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Preganglionic fibres of the adrenal medulla and cervical sympathetic ganglia: differential involvement during experimental American trypanosomiasis in rats

E.R.S. CAMARGOS, L.R.M. HAERTEL AND C.R.S. MACHADO
Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

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Summary. The acute phase of experimental Chagas' disease in rats is associated with early lesions of the post-ganglionic sympathetic nerve terminals of the heart, the varicosities being the target. In the superior cervical and stellate ganglia the preganglionic fibres showed no signs of lesion in the course of experimental Chagas' disease. In the adrenal medulla, however, the acute phase of the *Trypanosoma cruzi* infection induced a clear rarefaction of the acetylcholinesterase-positive nerve fibres (20 and 32 days after infection). Recovery of the normal pattern occurred in most animals by day 125 after infection. At days 20, 32 and 46 after infection, electron-microscopic studies demonstrated the occurrence of damage in cholinergic nerve terminals contacting the chromaffin cells. The signs of damage included dense bodies, clumps of synaptic vesicles and filaments, rarefaction of all organelles, vacuoles and irregular contour. The ultrastructural peculiarities of the sympathetic ganglia may explain the ganglionar microenvironment protective against the hazardous factors elicited by acute Chagas' disease.

Keywords: preganglionic sympathetic innervation, Chagas' disease, adrenal medulla, superior cervical ganglion, stellate ganglion

Chagas' disease (American trypanosomiasis) affects millions of people in South American countries (reviews in Brener 1980; Teixeira 1987). Its causative agent is a digenetic protozoan, *Trypanosoma cruzi*, transmitted by a reduviid bug. In the acute phase of the disease, trypomastigotes circulate in the blood stream and amastigotes proliferate in several tissues. Cardiac, skeletal and some smooth muscle cells are particularly susceptible (Bice & Zeledon 1970; Melo & Brener 1978), probably because they exhibit specific surface receptors for molecules on the parasite surface membrane (Zingales & Colli 1985). A long and asymptomatic phase (latent or indeterminate) follows the acute phase; many patients remain asymptomatic but some proceed to a later chronic symptomatic phase.

Death of autonomic neurones occurs during the acute phase of Chagas' disease, mainly in the parasympathetic ganglia of the heart and the myoenteric plexus (Köberle 1968; Tafuri 1970). Several studies in experimental models support this ganglionic depopulation (Alcantara 1959; Tafuri 1979, Oliveira 1985). The mechanism underlying the neuronal death remains to be elucidated, but direct neuronal parasitism has been discarded

Correspondence: C.R.S. Machado, Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Caixa Postal 486, 30161-901 Belo Horizonte, MG, Brazil.

(Köberle 1970, Tafuri 1979; Ribeiro dos Santos & Hudson 1981).

In the rat, the acute phase of the *T. cruzi* infection with Y strain induces a severe reduction of both sympathetic and parasympathetic post-ganglionic axons in several organs. After the acute phase, a gradual recovery of the autonomic innervation takes place (Machado *et al*. 1978; 1979; 1984; 1987). Regarding the sympathetic system, the degree of post-ganglionic denervation seems to depend on the severity of tissue parasitism and inflammatory processes (Machado & Ribeiro 1989). In the heart, this denervation is virtually total (Machado *et al*. 1978), and the disappearance of the sympathetic terminals is independent of detectable neuronal death (Camargos & Machado 1988). This finding indicates that these terminals are more susceptible to damage than the neuronal cell bodies in paravertebral ganglia. Ultrastructural studies showed that damage to the noradrenergic axons of the heart starts very early, the main target being the varicosities (Machado *et al*. 1994). Nothing is known, however, of the possible involvement of preganglionic sympathetic fibres in either human or experimental American trypanosomiasis.

The present paper describes our observations on the preganglionic fibres of cervical paravertebral ganglia and the adrenal medulla, during the experimental Chagas' disease in rats. As happens with the sympathetic ganglia, a rich plexus of preganglionic axons supplies the adrenal medulla (Coupland 1965). Our interest in studying, comparatively, sympathetic ganglia and adrenal medulla rests on differences in their microvasculature. In sympathetic ganglia, a partial blood–ganglion barrier (Depace 1982) creates a microenvironment which protects the neurones while in the adrenal medulla such a barrier is absent.

Material and methods

Animals

Female Holtzman rats aged 27–29 days were inoculated intraperitoneally with a single inoculum of 150 μl of mouse blood containing 3×10^5 trypomastigotes of *T. cruzi*, Y strain (Silva & Nussenzweig 1953). Examination of fresh blood sampled from the tail showed living trypomastigotes in all infected animals 10–12 days after inoculation. These animals and control litter-mates were killed at different periods after infection for histochemical and ultrastructural studies of sympathetic paravertebral ganglia (superior and inferior cervical ganglia) and adrenal medulla.

Light-microscopic histochemistry for evidence of acetylcholinesterase activity

A thiocholine method (Karnovsky & Roots 1964) modified for better demonstration of central cholinergic axons (Hedreen *et al*. 1985), provided a good visualization of the preganglionic fibres in both adrenal medulla and sympathetic ganglia. The animals were sacrificed at days 20 (3 controls; 4 *T. cruzi*-infected), 32 (4 controls; 6 infected) and 125 (6 controls; 6 infected) post infection. The animals were killed under chloral hydrate anaesthesia by intracardiac perfusion with 0.1 M phosphate buffer at pH 7.2 (50 ml) followed by 4% phosphate-buffered paraformaldehyde (100–150 ml). The organs remained in the fixative for 5 hours at 4°C, being then washed three times in 15% sucrose-phosphate buffer. Cryostat sections (16 μm thick) were incubated in a medium containing acetylthiocholine iodide as substrate (Karnovsky & Roots 1964) and 10^{-4} M tetraisopropyl pyrophosphamide (Iso-OMPA, Sigma) as inhibitor of non-specific cholinesterases. After washing in acetate buffer, the sections were treated according to Hedreen *et al*. (1985) for enhancing the intensity of the reaction.

Electron-microscopic methods

The animals were killed under chloral hydrate anaesthesia at days 20, 32 and 46 post-infection. At each period, three controls and three *T. cruzi*-infected rats were perfused via the left ventricle with saline followed by phosphate-buffered 1% paraformadehyde and 1% glutaraldehyde. Fragments of the cervical ganglia and adrenals remained in the fixative overnight at 4°C. Osmication was performed for one hour with 1% phosphate-buffered osmium tetroxide. All tissues were then processed for resin embedding (PolyBed 812, Polysciences). The ultrathin sections were stained with uranyl acetate and lead citrate.

Ultrastructural demonstration of AChE activity was also performed in tissues of two controls and two infected rats, at days 20 and 46 post-infection. After fixation as described above, the ganglia and adrenals remained in the fixative for one hour at 4°C. Vibratome slices (50 μm thick) were incubated in Karnovsky's (1964) medium for 30 minutes at 4°C. After washing, the slices were treated with 1% ammonium sulphide, post-fixed in osmium tetroxide, and then processed for embedding in PolyBed 812.

Results

Light microscopy

In the superior cervical ganglia, the density and

distribution of the AChE-positive nerve fibres were very similar to those of controls at all periods of *T. cruzi* infection (Figure 1a and b). The density of the varicose terminals around the principal ganglion neurons varies considerably, some neurons being clearly more innervated. In view of this heterogeneity, it was impossible to rule out completely the occurrence of some mild denervation in the infected animals.

The cholinergic innervation of the adrenal medulla comprised a rich network of AChE-positive nerve fibres, the delicate varicose terminals coursing between the chromaffin cells and around the few neuronal cell bodies (Fig. 1c). The adrenal capsule and cortex exhibited AChE-positive nerve branches that became very thin inside the medulla. At day 20 of infection, 4 out of 6 animals presented clear reduction of the varicose nerve terminals. Some of the remaining axonal terminals disclosed dilated varicosities. At day 32, all infected animals presented moderate to severe reduction of the AChE-positive nerve terminals (Figure 1d). At day 125, four infected animals presented cholinergic innervation similar to that of controls. In the two other animals, this innervation was still moderately reduced but all the varicose terminals were apparently normal.

Electron microscopy

In both superior and inferior cervical ganglia, the *T. cruzi* infection failed to provoke any detectable ultrastructural changes in the neuronal cell bodies, cholinergic synapses or unmyelinated axons in Schwann cell units. In control and infected rats, the perineuronal Schwann cell completely surrounded the neuronal cell bodies (Figure 2a) and all axo-dendritic (Figure 2b) and axo-somatic cholinergic synapses. Thin processes of Schwann cells also enveloped the single unmyelinated axons and those in Schwann cells (Figure 2c). Macrophage-like cells were seen in perivascular spaces (Figure 2a) and close to Schwann cell units. These cells or their processes could also approach the neuronal cell bodies (Figure 2a) and the axo-dendritic synapses (Figure 2b). In this location, however, extracellular space containing collagen fibrils always separated the macrophage membrane from the Schwann cell basal membrane.

In the ganglionic capsule of all infected animals, areas indistinct from those of controls alternated with others exhibiting infiltrating mononuclear cells that sometimes formed rows between the perineural cells (Figure 3a). Inflammatory processes inside the ganglia were rare. When present, they were confined to the subcapsular space. Moreover, in some subcapsular interstices, as well as near the small granule-containing endocrine cells and their fenestrated capillaries, the collagen fibrils were more disassociated from one another, as expected in oedematous tissue (Figure 3a and b). In contrast, no signs of oedema were present around continuous capillaries (Figure 2a) which by far outnumbered the fenestrated ones.

In the adrenal medulla, all capillaries presented a wide lumen and were fenestrated. Unmyelinated axons in Schwann cell units were found among chromaffin cells. In these nerve bundles, the Schwann cell processes seemed to surround all axons completely. Myelinated axons were rarely observed. The synaptic nerve terminals contained mainly agranular synaptic vesicles (Figure 4a), responding positively to the histochemical method for AChE activity. They could be partially involved by (Figure 4a) or totally devoid of Schwann cell processes (Figure 4b). In the latter case, the nerve terminal could be completely surrounded by a chromaffin cell (Figure 4b).

At days 20, 32 and 46 after *T. cruzi* infection, several synapses presented the morphology described for the controls. However, synaptic nerve terminals presenting signs of damage were frequent. These signs comprised dense bodies (Figure 4c), clumps of synaptic vesicles and filaments (Figure 4d and e), swollen and empty nerve terminals (Figure 4e) and vacuoles. Nerve endings labelled by the presence of AChE activity also exhibited these alterations (Figure 4c). In contrast, the small bundles of unmyelinated axons exhibited no ultrastructural changes (Figure 4e), even in oedematous areas. Macrophages were easily found at all periods after infection. They seemed activated, exhibiting large cytoplasmic volume rich in granular endoplasmic reticulum, polysomes and a population of vesicles and dense bodies very heterogeneous in size and aspect. Frequently, these cells or their processes were close to both damaged (Figure 5a) and undamaged (Figure 5b) nerve terminals.

Discussion

In the rat, the great majority of the sympathetic preganglionic fibres are unmyelinated (Gabella 1985). In humans the proportion of these unmyelinated preganglionic fibres varies from 5 to 80% in the superior cervical ganglion and from 20 to 65% in the adrenal medulla (Gabella 1976). The long varicose nerve terminals inside sympathetic ganglia and adrenal medulla are unmyelinated as usual for any kind of nerve terminal. At both adrenal medulla and sympathetic ganglia, the ultrastructural aspects of the preganglionic innervation are well known (Coupland

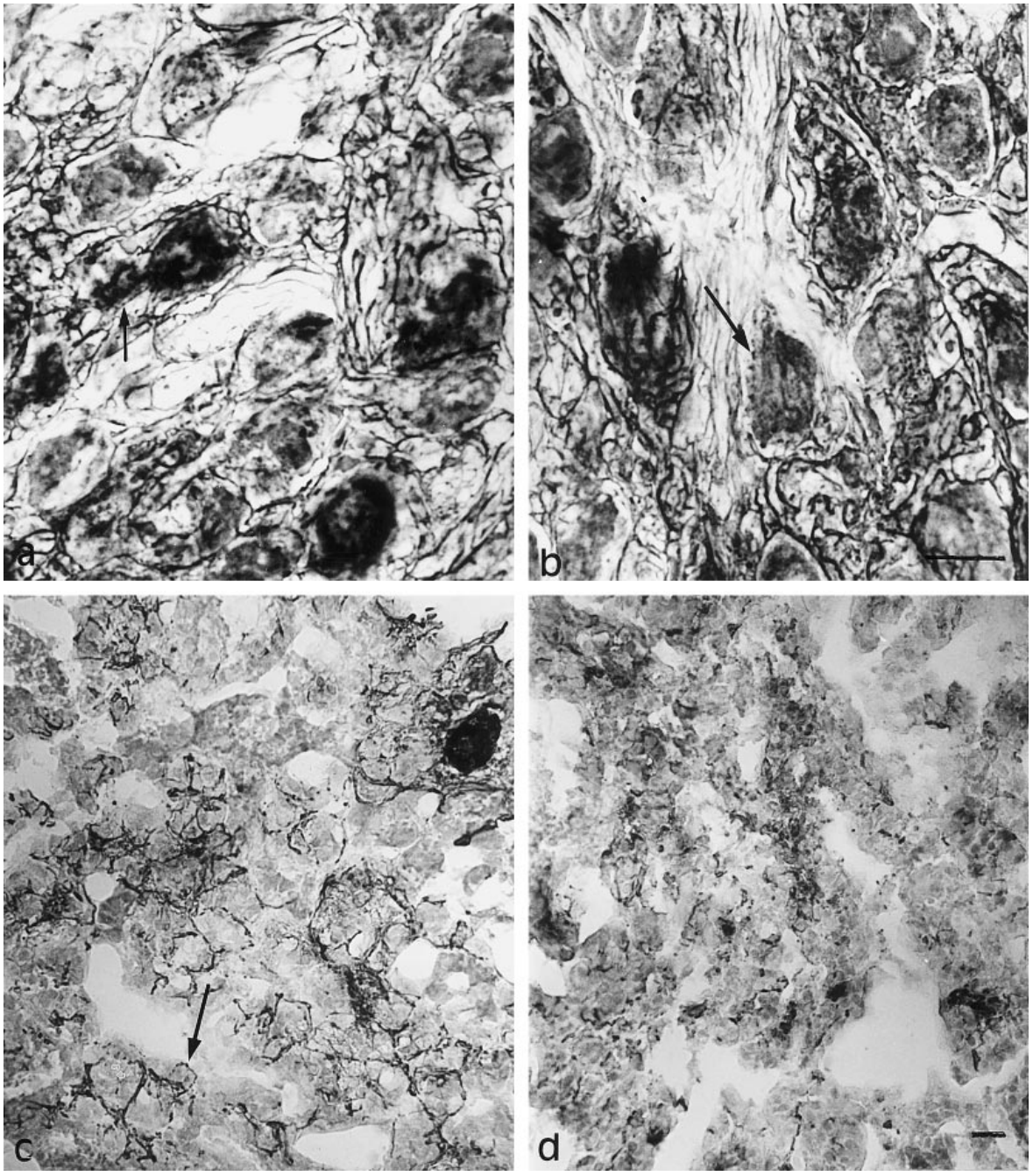

Figure 1. AChE-positive nerve fibres of a, b, the superior cervical ganglion and c, d, adrenal medulla of a, c, control and b, d, *T. cruzi*-infected rats at day 32 after infection. In the ganglion, the acute infection had no detectable effect on the density of the AChE-positive nerve fibres. Most nerve terminals surround darkly and light-stained neuronal cell bodies. In the adrenal medulla, the disease induced marked rarefaction of the preganglionic fibres. Some varicose nerve terminals are arrowed. Scale bar 20 μm.

1965; Gabella 1976; Kása *et al.* 1991) and our findings are in agreement with these previous studies.

The present results showed that the preganglionic fibres innervating the cervical sympathetic ganglia remained unchanged during the acute phase of the experimental American trypanosomiasis in rats. In the adrenal medulla, however, damage to the preganglionic axons occurred at their varicose endings. This damage caused moderate to severe reduction of the chromaffin cell innervation at the end of the acute phase, as histo-

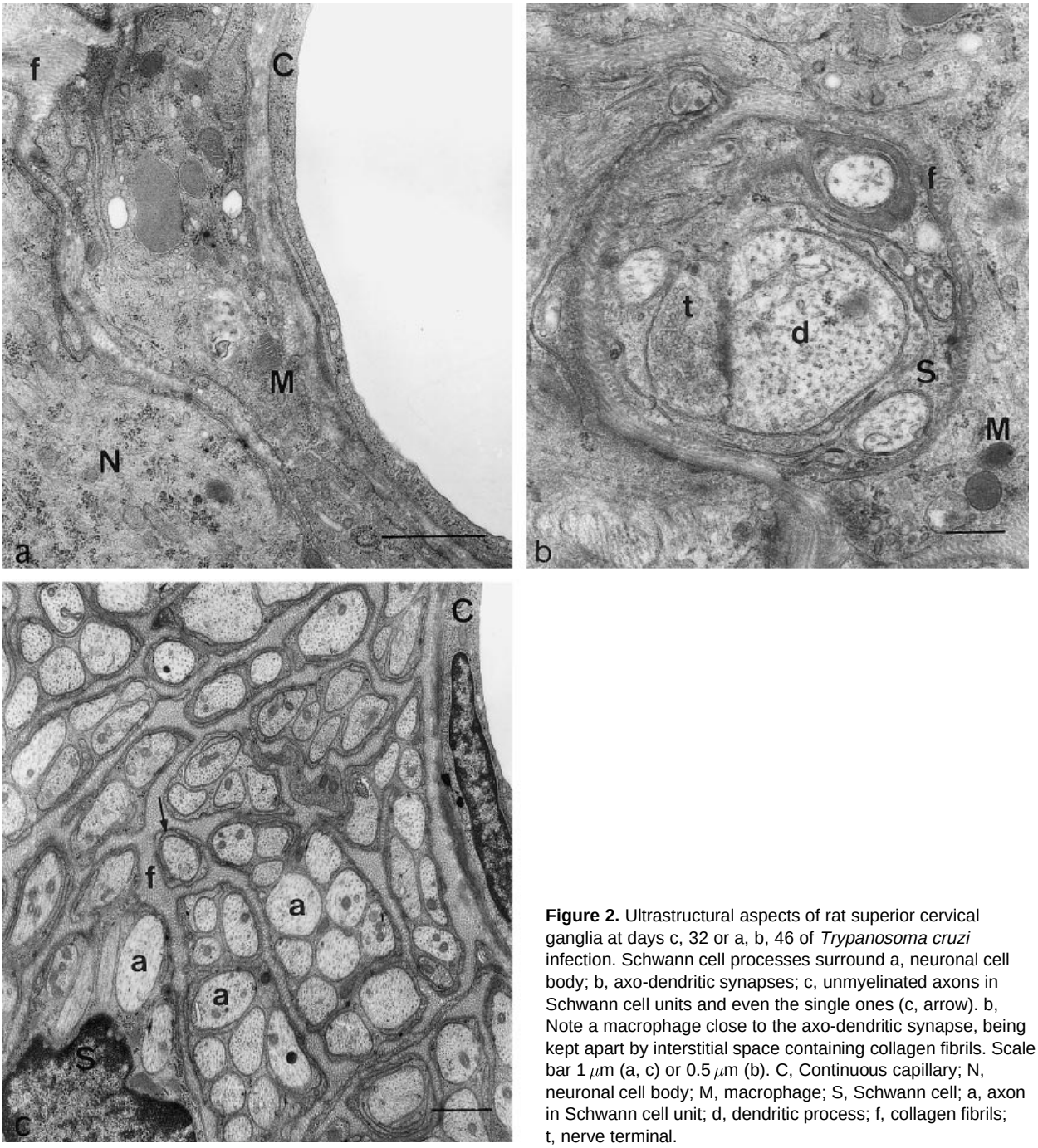

Figure 2. Ultrastructural aspects of rat superior cervical ganglia at days c, 32 or a, b, 46 of *Trypanosoma cruzi* infection. Schwann cell processes surround a, neuronal cell body; b, axo-dendritic synapses; c, unmyelinated axons in Schwann cell units and even the single ones (c, arrow). b, Note a macrophage close to the axo-dendritic synapse, being kept apart by interstitial space containing collagen fibrils. Scale bar 1 μm (a, c) or 0.5 μm (b). C, Continuous capillary; N, neuronal cell body; M, macrophage; S, Schwann cell; a, axon in Schwann cell unit; d, dendritic process; f, collagen fibrils; t, nerve terminal.

chemically depicted by light microscopy. The ultrastructural signs of lesion were similar to those found in both post and pre-ganglionic sympathetic fibres after axotomy (Hámori *et al.* 1968; Roth & Richardson 1969; Joó *et al.* 1971) or treatment with the toxic amine 6-hydroxydopamine (Tranzer *et al.* 1969). In the rat heart, *T. cruzi* infection induced the same signs of damage in the post-ganglionic sympathetic terminals (Machado *et al.* 1994).

The cardiac sympathetic post-ganglionic denervation is virtually total at the end of acute phase in all rats inoculated as in the present work (Machado *et al.* 1978, Machado & Ribeiro 1989). The parasympathetic post-ganglionic innervation of the heart is also greatly reduced

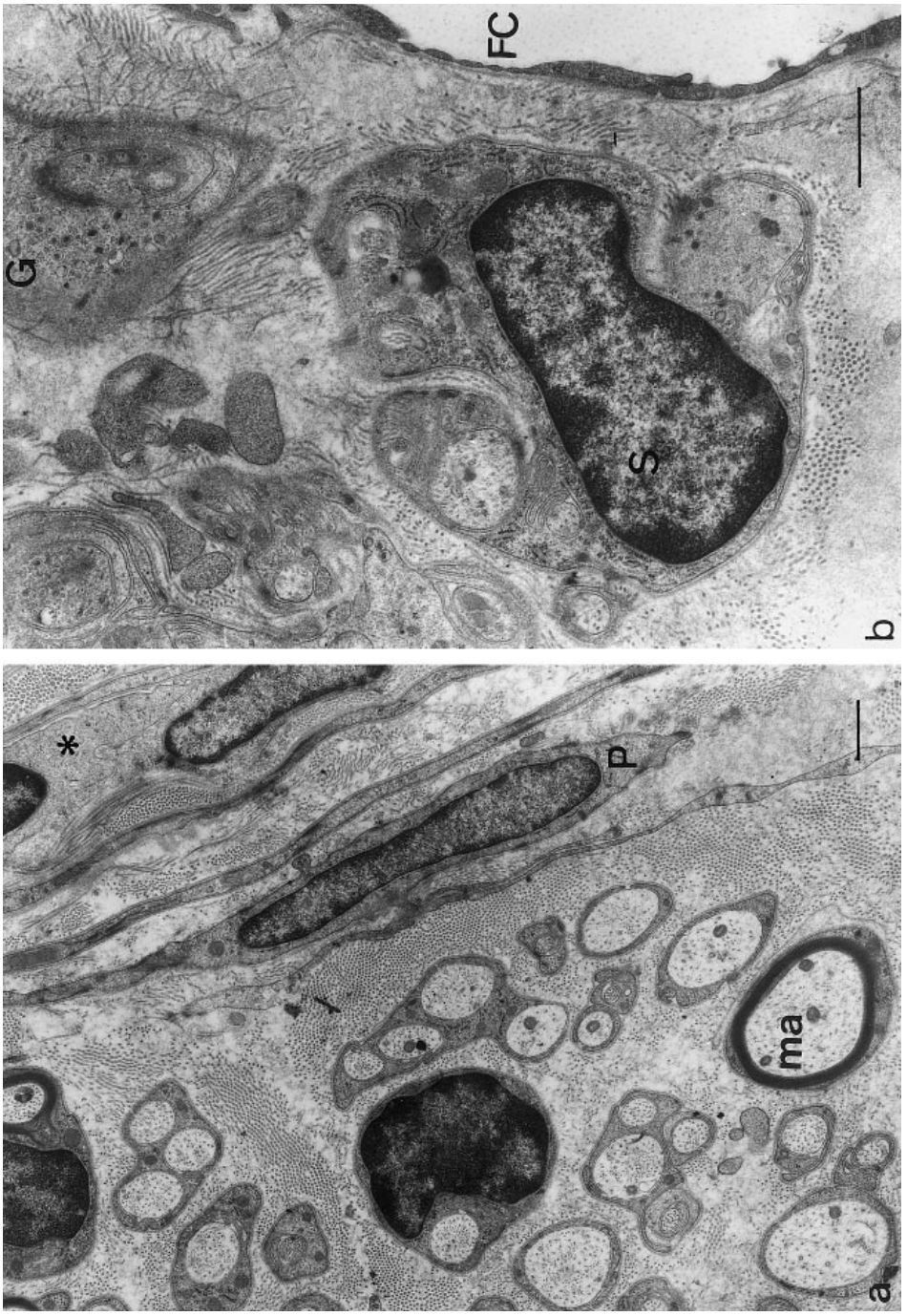

Figure 3. a, b, Superior cervical ganglia of *T. cruzi*-infected rats at day 20. The ganglionic capsule exhibits collagen fibrils, perineural cells and infiltrating cells (*); a, in a subcapsular region, the ganglionic interstice shows signs of oedema but the single fibres and Schwann cell units exhibit normal structure. b, An adjacent region close to a fenestrated capillary and a small granule-containing cell also shows oedema. Scale bar 1 μm. FC, Fenestrated capillary; G, small granule-containing cell; P, perineural cell; S, Schwann cell; ma, myelinated axon.

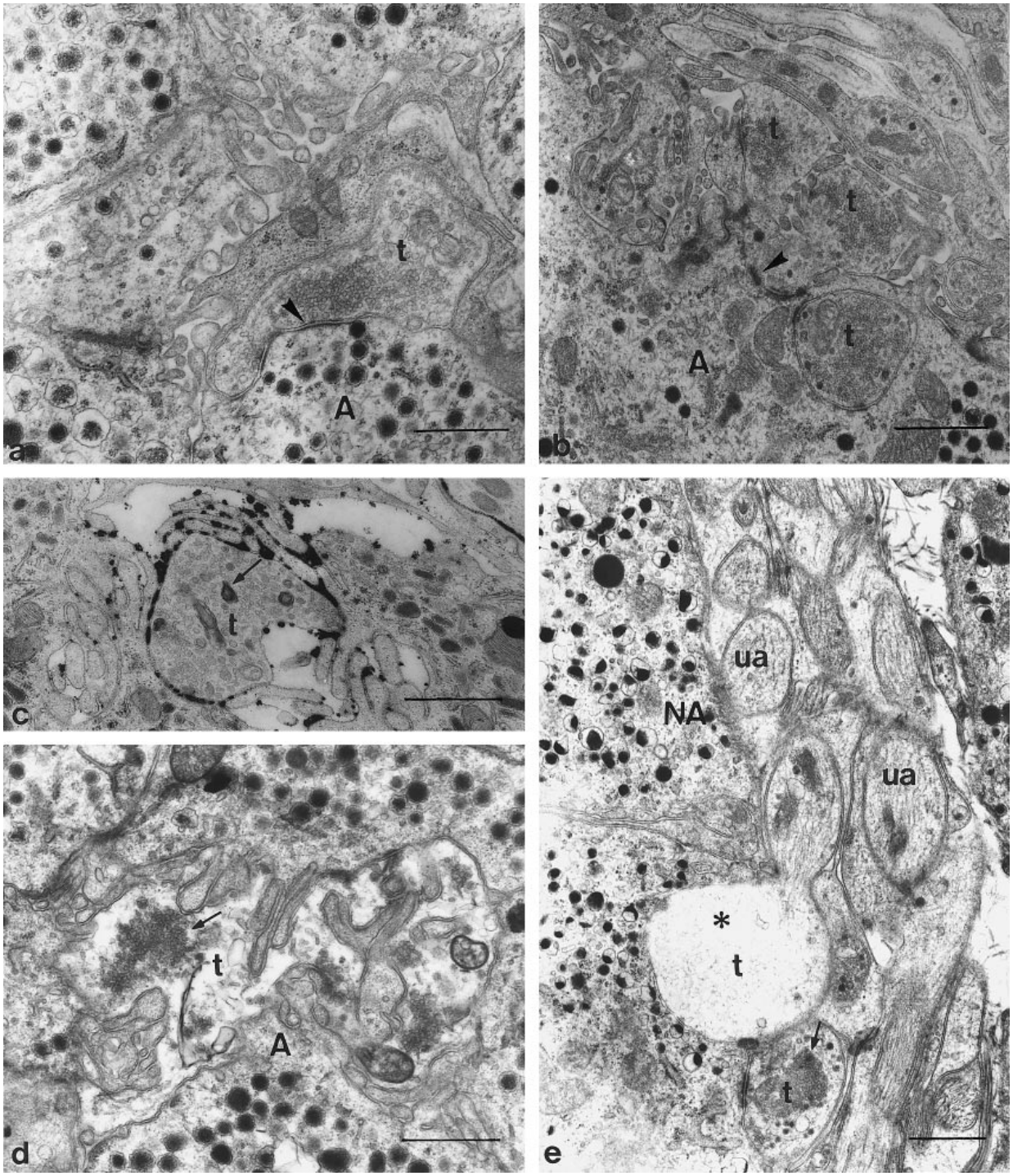

Figure 4. Adrenal medulla of control rats showing cholinergic synapses with active zones (arrowheads) a, partially involved by or b, totally devoid of Schwann cell processes. At days 32 and 46 after *T. cruzi* infection, note the damaged nerve terminals with c, dense bodies (arrow), d, e, clumps of synaptic vesicles (arrows); d, irregular contour and e, an almost empty axoplasm (*). In c, one nerve terminal is labelled by the histochemical demonstration of AChE activity. e, The unmyelinated axons in the Schwann cell unit remain undamaged. Scale bar 1 μm. A, Adrenaline-storing chromaffin cell; NA, noradrenaline-storing chromaffin cell; t, nerve terminal; ua, unmyelinated axon.

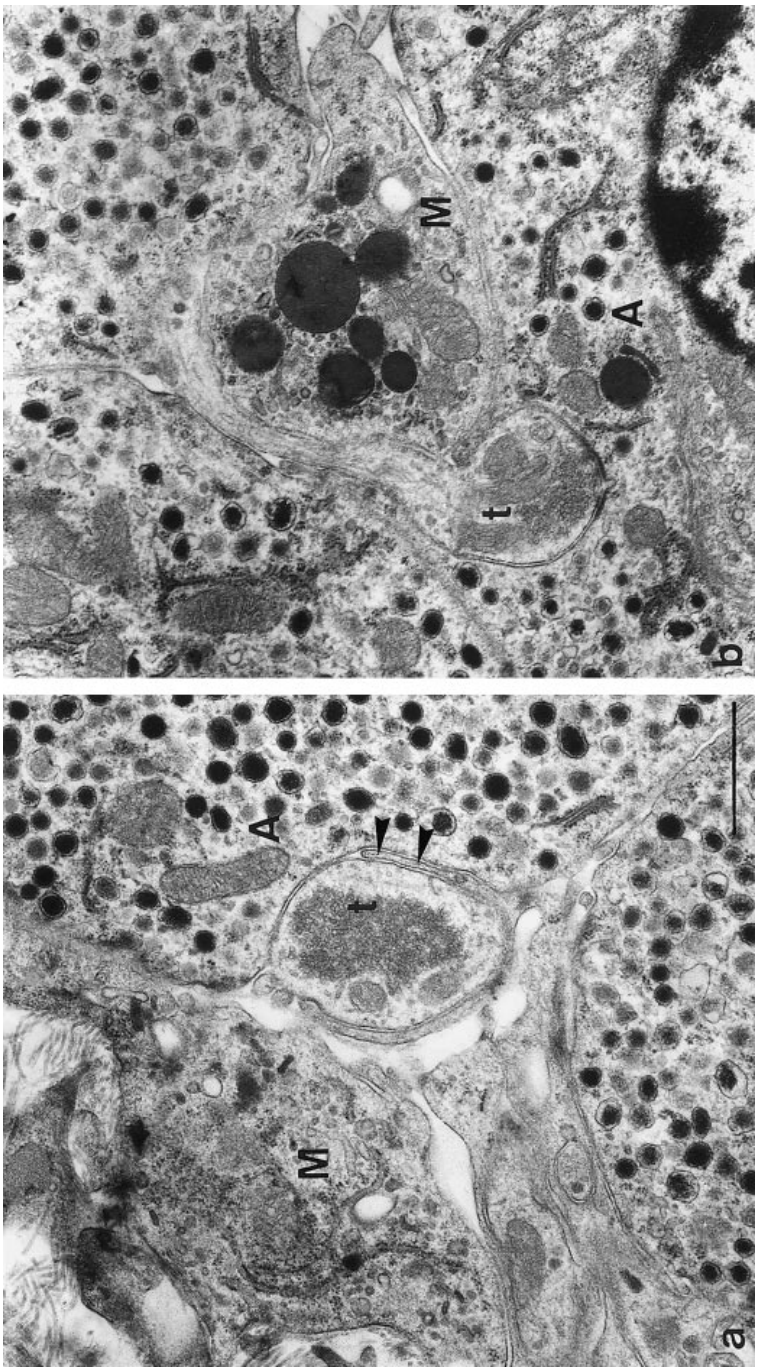

Figure 5. Adrenal medulla of *T. cruzi*-infected rats at day 20 of infection, showing macrophage processes close to a, a damaged and b, an undamaged nerve terminal. b, Note the close apposition of the macrophage to the axon and a, the cell process between the damaged nerve terminal and the chromaffin cell (arrowheads). Scale bar 1 μm. M, Macrophage process; A, adrenaline-storing chromaffin cells; t, nerve terminal.

at day 20 of infection (Machado *et al.* 1979). Previous studies have demonstrated that the level of parasitism and the inflammatory processes may have some role in determining the severity of denervation; both are high in the heart (Machado *et al.* 1978; Machado & Ribeiro 1989) and very low in the adrenal medulla (Machado *et al.* 1990) during the acute phase of the *T. cruzi* infection in rats. Moreover, in the submandibular gland, the rarefaction of both sympathetic and parasympathetic nerves is milder paralleling the low parasitism and discrete inflammatory processes (Alves & Machado 1984; Machado & Ribeiro 1989). As pointed out elsewhere (Machado & Ribeiro 1989), the denervation of poorly parasitized organs suggests that circulatory factors might be involved.

Light-microscopic studies have shown that, in spite of the virtual absence of parasitism in ganglionic tissues, the superior cervical ganglia of all rats at day 20 after *T. cruzi* infection exhibit moderate to intense periganglionitis, and most present focal ganglionitis without any detectable damage of neuronal cell bodies (Camargos & Machado 1988). Our ultrastructural results confirm the presence of periganglionitis in all ganglia of infected animals but ganglionitis was rarely observed. In spite of these inflammatory processes, the preganglionic fibres of both superior cervical and stellate ganglia remained undamaged. The absence of nerve ending lesion does not argue against the possible involvement of circulatory factors. Indeed, there are morphological peculiarities suggesting a ganglion protective microenvironment. The Schwann cell sheathing of all synapses, as happens with the non-varicose axons and neuronal cell bodies, may offer some protection against hazardous factors brought via the capillaries. Further protection might rest on a partial haematoganglionic barrier. The continuous capillaries are in part responsible for this barrier. Perivascular macrophages are also involved because they are able to quickly remove molecules that leave the capillaries (Depace 1982). In accordance with this view, our ultrastructural results showed the presence of oedema only near the few fenestrated capillaries related to the endocrine SIF-cells.

As demonstrated for the sympathetic denervation of the heart, the lesion of the adrenal preganglionic innervation seems to be independent of neuronal death, the varicosities being the target. The varicosities are synaptic sites known to be only partially surrounded by or even totally devoid of Schwann cell processes. The denuded varicosities could be naturally sensitive to the microenvironmental modifications induced by the acute *T. cruzi* infection or they could become sensitive after some kind of surface change. Several lines of investigation implicate immune reactions in the pathogenesis of neuronal death induced by acute *T. cruzi* infection. The mechanism could involve host autoreactive or cross-reactive antibodies (Khouri *et al.* 1979; Ribeiro dos Santos & Hudson 1981; Wood *et al.* 1982; Snary *et al.* 1983; Petry *et al.* 1988; Voorhis & Eisen 1989). Another possibility is the adsorption of *T. cruzi* antigens on the neuronal surface with subsequent binding of anti-*T. cruzi* antibodies (Williams *et al.* 1985). However, regarding the damage of sympathetic nerve terminals, it was possible to exclude a detectable participation of complement-dependent lytic antibodies (Machado *et al.* 1994).

Our results showed the presence of macrophages very close to or even contacting damaged and intact varicose nerve terminals. The possibility of actual participation of macrophages in the damaging mechanism is at present under investigation.

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