ml of packed infective T. canis eggs were suspended in 20 ml of phosphate buffered saline (PBS/0.15 M, pH 7.2-7.3) and disintegrated by sonic vibration for 45 to 60 minutes using a 10 kilocycle Raytheon Sonic Oscillator set at 1.25 amp. The remaining steps were identical with those used in the preparation of the adult antigen.

Hatching Fluid Antigen

After the infective eggs of Toxocara were hatched as described above, the contents (hatching fluid) of the flask were transferred to a 50 ml conical centrifuge tube and centrifuged at 1,000 X G for 10 minutes. The sediment containing the hatched larvae was saved to prepare Toxocara larval antigen as described below. The supernatant fluid containing the hatching fluid was collected and centrifuged at 10,000 X G for 30 minutes at 5°C. The supernatant fluid was removed, dialyzed, lyophilized, and stored as described above (Adult Antigen Preparation).

Toxocara Larval Antigen

After removal of the hatching fluid, the sediment containing the hatched larvae was washed five times with distilled water. One ml of washed, packed Toxocara larvae was suspended in 20 ml of PBS and disintegrated by sonic vibration for one hour using a Raytheon Sonic Oscillator which was set at an output of 1.25 amp. The remaining steps were identical with those used for preparation of adult antigen.

Various Ascaris Antigens

Similar methods were used to prepare infective eggs, hatching fluid, larval, and adult Ascaris antigens. The protein content of all Toxocara and Ascaris antigen preparations was estimated by the Lowry et al. method (1951) using bovine serum albumin as a standard.

Infection of Baboons

Before infection with Toxocara, and at intervals during the study, all baboons were checked for the presence of helminth eggs in feces by $ZnSO_4$ concentration method. No helminth eggs were found except

for small numbers of Trichuris eggs in some of them.

The fully developed Toxocara eggs were washed several times and suspended in distilled water. Egg estimations were made by taking the average of five counts of 0.05 ml aliquots of the egg suspension. The suspension was adjusted to the required number of eggs per ml on the basis of these estimates.

Ten baboons, two to six years old, were used in this study. They were kept in two connected chain link cages, an inside cage 8 x 15 x 5 feet high and an outside cage 8 x 15 x 9 feet high. They were housed at the Primate Behavior Laboratory in Norman, Oklahoma and fed commercial monkey chow (Purina). The animals were divided into five groups, two animals per group, and were infected orally by stomach tube after sedation with Sernylan, 1-(1-Phenylcyclohexylpiperdine) Hcl (Parke, Davis) given intramuscularly (1.1 mg/kg B W). Groups I, II, III, and V were males approximately 5 to 6 years old. Group IV consisted of two year old females. Each animal in Group I was given a single dose of one Toxocara infective egg/gm body weight. Animals in Groups II, III, and IV received 5, 10, or 20 eggs/gm body weight, respectively. The two animals in Group V were kept under the same conditions and served as normal controls. Mice and guinea pigs were infected and later sacrificed and digested to check the viability of all egg preparations used for baboon infections. Twelve weeks after the first infection, one baboon from each group (Group I through IV) was reinfected with the same size dose given initially. The total number of larvae which each animal received is summarized in Table 1.

TABLE 1

DOSE LEVELS OF INFECTIVE TOXOCARA CANIS EGGS GIVEN TO BABOONS

Animal Group	Weight (Kg)	Number of Larvae Given Per Gram Body Weight	
		First Infection	Second Infection
I			
A	40.00	1	0
B	24.50	1	1
II			
A	22.00	5	0
B	23.40	5	5
III			
A	20.00	10	0
B	17.17	10	10
IV			
A	5.35	20	0
B	5.35	20	20
V(Control)			
A	25.53	0	0
B	26.67	0	0

Bleeding and Collection of Sera

While they were under mild anesthesia with Sernylan (Parke, Davis), the baboons were bled from the femoral artery at weekly intervals during the first six weeks after infection and then every other week for an extended period of time. Total white blood cell counts, white blood cell differentials, absolute eosinophil count, serum protein analyses, and serological tests were done on each specimen.

The two baboons in the control group were housed under the same conditions and were bled at regular intervals.

Blood Cell Counts

Differential, absolute eosinophil, and total white cell counts were made on each animal using samples of femoral artery blood taken between 8:00 and 10:00 A.M. Blood smears were made on pre-cleaned microscope slides, allowed to air dry, covered with Wright's stain, and allowed to stand for one minute. An equal volume of phosphate buffer, pH 6.4, was then added and mixed by blowing. The mixture was then allowed to remain on the slide for 6 minutes. The smears were then rinsed with distilled water, air dried, and differential counts were made microscopically under oil immersion. At least 200 white blood cells were counted per slide and the percentages of each cell type was calculated.

For total white cell and absolute eosinophil counts, the Randolph (1944) method was used. Blood was drawn into a white cell pipette and diluted 1:20 with the diluting fluid. The diluting fluid was made prior to use by mixing an equal volume of solution No. 1 (0.1 per cent methylene blue, 50 ml propylene glycol, plus 50 ml distilled water) and solution No. 2 (0.1 per cent phloxine, 50 ml propylene glycol plus 50 ml distilled

water). The pipette was agitated for 30 seconds on a Yankee pipette shaker and then placed horizontally on the table. After 15 minutes the pipette was agitated again for 30 seconds. The first four drops were expelled from the pipette and both sides of a Levy Hemocytometer Chamber counting slide were filled from the remaining mixture. The filled slides were covered with inverted Petri dishes moistened with filter paper to prevent evaporation. Counts of both eosinophils and total white cells were begun 15 minutes after filling the chambers. An average of two readings was calculated for each blood sample.

Serum Protein Electrophoresis

The microzone cellulose-acetate electrophoresis system was used for separating the serum proteins. The microzone electrophoresis cell (Model R-101) the scanning attachment (Model R-102), and the Analytrol with B-5 cam (Beckman) were employed. The details of the electrophoresis technique were very similar to those described by Grunbaum et al. (1963), with minor modifications as suggested in the Beckman Instruction Manual. A drop of serum was placed on a nonwetable surface (Parafilm) and touched with the platinum loop of a sample applicator which picks up about 0.25 μl. of serum. The sample was transferred to the membrane. In the present study, the sample size was increased from 0.25 μl to 0.5 μl of serum to furnish enough protein for adequate separation. The membrane was then subjected to electrophoresis for 20 minutes at 250 volts and stained in Ponceau-S solution for 10 minutes. Excess stain was removed by washing three times with 5% acetic acid, followed by dehydration for 2 minutes in absolute ethanol. Subsequently, the membrane was placed in a clearing solution (25 ml of glacial acetic acid, 5 ml of distilled water, plus

70 ml absolute ethanol) and agitated gently for one minute. Excess solution was removed by gently applying a squeegee to the surface of the membrane. The membrane was fastened to a glass plate and dryed in an oven at 90-100°C for 15 minutes. After mounting the membrane in a plastic envelope it was scanned in the Analytrol.

The plot obtained after scanning was divided off into the various serum components and the integration counts for each component were calculated. The relative percentage concentration of each protein component was obtained by dividing individual integration counts by the sum of the counts. This value was then multiplied by the total protein, gm/ml, for each serum sample to calculate the concentration of each protein component in gm/100 ml of serum. The determinations were carried out in duplicate on each serum specimen and the result of the two tests were averaged. Total serum protein was measured by the method of Campbell et al. (1964), with minor modification. The size of the serum sample was 0.1 ml. The biuret reagent was prepared according to the method of Campbell et al. (1964) and human serum albumin was used as a standard.

Larval Recovery and Histological Studies

Approximately 4 gm of fresh liver, lung, kidney, brain, and heart from different sites of each organ from each of four animals were digested separately in pepsin. The total number of larvae in each organ from each animal was estimated by multiplying the number of larvae recovered/gm by the total weight of the organ.

For histological studies, selected tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 6 microns. They were

stained with hematoxylin and eosin, Gomori's trichrome, and Kinyoun's acid fast stains. Blocks were recut and serial sections were made as needed to demonstrate the three dimensional structure of the lesions.

Passive Hemagglutination Test

Jung and Pacheco's (1960) hemagglutination method was used, with certain modifications. The optimum concentration of each Toxocara antigen used to sensitize the red cells was determined by titrating varying concentrations of each antigen against varying dilutions of Toxocara hyperimmune rabbit serum. The hyperimmune serum was collected one week after the last infection, from a rabbit infected with 5,000, 5,000, 6,500, 8,000, and 10,000 infective T. canis eggs at 0, 12, 15, 19, and 22 weeks respectively. The antigen concentration giving the highest titer without non-specific reaction was selected for use with experimental baboon sera. Ascaris antigens were also standardized against hyperimmune serum of a rabbit infected with Ascaris infective eggs in a similar manner. The hyperimmune serum was collected, two weeks after the last infection, from a rabbit infected orally by stomach tube with 15,000, 25,000, and 100,000 infective Ascaris eggs at 0, 5, and 8 weeks, respectively.

All experimental sera were inactivated by heating them in a water bath at 56°C for 30 minutes. Each serum sample was mixed with one volume of washed, packed sheep red cells (Colorado Serum Co.) per 5 volumes of serum. The mixture was incubated at 37°C for one hour and stored overnight at 4°C. The suspension was centrifuged at 1,720 X G for 15 minutes and the supernatant serum was collected and stored in convenient volumes.

Initially, the standard tube technique was used, but later the micro technique was adopted exclusively when comparative studies indicated that there was no significant difference between the titers obtained with the two techniques. Thus, the microhemagglutination technique was used in this study because of the speed with which the microdilutions can be made and the economy of antisera and antigen. The microtiter apparatus of Cooke Engineering Co. was used.

Tanning of Sheep Red Cells

Various methods and compounds are available for coupling antigens to red cells for passive hemagglutination tests. Although tannic acid was the primary compound used for coupling of the antigen on the sheep red cells throughout this study, two other chemical compounds, bis-diazotized benzidine (BDB), Gordon et al. (1958) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ECDI), (Ott Chemical Co.), were also tried.

Sheep red cells preserved in Alsevers solution (Colorado Serum Co.) were stored at 4°C until used (within three weeks of bleeding date). One ml of stock sheep red cells yielded approximately 0.2 ml of packed red cells. The red cells were washed (1,400 X G for 5 minutes) three times with phosphate buffered saline (PBS/0.15 M, pH 7.2-7.3) using one ml of cell suspension per 5 ml of PBS. After the final washing and centrifugation, the packed cell volume was determined and a two per cent cell suspension was prepared in PBS. Commercial tannic acid (Mallinckrodt - Lot, 1764) was prepared as a one per cent stock solution in saline and kept at 4°C. Just prior to use the stock solution was diluted 1:20,000 in saline. One volume of the two per cent washed sheep red cell suspen-

sion was mixed with an equal volume of 1:20,000 tannic acid and mixed thoroughly by gently stirring with a Pasteur pipette, followed by incubation at 37°C for 15 minutes. The tanned cells were then washed once with PBS after centrifugation at 1,225 X G for 5 minutes.

Sensitization Process of Tanned Red Cells

1 ml of 2% tanned red cell suspension
+
Antigen (optimum concentration) in one ml PBS (0.15 M, pH 7.2-7.3)
+
3 ml of PBS. The cells were dispersed by gently stirring with Pasteur pipette.
↓
Incubated at either 37°C or room temperature for 30 minutes depending on the kind of antigen (See Results)
↓
Washed once, after centrifugation at 1,225 X G for five minutes, with 2 ml diluent (1% normal rabbit serum in PBS)
↓
Adjusted to 2% red cell concentration in diluent.

Control cells were treated in the same manner except that normal saline was substituted for the antigen.

Two-fold serial dilutions of each serum were made in diluent (1% normal rabbit serum in PBS), starting with a 1:80 dilution, with a final volume of 0.05 ml per V-shaped plate well. One drop (0.025 ml) of the 2 per cent sensitized cell suspension was added to each well. The plates were then shaken gently and covered by Parafilm to prevent evaporation. They were incubated at room temperature for three hours before results were read, or until the control cells settled to the bottom of the wells. Uniform, thin, translucent layers of cells covering the entire lower surface of the wells were considered positive, i.e. complete hemagglutination. On the other hand, a compact circular button of cells in

the bottom was interpreted as negative, i.e. no hemagglutination. Wells showing partial hemagglutination were considered as negatives. Controls were as follows:

A. Known positive, consisting of positive serum plus sensitized cells.

B. Normal serum plus sensitized cells.

C. Normal serum plus control cells (tanned but not sensitized).

D. Diluent plus sensitized cells.

E. Diluent plus control cells.

The test was considered valid only when all the controls, except the first one, were negative.

CHAPTER IV

RESULTS

Ten baboons were divided into 5 groups, each containing 2 animals. The animals were infected with varying levels of Toxocara as indicated in Table 1. One animal each in Groups I, III, and IV received the second infective dose 12 weeks after the first infection while one animal in Group II was reinfected the sixteenth week after the first infection. Blood was collected from each animal at weekly intervals during the first 6 weeks, and then every other week of the initial infection for the study period. Total white cell counts, absolute eosinophil counts and differential cell counts were made on each blood sample. Serum was saved for subsequent antibody and protein analyses.

Cellular Response

The cellular responses of each animal are summarized in Figures 1 and 2. The white cell count and percentage of eosinophils (absolute eosinophil count divided by total white cell count x 100) reached a peak the second or third week after infection. The white cell counts returned to almost normal levels within 5 to 6 weeks after infection (Figure 1). The eosinophil response showed a similar drop during this period but remained above pre-infection levels during the entire study period in most of the animals (Figure 2). The average maximum white cell

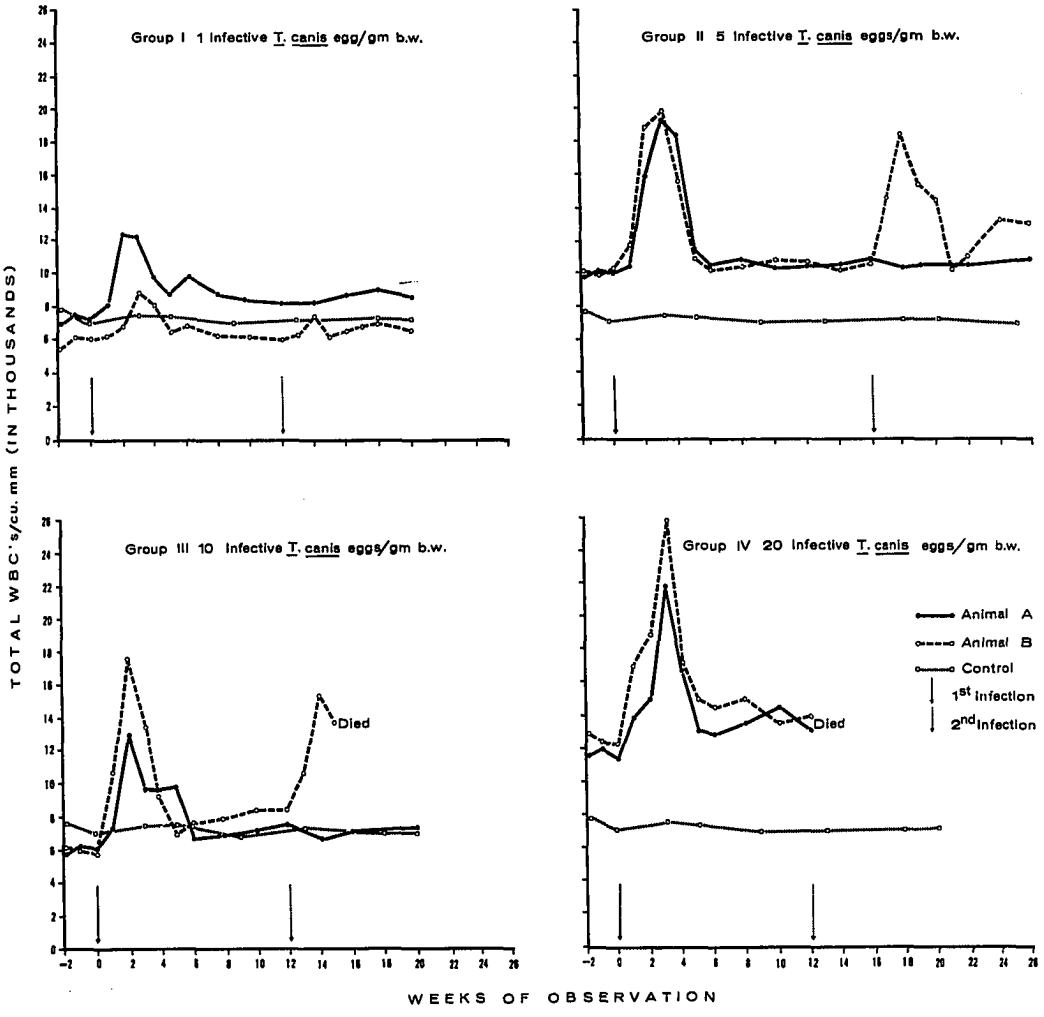


Figure 1. The total white cell response in baboons given infective Toxocara canis eggs. The control represents the average of the two uninfected animals.

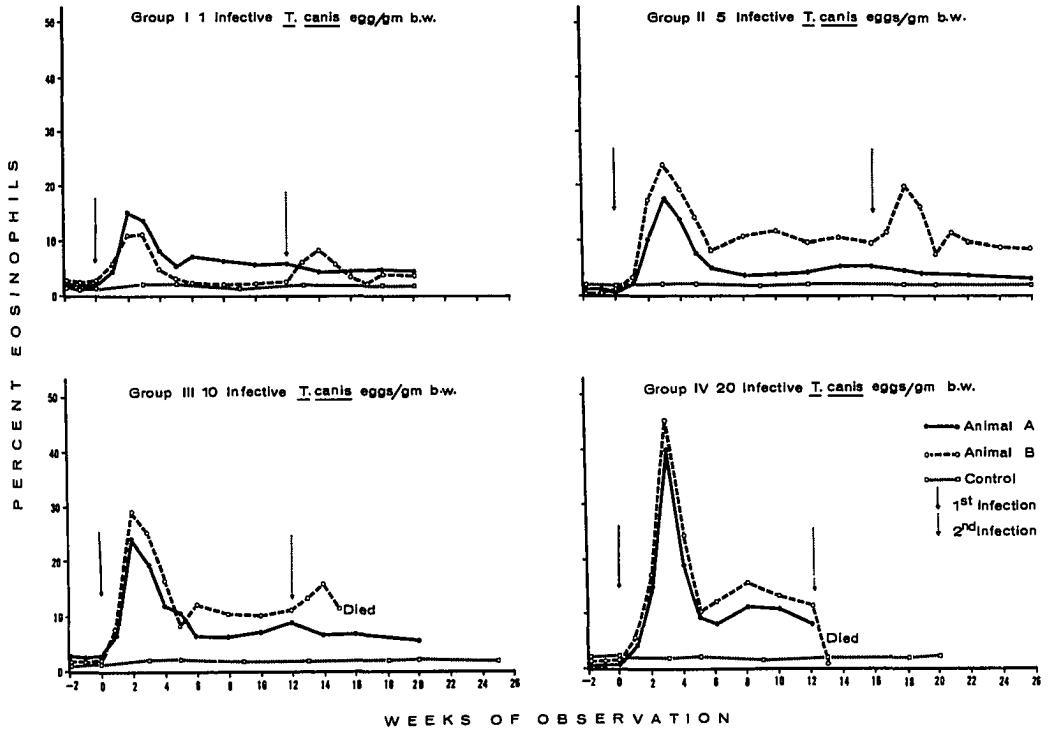


Figure 2. Eosinophil percent in baboons given different levels of infective Toxocara canis eggs. The control represents the average of the two uninfected animals.

and eosinophil responses of the 2 animals in each group are shown in Table 2. Cell counts increased as the infection level increased. This was especially evident in the case of the eosinophil response. Differential counts revealed a slight lymphocytosis with no apparent change in monocytes or basophils. In most of the infected animals an increase in eosinophils was accompanied by a decrease in neutrophils. The eosinophil values (percentage) obtained from the differential cell count agreed closely with thos obtained by absolute eosinophil counts.

Animals given a second infection showed another rise in total white cells and eosinophils. However, the responses never reached the levels seen during the first infection (Figure 1 and Figure 2). Two animals apparently died as a direct result of the second infection. The first, Animal B of Group III, died twenty days after the second infection with a high temperature and symptoms of respiratory distress. Several days prior to death the animal became lethargic. A temperature of 107°F was recorded 1 day prior to death. The second, Animal B of Group IV, died eight days after the second infection, also exhibiting a high temperature and rapid respiration. The gross pathology, microscopical finding as well as larval recoveries from the heart, lung, brain, liver, spleen, thyroid, and other organs are presented in detail under the histopathology and larval recovery sections. It is interesting to note that the second animal showed an extremely low eosinophil response one day before death.

Antibody Response

Preliminary studies using Jung and Pacheco's (1960) hemagglutination procedure gave negative results when adult or larval Toxocara

TABLE 2

THE MAXIMUM WHITE CELL AND EOSINOPHIL RESPONSES IN BABOONS INFECTED WITH VARIOUS LEVELS OF INFECTIVE *TOXOCARA CANIS* EGGS

Number of Infective Eggs/gm Body Weight (Animal Group)	Average Total White Blood Cells/CMM[a]			Average Percent Eosinophil (From the Absolute Count)[a]	
	Preinfection Count	Maximum Count After Infection	Increase	Preinfection Percentage	Maximum Percentage After Infection
1 (Group I)	6,214	10,625	4,411	1.4	13.0
5 (Group II)	10,066	19,350	9,284	1.0	20.6
10 (Group III)	6,018	15,800	9,782	1.6	26.0
20 (Group IV)	11,958	23,825	11,867	1.0	43.0
0 (Group V, Control)	7,512	7,650	136	1.7	2.0

[a]Each value is the average of two animals.

antigens were tested with antisera from rabbits heavily infected with Toxocara. However, positive results were obtained with the adult Toxocara antigen when certain modifications were introduced (See Materials and Methods). The modified method continued to give negative results with antigens of larvae, hatching fluid, and infective eggs of Toxocara. The above negative results were obtained with both the standard tube test and with the microhemagglutination technique (which was used exclusively for the analyses of baboon antisera described below).

Many factors were considered which may have caused these negative results. The pH of the buffer and the length of exposure of tanned cells to antigen were not criticial when red cell sensitization was done at room temperature. However, positive results were obtained with antigens of the immature stages when the tanned red cells were exposed to the antigen at 37°C. Table 3 shows the results of microhemagglutination tests using various Toxocara and Ascaris extracts when the sheep red cells were coated for at either 37°C or room temperature. The 37°C treatment during red blood cell sensitization greatly increased the sensitivity of the hemagglutination test in the homologous system with the antigens of Toxocara larvae, hatching fluid, or infective eggs but not with antigens of the adult stage. No significant improvement was noted when antigens of various Ascaris stages were similarly treated and reacted against the homologous antisera.

Experiments were done to see if the increased sensitivity caused by 37°C treatment was due to changes in antigen, changes in the tanned red cells, or both. When the tanned cells alone or the antigen alone were heated for 30 minutes at 37°C, then cooled to room temperature and

TABLE 3

THE EFFECT OF TEMPERATURE OF ANTIGEN-RED CELL SENSITIZATION ON THE SENSITIVITY OF THE HEMAGGLUTINATION TESTS USING ANTISERA FROM RABBITS INFECTED WITH TOXOCARA CANIS OR ASCARIS SUUM

Antigen	Reciprocal of Hemagglutination Titer[a]	
Toxocara[b]	Room Temperature	37°C
Larva	0-160	20,480
Infective Egg	0	2,560
Hatching Fluid	0	320-640
Adult	320-640	640
Ascaris[c]		
Larva	2,560-5,120	5,120-10,240
Infective Egg	2,560	5,120
Hatching Fluid	320	640-1,280
Adult	320-640	320-640

[a]Each value is the result of 3 replicate experiments (range is given when results varied)

[b]Tested against antisera from rabbit infected with T. canis.

[c]Tested against antisera from rabbit infected with A. suum.

reacted with the other component (either previously heated or non-heated in a similar manner at room temperature, very little increase in sensitivity occurred. Sensitivity increased only when both components were reacted together at 37°C. A similar increase in sensitivity was observed when both components were incubated at 37°C for 15 minutes or at 56°C for either 10 or 30 minutes.

The increased sensitivity seen when red blood cell sensitization was done at 37°C also occurred with tests using anti-Toxocara sera from infected baboons. Heat treated antigens of Toxocara larvae or infective eggs showed greater sensitivity than their unheated counterparts (Figure 3). In the studies reported below the antigens of all stages were heated during the sensitization process.

Using anti-Toxocara and anti-Ascaris infection antisera prepared in rabbits, the antigens of Toxocara and Ascaris larvae, infective egg, hatching fluid, and adult stages were tested in homologous and heterologous systems (Table 4). Cross-reactions occurred with all antigen stages. Greatest sensitivity occurred with the larval antigens. The most marked differences between homologous and heterologous systems occurred with larval and infective egg antigens. In each system the use of the homologous antigen gave much higher titers. On the other hand, the adult extracts of both species gave similar reactions in homologous and heterologous systems, indicating complete lack of specificity.

The antibody responses of baboons (Animal B of each Group) infected with either 1, 5, 10, or 20 infective Toxocara eggs/gm body weight are summarized in Figure 4. Circulating antibodies were detected in the sera of all animals one week after infection with Toxocara canis

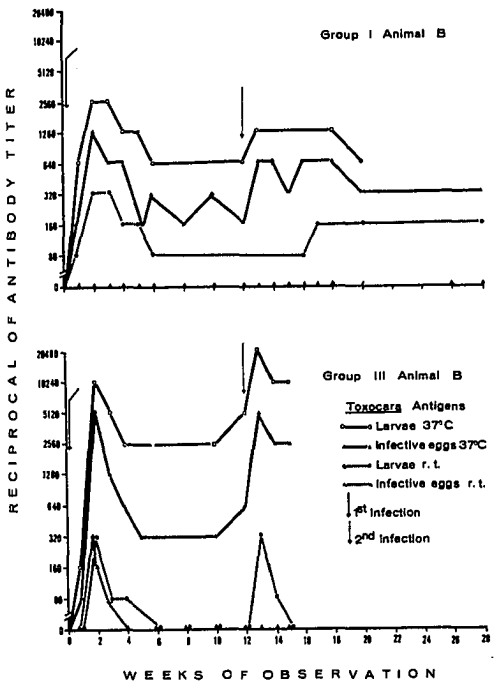


Figure 3. The effect of the temperature of antigen-red cell sensitization on the sensitivity of the hemagglutination tests using antisera from baboons infected with Toxocara canis.

TABLE 4

HEMAGGLUTINATION TITERS OF ANTI-*TOXOCARA* AND ANTI-*ASCARIS* RABBIT SERA USING VARIOUS ANTIGEN STAGES

Antigen	Reciprocal of Hemagglutination Titer[a] Antiserum	
Toxocara	Anti-Toxocara	Anti-Ascaris
Larvae	20,480-40,960	1,280
Infective Eggs	2,560	160
Hatching Fluid	640	0-80
Adult	640	640
Ascaris		
Larvae	320	10,240-20,480
Infective Eggs	160	2,560
Hatching Fluid	80	640-1,280
Adult	640	640

[a]Each value is the result of three replicate experiments (range is given when results varied).

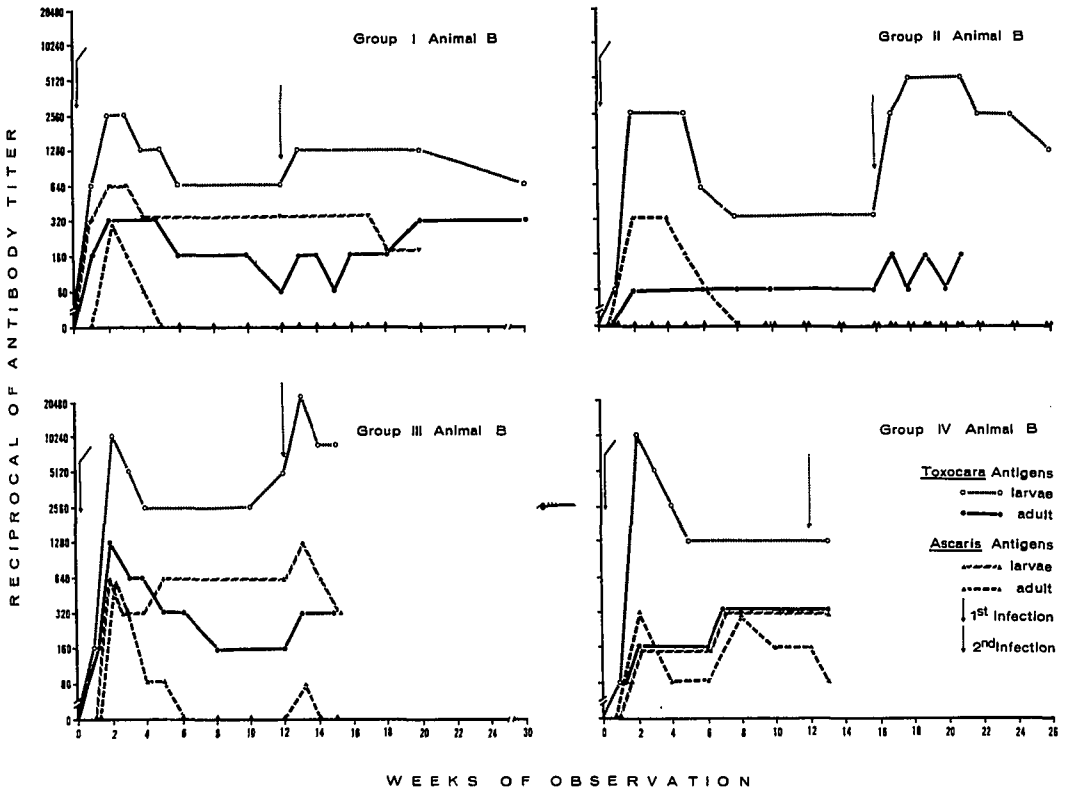


Figure 4. Hemagglutination titers in sera from baboons infected with Toxocara canis, using red cells sensitized with various homologous and heterologous antigens.

and reached a peak the second or third week. Antibodies to Toxocara larval antigens were still detectable 30 weeks after infection. In every instance, the systems using antigen of Toxocara larvae detected higher antibody titers than systems using Toxocara adult or Ascaris larval or adult extracts. With the exception of the Toxocara larval extract, none of the other antigens demonstrated consistently greater sensitivity when tested with the baboon antisera. Similar results were obtained with antisera from four additional baboons infected with Toxocara. These results are summarized in Table 5. In no instance were antibodies detected in sera taken from these eight baboons before infection.

The animals depicted in Figure 4 were reinfected either 12 or 16 weeks after the first infection. One animal (Group IV) died eight days after reinfection and serum was not available for testing. The other 3 animals showed a rise in antibody titer to the Toxocara larval antigen. In two animals, the anamnestic response exceeded that achieved during the primary response.

Serum Protein Electrophoresis

No significant changes in the alpha 1, -2, and beta globulins were detected in the sera of the infected baboons. The changes that occurred in the albumin, gamma globulin, and total protein fractions in each animal are summarized in Tables 6 through 10.

Generally, there was an increase in the total protein in all infected animals during the first infection as well as after the second infection. This increase reached a maximum between two to five weeks after infection. The increase was especially apparent in Animal B of Group II (Table 7). With the exception of Animal A of Group IV (Table 9)

TABLE 5

HEMAGGLUTINATION TITERS IN SERA FROM BABOONS INFECTED WITH *TOXOCARA CANIS*, USING RED CELLS SENSITIZED WITH VARIOUS HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

Weeks After Infection	RECIPROCAL OF HEMAGGLUTINATION TITERS							
	Group I (A)[a] Antigen Used For Sensitization				Group II (A)[b] Antigen Used For Sensitization			
	Toxocara		*Ascaris*		*Toxocara*		*Ascaris*	
	Larva	Adult	Larva	Adult	Larva	Adult	Larva	Adult
1	160	80	0	0	160	80	0	0
2	5120	1280	0	320	2560	320	320	320
3	5120	640	0	0	5120	160	320	320
4	2560	640	0	320	2560	160	320	320
5	1280	320	0	80	1280	160	160	160
6	1280	320	0	160	1280	160	160	80
8	640	320	0	80	1280	160	80	0
10	640	320	0	80	1280	80	0	0
12	640	160	0	80	1280	80	0	0
14	640	160	0	0	2560	0	0	0
16	640	160	0	0	1280	0	0	0
18	640	160	0	0	-	-	-	-
19	-	-	-	-	2560	0	0	0
20	640	160	0	0	-	-	-	-
22	-	-	-	-	1280	0	0	0
24	-	-	-	-	-	-	-	-
26	-	-	-	-	2560	0	0	0

[a] Infected with 1 infective *Toxocara* egg/gm body weight.

[b] Infected with 5 infective *Toxocara* eggs/gm body weight.

TABLE 5 -- Continued

HEMAGGLUTINATION TITERS IN SERA FROM BABOONS INFECTED WITH TOXOCARA CANIS, USING RED CELLS SENSITIZED WITH VARIOUS HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

Weeks After Infection	RECIPROCAL OF HEMAGGLUTINATION TITERS							
	Group III (A)[a] Antigen Used For Sensitization				Group IV (A)[b] Antigen Used For Sensitization			
	Toxocara		Ascaris		Toxocara		Ascaris	
	Larva	Adult	Larva	Adult	Larva	Adult	Larva	Adult
1	160	80	80	0	80	80	0	0
2	5120	640	160	320	2560	320	80	320
3	2560	320	320	80	2560	640	160	640
4	1280	320	160	0	1280	320	80	160
5	1280	320	160	0	640	160	80	80
6	1280	320	160	0	640	160	80	80
8	1280	320	320	0	640	160	160	160
10	1280	320	320	0	640	160	160	160
12	1280	160	320	0	640	160	160	160
14	1280	160	320	0	-	-	-	-
16	1280	320	320	0	-	-	-	-
18	-	-	-	-	-	-	-	-
20	2560	160	320	0	-	-	-	-
28	1280	160	160	0	-	-	-	-

[a] Infected with 10 infective Toxocara eggs/gm body weight.

[b] Infected with 20 infective Toxocara eggs/gm body weight.

TABLE 6

SERUM PROTEIN ANALYSES IN BABOONS RECEIVING ONE INFECTIVE *TOXOCARA CANIS* EGG PER GRAM BODY WEIGHT

	Group I					
	Animal (A)[a]			Animal (B)[ab]		
Weeks of Observation	Total Protein	Albumin	Gamma-Globulin	Total Protein	Albumin	Gamma-Globulin
Preinfection	7.41	3.05	1.71	7.40	4.20	1.09
1	7.49	3.53	1.54	7.40	3.93	1.21
2	7.90	3.21	1.94	7.42	4.09	1.18
3	7.81	3.58	1.58	7.60	3.80	1.24
4	8.05	3.60	1.64	7.72	3.68	1.46
5	8.15	3.76	1.62	8.25	3.62	1.37
6	7.80	3.57	1.56	7.65	3.68	1.42
8	7.62	3.16	1.70	7.40	3.52	1.40
10	8.22	3.05	1.99	7.42	3.84	1.23
12	7.85	3.09	1.83	7.72	3.66	1.35
13	-	-	-	7.50	4.13	1.10
14	8.13	3.31	1.78	8.00	4.23	1.26
15	-	-	-	8.35	4.36	1.35
16	7.96	3.03	1.94	8.60	4.44	1.35
17	-	-	-	7.80	3.76	1.27
18	8.42	3.41	1.96	8.25	3.28	1.66
20	-	-	-	8.00	3.66	1.41

[a]Each value (gm/100 ml) is average of two determinations.

[b]Received second infection (1 infective *Toxocara* egg/gm body weight) the 12th week.

TABLE 7

SERUM PROTEIN ANALYSES IN BABOONS RECEIVING FIVE INFECTIVE *TOXOCARA CANIS* EGGS PER GRAM BODY WEIGHT

	Group II					
	Animal (A)[a]			Animal (B)[ab]		
Weeks of Observation	Total Protein	Albumin	Gamma-Globulin	Total Protein	Albumin	Gamma-Globulin
Preinfection	7.80	3.66	1.51	7.05	3.81	1.02
1	8.00	3.07	2.08	7.10	3.46	1.30
2	8.10	3.03	2.21	8.05	3.31	1.76
3	8.15	3.04	2.38	7.30	3.70	1.45
4	8.35	3.39	2.15	8.00	3.39	1.96
5	8.60	3.65	2.02	7.50	3.57	1.43
6	8.50	3.36	2.12	7.40	3.57	1.43
8	8.05	3.42	1.84	7.40	3.54	1.43
10	8.15	3.06	2.21	7.58	3.80	1.32
12	8.15	3.26	1.95	7.58	3.97	1.30
14	8.20	3.52	1.78	7.50	3.61	1.47
16	8.25	3.23	1.90	7.40	3.68	1.47
17	-	-	-	7.60	3.35	1.78
18	-	-	-	7.72	3.38	1.88
19	8.50	3.24	1.99	7.30	3.06	1.67
20	-	-	-	7.60	3.50	1.36
21	-	-	-	7.95	3.68	1.61
22	8.20	3.24	1.82	7.90	3.46	1.68
24	-	-	-	7.60	3.18	1.64
26	8.25	3.09	1.81	7.85	2.87	1.82

[a]Each value (gm/100 ml) is the average of two determinations.

[b]Received second infection (5 infective *Toxocara* eggs/gm body weight) the 16th week.

TABLE 8

SERUM PROTEIN ANALYSES IN BABOONS RECEIVING TEN INFECTIVE, *TOXOCARA CANIS* EGGS PER GRAM BODY WEIGHT

	Group III					
	Animal (A)[a]			Animal (B)[ab]		
Weeks of Observation	Total Protein	Albumin	Gamma-Globulin	Total Protein	Albumin	Gamma-Globulin
Preinfection	7.40	3.59	1.29	7.40	3.64	1.24
1	7.40	3.25	1.38	7.40	2.92	1.61
2	8.20	3.15	1.90	7.60	2.50	2.26
3	8.35	3.16	1.93	7.85	3.38	1.79
4	8.15	3.07	1.88	7.95	3.21	1.72
5	8.50	3.43	1.86	7.85	3.47	1.57
6	8.35	3.35	1.81	8.00	3.43	1.60
8	7.95	3.28	1.55	7.85	3.14	1.71
10	7.95	3.43	1.65	7.95	3.95	1.26
12	8.15	3.59	1.60	7.95	3.71	1.44
13	-	-	-	7.85	3.00	1.88
14	7.85	3.41	1.58	8.10	3.28	1.88
15	-	-	-	7.45	3.02	2.11
16	7.85	3.47	1.58	-	-	-
20	-	-	-	-	-	-
26	7.95	3.14	1.85	-	-	-

[a]Each value (gm/100 ml) is the average of two determinations.

[b]Received second infection (10 infective *Toxocara* eggs/gm body weight) the 12th week.

TABLE 9

SERUM PROTEIN ANALYSES IN BABOONS RECEIVING TWENTY INFECTIVE TOXOCARA CANIS EGGS PER GRAM BODY WEIGHT

	Group IV					
	Animal (A)[a]			Animal (B)[ab]		
Weeks of Observation	Total Protein	Albumin	Gamma-Globulin	Total Protein	Albumin	Gamma-Globulin
Preinfection	7.40	2.75	1.47	7.55	3.25	1.36
1	7.40	2.55	1.66	7.53	3.35	1.26
2	7.40	2.25	1.83	7.50	2.83	1.52
3	7.70	2.15	2.00	7.30	2.64	1.53
4	7.70	2.32	1.82	7.14	2.83	1.45
5	7.10	2.35	1.74	7.20	2.85	1.36
6	7.10	2.26	1.75	7.50	2.85	1.52
8	7.48	2.51	1.77	7.70	2.57	1.91
10	7.70	2.47	1.86	7.85	2.57	1.86
12	7.80	2.49	1.96	7.95	2.45	1.86

[a]Each value (gm/100 ml) is the average of two determinations.

[b]Received second infection (20 infective Toxocara eggs/gm body weight) the 12th week.

TABLE 10

SERUM PROTEIN ANALYSES OF NON-INFECTED BABOONS

	Group V					
	Animal (A)[a]			Animal (B)[a]		
Weeks of Observation	Total Protein	Albumin	Gamma-Globulin	Total Protein	Albumin	Gamma-Globulin
0	7.85	3.52	1.78	7.60	4.40	1.10
3	7.60	3.34	1.71	7.85	4.33	1.28
5	7.60	3.59	1.61	7.50	3.92	1.29
9	7.60	3.47	1.69	7.35	3.62	1.30
13	7.70	3.69	1.61	7.40	4.06	1.14
18	8.00	3.56	1.59	7.60	3.93	1.31
20	8.00	3.40	1.79	7.45	4.09	1.13

[a]Each value (gm/100 ml) is the average of two determinations.

the increase in serum protein was due primarily to an increase in the globulin fraction. From these results it can be seen that the seven of eight animals exhibited an apparent increase in gamma globulin. The possible exception was Animal A, Group I (Table 6). In general the peak increase occurred three to five weeks after the first infection and then began to drop, although in Animal B, Group IV (Table 9), the gamma globulin increased markedly only after eight weeks of infection in most of the animals. Accompanying the increase in gamma globulin was a slight decrease in the albumin which reached its lowest level between the second and third week after infection in all animals except Animal A of Group I (Table 6). This animal showed a slight increase during the first eight weeks and also during the latter part of the infection period. There was no clear-cut relationship between the infection dosage level and the extent of changes in serum fractions.

As shown in Table 10, the control animals (non-infected) showed no significant changes in total protein, albumin, or gamma globulin during the entire study.

Gross Post Mortum Studies and Larval Recoveries

Although they were not planned as part of the original objective, post-mortum and larval recovery studies were included when two of the experimental animals died shortly after the second infection. Apparently these two animals died as a result of the Toxocara infection. Similar post mortum and larval recovery studies were made on the remaining animals after the termination of the major portion of the study.

Grossly, multiple, white, pinhead-sized nodules were seen in the liver (beneath the capsule and in the parenchyma), the lungs (beneath the

pleural surface and also in the parenchyma), and the kidney. The number of nodules was directly related to the size of the dose of infective eggs.

Granulomata were seen in various organs in two animals that died after receiving a first and second infection. It was difficult to distinguish the lesions of the first and the second infection with precision. One of these animals, a female (Animal B, of Group IV), died eight days after the second infection with high temperature and rapid respirations. Post-mortem examination revealed consolidation of the entire right lung, with purulent pericarditis and purulent meningitis. Larvae were demonstrated in the pericardial sac of this animal but not in the meninges, although larvae were recovered from the brain by pepsin digestion (Table 11). The second animal, a male baboon (Animal B of Group III), died 20 days after the second infection, also with high temperature and symptoms of respiratory distress. Grossly, the lungs were extremely congested and contained multiple areas of hemorrhage. Microscopically, the changes were predominantly those of congestion with intra-alveolar hemorrhage and edema. A few areas of focal broncho-pneumonia were noted.

Larval recoveries were made from certain organs which are described in the Materials and Methods. The results are summarized in Table 11. From these results it can be seen that the larval recovery was related to the number of larvae given to each animal, i.e., the higher the dose the greater the number of larvae recovered. The percentage of larvae recovered in relation to total number given ranged from 14.6% to 25%. Although more larvae were recovered (per gram of organ weight) from the liver than from any other organ, the number of larvae recovered from the lungs was of a similar magnitude in two of four animals studied.

TABLE 11

LARVAL RECOVERIES FROM ORGANS OF BABOONS INFECTED WITH TOXOCARA CANIS

Animal[a] Group	Number of Larvae Given (Gm B W)	Time After Infection[b]	Number of Larvae/Gm Organ Weight (% Recovery of Total Larvae Given) Liver	Lung	Brain	Kidney	Heart
Group I-B	2	25	26	0	6.6	0	6.6
			(22.4)	(0%)	(2.2)	(0%)	(2.4)
Group II-B	10	32	26.4	22.4	26.0	ND	20
			(6.8)	(4.7)	(1.9)	ND	(1.2)
Group III-A	10	24	53.3	32	15	15	10.5
			(15.6)	(5.9)	(2.1)	(0.98)	(0.88)
Group IV-B	40	13	84	77	44.4	75	ND
			(8.8)	(5.5)	(2.7)	(1.4)	ND

ND - Not done.

[a]No larvae were recovered from the organs of the control group.

[b]Infection schedule given in Table 1.

Histopathological Studies

Sections were made of the brain, lung, liver, pancreatic capsule, thyroid, heart, and mesenteric lymph nodes of animals from each group. The results are described below.

Brain

The predominant cells were of the large mononuclear and giant variety. Many punctate lesions, characterized by small areas of coagulation necrosis, and infiltrated by one or two inflammatory cells, were commonly seen in the brain adjacent to granulomatous-like lesions, but lacking thick fibrous capsules that were seen in other organs (Figure 5).

Lung

Another rather characteristic feature of Toxocara infection appeared to be the presence of numerous hemosiderin laden macrophages adjacent to the small pulmonary arteries, as shown in Figure 6. These clusters were seen in all but one of the infected animals, in none of the controls, and the numbers were directly proportional to the number of infective eggs in the inoculum.

Liver

Typical granulomata with thick fibrous capsules were seen in sections of the liver 12 weeks after a single infection. They were characterized by a large central region containing giant cells, amorphous reddish debris, and thick eosinophilic fibrillae which usually resembled the larvae in shape and presumably represented degenerating larvae. This central region was surrounded by fibrous tissue which was infiltrated with moderate numbers of polymorphonuclear leukocytes, eosinophils, lymph-

cytes, and plasma cells, as shown in Figure 7. In sections made from an animal 22 weeks after a single infection, the fibrous tissues in the capsule was more dense and infiltrated with a smaller number of inflammatory cells, most of which were lymphocytes and plasma cells. Intact larvae were seen, along with large mononuclear cells and giant cells. The latter had foamy cytoplasm which frequently contained slivers of bright red, doubly refractile, chitinous-like material.

More recent lesions were observed in the male that died 20 days after the second infection and they showed extensive necrosis. These lesions, apparently from the second infection, contained deeply stained eosinophilic fibrillae surrounded by many polymorphonuclear leukocytes and eosinophils. A variable number of lymphocytes and plasma cells were present, usually peripherally (Figure 8, 9). Moreover, lesions in the myocardial sections of this animal showed intact larvae, with only moderate necrosis and minimal inflammatory reaction (Figure 10).

Pancreatic Capsule

In this organ, deeply stained eosinophilic fibrillae were seen encasing a larva which was surrounded by eosinophils and polymorphonuclear leukocytes with no evidence of fibrous capsule (Figure 11). Adjacent to this larva were giant cells with foamy cytoplasm and chitinous-like slivers, resembling the central portion of granuloma.

Thyroid Gland

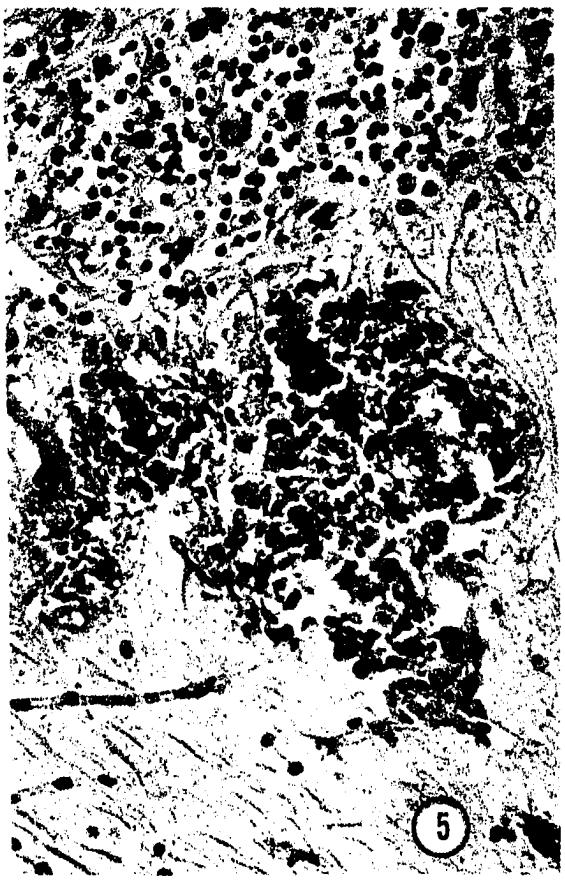
The granuloma observed in this organ was small and was surrounded by a mature fibrous capsule which was infiltrated with a few lymphocytes. Giant cells and large mononuclear cells with cytoplasmic chitinous slivers

Figure 5. Animal B, Group III. Cerebellum, H & E x 128. The lesion consists of an area of liquefaction necrosis infiltrated by numerous mononuclear cells, reactive astrocytes, and several giant cells.

Figure 6. Animal B, Group IV. Lung, H & E x 128. Multiple hemosiderin ladened macrophages adjacent to small pulmonary artery.

Figure 7. Animal A, Group IV. Liver, H & E x 50. A three month lesion showing central thick eosinophilic fibrils, apparently larval remnants, and occasional giant cell. The fibroblasts in the capsule are plump, randomly alligned, and infiltrated by a moderate number of lymphocytes and plasma cells.

Figure 8. Animal B, Group III. Liver, H & E x 128. Recent lesion consisting of numerous thick eosinophilic fibrils surrounded by polymorphonuclear leukocytes, eosinophils, lymphocytes, and plasma cells. A few fibroblasts are present among the inflammatory cells, but there is no recognized capsule.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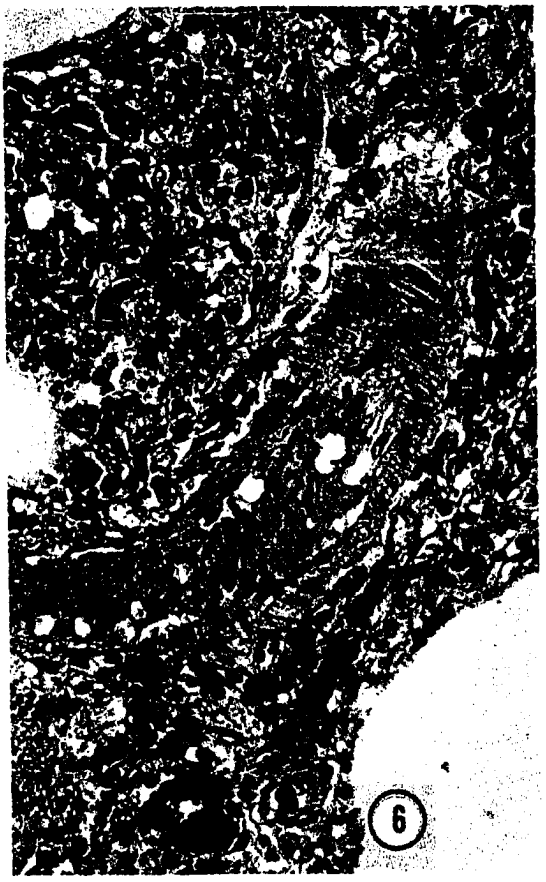
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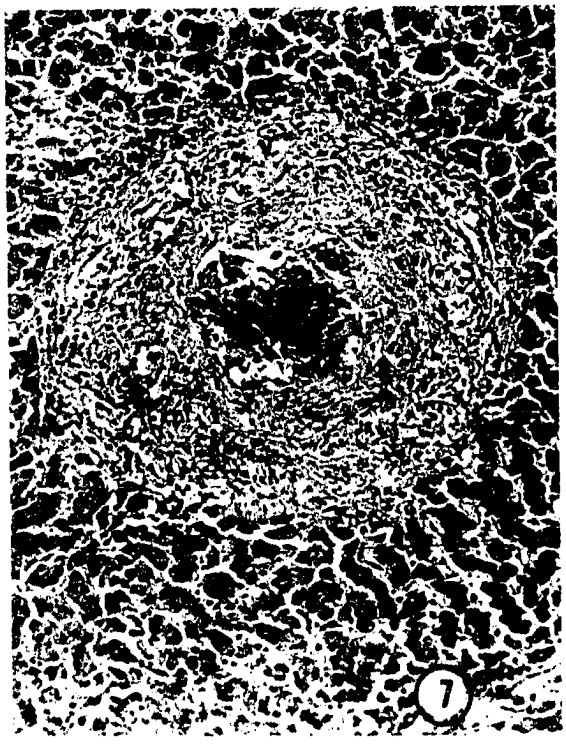
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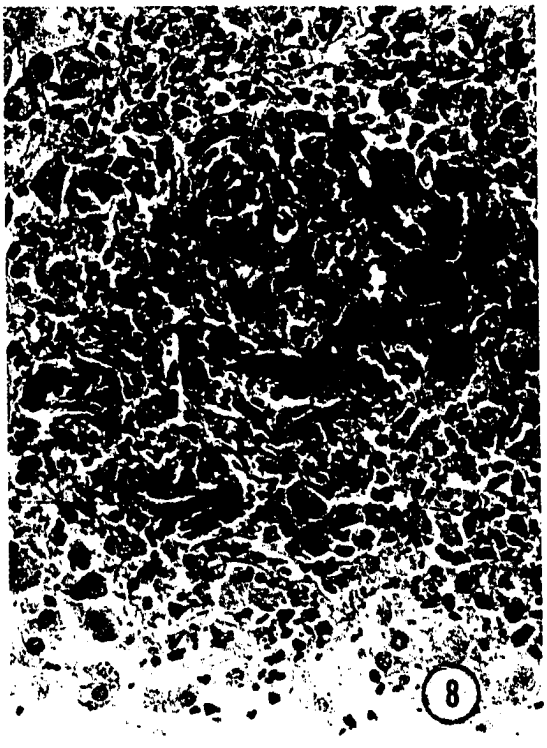
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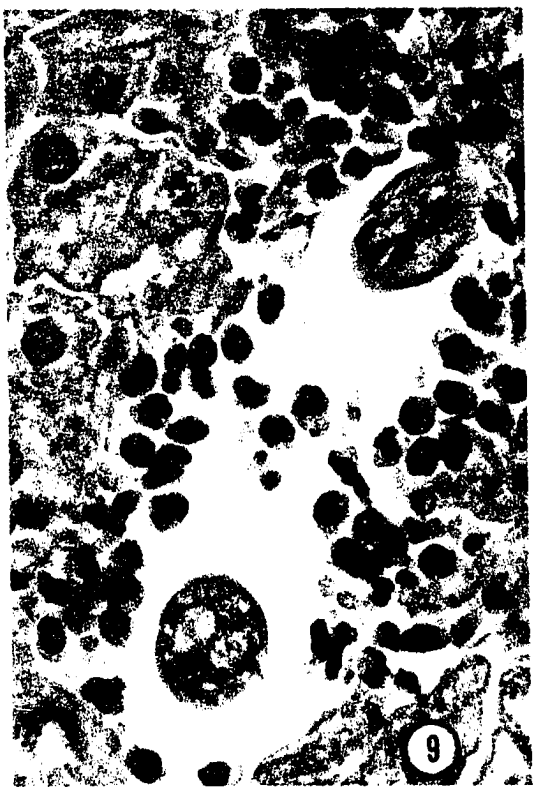
Figure 9. Animal B, Group III. Liver, H & E x 320. An apparent recent lesion showing cross-section of Toxocara larva in hepatic sinusoid surrounded by polymorphonuclear leukocytes and eosinophils.

Figure 10. Animal B, Group III. Heart, H & E x 128. Recent lesion containing two intact larvae, infiltrated with lymphocytes and plasma cells. An occasional fibroblast is evident.

Figure 11. Animal A, Group III. Pancreatic capsule, H & E x 128. Six months after infection with Toxocara canis. Toxocara larva is present on the right surrounded by deeply staining eosinophilic fibrils suggesting acute larval necrosis. The circulate lesion at the left resembles the center of a well-developed granuloma, although no fibrous capsule is seen. The surrounding tissue is infiltrated with polymorphonuclear leukocytes, eosinophils, lymphocytes, plasma cells, and a few fibroblasts.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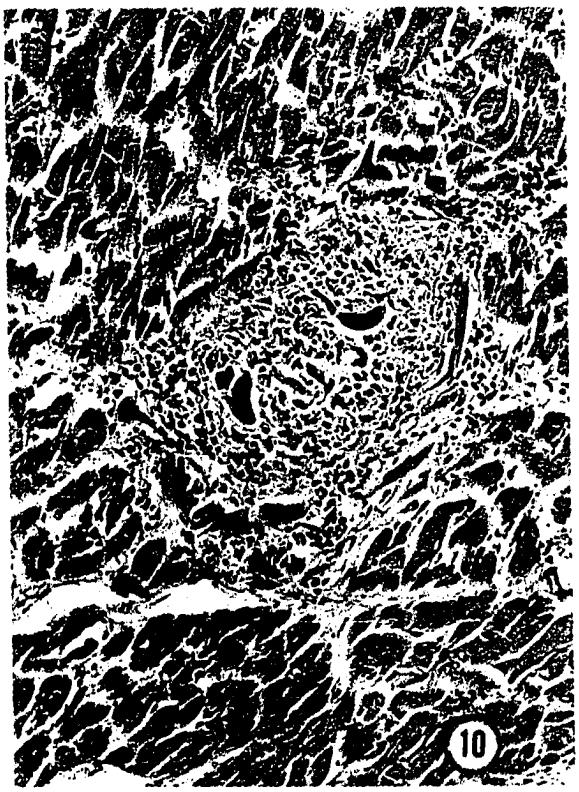
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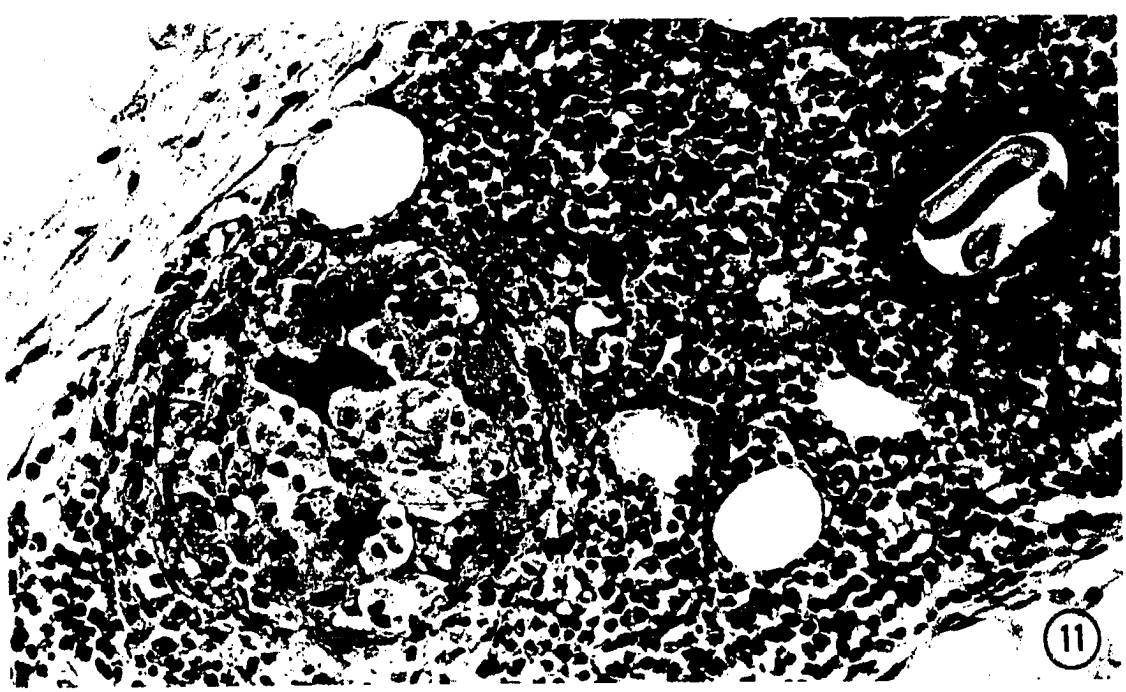
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were present and intact larvae were seen (Figure 12).

Heart and Pericardial Sac

Larvae were demonstrated in the myocardial and pericardial sections of the two animals that died after receiving a first and second infection. These lesions apparently were from the second infections given 20 and 8 days prior to death (Figure 10 and 13). The inflammatory reaction was minimal.

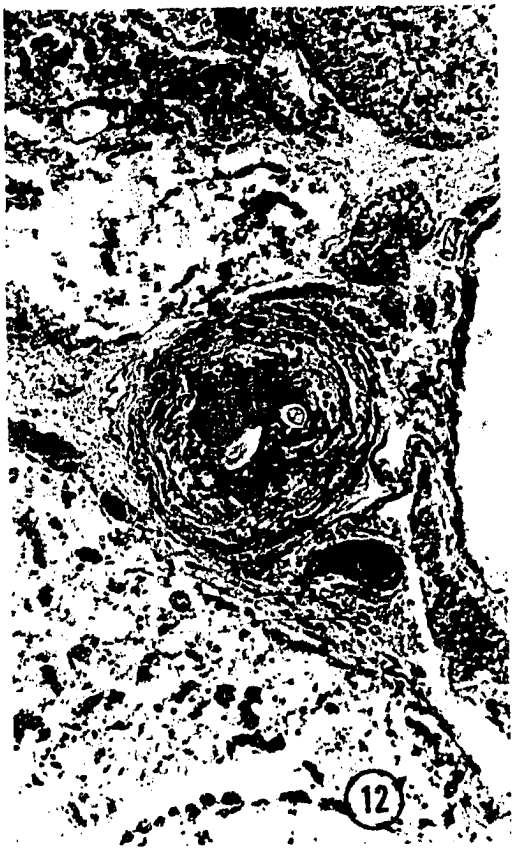
Mesenteric Lymph Nodes

Typical granulomata were seen in the female that died eight days after the second infection (Figure 14). The lesions were similar to those seen in other tissues in animals given a single infection of three months duration.

Figure 12. Animal A, Group II. Thyroid, trichrome x 50. Eight month granuloma characterized by contracted, mature capsule and central giant cells containing chitinous slivers. Transverse section of the larva is also seen.

Figure 13. Animal B, Group IV. Pericardial sac, trichrome x 128. Recent lesion showing cross-section of an intact larva surrounded by lymphocytes, plasma cells, a few polymorphonuclear leukocytes, and numerous plump fibroblasts.

Figure 14. Animal B, Group IV. Mesenteric lymph nodes, H & E x 50. Three months granuloma with a fairly well-developed fibrous capsule. The central zone contains giant cells, amorphous debris, and an intact larva.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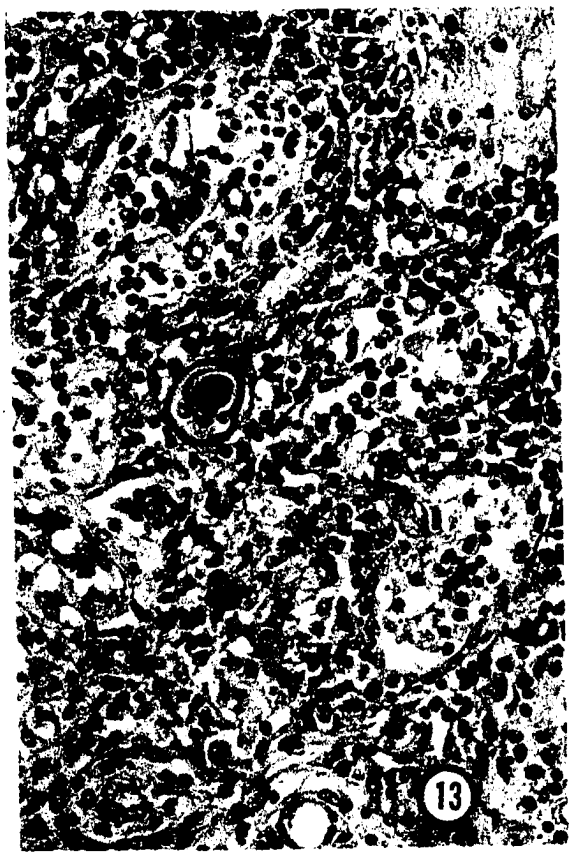
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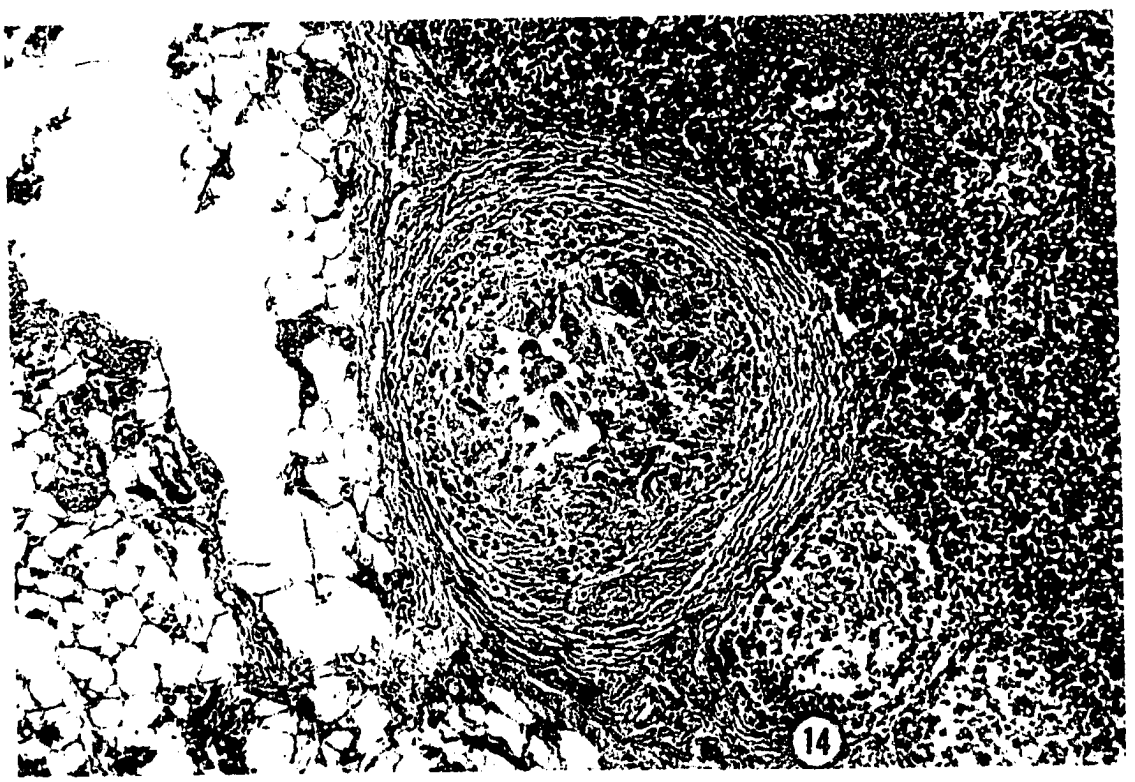
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CHAPTER V

DISCUSSION

The baboons involved in this study showed a leukocytosis and an eosinophilia as early as one week after infection with Toxocara. The cell responses reached a peak the second or third week after infection, declined gradually thereafter, and increased again following the second infection (Figure 1 and 2). A relationship seemed to exist between the size of the infective dose and the intensity of the response. The increase in cell counts in infected baboons generally agreed with previous observations in both man and experimental animals. Several workers have shown that the maximum eosinophilic response usually occurs within two to three weeks after infection with nematodes, e.g. T. canis in rabbits, (Sadun, et al. 1957); D. viviparus in cattle, (Weber and Robin, 1958); T. canis in guinea pig and man, (Chaudhuri and Saha, 1959); Trichinella in rats (Scardino and Zaiman, 1962); and T. canis in guinea pigs, (Olson and Schulz, (1963). Furthermore, an increased eosinophilic response has also been noted by Sadun et al. (1957) and Olson and Schulz (1963) after a second infection with Toxocara.

The eosinophilia in humans differs from that observed in experimental animals in that the response lasts longer. Smith and Beaver (1953) reported that the eosinophilia peaked after one to three months and remained between 17 to 30% for 13 months in two children infected orally with

200 infective T. canis eggs. Chandhuri and Saha (1959) noted a maximum eosinophilia of 62% on the thirtieth day after infection and thereafter the level remained between 40 to 50% for the five months that an infected volunteer was observed. In the present studies, however, the maximum eosinophil level in baboons occurred two to three weeks after infection and dropped rather rapidly by the fourth or fifth weeks (Figure 2).

There is no widely accepted explanation for the observed differences in eosinophilic responses of humans and other animal species infected with T. canis. Olson and Schulz (1963) suggested that the decline in eosinophil count in guinea pigs after the second week of infection with T. canis may be related to the negative larval recoveries from animals necropsied on day 14. In the present study, however, larvae were either recovered by pepsin digestion or demonstrated in tissue sections of baboons eight months after they were infected with T. canis. Moreover, when guinea pigs were used to check the viability of Toxocara eggs given the baboons approximately 80% of the larvae were recovered four weeks after the guinea pigs were infected. Also, Beaver (1962) reported that 26.4$ of the larvae were recovered from guinea pigs one year after they were infected with T. canis. Ivey (1965) attained even higher larval recoveries from the livers of guinea pigs 10 weeks after infection with T. canis.

The present finding that the eosinophilic response is dose related tempts one to speculate on the reasons for such an observation. Possibly the larvae provoked histamine release by direct cell damage during migration, or possibly the excretions and secretions of the larvae provoke an eosinophilic response. Vaughn (1952) demonstrated an eosino-

philia in guinea pigs injected with an A. suum extract. The eosinophilia disappeared after an anti-histamine was given. Archer and Hirsch (1963) and Litt (1964) have shown that antigen-antibody complexes provoke eosinophilia. Possibly a similar circumstance occurred after an infected animals produce antibody to Toxocara. However, until more is known about the basic function of the eosinophil the above possibilities cannot be adequately investigated.

In the present studies specific antibodies were produced in baboons following infection with T. canis. Antibodies were detectable in a reproducible manner by the microhemagglutination technique one week after infection, reached a peak the second or third week, and were still detectable 30 weeks after infection using antigen prepared from Toxocara larvae. In general, there was no relationship between the size of the infective dose given and the antibody titer. Additional evidence in favor of an antibody response, although indirect, was the apparent increase in gamma globulin seen in the baboons infected with Toxocara (Table 6 and 9).

The increase in gamma globulin after infection with Toxocara has also been reported in humans (Beaver, 1956; Huntly and Costas, 1965, and Snyder, 1961). Furthermore, Beaver (1962) reported that there was a direct relationship between the degree of eosinophilia and hyperglobulinemia in most, but not all, cases diagnosed as visceral larva migrans.

A hypergammaglobulinemia has been reported in experimental animals infected with Toxocara. Sadun et al. (1957) found an increase in the gamma globulin of infected rabbits. Nash (1965) found an increase in the gamma globulin of mice infected with T. canis and suggested that

this increase was not due to production of antibodies, but probably due to bacterial invasion of the extra-intestinal tissues following penetration of larvae. He based this interpretation on antibody-antigen absorption studies, using antigens of infective eggs, undeveloped eggs, and adult worms.

Technical factors play an important role in the sensitivity of the hemagglutination test for detecting antibody against Toxocara. Earlier workers used red cell sensitization techniques that required three to four fluid volumes of antigen per volume of packed red cells. In the present study, a ratio of one volume of antigen per volume of red cell suspension markedly increased the sensitivity of the hemagglutination test. The significance of this minor modification, in terms of earlier results, is difficult to determine at present. In the present study, the most important technical factor was the temperature at which the red cells were sensitized. With extracts of Toxocara larvae, sensitization at 37°C increased the sensitivity of the hemagglutination test (Table 3 and Figure 3). However, this was not a general phenomenon in that with the adult Toxocara extracts or any of the Ascaris extracts sensitization at 37°C failed to increase sensitivity significantly. Previous workers sensitized red cells at room temperature and used adult extracts only. Such a system resulted in low titers, similar to those observed in the present study. The lack of reports using larval or egg extracts in earlier studies may reflect either the scarcity of the antigenic material or the failure to get positive reactions when sensitizations were done at room temperature. Ivey (personal communication) tested extracts of infective eggs in this manner in 1963 but without

success.

Although the above technical modifications greatly increased the sensitivity of microhemagglutination tests using antigens of the immature stages, cross-reactions with Ascaris were common. Using sera from rabbits infected with either Toxocara or Ascaris, cross reactions were evident with antigens of immature stages and adult stages (Table 4). However, the extracts of the larval stages detected significantly higher antibody titers in the homologous systems, whereas the extracts of the adult stages gave identical titers in homologous and heterologous systems, indicating complete lack of specificity. The cross reactions seen with extracts of adult Toxocara and Ascaris were to be expected since antigenic analysis of Toxocara and Ascaris whole worms by agar-diffusion studies have shown that they have five to nine common antigenic components (Kagan, 1957). Furthermore, Kagan (1958), Kagan et al. (1959), and Jung and Pacheco (1960) reported cross-reactions between Ascaris and Toxocara systems by the indirect hemagglutination test.

Several workers have detected antibody against Toxocara by using other serological techniques, e.g. bentonite flocculation (Sadun et al. 1957); in vitro larval precipitin test, (Olson, 1960); Schultz-Dale reaction (Sharp and Olson, 1962); agar-diffusion precipitin tests (Huntly and Mooreland, 1963); fluorescence-antibody tests (Mitchell, 1964); passive cutaneous anaphylaxis (Ivey, 1967); and complement-fixation test (Fernando, 1968). All of the investigators reported cross reactions. However, Hogarth-Scott (1966), using the direct immunofluorescence technique, found no cross-reactions when sera from

individuals infected with other nematodes were tested with Toxocara larvae.

The greater sensitivity of extracts of the Toxocara larval stage and, to a lesser extent, the Toxocara infective egg, was not unexpected since Toxocara infection manifests itself as a larval infection in all hosts except the dog. Hence, the larval stages should contain infection stage specific antigen. Antigenic analysis of larval and adult stages by immunoelectrophoresis showed that the larval stages of Ascaris have antigens not present in the adult Ascaris (Justus, 1968). If the same is true of Toxocara, then perhaps the antigens unique to the larval stage account for their greater sensitivity. Schultz-Dale desensitization studies by Ivey (1965) indicated that Toxocara larvae posses antigens not present in Toxocara adults. In the present studies, the results with rabbit antiserum (Table 4) and baboon antisera (Figure 4 and Table 5) all point toward the greater sensitivity of the larval extracts.

The greater sensitivity of the Ascaris larval extract in detecting antibody against Ascaris was expected because in the rabbit the parasite does not develop to the adult stage. Hence, this host is exposed only to the antigens of the immature stages. In the human host, however, both larval and adult stages would occur and antibody to both would be expected. It would be interesting to determine the serological reaction of sera from Ascaris-infected human hosts.

The lack of specificity shown by antigens of Toxocara indicates that further purification will be required to remove the nonspecific moeity. Since the present study demonstrated that Toxocara larval extract

was superior to other Toxocara extracts, attempts at isolation of a specific larval antigen by fractionation procedures should be done using antigens of the larval stages. Although a completely specific antigen is lacking, the microhemagglutination test, as used here, still may prove of great value because of the much higher titers achieved in the homologous system using antigens of the larval stage.

Autopsy of infected baboons revealed visible, pinhead-sized lesions in the liver, lung, and kidney. The number of lesions appeared to be dose related. Larval recovery studies (Table 11) confirmed this observation. No gross lesions were noted in the brain or heart, but larvae were recovered by digestion. Several workers have reported recovery of larvae from various organs of experimental animals and humans e.g. Sprent (1952, 1955), Smith and Beaver (1953); Lee (1960); Oshima (1961), Done et al. (1960); Beaver (1962), Sinha (1966), and Fernando (1968). Furthermore, Dent et al. (1956) reported a necropsy findings which revealed granulomatous lesions with and without the larvae of Toxocara. Lesions were widely scattered throughout the viscera and the larvae were recovered most abundantly from the liver, brain, and lung.

Larval granulomata were found in various organs. In the brain, however, thick fibrous capsules, similar to those in other organs, were not seen. Furthermore, in the brain, the predominant cells were of the large mononuclear and foreign body giant cells. Many punctate lesions were observed adjacent to parasitic lesions and these were thought to be "tracts" left by the larvae as they migrated freely and extensively through the brain tissue. Done et al. (1960) believed that the neurological symptoms associated with Toxocara infections are caused by the

reaction to static larvae rather than by the damage resulting from active migration of the larvae. Another rather characteristic feature of Toxocara infection in baboons appeared to be the presence of numerous hemosiderin laden macrophages adjacent to the small pulmonary arteries. These clusters were seen in all but one of the infected animals, in none of the controls, and were directly proportional to the dosage of infective eggs.

Beaver (1956, 1962) stated that the organs most severely affected in various experimental animals were the liver, lung, brain, and kidney. In the present study more larvae were recovered from the liver than any other organ but larval recoveries from the lungs were of similar magnitude in two of the four animals studied. In organs other than the brain the larvae eventually became encapsulated. Higashikawa (1961) also reported that in mouse brain Toxocara larvae were free and cellular reactions were not pronounced. However, Moore (1962) reported a necropsy case with numerous larvae in the brain, mostly in granulomata, and no larvae in the liver.

As previously mentioned, granulomata with thick fibrous capsules were more frequent in the liver, lung, kidney, thyroid glands, and mesenteric lymph nodes. On the other hand, a less intense cellular response was noted in the heart--tissue, pericardial sac, and even the liver of the two animals that died shortly after the second infection (Figure 8, 9, 10, and 13). These recent lesions lacked a well consolidated tissue reaction and were probably caused by larvae of the second infection. Lesions showing central necrosis were more common in the liver of one of these two animals (Figure 8). Dent et al. (1956) stated that fibrinoid

necrosis occurred in the central zone of recent lesions, while older lesions showed fibrous encapsulation. In contrast, in the present study a recent lesion also was found in a section from an animal that received a single infection 6 months previously (Figure 11). In this lesion, which was adjacent to the one that lacked a larva, an intact larva was seen surrounded by eosinophils and polymorphonuclear leukocytes, with no evidence of fibrous capsule. Since this animal received only a single infection, the fresh lesion possibly suggests that a larva escaped from the granuloma, migrated to the new site, and was in the process of being re-encapsulated again. Beaver (1962) reported similar lesions in a monkey infected with Toxocara for 18 months and he reached similar conclusions.

The two animals that received high doses of infective eggs became ill and died shortly after the second infection. The animals showed extensive intra-alveolar hemorrhage and edema which resulted from an increased host response and/or larger numbers of larvae reaching the lungs.

CHAPTER VI

SUMMARY

Ten baboons, two to six years old, were divided into five groups, two in each group, and were infected orally with a single dose of either one, five, ten, or twenty infective Toxocara canis eggs/gm body weight. Control animals were not infected. The baboons were bled at weekly intervals during the first six weeks, and then every other week during the initial infection. One animal in each group received a second infection either twelve or 16 weeks after the first infection. Total white cell counts, absolute eosinophil counts, and differential cell counts were made on each blood sample. Sera were saved for subsequent antibody and protein analysis.

The white cell counts and percentage of eosinophils reached a peak the second or third week after infection. The white cell counts returned to almost normal levels within five to six weeks after infection. The eosinophil response showed a similar drop during this period but remained above preinfection levels during the entire study period in most of the animals. The extent of the eosinophilia and leukocytosis appeared to be dose dependent. Animals given a second infection showed a second rise in total white cells and eosinophils. However, the responses never attained the peaks reached during the first infection.

Two animals apparently died as a direct result of the second infection.

Jung and Pacheco's (1960) hemagglutination procedure gave positive results with adult Toxocara antigen when a modified ratio of antigen to red cells was used for sensitization. However, significantly greater sensitivity was obtained with the antigens of the immature stages when the tanned red cells were exposed to the antigens at 37°C for 30 minutes instead of at room temperature, as was done in most of the earlier studies. The 37°C treatment during red cell sensitization greatly increased the sensitivity of the test in the homologous system with the antigens of Toxocara larvae, hatching fluid, and infective eggs, but not adult antigen. No significant improvement was noted when antigens of various Ascaris stages were similarly treated and reacted against homologous antisera.

Cross reactions between Ascaris and Toxocara antigens occurred when they were tested with antisera prepared in rabbits. Greater sensitivity was noted with the antigens of the larvae. On the other hand, the adult extracts of both species gave similar reactions in homologous and heterologous systems.

Using the above modified hemagglutination test, circulating antibodies were detected in the sera of all baboons one week after infection with Toxocara and they reached a peak during the second or third week. Antibodies were still detectable 30 weeks after infection when antigen prepared from Toxocara larvae was used. In every instance, the system using antigen of Toxocara larva detected higher antibody titers than systems using Toxocara adult or Ascaris larval or adult extracts.

The microzone cellulose electrophoresis system was used for

analyzing serum proteins changes. No significant changes in the alpha-1, -2, and beta globulins were detected. Generally, there was an increase in the total protein, and slight decrease in the albumin, and an apparent increase in the gamma globulin in the infected animals.

At autopsy, grossly, multiple, white, pinhead-sized nodules were seen in the liver, lungs, and the kidney of infected animals. The number of lesions was directly related to the size of the dose. Using pepsin digestion, Toxocara larvae were recovered from the liver, lung, kidney, brain, and heart. Granulomata with thick capsules were frequent in the liver, lung, kidney, thyroid glands, spleen, and mesenteric lymph nodes. In the brain, however, the granulomas lacked thick, fibrous capsules.

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