High susceptibility of activated lymphocytes to oxidative stress-induced cell death

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ABSTRACT

The present study provides evidence that activated spleen lymphocytes from Walker 256 tumor bearing rats are more susceptible than controls to tert-butyl hydroperoxide (t-BOOH)-induced necrotic cell death in vitro. The iron chelator and antioxidant deferaxamine, the intracellular Ca2+ chelator BAPTA, the L-type Ca2+ channel antagonist nifedipine or the mitochondrial permeability transition inhibitor cyclosporin A, but not the calcineurin inhibitor FK-506, render control and activated lymphocytes equally resistant to the toxic effects of t-BOOH. Incubation of activated lymphocytes in the presence of t-BOOH resulted in a cyclosporin A-sensitive decrease in mitochondrial membrane potential. These results indicate that the higher cytosolic Ca2+ level in activated lymphocytes increases their susceptibility to oxidative stress-induced cell death in a mechanism involving the participation of mitochondrial permeability transition.

Key words: cell death, free radicals, immune response, lymphopenia, mitochondrial permeability transition, spleen lymphocyte.

INTRODUCTION

Lymphocyte activation is an essential step in the immune response that generally occurs during tumor development; it requires T-cell recognition of specific tumor antigens through the interaction of the lymphocyte receptor with the major histocompatibility complex of antigen-presenting cells (Hermiston et al. 2002, Roy 2003, Grossman et al. 2004). Elevations in cytosolic Ca2+ concentration ([Ca2+]cyt) and reactive oxygen species (ROS) production are characteristically observed in such activated lymphocytes, and are important factors associated with either a high rate of cell proliferation or mechanisms that lead to cell death (Buttke and Sandstrom 1995, Williams and Kwon 2004, Feske 2007). It is proposed that a high expression of anti-apoptotic factors, including interleukin-2, mitochondrial uncoupling protein-2, and members of the Bel-2 family, increases the resistance of activated lymphocytes to cell death, pushing the cell towards proliferation (Quintana et al. 2005, Arnold et al. 2006, Degasperi et al. 2006a, Krammer et al. 2007).

In the present study, the effects of the tert-butyl hydroperoxide (t-BOOH), a well known prooxidant, were evaluated on proliferation and death of activated spleen lymphocytes from Walker 256 tumor bearing (TB) rats (Quintana et al. 2005, Degasperi et al. 2006a, b) and lymphocytes from control (CT) rats. t-BOOH induces oxidative stress by exhausting cellular GSH and NADPH, substrates of the antioxidant enzymes glutathione peroxidase and glutathione reductase, respectively, thus favoring the accumulation of H2O2 (Jocelyn and Dickson 1980, Bernardes et al. 1986, Castilho et al. 1995). In addition, t-BOOH promotes a Ca2+-stimulated generation of methyl, t-butoxy, and t-butyldihydroxy radicals (Kennedy et al. 1992, Castilho et al. 1995). Previous
results from our group indicated that the enhanced and continuous production of ROS by mitochondria treated with Ca\(^{2+}\) plus t-BOOH leads to a form of non-selective permeabilization of the inner mitochondrial membrane known as the mitochondrial permeability transition (MPT) (Castilho et al. 1995, Kowaltowski et al. 2001). The results presented here demonstrate that activated TB rat lymphocytes are more susceptible to necrotic death induced by t-BOOH-mediated oxidative stress than are control cells.

**MATERIALS AND METHODS**

**CHEMICALS**
The tert-butyl hydroperoxide (t-BOOH), carbonyl cyanide m-chloro phenyl hydrazone (CCCP), cyclosporin A (CsA), oligomycin, deferoxamine, nifedipine and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). BAPTA-AM (1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester), 3,3′-dihexyloxacarbocyanine iodide (DioC\(_6\)), Fluo-3AM and Pluronic acid F-127 were purchased from Molecular Probes Inc. (Eugene, OR, USA). Ficoll-Paque\(^{TM}\) PLUS was obtained from Becton Dickinson Biosciences (San Jose, CA, USA), RPMI 1640 (Roswell park medium) from Cultlab (Campinas, Brazil), and FK-506 from LC Laboratories (Woburn, MA, USA). Annexin V was purchased from the Laboratory of Immunobiology of the University of São Paulo (São Paulo, Brazil). All other chemicals were standard commercial products of reagent-grade quality.

**ANIMAL TREATMENT**
Nine-week-old male Wistar rats (Rattus norvegicus albinos) were obtained from the UNICAMP Central Animal Breeding Center. Rats were kept under standard laboratory conditions (20-22°C and 12 h/12 h light/dark cycle) with free access to a standard diet (Labina/Purina, Campinas, SP, Brazil) and tap water. Animal experiments followed the University guidelines for the use of animals in experimental studies (protocol no. 487-1, approved by the UNICAMP Ethics Committee in 2002) and followed the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised in 1996).

The Walker 256 tumor cell line (originally obtained from the Christ Hospital Line, National Cancer Institute Bank, Cambridge, USA) is kept frozen in liquid nitrogen. For experimental procedures, Walker 256 cells were maintained by consecutive intraperitoneal inoculums, each with 20 × 10\(^6\) tumor cells in phosphate-buffered saline solution (PBS). Walker 256 tumor cells with viability > 98% isolated from the ascitic fluid were used for intraperitoneal inoculums, with viability estimated by the trypan blue exclusion method.

**SPLUNK CEYTOCYTE ISOLATION**
Rat spleens were gently homogenized in a manual Dounce homogenizer. Spleen homogenates were overlaid onto a Ficoll-Paque\(^{TM}\) PLUS layer, with density adjusted to 1.076 g/ml, and centrifuged at 1000 g at room temperature for 25 min. The interface cell layer containing lymphomonocytes was recovered by Pasteur pipette, washed twice in PBS, and centrifuged at 500 g for 10 min (Boyum 1976, Degasperi et al. 2006a, b). Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was greater than 98%.

**CELL CULTURE AND TREATMENTS**
The in vitro lymphocyte cultures were obtained by seeding isolated spleen lymphocytes at an initial density of 10\(^6\) cells/ml in RPMI 1640 in 1.5 cm\(^2\) plate wells in a humidified atmosphere (5% CO\(_2\) at 37°C). After 1 h, lymphocytes from TB rats showed a more rapid growth rate than those of the CT rats. At this time, the TB rat lymphocytes were again counted and diluted to 10\(^6\) cells/ml in RPMI 1640. CT and TB rat lymphocytes were then treated with t-BOOH in the presence of deferoxamine, nifedipine, cyclosporin A or FK-506, as indicated in the figure legends.

For BAPTA loading, the lymphocytes were pre-incubated with 20µM BAPTA-AM, after 1 h, the lymphocytes were washed, counted and diluted to 10\(^6\) cells/ml in RPMI 1640. BAPTA-loaded CT and TB rat lymphocytes were then incubated under control conditions or treated with t-BOOH for up to 4 h.

**FLOW CYTOMETRY**
The samples (isolated CT and TB rat spleen lymphocytes) were analyzed in a FACSCalibur flow cytome-
ter equipped with an argon laser and CellQuest software (Becton Dickinson, San Jose, CA, USA). Ten thousand events were acquired from each sample. The lymphocyte populations were identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for intensity of the fluorescent probe signal (Degasperi et al. 2006a).

**ANALYSIS OF CELL VIABILITY BY ANNEXIN-V AND PI STAINING**

Lymphocytes were labelled with annexin-V following the manufacturer’s instructions (Brumatti et al. 2003). Briefly, 10⁶ cells were harvested at each point in time, washed twice with PBS and resuspended in a binding buffer containing annexin V-FITC (1:500). After 20 min of incubation at room temperature, the lymphocytes were centrifuged at 1000 g for 5 min and resuspended in binding buffer containing PI (1:50). Quantitative determinations of apoptotic and necrotic cells were carried out as previously described (Martin et al. 1995).

**MEASUREMENT OF CYTOSOLIC FREE Ca²⁺ CONCENTRATIONS**

Cytosolic free Ca²⁺ concentrations ([Ca²⁺]_{cyt}) were monitored with the green fluorescent probe Fluo-3AM (5μM), a single wavelength indicator, by flow cytometry. Spleen lymphocytes (10⁶ cells/mL) in RPMI 1640 medium were loaded with 5μM Fluo-3AM containing 1μM pluronic acid F-127 (for proper dispersal) and 30μg/mL BSA in a humified CO₂ incubator (5% CO₂) at 37°C for 40 min. Nonhydrolyzed Fluo-3 AM was removed by washing the cells with medium just before fluorescence acquisition. Calibration was performed at the end of each experiment. [Ca²⁺]_{cyt} was calculated considering K_d of the Ca²⁺-Fluo-3 complex 390 nM (Mather and Rottenberg 2002).

**DETERMINATION OF MITOCHONDRIAL MEMBRANE ELECTRICAL POTENTIAL (ΔΨ_m) USING FLOW CYTOMETRY**

After experimental incubation, lymphocytes (10⁶ cells/ml) were washed in PBS buffer and incubated with 0.2 nM DioC₆(3) in 400μl of RPMI 1640 medium for 30 min at 37°C in a humified CO₂ incubator (5% CO₂). One half of each cell sample (200μl) was separated into a new tube, with the addition of 50μM CCCP a protonophore that dissipates ΔΨ_m (Rottenberg and Wu 1998, Campos et al. 2004). Both samples were incubated for a further 30 minutes. Oligomycin (1μg/ml) was added during the last 10 min prior to FACS analysis, as indicated in the figure. In non-saturated concentrations, DioC₆(3) binds preferentially to mitochondria, since electrical potential of the mitochondrial (ΔΨ_m) is much higher (~180 mV) than that of the plasma membrane (~60 mV). Results were normalized using the F/F_{CCCP} ratio, where F is the mean fluorescence intensity of DioC₆(3) (maximum fluorescence) and F_{CCCP} is the mean fluorescence in the presence of CCCP (minimum fluorescence).

**STATISTICAL ANALYSIS**

Three independent experiments were performed, each in triplicate, with results displayed as average ± SD; significance was assessed by ANOVA and a post-hoc Tukey test. The results of cell viability estimated by annexin-V and propidium iodide staining were analysed by a Mann-Whitney U test. Level of significance was set at p<0.05 using SAS software (Statistical Analysis System 6.12/SAS Institute Inc, 1989-1996, Cary, NC, USA).

**RESULTS**

**HIGH SUSCEPTIBILITY OF ACTIVATED LYMPHOCYTES TO T-BOOH-INDUCED CELL DEATH**

The experiments presented in Figure 1A show that TB rat lymphocytes exhibited a higher rate of proliferation than did CT rat lymphocytes, although the presence of t-BOOH decreased the rate of proliferation, especially for the TB rat lymphocytes. The susceptibility of CT and TB rat lymphocytes to t-BOOH was also analyzed as the percentage of viable cells considering their respective controls at each time point (Fig. 1B). After 4 h of incubation in the presence of t-BOOH the percentage of viable cells was reduced to nearly 20% for TB rat lymphocytes whereas 60% of CT rat lymphocytes continued viable (Fig. 1B). The decrease in rate of proliferation for both cell types upon t-BOOH treatment was accompanied by an increase in the number of trypan blue-positive cells (Fig. 1C), with the number of dead cells in TB rat lymphocytes being more than double that of the control lymphocytes. Furthermore, in the presence of t-BOOH the number of lymphocytes positive to propidium iodide...
Fig. 1 – Increased susceptibility to t-BOOH-induced necrotic cell death of lymphocytes from tumor bearing rats (TBR) over that of control rats (CTR). (A) Lymphocytes were incubated for 4 h in the absence of 500μM t-BOOH (CTR; △: TBR) or in its presence (■: CTR + t-BOOH; ▲: TBR + t-BOOH). *p < 0.05 vs. CTR without t-BOOH; #p < 0.05 vs. respective group without t-BOOH. (B) Effect of t-BOOH on the percentage of viable cells considering their respective controls at each time point (■: CTR + t-BOOH; ▲: TBR + t-BOOH). *p < 0.05 vs. CTR + t-BOOH. (C) Death of lymphocytes in the absence of 500μM t-BOOH (CTR: white bars; TBR: white hatched bars) or in its presence (CTR + t-BOOH: gray bars; TBR + t-BOOH: gray hatched bars). Viability was estimated by the trypan blue exclusion method repeated hourly for 4 h. *p < 0.05 vs. respective group without t-BOOH. (D) Determination of apoptotic and necrotic death of lymphocytes incubated in the presence of t-BOOH. After 4 h, samples of 10⁶ cells/ml were annexin V-FITC labeled (AV; gray bars) and propidium iodide stained (PI; black bars) and then analyzed by flow cytometry. *p < 0.05 vs. respective CTR.
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was much larger than that of those positive to annexin-V (Fig. 1D), indicating that cell death under these conditions was predominantly necrotic.

T-BOOH-INDUCED LYMPHOCYTE DEATH IS PREVENTED BY DEFEROXAMINE

The involvement of oxidative stress in the process of t-BOOH-induced spleen lymphocyte death was assessed in vitro, by testing the effect of deferoxamine on cell proliferation and viability (Fig. 2). In the presence of this antioxidant/iron chelator (Halliwell 1989, Bartesaghi et al. 2004), t-BOOH did not significantly decrease the proliferation rate of CT and TB rat lymphocytes (Fig. 2A). Moreover, deferoxamine decreased at least 2.5-fold the number of trypan blue-positive cells after 4 h of incubation in the presence of t-BOOH (Fig. 2B) indicating that lymphocyte death under these conditions is mediated by oxidative stress.

INCREASED SUSCEPTIBILITY OF TB RAT LYMPHOCYTES TO T-BOOH-INDUCED DEATH IS MEDIATED BY HIGH [Ca$^{2+}$]$_{cyt}$

The experiments presented in Figure 3 show that [Ca$^{2+}$]$_{cyt}$ in TB rat spleen lymphocytes is at least double that of CT rat lymphocytes when both cells were incubated during 3 h in RPMI 1640 medium. When lymphocytes were incubated in a medium containing 10µM nifedipine, a L-type Ca$^{2+}$ channel antagonist, no such significant difference in [Ca$^{2+}$]$_{cyt}$ was observed.

Fig. 2 – t-BOOH-induced death of CTR and TBR lymphocytes is prevented by deferoxamine (DFO). (A) Growth of DFO-treated CTR (white and gray bars) and TBR lymphocytes (white hatched and gray hatched bars) incubated for 4 h in the absence of 500µM t-BOOH (white and white hatched bars) or in its presence (gray and gray hatched bars). (B) Death of DFO-treated CTR and TBR lymphocytes incubated for 4 h in the absence of 500µM t-BOOH or in its presence. *p < 0.05 vs. respective group without t-BOOH in the absence of DFO. #p < 0.05 vs. respective group treated with t-BOOH in the absence of DFO.

Fig. 3 – The increase in cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{cyt}$) in TBR lymphocytes is prevented by nifedipine. CTR (white bars) and TBR lymphocytes (hatched bars) were incubated for 3 h in RPMI 1640 medium in the absence or presence of 10µM nifedipine as indicated in the figure. *p < 0.01 vs. CTR.

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t-BOOH-induced death of CT and TB rat lymphocytes incubated in the presence of nifedipine (Fig. 4) or loaded with the Ca\(^{2+}\) chelator BAPTA (Fig. 5) was also investigated. The cytoprotective effects of nifedipine and BAPTA were similar to those obtained with deferoxamine. Thus, these findings indicate that [Ca\(^{2+}\)]\(_{\text{cyst}}\) plays an important role in the higher susceptibility of TB rat lymphocytes to t-BOOH.

**T-BOOH-Induced Lymphocyte Necrosis is Mediated by MPT**

Both cyclosporin A and FK-506 inhibit calcineurin, but only cyclosporin A inhibits MPT (Crompton et al. 1988, Griffiths and Halestrap 1991, Galat 2003). Here, the presence of cyclosporin A (Figs. 6A-C) almost completely prevented the decrease in cell proliferation and viability, as well as the increase in the number of trypan blue-positive cells induced by t-BOOH. A possible decrease in cell proliferation by the immunosuppressant drug cyclosporin A was not observed probably because the activated lymphocytes already contained high levels of IL-2 during the time course of our experiments. In contrast, protective effects were not observed in the presence of FK-506 (Figs. 6D-F). Moreover, the results shown in Figure 7 indicate that incubation in the presence of t-BOOH for 4 h induces a decrease in \(\Delta\Psi_m\) especially in TB rat lymphocytes. CT rat lymphocytes treated with t-BOOH showed only a \(\sim 20\%\) decrease in the F/F\(_{\text{eccp}}\) ratio during this incubation time (results not shown). The t-BOOH-induced decrease in \(\Delta\Psi_m\) was prevented by simultaneous incubation in the presence of cyclosporin A plus oligomycin, but not in that of oligomycin alone, supporting the notion that t-BOOH promotes MPT in these cells.

**DISCUSSION**

The present study analyses the effects of t-BOOH on the proliferation and death of lymphocytes obtained from TB and CT rats. The TB rat lymphocytes have been activated as seen by the presence of high levels of interleukin 2 (IL-2) (Degasperi et al. 2006a). Cell proliferation was stimulated doubling the number of activated lymphocytes in four hours (Fig. 1A). The activated TB rat lymphocytes overexpress the anti-apoptotic protein Bcl-2 and mitochondrial uncoupling protein-2 (Degasperi et al. 2006a) which protects the cells by preventing the conversion of high levels of ROS and [Ca\(^{2+}\)]\(_{\text{cyst}}\) into a pathway leading to mitochondrial dysfunction and cell death. Despite the protective effect of these proteins, TB rat lymphocytes display a higher susceptibility to cell death induced by t-BOOH than do the control cells (Fig. 8). t-BOOH induces oxidative stress by exhausting cellular GSH and NADPH, and promotes a Ca\(^{2+}\)-stimulated generation of methyl, \(\text{t-butoxyl}\), and \(\text{t-butylperoxyl}\) radicals as detected by electron paramagnetic resonance (EPR) signals (Kennedy et al. 1992, Castilho et al. 1995). Both nifedipine or BAPTA, that decrease [Ca\(^{2+}\)]\(_{\text{cyst}}\) of control and activated lymphocytes render them equally resistant to the toxic effects of t-BOOH. This indicates that the higher [Ca\(^{2+}\)]\(_{\text{cyst}}\) in activated lymphocytes renders these cells more susceptible to exogenous prooxidants.

Although both cyclosporin A and FK-506 inhibit the activity of peptidylprolyl cis/trans isomerases or immunophilins, only cyclosporin A is an inhibitor of MPT (Crompton et al. 1988, Griffiths and Halestrap 1991, Friberg et al. 1998, Galat 2003). Therefore, the reduction in t-BOOH-induced necrotic lymphocyte death by cyclosporin A, but not by FK-506, indicates that MPT is involved in the mechanism of cell death and excludes activation of the calcineurin pathway. Inner membrane permeabilization caused by MPT results in loss of matrix components, impairment of mitochondrial function and substantial mitochondrial swelling, with consequent outer membrane rupture and release of pro-apoptotic mitochondrial proteins (Zoratti and Szabo 1995, Green and Reed 1998, Lemasters et al. 1998, Crompton 1999, Kowaltowski et al. 2001, Green and Kroemer 2004). As a result, MPT actively participates in events that initiate either necrotic or apoptotic cell death.

The protection conferred by deferoxamine against t-BOOH-induced lymphocyte death indicates the participation of ROS under these conditions. Deferoxamine, approved by FDA for the removal of iron in conditions involving iron overload, such as \(\beta\)-thalassemia (Giardini et al. 1993), has been used primarily as a metal chelating agent to block the iron-dependent hydroxyl radical (Goldstein and Czapski 1990). It may involve an additional or alternative antioxidant mechanism which would directly scavenge free radicals, such as superoxides (O\(_2^-\)) (Goldstein and Czapski 1990), hydroxyls (• OH) (Hoe et
Fig. 4 – t-BOOH-induced death of CTR and TBR lymphocytes is prevented by nifedipine. (A) Growth of nifedipine-treated CTR (white and gray bars) and TBR lymphocytes (white hatched and gray hatched bars) incubated for 4 h in the absence of 500 µM t-BOOH (white and white hatched bars) or in its presence (gray and gray hatched bars). (B) Death of nifedipine-treated CTR and TBR lymphocytes incubated for 4 h in the absence of 500 µM t-BOOH or in its presence. *p < 0.05 vs respective group without t-BOOH in the absence of nifedipine. #p < 0.05 vs. respective group treated with t-BOOH in the absence of nifedipine.

Fig. 5 – t-BOOH-induced death of CTR and TBR lymphocytes is prevented by intracellular Ca²⁺ chelation. (A) Growth of BAPTA-loaded CTR (white and gray bars) and TBR lymphocytes (white hatched and gray hatched bars) incubated for 4 h in the absence of 500 µM t-BOOH (white and white hatched bars) or in its presence (gray and gray hatched bars). (B) Death of BAPTA-loaded CTR and TBR lymphocytes incubated for 4 h in the presence of 500 µM t-BOOH or in its absence. *p < 0.05 vs. respective group without t-BOOH, in the absence of BAPTA. #p < 0.05 vs. respective group treated with t-BOOH, in the absence of BAPTA.

al. 1982), peroxyls (Hartley et al. 1990), peroxynitrite-derived carbonate species (CO₃⁻) or nitrogen dioxide (NO₂) radicals (Bartesaghi et al. 2004). Indeed, it is known that under conditions of t-BOOH-mediated oxidative stress deferoxamine inhibits death of hepatocytes (Niemenen et al. 1997), thymocytes (Bartoli et al. 1994),

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neurons and astrocytes (Abe and Saito 1998) or cardiac myocytes (Daly et al. 1991).

These results indicate that activated lymphocytes are more susceptible to oxidative stress-induced death than are controls. This observation may help the understanding of the mechanisms underlying activated lymphocytes death in vivo in patients treated by the anthracyclines doxorubicin or daunorubicin. The lymphopenia caused by these antineoplastic agents is associated with increased susceptibility to opportunistic infections (Mackall et al. 1994, Sthanke et al. 2001). In addition to interacting with DNA, these compounds also give rise to ROS, since they undergo intracellular redox-cycling (Doroshow 1983, Wallace 2003, Conklin 2004). Therefore, it seems that activated lymphocytes would be more susceptible to death caused by antineoplastic agents such as anthracyclines, alkylating agents, or camptothecins (Conklin 2004).

In conclusion, the present study has demonstrated that activated lymphocytes from Walker 256 tumor-bearing rats are more susceptible to in vitro t-BOOH-mediated oxidative stress than are control cells. This cytotox-
Fig. 7 – Mitochondrial permeability transition mediates t-BOOH-induced ΔΨm decrease in TBR lymphocytes. ΔΨm in CTR and TBR lymphocytes (10^6 cells/ml) was determined after 4 h incubation in the presence of 500 µM t-BOOH. Where indicated, 1 µM oligomycin (Oligo) and/or 1 µM cyclosporin A (CsA) were added to the incubation medium. ΔΨm was determined as the ratio of DioC6(3) fluorescence (F) in the absence of 50 µM CCCP to that in its presence (F/F CCCP). *p < 0.05 vs. respective CTR group; #p < 0.05 vs. lymphocytes treated only with oligomycin.

Fig. 8 – Summary of the effects of t-BOOH-induced oxidative stress on pathways involved in proliferation and death of activated lymphocytes. The process of lymphocyte activation involves both ROS overproduction and increases in [Ca^{2+}]_{cyt}. Treatment with agents that induces cellular oxidative stress (e.g., t-BOOH) leads to a conversion of the physiological signals from proliferation to death of activated lymphocytes. t-BOOH induces oxidative stress by exhausting cellular antioxidants and promoting free radicals generation. t-BOOH results in necrotic death mediated by mitochondrial permeability transition (MPT) induction.
icity seems to be mediated by increased $[\text{Ca}^{2+}]_{\text{cyt}}$ and MPT (Fig. 8). These findings may contribute to the understanding of increased fragility of the immunological system to chemotherapy-generated oxidative stress.

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