Trypanosoma cruzi does not induce apoptosis in murine fibroblasts

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(*Received 29 April 1998; revised 28 July 1998; accepted 29 July 1998*)

SUMMARY

The intracellular cycle of *Trypanosoma cruzi* in mammalian host cells involves the differentiation of dividing amastigote forms into flagellated trypomastigote forms. The mechanism(s) regulating the growth and differentiation of the intracellular parasites is (are) not known. The number of parasites in infected cells can be several hundred and may be enough to induce apoptosis, a suicide-like death programme, generating products (e.g. nuclear proteins) that could function as signals to initiate the differentiation of amastigotes into trypomastigotes. Murine fibroblasts infected with *T. cruzi* were examined during a 5-day course of infection for evidence of apoptosis. However, characteristics of apoptosis, including degeneration of nuclear structure, condensation of chromatin, loss of plasma membrane integrity, or the cleavage of DNA into nucleosomal fragments, were not observed. Therefore, it is unlikely that products resulting from host cell apoptosis function to induce parasite differentiation. The possibility that *T. cruzi* might inhibit host cell apoptosis by increasing intracellular levels of Bcl-2, an endogenous inhibitor of apoptosis, was then investigated. Analysis of infected cells by flow cytometry did not demonstrate a significant amount of intracellular Bcl-2. This suggests that if the parasite is inhibiting host cell apoptosis, it is by a method that does not involve increasing levels of Bcl-2.

Key words: *Trypanosoma cruzi*, apoptosis, cell death, murine fibroblasts.

INTRODUCTION

Trypanosoma cruzi is transmitted to mammals through mucosal membranes or an open wound via infective faeces of reduviid bugs. On invasion of the mammalian host, the parasite invades a cell, differentiates into the amastigote form, undergoes binary fission and, after 4–7 days, differentiates back to the trypomastigote form then leaves the host cell to infect other host cells. At the end of the cycle, the trypomastigotes begin actively moving in the host cell cytoplasm, probe the cell membrane with their flagella and escape by bursting out of the host cell (Dvorak & Hyde, 1973). It has not been determined whether or not the intracellular amastigotes are induced to differentiate in trypomastigotes by chemical signals or if the parasite has an internal clock that programmes differentiation after 9 divisions, as suggested by Dvorak & Hyde (1973).

Alternatively, it has been suggested that an increase in cyclic adenosine monophosphate (cAMP) plays a role in *T. cruzi* differentiation (DeCastro, De Nazareth & Meirelles, 1987; Gonzales-Perdomo, Romero & Goldenberg, 1988; Heath, Hiney & Sher, 1990; Zavala-Castro, Guzman-Marin & Zavala-Velaquez, 1995). Increased levels of cAMP have been reported to stimulate *in vitro* differentiation of *T. cruzi* extracellularly, from epimastigotes to metacyclic trypomastigotes (DeCastro *et al*. 1987; Gonzales-Perdomo *et al*. 1988), and intracellularly from amastigotes to trypomastigotes (Zavala-Castro *et al*. 1995). In addition, it has been shown that exogenously supplied cAMP induces the expression of the TC26 gene which is associated with *in vitro* differentiation of epimastigotes to metacyclic trypomastigotes (Heath *et al*. 1990).

Various signals originating from the host cell may function to induce the change from amastigotes to trypomastigotes as the host cell approaches death and it is possible that the infected host cell undergoes apoptosis as a result of the massive numbers of intracellular parasites that develop during the course of infection. Characteristics indicative of apoptosis including nuclear degeneration, breakdown of mitochondria, changes in the plasma membrane, and chromatin condensation within the nucleus have been observed in *T. cruzi*-infected cells *in vitro* (Dvorak & Hyde, 1973).

The study described herein was done to determine whether or not *T. cruzi* induces apoptosis in infected murine fibroblasts and if apoptotic events may be correlated with the intracellular differentiation of *T. cruzi* from amastigotes to trypomastigotes.

MATERIALS AND METHODS

Cell cultures

L cells, a murine fibroblast cell line, were used as host cells unless stated otherwise (a gift from Dr R. J. Spolski, Wake Forest University). Cell cultures

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https://doi.org/10.1017/S0031182098003631 Published online by Cambridge University Press

were maintained in RPMI-1640 medium (Sigma Chemical Co., St Louis, MO) containing 20 mM HEPES (Sigma) and supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Atlanta Biologics, Norcross, GA), 10^{-5} M 2-mercaptoethanol (Sigma), 20 units/ml penicillin, 20 μm/ml streptomycin, and 40 μm/ml neomycin (RPMI-C). Cultures were maintained at 37 °C in an atmosphere of 5 % CO_2 with 70–80 % relative humidity.

Parasites

A Brazil strain of *Trypanosoma cruzi* which has been maintained in our laboratory for more than 25 years was used in all experiments. The parasites are grown as culture forms in liver infusion tryptose medium (LIT) (Castellani, Ribeiro & Fernandes, 1967) and as fibroblast-derived trypomastigotes (FDTs) in murine fibroblast cultures (Davis & Kuhn, 1990). For use in experiments, recently released FDTs were obtained by washing infected fibroblast cultures 3 times with warm (37 °C) RPMI-C, to remove extracellular parasites, followed by incubation with fresh medium. FDTs that were released from fibroblasts after 24 h were collected and utilized in experiments.

Experiments involving simultaneously infected fibroblast cultures were prepared by incubating fibroblasts with FDTs for 3 h at a 10:1 parasite to host cell ratio. Approximately 25 % of the host cells were infected when exposed to trypomastigotes in this manner (data not shown). Parasites which had not penetrated host cells were then removed by 3 washes with warm (37 °C) RPMI-C medium. Control fibroblasts were treated identically but without parasites. The time of removal of free FDTs from the culture medium was considered 0 h post-infection.

Quantification of host cell viability by use of fluorescent dyes

L cells (4×10^4 cells in 2 ml) were plated on 1-chambered slides (Nunc Inc., Naperville, IL) in RPMI-C and infected as described above. At 24, 48, 72, 96, and 120 h post-infection, duplicates of experimental and control slides were treated with DNA-binding dyes and examined by fluorescence microscopy on a Zeiss Axioplan. A working solution of 100 μg/ml ethidium bromide (Sigma) and 100 μg/ml acridine orange (Sigma) in phosphate-buffered saline (PBS; 4 mM Na_2HPO_4, 0·955 mM KH_2PO_4, 145 mM NaCl) was utilized. A volume of 5 μl of the dye mixture was placed in the medium of each chamber slide at the appropriate time for approximately 1 min. The medium was then drawn off, the plastic chamber and rubber adhesive on each slide were removed and replaced with a 22 × 50 mm coverslip. Photographs were taken of the cells under a × 40 objective and epi-illumination with a filter combination appropriate for visualizing fluorescein. The photographs were used later to determine numbers of live and dead infected cells and for characterization of the parasite population within infected host cells. Infected host cells were scored for approximate number of parasites within the cell, the stage(s) of the parasites, and for the approximate percentage of host cell cytoplasm occupied by the parasites.

Quantification of host cell detachment during infection

Two ml of L cell suspension (2×10^4 cells/ml) were plated into 1-chambered glass slides (Nunc Inc.) in RPMI-C. Triplicates of experimental and control slides were set up as described above. At the appropriate time post-infection, the supernatant fluid containing detached cells was removed from each slide and spun at 500 ***g*** for 5 min. The pelleted cells were resuspended in 100 μl of RPMI-C with 2 μl of the acridine orange and ethidium bromide fluorescent dye mixture previously mentioned. A 10 μl aliquot of each sample was used to make microscope slides which were then viewed on a Zeiss Axioplan under epi-illumination. Detached cells were examined similarly at 0, 24, 48, 72, 96 and 120 h post-infection.

Kinetics of cell growth

L cells were plated at a concentration of 4×10^4 cells/ml in 25 cm^2 tissue culture flasks (Corning, NY) in triplicate. Experimental and control cultures were prepared as described above. At 0, 24, 48, 72, 96 and 120 h after infection, infected and uninfected L cells were harvested using warm (37 °C) 0·02 % EDTA in PBS and spun at 500 ***g*** for 10 min. The cell pellet was then resuspended in fresh medium, and cell numbers were determined using a haemocytometer. Percentage viability in each sample was determined by trypan blue exclusion (0·04 % trypan blue in PBS).

Detection of apoptosis by ELISA

Quantification of the degree of DNA fragmentation in infected and control cells was determined by the Cell Death Detection ELISA kit (Boehringer Mannheim, Indianapolis, IN; cat. no. 1544 675). The kit employs a sandwich enzyme immunoassay technique using mouse monoclonal antibodies against DNA and against histones to detect mono- and oligonucleosomes present in the cytoplasmic fraction of cell lysates. These nucleosomes are present as products of the apoptotic process. Samples for utilization in the kit were obtained by plating 5×10^4 L cells with 250 μl of RPMI-C in a flat-

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bottomed 96-well plate (Nunc Inc.). Experimental and control wells were established as previously described. Lysates were prepared from experimental and control wells at 0, 24, 48, 72, 96 and 120 h post-infection. Lysates from adhered cells in the wells were pooled with lysates obtained from detached cells in the supernatant fluid. The ELISA was performed according to manufacturer's instructions.

Electrophoresis of DNA

Cell samples were harvested using 0·02 % EDTA in PBS and washed once in warm (37 °C) PBS by centrifugation (500 ***g*** for 5 min). Whole cell lysates were prepared by incubating the cells overnight in TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0) with 0·1 % sodium dodecyl sulfate (SDS) containing 50 μg/ml Proteinase K (Sigma) in a 37 °C water bath. Lysis was followed by phenol–chloroform extraction of DNA. The DNA was precipitated by adding 0·1 volume of 3 M sodium acetate and 2 volumes of 100 % EtOH. Samples were placed at 20 °C for 10–15 min and then centrifuged for 2 min at 16000 ***g***. The DNA pellet was washed with 70 % EtOH and resuspended in TE buffer. This was followed by a 30 min incubation with 10 μg/ml RNase in a 37 °C water bath. DNA concentration was determined by measuring the absorbancy at 260 nm of a diluted sample (1/1000) in water. Approximately 6 μg of DNA per lane was run on a 1·5 % agarose gel at 60 V with ethidium bromide added to the electrophoresis buffer (0·4 M Tris, 2 mM EDTA). Lambda DNA/*Hin*d III markers (Promega, Madison, WI) were used as standards. The gel was rinsed in deionized water for 15 min before photographing under UV light.

To obtain DNA from infected and control cells, 2×10^6 L cells were plated in 75 cm^2 tissue culture flasks (Corning) and experimental and control cultures were prepared as described above. Infected and control cells for time 0 were immediately harvested with 5 ml of 0·02 % EDTA in PBS, all other flasks were provided 10 ml of RPMI-C and incubated until the appropriate time of 24, 48, 72, 96 or 120 h post-infection. At these times, infected and control cells were harvested, counted, lysed and the DNA extracted.

Flow cytometric analysis of Bcl-2 content

L cells at various levels of infection were analysed for Bcl-2 content. AG10 cells were used as a positive control (a gift from Dr R. J. Spolski, Wake Forest University). AG10 cells are Abelson murine leukaemia virus-transformed cells derived from thymic tumours (Spolski *et al*. 1988). *T. cruzi* -infected L cells were harvested using 0·02 % EDTA in PBS. Single-cell suspensions of infected L cells and AG10 cells were washed in PBS and permeablized by resuspending in ORTHO PermeaFix (Ortho Diagnostic Systems, Inc., Raritan, NJ) at a concentration of 10^6 cells/ml. After a 40 min incubation at room temperature, permeablized cell samples were spun at 400 ***g*** for 5 min and the supernatant fluids removed via aspiration. The cells were then washed by resuspension in 1 ml of wash buffer (2 % bovine serum albumin and 0·1 % sodium azide in PBS) and a 10 min incubation at room temperature. Next, cells were pelleted by a 5 min spin at 400 ***g*** and then resuspended in a 100 μl of 1 μg/10^6 cells of hamster anti-mouse Bcl-2 IgG (PharMingen, San Diego, CA., cat. no. 15021A) or 1 μg/10^6 cells of hamster anti-mouse CD3-epsilon IgG (PharMingen, cat. no. 01081D) and incubated at 4 °C overnight. The primary antibody was removed by a 10 min incubation in wash buffer followed by a 5 min spin at 400 ***g***. Cells were then incubated at 4 °C for 8 h with FITC-conjugated goat anti-hamster IgG (Southern Biotechnology Associates, Inc., Birmingham, AL; cat. no. 6061-02) at a concentration of 1 μg/10^6 cells in a 100 μl volume. The secondary antibody was removed by a 10 min incubation in wash buffer followed by centrifugation (400 ***g*** for 5 min). Samples prepared to determine autofluorescence were incubated throughout in wash buffer only. Stained cells were then resuspended in wash buffer at a concentration of 10^6 cells/ml and stored at 4 °C in the dark before being analysed by flow cytometry (Coulter Epics XL flow cytometer).

Statistics

Student's *t*-test was used to determine statistical significance and differences were considered significant at $P < 0{\cdot}05$.

RESULTS

Cell growth kinetics of uninfected and infected L cells

In order to determine whether or not apoptosis may occur in infected murine cells, a number of characteristics of the infection needed to be defined in order to determine the time at which apoptosis may occur. First, the growth rate and the viability of infected and uninfected fibroblasts were determined. On each day post-infection, cells were harvested, counted and the viability determined. Cell numbers on the first 2 days following infection were identical in infected and uninfected cultures (Fig. 1). After day 2, the number of infected cells continued to increase but at a much slower rate than the uninfected cells. The number of infected cells remained lower than the infected cells throughout the 5-day period of examination. By day 5, there was approximately a 3-fold difference between the number of uninfected and infected cells. However, there was no difference in the viability of infected and uninfected cultures on

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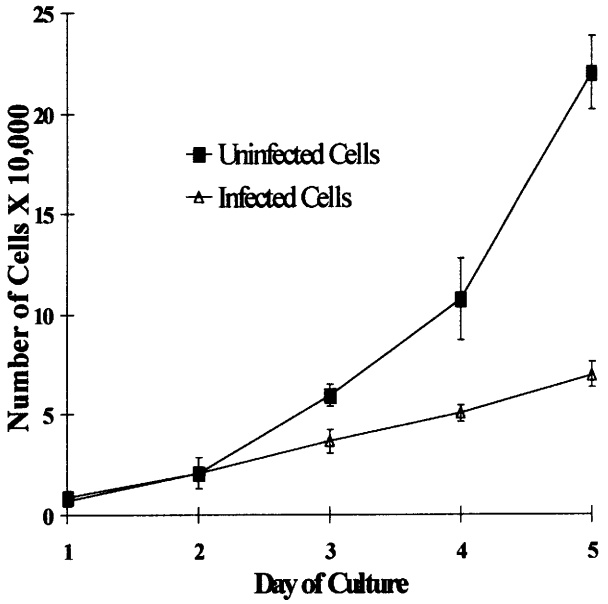

Fig. 1. Growth of uninfected and infected L cells. On each day post-infection the number of cells and the percentage of viable cells were determined in infected and uninfected cultures. Viability was determined by trypan blue exclusion. Error bars represent the standard error of the mean.

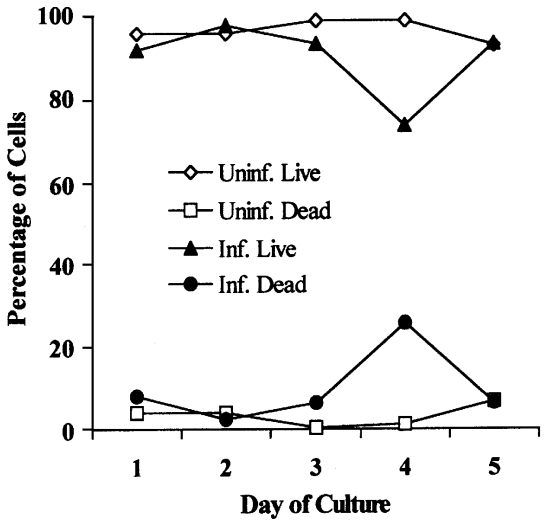

Fig. 2. Viability of uninfected and infected L cells during the 5-day infection period. Viability was determined by staining cells with the fluorescent DNA binding dyes acridine orange and ethidium bromide (for uninfected cells $n > 200$ except day 1 where $n = 74$, and for infected cells $n > 100$ except days 1 and 2 where $n = 39$ and 88, respectively).

any day. The viability of cells harvested was consistently $\geqslant 90\,\%$ in uninfected and infected cultures.

Quantification of host cell viability using fluorescent dyes

To quantify the degree of host cell death on each day of infection, cell cultures prepared on 1-chambered

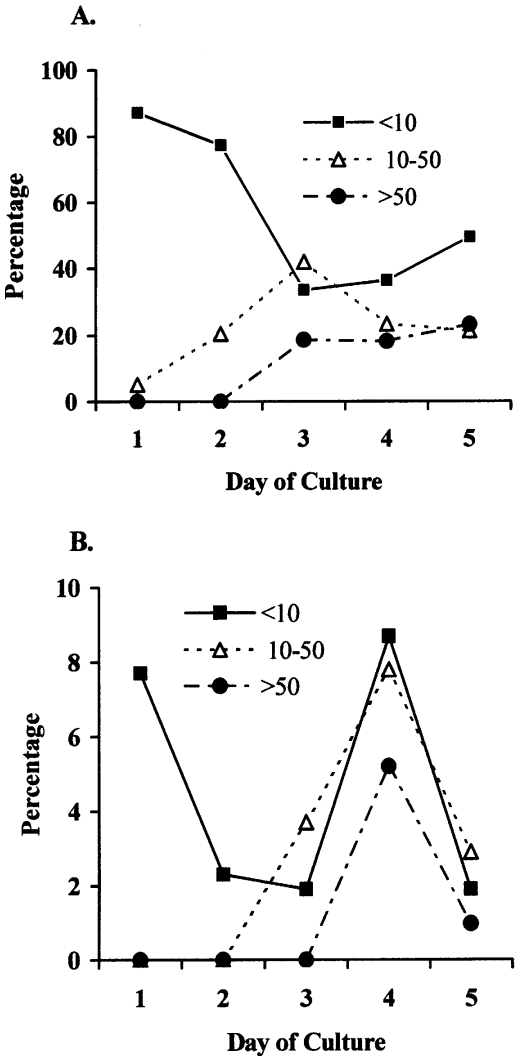

Fig. 3. Degree of infection of live (A) and dead cells (B) indicated by the number of parasites per infected host cell during 5 days of culture. The results are expressed as the percentage of the total number of infected cells counted (for uninfected cells $n > 200$ except day 1 where $n = 74$, and for infected cells $n > 100$ except days 1 and 2 where $n = 39$ and 88, respectively).

slides were examined after staining with acridine orange and ethidium bromide. Acridine orange is permeable to viable and non-viable cells and intercalates into the DNA, giving it a green appearance under fluorescent illumination. It binds to RNA but does not intercalate, resulting in a bright orange appearance. Viable cells, therefore, have a green nucleus and orange cytoplasm. Ethidium bromide, however, is only permeable to non-viable cells and also intercalates into the DNA. This results in a bright orange nucleus. Ethidium bromide staining will overpower staining by acridine orange. Stained viable cells have green nuclei and orange cytoplasm and non-viable cells have orange nuclei (McGahon *et al.* 1995). On each day post-infection, with the exception of day 4, more than 90 % of uninfected and infected cells were living (Fig. 2). Day 4 post-infection was the only day in which there were more

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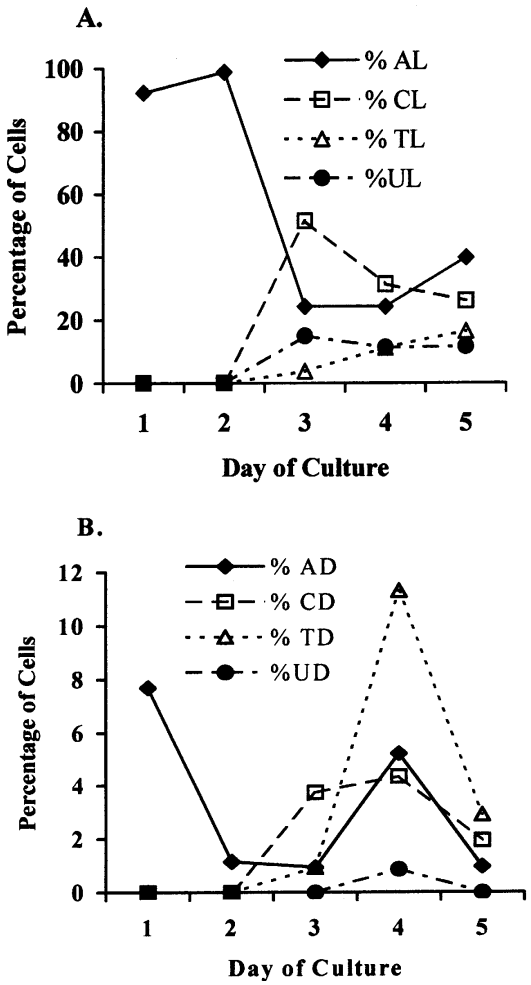

Fig. 4. Stages of parasites in live (A) and dead (B) infected host cells. Abbreviations for parasite stages are as follows: AL, amastigotes only; CL, combinations of amastigotes, transitional shapes, and/or trypomastigotes; TL, trypomastigotes only; UL, parasite stage was undeterminable. The results are expressed as the percentage of the total number of infected cells counted ($n > 100$ except for days 1 and 2 in which $n = 39$ and 88, respectively).

dead cells in infected cultures than in uninfected cultures. However, no morphological evidence of apoptosis, such as cell shrinkage, chromatin condensation, or increased membrane permeability, was observed in infected or uninfected cells.

Characterization of infected cell populations on each day of infection

Infected cells cultured on 1-chambered slides were stained with ethidium bromide and acridine orange and scored for the following criteria: (1) number of intracellular parasites, (2) the stages of the parasites (i.e. amastigotes only, combinations of amastigotes, transitional shapes, and trypomastigotes, trypomastigotes only, or the stage was undeterminable), and (3) an estimate of the percentage of host cell cytoplasm occupied by the parasites. On the first day post-infection approximately 93% of infected cells were alive and contained only amastigotes. As expected, on the first 2 days after infection the majority of infected cells (*ca* 90%) had less than 10 parasites and these were all amastigotes (Figs 3 and 4). In most of the infected cells the parasites occupied 50% or less of the host cell cytoplasm. On day 3 approximately one-third of the infected cells contained more than 50 parasites (Fig. 3) and the parasites occupied equal to or greater than 75% of the host cell cytoplasm. By day 3, differentiation from amastigote to trypomastigote had begun in a majority of infected cells (Fig. 4).

The largest percentage of dead, infected cells (26%) was observed on day 4 post-infection (Fig. 2). The majority of these dead cells contained more than 10 parasites which occupied 10–75% of the cytoplasm (data not shown). The parasites in over 50% of these cells had begun or had completed differentiation from amastigote to trypomastigote forms (Fig. 4). Living, infected cells on day 4 post-infection exhibited a similar pattern, with the exception that a larger portion (19%) of living cells had more than 50 intracellular parasites (Fig. 3).

On day 5 post-infection the percentage of live, infected cells with only amastigotes increased to about 40% (Fig. 4). This was likely due to recently released trypomastigotes infecting previously uninfected cells. Approximately half of the live, infected cells had 10–75% of the cytoplasm occupied by parasites (data not shown). Most of the dead, infected cells observed on day 5 had between 10 and 50% of the cytoplasm occupied by parasites and the parasites had begun or completed amastigote to trypomastigote transformation (Fig. 4). During the 5 days of observation, only a small percentage of cells contained parasites which occupied more than 75% of the host cell cytoplasm.

Cell detachment during infection

Detachment of L cells from the monolayer has been defined as one of the terminating events of apoptosis (Barbiero *et al.* 1995). It was of interest, therefore, to examine the populations of detached cells in the medium of cultures. It was possible that a significant population of dead cells had detached and were in the culture medium. The population of detached cells was not examined when the percentages of dead cells were determined each day post-infection (see above). The population of adherent cells, therefore, may not have accurately represented the status of the total cell population (adherent and detached cells) in the cultures.

The total number of detached cells in infected cultures was consistently greater than in uninfected cultures during the 5 days of culture. In the infected cultures, the number of infected, dead cells that were detached was significantly greater than the number

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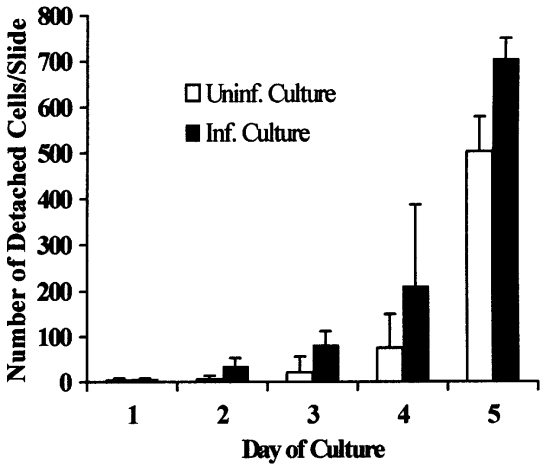

Fig. 5. Total number of detached cells per slide from cultures of infected and uninfected cells on each day post-infection. Error bars represent the standard error of the mean.

of uninfected, dead cells that were detached (Fig. 6B). Approximately the same number of live detached cells was found in infected cultures and uninfected cultures. The majority of non-adherent live cells in the medium of infected cultures were infected (Fig. 6C). There were no differences between the number of live and dead infected cells in the culture medium on any day (Fig. 6B).

In uninfected cultures, cell detachment increased after day 3 (Fig. 6A). There was approximately a 9-fold increase in the number of detached cells in the uninfected cultures between days 4 and 5. There was no significant difference in the number of live and dead cells that were detached in uninfected cultures until after day 4. On day 5, there were more live cells than dead cells which were detached in uninfected cell cultures (Fig. 6A).

No evidence of apoptosis based on DNA gel electrophoresis

One characteristic of apoptosis is the activation of endonucleases that cleave nuclear DNA between nucleosomes. When run on an agarose gel, these oligonucleosomes create the DNA ladder pattern characteristic of apoptotic cells. The results of electrophoresis of DNA from normal L cells, FDTs, and infected L cells at 0, 24, 48, 72, 96 and 120 h post-infection did not reveal the typical ladder formation characteristic of oligonucleosomal fragments (data not shown). DNA smears were observed in DNA from normal L cells, *T. cruzi* DNA, and DNA from infected L cells indicating endonuclease activity resulting in non-specific fragmentation of DNA.

No evidence of apoptosis based on ELISA

Apoptosis in a population of cells can be measured in a lysate sample by using monoclonal antibodies against DNA and histones. These antibodies detect oligo- and mononucleosomes present in the lysate of eukaryotic cells. A commercial ELISA kit (see Materials and Methods section) was used for these

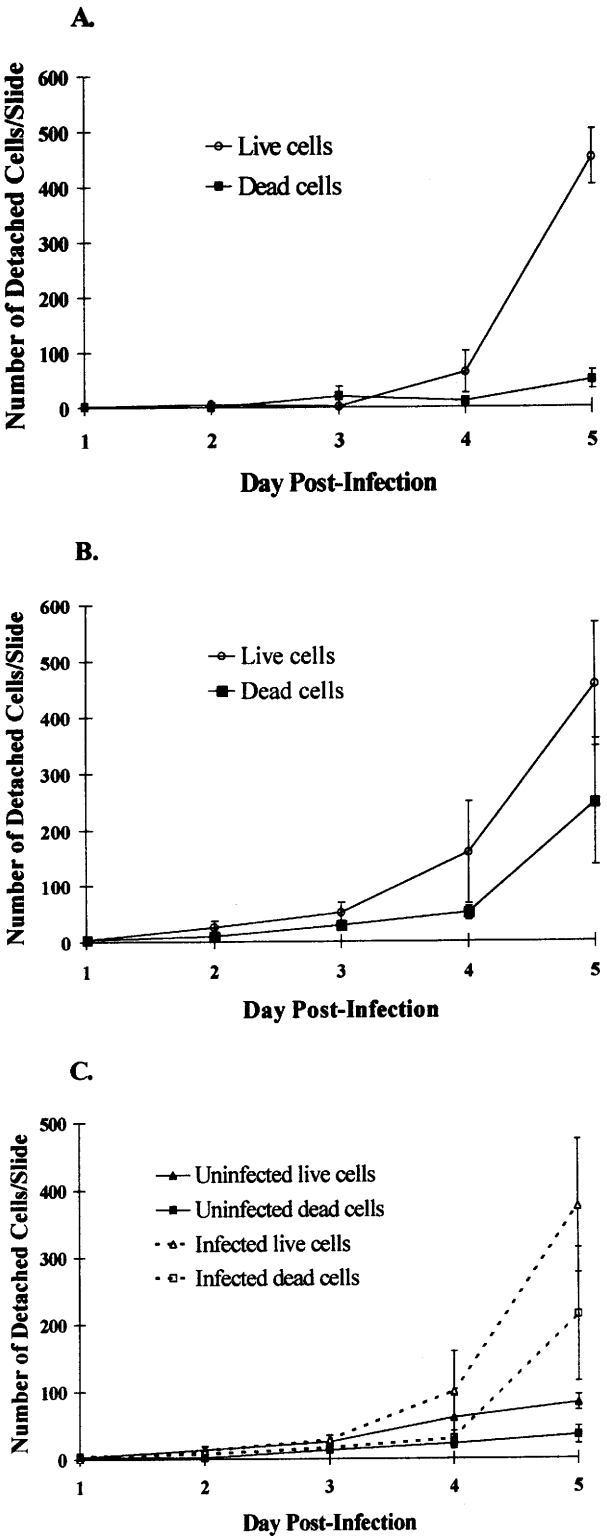

Fig. 6. Live and dead detached cells in the supernatant fluid in cultures of (A) uninfected cells and (B) infected cells. The number of live and dead cells which are detached in infected cell cultures are grouped as infected or uninfected in (C). Error bars represent the standard error of the mean.

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Table 1. Detection of apoptosis by ELISA
(Cell lysates from infected and uninfected cultures were collected and run according to manufacturer's instructions. The results represent the absorbance of samples at 405 nm after 10 min (±standard deviation).)

Day post-infection	Uninfected cells	Infected cells
1	1·52±0·17	1·20±0·24
2	1·00±0·17	0·99±0·13
3	0·94±0·13	0·73±0·20
4	0·73±0·03	1·01±0·70
5	0·31±0·04	1·18±0·42

assays. The results from this experiment were consistent with the results from examining cells for DNA ladders by electrophoresis (see Table 1). There were no significant differences found in the amount of apoptosis between infected and uninfected cells on days 1–4 of infection. On day 5 post-infection, the results indicate a significant decrease of apoptosis in uninfected samples. Results for infected cells on day 5 post-infection are not significantly different from samples on other days.

No detection of Bcl-2 in infected cells by flow cytometry analysis

The experiments described above examined the presence of apoptosis based on internucleosomal DNA cleavage, one of the concluding events in the apoptotic pathway. The results indicated that apoptosis was not occurring, even during the later stages of *T. cruzi* infection in cells and notwithstanding the enormous parasite burden in these cells. It was, therefore, of interest to analyse the possibility that *T. cruzi* may be inhibiting apoptosis in infected cells by stimulating host cell production of Bcl-2. Bcl-2 is a mitochondrial protein that functions to inhibit apoptosis in mammalian cells and, thereby, prolongs cell survival. Using immunostaining and flow cytometric techniques an increased level of Bcl-2 in infected cells was not found. A shift in staining intensity in the positive control, AG10 cells, was observed showing that the assay functioned appropriately. Therefore, apoptosis does not appear to be inhibited in host cells by parasite-induced increases in Bcl-2 levels.

DISCUSSION

It is thought that there are 2 basic mechanisms of cell death, necrosis and apoptosis. Necrosis is considered accidental cell death and is the result of sudden or severe injury (Cohen, 1993; Farber, 1994; Molloy *et al*. 1994; Stewart, 1994; Majno & Joris, 1995). Apoptosis is a suicide-like programme initiated by the cell itself and has several important roles, one of which is to combat intracellular parasites. Examples of intracellular parasites that induce apoptosis of host cells include viruses (Terai *et al*. 1991), bacteria (Kato *et al*. 1995; Molloy *et al*. 1994; Monack *et al*. 1996; Rogers *et al*. 1996) and *Toxoplasma gondii* (Hisaeda *et al*. 1997).

Characteristic events of apoptosis have been observed in *T. cruzi*-infected cells. These include breakdown of mitochondria, nuclear degeneration, chromatin condensation, and changes in the cytoplasm and plasma membrane (Dvorak & Hyde, 1973). In the fibroblast cell line used in this study, the transformation from amastigotes to trypomastigotes occurred after the third day of infection. This transformation was marked by the occurrence of infected host cells with combinations of parasite stages (amastigotes, transitional shapes, and trypomastigote forms) and were easily observed by staining cells with ethidium bromide and acridine orange. This staining method also allowed for the observation of any gross morphological changes in the host cell at the time of parasite differentiation. Changes characteristic of apoptosis, such as condensation of chromatin within the nucleus, cell shrinkage, and loss of integrity of the plasma membrane were not observed in *T. cruzi*-infected cells. The absence of morphological changes indicative of apoptosis at the time of differentiation indicates that products associated with those characteristics do not function to induce parasite differentiation.

Another characteristic of apoptosis that was not detected in *T. cruzi*-infected cells was the presence of nucleosomal fragments. During apoptosis, endonucleases are activated which cleave DNA at internucleosomal regions (Cohen, 1993; Farber, 1994; Majno & Joris, 1995; Molloy *et al*. 1994; Stewart, 1994). The resulting fragments appear as a DNA ladder when examined by electrophoresis in an agarose gel. The DNA extracted from *T. cruzi*-infected cells did not show this DNA ladder. Likewise, an ELISA assay for detecting nucleosomes revealed no significant increase in the amount of nucleosomal fragments in the cytoplasm of infected cells over the 5-day course of infection. The absence of nucleosomal fragments was another indication that *T. cruzi*-infected cells do not undergo apoptosis and, therefore, products of host cell apoptosis do not function to initiate parasite differentiation.

Because detachment from the substratum is considered another characteristic of apoptosis in L cells (Barbiero *et al*. 1995), it was of interest to look for signs of apoptosis in the population of cells which had detached. There were significantly more detached cells in the medium of infected cell cultures than in uninfected cell cultures. However, the integrity of the plasma membrane and nuclear morphology of detached cells did not demonstrate a significant cell population undergoing apoptosis.

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Therefore, the increase in the number of detached cells was not a result of cells undergoing apoptosis.

Although there were no signs characteristic of apoptosis in the *T. cruzi*-infected L cells, there were effects observed that were presumed to be the result of *T. cruzi* infection. The number of uninfected cells increased at a greater rate than infected cells after the third day post-infection. Infected cells also showed decreased adherence to the plastic of the tissue culture flask compared to uninfected cells. It is known that *T. cruzi* disrupts the mitotic apparatus of the host cell (Dvorak & Hyde, 1973) which could contribute to a decreased rate of division and, therefore, a lower number of infected cells compared to uninfected cells after 3 or more days in culture. It may also be that the progressive cytoskeletal damage caused by *T. cruzi* invasion and replication (Low, Paulin & Keith, 1992) restricts the host cell's ability to divide as well as adhere to the substratum.

Another possible effect of *T. cruzi* infection on the host cell may be the inhibition of apoptosis. This hypothesis was generated by the observation that on the third day post-infection approximately 35% of infected cells had over 50% of the cytoplasm occupied by parasites, yet these heavily infected cells were alive. It has been reported that *Leishmania donovani*, an intracellular protozoan parasite closely related to *T. cruzi*, inhibits apoptosis of its host cell (Moore & Matlashewski, 1994). A mammalian protein, Bcl-2, functions as an inhibitor of apoptosis in many cell types. When overexpressed, Bcl-2 prolongs the life of a cell that would normally undergo apoptosis (Cohen, 1993; Zamzami *et al.* 1996). However, it could not be demonstrated by flow cytometry that Bcl-2 was overexpressed in *T. cruzi*-infected L cells. Therefore, if the parasites do inhibit apoptosis of host cells, it is apparently not as a result of an increase in the intracellular level of Bcl-2.

It is not known how intracellular or extracellular *T. cruzi* differentiation is controlled. It is possible that host cell molecules function to signal the induction of intracellular parasite differentiation from amastigotes to trypomastigotes. However, the data presented herein suggest that it is unlikely that host cell products generated as a result of apoptosis act as signals to induce parasite differentiation. The results from the present study suggest that under the experimental conditions used, the host cell remains alive throughout the course of *T. cruzi* infection and, therefore, does not undergo apoptosis.

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