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N-Acetylcysteine Increases Apoptosis Induced by H_2O_2 and mo-antiFas Triggering in a 3DO Hybridoma Cell Line

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N-Acetylcysteine (NAC) has been used as an antioxidant to prevent apoptosis triggered by different stimuli in different cell types. It is common opinion that cellular redox, which is largely determined by the ratio of oxidized and reduced glutathione (GSH), plays a significant role in the propensity of cells to undergo apoptosis. However, there are also contrasting opinions stating that intracellular GSH depletion or supplemented GSH alone are not sufficient to lead cells to apoptosis or conversely protect them. Unexpectedly, this study shows that NAC, even if it maintains the peculiar characteristics of an agent capable of reducing cell proliferation and increasing intracellular GSH content, increases apoptosis induced by H_2O_2 treatment and mo-antiFas triggering in a 3DO cell line. We found that 24 h of NAC pre-treatment can shift cellular death from necrotic to apoptotic and determine an early expression of FasL in a 3DO cell line treated with H_2O_2.

KEY WORDS — NAC; H_2O_2; apoptosis

INTRODUCTION

Apoptosis, an active form of cell suicide, plays an essential role in various development stages and many physiological situations where deletion of cells is required.[1] Potential exogenous apoptosis stimuli range from growth factor withdrawal to ligand- or antibody-mediated engagement of specific cell surface proteins, capable of transducing a lethal signal. The Fas (APO-1; CD95)/Fas ligand (FasL) system has emerged as an important cellular pathway regulating the induction of apoptosis in a wide variety of tissues.[2,3] The biological importance of the Fas/FasL system has been extensively studied in T cells, where it plays a critical role in clonal deletion of autoreactive T cells and activation-induced suicide.[4,5]

Many stimuli able to induce apoptosis also imply an oxidative stress. There is now much evidence showing that controlled levels of reactive oxygen intermediates (ROIs) modulate cellular functions and are necessary for signal transduction pathways, including those mediating apoptosis.[6]

Disturbance in the cellular redox potential is evident before cells demonstrate gross features of nuclear apoptosis such as chromatin condensation, oligonucleosomal DNA fragmentation or nuclear DNA loss.

Recent studies have demonstrated that H_2O_2, utilized at appropriate concentrations, can induce a variety of cell types to die through an active programme of self destruction, which is completely different from necrosis and has no implications for the neighbouring cells.[7] In fact it is well known that maintaining the oxidative balance is necessary not only for the homeostasis of the bone marrow microenvironment, but also in the peripheral cells where many cellular metabolic processes involving proliferation and/or differentiation occur.[8]

In inflammatory diseases for example, a central feature of the process is the recruitment of phagocyte cells (e.g. neutrophils and macrophages) and

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as a result of the respiratory burst and activity of the NADPH oxidase enzyme, these cells produce superoxide, hydrogen peroxide and hypochlorite, primarily as an antibacterial killing mechanism. However, this final product of cellular metabolism can itself be a source of local tissue damage.

The concept of blanket protection against damaging free radical oxidation such as antioxidant therapy is attractive, and agents that can supplement the natural antioxidant systems are constantly being discovered. It has been suggested that reduced GSH plays a role in rescuing cells from apoptosis, by buffering an endogenous oxidative stress mechanism.[9] On the other hand, Ghibelli[10] showed that GSH depletion alone is not enough to lead cells to apoptosis and Van den Dobbelsteen[11] showed that the supplemented intracellular GSH does not protect the Jurkat cell line against apoptosis induced by anti-Fas/APO 1 antibody. Packham[12] found that myeloid programmed cell death by cytokine withdrawal, even if effectively suppressed by the antioxidant, is not associated with an increase in reactive oxygen intermediate species.

Though current opinion holds that oxidative stress induced by ROI is related to apoptosis, some authors show that overproduction or decreased elimination of O_2^-, the primary ROI generated by normal cellular metabolism, can be an advantage for survival since it blocks Fas-triggered apoptosis in a cell population with a high metabolic rate.[13] Apoptotic death or rescue from apoptotic death seem to be phenomena which are closely linked to unbalanced redox status in the cells.

Using a lymphocytic hybridoma murine 3DO cell line we investigated whether pre-treatment with NAC, an agent which increases GSH levels and known to be used as an antioxidant to prevent apoptosis in lymphocytes,[14–16] could modify apoptosis induced by Fas triggering or H_2O_2 treatment.

MATERIAL AND METHODS

Cell Culture

3DO (murine lymphocytic hybridoma)[17] cells were grown in 5 per cent CO_2 in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10 per cent FCS (Gibco, Grand Island, NY), 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 2 mM L-glutamine, 2×10^{-5}-mercaptoethanol, 1 per cent sodium pyruvate, 25 mM Hepes buffer and 1 per cent gentamycin. Cell viability was analysed by the Trypan Blue method.

Reagents

N-Acetylcysteine (NAC), buthinine sulfoximine (BSO) and H_2O_2 were purchased from Sigma. Purified and PE-labelled hamster IgG anti-Fas (clone Jo2), purified hamster IgG isotypic control (clone UC8-4B3), purified antiFasL (clone Kay-10), FITC-conjugated mouse anti rat (IgG_{2a} second antibody and mouse IgG isotype control (clone G155-178) were purchased from Southern Biotechnology Associates, Inc (Birmingham, AL, USA).

Flow Cytometry

Approximately 1×10^6 cells per sample (< 5 per cent dead cells) were pelletted in a round-bottom centrifuge tube at 200 $g \times 5$ min. The pellet was resuspended in 10 ml of a predetermined dilution of Ab and incubated at 4°C for 20 min in the dark. The cells were washed twice and resuspended in $1 \times$ PBS. The samples were then analysed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Dead cells were gated out by size (FCS). The percentage of positive cells was calculated after subtraction of background present in the isotype control sample.

Apoptosis Evaluation by Propidium Iodide Solution

Apoptosis was measured by flow cytometry as described elsewhere.[18] Briefly, after culturing, cells were centrifuged and the pellet gently resuspended in 1·5 ml hypotonic propidium iodide solution (PI, 50 mg ml^{-1} in 0·1 per cent sodium citrate plus 0·1 per cent Triton X-100; Sigma). The tubes were kept at 4°C in the dark overnight. The PI-fluorescence of individual nuclei was measured by flow cytometry with standard FACS equipment (Becton Dickinson). The nuclei transversed a 488-nm Argon laser light beam. A 560-nm dichroid mirror (DM 570) and a 600-nm band pass filter (band with 35 nm) were used to collect the red fluorescence from PI DNA staining, and the logarithmic data was recorded using a Hewlett Packard (HP 9000, model 310; Palo Alto, CA) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II).

Glutathione Determination

Measurement of intracellular glutathione was carried out for 24 h on 3DO cell cultures maintained

on six-well dishes at the same concentrations used for apoptosis evaluation (1×10^6 cells m^{-1}). Cells were then washed twice with phosphate buffered saline (PBS), lysed with 0·2 ml of 3 per cent perchloric acid for 20 min at 4°C, centrifuged at 400 *g* for 5 min and the supernatant neutralized with 1·8 ml of 0·1 M NaH_2PO_4 + 5 mM EDTA, pH 7·5.

Intracellular samples were prepared for derivatization according to Ubbink *et al.*[19] Briefly, to 100 µl samples, 10 µl of a 10 per cent solution of Tri-*N*-butylphosphine in dimethylformamide, was added. This mixture was chilled at 4°C for 30 min to reduce thiols and deconjugate them from proteins. After reduction, 100 µl 10 per cent trichloroacetic acid containing 1 mM EDTA was added to the mixture and the sample mixed on a vortex mixer for 1 min. To 100 µl of the supernatant was added a mixture of 20 µl 1·55 M NaOH solution, 250 µl 0·125 M borate buffer, pH 8, containing 4 mM EDTA, 20 µl ABDF solution (1 mg m^{-1} in borate buffer). This was incubated at 70°C for 10 min to derivatize the thiols and then cooled to room temperature.

HPLC

A Gilson pump, Rheodyne injector was connected to a Varian fluorescence spectrophotometer mod. Fluorichrom and a Hewlett Packard integrator. The excitation wavelength was 385 nm and emission wavelength 515 nm. The mobile phase, pumped at 1·5 m min^{-1}, was 0·05 M potassium dihydrogenphosphate (adjusted to pH 3 with orthophosphoric acid) containing a 10 per cent concentration of acetonitrile.

RESULTS

Figure 1 shows the number of viable cells of 3DO, a murine lymphocyte hybridoma cell line measured after 24 h pre-treatment with the thiol-containing antioxidant NAC or the GSH-depleting agent BSO. Both agents decreased the number of viable cells evaluated by Trypan Blue exclusion, even though viability of the two agents was ⩾98 per cent at all concentrations used (data not shown). This contradictory evidence could be due to the probable induction of apoptotic death by NAC and BSO as Tsai demonstrated.[20]

The 3DO intracellular GSH content was also evaluated by HPLC (Figure 2) after 24 h pre-treatment with NAC or BSO at two different concentrations. Both 5 and 50 µM of BSO dramatically

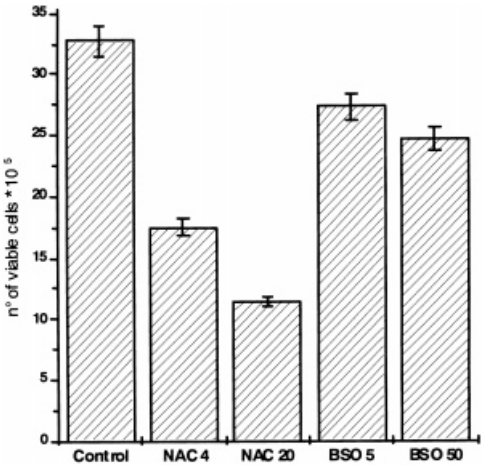

Figure 1. Number of viable cells measured by Trypan Blue exclusion after 24 h of NAC (4 mM, 20 mM) or BSO (5 µM, 50 µM) treatment. At any concentration of NAC or BSO used the viability was ⩾98 per cent. Values are means ± SEM of three independent experiments.

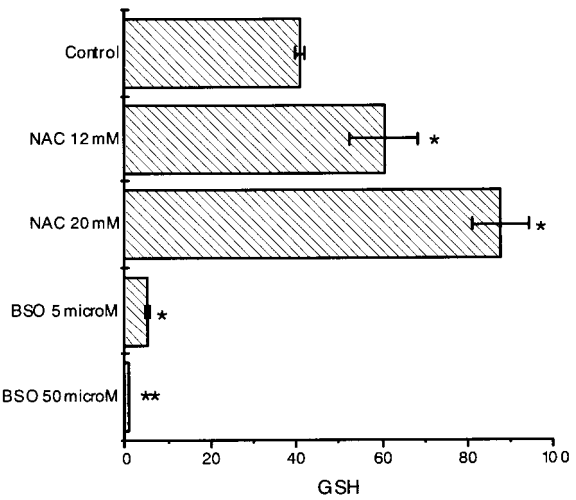

Figure 2. The effect of 24 h exposure to NAC or BSO on intracellular GSH content (µM) per 10^6 cells. Values are means ± SEM of six experiments. Marked values were: * $p \leqslant 0{\cdot}01$; ** $p \leqslant 0{\cdot}005$ significantly different with respect to the control (Student's *t*-test).

decreased the intracellular GSH content, whereas 12 and 20 mM NAC increased it by 1·48 and 2·1-fold respectively.

It is well known that low H_2O_2 concentrations can induce cell death by apoptosis rather than necrosis.[21] We tested (Figure 3) whether treating a 3DO cell line with 20 mM NAC or 50 µM BSO for 24 h could modify apoptosis, which occurs in the

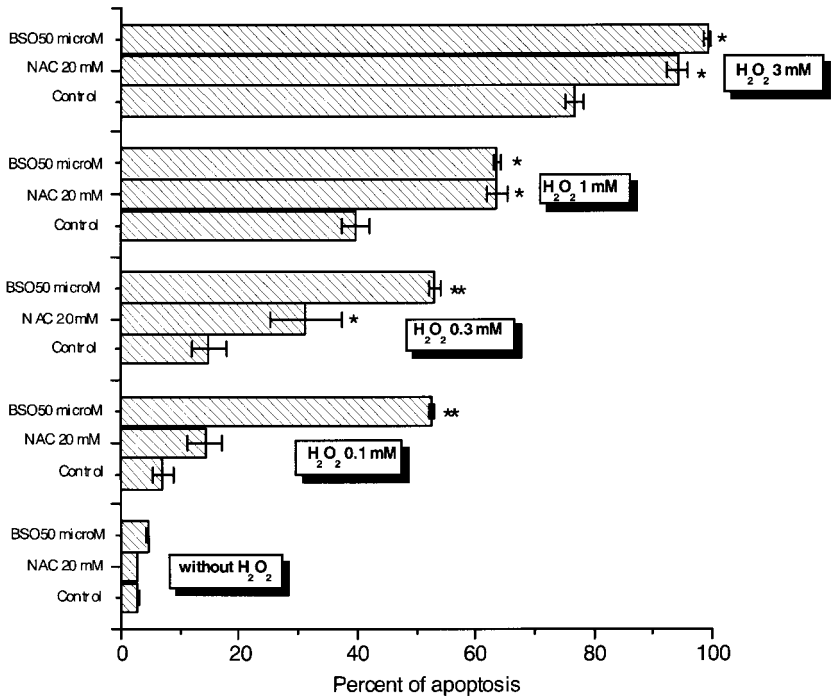

Figure 3. Pre-treatment with NAC or BSO for 24 h before addition of H_2O_2 for 1 h. After H_2O_2 washing, the cultures were kept for another 24 h before determining apoptosis by the PI method. Values are means ± SEM of three different experiments. Marked values were: * $p \leqslant 0{\cdot}01$; ** $p \leqslant 0{\cdot}005$ significantly different with respect to the control (Student's *t*-test).

cells after 1 h of H_2O_2 treatment. Surprisingly and in contrast with other findings[22–24] regarding the well-documented radical scavenger NAC activity, we demonstrated that 24 h pre-treatment with 20 mM NAC significantly increased apoptosis in 3DO cells after 1 h exposure to H_2O_2 at 0·3, 1 and 3 mM. Nevertheless these apoptotic values were lower than those revealed after 24 h pre-treatment with 50 μM BSO. The apoptotic value was measured 24 h after H_2O_2 treatment and neither NAC nor BSO when used alone enhanced apoptosis.

To verify if the modality of NAC treatment is important for modifying its capacity to enhance H_2O_2 apoptosis, a 3DO cell line was placed in contact with H_2O_2 or NAC for different exposure times before measuring apoptosis by the PI method. When NAC was added after H_2O_2 treatment, there was always an increase in the percentage of hypodiploid nuclei. After lengthy treatment with H_2O_2 the value of apoptosis was higher than after 1 h treatment at the same concentrations. When NAC was added together with H_2O_2 there was no change or a slight decrease in the apoptotic value. In the latter case NAC could have exercised its direct reducing role on H_2O_2.

We also determined if the other mechanism of apoptotic lymphocyte death due to Fas–FasL triggering could also be modified by 24 h pre-treatment with NAC or BSO. Figure 4 shows that 3DO cell line apoptosis, determined by the PI method evaluated after 24 h mo-antiFas engagement, was enhanced by pre-treatment with either 20 mM NAC or 50 μM BSO.

For this reason we determined if NAC could modify the expression of Fas or FasL in these cells. The histograms in Figure 5 show that neither the 24 h pre-treatment of 3DO cells with NAC or BSO modifies the expression of Fas or FasL in these cells. Conversely 1 h of H_2O_2 treatment can upregulate the FasL expression evaluated 24 h after the 1 h H_2O_2 contact.

The kinetics of FasL expression on 3DO cells (Figure 6) shows that 24 h pre-treatment with 20 mM NAC can enhance FasL expression in the cells treated for 1 h with H_2O_2 3 mM. The apoptotic value was only evaluated 3 h after H_2O_2 treatment. The FasL expression remained higher in the NAC pre-treated samples than in samples treated with 3 mM H_2O_2 alone, when FasL expression was evaluated both at 6 and 10 h after H_2O_2 treatment. This early

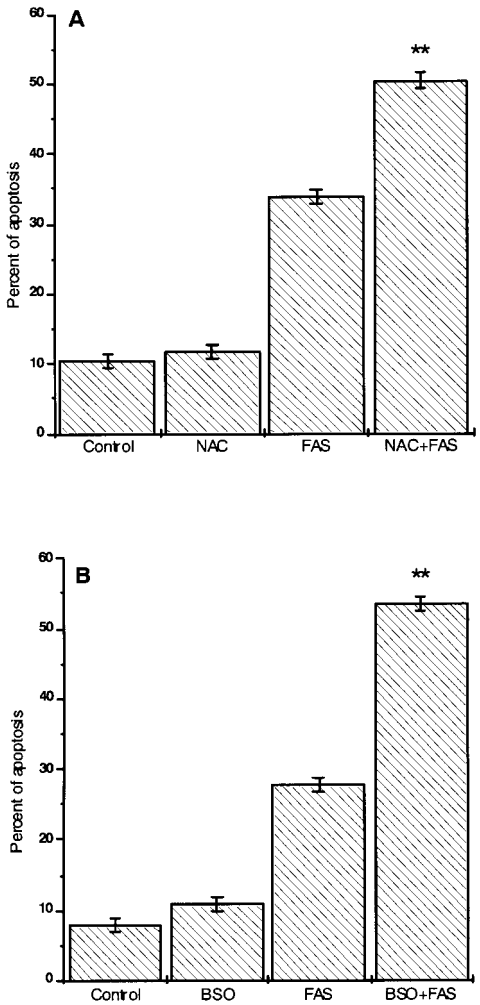

Figure 4. Apoptosis by PI method in a 3DO cell line pre-treated with 20 mM NAC (A) or 50 μM BSO (B) 24 h before anti-Fas- moAbJo-2. Values are means $\pm$ SEM of three different experiments. Marked values were: $^{**}p \leqslant 0{\cdot}005$ significantly different with respect to the control (Student's *t*-test).

FasL expression after pre-treatment with NAC could account for the enhanced apoptotic death, which was the result in this study.

DISCUSSION

Apoptosis is not a traumatic physiological means by which the cells die without any damage to the neighbouring cells. The apoptotic process can be divided into at least three functionally distinct phases: initiation, effector and degradation.[24] Many heterogeneous death-inducing stimuli during the

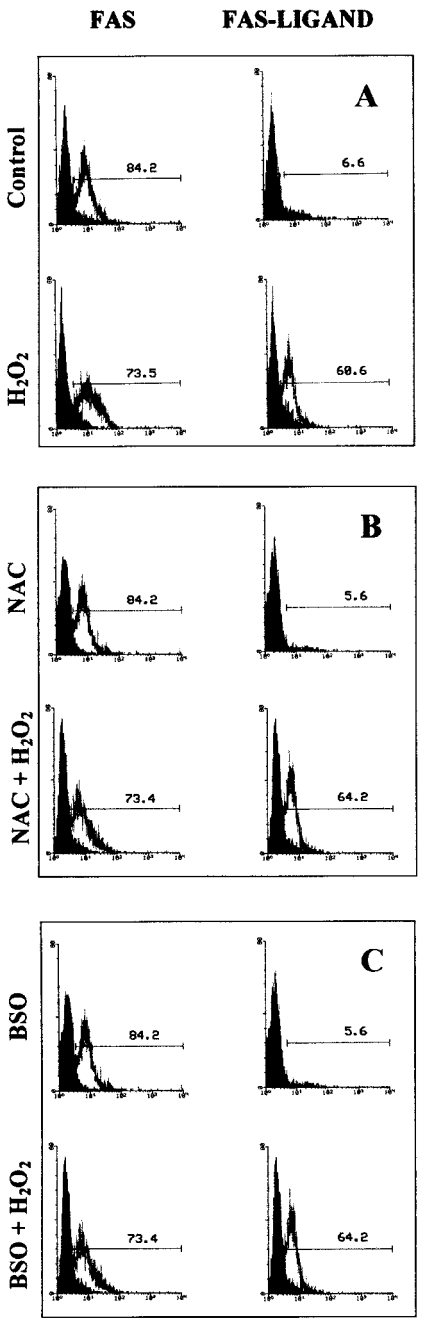

Figure 5. Fas and FasL expression in a 3DO cell line, 24 h pre-treated with 20 mM NAC or 50 μM BSO before 1 h 1 mM H_2O_2 treatment. Evaluation of Fas and FasL expression was done 24 h after H_2O_2 treatment using PE-labelled anti-Fas moAb and purified anti-FasL plus FITC mouse antirabbit IgG. Histogram overlay from three independent experiments, represents fluorescence data of Fas or FasL (white) versus negative control (black) staining. Data are representative of three independent experiments with similar results.

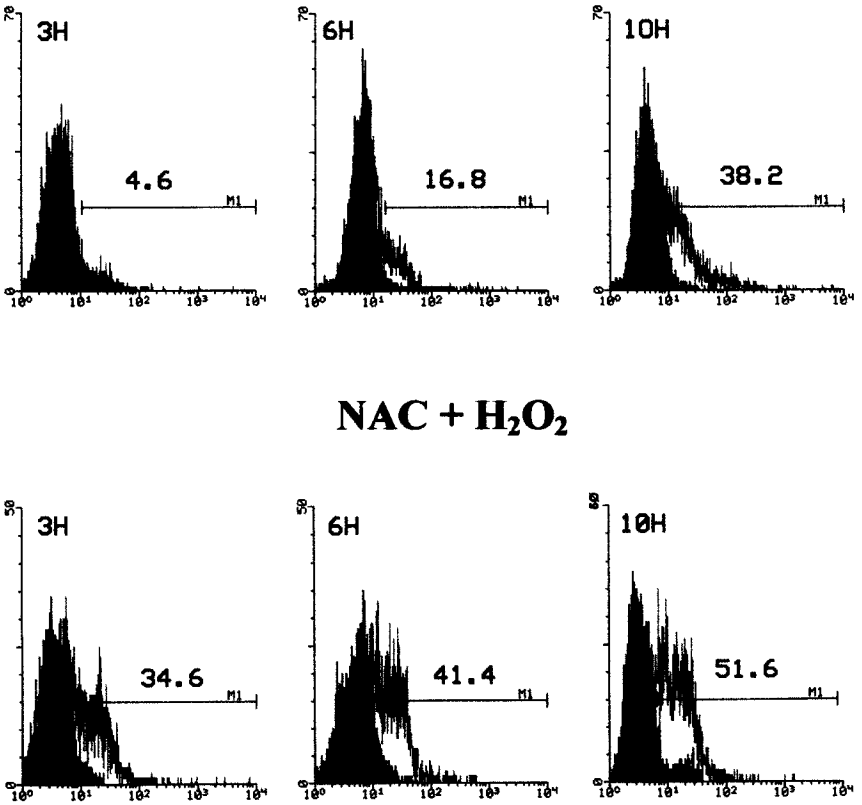

Figure 6. Kinetics of FasL expression in a 3DO cell line 24 h pre-treated with 20 mM NAC before 1 mM H_2O_2 treatment. Purified anti-Fas and FITC anti-rabbit IgG were added 3, 6 and 12 h after H_2O_2 treatment. Data are expressed in the form of a fluorescence histogram overlay depicting specific FasL plus FITC mouse antirabbit IgG staining (white) versus only FITC anti-rabbit IgG (black) control staining. Data are representative of three independent experiments with similar results.

initiation phase can contribute to triggering this programmed cell death and today there is agreement about the importance of the cellular oxidoreductive balance status which permits or inhibits this planned cell death.

It is recognized that oxidative stress may be a common mediator of apoptosis.[25,26] Many treatments known to elicit oxidative stress can also induce apoptosis and one of these is exposure in a variety of cell types to low doses of H_2O_2.[21] Certainly, the capacity of H_2O_2 to induce apoptotic death is significant when the level of H_2O_2 is physiologically relevant, such as during a local immune response in which many neutrophils and macrophages, normally secreting H_2O_2, might be recruited into a small area.

The Fas–FasL system is an important apoptosis mechanism because of its role in regulating the normal immune response and maintaining self-tolerance. Fas was originally described as a cell surface molecule which could mediate apoptotic cell death in transformed cells but today the main studies are on its expression in immunological cells. Stimulation of previously activated T cells through the T cell-receptor complex by viral antigen induces the expression of the FasL gene. FasL expression on the surface of the effector cells will be important for binding Fas on the target cells and to induce them to undergo apoptotic cell death, in a process termed activation induced cell death (AICD). T cells from either lpr/lpr or gld/gld mice, defective in genes encoding for Fas and FasL respectively, do not undergo AICD and therefore the Fas–FasL system is implicated in this process.[27] It has been suggested that AICD is important not only for eliminating autoreactive T cells that have escaped thymic selection, but also in the course of the effector cell response and later in eliminating lymphocytes, which are no longer needed.[28]

A large panel of stimuli (antigen and co-receptor-

mediated activation, cytokines, hormones, cytotoxic drugs, physical damage, etc.) that deplete lymphocytes *in vivo* also cause lymphocyte apoptosis *in vitro*.[29,30] It has been shown that scavengers of free radicals, intracellular thiol reductants, thiol antioxidants and membrane peroxidation inhibitors all inhibit apoptosis.[2]

In disagreement with data obtained by others we found that pre-treatment with NAC, even though increasing intracellular GSH content (Figure 2) and inhibiting cell proliferation (Figure 1), like other antioxidants,[31] does not protect against H_2O_2-induced apoptosis, but on the contrary increases it. The different conditions for NAC or H_2O_2 treatment and the different types of cells used could explain these contrasting data. However, under the same conditions, pre-treatment with BSO, an agent that completely inhibits GSH intracellular content, increased H_2O_2 apoptosis in 3DO cells.

For this reason we suggest that intracellular GSH content is not important for the NAC mechanism which increases apoptotic death. It has been shown that NAC can have different effects on apoptosis, independent of changes in either the number of T cells or the redox state of intracellular GSH.[32] Our data suggest that NAC is less toxic when used together with H_2O_2 or if it remains in the unwashed culture medium after 24 h of treatment (data not shown), thus demonstrating its direct reducing effect on H_2O_2 as described elsewhere.[33]

The possible NAC interaction with the apoptotic death mechanism triggered by crosslinking with Fas antibody was evaluated. Not even in this case were these antioxidant drugs able to inhibit apoptosis in 3DO cells (Figure 4).

This study shows that NAC could change the kind of 3DO cell death from necrotic to apoptotic. The number of dead cells counted by Trypan Blue assay was the same as in samples pre-treated with NAC or those treated with H_2O_2 alone (data not shown). For this reason the possibility that NAC and H_2O_2 treatment might influence the expression of Fas or FasL mediators of apoptotic death was investigated. FasL expression is of fundamental importance for eliminating activated T cells no longer needed in the course of immune response.[28]

The data suggest that neither NAC nor BSO when used alone, modify Fas or FasL expression. On the contrary 1 h of H_2O_2 treatment can upregulate FasL expression in 3DO cells tested after 24 h. Therefore the function of H_2O_2 produced by polymorphonucleated cells during infection could also, among other functions, be responsible for the integration with phospholipase A_2. This would make the cells accessible through a new mechanism[30] and also modulate apoptosis induced by T lymphocytes *in situ*, bypassing the activation process. This apoptosis mechanism could be useful for eliminating the activated but useless T lymphocytes. The data from analysis of FasL kinetic expression on 3DO cells showed that, 24 h of NAC pre-treatment resulted in an early expression of FasL on the membrane, i.e. after only 3 h H_2O_2 exposure. The 34 per cent of cells expressing FasL so early could be a possible explanation for the higher apoptotic value observed. This interpretation would agree with some of the present treatments used to reduce inflammation by antioxidant drugs,[34] which operating in an H_2O_2-rich environment could change necrotic death to the safer apoptotic death of immune cells which are no longer necessary.

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