

Enhancement of Chronic *Trypanosoma cruzi* Myocarditis in Dogs Treated With Low Doses of Cyclophosphamide

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An enhancement of chronic myocarditis was obtained in dogs chronically infected with *Trypanosoma cruzi* protozoa soon after they were submitted to treatment with low doses of cyclophosphamide (50 mg/sq m bs three times a week for 3 weeks). Such treatment did not cause immunodepression. Myocarditis varied in intensity, but was quite severe and diffuse in some animals, with focal fibrinoid, coagulative, and lytic necrosis and invasion of disintegrating myocardial fibers by the

mononuclear inflammatory cells. Untreated infected controls exhibited mild focal myocarditis, usually represented by accumulation of lymphocytes in the interstitial connective tissue. It is suggested that the administration of low doses of cyclophosphamide interfered with the immunologic suppressor network that is thought to maintain the chronic indeterminate (or latent) phase of *T cruzi* infection. (Am J Pathol 1987, 127:467-473)

MOST SUBJECTS infected with *Trypanosoma cruzi*, the protozoon causing Chagas' disease, are asymptomatic. They may remain so for prolonged periods, usually from 5 to 30 years. In about 30% of them will eventually develop the picture of progressive cardiac failure due to a chronic diffuse myocarditis that is thought to develop from a delayed hypersensitivity reaction to parasite-related antigens^{1,2} or to autoimmunity.^{3,4} The reason for the prolonged period of latent infection is unknown.

The experimental approach to study of this problem depends on the identification of a suitable model. So far only in the dog has it been possible to observe the spontaneous development of congestive cardiac failure, with cardiomegaly, arrhythmias, and diffuse myocarditis after a prolonged period of chronic asymptomatic *T cruzi* infection.⁵⁻⁷ However, the rarity and unexpectedness with which diffuse progressive myocarditis spontaneously develops in chronically *T cruzi*-infected dogs represents a limitation to the utilization of such a model.⁸

In this present study we observed the regular development of severe myocarditis in dogs with chronic, apparently latent, *T cruzi* infection after they were treated with low doses of cyclophosphamide.

It has been claimed that low doses of cyclophosphamide may exacerbate cell-mediated lesions, probably by selectively destroying suppressor T lymphocytes, their precursor cells, or other elements in the host-immune suppressor network.⁹⁻¹²

Materials and Methods

Twenty-one young (2-3 months old) mongrel dogs of both sexes were used in the experiments. Infection was accomplished by means of blood forms of *T cruzi* taken from infected mice. These were outbred Swiss mice, infected intraperitoneally with 1×10^5 trypomastigotes of the 12SF and Colombian strains of *T cruzi*, which have been maintained by serial passage in mice (see Andrade et al¹³ for details on strain characterization). Mice were bled at the height of parasite-

Supported by the Brazilian Scientific Council (CNPq) PIDE VI (Grant 40.0631/85).

Accepted for publication January 22, 1987.

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mia, and the citrated blood was collected from the axillary plexus. Trypomastigotes were counted by means of Brener's method.¹⁴ Inocula were adjusted according to the weight of the animals, and the infection of the dogs was done by the intraperitoneal route. The different sets of experiments are listed below.

The ten dogs from the first two experiments had been infected and/or vaccinated with *T cruzi* and were under observation for more or less prolonged periods, showing no apparent external signs of disease, with normal electrocardiograms and negative parasitemia, when it was decided to subject them to treatment with cyclophosphamide.

1. In 6 of those dogs infected with *T cruzi* trypomastigotes, acute infection (positive parasitemia) developed 16 to 30 days after inoculation, but the dogs recovered after 2–4 weeks, when the electrocardiographic changes returned to normal and trypomastigotes could not be found by direct examination of peripheral blood (Table 1). One of the dogs developed severe disease during the acute phase of the infection and had to be treated with nitroimidazole and corticoids as previously described.¹⁵

2. Four dogs were vaccinated with a living non-replicating vaccine¹⁶ given three times subcutaneously, in 10-day intervals. Each dose, administered in 1 ml, contained 1×10^8 epimastigotes. Two of these vaccinated animals were challenged 1 week after the last immunizing dose with 60,000 and 82,000 blood trypomastigotes, respectively, given intraperitoneally. The two other dogs were not infected and remained as vaccine controls (Table 2), although they were also treated with cyclophosphamide later on.

3. Five dogs were used in another similar experiment. They were likewise infected as the previous 6 dogs were, developed acute infection, and were observed until they reached the chronic stage of the infection. Then 3 dogs were treated with low-dose cyclophosphamide under the same schedule as the others, and 2 were left as untreated controls. To evaluate the type and degree of myocarditis in these animals, we coded the microscopical slides and the three

pathologists who examined them separately did not know to which group the slides belonged (Table 3).

4. From the files of the Department the slides containing heart tissue from 5 dogs which had been infected but not treated with cyclophosphamide were examined for illustration of the type of myocarditis which is usually found in dogs chronically infected with *T cruzi* (Table 4). These cases were selected because the duration of infection was comparable to that of the other experiments; the *T cruzi* strains used were also the same.

Sera were obtained prior to inoculation and at different time intervals thereafter. The presence of anti-*T cruzi* antibodies were detected by immunofluorescence, according to Camargo's methods.¹⁷

Classic 12-lead electrocardiograms (ECGs) were taken with the dogs under light anesthesia with Nembutal. The ECGs were taken before inoculation, weekly during the acute phase, and at various intervals (at least monthly) during the chronic phase of the infection. A single-channel electrocardiograph was used, with small metal needles for subcutaneous placement instead of the usual electrodes.

Cyclophosphamide (Enduxan, Pravaz-Abbott Laboratories, Brazil, Ltd.) was administered intraperitoneally in the dose of 50 mg/sq m of body surface, three times a week, for 3 weeks. The dose was calculated to be comparable with that utilized by Colley et al¹⁰ for the mouse. Four days after the last injection of cyclophosphamide, all the animals were killed and submitted to autopsy. All the organs were examined except for the central nervous system. Fragments of the organs were fixed in 10% buffered formalin and embedded in paraffin, and the sections stained with hematoxylin and eosin (H&E). The entire heart was fixed for the proper examination of the sinus node by Hudson's technique¹⁸ and the atrioventricular conducting system by Lev's method.¹⁹

In addition to the routine H&E, the heart sections were stained by the Masson's trichrome, periodic acid-Schiff (PAS), and Picro-Syrius red methods for collagen.²⁰

Table 1—Data on Young Dogs Infected With *T cruzi* and Treated During the Chronic Stage of the Infection With Low-Dose Cyclophosphamide (Cy)

Dog	<i>T cruzi</i> strain	Number of parasites in inoculum	Duration of infection (days)	Antibody titer after Cy	Myocarditis	
					Focal	Diffuse
1	Colombian	5×10^4	338	1:1280	+	+
2	Colombian	5×10^4	338	1:1280	+++	+++
3	Colombian	5×10^4	338	1:1280	0	++
4	Colombian	5×10^4	186	1:2560	++	+++
5	12 SF	5×10^4	122	1:6120	+++	+++
6	12 SF	82×10^3	122	1:2560	+++	+++

Table 2—Data on Dogs Vaccinated Against *T cruzi*, Challenged or Not, and Treated With Low-Dose Cyclophosphamide (Cy)

Dog	Status	Strain	Inoculum	Duration of infection or vaccination (days)	Antibody titer after Cy	Myocarditis	
						Focal	Diffuse
7	Vaccinated and challenged	12 SF	6×10^4	122	1:5120	+++	++
8	Vaccinated and challenged	12 SF	82×10^3	122	1:2560	+	++
9	Vaccinated only	—	—	122	1:1280	0	0
10	Vaccinated only	—	—	122	1:640	0	0

Results

General Data

General clinical, immunologic, and parasitologic data appear in Tables 1, 2, 3, and 4.

Electrocardiographic Findings

During the acute phase of the infection, the electrocardiograms disclosed some alterations in most of the animals. These ranged from sinus bradycardia, inversion of T waves, and irregularities in the S-T segments, to premature beats and right bundle branch block. After resolution of the parasitemia, the ECG abnormalities gradually disappeared, and during the stabilized chronic stage repeated ECGs were within normal limits. Four days after the last injection of cyclophosphamide the treated animals were found again to have some mild ECG changes, such as alterations in the S-T segments, increased P-R intervals, sinus arrhythmias, and one instance of intraventricular conduction defect.

Pathologic Findings

Gross findings at autopsy were not remarkable, except for moderate dilatation of the right chambers of the heart in a few animals.

Table 3—Data on 5 Dogs All Infected at the Same Time With 12 SF Strain of *T cruzi**

Dog	Cy treatment	<i>T cruzi</i> antibody titers		Myocarditis	
		before Cy	After Cy	Focal	Diffuse
11	Yes	1:320	1:320	++	++
12	Yes	1:160	1:80	+++	++
13	Yes	1:160	1:320	++	+
14	No	1:320	1:160	+	0
15	No	1:640	1:320	+	+

*At the chronic stage of the infection 3 dogs were treated with low doses of cyclophosphamide (Cy), and 2 were left untreated. The animals were sacrificed 4 days after the last Cy infection.

Inoculum: 1×10^5 parasites per kilogram body weight. Duration of infection: 213 days.

Microscopically, the main changes were limited to the heart. All the infected animals exhibited variable degrees of chronic myocarditis. In the first experiment, those animals treated with cyclophosphamide showed marked myocardial inflammation, with focal necrosis of myocardial fibers (Figure 1). A focal and diffuse infiltration of macrophages, lymphocytes, and plasmocytes dissociated the myocardial fibers and extended to the endocardium and to the epicardial fibroadipose tissue. Sometimes the mononuclear inflammatory cells seemed to be invading, destroying, and replacing the myocardial fibers (Figure 2). Within areas where the inflammation became more accentuated, it was not uncommon to observe fibrinoid, coagulative, and/or lytic necrosis of the myocardial fibers, usually followed by collapse of the connective tissue framework (Figure 3). Throughout the myocardium there were patchy areas of interstitial edema and fibrosis, especially around the vessels. No overt vascular involvement was detected. The sinus node and the atrioventricular conducting tissues were likewise involved (Figure 4). The inflammation was more severe at the right atrial walls and reached the autonomic nervous ganglia and other nervous structures in the proximity of the sinus-atrial node. The His main bundle and its right and left branches were infiltrated by mononuclear inflammatory cells, but its continuity was maintained.

No parasites could be detected within the cardiac fibers, even after a thorough search. Extracardiac changes were not remarkable.

Table 4—Data on 5 Young Dogs Infected With *T cruzi* and Killed During the Chronic Stage of the Infection

Dog	<i>T cruzi</i> strain	Inoculum	Duration of infection (days)	Myocarditis	
				Focal	Diffuse
16	12 SF	5×10^4	425	+	0
17	12 SF	5×10^4	425	+	0
18	12 SF	6×10^4	94	+	0
19	Colombian	1×10^6	274	+	0
20	Colombian	1×10^6	274	+	0
21	Colombian	1×10^6	274	+	0

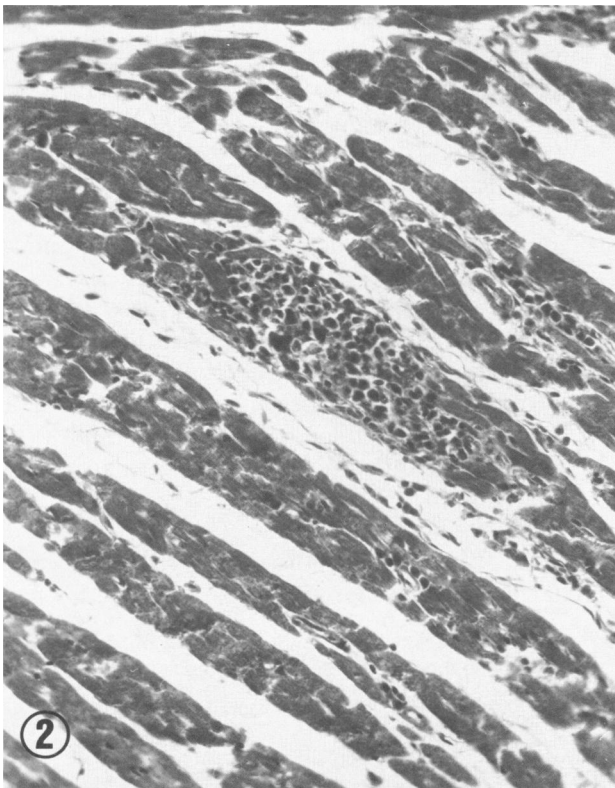
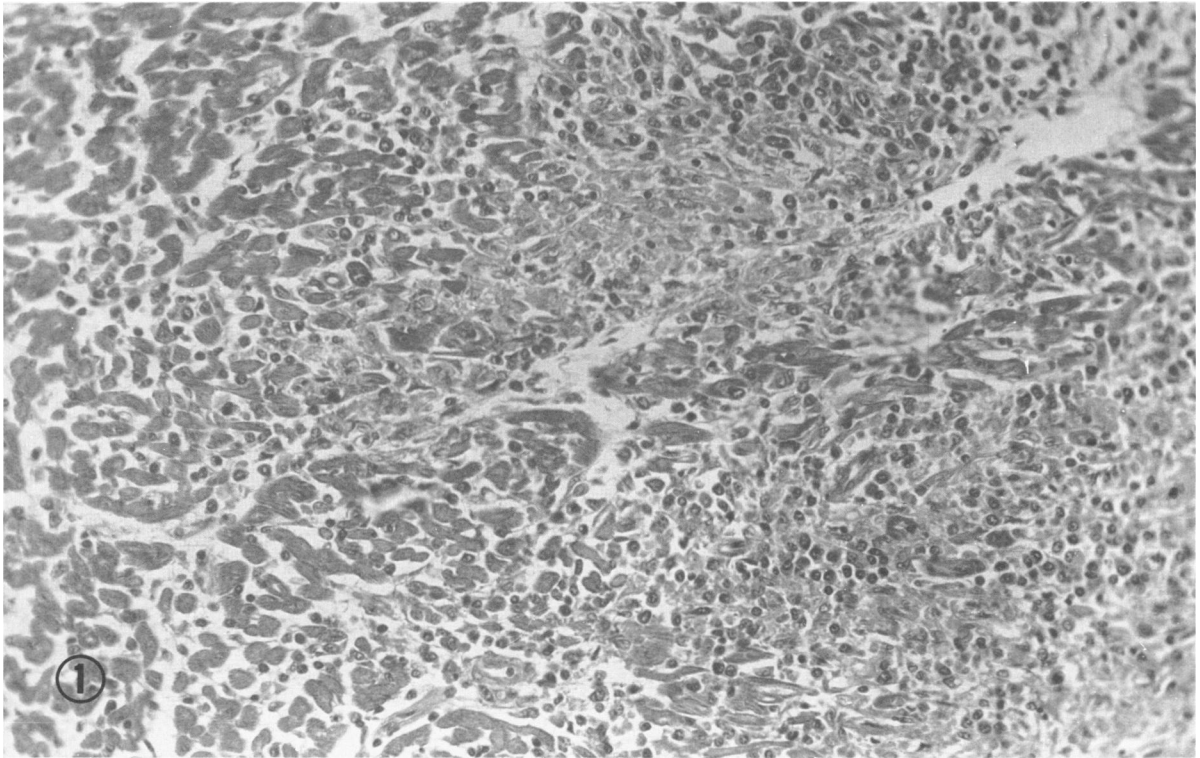


Figure 1—Severe myocarditis in a dog with prolonged *T. cruzi* infection and treated with low-dose cyclophosphamide. There is diffuse infiltration with mononuclear cells, dissociation, fragmentation, and necrosis of myocardial fibers. (H&E, $\times 200$) **Figure 2**—Mononuclear cells are seen inside a degenerating myocardial fiber. Dog treated with cyclophosphamide. (H&E, $\times 150$) **Figure 3**—Myocarditis in a dog treated with cyclophosphamide. Presence of focal lytic necrosis of myocardial fibers. (H&E, $\times 120$)

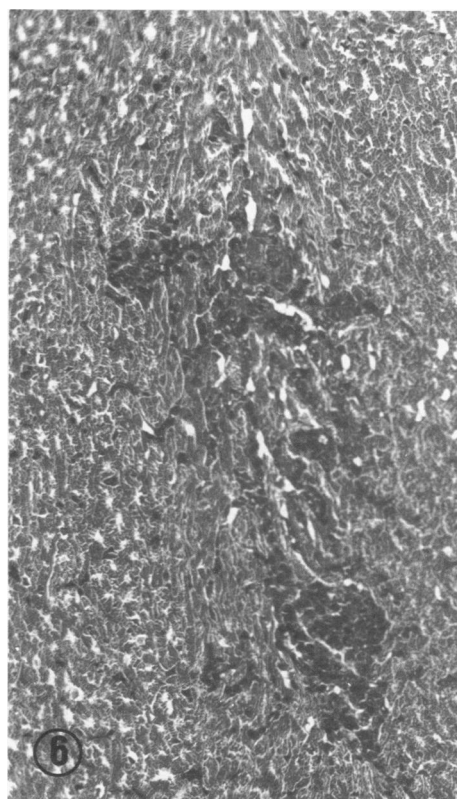
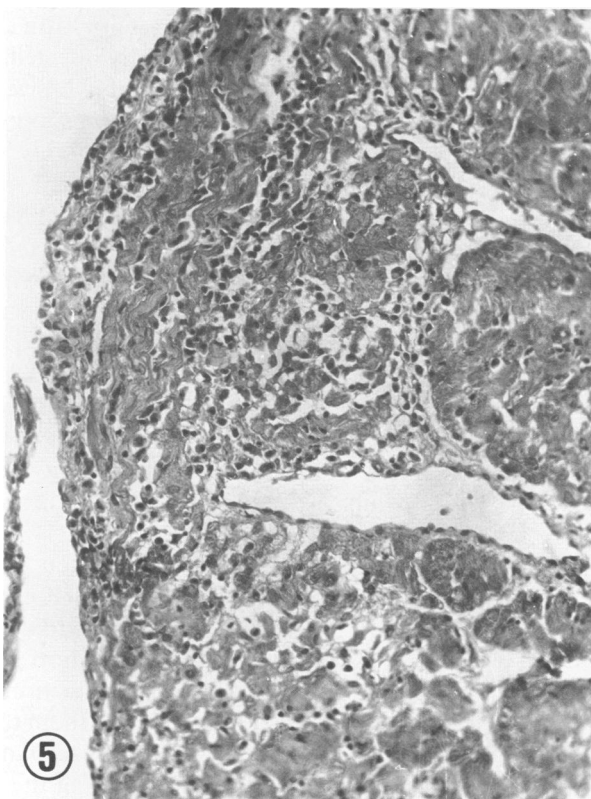
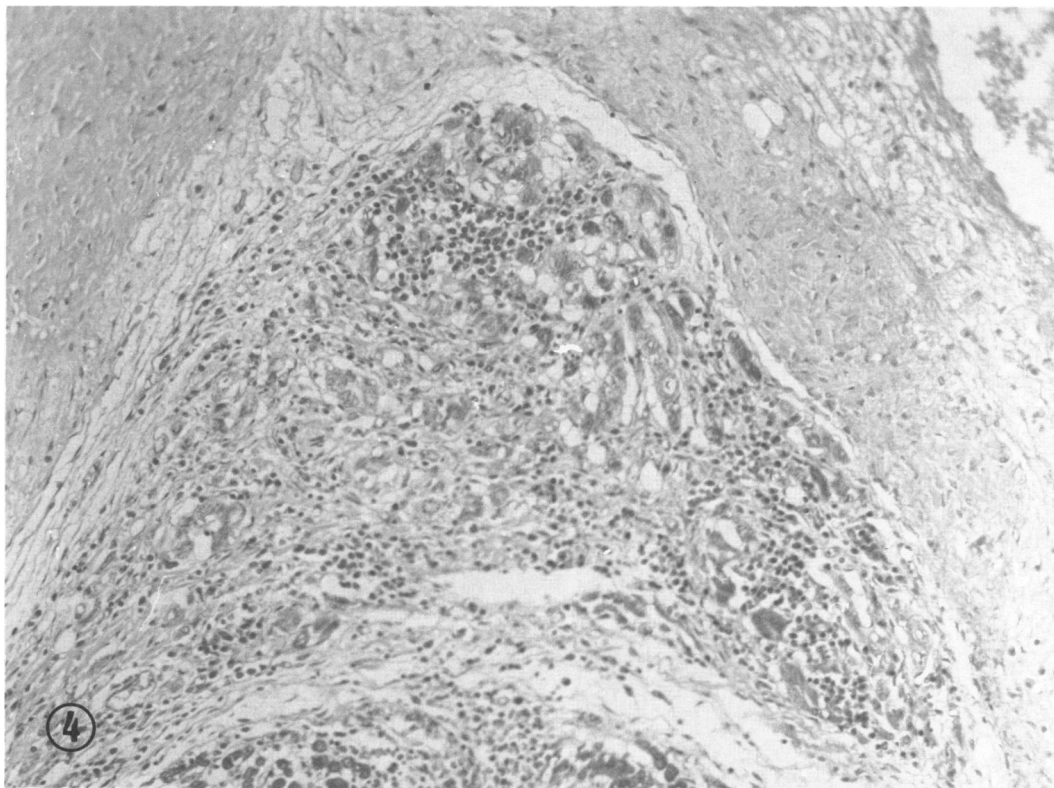


Figure 4—Severe myocarditis involving the His main bundle in a *T. cruzi*-infected dog treated with cyclophosphamide. There are inflammatory mononuclear cells, edema, and "dropping-out" of isolated conducting fibers. (Masson's trichrome, $\times 200$) **Figure 5**—Representative section from the heart of a dog treated with cyclophosphamide and showing only a moderate degree of myocarditis. The diffuse inflammatory infiltrate and the lytic necrosis of isolated myocardial fibers were taken as signs of activity of the myocarditis. (H&E, $\times 150$) **Figure 6**—Control *T. cruzi*-infected and untreated dog. There is an accumulation of lymphocytes in the interstitium of the myocardium, which otherwise appears normal. (H&E, $\times 150$)

In the second experiment, the two vaccinated and challenged animals exhibited severe myocarditis, which did not differ from that described above. The two vaccinated but not challenged dogs did not present any heart changes.

In the third experiment, the myocarditis observed in the three treated dogs was less intense than that described above. It was, however, more marked and diffuse than the mild focal myocarditis found in the two untreated dogs. In addition, the mononuclear infiltration in the treated animals exhibited signs of activity not found in those untreated. These consisted of the presence of focal necrosis of myocardial fibers and invasion of the fibers by inflammatory cells (Figure 5). By looking at the heart sections, three observers were independently able to separate the slides from cyclophosphamide-treated dogs from those untreated.

The sections from the hearts of dogs killed during the chronic stage of the infection and which were taken from the files and examined, showed a uniform microscopic picture of mild focal myocarditis. The focal infiltrates tended to be predominantly lymphocytic and to accumulate in the interstitial tissue rather than to involve the myocardial fibers (Figure 6).

In none of the animals included in the present study did congestive cardiac failure develop at any time during the experiments.

Discussion

The administration of repeated low doses of cyclophosphamide was seen to cause an exacerbation of the usually mild myocarditis found in dogs chronically infected with *T cruzi*. The extraordinary findings of the first two experiments here described could not be entirely duplicated in the third one. However, the independent assessment by three different examiners did agree that the treated animals disclosed a more intense myocarditis, which showed unequivocal signs of activity.

In the recent past we have examined several dogs with chronic *T cruzi* infection,^{8,12} and only once⁷ was it possible to see the spontaneous development of a chronic active myocarditis, such as has been described by others.^{5,6} Generally our animals have remained in the chronic latent (indeterminate) form of the disease, with only a mild residual myocarditis, as described by Goble.²²

We did not determine why cyclophosphamide enhanced the *T cruzi* myocarditis in dogs. Apparently, it did not cause *T cruzi*-specific immunodepression. The antibody titers remained elevated, and no evi-

dence of exaggerated parasite multiplication could be observed. At least the extensive and severe myocarditis found in cyclophosphamide-treated dogs could not be directly correlated with *T cruzi* multiplication when the tissue sections from the heart were examined. However, when *T cruzi* chronically infected dogs are treated with large doses of immunosuppressive drugs, the acute infection reappears, with numerous trypomastigotes directly demonstrated in the peripheral blood.²³

On the other hand, it has been shown that asymptomatic *T cruzi*-infected individuals may develop specific as well as autoimmune antibodies and sensitized immunologically competent cells.²⁴ It has been claimed that these cells can adhere to and destroy cardiac cells *in vitro*.^{3,25} Therefore, it is possible that a suppressed immunologically mediated destructive reaction against the cardiac tissue, which is suppressed during the indeterminate period of the infection, may be triggered during chronic progressive Chagas' myocarditis.

Experimental data from the literature are controversial regarding a specific immunologic suppression of the *T cruzi*-induced lesions.^{26,27} It has been demonstrated that a specific suppression of delayed hypersensitivity to *T cruzi* antigens, but not to an unrelated antigen, occurs in mice chronically infected with the parasite.²⁸ Further studies on chronic *T cruzi* mouse infection have also shown a persistent suppression of responses by immunoglobulin G antibody, which is mediated by suppressor T lymphocytes.²⁹

Studies along these lines in the dog are highly desirable, because the cardiac form of Chagas' disease, with diffuse myocarditis, cardiomegaly, arrhythmias, and progressive heart failure, has so far been experimentally obtained only in dogs. Similar to what has been observed in human Chagas' disease,^{1,24} in the dog treated with low-dose cyclophosphamide, active myocarditis occurs without any indication of interference with the established partial immunity against the parasite, which is present during the chronic latent form.

An interesting collateral observation was made with the vaccinated animals. In the first experiment we decided to give cyclophosphamide to all the dogs we had at the moment under observation and which had presented prolonged *T cruzi* infection without any external signs of disease. Among them there were 4 dogs which had been submitted to vaccination. The vaccine did prove efficient in avoiding the development of acute disease when the animals were challenged with virulent *T cruzi*, although it did not prevent the development of chronic infection. Cyclophosphamide treatment indicated that the vaccine

did not protect against the development of chronic disease. On the other hand, although the living vaccine by itself induced specific antibody response, it did not cause infection, nor did it seem to sensitize the heart tissue for a hypersensitivity reaction in the non-challenged dogs, despite the fact that they also received cyclophosphamide treatment. Although these observations were made on a rather small number of animals and say nothing about the efficacy of the vaccine used, they suggest that future studies may be useful to clarify whether the administration of low doses of cyclophosphamide may be of help in indicating whether a given experimental vaccine is prone to cause autoimmunity or other delayed type hypersensitivity reactions.

The results observed in the present study justify further attempts with the canine model to investigate problems related to the immunopathology and pathogenesis of Chagas' disease.

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Acknowledgments

Thanks are due to Drs. B. Enders, O. Zwissler, and Mr. K. Wiegand, of the Behring Institute, Marburg, Germany, for their help with the *T. cruzi* vaccine utilized in this investigation.