A STUDY OF THE TRANSMISSION OF CANINE LEISHMANIASIS BY THE TICK, *RHIPICEPHALUS SANGUINEUS* (LATREILLE), AND AN ULTRASTRUCTURAL COMPARISON OF THE PROMASTIGOTES

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CHAPTER I

INTRODUCTION

The genus *Leishmania* Ross was named in honor of William Leishman by Ross in 1903 (Hoare, 1972). Leishman discovered that the cause of kala-azar was a protozoan parasite (*L. donovani*) which he recovered from a spleen smear of a soldier that died from the disease (Faust et al., 1975). Since that time, forms of *Leishmania* have been found in the subtropical and tropical zones of the Old World. Wherever vectors for the organisms exist there is a potential for infections with these highly debilitating parasites. Even if victims of the disease live, they may be mildly to seriously disfigured. It is important to identify the presence of both the parasites and the vectors that transmit them in order to control spread of the disease. The present investigation dealt with leishmaniasis in dogs in Oklahoma and possible new vectors for the disease. The objectives of this investigation were: to investigate the potential of the tick, *Rhipicephalus sanguineus* Latreille, as a suitable vector for the disease and to further characterize the Oklahoma *Leishmania* isolate.

Review of Literature

Taxonomy of Leishmania

The group of organisms comprising the genus Leishmania was classified according to Hoare (1972) as follows:

Phylum PROTOZOA: unicellular, eucaryotic organisms.

Class ZOOMASTIGOPHOREA: chromatophores absent.

Order KINETOPLASTORIDA: kinetoplast and one to four flagella.

Family TRYPANOSOMATIDAE: leaf-like body in the trypomastigote stage.

Genus Leishmania: have amastigote and promastigote life cycle stages.

Levine (1973) stated, "All species of Leishmania look alike, although, there are size differences..." It is difficult to differentiate strains or species of these parasites on the basis of gross morphology. Several methods have been developed to identify leishmanial parasites. Biochemical and serological methods were used by Chance et al. (1978). With the use of various intrinsic factors they separated 68 isolates of Leishmania into 6 distinct groups (designated as groups 1-6). The intrinsic factors included chromatographic variants of the enzyme glucose-phosphate isomerase, nucleic acid and kinetoplast buoyant density and excretory factor serotyping. All of these techniques were too complicated for use in routine laboratory identification.

Amastigotes sizes as determined by light microscopy, overlap such that species cannot be differentiated by this characteristic alone. Ultrastructural characteristics have been studied for identification of leishmanial forms. Descriptions of amastigotes and promastigotes in which a transverse section through the nucleus were made proved to be somewhat reliable to differentiate between the Leishmania species (Hommel, 1978). Cell diameter, number of subpellicular microtubules and distance between the microtubules were the most reliable characteristics used to differentiate Leishmania sp. according to Garnham (1971a), Gardener et al. (1977), Croft and Molyneux (1979) and Kocan et al. (1983) used electron microscopy to differentiate various leishmanial forms and demonstrated that ultrastructure was only useful to distinguish between visceral and cutaneous forms.

Morphology

Leishmanial parasites occur in amastigote and promastigote forms. The amastigotes are intracellular and are usually found in phagocytic cells within the definitive host. They are small, ovoid or rounded and measure 2.5-5.0 μ by 1.5-2.0 μ. The nucleus and kinetoplast are visible by light microscopy in specimens prepared with Romanowsky stains (Hewitt, 1940). The basal body and kinetoplast, from which the flagellum arises can be observed using electron

microscopy (Levine, 1973). Promastigote forms are extracellular and are only seen in cultures and arthropod vectors. Promastigotes are elongate measuring 14-20 μ by 1.5-3.5 μ. The nucleus, kinetoplast and flagellum can be viewed by light microscopy in Romanowsky-stained preparations and ultrastructurally by electron microscopy.

Life Cycle

The life cycle of *Leishmania* is indirect involving vectors. In a vertebrate host, such as a dog, the amastigotes are found in macrophages and other phagocytic cells of the reticuloendothelial system. Infected phagocytes may be located in skin, spleen, liver, bone marrow, and lymph nodes, but rarely in peripheral blood leucocytes (Levine, 1973). Amastigotes divide within cells by binary fission (Levine, 1973; Olsen, 1974).

The primary invertebrate hosts for leishmanial parasites are sandflies of the genera *Phlebotomus* and *Lutzomyia*. Hommel (1978) described the life cycle as having six phases: (1) amastigotes are ingested by a susceptible sandfly; (2) the amastigotes transform into promastigotes in the stomach of the fly; (3) the promastigotes break through the peritrophic membrane and invade the midgut where they start dividing; (4) the newly divided promastigotes migrate towards a cuticle covered area of the gut where they anchor themselves; (5) from the anchored zone the parasites further

migrate towards the buccal cavity and the proboscis; and (6) the parasites infect a new host when the fly takes a blood meal or is crushed onto the skin of an appropiate host.

Other vectors which have been reported for leishmaniasis are: Culicoides (C. forcipomyia utae and C. f. towsendi) and Rhipicephalus sanguineus. Culicoides is a ceratopogonid midge which feeds on the blood of vertebrates. It is a proven vector of filarial worms, several protozoan parasites and certain viral diseases (Cheng, 1973). Hommel (1978) reported that Leishmania promastigotes could survive for 2-3 weeks in larvae of the bee-moth, Galleria mellonella. He also reported that Stomoxys, lice and ticks may become infected with Leishmania when feeding on infected hosts and in some instances serve as mechanical vectors. Rhipicephalus sanguineus, the brown dogtick, is a suitable vector of several protozoan and rickettsial diseases, the most prominent of which is Rocky Mountain Spotted Fever (Hardwood and James, 1979). Sherlock (1964) postulated that Rhipicephalus sanguineus may be a principal vector of leishmaniasis in dogs in Brazil. He observed that canine leishmaniasis occurred in areas where P. longipalpis was not found. Upon examining several specimens of R. sanguineus he further showed that some ticks contained leishmanial promastigotes.

Cultivation

Promastigotes of *Leishmania* can be cultured *in vitro* at 25 C using a variety of media (Steiger and Black, 1980). One such medium is NNN (Novy McNeal and Nicolle) medium (Novy and McNeal, 1903). This is a blood-based nutrient agar medium to which a saline overlay is added. Amastigotes can also be grown *in vitro* by continuous culture with phagocytic cell lines maintained at 37 C. (Berens and Marr, 1979).

Disease in Canidae

Leishmaniasis is commonly a disease of humans beings and dogs (Hardwood and James, 1979), but it has also been found in foxes, wolves, sloths, rodents, raccoons, and armadillos (Zuckerman and Lainson, 1977), although many of the latter hosts probably serve as reservoirs of the infection.

There are three forms of leishmaniasis observed in humans and they are commonly classified as visceral, cutaneous or mucocutaneous disease (Garham, 1971). Visceral leishmaniasis caused by *Leishmania donovani* is characterized by finding organisms in phagocytes in almost all body organs. In cutaneous and mucocutaneous leishmaniasis the organisms localize in tissue macrophages and erode skin or cartilage but do not normally visceralize. In the Mediterranean area and in the orient, India and

Africa, the cutaneous form of the disease is caused by *L. tropica*. In the Americas the causative agents for cutaneous and mucocutaneous leishmaniasis are *L. mexicana* and *L. brasiliensis*, respectively (Faust, 1975; Garnham, 1971; and Marsden, 1979). Other species of *Leishmania* have also been associated with visceral and cutaneous leishmaniasis.

The clinical signs observed in canine leishmaniasis include generalized muscle atrophy, scabs, ulcerations, ocular lesions and long toe nails (McConnell et al., 1970; Anderson et al., 1980). Garett (1978) stated that dogs with visceral leishmaniasis most often appear normal.

Pathological signs in dogs include enlargement of the spleen, liver and lymph nodes, and anemia, dermal nodules (depending on invading species), and bleeding from the mucosa of the gums, nose, lips, and intestine. Preliminary diagnoses can be made from serology or gross pathological findings but must be confirmed by demonstration of the parasites in the tissues by histological or by culture methods.

Rhipicephalus Sanguineus Latreille

Ticks of the genus *Rhipicephalus* are cosmopolitan in their distribution and infest several species of mammals. *Rhipicephalus sanguineus* is known as the brown dogtick and the dog is its principal host; however, it is occasionally found on other mammals including man, cats, cattle, and

sheep (Harwood and James, 1979). Brown dogticks are common in Oklahoma, and as adults, they perfer to attach in the ears and between the toes of dogs (Hair et al., O.S.U. extension publication No. 7001).

Taxonomy

The following classification of Rhipicephalus sanguineus (Latreille) (Bequaert, 1945) was reported:

Phylum ARTHROPODA: Characterized by segmented body, jointed appendages and an exoskeleton of chitin.

Class ARACHNIDA: Abdomen lacking, adults have four pair of legs, nymphs and larvae have three pair of legs.

Order ACARINA: Head, thorax and abdomen are fused.

Family IXODIDAE: Scutum covers dorsal surface in male and anterior portion only in the female. Capitulum placed anterior and one or more coxae with spurs projects anteriorly and is visible from above.

Genus Rhipicephalus: Hind margin of shield divided into festoons, post-anal groove distinct.

Ticks as Disease Vectors

In general ticks are efficient vectors of disease because they are persistent, slow-feeding blood suckers which provides a long period for them to either ingest or transmit a pathogen. During the course of their life they

usually feed on multiple hosts which enhances their chances of ingesting a pathogen. In addition, some organisms may undergo transovarial transmission in ixodid ticks insuring that parasites are passed to the next generation; thus larval, nymphal and adult stages may be infected giving the parasite great potential to reach a susceptible host. The latter characteristics plus resistance to adverse environmental conditions and high reproductive potentials confirms their importance as potential disease transmitters (Harwood and James, 1979; and Balashov, 1968).

Life Cycle

The typical life cycle of *R. sanguineus* involves three hosts. The larvae hatch from an egg and attach to small ground animals or dogs. After feeding the larvae drop to the ground and molt into nymphs. The nymphs attach to the skin in the region of the neck and are hidden by the long neck hairs of the host. The dog is the principal host for both the nymphal and adult stages. After engorgment the nymphs again leave the host animal and molt to the adult stage. Adult male and female ticks then attach to a dog and mate while feeding. Once the females copulate and are replete with blood, they drop from the dog to the ground. The engorged *Rhipicephalus* females crawl upward and deposit eggs in the cracks of kennel roofs or other concealed locations. After the female has laid her eggs she dies;

males normally live several more weeks than the females. In 20 to 30 days the eggs embryonate and hatch into larvae. Detailed Rhipicephalus life cycles were reported by Balashov (1968) and Harwood and James (1979).

Several patterns of pathogen transmission occur when a tick takes a blood meal. Microrganisms can be passed to the tissue of the vertebrate host by way of the tick mouth-parts during feeding activities, through contamination with tick feces or by ingestion of the tick by the host (Cheng, 1973).

Tick-Transmitted Diseases

As discussed previously, ticks have been shown to be important vectors of disease. According to Balashov (1968), they are able to transmit viruses, rickettsiae, bacteria, spirochetes, and piroplasms. Other findings indicate that ticks transmit a filarid helminth in reptiles (Cheng, 1973). Balashov (1968) reported that 13 species of fungi (Aspergillus and Penicillium) have been isolated from ticks. He pointed out that fungal transmission was strictly mechanical. Protozoa of the family Trypanosomatidae have also been reported to be transmitted by ticks. Balashov (1968) indicated that Crithidia hyalommae has been found in the hemolymph and eggs of both Hyalomma sp. and Boophilus calcaratus, and earlier Cross (1923) showed that Trypanosoma evansi could be transmitted by Ornithodoros crossi. Krinsky

and Burgdorfer (1976) reported that a trypanosome, presumablly of white-tailed deer, was found in hemolymph of a dissected tick. This trypanosome appeared to be similar to T. theileri of ruminants. Burgdorfer et al. (1973) described developmental stages of trypanosomes from cattle in Rhipicephalus pulchellus and Boophilus decoloratus in Ethiopia. They reported that the trypanosomes, thought to be T. theileri, were seen in all tick tissues examined. Sherlock (1964) reported the possiblity of tick-transmission of Leishmania sp. in Brazil. He found that Rhipicephalus sanguineus which had been removed from dogs with leishmaniasis contained large numbers of promastigotes.

The Research Problem

Clinical leishmaniasis was recently described in a hunting dog from a private kennel in Oklahoma (Anderson et al., 1980). Preliminary investigations (MacVean et al., 1979) at the Oklahoma locality resulted in isolation of leishmanial organisms from three dogs and serologic evidence of possible infections in cats and several wild animals trapped in the vicinity. Several horses on the premises also had high serum titers; the latter samples were examined by the Centers of Disease Control (CDC) in Atlanta, Georgia, using a direct agglutination.

The lack of travel by these dogs to regions where leishmaniasis has been found or of contact with known

infected animals suggested that the disease was endemic in central Oklahoma. Seroconversions in and *Leishmania* isolations from several dogs between 1978 and 1983 provided further evidence of endemicity in Oklahoma (Fox, unpublished data). The Oklahoma cases were the first reports of locally-acquired leishmaniasis in dogs in the United States (Anderson et al., 1980).

A dog brought to Oklahoma from a kennel in Kansas in May, 1981, was found to be infected with *Leishmania* only one week after arrival. Having no history of previous travel to Oklahoma, this dog provided evidence for the presence of a second endemic focus of canine leishmaniasis in North America. It is probable that the Oklahoma and Kansas foci may have a common origin (Fox, unpublished data).

Because dogs in Oklahoma have continued to become infected with the parasite and develop clinical leishmaniasis, *Leishmania*-infected animals have been isolated and euthanitized to prevent further transmission of the disease. Continual monitoring and removal of infected dogs from the kennel has not curtailed spread of the disease. Transmission from the reservior hosts by some vectors other than sandflies must be considered because of a lack of the normal vectors. Therefore, the possibility of transmission by ticks, specifically *Rhipicephalus sanguineus*, was studied. The objectives for the investigation were as follows:

To demonstrate survival of *Leishmania* in experimentally infected ticks.

To demonstrate transtadial carry-over of *Leishmania* from the nymph to the adult stage of the tick.

To determine whether ticks could serve as either biological or mechanisal vectors of the Oklahoma dog (OKD) and Kansas dog (KSD) isolates of *Leishmania* in dogs.

To compare the ultrastructure of culture forms of *Leishmania* isolated from dogs and ticks with other species of *Leishmania*.

CHAPTER II

MATERIALS AND METHODS

Infections in Donor Dogs

Two foxhounds with naturally acquired leishmanial infections were used as source animals. Both donor dogs were obtained from a kennel in Central Oklahoma; one was born and raised at the kennel, whereas the other came to Oklahoma from a kennel in Kansas. The disease was diagnosed in the latter animal within 2 weeks after arrival in Oklahoma. Both animals were maintained in isolation facilities at the Oklahoma State University College of Veterinary Medicine from the time they were identified until the time they died (2-3 years).

Maintenance of Experimental Dogs

Thirteen puppies from two different litters (German Shepherd-Labrador Retriever crosses) were purchased at 4-6 weeks of age from an Oklahoma City breeder for tick transmission studies.

Four dogs were selected at random as control animals and were taken to the Boren Veterinary Medicine Teaching Hospital to be housed in a special research area. The

animals were fed 2 cups of dry dog food twice each day and were allowed to play together for 5 hours during the day. Females were spayed at maturity to prevent mating. At night each animal was placed in a stainless steel cage lined with absorbent paper.

The principal animals were housed in an isolated loft in a separate building. These dogs had automatic feeder and food was available *ad libitum*. The animals were allowed to run within the room for one hour each morning and night and during examination periods. They were kept in 4' by 6' pens with wood shavings as bedding. Animal care personnel cleaned all animal pens twice daily; shavings were replaced once each morning.

Upon arrival at the laboratory all 13 pups were treated with pyrantel pamoate (1cc/20 lb) to elimate helminths, and vaccinated with rabies vaccine (Norden Laboratories, Lincoln, NB) administered in the hamstring muscle of the back leg and with Norden's distemper, hepatitis, leptospirosis, parainfluenza and parvo vaccine (DHLPP) given SC over the loin (1 dose/dog). In addition, the pups were tested serologically for antibodies to *Leishmania* sp. and *Dirofilaria immitus*. Hematologic examinations (total and differential blood counts) and blood chemistry examinations were done as described later.

Initial hematological examinations were followed with periodic exams designed to detect any changes that developed

during the course of the experiment. Rectal temperatures, heartrates (pulse) and respiration rates were monitored daily along with general observations of each dog's condition and attitude. The guidelines followed in evaluating general animal health and for recording observations were similar to those of Blood and Henderson (1979).

Diagnostic Techniques

Monitoring Vital Signs

All experimental dogs were monitored daily for rectal temperature, heartrate and respiration rate. Rectal temperatures were taken by inserting a thermometer into the rectum for 2 minutes. Respiration rates and heartrates were obtained with the aid of a stethoscope. The number of breaths and heartbeats were counted for 10 seconds and multiplied by 6 for total per minute.

Serology

Serology was used to monitor antibody responses in the pups. The FIAX[R] Immunoassay is a fluorescent immunoassay system that is capable of detecting and quantitating minute amounts of flouresence.

Equipment and Supplies. The FIAX[R] System (International Diagnostic Technology, Santa Clara, CA) included a fluorometer, microdiluter, shaker and microcomputer. Other

secondary materials needed were serum tubes, a hand pipettor, StiQ[R] Samplers (IDT, Santa Clara, CA), a clip to move the StiQs from one solution to another and a timer. Antibody responses were measured through detection of antibody which reacted with antigens adsorped to the surface of the StiQ sampler.

Antigen preparation. Leishmanial promastigotes isolated from the Oklahoma dog (OKD) #11 were inoculated onto 25 cm culture flasks of MaeKelt's blood agar medium with a saline overlay and allowed to grow for one week at 25 C. The cultures were then transferred to a 75 cm culture flask containing MaeKelt's medium without blood; one large flask was inoculated with the overlay from two small flasks. The large flasks were then allowed to incubate at 25 C for two weeks to obtain maximum growth. After incubation, the overlay containing the organisms was poured into 50 ml centrifuge tubes and centrifuged at 10,000 r.p.m. in a Sorval RC2B Centrifuge (Dupont, Newtown, CN) for 20 minutes. The supernatant was then decanted and the parasites were washed three times in sterile saline with centrifugation. After the final washing, the supernatant was again removed, and the packed cells were measured and resuspended to 10 times in volume with a 0.1% solution of sodium azide in distilled water. The preparation was then freeze-thawed three times and homogenized for 3 minutes using a Brinkman Polytron (Brinkman Instruments, Newbury,

NY) at the maximum setting (14,000 r.p.m.). The antigen was titrated in 2-fold dilutions against control antisera to determine the optimum test concentration. The titrated antigen was kept frozen at -20 C until needed.

<u>FIAX</u> <u>test.</u> Sera collected from experimental animals were tested using FIAX serology. Antigen (25 µl at optimum concentration) was applied to the surface of the StiQ samplers and allowed to air dry for 3.0 hours or overnight. StiQs with antigen were incubated for 30 minutes at 25 C in 1.0 ml of pre-diluted serum (1:100 in phosphated buffered saline at pH 7.3 containing 0.15% TWEEN 20). The StiQs were then washed for 10 minutes in 0.6 ml of Tween 20-buffer and transferred for 20 minutes to 0.5 ml of a 1:200 dilution of fluorescein-conjugated, rabbit anti-dog IgG serum (Cappel Laboratories) diluted in Tween 20-buffer. After a final 10 minute wash in Tween 20-buffer, the StiQs were inserted into the FIAX 100 Fluorometer to determine the fluorescent signal units (FSUs) from bound conjugate. Control sera from naturally infected dogs were used to obtain FSU measurements to be used as control FIAX values. Standard curves were calculated after each test by plotting FSU values of control sera against the assigned FIAX values. Values for unknown samples were then extrapolated from the standard curve using regression analysis. The FIAX values for serum samples were then used as a measurement of antibody levels.

Culture Methods

Culture methods for trypanosomes have been described by Levine (1973), Hoare (1972), and Novy and McNeal (1903). Many are used successfully for diagnostic purposes.

Media preparation. Tissue culture flasks with MaeKelt's enriched blood agar, Novy, MacNeal, Nicolles' blood agar (NNN), and Schneider's Drosophila media were used for culturing the isolates of *Leishmania* sp. and *Trypanosoma cruzi*. A 2.0 ml saline overlay containing 0.5% peptone and 0.4 mg of vitamin B-12 was placed over the agar. Also, 0.2 ml of a solution containing 10,000 units of penicillin and 25 mg streptomycin per ml was added to the overlay to inhibit bacterial growth, and 0.5 ml of sterile fetal calf serum was added to enhance protozoal growth.

Culture maintenance. Additional Schneider's medium was periodically added to the overlay solution as a source of fresh nutrients in order to keep the parasites in an exponential growth phase. Culture media were inoculated within a laminar flow hood to prevent contamination.

Biopsies and Impression Smears

Samples of bone marrow, lymph node and skin were obtained from experimental animals and examined for the presence of parasites.

Bone marrow aspirates. Dogs were anesthetized by administering sodium pentabaritol IV (10mg/lb). A sterile

bone marrow punch (16 gauge) was inserted into the trochanteric fossa of the femur. Approximately 10 ml (amount varied according to needs) of bone marrow was withdrawn using a 12 ml sterile syringe containing 1.0 ml of 12.0% sodium citrate in saline. Bone marrow (approximately 2.0 ml) was then inoculated into flasks containing NNN, MaeKelt's, and Schneider's media. The cultures were examined daily for 20 days using an inverted microscope to search for swimming or moving parasites.

Lymph node aspirates. A 1.0 ml tuberculin syringe equipped with a 25 gauge needle was filled with 0.5 ml of sterile saline. Holding the dog in a standing position, the popliteal lymph node was palpated and held in place with the thumb and forefinger. After the needle was inserted into the lymph node, 0.1 to 0.2 ml of saline was injected while moving the needle from side to side to disrupt the tissue. The saline with tissue and lymph was drawn back into the syringe and later inoculated onto culture media as described above.

Skin biopsies. To obtain a skin biopsy, the animal was anesthetized with sodium phenobaritol (10.0 mg/lb) injected intravenously. A 2.5 cm ellipical skin sample was removed from the dorsal, cervical region of the neck or from a place where ticks had been feeding. The wounds were then sutured and observed until healed. The sutures were removed 10 days later and no antibiotics were given. One portion of

each skin sample was fixed in 10.0% formalin for histophathologic examinations, and a second was cut into 0.5 mm pieces and placed in cold 2.0% glutaraldehyde for electron microscopy.

Smears and Stains. Tissue impression smears were made by first cutting the tissue with a sharp razor blade to produce a fresh cut surface. The cut surface was then blotted on a clean paper towel and then on a clean microsçope slide. The impression smear was allowed to air dry. Smears were stained with Diff-Quik Stain (American Scientific Products, Grand Prairie, TX) by leaving the slide in each of the 3 staining solutions for 30 seconds each followed by a gentle rinse with tap water. After the slides dried they were viewed by oil immersion microscopy without coverslips.

Bone marrow impression smears were made by depositing blood containing flecks of bone marrow onto a clean slide. A cover slip was then added and pressure applied with the thumb to flatten the flecks. The clover slip was then pulled across the slide to produce the smear. Once the smears were dry they were stained with a Diff-Quik stain.

Histopathological Examinations

Histopathological examinations were done by staff in the Veterinary Pathology department at Oklahoma State University. All tissues were cut into 25 cm squares, fixed

in 10.0% formalin, thin sectioned and stained with hemotoxylin-azureosin (H&E) stain.

Several ticks which had fed on the naturally infected dogs until replete were incubated at 25 C for 5 days, fixed in 10.0% formalin and examined histopathologically. The exposed ticks were serially sectioned with a microtome and every 20th section placed on a slide and stained with H and E. The sections were then examined microscopically to locate the position of leishmanial organisms within the tick.

Hematology and Blood Chemistry

Hematology and blood chemistry examinations were used to monitor infections in the dogs. Blood was collected by syringe and placed in serum tubes with and without EDTA. All dogs were tested on weeks -3, -2, -1, on the day of exposure, and on weeks 1 and 2 post-exposure. Additional samples were collected on all dogs the day of splenectomy and weekly for the following 2 weeks. Blood samples were collected on the week before corticsteroid treatment began and weekly for 3 weeks thereafter.

Blood counts. Blood counts were made by the Clinical Pathology staff of the Department of Veterinary Pathology. Complete leukocyte counts were done on the Beckman Cell Counter and differential cell counts were made on blood smears stained with Diff-Quik.

Blood chemistry. Blood examinations were also done by the Clinical Pathology staff. Samples were analyzed with a Beckman Chem-20 Analyzer. The following chemistry values were obtained as outlined by Schalm (1965): potassium, sodium, glucose, urea nitrogen (BUN), glutamic pyruivic transaminase (SGPT) and alkaline phosphatse (SAP).

Xenodiagnosis Using Ticks

Dogs were placed into stainless steel isolation cages with tick barriers made with masking tape. _Rhipicephalus sanguineus_ nymphs (250) and adults (250) were placed on each dog. The ticks that attached were allowed to feed to repletion, after which they were removed and placed in cardboard specimen boxes covered with plastic wrap. Guts were dissected out of the ticks into 1.0 ml of sterile M-199 tissue culture medium (GIBCO, Grand Island, NY), teased apart and subsequently inoculated onto NNN, MaeKelt's, and Schneider's media with saline-peptone overlays as previously described. Once the ticks were removed, the dogs were dipped in a Paramite (VetKem, Oklahoma City, OK) solution (8.0 ml/liter of water) until soaked in order to kill any remaining ticks. The tick-gut cultures were then examined on a daily basis using an inverted microscope to detect the presence of swimming or moving parasites.

Hamster Inoculations

Golden hamsters were inoculated with bone marrow aspirates and spleen homogenates as a means of detecting parasites in experimental dogs and as an alternate method for propogation of leishmanial isolates in the laboratory. All aspirates and homogenates were injected intraparitoneally (IP) and usually consisted of 1.0 to 3.0 ml of inoculum. Hamsters were killed after 4 to 6 months. Spleen impression smears and bone marrow smears were stained with Diff-Quik and examined by light microscopy for the presence of amastigotes in macrophages. If parasites were found on direct microscopic examination, spleen homogenate was either transferred to culture media or subinoculated into another set of hamsters.

Necropsies

At the completion of the study, blood samples were drawn with a 21 guage needle and a 10 ml syringe to be used for blood chemistry and serology. All dogs were then euthanitized by IV injection of a lethal dose of sodium pentabaritol (25 mg/lb). Samples of bone marrow, liver, lung, skin, lymph nodes, and small intestine were removed and fixed in 10.0% formalin for histopathologic examination. Impression smears and cultures were made from the bone marrow and lymph nodes. Bone marrow was removed by fracturing the femur and scraping the bone marrow into

sterile saline. Hamsters and culture media were inoculated with the bone marrow mixture. The general conditions of the dogs were recorded.

Electron Microscopy

Electron microscopy was used in an attempt to compare the leishmanial promastigotes isolated in cultures from ticks, experimental dogs, and naturally infected dogs. Epimastigotes of *Trypanosoma cruzi* were included for comparative purposes because this parasite may be confused with *Leishmania* sp. when found in tissues.

Promastigote cultures. Promastigotes were inoculated onto 25 cm flasks of MaeKelts's medium with rabbit blood. After 5 days, cultured organisms were transferred to a 75 cm flask of MaeKelt's medium without blood and allowed to grow for 5 additional days. When abundant single organisms were present without clumps they were transferred to a sterile centrifuge tube and washed three times with sterile saline.

Transmission electron microscopy. After the last washing, the parasites were fixed in cold 2.0% glutaraldehyde in 0.27 M sodium cacodylate buffer (SCB), fixed in 2.0% osmium tetroxide in 0.27 M SCB and processed for electron microscopy according to the precedures of Kocan et al. (1978).

Ultrathin, silver-reflective, sections (500 Å) were cut with an ultramicrotome (Porter-Blum MT-2) and a glass

knife. The sections were collected on 300-mesh copper grids, stained with uranyl acetate and lead citrate (Venable and Coggeshal, 1965), and observed and photographed with a Philips 200R Electron Microscope.

Approximately 20 electron micrographs were taken of each leishmanial isolate and of T. cruzi. Organisms to be photographed were selected on the basis that cell cross-sections contained a portion of the nucleus. This was done in order to standardize comparison of the various species examined.

Scanning electron microscopy. Polylysine, a molecular glue, was placed on a 0.5 cm coverslip. One drop of washed prostigote suspension was added to a coverslip for each isolate and allowed to stand for 20 minutes. After the cells had adhered to the coverslip they were placed into an 8.0% glutaraldehyde solution for 15 minutes to cause the polylysine to bind with the parasites and permanently fix them to the slide. The samples were then dehydrated in 20 minute steps through 50, 70, 90, 95, and 100% ethyl alcohol (EtOH). The specimens were then critical point dried, outgassed, coated with gold and palladium and examined with a JEOL JSM 35-U scanning electron microscope.

Special Treatments

Post-Exposure Splenectomy

Hudson (1973) stated that animals which were splenectomized prior to or following exposure to protozoan parasites were more apt to succumb to the infection. In an attempt to stress the exposed pups with the aim of allowing the parasites to multiply and become more apparent, all experimental pups (11 in total) were splenectomized 7 months following exposure. Blood samples were drawn and acepromizine (3.0 mg/dog) administered IM as a pre-surgery anesthestic. The cephalic vein at the foreleg of each pup was then catheterized with indwelling catheters to facilitate the administering of pentabaritol (10.0 mg/kg).

The abdomen of each dog was shaved and then scrubbed three times with Betadine . Each dog was then moved from the preparation area to a surgery area where the dogs were again scrubbed with Betadine solution followed by the application of 70% EtOH which was allowed to dry. The dogs were incubated and given further anesthesia using halothane gas. Each dog was draped with a sterile plastic drape and surgically incised along the ventral midline. The spleen was removed and placed into a sterile plastic bag for subsequent examinations. The dogs were then sutured and given 3.5 mls of penicillin (300,000 units/ml) injected SC. The dogs were maintained in a recovery area until they

regained consciousness. Additional penicillin injections (2.5 ml) were administered under the skin over the lumbar region morning and night for five days following sugery. The sutures were removed 10 days after the surgery.

Once the spleen was removed, it was aseptically cut into pieces. One portion of each spleen was fixed in 2.0% glutaraldehyde in 0.027 M sodium cacodylate buffer in preparation for subsequent electron microscopy, and another sample was fixed in 10.0% formalin for histological examinations. Spleen impression smears were made and examined as previously described.

A separated portion of the spleen was placed in a sterile plastic bag with 2.0 ml of sterile saline and homogenized in a Stomacher Homogenizer (Tekmar, Cincinnati, OH) for 30 seconds. The spleen homogenate was then used to inoculate NNN, MaeKelt's, and Schneider's media (0.2 ml/flask). Two hamsters were inoculated IP with 2.5 ml of spleen homogenate each dog. The cultures and hamsters were subsequently examined as previously described.

Treatments with Corticosteroids

Prednisolone (9-fluoro-prednisolone; Upjohn) was administered in an attempt to induce parasite growth. The prednisolone was administered in the hamstring muscles of the back legs at a dosage of 2 mg/dog each day for the first week, alternate days for the second week, and daily

thereafter until the experiment was terminated. Injection sites and routes were alternated (IM and SC) to reduce trauma at injection sites.

Infections in Ticks

Laboratory Maintenance of Ticks

Ticks were propagated and maintained at the Oklahoma Agricultural Experiment Station, Entomology Tick Laboratory at Oklahoma State University according to the methods of Patrick and Hair (1975). Larval *Rhipicephalus sanguineus* were fed on rabbits and allowed to molt to the nymphal stage.

Exposure of Ticks to Leishmania

The nymphal ticks were infected by feeding them on *Leishmania*-infected dogs until they fully engorged and detached. The ticks were then maintained in a humidity chamber at 25 C in 90-98% relative humidity and with a 14-hour photophase until one month post-molting.

Analysis of Tick Infections

Gut tissues were cultured from five different ticks each week using NNN, MaeKelt's, or Schneider's Drosophilia media to determine if they contained flagellates. One month after development to the adult stage, representative ticks were prepared for examination by electron microscopy in

order to confirm the presence of promastigotes.

The ticks to be cultured were dipped in 70% EtOH and the alcohol was allowed to evaporate for one minute. The ticks were then placed onto a piece of paraffin that had 2 ml of M-199 tissue culture medium on its surface. The anterior end of the tick was severed using a sharp razor blade and the guts were expressed, quickly picked up with sterile forceps and placed into the overlay solution in the culture vesels. The cultures were incubated at 23-25 C and examined for promastigotes using an inverted microscope. Once promastigotes were detected, identification was confirmed by examination of Giemsa-stained smears by light microscopy (Hewitt, 1940).

Experimental Transmission of *Leishmania* by Ticks

The experiments were divided into five general categories as follows: 1) transfer of infected bone marrow; 2) mechanical transmission by interrupted tick feeding; 3) biological transmission through infected ticks; 4) inoculations with incubated infected tick guts; and 5) inoculations with unincubated infected tick guts (Fig. 1). General information about each experimental group can be seen in Table 1. There were three dogs in each of the tick transmission categories and only one received a direct bone marrow transfer. Dogs 0464 and 0444 served as uninfected

Figure 1. Experimental design for a *Leishmania* transmission study in dogs using *Rhipicephalus sanguineus*.

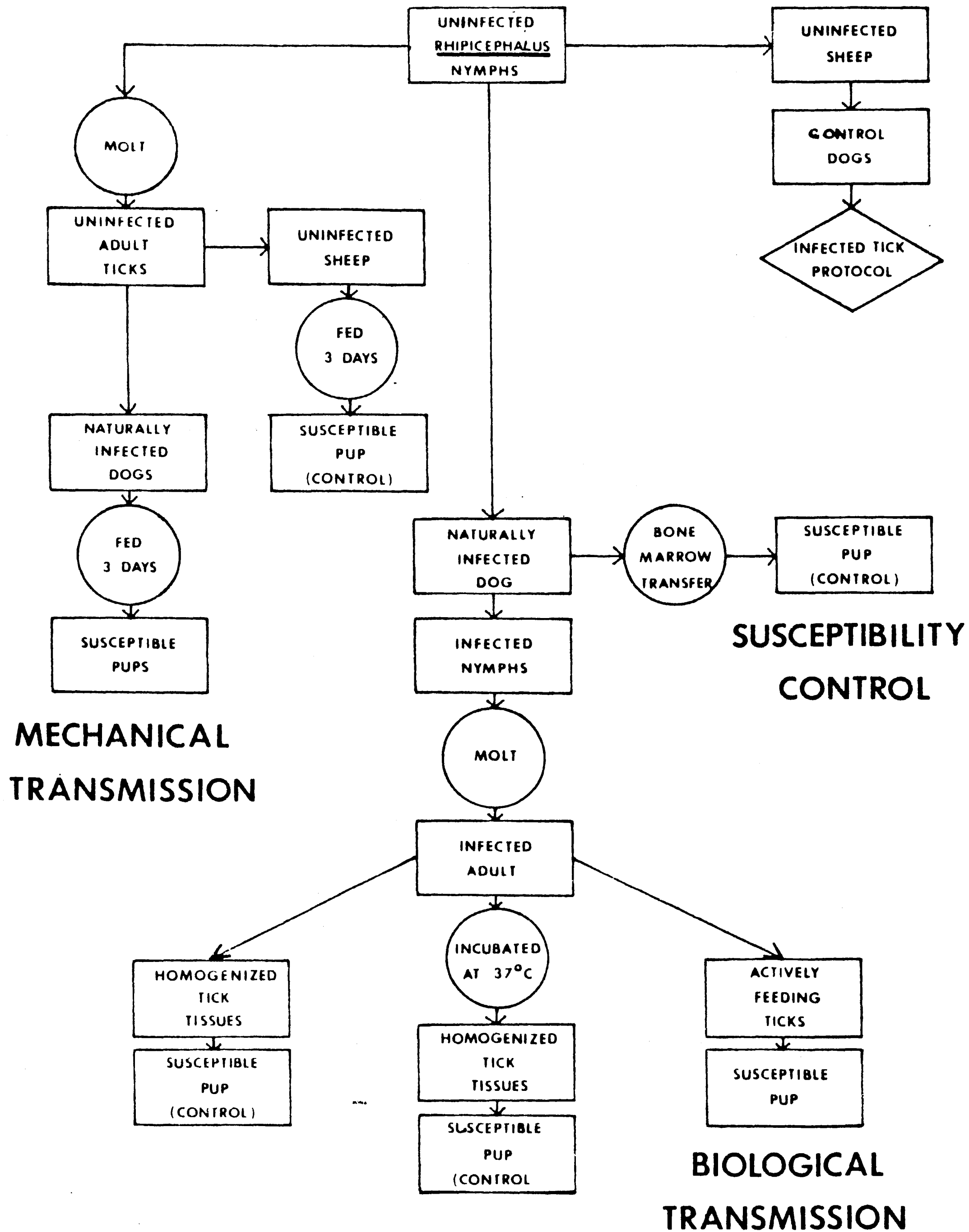

EXPERIMENTAL DESIGN
UNINFECTED RHIPICEPHALUS NYMPHS
UNINFECTED SHEEP
CONTROL DOGS
INFECTED TICK PROTOCOL
MOLT
UNINFECTED ADULT TICKS
UNINFECTED SHEEP
FED 3 DAYS
SUSCEPTIBLE PUP (CONTROL)
NATURALLY INFECTED DOGS
FED 3 DAYS
SUSCEPTIBLE PUPS
MECHANICAL TRANSMISSION
NATURALLY INFECTED DOG
BONE MARROW TRANSFER
SUSCEPTIBLE PUP (CONTROL)
SUSCEPTIBILITY CONTROL
INFECTED NYMPHS
MOLT
INFECTED ADULT
HOMOGENIZED TICK TISSUES
SUSCEPTIBLE PUP (CONTROL)
INCUBATED AT 37°C
HOMOGENIZED TICK TISSUES
SUSCEPTIBLE PUP (CONTROL
ACTIVELY FEEDING TICKS
SUSCEPTIBLE PUP
BIOLOGICAL TRANSMISSION

TABLE I

PROTOCOL FOR A TICK TRANSMISSION STUDY OF LEISHMANIASIS IN DOGS USING INFECTED *RHIPICEPHALUS SANGUINEUS* AND PARENTERAL INOCULATIONS OF HOMOGENIZED INFECTED TICK GUTS

Group	Dog No.	Sex	Source	No. of Ticks/ Inoculation	Time Infected (Days)
Biological Transmission	0438	F	OKD	50 ticks	511
	0447	M	KSD	50 ticks	511
	0464	M	Control	50 ticks	365
Mechanical Transmission	0440	F	OKD	50 ticks	511
	0441	M	KSD	50 ticks	0
	0444	M	Control	50 ticks	511
Injection Incubated Tick Gut	0439	F	OKD	6 ml	511
	0446	M	KSD	6 ml	365
	0465	F	Control	6 ml	365
Injection Unincubated Tick Gut	0443	F	OKD	6 ml	511
	0445	M	KSD	6 ml	-
	0467	F	Control	6 ml	511
Direct Bone Marrow (KSD) Transfer	0466	M	KSD	1.5 ml	365

controls after uninfected ticks were allowed to feed on them.

Bone Marrow Transplants

Figure 1 contains a diagram showing the experimental design for experiments to test the ability of ticks to transmit leishmaniasis in dogs. Two dogs were used as infected bone marrow controls (susceptibility controls). In order to obtain bone marrow infected with *Leishmania* for transplantation, the KSD donor dog was anestheized with pentabaritol. A sterile bone marrow punch was inserted into the trochanteric fossa of the femur and 10 ml of infected bone marrow was withdrawn and examined for the presence of parasites. An uninfected pup was anestheized and prepared in the same manner, and 5 ml of blood and bone marrow were removed. Infected bone marrow (1.5 ml) from the infected dog (KSD) was transferred to the control pup 0466 (Table 1) in an attempt to produce a control infection. Some of the remaining bone marrow from the infected dog was cultured on NNN media along with bone marrow from the recipient pup to determine whether parasites were present. In addition, bone marrow smears were made for both dogs and examined for the presence of paraistes. Both dogs were monitored for changes in blood parameters and vital signs, and serum antibody titers to *Leishmania* were monitored weekly.

Inoculations with Tick-Gut Homogenates

Rhipicephalus ticks were used for transmission studies one month after molting to the adult stage. Ticks were exposed to parasites as nymphs by allowing them to feed on dogs that had natural infections with *Leishmania*. Uninfected control ticks were fed as nymphs on an uninfected rabbit to induce molting to the adult stage. Four types of tick-gut homogenates were prepared for dog inoculations. One inoculum was prepared from uninfected ticks and another from infected ticks which were incubated at 37 C for 2.5 days prior to removal of the guts. Two other inocula were prepared similarly from ticks which were kept at 25 C. The inocula were prepared by removing gut tissues from 50 ticks of each type (described above) and placing them into a clean tissue grinder with 3.0 ml of RPMI 1640 medium as described by Kocan et al. (1982). The tissue was ground for 3 minutes, and the total volume was adjusted to 6.0 ml. The homogenate was then placed in syringes and inoculated into dogs. Five inoculation routes and dosages were used as follows: 1.0 ml intravenously (IV) in foreleg vein, 2.0 ml intradermally (ID) at four different locations in the abdominal skin near the genitalia, 1.0 ml subcutaneously (SC) on the non-hairy part of the abdomen, 1.0 ml intrafemorally (IF) into bone marrow, and 1.0 ml intraperitoneally (IP). Pre- and post-inoculation blood samples were obtained from all dogs at one-week intervals

for the duration of the experiment. Similar preparation of unfed unincubated ticks were inoculated in similar manner into control dogs. Dogs were monitored by serology, lymph node aspirate cultures, bone marrow cultures, and spleen biopsy for evidence of infections.

Ultrastructural Study of Culture Forms

Special Studies

Several different isolates of *Leishmania* spp. and *Trypansoma cruzi* were maintained in the laboratory. The different leishmanial strains were: *Leishmania tropica* (LT), *L. donovani* (LD), *L. mexicana* (Texas; LMT), *L. mexicana* (British Honduras; LMB), *L. brasiliensis* (LB), Oklahoma isolate 1 (OKD-11), Oklahoma isolate 2 (OKD-5), Kansas isoalte 1 (KSD-1), experimental tick isolate (PK), experimental dog bone marrow isolates (38B and 66B), experimental tick isolates (38T and 66T), and naturally infected Wakefield tick isolate (WK). All the leishmanial isolates and *Trypanosoma cruzi* (TC) were cultured in small flasks of MaeKelt's blood agar medium as previously described.

Comparison of Forms

Transmission electron microscopy. Leishmanial isolates of LT, LD, OKD, KSD, PK, 38B, 38T, 66B, 66T, and TC were

grown on culture media and fixed in glutaraldehyde and osmium tetroxide as previously described.

The microtubules found beneath the plasma membrane of the cell were counted for all isolates. The range, mean, standard deviation, variance, and standard error of the mean for microtubule counts were calculated and depicted in Dice-Lerass diagrams for comparison.

Gut tissues from infected nymphal and adult ticks were fixed and processed for subsequent electron microscopy to study promastigote development in the tick.

Scanning electron microscopy. *Leishmania donovani*, OKD:CH, and *Trypanosoma cruzi* were prepared for SEM as previously described and were viewed on the scanning electron microscope.

CHAPTER III

RESULTS

Survival of *Leishmania* in Ticks

Laboratory-reared nymphal ticks were were fed on naturally infected dogs and examined for *Leishmania* for a 220 day period to measure the survival potential of *Leishmania* in ticks.

Cultures

Table 2 shows the results of the tick-gut cultures. Ticks that were fed on dog OKD were culture-positive by day 3 and remained positive through day 95. Nymphal ticks fed on KSD were also positive by day 3 but leishmanial parasites were detected through day 160 post-feeding. The infected ticks carried the parasites through a molt from nymphal to the adult stages; the molting occurred between days 24 and 35. After the molt it was not possible to culture the parasites from ticks by serial passages. On day 50, one tick from the control group was found positive.

Histopathology

Ticks that were fed on a dog (OKD) were fixed in 10.0%

TABLE II

LEISHMANIAL CULTURES OF GUTS FROM *RHIPICEPHALUS SANGUINEUS* NYMPHS AND ADULTS FED UPON *LEISHMANIA* INFECTED DOG

Stage	Day Post-feeding	Culture Results (5 ticks)		
		Low (OKD[1]) Dog	High (KSD[2]) Dog	Rabbit[3]
Nymphs	3	+	+	–
	4	+	+	–
	5	+	+	–
	6	+	+	–
	7	+	+	–
	14	+	+	–
	20	+	+	–
	24	+	+	–
Adults	35	+	+	–
	40	+	+	–
	45	ND[4]	+	–
	50	+	+	+
	55	+	+	–
	60	–	+	–
	65	+	+	–
	70	–	+	–
	75	–	+	–
	80	+	–	–
	85	+	+	–
	90	+	–	–
	95	+	+	–
	100	–	+	–
	105	–	+	–
	120	–	–	–
	135	–	+	–
	145	–	+	–
	160	ND	+	–
	175	ND	–	–
	190	ND	–	–
	205	ND	–	–
	220	ND	–	–

[1] Naturally infected dog from central Oklahoma.

[2] Naturally infected dog from eastern Kansas.

[3] Uninfected control rabbit.

[4] Not done.

formalin and serially sectioned. Every 20th section was examined for parasites, but leishmanial organisms were not found in the tissues or the hemolymph in the H and E stained sections.

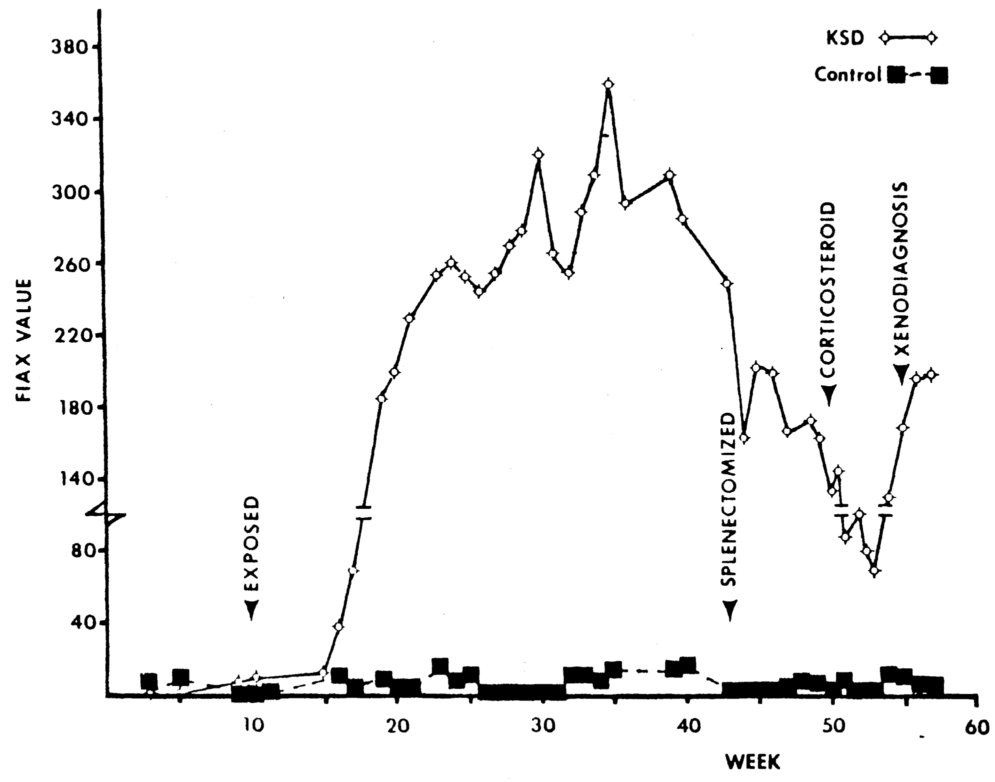
Transmission of *Leishmania* Using Infected Bone Marrow

One pup was inoculated intrafemorally with 1.5 ml of bone marrow from an infected dog (KSD) to test animal susceptibility and provide baseline data for an infected animal.

Serology

Figure 2 shows serum antibody titers (FIAX values) during the course of the study. The peak serological response occurred in the infected dog at week 25 post-inoculation (PI). The antibody levels rapidly declined in the infected animal until splenectomy. A slight increase in antibody level occured one week following splenectomy after which the antibody levels again began to fall. Once corticosteroid treatments were initiated on week 50 the antibody levels dramatically rose to a peak measured the day the animal was euthanitized on week 57. Antibody levels in the control dog did not change significantly during the course of the study.

Figure 2. FIAX serum antibody titers to *Leishmania* before and after splenectomy and corticosteroid treatments in a dog given a bone marrow transfer from a dog from Kansas (KSD) which was naturally infected with *Leishmania* sp.


KSD
Control
380
340
300
260
220
180
140
80
40
FIAX VALUE
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
XENODIAGNOSIS
10
20
30
40
50
60
WEEK

Rectal Temperature, Respiration, and Heartrate

Rectal temperatures for the control dogs are shown in Figure 3. The infected dog maintained a chronic low-grade temperature which was significantly higher than that of the control dog. Figures 4 and 5 show respiration rates and heartrates, respectively. There were no significant differences in the latter measurements for the infected and non-infected dogs during the course of the study.

Blood Parameters

Total blood counts and hemoglobin values are listed in Table III. Total white blood cell counts and hemoglobin values increased after splenectomy and during corticosteroid treatments. Table IV contains results of the blood chemistry tests. The SGPT and the SAP values changed noticeably during corticosteroid treatments, but these parameter changes did not appear to be related to infection. All other blood parameters remained within normal ranges.

Transmission of *Leishmania* Through Uninoculated Tick-Gut Homogenate

Nymphal ticks were fed on dogs with natural *Leishmania* infections and allowed to molt. Guts were extracted from 50 ticks, homogenized and inoculated parentally into recepient

Figure 3. Rectal temperatures before and after splenectomy and corticosteroid treatments in a dog given a bone marrow transfer from a dog from Kansas (KSD) which was naturally infected with *Leishmania* sp.

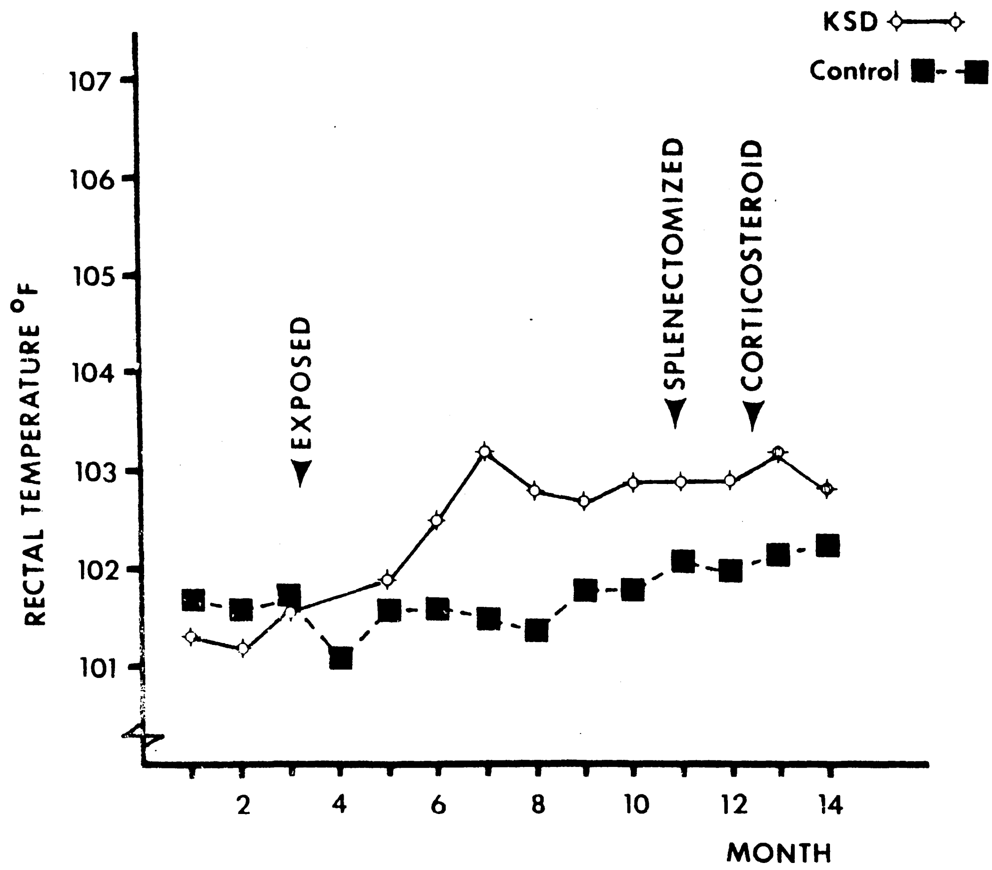

KSD
Control
RECTAL TEMPERATURE °F
107
106
105
104
103
102
101
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

Figure 4. Respiration rates before and after splenectomy and corticosteroid treatments in a dog given a bone marrow transfer from a dog from Kansas (KSD) which was naturally infected with *Leishmania* sp.

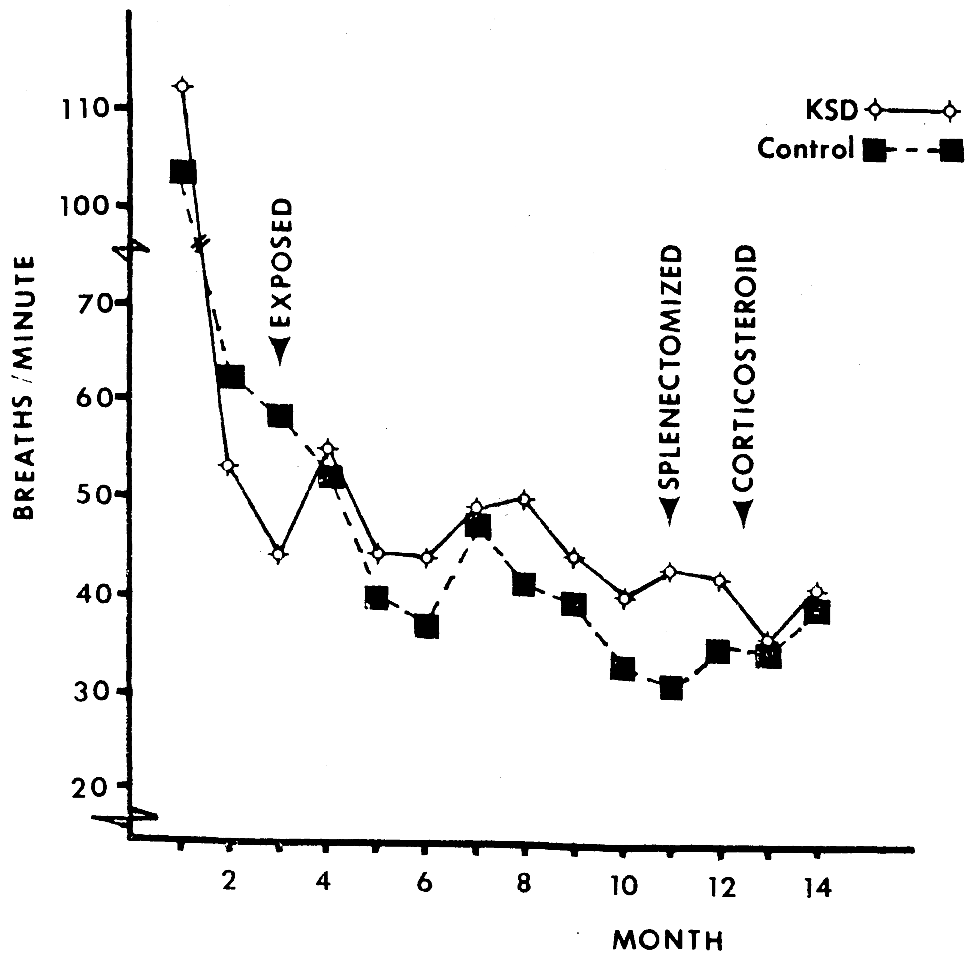

KSD
Control
BREATHS / MINUTE
110
100
70
60
50
40
30
20
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

Figure 5. Heartrates before and after splenectomy and corticosteroid treatments in a dog given a bone marrow transfer from a dog from Kansas (KSD) which was naturally infected with *Leishmania* sp.

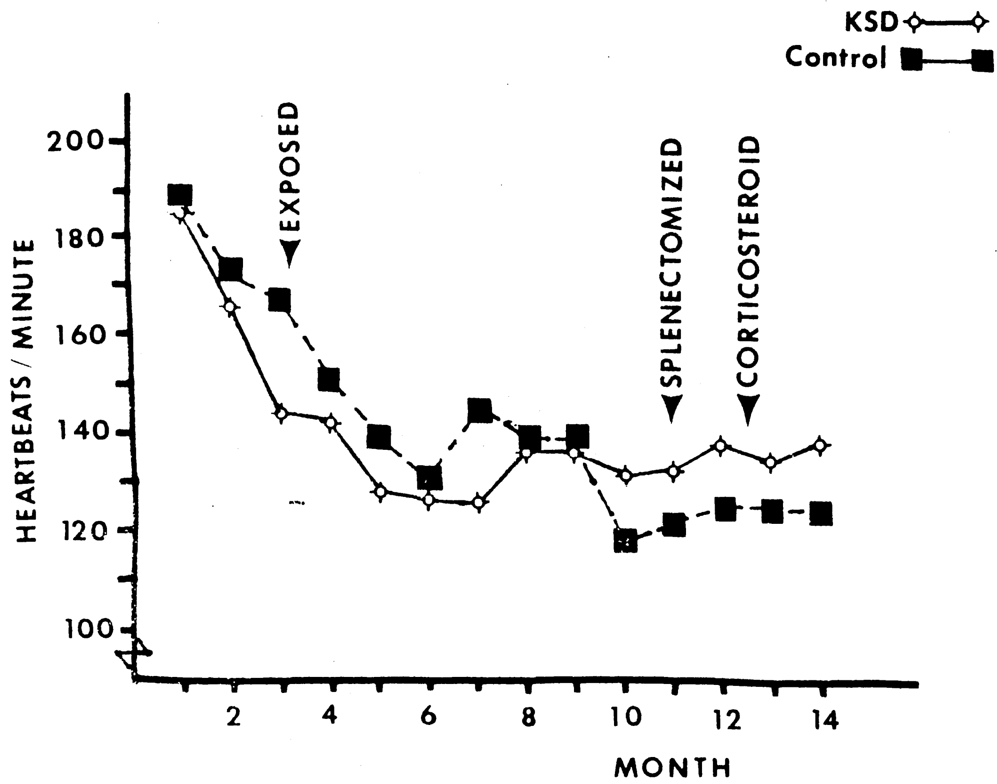

KSD
Control
HEARTBEATS / MINUTE
200
180
160
140
120
100
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

TABLE III

MEAN BLOOD COUNTS AND HEMOGLOBIN VALUES BEFORE AND AFTER SPLENECTOMIES AND PREDNISOLONE TREATMENTS IN DOGS EXPERIMENTALLY INFECTED WITH LEISHMANIA SP. BY THE INTERRUPTED AND UNINTERRUPTED FEEDING OF INFECTED TICKS OR INOCULATION OF HOMOGENIZED INFECTED TICK GUTS

Blood Parameters	Bone Marrow Controls		Feeding Ticks				Tick-Gut Homogenates			
			Interrupted		Uninterrupted		Incubated		Unincubated	
	Infected	Uninfected	Infected	Control	Infected	Control	Infected	Control	Infected	Control
Leucocytes ($X10^3$)										
Before Treatment	13.2	12.9	11.7	8.3	11.6	12.4	15.1	12.9	12.7	9.6
After Treatment	22.7	18.9	19.5	22.0	20.5	24.5	20.2	18.9	30.1	16.3
Hematocrit (%)										
Before Treatment	38.5	38.0	34.6	36.4	36.8	36.0	36.4	38.0	36.0	37.3
After Treatment	40.7	39.0	35.7	42.0	37.65	37.7	39.0	39.0	35.3	39.7
Hemoglobin gm/dl										
Before Treatment	13.0	12.9	12.2	12.7	12.5	12.2	12.5	12.9	12.4	13.0
After Treatment	14.1	13.2	12.2	14.5	12.9	13.0	13.8	13.2	12.3	13.9

TABLE IV

MEAN BLOOD CHEMISTRY VALUES BEFORE AND AFTER SPLENECTOMIES AND PREDNISOCONE TREATMENTS IN DOGS EXPERIMENTALLY INFECTED WITH LEISHMANIA SP. BY THE INTERRUPTED AND UNINTERRUPTED FEEDING OF INFECTED TICKS OR INOCULATION WITH HOMOGENIZED INFECTED TICK GUTS

Blood Parameters	Bone Marrow Controls		Feeding Ticks				Tick-Gut Homogenates			
			Interrupted		Uninterrupted		Incubated		Unincubated	
	Infected	Uninfected	Infected	Control	Infected	Control	Infected	Control	Infected	Control
Total Protein										
Before Treatment	5.5	5.4	5.8	5.6	5.5	5.2	5.5	5.4	5.5	5.6
After Treatment	6.9	6.3	6.8	6.7	6.5	6.5	6.5	6.3	6.8	6.5
BUN* mg/dl										
Before Treatment	11.0	12.8	17.2	17.3	17.8	12.5	17.5	12.8	19.8	17.8
After Treatment	13.0	32.0	27.0	22.0	18.5	24.0	17.5	32.0	20.0	22.0
Glucose mg/dl										
Before Treatment	134.0	116.8	98.3	105.3	104.7	126.3	103.7	116.8	107.9	104.4
After Treatment	98.0	95.0	80.0	81.0	75.0	99.0	87.5	95.0	77.0	129.0
Potassium mg/dl										
Before Treatment	5.3	5.2	5.3	5.3	5.2	5.3	5.7	5.2	5.2	5.1
After Treatment	4.6	4.5	4.6	3.9	4.4	4.4	4.3	4.5	4.4	4.0

TABLE IV (continued)

Blood Parameters	Bone Marrow Controls		Feeding Ticks				Tick-Gut Homogenates			
			Interrupted		Uninterrupted		Incubated		Unincubated	
	Infected	Uninfected	Infected	Control	Infected	Control	Infected	Control	Infected	Control
Sodium mg/dl										
Before Treatment	145.0	142.4	146.2	146.8	144.3	143.0	143.2	142.4	142.5	145.6
After Treatment	146.0	154.0	144.5	149.0	142.0	150.0	145.5	154.0	146.0	147.0
SGPT+ mg/dl										
Before Treatment	17.0	16.8	20.7	20.5	23.4	19.8	23.2	16.8	20.0	22.6
After Treatment	300.0	360.0	112.0	150.0	238.0	200.0	238.0	360.0	235.0	150.0
SAP++ mg/dl										
Before Treatment	50.0	40.0	29.0	41.0	46.5	43.0	36.0	40.0	20.0	20.0
After Treatment	369.0	214.0	124.7	73.0	167.6	133.7	56.9	214.0	91.0	74.0

* BUN = Urea nitrogen
+ SGPT = Glutamiepyrivictransaminase mu/dl
++ SAP = Alkaline phosphatase mu/dl

animals to test for infective stages within the ticks.

Serology

The serological responses for dogs inoculated with unincubated homogenate are shown in Figure 6. The dog inoculated homogenated (OKD) showed a peak antibody response at 25 weeks PI after which the titers slowly declined. A second peak occurred after the corticosteroid treatments were begun on week 40 PI and continued to increase until the dog was euthanitized. Antibody levels were lower in the dog inoculated with infected tick homogenate than in the dog that received infected bone marrow (Figure 1). Although it had been previously vaccinated, the dog inoculated with the KSD tick gut homogenate died at week 15 as a result of a parvovirus infection.

Rectal Temperatures, Heartrates, and Respiration Rates

Rectal temperatures (Fig. 7) showed that the dogs exposed to infected homogenate maintained higher average temperatures than the control animal inoculated with non-infected homogenate. The dog exposed to infected tick homogenate (KSD) suddenly developed a high fever apparently as a result of parvovirus infection, and died on week 8. Heartrates and respiration rates did not vary appreciably in any of these animals during the course of the infection

Figure 6. FIAX antibody titers to *Leishmania* before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from unincubated infected adult *Rhipicephalus sanguineus* previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.

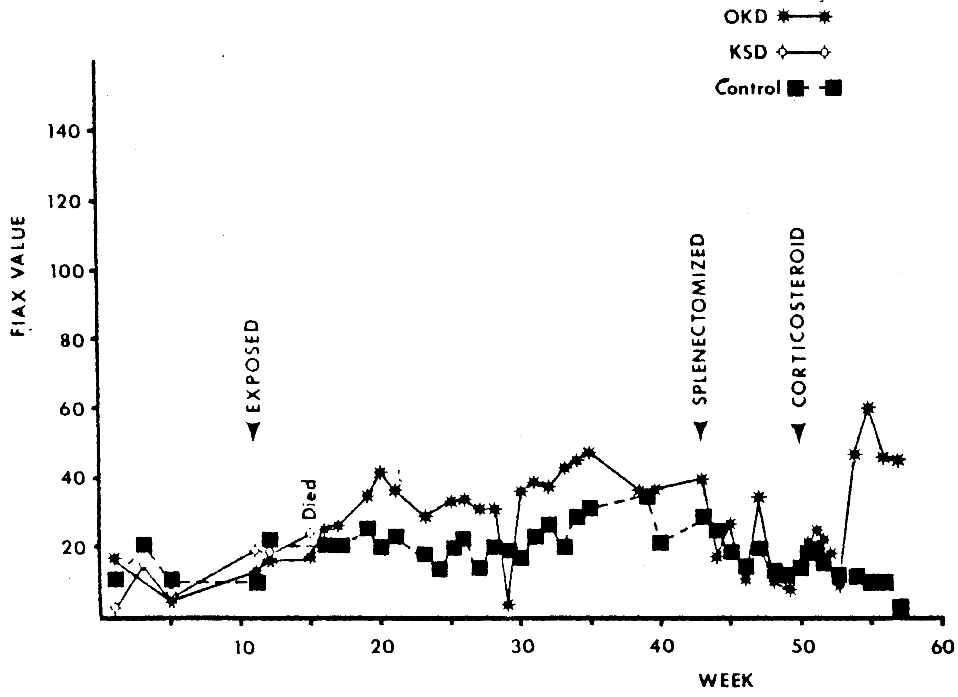
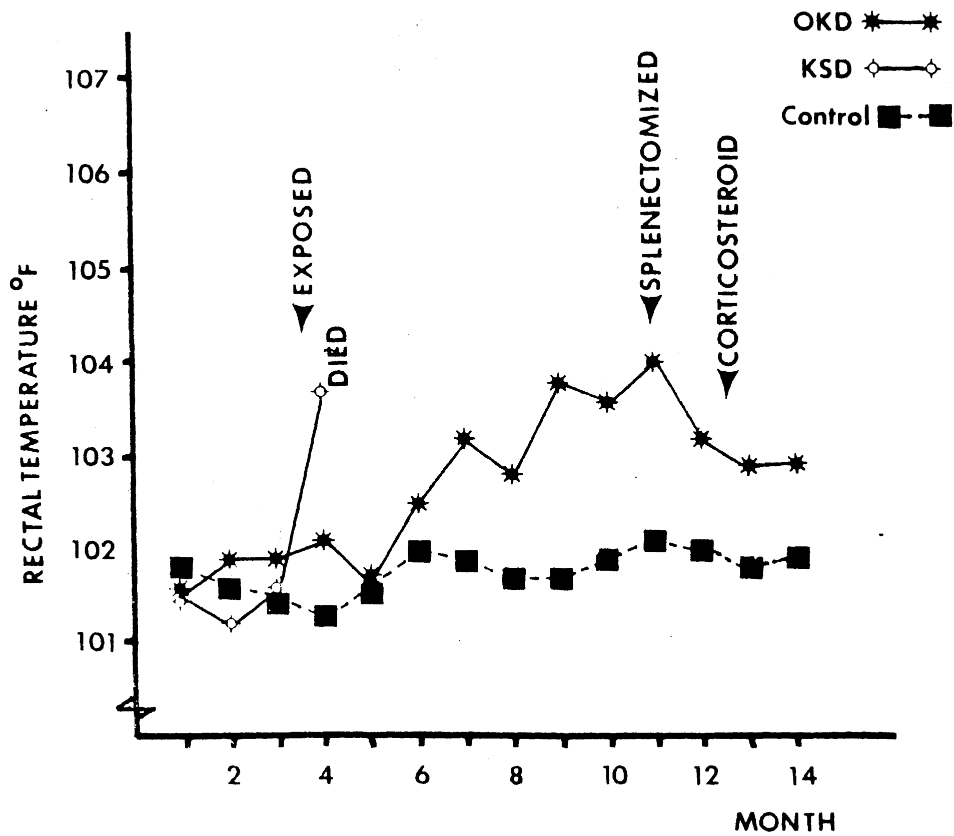

OKD
KSD
Control
140
120
100
80
60
40
20
FIAX VALUE
EXPOSED
Died
SPLENECTOMIZED
CORTICOSTEROID
10
20
30
40
50
60
WEEK

Figure 7. Rectal temperatures before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from unincubated infected adult *Rhipicephalus sanguineus* previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.


OKD
KSD
Control
RECTAL TEMPERATURE °F
107
106
105
104
103
102
101
EXPOSED
DIED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

(Fig. 8 and Fig. 9).

Blood Parameters

Treatments with the corticosteroid prednisolone resulted in elevated WBC counts, but hemoglobin and packed cell volume remained within normal ranges (Table III). Increased levels of SGPT and SAP were observed in both the exposed and control pups after corticosteroid treatments were begun.

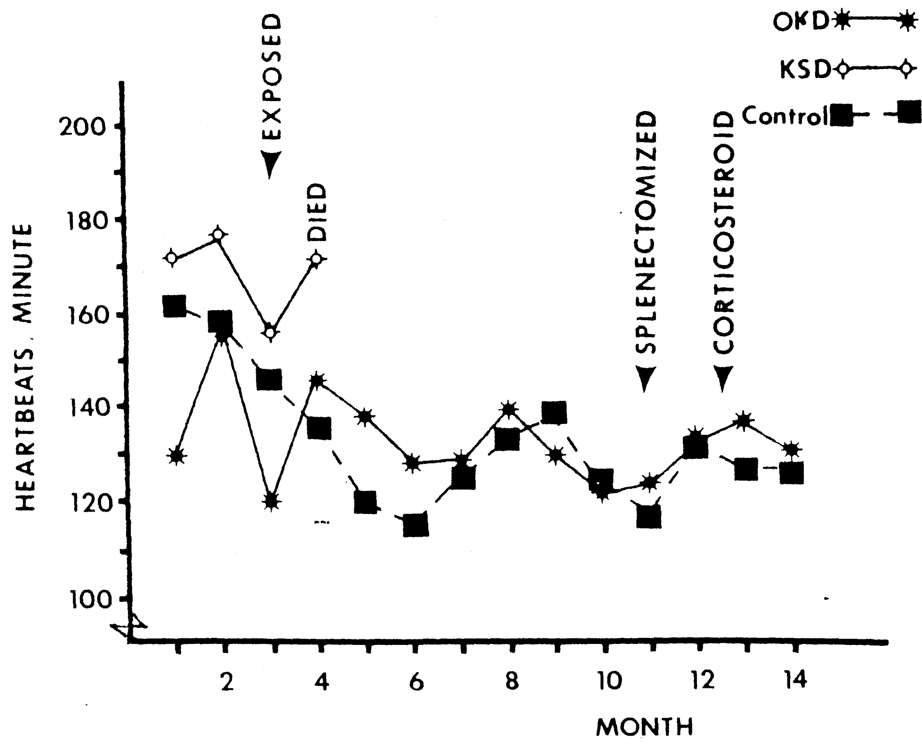
Transmission of Leishmania Through Incubated Tick-Gut Homogenate

Three dogs were inoculated with incubated infected tick-gut homogenates to see if elevated tick incubation temperatures (35 C for 2.5 days) would alter the infectivity of the parasites.

Serology

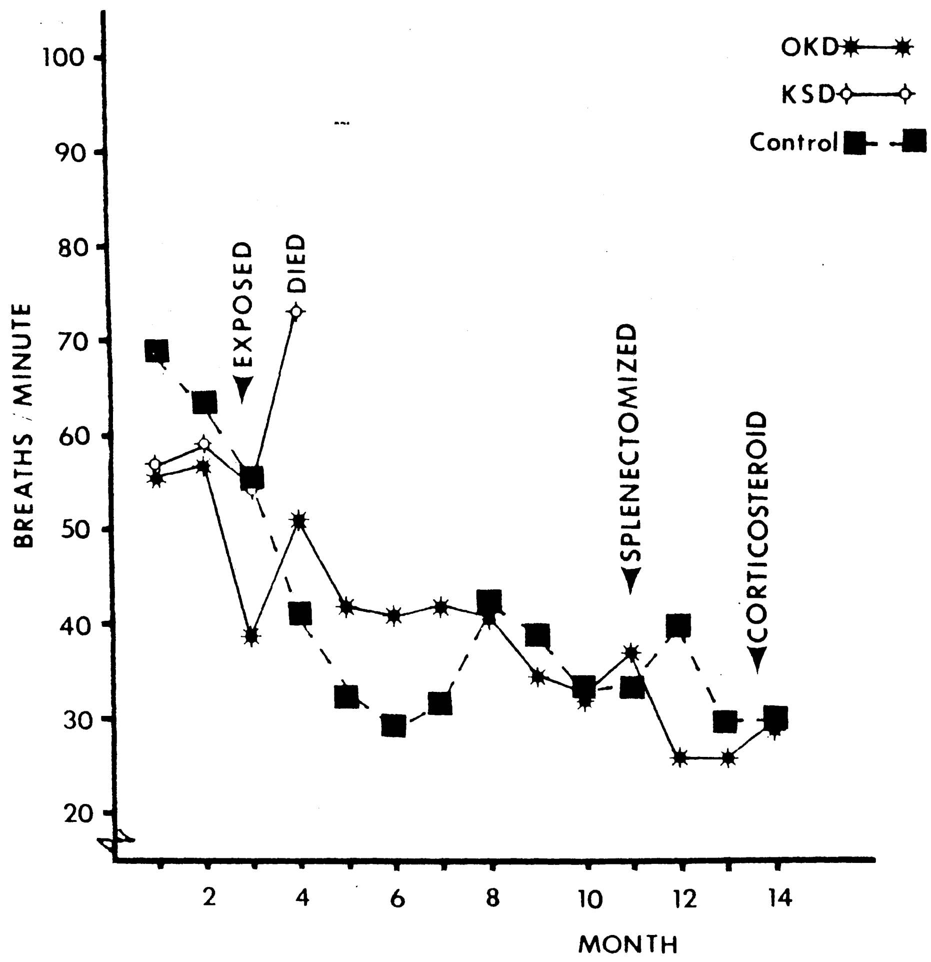
Figure 10 shows that before corticosteroid treatments were begun, serum antibody titers were not appreciably different between the principal (OKD and KSD) and control pups. Low FIAX values were obtained for all dogs; the OKD-exposed dog had slightly higher values. After corticosteroid treatments were begun, the antibody titers in the OKD-exposed dog increased markedly as though seroconversion had occurred at that time.

Figure 8. Heartrates before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from unincubated infected adult *Rhipicephalus sanguineus* previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



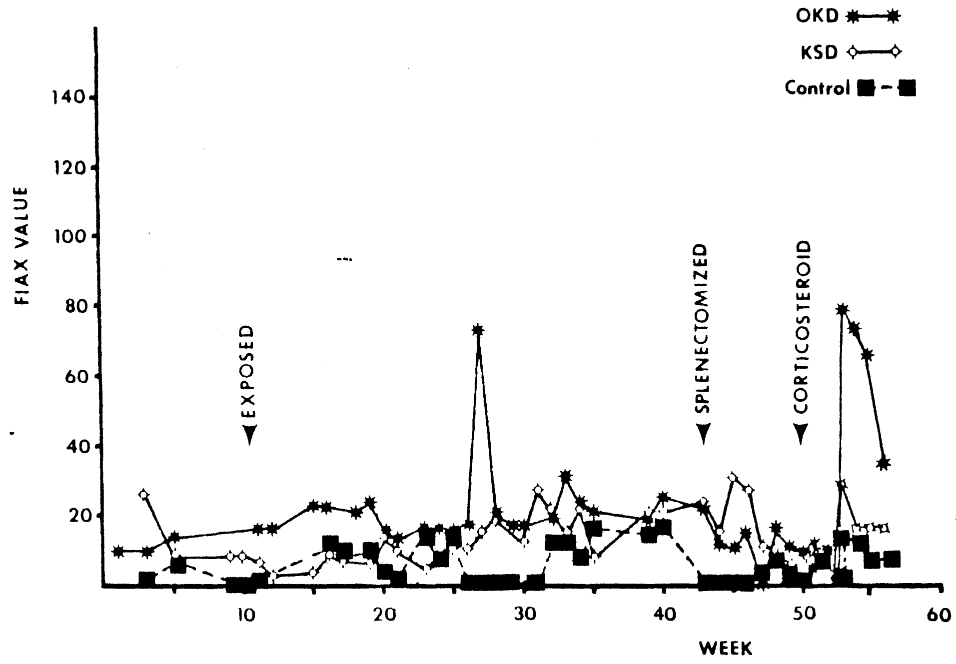
OKD
KSD
Control
EXPOSED
DIED
SPLENECTOMIZED
CORTICOSTEROID
HEARTBEATS. MINUTE
200
180
160
140
120
100
2
4
6
8
10
12
14
MONTH

Figure 9. Respiration rates before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from unincubated adult *Rhipicephalus sanguineus* previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



OKD
KSD
Control
BREATHS / MINUTE
100
90
80
70
60
50
40
30
20
EXPOSED
DIED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

Figure 10. FIAX serum antibody titers to *Leishmania* before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from incubated infected adult *Rhipicephalus sanguineus* (37 C for 2.5 days) previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



OKD
KSD
Control
140
120
100
80
60
40
20
FIAX VALUE
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
10
20
30
40
50
60
WEEK

Rectal Temperatures, Heartrates, and Respiration Rates

Figure 11 shows that the dogs that received the injected homogenate maintained a higher body temperature than the control animal with the KSD-exposed pup showing the highest temperature. Heartrates (Fig. 12) did not differ in treatment groups; however, the respiration rates (Fig. 13) were slightly higher in the animals inoculated with the infected tick homogenate.

Blood Parameters

Table III lists the changes observed in blood cell counts and hemoglobin values for dogs inoculated with incubated tick homogenates. After corticosteroid treatments were begun white blood cell counts increased markedly in all animals. Blood chemistry values (Table IV) show that SGPT and SAP levels also increased in all dogs during steroid treatments. The BUN of the only non-infected bone marrow control animal increased slightly during steroid treatments, whereas other values for all other animals remained within normal ranges.

Transmission of *Leishmania* Through Interrupted Feeding of Infected Ticks

Groups of laboratory-reared, adult *Rhipicephalus*

Figure 11. Rectal temperatures before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from incubated infected adult *Rhipicephalus sanguineus* (37 C for 2.5 days) previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.

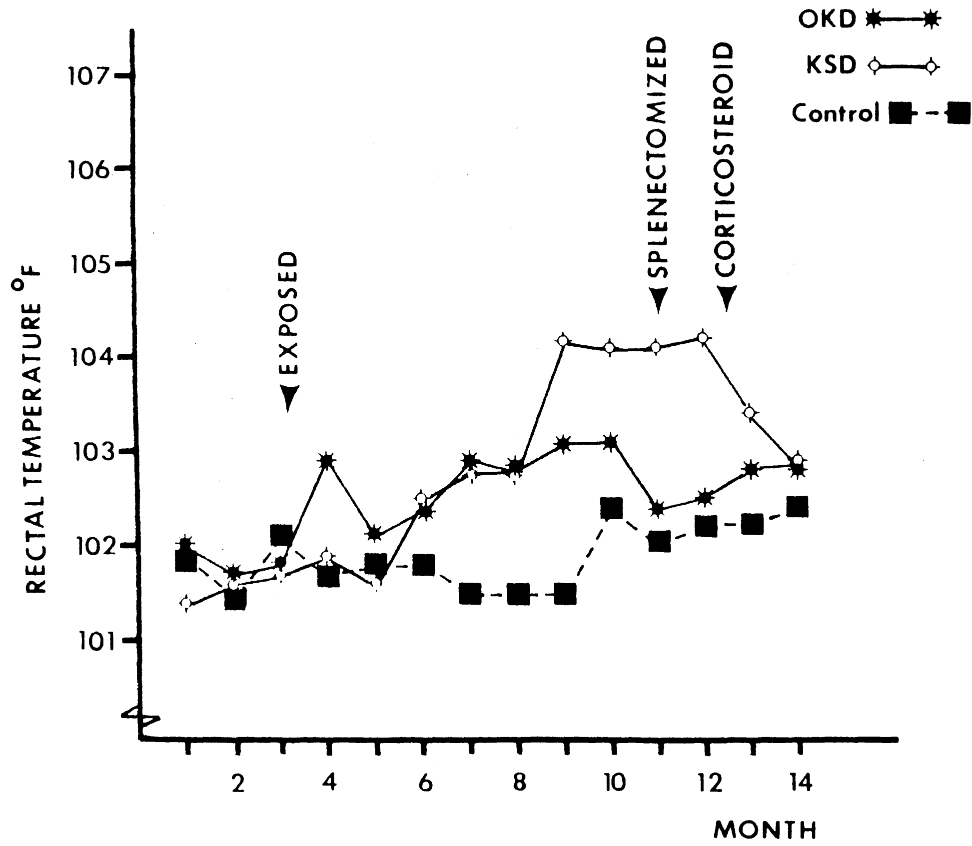

OKD
KSD
Control
SPLENECTOMIZED
CORTICOSTEROID
EXPOSED
RECTAL TEMPERATURE °F
107
106
105
104
103
102
101
2
4
6
8
10
12
14
MONTH

Figure 12. Heartrates before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from incubated infected adult _Rhipicephalus sanguineus_ (37 C for 2.5 days) previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with _Leishmania_ sp.

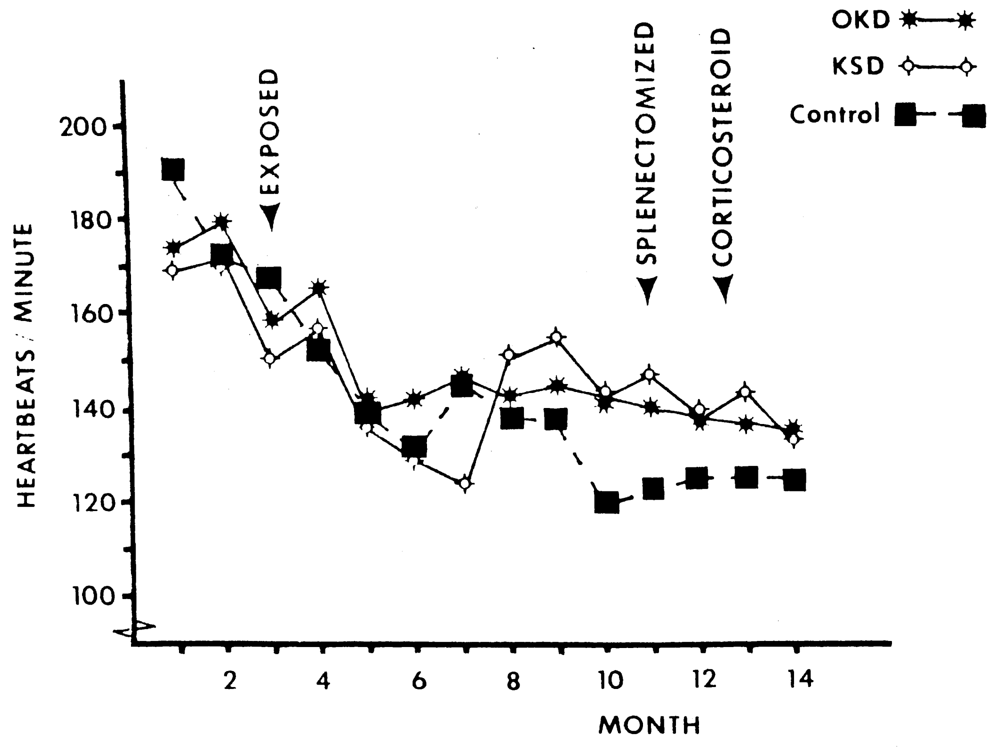
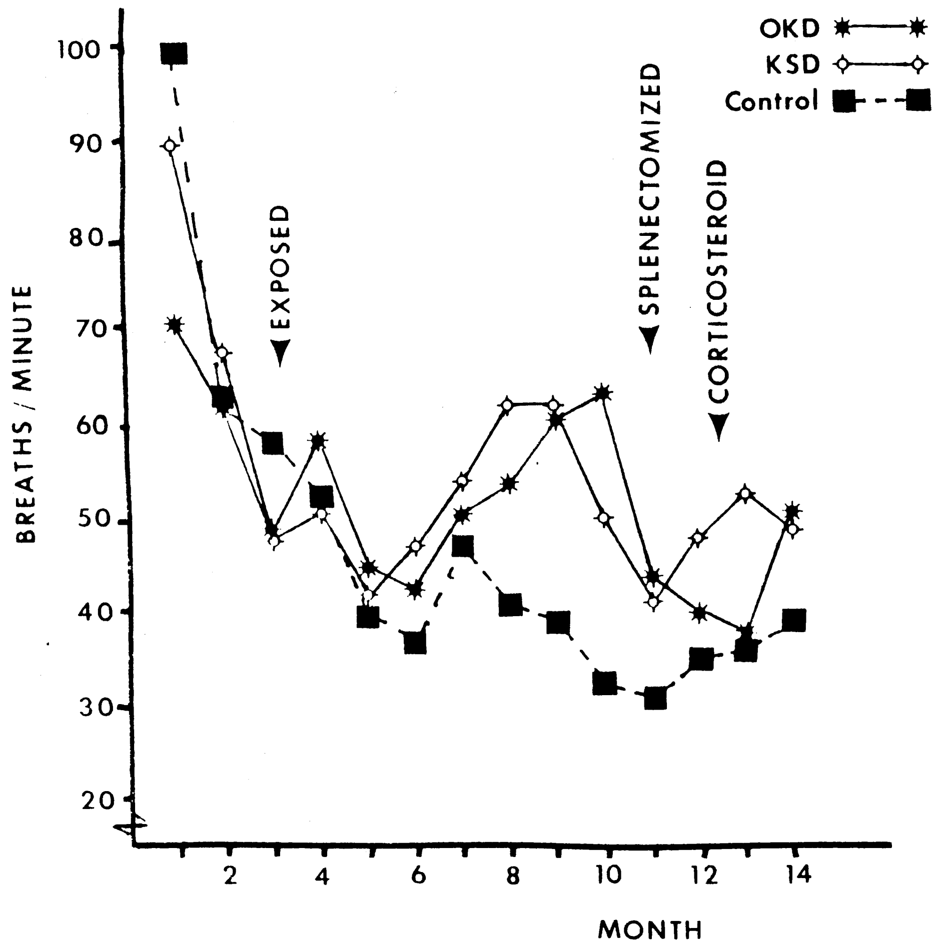

OKD
KSD
Control
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
HEARTBEATS / MINUTE
200
180
160
140
120
100
2
4
6
8
10
12
14
MONTH

Figure 13. Respiration rates before and after splenectomies and corticosteroid treatments in dogs inoculated with homogenized guts from incubated infected adult *Rhipicephalus sanguineus* (37 C for 2.5 days) previously fed on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.


OKD
KSD
Control
100
90
80
70
60
50
40
30
20
BREATHS / MINUTE
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

sanguineus were allowed to feed on 2 naturally infected dogs for 3 days, detached by hand before feeding was completed and placed directly on a non-infected dog where they were allowed to reattach and feed to repletion in order to determine if leishmanial parasites could be transmitted mechanically by ticks.

Serology

Serological responses and seroconversion (Figure 14) could not be detected by FIAX serology in any of the animals exposed by interrupted tick feeding. The dog which was exposed to KSD-exposed ticks died shortly after exposure as a result of a parvo virus infection. A slight antibody response was detected during corticosteroid treatments in the dog exposed to OKD ticks.

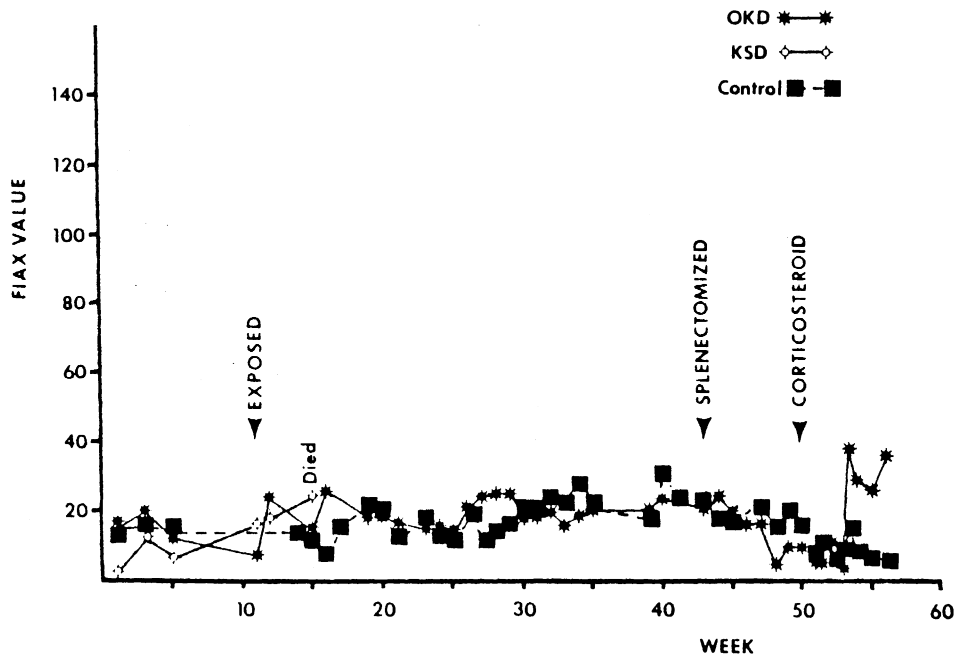
Rectal Temperature, Heartrates, and Respiration Rates

The dog exposed to OKD-ticks had a slightly higher rectal temperature than the control dog during the course of the experiment (Fig. 15). Heartrates (Fig. 16) and respiration rates (Fig. 17) did not differ appreciably among animals.

Blood Parameters

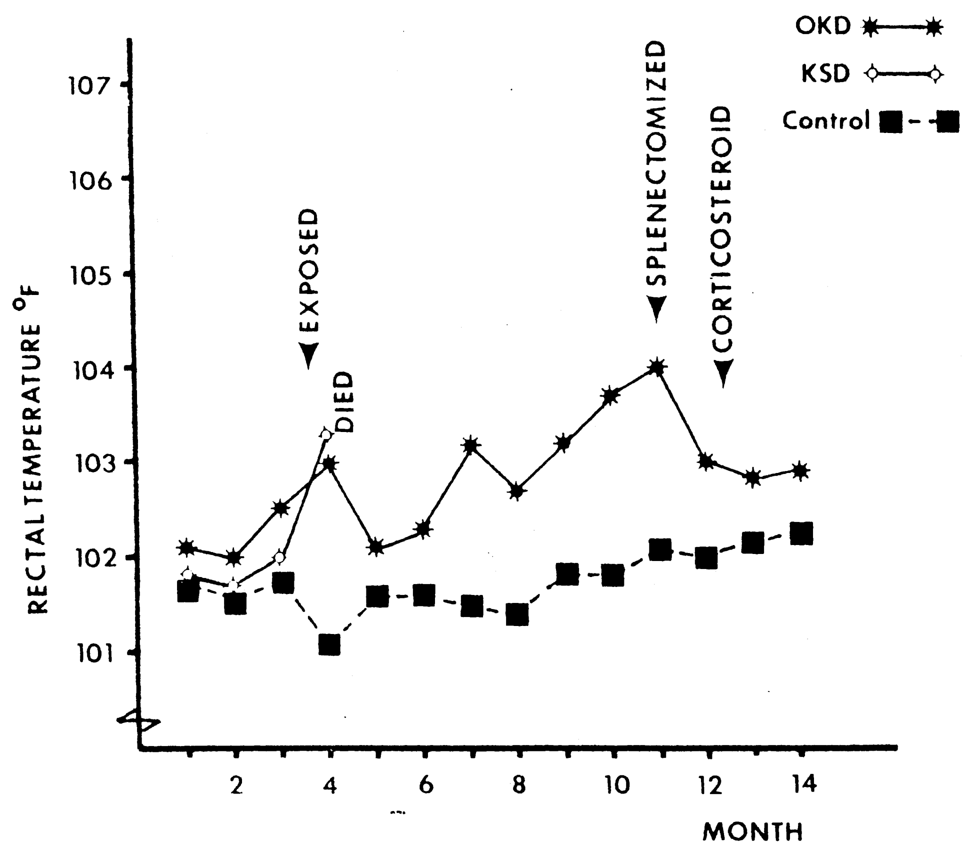
Total white blood cell counts increased significantly

Figure 14. FIAX serum antibody titers to *Leishmania* before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* previously fed (interrupted and immediately transferred) on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



OKD
KSD
Control
FIAX VALUE
140
120
100
80
60
40
20
EXPOSED
Died
SPLENECTOMIZED
CORTICOSTEROID
10
20
30
40
50
60
WEEK

Figure 15. Rectal temperature before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* previously fed (interrupted and immediately transferred) on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



OKD
KSD
Control
107
106
105
104
103
102
101
RECTAL TEMPERATURE °F
EXPOSED
DIED
SPLENECTOMIZED
CORTICOSTEROID
2
4
6
8
10
12
14
MONTH

Figure 16. Heartrates before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* previously fed (interrupted and immediately transferred) on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.

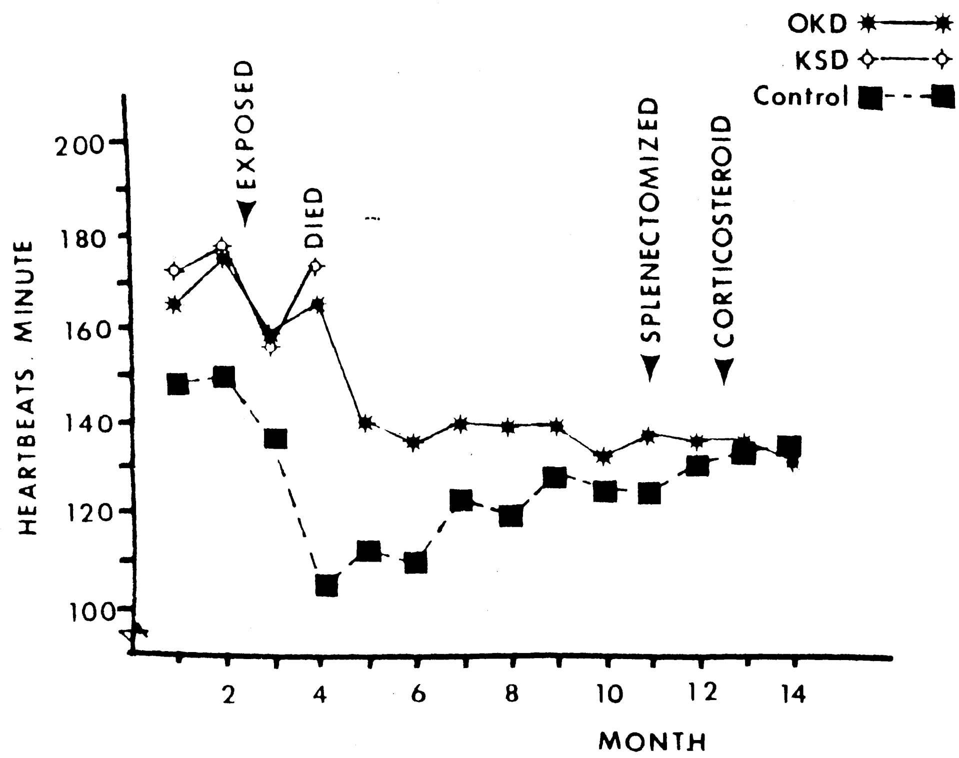
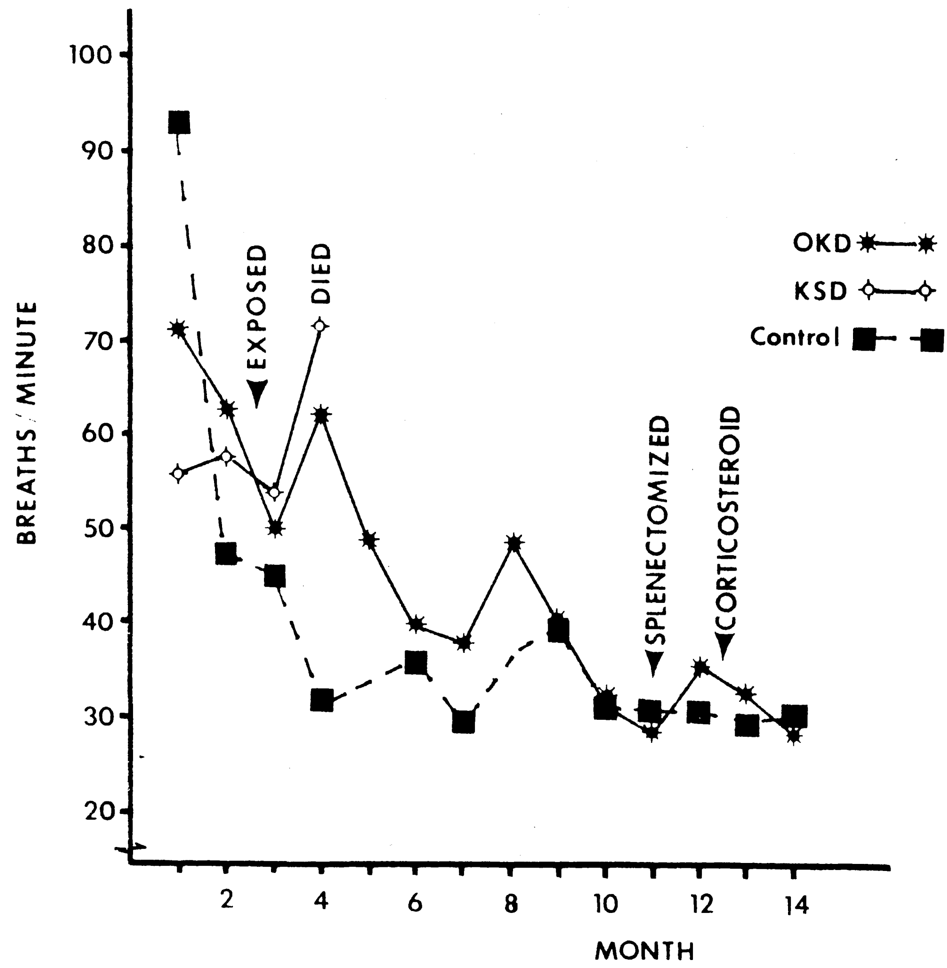

OKD
KSD
Control
EXPOSED
DIED
SPLENECTOMIZED
CORTICOSTEROID
HEARTBEATS MINUTE
200
180
160
140
120
100
2
4
6
8
10
12
14
MONTH

Figure 17. Respiration rates before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* previously fed (interrupted and immediately transferred) on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.


100
90
80
70
60
50
40
30
20
BREATHS / MINUTE
EXPOSED
DIED
SPLENECTOMIZED
CORTICOSTEROID
OKD
KSD
Control
2
4
6
8
10
12
14
MONTH

in all the interrupted tick feeding animals while corticosteroid treatments were administered. The hemoglobin and hematocrit measurements did not change markedly (Table III). Data in Table IV show that the SGPT and SAP enzymes reached significantly high levels during steroid treatments, whereas total protein and BUN levels increased only slightly. Other blood chemistry values remanined within normal limits.

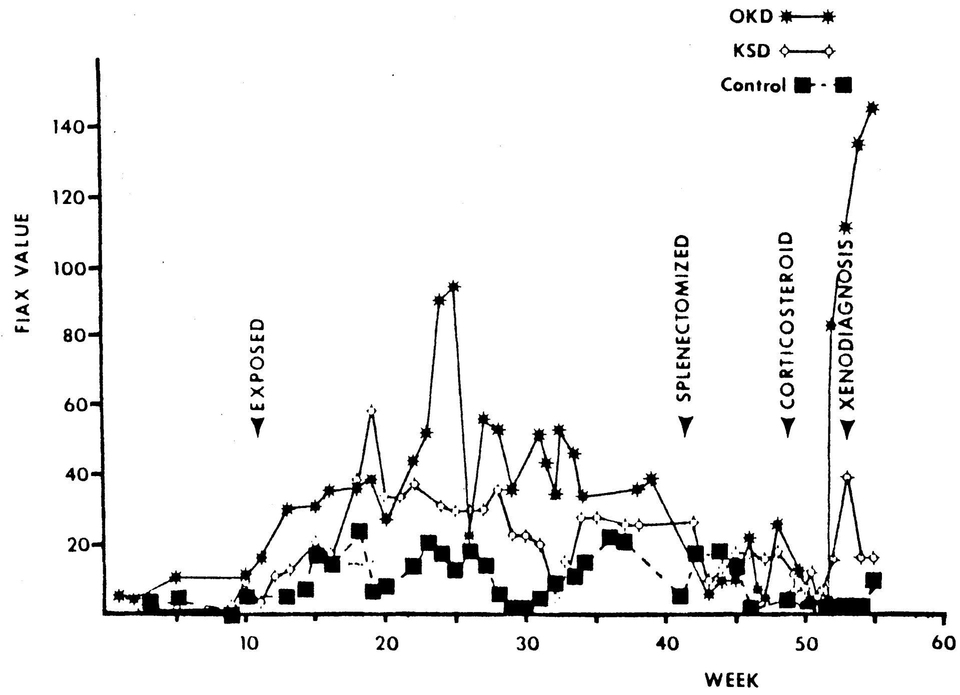
Transmission of Leishmania Through Feeding of Infected Ticks

Nymphal *Rhipicephalus sanguineus* were allowed to feed on 2 naturally infected dogs and molt. After molting the ticks were placed on separate uninfected dogs and allowed to feed to determine if ticks could transmit the disease by normal feeding process.

Serology

Both dogs exposed to the natural feeding process of infected adult ticks showed serum antibody responses to *Leishmania* after the infected ticks had fed on them (Fig. 18). The peak antibody titer in the KSD-exposed dog occurred 7 weeks after the ticks fed and at 15 weeks in the OKD dog. After reaching a peak, the antibody levels declined even after splenectomy until the corticosteroid treatments were started. The corticosteroid treatments

Figure 18. FIAX serum antibody titers to *Leishmania* before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* (30 days post-molting) previously fed as nymph on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.


OKD
KSD
Control
FIAX VALUE
140
120
100
80
60
40
20
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
XENODIAGNOSIS
10
20
30
40
50
60
WEEK

resulted in a dramatic increase in antibody levels in the OKD-exposed dog but not in the others.

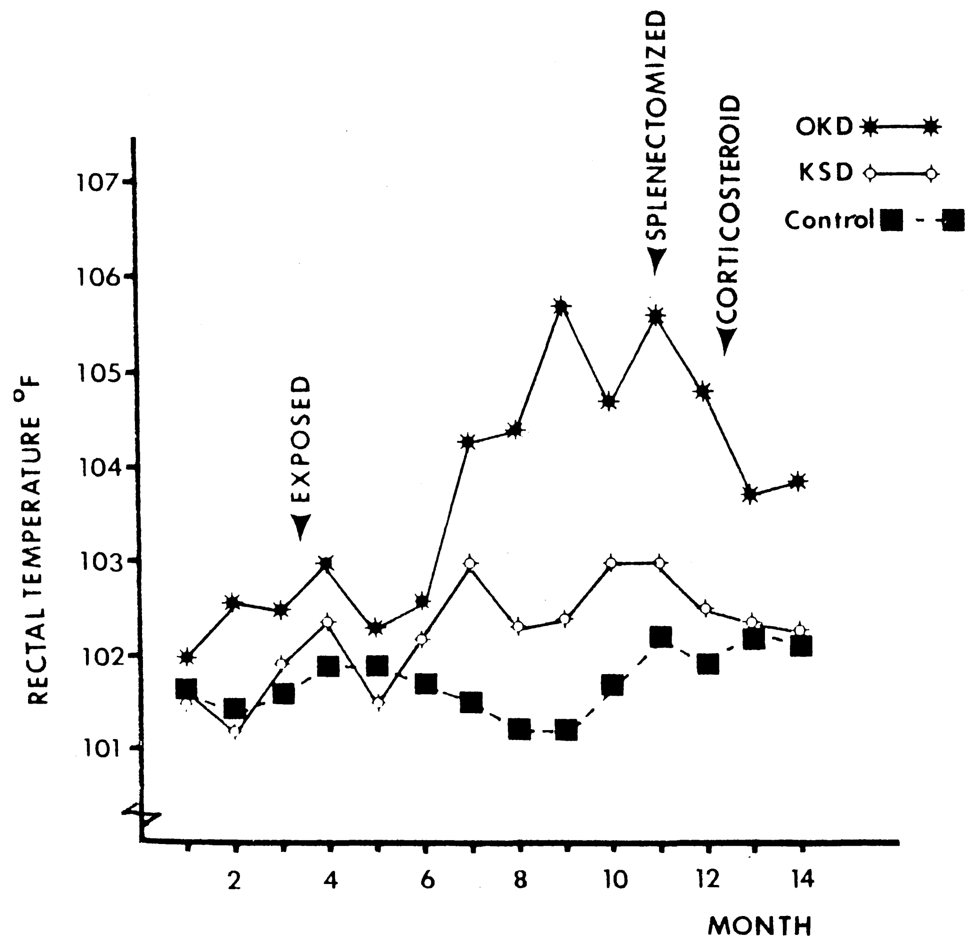
Rectal Temperature, Heartrates and Respiration Rates

Both of the dogs exposed to infected ticks experienced elevated body temperatures (Fig. 19); the OKD-exposed dog generally maintained a higher body temperature than the KSD-exposed dog. Figure 20 shows that the heartrates for all 3 dogs were comparable and showed no significant differences. Figure 21 shows that the OKD-exposed dog maintained a slightly higher respiration rate than the other animals exposed by uninterrupted feeding ticks.

Blood Parameters

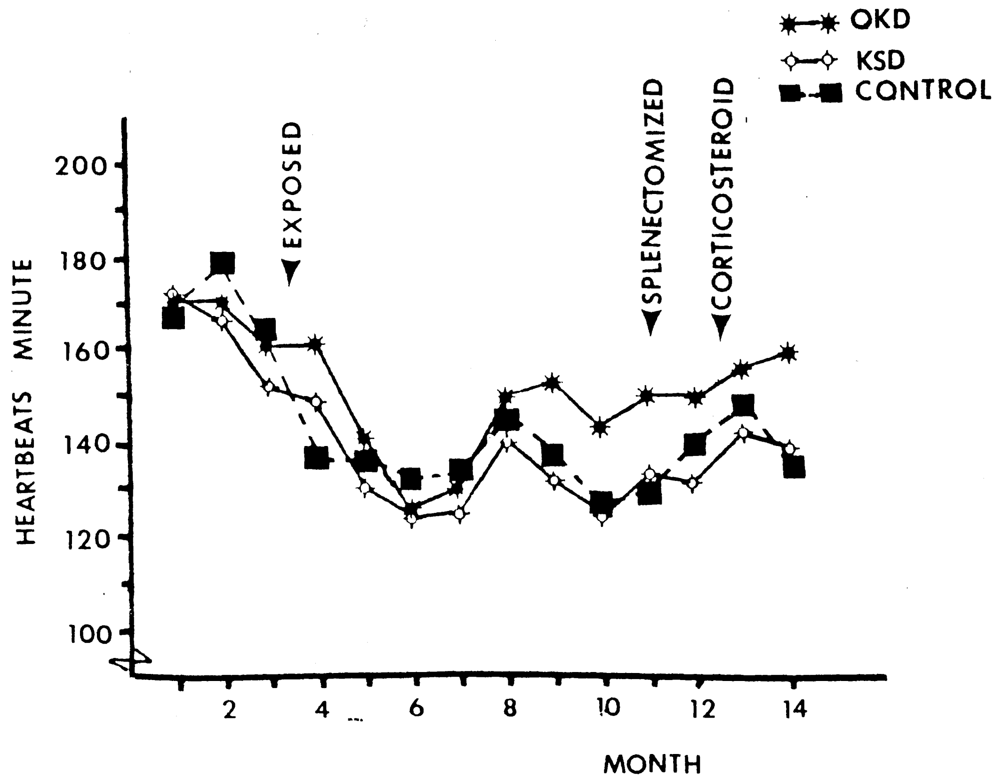
Total WBC counts for dogs in the uninterrupted tick feeding experiment increased markedly during the period of corticosteroid treatments (Table III); hemoglobin and hematocrit values were not significantly altered. The SGPT and SAP levels increased significantly during corticosteroid treatments with only a slight elevation in the BUN level observed. All other blood parameters levels remained within normal ranges.

Figure 19. Rectal temperatures before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* (30 days post-molting) previously fed as nymph on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



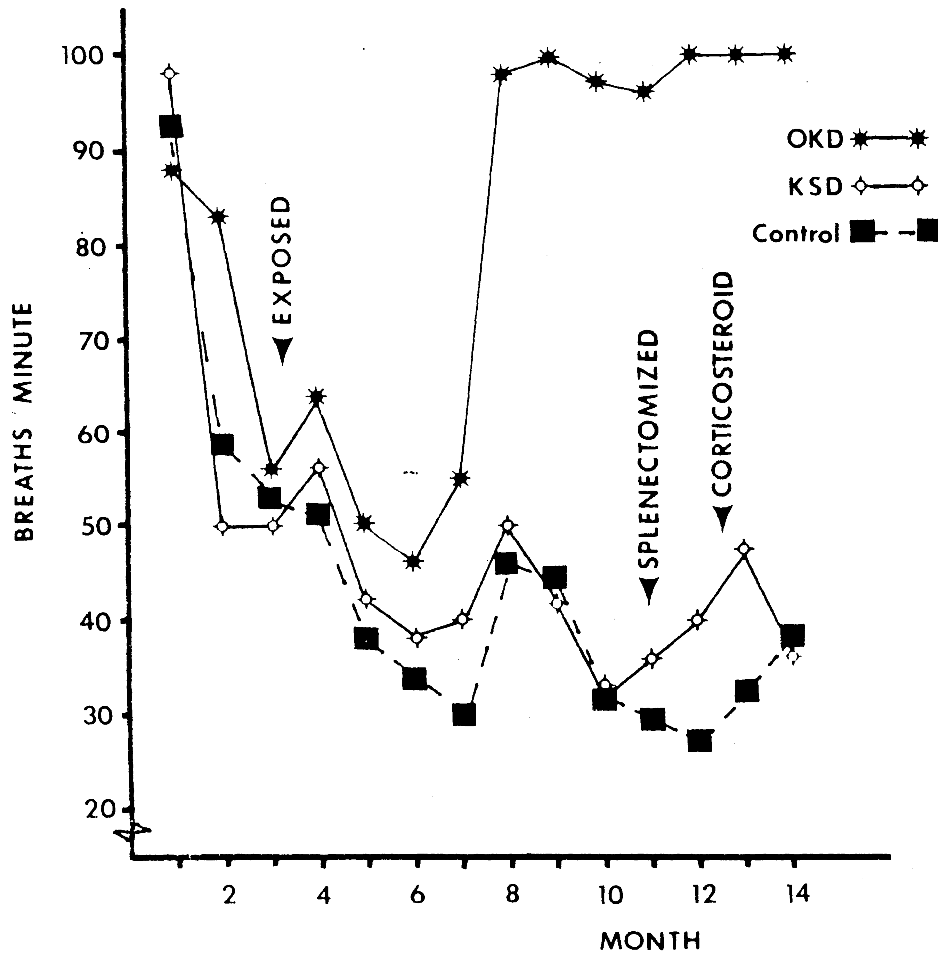
RECTAL TEMPERATURE °F
107
106
105
104
103
102
101
2
4
6
8
10
12
14
MONTH
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
OKD
KSD
Control

Figure 20. Heartrates before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* (30 days post-molting) previously fed as nymph on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.



OKD
KSD
CONTROL
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
HEARTBEATS MINUTE
200
180
160
140
120
100
2
4
6
8
10
12
14
MONTH

Figure 21. Respiration rates before and after splenectomies and corticosteroid treatments in dogs fed upon by adult *Rhipicephalus sanguineus* (30 days post-molting) previously fed as nymph on a dog from Kansas (KSD) or a dog from Oklahoma (OKD) which were naturally infected with *Leishmania* sp.


100
90
80
70
60
50
40
30
20
BREATHS / MINUTE
EXPOSED
SPLENECTOMIZED
CORTICOSTEROID
OKD
KSD
Control
2
4
6
8
10
12
14
MONTH

Examination of Tissues Following Splenectomies And Corticosteroid Treatments in Exposed Dogs

Occassionally lymph node aspirates were examined in an attempt to isolate the leishmanial organisms from parasite exposed dogs. In addition, all the exposed and control animals were splenectomized and treated with corticosteroids in an attempt to promote parasite reproduction and to improve the chances to reisolate the parasite from the exposed animals.

Impression Smears and Lymph Node Aspirates

Parasites were not found in spleen impression smears from any of the dogs at the time of surgery. Smears of lymph node aspirates made for all dogs before and after splenectomy and during steroid treatments were also negative.

Cultures

All cultures made from spleen homogenates and lymph node aspirates using NNN, MaeKelt's, and Schneider's Drosophila media were negative.

Hamster Inoculations

All hamsters that were inoculated with a spleen

homogenate from dogs were negative for *Leishmania* when examined at necropsy 3 and 6 months post-inoculation.

Xenodiagnosis Using Ticks

After corticosteroid treatments had been administered for 4 weeks, nymphal and adult *Rhipicephalus sanguineus* were placed on the two dogs exposed through uninterrupted feeding of infected ticks and the dog which received a transfer of infected bone marrow. These dogs were chosen because they exhibited high serum antibody titers at some point during the course of the study. Once the ticks had fed to repletion and detached, cultures of the tick-gut were made. Cultures from both nymphal and adult ticks were *Leishmania*-positive for the OKD-tick exposed dog and the dog that received a direct transfer of parasite-infected bone marrow. The ticks from the dog exposed to KSD-ticks were culture negative. It was 5 days before the leishmanial promastigotes were observed in the cultures.

Examination of Tissues From Experimental Dogs

The experiment was terminated after observing increasing antibody levels and results from the dog exposed to uninterrupted feeding of OKD-infected ticks and the dog which received the direct transfer of infected bone marrow. Tissues from the experimental animals were examined for the

presence of the parasites.

Impression Smears

Parasites could not be found in any of the smears of mesenteric lymph nodes, liver or bone marrow from all dogs.

Cultures

Cultures of lymph nodes and bone marrow were made using NNN, MaeKelt's and Schneider's Drosophila media; only the dog which was exposed to infected feeding ticks (OKD) and the dog which received infected bone marrow (KSD) were culture-positive for *Leishmania*. In both cases the bone marrow was culture positive.

Hamster and Dog Subinoculations

Six hamsters were inoculated with bone marrow from the culture positive dogs and killed at 3 and 6 months post-inoculation. Parasites were not found in either the impression smears or cultures of liver, spleen and bone marrow.

A splenectomized dog was inoculated intrafemorally with 10 ml of bone marrow from the dog which received infected bone marrow in an attempt to propagate the parasites. The dog never showed signs of infection.

Electron Microscopy

Subpellicular microtubule counts were used to compare promastigotes of several *Leishmania* isolates and the epimastigotes of *Trypanosoma cruzi*.

Comparison of Microtubule Counts

Dice-Lerass diagrams depicting increasing mean microtubule counts are shown in Figure 22. *Leishmania brasiliensis* (LB) had the lowest microtubule counts and OKD:PKt had the highest. All OKD isolates had similar mean counts and standard errors with the exception of the OKD:66bm and OKD:5 isolates which showed less variation than the other canine isolates.

Dice-Lerass diagrams of microtubule counts arranged from the lowest minimum count to highest minimum count are shown in Figure 23. *Trypanosoma cruzi* had the lowest minimum counts and OKD:66t had the highest. With this arrangement the OKD isolates showed no trend for grouping. When microtubule counts were arranged according to smallest range to the largest range (Fig. 24), the largest range was seen in KSD:CH and the smallest was in LB. With the latter grouping, the tick isolates tended to group together with similar variations in ranges, but the dog isolates showed no tendency toward grouping.

Figure 25 shows typical cross-sections of *Leishmania donovani* (LD), *L. brasiliensis* (LB), *L. mexicana* (Texas;

Figure 22. Dice-Lerass diagrams of microtubule counts for promastigotes of _Leishmania brasiliensis_ (LB), _L. tropica_ (LT), _L. donovani_ (LD), _L. mexicana_ (Texas; LMT), _L. mexicana_ (British Honduras; LMB), _Leishmania_ sp. (Kansas; KSD:CH), Oklahoma dog isolates (OKD:5; OKD:66bm; OKD:38bm; OKD:11), Oklahoma tick isolates (OKD:WKt; OKD:38t; OKD:66t; OKD:PKt) and _Trypanosoma cruzi_ (TC) arranged according to increasing mean microtubule counts. Verticle lines represent the mean; horizontal lines, the range; black areas, the standard deviation; and white areas, the standard error.

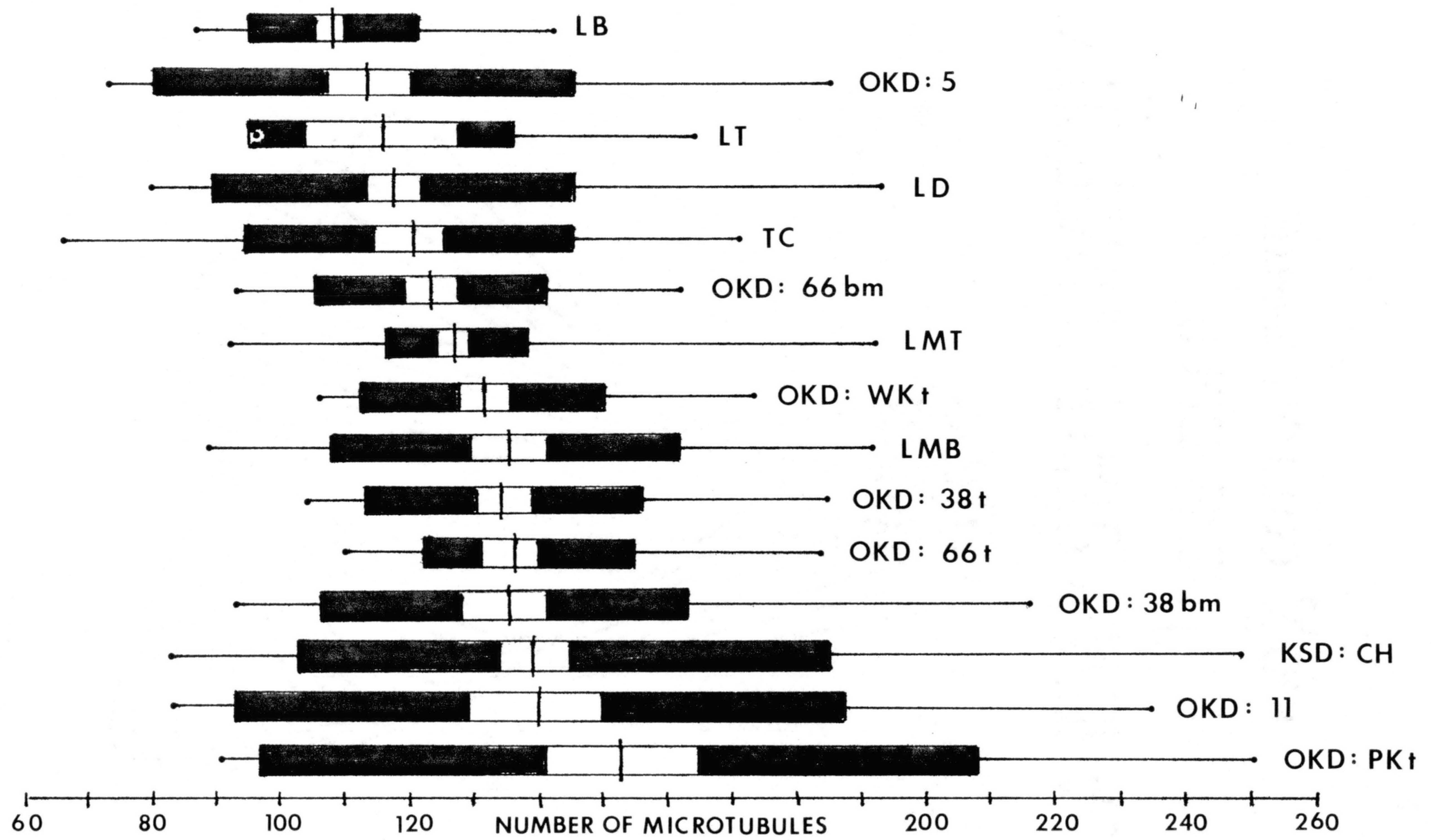
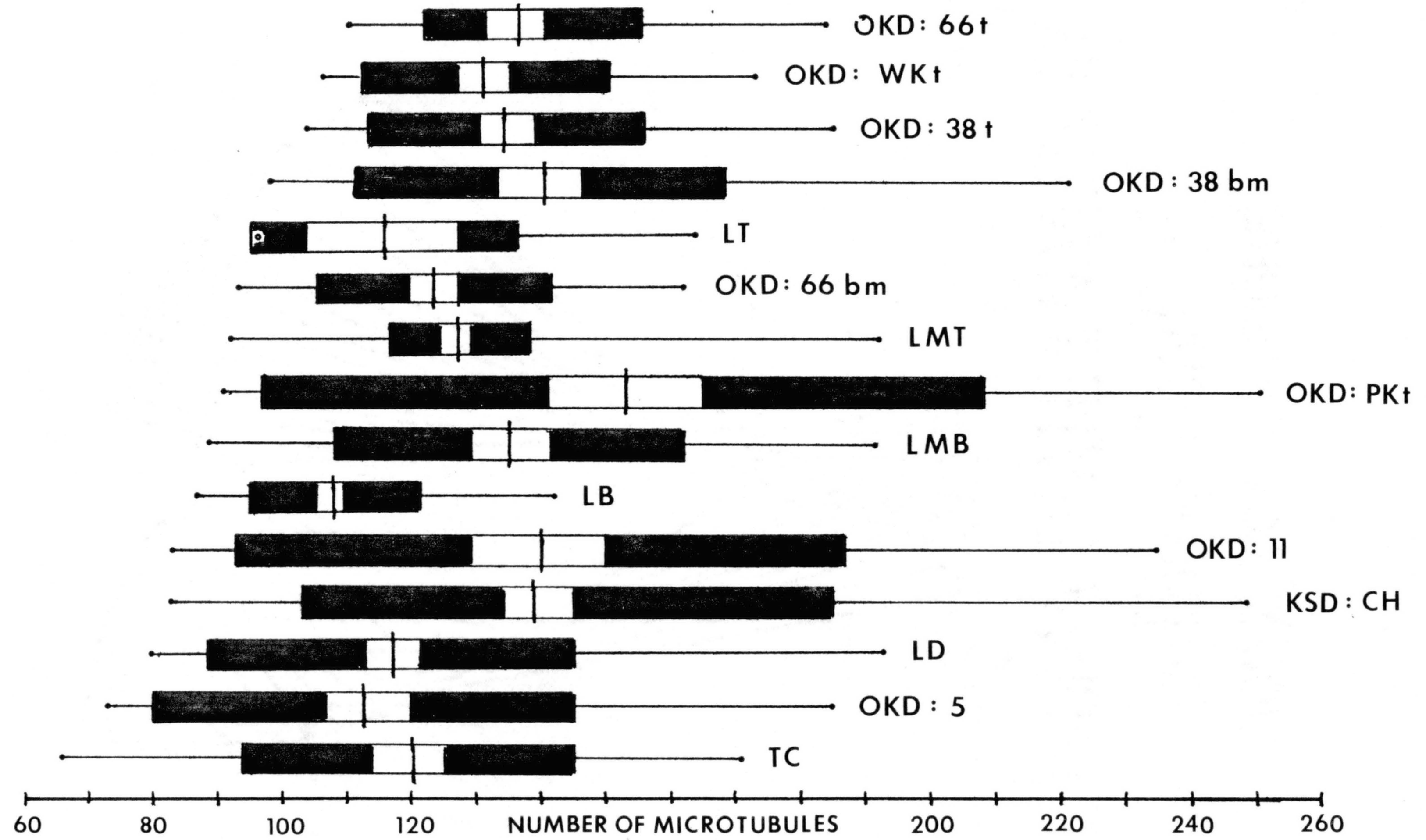

LB
OKD: 5
LT
LD
TC
OKD: 66 bm
LMT
OKD: WK t
LMB
OKD: 38 t
OKD: 66 t
OKD: 38 bm
KSD: CH
OKD: 11
OKD: PK t
60
80
100
120
NUMBER OF MICROTUBULES
200
220
240
260

Figure 23. Dice-Lerass diagrams of microtuble counts for promastigotes of *Leishmania brasiliensis* (LB), *L. tropica* (LT), *L. donovani* (LD), *L. mexicana* (Texas; LMT), *L. mexicana* (British Honduras; LMB), *Leishmania* sp. (Kansas; KSD:CH), Oklahoma dog isolates (OKD:5; OKD:66bm; OKD:38bm; OKD:11) Oklahoma tick isolates (OKD:WKt; OKD:38t; OKD:66t; OKD:PKt) and *Trypanosoma cruzi* (TC) arranged according to the lowest number of microtubules. Vertical lines represent the mean; horizontal lines, the range; black areas, the standard deviation; and white areas, the standard error.



OKD: 66t
OKD: WKt
OKD: 38t
OKD: 38bm
a
LT
OKD: 66bm
LMT
OKD: PKt
LMB
LB
OKD: 11
KSD: CH
LD
OKD: 5
TC
60
80
100
120
NUMBER OF MICROTUBULES
200
220
240
260

Figure 24. Dice-Lerass diagrams of microtubule counts for promastigotes of *Leishmania brasiliensis* (LB), *L. tropica* (LT), *L. donovani* (LD), *L. mexicana* (Texas; LMT), *L. mexicana* (British Honduras; LMB), *Leishmania* sp. (Kansas; KSD:CH), Oklahoma dog isolates (OKD:5; OKD:66bm; OKD:38bm; OKD:11), Oklahoma tick isolates (OKD:WKt; OKD:38t; OKD:66t; OKD:PKt) and *Trypanosoma cruzi* (TC) arranged according to variation in ranges of microtubule counts. Verticle lines represent the mean; horizontal lines, the range; black areas, the standard deviation; and white areas, the standard error.

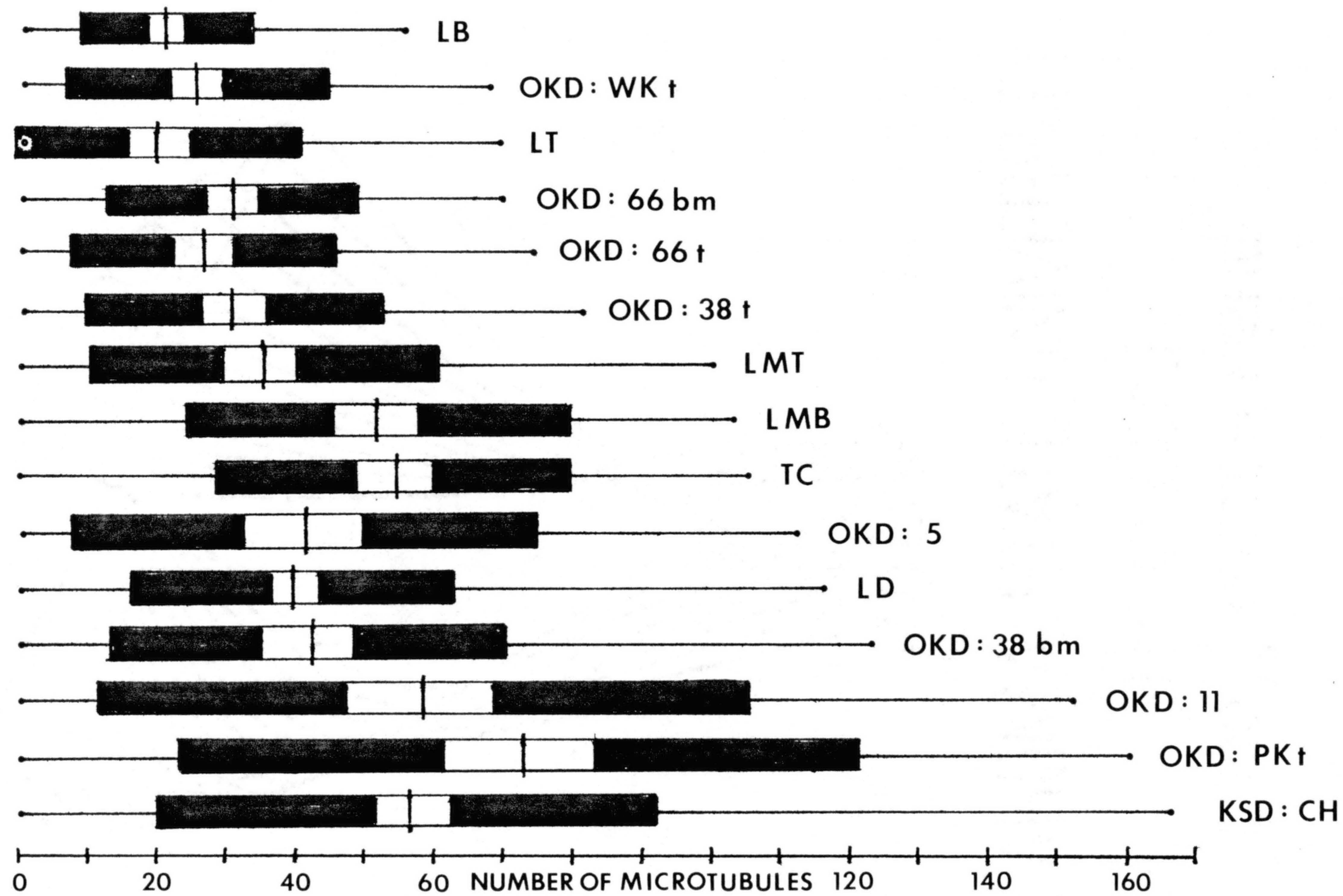
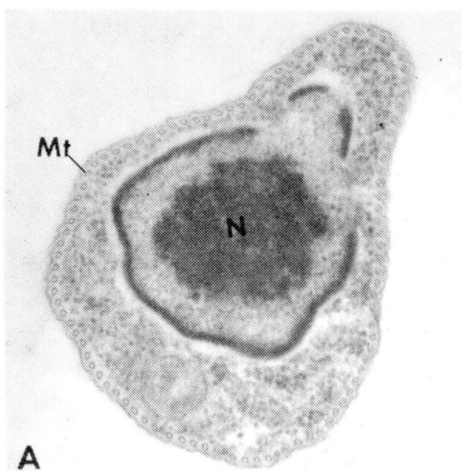
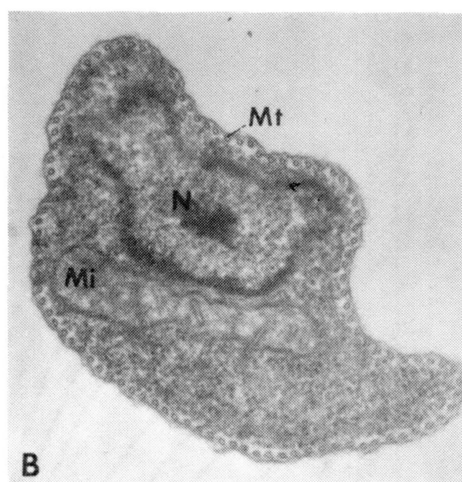
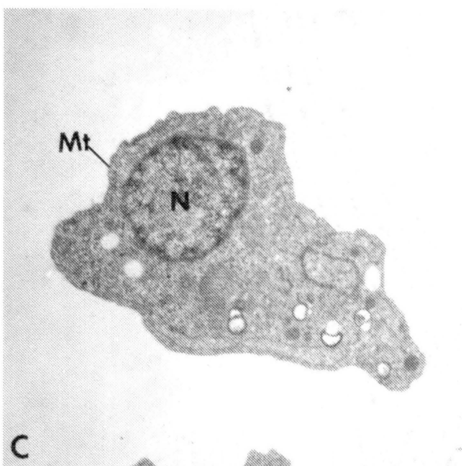
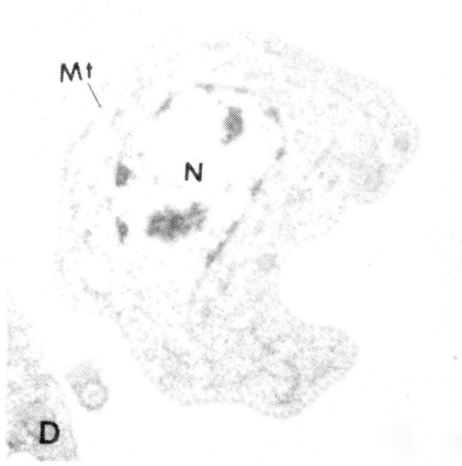

LB
OKD : WK t
LT
OKD : 66 bm
OKD : 66 t
OKD : 38 t
LMT
LMB
TC
OKD : 5
LD
OKD : 38 bm
OKD : 11
OKD : PK t
KSD : CH
0
20
40
60
NUMBER OF MICROTUBULES
120
140
160

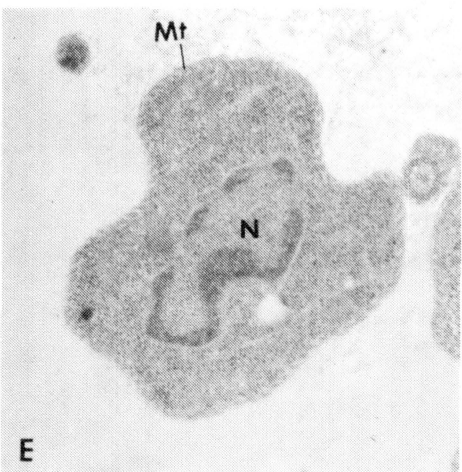
Figure 25. Electron micrographs of promastigotes of *Leishmania donovani* (A), *L. tropica* (B), *L. mexicana* (Texas; C), *L. mexicana* (British Honduras; D), *L. brasiliensis* (E) and *Trypanosoma cruzi* (F). Cross-sections of the promastigotes and epimastigotes show nucleus (N), mitochondria (Mi), and subpellicular microtubules (Mt). Micrograph magnifications: (A) and (B) x 30,500; (C) and (E) x 23,000; (D) and (F) x 17,000.

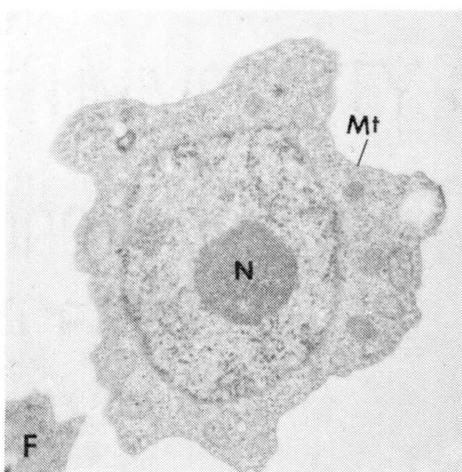

Mt
N
A


Mt
N
Mi
B


Mt
N
C


Mt
N
D


Mt
N
E


Mt
N
F

LMT), L. mexicana (British Honduras; LMB), L. tropica (LT), and Trypanosoma cruzi (TC) similar to those from which microtubule counts were made. Figure 26 shows cross-sections of the canine isolates (OKD:5, OKD:11, OKD:66bm, OKD:38bm, and KSD:CH) and Figure 27 shows the isolates from ticks removed from infected dogs (OKD:WKt, OKD:38t, OKD:66t, and OKD:PKt).

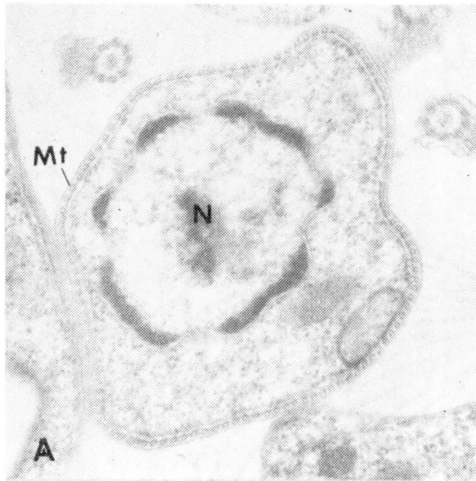
General Morphology

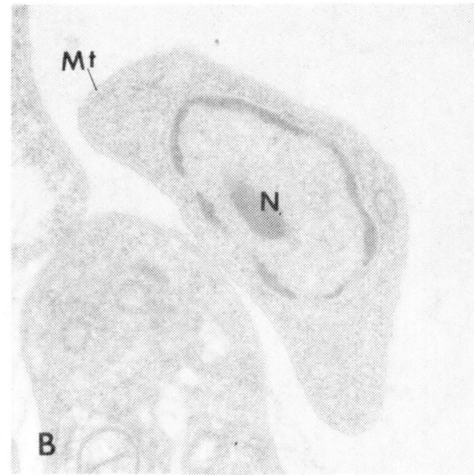
Cytoplasmic membranes can be seen in promastigotes of LMB and epimastigotes of TC (Fig. 28). Though mitochondria were seen in all cells, the size, shape and location varied greatly (Fig. 29). The kinetoplast (K), blephoblast (Bl), flagellar pocket (FP) and flagellum (F) are visible in organisms in Figure 30. Euchromatin and heterochromatin can be seen in the nucleus and occassionally on nuclear membranes (NM) such as those in Figure 31. The 9+2 arrangement of microtubules can be seen in the flagella (Fig. 31). A high degree of variation in dividing organisms was observed by both TEM and SEM (Figure 32). In general Leishmania were more variable in size, shape and flagellar length than Trypanosoma cruzi (TC).

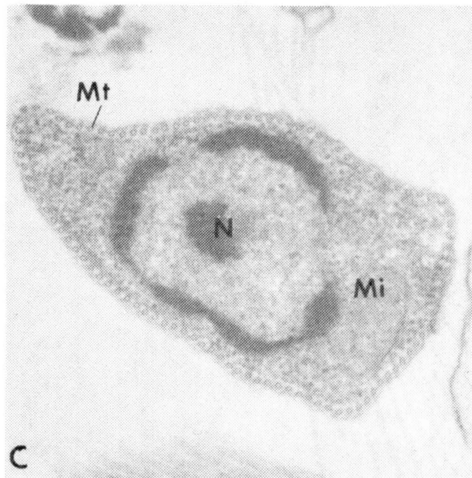
Scanning Electron Microscopy

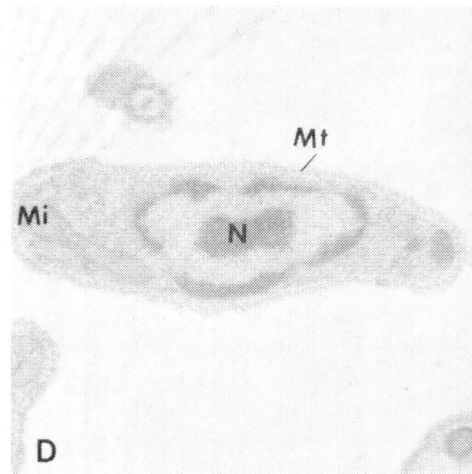
When the leishmanial isolates were compared to Trypanosoma cruzi by scanning electron microscopy, definite

Figure 26. Electron migcrographs of promastigotes of Oklahoma dog isolates OKD:38bm (A), OKD:66bm (B), OKD:5 (C), OKD:11 (D), and a Kansas dog isolate KSD:CH (E). Cross-sections of promastigotes show nucleus (N), mitochondria (Mi), and subpellicular microtubules (Mt). Micrograph magnifications: (A) and (C) x 30,500; (B), (D), and (E) x 23,000.


Mt
N
A


Mt
N
B


Mt
N
Mi
C


Mt
Mi
N
D

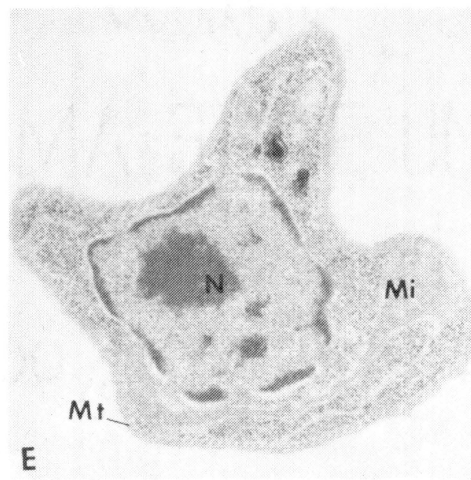
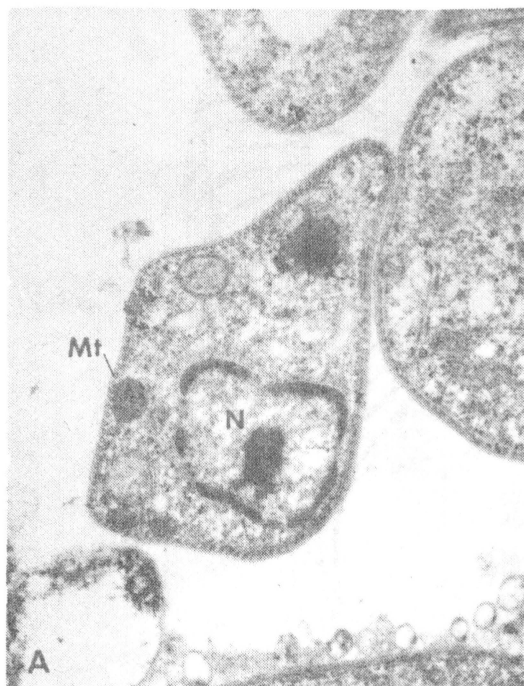
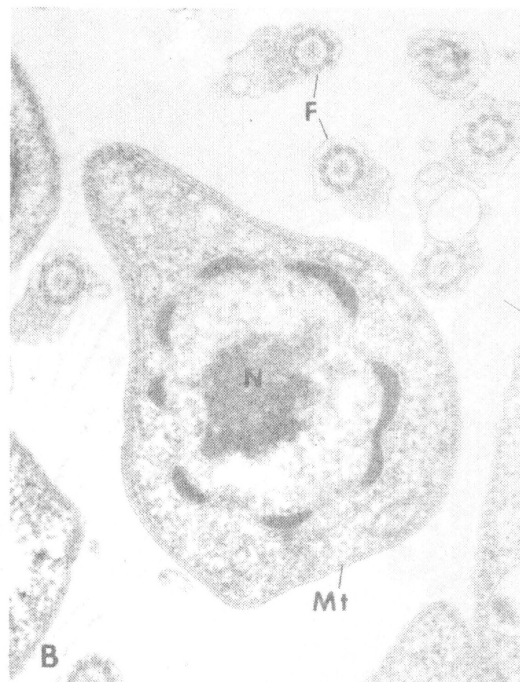
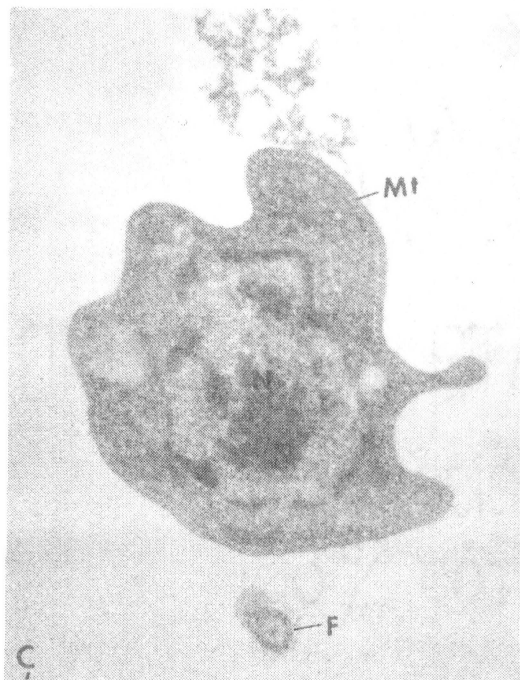

N
Mi
Mt
E

Figure 27. Electron micrographs of leishmanial promastigotes isolated from ticks: OKD:38t (A), OKD:66t (B), OKD:PKt (C), OKD:WKt (D). Cross-sections of promastigotes show nucleus (N), mitochondria (Mi), subpellicular microtubules (Mt), and flagella (F). Micrograph magnifications: (A), (C), and (D) x 23,000; (B) x 30,500.


Mt
N
A


F
N
Mt
B


Mt
N
F
C

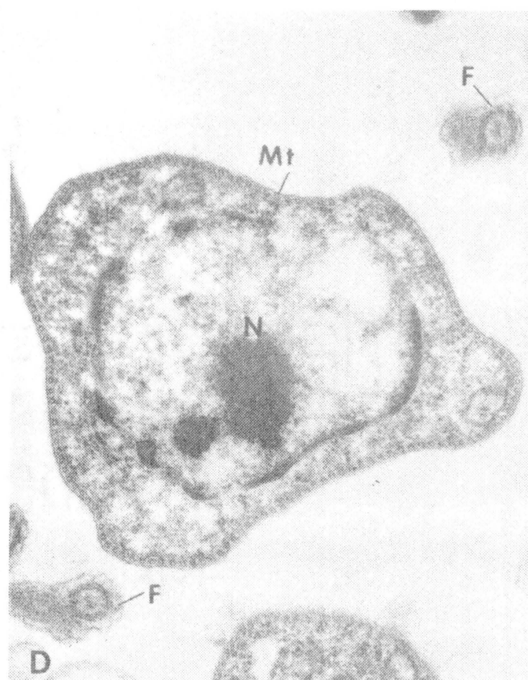
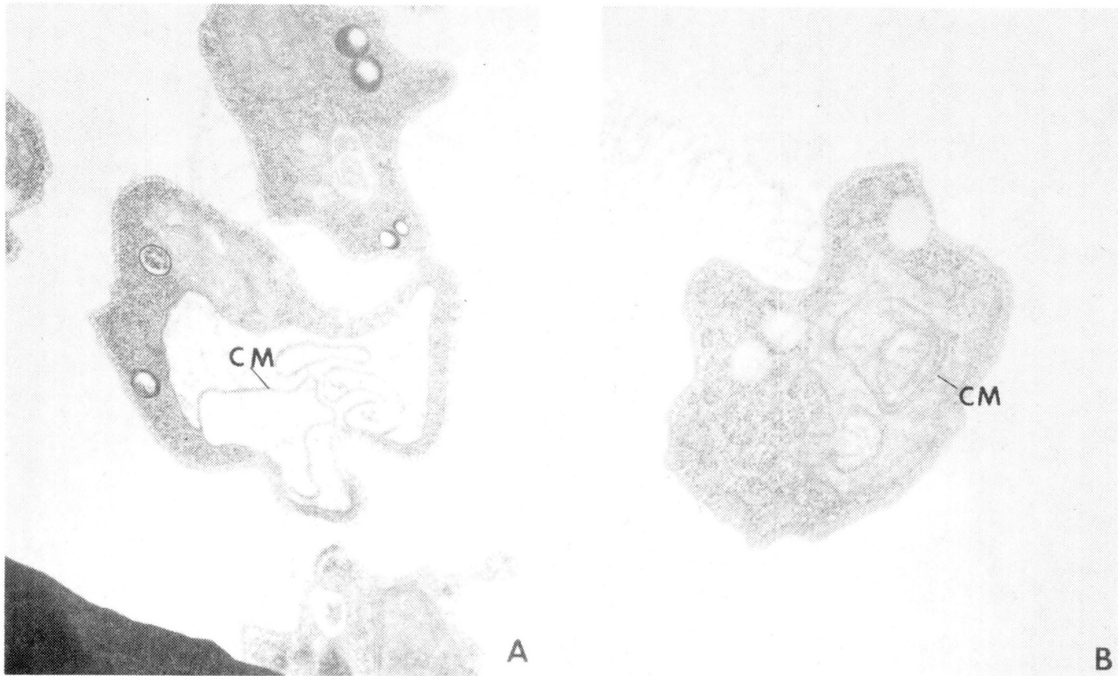

F
Mt
N
F
D

Figure 28. Electron micrographs of promastigotes of *Leishmania mexicana* (British Honduras), (A) and (B) and an epimastigote, *Trypanosoma cruzi* (C), showing cytoplasmic membranes (CM) and a longitudinal section of a flagellum (F). Total magnification: A x 17,000; B x 23,000; C x 30,500.


CM
A
CM
B

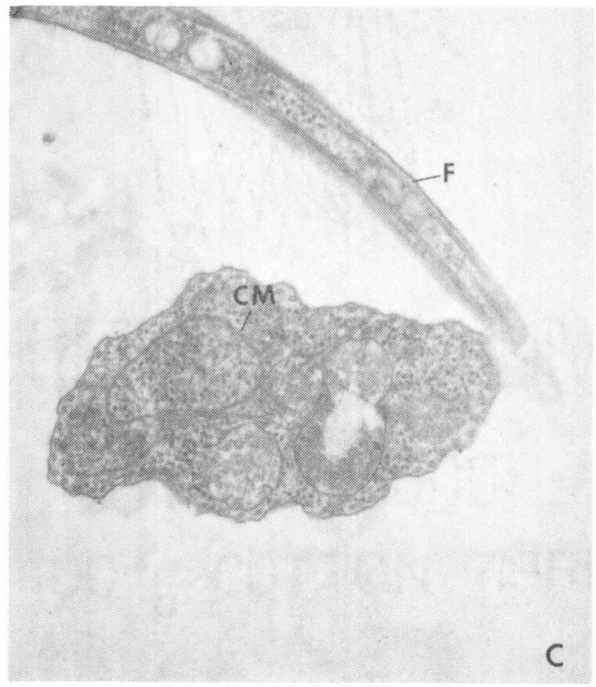
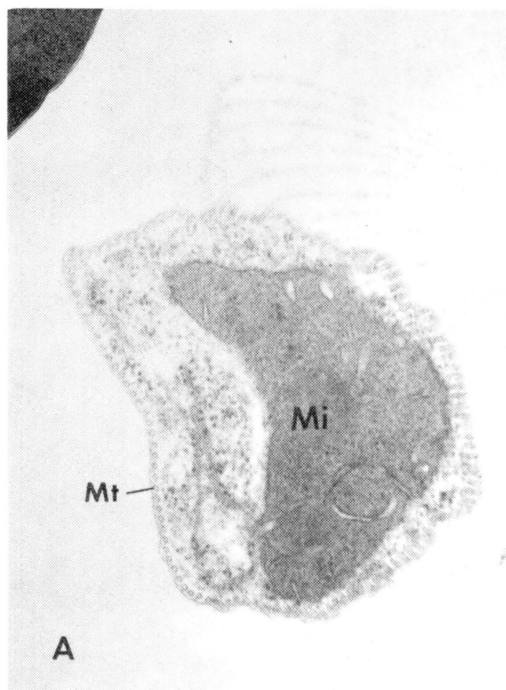
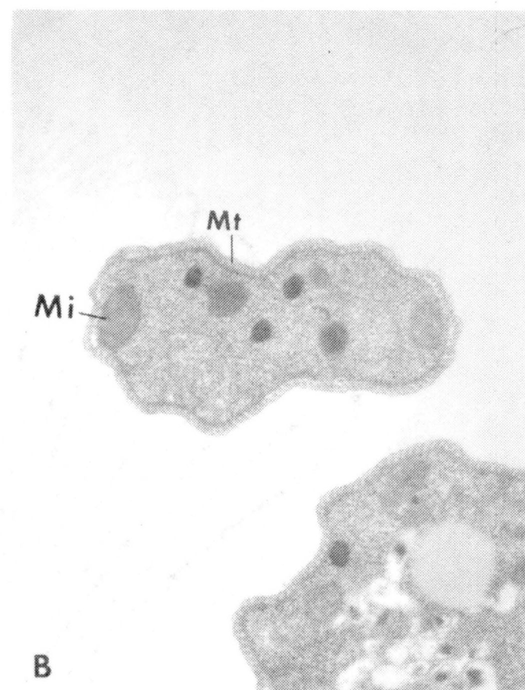

F
CM
C

Figure 29. Electron micrographs of promastigotes of *Leishmania mexicana* (British Honduras) (A) and (C), and *Leishmania* sp. (KSD:CH) (B) showing mitochondria (Mi) and microtubules (Mt). Total magnification: (A) and (B) x 23,000; (C) x 30,500.


Mi
Mt
A


Mt
Mi
B

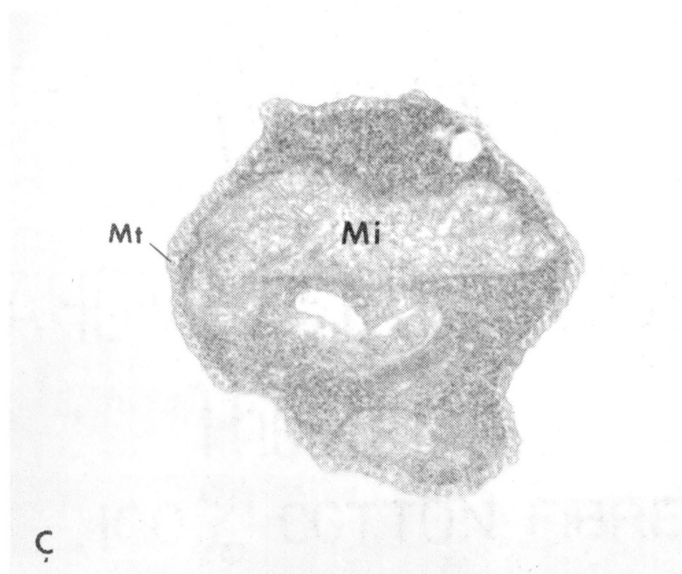
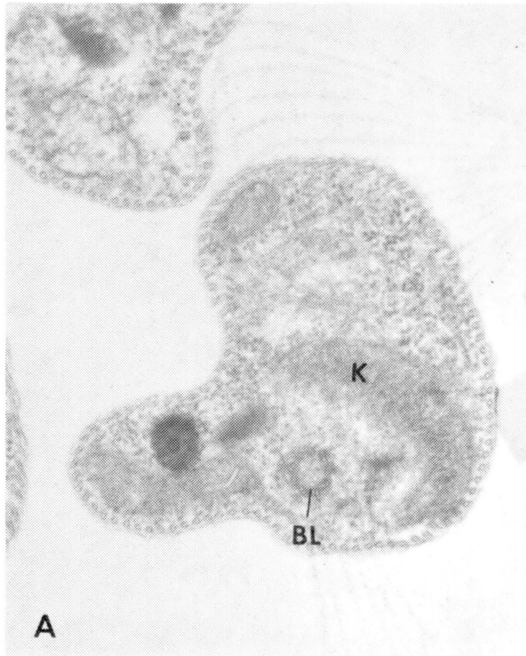
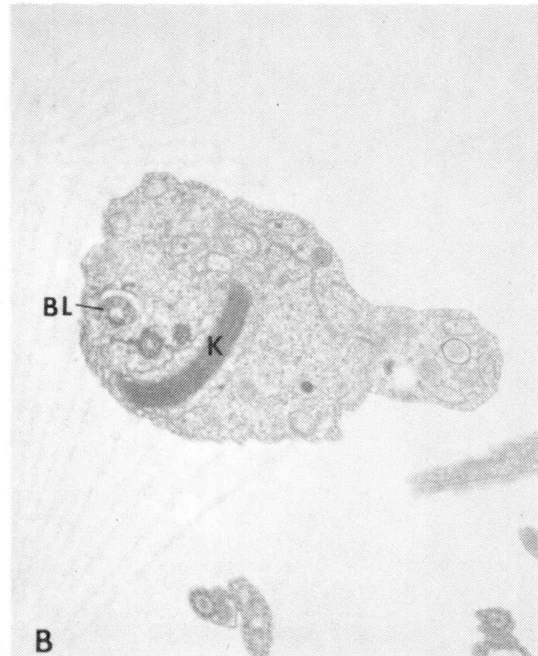
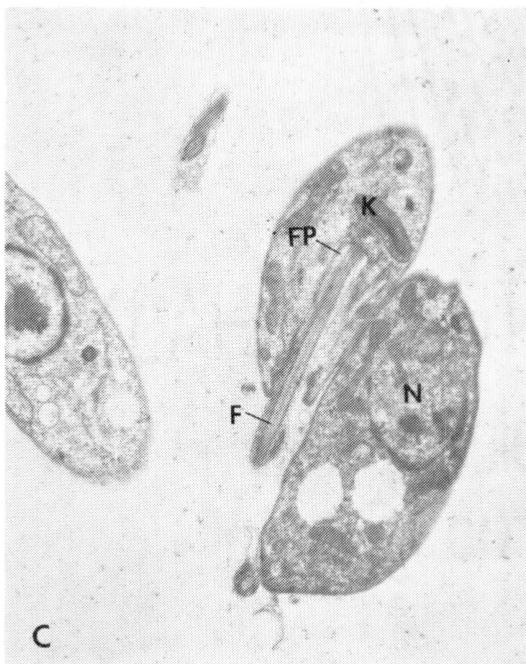

Mt
Mi
Ç

Figure 30. Electron micrographs of promastigotes of *Leishmania donovani* (A), *L. mexicana* (Texas) (B), *Leishmania* sp. (KSD:CH) (C) and an epimastigote *Trypanosoma cruzi* (D) showing kinetoplast (K), blephoplast (Bl), nucleus (N), flagellar pocket (FP), and a flagellum (F). Total magnification: (A) and (D) x 17,000; (B) and (C) x 12,500.


K
BL
A


BL
K
B


K
FP
N
F
C

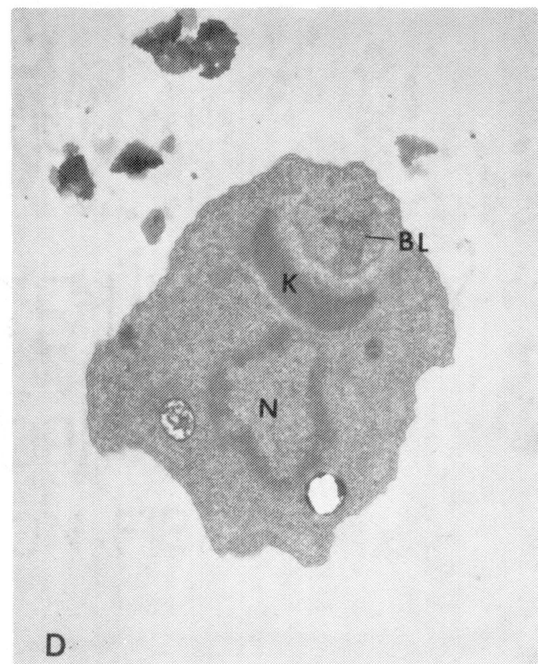
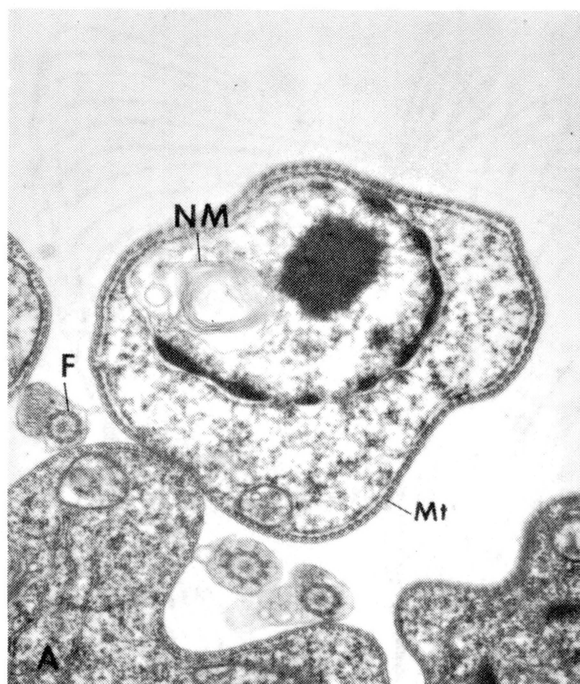

BL
K
N
D

Figure 31. Electron micrographs of promastigotes of *Leishmania* sp. OKD:66t (A) and OKD:66bm (B) showing nuclear membranes (NM), microtubules (Mt), and a flagellum (F). Total magnification: (A) x 23,000; (B) x 30,500.


NM
F
Mt
A

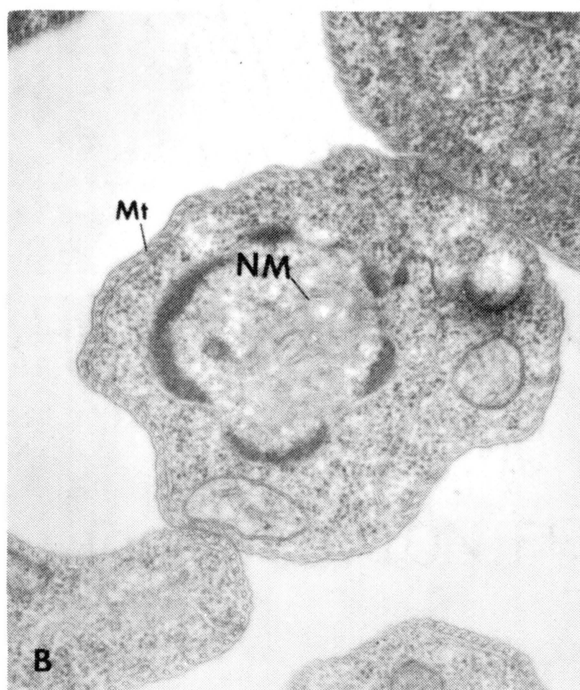
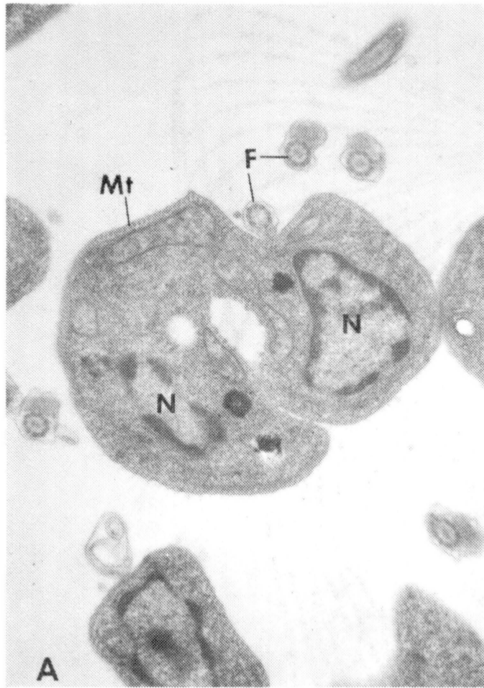
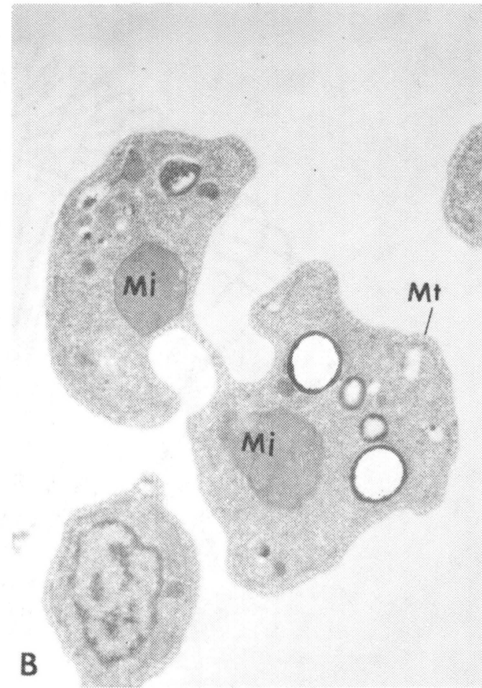
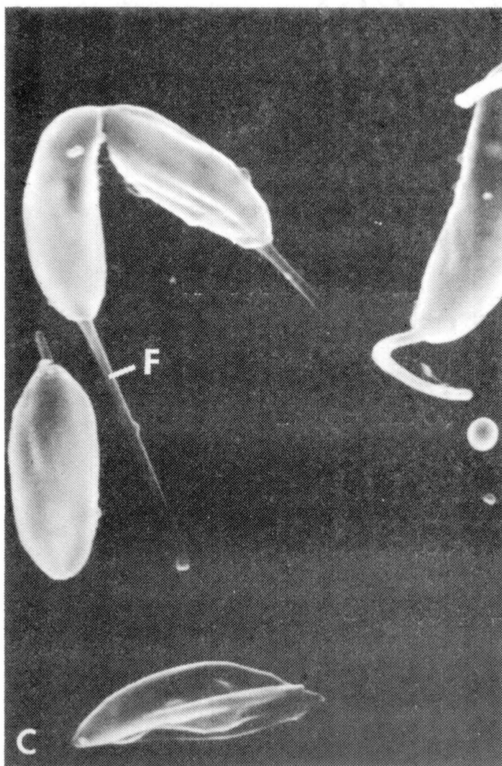
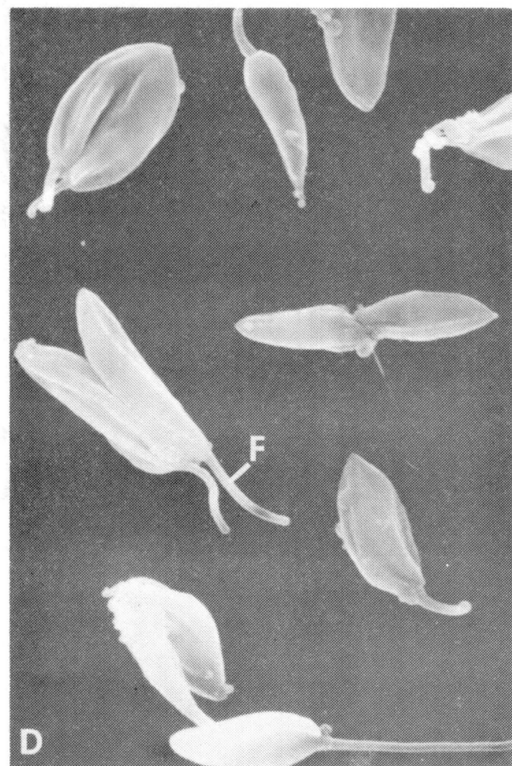

Mt
NM
B

Figure 32. Transmission electron micrographs (A) and (B) and scanning electron micrographs (C) and (D) of promastigotes of *Leishmania donovani* (LD) dividing by binary fission showing nucleus (N), mitochondria (Mi), microtubules (Mt), and flagella (F). Total magnification: A x 17,000; B x 12,500; C x 5,400; D x 4,000.


Mt
F
N
N
A

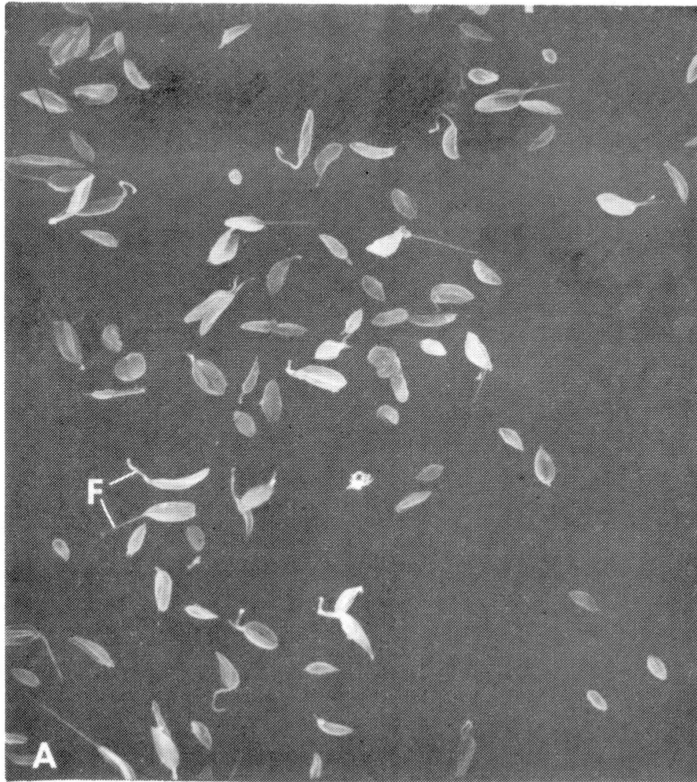

Mi
Mt
Mi
B


F
C


F
D

morphological differences were observed (Fig. 33). The *Leishmania* isolates exhibited a high degree of morphological variation which suggested that unequal binary fission occurred. The presence of both small and large forms indicated a high rate of replication. *Trypanosoma cruzi* epimastigotes (Fig. 34) were highly uniform in length, size and shape. Size consistency indicated a more equal binary fission. In addition, the *T. cruzi* epimastigotes exhibited an undulating membrane which is not found on leishmanial promastigotes. Thus, culture forms of *Leishmania* sp. and *Trypanosoma cruzi* can be easily differentiated using SEM.

Figure 33. Scanning electron micrographs of promastigotes of *Leishmania donovani* (LD) showing size and shape variations and flagella (F). Total magnification: (A) x 500; (B) x 6,000.


F
A

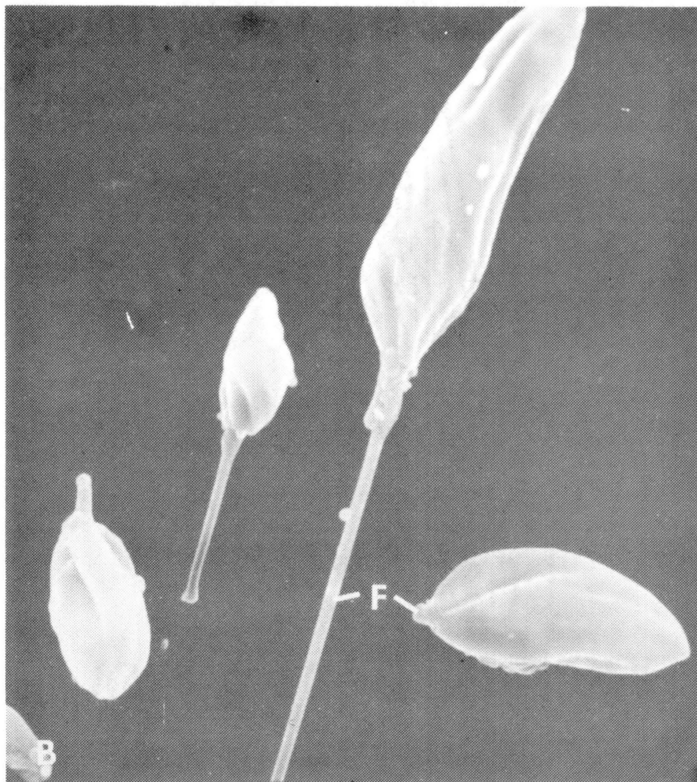
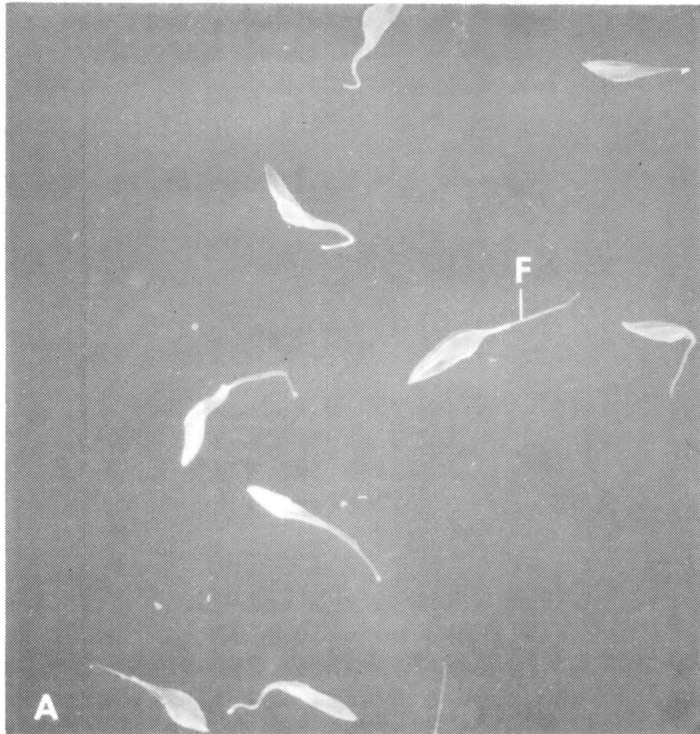
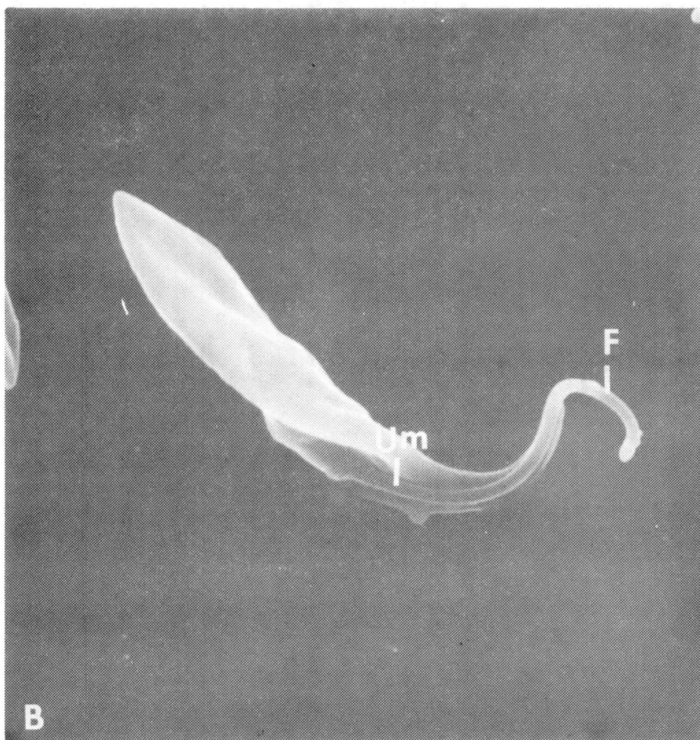

F
B

Figure 34. Scanning electron micrographs of epimastigotes of *Trypanosoma cruzi* showing size and shape consistency, undulating membrane (UM), and flagellum (F). Total magnification: (A) x 510; (B) x 6,000.


F
A


F
Um
B

CHAPTER IV

DISCUSSION

Survival of *Leishmania* Species in Ticks

Under normal circumstances leishmaniasis is transmitted by the sandflies *Phlebotomus* and *Lutzomyia* however, Lewis (1974) reported that several species of the Trypanosomidae may be transmitted by ticks. Krinsky and Burgdorfer (1976) observed trypanosomes of an unknown species in the hemolymph in *Amblyoma americanum* while looking for rickettsiae; also, Hoare (1972) observed trypanosomes (presumably *Trypanosoma theileri*) in ticks which had previously fed on cattle. Cross (1923) reported the successful transmission of *Trypanosoma evansi* in laboratory rats and cattle using *Ornithodoros crossi*. He further observed that these parasites appeared to undergo certain morphological changes within the tick.

Sherlock (1964) found leishmanial promastigotes in engorged *Rhipicephalus sanguineus* which he removed from infected dogs in Brazil. The survival of *Leishmania* in ticks for long periods, and through molting, was demonstrated in the present study (Table II). This represents the first observation of transstadial transmission of *Leishmania* in ticks to be documented.

Promastigotes of the canine *Leishmania* isolates (Oklahoma and Kansas) could be found in the experimentally infected ticks up to 160 days post-feeding (exposure) and 130 days post-molting. Under field conditions 130 days would allow ample time for the infected ticks to locate a new host, feed and possibly transmit the disease. This suggests that under certain conditions *Rhipicephalus sanguineus* might be a suitable vector for some *Leishmania* species.

It was not determined whether the leishmanial parasites underwent developmental changes in the tick. Because transmission from ticks to dogs occurred in the present study, developmental changes such as those described for sandflies (Lewis, 1974; Olsen, 1974; Hommel, 1978) may have taken place. Neither was it determined whether the parasites multiplied within the ticks; in fact, it appeared that the number of organisms decreased based on increased difficulty in finding positive cultures as time passed. It is possible that multiplication roles may have decreased as the blood in the gut was digested creating a lack of nutrients for parasite growth.

Table II also shows that the ticks which were fed on the naturally infected dog from Kansas carried the parasites longer (160 days total) than those that fed on the dog from Oklahoma (95 days). The Kansas dog appeared to carry more parasites within its tissues as was assessed by serological

responses, the numbers of infected macrophages in bone marrow smears, and the ability to culture organisms from bone marrow and lymph node aspirates. The ticks which fed on the Kansas source dog probably ingested more organisms with blood meals than did those which fed on the dog from Oklahoma. In both cases it became more difficult to find the organism in cultures as time passed. By day 75 few organisms were seen and the multiplication rate in culture was slow and all cultures were negative by day 160.

One of the control-tick cultures (day 50 post-exposure) contained a flagellate. It was not determined if it was a leishmanial promastigote because only one organism was observed. It is possible that an infected tick (or infected gut material) was mixed with control ticks, however, this occurred only once during the 220 days that cultures were examined and was considered an incidental finding.

The technique of culturing gut material from ticks proved to be a valuable method for diagnosis of _Leishmania_ in dogs. Because ticks appeared to contain progressively fewer promastigotes as time passed, it was usually necessary to examine cultures for a longer period to find organisms. It was also necessary to observe cultures on a daily basis to avoid missing organisms.

Transmission From Ticks to Dogs

It was evident that _Leishmania_ infections were

successfully transmitted from ticks to dogs in at least one experiment. Infections were monitored in dogs using serology, xenodiagnosis with ticks and direct culture of bone marrow and lymph node aspirates.

Serology

The FIAX serological assay proved to be a reliable test for detecting antibody responses to *Leishmania* in dogs by using antigens prepared from an Oklahoma isolate of canine *Leishmania* (OKD #11). Seroconversions in exposed dogs were observed (Fig. 2) with the infected control dog producing the strongest antibody response to the leishmanial parasites. The prepatent period was approximately 6 weeks after which time seroconversion was evident. The peak antibody response was measured approximately 19 weeks after the infected bone marrow was given. This suggested that the antibodies were most likely produced against living parasites, and not just those that were present in the inoculum (Bryceson, 1972). Tizard (1982) stated that the normal response time for an inoculation with *Leishmania* (using dead or attenuated organisms) was approximately 1 week with the peak antibody response occurring 10 to 14 days later. The peak was always followed by a rather rapid decline in the amount of antibody. Tizard concluded that the amount of antibody produced was directly proportional to the amount of antigenic stimulus provided.

A dog which had infected ticks placed upon it (uninterrupted feeding, Fig. 18) and the dog that received infected bone marrow (Fig. 2), had similar antibody responses (Fig. 18). The antibody response in the tick exposed dog suggested that transmission of Leishmania had occurred through infected ticks. Appreciable antibody levels were observed in both of the dogs that were exposed to ticks which had previously fed on naturally infected dogs (KSD or OKD); the highest titers were in the pup exposed to the OKD organisms. This was unexpected as the ticks that fed on the KSD source animal were culture-positive for a longer period.

The serological patterns observed in the dogs exposed to infected ticks closely followed the pattern in the infected control dog; however, antibody levels were never as high as in the infected control animal. In the dog used for uninterrupted feeding by infected ticks peak antibody response occurred at approximately 15 weeks post-inoculation as compared to 19 weeks for the infected bone marrow control. In all animals the peak titers were followed by a rapid decline in antibody levels. Some of the dogs that were inoculatd with infected tick-gut homogenates also appeared to serconvert (Figs. 6 and 10) although these antibody levels were lower than the infected control or the uninterrupted tick feeding dogs.

Dogs in which transmission was attempted by interrupted

feeding of exposed ticks failed to demonstrate increased antibody levels to *Leishmania*. Possible explanations for the lack of seroconversion include that the ticks might have been damaged in the transfer or that such low numbers of *Leishmania* were transmitted that the parasites failed to establish. It is also possible that the 3 days that the ticks fed on the infected dog was not enough time for the ticks to pick up sufficient numbers of parasites to successfully transmit the disease.

The dog inoculated with homogenate from incubated ticks (Fig. 6) exhibited a low level antibody peak that occurred at 25 weeks post-inoculation. The peak response was greatly delayed when compared to the 19-week peak for the infected bone marrow control dog (Fig. 2) and 15 weeks for the dog on which infected ticks were fed without interruption (Fig. 15). The peak antibody response was followed by a gradual decline.

An animal inoculated with homogenate from unincubated, infected ticks did not exibit seroconversion. Infected ticks were incubated at 37 C for 2.5 days prior to removing the guts. This was done to determine if an incubation temperature of 37 C would enhance infectivity. Kocan et al. (1982) showed that incubation temperature of 37 C affected the infectivity of *Anaplasma marginale* in cattle. The higher incubation temperature shortened prepatent periods and apparently enhanced infectivity. Young (1979) using

Rhipicephalus appendiculatus, demonstrated that a 37 C incubation of temperature enhanced transmission and infectivity of *Theileria parva* for cattle.

Incubation of ticks infected with *Leishmania* at 37 C did not seem to enhance the infectivity to dogs as an antibody titer could not be measured in this animal (Fig. 10) nor were any parasites recovered from this animal. Because all the incubated, interrupted and uninterrupted exposed dog groups had higher antibody titers than the dogs inoculated with homogenates from incubated ticks; it is possible that the elevated incubation temperature was harmful to the parasites. This would not be unexpected when it is considered that promastigotes develop in the normal vectors and *in vitro* cultures at temperatures of 22-25 C. When *in vitro* cultures are incubated at 37 C the parasites revert to non-motile amastigote forms which may not be infective (Hoare, 1972).

When the antibody titers began to decline all animals were splenectomized in an attempt to stress them sufficiently to encourage parasite development and facilitate recovery of the organisms from the dogs. When splenectomization failed to produce increased titers, the dogs were started on a prolonged corticosteroid treatments. The corticosteroid treatments caused an increase in the serum antibody titers and, presumably, the parasite levels. Following 3 weeks of daily corticosteroid treatments it was

possible to recover parsites from bone marrow and lymph nodes of two of the of the exposed animals. Slightly elevated antibody titers were seen in most dogs, but significant increases occurred only in the dog exposed by uninterrupted tick feeding and the infected control dog.

Xenodiagnosis

Xenodiagnosis was first described for diagnosis of Chagas' disease by Faust et al. (1975). They allowed laboratory-reared triatomid bugs (Panstrongylus magistus, Triatoma infestans, Triatoma dimidiata and Rhodnius prolixus) to feed on a patient and infected epimastigotes of Trypanosoma cruzi could be found in the intestinal contents of the bugs after 10 days. Xenodiagnosis using ticks proved useful in the present study for detecting low numbers of leishmanial parasites in infected dogs.

Prior to termination if the study, xenodiagnosis was attempted on selected dogs (infected bone marrow control and those exposed to uninterrupted feeding of infected ticks). Both nymphal and adult stages of Rhipicephalus sanguineus contained Leishmania when cultured, whereas none were found in unexposed control ticks or ticks which fed on the KSD-exposed dog.

Organisms were usually observed in the cultures of tick-gut material within 5 days after detachment. This appeared to be a more reliable means of diagnosis than bone

marrow and lymph node aspirate cultures. The latter methods usually required 9 or more days to find the organisms in cultures. Apparently ticks were able to ingest and facilitate parasite multiplication which made it easier to detect them in culture.

Berman et al. (1979) stated that Leishmania sp. have the ability to enter macrophages and prevent the fusion of the phagosome with the lysosome. This allows the parasite to multiply within the phagosome without being killed. Anderson et al. (1980) noted numerous infected macrophages within the skin of a clinically ill foxhound from Oklahoma. Inflammatory responses caused by prolonged tick feeding may possibly attract infected macrophages into the area where they may be ingested by the ticks. This may be the reason xenodiagnosis was effective. Balashov (1968) reported that ticks are slow and persistent blood feeders, and this would give infected macrophages time to be attracted into the area and be ingested.

Vital Signs

Marsden (1979) observed that humans with visceral leishmaniasis exhibit intermittent fevers, and Hommell (1978) described chronic fevers in dogs with leishmaniasis. During the course of this study high temperatures were observed in all of the exposed dogs.

The highest temperatures (106 F) were seen in the dogs

exposed by uninterrupted feeding of infected ticks (Fig. 19). Temperatures of 104 F were also observed at various times in dogs inoculated with unincubated infected tick homogenate (Figs. 7 and 11), incubated tick homogenate (Fig. 11) and a dog exposed by interrupted feeding of infected ticks (Fig. 15). The lowest temperature of 103 F was observed in the infected control dog (Fig. 3). The animal exposed to OKD organisms through ticks exhibited higher temperatures than did the KSD-exposed dogs. It was interesting to note that the dogs from which parasites were isolated had both the highest (106 F) and lowest (103 F) temperatures. Normal rectal temperatures in dogs as reported by Blood and Henderson (1979) range from 100 F to 103 F. In this study temperatures were elevated only in pups which had been exposed to Leishmania (Figs. 3 and 19). The elevated temperatures may have resulted from environmental influences because the exposed dogs were housed in different facilities than the control dogs.

Respiration rates did not differ appreciably in the experimental dogs except for one animal that was exposed by uninterrupted feeding (Fig. 20). In this dog the respiration rate increased at month 7 and remained high until the end of the experiment. The cause of the higher respiration rate was not determined. No significant changes in heartrates were observed.

Blood Parameters

Blood parameter values were not useful for detecting the early stages of leishmaniasis in the present investigation. Garett (1978) stated that blood chemistry values and complete blood counts remained within normal ranges in a 4-year-old male dog with clinical leishmaniasis. On the other hand Anderson et al. (1980) reported anemia, a slightly elevated leukocyte count, neutrophilia and elevated serum protein levels, whereas glucose, BUN, and SGPT were within normal limits. Blood parameters were useful, however, for measuring the effects of steroid treatments.

Culture Methods

Bone marrow and lymph node cultures were done on all experimental dogs at necropsy, and cultures from the infected bone marrow control dog and a dog exposed to infected ticks (OKD) by uninterrupted feeding were positive for leishmanial parasites. All other dogs failed to exhibit the parasite.

Impression smears failed to demonstrate the presence of parasites in the exposed dogs. Anderson et al. (1980) and Garett (1978) reported impression smears as useful tools in diagnosing leishmaniasis. In the present investigation cultures of tissues or tick-gut contents were the most reliable diagnostic tests.

Two dogs died during the course of the study; one which received unincubated tick-gut homogenate and the other was exposed by interrupted tick-feeding. The deaths of these animals resulted from parvovirus infections. All dogs were vaccinated once but did not receive a booster. Six of the dogs contracted the disease and were immediately isolated from the remaining animals. The sick dogs were treated with antibiotics and IV drips with lactated ringer solution. The source of the outbreak was not known.

Examination of Tissues Following Splenectomies and Corticosteroid Treatments in Exposed Dogs

Leishmainasis was diagnosed in a 7-year-old female foxhound by using impression smears of bone marrow and prescapular lymph nodes (Anderson et al., 1980). In the present investigation, organisms were not found in impression smears of these tissues. Schnur et al. (1973) reported that dissemination of the parasites in a Syrian hamster took 3 to 5 months. Parasites were not numerous in the experimental dogs even after a full year. Because seroconversion occurred at about 3-6 months it is possible that infections were under control in these animals. This raises a question concerning the susceptibility of healthy dogs.

Several kinds of laboratory animals such as hamsters,

mice, and guinea pigs (Wilson et al, 1979; Scott and Farrel, 1982; and Levine, 1973) have been used for *in vitro* cultivation of various *Leishmania* species. Cotton rats, gerbils, mice and hamsters have been shown to be susceptible to the Oklahoma canine isolates of *Leishmania* (McKenzie and Fox, unpublished data). In the latter observations, cotton rats, gerbils and white mice remained infected more than 1 year in the laboratory. Hamsters inoculated with spleen homogenate and bone marrow from the exposed dogs failed to develop leishmaniasis. This was possibly due to limited dissemination of organisms in the dog. Kreutzer et al. (1983) using multiple isozyme analysis classified the dog isolate as a strain of *L. mexicana mexicana*. If his classification was correct, then it may be that the Oklahoma leishmanial isolates would most likely be found in the cutaneous tissues and not the visceral organs.

A bone marrow transfer from the infected bone marrow control dog, which showed a high antibody titer and was culture positive, failed to produce an infection in a splenectomized recipient dog. According to Brown and Nova (1983) *L. m. mexicana* readily metastaizes to produce satellite lesions or subcutaneous nodules. If the Oklahoma dogs were infected with *L. m. mexicana* as postulated by Kreutzer et al. (1983), the parasites may have been located in the skin. Bone marrow transfer of infection from naturally infected dogs to hamsters and recipient dogs was

accomplished several times previously with the Oklahoma *Leishmania* sp.

Electron Microscopy

Transmission Electron Microscopy

Gardener et al. (1977) showed that subpellicular microtubule counts for amastigotes of *L. donovani* in macrophages (visceral disease) differed significantly from those of *L. mexicana* and *L. m. amazonesis* (cutaneous disease). Microtubule counts for *L. donovani* ranged from 58 to 120 (mean = 81), whereas the cutaneous forms ranged from 86 to 160 (mean = 118). The large differences in mean microtubule counts seemed to provide a reliable method for distinguishing between visceral and cutaneous forms of leishmaniasis.

Veress et al. (1980) compared microtubule counts of what were thought to be visceral and cutaneous forms of leishmaniasis from the Sudan. He found that the organism, which produced various types of human disease, were all visceral forms based on consistently low microtubule counts (range 90 to 93). In addition, Kocan et al. (1983) reported on the amastigote ultrastructure for the *Leishmania* species found in dogs from Oklahoma (OKD). The microtubule counts for the OKD isolate ranged from 52 to 81 (mean = 67), thus it was concluded that the *Leishmania* species in dogs in Oklahoma were morphologically most similar to *L. donovani*, a

visceral form. This conflicts with Kreutzer's (1983) classification of the Oklahoma dog isolate which he determined to be L. mexicana mexicana.

In the present investigation, promastigote subpellicular microtubules were counted for several species. When the isolates were compared using mean microtubule counts (Fig. 22), L. brasiliensis had the lowest mean counts while a tick isolate (OKD:PKt) had the highest. Trypanosoma cruzi also had a low mean microtubule counts.

The tick isolates (OKD:WKt, OKD:38t, and OKD:66t) with the exception of OKD:PKt tended to have mean microtubule counts of approximately 130 (Fig. 27). The OKD:PKt isolate had a mean microtubule count (150) which was considerably higher than the others. This may have rulted from the fact that OKD:PKt had been in culture for more than a year longer than the other tick isolates. Most of the tick isolates were statistically similar to L. mexicana (Fig. 20) when compared according to increasing mean microtubule counts.

The isolates obtained directly from dogs (OKD:38bm, OKD:11, OKD:66bm, OKD:5 and KSD:CH) had dissimilar mean microtubule counts (Fig. 26). Isolate OKD:5 had the mean count and was statistically similar to L. brasiliensis and L. tropica. The OKD:66bm isolate had an intermediate mean microtubule count and was more similar to Trypanosoma cruzi and L. mexicana from Texas. The OKD:38bm, OKD:11 and KSD:CH isolates had high mean microtubule counts and were most

similar to OKD:PKt.

Isolates of *L. brasiliensis*, *L. tropica*, *L. donovani*, *L. mexicana* from Texas and *L. mexicana* from British Honduras had high mean microtubule counts (Fig. 25). *Leishmania donovani*, *L. tropica*, and *Trypanosoma cruzi* were statistically similar, as were *L. mexicana* from Texas and *L. mexicana* from British Honduras.

When the isolates were compared by minimum number of microtubules (Fig. 23), the isolates were in different positions as compared to the arrangement observed with mean microtubule counts. *Trypanosoma cruzi* had the lowest minimum number microtubules and OKD:66t had the highest. The tick isolates, except OKD:PKt, were located together in the upper range of minimum counts and were statistically similar to the dog isolate OKD:38bm. Isolate OKD:PKt was in the middle range of minimum counts and was statistically similar to OKD:11 and KSD:CH.

Those dog isolates that had been subcultured for the longest period (OKD:11, OKD:5, and KSD:CH) had the lowest minimum microtubule counts (approximately 80), whereas the more recent isolates (OKD:38bm and OKD:66bm) had higher minimum counts (approximately 90). OKD:1 and KSD:CH were statistically comparible to *L. mexicana* from British Honduras. When using the minimum microtubule counts (OKD:5) was more similar to *T. cruzi* and *L. donovani*; OKD:38bm was similar to *L. mexicana* from British Honduras; and OKD:66bm

was similar to *L. tropica* and *L. mexicana* from Texas.

Based on variations in range (Fig. 24), the ranking showed that *L. brasiliensis* had the more narrow range and KSD:CH had the widest. *Trypanosoma cruzi* was intermediate in range as ranked with the leishmanial isolates. The wider ranges were observed in OKD:11, OKD:PKt, KSD:CH. This may have resulted from the extended length of time that these isolates had been maintained in culture and were adapted for rapid growth in culture. The more recent isolates (OKD:WKt, OKD:66bm, OKD:66t, OKD:38t) with the exception of OKD:38bm had much shorter ranges in microtubule numbers indicating that they possibly grew slower in culture.

An additional difference in the isolates can be seen on Figure 24 when they were grouped as slow or fast growers on artificial nutrient medium. The slow growers (*L. brasiliensis*, OKD:WKt, *L. mexicana* from Texas, and *L. mexicana* from British Honduras) tended to have small variation in range whereas fast growers (OKD:11, OKD:PKt, KSD:CH) had a large variation in range.

The use of promastigotes to differentiate species of *Leishmania* was not possible due to overlapping microtubule counts for the various species. Even the epimastigotes of *T. cruzi* could not be differentiated from *Leishmania* sp. on the basis of transmission electron microscopy and the ultrastructural characteristics.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) appeared to be useful for differentiation of *Leishmania* parasites from *T. cruzi* (Figs. 33 and 34). Three forms of *Leishmania* (rounded with no flagellum, oval with short flagellum and elongate with long flagellum) could be observed in all leishmanial cultures. *Trypanosoma cruzi* was more consistent in size and shape. Perhaps the differences in form may be attributed to the characteristic unequal binary fission in *Leishmania* as reviewed by Levine (1973) and equal binary fission in *Trypanosoma* sp. (Hoare, 1972).

The SEM micrographs of leishmanial promastigotes (Fig. 32) supported the observation that short and long forms divided. Perhaps this was one explanation for considerable variation in microtubule counts.

Conclusions

This study showed that ticks were able to transmit canine leishmaniasis. It was demonstrated that the ticks were able to harbor the parasites for long periods and through a molt. The ticks were able to transmit the organisms to susceptible pups during normal feeding processes which resulted in an infection. *Leishmania* were re-isolated from two of the exposed dogs and from tick-gut contents by culturing.

The FIAX serology was useful in detecting antibody levels in the naturally infected and exposed dogs. Corticosteroid treatments enhanced parasite development and elevated antibody titers where splenectomy failed to do so.

Electron microscopy study revealed only minor differences among the leishmanial isolates. Transmission electron microscopy was useful in studying the internal morphology of the parasite, and scanning electron microscopy could be used to distinguish between *Leishmania* species and *Trypanosoma cruzi*. SEM preparation demonstrated three different forms of the leishmanial promastigotes. *Trypanosoma cruzi* exhibited only one form which was consistent in its size and shape.

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Thesis: A STUDY OF THE TRANSMISSION OF CANINE LEISHMANIASIS BY THE TICK *RHIPICEPHALUS SANGUINEUS* (LATREILLE) AND AN ULTRASTRUCTURAL COMPARISON OF THE PROMASTIGOTES.

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