Effect of treatment with cyclophosphamide in low doses upon the onset of delayed type hypersensitivity in mice chronically infected with Trypanosoma cruzi: involvement of heart interstitial dendritic cells

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Acute infection with Trypanosoma cruzi results in intense myocarditis, which progresses to a chronic, asymptomatic indeterminate form. The evolution toward this chronic cardiac form occurs in approximately 30% of all cases of T. cruzi infection. Suppression of delayed type hypersensitivity (DTH) has been proposed as a potential explanation of the indeterminate form. We investigated the effect of cyclophosphamide (CYCL) treatment on the regulatory mechanism of DTH and the participation of heart interstitial dendritic cells (IDCs) in this process using BALB/c mice chronically infected with T. cruzi. One group was treated with CYCL (20 mg/kg body weight) for one month. A DTH skin test was performed by intradermal injection of T. cruzi antigen (3 mg/mL) in the hind-footpad and measured the skin thickness after 24 h, 48 h and 72 h. The skin test revealed increased thickness in antigen-injected footpads, which was more evident in the mice treated with CYCL than in those mice that did not receive treatment. The thickened regions were characterised by perivascular infiltrates and areas of necrosis. Intense lesions of the myocardium were present in three/16 cases and included large areas of necrosis. Morphometric evaluation of lymphocytes showed a predominance of TCD8 cells. Heart IDCs were immunolabelled with specific antibodies (CD11b and CD11c) and T. cruzi antigens were detected using a specific anti-T. cruzi antibody. Identification of T. cruzi antigens, sequestered in these cells using specific anti-T. cruzi antibodies was done, showing a significant increase in the number of these cells in treated mice. These results indicate that IDCs participate in the regulatory mechanisms of DTH response to T. cruzi infection.

Key words: Trypanosoma cruzi - murine infection - chronic myocarditis - delayed hypersensitivity - cyclophosphamide - heart dendritic cells

Infection with the protozoan Trypanosoma cruzi is an important cause of chronic myocardopathy in endemic areas of South and Central America. Survivors of the acute infection usually pass into an asymptomatic phase: the so-called indeterminate form of Chagas disease. Approximately 30% of T. cruzi infection will later progress to the chronic cardiac form, characterised by chronic fibrotic myocarditis (Andrade 1991). Pathogenesis of the chronic cardiac form of Chagas disease is considered to be dependent on the development of delayed type hypersensitivity (DTH) (Tarleton 1995, 2001, Tarleton et al. 1996, dos Reis 1997), which is responsible for the fibrotic inflammatory cardiac lesions (Andrade 1999). A previous study conducted in chronically infected dogs demonstrated that low doses of cyclophosphamide (CYCL) caused a rapid evolution from the indeterminate form of Chagas disease to active chronic myocarditis (Andrade et al. 1987). The responses to low doses of CYCL are attributed to the suppression of humoral and cellular responses, the result of selective destruction of suppressor T lymphocytes, their precursor cells or other elements in the host-immune suppressor network (Turk et al. 1972, Askenase et al. 1975, Schwartz et al. 1978, Andrade et al. 1997, Shevach et al. 2001, Murata et al. 2004, Lutsiak et al. 2005). These cells are now identified as regulatory T cells (T-regs), which express CD4+CD25+ markers and are involved in the regulation and suppression of DTH (Shevach et al. 2001).

A significant increase in the number of the IDCs in acute and chronic myocarditis compared to normal controls or to the indeterminate form of disease has also been demonstrated (Andrade et al. 2000) through a quantitative evaluation of heart interstitial dendritic cells (IDCs) in the aforementioned CYCL-treated dogs. This suggests a direct relationship between the number IDCs and the intensity of inflammatory infiltration. The importance of these IDCs in the evolution of cardiac lesions and in the pathogenesis of chronic myocarditis has also been evaluated (Andrade et al. 2000). These cells are “antigen presenting cells” (APCs) and exhibit the capacity to bind to antigens and to stimulate T lymphocyte responses. Migration of the IDCs to the T-cell zone of the spleen may give rise to a continuous sensitisation of the heart to DTH.

According to Lutsiak et al. (2005), low doses of CYCL not only decrease the number of T-regs, but also lead to decreased functionality. CYCL treatment enhances apoptosis and decreases homeostatic proliferation of T-regs.
In this study, we investigated the effects of low-dose CYCL treatment on the evolution of the chronic form of Chagas disease in mice. We administered a skin test for DTH response, evaluated the intensity of myocarditis, quantified T cells infiltrates (CD4/CD8) and evaluated the participation of the antigen presenting IDCs.

**MATERIALS AND METHODS**

**Experimental animals** - One hundred inbred BALB/c mice weighing between 15-20 g and raised in the animal facilities of the Gonçalo Moniz Research Centre, Oswaldo Cruz Foundation were used. The mice were maintained in accordance with the ethical guidelines established by the Ethical Committee for the Use of Experimental Animals. Thirty of these mice were used as normal controls and 70 were infected with *T. cruzi*.

**T. cruzi strain** - The Colombian strain classified as Biodeme Type III (Andrade & Magalhães 1997), corresponding to the taxa *T. cruzi* I (Anonymous 1999, Zingales et al. 2009), was used in this study.

**Inoculum** - Blood forms of trypomastigote at 1 x 10^7 were obtained from infected mice that were inoculated by intraperitoneal injection.

**Experimental groups** - I: the infected animals were followed up to the chronic phase of infection (180 days). The survivors (60) were divided into two experimental groups: first group - 30 infected untreated mice, and second group - 30 infected mice treated with low-dose CYCL; II: 30 uninfected mice were maintained as controls.

**Treatment with CYCL** - CYCL was used in the commercial form Genuxal, Lot OM 105, ASTA Medical AG, Frankfurt, Germany.

**Treatment schedule** - The drug was intraperitoneally administered three times a week over four weeks at a dose of 20 mg/kg body weight.

**Skin tests** - To evaluate the cellular immune responses, a DTH skin test was performed using antigen culture forms (Warren medium) of *T. cruzi* with 90% epimastigotes. The cells were washed in phosphate buffered saline (PBS) (pH 7.2) with centrifugation. The pellet contained the culture forms and was frozen and thawed several times in liquid nitrogen. The antigenic extract was then filtered in a Milipore 0.22 µm filter. Protein dosage was performed using the bicinchoninic acid assay (BCA) with a BCA Protein Assay Kit (Pierce Catal. 2161297A). The protein concentration for the sample used was adjusted to 2 mg/mL.

The skin test was performed in 15 mice from each experimental group at two time points: 24 h and 15 days after the CYCL treatment. The antigen was administered in a dose of 25 µL (50 µg of protein) intradermally in the right hind-footpad. The same volume of PBS was injected in the left hind-footpad and served as a control. Footpad thickness was measured with a digital calliper (Fisherbrand Digital Callipers, Traceable, Fisher Scientific) at 24 h, 48 h and 72 h after the antigen injection.

**Histopathological studies** - For each footpad measurement time point, three mice were sacrificed by exsanguination after anaesthesia with sodium pentobarbital. Blood was collected for serological tests. All of the sacrificed animals were necropsied and sections of the heart and skeletal muscle were divided and fixed in 10% formalin or cryopreserved in liquid nitrogen for histochemical studies. morphometric evaluation of inflammatory cells - Quantification of the inflammatory cells was performed in fixed sections stained with haematoxylin and eosin (H&E) and in cryostat sections histochemically stained for CD4 and CD8. Using a Zeiss optical microscope with 10X ocular and 40X objective lenses, an area of 60 mm^2 was selected at a thickness of 5 µm and mounted on polylysine-prepared slides and were then fixed with dehydrated acetone. Blocking of endogenous peroxidase was performed using a 3% solution of hydrogen peroxide plus methanol. The slides were washed twice in PBS and incubated with the primary antibodies (anti-CD4, anti-CD8). After washing three times with PBS, slides were incubated with the respective biotinylated secondary antibodies for 30 min. The slides were then washed in PBS and treated with streptavidin peroxidase (Sav-HRP Biosciences Pharmingen Catalogue 550946). After washing in PBS, the slides were incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB). Nuclear staining was performed using methyl green pyronin stain and the slides were coverslipped using Permount resin.

**Immunohistochemistry** - Immunolabelling of the CD4 + and CD8 + T-cell infiltrates was performed on cells from the cryopreserved heart, skeletal muscle and footpad sections. The immunolabelling was performed using the monoclonal antibodies anti-CD4 and anti-CD8 as primary antibodies (BD Biosciences Pharmingen). Biotin-conjugated IgG2a (Clone A92-1 anti-rat, Catalogue 553909, BD Biosciences) was used as a secondary antibody. Cryopreserved sections of the heart were sectioned at a thickness of 5 µm and mounted on polylysine-prepared slides and were then fixed with dehydrated acetone. Blocking of endogenous peroxidase was performed using 3% solution of hydrogen peroxide plus methanol. The slides were washed twice in PBS and incubated with the primary antibodies (anti-CD4, anti-CD8). After washing three times with PBS, slides were incubated with the respective biotinylated secondary antibodies for 30 min. The slides were then washed in PBS and treated with streptavidin peroxidase (Sav-HRP Biosciences Pharmingen Catalogue 550946). After washing in PBS, the slides were incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB). Nuclear staining was performed using methyl green pyronin stain and the slides were coverslipped using Permount resin.

**Morphometric evaluation of inflammatory cells** - Quantification of the inflammatory cells was performed in fixed sections stained with haematoxylin and eosin (H&E) and in cryostat sections histochemically stained for CD4 and CD8. Using a Zeiss optical microscope with 10X ocular and 40X objective lenses, an area of 60 mm^2 corresponding to five non-successive 12 mm^2 fields was examined. The images were captured and evaluated using the Axion Vision program. The sectional areas of inflammation were measured directly and the total number of cells in the examined area was calculated. Statistical analysis was performed using the non-parametric Mann-Whitney U test (significance p < 0.05)

**Immunolabelling of T. cruzi antigens in heart IDCs,** parasites and parasite debris - Immunohistochemical staining of parasite antigens as intracellular parasites, extracellular particulate debris or deposits in the membranes of heart IDCs was performed on 5-µm-thick paraffin sections after deparaffinisation and hydration. Blocking of endogenous peroxidase was performed using a solution of hydrogen peroxide (3%) diluted in methanol. After washing in distilled water and PBS, sections were incubated for 15 min in 10% skim milk to block non-specific binding. Sections were treated with the primary antibody, a purified, specific anti-*T. cruzi*
IgG produced in rabbits, at a 1:600 dilution in PBS with 2% Tween-20 for 30 min at 37°C. After washing in PBS and 2% Tween-20, sections were incubated in normal goat serum (Vectastain - Elite) for 20 min for additional blocking of non-specific binding. After washing in PBS, the slides were incubated for 30 min at 37°C with the secondary antibody: goat anti-rabbit IgG-peroxidase conjugate (Sigma) at a 1:800 dilution in PBS. The sections were developed with 2.4% DAB and 1% H₂O₂ plus 1% dimethyl sulphoxide (Sigma) at room temperature (RT). Sections were counterstained with 1% methyl-green for 15 min, dehydrated and mounted with Canadian balsam. Sections of hearts obtained from non-infected controls were subjected to the same treatment and staining for use as negative controls. Paraffin-embedded sections of the hearts of acutely infected mice that contained several parasite nests were processed for immunohistochemistry following the same steps described above, but using a polyclonal anti-T. cruzi antibody.

**Immunohistochemical identification of IDCs** - A purified rat anti-mouse monoclonal antibody for CD11b (BD Pharmingen) and a purified hamster anti-mouse CD11c antibody (BD Pharmingen) were used. Sections of the hearts from infected mice of both untreated and treated groups and from non-infected controls were examined. Immunolabelling with the rat anti-CD11b monoclonal antibody was conducted on cryopreserved sections of the heart embedded in Tissue-Tek OCT compound (Miles Inc, Diagnostic Division Elkhart). After washing in PBS, the slides were treated with a sheep-anti-rat Ig-POD (peroxidase-conjugated) Fab fragment secondary antibody (Boehringer Mannheim Biochemicals) at a 1:300 dilution. Sections treated with the hamster anti-mouse CD11c primary antibody were treated with a goat anti-Syrian hamster IgG-POD secondary antibody (Jackson Immuno-Research) at a 1:500 dilution for 30 min at 37°C. The sections were developed with 2.4% DAB and 1% H₂O₂ plus 1% dimethyl sulphoxide (Sigma) at RT. Sections were counterstained with 1% methyl-green for 15 min, dehydrated and mounted with Canadian balsam.

**Quantitative evaluation of the number of IDCs presenting T. cruzi antigen by immunolabelling with specific antibodies** - The number of IDCs was evaluated in 5-μm-thick paraffin-embedded sections by counting the cells in 10 non-successive microscopic fields using a Zeiss Standard 18 microscope with 10X ocular and 40X objective lenses. The mean and standard deviation (SD) for the number of cells was established for each group and calculated for 1 mm². Heart sections of non-infected controls were processed in the same manner.

**RESULTS**

**Parasitaemia** - In the acute phase of infection with the Colombian strain, parasitaemia is indicative of the Biodeme Type III (T. cruzi I) infection pattern. A peak in parasitaemia at the 29th day post-infection was observed, followed by a progressive decrease until the chronic phase (60 days), when parasitaemia became negative (Fig. 1A).

**Cumulative mortality** - The cumulative mortality from the acute phase until 180 days post-infection was 40%. The cumulative mortality of these groups from the first day until the 30th day of treatment was also evaluated, which totalled 33% of the untreated controls and 46.6% in CYCL-treated group (Fig. 1B).

**Skin test (DTH)** - The evaluation of the results of the skin test was performed at two time points after the end of the treatment: the first evaluation occurred at 24 h and the second at 15 days after the end of treatment. In both cases, measurement of footpad thickness was performed at 24 h, 48 h and 72 h after antigen injection (Fig. 2A, B). The footpads injected with PBS did not present alterations when compared with normal controls. In Fig. 1A: parasitaemic profiles in mice with acute infection with the Colombian strain reproduces the patterns of the Biodeme Type III (Trypanosoma cruzi I). After treatment (180 days) a negativation of parasitaemia was observed; B: cumulative mortality evaluated from the acute phase until the 180th day of infection was of 40%. During the treatment with cyclophosphamide (CYCL) from the first day until the 30th day of treatment the mortality was of 33% for the untreated controls and 46.6% in the group treated with CYCL.
contrast, the footpads injected with antigen displayed increased thicknesses in both the untreated and CYCL-treated mice. The maximum reaction was observed 24 h after the antigen injection, decreased progressively and disappeared by 72 h. Footpad measurement revealed a predominance of the revealed greater thickness in the group treated with CYCL at the three time points. Statistical analysis using the Mann-Whitney U test showed significant differences at 24 h after antigen injection in both the group injected 24 h after treatment with CYCL (p = 0.0049) (Fig. 2A) and the group injected 15 days after CYCL treatment (p = 0.0117) (Fig. 2B).

**Histopathological evaluation of the skin test - First group (infected untreated mice):** 24 h after antigen injection, a diffuse mononuclear infiltration involving small vessels was present in the dermis (Fig. 3A, B); second group (infected mice treated with CYCL): necrotic areas were present in the perimuscular subdermic conjunctive tissue, along with polymorphonuclear neutrophils, diffuse mononuclear infiltration and arteritis (Fig. 3C). In the dermis, the presence of diffuse mononuclear cell infiltration with accumulation around small vessels and cutaneous annexes was observed (Fig. 3D-F); third group (uninfected control mice): 24 h after antigen injection in the footpad, mild perivascular and subdermal mononuclear cell infiltration was observed. This infiltration involved the muscles, with an absence of inflammatory infiltrations in the dermis.

**Histopathological evaluation of the myocardium and skeletal muscles - First group (infected untreated mice)** myocardium: moderate, diffuse and focal mononuclear infiltrates in the atria, sub-epicardial and perivascular spaces was observed, along with the presence of mild, interstitial fibrosis (Fig. 4A). Skeletal muscles: inflammatory lesions varied from mild to moderate and were limited to focal perivascular mononuclear cell infiltration with involvement of the small arteries (Fig. 4B); second group (infected mice treated with low-dose CYCL) myocardium: the presence of moderate necrotic-inflammatory lesions with focal and diffuse mononuclear infiltrations was present in most of the cases (10/16). Intense lesions in the myocardium were present in three/16 cases, with large areas of necrosis of cardiac myocells, diffuse

![Fig. 2A: skin test. Comparison between the means of footpads thickness in treated mice, 24 h, 48 h and 72 h after antigen injection, comparing with untreated controls showing significant difference in the 24 h point (p = 0.0049); B: delayed type hypersensitivity test performed 15 days after the end of treatment with cyclophosphamide revealed significant difference between the means of footpads thickness in treated mice and untreated controls 24 h after the antigen injection (p = 0.0117).](image)

![Fig. 3: histopathology of the delayed type hypersensitivity (DTH) skin tests. Untreated mouse chronically infected with Colombian strain of Trypanosoma cruzi. A, B: sections of the skin with mild interstitial diffuse and perivascular mononuclear infiltrations; C: mouse treated with cyclophosphamide. Necrosis of the intradermal connective tissue and intense perivascular infiltration with mononuclear cells. Sections of the DTH skin test in untreated mouse; D-F: diffuse mononuclear infiltration in the dermis and involvement of the small vessels and cutaneous annexes. H&E 400X.](image)
mononuclear infiltration and matritial fibrillar deposits (Fig. 4C, D). The atrial wall was predominantly involved, showing myocardial destruction with mononuclear cell infiltration and arteriolar involvement (Fig. 4E, F).

**Skeletal muscle** - Lesions were mild in most of cases, represented by focal perivascular mononuclear infiltrates and arteritis. Diffuse interstitial infiltration was present in some cases.

**Morphometric evaluation of inflammatory cells** - The numbers of mononuclear inflammatory cells in the heart and skeletal muscle sections were significantly higher in the CYCL-treated group as compared with those in the untreated group, as assessed by H&E staining (Fig. 5). The statistical analysis indicated that the differences in mononuclear cell numbers were significant in both the heart (p = 0.0191) and the skeletal muscle (p = 0.0149).

**Immunohistochemical labelling of TCD4 and TCD8 cells in the myocardium** - TCD4 and TCD8 cells were observed in both the interstitium and the perivascular areas (Fig. 6A, B).

**Morphometric evaluation of the numbers of TCD4 and TCD8 cells** - Assessment of the CD4/CD8 relationship in both untreated and treated mice revealed a predominance of CD8 cells; this difference was not significant for the untreated mice (p = 0.0809), but was significant for those treated with CYCL (p = 0.0495) (Fig. 7A, B).
Immunolabelling of heart IDCs - Immunolabelling was performed using the monoclonal antibodies anti-CD11b and anti-CD11c, as shown in Fig. 8A, B. The IDCs (arrows) appeared elongated and isolated, with compact cytoplasm, a central round pale nucleus and two or three fine cytoplasmic processes. For identification of *T. cruzi* antigens in IDCs, immunolabelling was performed using an anti-*T. cruzi* antibody in the heart tissue of chronically infected mice not treated with CYCL (Fig. 9A, B) and in the heart tissue of mice treated with low-dose CYCL (Fig. 10C, D). Parasite antigens were visible as dense granular deposits in the IDC membranes.

Morphometric evaluation of IDCs - As seen in Fig. 10, a significant increase ($p < 0.05$) in the IDC numbers in the myocardia of CYCL-treated mice were significantly increased compared to the numbers in untreated mice.

**DISCUSSION**

In this study, low-dose treatment with CYCL determined the development of a DTH response in mice chronically infected with *T. cruzi*. The DTH response was characterised by the presence of a positive skin test following intradermal injection of *T. cruzi* antigens, which showed significant enlargement of the injected footpad and focal, perivascular, intradermic inflammatory lesions. An exacerbation of the chronic inflammatory response in the heart, with predominance of TCD8 cells, was observed concomitantly with significantly
increased IDC numbers. Dendritic cells (DCs) are a major accessory cell for the activation of both T-cell subpopulations. Antigen-specific CD8+CD4- cells can be induced to proliferate and become killer cells (Inaba et al. 1987). In this study, we demonstrated IDC stimulation in the myocardium by quantitative evaluation and observed a significant increase in the number of these cells expressing *T. cruzi* antigens in the hearts of treated mice. Previous studies based on a canine model of experimental Chagas disease (Andrade et al. 2000) have shown the evolution of the indeterminate form of the disease to chronic diffuse myocarditis after the use of low-dose CYCL, which was characterised by intense inflammatory infiltration and focal damage of myocardiocytes. The presence of a significant number of *T. cruzi* antigen-expressing IDCs upon CYCL treatment confirms the importance of these cells as APCs. With this function, these cells can migrate to the T zone of the spleen and can stimulate the CD4/CD8 response in the heart.

In low doses, CYCL functions as an immunostimulatory drug, as has been shown in studies in the field of cancer immunotherapy (Sistigu et al. 2011). CYCL markedly influences IDC homeostasis and promotes interferon (IFN)γ secretion, contributing to the induction of anti-tumour cytotoxic T-lymphocytes and the proliferation of CD4 T-cells. This eventually affects the T-reg/T effector ratio in favour of tumour regression.

The immune response to infection with *T. cruzi* is dependent on several factors and is initiated by the DCs (Van Overtvelt et al. 2002, Chaussabel et al. 2003). *T. cruzi* downregulates lipopolysaccharide-induced major histocompatibility (MHC) class I presentation in human DCs and impairs antigenic presentation to specific TCD8 lymphocytes (Van Overtvelt et al. 2002, Chaussabel et al. 2003, Soto et al. 2003). Splenic white pulp was severely depleted of both CD4 and CD8 T-cells at the peak of *T. cruzi* infection. CYCL acts directly on IDCs to initiate maturation, to process the antigens and to present to the CD4+ and CD8+ lymphocytes in the spleen. In vivo infection with *T. cruzi* modulates APC functionality in a strain-dependent manner (Steinman 1991); highly virulent strains downregulate MHC II expression on splenic DCs and inhibit its induction on peritoneal macrophages and splenic B cells (Soto et al. 2003).

During the indeterminate phase of Chagas disease, development of DTH is inhibited. Several factors are involved in this process, including parasite direct action and the inhibition of heart IDCs. DCs mediate T-helper 1 development and IFNγ production (Heufler et al. 1996). These cells reside in the tissues in immature forms and respond to various chemo-attractants from sites of inflammation. The cells can be activated by microorganisms and inflammatory stimuli, after which they complete their maturation process and become potent stimulators of T-cells. Specific cytokines upregulate both the presentation and sensitisation functions of accessory cells, which require the action of interleukin-1, a DC activator (Heufler et al. 1996). The impairment of the DTH determined by suppressor cells is responsible for the persistence of the infection until the chronic indeterminate form.

Fig. 9A, B: histochemistry. Sections of the heart of mouse chronically infected with *T. cruzi*, treated with cyclophosphamide in low dose. Immunolabelling of interstitial dendritic cells with anti-*Trypanosoma cruzi* antibody. H&E: 1,000X.

Fig. 10: morphometric evaluation of interstitial dendritic cells (IDCs) in mice infected with the Colombian strain of *Trypanosoma cruzi* treated or untreated with cyclophosphamide in low doses. A significant increasing of the IDCs was seen (p < 0.05).
A murine model has recently been used (Portella & Andrade 2009) to investigate the participation of heart IDCs carrying T. cruzi antigens in the maintenance of residual inflammatory infiltrates in chronically infected mice treated with benznidazole. The elimination of parasites upon benznidazole treatment led to a regression of inflammatory lesions and a decrease in IDCs. Residual inflammatory infiltrates persisted in treated mice and were correlated with the presence of IDCs carrying parasite antigens (Portella & Andrade 2009). This observation raised the possibility that regression of the cellular immune response after specific chemotherapy with benznidazole, followed by parasitological cure, may depend on the clearance of the processed antigens expressed on the membranes of IDCs.

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