Interferon- γ induced nitric oxide mediates *in vitro* neuronal damage by *Trypanosoma cruzi*-infected macrophages

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Neuronal lesions and peripheral denervation in Chagas' disease are related to local inflammation; however, the pathogenic mechanisms of neuronal lesions in the heart and megavisceras are still unclear. We investigated the involvement of nitric oxide (NO) on neuronal lesion in co-cultures of neurons and macrophages. *Trypanosoma cruzi*-infected and interferon- γ (IFN- γ)-activated co-cultures of neurons and wild-type (WT) macrophages showed significant reduction of both neuronal survival and neurite density. These findings correlated with the levels of NO and the expression of inducible nitric oxide synthase (iNOS). Accordingly, neuronal survival rate in the co-cultures was recovered to control levels by treatment of the cultures with the iNOS inhibitor, aminoguanidine. Moreover, neither neuronal survival nor the neurite density was affected in the co-cultures when the macrophages were harvested from iNOS-deficient mice. These results demonstrate that iNOS-derived NO is the major molecule involved in neuronal damage mechanism in our *in vitro* model of Chagas' disease neuropathology.

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Introduction

Despite recent progress in the control of Chagas' disease, approximately 20 million individuals are still chronically infected with its causative agent, *Trypanosoma cruzi*. Indeed, Chagas' disease is a significant cause of morbidity and mortality in Latin America (Moncayo, 2003). Infection with *T. cruzi* presents initially

as an acute phase, during which the parasite infects a wide variety of tissues. In most patients, specific host defenses develop and the acute phase subsides without any serious sequelae. The acute phase passes into a chronic phase, in which a few parasites remain in the circulating blood and body tissues, though most patients remain asymptomatic at this stage. However, some individuals develop clinical manifestations 10 to 30 years after the onset of infection (Rossi and Bestetti, 1995). These typically present as cardiac arrhythmias, cardiomyopathy or disturbances in esophageal and colonic motility (Köberle, 1968) involving hypertrophy and dilatation of the organ, i.e., mega esophagus and mega colon. The underlying anatomical abnormality in the organs appears to be a reduction in ganglion cell number (Köberle, 1970). In the past, several hypotheses were investigated in order to explain the mechanism of neuronal damage. Indeed, the existence of a neurotoxic agent liberated by the disintegration of the amastigote forms (Köberle, 1956; Alvarenga, 1960; MacClure and Poche, 1960) and the direct destruction of nerve cells through parasitism (Okumura, 1966; Köberle, 1968) were proposed but never proven. Similarly, myenteric denervation has been attributed to an intense inflammatory reaction around the ruptured pseudocysts (Tafari, 1970).

Recently, we described the loss of enteric ganglia in *T. cruzi*-triggered tissue inflammation in a mouse model of Chagas' disease. Our *in vivo* studies of mice lacking the inducible nitric oxide synthase (iNOS) gene indicated that the acute phase of *T. cruzi* infection led to myenteric denervation due to IFN- γ elicited nitric oxide (NO) production resulting from iNOS activation in the inflammatory foci along the intestinal wall (Arantes et al., 2004). We interpreted these data as an indirect evidence for the role of iNOS in the mechanism of neuronal damage in this disease. However, interactions between inflammatory cells and nerves are complex and difficult to assess *in vivo*. In particular, the specific sources of the several potential mediators of neuronal cell damage that are liberated in these sites of inflammation cannot be clearly resolved.

The induction of iNOS by diverse stimuli, such as microbial and/or cytokines, is Ca²⁺ independent and results in the generation

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of high and enduring NO levels (Nathan and Xie, 1994; Stuehr, 1999) in many cell types including macrophages, muscle cells, hepatocytes, fibroblasts, astrocytes and endothelial cells (reviewed in Nathan and Shiloh, 2000). It has been demonstrated that resistance to *T. cruzi* infection is associated with the capacity of lymphocytes to generate IFN- γ , which in turn can activate macrophages to produce NO, the main effector molecule that controls intracellular *T. cruzi* multiplication (Gazzinelli et al., 1992; Vespa et al., 1994). Studies have suggested that NO also plays a role in the tissue damage seen in Chagas' disease (Garcia et al., 1999; Vespa et al., 1994; Ny et al., 1999; Pinto et al., 2002; Chandra et al., 2002), but a direct role of macrophage-derived NO in mediating the neurotoxicity of *T. cruzi* was never definitively demonstrated.

Earlier, we described a co-culture model of sympathetic cervical ganglia (SCG) neurons and macrophages, shown to be a useful system for clarifying the roles of inflammatory cells and their products in neuronal injury (Arantes et al., 2000). Using this co-culture model, we have now investigated the direct effect of NO derived from *T. cruzi*-infected and IFN- γ -activated macrophages on neuronal structure *in vitro*. Peritoneal macrophages from either normal mice or iNOS knockout (iNOS^{-/-}) mice were activated *in vitro* to precisely evaluate the role of macrophage derived nitric oxide in our model of neuronal injury.

Materials and methods

Culture of SCG neurons

Pure primary cultures of SCG neurons were prepared as previously described (Arantes et al., 2000; Blennerhassett and Bienenstock, 1998). Briefly, SCG were removed from 2- to 3-day-old C57BL/6 (WT) mice and enzymatically dissociated in 0.25% trypsin II (Sigma Chemical Company, MO). Isolated sympathetic neurons in a final concentration of 10^4 cells/well were cultured on 96-well plates (Sarstedt) coated with Matrigel (Becton Dickinson, NJ) in 50 ng/ml NGF (NGF, Alomone, Israel), 10% fetal bovine serum (FBS)/minimal essential medium (MEM) with 2×10^{-6} M cytosine arabinoside (Ara-C, Sigma Chemical Company, MO) to remove glial cells. The remaining accessory cells represented less than 5% of cells in the cultures as shown in Figs. 1A and H (low magnification fields). The cultures were maintained at 37°C in a humidified 5% CO₂ incubator for 48 h before interventions were made. The identity of neurons was confirmed using the pan neuronal antibody 9.5 Protein Gene Product (PGP 9.5) as described below.

Neuron and macrophage co-cultures

Neuronal cultures, obtained as described above, were maintained for 48 h before macrophages were added in order to establish the co-cultures. Macrophages were harvested from the peritoneal cavity of adult WT and iNOS^{-/-} mice and centrifuged at 400×g for 10 min. The pellet was resuspended in medium and a single hypotonic lysis was performed as previously described (du Manoir et al., 2002) to remove erythrocytes. After another centrifugation, the cells were resuspended in complete medium and added to the neuronal cultures in a ratio of 10 macrophages per neuron. After 2 h, the cultures were washed to remove non-adherent cells, leaving a 97% pure population of adherent macrophages.

Parasites

The Y strain of *T. cruzi* was used for all experiments. Trypomastigote forms were cultured and purified from monkey kidney epithelial cell line VERO, as previously described (Braga et al., 1993). Parasites were harvested after 6 days in culture, centrifuged at 150×g for 10 min at room temperature, counted in a Neubauer chamber, centrifuged at 450×g for 10 min at 4°C, suspended in 10% FBS supplemented 199 medium and used for infection of cultures and co-cultures.

T. cruzi infection

Y strain *T. cruzi* trypomastigotes were added in a parasite-to-macrophage ratio of 10:1 to neuronal cultures or co-cultures. The cells were incubated at 37°C and 5% CO₂ in the presence or absence of 200 UI/ml of recombinant murine IFN- γ .

Blockade of iNOS activity

In order to define the role of NO derived from iNOS in neuronal lesions, we obtained macrophages from iNOS^{-/-} mice similarly as described above. In some experiments, the iNOS inhibitor aminoguanidine (30 and 100 μ M, RBI, Natick, MA) was used at to block iNOS activity of WT macrophages (Misko et al., 1993).

Assessment of neuronal damage

Cultures were fixed in 10% neutral-buffered formalin solution after different time intervals post-infection (p.i.) and stained with 10% Giemsa (Doles Reagents, Brazil). Neuronal death was quantified by counting the total number of surviving neurons in the whole area of each well and expressed as the percentage of surviving neurons in controls. The neuronal damage was assessed using the fluorescent probe Hoechst 33342 (0.2 μ g/ml; Molecular Probes). This fluorochrome binds specifically to DNA and is used to evaluate nuclear appearance, detecting changes such as DNA clumping into apoptotic bodies. In addition, apoptotic nuclei were detected with a terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) detection kit (TdT-FragEL, DNA Fragmentation Detection Kit, QIA33, Oncogene Research Products). Data obtained were representative of 3–4 independent experiments in triplicates.

Immunocytochemistry and immunofluorescence

Cultured cells were washed in PBS and permeabilized with 0.25% Triton X-100 for 10 min at room temperature. For immunocytochemistry, endogenous peroxidase activity was abolished with 3.5% H₂O₂ for 30 min, followed by incubation with 1:40 normal goat serum (NGS) for 30 min at room temperature. The cells were incubated with the primary rabbit anti-PGP 9.5 (1:1000; Ultraclone, UK) or primary mouse anti-CD68 (1:150, 556059, BD Biosciences, USA) antibodies for 2 h at room temperature. Secondary biotinylated antibodies were goat anti-rabbit and anti-mouse antibodies, followed by streptavidin–peroxidase complexes (DAKO-LSABR 2 system; DAKO, Carpinteria, CA, USA). The reaction was visualized by incubating the cultures with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Company, MO). For immunofluorescence, cells were incubated with 1:40 NGS and 2% bovine serum albumin (BSA) for 30 min each at room

temperature. Macrophages and iNOS expression were detected with 1:500 dilution of the FITC-conjugated primary rat anti-F4/80 (MCA497F, Serotec, USA) antibody and 1:50 dilution of primary rabbit anti-iNOS (AB1631, Chemicon, EUA) and Alexa 568-conjugated secondary antibodies (1:500; Molecular Probes), respectively. Secondary antibodies alone were used as specificity controls and uniformly resulted in very low background levels of

reactivity. The cells were examined by microscopy and digital images were acquired for documentation.

Neurite density assessment

As an additional assessment of neuronal damage, the neurite area were measured in control cultures and at 48 h p.i. following

immunocytochemistry for PGP 9.5. Captured video images of culture dishes were measured for the neurite area using the KS300 program (Version 2.0, Zeiss, Germany) and neurite density was expressed as the percentage of neurite area relative to control. Data obtained were representative of 3–4 independent experiments in triplicates.

Evaluation of NO^{-2} production by macrophages

Culture supernatants were collected at different times points p.i., stored at -20°C and assayed for the nitrite concentration by mixing 0.1 ml of culture supernatant with 0.1 ml of (0.1% naphthylethylene diamine dihydrochloride and 1% sulfanilamide plus 2.5% phosphoric acid) as previously described (Green et al., 1981). Absorbance at 540 nm was read 10 min later, and NO^{-2} concentration was determined by reference to a standard curve of 1 to 200 μM NaNO_2 .

Statistical analysis

The results were expressed as the average and SEM of at least three independent experiments and analyzed for statistical significance with Mann–Whitney U test. Probability values (P) of 0.01 or less were considered significant.

Results

Aspects of SCG neurons in cultures and co-cultures

The neurons *in vitro* conserved their morphological characteristics and displayed extensive neurite outgrowth with visible varicosities at 48 h post-plating (Fig. 1A), which was evident by the immunoreactivity with the pan-neuronal marker PGP 9.5. Efficient inhibition of glial cell proliferation was maintained by ARA-C.

The addition of pure populations of peritoneal macrophages to neuronal cultures resulted in a homogenous pattern of distribution of CD68 (Fig. 1B) and F4/80 (Fig. 1B, insert) immunopositive macrophages that were dispersed among the neurites.

T. cruzi induced macrophage activation causes neuronal death in co-cultures

Treatment of neuronal cultures by addition of *T. cruzi* indicated a lack of direct action of the parasite on neuronal cultures (Fig. 2B). Because no significant neuronal death was observed, we next assessed macrophages for their potential to cause neuronal damage *in vitro* following infection with *T. cruzi* and activation with IFN- γ . This caused progressive and extensive neuronal death, with 73%,

49% and 40% of neuronal survival relative to controls at 24, 48 and 72 h p.i., respectively (Figs. 2A–C). Treatment of co-cultures by addition of *T. cruzi*-infected or IFN- γ -activated macrophages and treatment of neuronal cultures by IFN- γ (Figs. 2A–C) did not significantly affect either neuronal morphology or survival relative to pure neuronal cultures controls at any time points.

In *T. cruzi*-infected and IFN- γ -activated co-cultures of neurons and WT macrophages, we observed shrinkage of cellular bodies and irregularities of cell membrane associated with pycnotic nuclei stained by Hoechst 33342 (Fig. 1D) compared to the control aspect showed in Fig. 1C. In addition, apoptotic nuclei were detected by TUNEL labeling (Fig. 1D, insert). In parallel cultures, the identity of each cell was detected as described above (Figs. 1A and B). In these co-cultures, we frequently also identified necrotic and degenerative neuronal changes characterized by cell vacuolization and swollen nuclei, cytoplasmic dissolution and the presence of cellular debris (Fig. 1E). These very intense alterations led to loss of morphological aspects of the cell types in co-culture (Figs. 1D and E). In contrast, these changes were never seen in pure neuronal cultures or in any of our other co-cultures conditions (Fig. 1C). Close proximity between damaged neurons and parasitized macrophages immunopositive for iNOS was often found (Fig. 1G), suggesting a link between NO generation and subsequent neuronal damage. This physical proximity of neurons and activated macrophages in our model mimics natural proximity of inflammatory cells and tissue damage described in human and experimental Chagas' disease (Tafuri, 1970; da Silveira et al., 2005).

Neurite density

To evaluate the possibility of a differential effect of macrophage-mediated damage on neuronal cell body vs. neurites under our experimental conditions, neurite area was quantified at 48 h p.i. (Fig. 3). Neurite density was reduced to 40% of control neurite area in simultaneously *T. cruzi*-infected and IFN- γ -activated co-cultures of neurons and macrophages (Figs. 1H and I). The direct addition of either *T. cruzi* or IFN- γ to neuronal cultures had no effect on neurite density by 48 h post-treatment, and again ruled out a direct effect of these agents on neuronal structure (Fig. 3).

Nitric oxide production correlates to neuronal death

Nitrite levels in culture supernatants were measured by the Griess reaction as an indicator of NO production in our system. The addition of alternatively *T. cruzi*, IFN- γ or peritoneal macrophages to neuronal cultures and treatment of co-cultures by addition of *T. cruzi*-

Fig. 1. (A) Aspects of SCG neurons in cultures stained with the pan-neuronal marker PGP 9.5. Neuronal cell bodies (arrows) and varicosities (*). Original magnification: $\times 35$. (B) Distribution of neurons and WT macrophages in co-cultures. CD68 (open arrowheads)-immunopositive WT peritoneal macrophages in co-culture with non-stained SCG neurons (arrows). Insert: Immunofluorescence of macrophages with F4/80 (green, open arrowheads). Original magnification: $\times 65$. (C and D) Nuclear staining by Hoechst fluorescent probe 33342 (blue) of pure neuronal control cultures (C) and of WT *T. cruzi*-infected and IFN- γ -activated co-cultures (D). Compare condensed and fragmented nuclei, suggesting apoptotic changes in neurons in panel D (open arrow heads) with normal nuclei aspect in control (C, open arrow heads). Notice the presence of cell debris in WT *T. cruzi*-infected and IFN- γ -activated co-cultures (D, white arrows). Insert: Apoptotic neuronal death in co-culture demonstrated by TUNEL labeling of some neurons (black arrows) in WT *T. cruzi*-infected and IFN- γ -activated co-cultures. Original magnifications: $\times 35$ (C and D); $\times 65$ (D, insert). (E) Some intact (arrow) and intensely degenerated neurons (arrowheads) in close proximity to intracellular amastigotes (long arrow) and cell debris. Giemsa staining in WT *T. cruzi*-infected and IFN- γ -activated co-cultures. Original magnification: $\times 65$. (F) F4/80 (green) and iNOS (red) immunopositivity in WT *T. cruzi*-infected and IFN- γ -activated macrophages were merged in lower panel. Original magnification: $\times 125$. (G) Nuclear staining by Hoechst fluorescent probe 33342 (long arrow, blue) and iNOS immunopositivity (arrow, cytoplasmatic staining in red) of WT *T. cruzi*-infected and IFN- γ -activated macrophages. Notice H33342 staining of amastigotes DNA inside macrophages (blue dots, open arrowheads). Original magnification: $\times 125$. (H and I) Representative aspects of neurites stained with the pan-neuronal marker PGP 9.5 in control cultures, normal density (H) and in WT *T. cruzi*-infected and IFN- γ -activated co-cultures, decreased density (I). Original magnification: $\times 35$.

infected or IFN- γ -activated macrophages did not cause a significant increase in NO production at 24, 48 and 72 h post-treatment (Fig. 4). However, the addition of IFN- γ to the *T. cruzi*-infected WT co-cultures resulted in significantly higher levels of NO production at

Fig. 2. Decreased neuronal survival in *T. cruzi*-infected and IFN- γ -activated co-cultures of neurons and WT macrophages (Neuron+Mac+IFN- γ +*T. cruzi*) 24 h (A), 48 h (B) and 72 h (C) p.i. We did not detect significant neuronal survival decrease in controls (Neuron, Neuron+IFN- γ) nor experimental (Neuron+Mac, Neuron+Mac+IFN- γ , Neuron+Mac+*T. cruzi*, Neuron+*T. cruzi*, Neuron+*T. cruzi*+IFN- γ) conditions. $N=3$ independent experiments in triplicates per experimental condition, except for Neuron+*T. cruzi*+IFN- γ ($N=3$). N.D., not done. * $p<0.001$.

Fig. 3. Decreased neurite density in *T. cruzi*-infected and IFN- γ -activated co-cultures of neurons and WT macrophages (Neuron+Mac+IFN- γ +*T. cruzi*) compared to normal density in controls (Neuron, Neuron+IFN- γ) and experimental (Neuron+*T. cruzi*) condition at 48 h p.i. $N=3$ independent experiments in triplicates per experimental conditions. * $p<0.001$.

24–72 h p.i. (Fig. 4) and induced expression of iNOS in parasitized (Fig. 1G) and F4/80-positive macrophages (Fig. 1F), allowing us to confirm the source of iNOS derived NO in our system.

Macrophage-mediated neuronal damage requires iNOS activity.

In order to evaluate the role of NO in neuronal death in this model, we isolated macrophages from iNOS^{-/-} mice and repeated the experiments above. Addition of iNOS^{-/-} macrophages alone did not interfere with neuronal survival. Indeed, infection of iNOS^{-/-} macrophages with *T. cruzi* and activation with IFN- γ in co-cultures failed to reproduce the neuronal death seen with WT cells (Fig. 5A), with no significant morphological alterations observed when compared to the control. As expected, NO production was not significant at all time points studied (Fig. 4). Further, the neurite density was similar to control (data not shown).

These results strongly indicated a critical role for IFN- γ -induced nitric oxide in neuronal damage, and this was assessed further through the pharmacological inhibition of iNOS in WT macrophages using aminoguanidine (100 μ M), which reduced NO amounts to an undetectable level (Fig. 5B) and failed to reproduce the neuronal death (Fig. 5A). We conclude that NO production from activated macrophages is a primary mechanism of neuronal damage in this model.

Discussion

We have developed a tissue culture model for the analysis of the cellular mechanisms responsible for the neuronal damage that occurs upon infection with the protozoan parasite, *T. cruzi*.

Sympathetic neurons maintained *in vitro* with NGF survive and develop well. In our system, the incubation of SCG neuron and macrophage co-cultures with *T. cruzi* infective forms (trypomastigotes) resulted, as expected, in efficient macrophage invasion, but neurons were systematically non-infected, regardless of the

presence or absence of concomitant IFN- γ treatment. The percentage of surviving neurons was not altered in pure neuronal cultures exposed to *T. cruzi* for 48 h (Fig. 2B) when compared to controls. In addition, this experimental condition did not result in NO production by 24, 48 and 72 h p.i. (Fig. 4). The addition of IFN- γ and *T. cruzi* to pure neuronal cultures did not change the parameters described above (data not shown), nor the morphological aspect of these cultures when compared to the control cultures. Our findings suggest that neurons, in contrast to most mammalian cells, are resistant to parasite invasion. Our *in vitro* findings are in agreement with intensive and thorough studies that have shown a very small number of parasitized cells among the hundreds of thousands of ganglion cells in mice, rats and dogs experimentally infected with *T. cruzi* (Köberle, 1968). The mechanisms of neuronal resistance to *T. cruzi* invasion are currently under investigation.

We found that addition of normal/resting macrophages did not substantially alter neuronal survival, but that addition of a similar number of macrophages and the following concomitant infection with *T. cruzi* and activation by IFN- γ led to significant and reproducible neurite loss and neuronal cell death. This clearly identifies the interaction between the macrophage and *T. cruzi* as a specific primary cause of neuronal damage, potentially responsible for the progressive neuronal damage and motility impairment seen in Chagas' disease.

Although iNOS is not considered essential for the control of *T. cruzi* infection (Cummings and Tarleton, 2004), it has been shown that resistance to *T. cruzi* is associated with the activation of macrophages by lymphocyte-derived IFN- γ to produce nitric oxide. NO is paradoxically implicated in controlling intracellular *T. cruzi* multiplication (Gazzinelli et al., 1992; Vespa et al., 1994) but also plays a role in the tissue damage seen in Chagas' disease (Garcia et al., 1999; Vespa et al., 1994; Ny et al., 1999; Pinto et al., 2002; Chandra et al., 2002).

We focused on iNOS-derived NO as the important mediator of neuronal lesions seen in our model. This unique co-culture model reproduces the *in vivo* proximity and interaction between neurons and macrophages. SCG neurons in culture display electrophysiological and molecular characteristics that resemble those *in vivo* (Blennerhassett and Bienenstock, 1998; Janiszewski et al., 1990). Our observation of the co-cultures showed that the macrophages were dispersed uniformly, representing ratio of approximately 10 macrophages per neuron. We believe that the levels of NO and subsequent neuronal damage obtained here are expected to mimic the conditions of natural *T. cruzi* infection. The neuronal loss of 60% at 72 h p.i. obtained in our study is in accordance with the myenteric neuronal loss described in experimental models (Arantes et al., 2004) and human studies (Köberle, 1968; Adad et al., 2001). Neuronal death is mediated by one or several interlinked death-initiating pathways resulting in activation of two major final

Fig. 4. Increased NO₂⁻ production in *T. cruzi*-infected and IFN- γ -activated co-cultures of neurons and WT macrophages (Neuron + WT Mac + IFN- γ + *T. cruzi*) compared to *T. cruzi*-infected and IFN- γ -activated co-cultures of neurons and iNOS^{-/-} macrophages (Neuron + iNOS^{-/-} Mac + IFN- γ + *T. cruzi*) at 24 (A), 48 (B) and 72 (C) h p.i. We did not detect significant NO₂⁻ production in controls (Neuron, Neuron + IFN- γ) nor experimental WT conditions (Neuron + *T. cruzi*, Neuron + WT Mac, Neuron + WT Mac + IFN- γ , Neuron + WT Mac + *T. cruzi*). *N* = 3 independent experiments in triplicates per experimental conditions. **p* < 0.001 (A, B) and **p* < 0.01 (C).

Fig. 5. Neuronal survival (A) and NO₂⁻ production (B) in *T. cruzi*-infected and IFN-γ-activated co-cultures of neurons and WT or iNOS^{-/-} macrophages. Recovered neuronal survival rate (A) and impaired NO₂⁻ production (B) in aminoguanidine-treated *T. cruzi*-infected and IFN-γ-activated WT macrophages (Neuron+WT Mac+IFN-γ+*T. cruzi*+AG 100 μM) and *T. cruzi*-infected and IFN-γ-activated iNOS^{-/-} macrophages (Neuron+iNOS^{-/-} Mac+IFN-γ+*T. cruzi*) compared to *T. cruzi*-infected and IFN-γ-activated WT macrophages (Neuron+WT Mac+IFN-γ+*T. cruzi*) co-cultures at 48 h p.i. *N*=4 independent experiments in triplicates. **p*<0.001.

common pathways that lead to cell death (Leist and Jäätelä, 2001). NO-mediated neuronal damage has been described in many pathological conditions (Wang et al., 2002; Carreno-Muller et al., 2003; Eskes et al., 2003; Eyupoglu et al., 2003; Katsuse et al., 2003; Liu et al., 2003; Rozenfeld et al., 2003) and involves apoptosis (McCarthy et al., 1997; Stefanis et al., 1998; Yu et al., 2003). Necrosis is a catastrophic loss of ionic integrity caused by membrane disruption or loss of energy supply and was

morphologically detected in association with apoptosis contributing to decreased neuronal survival in our model.

The *in vivo* source of IFN-γ in natural infection is lymphocytes (Gazzinelli et al., 1992; Vespa et al., 1994). In our system, we provided IFN-γ directly to the *T. cruzi*-infected macrophages reproducing the mechanism of macrophage activation described elsewhere (Drapier et al., 1988). The addition of IFN-γ to *T. cruzi*-infected co-cultures of neurons and macrophages increases NO production by macrophages between 24 and 72 h p.i. Detection of enhanced levels of nitrite, a stable degradation product of NO, and macrophage iNOS expression are correlated to significant neuronal damage assessed as decreased neuronal survival and decreased neurite density. The inability for NO production by iNOS^{-/-} macrophages was able to prevent development of neuronal damage observed in our system 24 to 72 h p.i., confirming the role of NO in the neuronal death in this model of *T. cruzi*-infected and IFN-γ-activated co-cultures. Neuronal death was additionally prevented by aminoguanidine, a specific iNOS inhibitor (Misko et al., 1993).

Although we have earlier experimental indirect evidence associating tissue damage with inflammation in mice in an experimental model of Chagas' disease (Arantes et al., 2004), this is the first demonstration of a direct effect of macrophage-derived NO in *T. cruzi*-induced neuronal damage. Besides macrophages, in *T. cruzi* and other infections, NO is produced by a wide variety of cell types, including NK cells, dendritic cells, neutrophils, endothelial cells, myocytes and fibroblasts (Hibbs et al., 1987; Ding et al., 1988; Kilbourn and Belloni, 1999; Werner-Felmayer et al., 1990; Nussler and Billiar, 1993; Vespa et al., 1994). During *in vivo* *T. cruzi* infection, different leukocyte sub-types are present in the inflammatory infiltrate, affecting tissue integrity. This makes the discrimination of the cellular sources of cytotoxic mediators to neural tissue difficult. Furthermore, in the *in vivo* model (Arantes et al., 2004), we could not also rule out the participation of glia cells as source of NO. In the current *in vitro* model, we eliminated viable glia cells in order to obtain pure neuronal cultures. If present, remaining glia represented less than 5% of cells and contribute to non-significant levels of NO that were detected in pure neuronal controls (Fig. 4), indicating that in this system glia cells should not be considered a relevant source of NO. The co-culturing of SCG neurons and WT macrophages for 24, 48 and 72 h showed, respectively, 95%, 81% and 81% of neuronal survival related to controls confirming our previous results (Arantes et al., 2000). These degrees of loss of neuron survival and the NO levels measured in this condition are not significant when compared to control. On the other hand, NO is being generated in small amounts in the co-culture conditions (Neuron+Mac, Neuron+Mac+IFN-γ or Neuron+Mac+*T. cruzi*) as attested by Fig. 4. In agreement with our arguments, this may contribute to some degree for decreased, though not statistically significant, neuronal viability showed in these conditions, in Fig. 2. However, this contribution is not as significant as *T. cruzi*-infected and IFN-γ-activated co-cultures.

We were able to correlate the enhanced levels of NO production not only with decreased neuronal survival but with neurite damage as well. Although ganglion cells are well visualized in murine and in human intestinal walls, the nerves are not routinely observed without specific staining. This can explain the focus of relevant investigations in counting neuronal body numbers (Adad et al., 2001; da Silveira et al., 2005; Köberle, 1970) without addressing axonal damage. In the context of Chagas' disease, however, the

participation of inflammatory-induced axonal damage and synaptic alterations in the pathogenesis of motility disturbances and mega visceral syndromes are highly relevant and deserve further investigation. Acute and chronic damaging mechanisms could affect diverse regions of the ganglion cells, including the axon and its functional synapses, and could reflect the different degrees of dysfunctions clinically reported (Madigan et al., 1996; Meneghelli, 2004). One of the merits of this *in vitro* approach is to highlight the importance of studying the nerve–effector cells interaction in Chagas' disease, which we intend to pursue.

In conclusion, our findings add to the understanding of the mechanisms involved in the development of cardiac/digestive pathologies and contribute new insights into prevention and treatment of symptomatic forms of Chagas' disease.

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