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Modulation of glutathione intracellular levels alters the spontaneous proliferation of lymphocyte from HTLV-1 infected patients

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ABSTRACT

The human T-cell lymphotropic virus type 1 (HTLV-1) is a retrovirus associated with neoplasias and inflammatory diseases, such as adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1-infected individuals present a spontaneous T lymphocyte proliferation. This phenomenon is related to the HTLV-1-proviral load and the persistence of the infection. Viral proteins induce many cellular mediators, which can be associated with the abnormal cellular proliferation. The intracellular levels of glutathione (GSH) are important to modulate the cellular proliferation. The aim of this study was to investigate the correlation between the modulation of intracellular GSH levels and the spontaneous lymphocyte proliferation during the HTLV-1 infection. Intracellular GSH level can be modulated by using DL-buthionine-[S,R]-sulfoximine (BSO, GSH synthesis inhibitor) and N-acetylcysteine (NAC, peptide precursor). Our results demonstrated that BSO was capable of inducing a decrease in the spontaneous proliferation of PBMC derived from HTLV-1 carriers. On the other hand, the GSH precursor induces an increase in mitogen-stimulated cellular proliferation in infected and uninfected individuals. Similar results were observed by the inhibition of ABCC1/MRP1 protein, augmenting the mitogen-induced proliferation. This effect can be related with an increase in the GSH levels since ABCC1/MRP1 transports GSH to the extracellular medium. There was a significant difference on the expression of CD69 and CD25 molecules during the lymphocyte activation. We did not observe any alterations on CD25 expression induced by BSO or NAC. However, our results demonstrated that NAC treatment induced an increase in CD69 expression on unstimulated $CD8^+$ T lymphocytes obtained from HTLV-1 infected individuals, healthy donors and HTLV carriers. Therefore, our results suggest that the cellular proliferation promoted by the infection with HTLV-1 and the activation phenotype of $CD8^+$ T lymphocytes can be regulated by changing the intracellular GSH levels; suggesting the modulation of these intracellular levels as a new approach for the treatment of pathologies associated with the HTLV-1 infection.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiological agent of the adult T-cell leukemia/lymphoma (ATLL) malignancy (Uchiyama et al., 1977) and the HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985), a chronic progressive disabling disease characterized by demyelination, axonal loss, neuronal degeneration and gliosis. The main site of neurodegeneration is the thoracic spinal cord; this leads to a slowly progressive spastic paraparesis with low back pain, and bowel, urinary and sexual dysfunction (Leite et al., 2004; Araujo and Silva, 2006). High prevalence areas include Latin America,

Abbreviations: HTLV-1, human T-cell lymphotropic virus type 1; HAM-TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; ATLL, adult T-cell leukemia/lymphoma; GSH, glutathione; ABCC1/MRP1, multidrug resistance protein; ROS, reactive oxygen species; BSO, DL-Buthionine-[S,R]-sulfoximine; NAC, N-acetylcysteine; PBMC, peripheral blood mononuclear cells; INDO, Indomethacin; CTL, cytotoxic T lymphocyte.

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Southern Japan, Central and Western Africa (de Thé and Bomford, 1993; Gonçalves et al., 2010). In Brazil, the prevalence of HTLV-1 infection among blood donors is approximately 0.45% (Araújo and Andrada-Serpa, 1996; Proietti et al., 2005), which represents about 2 million infected people.

$CD4^+$ T lymphocytes are the main targets of HTLV-1 infection, but it has been shown that $CD8^+$ T cells are also infected (Catovsky et al., 1982; Richardson et al., 1990). The HTLV-1 infection induces the activation of $CD4^+$ T lymphocytes, leading to spontaneous proliferation (Prince et al., 1994). Sibon et al. (2006) showed that both $CD4^+$ and $CD8^+$ infected clones display a significantly higher level of spontaneous proliferation than their uninfected lymphocytes. This phenomenon is correlated with Tax mRNA levels. HTLV-1 soluble Tax protein increases the proliferation of uninfected human peripheral blood lymphocytes stimulated by mitogen (Marriott et al., 1991). Moreover, the HTLV-1 infection promotes the expression of various cytokines and their receptors (Nishiura et al., 1996; Higuchi et al., 1997), and it induces abnormal expression of genes involved in apoptosis, cell cycle and transcription factors (Sun and Yamaoka, 2005).

Cell cycle progression is regulated by a large number of intracellular signals, including a slight increase of reactive oxygen species (ROS) (Trachootham et al., 2009). HTLV-1 proteins have been related with ROS production. In 2010, Kinjo et al. demonstrated that Tax induces ROS generation, causing DNA damage and cellular senescence. HTLV-1 protein p13 is involved in mitochondrial ROS production (Silic-Benussi et al., 2009). The authors suggested this effect was associated with the persistence of the virus in normal infected T lymphocytes, promoting an expansion of the infected cells (Silic-Benussi et al., 2010).

Glutathione (GSH) has an important role in cellular physiology and metabolism, including antioxidant activity and induction of cell cycle progression in lymphocyte proliferation (Messina and Lawrence, 1989; Hamilos et al., 1989; Suthanthiran et al., 1990; Kubbies et al., 1991; Martin et al., 2000). GSH is a tripeptide synthesized in all mammalian cells from the amino acid precursors L-glutamate, L-cysteine and glycine, through the reactions catalyzed by γ-glutamylcysteine and GSH synthetase (Wang and Ballatori, 1998). Physiologically, 98% of intracellular glutathione is found in reduced form, and only 2% is detected under oxidized form (GSSH) or joined with other molecules. HTLV-1 Tax mediates the prooxidant state and is involved in the decrease of intracellular GSH levels (Los et al., 1998).

Moreover, GSH is related with the transport of endogenous and exogenous molecules to extracellular medium. Glutathione is a physiological substrate of ABCC1/MRP1, and Ishikawa et al. (1994) observed an increase of the intracellular GSH level in drug-resistant tumor cells. Multidrug resistance related protein 1 (ABCC1/MRP1) transports several compounds in a GSH-dependent manner; its activity could be stimulated by the GSH intracellular levels (Cole and Deeley, 2006). The members of the ABCC/MRP family are ATP-dependent efflux pumps, belonging to the ABC family of transport proteins, and they are also involved in resistance against anticancer drugs (Cole et al., 1992). ABCC1/MRP1 is expressed in tumor cells (Cole et al., 1992) and normal tissues, such as brain (Regina et al., 1998), liver (Keppler et al., 1996) and mature lymphocytes (Legrand et al., 1996).

Our group demonstrated that $CD4^+$ and $CD8^+$ T lymphocytes from HAM/TSP asymptomatic and symptomatic individuals presented a reduced ABCC1/MRP1 expression and activity when compared to uninfected ones (Echevarria-Lima et al., 2007). However, a lower ABCC1/MRP1 expression was detected in $CD4^+$ T lymphocytes from symptomatic patients. This result was directly correlated to the proviral load; a lower expression of ABCC1/MRP1 was observed in patients with higher proviral load (Echevarria-Lima et al., 2007). Thus far, the relationship between HTLV-1 infection and GSH during HAM/TSP disease is poorly understood. The important role of GSH on lymphocyte proliferation led us to investigate the effects of its modulation on proliferation, activation, and ABCC1/MRP1 expression and activity of peripheral blood mononuclear cells obtained from HTLV-1 infected individuals.

Table 1
HTLV-1 infected individuals.

Subject	Gender	Age (years)	Clinical status	Proviral load
1	F	62	OLIGO	11.43
2	F	48	AS	ND
3	F	19	Inf. Dermatitis	6.89
4	F	70	OLIGO	1.83
5	F	42	AS	4.91
6	F	61	AS	0.62
7	F	60	HAM/TSP	4.32
8	M	60	OLIGO	6.58
9	F	65	AS	5.42
10	M	51	HAM/TSP	9.49
11	F	57	HAM/TSP	9.43
12	M	76	OLIGO	2.93
13	F	53	AS	1.33
14	M	52	AS	0.69
15	M	66	AS	6.81
16	F	64	AS	1.81
17	M	52	OLIGO	10.38
18	F	58	HAM/TSP	6.60
19	M	59	OLIGO	ND
20	F	53	AS	ND
21	F	52	HAM/TSP	10.38
22	M	53	HAM/TSP	14.78
23	M	40	HAM/TSP	11.79
24	F	58	HAM/TSP	21.62
25	F	69	HAM/TSP	22.76
26	M	50	HAM/TSP	0.76
27	F	46	HAM/TSP	4.53
28	F	65	HAM/TSP	4.57
29	F	45	HAM/TSP	8.50
30	F	61	HAM/TSP	ND
31	F	62	AS	4.62
32	F	43	HAM/TSP	3.84
33	M	50	HAM/TSP	12.47
34	F	60	ATLL	16.39
35	M	45	HAM/TSP	7.63

AS: asymptomatic; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; ATLL: adult T-cell leukemia/lymphoma; OLIGO: oligosymptomatic; ND: undeterminated. Proviral load % of peripheral blood cells.

Materials and methods

Subjects

The Ethics Committee of the Instituto de Pesquisa Clínica Evandro Chagas (IPEC) approved the study protocol, and informed consent was obtained from HTLV-1 infected individuals (Table 1) attending the HTLV-1 outpatient clinic at IPEC, Rio de Janeiro, Brazil. This study involved HAM/TSP, asymptomatic carriers and uninfected individuals. The diagnosis of HAM/TSP was made according to the World Health Organization criteria. Uninfected controls were matched according to age and gender.

Cellular proliferation

A 20 ml peripheral blood sample was obtained from volunteers using sodium heparin (Roche, Brazil). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque density gradient centrifugation (GE, USA), and then washed three times with saline solution. The cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Rockville, MD), 50 μM β-mercaptoethanol, 60 mg/ml penicillin and 100 mg/ml streptomycin (Sigma), pH 7.4.

PBMC were seeded at a concentration of 1.25×10^6 cells/ml with or without 5 μg/ml PHA (Sigma) in the presence or absence of BSO (0.1–2 mM; Sigma), NAC (0.1–5 mM; Sigma), or MK571 (25 μM; Merck) for 72 h in 96-well plates, at 37 °C in a 5% CO_2 atmosphere. Thymidine incorporation was measured after a 6 h pulse [^{3}H] thymidine (1 μCi/well, Amersham Life Science), and harvested later. [^{3}H] Thymidine incorporation was evaluated by a TriCarb 1600CA liquid scintillation counter (Packard, Inc.).

Viability assay

PBMC (1.25×10^6 cells/ml) were seeded at a 96-well cell culture plate and incubated under the same conditions described above. Cell viability was assessed with an additional 20 μl/well of MTT (5 mg/ml; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) as described earlier (Mosmann, 1983). The plates were read on microplate reader (Sunrise-Basic TECAN) at a 490 nm wavelength.

ABCC1/MRP1 expression and activity

1.25×10^6 cells/ml were seeded at a 24-well cell culture plate. Cells were incubated in the presence or absence of BSO 1 mM, NAC 5 mM with or without PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere. 72 h later, cells were permeabilized using a FACS™ Lysing Solution (Becton & Dickinson – BD, San Jose, CA) following the manufacturer's instructions. After 10 min at room temperature (RT), cells were washed with phosphate-buffered saline (PBS) and labeled with mouse anti-human ABCC1/MRP1 antibody (1:80, v/v; Monosan) for 30 min at RT. Cells were washed again and incubated with rat anti-mouse-IgG secondary antibody (1:200, v/v; BD) for 20 min at RT. Cells were washed once more, and the fluorescence was examined by FACScan flow cytometry (BD). 10,000 events were acquired for the experimental data, and a gate based on forward (FSC) and side (SSC) scatter parameters was made to analyze only viable lymphocytes. The analysis was accomplished using WinMDI 2.8.

ABCC1 related transport activity was investigated using Fluo 3-AM fluorescent probe in an efflux assay (Sommer et al., 1994). 1.25×10^6 cells/ml were seeded at a 96-well cell culture plate and incubated in the presence or absence of BSO 1 mM, NAC 5 mM with or without PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere. After 72 h, cells were harvested, loaded with 5 μg/ml Fluo3-AM (Molecular Probes, Eugene, OR) in the presence or absence of ABCC1 inhibitors, Indomethacin (INDO) from Sigma or MK571, and incubated for 30 min at 37 °C. Then, cells were washed and re-incubated in medium with or without inhibitors under the same conditions. Finally, cells were washed with PBS, and the retained fluorescence was examined by flow cytometry.

CD69 and CD25 expression

1.25×10^6 cells/ml were seeded in 96-well plates with or without 5 μg/ml PHA (Sigma) in the presence or absence of BSO (1 mM) or NAC (5 mM) for 24 h for the detection of CD69, or 72 h for the analysis of CD25, at 37 °C in a 5% CO_2 atmosphere. After that, cells were harvested and washed with PBS + FBS 5%. They were labeled with the monoclonal antibodies (Becton & Dickinson – BD), anti-human-CD69 PE (1:100; v/v) or anti-human CD25 PE (1:100; v/v), with anti-human-CD4 FITC (1:100, v/v) and anti-human-CD8 PE-Cy5 (1:100; v/v) for 20 min at 4 °C. Then, cells were washed with PBS, and the fluorescence was examined by flow cytometry. 10,000 cells were acquired based on forward and side scatter, and data analyses were performed using Summit 4.3 software.

Detection of intracellular glutathione (GSH)

The intracellular glutathione detection was performed as described earlier by Berendji et al. (1999). PBMC (10^7 cells) were isolated from HTLV-1 infected and uninfected individuals and centrifuged at $200 \times g$ for 7 min. The pellet was centrifuged in 1 ml of PBS at $5000 \times g$ for 5 min at 4 °C. Then, it was resuspended in 200 μl of ultrapure water and submitted to heat shock. After that, $HClO_4$ (2 M) plus EDTA (1 M) was added to sample, and then centrifuged at the same conditions. The supernatant was collected and neutralized with KOH (2 M) plus MOPS (0.3 M) at pH = 7. The samples were centrifuged at the same conditions, and 100 μl of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma) was added in 100 μl of supernatant. The optical density was determined at 414 nm wavelength using a microplate reader (Sunrise-Basic, TECAN). The GSH quantification was performed using a GSH standard curve (0–1000 μM).

Proviral load

A real-time PCR (SmartCycle, Cepheid) assay was performed using Puregene DNA Isolation Kit (Gentra, Minneapolis, MN) and TaqMan system (Applied System, Boston, MA). Standard curves were generated by the amplification of a β-globin gene fragment; and for the HTLV-1 provirus, a pX region (Tax gene) fragment from a cell line containing a single copy of it (TARL-2) (Tateno et al., 1984). The primer set for HTLV-1 pX region was 5′-CGGATACCCAGTCTACGTGT-3′ and 5′-GAGCCGATAACGCG-TCCATCG-3′, and for β-globin, it was 5′-GCAAGAAAGTGCTCGG-TGC-3′ and 5′-TCACTCAGTGTGGCAAAGGTG-3′. TaqMan fluorescent probe for HTLV-1 pX region was 5′-FAMACGCCCTACTGG-CCACCTGTC-TAMRA-3′, and for β-globin, 5′-FAM–TAGTGATGG-CCTGGCTCACCTGGAC–TAMRA-3′. PCR was performed using 200 ng of DNA from peripheral blood cells with 12.5 μl universal master mix (Applied Biosystems), 15 pmol for pX primers, and 30 pmol for the β-globin, besides 5 pmol and 2.5 pmol of the fluorescent probes, respectively. The HTLV-1 proviral load was calculated as: copy per 100 peripheral blood cells = {Tax copies (β-globin copies/2)} × 100. The lowest limit of detection was 1 copy per 10^4 cells.

Statistical analysis

Statistical analysis was performed by Mann–Whitney *U* test. Values of $P \leq 0.05$ were considered statistically significant, using Prism 5 software.

Results

Analysis of intracellular glutathione in PBMC

We evaluated the total intracellular GSH levels (reduced and oxidized) in PBMC obtained from HTLV-1 infected and uninfected donors. This analysis demonstrated similar levels of intracellular glutathione obtained from PBMC from HTLV-1-infected individuals and from healthy donors (Fig. 1).

BSO inhibits HTLV-1 spontaneous and stimulated cellular proliferation

Intracellular GSH levels can be pharmacologically modulated, reduced using an γ-glutamylcysteine synthetase inhibitor, L-buthionine-(R,S)-sulfoximine (BSO), or increased by the GSH precursor N-acetyl-l-cysteine (NAC) (Chiba et al., 1996). We analyzed whether the modulation of GSH levels alters the mitogen-stimulated (PHA) cellular proliferation of PBMC from uninfected individuals in the presence and absence of BSO or NAC. After

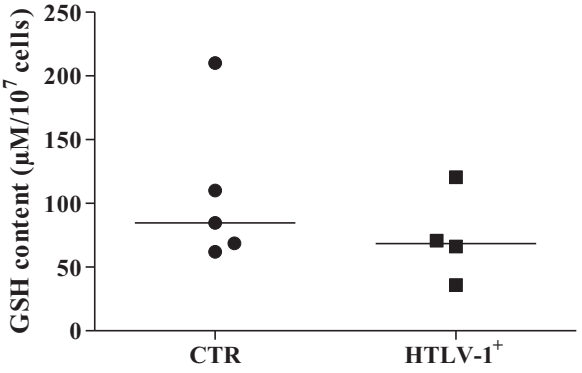

Fig. 1. Comparison of GSH levels in PBMC from HTLV-1 infected and uninfected donors. 10^7 PBMC obtained from uninfected and HTLV-1 infected individuals were centrifuged at 200 × *g* for 7 min, and 5000 × *g* for 5 min at 4 °C. The pellet was resuspended in ultrapure water and submitted to heat shock. After that, $HClO_4$ + EDTA was added and centrifuged at the same conditions. The supernatant was neutralized with KOH + MOPS, and the samples were centrifuged at the same conditions. Then, DTNB was added, and the optical density was determined at a 414 nm wavelength. The GSH quantification was performed using a GSH standard curve (0–1000 μM). Each point represents the result for one donor. The traces in these graphs represent the median values.

72 h, we observed that 1 mM and 2 mM BSO caused an important decrease in the proliferation of activated cells (Fig. 2B). On the other hand, NAC induced a significant increase in the proliferation of the cells activated with PHA (Fig. 2D). Thus, these results confirmed the literature data regarding the modification of the PBMC proliferation due to the modulation of intracellular GSH levels.

The results led us to analyze the effects of modulation of intracellular GSH levels on spontaneous and mitogen-stimulated proliferation of PBMC obtained from HTLV-1-infected individuals. The decrease of intracellular GSH levels induced by BSO promoted a significant reduction of spontaneous proliferation in cells from HTLV-1-infected patients (Fig. 3A). Moreover, BSO reduced the proliferation stimulated by PHA, inducing a similar effect to that observed in PBMC obtained from uninfected donors (Fig. 3B). However, the incubation of PBMC in the presence of NAC (5 mM) did not alter the spontaneous proliferation induced by the HTLV-1 infection (Fig. 3A); it induced an increase in the mitogen-stimulated proliferation in PBMC obtained from HTLV-1-infected individuals (Fig. 3B).

BSO did not modify cellular viability

To exclude the hypothesis that alterations observed to the cellular proliferation induced by the modulation of GSH levels were a consequence of a cell death promoted by BSO toxicity, we analyzed the effects of these substances on the PBMC viability. PBMC incubation with BSO in the presence or absence of PHA did not alter the cellular viability evaluated by MTT assay, except for the highest concentrations of BSO (5 and 10 mM) in PHA activated cells (Fig. 4). A similar result was observed when we analyzed the cellular viability by annexin-V (data not shown), suggesting that the effects

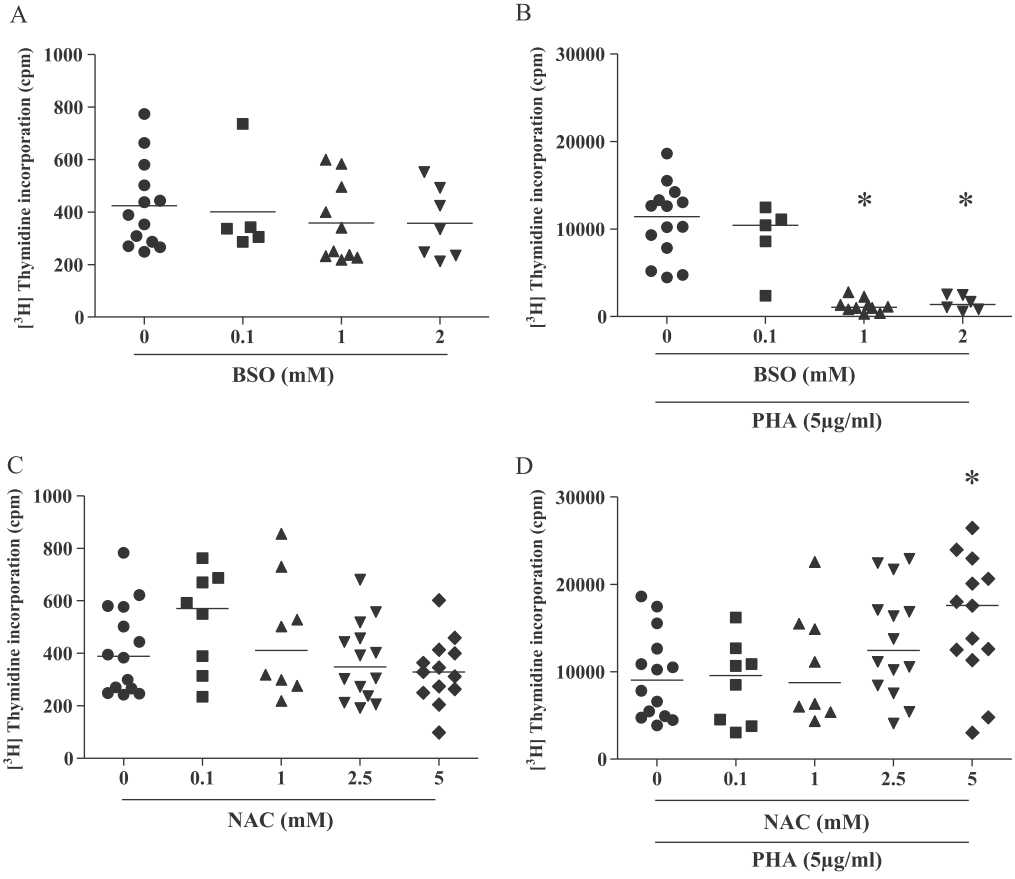

Fig. 2. Effects of intracellular GSH modulation in cellular proliferation. 1.25 × 10^6 cells/ml were seeded at a culture plate. Cells were incubated with or without PHA (5 μg/ml) in the presence or absence of BSO (0.1–2 mM) (A and B) or NAC (0.1–5 mM) (C and D) at 37 °C in a 5% CO_2 atmosphere. After 72 h, these cells were incubated with ^{3}H-thymidine for 6 h at the same conditions. Then, cells were collected and washed, and the radioactivity was measured in a liquid scintillation analyzer. The graphs above represent the [^{3}H] thymidine incorporation. *Significantly different from the control ($P < 0.05$). Each point represents the result for one uninfected donor. The traces in these graphs represent the median values.

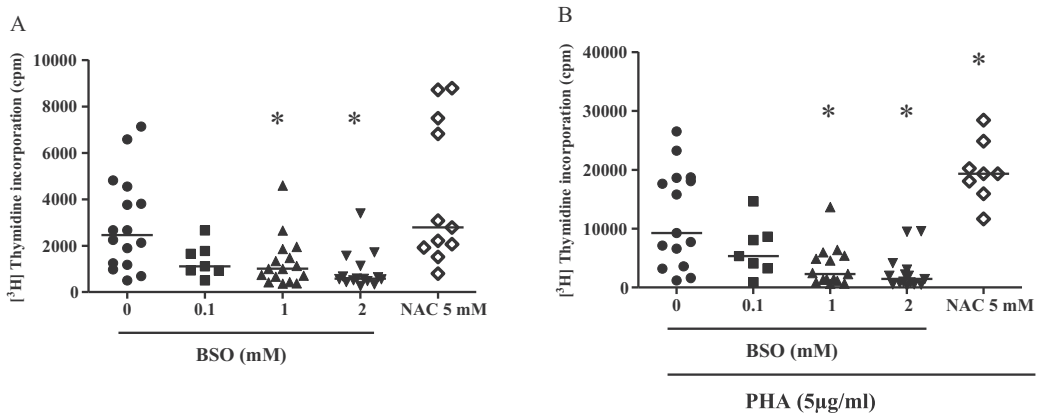

Fig. 3. Intracellular GSH modulation alters HTLV-1 spontaneous and stimulated cellular proliferation. 1.25×10^6 cells/ml obtained from HTLV-1 infected patients were seeded at a culture plate. Cells were incubated with or without BSO (0.1–2 mM) or NAC (5 mM) in the absence (A) or presence (B) of PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere. After 72 h, these cells were incubated with ^{3}H-thymidine for 6 h at the same conditions. Then, cells were collected and washed, and the radioactivity was measured in a liquid scintillation analyzer. The graphs above represent the [^{3}H] thymidine incorporation. *Significantly different from the control ($P < 0.05$). Each point represents the result for one HTLV-1 infected donor. The traces in these graphs represent the median values.

promoted by BSO were not correlated with the reduction of cell viability.

Effect of ABCC/MRP inhibition in cellular proliferation

The physiopathology relevance of ABCC/MRP protein led some researchers to study the ability of some drugs to inhibit it; among them, non-steroidal anti-inflammatory drugs such as Indomethacin and MK571 (Duffy et al., 1998). Furthermore, INDO at therapeutic concentrations enhanced the proliferation of mitogen-stimulated human lymphocytes (Kalmár et al., 1986).

We evaluated the effect of ABCC/MRP inhibition in cellular proliferation of PBMC obtained from HTLV-1-infected individuals. After 72 h, we did not observe any effect of ABCC/MRP inhibitor MK571 on spontaneous proliferation, but it had improved the PHA-induced proliferation (Fig. 5). A similar effect was observed on NAC incubation.

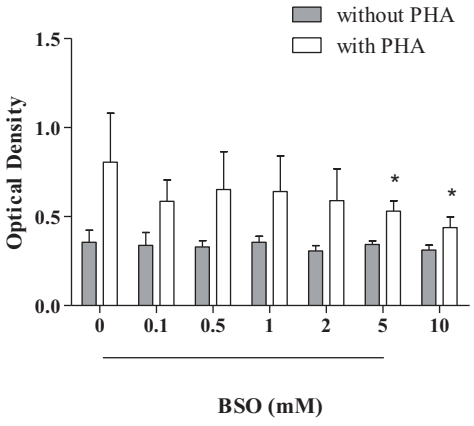

Fig. 4. BSO did not modify cellular viability. 1.25×10^6 cells/ml obtained from uninfected individuals were seeded at a culture plate. Cells were incubated with or without BSO (0.1–10 mM) in the absence or presence of PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere. After 72 h, these cells were incubated with MTT for 3 h at the same conditions. Cells were centrifuged for 7 min at 200 × *g*, and supernatant was discarded. Then, DMSO was added to dissolve the crystals formed by the MTT reduction. The optical density was acquired at 490 nm wavelength. The graphs above represent the optical density. Results were the mean ± SD of 8 experiments. *Significantly different from the control ($P < 0.05$).

BSO and NAC effect on ABCC1/MRP1 transporter expression

The results of ABCC/MRP inhibition on lymphocyte proliferation led us to investigate whether the GSH modulation was capable of regulating ABCC1/MRP1 expression and activity. We did not observe any alteration to ABCC1/MRP1 expression in non-activated (Fig. 6A) or activated (Fig. 6B) cells from uninfected donors incubated in the presence or absence of BSO or NAC. Similar results were obtained when we investigated the ABCC/MRP activity. Pre-incubated PMBC in the presence or absence of BSO or NAC did not modify the ABCC/MRP activity (data not shown). These results demonstrated that the modulation of intracellular GSH levels did not alter the ABCC1/MRP1 expression or activity in mononuclear cells.

GSH modulation alters CD69 expression in CD8$^+$ T lymphocytes from HTLV-1 infected individuals

The intracellular ROS levels are involved in cell signaling, inducing or blocking the expression of some molecules. Considering the

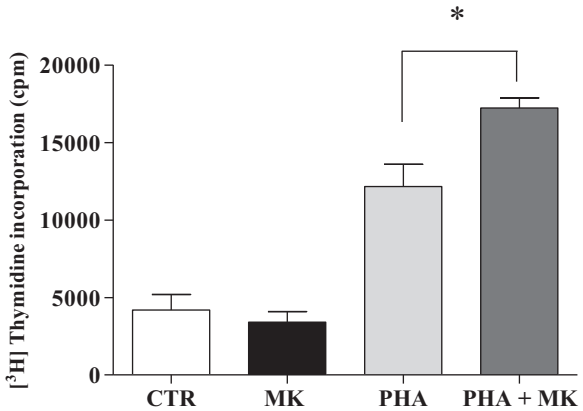

Fig. 5. Effect of ABCC/MRP inhibition on cellular proliferation. 1.25×10^6 cells/ml obtained from HTLV-1-infected individuals were seeded at a culture plate. Cells were incubated with or without MK571 (25 μM) in the absence or presence of PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere. After 72 h, these cells were incubated with ^{3}H-thymidine for 6 h at the same conditions. Then, cells were collected and washed, and the radioactivity was measured in a liquid scintillation analyzer. The graphs above represent the [^{3}H] thymidine incorporation. Results were the mean ± SD of 6 experiments. *Significantly different from the cells stimulated with PHA ($P < 0.05$).

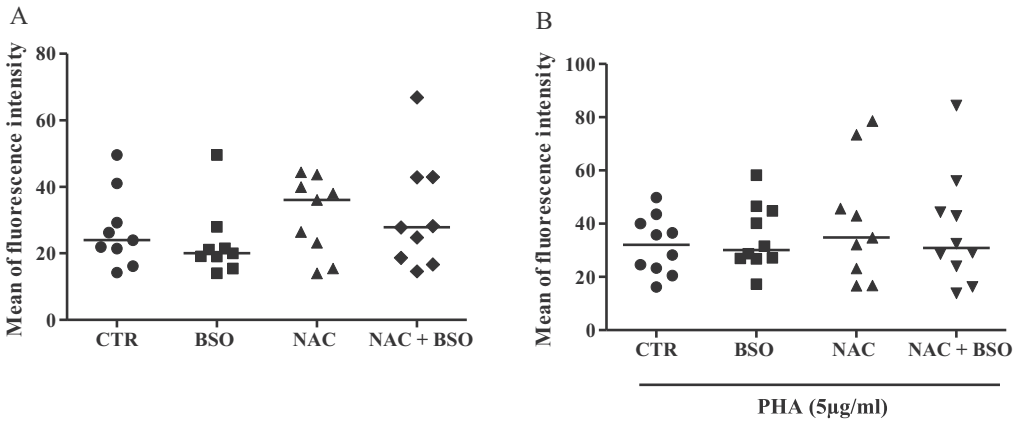

Fig. 6. ABCC1/MRP1 expression. 1.25×10^6 cells/ml obtained from uninfected individuals were seeded at a culture plate. Cells were incubated with or without BSO (1 mM) or NAC (5 mM) in the absence or presence of PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere for 72 h. To evaluate the ABCC1/MRP1 expression (A and B), cells were permeabilized using FACS Lysing Solution and stained with mouse anti-human-ABCC1 for 30 min at room temperature. Then, cells were washed and incubated with anti-IgG-FITC for 30 min at room temperature. Cells were washed and acquired using flow cytometer.

activated state of the lymphocytes from HTLV-1-infected individuals, we analyzed the effects of GSH modulation on the CD69 and CD25 expression in $CD4^+$ and $CD8^+$ T-cells. After a 72 h culture, the percentage of $CD4^+/CD25^+$ and $CD8^+/CD25^+$ non-activated lymphocytes from HTLV-1-infected patients was higher than from healthy donors (Fig. 7). PHA activation induced an increase in the percentage of $CD4^+/CD25^+$ and $CD8^+/CD25^+$ lymphocytes from uninfected and HTLV-1-infected individuals (Fig. 7). However, PBMC treatment with BSO or NAC in the presence or absence of mitogen did not alter the percentage of $CD4^+/CD25^+$ or $CD8^+/CD25^+$ lymphocytes.

We also investigated the percentage of $CD4^+/CD69^+$ and $CD8^+/CD69^+$ naive lymphocytes. And we observed an increase in the percentage of $CD8^+/CD69^+$ from HTLV-1-infected patients incubated with NAC when compared with control cells (Fig. 8B), suggesting that the GSH precursor stimulates the CD69 expression in $CD8^+$ T-cells. The mitogenic activation induced an increase in the percentage of $CD4^+/CD69^+$ and $CD8^+/CD69^+$ lymphocytes from uninfected and HTLV-1 infected individuals, but this effect was not altered by BSO or NAC (Fig. 8C and D).

Discussion

Some research groups have been studying total whole blood glutathione levels or PBMC from patients infected with virus such as human immunodeficiency virus (HIV), influenza, hepatitis and herpes simplex virus-1 (Beck et al., 2000). Fraternale et al. (2009) revised that depletion of GSH levels characterizes several viral infections and viral associated-disease progression. It was recently demonstrated that glutathione was down-regulated in HTLV-1 transformed cells (Sripadi et al., 2010). In our study, we did not find a decrease in total glutathione levels; we observed a slightly lower level of total glutathione in mononuclear cells from HTLV-1 infected individuals compared with levels from healthy donors.

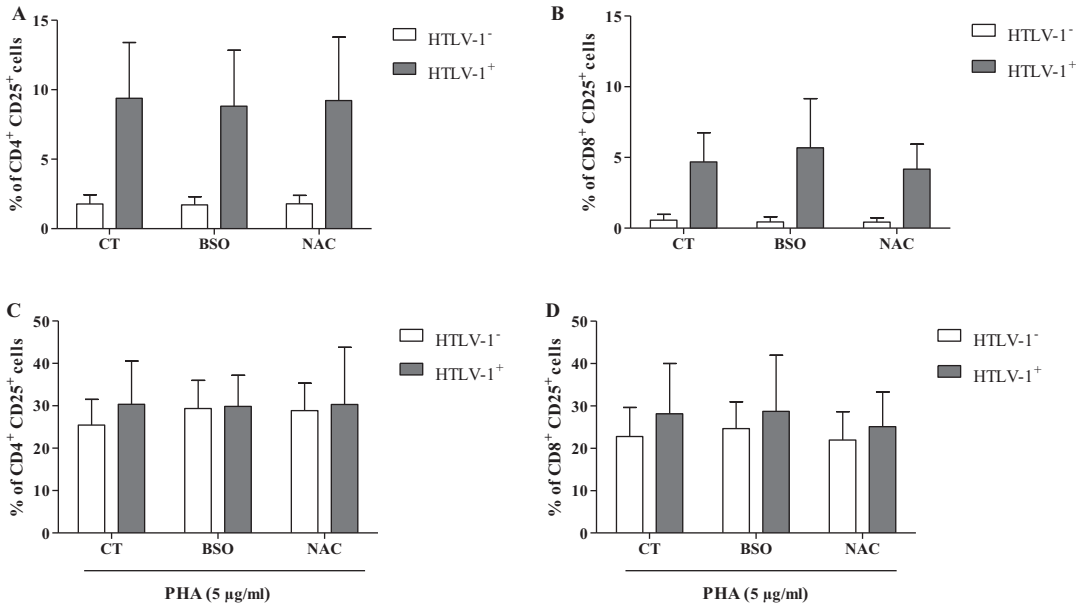

Fig. 7. CD25 expression. 1.25×10^6 cells/ml obtained from uninfected and HTLV-1-infected individuals were seeded at a culture plate. Cells were incubated with or without BSO (1 mM) or NAC (5 mM) in the absence (A and B) or presence (C and D) of PHA (5 μg/ml) at 37 °C in a 5% CO_2 atmosphere. After 72 h, cells were harvested, washed and labeled with the monoclonal antibodies anti-human-CD25 PE, anti-human-CD4 FITC and anti-human-CD8 PE-Cy5 for 20 min at 4 °C. After that, cells were washed, and the fluorescence was examined by flow cytometry. The graphs above represent the % of $CD4^+/CD25^+$ (A and C) or $CD8^+/CD25^+$ (B and D). Results were the mean ± SD of 4 experiments.

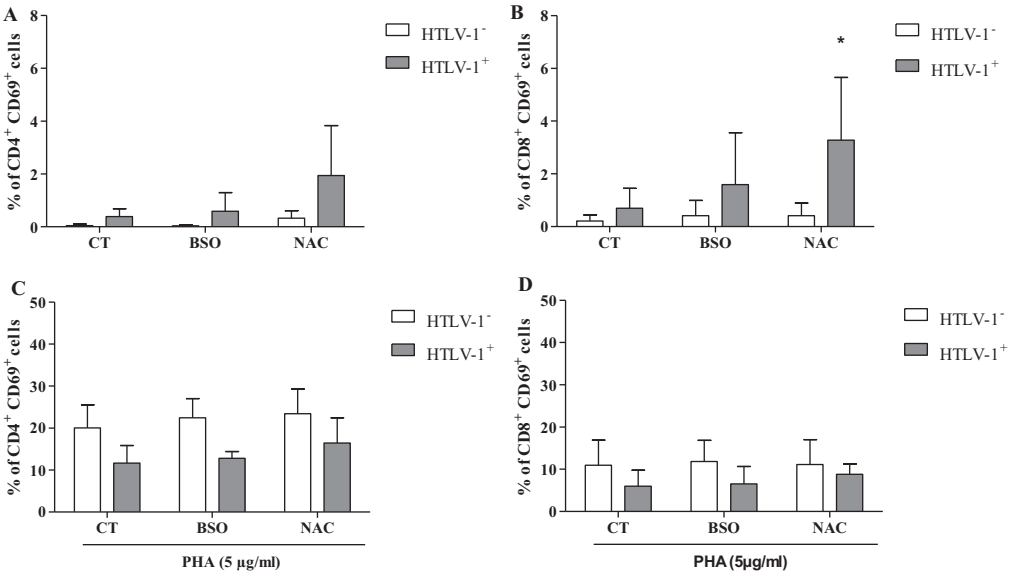

Fig. 8. GSH modulation alters CD69 expression in $CD8^+$ T lymphocytes from HTLV-1-infected patients. 1.25×10^6 cells/ml obtained from uninfected and HTLV-1-infected individuals were seeded at a culture plate. Cells were incubated with or without BSO (1 mM) or NAC (5 mM) in the absence (A and B) or presence (C and D) of PHA (5 µg/ml) at 37 °C in a 5% CO_2 atmosphere. After 24 h, cells were harvested, washed and labeled with the monoclonal antibodies anti-human-CD69 PE, anti-human-CD4 FITC and anti-human-CD8 PE-Cy5 for 20 min at 4 °C. Then, cells were washed, and the fluorescence was examined by flow cytometry. The graphs above represent the % of $CD4^+/CD69^+$ (A and C) or $CD8^+/CD69^+$ (B and D). Results were the mean ± SD of 5 experiments. *Significantly different from the control ($P < 0.05$).

This result can be related with the increase of consumption during the proliferation induced by HTLV-1 proteins.

Our results indicate that the spontaneous proliferation induced by HTLV-1 infection depends on intracellular GSH levels. For the first time, data showed that the GSH synthesis inhibition by BSO impaired the spontaneous proliferation induced by HTLV-1. Many findings have demonstrated that GSH regulates the activation and proliferation of T-cells (Messina & Lawrence, 1989; Suthanthiran et al., 1990; Franklin et al., 1990; Smyth, 1991; Iwata et al., 1994; Walsh et al., 1995; Hadzic et al., 2005; Yan et al., 2010). An increase in intracellular GSH levels is induced during the activation of peripheral blood mononuclear cells (Messina and Lawrence, 1989; Hadzic et al., 2005). Moreover, the modulation of intracellular GSH levels, using BSO or NAC, altered the IL-2 production by activated T-cell. Hadzic et al. (2005) described that BSO impaired the IL-2 secretion, and NAC induced an augmentation of the IL-2 levels. These effects might be correlated with the phenomenon we observed, since the spontaneous proliferation induced by HTLV-1 depends on the increase in IL-2 production and CD25 expression (Kimata and Ratner, 1991; Nishiura et al., 1996). Our data suggested that BSO promoted an impairment of IL-2 production induced by HTLV-1 proteins.

The cell treatment with NAC induced an increase in mitogen-stimulated cellular proliferation. The spontaneous proliferation observed in PBMC obtained from HTLV-1-infected individuals was not modulated by NAC. Furthermore, we found similar results using ABCC/MRP inhibitor MK571, which suggested that the ABCC/MRP inhibition induced an intracellular increase of metabolites involved in the modulation of cellular proliferation, such as the GSH. Altogether, these results indicate that NAC effects are related to responses for exogenous stimulus to T-cell proliferation.

Al-Fahim et al. (1999) demonstrated that the percentage of fresh and activated T lymphocytes expressing CD25 obtained from HAM/TSP patients was higher than from uninfected individuals. We observed a higher percentage of $CD4^+CD25^+$ and $CD8^+CD25^+$ in unstimulated cells from infected individuals than from healthy donors, corroborating Al-Fahim et al. findings. This feature leads us to investigate the effects of GSH modulation on CD69 and CD25 expression in $CD4^+$ and $CD8^+$ T lymphocytes from uninfected and HTLV-1-infected individuals. Our results demonstrated for the first time that NAC (5 mM) treatment induced an increase in CD69 expression on unstimulated $CD8^+$ T lymphocytes obtained from HTLV-1-infected individuals. This result suggested that the increase in CD69 expression on $CD8^+$ lymphocytes from HTLV-1 infected donors was correlated with an augmentation of GSH. Moreover, the elevation in $CD8^+CD69^+$ could be related to the increase of the cytotoxicity capacity of HTLV-1 specific CTLs. Similar results were found by Hadzic et al., who showed an increase in $CD69^+CD25^+$ T-cells stimulated with anti-CD3 in the presence of NAC (20 mM).

Although our data indicate that spontaneous proliferation depends on GSH, this thiol is involved in the acquisition of activated phenotype, which stimulates CD69 expression in $CD8^+$ T-cells. Thus, increases in GSH levels could be beneficial to the activation of HTLV-1-specific $CD8^+$ T-cell and to the elimination of HTLV-1-infected cells. In addition, HTLV-1-specific $CD8^+$T-cells are generated when the virus antigen expression increases (Yamano et al., 2002). Therefore, our results suggested that the expansion of HTLV-1 can occur during GSH replacement, but this effect can activate CTL, which is responsible for infection control (Kattan et al., 2009). Many studies aim to correlate the glutathione levels with immune response, and suggest adding the glutathione-replenishment to highly active antiretroviral treatment (Harakeh and Jariwalla, 1991; Jariwalla et al., 2008; Fraternale et al., 2009). The GSH replacement was linked to the improvement of lymphocyte proliferative response and the attenuation of AIDS symptoms (Jariwalla et al., 2008; Herzenberg et al., 1997). However, GSH replacement treatment, using NAC or another drug, has not been reported in HTLV-1 clinical trials yet; thus, we propose the modulation of GSH levels as a therapeutic target in HTLV-1-associated pathologies.

Conflict of interest

The authors declare no conflict of interests.

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