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# Involvement of oxygen radicals in cytarabine-induced apoptosis in human polymorphonuclear cells

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**Abstract**

We investigated apoptosis in polymorphonuclear neutrophils (PMNs) induced by cytarabine (Ara-C). This drug increased apoptosis by 100% with respect to the controls after 3 hr of incubation. This increase was inhibited by *N*-acetyl-L-cysteine (NAC) or diphenyleneiodonium chloride (DPI). Ara-C alone caused an early increase (after a 30-min incubation) in intracellular oxidant generation (inhibitable by rotenone, fumonisin b1, and DPI) and in protein tyrosine phosphorylations (inhibitable by NAC). The drug also affected the observed reduction of dimethylthiazol diphenyltetrazolium bromide (MTT). No extracellular release of reactive oxygen species (ROS) was elicited by the addition of Ara-C, while the drug increased the release of ROS by *N*-formyl-leucyl-phenylalanine-(f-MLP) but not phorbol 12-myristate 13-acetate-stimulated PMNs. This phenomenon was abolished by the addition of genistein, whereas such an effect was not observed following the addition of 1-(5-isoquinolynilsulfonyl)-2-methylpiperazine (H7). Ara-C induced ROS release from PMNs in the presence of subthreshold concentrations of f-MLP (priming effect). These results indicate that intracellular ROS production from mitochondria promotes Ara-C-induced apoptosis. Ara-C primes plasma membranes by a mechanism involving protein tyrosine phosphorylations and may also contribute to ROS generation from the granules. 

*Keywords:* Antileukemic drugs; Tyrosine phosphorylations; Mitochondria

## 1. Introduction

Apoptosis is a form of physiological cell death regulated by a cell-intrinsic machinery [1]. Morphological findings include cell shrinkage, condensation and fragmentation of the nucleus, and cleavage of chromosomal DNA, resulting in generation of a characteristic pattern of DNA fragmentation on electrophoresis. Blebs occur on the cell surface and phosphatidylserine is exposed on the outer surface of the plasma membrane, providing the recognition signal for the engulfment by phagocytes of superfluous or damaged cells. The process is activated in response to specific stimuli or following various forms of cell injury or stress [2]. Several physiological stimuli, including tumor necrosis factor receptor, Fas, and heat shock proteins, have been demonstrated to induce apoptosis in different cell types by initiating a cascade in which caspases are activated by proteolysis, leading to enzyme cleavage and nuclear DNA fragmentation [2,3].

A variety of evidence indicates that ROS may be involved in the apoptotic processes. Indeed, the external signals leading to apoptosis are known to induce oxidative stress. Ionizing and ultraviolet irradiations or anticancer drugs are able to induce apoptosis and to generate ROS. The type of ROS involved and the site of their generation are not completely known. The mechanism(s) by which ROS are involved in apoptosis activation is controversial. ROS have

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*Abbreviations:* ROS, reactive oxygen species; PMNs, polymorphonuclear neutrophils; SOD, superoxide dismutase; Ara-C, cytarabine; Ara-CTP, cytarabine 5′-triphosphate; $O_2^-$, superoxide anion; $H_2O_2$, hydrogen peroxide; f-MLP, *N*-Formyl-Met-Leu-Phe; PMA, phorbol 12-myristate 13-acetate; cyt *c*, cytochrome *c*; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue); MoAb, monoclonal antibody; NAC, *N*-acetyl-L-cysteine; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein; DCFH, dichlorofluorescin; and DPI, diphenyleneiodonium chloride.

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been proposed to induce apoptosis directly or through the redox status of cells [4–7].

Mature PMNs can undergo apoptosis without requiring any apparent inductive stimuli. Moreover, when PMNs are damaged by infectious agents, drugs, irradiations, or cytokine activation, they may die a programed death [8]. PMN apoptosis may also be regulated by cytokines such as granulocyte-macrophage colony-stimulating factor or by materials derived from platelets or red cells [9,10]. Since PMNs generate large amounts of ROS, several recent studies have focused on the role of oxidative stress in inducing apoptosis in these cells. PMNs generate ROS in the course of the respiratory burst, a process that is associated with oxygen consumption and involves production of $O_2^-$ by NADPH oxidase [11]. This radical dismutates in $H_2O_2$ and forms toxic agents such as hypochlorous acid and chloramine. In resting cells, three components—p40phox, p47phox and p67phox—exist in the cytosol. Two other components—p22phox and p91phox—are located on the membranes of specific granules and secretory vesicles, e.g. a flavohemoprotein known as cytochrome $b_{558}$ [11]. When the cells are stimulated, the cytosolic complex migrates to the membrane, where it is associated with cytochrome $b_{558}$. Apoptosis is, in fact, blocked in neutrophils treated with flavoenzyme inhibitors or in PMNs from patients affected with chronic granulomatous disease [8]. Moreover, activated PMNs use $H_2O_2$ formed by NADPH oxidase to commit suicide [12]. PMN apoptosis is inhibited under hypoxia, which decreases ROS generation [13]. It has been shown in myeloid leukemic cells that ROS derived from the respiratory burst play a role in chemotherapeutic drug-induced apoptosis [14]. SOD and catalase have been shown to delay spontaneous apoptosis of PMNs [15], and it has recently been reported that intracellular generation, but not extracellular release of ROS, leads to apoptosis of PMNs [12]. Activation of caspase via ROS generation has been demonstrated in oral neutrophils [16]. On the other hand, it has been reported that oxidative metabolism is decreased during apoptosis of PMNs [17]. It has been suggested that excessive oxidative stress can prevent caspase activation, and that NADPH oxidase-derived oxidants may indeed prevent the activation of these proteases [18]. Caspase possesses an active site cysteine that is predicted to be susceptible to oxidation [19], even if the presence in these cells of a novel oxidant-dependent mechanism of programed death independent of caspase activation could not be ruled out. In apparent contrast with data reported previously, acceleration of apoptosis is observed in PMNs exposed to externally produced ROS, as compared to those undergoing spontaneous apoptosis. It has been suggested that hydroxyl radicals, generated by the reaction of $H_2O_2$ with iron, may mediate apoptosis [20]. Moreover, it is also possible that reductive stress may constitute a signal for apoptosis in PMNs, as has been shown in $H_2O_2$-mediated apoptosis [21]. Since aerobic cells may produce ROS via mitochondrial respiratory enzymes or many other types of enzyme systems, such as the arachidonic acid pathway, microsomial cytochrome systems, and DNA synthesis, the source of oxidants that participate in apoptosis of PMNs demands further investigation.

Ara-C is the most important antimetabolite used for induction of remission in acute leukemia in children and adults. It is the single most effective agent for induction of remission in the therapy of acute myeloid leukemia. As Ara-C is primarily toxic to rapidly dividing tissues, myelosuppression and gastrointestinal toxicity represent the major side effects of this agent. Cellular responses to the effects of Ara-C on DNA replication in tumor cells include the induction of apoptosis [22].

The first aim of the present paper was to study whether Ara-C could also induce apoptosis in non-dividing cells. Secondly, we investigated whether Ara-C-induced apoptosis is mediated by ROS. The apoptosis of human PMNs triggered by this drug could be used as a model for addressing these points. We demonstrate herein that Ara-C triggers a ceramide-mediated ROS production from mitochondria. ROS, in turn, induce tyrosine phosphorylations. In this study, we also observed that Ara-C primes the respiratory burst of PMNs to produce $O_2^-$ upon f-MLP stimulation, possibly via tyrosine phosphorylations.

## 2. Materials and methods

### 2.1. Reagents

Ara-C was obtained from Upjohn. PBS pH 7.4, Ara-CTP, PMA, f-MLP, hystopaque 1077 and hystopaque 1119, cyt *c* from horse heart, SOD (EC 1.15.1.1) from horseradish, H7, genistein, fumonisin b1, rotenone, DPI, RPMI-1640, fetal bovine serum, L-glutamine, penicillin, streptomycin, NAC, MTT, propidium iodide, Triton X-100, Tween 20, ribonuclease A, Hanks' balanced salt solution, 5-fluorouracil, $NaN_3$, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were all purchased from Sigma Chemical Co. DCFH-DA was obtained from Molecular Probes. All reagents for SDS–PAGE were obtained from Bio-Rad. Purified antiphosphotyrosine MoAb 4G10 and peroxidase-conjugated anti-mouse antibody (NA 931) were purchased from Amersham Life Science.

### 2.2. Isolation and incubation of PMNs

PMNs were isolated from the venous blood of consenting healthy volunteers by a double-gradient Ficoll-Hypaque and hypotonic lysis of contaminating red blood cells, as previously described [23,24]. PMN viability (95%) was assessed by trypan blue exclusion. PMNs were resuspended in RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 $\mu$g/mL of streptomycin, and incubated at a density of $10^6$/mL at 37° in a humidified incubator contain-

ing 5% $CO_2$ in air for various times, in the absence (control) or the presence of $10^{-5}$ M Ara-C. Where appropriate, PMNs were preincubated with the indicated concentrations of inhibitors 30 min prior to the addition of Ara-C. At stated time points, aliquots of cell suspensions containing $10^6$ PMNs were withdrawn, and cells were then washed twice and resuspended in PBS before testing.

### 2.3. Flow cytometric analysis of DNA fragmentation (apoptosis)

Samples of PMNs incubated in the presence or absence of Ara-C were withdrawn at time intervals of 3, 6, and 24 hr. Apoptosis was determined by flow cytometry as previously described [25]. Briefly, the washed cells were treated overnight at 4° in the dark with hypotonic fluorochrome solution containing 50 $\mu$g/mL of propidium iodide, 250 $\mu$g/mL of ribonuclease A, 0.1% Triton X-100, and 0.1% sodium citrate. The resulting fluorescence of individual nuclei was analyzed by a flow cytometer (FACScan, Becton Dickinson). The data were analyzed using the LYSYS II software (Becton Dickinson).

### 2.4. Evaluation of intracellular DCFH oxidation in PMNs

At stated time points, samples of PMNs incubated in the presence or absence of Ara-C were withdrawn to detect the intracellular ROS production by the DCFH method. PMNs were loaded with DCFH-DA [26]. This assay depends on the incorporation of DCFH-DA into hydrophobic lipid regions of the cell, where the acetate moieties are cleaved by esterases yielding the non-fluorescent molecule DCFH. DCFH is trapped due to its polarity within the intracellular granules (myeloperoxidase-positive) and the cytoplasm. ROS are able to oxidize the trapped 2′,7′-dichlorofluorescin to DCF, which is fluorescent in the green area of the spectrum (525 nm). Cells ($10^4$/sample) were analyzed by a flow cytometer. All measurements were done at 510–540 nm after excitation at 488 nm (argon ion laser, 15 mW output) using a 10-nm slit. The collected parameters included forward light scatter (FSC), 90° light scatter (SSC), and green fluorescence (FL1). The data were analyzed using LYSYS II and Consort 30 softwares.

### 2.5. Cytotoxicity assay (mitochondrial dehydrogenase activity)

Samples of PMNs incubated in the presence or absence of Ara-C were withdrawn after 30 min. MTT was dissolved at 5 mg/mL in PBS and used as previously described [27]. Briefly, 20 $\mu$L of MTT solution was added to each well, and the microplates were further incubated at 37° for 4 hr. Supernatants were then discarded, and 200 $\mu$L of acidified isopropanol was added to the incubation mixtures and mixed to thoroughly dissolve the dark blue crystal of formazan. Formazan quantification was performed using an automatic plate reader with a 570-nm test wavelength.

### 2.6. Immunoblot for tyrosine phosphorylations

PMNs incubated with Ara-C for 30 min were washed twice in PBS, lysed in ice-cold lysing buffer (25 mL Dulbecco's phophate buffer, 25 mL of 20% Triton X-100, 5 mL of 10% SDS, 0.5 g $NaN_3$, 12.5 mL NaCl 4 M, phenylmethylsulfonyl fluoride 0.1 M, aprotinin 5 $\mu$g/mL, and leupeptin 10 $\mu$g/mL), sonicated and centrifuged at 13,000 *g* at 4° for 10 min, and assayed for protein concentration (Bio-Rad). Equivalent protein amounts (50 $\mu$g) were loaded for SDS–PAGE on 10% acrylamide gel. Following electrophoresis, samples and molecular weight standards were electrophoretically transferred to nitrocellulose paper and incubated overnight at 4° in a blocking solution containing 5% dry milk in Tris-buffered saline. The blot was then incubated with blotting solution containing a 1:500 dilution of affinity-purified MoAb antiphosphotyrosine 4G10 for 2 hr while shaking at room temperature. The blot was washed three times with Tris-buffered saline containing 0.1% Tween 20. A second incubation was made for 2 hr in PBS containing a 1:10,000 dilution of peroxidase-conjugated anti-mouse antibody and 0.05% Tween 20. Detection of phosphoproteins was performed by the enhanced chemiluminescence system (Amersham). The molecular weights and the amount of the proteins were analyzed by a densitometer (Bio-Rad GS 700) with the Bio-Rad Molecular Analyst program. The specificity of the MoAb was confirmed by demonstrating inhibition of the binding by 1 mM phosphotyrosine but not by phosphoserine or phosphothreonine.

### 2.7. Evaluation of $O_2^-$ release by PMNs (SOD-inhibitable cyt c reduction)

PMNs incubated with Ara-C for 30 min in the presence or absence of genistein (10 $\mu$g/mL) or H-7 (100 $\mu$M) were stimulated with f-MLP ($10^{-7}$ M) or PMA (1 $\mu$g/mL). Extracellular $O_2^-$ production was assessed spectrophotometrically by means of SOD-inhibitable cyt *c* reduction, and a continuous assay was performed in a double-wavelength spectrophotometer (Beckman DU 65) equipped with a thermostated cuvette holder (37°) [28]. Briefly, washed cells were resuspended in Hanks' balanced salt solution, pH 7,4, before they were added to 1-mL cuvettes containing 80 $\mu$M cyt *c* with a final volume and concentration of 0.995 mL and $10^6$ cells, respectively. Reduction of cyt *c* was measured at 550 nm with a reference wavelength of 540 nm. Either f-MLP or PMA was added to the reaction mixture in a final volume of 1 mL. The time-course of cyt *c* reduction was followed on the recorder built into the spectrophotometer. Extracellular $O_2^-$ production was calculated as nmol/$10^6$ PMNs/min.

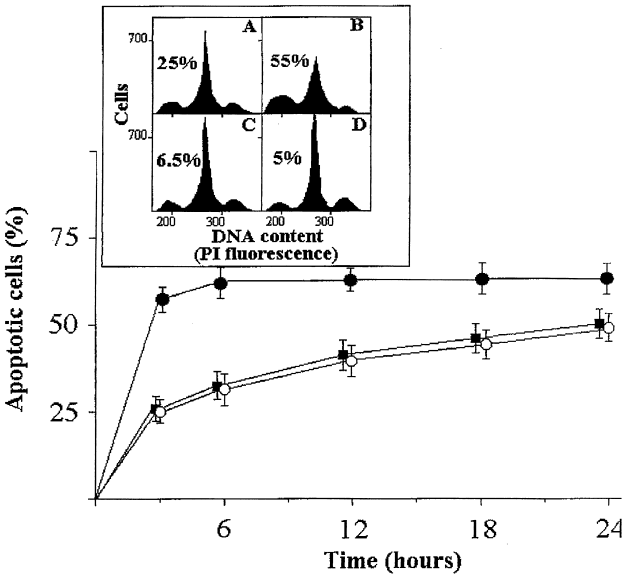


Fig. 1. Induction of apoptosis in PMNs exposed to Ara-C at various times as measured by DNA fragmentation. Spontaneous apoptosis of PMNs (open circles); apoptosis of PMNs in the presence of Ara-C ($10^{-5}$ M) (closed circles) or the presence of Ara-CTP ($10^{-5}$ M) (closed squares). The inset shows the effect of Ara-C on apoptosis of PMNs analyzed after 3 hr of incubation. Panel A: spontaneous apoptosis of PMNs; panel B: PMNs incubated with Ara-C ($10^{-5}$ M); panel C: PMNs treated with Ara-C in the presence of DPI (20 $\mu$M); panel D: PMNs treated with Ara-C in the presence of NAC ($10^{-5}$ M). The percentages of hypodiploid nuclei are reported for each condition. The results are expressed as the means ± SEM (N = 10). The concentration of PMNs was $10^6$ cells/mL in all experiments.

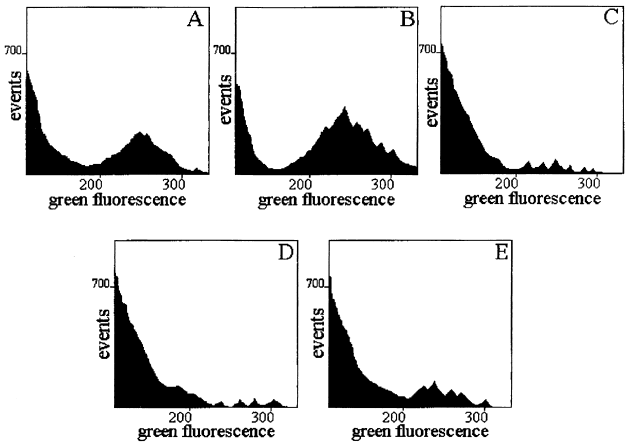


Fig. 2. Cytofluorimetric analysis of intracellular production of ROS by PMNs. Panel A: DCFH fluorescence of resting PMNs; panel B: DCFH fluorescence of PMNs incubated with Ara-C ($10^{-5}$ M) for 30 min; panel C: DCFH fluorescence of PMNs incubated with Ara-C for 30 min in the presence of rotenone (100 $\mu$M); panel D: DCFH fluorescence of PMNs incubated with Ara-C for 30 min in the presence of fumonisin b1 (100 $\mu$M); panel E: DCFH fluorescence of PMNs incubated with Ara-C for 30 min in the presence of DPI (20 $\mu$M). The concentration of PMNs was $10^6$ cells/mL in all experiments. By flow cytometry, $10^4$ events per condition were analyzed. The figure presented is representative of 5 independent experiments with similar results.

### *2.8. Statistics*

The analysis of histograms of fluorescence intensity was performed using the Kolmogorov–Smirnov (K–S) two-sample test [29]. The computer program included calculation of the median and, when appropriate, the median test was calculated.

## 3. Results

### *3.1. Identification of apoptotic cells*

Incubation with Ara-C triggered PMN apoptosis. In fact, the cytofluorimetric analysis demonstrated that approximately 55% of PMNs showed the DNA fragmentation characteristic of apoptosis in the first 3 hr of incubation (Fig. 1, panel B of the inset). At the 3-hr time point, only about 25% of control cells had undergone spontaneous apoptosis (Fig. 1, panel A of the inset). The rate of PMN constitutive apoptosis shown here is within the range reported by others [8]. At 6- and 24-hr time points of incubation with Ara-C, the percentage of cells showing hypodiploid DNA content was not significantly changed (Fig. 1). The incubation of cells with the impermeable form of Ara-C (Ara-CTP) did not promote apoptosis (Fig. 1). An inhibition of the apoptotic index was observed when PMNs were incubated with Ara-C in the presence of 20 $\mu$M DPI (Fig. 1, panel C of the inset) or $10^{-5}$ M NAC (Fig. 1, panel D of the inset). As a control, we studied the effect of 5-fluorouracil on PMNs in the apoptotic index after the 3-hr incubation with this drug. Under this experimental condition, there was an increase of 10% in the apoptotic index of PMNs compared with controls. The addition of NAC reverted this phenomenon (N = 3) (data not shown).

### *3.2. Detection of ROS production*

The above-reported data suggest a role for the intracellular generation of ROS in PMN apoptosis. As shown in Fig. 2, panel B, incubation of PMNs with Ara-C for 3 hr caused a significant increase in the number of cells reacting with the fluorochrome. Moreover, a shift in the median fluorescence intensity (242 for resting cells vs 252 for Ara-C-treated cells; $P < 0.002$, median test) was observed, indicating an increase in intracellular ROS production. The statistical significance of the change in the fluorescence peak was also confirmed by the K–S two-sample test: $\alpha < 0.001$. When the incubation was performed in the presence of 100 $\mu$M rotenone, this shift was inhibited (Fig. 2, panel C). Similar results were observed when cells were incubated in the presence of 100 mM fumonisin b1 (Fig. 2, panel D). Finally, the addition of 20 $\mu$M DPI decreased DCFH fluorescence (Fig. 2, panel E). Under our experimental conditions, ROS generation was an early event, since low concentrations of ROS could be detected at the 6- and 24-hr

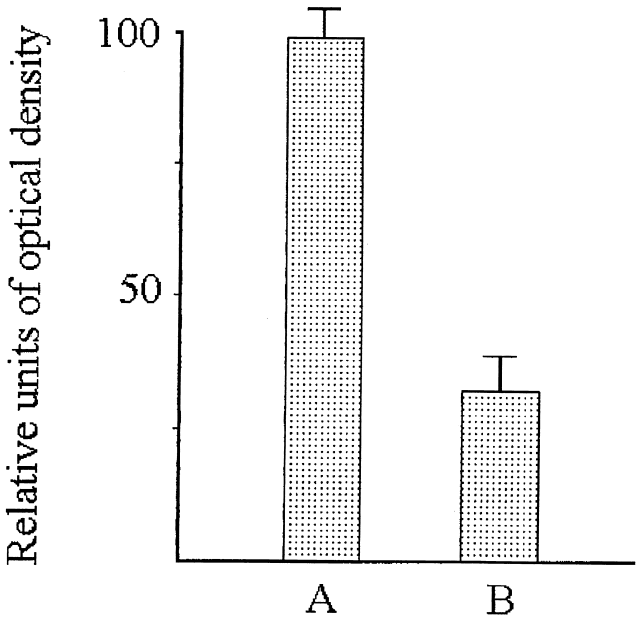


Fig. 3. Cytotoxicity assay (mitochondrial dehydrogenase activity). Mitochondrial activity was studied by measuring the MTT reduction in PMNs ($10^6$ cells/mL). A: resting cells; B: MTT reduction after 30 min of incubation with Ara-C ($10^{-5}$ M). Results are expressed in relative units of optical density. Error bars represent the standard error based on at least five independent experiments.

time points (data not shown). The effect of fumonisin, an inhibitor of ceramide synthase, suggested a role of the ceramide in Ara-C-induced apoptosis in PMNs.

### 3.3. Mitochondrial activity

Mitochondrial activity was studied by measuring MTT reduction in PMNs. In preliminary studies, we observed that only 10% of MTT reduction was SOD-inhibitable, consistent with data from the literature [30]. Ara-C induced an early (after 30 min) and strong diminution in the MTT reduction (Fig. 3).

### 3.4. Western blot analysis of tyrosine phosphorylations

Exposure of PMNs to Ara-C for various times stimulated tyrosine phosphorylations or dephosphorylations (Fig. 4, lane B). A kinetic study revealed that tyrosine phosphorylations of these proteins was apparent after 30 min of incubation, and then declined toward baseline. The enhanced tyrosine phosphorylations were abolished by the presence in the incubation mixture of 10 $\mu$g/mL of genistein (data not shown). The phosphorylations of proteins, in particular those of 50 and 97–100 kDa, were also decreased by the presence of $10^{-5}$ M NAC (Fig. 4, lane C). Interestingly, the increase in these phosphorylations was associated with a decrease in the tyrosine phosphorylations of the proteins of 45–47 kDa.

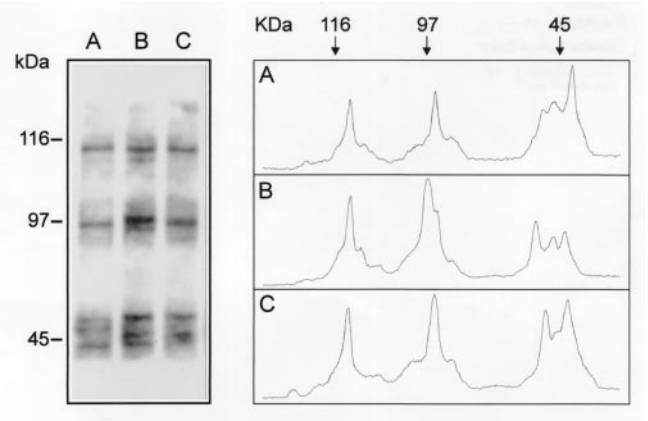


Fig. 4. Western blot analysis of tyrosine phosphorylation of PMNs. Cells ($10^6$/mL) were lysed in ice-cold buffer. Protein samples (50 $\mu$g) were determined in the lysates and subjected to gel electrophoresis and immunoblotting. SDS–PAGE was performed on 10% acrylamide. Following electrophoresis, the samples and molecular weight standards were electrophoretically transferred to nitrocellulose paper. The blot was then incubated with blotting solution containing the affinity-purified MoAb antiphosphotyrosine 4G10. After a second incubation with peroxidase-conjugated anti-mouse antibody, detection of phosphoproteins was performed by the enhanced chemiluminescence system. Lane A: resting PMNs; lane B: PMNs after a 30-min incubation with Ara-C ($10^{-5}$ M); lane C: PMNs after a 30-min treatment with Ara-C and in the presence of NAC ($10^{-5}$ M).

### 3.5. Release of $O_2^-$

To detect whether the extracellular release of ROS plays a role in the proapoptotic effect of Ara-C, we assayed SOD-inhibitable cyt *c* reduction. $O_2^-$ release was recorded in Ara-C-incubated PMNs following activation either by f-MLP (which mainly induces extracellular ROS release via a receptor-triggered pathway) or by PMA (which mainly induces intracellular release of ROS in a receptor-independent manner) in order to study whether the drug affects NADPH oxidase. The addition of Ara-C to PMNs did not induce SOD-inhibitable cyt *c* reduction, indicating that extracellular $O_2^-$ was not released from the cells. The drug was able, instead, to increase the $O_2^-$ release by f-MLP- but not PMA-stimulated PMNs. The increase in $O_2^-$ production was completely abolished by the addition to the incubation mixture of 10 $\mu$g/mL of genistein, a powerful tyrosine kinase inhibitor, but not by the addition of 100 $\mu$M H-7, an inhibitor of protein kinase C (Fig. 5). The incubation of PMNs with Ara-C also induced ROS release from the cells when they were stimulated with subthreshold concentrations of f-MLP (priming effect) (Fig. 6) (N = 3).

## 4. Discussion

The most effective agent in the treatment of acute myelogenous leukemia is Ara-C [22]. This agent incorporates into leukemic cell DNA. In cells, Ara-C is phosphorylated to Ara-CTP and incorporated into DNA, where it acts as

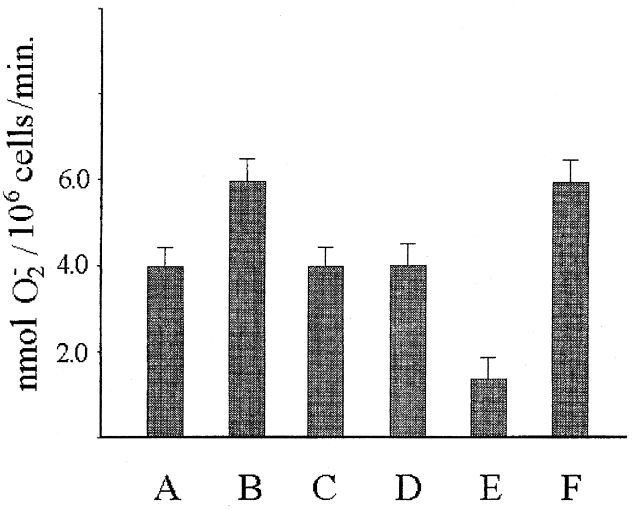


Fig. 5. $O_2^-$ release from PMNs as assessed by SOD-inhibitable cyt *c* reduction. A: $O_2^-$ release (nmol/$10^6$ cells/min) from PMNs stimulated by $10^{-7}$ M f-MLP. B: $O_2^-$ release from PMNs incubated with Ara-C ($10^{-5}$ M) for 30 min and then stimulated by f-MLP. C: $O_2^-$ release from PMNs stimulated by PMA (1 $\mu$g/mL). D: $O_2^-$ release from PMNs incubated with Ara-C for 30 min and then stimulated by PMA. E: $O_2^-$ release from PMNs treated with Ara-C for 30 min, in the presence of 10 $\mu$g/mL of genistein and then stimulated by f-MLP. F: $O_2^-$ release from PMNs treated with Ara-C for 30 min, incubated with 100 $\mu$M H7, and then stimulated by f-MLP. Error bars represent the standard error based on five independent experiments.

chain terminator. A strong association has been found between incorporation and cell kill. Cellular responses to the effects of Ara-C on DNA replication include the induction of apoptosis [31]. On the other hand, it has been demonstrated that NAC protects cells against damage to DNA, possibly by neutralizing the toxic effects of radical species. This observation provides indirect evidence that toxic ROS are generated in cells following Ara-C-induced damage to DNA. Here, we have shown that Ara-C-treated PMNs undergo apoptosis within 3 hr of incubation. The effect on fully differentiated and growth-arrested cells suggests that Ara-C may also affect the cells by a mechanism independent of replication. The rate of apoptosis was completely reverted by the addition of NAC, a thiol-containing compound used in clinical practice for the treatment of congestive and obstructive lung disease associated with hypersecretion of mucus. Cysteinyl residues of this drug account for the redox-sensing properties, and are also involved in the thiol–disulphide exchange reaction. The inhibition of apoptosis by NAC has generally been attributed to its scavenging effect [32,33]. The impermeable form of Ara-C (Ara-CTP) did not increase the apoptotic index, either suggesting a role for the phosphorylations involved in the metabolism of the drug or indicating an intracellular localization of the drug target agents for apoptosis [22]. The incubation of PMNs with Ara-C increased the generation of ROS, which was inhibited by rotenone and fumonisin. The use of DCFH as a measure of ROS has been widely employed [26,34,35]. In fact, since DCFH oxidation may be derived from several reactive intermediates, the interpretation of specific ROS involved in biological systems should be approached with caution. The inhibition of ROS production in the presence of rotenone indicates a role for mitochondria. Oxidative phosphorylations are a major source of ROS, since when the electron transport chain is disrupted, the electrons accumulate in the early stages of the electron chain where they can be donated to molecular oxygen to give $O_2^-$, which is, in turn, converted to $H_2O_2$ [36]. The mitochondrial inner membrane contains a number of cell death-promoting factors including cyt *c* and caspases [18]. Opening of the mitochondrial permeability transition pores leads to the release of the death-promoting factors [37]. It has been reported that this may be initiated by excessive uptake of calcium, decline of energy production, and increased exposure to ROS [38]. Our results unequivocally show that an increase in the production of ROS from the mitochondria is an early event after the incubation of PMNs with Ara-C. An impairment of the general mitochondrial activities in PMNs induced by this anticancer drug could be detected by the MTT assay. This assay, which is widely used to measure cell proliferation and to screen for anticancer drugs, is based on the reduction of the tetrazolium salt [38,39]. Despite broad acceptance of this assay, neither the enzyme system(s) that reduce MTT nor the subcellular localization of MTT-reducing activity is well defined. However, it has been shown that MTT reduction is largely dependent on reduced pyrimidine nucleotides rather than succinate [39]. An alteration of the cellular redox status induced by Ara-C may represent a critical step in its proapoptotic effects. The addition of fumonisin inhibited DCF fluorescence of PMNs incubated with Ara-C, indicating a role of ceramide in the ROS production. The prominent role of mitochondria in ROS production is in keeping with recent data [40]. However, a late ROS production from the respiratory burst may be involved in apoptosis, as DPI inhibits ROS production in Ara-C-treated PMNs. DPI is indeed a flavoenzyme inhibitor

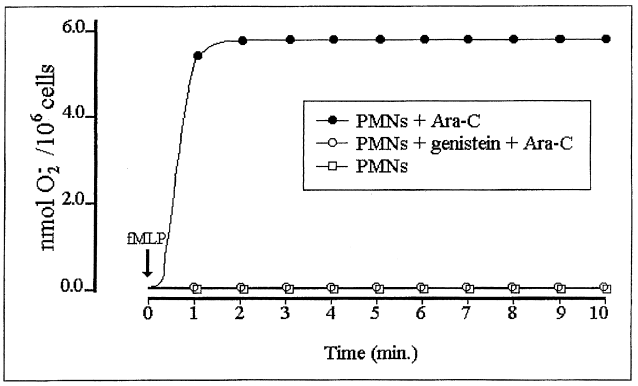


Fig. 6. Genistein inhibition of the priming effect by Ara-C on f-MLP ($10^{-9}$ M)-induced $O_2^-$ production. When indicated, PMNs ($10^6$ cells/mL) were preincubated in the absence or presence of genistein (10 $\mu$g/mL) with or without Ara-C ($10^{-5}$ M) for 30 min. The figure presented is representative of 3 independent experiments.

and in PMNs its effect is generally considered as an indicator of NADPH oxidase inhibition. It must be stressed that the activation of the granule pool of NADPH oxidase has recently been shown to induce intracellular ROS production, while the stimulation of the membrane-bound oxidase mainly generates extracellular release of ROS [12]. The activation of NADPH oxidase may be triggered by a pathway involving cross-talking between ROS and tyrosine phophorylations, since these were inhibited by NAC. Indeed, the preincubation of PMNs with Ara-C significantly enhanced extracellular $O_2^-$ release when f-MLP, a receptor-dependent agonist, but not when PMA, was used as activator. Moreover, Ara-C showed a priming effect on f-MLP-induced $O_2^-$ cell production. Proposed mechanisms involved in synergistic and/or priming effects include the modulation of the number of receptors or their affinity for agonists, covalent modification of specific PMN proteins, elevation of basal or peak calcium, and modulation of protein tyrosine phosphorylations. The synergistic augmentation of $O_2^-$ production by f-MLP and Ara-C was, in fact, reduced by genistein, a specific inhibitor of protein tyrosine phosphorylations, but not by H7, a protein kinase C inhibitor. Moreover Ara-C elicited changes in tyrosine phosphorylations of various proteins. Taken together, these data indicate a crucial role for tyrosine phosphorylations triggered by Ara-C. A number of protein phosphorylations have been described in cells undergoing apoptosis mediated by monoclonal antibodies, Fas, ionizing radiations, and anticancer drugs [3,41]. The nature of these proteins has not yet been determined. We have demonstrated here that the phosphorylations lasted at least 30 min after the addition of Ara-C to PMNs. It is tempting to speculate, in particular, that phosphorylations or dephosphorylations of proteins could play an important role in the synergistic and priming effect of Ara-C.

Our data show that endogenous ROS released from mitochondria may activate downstream signals such as tyrosine phosphorylations. These early events may contribute to the proapoptotic effects of Ara-C on PMNs. ROS generated by PMNs themselves, beside their contribution to tissue damage, may also regulate inflammatory processes by triggering apoptosis and limit the injury produced by the cell activation. Our data could also be useful in finding agents able to modulate the toxic effects produced by Ara-C in normal cells. The observation that another nucleoside-type cytotoxic agent, 5-fluorouracil, may induce apoptosis in non-proliferating PMN by ROS generation bears further investigation. Potential side effects observed with this type of chemotherapeutic agent could arise through oxidation(s).

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