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N-Acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells

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Apoptosis or programmed cell death (PCD) is a type of death occurring in various physiological processes. Several data suggest that: (1) apoptosis may play a critical role in AIDS pathogenesis; (2) an increase of endocellular free radical levels can be associated with activation of previously latent HIV virus. Tumor necrosis factor (TNF), a cytokine capable of inducing oxygen free radicals and apoptosis, appears also to be involved in HIV activation. The present findings, which elucidate a relationship between the percentage of apoptotic cells, reduced glutathione (GSH) depletion and an increase of p24 antigenemia, suggest that pretreatment with *N*-acetylcysteine (NAC) is capable of decreasing the above-mentioned phenomena in HIV-infected U937 cells.

Apoptosis; Human immunodeficiency virus (HIV); Tumor necrosis factor (TNF); Reactive oxygen intermediate (ROI); Reduced glutathione (GSH), *N*-Acetylcysteine (NAC)

1. INTRODUCTION

The progression to the more advanced stages of AIDS (acquired immunodeficiency syndrome) is characterized by increased: (a) viremia, (b) p24 antigenemia, (c) number of infected cells, and (d) immune dysfunction [1]. These findings suggest that the evolution of HIV infection is associated with an increased activation of latent virus [2]. It has also been postulated that endocellular free radical production, especially reactive oxygen intermediates (ROI), can be involved in HIV activation and consequent replication via the NF-kB transcription enhancer factor in chronically infected T cells [3]. Various substances, including tumor necrosis factor (TNF), a cytokine also involved in ROI production and programmed cell death (PCD) [4], are responsible for NF-kB activation. PCD, also termed apoptosis, is a physiological type of death alternative to necrosis and occurs when the cell actively participates in its own death process [5]. In contrast to necrosis, apoptosis is accompanied by membrane and organelle integrity, condensation and fragmentation of nuclear chromatin in multiples of 200 base pairs [6]. Recently, it has been hypothesized that PCD may play a critical role in AIDS pathogenesis [7]. The aim of the present work was to investigate: (1) the relationship between PCD, oxidative stress and the increase of viral progeny in HIV-chronically infected cells; (2) the contribution of viral particles to PCD; (3) the role of an antioxidant, such as *N*-acetylcysteine (NAC), in the modulation of the above-mentioned phenomena.

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2. MATERIALS AND METHODS

2.1. *Cells*

Uninfected and HIV-infected U937 cells, a monomyelocytic human cell line, were grown at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland, UK), 1% non-essential amino acids, 5 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were subcultured in 25 cm^2 Falcon plastic flasks or wells at a density of approximately 10^5 cells/ml and placed in a 37°C incubator containing a 95% air/5% CO_2 atmosphere. Cells were infected with HIV virus as previously reported [8].

2.2. *Treatments*

Uninfected and HIV-infected cells were treated with 50 U/ml of TNF-α (Sigma, final purity ≥ 95%) for 24, 48, or 72 h. For the NAC studies, U937 cells were pretreated with 15 mM of this antioxidant for 2 h and left in the medium for the entire time of TNF treatment. In other cases, in addition to the 2 h pretreatment with NAC and in order to make certain that this agent did not lose its strength, NAC (15 mM) was again added to the cells at 24 h and 48 h. In order to evaluate differences between necrosis and apoptosis on DNA fragmentation, two agents able to induce necrosis (200 μM menadione for 2 h and 5% *N*-methylformamide for 2 h) were used (negative controls).

2.3. *Biochemical analyses*

2.3.1. DNA gel electrophoresis

In order to separate high molecular weight chromatin from the nucleosomal DNA fragments, cells were washed in PBS and lysed in 0.5 ml of lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA pH 8.0, 0.2% Triton X-100) containing Proteinase K (100 μg/ml) for 1 h at room temperature and were centrifuged at 13,000 rpm for 15 min. DNA in the supernatant was precipitated with 5 N NaCl in 1 vol. of isopropilic alcohol for 1 h at −20°C. The DNA precipitates were recovered by centrifugation at 13,000 rpm for 15 min, were air-dried for 15 min at room temperature, resuspended in TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA, pH 8.0) containing RNAse (100 μg/ml) and incubated

for 1 h at 37°C. Before electrophoresis, loading buffer (2% SDS, 15 mM EDTA, 0 25% w/v Bromophenol blue, 50% v/v glycerol) was added to each sample in a 1:5 ratio. DNA was separated overnight by electrophoresis on 1% agarose gel containing 0.5 μg/ml ethidium bromide at 25 V in TBE buffer (2 mM EDTA, pH 8.0, 89 mM Tris, 89 mM boric acid). A *Hin*dIII digest of λ-DNA provided molecular weight standards. Following electrophoresis, DNA was visualized by transilluminator and photographed by a Polaroid camera.

2.3.2. Determination of reduced glutathione (GSH)
Intracellular GSH level was measured as acid-soluble thiols using a colorimetric assay [9].

2.3.3 Determination of p24 antigenemia
Supernatants were tested for HIV p24 antigen by an antigen capture ELISA kit (American Bio-Tecnologies Inc., Cambridge, USA).

2.4. *Fluorescence microscopy*
In order to evaluate PCD, in addition to gel electrophoresis, the chromatin dye Hoechst 33258 was used to evaluate DNA fragmentation [10]. After washing, all the samples were mounted with glycerol/PBS (1 1) and observed with a Nikon Microphot fluorescence microscope. Quantitative evaluation of apoptotic cells was performed by counting 200 cells at a 50× magnification.

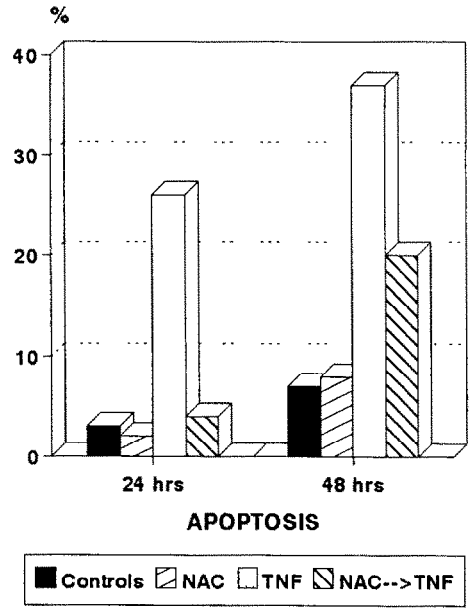

Fig. 2. Percentage of apoptosis in U937-infected cells after 24 and 48 h of exposure to NAC and TNF.

3. RESULTS

3.1. *PCD analysis*

In the experimental conditions described above, TNF was able of inducing significant PCD only in the HIV-infected U937 cultured cells as detected by gel electrophoresis (Fig. 1). In fact, after 24 and 48 h, when a quantitative evaluation of apoptotic cells was performed by light microscopy, the percentages of apoptotic cells in the samples treated with TNF alone were, respectively, 25% and 38% of the total cell number (Figs. 2, 3a, 3b). Furthermore, at the concentrations used, TNF was probably capable of inducing apoptosis in all cells after 72 h. However, the presence of numerous apoptotic bodies together with cell debris rendered the evaluation of the exact number of apoptotic cells by light microscopy impossible at this time period (Fig. 3c). In contrast, uninfected U937 cells did not show significant PCD, which in fact remained under 5%. In addition, it was evident that pretreatment with 15 mM NAC for 2 h markedly inhibited the progression of PCD in samples exposed to TNF for 24 and 48 h (Fig. 2). Such a 'protection' could not be observed after 72 h in view of the difficulties described above in the quantification of apoptotic cells in the control samples. However, intact nuclei in these samples were about 30%.

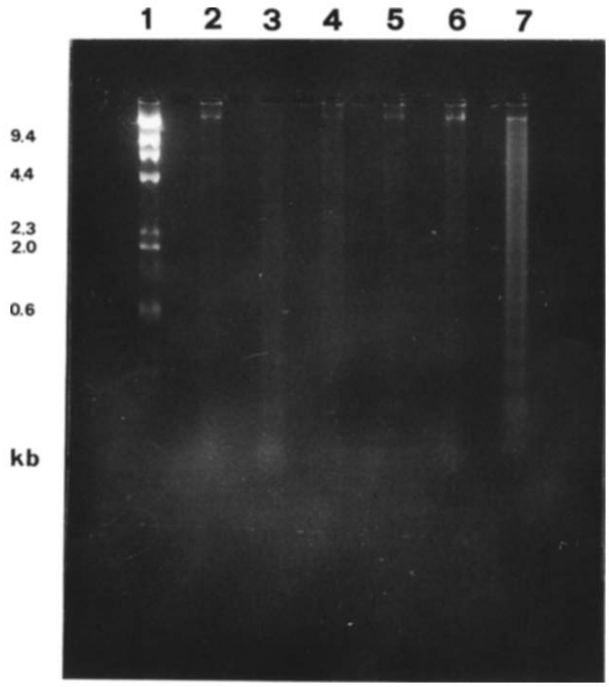

Fig 1. DNA fragmentation in U937-infected cells. Low molecular weight DNA was isolated from the supernatant obtained after a 13,000 rpm centrifugation of untreated cells (lane 4), NAC 24 h (lane 5), NAC 2 h→TNF 22 h (lane 6) and TNF 24 h (lane 7). Cells exposed to menadione (lane 2) and *N*-methylformamide (lane 3) were used as negative controls A *Hin*dIII digest of λ-DNA provided molecular weight standards (lane 1).

3.2. *Intracellular GSH level*

In view of the specific effect of the antioxidant NAC, considering the role of oxygen free radical production in TNF subcellular activity [3], and in order to obtain information about the redox-state of cells, specific measurements of intracellular soluble thiols was conducted. The results obtained indicate that intracellular thiol levels appeared to be decreased slightly with respect to those of controls when TNF-treated U937-infected cells were preloaded with NAC. However, cells treated with TNF alone showed a marked decrease of thiol groups, particularly at 48 h (Fig. 4).

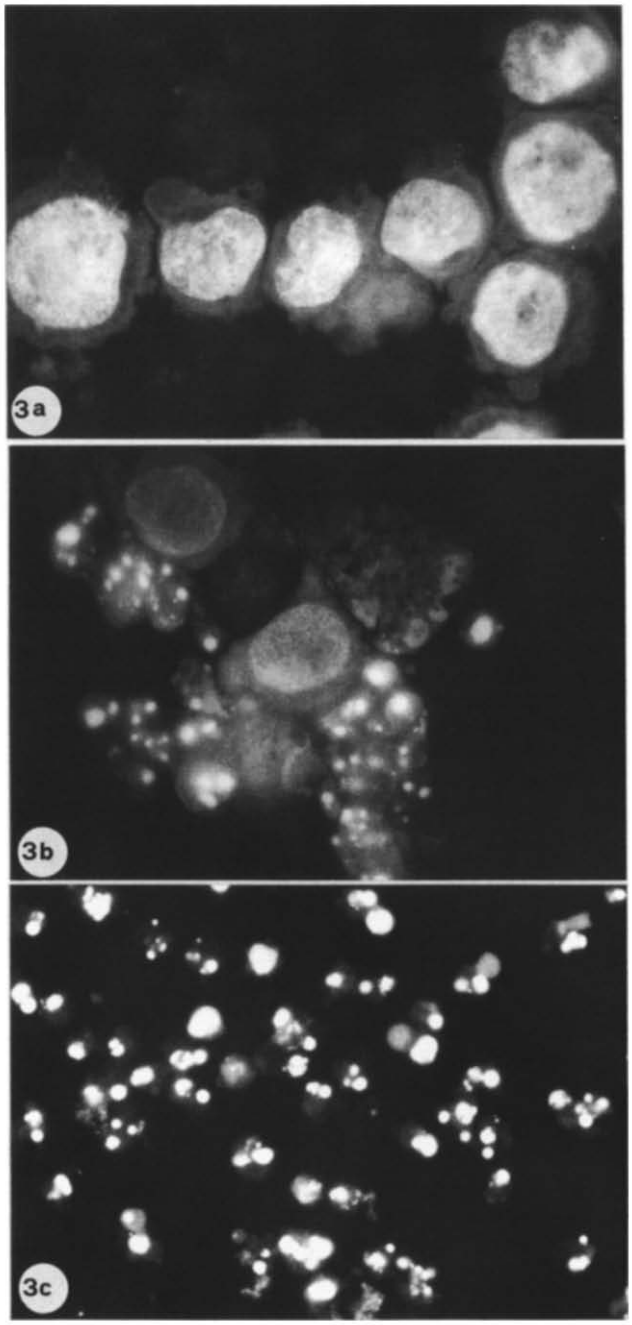

Fig. 3. Evaluation of TNF-induced apoptosis in U937-infected cells by fluorescence microscopy using the chromatin dye Hoechst 33258. (a) Untreated cells; (b) 48 h TNF; (c) 72 h TNF. As can be observed, treated cells undergoing apoptosis show typical condensation and fragmentation of chromatin.

3.3. *Evaluation of HIV particles*

The results reported above are well-substantiated by the parallel evaluation of p24 antigenemia (the number of HIV mature viral particles produced in a fixed number of U937 cultured cells). In fact, a significant decrease of this viral protein was found only in cells treated daily with NAC, especially after 48 h of treatment, when compared to cells exposed to TNF alone (Fig. 5).

4. DISCUSSION

The association between the endocellular production of reactive oxygen intermediates and activation of HIV replicative machinery was previously hypothesized [11]. ROI are in fact capable of potently and rapidly activating the multisubunit transcription factor NF-kB which is present in the cytoplasm in a non-DNA-binding form [3,12]. The replication of HIV is under the control of NF-kB [2,13] and the activation of this factor can be induced by some oxidizing agents as well as by many other substances, including the cytokine TNF [4,14]. This cytokine which induces cell injury by production of ROI [15], has also been shown to induce an increase in HIV expression [16]. GSH is an important intracellular defense against ROI production and an excess of these may result in GSH depletion. Thus, the previously hypothesized thiol suppression of HIV replication [17] could be related to the 'protection' induced by NAC reported in this paper. It may be the result of such thiol group restoration, the renewed supply of which may counteract the effect of oxygen free radicals produced by TNF and activation of HIV via NF-kB.

TNF was also demonstrated to be capable of inducing apoptosis [18], the programmed cell death recently associated with HIV pathogenesis [7]. Our experimental model seems to indicate that antioxidant preloading can 'protect' cells from thiol intracytoplasmic imbalance and contribute to an impairment of the apoptotic program. This was previously suggested by using other experimental models [19] and could probably be ascribed to the specific role of ROI in DNA damage as well as in metabolic changes due to the depletion of intracellular thiols [20]. In conclusion, our findings seem to indicate that: (1) oxidative imbalance can play an

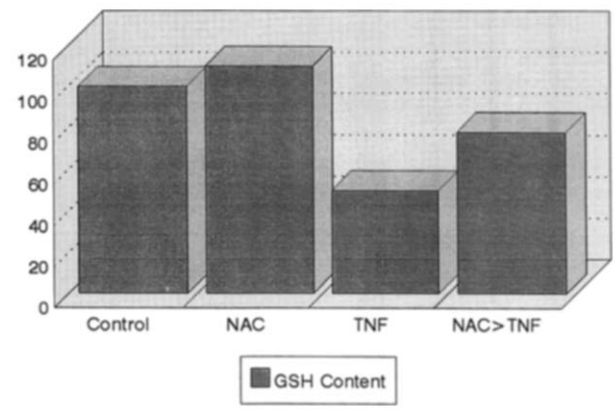

Fig. 4. Evaluation of relative GSH content in U937-infected cells. Results are expressed as percentage of controls (32.4 nmol/10^6 cells), which, as can be seen, are considered as 100%. The means of three separate experiments are represented. Variations between each experiment were less than 10%.

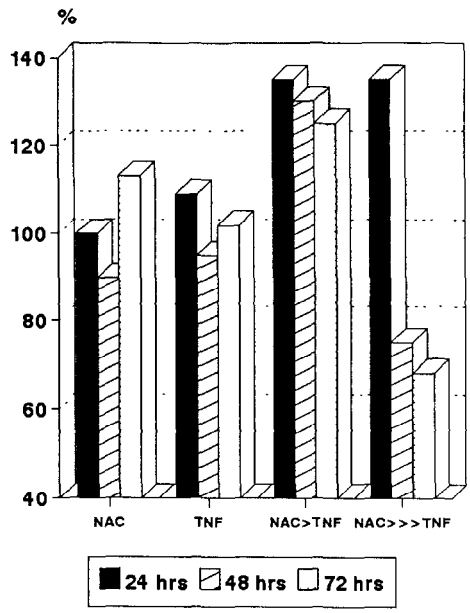

Fig. 5. Evaluation of p24 antigenemia of supernatant of U937-infected cells after 24, 48 and 72 h of treatment using an antigen capture ELISA kit. Control values were 52, 88, 102 ng/ml, respectively. Each data point is expressed as the percentage of its respective control which was considered as 100% The means of three separate experiments are represented. Variations between each experiment were again less than 10%. NAC >TNF: 2 h pretreatment with NAC; NAC >>> TNF: 2 h pretreatment plus daily exposures to NAC.

important role in TNF-mediated apoptosis, (2) this imbalance can be essential for HIV viral progeny formation, and that (3) a partial interference with this complicated machinery can be obtained by using specific antioxidative agents.

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