Skeletal muscle regeneration and
Trypanosoma cruzi-induced myositis in rats

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Summary. Although Chagas’ disease is known to provoke severe acute myositis, information on muscle regeneration is missing. The current paper shows that during T. cruzi infection in rats, skeletal muscle parasitism and the consequent inflammatory process are higher in muscle with a high proportion of type-I myofibres (soleus and diaphragm). Immuno-histochemistry showed an acute inflammatory process characterized by ED1+ and ED2+ macrophages, CD8+ lymphocytes, and NK cells. Parasite-nest rupture provoked segmental degeneration of myofibres followed by regeneration. These phenomena were observed at both light and transmission electron microscopy levels. Myofibre regeneration involved activation of satellite cells assessed by the expression of MyoD, a muscle-specific transcription factor. Ultrastructural evidence of fusion of myoblast-like cells with the intact segment of degenerating fibres has been provided. At the chronic phase no signs of fibrosis were found, but sparse and small inflammatory foci were found. Our results argue against the relevant participation of autoimmunity phenomena in both acute and chronic phases and furnish a new view for explaining histopathological findings in human patient muscles.

Key words: Muscle regeneration, Satellite cells, MyoD expression, Macrophages, Chagas’ disease

Introduction

Chagas’ disease (American trypanosomiasis) affects nearly 18 million people in Central and South America. Its etiological agent, the protozoan Trypanosoma cruzi, is highly pleomorphic and requires invertebrate, a reduvid bug, and mammalian hosts. In the latter, the infective trypomastigotes circulate in the blood and amastigotes proliferate inside cells. During the acute phase amastigote nests occur mainly in skeletal, cardiac and smooth muscles. The life-long chronic phase has three clinical forms: latent or indeterminate, cardiac (chagasic cardiomyopathy) and digestive (megacolon and/or megaoesophagus). Around 70% of infected people stay in the indeterminate form characterized by positive serology in absence of symptoms and presence of sparse inflammatory foci and rare amastigote nests in autopsied tissues (reviews by Köberle, 1968; Tanowitz et al., 1992; Prata, 2001). Molecular methods have confirmed parasite persistence in affected tissue of chronic patients (Jones et al., 1993, Vago et al., 1996). Mechanisms responsible for the chronic-phase manifestations (cardiac and/or digestive forms) have not been completely elucidated, but could involve autoimmunity and/or parasite persistence (reviews in Kierszenbaum, 1999; Soares et al., 2001).

Muscular pain and weakness are frequent symptoms in chagasic patients (Köberle, 1968). Muscle biopsies have shown histological and ultrastructural changes in myofibres (Laguens et al., 1975). Several studies in murine models of Chagas’ disease have shown myofibre parasitism and myositis as well as degeneration and necrosis of myofibres during both acute and chronic phases (Bijovsky et al., 1983; Molina et al., 1987; Losavio et al., 1989; Monteón et al., 1996). Even the replacement of muscle by adipose tissue has been reported (Bijovsky et al., 1983). However, histological normality has been described in the chronic phase, despite the occurrence of myositis, and the evidence of motor denervation during the acute phase (Brennessel et al., 1985). In rats myositis severity and duration may depend on T. cruzi population (Franco et al., 2003).

Skeletal muscles have a remarkable regenerative capacity after damage induced by several procedures such as heat, cold, scratches, sustained exercise, and transplantation (reviewed by Grounds, 1991). Surprisingly, in all the previous studies on both human and experimental Chagas’ disease, there is no information on the possibility of skeletal muscle regeneration. Even the process of myocyte degeneration
in *T. cruzi* infection has not been fully described.

In the present paper, skeletal muscles were studied with regard to muscular parasitism, inflammatory processes, myocyte damage and regeneration in rats infected with three distinct *T. cruzi* isolates, Y strain, Col1.7G2 and CL-Brener clones. All these *T. cruzi* populations have already been tested in rats, with regard to parasitaemia and myocarditis (Camargos et al., 2000). Y-strain infection has been the most studied in our previous papers. It provokes severe acute myocarditis and rare inflammatory foci during the chronic phase due to parasitaemia and myocarditis (Machado and Ribeiro, 1989; Melo and Machado, 1998; Guerra et al., 2001). The CL-Brener clone is the most virulent and provokes intense and diffuse acute myocarditis (Camargos et al., 2000) and myocarditis (Franco et al., 2003) but normality is reached in the surviving animals after 4 months. Col1.7G2 clone is the least virulent and causes mainly focal and milder acute myocarditis which, although reduced, is sustained during the chronic phase (Camargos et al., 2000).

**Material and methods**

**Parasites and infections**

Juvenile Holtzman rats aged 27-29 days were inoculated intraperitoneally with 0.1 ml of mouse blood containing 300,000 trypomastigotes of Y strain as previously reported (Teixeira-Jr et al., 2001 and references therein). At different periods of the acute (12, 20, 32 days) and chronic phases (50 and 100 days), *T. cruzi*-infected and age-matched control rats were killed under chloral hydrate/ether anaesthesia for histological, histoquantitative, ultrastructural and immunohistochemical studies. To further test the muscle regeneration hypothesis two other *T. cruzi* populations, the CL-Brener clone and the Col1.7G2 clone, were used. These two populations have a very different virulence and pathogenic ability in rats (Camargos et al., 2000). Animals infected with 1,000 trypomastigotes of CL-Brener or 10,000 trypomastigotes of Col1.7G2 were killed at the end of patent parasitaemia period (30 days for the CL-Brener clone, and 30 and 42 days for the Col1.7G2 clone) for histological analysis.

**Histological and histoquantitative methods**

The following muscles were dissected out: diaphragm, extensor digitorium longus, soleus, pectineus and psoas. In the CL-Brener clone- and Col1.7G2 clone-infected rats, only the diaphragm and soleus were analysed. Muscle fragments were fixed in 0.1 M phosphate-buffered 4% paraformaldehyde at pH 7.4 for 24 hours at room temperature, and processed for embedding in glycol methacrylate (Technovit 7100, KULSER), with the object of providing longitudinal and transversal sections. 5-µm thick sections at 25-µm intervals were stained with toluidine blue or erythrosin/toluidine blue (Dominici’s technique).

Amastigote nests were counted in longitudinal sections at a magnification of 400 (100 microscopic fields/animal) at days 12 and 20 of Y-strain infection. For comparing the number of parasite nest in several muscles (Table 1) the Student t test was used with significance level at 0.05.

**Electron-microscopic methods**

Only diaphragm and soleus from control and Y-strain-infected rats at days 20 post-inoculation were analysed (3 control and 3 infected animals). The animals were killed under anaesthesia during left ventricle perfusion with saline (50 ml) followed by 120 ml of the fixative (1% paraformaldehyde, 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2). Muscle fragments remained in the fixative overnight and then were routinely processed for post-fixation in potassium ferrocyanide-reduced osmium tetroxide, for block staining with uranyl acetate and for embedding in Epon resin. Semithin sections stained with toluidine blue allowed the selection of the best areas for ultramicrotomy. Ultrathin sections were routinely stained with uranyl acetate and lead citrate and examined in a Zeiss EM10 microscope.

**Immunohistochemical identification of inflammatory cells**

Diaphragm fragments obtained from 3 Y-strain-infected and 3 control rats at days 12, 20, 36, and 50 post inoculation were embedded in OCT (Tissue-Teck, Sakura Finetek, Torrance, CA), frozen in liquid nitrogen and stored at -70 ºC. From each fragment, 5-µm thick cryostat sections were fixed in acetone, washed in PBS and incubated in 0.1M sodium azide/0.1%hydrogen peroxide, for endogenous peroxidase inhibition. After incubation with primary antibodies (Serotec, Kidlington, England) at previously tested dilutions (ED1, 1:400; ED2, 1:400; CD8, 1:100; CD4, 1:100 and NKR/CD161, 1:200), the sections were washed and incubated with peroxidase-labelled secondary antibody (Pharmigen, San Diego, CA) diluted to 1:50. The peroxidase activity was revealed by 3,3'-diaminobenzidine (Sigma, St. Louis, MO) in PBS buffer with 0.1% hydrogen peroxide for 10 minutes.

The mean area (µm²) occupied by the immunolabelled cells was estimated by using a computer-assisted morphometric program (KS 400, Kontron Elektron Imaging System) coupled to Zeiss Axioplan 2 microscope. The values were expressed as µm²/mm². For statistical comparison, the Student-Newman-Keuls one-way analysis of variance was used as indicated by the SigmaStat software (Sigma, St. Louis, MO, USA) with significance level at 0.05.

**Myoblast immunolabelling**

Activated satellite cells were identified through
Results

Tissue parasitism, inflammatory processes and myocyte damage

Amastigote nests were found in all muscles withdrawn from Y-strain-infected rats during the acute phase (12 and 20 days) where they could occupy large myofibre portions (Fig. 1a). The nests became rare at day 32 post-inoculation, but even at day 100 they could be found sporadically. Higher myocyte parasitism occurred in the diaphragm and soleus (Table 1). In the diaphragm, the parasitism dropped 40% at day 20. In the soleus the 20-day values were about twice the 12-day values. In the other muscles the values remained about the same at both time periods. One out of five animals at day 20 showed heavy parasitism in all tested muscles, the number reaching 122 nests/100 microscopic fields in psosas, the least affected muscle. This agonizing animal was eliminated from all analyses. During the acute phase, predominantly focal inflammatory processes with mononuclear cells and some neutrophils occurred in all five tested muscles of Y-strain-infected rats, being more severe in the diaphragm and soleus at days 12 and 20. It became clearly reduced at day 32. The bulk of the infiltrating cells in each focus could be inside a myocyte segment or in the endomysium (Fig. 1b). By day 100, rare inflammatory foci occurred in the Y-strain-infected animals but no signs of fibrosis or replacement by adipose tissue were seen. The soleus and diaphragm of CL-Brener clone-infected rats showed amastigote nests and severe focal or diffuse myositis at day 30 post-inoculation. In the Col 1.7G2 clone-infected rats, only the diaphragm showed sparse and small inflammatory foci at day 30 which became rare by day 42. In the acute myositis induced by all three tested T. cruzi populations, myocyte segmental degeneration occurred (Fig. 1b), but no evidence of whole myocyte damage was seen.

Myofibre regeneration

At light microscopy, all tested muscles from Y-strain-infected rats, mainly the diaphragm and soleus, showed evidence of muscle regeneration in parallel with acute myositis and myocyte segmental degeneration. The same occurred in the two tested muscles of the CL-Brener clone-infected animals, but such evidence was hardly seen in the Col 1.7G2 clone infection, in accordance with the poor inflammation and myocyte degeneration signs. In the Y-strain-infected rats, regeneration signs were observed from day 12 onwards and the best evidence was the occurrence of basophilic myotube-like structures in inflamed (Fig. 1c) or normal stroma (Fig. 1e). Some of them were clearly growing processes from healthy myofibre segments (Fig. 1c,d). They exhibited several central, pale-stained nuclei. Elongate and basophilic cells with pale-stained nuclei were also found in the endomysium and close to regenerating myofibres (Fig. 1d,e). In addition, at days 12 and 20 of infection, basophilic mitotic cells occurred in close apposition to myofibres in the non-inflamed endomysium (Fig. 1f) or inside the inflammatory foci. Those located in the muscle surface concavity (Fig. 1f) could well be activated satellite cells. Striated myofibres with ectopic (central) nuclei were rare in muscles of younger control animals and they were absent from the older chronic-phase control muscles. Ectopic nuclei were easily found at day 32 post inoculation (Fig. 1g) and could form long chains (Fig. 1h). Those very close to each other were ovoid, pale-stained and with evident nucleoli (Fig. 1g). Those maintaining a longer distance from each other were elongated and denser. By day 100 some dense ectopic nuclei were still seen. Thinner myofibres and the occurrence of split fibres were also observed in the chronic phase.

In muscles of younger control rats rare nuclei expressed the MyoD antigen (Fig. 2a). At day 20 post inoculation, immunolabelling showed numerous MyoD+ cells. They could be in close apposition to healthy myocytes (Fig. 2b) or be among healthy, damaged or regenerating myocytes. At day 12 such labelling was virtually absent from the soleus but occurred in the diaphragm. At day 50 no MyoD+ cells were identified.

Electron-microscopic analyses at day 20 confirmed the invasion of parasitized myocyte (ruptured nests) by mononuclear cells and neutrophils. Cell debris and/or leukocytes (Fig. 3a) could fill the degenerated segment.

Table 1. Amastigote-nest numbers in skeletal muscle during the acute phase of T. cruzi infection with 3×10⁵ trypomastigotes of Y strain. Four animals in each period. Values are mean ± SD

<table>
<thead>
<tr>
<th>MUSCLES</th>
<th>DAYS AFTER INOCULATION</th>
<th>12</th>
<th>20</th>
</tr>
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<tbody>
<tr>
<td>Diaphragm</td>
<td>32.5±3.1a</td>
<td>13.75±0.95ab</td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>6.75±1.25a</td>
<td>14.50±2.6ab</td>
<td></td>
</tr>
<tr>
<td>Pectineus</td>
<td>2.75±0.95</td>
<td>3.0±0.8</td>
<td></td>
</tr>
<tr>
<td>Ext. dig. longus</td>
<td>2.25±0.5</td>
<td>2.50±0.6</td>
<td></td>
</tr>
<tr>
<td>Psosas major</td>
<td>1.25±0.5</td>
<td>1.75±0.5</td>
<td></td>
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</tbody>
</table>

a: different from others in the column (p<0.01); b: different from 12-day values (p<0.01)
Fig 1. Light microscopy view of diaphragm (excepting a) of T. cruzi (Y strain)-infected rats. a. Portion of a long amastigote nest in psoas myofibre at day 12 of infection. b. Mononuclear cell infiltration with some neutrophils. Segmental degeneration of a myofibre(*) and a myofibril alteration in another myofibre at day 12. c. Coexistence of an inflammatory process and myotube-like structures at day 12. One of them (arrow) is growing from an intact myofibre segment. d. Detail of the arrowed structure in c. Note the basophilic cells in concavities of the regenerating fibre. e. Basophilic elongated cell (arrow) close to a myotube among myofibres at day 32 in absence of inflammatory cells. f. Semithin section showing two mitotic cells (arrows) at day 12, one is in close apposition to a myofibre and the other is in the endomysium. g. Cross-section showing ectopic nuclei at day 32 and several thinner myofibres in an area already free of inflammatory process. h. A row of ectopic nuclei close together in a striated myofibre at day 32. Dominici staining. a, c x 230; b, d-h, x 590
Almost empty intervals between intact segments were also seen, indicating the scavenging action of infiltrating cells. Sometimes only the basal membrane encircled the infiltrating cells. In control animals, ultrastructural analyses allowed the identification of satellite cells by their undifferentiated aspect and location beneath the myocyte basal membrane in both control and infected animals. Fusion and engulfment of presumptive myoblasts in healthy myocyte segments were observed (Fig. 3b,c).

Leukocyte phenotypic characterization

In younger and older control rats, immunohistochemistry showed rare CD8+ and CD4+ cells and sparse NK cells among the myofibres. ED2+ (resident) macrophages outnumbered by far the ED1+ (recruited) macrophages as confirmed by our histoquantitative analysis (Fig. 4). In Y-strain-infected animals, the amount of ED1+ macrophages and NKR+ and CD8+ cells increased significantly at day 12 of infection (Fig. 4). The mean values remained about the same at day 20 and dropped markedly at day 36. At day 50, ED1 values were still higher than those of control rats. The amount of ED2+ macrophages remained as in the control muscle except for day 20. ED1+ cells had both focal and diffuse distribution. They were the most frequent mononuclear cell type inside the parasitized (ruptured nests) or degenerating myofibres. ED2+ macrophages with a diffuse distribution occurred only in the endomysium. At day 20, they were so numerous that they could touch each other along the myocytes. NKR+ and CD8+ cells occurred at the inflammatory foci.

Discussion

In Holtzman rats, the T. cruzi populations used in this work have high tropism for cardiac tissue and very low, if any, tropism for intestinal smooth muscle or striated oesophageal muscle (Camargos et al., 2000). Now we have demonstrated that Y strain and CL-Brener clone provoked severe acute myositis in rat skeletal muscles. However, skeletal muscles were largely spared in Col 1.7 G2 infection. This clone provokes milder and sustained myocarditis (Camargos et al., 2000).

Most rat skeletal muscles have type I (slow oxidative), IIA (fast oxidative/glycolytic), IID/X (fast oxidative/glycolytic) and IIB (fast glycolytic) myofibres. Type II predominates in most rat muscles (Delp and Duan, 1996). Because of evidence favouring greater parasitism in type I fibres in mice (Teixeira and Dvorak, 1985), we tested skeletal muscles presenting a distinct proportion of type I myofibres as follows: 84% in the soleus, 44% in the diaphragm, 12% in the pectineus, 4% in the extensor digitorum longus and 2% in the psoas major (Delp and Duan, 1996). The diaphragm and soleus were the most affected by T. cruzi infection and this probably reflects their richness in type I fibres. Accordingly, the psoas major was the least affected. Interestingly, the diaphragm exhibited significantly more parasite nests at day 12 when compared to the soleus. The intraperitoneal inoculation of trypomastigotes could favour the direct invasion of the diaphragm.

Amastigote nest rupture provokes inflammatory process characterized by the predominance of mononuclear cells. Our quantitative analysis showed a significant increase of CD8+ lymphocytes, NK cells and macrophages. These cells are involved in the control of acute phase T. cruzi infection. Macrophage-derived nitric oxide has been implicated in such a control (Vespa et al.,...
Fig. 3. Diaphragm electron micrographs at day 20 of *T. cruzi* infection. **a.** A myofibre segment completely filled with neutrophils, macrophages and lymphocyte-like cells lies between inflamed stroma and normal myofibre x 3,060. **b.** A presumptive myoblast fusion with an intact myofibre segment x 15,000. Insert shows a detail of the arrowed filamentous organelle suggestive of myofibril assembly x 37,000.
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1994; Aliberti et al., 1999). Lymphocyte depletion exacerbates the myocarditis and parasitism in a mouse model of Chagas’ disease (Tarleton et al., 1994). In our rat model, granulocyte and lymphocyte depletion also exacerbates tissue parasitism, but were unable to block degenerative phenomena such as sympathicodenervation (Melo and Machado, 1998). The denervation is prevented if macrophages are also depleted (Guerra et al., 2001).

Despite the severity of Y-strain-induced acute myositis in the diaphragm and soleus, the surviving animals showed slight histopathological alteration 3 months after inoculation, as demonstrated for the heart. Parasite persistence was demonstrated in the hearts of rats infected with the Y strain (Machado and Ribeiro, 1989; Guerra et al., 2001) and Col 1.7G2 clone (Camargos et al., 2000). Here we have shown persistence of parasites in skeletal muscle either by the presence of some small inflammatory foci or by the rare amastigote nests. In this aspect the rat is a good model for the asymptomatic indeterminate chronic form of Chagas’ disease as discussed previously (Franco et al., 2003). There is no evidence for autoimmunity-induced damage in our experimental model of Chagas’ disease as indicated by the recovery from myocarditis and cardiac autonomic denervation (reviewed in Melo and Machado, 1999; Camargos et al., 2000) as well as by the skeletal muscle regeneration here demonstrated. Probably, some kind of parasite-host equilibrium is reached in both asymptomatic chagasic patients and infected rats.

The present paper has shown that nest rupture provokes segmental degeneration of myofibres in parallel with myositis. Segmental regeneration of myofibres was clearly observed and involved the activation of satellite cells, as indicated by expression of Myo D antigen, a marker of proliferative myoblasts. Myo D+ nuclei occurred in close contact with healthy segments and in both inflamed and normal endomysium in accordance with the location of basophilic and elongate cells. Myotube-like structures were identified by their basophilic cytoplasm and several central nuclei. In addition, ultrastructural analysis showed undifferentiated cells fusing with intact myofibre segments. From day 32 on the main signs of regeneration were the ectopic nuclei. Activation of muscle satellite cells has been associated with expression of insulin-like growth factor gene that can undergo alternative splicing to generate different gene products with varying functions. The initial pulse of one of these products, the mechano growth factor, seems to be responsible for satellite cell activation (Hill and Goldspink, 2003).

It is well known that myoblast proliferation and myotube formation after muscle damage is associated with neutrophils and macrophage infiltration. In rats, the ED1 antibody recognizes phagolysosomes in monocytes and hematogenous infiltrating macrophages (van der Berg et al., 2001). In freeze-damaged skeletal muscle, ED1+ macrophages appear within a few hours after damage and become abundant until necrotic tissues have been removed, a necessary step for muscle regeneration (Mc Lennan, 1993, 1996). In our experimental condition, ED1+ macrophages, CD8+ lymphocytes and NK cells became significantly more numerous at day 12 post inoculation. This period corresponds to the parasitaemia peak (Teixeira Jr et al., 2001). A marked drop occurred at day 32, reflecting the control of parasite proliferation in tissues. Besides their location on stromal inflammatory foci, ED1+ macrophages were the predominant leukocyte inside the ruptured or degenerating myofibre segments. Histological and electron-microscopic analysis also showed the presence of some neutrophils at both locations. So, in parallel with parasite control, these phagocytes propitiate segmental regeneration by removing damaged cytoplasm. Interestingly, muscle regeneration occurred in the presence of NK cells and CD8+ lymphocytes that are involved in innate and cellular immune responses with possible implications in tissue damage (Reis et al., 1993, 2001).

A significant increase in the ED2+ population occurred at day 20 post inoculation. Expression of the ED2 antigen is specific for resident macrophages. However ED2+ macrophages comprise different subpopulations and there is evidence that one of these may become proliferative in inflamed tissue (Mueller et al., 2001, 2003). Physiological turnover of resident macrophages by blood-derived cells is also a possibility (Mueller et al., 2001). On the other hand, ED2+ macrophages have been associated to muscle regeneration (McLennan, 1993, Merly et al., 1999). Also, in vitro studies showed an increase in myoblast proliferation by ED2+ macrophages (Massimino, 1997).

As far as we know this is the first work showing muscle regeneration in parasite-induced muscle damage. Our study does not support the notion that autoimmunity has an important role in skeletal muscle...
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