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Studies of immediate hypersensitivity responses
to toxocariasis in the dog

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by

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A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
MASTER OF SCIENCE

Major Subject: Veterinary Pathology (Parasitology)

Signatures have been redacted for privacy

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1971

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INTRODUCTION

Around the turn of the century, the idea that nematodes stimulated immunological resistance to reinfection was not a widely accepted concept. Many facts, such as the inability to passively transfer parasitic immunity with serum and the inability to produce parasitic immunity with extracted antigens, accounted for the difficulty in elucidating the importance of immunity in the host-parasite relationship. Since then, better immunological techniques allowed the discovery of complement-fixing, hemagglutinating, and homocytotropic antibodies which react with parasitic antigens in parasitized animals. Host resistance causes reduced fecundity of female nematodes, structural changes in developing parasites, and even prevents reinfection in some instances. Host immune responses have also been utilized in the diagnosis of parasitism; for example, hemagglutination test, complement fixation test, and dermal test have all been used to aid in the diagnosis of parasitism. However, the role of antibodies in functional host resistance has not been fully elucidated.

Homocytotropic antibodies are produced during Toxocara canis (Werner, 1782) infections in rabbits (34), rats (17), and man (102); but their production in the definitive canine host has not been reported. Several facts suggest the dog may produce homocytotropic antibodies in response to T. canis infection. In older dogs second-stage juveniles localize in

eosinophilic granulomas. Booth (6) demonstrated anti-Ascaris suum IgE antibodies in mongrel dogs. Fernando (23) found that mongrel dogs went into anaphylactic shock when injected intravenously with T. canis extract and that self-cure occurs in T. canis superinfection of puppies.

Demonstration of immediate hypersensitivity mediated by canine IgE in dogs infected with T. canis would help elucidate the host-parasite relationship involved in canine Toxocara infections. The liberation of histamine could stimulate the formation of eosinophilic granulomas since histamine activates the reticuloendothelial system (51).

The purpose of this study was to demonstrate the presence or absence of immediate hypersensitivity in dogs infected with T. canis. Homologous passive cutaneous anaphylaxis, Schultz-Dale, and hemagglutination tests were used to measure the immune response in Toxocara-infected dogs.

LITERATURE REVIEW

Life Cycle of Toxocara canis

The life cycle of T. canis has been described by Fülleborn (26, 27, 28, 29), Koutz et al. (52), Noda (59, 60, 61), Schacher (77), Sprent (87), and Webster (101). In its simplest form the life cycle is direct, and the adults live in the canine small intestine. Unembryonated eggs are passed in the feces of the definitive host. Under optimal environmental conditions, the eggs embryonate in 3 to 4 weeks. When embryonated eggs are ingested, the infective (second-stage) juveniles hatch in the upper small intestine. They penetrate the wall of the ileum and cecum and enter the peritoneal cavity. In puppies less than 6 weeks of age, the juveniles may migrate through numerous organs; but most of them eventually reach the lung. In the lung, the juveniles molt to third-stage juveniles and penetrate into the lumen of the alveoli. After migrating up the respiratory tract, they are coughed up and swallowed. In the jejunum, the juveniles complete their development to adult nematodes. The prepatent period in oral infection is about 30 days.

In dogs older than 6 weeks, age resistance alters the migratory pathways of T. canis juveniles. The majority of the second-stage juveniles migrate into various tissues and become surrounded by eosinophilic granulomas. Second-stage juveniles remain in this semiquiescent, undeveloping stage

until proper environmental conditions (such as pregnancy) instigate further development (87).

In utero infection is very common and occurs when juveniles in maternal tissue are activated during pregnancy and begin to migrate extensively in maternal tissues (31). Juveniles begin to appear in fetal tissue during the 42nd day of gestation. They migrate to the liver and lungs of the fetus and molt to third-stage juveniles before parturition. After parturition, the third-stage juveniles migrate up the respiratory tract and are swallowed. Puppies infected prenatally have a shorter prepatent period (21 days) than puppies infected orally (87). After parturition, second-stage juveniles have been found in bitch's milk (92). The importance of transcolostral transmission has not been determined.

Paratenic hosts such as mice (83, 100), rabbits (39), pigs (18), sheep (78), opossum (95), and chickens (30) afford another ramification in the life cycle of T. canis. Sprent (87) attempted to demonstrate that when T. canis second-stage juveniles in paratenic hosts were ingested by carnivorous dogs, the juveniles did not migrate in somatic tissue of the dog but matured to adults in the small intestine. In contrast, Warren (100) found somatic and tracheal migration was still essential for T. canis juvenile maturation in the definitive host (dog) when T. canis visceral larva migrans second-stage juveniles in a paratenic host were ingested.

Parasitic Antigens

In the host, parasites are complex organisms which do not possess a high degree of immunological specificity. They consist of many cells and tissues which change during maturation and may antigenically mimic host tissue (12). Soulsby (84) found that antigen extracted from adult parasites does not elicit the same immunological response as a natural infection. He theorized extraction procedures used either destroyed the functional antigens, or they were excretory or secretory in nature and were present in minute levels in extracts. Being present in low levels in extracts, they were camouflaged in antigenic competition.

An array of antigens is present in T. canis. Heterophile antigen was found by infecting rabbits with T. canis (21). Genus-specific antigens have been isolated from the cuticle, ovarian tissue, and other tissues (41, 42, 44). Desoxyribonucleic acid has been detected in muscle nuclei of Toxocara which leaves open the possibility of finding nucleic acid-agglutinating antibodies in the canine host, as has been reported in Plasmodium infections in mice (53, 106). Antigenic extracts of adult T. canis (37, 48, 50, 80, 102, 103, 105) and of infective-stage juveniles (2, 4, 39, 64, 68) have been used in serological tests. Results of these tests indicate genus-specific antigens are present in both second-stage juveniles and adult T. canis.

Host Resistance to Parasites

Hosts resist helminthic infections with several types of immunity. Innate resistance prevents patent T. canis infection from developing in noncanine hosts; however, the juveniles survive in eosinophilic granulomas and initiate an antibody response (1, 2, 22, 86). Physiological factors may be more important in innate resistance than circulating immunoglobulins. The function of serum-precipitating antibodies (IgG and IgM) is not clear. Ancylostoma and Toxocara juveniles exposed in vitro to immune serum develop plugs around the excretory pore, oral cavity, and anus and have a reduced infectivity. Parasites such as ascarids, Dictyocaulus, and Ancylostoma which migrate into host tissue develop a better immune response than lumen dwellers (86). Passive immune experiments have shown that serum immunoglobulins are important in parasitic resistance (for example, canine Ancylostoma infections) (54). Immunity to invading parasites may be manifested by decreased numbers of parasites, decreased fecundity, or even reduction of parasitic organelles, such as occurs in Nippostrongylus infections in mice (82).

Jeska (43) has demonstrated that leukocyte adhesion is an important reaction in a host. Cells sticking to parasites in gnotobiotic animals are probably nonspecific, but they are important in initiating an immune response. The cells adhering to parasites inoculated into parasite-free animals were not producing immunoglobulins but were probably macrophages

processing antigens for immunocompetent cells. In contrast, numerous pyroninophilic cells were sticking to parasites in immune animals. Adhering cells in this case may be producing protein (immunoglobulins) and represent immune adherence.

Self-cure was first described by Stoll (91) to designate a phenomenon found in lambs infected with Haemonchus contortus. When lambs were subjected to reinfection, the number of eggs passed in the feces fell precipitously. At necropsy, the majority of the worms were found to have been eliminated. Hogarth-Scott (35) demonstrated self-cure was due to homocytotropic antibodies. These antibodies sensitize mast cells and cause histamine release when exposed to parasitic allergens. This would account for the rise in blood histamines reported by Stewart (89) in sheep during self-cure. Although blood histamine level was elevated, the level of histamine in the intestinal wall was within normal levels (90). Parasitic antigens which elicit histamine release in natural infections do not release histamine when extracted from parasites and given orally (89). This has led some investigators to believe allergens are excretory products produced during exsheathment and are not absorbed from the intestine (84). The phenomenon of self-cure also has been demonstrated in a number of other host-parasite relationships such as: Trichostrongylus species infections in sheep, Dictyocaulus infections in cattle (85), Toxocara infections

in dogs (23), and Nippostrongylus infections in rats and mice (82).

Definitive (canine) hosts have an innate age resistance which is independent of prior exposure (24, 29). Age resistance prevents T. canis juveniles from developing beyond the second stage in canine tissues except under specific circumstances such as pregnancy. After exposure to infective juveniles, an eosinophilia occurs. Eosinophilia is commonly believed to be indicative of an allergic state. Fernando (23) found complement-fixing antibodies in serum of T. canis-infected dogs and a loss of adult Toxocara from the intestine after a challenge with infective juveniles. The loss of adults was attributed to a self-cure reaction as described by Stoll (91). Dogs inoculated intravenously with T. canis extracts go into anaphylactic shock, which is clinically manifested by salivation, defecation, urination, and erection of the hair on the back (23). Although homocytotropic antibodies against Toxocara have not been demonstrated, homocytotropic antibodies against a closely related ascarid (A. suum) have been demonstrated in canine blood (6).

Canine Antibody Production

Dennis et al. (15) found that puppies are anatomically immunologically mature at birth and capable of producing immunoglobulins. The thymus is well developed by the 40th day of gestation. Lymphogenesis begins in the thymus at

about this time. Migration of lymphocytes from the thymus occurs before parturition; nodal, splenic, and gastrointestinal lymphoid tissues are well developed at birth. Although the lymphoid tissues are well developed at birth, immunological competence to a series of antigens develops sequentially in the dog as in other species. When bacteriophage ϕ X-174 (an antigen used for probing the capabilities of ontogenically primitive lymphoid cells) is injected into a 40-day-old fetus, an antibody response is elicited (38). Skin allografts on fetal puppies (48-day-old) survive longer than skin allografts on neonatal puppies, indicating a weaker immune response and/or tolerance in neonates (16). Only a small portion of dogs at 6 weeks of age produce antibodies to bovine serum albumin, but most adults have a good immune response, which indicates a persistent immunological immaturity of young puppies rather than an antigenic weakness of bovine serum albumin in the dog (38).

Immunoglobulins produced by antigenic stimulation are similar to other species. Six classes of immunoglobulins have been reported in canine serum (45, 46). IgG (γ_2a and γ_2b), IgM, IgA (intermediate γ_1), and IgE appear to be similar to their respective human analogues (67, 98). IgD, as described in man, has not been identified in canine serum; but the characteristics of γ_2c (45) have not been elucidated in dogs. Canine IgG and IgM are the precipitating and complement-fixing antibodies produced in response to most

antigens. IgM production is high initially after antigenic stimulation and wanes as the production of IgG increases. IgA is a nonprecipitating antibody which produces some passive immunity and will inhibit passive anaphylaxis resulting from canine reaginic antibody (67). Canine colostrum and fecal immunoglobulins are predominantly IgA (72, 73, 74), although small amounts of IgG and IgM are also present.

Canine Reaginic Antibodies

Patterson (66) described homocytotropic antibodies in dogs sensitized to ragweed. He concluded that canine and human IgE are homologous on the basis of results of the precipitin test. The characteristics of canine IgE are that it will passively transfer to normal dog, human, or monkey skin, but not to rabbit or guinea pig skin. Heating at 56 C. for 4 hours destroys its ability to sensitize homologous skin. Canine reaginic antibodies do not precipitate with their respective allergens in vitro and persist at dermal transfer sites for at least 2 weeks. Exposure to 2-mercaptoethanol (with or without alkylation) markedly reduces the ability of reaginic antibodies to sensitize dermal sites. Patterson postulated that a covalent bond might be formed between reaginic antibodies and dermal cells by disulfide interaction which then participates in the release of vasoactive amines. Canine homocytotropic antibodies are soluble in 33.3% saturated ammonium sulfate. Electrophoretically canine IgE

migrates slower than IgA on zone electrophoresis. In a density gradient ultracentrifuge IgE sediments more rapidly (8S to 9S) than IgG (7S) (75).

Anaphylaxis

Laboratory demonstration

Anaphylactic reactions are based on two underlying physiological responses: an increased vascular permeability and smooth muscle contraction. Commonly used methods of measuring anaphylactic changes are cutaneous anaphylaxis test (direct and passive), histamine analysis, and the Schultz-Dale test. In direct cutaneous anaphylaxis, small amounts of allergen are injected intradermally into a hypersensitive individual; and 15 to 30 minutes later, the reaction site is examined for the presence of a wheal-and-flare. When homologous passive cutaneous anaphylaxis is used, serum antibodies from a sensitized animal are transferred to dermal sites on a normal animal. After an incubation period, the reaginic antibodies attach to dermal mast cells. The antigen is then injected either intravenously or intradermally, and the dermal sites are examined 15 and 30 minutes later for wheal-and-flare reaction. Intravenous injection eliminates nonspecific reactions caused by irritating allergens.

The Schultz-Dale test measures the amount of smooth muscle contraction that occurs when tissues from a sensitized animal are exposed to a homologous allergen. Tissues are

removed from a euthanatized animal and placed in Tyrode's solution at 37 C. Muscular contractions are then measured by a myograph when allergens are added to the bath. Historically guinea pig ileum was used in the Schultz-Dale test. Numerous other tissues (bronchi, stomach, uterus) have been used, but variation in smooth muscle response to vasoactive amines has been detected.

The amount of vasoactive amines released may also serve as an anaphylactic index. In vivo the level of blood histamine rises during anaphylaxis and self-cure (89). In vitro the intensity of reaginic reactions (Schultz-Dale) has been quantitated by measuring the amount of histamine liberated from tissue mast cells during exposure to allergens (76).

Mediators

Although histamine is not the only mediator of anaphylaxis, its physiological role has been studied more than other mediators (serotonin, kinins, complement anaphylatoxin, and enzymes from neutrophil granules). The importance of other anaphylatoxins is exemplified by the finding that kinins are released in wheal-and-flare reactions in sensitized human beings (56). Contraction of the sensitized guinea pig ileum in vitro is only partially dependent upon histamine, since antihistamines only partially block the Schultz-Dale reaction (5). This indicates other agents besides histamine

are being released in anaphylactic reactions to stimulate smooth muscle.

Release of mediators

The release of anaphylactic mediators is not well delineated. In man most of the available evidence indicates IgE antibody releases vasoactive amines (10). The ability of human serum to sensitize human leukocytes parallels the ability of serum to sensitize skin. Secondly, antibodies produced in the rabbit against human IgE antibody evoke a wheal-and-flare reaction when injected intradermally into human skin, while rabbit antiserum specific for other human immunoglobulins fails to elicit a reaction when injected intradermally (36). Elevated levels of IgE are found not only in atopic individuals, but also in other conditions such as: celiac diseases, idiopathic pulmonary hemosiderosis, Laennec's cirrhosis, and clinically healthy patients with no history of allergy. Therefore, increased serum levels of IgE do not necessarily indicate an atopic condition (32).

Recent work has demonstrated that all homocytotropic antibodies are not IgE. Reid (69) found two homocytotropic antibodies in human cord serum which will initiate histamine release. One was an IgE, and the other was an IgG. Two homocytotropic antibodies have also been found in mice (8, 55, 71). In contrast, guinea pigs have only one homocytotropic antibody and two classes of complement-fixing antibodies

which participate in anaphylaxis (63). Patterson (66) found that the injection of heterologous serum into dogs produced an anaphylaxis which was not mediated by canine IgE antibody.

Complement may release anaphylatoxic reagents by several means. Enzymes such as trypsin may split complements 3 and 5 and release anaphylatoxin (99). The same anaphylatoxins are produced when the several complement components sequentially attach to antigen-antibody complexes of IgG or IgM. The conglomerate thus formed has a tendency to adhere to platelets, leukocytes, and other blood elements (25). Immune complexes (antigen-antibody) release histamine from leukocytes and platelets in some species (79).

Immune complexes are also detrimental in other ways (33, 58). In rabbits they form microemboli, which obstruct small pulmonary arteries. Neutrophils and platelets are then attracted, and the immune complexes are phagocytized. During the process lysosomal contents of neutrophils are released, producing a rise in plasma acid proteases and other hydrolases. Although the whole process is similar to Arthus' phenomenon, the physiological effects of increased vascular permeability and smooth muscle contraction are seen immediately.

Canine anaphylaxis

Several mediators may be involved in canine anaphylaxis. Systemic anaphylaxis is manifested clinically by urination,

defecation, salivation, and erection of the hair on the dorsal midline (66).

Pathologic changes in canine anaphylaxis have been described by Dean and Webb (14) and Patterson (66). The major change is marked engorgement of the liver, which is the shock organ. If the liver is removed, anaphylaxis is less severe. Multiple hemorrhages appear on the mucosal surface of the gall bladder, small intestine, and colon. Microscopically centrilobular necrosis, sinusoidal congestion, and focal areas of hemorrhage are seen in the liver. Submucosal hemorrhages are seen in the wall of the gall bladder. The small intestinal and colonic lesions consist of focal areas of hemorrhage, focal areas of necrosis, and sloughing of mucosal epithelial cells.

Patterson (66) also described physiologic changes seen with anaphylaxis. A significant rise in blood histamine level is present. Heparin levels of the blood rise and produce a prolonged clotting time. While the roles of heparin and histamine have been demonstrated in canine anaphylaxis, the roles of such mediators as slow-reacting substance, hypotensive polypeptides, catecholamines, proteases, and anticoagulants have not been elucidated.

Tissue Histamine

Dermal mast cells are almost totally responsible for histamine in the skin of the dog. Histamine content of the skin fluctuates as a result of release stimulated by such agents as: physical irritation, inflammation, venoms, noxious agents, trypsin, heat, cold, cutaneous allergy, bacterial endotoxins and exotoxins, and basic compounds (ammonia and thiamine). Dermal levels of histamine vary from one part of the body to another. Higher levels are present in the skin of the ears, face, and feet of the dog (11). An age variation in canine dermal histamine also is evident. At 3 days of age, the average histamine concentration of the skin is between 4.2 to 5.2 $\mu\text{g./Gm.}$ of tissue. A gradual increase in histamine content continues for the first 3 to 5 weeks of life and reaches a peak in excess of 11.5 $\mu\text{g./Gm.}$ of skin. Values then gradually recede to normal adult limits (1.4 to 9.0 $\mu\text{g./Gm.}$ of tissue) by 9 weeks of age (11). The half-life of histamine in the skin is about 15 days; and if the skin histamine is depleted, restoration takes 2 to 3 weeks (51).

Gastrointestinal histamine concentration also varies according to location. The histamine concentration of the gastric wall is higher in the fundic region (120 to 130 $\mu\text{g./Gm.}$ of tissue) than in the pyloric region (24 to 80 $\mu\text{g./Gm.}$ of tissue). The distribution of histamine within the gastric mucosa shows two areas of concentration, one in the region of the parietal cells and the other in the region of the

muscularis mucosae. Levels of intestinal histamine are highest in the duodenum (40 to 100 $\mu\text{g./Gm.}$) and gradually decrease distally to the colon (104). An overall mean of 50 to 54 $\mu\text{g./Gm.}$ is given by Eichler and Farah (19) for the small intestine. Unlike dermal histamine, a major portion of small intestinal histamine is not found in mast cells and is not released during anaphylaxis (51).

EXPERIMENTAL TECHNIQUES

Source and Preparation of Antigens

Toxocara canis adults were obtained from pound dogs after administration of piperazine. The narcotized adult nematodes were collected from the excreta in the dog runs and were washed four times in sterile physiological saline. Eggs were removed from females by severing the anterior end of each worm and squeezing out the uterus and eggs into a container.

Uteri were cut into small pieces to facilitate egg removal. Eggs were suspended in 0.525 molar sodium hypochlorite and shaken for 5 minutes. The supernatant fluid was poured off after a 20-minute sedimentation period. The eggs were washed three times with distilled water, allowing a 20-minute sedimentation period between washings. Eggs were placed in large covered petri dishes and immersed in 2.5 cm. of 0.1 N sulfuric acid. They were incubated for 45 days at room temperature with continuous aeration to allow the development of infective juveniles (20).

After embryonation, the eggs were hatched using the technique of Jaskoski and Colucci (40). The eggs were rinsed twice with tap water, and a 40 ml. suspension of eggs was placed in a 250 ml. stoppered hatching flask with 100 glass beads. Air in the flask was replaced by a mixture of 50% CO₂ and 50% air. The flask was placed on a rotating mill with a heat lamp shining on it from about 1 foot away. The mill was

rotated for 20 minutes, or until 80 to 90% of the embryonated eggs had hatched.

A cotton filter was used to remove egg shells and debris from viable juveniles. Wire mesh was placed in a glass funnel with a test tube connected to the bottom of the funnel. The mesh was covered with a 2 mm.-thick layer of teased cotton. Water was then added to the system until it touched the wire mesh. Juveniles and debris were carefully placed on the cotton until it was covered. Viable juveniles were allowed 4 hours to migrate through the cotton and into the test tube below (40).

After filtration, the juveniles were washed in physiological saline, placed in a Ten Broeck grinder, and covered with 0.1 molar trishydroxychloride buffer. Grinding was continued until all of the juveniles were ruptured. The broken juveniles were placed in 0.1 molar trishydroxychloride solution on a magnetic stirrer at 4 C. for 24 hours to extract the antigens (41).

Frozen adult T. canis were cut into pieces and placed in a Servall-Omnimixer¹ canister containing an equal volume of nematodes and absolute ethanol. The canister was immersed in a dry ice-alcohol bath, and nematodes were homogenized for 30 minutes at top speed. After homogenization, chilled butanol was added (20% by volume) to the macerate-ethanol mixture and

¹Ivan Sorvall, Inc., Norwalk, Connecticut.

placed in the freezer for 30 minutes to remove lipids (57). The mixture was then centrifuged at 5,000 rpm for 10 minutes at 0 C., and the supernatant fluid was decanted. The sediment was resuspended in 0.1 molar trishydroxychloride buffer (pH 8.2) and centrifuged again. A layer of alcohol was siphoned off the top before the rest of the supernatant fluid and sediment were placed in a large beaker on a magnetic stirrer at 4 C. for 24 hours. The solution was centrifuged at 48,000xG for 45 minutes in a Servall centrifuge¹ at 0 C., and the supernatant fluid (containing the antigen) was saved (41).

Antigen extracts of both the juveniles and adults were dialyzed overnight in a trishydroxychloride buffer. They were then frozen in a dry ice-alcohol bath and stored in a freezer at -20 C.

After extraction, the protein content was determined with the biuret method (9).

Source and Infection of Dogs

Mongrel dogs were obtained from various pounds, but all of the dogs were young mature animals. Serum and fecal samples were taken from each dog before they were put in a barn with an outside run. Each dog was given 1,500 embryonated T. canis eggs orally each week for 3 weeks. Serum samples were then collected weekly for an additional 3 weeks. Each

¹Ibid.

sample was frozen in a dry ice-alcohol bath and stored at -20 C. until used.

Six ascarid-naive dogs, 10 weeks of age, were obtained from Iowa State University Veterinary Medical Research Institute to be used as controls. This age of dog was selected because the dermal histamine content should have receded to the normal range for adult dogs (11).

Histamine Analysis

A rectangular area of skin on the lateral side of the dog immediately posterior to the pectoral limb and halfway between the dorsal and ventral midlines was chosen for injecting the solutions. The area was 3 inches by 2 inches, with the 2-inch width being in a dorsoventral direction. This area was clipped with a number 40 electric clipper and further divided into six 1-inch squares. Each solution was randomly assigned a square injection site, and the remaining site (6th) was used as an uninjected control.

After anesthetizing the dog with pentobarbital, 0.1 ml. of each respective solution--0.1 molar trishydroxychloride buffer, bovine serum albumin, physiological saline, Toxocara extract (60 mg. protein/100 ml.) in 0.1 molar trishydroxychloride buffer, and Ascaris extract (120 mg. protein/100 ml.) in 0.1 molar trishydroxychloride buffer--was injected intradermally in its designated square of skin. Ten minutes later the squares of skin were quickly removed and frozen in a dry

ice-alcohol bath. Then they were placed in a freezer at -20 C. until they could be analyzed for histamine content.

The abdominal cavity was opened surgically, and a section of jejunum was exposed. Sites were selected for subserosal injection of each of the respective preparations used in the intradermal injections. Ten minutes after injection, the injected sections of jejunum were excised and frozen in a dry ice-alcohol bath. They were stored at -20 C. until a histamine analysis could be performed.

Histamine content was analyzed by Shore's technique (81). Individual tissue samples were homogenized in 9 volumes of 0.4 N perchloric acid using a motor-driven glass homogenizer. The homogenate was allowed to stand for 10 minutes and then centrifuged. A 4 ml. aliquot of the supernatant fluid was placed in a 25 ml. glass-stoppered shaking tube containing 0.5 ml. 5 N sodium hydroxide, 1.5 Gm. sodium chloride, and 10 ml. butanol. The tube was agitated for 5 minutes to extract the histamine. After centrifugation for 5 minutes at 5,000 rpm, the aqueous layer was removed by aspiration. Five ml. of 0.1 N sodium hydroxide saturated with sodium chloride were added to the sample and agitated for 1 minute to remove the histidine which would interfere with the analysis of histamine. After centrifugation for 5 minutes at 5,000 rpm, an 8 ml. sample was transferred to a 40 ml. glass-stoppered shaking tube containing 4.5 ml. of 0.1 N hydrochloric acid and 15 ml. N heptane. Agitation for 5 minutes in this

solution returned the histamine to the aqueous phase, which could be analyzed fluorometrically.

Two ml. of the aqueous extract from each tissue sample were added to 0.4 ml. of a 1 N sodium hydroxide solution to produce a strongly alkaline solution. Then 0.1 ml. of orthophthalaldehyde reagent was added to the tube and mixed thoroughly. Four minutes were allowed for the fluorescent conjugation to occur. Then 0.2 ml. of 3 N hydrochloric acid was added to stabilize the conjugate. The solution was transferred to a cuvette. An activation wave length of 360 μ was used to produce a fluorescence with a wave length of 450 μ which was monitored by a Beckman spectrofluorometer¹.

Histamine standards, consisting of 1 ml. of 10 μ g./ml., 0.5 μ g./ml., 0.1 μ g./ml., and 0.05 μ g./ml., were run with each group of tissues analyzed. Reagents injected into the sites--such as the antigen extracts of Toxocara and Ascaris, bovine serum albumin, trishydroxychloride buffer (0.1 molar), and physiological saline--were checked for fluorescing properties.

After a fluorometric reading was obtained for the standards and tissue samples, the histamine concentration/ml. of tissue was computed. The concentration of histamine in the unknown tissue sample was calculated according to the following formula (81):

¹Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, California.

$$\text{Histamine concentration in tissue} = \left[\frac{\text{Reading of sample}}{\text{Reading standard}} \right] \text{Concentration standard}$$

Direct Dermal, Subserosal, and Submucosal Tests

Direct dermal, subserosal, and submucosal tests were conducted using procedures similar to those used in histamine analysis. Dermal, subserosal, and submucosal sites were prepared using identical procedures for each. Antigens of three different protein concentrations (240 mg./100 ml., 120 mg./100 ml., and 60 mg./100 ml.) were used in the direct tests. To facilitate evaluation of the reaction, 1 ml. of 5% Evans' blue was injected intravenously prior to the intradermal injection of the various reagents. After injection, the sites were observed for 30 minutes.

Anaphylaxis

Passive cutaneous anaphylaxis

The production of homologous passive cutaneous anaphylaxis was attempted using dogs, rabbits, and rats infected with T. canis. Patterson's (66) technique was used in passive cutaneous transfer in dogs. Serum was collected from infected dogs and frozen until used. The serum samples to be tested for Toxocara homocytotropic antibodies were divided into two equal parts. Half of it was heated at 56 C. for 4 hours to destroy canine homocytotropic antibody. Then 0.1 ml. of both heated and unheated samples were injected intradermally into

ascarid-naive dogs. Forty-eight hours later, 2 ml. of 1% Evans' blue was injected intravenously, and 0.1 ml. of 60 mg./100 ml. antigen was injected intradermally into two Toxocara-naive dogs in sites previously injected with serum. To eliminate nonspecific injection reactions in the four remaining Toxocara-naive dogs, 2 ml. of 600 mg./100 ml. T. canis extract were injected intravenously (instead of intradermally) with the 2 ml. of 1% Evans' blue.

Homocytotropic antibody transfer in rabbits (70, 93, 94) and rats (17) was tried to demonstrate the presence of Toxocara allergens in the prepared antigens. A modification of Hogarth-Scott's (34) technique was used to sensitize rabbits. Fifteen hundred embryonated T. canis eggs were given via stomach tube to two rabbits. Thirty days later serum was collected from the rabbits and pooled. One aliquot was heated at 56 C. for 3 hours to destroy the homocytotropic antibodies present. One-tenth ml. each of heated and unheated samples were injected intradermally into a nonsensitized rabbit. After a 48-hour sensitization period, 1 ml. of Evans' blue and 1 ml. of 600 mg./100 ml. adult T. canis antigen were injected intravenously via an ear vein. The intradermal injection sites were observed for 20 minutes after intravenous injection.

Homocytotropic antibodies were demonstrated in rats, using the technique of Dobson et al. (17). Six rats were infected orally with 4,000 embryonated T. canis eggs in 1 ml.

of fluid. Blood samples were obtained from the rats 30 days later and the serum collected and frozen until used. Two ml. of serum from each rat were heated at 56 C. for 4 hours to destroy the homocytotropic antibody (88). Then 0.1 ml. of both heated and unheated serum samples from each rat were injected intradermally into noninfected rats. After a 48-hour sensitization period (17), 0.5 ml. of 1% Evans' blue and 0.5 ml. of 600 mg./100 ml. T. canis extract were injected intravenously. Fifteen minutes later the intradermal injections were observed and the results recorded.

Heterologous passive cutaneous anaphylaxis was performed in guinea pigs, using dog, rabbit, and rat serum (65). Samples of serum from each dog, pooled rabbit serum, and pooled rat serum were divided in half. One-half was heated at 56 C. for 30 minutes (dog), 3 hours (rabbit), and 4 hours (rat) respectively. Then 0.1 ml. each of heated and unheated samples were injected intradermally into guinea pigs. After a 5- to 8-hour sensitization period, 0.5 ml. of 600 mg./100 ml. T. canis antigen was injected intravenously with 0.5 ml. of 1% Evans' blue. The guinea pigs were observed for 30 minutes and the results recorded. Thirty minutes after the guinea pigs were injected intravenously, they were euthanatized and skinned so the reactions could be observed from the dermal side.

Systemic anaphylaxis

Systemic anaphylaxis was produced in T. canis-infected dogs. One ml. of 600 mg./100 ml. Toxocara extract was inoculated intravenously. Each dog was observed for 30 minutes after the intravenous inoculation of T. canis antigen.

Schultz-Dale Test

Schultz-Dale tests were run on sections of dog stomach, duodenum, and jejunum. Organs were removed from euthanatized dogs and placed in an Erlenmeyer flask containing Tyrode's solution and aerated by a continuous stream of small oxygen bubbles emerging near the bottom of the solution. The flask was immersed in a precision water bath held at 37 C. to maintain tissue viability until used in the Schultz-Dale test. An aerated, cylindrical, 20 ml., thermostatically-controlled Tyrode's solution bath, with a hook in the bottom to hold one end of the tissue, was used to run the Schultz-Dale test. The other end of the tissue strip was attached via a fine string to a myograph above the bath. A projector physiograph (model PMP-48) was used to record muscular contractions (19).

One ml. of 0.001% acetylcholine, histamine (0.1% to 10%), or antigen was added to the 20 ml. bath containing the test tissue. A contact time of 5 minutes was allowed for each solution, unless a contraction occurred prior to 5 minutes. After a contraction or 5 minutes' contact time, the tissue was washed three times with fresh Tyrode's solution; and a

rest period of 1 minute was allowed before another test solution was added to the bath (19).

Hemagglutination

Sheep erythrocytes were prepared for tanning by washing three times in Veronal¹ buffer (pH 7.2) and by making a 2.5% red blood cell suspension in Veronal buffer. One ml. of the 2.5% cell suspension was transferred to a round-bottomed tube, and an equal volume of 1:20,000 buffered tannic acid was added. The cells and tannic acid solution were incubated for 8 to 10 minutes, centrifuged, and washed twice with Veronal buffer. The cells were then ready to absorb the T. canis antigen. An equal volume of the appropriate antigen concentration and 2.5% solution of tannic acid-treated cells were placed in a tube and incubated at room temperature for 15 minutes. The tanned-coated cells were washed once and re-suspended in a buffer containing 1% inactivated guinea pig serum. Coated cells were used immediately in a doubling dilution hemagglutination plate test (49).

To remove the hemolysin contained in T. canis adult antigen, the antigen was run through a Sephadex-200² column. The first peak contained the hemolysin, and the last two peaks

¹Ten liters of the buffer were made up as follows: 3.75 Gm. sodium diethyl barbituate, 1.68 Gm. magnesium chloride, 2.8 Gm. calcium chloride, 85 Gm. sodium chloride, and 5.75 Gm. barbituric acid in distilled water.

²Pharmacia Fine Chemicals, Inc., 800 Centennial Ave., Pascataway, New Jersey.

were pooled and used to coat the sheep erythrocytes. Juvenile antigen did not contain a hemolysin and was used in a 1:8 buffered dilution to tan sheep erythrocytes.

RESULTS

Protein Concentration of Antigen and Canine Fecal Flotation

The protein concentrations of the adult and infective-stage Toxocara antigens were approximately the same. Adult T. canis extract was 600 mg. protein/100 ml. of extract, while the infective-stage juvenile was 650 mg. protein/100 ml. of extract as determined by the biuret method.

Fecal flotation on the mongrel dogs revealed they were all infected with T. canis when they were obtained. Toxocara canis eggs in the feces were not numerous, but a few were present in each sample. In contrast, no T. canis eggs were found in feces from the six T. canis-naive dogs.

Histamine Analysis

Histamine concentrations in the skin injected with physiological saline, bovine serum albumin, trishydroxychloride buffer, Toxocara extract, Ascaris antigen, and uninjected dermal sites 10 minutes post injection are given in Table 1. The variation of histamine between dermal sites on different dogs injected with the same solution was just as great as the variation between sites on one dog injected with different solutions. An "F" test was used to evaluate the histamine concentration of dermal sites injected with the respective solutions and antigens. It was shown that, on the strength of the evidence present, the probability of finding a

Table 1. Micrograms of histamine/ml. of skin after injection

| Dog No. | PSS ^a | BSA ^b | Tris-Hcl ^c | <u>Toxocara</u> antigen | <u>Ascaris</u> antigen | Uninjected control | Range | Mean | S.D. ^d |
|---------|------------------|------------------|-----------------------|----------------------------|---------------------------|-----------------------|----------|------|-------------------|
| 44 | 11.8 | 6.4 | 10.9 | 9.0 | 13.6 | 7.2 | 7.2-13.6 | 9.8 | 2.8 |
| 45 | 5.9 | 6.1 | 4.9 | 5.5 | 4.4 | 6.3 | 4.4- 6.3 | 5.5 | 0.8 |
| 46 | 2.8 | 1.9 | 4.0 | 1.3 | 1.4 | 2.2 | 1.3- 2.8 | 2.2 | 1.0 |
| 48 | 2.6 | 2.8 | 3.4 | 4.0 | 3.5 | 3.4 | 2.6- 4.0 | 3.3 | 0.5 |
| 49 | 2.9 | 2.5 | 1.6 | 2.3 | 2.9 | 2.1 | 1.6- 2.1 | 2.4 | 0.5 |
| 50 | 3.2 | 2.2 | 2.6 | 2.1 | 1.1 | 2.0 | 1.2- 3.2 | 2.2 | 0.8 |
| 75 | 4.2 | 3.4 | 4.0 | 4.1 | 3.9 | 4.3 | 3.4- 4.3 | 4.0 | 0.3 |
| Mean | 4.8 | 3.6 | 4.5 | 3.9 | 4.4 | 3.9 | -- | 4.2 | -- |

^aPhysiological saline.

^bBovine serum albumin.

^cTrishydroxychloride buffer.

^dStandard deviation.

difference between the histamine levels in the control site and test site was less than 0.05.

Histamine analyses of jejunum injected with 0.1 ml. of physiological saline, bovine serum albumin, 0.1 molar tris-hydroxychloride buffer, Toxocara antigen (60 mg. protein/100 ml. of buffer), A. suum (120 mg. protein/100 ml. of buffer), and an uninjected site are summarized in Table 2. The means, range, and standard deviation of each dog's jejunal injection sites are listed. An analysis of variance was performed on the histamine values obtained for each respective solution injection site. The "F" test indicated that the probability (P) of finding a difference between the histamine levels in the control site and the test site was small ($P < 0.05$).

Direct Dermal, Subserosal, and Submucosal Tests

The antigen extracts of T. canis adults (120 mg./100 ml. and 60 mg./100 ml.) failed to elicit either a direct dermal reaction when injected intradermally or tonus rings when injected submucosally into two puppies 1 month old. When the puppies were necropsied, adult Toxocara were found in the intestine. Two litter mates yielded different results when they were injected intradermally at 8 weeks of age. Antigen extracts of adult Toxocara (120 mg./100 ml. and 60 mg./100 ml.) produced reaction in both the skin and subserosa of 8-week-old litter mates. Fifteen minutes after the

Table 2. Micrograms of histamine/ml. of small intestinal tissue after injection

| Dog No. | PSS ^a | BSA ^b | Tris-Hcl ^c | <u>Toxocara</u> antigen | <u>Ascaris</u> antigen | Uninjected control | Range | Mean | S.D. ^d |
|---------|------------------|------------------|-----------------------|----------------------------|---------------------------|-----------------------|-----------|------|-------------------|
| 44 | 51.8 | 60.0 | 58.7 | 41.8 | 47.2 | 74.6 | 41.8-74.6 | 55.7 | 11.5 |
| 45 | 65.4 | 69.0 | 81.0 | 72.4 | 63.6 | 90.0 | 63.6-90.0 | 73.6 | 10.1 |
| 46 | 28.6 | 22.2 | 12.6 | 41.4 | 8.2 | 15.0 | 8.2-41.4 | 21.1 | 9.6 |
| 48 | 29.4 | 25.3 | 28.4 | 30.5 | 31.0 | 27.0 | 27.0-31.0 | 28.6 | 2.1 |
| 49 | 22.6 | 13.6 | 27.2 | 29.5 | 29.5 | 24.0 | 13.6-29.5 | 24.2 | 6.0 |
| 50 | 33.2 | 42.3 | 41.4 | 54.4 | 36.4 | 34.1 | 33.2-54.4 | 40.3 | 7.8 |
| 75 | 43.0 | 38.4 | 40.5 | 49.6 | 48.0 | 45.2 | 38.4-49.6 | 44.1 | 4.3 |
| Mean | 39.3 | 38.7 | 41.4 | 45.6 | 37.7 | 44.3 | -- | 41.1 | -- |

^aPhysiological saline.

^bBovine serum albumin.

^cTrishydrochloride buffer.

^dStandard deviation.

intravenous injection of Evans' blue and the intradermal injection of 0.1 ml. of the respective antigen concentration, a slightly raised blue area, 1.5 cm. in diameter, was present at the antigen-injected dermal sites. Two dermal control sites injected with trishydroxychloride buffer (0.1 molar) and physiological saline were negative. Subserosal injection of the above antigens caused the formation of a dark blue tonus ring 1 cm. wide at the site of injection. A similar dark blue thickened area, 1 cm. in diameter, was produced when the antigens were injected submucosally.

Typical results of direct dermal tests are illustrated in Figures 1-4. Three different concentrations of adult Toxocara antigen and one concentration of juvenile antigen were injected intradermally in 15 young, mature, T. canis-infected dogs. All concentrations of antigen used produced a wheal-and-flare (dark blue area 1 to 1.5 cm. in diameter) within 5 to 10 minutes in T. canis-infected dogs. In control sites injected with trishydroxychloride or physiological saline the initial bleb produced by the injection disappeared within 15 minutes, and no dermal reaction developed.

Subserosal injection of antigen was tried in nine mature T. canis-infected dogs. Three different concentrations of adult Toxocara antigen were injected beneath the serosal surface (240 mg./100 ml., 120 mg./100 ml., and 60 mg./100 ml.). The extract with 240 mg. protein/100 ml. of buffer produced a dark blue tonus ring 1 cm. wide in all nine dogs.

Figure 1. Skin of *T. canis*-infected dog 15 minutes after intradermal injection with 0.1 ml. of each reagent. Dark foci represent capillary leakage of Evans' blue at the sites of wheal-and-flare reactions produced by the antigens

- AD 1 = adult *Toxocara* extract with 240 mg. protein/100 ml. of buffer
- AD 2 = adult *Toxocara* extract with 120 mg. protein/100 ml. of buffer
- AD 3 = adult *Toxocara* extract with 60 mg. protein/100 ml. of buffer
- LA = second-stage juvenile *Toxocara* extract with 60 mg. protein/100 ml. of buffer
- TRIS = 0.1 molar trishydroxychloride buffer, pH 8.2
- PSS = physiological saline

Figure 2. Skin of *T. canis*-infected dog 30 minutes after intradermal injection with 0.1 ml. of each reagent. Dark foci represent capillary leakage of Evans' blue at the sites of wheal-and-flare reactions produced by the antigens

- AD 1 = adult *Toxocara* extract with 240 mg. protein/100 ml. of buffer
- AD 2 = adult *Toxocara* extract with 120 mg. protein/100 ml. of buffer
- AD 3 = adult *Toxocara* extract with 60 mg. protein/100 ml. of buffer
- LA = second-stage juvenile *Toxocara* extract with 60 mg. protein/100 ml. of buffer
- TRIS = 0.1 molar trishydroxychloride buffer, pH 8.2
- PSS = physiological saline

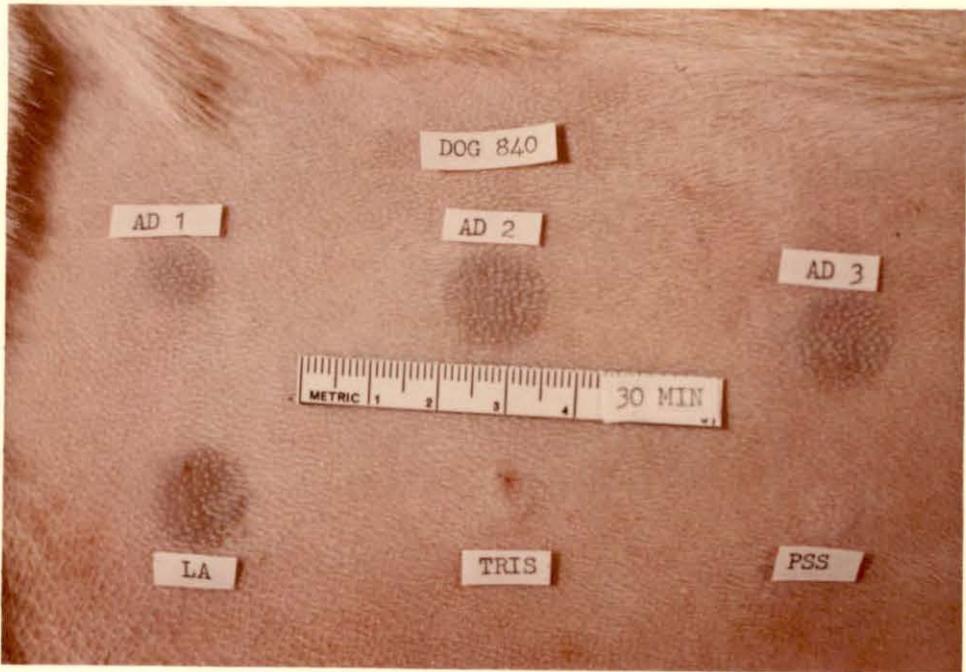


Figure 3. Skin of T. canis-infected dog 15 minutes after intradermal injection with 0.1 ml. of each reagent. Dark foci represent capillary leakage of Evans' blue at the sites of wheal-and-flare reactions produced by the antigens

AD 1 = adult Toxocara extract with 240 mg. protein/
100 ml. of buffer

AD 2 = adult Toxocara extract with 120 mg. protein/
100 ml. of buffer

AD 3 = adult Toxocara extract with 60 mg. protein/
100 ml. of buffer

LA = second-stage juvenile Toxocara extract with
60 mg. protein/100 ml. of buffer

TRIS = 0.1 molar trishydroxychloride buffer, pH 8.2

PSS = physiological saline

Figure 4. Skin of T. canis-infected dog 30 minutes after intradermal injection with 0.1 ml. of each reagent. Dark foci represent capillary leakage of Evans' blue at the sites of wheal-and-flare reactions produced by the antigens

AD 1 = adult Toxocara extract with 240 mg. protein/
100 ml. of buffer

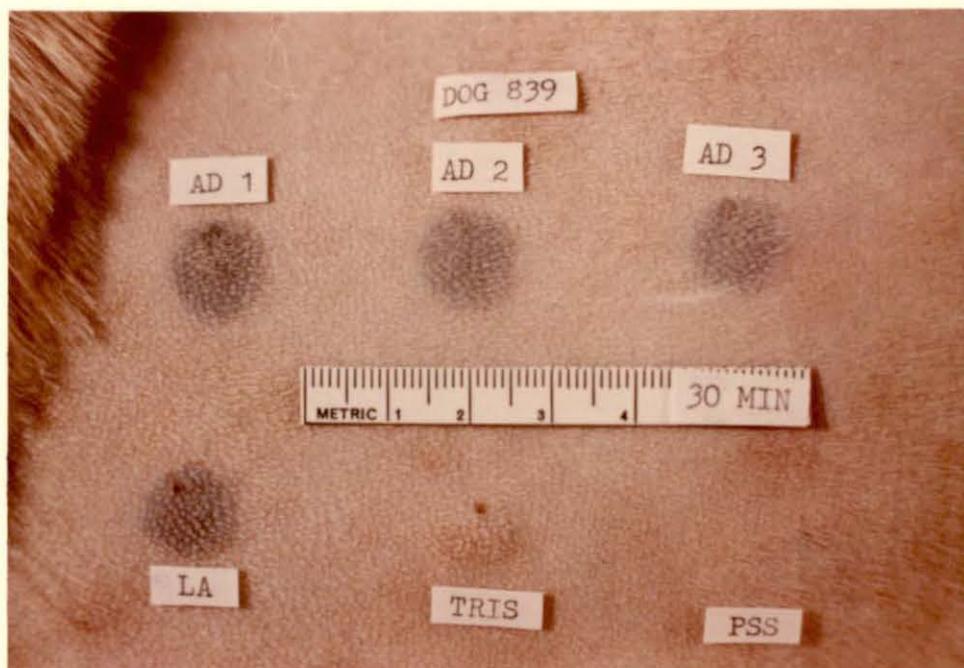
AD 2 = adult Toxocara extract with 120 mg. protein/
100 ml. of buffer

AD 3 = adult Toxocara extract with 60 mg. protein/
100 ml. of buffer

LA = second-stage juvenile Toxocara extract with
60 mg. protein/100 ml. of buffer

TRIS = 0.1 molar trishydroxychloride buffer, pH 8.2

PSS = physiological saline



Lower concentrations of antigens produced slightly smaller tonus rings as shown by antigen two (AG 2) and antigen three (AG 3) in Figure 5.

Submucosal injections of antigen were performed on several dogs. Results obtained were similar to subserosal injection. They consist of a thickened, blue-tinged intestinal wall due to smooth muscle contraction and leakage of serum and Evans' blue out of the vessels (Figure 6). Lesions seen mimic the action of a vasoactive amine such as histamine.

Anaphylaxis

Passive cutaneous anaphylaxis

Two Toxocara-naive dogs sensitized with heated and unheated serum samples from Toxocara-infected dogs received 48 hours later 0.1 ml. intradermal injections of T. canis antigen (60 mg./100 ml.) at each dermal site. Each of these antigen-injected sites, including a control antigen site not previously sensitized with serum, were marked 15 minutes after intradermal injection by a dark blue wheal due to the leakage of serum and Evans' blue from capillaries and venules. The average wheal was 2 mm. in diameter. Control antigen sites were 3 mm. and 4 mm. in diameter on the right side and 2 mm. on the left side. The range of the wheals was from 2 mm. to 4 mm. No difference between sites sensitized by heated and unheated serum could be seen. Control sites injected with

Figure 5. Small intestine of *T. canis*-infected dog 30 minutes after antigenic and control reagents were injected subserosally. The dark blue tonus rings represent foci where *T. canis* antigens were injected

AG 1 = adult *Toxocara* extract with 240 mg. protein/
100 ml. of buffer

AG 2 = adult *Toxocara* extract with 120 mg. protein/
100 ml. of buffer

AG 3 = adult *Toxocara* extract with 60 mg. protein/
100 ml. of buffer

BUFF = 0.1 molar trishydroxychloride buffer, pH 8.2

PSS = physiological saline

Figure 6. Small intestine of *T. canis*-infected dog 30 minutes after submucosal injection of antigenic and control reagents. The two dark foci represent areas where antigen was injected and allowed leakage of Evans' blue

AG 2 = adult *Toxocara* extract containing 240 mg.
protein/100 ml. of buffer

BUFF = 0.1 molar trishydroxychloride buffer, pH 8.2

PSS = physiological saline



bovine serum albumin, physiological saline, or trishydroxychloride buffer were not visible 15 minutes after injection.

To evaluate the possibility that nonspecific dermal reaction had occurred when antigen was injected intradermally into the first two Toxocara-naive dogs, the same antigen was injected intravenously with Evans' blue into four other Toxocara-naive dogs after the 48-hour dermal sensitization period. In these dogs no systemic or dermal reactions were observed 15 or 30 minutes after intravenous injection (Figures 7, 8). This indicated that reaginic antibodies capable of sensitizing homologous canine dermal sites and persisting for 48 hours were not present in the dogs checked, and that the reactions seen in the first two dogs were nonspecific reactions.

In an attempt to demonstrate allergens in the extracts prepared, homologous passive cutaneous anaphylaxis was performed using serum from Toxocara-sensitized rats and rabbits. In rats sensitized dermally with atopic rat serum, only the unheated atopic serum induced a wheal-and-flare reaction after antigen and Evans' blue were injected intravenously. Both adult and second-stage juvenile antigens (Figures 9, 10) initiated the production of a wheal-and-flare reaction in sensitized rats. In rabbits sensitized by the intradermal injection of atopic serum, a wheal-and-flare reaction 1.5 cm. in diameter was present at each dermal site injected with unheated atopic serum. Smaller wheal-and-flare reactions (0.7

Figure 7. Skin of Toxocara-naive dog, 48 hours after 0.1 ml. of serum from a Toxocara-infected dog had been injected intradermally into each square and 15 minutes after 2 ml. of Toxocara adult extract (600 mg. protein/100 ml. of buffer) had been injected intravenously. Wheal-and-flare reactions are not seen in any of the squares

Figure 8. Skin of Toxocara-naive dog, 48 hours after 0.1 ml. of serum from a Toxocara-infected dog had been injected intradermally into each square and 15 minutes after 2 ml. of Toxocara adult extract (600 mg. protein/100 ml. of buffer) had been injected intravenously. Wheal-and-flare reactions are not seen in any of the squares

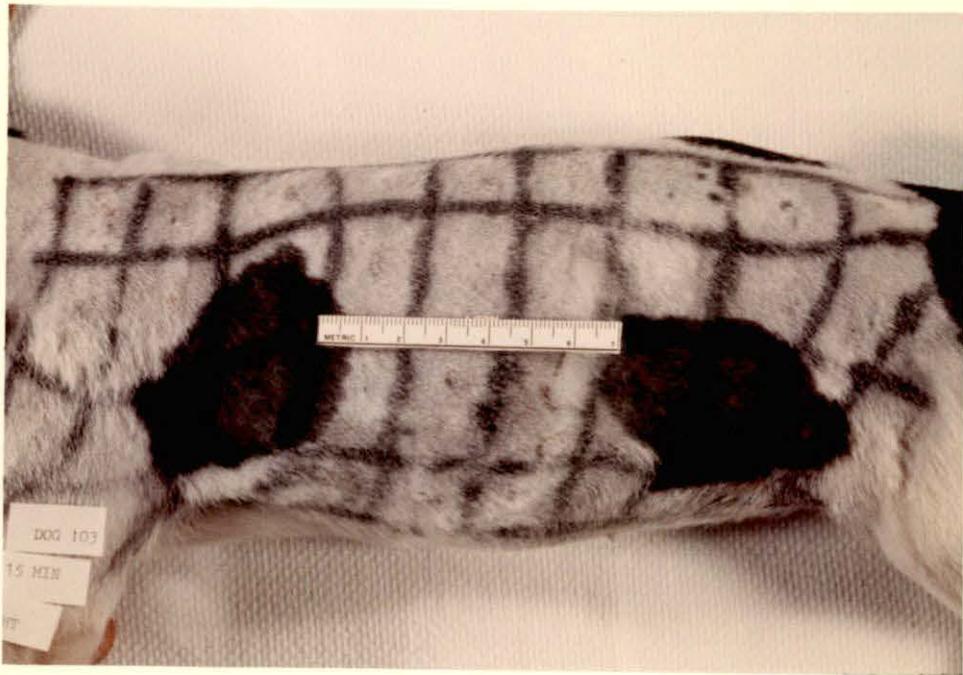
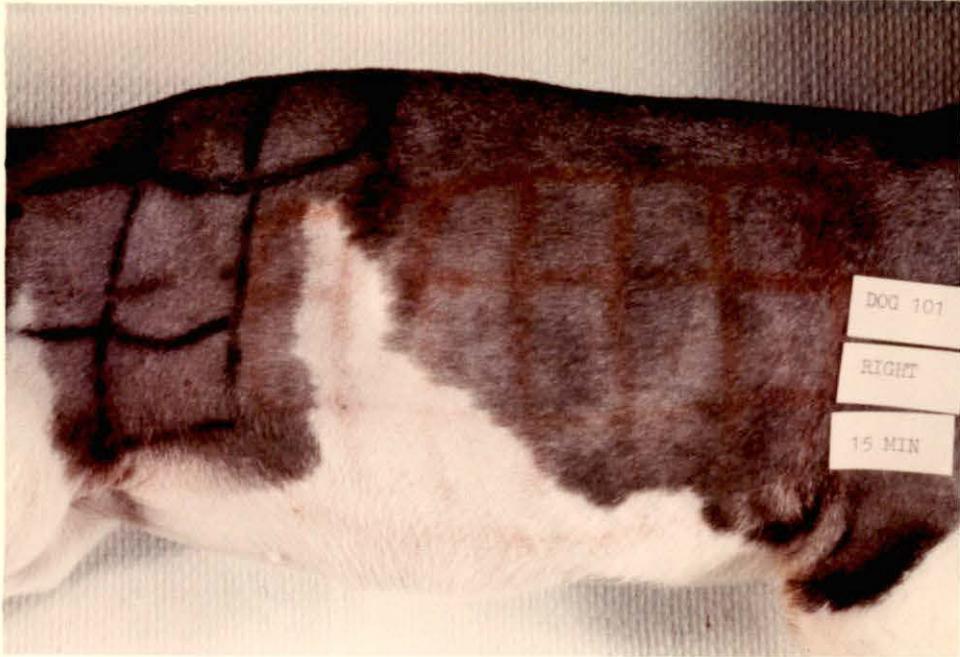


Figure 9. Skin of rat 48 hours after intradermal injection of serum from rats before and after Toxocara sensitization and 15 minutes after the intravenous injection of Toxocara juvenile antigen and Evans' blue. The dark blue foci are due to the presence of homocytotropic antibodies which initiate histamine release

- 1-UH = unheated serum from rat prior to sensitization
- 2-UH = unheated serum from rat prior to sensitization
- 3-UH = unheated serum from rat prior to sensitization
- 2I-H = heated serum from rat after sensitization
- 3I-H = heated serum from rat after sensitization
- 2I-UH = unheated serum from rat after sensitization
- 3I-UH = unheated serum from rat after sensitization

Figure 10. Skin of rat 48 hours after intradermal injection of serum from rats before and after Toxocara sensitization and 15 minutes after the intravenous injection of adult Toxocara antigen and Evans' blue. The antibody responsible for the passive cutaneous anaphylaxis is a homocytotropic antibody

- 1-UH = unheated serum from rat prior to sensitization
- 2-UH = unheated serum from rat prior to sensitization
- 3-UH = unheated serum from rat prior to sensitization
- 2I-H = heated serum from rat after sensitization
- 3I-H = heated serum from rat after sensitization
- 2I-UH = unheated serum from rat after sensitization
- 3I-UH = unheated serum from rat after sensitization



cm. in diameter) were present at each dermal site injected with heated atopic rabbit serum (Figure 11).

Heterologous passive cutaneous reactions in guinea pigs varied with the Toxocara-infected host from which the serum was obtained. No reaction was obtained with an 8-hour sensitization period using serum from dogs. When serums from infected rats or rabbits were used for sensitization of guinea pig skin, a wheal-and-flare reaction was obtained when Evans' blue and Toxocara antigen were injected intravenously. Both heated and unheated samples produced comparable reactions. The rabbit serum produced a dark blue wheal 1.5 cm. in diameter, while rat serum produced a wheal 2 cm. in diameter (Figure 12). Since both heated and unheated samples produced similar reactions, the antibody responsible for guinea pig sensitization was a heat stable antibody such as IgG.

Systemic anaphylaxis

Toxocara canis-sensitized dogs developed classical anaphylaxis after the intravenous injection of 1 ml. of Toxocara antigen (600 mg./100 ml.). Clinical symptoms of anaphylaxis occurred within 5 to 10 minutes after antigen injection. Symptoms consisted of gastrointestinal reaction with vomition and defecation, salivation, and erection of hair on the dorsal midline. Two of the dogs collapsed and died, while the other dogs recovered within a few hours. When necropsies were

Figure 11. Rabbit skin 48 hours after being sensitized with 0.1 ml. of serum from a rabbit sensitized to Toxocara and 15 minutes after intravenous injection of Evans' blue and adult Toxocara antigen. The two dark foci in the squares on the far left are areas where unheated atopic serum was injected. Four smaller dark foci on the right represent areas where atopic serum heated for 3 hours was injected. The antibody responsible for the homologous passive cutaneous anaphylaxis is a homocytotropic antibody

Figure 12. Guinea pig skin 8 hours after being sensitized with atopic serum from rats and rabbits and 15 minutes after the intravenous injection of Evans' blue and Toxocara antigen. Atopic rat serum was injected into the two dark foci on the left, and atopic rabbit serum was injected into the four dark foci on the right. The three lower foci were atopic serums heated prior to injection, while the three upper ones were not heated. The antibody responsible for heterologous passive cutaneous anaphylaxis is an IgG



performed on the two dogs, the livers were dark and two to three times their normal size as a result of severe congestion.

Schultz-Dale Test

The Schultz-Dale test was performed to determine if homocytotropic antibodies were present in dogs infected with T. canis. Sections of smooth muscle from the stomach, duodenum, jejunum, and ileum were used in each experimental trial. In the initial trial, the antigen was still suspended in tris-hydroxychloride buffer (0.1 molar). After satisfactory muscle contractions had been obtained with histamine and acetylcholine, 1 ml. of Toxocara antigen (600 mg./100 ml.) was added to the water bath. The muscle strip reacted to the antigen as well as it did to histamine and acetylcholine. Contractions did not decrease when successive aliquots of antigen were added to the tissue bath. A trishydroxychloride (1 ml. of 0.1 molar) buffer control was run to determine its effect on the muscle strip. The buffer alone produced a muscle contraction equal in amplitude to the histamine, acetylcholine or antigen (in trishydroxychloride buffer) (Figure 13).

Before the next trial was run, the antigen was dialyzed overnight against phosphate-buffered saline to remove the trishydroxychloride. After dialysis, neither the antigen preparation nor the phosphate buffer control produced muscular contraction (Figure 14) of isolated gastric muscular tissue.

Figure 13. Schultz-Dale test on a section of stomach from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

HIST = histamine

ACET = acetylcholine

AG = adult Toxocara antigen in trishydroxychloride buffer

TRIS = 0.1 molar trishydroxychloride buffer, pH 8.2

Figure 14. Schultz-Dale test on a section of stomach from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

ACET = acetylcholine

HIST = histamine

PO₄ = phosphate-buffered saline, pH 7.2

AG = adult Toxocara antigen in phosphate-buffered saline

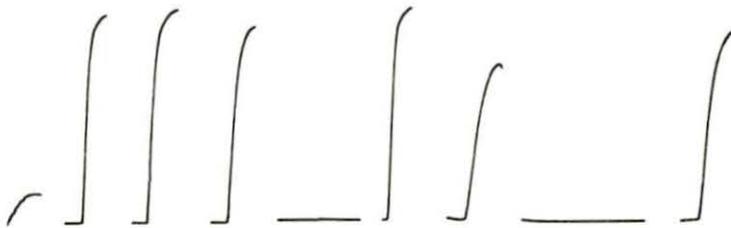
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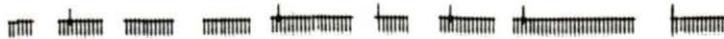
HIST ACET AG HIST TRIS AG



840 STOMACH



ACET HIST ACET PO4 HIST ACET AG



The gastric muscle was more sensitive to histamine than smooth muscle of the duodenum, jejunum, or ileum. It gave reproducible results. Contractions of gastric wall strips initiated by histamine and acetylcholine could be standardized by varying the concentrations of histamine or acetylcholine (Figures 13-19). Although standardizing the length of contractions was possible, it was not necessary to get reproducible results. Muscular contractions were readily produced by acetylcholine and histamine, but Toxocara antigen in phosphate buffer would not stimulate gastric muscle contraction in vitro using gastric tissue from Toxocara-infected dogs (Figures 14-17) or from Toxocara-naive dogs (Figures 18, 19).

The canine small intestine reacted satisfactorily to acetylcholine, but erratically to histamine. Only one small contraction was obtained using histamine on duodenal strips in vitro. One ml. of a 1% histamine solution (Figure 21) initiated a small contraction or, more usually, no contraction (Figure 20). Sections of jejunum also reacted consistently to 1 ml. of a 0.001% acetylcholine solution but only sporadically, if at all, to histamine (1 ml. of 1% solution) (Figures 22-24). Even the ileum, which is very reactive to histamine in the guinea pig, did not respond consistently to 1 ml. of 1% histamine added to the tissue bath (Figures 25, 26). Toxocara antigen did not stimulate an in vitro muscular contraction in any section of canine small intestinal muscle (Figures 20-26).

Figure 15. Schultz-Dale test on a section of stomach from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

HIST = histamine

ACET = acetylcholine

AG = adult Toxocara antigen in phosphate-buffered saline

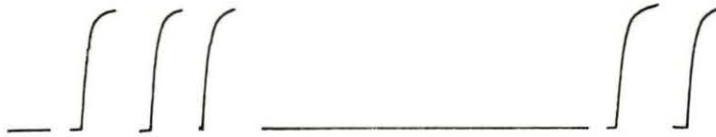
Figure 16. Schultz-Dale test on a section of stomach from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

ACET = acetylcholine

AG = adult Toxocara antigen in phosphate-buffered saline

HIST = histamine

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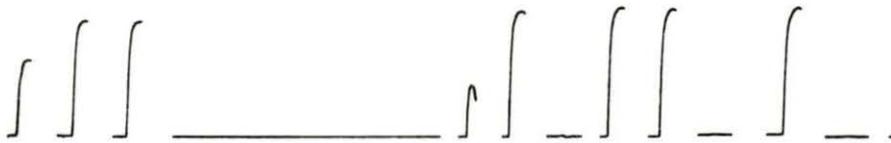


HIST
ACET
ACET
AG

HIST
ACET



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ACET
ACET
AG

HIST
ACET

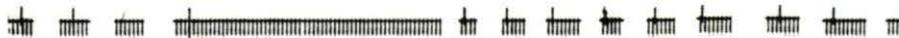


Figure 17. Schultz-Dale test on a section of stomach from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- AG = adult Toxocara antigen in phosphate-buffered saline
- HIST = histamine

Figure 18. Schultz-Dale test on a section of stomach from a Toxocara-naive dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- HIST = histamine
- AG = adult Toxocara antigen in phosphate-buffered saline

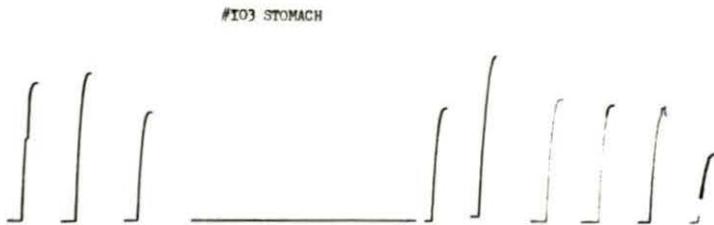
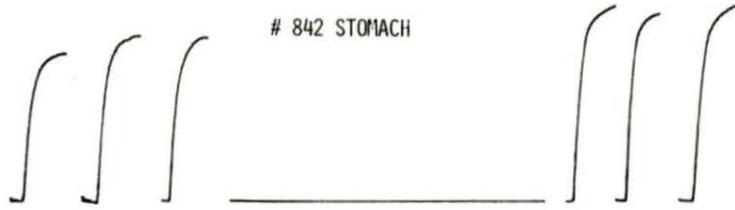


Figure 19. Schultz-Dale test on a section of stomach from a Toxocara-naive dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- HIST = histamine
- AG = adult Toxocara antigen in phosphate-buffered saline

Figure 20. Schultz-Dale test on a section of duodenum from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- HIST = histamine
- ACET = acetylcholine
- AG = adult Toxocara antigen in phosphate-buffered saline

105 STOMACH



850 DUOD

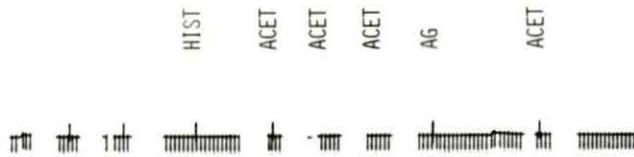


Figure 21. Schultz-Dale test on a section of duodenum from a Toxocara-naive dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- HIST = histamine
- AG = adult Toxocara antigen in phosphate-buffered saline

Figure 22. Schultz-Dale test on a section of jejunum from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- HIST = histamine
- AG = adult Toxocara antigen in phosphate-buffered saline

102 DUODENUM



ACET ACET HIST ACET AG ACET



844 JEJUNUM



ACET ACET ACET ACET HIST ACET AG ACET



Figure 23. Schultz-Dale test on a section of jejunum from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- HIST = histamine
- AG = adult Toxocara antigen in phosphate-buffered saline

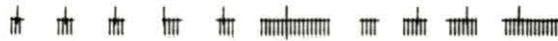
Figure 24. Schultz-Dale test on a section of jejunum from a Toxocara-naive dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

- ACET = acetylcholine
- AG = adult Toxocara antigen in phosphate-buffered saline
- HIST = histamine

850 JEJUNUM



ACET ACET HIST ACET AG ACET ACET ACET



102 JEJUNUM



ACET ACET AG HIST



Figure 25. Schultz-Dale test on a section of ileum from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

ACET = acetylcholine

HIST = histamine

AG = adult Toxocara antigen in phosphate-buffered saline

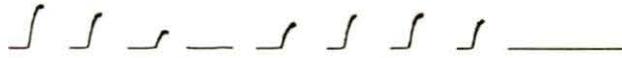
Figure 26. Schultz-Dale test on a section of ileum from a Toxocara-infected dog. The lower vertical lines represent 5-second intervals of time. The upper vertical lines represent in vitro muscular contraction which occurred when the corresponding reagents below were added to the tissue bath:

ACET = acetylcholine

HIST = histamine

AG = adult Toxocara antigen in phosphate-buffered saline

840 ILEUM



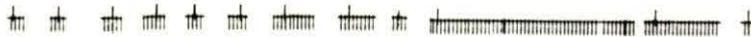
ACET HIST AG HIST ACET ACET ACET AG



#842 ILEUM



ACET ACET ACET HIST HIST ACET AG



Hemagglutination

Hemagglutination tests, using tanned sheep erythrocytes absorbed with Toxocara extracts, were performed on serums from infected and Toxocara-naive dogs and on serum from infected rabbits. The Toxocara adult antigen initially contained a hemolytic substance that had to be removed by passage through a Sephadex-200 column. The hemolysin was located in the first of three peaks which came off the column. The last two peaks were pooled and absorbed with the tanned sheep erythrocytes. Sheep erythrocytes were also tanned and absorbed with infective-stage Toxocara juvenile antigens.

The results of hemagglutination tests are summarized in Table 3. Anti-T. canis serum titers in Toxocara-naive dogs were very low in comparison to titers of infected dogs. This suggests the infected dogs were producing precipitating antibodies against T. canis.

A hemagglutination test was run on a pooled serum sample from the two infected rabbits. The titer of antibodies present was $1/2048$ using tanned sheep erythrocytes absorbed with Toxocara-infective juvenile antigen and $1/1024$ using tanned cells absorbed with Toxocara adult antigen. The difference of one dilution is probably not significant.

Table 3. Doubling serial dilution hemagglutination tests
with sheep erythrocytes tanned with antigen
from T. canis

| Dog number | Source of antigen | |
|------------|---------------------------|-------|
| | Infective-stage juveniles | Adult |
| 100 | + 1 | + 1 |
| 102 | + 1 | + 1 |
| 103 | + 1 | + 1 |
| 104 | + 1 | + 1 |
| 105 | + 1 | + 1 |
| 838 | + 7 | + 6 |
| 839 | + 7 | + 8 |
| 840 | + 8 | + 9 |
| 844 | + 9 | + 8 |
| 849 | + 7 | + 7 |
| 850 | + 9 | + 9 |
| 51 | + 8 | + 8 |
| 85 | + 10 | + 10 |

DISCUSSION AND CONCLUSIONS

The life cycle of T. canis is intricate, and the effect of acquired immunity upon different facets of the life cycle has not been reported. Fernando (23) reported that complement-fixing antibodies were produced in response to T. canis infections in dogs, but he did not demonstrate that those antibodies were functional in affecting the life cycle. In his work with T. canis superinfections, dogs had to be given a tranquilizer to prevent vomiting, initiated by juvenile allergens, when T. canis infective-stage juveniles were given orally. After oral administration of infective-stage juveniles, adult T. canis in the small intestine were eliminated from the definitive host. The loss of adult nematodes was attributed to self-cure mediated by homocytotropic antibodies.

Patterson (66) found and described the properties of canine homocytotropic antibody in ragweed-sensitive dogs. Although proof does not exist that canine homocytotropic antibody is produced in response to T. canis infections in dogs, homocytotropic antibodies which react with A. suum antigens have been found in dogs.

Direct dermal tests are a quick, easy screening method for detecting the presence of homocytotropic antibodies, but nonspecific reactions occur and produce false positive test results. All T. canis-infected dogs in this experiment gave positive direct dermal test results when injected

intradermally with 0.1 ml. of Toxocara antigen (60 mg./100 ml.). In contrast, only small, insignificant dermal reactions were produced when antigen was injected intradermally into Toxocara-naive dogs. The small reactions seen in the control dogs may have been the result of toxic substances in the antigen (13), or to vascular damage produced by the needle used for injection (102). A high concentration of antigen also can produce the nonspecific reaction in the Toxocara-naive dogs (7, 37), but the antigen used in this instance was the lowest concentration which would give consistent results in the infected dogs.

Several features indicate the dermal reactions obtained in T. canis-infected dogs were due to immunologic responses and outweigh the possibility that the dermal reactions were nonimmune responses. Two puppies 1 month of age failed to give positive direct dermal test results when injected intradermally with antigen, while two litter mates 4 weeks later gave positive direct dermal results. The difference in the dermal tests indicates that the two older puppies started responding immunologically to T. canis infection between 4 to 8 weeks of age. Furthermore, the sizes of the reactions produced by the intradermal antigen injections in Toxocara-naive and Toxocara-infected dogs were significantly different, indicating that prior exposure determined the type of reaction obtained when T. canis antigen was injected intradermally.

Results of the direct subserosal and submucosal tests also indicated that the release of vasoactive amines, which initiated the formation of a tonus ring, were due to prior antigenic stimulation. Increased vascular permeability and smooth muscle contraction were seen in T. canis-infected dogs after antigen injection, while no reaction was obtained in Toxocara-naive dogs. Smooth muscle contraction initiated by the trishydroxychloride buffer in the antigen, which occurred in the Schultz-Dale test, was ruled out when a buffer control failed to initiate any response.

Histamine analyses of dermal and intestinal sites 10 minutes after the injection of antigen failed to substantiate the release of vasoactive amines as found by Stewart (90) in sheep during self-cure. Statistical analysis of the histamine data revealed that, on the strength of present evidence, the mean histamine values from control and injected sites (dermal or intestinal) were probably from the same normal distribution; therefore, no significant difference was present. Normal levels of histamine vary widely from 1.4 to 9.0 $\mu\text{g./Gm.}$ of skin (11). Numerous samples would have been required to detect small changes in tissue histamine. Therefore, the number of samples evaluated in the present study may have been insufficient, or the amount of histamine required to produce a dermal reaction may have been too small to be detected using Shore's (81) technique.

Histamine is one of the mediators of canine systemic anaphylaxis (66). Dogs infected with T. canis developed anaphylaxis when injected intravenously with Toxocara antigen. Defecation, urination, and salivation were seen in the Toxocara-infected dogs. In contrast, the Toxocara-naive dogs remained clinically normal after intravenous injection of Toxocara antigen.

Two types of systemic anaphylaxis mediated by vasoactive amines have been reported in dogs (66). One, serum sickness, occurs when heterologous serum is injected into a dog for the second time. Patterson (66) did not describe the antibody responsible for serum sickness, but stated it was not canine IgE. The other type is mediated by canine homocytotropic antibody and has been described only in dogs sensitive to ragweed.

Anti-Toxocara canine IgE was not found in T. canis-infected dogs using two different laboratory techniques. Homologous passive cutaneous anaphylaxis was attempted in six Toxocara-naive dogs. One-tenth ml. serum from Toxocara-infected dogs was transferred to the skin of Toxocara-naive dogs. After an incubation period to allow the canine IgE to attach to the dermal mast cells via a covalent bond, T. canis antigen was injected intravenously. Intravenous injection of antigen eliminated the small nonspecific reactions which were seen when the antigen was injected intradermally. No passive cutaneous anaphylactic reactions were observed. Canine IgA

has been reported (66) to block passive cutaneous anaphylaxis when the antigen is injected intravenously; but large amounts of antigen, as used in the present experiment, would overcome the blocking effect of canine IgA.

If the dog has a second (i.e., non-IgE) homocytotropic antibody with a short sensitization period as reported in mice (71), it would not have been detected using the long 48-hour sensitization period used in this study for canine IgE detection (66). However, the Schultz-Dale test should have detected non-IgE homocytotropic antibody. Gastric muscle was the most sensitive to histamine, but it failed to contract in the presence of T. canis antigen, indicating that anti-Toxocara homocytotropic antibodies were not present in gastric tissue.

Humoral antibodies and complement, both of which may initiate release of histamine, would have been washed out of the tissue and would not have been available to initiate histamine release in the Schultz-Dale test (25).

Hemagglutinating antibodies were found in the serum of dogs infected with Toxocara. Hemagglutinating titers in Toxocara-infected dogs were significantly higher than titers found in Toxocara-naive dogs. The low titers in Toxocara-naive dogs probably represented blood group antibodies which were reacting with blood group antigens found in T. canis (41).

The hemagglutinating titers may represent a desensitization of the infected dogs, although hemagglutinating titers found in the infected dogs were about the same as those found in the serum of the infected rabbits which had homocytotropic antibodies in the serum. If the immune response of the dog is like that of the rat, the serum level of IgE declines when the level of IgG (which reacts with the same antigen) increases (62, 96, 97). The act of desensitization in man is a long, tedious process, and it is not probable that the three weekly oral administrations of infective-stage juveniles desensitized the dogs in the present study. Such a regimen would be more likely to stimulate an anamnestic response and increase the titer of homocytotropic antibodies. Systemic anaphylaxis still occurred when antigen was injected, indicating the dogs had not been desensitized.

The inability to find homocytotropic antibodies may have been due to the destruction of T. canis allergens during the extraction process. Keeping the nematodes cold during the grinding process and using butanol as a delipidizing agent may have prevented denaturation of delicate enzymes (57). If the allergen in T. canis is a glycoprotein, as reported in A. suum (3), the extraction process probably would not have denatured it.

Both rats and rabbits, which produce homocytotropic antibodies during T. canis infections, were used to test for the presence of allergens in the T. canis antigen. Allergens

that would initiate a wheal-and-flare reaction in sensitized rats or rabbits were present in the extract. Although allergens active in rat, rabbit, or dog may be different from one another, the presence of rat and rabbit allergens in the antigen indicates the extraction procedure did not destroy allergens.

In conclusion, neither the Schultz-Dale test nor passive cutaneous anaphylactic test demonstrated the presence of homocytotropic antibodies in dogs infected with T. canis. The results of direct dermal tests, submucosal tests, and systemic anaphylactic tests in Toxocara-infected dogs indicate that T. canis infections in the dog initiate an antibody response, but this antibody was detected only with the hemagglutination test. The systemic anaphylaxis initiated by intravenous injection of antigen may be similar to serum sickness and mediated by an antigen-antibody-complement system similar to that which occurs in guinea pigs (63). The inability to find anti-Toxocara homocytotropic antibody does not eliminate the possibility of self-cure occurring in the dog, as reported by Fernando (23). Self-cure in the dog may be mediated by another class of antibody, such as occurs in the rat (47).

SUMMARY

Mongrel dogs, naturally infected with Toxocara canis, were obtained from dog pounds to study the occurrence of immediate hypersensitivity in canine ascariasis. Serum was collected weekly for 10 weeks. For the first 3 weeks, each dog was given 1,500 embryonated eggs per os weekly.

Positive direct dermal and submucosal test results were seen when 0.1 ml. of crude Toxocara extract (60 mg./100 ml.) was injected into Toxocara-sensitized dogs. Histamine analyses of injected skin and intestine did not show a significant change in histamine content. A Schultz-Dale reaction could not be obtained on isolated strips of stomach, duodenum, jejunum, or ileum. Using serum from sensitized dogs, passive cutaneous anaphylactic trials were unsuccessful in ascarid-naive dogs.

The antigen used produced systemic anaphylaxis when injected intravenously into infected dogs. It also produced positive cutaneous anaphylaxis in rabbits and rats, indicating allergens were present in the antigenic extract.

Hemagglutination titers on ascarid-naive dog serums were insignificant. In contrast, serums from infected dogs had high hemagglutination titers, indicating that hemagglutinating antibodies were produced in dogs infected with T. canis.

Positive direct dermal test results and systemic shock were produced in infected dogs by the use of Toxocara

antigens. However, the inability to passively transfer serum components to homologous canine skin or to obtain a Schultz-Dale reaction indicated the antibody responsible for systemic shock was not homocytotropic antibody.

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ACKNOWLEDGMENTS

My grateful appreciation is hereby expressed to the members of my graduate committee--Dr. J. H. Greve, Dr. E. L. Jeska, Dr. F. K. Ramsey, and Dr. E. A. Hicks--for their assistance, advice, criticism, suggestions, support, and words of encouragement in the various aspects of this study.

A big "thank you" to my family--to my wife, Arline, for her typing and to my son, Arlie, for his devotion to his daddy throughout the course of my studies and research.