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EXPERIMENTAL AMERICAN TRYPANOMIASIS IN RATS: SYMPATHETIC DENERVATION, PARASITISM AND INFLAMMATORY PROCESS

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Tissue parasitism, inflammatory process (histologic methods) and sympathetic denervation (glyoxylic acid-induced histofluorescence for demonstration of catecholamines) were studied in the heart (atrium and ventricle) and the submandibular gland of rats infected with the Y strain of Trypanosoma cruzi. *In the heart paralleling intense parasitism and inflammatory process, the sympathetic denervation started at day 6 of infection and at the end of the acute phase (day 20) practically no varicose nerve terminals were found in both myocardium and vessels. In the submandibular gland, in spite of the rarity of amastigote pseudocysts and the scarcity of inflammatory foci, slight to moderate (days 13-15 of infection) or moderate to severe denervation (day 20) was found. At day 120 of infection both organs exhibited normal pattern of sympathetic innervation and only the heart showed some inflammatory foci and rare pseudocysts (ventricle). Our data suggest the involvement of circulating factors in the sympathetic denervation phenomena but indicate that local inflammatory process is, at least, an aggravating factor.*

Key words: heart – submandibular gland – Chagas' disease – *Trypanosoma cruzi* – sympathetic denervation – inflammation – parasitism – nerve terminal lesions – myocarditis

Neuron cells death has been fully demonstrated to occur in autonomic ganglia during the acute phase of the human and experimental Chagas' disease (Koberle, 1968; Tafuri, 1970; Oliveira, 1985). The mechanisms underlying this neuronal death remain to be elucidated. Neuronal parasitism (Koberle, 1968; Tafuri, 1970) and toxic substances released by the parasites (Teixeira et al., 1980) are no longer regarded as important factors. Alternatively, parasitism of glial cells and the resulting inflammatory response has been proposed (Tafuri, 1970). More recently, several evidences are pointing to the involvement of immune reactions through auto-reactive or cross-reactive antibodies or through the adsorption of *Trypanosoma cruzi* antigens to the neuronal surface membrane (Santos-Buch, 1979; Ribeiro-Santos & Hudson, 1980; Wood et al., 1982; Williams et al., 1985). The lesion of neuron cell bodies may have irregular and unpredictable distribution (Tafuri, 1970) or may be even absent, especially in the sympathetic ganglia (reviewed by Camargos & Machado, 1988). In contrast, in peripheral organs of young rats, the sympathetic nerve terminals are always greatly reduced or even completely lost during the acute phase of the *T. cruzi*-infection (Machado et al., 1975, 1978, 1984) most probably by destruction of nerve terminals (Camargos & Machado, 1988).

A severe acute myocarditis occurs in the *T. cruzi*-infected rats (Scorza & Scorza, 1972; Machado et al., 1975) and a correlation between cardiac denervation and this inflammatory process has been proposed (Machado et al., 1975). However, in the submandibular gland no inflammatory exudate was found at the end of the acute phase when the sympathetic innervation was greatly reduced (Machado et al., 1984). In order to gain further insight on the relationship between these phenomena, the time course of tissue parasitism inflammatory process and sympathetic denervation was studied in the heart and submandibular gland of *T. cruzi*-infected rats. Since the atrial and ventricular muscle fibers are functionally and morphologically different (Gorza et al., 1982), the left atria and left ventricle were studied separately.

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MATERIAL AND METHODS

Holtzman rats of both sexes aged 25-28 days were inoculated intraperitoneally with 0.15 ml of mouse blood containing 300,000 trypomastigotes of the Y strain of *T. cruzi*. Littermates were kept as control.

The infection was confirmed by fresh blood examination before or at day 13 of infection. At different periods of infection (Table I), the submandibular glands and the hearts of control and *T. cruzi*-infected animals were dissected out under ether anaesthesia for histological studies (parasitism and inflammatory exudate) and for the histochemical demonstration of sympathetic innervation.

For histological study the left auricular appendage, a transverse fragment of the base of the left ventricles, and a lengthwise slice of the left submandibular glands were fixed in 25% Bouin's fluid for 20 hours followed by fixation in 10% formalin for 4 hours. After routine procedure for paraffin embedding, 5 μm thick sections were stained by haematoxylin and eosin.

Tissue parasitism was estimated in sections separated by 70 μm intervals to avoid recounting of amastigote nests (Vichi, 1964; Hanson & Roberson, 1974). For each tissue fragment of all animals, 150 microscopic fields were examined at a magnification of 500X and all amastigote nests were counted in every field.

The sections used in the study of tissue parasitism as well as sections from control animals were examined at 256X for a semi-quantitative evaluation of the inflammatory process. Each microscopic field received a value for the inflammatory exudate from 0 to 4 according to the following gradation: 0 = none; 1 = slight and with diffuse or focal distribution; 2 = moderate and with focal distribution; 3 = either moderate and diffuse or intense and focally distributed; 4 = intense and diffuse. This gradation is similar to that used by Kulmar et al. (1969). Fifty microscopic fields were scored for each tissue fragment of all control and *T. cruzi*-infected animals and an inflammatory index was obtained by summing the values given to each field.

For histochemical estimative of the catecholamine-containing nerves, the right auricular appendage, a second fragment of the base of the left ventricles and a lengthwise slice of the right submandibular glands were taken from each control and infected animals at days 6, 13, 15, 20, 32 and 120 of infection. Cryostat sections (16 μm thick) were submitted to a glyoxylic acid-induced fluorescence method according to the la Torre (1980).

The values for tissue parasitism and inflammatory process obtained at days 6, 10, 13, 15 and 20 of infection were treated statistically by the analysis of variance and least significant difference, using logarithmic transformation when necessary.

RESULTS

Heart – Our results on tissue parasitism, inflammatory process and sympathetic innervation are shown in Tables I (atria) and II (ventricles).

During the acute phase of the experimental Chagas' disease, the heart was heavily parasitized although great variation had occurred among the animals. Amastigote nests inside cardiac muscle cells by far outnumbered those identified in connective tissue cells, including macrophage-like ones. Sometimes macrophages were seen inside ruptured muscle pseudocyst (Fig. 1).

Amastigote nests could be detected in both atria and ventricles as early as 4 days after inoculation. In atrial tissue, the pseudocyst number became maximal at day 10 of infection. However, for ventricle tissue no statistical difference was found among the parasitism values from day 6 to day 15 of infection. In spite of that, an abrupt and significant fall occurred at day 20 of infection. In both heart regions, amastigotes practically disappeared after day 20 of infection but even at day 120 of infection one amastigote nest per 450 microscopic field could be detected in ventricle tissue.

Inflammatory process was an outstanding feature of the heart during the acute phase of the experimental Chagas' disease. The cellular infiltrate was characterized by a predominance of mononuclear cells.

TABLE I

Rat auricular appendages at different periods of *Trypanosoma cruzi* infection: tissue parasitism (number of amastigote nests/150 microscopic fields at 500X); inflammatory process (index/50 microscopic fields at 256X) and density of sympathetic fluorescent nerve fibers. The number of rats are given in parenthesis

Days after inoculation	Parasitism		Inflammation		Innervation
	Mean	Range	Mean	Range	
Controls (17)	–	–	4.13	1-10	Normal pattern
4 (3)	1.00	0-3	21.67	9-32	Not done
6 (6)	16.33*	0-49	40.33**	20-53	Slight to moderate denervation except for 1 animal
10 (6)	141.67*	3-401	78.17**	36-119	Not done
13 (6)	12.33*	1-26	48.67**	36-68	Moderate to complete denervation
15 (6)	32.33*	1-114	77.67**	53-98	Moderate to complete denervation
20 (6)	1.50*	0-5	44.33**	42-51	Complete or almost complete denervation
24 (3)	0.67	0-2	49.33	42-60	Not done
32 (4)	0	–	37.00	30-41	Almost complete denervation. Only one animal with moderate denervation
120 (3)	0	–	7.67	3-14	Similar to controls

* $P < 0.01$; least significant difference (1sd) at the 1% level = 108.71.
** $P < 0.05$; 1sd at 5% level = 21.94; 1sd at 1% level = 29.68.

TABLE II

Rat ventricle at different periods of *Trypanosoma cruzi* infection: tissue parasitism (amastigote nests/150 microscopic fields at 500X), inflammatory process (index/50 microscopic fields at 256X) and density of sympathetic fluorescent nerve fibers. The number of rats are given in parenthesis

Days after inoculation	Parasitism		Inflammation		Innervation
	Mean	Range	Mean	Range	
Controls (17)	–	–	5.30	0-10	Normal pattern
4 (3)	2.00	0-5	24.00	5-43	Not done
6 (6)	18.00*	0-52	41.67**	24-57	Moderate denervation
10 (6)	41.33*	0-125	50.00**	12-63	Not done
13 (6)	26.50*	9-79	51.16**	30-78	Moderate to complete denervation
15 (6)	72.33*	7-358	46.67**	31-55	Moderate to complete denervation
20 (6)	2.67*	0-10	52.50**	42-66	Complete denervation
24 (3)	0.33	0-1	34.00	28-38	Not done
32 (4)	0.25	0-1	29.75	19-37	Complete denervation
120 (3)	0.33	0-1	15.33	3-22	Similar to controls

* $P < 0.05$ after logarithmic transformation, when the means were 0.807, 1.221, 1.3116, 1.4136, and 0.426; least significant differences at 5% level = 0.7189.
** $P > 0.05$.

Inflammatory exudate could already be detected at day 4 of infection as occasional and very discrete focal infiltrates, in both atria and ventricles. At day 6 of infection either focal or diffuse inflammatory infiltrates were clearly present in the heart (atria and ventricle) of all *T. cruzi*-infected animals. Thereafter, the myocarditis became markedly more severe in atrial tissue where it was maximal at days 10 and 15 of infection (Fig. 3). At day 32 of infection, discrete to moderate infiltrates were still present. In ventricular tissues, the inflammatory process remained about the same from day 6 to 20 of infection. Thereafter, a tendency to resolution was observed but till day 120 of infection some focal discrete infiltrates were found.

The involvement of the heart sympathetic innervation of atria and ventricles was quite

TABLE III

Rat submandibular gland at different periods of *Trypanosoma cruzi* infection: tissue parasitism (amastigote nests/150 fields at 500X); inflammatory process (index/50 fields at 256X), and density of sympathetic fluorescent nerve fibers. The numbers of rats are given in parenthesis

Days after inoculation	Parasitism		Inflammation		Innervation
	Mean	Range	Mean	Range	
Controls (17)	–	–	0.77	0-3	Normal pattern
4 (3)	0	–	1.33	0-4	Not done
6 (6)	0.67*	0-3	2.50*	0-4	Normal pattern
10 (6)	0.50*	0-2	6.50*	1-16	Not done
13 (6)	0.50*	0-1	3.50*	1-7	Slight to moderate denervation
15 (6)	1.00*	0-4	8.67*	0-21	Slight to moderate denervation
20 (6)	0*	–	6.50*	3-12	Moderate to severe denervation
24 (3)	0	–	2.33	2-3	Not done
32 (4)	0	–	2.75	2-3	Slight to moderate denervation
120 (3)	0	–	0.67	0-1	Similar to controls

* $P > 0.05$ after logarithmic transformation.

similar during the course of the experimental disease. Clear signs of denervation were already present in most animals at day 6 of infection. All animals killed at days 13 and 15 of infection exhibited moderate to complete sympathetic denervation (Figs 5 and 6). Practically no fluorescent varicose nerve fibers could be detected at day 20 of infection in both myocardium and vessels. The virtual absence of fluorescent fibers remains till day 32 in the ventricle. However, a few varicose nerve fibers were present in atria of most animals by this time. Complete recovery of the vascular and myocardial sympathetic innervation was observed in atria and ventricles at day 120 of infection.

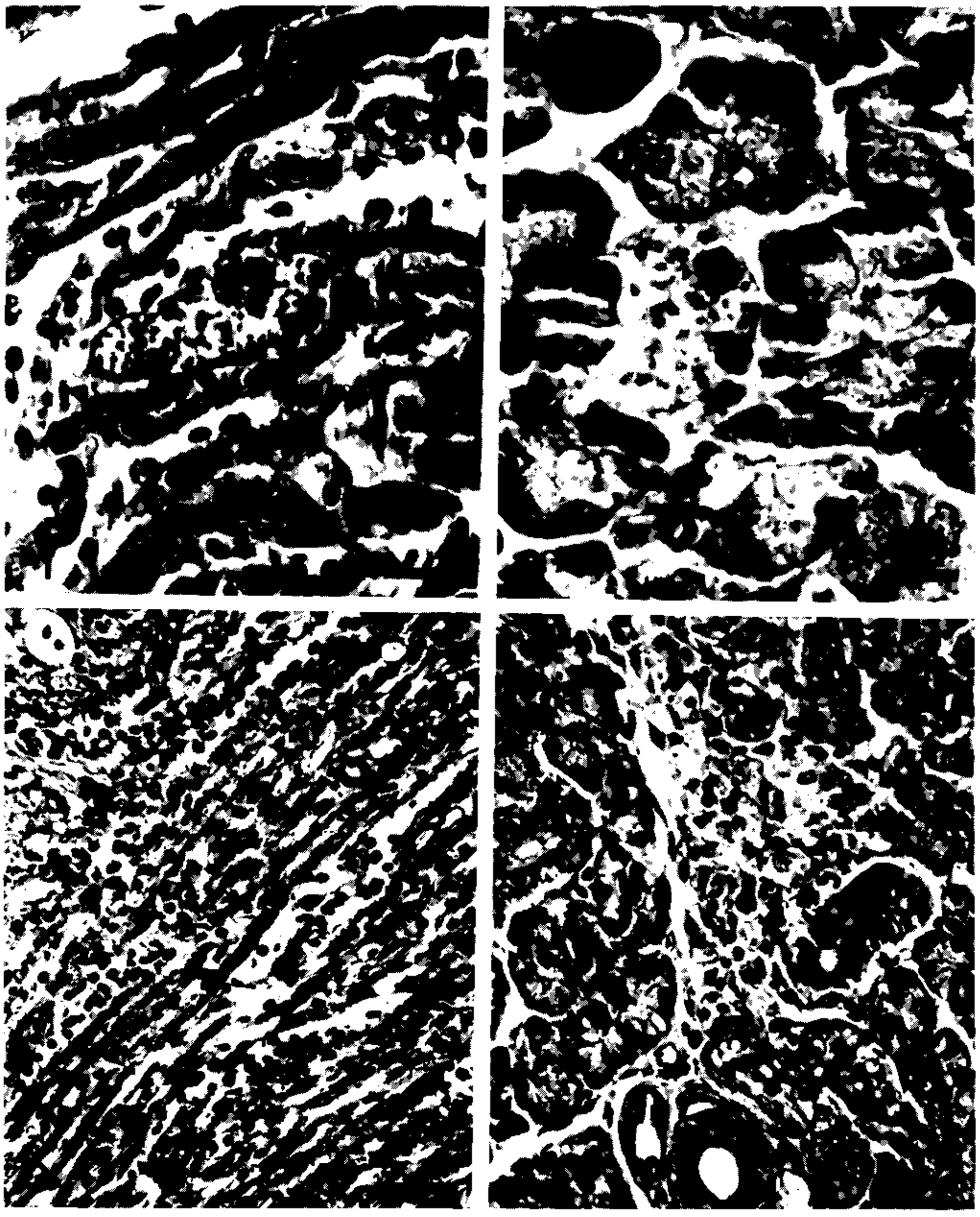
Submandibular gland – Our results are summarized in Table III. Amastigote nests were rarely observed in the submandibular gland from day 6 to day 15 (only 16 nests in 3,750 microscopic fields at 500X; 25 animals). These few nests were observed in acinar cells (Fig. 2) or in connective tissue cells, without any significant difference among the values found for each period of infection.

Inflammatory process, when present, was very discrete and confined to a few small areas, mainly in the connective tissues around some ducts and blood vessels. At day 4 of infection no inflammatory process was found, and at day 6 only one infected animal exhibited a discrete accumulation of inflammatory cells in a few areas. About 50% of the animals killed at day 10 and 13 and 70-85% of those killed at days 15 and 20 of infection exhibited such small and sparse areas with inflammatory cells (Fig. 4). However, no significant difference was found among the inflammatory indexes at these periods of infection.

The sympathetic innervation of the submandibular gland was not appreciably altered by the experimental disease till day 13 of infection when slight to moderate denervation was observed in most animals (Figs 7 and 8). At day 20 of infection moderate to severe denervation was present in all infected animals. However, some recovery was already present ad day 32 of infection, becoming total at day 120.

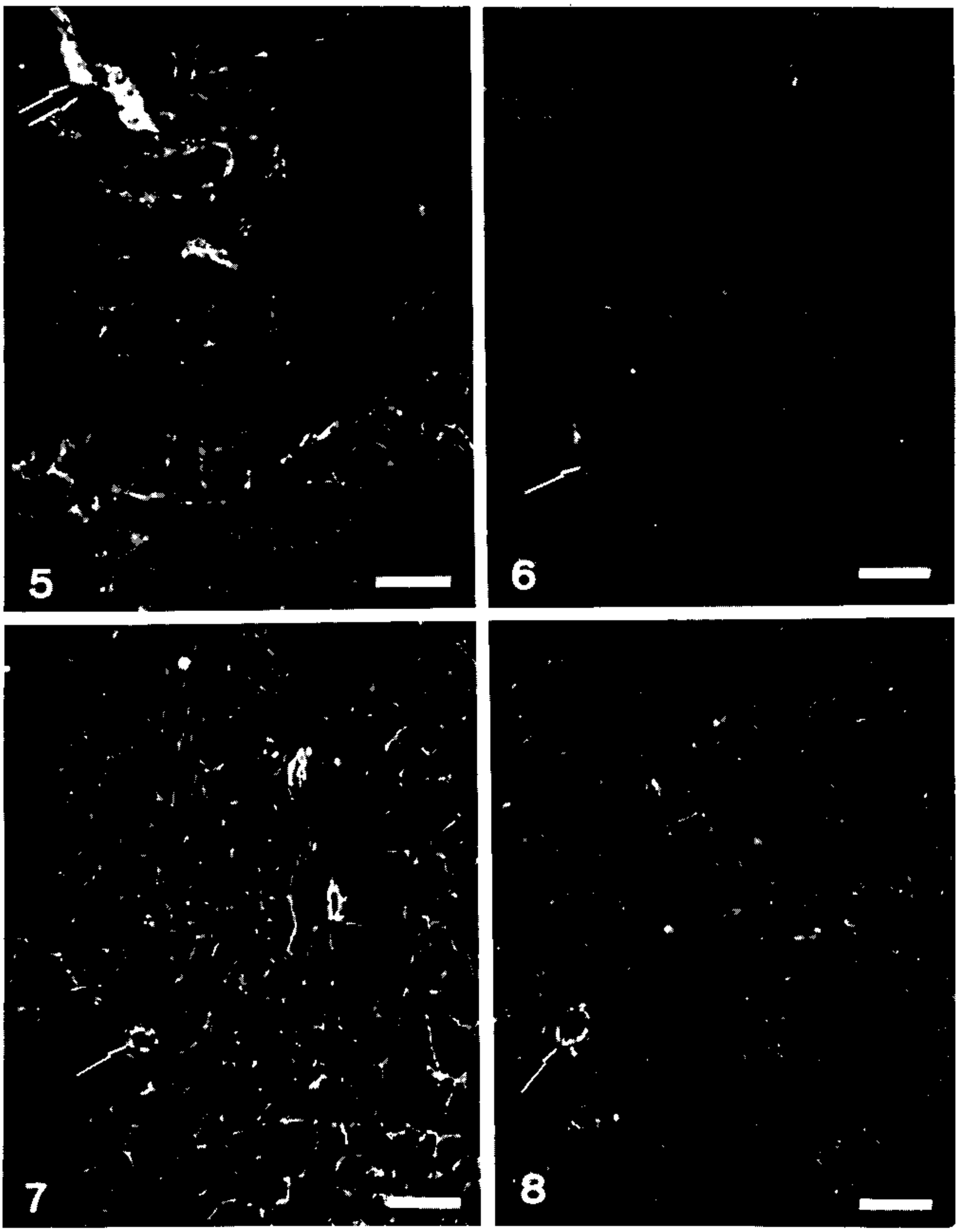
DISCUSSION

Adult rats are naturally resistent to infection by *T. cruzi* (Dias, 1934) but young rats develop a typical acute phase with mortality diminishing as the animals age (Kolodny, 1939; Scorza & Scorza, 1972; Sogayar, 1978). During the acute phase of the disease in rats, several organs are parasitized with tissue tropism varying according to the parasite strain (Sogayar, 1978) as demonstrated for mice (Melo & Brener, 1978). With the Y strain, myotropism is observed with the highest parasitism values in the heart (Sogayar, 1978). As described for the human and murine experimental acute disease (Koberle, 1968; Tafuri, 1970), inflammatory process is an outstanding finding in the rat heart and its parasympathetic ganglia (Scorza & Scorza,



Hematoxylin-eosin stained sections of heart and submandibular gland of *Trypanosoma cruzi*-infected rat killed at 10 days (2, 3) and 17 (1) after inoculation. Fig. 1: observe an amastigote nest in a ventricular muscle fiber invaded by a macrophagelike cell and several lymphocytes along some cardiac muscle fibers (arrows). Fig. 2: submandibular gland showing amastigotes in acinar cells and absence of inflammatory cells. Fig. 3: diffuse and intense inflammatory process in atrial tissue with predominance of mononuclear cells. Fig. 4: a focal and discrete inflammatory process in the submandibular gland. Bars = 18 μm (1), 11 μm (2), 36 μm (3), 29 μm (4).

1972). Our findings on heart parasitism and inflammatory process paralleled those previously described (Scorza & Scorza, 1972) except for the early detection of amastigote pseudocysts and inflammatory foci. The discrepancy may be explained by the younger age of our animals that were also inoculated with a higher amount of trypomastigotes. Our histologic findings on the heart at day 120 after inoculation are very similar to those obtained in organs of chagasic patients that survived the acute phase passing to an asymptomatic chronic phase (latent or indeterminate phase). When some of these patients died in consequence of another disease



Sympathetic innervation of auricular appendages (Figs 5, 6) and of submandibular gland (Figs 7, 8) of control and *Trypanosoma cruzi*-infected rats killed at day 13 of infection. Note that in the infected animals (6, 8) the fluorescent varicose nerve terminals are practically absent in the heart (6) or moderately reduced in the submandibular gland (8). Arrows indicate vascular innervation. Bar = 83 μm.

or accident, no characteristic feature was found at autopsy. However, as reported by Koberle (1968) "a painstaking examination of many histologic sections revealed some chronic inflammatory foci and, exceptionally a pseudocyst".

In good agreement with our results, tissue parasitism and inflammatory process have not been found or were rarely reported in salivary glands of *T. cruzi*-infected mice (Collier et al., 1942; Bice & Zeledon, 1970; Hanson & Roberson, 1974).

An extensive destruction of parasympathetic neurons has been described in the heart (Alcantara, 1959) and gastro-intestinal tract (Alcantara & Oliveira, 1964) of the

T. cruzi-infected rats. However, the parasympathetic denervation is followed by recovery through axonal sprouting from neurons left intact (Machado et al., 1987). In the sympathetic nervous system, however, only the nerve terminals in peripheral organs are consistently lesioned during the acute infection in rats and the recovery observed is probably due to axonal regrowth (Machado et al., 1978, 1980; Camargos & Machado, 1988).

It has been demonstrated that the rat cardiac noradrenaline content drops to about 50% at day 12 and to undetectable values at day 18 of the infection (Machado et al., 1975). Using a very sensitive histochemical method, we demonstrated now that the catecholoamine-containing nerve terminals begin to disappear from both atrium and ventricle as early as 6 days after inoculation when discrete to moderate inflammatory process is also present. In the submandibular gland however, the sympathetic denervation starts with a delay of about one week in relation to that of the heart, and was always less severe. Our findings on tissue parasitism and inflammation showed striking difference between heart and submandibular gland. Therefore the ansynchronism of the denervation phenomena as well as its severity may be correlated with the parasitism and resulting inflammatory process. In mice and humans, the destruction of nerve cells in para-sympathetic and sympathetic ganglia are always associated with inflammatory exudate (Tafuri, 1970). Our data suggest that although intense parasitism and inflammatory process are not necessary for sympathetic denervation, they seem to be at least aggravating factors. It is also important to remember that nerve terminals in peripheral organs are supposed to be more accessible to lytic factors than the nerve cell bodies inside the sympathetic ganglia as discussed elsewhere (Camargos & Machado, 1988).

The occurrence of sympathetic denervation in the submandibular gland in the absence of appreciable inflammatory infiltrate at any period of the acute infection points to the possibility of involvement of circulating factors in the denervation phenomenon. This possibility is supported by studies showing circulating antibodies reacting with unmielinated autonomic nerves (Khoury et al., 1979). However, the possibility of deleterious influence of a few inflammatory foci on nerves that penetrates the salivary gland through blood vessel can not be ruled out.

RESUMO

Desnervação simpática, parasitismo e processo inflamatório durante a doença de Chagas experimental, em ratos – Parasitismo tecidual, processo inflamatório (métodos histológicos) e desnervação simpática (histofluorescência induzida por ácido glioxílico para demonstração de catecolaminas) foram estudados no coração (átrio e ventrículo) e na glândula submandibular de ratos infectados com cepa Y de *Trypanosoma cruzi*. No coração, em paralelo com intenso parasitismo e processo inflamatório, a desnervação simpática iniciou-se no 6º dia de infecção e ao fim da fase aguda (20º dia) praticamente nenhuma terminação nervosa varicosa foi encontrada tanto no miocárdio como em vasos. Na glândula submandibular, apesar da raridade de ninhos de amastigotas e da escassez de focos inflamatórios, encontram-se discreta e moderada 13º-15º dia de infecção) ou moderada a severa (20º dia) desnervação. Aos 120 dias de infecção, ambos os órgãos exibiram padrão normal de inervação e somente o coração mostrou alguns focos inflamatórios e raros pseudocistos (ventrículos). Nossos dados sugerem o envolvimento de fatores circulantes no fenômeno de desnervação simpática mas indicam que processo inflamatório local é, pelo menos, um fator agravante.

Palavras-chave: coração – glândula submandibular – doença de Chagas – *Trypanosoma cruzi* – desnervação simpática – inflamação – parasitismo – lesão de terminações nervosas

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