Rev. Inst. Med. trop. São Paulo
22(5):219-230, *setembro-outubro*, 1980

UDC 593.161.13
616.937.3-092.9

THE EFFECT OF IRRADIATED TRYPANOSOMA CRUZI ON THE PATHOGENESIS OF CHAGAS' DISEASE IN DOGS

Michael J. TOMLINSON (1), Willie L. CHAPMAN Jr. (1), William L. HANSON (2) and Arthur J. GOVEN (2)

SUMMARY

Dogs were given 5 weekly injections of 3.26-8.42 x 10^8 irradiated **T. cruzi** from cell culture via the intraperitoneal route. The irradiated parasites were not capable of producing infections in either Vero cell cultures or C_3H mice. One week after the last injection of irradiated parasites, the dogs were challenged by intravenous injection of 5.0 x 10^4 virulent **T. cruzi** in mouse blood per kg of body weight. Although dogs receiving irradiated parasites had a significant response to **T. cruzi** antigen as measured by the lymphocyte blast transformation test, they were not significantly protected against a challenge infection as indicated by death of 4 or 5 dogs. Neither cardiac dysfunction nor other clinical signs were observed prior to sudden death of challenged dogs. Maximum mean parasitemia in dogs given irradiated parasites was 1.6 x 10^4 trypomastigotes per ml blood 28 days after challenge and was 8.8 x 10^4 trypomastigotes per ml blood 21 days after challenge in nonvaccinated control dogs. Antibody titers determined by direct agglutination test were 1:64 or less in dogs receiving irradiated parasites but no challenge infection. In dogs receiving irradiated parasites and challenge infections, titers ranged from 1:512 to 1:2048. In dogs receiving only challenge infections, titers ranged from 1:16 to greater than 1:4096. In dogs surviving acute **T. cruzi** infection, maximum titers were greater than 1:4096 in 3 dogs and 1:512 in one dog. Mean serum creatine phosphokinase activities were elevated during the acute infection in dogs receiving irradiated parasites prior to challenge. In dogs receiving only challenge infections, mean serum creatine phosphokinase and mean serum aspartate aminotransferase activities were also increased during the acute infections. The primary necropsy finding in all dogs challenged with the virulent parasites was a hypertrophied, dilated heart with hemorrhages. These changes were most severe in the right ventricle. All dogs dying with acute **T. cruzi** infection had severe, necrotizing granulomatous myorcarditis, but cardiac muscle contained few intracellular clusters of amastigotes of **T. cruzi.**

INTRODUCTION

Chagas' disease, caused by the stercorarian hemoflagellate **Trypanosoma cruzi**, is a widespread zoonosis which extends from Argentina and Chile into the United States [36]. In the United States, **T. cruzi** has been isolated from either insect vectors **(Triatoma spp.)** or animal hosts in California [34], Texas [27], Alabama [22], Florida and Georgia [19,24] and Maryland [32]. Although some strains of the parasite isolated from vectors in the United States were found to be of low virulence in laboratory animals [13, 34] at least two indigenous cases of Chagas' disease in man have occurred in the United States [9,35].

Fatal, naturally occurring Chagas' disease in dogs has been reported from Indiana [31], South Carolina [21], and Texas [33]. In some endemic areas

(1) Departments of Veterinary Pathology and
(2) Parasitology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, U.S.A.

of South America, particularly those in which **Triatoma infestans** is the primary vector, dogs are recognized as an important peri-domiciliary host [36]. Chagas' disease in dogs is thus of considerable significance because of the severe disease in the dog as well as the public health significance due to vector transmission from infected dogs to man.

Many antibiotic and synthetic compounds, including drugs with activity against African trypanosomes and the leishmanias, have been used in the treatment of Chagas' disease. While some drugs, such as nitrofurtimox and primaquine, have some **in vivo** activity against **T. cruzi**, no satisfactory curative drug is currently available [25].

A variety of preparations have been used in attempts to induce protective immunity in experimental animals. These have been recently reviewed by HANSON (1976) [10] who indicated that these attempts had been generally unsatisfactory. Significant protection against Chagas' disease in mice has been achieved by intraperitoneal administration of irradiated trypomastigotes and amastigotes grown in cell culture [11]. Because of the success observed in mice with this approach, studies were conducted in dogs to determine: (1) if the irradiated vaccine would stimulate protective immunity in another species, (2) if the dog would develop chronic Chagas' disease as previously reported [3], and (3) the effect of irradiated **T. cruzi** on the development of chronic Chagas' disease in dogs.

MATERIALS AND METHODS

Animals — Female CF_1 albino mice (Carworth Farms, Portage, Mich.) were used for maintenance of **T. cruzi.** Female C_3H mice (Flow Laboratories, Dublin, Va.), 4-8 weeks of age, were used for detection of virulent **T. cruzi** in the irradiated parasite suspensions as described by HANSON et al. [11]. All mice were housed in solid-bottom plastic cages equipped with one-quarter inch wire mesh lids and containing wood chip bedding (Aspen Bedding, American Excelsior Co., Woodland, Ga.). Mouse chow (Ralston Pruina Co., St. Louis, Mo.) and tap water in drinking bottles were provided **ad libitum.** All mice receiving either irradiated or nonirradiated **T. cruzi** were maintained within screened enclosures to minimize exposure to blood sucking arthropods.

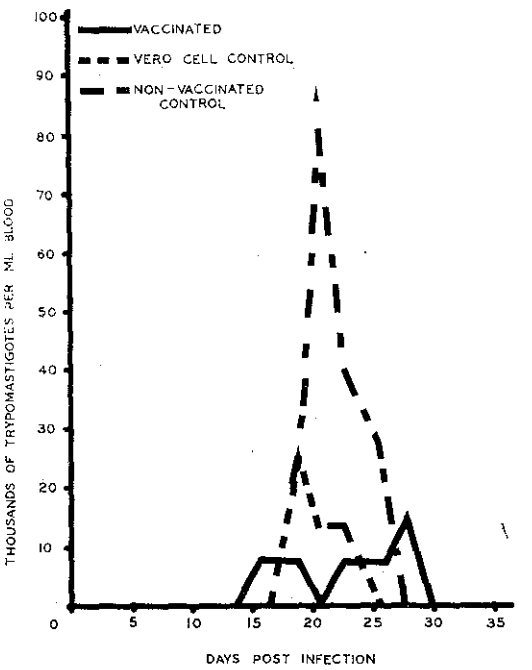

Fig. 1 — Parasitemia in dogs infected with T. cruzi

Pregnant mongrel bitches were obtained locally (Laboratory Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, Ga.*) and allowed to whelp in outdoor pens. Puppies were reared until weaning in these pens when they were moved to indoor cages and conditioned prior to being placed on experiment. Two additional conditioned, juvenile, male dogs were obtained from the same source about one month before being placed on experiment. During the experiment, dogs were maintained individually in stainless steel cages, and were provided tap water and dry dog food (Wayne Dog Food, Allied Mills, Inc., Chicago, Il.) **ad lib.** Rooms housing mice and dogs were at a temperature of approximately 25°C and relative humidity of approximately 40%. The dogs were apportioned into five experimental groups as indicated in Table I.

Maintenance and mass production of parasites — A Brazil strain of **T. cruzi,** maintained in this laboratory since 1967, was used. The parasite was maintained in female CF_1 mice by passage of blood from donors with infections of 14-18 days duration. These parasites were

(*) These dogs were obtained from several dog pounds in Georgia by Laboratory Animal Medicine, and conditioned before being assigned to investigators.

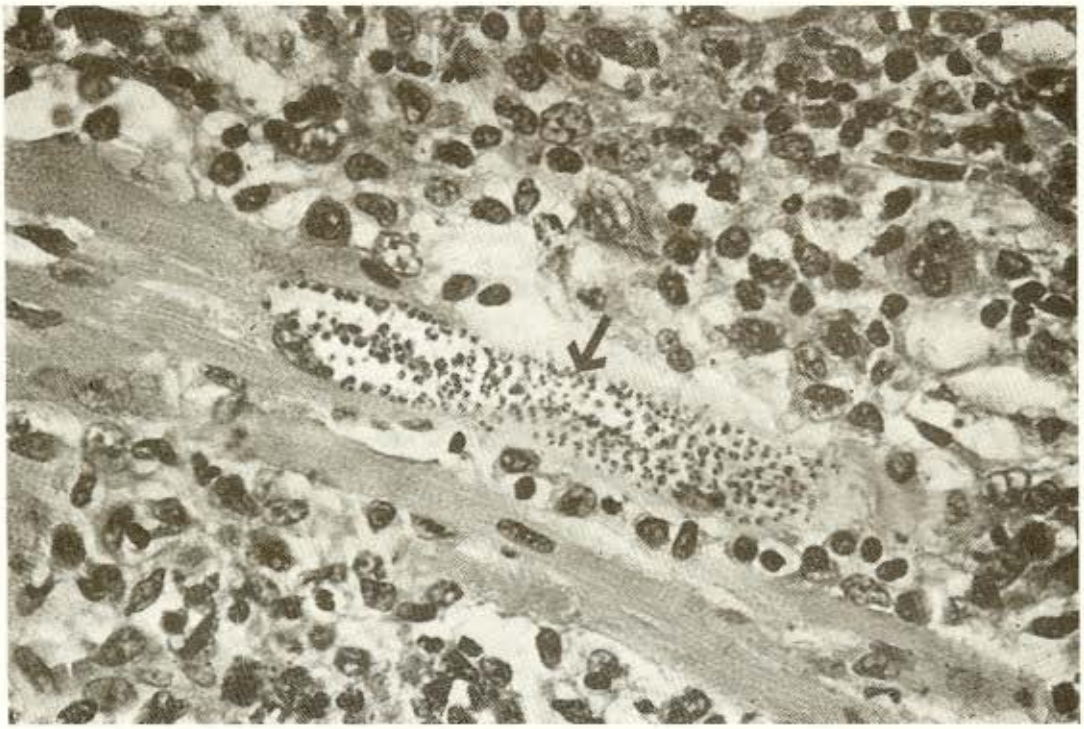

Fig. 2 — Myocardium from dog infected with **T. cruzi** with intracellular cluster of **T. cruzi** amastigotes (arrow) and mononuclear cell infiltrate in the interstitium (H. & E., 408 X)

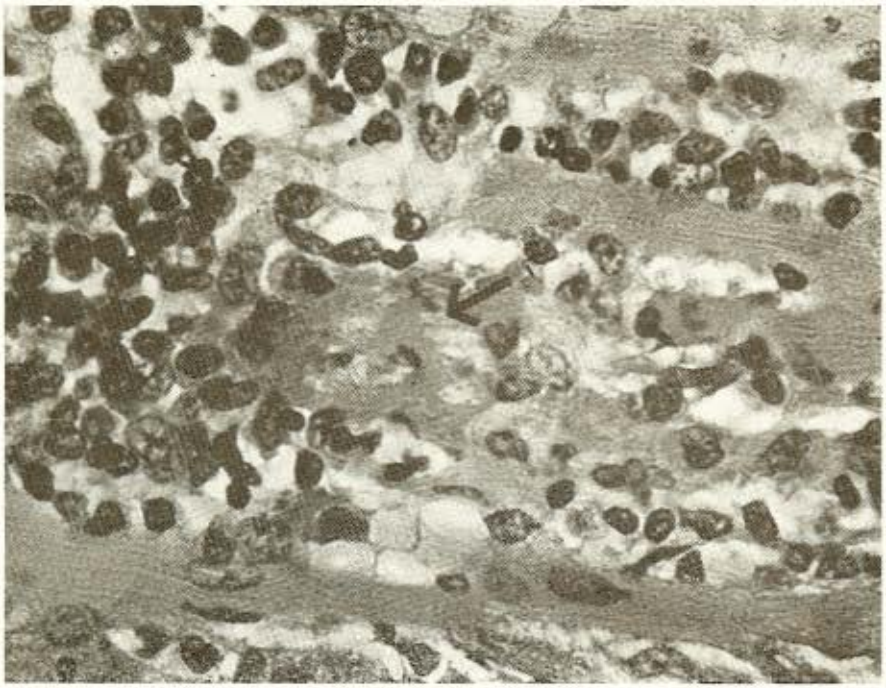

Fig. 3 — Myocardium from dog infected with myocardial necrosis (arrow) and mononuclear cell infiltrate in the interstitium (H. & E., 408 X)

TABLE I

Treatment and number of dogs in each experimental group

Group	Treatment	No. Males	No. Females	Total Dogs per Group
I	Irradiated **T. cruzi**, **T. cruzi** infection	2	3	5
II	Irradiated **T. cruzi**	1	2	3
III	Irradiated Vero cells, **T. cruzi** infection	1	2	3
IV	Irradiated Vero cells	1	2	3
V	**T. cruzi** infection	2	3	5

used for infecting Vero (African green monkey kidney) cell cultures as previously described [11] and for infecting dogs. Vero cell cultures were maintained at 37°C in Falcon tissue culture flasks (75 cm^2, Falcon Plastics, Los Angeles, CA) and cell production roller vessels (Belco Glass, Inc., Vineland, N. J.). Parasites were harvested by centrifugation of culture medium from infected culture vessels at 8000 X g at 4°C for 15 minutes in a Sorval RC2-B refrigerated centrifuge. The supernatant was decanted and the parasites washed once in fresh Eagle's Minimal Essential Medium (MEM, instant tissue culture powder Gibco, Grand Island, N. Y. with 2% heat inactivated dog serum added) and used as described in subsequent sections.

Irradiation of T. cruzi and vaccination of dogs — The washed cell culture derived parasites were quantitated as previously described [23], and the ratio of trypomastigotes to amastigotes determined. After adjusting the concentration of parasites to approximately 20.0×10^7 trypomastigotes per ml, the suspension was exposed to 300 kR irradiation from ^{60}Co in a Gamma cell 200 Irradiator. These parasites will be referred to subsequently as irradiated parasites. Parasites of this strain so irradiated do not establish infections in either C_3H mice or Vero cell cultures [11,12]. Suspensions containing $3.26 - 8.42 \times 10^8$ irradiated parasites were administered by intraperitoneal injection at weekly intervals for a total of five injections. Control groups of dogs were given irradiated (300 kR), uninfected Vero cells, in the numbers shown in Table II, by intraperitoneal injection of the cell suspensions.

TABLE II

Number of parasites with percent trypomastigotes and number of Vero cells given in each dose of irradiated **T. cruzi** vaccine

Days prior to **T. cruzi** infection	Total Parasites x 10^8	Number Amastigotes x 10^8	Number Trypomastigotes x 10^8	Percent Trypomastigotes	Number Vero Cells x 10^6
35	3.90	1.74	2.26	58	9.83
28	3.26	.66	2.60	79.8	4.59
21	7.29	2.29	5.00	68.6	11.25
14	8.42	3.42	5.00	59.4	5.5
7	6.27	1.27	5.00	79.8	4.5

Infection of dogs and counting of parasites in the blood — One hundred, four week old, female CF_1 mice were infected with 5.0×10^4 **T. cruzi** in blood from donor mice. On day 17 of infection, they were anesthetized with ether and exsanguinated by cardiac puncture. The blood was heparinized and pooled, and the parasites quantitated by a method previously described [23]. Dogs were then infected by intravenous administration of 5.0×10^4 virulent **T. cruzi** per kg of body weight in heparinized whole mouse blood. At predetermined intervals during the experiment, numbers of parasites in the blood of the dogs were determined as previously described [23].

Blood cultures — On day 28 post-infection (PI), one-half ml of fresh whole blood from infected dogs was cultured in each of three screw-cap tubes containing Yeager's liver infusion-tryptose broth (LIT) [17] overlain on blood agar slants. These were incubated at 20-22°C

for 42 days when a drop of LIT from each tube was examined by phase contrast microscopy for the presence of parasites.

Direct agglutination tests — Serum antibody titers to **T. cruzi** were determined by a modification of the direct agglutination procedure of Vattuone and Yanovsky (1971). The concentration of parasites in the final antigen suspension was modified to 1 x 10^8 epimastigotes per ml and the tests were read on a microtiter mirror (Cooke Engineering Co., Alexandria, VA) in a dark room with a 15 watt fluorescent light behind the mirror. Control sera were from a puppy whelped and reared by the investigators, and from an adult female dog which had been infected with 5 x 10^4 virulent **T. cruzi** in blood from a donor mouse.

Preparation of antigens and performance of delayed-hypersensitivity tests — Antigens for delayed-hypersensitivity (DH) tests were prepared from a suspension of parasites grown in Vero cell cultures or from uninfected Vero cells, prepared as described in a preceding section. The suspension, which contained 29 x 10^6 epimastigotes, 9 x 10^6 trypomastigotes and 11.9 x 10^6 amastigotes per ml, was divided into 4 equal aliquots for antigen preparation. One aliquot was exposed in a water bath to 56°C for 10 minutes and used as heat-killed whole parasite antigen. Remaining aliquots were then exposed to 300 kR irradiation from ^{60}Co, and one aliquot was set aside for use as irradiated whole parasite antigen. Remaining aliquots were subjected to three freeze-thaw cycles, and an aliquot taken for use as freeze-thaw antigen. The unused frozen and thawed parasite suspension was sonicated in a 100 watt Ultrasonic Disintegrator at 4°C by 3 bursts for 30 seconds each at 50 watts, with a one-minute interval between bursts. The sonicated suspension was centrifuged at 4000 X g for 10 minutes, and one-half of the supernatant removed for use as soluble antigen. Sedimented fragments of disrupted parasites were resuspended in the remaining supernatant and used as insoluble antigen. The Vero cell antigen consisted of irradiated, uninfected Vero cells of a concentration of 1.7 x 10^6 per ml, prepared as indicated in a preceding section.

DH tests were done on 10 dogs 162 days after the last administration of irradiated parasites using a standard technique with .1 ml of each antigen. Injection sites were examined for erythema and induration at 48 hours after injection.

Lymphocyte blast transformation tests — Canine peripheral blood lymphocytes (PBL) were isolated by centrifugation of heparinized venous blood on a sodium diatrizoate-Ficoll gradient (Isolymph, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) at 400 X g for 40 minutes at 18-21°C. The isolated lymphocytes were suspended in MEM with 10% heat inactivated fetal calf serum, 100 μ/ml penicillin, and 100 μg/ml streptomycin to a concentration of 2 x 10^6 cells per ml. Blast transformation tests were done using standard microtechnique with .1 ml cell suspension per culture [26]. Cultures of control cells, and both mitogen and antigen stimulated cells were prepared in triplicate. Mitogens used were Concanavalin A and phytohemagglutinin (Con A and PHA, Difco) at .25 μ g per culture. Triplicate antigen stimulated wells received 22 μ g protein [18] of soluble **T. cruzi** antigen in .1 ml MEM. After incubation and pulse labeling with ^3H-labeled thymidine, the cells were harvested and radioactivity measured as counts per minute in a Beckman 7000 liquid scintillation counter.

Laboratory tests — Blood for complete blood counts and serum enzyme determination was collected at the same time of the day on predetermined days before and during the experiment. Total leukocyte counts were done using a commercially available method (Unopette Test 5856, Becton Dickinson and Co., Rutherford, N.J.). Microhematocrits and differential leukocyte counts (Wright-Leishman stained smears) were done by standard procedure.

Total serum proteins were quantitated by the biuret method [8] and electrophoresis was performed at 180 volts for 15 minutes on cellulose acetate plates using tris-barbitol-sodium-barbitol buffer, pH 8.8. The plates were stained with Ponceau-S, rinsed in 5% acetic acid, dehydrated in methanol, cleared in 20% acetic acid in methanol and dried. They were scanned at 525 nm by a calculating densitometer (Auto Scanner Flur Vis, Quik Quant II, Helena Labs, Beaumont, TX).

Serum aspartate aminotransferase (AST) and creatine phosphokinase (CPK) levels in selected sera were determined by commercially

available spectrophotometric methods (Worthington Statzyme-GOT and Statzyme CPK-n-1, Worthington Diagnostic Division of Millipore Corp., Freehold, N. J.).

Standard 10-lead electrocardiograms (ECG) were done on dogs prior to initiation of the experiment and at selected intervals during the acute infection. ECG's were obtained from unanesthetized dogs when possible. If chemical restraint was necessary, acepromazine maleate (Ayerst Labs, New York, N. Y.) and sodium pentobarbital (W. A. Butler, Co., Columbus, OH) were used to effect.

All dogs that died during the experiment were examined by standard necropsy technique. The body cavities were opened and all organs were examined with the exception of the spinal cord. Representative sections from all major organs, tongue, diaphragm, psoas minor muscle, and sciatic nerve were fixed in 10% neutral buffered formalin, as were both intact eyes with attached extrinsic muscles and the brain. After fixation, tissues were trimmed, dehydrated, infiltrated and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin for light microscopy by standard procedures.

RESULTS

None of the irradiated parasite suspensions used in these studies produced infection in Vero cell cultures or C_3H mice.

Parasitemia and mortality — Among the three groups (I, III, V) of dogs receiving challenge infections, the prepatent period in those in group I, previously given irradiated parasites, was 16 days. Parasitemia in these dogs persisted until PI day 28 (12 days) with the exception of PI day 21 when no parasites were detected. Maximum mean parasitemia in this group was 1.6×10^4 trypomastigotes per ml blood on PI day 28. The dogs in group III, receiving irradiated Vero cells prior to challenge, had a detectable parasitemia from PI days 19 to 23. A maximum mean parasitemia of 2.67×10^4 **T. cruzi** per ml blood occurred on PI day 19 in dogs in group III. The dogs in group V, given only challenge infection, had a prepatent period of 19 days and were parasitemic until PI day 26. Maximum mean parasitemia was 8.8×10^4 **T. cruzi** per ml blood on PI day 21 in this group. Statistical significance of differences in mean parasitemia between dogs in group I and group V were evaluated by the pooled-t test[5]. Dogs which received irradiated parasites prior to challenge had significantly ($P < .05$) lower mean parasitemia on days 19, 21, 23 and 26 PI, than did dogs given no treatment prior to challenge.

Between PI days 31 and 34, 3 of 3 females and 1 of 2 males given irradiated parasites prior to infection had sudden, unexpected deaths. All 3 dogs given irradiated Vero cells before infection died on PI days 26 or 27. Two females in group V, given only challenge infections died; one each on PI days 24 and 25.

Neither parasitemia nor death occurred in uninfected dogs given irradiated parasites or irradiated Vero cells in this experiment.

Blood Cultures — Blood from dogs in groups I and V was cultured in triplicate on PI day 28. Three of the 5 dogs in group I had positive cultures, and all 3 of these dogs subsequently died. The single dog in this group which survived the acute infection was negative in all cultures. Blood from the 3 dogs in group V which survived the acute infection was cultured. Blood from one of these dogs was positive.

Direct agglutination tests — The surviving dog in group I had a positive DH reaction only to the freeze-thaw parasite antigen. Dogs in group V, receiving only virulent **T. cruzi**, had positive DH responses to all **T. cruzi** antigens tested. Insoluble antigen resulted in the least response, with only 1 of 3 dogs having a positive reaction. Freeze-thaw antigen elicited a positive response in all 3 dogs tested in group V. No positive DH tests occurred in dogs which received only irradiated parasites (group II) or only irradiated Vero cells (group IV).

Lymphocyte blast transformation tests — For evaluation of lymphocyte blast transformation (LBT) data, the dogs were apportioned as indicated in Table III. By realigning the groups in this manner, the effect of irradiated parasites on the response of PBL to **T. cruzi** antigen in LBT tests prior to infection with virulent **T. cruzi** could be evaluated in larger groups of dogs. Statistical significance of these responses was determined by analysis of variance for a completely randomized design with 95% confidence intervals[5]. When stimulation

indices of PBL prior to injection of irradiated parasites were used as control values, statistically significant ($p < .05$) stimulation of PBL by soluble **T. cruzi** antigen was observed in dogs after they had received the third and fifth injections of irradiated parasites. However, no significant difference in stimulation was observed when the third injection was compared with the fifth injection of irradiated parasites.

T A B L E III

Mean Stimulation Indices, ± 1 S.D. of Lymphocyte Blast Transformation Tests by Group

Group	Dogs per Group	Response to Soluble T. cruzi Antigen
I	8	1.48 ± 1.35
II	8	(*)5.09 ± 1.73
III	8	(*)4.91 ± 2.12
IV	4	2.71 ± 1.87
V	6	1.49 ± .75
VI	4	1.30 ± .26

Group I: All dogs prior to administration of irradiated parasites or Vero cells to any dog (pre-vaccination control).

Group II: Dogs receiving irradiated parasites, 6 days after the third dose of parasites.

Group III: Dogs receiving irradiated parasites, 6 days after the fifth dose of parasites.

Group IV: Dogs receiving irradiated Vero cells, 6 days after the third dose of cells.

Group V: Dogs receiving irradiated Vero cells, 6 days after the fifth dose of cells.

Group VI: Dogs receiving neither irradiated parasites nor Vero cells, at the same time as dogs in groups II and IV.

(*) Significant at $P < .05$ when compared to prevaccination control values.

Laboratory tests — Total and differential leukocyte counts and packed cell volumes were normal for all dogs through PI day 90.

Pre and postinfection serum protein values obtained by both chemical and electrophoretic methods were normal (TP: 5.3-7.8 g/dl, A: 2.3-4.3 g/dl, G: 1.0-5.5 g/dl).

Mean values for serum AST and CPK for dogs in groups I and V are given in Table IV. In dogs in group I, AST values were within the normal range throughout the experiment while CPK was elevated to 93.0, 225.5, 75 and 77 IU/L on PI days 28, 33, 37, and 43 respectively. AST values in dogs in group V were within the normal range except on PI days 23 and 28 when they were 69.4 and 49 IU/L, respectively. Elevated CPK values in dogs in group V were 60.7, 189.2, 126, 132.3, and 62 on PI days 19, 23, 28, 33, and 37 respectively.

T A B L E IV

Mean serum AST and CPK values in Group I and Group V dogs in IU/L

Days Post-infection	AST		CPK	
	I	V	I	V
Pre	16.1	20.5	101.4	82.4
6	22.0	18	45.8	57.8
12	18.3	18.5	50.4	40.2
19	18.4	24.3	48.2	60.7
23	14.2	69.4	46.8	189.2
28	31.3	49	93.0	126
33	39.5	39	225.5	132.3
37	24(*)	22	75(*)	62
43	24(*)	39	77(*)	53.7
53	19(*)	16	49(*)	44.5
62	15(*)	23.6	87(*)	49
90	16(*)	16	30(*)	31.3

(*) Value from one animal
Range of normal values: AST — 0-40 IU/L
CPK — 8-60 IU/L

ECG's completed on all dogs prior to initiation of the experiment were interpreted as normal. One dog, receiving irradiated parasites prior to challenge with **T. cruzi** had premature ventricular contraction on PI day 32. This dog died 2 days after the ECG was obtained. Electrocardiographic abnormalities were not detected during acute infections in other dogs.

The rectal temperature, obtained throughout the experiment, was elevated above the normal range (37.8-39.2°C) on at least one day in 3 of 5 dogs in group I, but the mean value for dogs in this group was never abnormally elevated. A febrile response occurred in 2 of 3 dogs receiving irradiated Vero cells (group III) prior to challenge, and in all dogs receiving only virulent **T. cruzi.**

No gross lesions were observed in dogs in groups II and IV which had not received virulent parasites. In dogs which had received virulent parasites, the right ventricle of the heart was thickened and dilated in the 4 dogs which had been irradiated parasites (group I). Primarily in the right heart and to a lesser extent in the left heart, the myocardium had

irregular, mottled yellow-tan areas, and the endocardium and epicardium contained petechial and ecchymotic hemorrhages. Two of these 4 dogs had wet, heavy lungs. Generalized lymph node and splenic enlargement occurred in all of these dogs. One dog had a non-perforated gastric ulcer.

Dogs given irradiated Vero cells had cardiac and pulmonary changes similar to those in dogs given irradiated parasites. In addition to those cardiac changes, 2 of these dogs had 30-40 ml of clear, straw-colored fluid in the pericardial sac. Enlarged lymph nodes were present in 1 dog, while splenomegaly occurred in 2 dogs of this group.

Cardiac changes, pulmonary changes and splenomegaly were similar in dogs not receiving irradiated **T. cruzi** to those changes in dogs which did receive irradiated parasites.

Incidental findings consisted of **Ancylostoma caninum** in 1 dog in group I, and **Dipylidium caninum** in 1 dog in group V.

Lesions were observed in the myocardium, extrinsic ocular muscle, esophagus, lymph node, spleen, and brain by light microscopic examination of tissues from dogs in each group. The myocardium contained a multifocal to diffuse mixed cellular infiltrate in which lymphocytes, plasma cells, and macrophages predominated. Small numbers of eosinophils were scattered throughout the myocardium. Occasional multinucleated giant cells were randomly distributed in the myocardium, but these cells were not observed in the hearts of all dogs. Individual cardiac myofibers were fragmented and granular, had no visible striations and were intensely eosinophilic. Neutrophils were present in small numbers around these degenerate or necrotic myofibers. Intracellular clusters of **T. cruzi** amastigotes were randomly distributed within myofibers of all infected dogs. These were, however, often not within areas of cellular infiltration, and were observed perineurally, intraneurally and perivascularly in the adipose and loose connective tissues of the coronary groove. Extrinsic ocular muscles from all dogs challenged with **T. cruzi** were infiltrated by lymphocytes, plasma cells and macrophages similar to infiltrations in the myocardium, but intracellular clusters of **T. cruzi** amastigotes were observed in ocular muscles from only 2 dogs. The esophagus from 8 of the 9 dogs dying as the result of acute Chagas' disease had foci of lymphocytes, plasma cells, and macrophages in the muscularis. Most of these foci were located perineurally among parasympathetic ganglion cells. However, mucous glands in the esophagus of two dogs contained foci of mononuclear inflammatory cells. Small microglial nodules and occasional perivascular lymphocytic cuffing were present in basal ganglia, midbrain and medulla of 4 of the dogs which died. Follicular lymphoid hyperplasia occurred in lymph nodes from all dogs, spleen from 4 dogs and Peyer's patch from 1 dog which died as a result of acute Chagas' disease.

Tongue, psoas minor muscle, diaphragm, prostate and urinary bladder, from 1 or 2 dogs each, contained small foci of lymphocytes and plasma cells which were not associated with muscle degeneration or necrosis. Intracellular clusters of amastigotes occurred in psoas minor muscle and diaphragm from 1 dog each, but were not associated with inflammation. Lung from one dog contained smooth muscle hyperplasia in the interstitium and nematode larvae in peripheral alveoli. The larvae were identified as **Filariodes sp.**[6].

DISCUSSION

Immunization of female CF_1 mice against acute Chagas' disease by weekly injections of irradiated parasites grown in Vero cell culture has been previously reported[11]. Evidence of immunization consisted of significantly reduced parasitemia and mortality in mice receiving irradiated parasites prior to infection as compared to control mice. Extending these studies to another species seemed appropriate in evaluating the immunizing effect of irradiated **T. cruzi.**

Stimulation of an immune response against **T. cruzi** in the dogs in this experiment by a series of injections of irradiated parasities was indicated by significant stimulation of lymphocytes in LBT tests and decreased parasitemia in challenged dogs. However, other criteria of immunization used in this experiment clearly indicated that any immune response induced in these dogs by irradiated parasites did not protect against acute Chagas' disease. Female CF_1 mice infected with **T. cruzi** were significantly protected against death by 5 weekly injections of either 2.0×10^6 or 20.0×10^6 irra-

diated parasites while 50% or greater mortality occurred in control mice [11]. In the present studies, 4 of 5 dogs receiving irradiated parasites prior to challenge with virulent **T. cruzi** died as the result of acute Chagas' disease. Three of 3 dogs receiving irradiated Vero cells, but only 2 of 5 dogs receiving no treatment prior to challenge died as a result of acute Chagas' disease. In 4 dogs receiving irradiated parasites and 2 dogs receiving no treatment prior to challenge with **T. cruzi** which died due to acute Chagas' disease, DA titers in serum obtained from each dog within 3 days of death ranged from 1:16 in 1 dog to 1:512 or higher in the remaining 5 dogs. One of the dogs receiving no treatment prior to challenge had a titer of greater than 1:4096 before death. Four dogs survived acute **T. cruzi** infection and maximum titers were greater than 1:4096 in 3. The fourth dog had been given irradiated parasites prior to challenge but had a maximum DA titer of 1:512. In 3 dogs given irradiated parasites but no challenge, the maximum DA titer occurred 50 days after the last injection of irradiated parasites and was 1:64. Direct agglutination titers of less than 1:512 are not specific for antibody against **T. cruzi** [1]. These data indicate that in animals challenged with **T. cruzi**, factors other than antibody measured by the DA test play a role in protection against death.

Clinical signs, including those of cardiac dysfunction, were limited in dogs receiving **T. cruzi**. One dog out of 6 in which an ECG was obtained during acute infection had premature ventricular contraction on PI day 32. This dog was in the group receiving irradiated parasites prior to challenge and died as the result of acute myocarditis 2 days after the ECG was obtained. Electrocardiograms taken on other dogs during the acute infection were interpreted as normal. This is in contrast to a previous study of Chagas' disease in dogs in which 20 of 31 dogs had electrocardiographic changes during the first 90 days of **T. cruzi** infection [3]. Changes reported in that study were rate and conduction disturbances including bundle branch block and premature ventricular contraction.

Mean serum CPK activities were increased as much as 2.5 fold in dogs given irradiated parasites prior to infection, but mean AST activity was not increased. In dogs infected with **T. cruzi**, but not receiving irradiated parasites, mean serum AST increased as much as 1.5 fold and mean CPK activity increased as much as 3 fold. Increased enzyme activity in both groups of dogs occurred during and just after the period of detectable parasitemia, and indicated either alteration of myocardial cell membrane permeability or myocardial necrosis. The febrile response in these dogs before and during the period of enzyme elevation was not specific for cardiac disease but suggestive of necrosis or an inflammatory response.

Significant necropsy findings were confined to the heart, and consisted of a thickened, pale right ventricle with subendocardial and subepicardial hemorrhages. Histopathologic lesions (myocarditis, perineuritis and neuritis in the coronary groove, lymphoid nodules in esophageal muscularis, non-suppurative encephalitis, and lymphoid hyperplasia) are consistent with lesions previously reported in humans and dogs with Chagas' disease [4,14,20,33]. The lesions of acute canine Chagas' disease reported by KRAMER [14] were more severe and extensive than those occurring in the dogs in this study, possibly because his dogs were younger and were infected with a 10 fold large number of parasites per kg body weight than were the dogs in our study.

A variety of theories have been suggested to explain the pathogenesis of the cardiomyopathy associated with Chagas' disease and have been reviewed by ANSELMI & MOLEIRO [2]. These include mechanical destruction of cardiac fibers, destruction of cardiac fibers by toxic products of the parasite, destruction of cardiac adrenergic innervation by the parasite and the allergic theory. The allergic or immune-mediated theory has been supported by the low number of invading parasites relative to the intensity of the inflammatory reaction, and the predominance of lymphocytes and plasma cells in the inflammatory infiltrate [2]. Antibodies which react with endocardium, vascular structures, interstitium of striated muscle and peripheral nerves in the serum of up to 95% of patients with Chagas' cardiomyopathy and absorption of this antibody activity from serum by lypholized **T. cruzi** epimastigotes have been demonstrated [7,29]. A relationship between these antibodies and the pathogenesis of cardiac muscle damage in Chagas' disease has been suggested [16,28]. Destruction of parasitized syngeneic mouse fibroblasts **in vitro** by spleen

cells from mice with **T. cruzi** infections has suggested that cell-mediated immunity may have a role in the pathogenesis of the cardiomyopathy of Chagas' disease [15]. KUHN'S [15] study demonstrated that uninfected fibroblasts were not destroyed by sensitized spleen cells, but it was not determined if addition of antibody to **T. cruzi** would have resulted in destruction of the uninfected fibroblasts by either sensitized or unsensitized spleen cells.

Significant protection against Chagas' disease in dogs was not achieved by administration of irradiated **T. cruzi** grown in cell culture. Injection of irradiated parasites did not induce significant levels of antibody against **T. cruzi** as measured by DA test in dogs even by 50 days after the last injection. With one exception, dogs receiving irradiated parasites had lower maximum antibody levels than did dogs not receiving irradiated parasites prior to challenge. The dog has been indicated to be an appropriate model for Chagas' disease by previous studies [3,14,20], and by the observations of this study. The low number of **T. cruzi** amastigotes within the myocardium relative to the severe inflammatory reaction in the myocardium in which lymphocytes and plasma cells predominated was consistent with an immune-mediated mechanism of this cardiomyopathy. However, this finding does not exclude a non-immune-mediated inflammatory mechanism of this lesion.

RESUMO

O efeito de Trypanosoma cruzi **irradiado na patogênese da doença de Chagas em cães**

Cães foram injetados semanalmente com doses de 3,26-8,42 X 10^8 **T. cruzi** irradiados de cultura de célula por via intraperitoneal. Os parasitas irradiados não foram capazes de produzir infecções em cultura de células Vero ou camundongos C_3H. Uma semana após a última injeção de parasitas irradiados os cães foram testados por injeção intravenosa de 5,0 X 10^4 **T. cruzi** virulentos em sangue de camundongo por kg de peso. Apesar de os cães que receberam parasitas irradiados terem resposta significante do antígeno de **T. cruzi** como foi demonstrado pela prova de transformação blástica de linfócito, não foram significantemente protegidos contra uma infecção teste como indicado pela morte de 4 a 5 cães. Nem disfunção cardíaca nem outros sinais clínicos foram observados antes da morte súbita dos cães testados. A média máxima de parasitemia em cães que receberam parasitas irradiados foi de 1,6 X 10^4 tripomastigotas por ml de sangue 28 dias após teste e foi de 8,8 X 10^4 tripomastigotas por ml de sangue 21 dias após teste em cães controles não vacinados. Títulos de anticorpos determinados pela prova de aglutinação direta foram de 1/64 ou menos em cães recebendo parasitas irradiados mas não infecção teste. Em cães recebendo parasitas irradiados e infecção teste, os títulos variavam de 1/512 a 1/2048. Em cães recebendo somente infecções testes os títulos variavam de 1/16 maiores que 1/4096. Em cães sobrevivendo à infecção aguda por **T. cruzi**, títulos máximos eram maiores que 1/4096 em três cães e 1/512 em um cão. Atividades médias de creatinina fosfoquinase sérica foram elevadas durante a infecção aguda em cães recebendo parasitas irradiados antes do teste. Nos cães recebendo somente infecções testes, as atividades médias de fosfoquinase e aspartato aminotransferase foram também aumentadas durante as infecções agudas. O achado primário de necrópsia em todos os cães testados com os parasitas virulentos foi um coração hipertrofiado, dilatado com hemorragias. Essas mudanças foram mais severas no ventrículo direito. Todos os cães que morreram com infecção aguda por **T. cruzi** tiveram miocardite severa, necrotizante granulomatosa, mas o músculo cardíaco continha poucos grupos intracelulares de amastigotas de **T. cruzi**.

ACKNOWLEDGEMENTS

The Authors express their appreciation to Dr. Clarence A. Rawlings for interpretation of electrocardiograms and to Ms. Kathy Hunt and Ms. Maribeth Ciernia for editorial assistance. Funding for this research was provided by a Veterinary Medical Experiment Station Grant (79-157), University of Georgia, Athens, Georgia, 30602, U.S.A. Prepared as Georgia Veterinary Medical Experiment Station Manuscript ≠1662.

REFERENCES

1. ALLAIN, D. S. & KAGAN, I. G. — An Evaluation of the Direct Agglutination Test for Chagas' Disease. **J. Parasitol.** 60: 179-184, 1974.

2. ANSELMI, A. & MOLEIRO, F. — Pathogenic Mechansims in Chagas' Cardiomyopathy. Ciba Foundation Sym-

posium No. 20, Trypanosomiasis and Leishmaniasis with Special Reference to Chagas' Disease. Associated Scientific Publishers, Amsterdam, 125-136, 1974.

3. ANSELMI, A.; PIFANO, G.; SUAREZ, A.; DOMINGUEZ, A.; VASQUEZ, A. D. & ANSELMI, G. — Experimental **Schizontrypanum cruzi** Myocarditis, Correlation between histopathologic and electrocardiographic findings in experimental Chagas' heart disease. **Am. Heart J. 70:** 638-656, 1965.

4. ANSELMI, A.; PIFANO, F.; SUAREZ, J. A. & GURDIEL, O. — Myocardiopathy in Chagas' disease. I. Comparative study of pathologic findings in chronic human and experimental Chagas' myocarditis. **Am. Heart J. 72:** 469-481, 1966.

5. BAHATTACHARYYA, G. K & JOHNSON, R. A. — Comparing Two Treatments, Design of Experiments and Analysis of Variance, in **Statistical Concepts and Methods.** New York, John Wiley and Sons, 1977, 286-333, 453-469.

6. CHITWOOD, M. & LICHTENFELS, J. R. — Identification of Parasitic Metazoa in Tissue Sections. **Exp. Parasitol. 32:** 407-519, 1972.

7. COSSIO, P. M.; LAGUENS, R. P.; DIEZ, C.; SZARFMAN, A.; SEGAL, A. & ARANA, R. M. — Chagas' Cardiomyopathy, Antibodies reacting with plasma membrane of striated muscle and endothelial cells. **Circulation 50:** 1252-1259, 1974.

8. GORNALL, A. G.; BARDAWILL, C. J. & DIVID, M. M. — Determination of serum proteins by means of the biuret reaction. **J. Biol. Chem. 177:** 751-766, 1949.

9. GREER, D. A. — Texas Public Health Reports, 1955.

10. HANSON, W. L. — Immunology of American Trypanosomiasis (Chagas' Disease) in: S. Cohen and E. Sadun (eds). **Immunology of Parasitic Infections.** Oxford, London, Edinburgh, Melbourne, Blackwell Scientific Publications, 1976, pp. 222-234.

11. HANSON, W. L.; CHAPMAN Jr., W. L. & WAITS, V. B. — Immunization of mice with irradiated **Trypanosoma cruzi** grown in cell culture: relation of numbers of parasites, immunizing injections and route of immunization to infection. **Int. J. Parasitol. 6:** 341-347, 1976.

12. HANSON, W. L.; CHEIN, J. J.; CHAPMAN Jr., W. L. & ROBERSON, E. L. — Current Status of Vaccination against Chagas' Disease: Use of Irradiated Parasites. Proceedings **VI International Congress of World Association for Advancement of Veterinary Parasitology.** "Parasitic Zoonoses — Clinical and Experimental Studies". New York, Academic Press, 1974, 117-131.

13. KAGAN, I. G. & NORMAN, L. — Immunological studies on **Trypanosoma cruzi.** III. Duration of acquired immunity in mice initially infected with a North American strain of **T. cruzi. J. Infect. Dis. 108:** 213-217, 1961.

14. KRAMER Jr., A. W. — Experimental Chagas' disease in purebred Beagle dogs acutely infected with **Trypanosoma cruzi** (B-strain). **Rev. Inst. Med. trop. São Paulo 14:** 291-300, 1972.

15. KUHN, R. E. & MURNANE, J. E. — **Trypanosoma cruzi:** Immune destruction of parasitized mouse fibroblasts **in vitro. Exp. Parasit. 41:** 66-73, 1977.

16. LAGUENS, R. P.; COSSIO, P. M.; DIEZ, C.; SEGAL, A.; VASQUEZ, C.; KREUTZER, E.; KHOURY, E. & ARANA, R. M. — Immunopathologic and morphologic studies of skeletal muscle in Chagas' disease. **Am. J. Path. 80:** 153-162, 1975.

17. LOGAN, L. L. & HANSON, W. L. — **Trypanosoma cruzi:** Morphogenesis in diffusion chambers in the mouse peritoneal cavity and attempted stimulation of host immunity. **Exp. Parasitol. 36:** 439-454, 1974.

18. LOWRY, O. H.; ROSEBROUGH, N. J.; RANDALL, R. J. & FARR, A. L. — Protein measurement with the folin phenol reagent. **J. Biol. Chem. 193:** 265-275, 1951.

19. McKEEVER, S.; GORMAN, G. W. & NORMAN, L. — Occurrence of a **Trypanosoma cruzi**-like organism in some mammals from southwestern Georgia and northwestern Florida. **J. Parasitol. 44:** 583-587, 1958.

20. MOTT, K. E. & HAGSTROM, J. W. C. — The pathologic lesions of the cardiac autonomic nervous system in chronic Chagas' myocarditis. **Circulation 31:** 273-281, 1965.

21. NISSEN, E. E.; ROBERSON, E. L.; LIPHAM, L. S. & HANSON, W. L. — Naturally occurring Chagas' disease in a South Caroline puppy. Presentation to the American Association of Veterinary Parasitologists, 114th Annual AVMA Meeting, Atlanta, GA, 1977.

22. OLSEN, P. F.; SHOEMAKER, J. P.; TURNER, H. F. & HAYS, K. L. — Incidence of **Trypanosoma cruzi** (Chagas) in wild vectors and reservoirs in east-central Alabama. **J. Parasitol. 50:** 599-603, 1964.

23. ROBERTSON, E. L.; HANSON, W. L. & CHAPMAN Jr., W. L. — **Trypanosoma cruzi:** Effects of anti-thymocyte serum in mice and neonatal thymectomy in rats. **Exp. Parasitol. 34:** 168-180, 1973.

24. SCHAFFER, G. D.; HANSON, W. L.; DAVIDSON, W. R. & NETTLES, V. F. — Hematotrophic parasites of translocated raccoons in the southeast. **J. Am. Vet. Med. Assoc. 173:** 1148-1151, 1978.

25. STECK, E. A. — The chemotherapy of protozoan disease. Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Published by U. S. Government Printing Office 0-462-573, Vol. II, 9.1-9.56, 1972.

26. STITES, D. P. — Laboratory Methods of Detecting Cellular Immune Function, in **Basic and Clinical Immunology.** Fudenberg, H. H., Stites, D. P., Caldwell, J. L., and Wells, J. V. (eds). Los Altos, CA, Lange Medical Publications, 1976, pp. 316-324.

27. SULLIVAN, T. D.; McGREGOR, T.; EADS, R. B. & DAVIS, D. J. — Incidence of **Trypanosoma cruzi,** Cha-

gas, in Triatoma (hemiptera, Reduviidae) in Texas. **Am. J. Trop. Med.** 29: 453-458, 1949.

28. SZARFMAN, A.; COSSIO, P. M.; ARANA, R. M.; URMAN, J.; KREUTZER, E.; LAUGUENS, R. P.; SEGAL, A. & COARASA, L. — Immunologic and Immunopathologic Studies in Congenital Chagas' Disease. **Clin. Immunol. Immunopathol.** 4: 489-499, 1975.

29. SZARMAN, A.; COSSIO, P. M.; KHOURY, E. L.; RITACO, V.; ARANA, R. M. & SCHMUNIS, G. A. — Tissue reacting Ig in children parasitemic with **Trypanosoma cruzi. Trans. R. Soc. Trop. Med. Hyg.** 71: 453, 1977.

30. VATTUONE, N. J. & YANOVSKY, J. F. — **Trypanosoma cruzi:** Agglutination activity of enzyme-treated epimastigotes. **Exp. Parasitol.** 30: 349-355, 1971.

31. VETERINARY PUBLIC HEALTH NOTES, Center for Disease Control, Public Health Service, Department Health, Education and Welfare, Atlanta, GA, 1979.

32. WALTON, B. C.; BAUMAN, P. M.; DIAMOND, L. S. & HERMAN, D. M. — The isolation and identification of **Trypanosoma cruzi** from raccoons in Maryland. **Am. J. Trop. Med. Hyg.** 7: 603-610, 1958.

33. WILLIAMS, G. D.; ADAMS, L. G.; YEAGER, R. G.; McGRATH, R. K.; READ, W. K. & BILDERBACK, W. R. — Naturally Occurring Trypanosomiasis (Chagas' Disease) in Dogs. **J. Am. Vet. Med. Assoc.** 171: 171-177, 1977.

34. WOOD, S. F. — **Trypanosoma cruzi:** New foci of endemic Chagas' disease in California. **Exp. Parasitol.** 38: 153-160, 1975.

35. WOODY, N. C. & WOODY, H. B. — American Trypanosomiasis: First indigenous case in the United States. **J. Am. Med. Assoc.** 159: 676, 1955.

36. ZELEDON, R. — Epidemiology, modes of transmission, and reservoir hosts of Chagas' disease. Ciba Foundation Symposium No. 20, Trypanosomiasis and Leishmaniasis with Special Reference to Chagas' Disease. Amsterdam, Associated Scientific Publishers, 1974, 51-85.

Recebido para publicação em 28/9/1979.