Biochemical Pharmacology, Vol. 51, pp. 1123–1129, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02240-9

N-Acetylcysteine Protects Lymphocytes from Nitrogen Mustard-Induced Apoptosis

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ABSTRACT. The ability of the antioxidant N-acetylcysteine to prevent apoptosis induced in lymphocytes by nitrogen mustard (HN2) was investigated. HN2 caused a concentration-dependent induction of apoptosis on C3H murine spleen cells, as identified by two criteria: morphological features revealed by microscopical observations and DNA fragmentation visualized by the characteristic "ladder" pattern observed upon agarose gel electrophoresis, as well as by hypodiploid DNA-containing cells revealed by the flow cytometric analysis of propidium iodide labelled cells. The antioxidant N-acetylcysteine (NAC) was found to markedly reduce the occurrence of HN2-induced apoptosis in these cells. This protective effect was still obtained when NAC was added 30 min after HN2. In contrast, the pretreatment of spleen cells with this antioxidant did not provide any significant protection. We also showed that lymphocytes protected by NAC are still able to respond to a mitogenic stimulation. To gain some insight into the mechanisms underlying the cytoprotective action of NAC against HN2, we tested whether or not poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30), a nuclear enzyme that participates in the triggering of apoptosis induced by alkylating agents, is involved. We report that 6(5H)-phenanthridinone, a potent PARP inhibitor, did not affect the ability of NAC to prevent HN2-induced apoptosis under our experimental conditions. Thus, the exact mechanism by which NAC protects lymphocytes from HN2 cytotoxicity has yet to be determined. BIOCHEM PHARMACOL 51;9:1123–1129, 1996.

KEY WORDS. nitrogen mustard; N-acetylcysteine; lymphocytes; apoptosis; poly(ADP-ribose) polymerase

Apoptosis is a form of cellular death requiring active participation of the cell and leading eventually to DNA fragmentation [1]. In addition to playing a physiological role in the deletion of autoreactive T cells and the elimination of senescent cells [2], apoptosis in lymphocytes easily can be triggered, accidentally or pathologically, by a series of physical, chemical, and biological stimuli. Thus, exposure to apoptotic agents, such as ionizing radiations, by depressing the immune defense beyond a critical level, could have dramatic consequences [3]. For these reasons, lymphocytes appear to constitute important cellular models, from a therapeutic point of view, for identifying chemical compounds capable of preventing or reducing apoptosis [4].

In the present study, we treated murine lymphocytes in culture with HN2,§ a potent and very toxic alkylating agent still in clinical use as an anticancer drug. As with other radiomimetic compounds, HN2 is highly toxic toward normal hematopoietic cells, in which it can induce apoptosis within hours of exposure [5, 6]. We show that NAC, an antioxidant widely used in human therapy, protects lymphocytes from this type of cell death. Moreover, preserved lymphocytes retain their capacity to be activated by a mitogenic stimulus.

We further investigated the role of PARP, an ubiquitous chromatin-bound enzyme critically involved in major DNA-linked processes [7], in the reduction by NAC of HN2-induced apoptosis. Indeed, PARP inhibition was previously found to sharply increase HN2 cytotoxicity in leukemia cell lines [8]. Thus, it was of interest to test whether NAC could also protect normal lymphocytes cotreated by HN2 and 6(5H)-phenanthridinone, a potent PARP inhibitor. We show here that the inhibition of PARP slightly reinforces the toxicity of HN2 toward lymphocytes, but does not affect its prevention by NAC.

MATERIALS AND METHODS

Chemicals

NAC, PI, ethidium bromide, Con A, RNAse type I-A, Tris-buffered phenol (pH 8), and agarose were purchased from Sigma Chimie (S.a.r.l., Saint Quentin Fallavier, France). HN2 was from Laboratoires Delagranges (Paris, France). HN2 is stored at room temperature as a stock

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§ *Abbreviations*: HN2, nitrogen mustard; ADP-ribose, adenosine 5′ diphospho-5-β-ribose; PARP, poly(ADP-ribose)polymerase; NAC, N-acetylcysteine; PI, propidium iodide; TE, Tris EDTA; TAE, tris acetate EDTA; Con A, concanavalin A.

Received 3 May 1995; accepted 1 December 1995.

solution of 5 mg/mL in triethyleneglycol. Its dilution with culture medium is performed just before being added to the cell culture. 6(5H)-phenanthridinone was purchased from Aldrich Chimie S.a.r.l. (Saint Quentin Fallavier, France). It was solubilized in DMSO and stored at room temperature as a 40 mM solution. [^{3}H]-Thymidine (6,7 mCi/mmol) was purchased from Du Pont-New England Nuclear (Hertfordshire, England).

Preparation of the Spleen Cell Suspensions

C3H mice between the ages of 6 and 8 weeks were used as lymphocyte donors. Spleens, thymus, and lymph nodes were removed aseptically and placed in cold RPMI 1640. Single-cell suspensions were prepared by gently teasing the spleens in RPMI 1640 medium before pushing them through a 60 mesh nylon membrane to remove aggregates. Erythrocytes were removed from suspensions by incubating 60 sec at 4°C in a solution referred to as ACT (Tris-HCl, 15 mM, pH 7.4 and NH_4Cl, 14 mM). After 2 washings, the cells were resuspended in the culture medium. In some experiments, red cells were eliminated by centrifuging cell suspension through Ficoll (MSL, Eurobio, Les Ulis, France). Cell number and viability were determined by counting Trypan Blue-excluding cells using a Neubauer hematocytometer.

Cell Culture

The cell culture medium used in our experiments is referred to as MC and consists of RPMI 1640 (Gibco BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (DAP, Vogelgrün, France), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 50 μg/mL gentamicin, and 50 μM β-mercaptoethanol (Gibco). Cells (1×10^7) were cultured in 2 mL MC in flat bottomed 24-well plates (Falcon, Becton-Dickinson, Le Pont-De-Claix, France) at 37°C in full humidity and 5% CO_2.

DNA Labelling and Flow Cytometry Analysis

Hypodiploid DNA in cells was measured according to the technique described by Nicoletti *et al.* [9]. Briefly, 1×10^6 cells were centrifuged at 200 × *g*, then fixed in 1 mL cold 70% ethanol at 4°C for 1 hr. The cells were then centrifuged, washed once with PBS, and resuspended in 1 mL of PBS containing 0.1 mg RNAse A and 0.1 mg of PI. The cells were incubated in the dark at 37°C for 30 min, then kept at 4°C until analysis within 24 hr. The fluorescence of 10,000 cells was analysed using a flow cytometer (Becton Dickinson).

Agarose Gel Electrophoresis

C3H spleen cells (2.5×10^6) prepared as described above were harvested by centrifugation at 200 × *g* for 10 min. The pellet was lysed with 0.2 mL of cold hypotonic lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) and centrifuged for 10 min at 13,000 × *g*. The DNA from the resulting supernatant was extracted with 1 volume of Tris pH 8-buffered phenol. After centrifugation for 10 min at 13,000 × *g* at 4°C, the aqueous phase was extracted with chloroform (v/v). The DNA was then precipitated overnight at −30°C in a mixture of 50% isopropanol and 3M ammonium acetate. The precipitates were pelleted by centrifugation for 20 min at 13,000 × *g*, rinsed with 70% ethanol, and dried under vacuum. The pellets were resuspended in 15 μL TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.4) containing 50 μg/mL of RNAse A (type I-A; Sigma), incubated for 10 min at 65°C, then by 15 min at 37°C. Loading buffer containing 50% glycerol, 5 × TAE (1 × + 40 mM Tris-acetate, pH 8.5, 2 mM EDTA,) and 0.1% Bromophenol Blue was added to the samples in a ratio of 1:4 (v/v). Electrophoresis was carried out in a 1.8% agarose gel at 10 V:cm in TAE buffer. DNA was visualized on an ultraviolet transilluminator after ethidium bromide staining.

Con A Stimulation Test

Activated T cells were prepared by incubating spleen cells (2×10^5/200 μL) in the presence of Con A (2.5 μg/mL) in flat-bottomed 96-well microplates (Falcon) for 48 hr at 37°C in a 5% CO_2 atmosphere. 20 μL of [^{3}H]-Thymidine (0.5 μCi) was then added to each well. Cells were harvested 6 hr later onto glass fiber strips using an automatic cell harvester (Titertek 530, Flow Laboratories, Les Ulis, France). The incorporation of [^{3}H]-Thymidine was measured by a beta counter (LKB-Wallac 1409, Pharmacia, Turku, Finland) and expressed in counts/min. The results were calculated as the mean ± SD of quadruplicate samples and expressed as percent of untreated control cells.

RESULTS

Induction of Apoptosis in Spleen Cells by HN2 and Reversal by NAC

C3H spleen cells were incubated in the presence of various concentrations of HN2. For times varying between 1 and 24 hr, they were harvested, their viability determined by the Trypan Blue exclusion test, and morphological examinations were performed. In the control, the number of viable mononuclear cells progressively decreased after the first hours of culture. Concomitantly, morphological observations and flow cytometry analyses revealed that an increasing proportion of these untreated cells, up to 27% at 18 hr of culture, underwent spontaneous apoptosis (Fig. 1). However, in treated groups, the proportion of cells presenting the characteristic morphological features of apoptosis was significantly higher than in the controls, as assessed by flow cytometry analyses. At higher

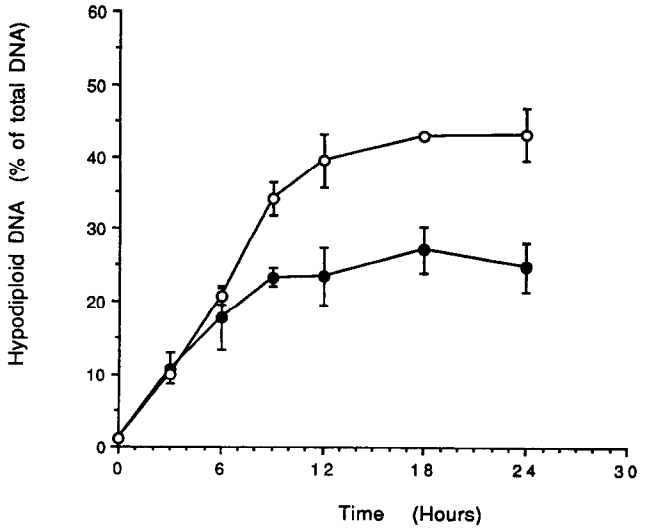

FIG. 1. Time course of hypodiploid DNA induced by nitrogen mustard (HN2) in murine spleen cells. C3H spleen cells were incubated in the presence of various concentrations of HN2. At various intervals of time, cells were recovered by centrifugation and labeled with propidium iodide (PI) as described in Materials and Methods. The percentage of cells containing hypodiploid DNA was determined by flow cytometry analysis. (●), untreated control cells; (○), cells treated with 2 μM of HN2. The graph shows the average results from 3 independent experiments. Error bars represent standard deviations from the mean.

concentrations (10 μM and more), a large amount of debris was observed, indicating a pronounced degree of cell disruption.

NAC was added to HN2-treated cells, at concentrations ranging from 0.1 to 10 mM. As shown in Fig. 2, the percentage of cells exhibiting hypodiploid DNA was significantly reduced and flow cytometric analysis revealed that the collapse of the 2n peak was partially prevented (not shown). Agarose gel electrophoresis of extracted DNA was also performed. In untreated cells, spontaneous DNA fragmentation was observed, as evidenced by the characteristic "DNA ladder" profile that is considered the hallmark of apoptosis (Fig. 3). HN2-treated cells displayed a sharp increase in the number of small DNA fragments whereas, in the presence of NAC, the degree of DNA fragmentation was not significantly different from that in untreated cells. It is interesting to note that, in cells treated with NAC alone, DNA fragmentation was found to be less pronounced than in untreated control cells.

Influence of the Lymphocyte Mode of Preparation in the Occurrence of HN2-Induced Apoptosis

In fresh spleen cell suspensions, only a very small percentage of cells present the features of apoptosis. However, the number of hypodiploid cells increases rapidly with time, perhaps due to the trauma consecutive to manipulations

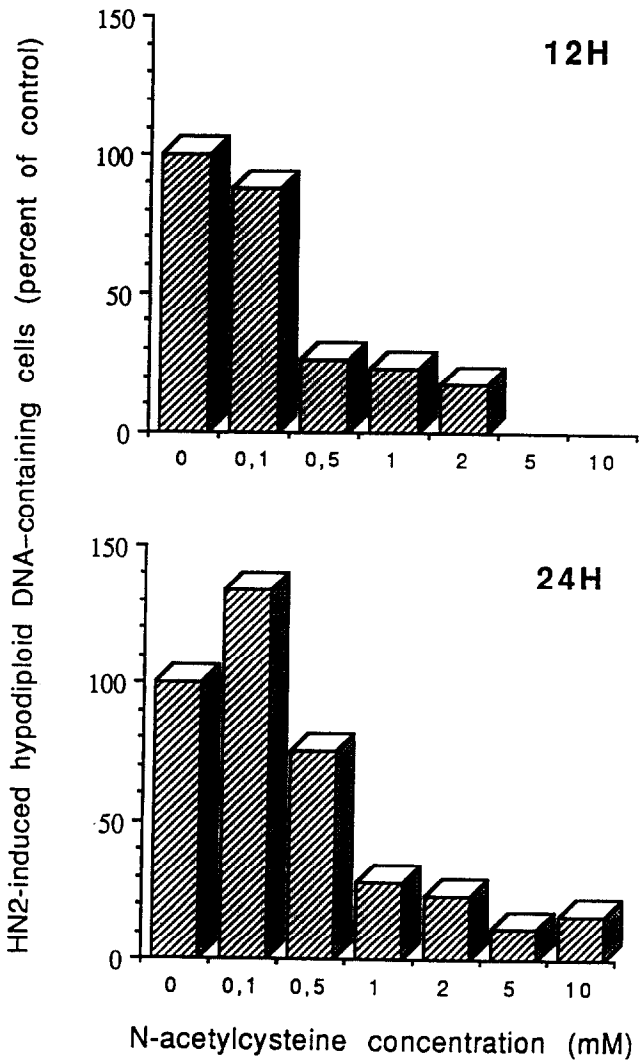

FIG. 2. Inhibition of HN2-induced DNA fragmentation by N-acetylcysteine (NAC). C3H spleen cells were incubated in the presence of HN2 (2 μM) and of various concentrations of NAC for 12 and 24 hr. The percentage of hypodiploid DNA in PI-labelled cells was determined by flow cytometry analysis. The controls correspond to the fraction of HN2-induced hypodiploid DNA of cells cultured with HN2 alone (i.e. in the absence of NAC). Control values (100%) correspond to 18% and 21% of the total cell population, respectively. Data are from one representative experiment.

and the passage from *in vivo* to *in vitro* conditions. In our experiments, we removed the red blood cells from our spleen lymphocyte preparations by incubation in ACT for 1 min at 4°C. However, this method, by inducing an osmotic fragility of membranes, could increase lymphocyte sensitivity to HN2 and, subsequently, contribute to the high spontaneous apoptosis rate recorded in cultured spleen cells [10]. To investigate this possibility, red cells were removed from the crude spleen cell preparation by centrifugation on a density gradient. As shown in Fig. 4, the HN2-induced DNA fragmentation and its prevention by NAC were similar in both lymphocyte preparations.

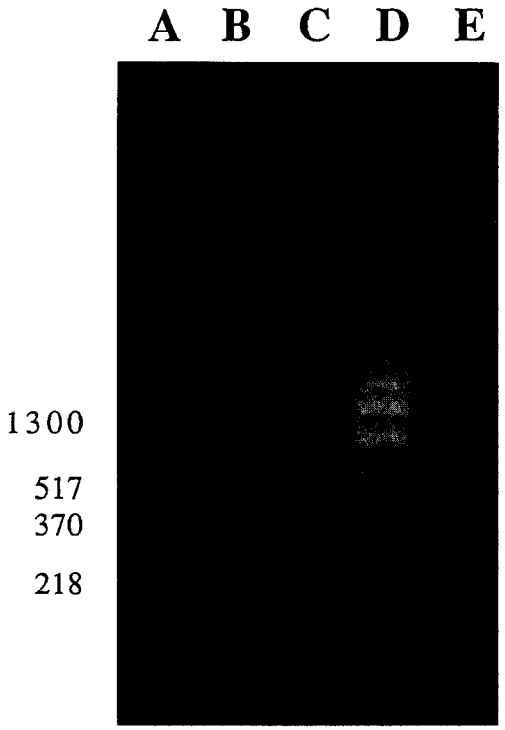

FIG. 3. Prevention of HN2-induced DNA-internucleosomal cleavage by N-acetylcystine. DNA extracted from the cytoplasmic fraction of 2.5 × 10^6 lysed C3H spleen cells was separated by electrophoresis on a 1.8% agarose gel. Lane A, molecular weight; lane B, untreated cells; lane C, cells incubated with NAC 2.5 mM; lane D, cells incubated with HN2 2 μM; lane E, cells incubated simultaneously with HN2 (2 μM) and NAC (2.5 mM). Untreated spleen cells (B) show a characteristic "ladder" pattern indicating a high level of spontaneous apoptosis that is reduced by the addition of 2.5 mM NAC (C). Treatment of lymphocytes by 2 μM HN2 (D) for 12 hr leads to a sharp increase in DNA fragmentation not observed in the presence of NAC (E). Similar results were obtained in 3 independent experiments.

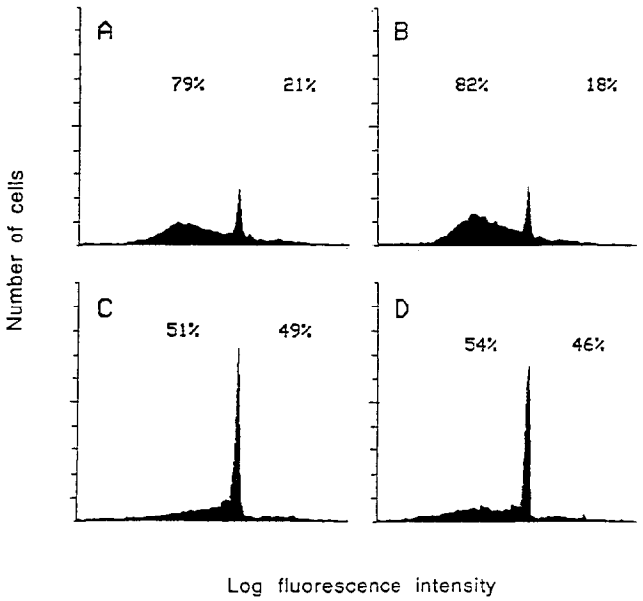

FIG. 4. Lack of influence of the mode of cell preparation on the induction of apoptosis by HN2. Flow cytometry analysis of C3H spleen cells prepared as described in Materials and Methods was performed. The crude spleen cell preparations were freed from erythrocytes, either by ACT treatment (A and C) or by centrifugation through a ficoll gradient (B and D). Cells (1 × 10^7/mL) were then incubated for 24 hr in the presence of HN2 alone at 2 μM (A and B) or together with 2.5 mN NAC (C and D). Values represent the percentage of hypodiploid (left) or diploid DNA (right).

Time Course Study

In further experiments, we attempted to determine whether or not NAC added at different times after the beginning of treatment by HN2 was still capable of protecting lymphocytes from apoptosis. Therefore, cells were incubated in the presence of HN2 with or without NAC for 30 min and were then washed and replaced in fresh medium for 12 hr. NAC was added concomitantly or either 30 or 60 min after the removal of HN2. Results presented in Fig. 5 show that the best protection is obtained when NAC is added at the same time as the alkylating agent. When added 30 min after the removal of HN2, NAC still provides good protection against HN2-induced apoptosis. However, this protective effect is lost when NAC is added 60 min later.

Response of Lymphocytes to Con A

Because NAC protects cells from apoptosis, but not from sublethal damages caused by HN2, some functional properties may no longer be fulfilled by lymphocytes. To investigatethis point, we verified whether or not lymphocytes treated by HN2 in the presence or absence of NAC could still undergo blastic transformation. Their response to Con A, a nonspecific T-cell mitogen, was measured by the incorporation of [^{3}H]-thymidine. As shown in Fig. 6, cells treated by HN2 in the presence of NAC are still capable of responding to Con A, indicating that their potential to be activated remained unaffected. In our experiments, we also noted that NAC alone was slightly mitogenic in unstimulated lymphocytes, as evidenced by a significantly higher [^{3}H]-thymidine incorporation (not shown).

Role of Poly(ADP-ribosylation) Processes

Poly(ADP-ribosyl)ation of nuclear proteins plays a major role in various DNA-related processes including apoptosis [10–13]. We used 6(5H)-phenanthridinone, a recently described poly(ADP-ribose)polymerase (PARP) inhibitor, [14] to detect a putative participation of PARP in the protection afforded HN2 by NAC. 6(5H)-phenanthridinone was added to the cells 2 hr before the addition of HN2 and NAC to ensure its penetration into the cells. NAC was found to prevent HN2-induced apoptosis, with or without 6(5H)-phenanthridinone, in spite of a slight reinforcement of HN2-cytotoxicity by the PARP inhibitor (Fig. 7). Similar

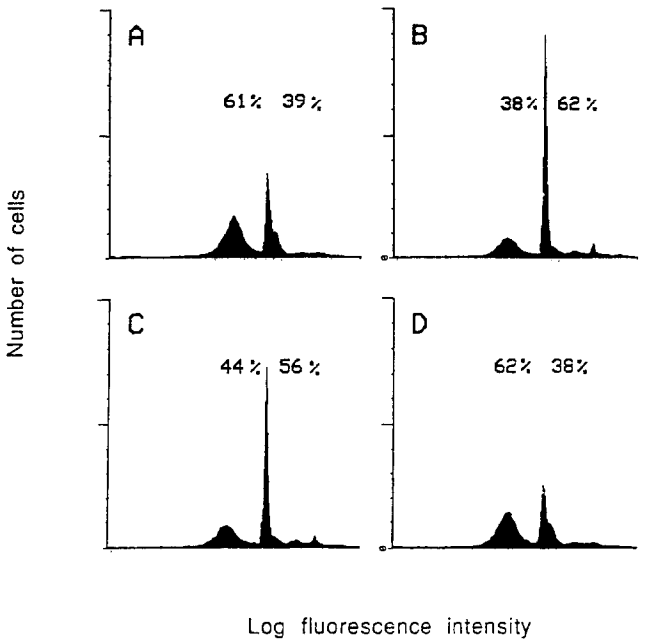

FIG. 5. Time course study of the protective effect of NAC against HN2-induced apoptosis. Cells were incubated in the presence of 2 μM HN2 for 30 min with (B) or without (A, C and D) 2.5 mM NAC. HN2 were then removed by changing the culture medium and NAC (2.5 mM) was added at the same time (B), 30 min (C), or 60 min (D) after the removal of HN2. After 12 hr of incubation, the cells were collected, labeled with PI, and analysed by flow cytometry as described in Materials and Methods.

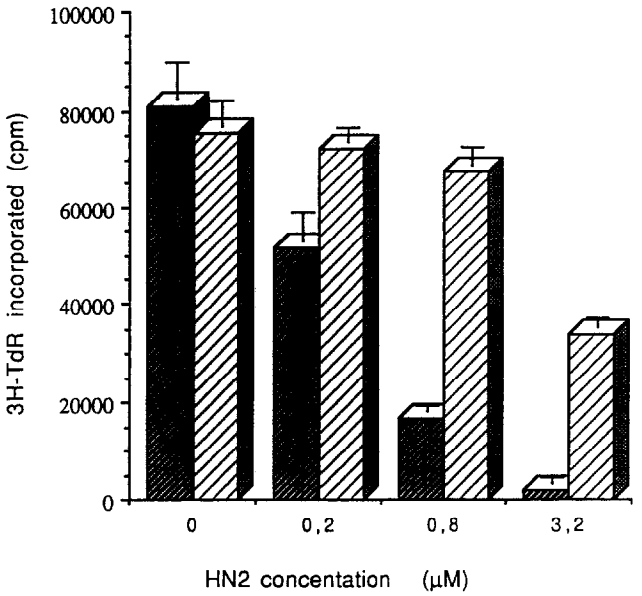

FIG. 6. NAC maintains the ability of spleen cells treated with HN2 to respond to a mitogenic stimulation. C3H spleen cells (1 x 10^6/mL) were stimulated by concanavalin A at 2.5 μg/mL and incubated for 48 hr in the presence of various concentrations of HN2 and with (clear hatched bars) or without (dark hatched bars) NAC 2.5 mM. The lymphocyte proliferation rate was assessed by [^{3}H]-thymidine incorporation and expressed in counts per minute (cpm). The data are from one representative experiment. Each point represents the mean ± SD of 4 replicate wells.

results were obtained with 3-aminobenzamide, another PARP inhibitor (not shown).

DISCUSSION

Our data indicate that NAC can prevent spleen cell apoptosis when these cells are treated *in vitro* with HN2 and that NAC-protected lymphocytes retain their capacity to undergo blastic transformation. We also demonstrate that lymphocytes can be partly rescued from a HN2 attack by posttreatment with NAC. However, the best protection is afforded when cells are treated continuously by this antioxidant agent.

Pharmacological interventions at various levels have been proposed to prevent apoptosis [4]. Among several classes of active chemical compounds, suppliers of sulhydryl groups occupy a place of choice in the antiapoptotic arsenal. Otherwise, these substances belong to the main class of antioxidants and radioprotectors. Indeed, by scavenging free radicals generated by ionizing radiation, they protect cells from DNA-damaging lesions that lead to clonogenic cell death. However, as far as alkylating agents such as HN2 are concerned, other mechanisms must be taken into account because their mode of actions differs somewhat from that of ionizing radiation. Indeed, DNA damage generated by alkylating agents results principally from the covalent binding of alkyl groups to N-7 guanine of DNA. However, HN2 toxicity is not strictly determined by the level of DNA damage or by the ability of the cell to remove alkylated bases or DNA cross-links. O'Connor [6] showed that the differential HN2 sensitivity of various Burkitt's lymphoma cell lines may be the result of processes that occur downstream of the DNA repair. Moreover, alkylation of other molecules, such as proteins, also occurs [15]. Thus, several hypotheses can be put forward to account for the effect of NAC. It may be postulated, that a chemical reaction between HN2 and NAC leads to the inactivation of HN2. Such a possibility cannot be ruled out, because sulfydryl groups constitute very good alkyl group acceptors [16] and thereby could react with the aziridinium ion which, in aqueous solution, results from the cyclization of HN2. However, it seems unlikely that this mechanism accounts for all NAC protective action. Second, NAC could also intercept signals involved in the triggering of apoptosis. This has already been demonstrated in oxidative stresses, where reactive oxygen intermediates (ROI), which participate in the transduction of apoptotic signals, can be scavenged by NAC [17]. Physiologically, such a role is played by intracellular glutathione. However, lymphocytes are particular, since they lack the ability to synthesize cysteine, a primary component of GSH as well as the ability to take up cystine. Thus, in these cells, the intracellular gluthatione level depends on the presence of extracellular cysteine [18], and NAC acts as a glutathione precursor. Third, NAC could also interfere downstream in the occurrence of apoptosis.

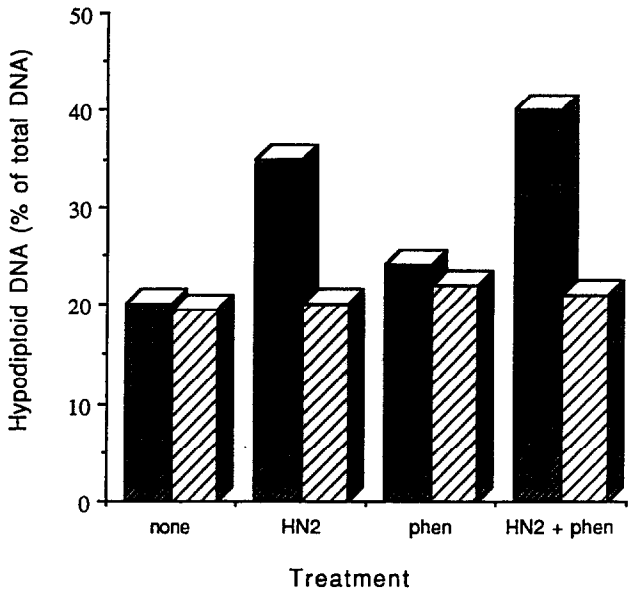

FIG. 7. Poly(ADP-ribose)polymerase does not interfere with the protective effect of NAC on HN2-induced apoptosis. C3H spleen cells were incubated for 24 hr in the presence of various compounds together with (clear hatched bars) or without (black hatched bars) NAC at 2.5 mM. The cells were recovered, labeled by PI, and their DNA content analysed by flow cytometry. The data are from one representative experiment.

Therefore, we investigated whether or not inhibitors of poly(ADP-ribose)polymerase could influence the course of apoptotic events, because this enzyme is involved in apoptotic processes, either enhancing [19] or reducing them [20] according to the experimental models used. Our results indicate that the inhibition of PARP by 6(5H)-phenanthridinone slightly reinforces HN2 toxicity but does not interfere with the protective effect of NAC. Finally, NAC could directly protect DNA from damage caused by HN2, as already demonstrated for fast neutrons [21] or 4-hydroperoxycyclo-phosphamide [22].

Independently of the precise mechanism by which it prevents cells from HN2-induced apoptosis, NAC represents one of the most attractive members of the thiol family. First, this compound is weakly toxic at active concentrations as compared with other thiol-containing compounds, such as WR-1065 or cysteine [23]. Second, NAC has proven its efficiency as a multipotent cellular protector in an increasing number of pathological situations. For instance, high doses (up to 300 mg/kg) of NAC are usually prescribed to counteract the hepatotoxicity caused by acetominophen overdosage [24]. Moreover, NAC enhances recovery from acute lung injury in man, a syndrome caused by toxic oxygen radicals [25] and, when topically applied, it protects against UVB-induced systemic immunosuppression [26]. NAC has also been proposed as a treatment for HIV-infected patients [27], because this virus could induce apoptosis in CD4 lymphocytes by generating oxidative stress. Unfortunately, clinical trials have been disappointing so far, possibly due to the inability of NAC to provide long-term protection against sustained contamination by the AIDS virus. Therefore, the development of NAC derivatives endowed with a protracted blood half-life or the design of pharmaceutical forms allowing a protracted action of this compound could be of great interest.

This work was supported from grants to P. Bischoff and J. Marchal from the Direction des Recherches et Etudes Techniques (DRET N° 93/50). We thank Honey Levallois for critical reading of the manuscript.

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