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Original Research

Induction of cellular proliferation in a human astrocytoma cell line by a *Trypanosoma cruzi*-derived antigen: a mechanism of pathogenesis?

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Abstract: *Trypanosoma cruzi* can compromise the human central nervous system (CNS) during acute infection or reactivation in immune-suppressed hosts. Astrocytes have been identified as targets of *T. cruzi's* CNS infection in humans. Despite a high degree of parasitism and cellular lysis by *T. cruzi in vitro* the number of astrocytoma cells did not change when compared to uninfected cultures. This work evaluated cellular proliferation, changes in Major Histocompatibility Complex (MHC) expression as a reflection of antigen processing, and cytokine (IL-6 & IL-8) secretion in a human astrocytoma cell line exposed to a trypomastigote-derived antigen. Light microscopy was used to evaluate the number of cells; MHC molecule expression, cell cycle and cytokine secretion were assessed by flow cytometry. The number of astrocytoma cells increased proportional to the amount of antigen used and the percentage of cells in G2/M phase was higher when compared to control cultures. Antigen exposure increased expression of MHC class II, but not MHC class I in comparison to cultures incubated without antigen. Astrocytoma cell secretion of IL-6 and IL-8 was unaffected by antigen exposure. These results suggest the participation of a trypomastigote-derived mediator that induces astrocytoma cell proliferation without an inflammatory response; which may contribute to the pathogenesis of neurologic Chagas disease.

Key words: *Trypanosoma cruzi*; Chagas disease; Central nervous system; Astrocytes.

Introduction

Several antigen-dependent mechanisms have been described in protozoan infection of mammalian host cells to foster parasite survival and replication (1, 2). In *Toxoplasma gondii* infection, for example, the parasite-derived proteins ROP16 and ROP18 down-regulate the synthesis of interferon-γ (IFN-γ) in macrophages, allowing for parasite survival and replication (1, 2). Similarly, *Leishmania spp.* parasites produce Gp63, a metalloprotease that cleaves transcription factors, which in turn inhibits intracellular signaling pathways responsible for inflammatory cytokine secretion (3).

Another infection in which the role of parasitic antigen-dependent mechanisms is coming to the forefront is that produced by *Trypanosoma cruzi*. This reduviid-transmited protozoan, which is the etiologic agent of Chagas disease, produces an acute infection with non-specific symptoms and, in approximately one third of affected individuals, a chronic disease manifesting with cardiac conduction alterations or gastrointestinal motility dysfunction (4). Antigen persistence has been associated with immune cellular dysfunction, which can in turn promote the pathogenesis of the chronic disease (5, 6).

T. cruzi can also affect the central nervous system (CNS) during the acute phase of infection or during reactivation in chronic chagasic patients who become immunosuppressed, but not in chronically infected immune-competent patients (7). Acute CNS infection by *T. cruzi* is seen primarily in children from endemic areas and adults over 60 years of age with a mortality ranging from 5 to 10% (8-10). Chagas disease reactivation secondary to HIV/AIDS accounts for 70 to 80% of cases in immunosuppressed individuals, with mortality reaching 85% (8, 11-15).

Histologic specimens from animals and humans with chagasic encephalitis have revealed intracellular amastigotes in astrocytes (10,16). *In vitro* exposure of human astrocytoma cells cultures to trypomastigotes led to a high percentage of infection as well as intracellular parasite replication with release of new infective forms (17). The infection triggers the secretion of pro-inflammatory cytokines such as interleukin 8 (IL-8) as well as the up-regulation Major Histocompatibility Complex (MHC) molecule expression (17). Interestingly enough, despite the high percentage of intracellular infection in this *in vitro* model, the total number of astrocytoma cells did not decrease as a function of time following infection (17). This indicates the possibility that *T. cruzi* induces astrocyte proliferation as a mechanism to secure host cells for replication. Furthermore, murine astrocytes exposed *in vitro* and *in vivo* to IFN-γ showed greater susceptibility to infection by *T. cruzi* (18).

The goal of this study was to assess cellular proliferation, MHC molecule expression and cytokine secretion of a human astrocytoma cell line after exposure to a *T. cruzi*-derived antigen. The characterization of the cellular response of human astrocytoma cells in response to *T. cruzi* will allow for a better understanding of the pathophysiologic process leading to CNS compromise in Chagas Disease.

Materials and Methods

Cell culture maintenance

American Tissue Culture Catalogue (ATCC) cell line CRL-1718 (Manassas, VA, USA), a human adherent cell line of neoplastic CNS origin was used in this study. Cells exhibit astrocyte-like morphology and express astrocyte the cell marker glial fibrillary acidic protein (GFAP). The cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis MO, USA) supplemented with 10% of fetal calf serum (FCS, Eurobio, Les Ulis, France) 2 mM of L-glutamine, 1.5 g/L of sodium bicarbonate, 4.5 g/L of glucose, 10 mM of HEPES, and 1.0 mM of sodium pyruvate (all from Gibco Life Technologies – Thermo Fisher Scientific, Waltham MA USA). The cells were cultured in T25 flasks (Corning Inc, Corning NY, USA) at 37°C in a 5% CO_2 environment. The cell monolayer was transferred by removing culture medium and adding a 0.25% trypsin-EDTA solution (Gibco) for 3 minutes at 37ºC, which was then neutralized by adding RPMI-1640 medium supplemented with 5% FCS. The cells were then transferred to a 15 mL tube (BD Bioscience, Franklin Lakes, NJ, USA) and centrifuged at 1,350 g for 5 min. After this they were used either for sub-culturing or for experiments as described below.

T. cruzi trypomastigote culture

T. cruzi trypomastigotes from the MHOM/CO/01/DA isolate (TcI) were kept in astrocytoma cell cultures or frozen in RPMI (Sigma-Aldrich) containing 10% FCS (Eurobio) and 10% DMSO (MP Biomedicals, Solon, OH, USA). Every third parasite outburst from host cells, trypomastigotes were harvested and transferred to a 15 mL tube, centrifuged at 1,350 g for 5 minutes with phosphate buffer saline solution 0.01 M pH 7.4 (PBS 1X) to wash away culture medium and collected for antigen preparation or infection assays.

T. cruzi antigen preparation and quantification

After spin-off, the parasite pellet was collected and frozen at -80°C. Harvested parasites were subjected to several cycles of thawing and refreezing in the presence of 10 µl/ml of a protease inhibitor cocktail (Sigma-Aldrich). The solution was then sonicated on ice with 20-second cycles of 20 kHz pulses for 5 minutes with a Sonics VCX75 device (Sonics & Materials, Newtown CT, USA). The resulting suspension was then sterilized by filtration with a 0.22 µm membrane (EMD Milipore – Merck, Darmstadt, Germany) and kept frozen at -80ºC. Protein concentration for the antigen was measured by fluorometry with a standard curve (Qubit 2.0 Fluorometer, Life technologies, CA, USA), after which it was stored at -80ºC. Lipopolysaccharide (LPS) content was measured with a limulus amebocyte lysate (LAL) assay (QCL-1000, Lonza Group, Walkersville, MD, USA) and was determined to be 0,69 EU/ml for 10 µg of antigen.

Cell culture infection assays and *T. cruzi*-derived antigen exposure

Astrocytoma cells from culture flasks (T25) were sub-cultured in 24-well ($1x10^5$ cells/well) plates or 48-well microplates ($5x10^4$ cells/well) (Falcon, Life Sciences, Tewksbury, MA). Cells were allowed to adhere for 24 hours before assays were carried out. Once the cells were attached they were exposed to 10 or 100 µg/mL of antigen. As a positive control, cultures were infected with trypomastigotes at a 1:1 cell: parasite ratio. After 72 hours of incubation at 37°C in an environment with 5% CO_2, the culture supernatant was removed and stored at -80ºC for cytokine analysis. Cells were then trypsin-treated, washed and centrifuged for cell counts or flow cytometry analysis. At least three independent experiments were carried out, each with a minimum of triplicates.

Surface molecule expression assessment with flow cytometry

Cells were labeled with 1 µl of anti-MHC class I (HLA-ABC, clone G46-2.6 BD Bioscience) and 2 µl of anti-MHC class II (HLA-DR, clone L243 BD Bioscience) antibodies for 20 minutes at 4°C, after which 2 µl of propidium iodide (PI, BD Bioscience) were added to each tube for 5 additional minutes. After this time, cells were washed, centrifuged, and the resulting cell pellet was reconstituted in PBS 1X. Flow cytometry reading was performed in a FACs Canto II flow cytometer and analysis was conducted with FACs DIVA software (BD Bioscience). The population gating strategy is shown in Supplementary Fig 1. The initial selection was made according to size (FSC) and granularity (SSC) characteristics (Supplementary Fig. 1A). Cell viability was determined according to the PI signal: cells negative for PI were considered viable (Supplementary Fig. 1B). Lastly, expression of MHC class I (HLA-ABC) and class II (HLA-DR) molecules was measured according to the signals from fluorochromes FITC and PE-Cy7-A respectively (Supplementary Fig. 1C). The percentage of expression was used for HLA class II molecules which are induce in this cell line, and the mean fluorescent intensity (MFI) was used for HLA class I molecules which are constitutively expressed.

Astrocytoma cell cycle assessment with flow cytometry

Astrocytoma cells were trypsin-treated and then an RNAse-trypsin inhibitor (CycleTest Plus - BD Bioscience) was added for 10 minutes each at room temperature before adding PI for 10 minutes at 4ºC. The resulting PI-stained nuclei were read in a FACs Canto II flow cytometer (BD Bioscience). FlowJo software (Tree Star Inc, Ashland, OR, USA) or FACS DIVA 6.1 (BD Bioscience) were used to determine each of the cell cycle phases (G0/G1, S and G2/M2) by constructing a PI histogram (See panels in figure 2A). Gating for each phase in the cell cycle was determined as described by Riggs *et al* (19).

Cytokine secretion assessment

Supernatants from all cultures were collected after 72 hours of incubation with the antigen. Interleukin-6 (IL-6) and IL-8 concentrations in the supernatants were measured using a BD cytometric bead array human chemokine kit (BD Biosciences). Chemokine concentrations were estimated using BD FCAP software v.1.0.1 (BD Biosciences). IL-6 and IL-8 assay ranges for the kits used were both 10-2,500 pg/ml.

Statistical analysis

The Kolgomorov-Smirnov test was used to determine the data's distribution. If it had a normal distribution, parametric tests (Student's *t* and ANOVA) were used; otherwise, non-parametric tests (Mann-Whitney U and Kruskal-Wallis) were employed. The median, interquartile range and percentages were calculated for collected data. SPSS v.20 was used to perform the calculations and $p < 0.05$ was considered statistically significant (IBM, Somers, NY, USA).

Results

Increased astrocytoma cell proliferation in the presence of antigen was detectable in cell counts and cell cycle analysis

Cell morphology in antigen-exposed astrocytoma cells did not change with respect to control groups (data not shown). The number of cells increased proportional to the amount of antigen added to the cultures. For all conditions, starting number of cells was $1x10^5$ cells in 1 ml of culture media. The median cell count, for control cells was 500,000 cells/ml (IQR 187,000), whereas for cells exposed to 10 µg of antigen it increased to 718,750 cells/ml (IQR 203,250) and for cells exposed to 100 µg it 1,025,000 cells/ml (IQR 468,750). The difference between the number of cells in control wells and in wells exposed to both 10 µg (p=0.002) and 100 µg (p= 0.0002) of antigen was significant, Fig. 1. The median percentage of cells in G2/M phase of the cell cycle was greater in cultures exposed to the antigen (7.99% and 10.15% with 10 and 100 µg/ml, respectively) than in cell cultures alone (6.5%, p=0.012 for 10 ug/ml p=0.018 for 100 ug/ml), Fig. 2.

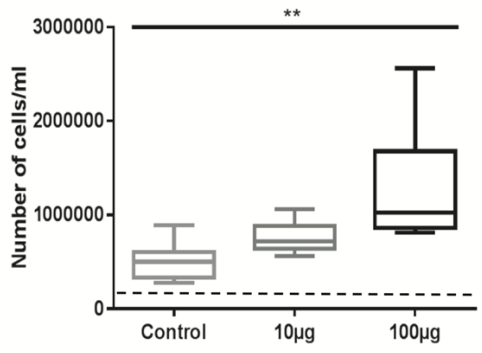

Figure 1. Manual cell count at day 3 post-exposure to antigen *p<0.05 by Mann-Whitney U test. Boxes represent the median and whiskers represent the IQR of three independent experiments with a minimum of three replicas. The observer responsible for counting was blind to each of the conditions read. Dotted line indicates the initial number of cells in the cultures.

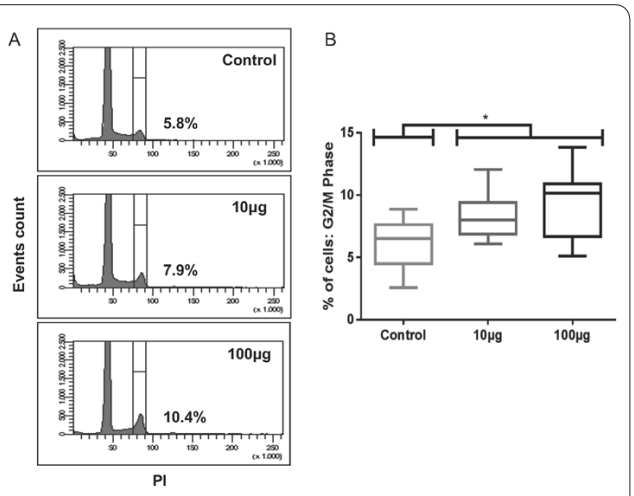

Figure 2. Propidium iodide (PI) histogram constructed from flow cytometry analysis (FACS DIVA) of control cells (upper), cells exposed to 10ug (middle) and 100ug of antigen (lower). Percentage of cells in G2/M phase of the cell cycle (B) *p<0.05 by Mann-Whitney U test. Boxes represent the median and whiskers represent the IQR of three independent experiments with a minimum of three replicas.

Antigen exposure induced selective up-regulation of class II MHC molecule expression

The expression of MHC molecules was evaluated as an indirect indicator of antigen-processing pathway activation in the astrocytoma cells. Since human astrocytes constitutively express MHC class I molecules and class II molecule expression is inducible, MFI was chosen as the outcome of interest for the former and percentage of expression for the latter. Surface expression of LPS receptor (CD14) in astrocytoma cells was also assessed by flow cytometry and was determined to be negative (data not shown), thus ruling out confounding by residual LPS in the *in vitro* system.

There were no changes in the amount of MHC class I molecules, which are constitutively expressed, with the exposure of astrocytoma cells to the *T. cruzi* antigen (Fig 3A). The median of mean fluorescent intensity (MFI) for unexposed cells was 1,744 arbitrary units (au; IQR 369) while cells exposed to 10µg and 100µg had 1,667 (IQR 346) and 1,672 (IQR 386) (p=0,96). MHC Class I molecule MFI of infected cells (10,360 IQR: 3,511) and cells exposed to IFN-γ (2,589 IQR: 132), both used as positive controls, increased with respect to control cells and antigen-exposed cells (Fig 3A) (p=$1.26x10^{-5}$).

In contrast, the median percentage of MHC class II expression (HLA-DR), which are not constitutively expressed, increased in cells exposed to the antigen (Fig 3B). Control cells had 1.9% (IQR 0.83%) of HLA-DR expression while cells exposed to 10µg had 7.2% (IQR 4,6%), and those with 100µg had 6.4% of expression (IQR 4.2%, Fig 3B) (p=0.02). Expression in *T. cruzi*-infected cells, in turn, was 11% (IQR 4%) and cells exposed to IFN-γ had the greatest expression at 66.9% (IQR 3%) (p=0.009; Fig 3B).

Cytokine secretion was unchanged by exposure of the trypomastigote-derived antigen.

IL-8 and IL-6 were detected in supernatants from all

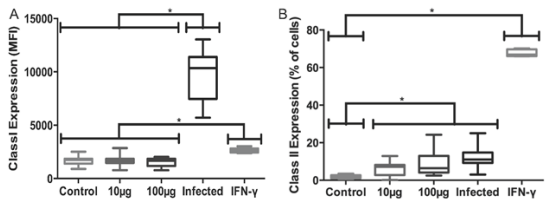

Figure 3. (A) Mean fluorescent intensity (MFI) of HLA class I molecule expression. *p<0.001 by Kruskal Wallis and Mann-Whitney U test. Boxes represent the median and whiskers represent the IQR of three independent experiments with a minimum of three replicas. (B) Percentage of HLA class II molecule expression. *p<0.05 by Kruskal Wallis and Mann-Whitney U tests. Boxes represent the median and whiskers represent the IQR of three independent experiments with a minimum of three replicas.

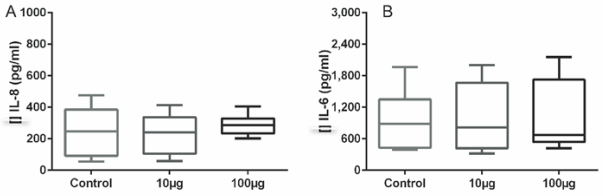

Figure 4. IL-8 (A) and IL-6 (B) secretion in culture supernatants after incubation with trypomastigote-derived antigen for 72 hours. Boxes represent the median and whiskers represent the IQR of three independent experiments with a minimum of three replicas. There were no differences in cytokine concentrations measured across the different groups.

culture wells. IL-6 is constitutively produced by astrocytes, and IL-8 is known to increase during *T. cruzi* infection *in vitro*. IL-8 concentration ranged from 100 to 400 pg/ml, and as expected in astrocytes, IL-6 secretion was higher than that of IL-8, ranging from 500 to 1,800 pg/ml. No differences were observed in IL-8 or IL-6 secretion between cells exposed to the antigen and control cells (p=0.66).

Discussion

Chagas disease experimental models in rodents have demonstrated infection of astrocytes (20, 21). They can host *T. cruzi* trypomastigotes of different parasite genotypes (DTUs) including TcI, TcII and TcVI during both acute and chronic infections (18, 22-24).

Previous work by our group demonstrated the capacity of *T. cruzi* to infect and replicate in human astrocytoma cells *in vitro*. However, despite reaching 90% parasitism after 96 hours, the intracellular infection does not reduce the number of viable cells when compared to uninfected cultures (17). This data prompted the question of whether a parasite-derived antigen has the capacity to induce cellular proliferation in astrocytoma cells. Here, an increase in astrocytoma cell counts and a greater percentage of cells in G2/M phase following exposure to a trypomastigote-derived antigen are documented.

It is possible that *T. cruzi* stimulates proliferation not only to provide more host cells for further infection, but also to create a glial barrier in order to hinder the migration of immune cells to the affected areas (2, 3). This possibility is supported by the histopathologic findings of gliosis with formation of glial nodules as well as areas of astrocytic proliferation encasing the necrotic foci in equines and rats infected by either *Trypanosoma evansi* or *T. cruzi* (22, 25).

Our results also suggest that the trypomastigote-derived antigen increases expression of MHC class II molecules while having no effect on class I expression. This contrasts with findings seen during astrocytoma cell infection by trypomastigotes, where expression of both HLA class I and class II molecules increases simultaneously (17). When comparing the effect of both positive controls used on MHC molecule expression; class I signal intensity increases much more in cells infected by *T. cruzi* than in those exposed to IFN-γ, whereas class II expression increases more in cells exposed to IFN-γ than in infected cells. This selective up-regulation of MHC molecules is consistent with the activation of differential antigen processing and presentation pathways (intracellular *versus* extracellular) (18, 26). Through MHC class II molecule expression, astrocytes can act as antigen presenting cells potentially activating $CD4^+$ lymphocytes, which are known to have a central role in autoimmune disease such as multiple sclerosis and its animal models (26-28). Furthermore enteroglial cells, the gastrointestinal counterpart of CNS astrocytes, are known to express MHC class II as well as co-stimulatory molecules in tissue biopsies from patients with chagasic megacolon (29).

Notably, the cellular changes described here were not accompanied by an increase in the secretion of IL-6 or IL-8. IL-6 is a relevant cytokine in the CNS, normally present in steady-state where it exerts a protective role in neuronal homeostasis; and up-regulated during neuroinflammation, parasitic infections (cerebral malaria), and HIV-associated neurocognitive disorders (HAND) (30, 31). Vargas-Zambrano, et al. (17) described an increase in the secretion IL-8 during astrocytoma cell infection by *T. cruzi in vitro*. This cytokine is chemotactic for neutrophils and has been implicated in the inflammation and neurodegenerative processes (31). The fact that astrocytes proliferate at a greater rate without releasing inflammatory cytokines in the presence of the antigen further supports the notion that these changes foster further infection.

Exposure of an astrocytoma cell line to a *T. cruzi*-derived antigen leads to increased cellular proliferation and differential changes in MHC molecule expression without an increase in the secretion of inflammatory cytokines. This suggests the exploitation of antigen-dependent mechanisms that could favor parasite survival by erecting a glial barrier to hinder the local immune response. Future work should be directed at characterization of this antigen and the molecular pathways it activates.

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