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REGULAR ARTICLE

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Host and parasite apoptosis following *Trypanosoma cruzi* infection in in vitro and in vivo models

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Abstract The mechanism of cell death which occurs during Chagas' cardiopathy is disputed. To address this issue we analyzed the molecular pathways implicated in the death of cardiomyocytes during *T. cruzi* invasion and found that they undergo apoptosis during both in vitro and in vivo infections. However, the death rates and onset were related to the parasite stocks belonging to different biodemes, which can be correlated to the different histological inflammation findings that have already been reported. Our in vitro data provide additional support for this hypothesis since higher levels and earlier apoptosis induction were noted during the interaction with the Dm28c (type I) as compared to the Y and CL stocks (type II). Modifications of the surface carbohydrates of the infected cardiomyocytes were observed and these molecular events may be acting as "eat me" tags for their final engulfment by macrophages and/or other non-professional phagocytes. The analysis of other host cell types showed that the in vitro infection of fibroblasts did not result in host apoptosis even when a highly infective stock was used. Conversely, infected macrophages undergo apoptosis but at a higher degree than cardiomyocytes. Apoptotic intracellular parasites were observed to varied extents depending on the *T. cruzi* stock, which was related to the parasite invasion and proliferation. In summary, our results show that during *T. cruzi* infection, the extent of apoptosis varies according to the host cell type and the parasite stocks. The apoptosis of both host and *T. cruzi* can contribute to the silent spreading and persistence of the parasite without triggering an exacerbated inflammatory response.

Keywords Cardiomyocytes · Biodemes · Apoptosis · *Trypanosoma cruzi* (Protozoa, Flagellata)

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Introduction

Host cell integrity may represent a valuable prerequisite for the survival and development of pathogens. The activation or prevention of cell death seems to be a critical factor in the outcome of an infection since it can favor or not favor the pathogen control and spreading. Apoptosis in the hosts can be managed during the infection with microorganisms, such as bacteria, viruses and protozoa (Lee et al. 2002; Lüder et al. 2001; Keane et al. 2000; Ullrich et al. 2000; Goebel et al. 1998, 1999). Pathogen-induced modulation of the host cell-death pathway may serve to eliminate key immune cells or evade host defenses that can act to limit the infection. Alternatively, the suppression of the death pathway may facilitate the proliferation of intracellular pathogens (reviewed in Weinrauch and Zychlinsky 1999).

During acute infection with protozoa such as *Plasmodium* sp., *Toxoplasma gondii*, *Leishmania donovani* and *Trypanosoma brucei*, apoptosis occurs in immune cells of the host possibly depressing the immune responses towards the antigens of the parasite (reviewed in Lüder et al. 2001; Quan et al. 1999; Das et al. 1998; Khan et al. 1996).

Our group has shown that apoptosis can be detected in the heart of *T. cruzi*-infected mice in a perforin-independent manner (Henriques-Pons et al. 2002). *T. cruzi* causes Chagas' disease, which affects over 17 million people in endemic areas of Latin America (WHO 1997), triggering

an important myocardial pathology that still requires more research on its physiopathological mechanism. Apoptosis of immune cells (including B and T cells) occurs during the course of *T. cruzi* infection. In experimental animal models infected by *T. cruzi,* there is a significant loss of uninfected $CD4^+$ T cells by the upregulation of Fas and Fas ligand expression, with a subsequent induction of apoptosis by activation-induced cell death (Lopes et al. 1995a, 1999).

Concerning the host cells that lodge the intracellular parasites, it has been reported that *T. cruzi* infection does not induce apoptosis in murine fibroblasts (Clark and Kuhn 1999) and inhibits Fas-mediated cell death in HeLa cells cultivated in vitro (Nakajima-Shimada et al. 2000). In addition, a molecule from the parasite known as trans-sialidase (TS), which is implicated in the process of invasion (Frasch 1994), modulates the survival of neuronal cells, preventing their apoptosis by the upregulation of the antiapoptotic protein Bcl-2 (Chuenkova and Pereira 2000), but triggers apoptosis in thymus, spleen and lymph nodes during the acute infection (Mucci et al. 2002). Moreover, proapoptotic activity has been reported with ceramide-containing glycolipids from the infective stages of the parasite. These lipids act in synergism to interferon-γ to promote apoptosis in macrophages (Freire-de-Lima et al. 1998). At present it is accepted that during chagasic myocardiopathy, heart cells can die by necrosis and also by apoptosis (Henriques-Pons et al. 2002; Zhang et al. 1999). Both types of cell death could then contribute in parallel to the progressive development of heart damage and failure.

Considering that the activation or prevention of cell death can be critical in the outcome of an infection, our aim was to analyze the extent and characteristics of the apoptotic death which occurs in heart cells during *T. cruzi* infection. Our present data showed that cardiomyocytes undergo apoptosis during *T. cruzi* invasion both in vivo and in vitro. The onset and death rates in cardiomyocytes were related to the parasite stock, occurring earlier and at higher levels during the interaction with parasites from the type I biodeme, which can be correlated to the reduced level of histological inflammation findings in different experimental models. We found remarkable differences regarding the rates of apoptosis between different host cell types (cardiomyocytes, fibroblasts and macrophages) besides detecting the occurrence of apoptosis in some intracellular parasites.

Materials and methods

Cell cultures

Primary cultures of embryonic cardiomyocytes (CM) were obtained as previously described (Meirelles et al. 1986). For fluorescent studies, CM were seeded at a density of 0.15×10^6 cells/well into 24-well culture plates containing gelatin-coated coverslips. Primary cultures of fibroblasts from skin (Corte-Real et al. 1995) or from skeletal muscles (Araújo-Jorge et al. 1986) were obtained by enzymatic dissociation of the mouse embryos and maintained in Eagle's medium as described in the cited references. Peritoneal mouse macrophages were also obtained as described elsewhere (Araújo-Jorge et al. 1989). All assays described here were run three to five times at least in triplicate. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals, resolution 242/99.

Parasites

Trypomastigotes of the Y strain and the CL-Brener clone (representatives of lineage II that circulate in the domestic biological cycle of *T. cruzi*, Zingales et al. 1997) and of the Dm28c clone of *T. cruzi* (representative of lineage I) were used throughout the experiments. Cell culture-derived trypomastigotes (Dm28c) were isolated from the supernatant of CM, which had previously been infected with metacyclic forms. Bloodstream trypomastigote forms from Y and CL stocks (type II biodemes) were harvested by heart puncture from *T. cruzi*-infected Swiss mice at the peak day of parasitemia, as described previously (Meirelles et al. 1982).

In vitro infection assays

After 24 h of plating, cell cultures were infected for another 24 h at 37°C with bloodstream or culture forms of *T. cruzi* employing a parasite:host cell ratio of 20:1. After host-parasite contact, the cultures were washed to remove free parasites and the infection monitored for up to 96 h.

In vivo infection and sample processing

Mice were infected by intraperitoneal injection of 10^4 trypomastigotes of the CL-Brener clone in Swiss male mice essentially as described previously (Waghabi et al. 2002). Age-matched uninfected mice were maintained under identical conditions compared to the infected ones. Mice were sacrificed at 14 days postinfection (dpi) and the hearts were quickly removed, frozen immediately in liquid nitrogen and stored at −70°C until processing for the fluorescent assays.

Apoptosis detection

The analysis of apoptotic events was carried out using different fluorescent microscopy approaches including the terminal deoxynucleotidyltransferase-mediated fluorescein dUTP nick end-labeling technique (TUNEL), methods using the fluorescent dyes 3, 3-dihexyloxacarbocyanine iodide ($DiOC_6$ from Molecular Probes Inc., Eugene, USA) and Hoechst 33342 (HO). TUNEL was performed using an apoptosis detection kit (Boehringer Mannheim, Mannheim, Germany). Cell cultures grown on coverslips were fixed for 20 min at 4°C with 4% paraformaldehyde solution (PFA), whereas cryostat sections (3–5 μm thick) were fixed in 2% PFA for 5 min. Samples were washed twice with phosphate-buffered saline (PBS) and then treated for 2 min/4°C with 0.1% Triton X-100 in 0.1% sodium citrate, washed and incubated with the TUNEL reaction mixture, followed by incubation for 60 min/37°C. For negative controls, cells were incubated in the absence of the terminal transferase and for positive controls the cultures were pretreated for 40 min at room temperature with 1 μg/ml DNase I prior to the TUNEL procedure. After staining, samples were further incubated with 10 μg/ml 4, 6-diamidino-2-phenylindole (DAPI) or 2 μg/ml propidium iodide (PI) for DNA staining, to enable visualization of parasites and heart cell nuclei and to allow direct quantification of the parasite infection levels. Samples were mounted with 2.5% 1, 4-diazabicyclo-(2, 2, 2)-octane (DABCO) and examined immediately using a Zeiss photomicroscope equipped with epifluorescence. Images were captured using the Quips Smart Capture (Vysis) software. For staining with $DiOC_6$, samples were fixed for 15 min with 0.25% PFA, washed twice with

PBS and incubated with 20 nM of the fluorescent probe (purchased from Molecular Probes, Inc.) for 5 min at 37°C in the dark, washed again and then incubated with a solution of PI and processed as described above. For DNA staining with the Hoechst 33342 dye, uninfected and infected fixed cultures were incubated with 2 μM HO for 30 min at 37°C. After three successive washes, samples were mounted using Vectashield to prevent fading and analyzed using a Zeiss photomicroscope equipped with epifluorescence.

Carbohydrate expression

To analyze the carbohydrate repertoire during *T. cruzi* invasion, CM were infected for 24 h with trypomastigotes (Dm28c stock) as described above and fixed for 5 min at room temperature with 2% PFA. After washing 3 times with PBS, samples were incubated for 30 min with 0.1 M PBS, 2% bovine serum albumin, 5 mM $CaCl_2$, followed by the fluorescein-conjugated lectin (50–250 μg/ml), for 1 h at 37°C. After washing, the cardiac cultures were further incubated with DAPI for DNA staining, and mounted with DABCO and examined as described above. Lectins from the following vegetal sources were used, with the respective specificities: *Canavalia ensiformis* (concanavalin A, Con-A, for α-D-mannosyl), *Triticum vulgaris* (WGA, for *N*-acetyl-D-glucosamine), and *Arachis hypogeae* (PNA, for *N*-acetyl-D-galactosamine). In all experiments, optimal lectin concentrations were determined by preliminary titration assays (ranging from 50 to 250 μg/ml), and to assure the specificity, competition assays were performed before the addition of the specific lectin, by incubating the cell cultures for 20 min at 37°C with 50–100 mM of the corresponding carbohydrates (Soeiro et al. 1999).

Results

Apoptosis of cardiac cells during in vivo infection

The heart is one of the most affected organs during Chagas' disease. We started by evaluating apoptosis in cryosections obtained from mice after 14 days of infection (dpi) with the myotropic CL-Brener clone of *T. cruzi* (Fig. 1). This parasite stock was chosen since it is the reference organism used in the *T. cruzi* genome project (Zingales et al. 1997) and develops a severe acute myocarditis (Waghabi et al. 2002). During the in vivo analysis, we performed both the TUNEL assay, which measures the extent of DNA fragmentation (Fig. 1a–f), and $DiOC_6$ labeling (Fig. 1g–l), a dye commonly used to monitor variations of the mitochondrial membrane potential during apoptotic events (Kluza et al. 2000). The results of the TUNEL assay indicated a higher number of apoptotic cells (stained green with FITC-nick end-dUTP in Fig. 1b, e, or yellow in Fig. 1c, f, for the superimposition of the green with the red staining due to propidium iodide) in the infected samples (Fig. 1d–f) as compared to the uninfected ones (Fig. 1a–c). The overlay of both tracers (FITC and PI) in the apoptotic cardiac nuclei (arrowhead) is confirmed in the graphic representation of the distribution of both fluorescences along the *xy*-axis (Fig. 1m). The figure displays a positive double-labeled cardiac nucleus (Fig. 1f, m, arrowhead) near to a non-apoptotic host nucleus (Fig. 1f, m, double arrowhead). The negative controls performed by the omission of terminal transferase displayed no positive labeling (data not shown). The analysis of heart cryosections from infected mice also showed an overall decrease in $DiOC_6$ staining at the host cytoplasm (Fig. 1j–l) as compared to uninfected samples (Fig. 1g–i), thus corroborating the TUNEL data. Our present results showed that during the in vivo infection with *T. cruzi,* cardiac cells undergo apoptosis, confirming and extending our previous TUNEL data concerning the infection of C57BL/6 mice with the Y strain (Henriques-Pons et al. 2002).

Apoptosis of cardiac cells during in vitro infection

Using the same approaches, we next investigated in more detail the induction of apoptosis during the interaction of cardiomyocytes with *T. cruzi* employing in vitro cultures (Soeiro et al. 2002; reviewed in Meirelles et al. 1999), which represent a powerful model to study the biology of this infection in the heart. *T. cruzi* stocks are grouped into different types or biodemes according to their various biological characteristics and histopathological findings in vivo (Zingales et al. 1999; Andrade and Magalhães 1996). They also exhibit different in vitro rates of infection and kinetics of invasion (Contreras et al. 1988; Meirelles et al. 1982). We then further explored the ability of different *T. cruzi* stocks to induce apoptosis in cardiomyocytes using Y and CL Brener clone (type II) and Dm28c (type I) parasites.

The interaction of CM with the Dm28c clone, which leads to an earlier invasion and higher proliferation rates as compared to the Y stock (Soeiro et al. 1995), showed a slight increase (6%) in the number of apoptotic cells (visualized by TUNEL labeling) as soon as after 2 h of parasite interaction (Table 1), which was undetectable when the Y stock was analyzed at the same time of infection (Table 1). After 24 and 48 h of host cell-parasite contact with Dm28c parasites, we observed a rise in the number of apoptotic cardiomyocytes (Fig. 2d–f) as compared to uninfected cultures (Fig. 2a–c), reaching levels of about 10% and 21% of increase, respectively (Table 1). Conversely, at the same time point, no significant increase was detected with Y stock (Fig. 2g–i, Table 1).

The analysis of the mitochondrial membrane potential variations in uninfected CM showed that the $DiOC_6$ stained intracellular compartments, mostly localized at the perinuclear region displaying a punctual pattern (Fig. 3a–c). After a short period of infection (24 h) with the Dm28c stock (Fig. 3d–f), we noted a decrease in the host cell labeling in infected culture.

The apoptosis of cardiomyocytes with the type II stocks (both Y and CL) could only be noted after 72 h of parasite contact by both TUNEL (Fig. 2j–l) and $DiOC_6$ analysis (Fig. 3g–i), where almost a loss of $DiOC_6$ staining could be noted in the highly infected cultures (Fig. 3h, i). The non co-localization of both fluorescent tracers (Fig. 3i, number 1) in the apoptotic cardiac nuclei (CM) is confirmed in the graph (Fig. 3j) of the distribution of both fluorescences along the *xy*-axis. In

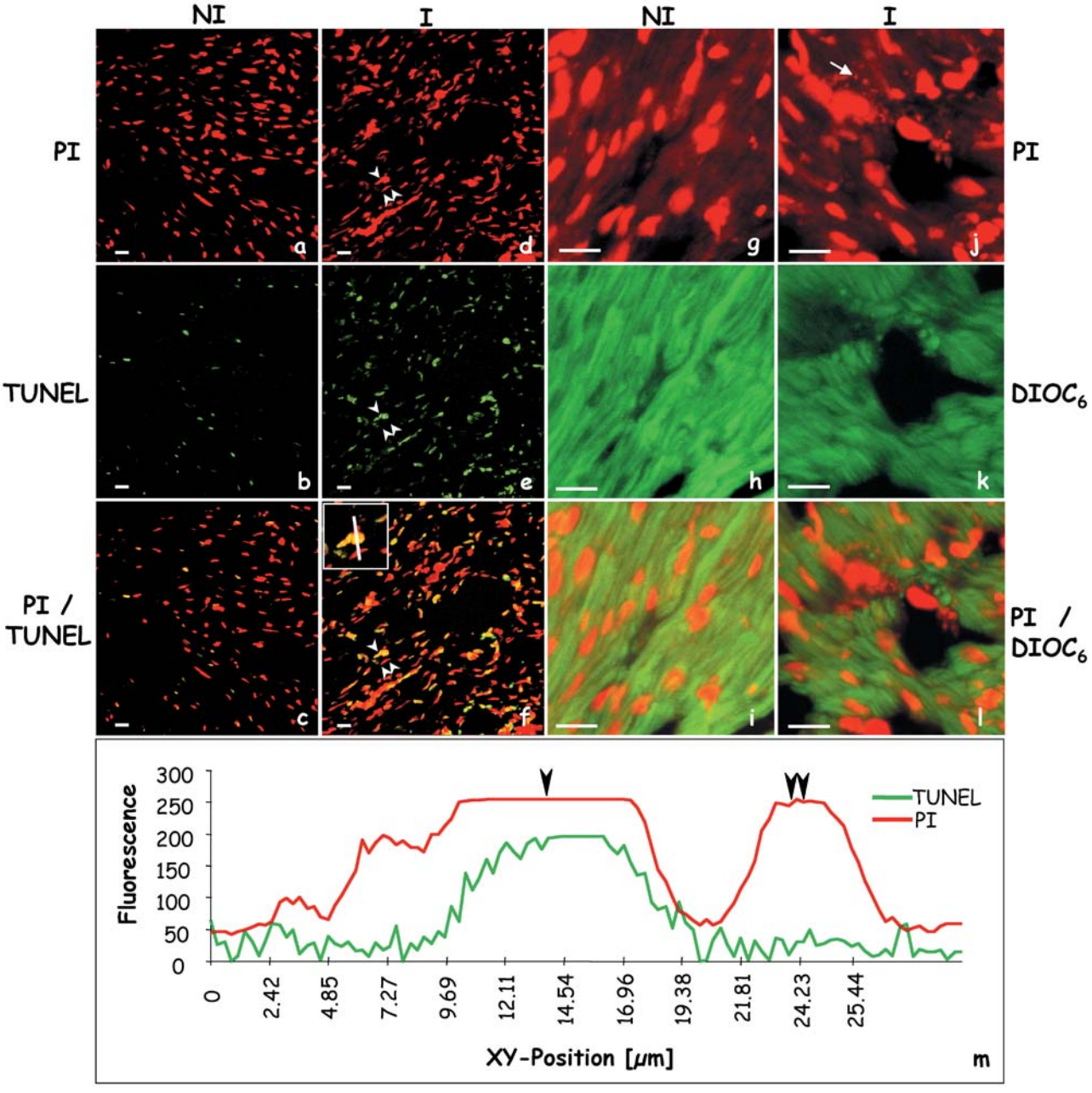

Fig. 1 Analysis by confocal scanning laser microscopy of apoptotic cell death in the myocardium of uninfected mice **(a–c, g–i)** and after infection **(d–f, j–l)** with *T. cruzi* (CL stock). Heart cryosections were probed with dUTP nick end labeling (*TUNEL*) **(a–f)** and with the $DiOC_6$ methods **(g–l)**. Propidium iodide (*PI*) stained the DNA **(a, d, g, j)** of the cardiac nuclei and the nuclei and kinetoplast of the intracellular parasites **(j,** *arrow*). The overlay of PI/TUNEL and PI/$DiOC_6$ is shown in **c, f, i** and **l**. The *graph* **(m)** represents the distribution of FITC and PI fluorescences along the *xy*-axis indicated in **f** (*inset, white bar*): an apoptotic cardiac nucleus (*arrowhead*) near to a non-apoptotic cardiac cell (*double arrowhead*). Note the increased number of apoptotic nuclei in the infected sample **(e, f)** as compared to the uninfected one **(b, c)**, and the lower $DiOC_6$ staining in the infected **(k, l)** as compared to uninfected heart **(h, i)** (*NI* and *I* indicate non-infected and infected host cells, respectively). *Bars* 20 µm

the same graph (Fig. 3j), some parasites (P, arrow) display a co-localization of $DiOC_6$ and PI at the kinetoplast (Fig. 3i–k, profiles 1, 2).

The apoptosis of the cardiomyocytes during their infection with *T. cruzi* was further analyzed through nuclear staining by the HO dye. As differently noted in uninfected cardiomyocytes (Fig. 3l), after infection with one or the other of the two parasite biodemes, the infected culture displayed cells with collapse of the nucleus chromatin into condensed masses characteristic of apoptosis. However, the kinetics were different. Apoptosis occurred at an earlier stage (as soon as after 2 h of infection) with the Dm28c clone (Fig. 3m, arrow) as compared to the Y strain (data not shown).

Table 1 Infection and apoptosis rates in cardiomyocytes, fibroblasts and macrophages after *T. cruzi* in vitro infection with Dm28c and Y stocks (mean results from at least three different experiments of each cell type). Apoptosis quantification from the cardiomyocytes, fibroblasts and peritoneal macrophages infected with *T. cruzi* was performed by the TUNEL reaction. The data show the percentage and the respective standard deviations of the apoptotic host cells after 2, 24 and 48 h of infection with trypomastigotes from Dm28c and Y stocks (1:20 host cell:parasite ratio) and the respective percentage of infection. *nd* not determined

Primary culture	Parameter	Hours after infection		
		2	24	48
Cardiomyocytes (Y strain)	% Infected cells	1±4.9	49±2	51±1.4
	% TUNEL + cells	1±0.2	1±0.7	1±0.7
Cardiomyocytes (Dm28c clone)	% Infected cells	47±4.3	59±2	63±5
	% TUNEL + cells	6±6	10±1	21±5
Skin fibroblasts (Dm28c clone)	% Infected cells	nd	70±1	72±11
	% TUNEL + cells	nd	0±0	0±0
Peritoneal macrophages (Dm28c clone)	% Infected cells	nd	76±5	80±8
	% TUNEL + cells	nd	75±7	90±9
	% Infected and TUNEL + cells	nd	52±5	66±7

Apoptosis of other host cells during in vitro infection

The primary cultures of cardiac cells contain a small population of fibroblast cells, which is important for the culture development (Meirelles et al. 1986). However, despite the fact that the fibroblasts were highly infected during the whole kinetics of infection, an intriguing characteristic of the TUNEL staining was the consistent lack of fibroblast labeling even at the later time points studied, employing the Dm28c parasites (data not shown). Then, to further explore whether the absence of labeling was related to the fibroblast origin, we prepared primary cultures of fibroblasts from other tissue sources, such as skin and skeletal muscle. Although both fibroblast cultures reached infection rates as high as 70% and 72% after 24 and 48 h of parasite contact, respectively (Table 1, showing data from skin fibroblasts), no positive TUNEL labeling was noted in infected fibroblast cultures, neither from skin nor from skeletal muscle. Since the inability of apoptosis induction in fibroblasts has already been reported in the literature (Clark and Kuhn 1999), contrasting the reported apoptosis in cells from the immune system (Freire-de-Lima et al. 1998), we next performed similar assays using resident peritoneal macrophages. After infection with the Dm28c stock, we found a striking TUNEL-positive labeling in the infected macrophage cultures reaching as high as 75% of positive cells after 24 h of infection (Table 1, Fig. 4a, b), while no apoptotic signals were found in the uninfected cultures (data not shown). These data contrast highly with the observed proportion of positive TUNEL cells in the infected cultures of cardiomyocytes (10%) and with the absence of staining in the fibroblast cultures (Table 1). However, in both macrophage and cardiomyocyte cultures infected by *T. cruzi* from both biodemes (type I and II), we noted (1) the presence of apoptotic uninfected cells in the infected cultures (data not shown), (2) highly infected cells not engaged in the apoptosis stage (Fig. 4a–e), and (3) apoptotic host cells providing lodging to both trypomastigotes (Fig. 2d–f) and/or amastigotes (Figs. 2j–l, 4a, b). The quantitative analysis obtained on TUNEL assays showed that the majority (70%) of the apoptotic cells are the infected ones (Table 1).

Carbohydrate expression at the surface of apoptotic cardiac cells

Cells undergoing apoptosis can display surface "eat me" tags such as sugar-altered expression (Dini et al. 2002). This consideration prompted us to evaluate the modifications at the carbohydrate cell surface in infected cardiomyocytes by using fluorescent lectins with different specificities. In the non-infected cultures, galactose detection through PNA-TRITC labeling only revealed a weak and almost absent binding at the cell surface (Fig. 5a), even at concentrations as high as 250 μg/ml, contrasting with the high positive staining in most infected cells (Fig. 5b). When uninfected cardiomyocytes were incubated with WGA-TRITC (100 μg/ml), sialic acid and/or *N*-acetyl-D-glucosamine residues were observed as a strong positive labeling at the cell surface of cardiomyocytes (Fig. 5c), which decreased 24 h after infection (Fig. 5d). With regard to mannose residues, Con-A-FITC (50 μg/ml) labeled uninfected cardiomyocytes at the cell surface and in intracellular vesicles around the nuclei, or surrounding it (Fig. 5e, f). In infected CM, although no significant difference in the host labeling was noted, the fluoresceinated lectin strongly bound to intracellular parasites (arrowhead, Fig. 5g, h). The previous treatment of cardiomyocytes with D-mannose, D-glucosamine, and D-galactose, specific for Con A, WGA, and PNA, respectively, before the incubation with the related lectin significantly reduced the binding (data not shown).

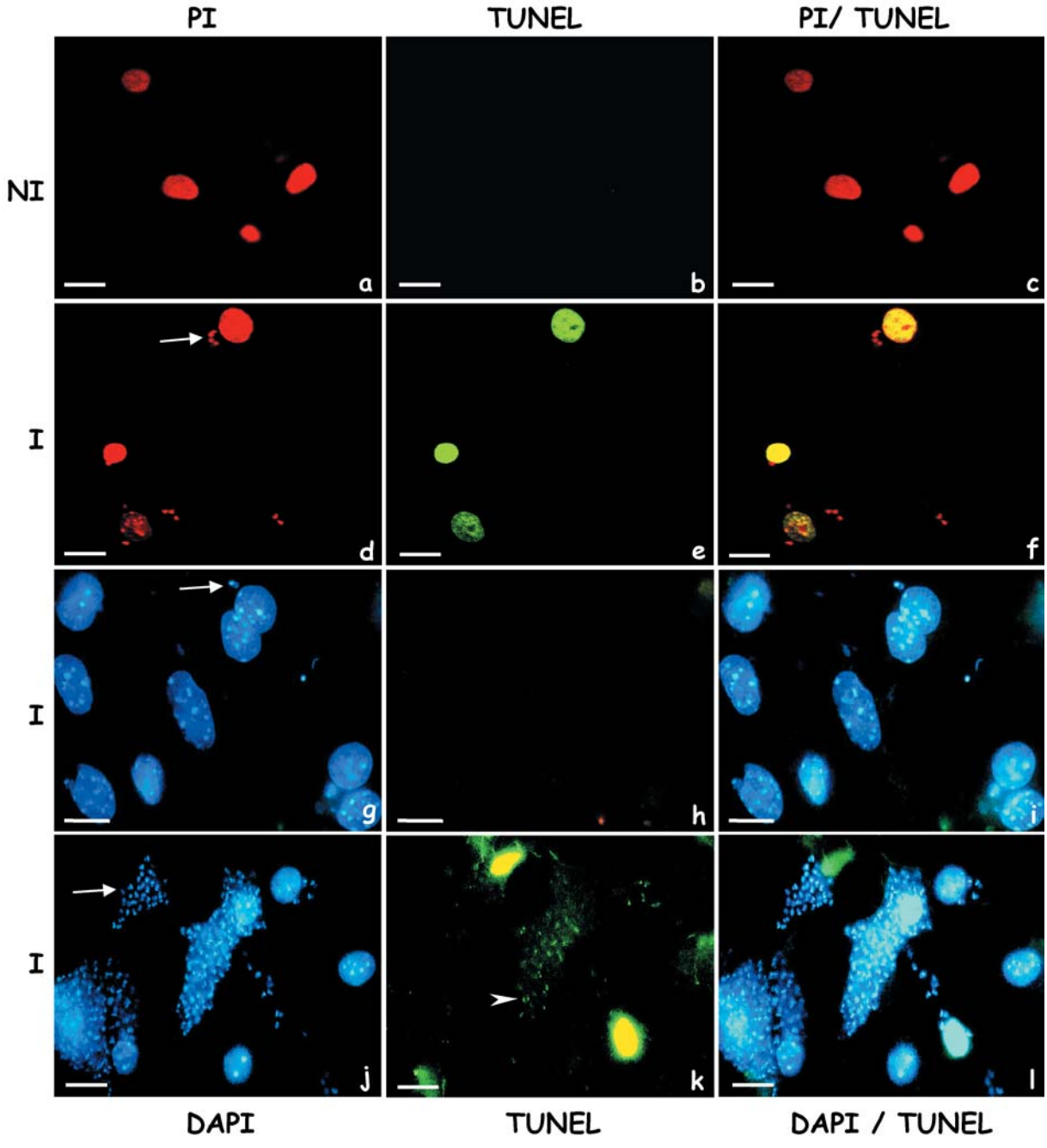

Fig. 2 Detection of apoptosis by TUNEL labeling in uninfected **(a–c)** and *T. cruzi*-infected cardiomyocyte cultures **(d–l)**. After 24 h of *T. cruzi* invasion, the apoptosis of the cardiomyocytes can be observed during the interaction with the Dm28c parasites **(d–f)** but not with the Y stock **(g–i)**, which could only be noted after 72 h of infection **(j–l)**. The nuclei of the host cells and the nuclei and kinetoplast of the parasites (*arrow*) were both stained by propidium iodide (*PI*) **(a, d)** for confocal scanning laser microscopy assays and by DAPI for visualization with the Zeiss microscope equipped with epifluorescence **(g, j)**. The double labeling with PI/TUNEL **(c, f)** and DAPI/TUNEL **(i, l)** shows the co-localization of both tracers. No apoptosis was detected in the uninfected cells **(b)**, whereas TUNEL-positive labeling was found in both host cell nuclei **(e, k)** and within the parasite kinetoplast (**k,** *arrowhead*) (*arrow* intracellular parasites, *NI* and *I* non-infected and infected host cells, respectively). *Bars* 20 μm

Apoptosis of *T. cruzi* during in vitro and in vivo infection

During the in vitro assays, some non-apoptotic as well as apoptotic host cells showed intracellular parasites that were stained positively by the TUNEL assay (Fig. 4a–e). In trypanosomatids, the mitochondrial genome is highly unusual, comprising a mass of catenated DNA called the kinetoplast. Each cell has one kinetoplast such that replication and segregation of this organelle must be carefully controlled during the cell cycle (Timms et al.

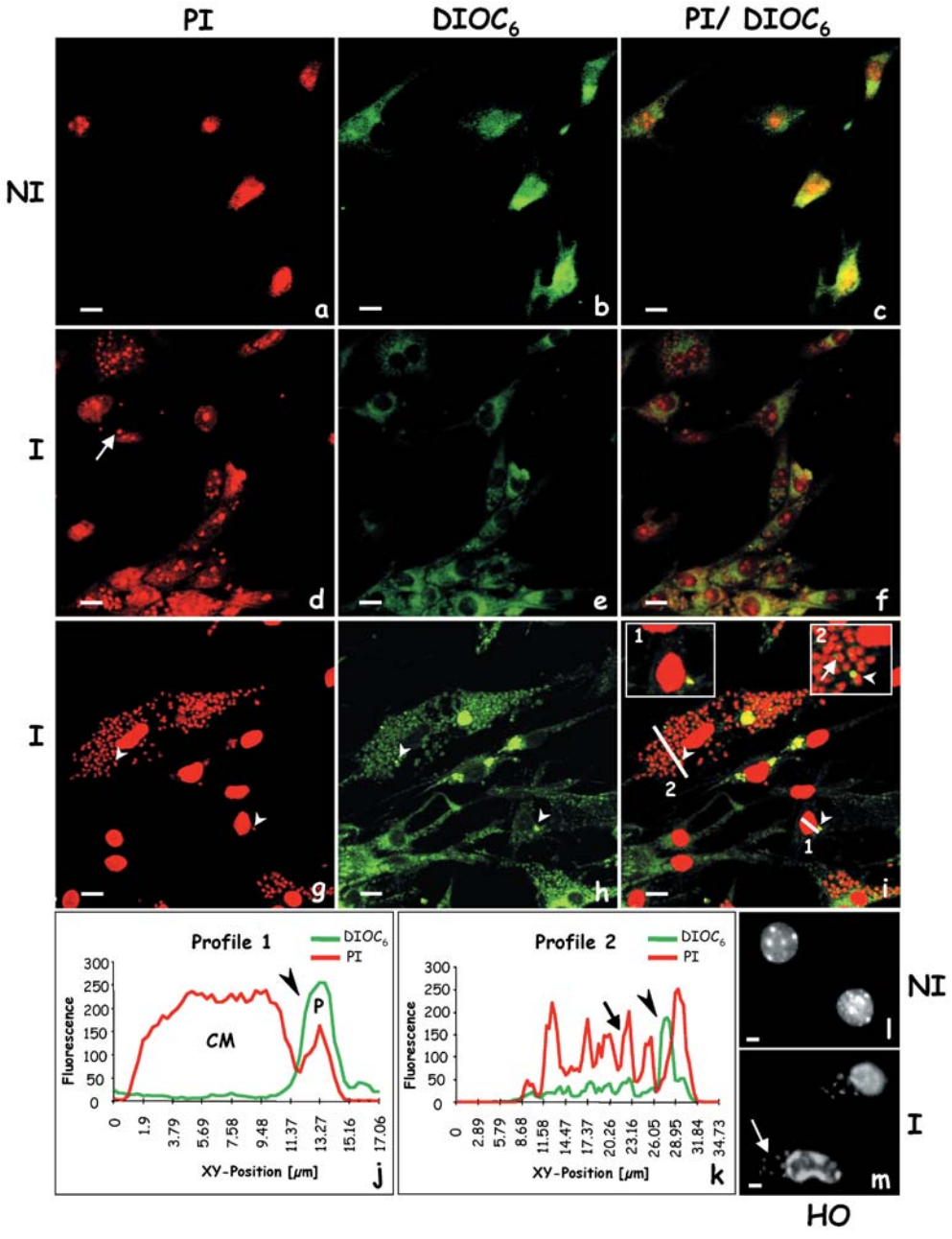

Fig. 3 Apoptosis detection by confocal scanning laser microscopy of $DiOC_6$ **(a–i)** and Hoechst 33342 (*HO,* **l, m**) staining in uninfected **(a–c, l)** and *T. cruzi*-infected cardiomyocytes **(d–i, m)**. The host nuclei **(a, d, g)** and both the nuclei and kinetoplast of the intracellular parasites (**d, g,** *arrow*) were labeled with propidium iodide (*PI*). After infection with the Dm28c **(d–f)** and Y **(g–i)** stocks for 24 and 72 h, respectively, a decrease in the $DiOC_6$ staining was noted in the infected cells **(e, h)** as compared to uninfected ones **(b)**, as well as in some intracellular parasites (**h,** *arrowhead*). Note the double labeling of PI and $DiOC_6$ in both cardiomyocytes **(c, f, i)** and within the parasites **(i)**. The distribution of FITC and PI fluorescences (**i,** *inset 1*) in both host cell (*CM*) and *T. cruzi* (*P*) is represented on the *xy*-axis of the *graph* (**j,** *profile 1*). In another host cell (**i,** *inset 2*), the distribution of FITC and PI fluorescences is represented in the *xy*-axis of the *graph* (**k,** *profile 2*), showing one intracellular parasite (*arrowhead*) near to others with reduced $DiOC_6$ labeling (*arrow*). The HO staining of infected cultures after 2 h of interaction with Dm28c parasites **(m)** shows the collapse of the host nucleus chromatin into condensed masses characteristic of apoptosis, which was not visualized in the uninfected cardiomyocytes **(l)**. *Arrow* indicates intracellular parasites (*NI* and *I* indicate non-infected and infected host cells, respectively). *Bars* 20 μm

2002). The TUNEL labeling was localized at the kinetoplast of the parasite, which was doubly stained with PI and FITC (Fig. 4e, inset). When probed with $DIOC_6$, we also found the staining in the kinetoplast of the parasites: the fluorescent dye surrounded it and the mitochondrial DNA could be co-localized with the PI staining (Figs. 3g–i, inset, 4i–k, inset, arrowheads). The co-localization of $DIOC_6$ and PI in the parasite kinetoplast (Fig. 3i) was further confirmed in the graph of the distribution of both fluorescences along the *xy*-axis (Fig. 3j, k, profiles 1, 2, respectively). In the same field we can note parasites with a higher $DIOC_6$ labeling (Figs. 3h, i, k, profile 2, 4j, k, inset, arrowheads), while others presented a reduced $DIOC_6$ fluorescent signal (Figs. 3h, i, k, profile 2, 4j, k, inset, arrow). The reduced fluorescent signal found at the parasite kinetoplast reveals variations in their mitochondrial membrane potential. These data prompted us to check the apoptosis of *T. cruzi* in vivo in a cryosection from infected heart, which was confirmed by TUNEL (arrow, Fig. 4f–h). We then quantified the extent of apoptosis in intracellular parasites stained by the TUNEL method (Table 2). The data showed that the apoptotic rates in *T. cruzi* also varied according to the parasite stock. Following infection for 24 and 48 h with Y strain, 44% and 58% of the intracellular parasites displayed positive labeling for TUNEL reaction, whereas parasites from the Dm28c clone displayed lower levels, around 25% and 26%, respectively. As expected, after 24 and

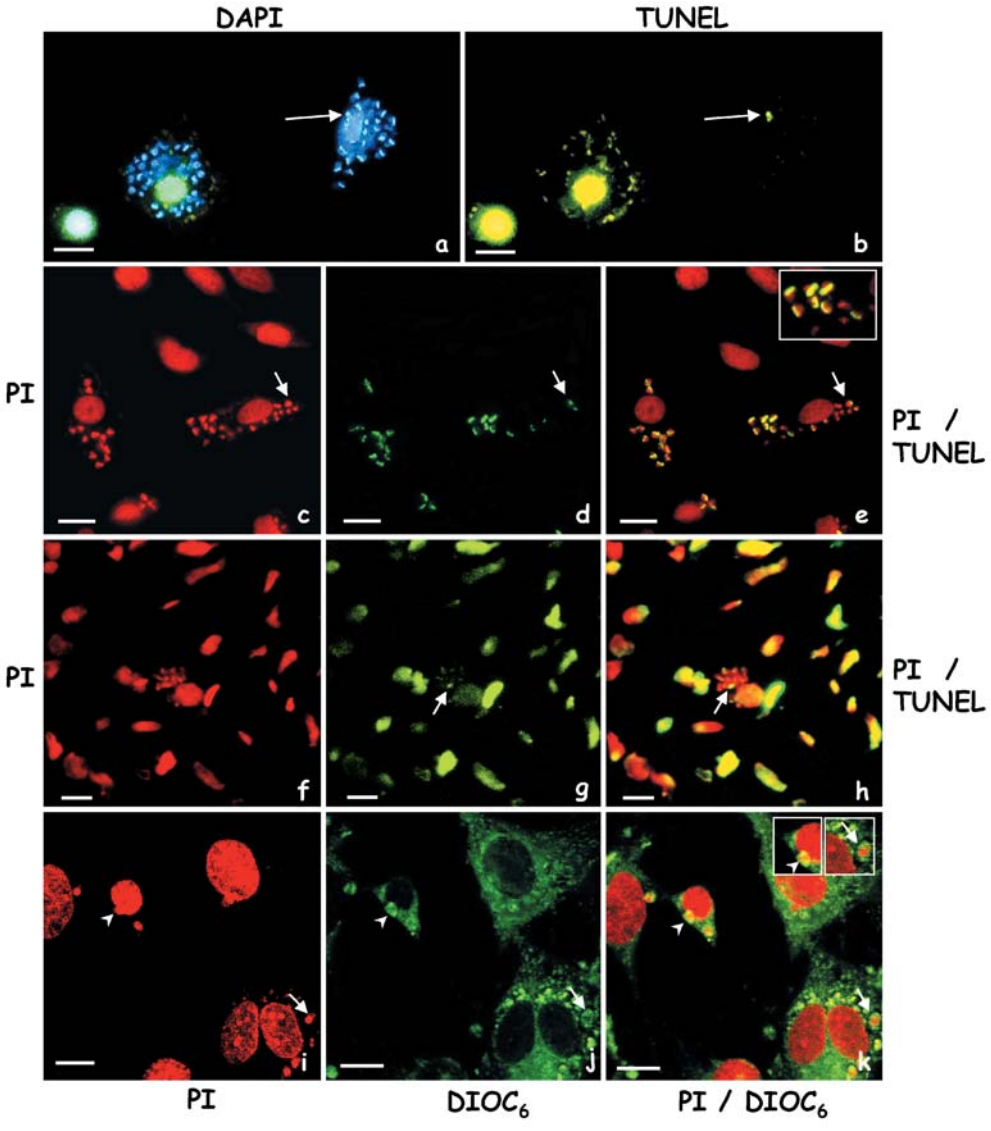

Fig. 4 Fluorescent analysis by both TUNEL **(a–h)** and $DiOC_6$ **(i–k)** methods of the apoptotic cell death in the intracellular forms of *T. cruzi* within peritoneal macrophages (**a, b,** 24 h of infection with Dm28c stock), cardiomyocyte cultures (**c–e, i–k,** 48 h of infection with Y stock) and heart tissues (**f–h,** 14 dpi with CL stock). TUNEL labeling shows the apoptosis in both infected host cell and intracellular parasites (*arrow*). The DNA of the macrophages and the *T. cruzi* (*arrow*) were labeled by the DAPI **(a)**. PI labeled the DNA in the host nuclei and within the nuclei and kinetoplast of the parasites **(c, f, i)** in confocal scanning laser microscopy analysis. In the same field we can visualize the co-localization of PI and TUNEL **(e, h)** and PI and $DiOC_6$ **(k)**. *Arrow* indicates the intracellular parasites positive for TUNEL reaction. Lower (**j, k,** *inset, arrow*) and higher (**j, k,** *inset, arrowhead*) $DiOC_6$ labeling can be visualized within the intracellular forms of *T. cruzi*. *Bars* 20 μm

Table 2 Apoptosis rates of intracellular forms of *T. cruzi* from Y and Dm28c stocks localized within cultured cardiomyocytes. Determination of the apoptosis levels in the intracellular forms of *T. cruzi* by the dUTP nick end-labeling (TUNEL) method. The mean number of intracellular parasites per infected cardiomyocytes as well as the apoptotic measurement of these parasites was performed employing the parasite/cardiomyocyte ratio of 20:01, after 24 and 48 h of infection. Data represent the mean number and the respective standard deviations obtained from at least three different in vitro assays

T. cruzi stock	Parameter	Hours after infection	
		24	48
Y	Mean number of intracellular parasites	4±0.5	5±3
	% TUNEL + parasites	44±5	58±4.3
Dm28c	Mean number of intracellular parasites	10±2	18±4.2
	% TUNEL + parasites	25±8	26±3

48 h of parasite interaction, the mean number of intracellular parasites per infected cardiomyocytes was higher with the Dm28c clone (10 and 18, respectively) as compared to Y strain (4 and 5, respectively).

Discussion

Parasites have evolved different strategies to induce or inhibit host cell apoptosis. The results reported here show for the first time that the infection of primary cultures with *T. cruzi* may or may not induce apoptosis according to the type of host cells: infected fibroblasts remain refractory, cardiomyocytes can undergo apoptosis at moderate levels and resident macrophages are highly affected by apoptosis induction. As the TUNEL-positive labeling does not directly mean apoptosis, but can also reflect other events such as DNA repair as reported in hypertrophied hearts (Kayakawa et al. 2002; Kanoh et al.

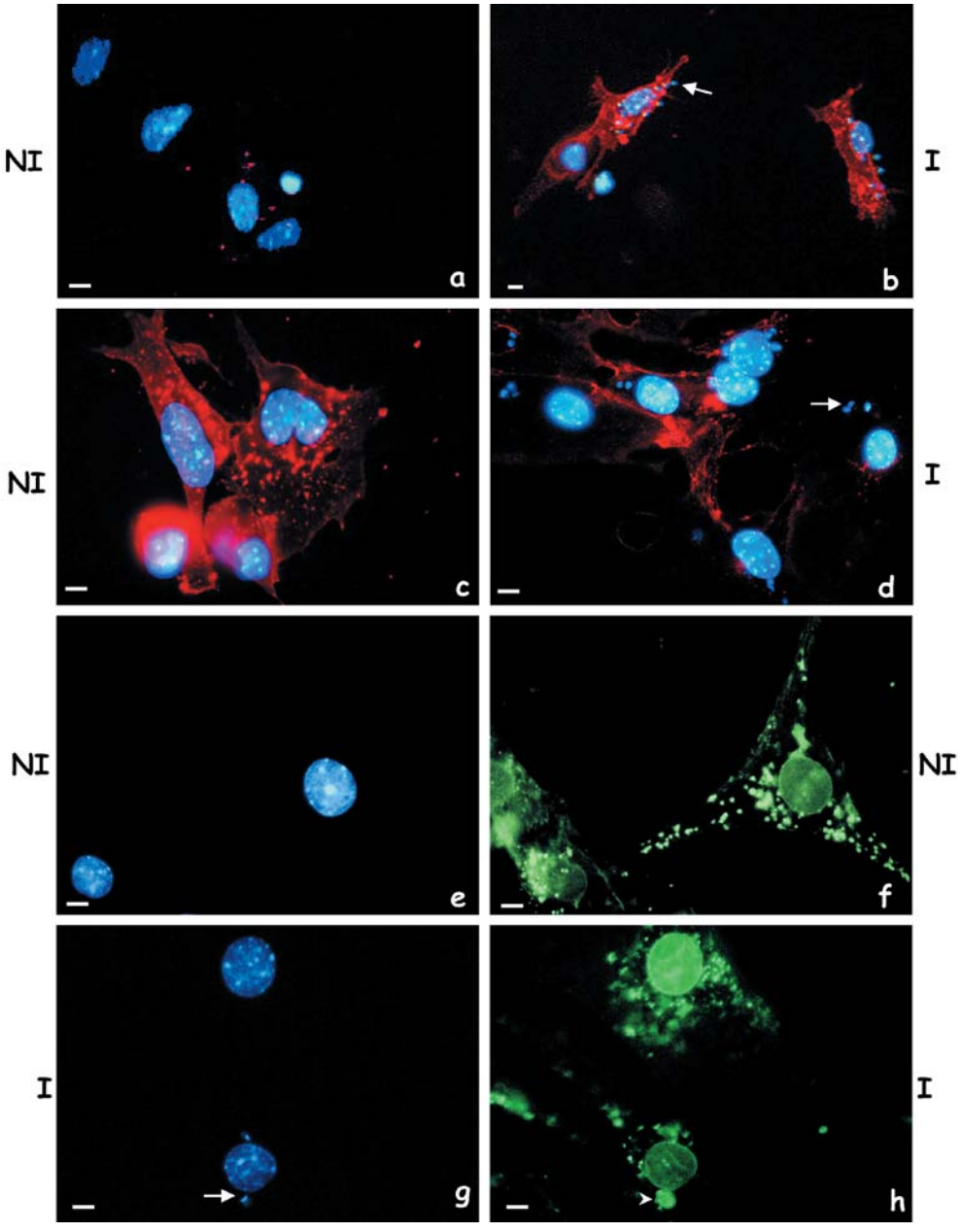

Fig. 5 Fluorescent analysis by lectin binding of the carbohydrate contents in the surface of uninfected **(a, c, e, f)** and *T. cruzi*-infected cardiomyocytes **(b, d, g, h)** after 24 h of interaction with the Dm28c stock. The nuclei of the host cells **(a–e, g)** and the nuclei and kinetoplast of the parasites **(b, d, g)** were both stained by DAPI. Double labeling of PNA-TRITC and DAPI **(a, b)** in the same photographic field showing the increase in galactosyl residues in infected CM **(b)** as compared to uninfected cells **(a)**. Co-localization of WGA-TRITC and DAPI labeling in uninfected **(c)** and *T. cruzi*-infected cardiomyocytes **(d)**. Note the marked decrease in the WGA-binding sites in the infected cardiac cells **(d)**. The labeling of Con-A-FITC in uninfected cardiac cells **(f)** shows a similar pattern to the *T. cruzi*-infected cells **(h)**. *Arrow* indicates the intracellular parasites and *arrowhead* shows parasite labeled by Con-A-FITC (*NI* and *I* indicate non-infected and infected host cells, respectively). *Bars* 20 μm

1999), we complemented this approach using the dyes $DiOC_6$ and Hoechst 33342 to measure the changes in the mitochondrial membrane potential and nuclear DNA condensation, respectively. The results of these experiments clearly demonstrated that apoptosis is induced in heart muscle cells after infection by *T. cruzi* both in in vivo and in vitro models. Thus, the aforementioned TUNEL-positive labeling is indeed related to apoptosis. As this phenomenon was not observed in the majority of the infected cardiomyocytes, our data confirmed that the apoptosis is not the predominant cell death mechanism operating in *T. cruzi* infection, but it can contribute to the overall inflammatory modulation, which occurs in the chagasic cardiomyopathy. Interestingly, a cytoskeletal breakdown (Pereira et al. 1993), a downregulation of α-actin mRNA (Pereira et al. 2000), a surface charge alteration (Soeiro et al. 1995), and an extracellular matrix degradation (Calvet et al., submitted), which are apoptotic characteristics, have already been described during the in vitro infection of cardiomyocytes with *T. cruzi.*

We found that the onset of the apoptosis in the infected cardiomyocytes is related to *T. cruzi* stock. The CM undergoes apoptosis earlier (as soon as after 2 h of parasite-host contact) during their interaction with trypomastigotes from the Dm28c stock as compared to the Y stock. It is known that both *T. cruzi* stocks behave differently during their interaction with CM (Soeiro et al. 1999; Contreras et al. 1988; Meirelles et al. 1986). The parasite stocks display different kinetics of internalization and infection levels, as well as different times required for

the intracellular differentiation of the parasite to the multiplicative amastigote forms and then differentiation again into the infective trypomastigotes, which are the main forms released after the death of the host cell. Therefore, the differences concerning the onset of apoptosis induction in cardiomyocytes after infection with Y and Dm28c parasites could be due to their different ability to invade and proliferate within those hosts. In fact, different modulations of cardiomyocyte physiology have been reported during *T. cruzi* infections (reviewed in Meirelles et al. 1999), and marked differences have been observed when comparing parasite stocks of various origins, usually with the Dm28c parasites displaying earlier and more pronounced alterations compared to other stocks (Soeiro et al. 1999, 1995). CM infected with Dm28c trypomastigotes display higher surface charge alterations (decreased surface anionogenicity) as compared to the Y stock (Soeiro et al. 1995), which as discussed above can denote another surface tag for the clearance of apoptotic cells such as described for apoptotic thymocytes (Morris et al. 1984).

Another result that deserves discussion is the differential induction of apoptosis in different types of host cells. The apoptosis of resident macrophages was attained by the majority of the infected cells as compared to the infected CM, even when the less infective stock (Y strain) was provided (data not shown). Also, in contrast to what was observed with cardiomyocytes, high apoptotic levels were noted when two to three parasites per infected macrophage were found as soon as after 24 h of parasite contact. On the other hand, when fibroblast cell cultures from different sources were employed, no apoptotic signals were noted although these hosts are highly susceptible to *T. cruzi* infection, reaching infection rates as high as those of phagocytes such as peritoneal macrophages. These observations corroborate the current literature data indicating that *T. cruzi* drives apoptosis in macrophages (Freire-de-Lima et al. 1998, 2000), but does not induce apoptosis in fibroblasts (Clark and Kuhn 1999). Considering that fibroblast cells are refractory to apoptotic induction by *T. cruzi*, it can be postulated that this cell type displays specific protective mechanisms preventing the cells from entering the apoptosis pathway. It is then possible that *T. cruzi* finds a more "silent" way to proliferate in cells in which it induces apoptosis than in refractory cell types, and this may be a mechanism implicated in the chronification of the infection.

Our data clearly show that apoptosis during Chagas' disease is not an all-or-nothing phenomenon and that *T. cruzi,* similarly to other pathogens, has evolved different ways to defeat and subvert the host defense systems by modulating the host apoptotic events (reviewed in Heussler et al. 2001). It drives the apoptosis of immune cells (including B, T cells and macrophages), which may contribute to many aspects, including: (1) the loss of $CD4^+$ T cells and thymocyte depletion affecting the autoimmune phenomenon, (2) control of inflammatory response and cell recruitment to the affected tissue due to the tumor growth factor-β production by phagocytic cells, which internalized apoptotic host cells and/or apoptotic parasites, and (3) control of the parasite load due to alteration of host metabolism towards putrescine production. Another mechanism for the escape of the parasite could be the prevention (as in fibroblasts) or induction (as in cardiomyocytes and macrophages) of the host cells undergoing apoptosis during their intracellular and proliferation stage until the parasite release, without affecting their survival and thus perpetuating the infection (reviewed in Lüder et al. 2001). We showed that in both cases an important proliferation is achieved and new trypomastigotes are released in the supernatant of the infected cultures. In infective pulmonary diseases, the apoptosis of inflammatory cells during infection with intracellular (e.g., mycobacterial infections) or extracellular pathogens (e.g., *P. cepacia*) may or may not also favor the host recovery, respectively (reviewed in Behnia et al. 2000). In this way, a successful host defense must establish an accurate balance between apoptotic and antiapoptotic events, associated with appropriate amounts and time course of immune mediator release and their effective cells leading to favorable parasite control and tissue repair. Clearly not only the direct parasite effect but also host-derived factors such as inflammatory mediators and their products such as tumor necrosis factor-α and nitric oxide production modulate the apoptosis in the host cells, interfering with the host susceptibility to the *T. cruzi* infection (reviewed in Roggero et al. 2002).

Our results employing cultured cardiomyocytes as host cells as well as in vivo analysis of heart from infected mice corroborate and extend the in vivo description data of Zhang et al. (1999) employing histological and ultrastructural analysis of the canine model of acute chagasic myocarditis. They found apoptosis in tissue cardiac myocytes even when these cells were uninfected and suggested that it could occur due to the presence of "toxic mediators" in the injured site. However, in our in vitro system we also observed apoptosis induction due solely to *T. cruzi* infection without the influence of immune cell populations and/or their mediators. The induction of apoptosis in the CM during the infection by *T. cruzi* could prevent the release of cytoplasmatic components, thereby avoiding high levels of inflammation. This is an attractive hypothesis for the infections with type I lineages of *T. cruzi* (such as Dm28c), which are infective but not pathogenic to mammals (Lopes et al. 1995b), besides inducing very little histological inflammation of the heart and consequently undetectable plasma creatine kinase (CK-mb) release during the acute phase (T.C. Araújo-Jorge, unpublished data). Our in vitro data comparing the infection with the different types of *T. cruzi* biodemes (I and II) provide additional support for this hypothesis since higher levels and an earlier apoptosis induction were noted during the interaction with the Dm28c (type I) as related to the Y and CL stocks (type II).

Our present work has still not answered the question of what is causing apoptosis in cardiomyocytes during *T. cruzi* invasion. However, a hypothesis could be envisaged including the known ability of CM to actively secrete

chemokines and cytokines after *T. cruzi* in vitro infection (Machado et al. 2000), which can contribute to the pathogenesis of chagasic cardiomyopathy seen in *T. cruzi*-infected mice. Alternatively, the apoptosis in infected CM could be related to the described molecules from the parasite (TS and the glycoinositolphospholipid), which are known to modulate apoptosis in immune cells (Leguizamon et al. 1999; Freire-de-Lima et al. 1998). These hypotheses are currently under investigation.

Since it is known that the recognition process of apoptotic cells is triggered by modifications of their surface (Dini et al. 2002; Platt et al. 1998), we searched for surface tags exposed by the apoptotic-infected cardiomyocytes such as the exposed surface sugars. We found substantial changes in the exposed carbohydrate residues occurring at the cardiomyocyte surface, exposing D-galactose residues which were normally hidden. The exposure of galactosyl residues, along with the lack of WGA-binding sites in the infected cardiomyocytes (including sialic acid residues) presently described, suggests a role for the parasite TS, which besides being implicated in the host apoptosis, has the ability to remove and transfer sialic acid from the host molecules to the parasite surface, sialylating important epitopes necessary for the successful invasion of *T. cruzi* (Schenckman et al. 1991). Our present results show the altered exposure of galactose epitope and the previously reported surface charge alterations (Soeiro et al. 1995) could act as "eat me" tags of the dead infected cardiac cells in a similar way to the phagocytosis of apoptotic cells by liver (Dini et al. 2002).

Another interesting result for discussion is the finding of apoptosis in intracellular parasites, observed both in vivo and in vitro. Parasite apoptosis has been described in obligate intracellular protozoans such as *Leishmania* species (reviewed by Arnoult et al. 2002; Lee et al. 2002), *T. gondii, Plasmodium berghei* (Al-Olayan et al. 2002) and *T. cruzi* (reviewed by Lee et al. 2002; Piacenza et al. 2001; Zhang et al. 1999). The number of these parasites in the infected cells can be several hundred and may be enough to induce apoptosis, a phenomenon that could be implicated in the parasite growth control, which seems also to be related to the parasites biodemes. Piacenza et al. (2001) evaluated L-arginine metabolism in epimastigotes of *T. cruzi* exposed to death stimuli and found that these parasites can undergo programmed cell death. Zhang et al. (1999) described TUNEL-positive labeling of intracellular parasites during canine acute infection. Parasite apoptosis could mean a mechanism for parasite evasion of the immune system by promoting the synthesis of anti-inflammatory mediators diverting the aggressive immune response from the sites of the parasite proliferation and permitting its silent spread. In fact, in infected tissues, although the inflammation is always related to the parasite and/or its antigens in the inflamed tissue, the level of inflammation does not necessarily correlate with the parasite load (Henriques-Pons et al. 2002; Waghabi et al. 2002; Palomino et al. 2000). We found that the Dm28c parasite stock, which shows lower apoptotic levels than the Y stock, also displayed higher proliferation rates. This suggests that apoptosis could then act as a clone population controller as has been proposed for other unicellular organisms, where the altruistic death may favor the clone populations of yeasts and trypanosomatids (reviewed in Al-Olayan et al. 2002). As discussed above, during the apoptotic cell death, the rapid endocytosis of apoptotic bodies by phagocytic cells prevents inflammation and tissue damage. Although it cannot be considered the main type of cell death found in the inflamed heart, the apoptosis of both cardiomyocytes and *T. cruzi* reported here can play a role in the silent spread and persistence of the infection throughout the acute and chronic phases of Chagas' disease, without triggering an exacerbated inflammatory response, besides being an interesting mechanism of parasite escape from the host immune response. The dual apoptotic activity of *T. cruzi* on the different host cell types that we noted might reflect a balance interchange between the parasite and the pro- and antiapoptotic signals of the host.

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