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Prevention of Nitrogen Mustard-Induced Apoptosis in Normal and Transformed Lymphocytes by Ebselen

Vincent Holl, David Coelho,* Luc Silbernagel,* Jean-François Keyser,* Caroline Waltzinger,*† Patrick Dufour* and Pierre L. Bischoff*‡*

*Laboratoire de Cancerologie Experimentale et de Radiobiologie (LCER), Institut de Recherche Contre les Cancers de l'Appareil Digestif (IRCAD), Hopitaux Universitaires, 67091 Strasbourg and †INSERM U184, Service Cytometrie, Institut de Genetique et de Biologie Moleculaire et Cellulaire (IGBMC), 67404 Illkirch, France

ABSTRACT. The alkylating agent, nitrogen mustard (HN2), is thought to cause apoptosis through production of free oxygen radicals. To explore the mechanism of HN2-induced apoptosis, we utilized ebselen, a selenoorganic compound with potent antioxidant activity. We examined whether ebselen would inhibit apoptosis in BALB/c mouse spleen lymphocytes and human MOLT-4 leukemia cells treated with HN2 (2.5 μM) *in vitro*. Non-toxic concentrations (<50 μM) of ebselen were found to prevent HN2-induced apoptosis of murine lymphocytes in a dose-dependent manner, as measured by cell viability, hypodiploid DNA formation, and phosphatidylserine externalization. However, ebselen was ineffective at preventing spontaneous apoptosis in these cells, pointing to the selectivity of its action. Furthermore, pretreatment with ebselen at 1–10 μM for 72 hr protected MOLT-4 cells from HN2-induced apoptosis and maintained cell viability and proliferation as monitored by the above-mentioned parameters. This was accompanied by the preservation of mitochondrial transmembrane potential and elevated glutathione levels and by a blockage of caspase-3 and -9 activation. *In vivo*, ebselen also had a marked protective effect against spleen weight loss associated with lymphocyte apoptosis in mice treated by HN2. Therefore, ebselen provides an efficient protection against HN2-induced cell death in normal and tumoral lymphocytes and might prove useful as an antidote against alkylating agents. BIOCHEM PHARMACOL **60**;11:1565–1577, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; ebselen; nitrogen mustard; lymphocytes; protection; oxidative processes; caspases

In anticancer treatment as in many other circumstances, the immune system can be exposed to a variety of physical and chemical aggressions. Among them, ionizing radiation and cytotoxic drugs such as alkylating agents are potent inducers of apoptosis in lymphocytes [1, 2]. Since these cells are the major components of the immune defense, their destruction could have serious deleterious consequences. For these reasons, lymphoid cells constitute important cellular models for identifying chemoprotector compounds capable of preventing or reducing apoptosis [3].

Nitrogen mustard (methyl-bis-(β-chloroethyl)methylamine, HN2§) is a valuable model for such studies, due to its radiomimetic properties. HN2 is a bifunctional DNA-reactive alkylating agent [4–6] still in clinical use as an anticancer drug in the treatment of Hodgkin's disease and Burkitt's lymphoma. Most of the cellular damage produced by this compound results from the production of free radicals (for review, see [7]), which directly react with biological molecules especially sensitive to free radical attack, and alter their activities, causing DNA alkylation and membrane lipid peroxidation, which lead to apoptosis [1, 8]. Second messagers or mediators are generated from membrane lipid peroxidation [9] or DNA strand breaks and are potent activators of the apoptotic signal.

Among the various processes that may protect cells from oxygen radical-induced apoptosis, the enzyme glutathione peroxidase appears to be particularly important. This enzyme catalyzes the reduction of peroxides by glutathione. The main function of GSH is indeed to protect tissue and DNA against the damaging effect of hydroperoxides, and the amount of cellular glutathione has been found to play a role in the defense against DNA damage induced by HN2 [10]. Recently, ebselen, a selenoorganic compound, was revealed to possess potent antioxidant properties that may result from glutathione peroxidase-like activity [11]. Selenium is an essential trace nutrient that provides oxidation–reduction properties to selenoproteins. Ebselen does not

‡ Corresponding author: Dr. Pierre L. Bischoff, Cancérologie Expérimentale/Radiobiologie, Université Louis Pasteur Strasbourg, I.R.C.A.D., B.P. 426 - 1 Place de l'Hôpital, F-67091 Strasbourg, France. Tel. +33 388 11 90 61; FAX +33 388 11 90 97; E-mail: Pierre.Bischoff@ircad.u-strasbg.fr

§ *Abbreviations:* Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Ac-LEHD-AMC, acetyl-Leu-Glu-His-Asp-amino-4-methylcoumarin; CHAPS, 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate; DTT, dithiothreitol; FBS, fœtal bovine serum; FITC, fluorescein isothiocyanate; HN2, nitrogen mustard; MCB, monochlorobimane; PI, propidium iodide; and ROS, reactive oxygen species.

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contain selenocysteine residue at its active site and, similar to glutathione peroxidase, catalyzes the glutathione-dependent reduction of peroxides [12–14]. Moreover, organoselenium compounds have also been found to be inducers of interferon gamma and tumor necrosis factor in human peripheral blood leukocytes [15] and to possess mitogenic potential correlated with the expression of interleukin-2 receptor in human T-lymphocytes [16]. Therefore, ebselen and organoselenides may be regarded as mitogenic cytokine and biological response modifiers. Nevertheless, ebselen, which has a glutathione peroxidase-like activity, has been found to possess potent antioxidant activity [17]. Moreover, it is thiol-dependent [18] and also has been shown to inhibit lipoxygenase [19], nitric oxide synthases [20], and protein kinase as well as to play a defensive role against peroxynitrite [21, 22]. The inhibitory effect of this synthesized glutathione peroxidase mimic on lipid peroxidation was well documented several years ago [23, 24]. It has also been reported to reduce cisplatin-induced nephrotoxicity [25]. In a recent study using mouse thymocytes, ebselen was found to inhibit radiation-induced apoptosis, apparently by reducing the levels of peroxides and interfering with membrane lipid peroxidation [26].

Here, we assessed the inhibitory capacity of ebselen on HN2-induced apoptosis in normal mouse lymphocytes and in the transformed human cell line, MOLT-4. Our results indicate that ebselen can prevent apoptosis induced in these *in vitro* models. Furthermore, a single dose of ebselen given 24 hr prior to HN2 injection in BALB/c mice prevented the spleen weight loss reflective of lymphocyte apoptosis.

MATERIALS AND METHODS

Reagents

Trypan blue, EDTA, EGTA, Tris, DTT, CHAPS, HEPES, NaCl, $MgCl_2$, PI, and RNase type I-A were purchased from Sigma Chemical Co. HN2 (Synthelabo) was stored at room temperature as a stock solution at 5 mg/mL. Its dilution with culture medium was performed just before being added to the cell culture. Ebselen (Calbiochem) was solubilized in DMSO and stored at room temperature as a 10-mM solution. MCB (Molecular Probes) was prepared as a 4-mM solution in ethanol 100% and stored in the dark at 4°. The caspase-3-like inhibitor (Ac-DEVD-CHO) and the fluorometric caspase substrates (Ac-DEVD-AMC and Ac-LEHD-AMC) were obtained from Neosystem and TEBU, respectively. Leupeptin, pepstatin A, aprotinin, and phenylmethyl sulfonylfluoride were purchased from Boehringer Mannheim. Alamar blue was purchased from Interchim.

Spleen Lymphocyte Isolation and MOLT-4 Culture

Female BALB/c mice were obtained from IFFA CREDO, France. All animals were provided with standard laboratory food and water *ad lib*. The mice (6 weeks old) were killed by cervical dislocation and their spleen aseptically removed. Spleens were rinsed in serum-free medium and homogenized by gently teasing the spleens with the plunger of a 2-mL syringue. Red cells were eliminated by lysis for 1 min at room temperature with NH_4Cl 0.83%, 10 mM Tris–HCl, pH 7.4. After two washings, the pellets were resuspended in culture medium made up of RPMI-1640-Glutamax (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (DAP), 1 mM sodium pyruvate, 1 mM non-essential aminoacids, and 50 μg/mL gentamicin (Life Technologies). Cell number and viability were determined by counting trypan blue-excluding cells using a Neubauer hemacytometer. Spleen cell concentrations were adjusted to 10^7 cells/mL and 2 mL was plated into 6-well plates (Costar) before treatment with ebselen and HN2.

MOLT-4 human leukemia T-cells were purchased from ATCC (Manassas, VA) and grown in suspension in the above-described culture medium. They were maintained at 37° in a humidified atmosphere containing 5% CO_2 and were harvested during exponential growth. Under these conditions, spontaneous mortality never exceeded 5% as assessed by trypan blue exclusion.

Animal Treatment

BALB/c mice, 6–8 weeks of age and weighing 20 to 23 g, were randomized into groups of 4 animals. Ebselen (4 mg/kg) was dissolved in a mixture of DMSO and PBS (1/4) and administered i.p. in a volume of 0.4 mL 24 hr before a single i.p. dose of HN2 (6 mg/kg) in 0.25 mL. Control groups were treated with ebselen or the vehicle. Another group was treated with a single i.p. injection of HN2. Twenty-four hours after HN2 administration, mice were sacrificed and spleen removed for weight and lymphocyte cellularity determination.

Ebselen and HN2 Treatment

Ebselen was diluted in culture medium before being added to the cell culture. Normal and tumoral cells were incubated in RPMI with 10% FBS containing different concentrations of ebselen, at 37° in a 5% CO_2 atmosphere, 1 hr or 2 days before addition of 2.5 μM HN2 depending on the experimental protocol. Control cells were brought to the concentration of DMSO or isotonic NaCl solution equivalent to that delivered with ebselen or HN2, respectively. Spleen lymphocytes or MOLT-4 cells were suspended in RPMI-1640 with 10% FBS (at 10^7 and 2×10^6 cells/mL), respectively and treated with a single concentration of HN2 (2.5 μM) 1 hr or 2 days after ebselen pretreatment.

Flow Cytometry Analysis of Apoptosis

To visualize phosphatidylserine expression on the outer leaflet of cellular membrane at the early stage of apoptosis, cells were stained with annexin V–FITC according to the manufacturer's procedures (Euromedex). PI (5 μg/mL) was added at the same time as annexin V–FITC to measure the

loss of membrane integrity and cell death [27, 28]. A total of 20,000 cells were analyzed by flow cytometry in a FACScan (Becton Dickinson) using the LYSIS II software (Becton Dickinson).

Hypodiploid DNA was measured according to Nicoletti [29]. Briefly, 10^6 cells were centrifuged and fixed in 1 mL cold 70% ethanol at 4° for 1 hr, washed once in PBS (Life Technologies), EDTA 2 mM, and resuspended in 1 mL of PBS containing 0.25 mg RNase A, EDTA 2 mM, and 0.1 mg of PI. After incubation in the dark at 37° for 30 min, cells were kept at 4° for less than 12 hr until analysis. The fluorescence of 20,000 cells was analyzed by flow cytometry.

To measure oxygen radical production, 1 μM of rhodamine 123 (Interchim) was added at 5×10^5 cells for 15 min in culture medium, at 37° and 5% CO_2. Cells were washed twice with PBS, collected by centrifugation at 200 *g*, and samples were analyzed by flow cytometry.

The level of cellular GSH per cell was detected by flow cytometry after staining with the cell-permeant MCB probe as previously described [30]. Cells (1×10^6) were preloaded with 200 μM MCB in culture medium. After 30 min of incubation at 37° in the dark, cells were washed in PBS, resuspended in PBS, and analyzed on an EPICS Elite flow cytometer (Beckman Coulter). MCB was excited at 351–363 nm with an Innova 90 laser argon and the blue fluorescence was collected with a 450-nm band pass filter. Analyses were performed on a total of 15,000 cells. The data were collected, stored, and re-analyzed with the CELLQuest software (Becton Dickinson).

Measurement of Tumor Cell Proliferation

Alamar Blue assay was performed according to the manufacturer's instructions. Briefly, after ebselen and HN2 treatment, MOLT-4 cells were seeded in triplicate at 5000 cells/well (200 μL) in a 96-well microplate. Each experimental assay was performed in triplicate. After 4 days of incubation, supernatants were replaced with fresh culture medium and 20 μL of Alamar Blue (Alamar) working solution was added to each well. After an additional 4-hr incubation at 37° in a humidified atmosphere containing 5% CO_2, for 4 hr, plates were read at 590 nm on a Fluorolite microplate reader (Dynex Technologies).

Fluorometric Caspase Assay

Twenty-four hours after the addition of HN2, cells were washed with cold PBS and lysed with cell lysis buffer (4×10^6 cells/200 μL in cell lysis buffer [50 mM HEPES (pH 7.4); 0.1% CHAPS; 1 mM DTT; 0.1 mM EDTA]). Lysates were spun at 1500 *g* at 4° for 10 min and supernatants were stored at −80° until analysis [31]. Seventy-five microliters of caspase assay buffer (50 mM HEPES (pH 7.4); 100 mM NaCl; 0.1% CHAPS; 10 mM DTT; 1 mM EDTA; 10% glycerol; supplemented with additional protease inhibitors: 1 μg/mL of leupeptin, 1 μg/mL of pepstatin A, 1 mM phenylmethyl sulfonylfluoride, 1 μg/mL of aprotinin) was added to 10 μL of the appropriate fluorimetric caspase substrate (1 mM of Ac-DEVD-AMC or Ac-LEHD-AMC) and to 25 μL of the appropriate equal amounts of protein from each supernatant sample into a microplate. Ninety-six-well plates were incubated for 6 hr at 37° and the fluorogenic substrate was excited at 350 nm and the fluorescence read at 450 nm on a fluorimetric reader.

Cell Staining for Morphologic Analysis

Cell permeability and cellular glutathione content were studied by fluorescence microscopy. Twenty-four hours after HN2 treatment, MOLT-4 cells were washed twice with PBS and incubated for 30 min at 37° in culture medium containing both MCB and PI antifade at a final concentration of 200 μM and 50 μg/mL, respectively. MOLT-4 cells were then washed and adjusted at 1×10^6 cells/mL in PBS. Cell deposits of about 40,000 cells were applied to glass slides by cytocentrifugation for 5 min at 200 *g*. Cell morphology was examined using an Eclipse E1000 contrast phase microscope (Nikon) equipped with a video SPOT 2 imaging system (Diagnostic Instruments). MCB was excited at 340–380 nm with a UV mercury lamp and the fluorescence was collected with a 435–485 nm band pass filter. (PI, excitation 540/25 nm; band pass filter 605/55 nm). Color pictures were taken at 80× magnification under fluorescent illumination and subsequently digitized for red–green–blue presentation using Adobe Photoshop.

RESULTS

Inhibition of HN2-Induced Apoptosis in Murine Lymphocytes by Ebselen

BALB/c spleen cells were incubated for 1 hr in the presence of various concentrations of ebselen, then HN2 at 2.5 μM was added to the culture. After 24 hr of further incubation, lymphocytes were harvested, their viability determined by trypan blue exclusion, and the loss of cellular membrane integrity measured by PI staining. In control groups, the number of viable spleen cells decreased after 24 hr of culture (Fig. 1). Concentrations of ebselen below 25 μM increased the cell viability of spleen lymphocytes treated with HN2. Morphological observations and flow cytometry analysis revealed that an increasing proportion of these cells, up to 25% at 24 hr of culture, underwent spontaneous apoptosis (Fig. 2A). Ebselen at 25 μM or below failed to reduce this spontaneous apoptosis. Moreover, in the presence of 50 μM ebselen, a large amount of debris was observed, indicating a pronounced degree of cell disruption correlated with a decrease in cell viability (data not shown), even though the hypodiploid peak was totally inhibited. Figure 2A shows that in HN2-treated groups, a 2-fold increase in hypodiploid DNA particle formation was observed at 24 hr of culture. The percentage of cells exhibiting subG_0-G_1 DNA was significantly reduced, and flow cytometry analysis revealed that the collapse of the 2N

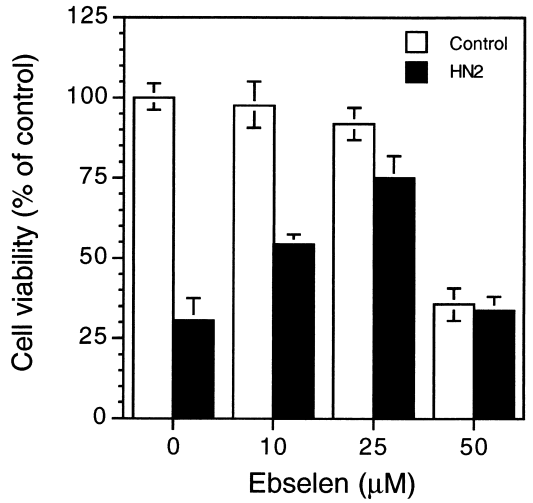

FIG. 1. Effect of ebselen on the viability of untreated or HN2-treated murine lymphocytes. Spleen cells from BALB/c mice were preincubated for 1 hr without or with various concentrations of ebselen and medium alone prior to HN2 (2.5 μM) addition. Cell viability was determined by trypan blue exclusion 24 hr thereafter. Values are means ± SD of four independent experiments.

peak was prevented by ebselen. The ratio between the percentage of hypodiploid cells and the percentage of cells in G_0-G_1 phase decreased almost linearly with the concentrations of ebselen (Fig. 2B). Interestingly, at non-toxic levels, ebselen concentration independently inhibited the DNA fragmentation induced by HN2. Phosphatidylserine, normally confined to the inner leaflet of the plasma membrane, is exported to the outer plasma membrane leaflet during apoptosis. Phosphatidylserine externalization, another hallmark of apoptosis, was analyzed by annexin V–FITC staining (Fig. 3). In ebselen-treated cells, a small increase in the amount of annexin V-positive cells was observed after 24 hr of culture, reaching a maximum at the toxic concentration of 50 μM. Apoptosis induced by HN2 was nevertheless clearly prevented by non-toxic concentrations of ebselen (as shown in Fig. 3).

Prevention by Ebselen of Apoptosis in MOLT-4 Cells Exposed to HN2

Next, we investigated the effects of ebselen preincubation in HN2-treated human MOLT-4 leukemia. In order to determine the cytotoxicity of ebselen on MOLT-4 cells, we evaluated the effect of different concentrations during 72 hr of continuous exposure. A cytotoxic effect was observed at concentrations higher than 10 μM (Table 1) as assessed by trypan blue exclusion. In subsequent experiments, MOLT-4 cells were therefore preincubated for 72 hr without or with non-toxic concentrations of ebselen (below 10 μM) and washed twice with RPMI medium to remove the compound. Cells were then resuspended in culture medium with 2.5 μM of HN2. The effects of ebselen on cell viability and morphological changes associated with apoptosis were evaluated at 24 hr. The data in Fig. 4A demonstrate that preincubation with ebselen inhibited the loss of cell viability induced by HN2 treatment. Furthermore, ebselen clearly protected MOLT-4 cells from an HN2-induced antiproliferative effect, as measured by the Alamar Blue assay (Fig. 4B). Hypodiploid particle formation was assessed by flow cytometry analysis of PI-labeled cells. As shown in Fig. 5, pretreatment with ebselen induced a significant dose-dependent inhibition of hypodiploid DNA particles in HN2-treated MOLT-4 cells. The production of ROS was evaluated by flow cytometry using the oxidation-sensitive fluorescent probe rhodamine 123. Dihydrorhodamine 123 is non-fluorescent, uncharged, and readily taken up by the cells, whereas rhodamine 123, the product of dihydrorhodamine oxidation, is fluorescent, positively charged, and binds selectively to the inner mitochondrial membrane of living cells [32]. The fluorescence of this dye is therefore an indicator of mitochondrial free radical production and membrane integrity. ROS contribute to the ability of HN2 to provoke cellular damage. The data in Fig. 6 demonstrate a significant increase in ROS levels in MOLT-4 cells 24 hr after the addition of HN2. Additionally, ebselen pretreatment diminished the ROS production after HN2 treatment in a concentration-dependent manner (Fig. 6). We next measured the amount of phosphatidylserine externalization in control and HN2-treated MOLT-4 cells (Fig. 7A). An 8-fold increase in annexin V–FITC-positive cells was detected at 24 hr in MOLT-4 cells treated with 2.5 μM HN2. Although 10 μM of ebselen pretreatment led to a 50% decrease in annexin V-positive cells and did not change the basal level of anionic phospholipid exposure, it did partially prevent phosphatidylserine redistribution of the apoptotic cells (Fig. 7). The number of annexin V-positive cells, 24 hr after 2.5 μM HN2 treatment, was 75% in non-treated cells and 35% in ebselen-pretreated cells. Interestingly, the PI versus annexin V–FITC staining pattern reveals an obvious difference between the HN2-treated cells in the absence or presence of ebselen (Fig. 7B). Simultaneous staining with PI and annexin V–FITC (Fig. 7B) allows the identification of an apoptotic population in HN2-treated cells (annexin V-positive and PI-negative) whose emergence is prevented by a pretreatment with ebselen. It is noteworthy that late apoptosis or necrotic cell death (annexin V-positive and PI-positive) induced by HN2 was also inhibited by pretreatment with ebselen. The cell-permeant MCB is an indicator of GSH level in whole cells. This dye is essentially non-fluorescent until conjugated. Flow cytometry analysis and fluorescence microscopy observations of GSH content and cell permeability were performed with simultaneous MCB and PI staining. A significant increase in the number of PI-permeable cells was observed at 24 hr after HN2 treatment (data not shown). Nevertheless, ebselen pretreatment inhibited apoptosis induced by HN2 and prevented cellular depletion of GSH pools (Fig. 8). These latter results were also confirmed by MCB and PI double staining cell microscopic observation at 24 hr after treatment (Fig. 9). We observed that ebselen impaired the

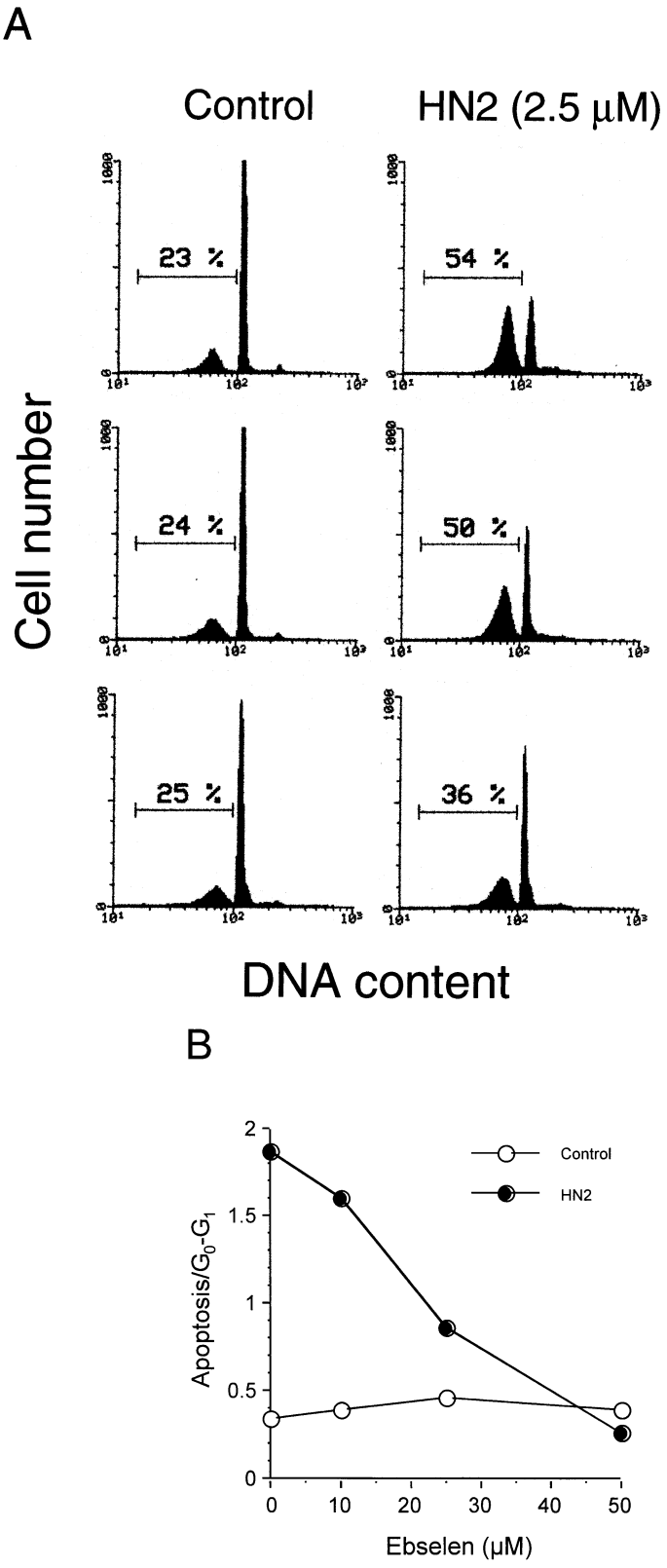

FIG. 2. Effect of ebselen on spontaneous and HN2-induced hypodiploid DNA particle formation in murine lymphocytes. (A) BALB/c splenocytes were preincubated for 1 hr in the presence of various concentrations of ebselen before the addition of 2.5 μM HN2. The cells were collected 24 hr thereafter, washed, fixed, and labeled with PI as described in Materials and Methods. The percentage of hypodiploid particles with subG_0-G_1 DNA content was determined by flow cytometry analysis. (B) Evolution of the ratio apoptosis/G_0-G_1 as a function of the ebselen concentrations. The ratios were calculated between the percentage of hypodiploid DNA population and the percentage of spleen cells in G_0-G_1 phase. Data are from one representative experiment. This experiment was repeated twice with similar results.

cytotoxicity of HN2, as well as the proportion of apoptotic cells (Figs. 8 and 9). Subsequently, to investigate whether caspase activity increases in response to HN2, cytosolic extracts from HN2 or ebselen plus HN2-treated MOLT-4 cells were subjected to a protease activity assay using the fluorogenic peptide substrates Ac-DEVD-AMC and Ac-LEHD-AMC. Enzymatic cleavage of Ac-DEVD-AMC and Ac-LEHD-AMC, which are specific to caspase-3-like and

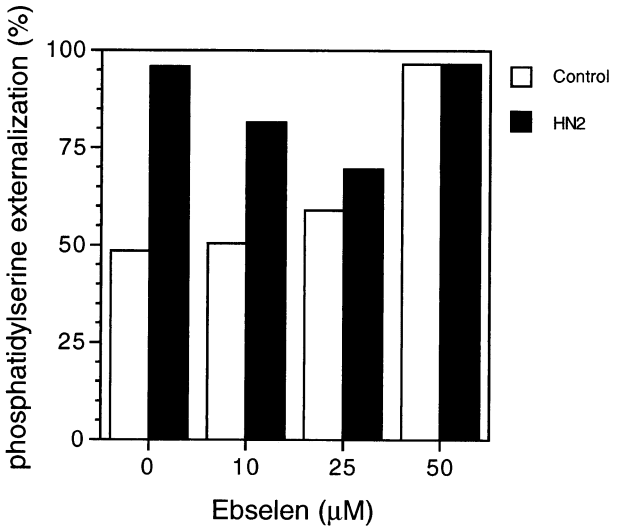

FIG. 3. Effect of ebselen on phosphatidylserine externalization in murine lymphocytes treated with HN2. BALB/c splenocytes were preincubated with different concentrations of ebselen for 1 hr and then treated with solvent vehicle alone or with 2.5 μM HN2. The percentage of annexin V-positive cells was measured 24 hr thereafter by flow cytometry analysis as described in Materials and Methods. Data are from one representative experiment. This experiment was repeated three times with similar results.

caspase-9-like proteases, respectively, became elevated 24 hr after the addition of HN2, whereas preincubation of ebselen considerably reduced the level of caspase activities in HN2-treated MOLT-4 cells (Fig. 10). On the other hand, treatment with a reversible and potent inhibitor of the caspase-3-like activity, Ac-DEVD-CHO (100 μM), 1 hr prior to HN2 incubation did not induce the enzymatic cleavage of Ac-DEVD-AMC (Fig. 10) or internucleosomal DNA fragmentation (data not shown). The inhibition of caspase-3-like protease by Ac-DEVD-CHO served as selective control inhibition of the effector caspase activation. Ac-DEVD-CHO at 100 μM blocked the activity of DEVD-sensitive proteases in control and HN2-treated cells with minimal effects on the activation of caspase-9, whereas ebselen less selectively inhibited both caspase activities, suggesting that caspase-9 is activated before the DEVD-

TABLE 1. Effect of ebselen alone on MOLT-4 cell viability

Incubation conditions	Viable cells (%)
DMSO	97 ± 3
NaCl 0.1%	98 ± 2
Ebselen (1.25 μM)	96 ± 5
Ebselen (5 μM)	97 ± 4
Ebselen (10 μM)	77 ± 8
Ebselen (25 μM)	5 ± 1
Ebselen (50 μM)	0 ± 1

MOLT-4 cells were incubated with the indicated concentrations of ebselen until viability was determined at 72 hr of continuous exposure. Cells excluding trypan blue were considered viable. Initial viability was 98 ± 2%. Data represent means ± SD of three independent measurements.

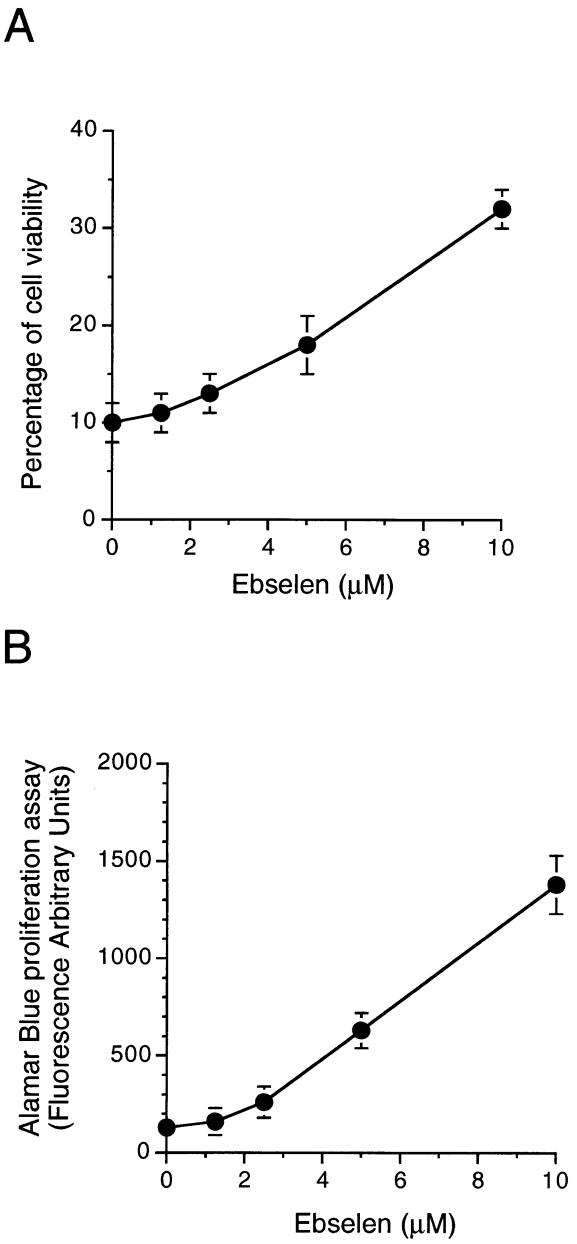

FIG. 4. Effect of ebselen on the viability and proliferation of HN2-treated MOLT-4. The cells were preincubated with different concentrations of ebselen for 72 hr, washed twice, and resuspended in fresh culture medium without ebselen immediately before the addition of 2.5 μM HN2. (A) Cell viability was determined 24 hr thereafter by trypan blue exclusion. Values are means ± SD of 3 independent experiments in duplicate. (B) Determination of cell proliferation by the Alamar Blue proliferation assay at 4 days after the addition of HN2. No significant difference was recorded in MOLT-4 cell growth between control and ebselen-treated cells. The assay was performed according to the manufacturer's instructions. Data represent means ± SD of three independent experiments.

sensitive proteases in HN2-treated cells. We also observed that ebselen inhibited, in a concentration-dependent manner, caspase activities in HN2-treated MOLT-4 cells (data not shown); however, no significant differences were observed between untreated MOLT-4 cells

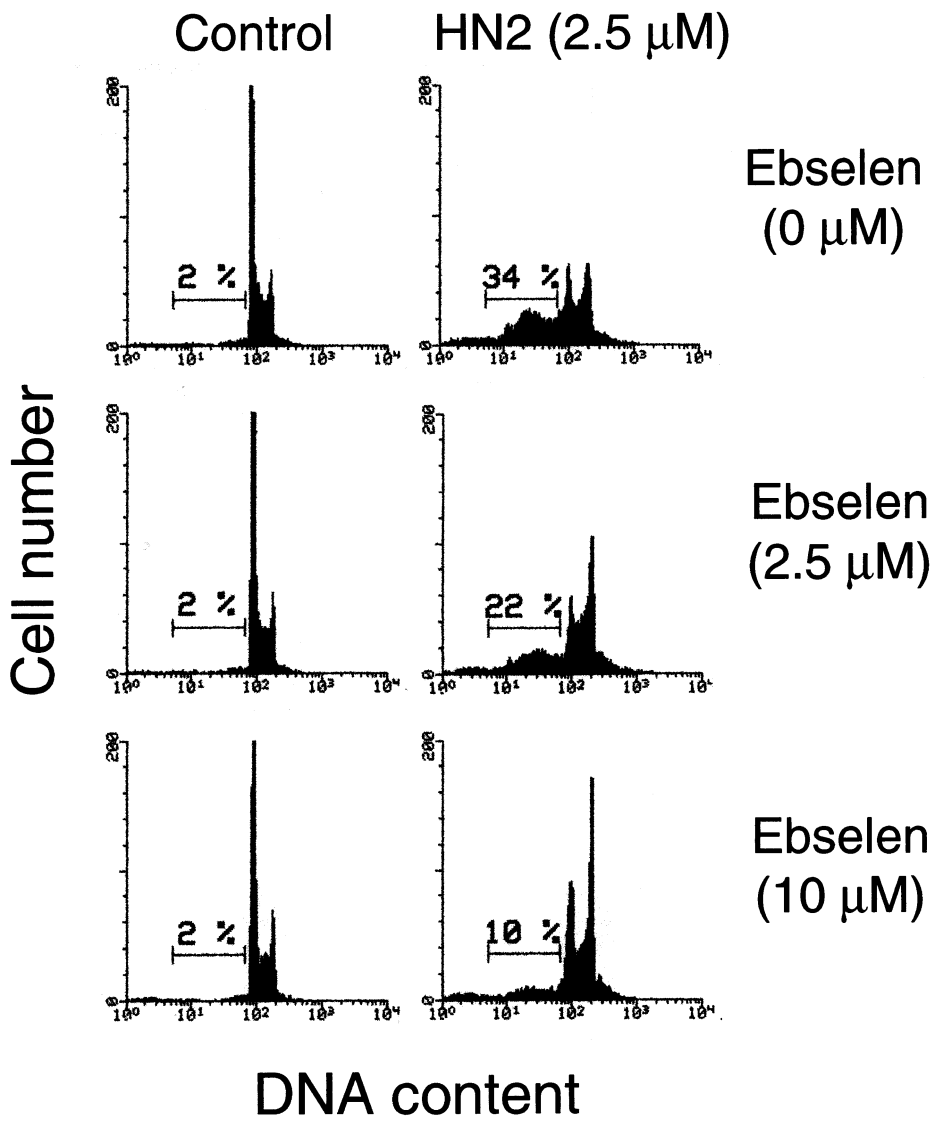

FIG. 5. Inhibition of hypodiploid DNA particle formation by ebselen in HN2-treated MOLT-4 cells. The cells were preincubated in the presence of various concentrations of ebselen for 72 hr, washed twice, and treated with 2.5 μM HN2. The cells were collected 24 hr thereafter, washed, fixed, and labeled with PI as described in Materials and Methods. The percentage of cells containing hypodiploid DNA was determined by flow cytometry analysis. Data are from one representative experiment. This experiment was repeated twice with similar results.

and ebselen-treated MOLT-4 cells. Taken together, our data demonstrate that ebselen impairs the mitochondrial initiation of apoptotic hallmarks triggered by HN2, which is associated with the maintenance of mitochondrial transmembrane potential and elevated intracellular GSH levels.

In vivo *Assessment of Ebselen Effect*

We reported previously that a single injection of HN2 provoked a marked reduction in splenic weight and cellularity in mice [33]. To examine a possible utilization of ebselen as antidote against nitrogen mustard, mice were treated by ebselen 24 hr prior to HN2 injection. The data in Fig. 11A show that 24 hr after HN2 administration, there was a significant loss of spleen weight, which was counteracted in ebselen-injected animals. Comparable results were observed for spleen lymphocyte cellularity (Fig. 11B). It is to be noted that ebselen alone (4 mg/kg) had no noticeable effect on spleen weight or cellularity. These results suggest that ebselen exerts a protective effect *in vivo*.

DISCUSSION

Taken together, the results presented here indicate that ebselen can prevent *in vitro* apoptosis induced by HN2 in both normal murine lymphocytes and MOLT-4 cells. This approach adds to other pharmacological interventions that have been proposed to prevent or inhibit apoptosis [3], e.g. *N*-acetylcysteine [34, 2] or trolox [35, 36], both of which were shown to inhibit apoptosis by scavenging ROS generated by ionizing radiation or HN2. Ebselen, a more recently identified antioxidant, was previously reported to protect cells from radiation-induced apoptosis by enhancing phospholipid hydroperoxide glutathione peroxidase activity in thymocytes [26]. In our experiments, we measured significant levels of hypodiploid DNA particles and phosphatidylserine externalization in untreated spleen lympho-

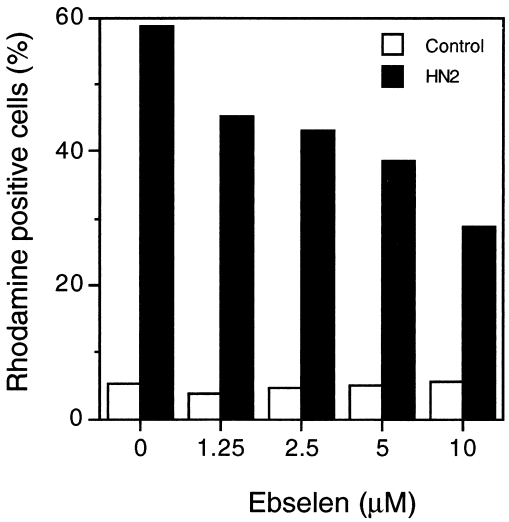

FIG. 6. Inhibition of mitochondrial reactive oxygen production by ebselen in HN2-treated MOLT-4 cells. The cells were preincubated with different concentrations of ebselen for 72 hr, washed twice, and treated with 2.5 μM HN2. The cells were collected 24 hr thereafter, and mitochondrial reactive oxygen species were quantified by flow cytometry using the rhodamine 123 probe as described in Materials and Methods. Results are representative of 3 separate experiments.

cytes. Spontaneous apoptosis, which appears shortly after the onset of culture [37], probably results from the loss of survival signals due to the disruption of the splenic microenvironment. In normal murine spleen cells treated with ebselen alone at non-toxic concentrations, the spontaneous hypodiploid DNA level was found to remain unchanged. In contrast, spontaneous apoptosis in mice thymocytes was found to be totally prevented by ebselen [26]. In this instance, oxidative damage induced by incubation conditions was blocked by the capacity of ebselen to inhibit peroxide production. This apparent discrepancy between our data and those of Ramakrishnan may reflect differences in apoptotic signal transduction between murine splenocytes and thymocytes [38]. Nevertheless, higher ebselen concentrations (50 μM), toxic for these cells under our experimental conditions, were effective at completely inhibiting spontaneous and HN2-induced apoptosis. In cells treated with 50 μM of ebselen alone or combined with nitrogen mustard, a large amount of debris was observed in PI-labeled spleen cells at 24 hr, indicating a pronounced degree of necrotic cell death correlated with the decrease in cell viability (data not shown). This relative inhibition of apoptosis after HN2 treatment is also truncated by the cytotoxic effect of ebselen preincubation. Moreover, our results indicate that at concentrations lower than 25 μM, ebselen reduces both hypodiploid particle formation and phosphatidylserine externalization in HN2-treated murine splenocytes.

A major concern in the clinical application of chemoprotectors is their intrinsic toxicity [12]. The toxicity of selenium compounds is thought to be caused by the bioavailability of their selenium moiety and their bioactivation to hydrogen selenide [39]. In contrast with most selenium compounds, the selenium moiety of ebselen is not available for incorporation into proteins and, moreover, ebselen is not converted into hydrogen selenide [39, 40]. However, our results indicate that ebselen is cytotoxic at concentrations higher than 25 μM toward both murine splenocytes and human MOLT-4 leukemia cells. At 25 μM, ebselen completely inhibited MOLT-4 proliferation and decreased splenocyte viability. Ebselen was recently found to be a competitive inhibitor of human thioredoxin reductase (K_i = 2.8 μM). A number of studies have shown that thioredoxin is responsible for the growth and transformed phenotype of some human cancer cells [41]. In HepG2 cells, higher concentrations (>50 μM) of ebselen induced apoptosis by depleting the intracellular pool of GSH [42]. Reduced glutathione and thioredoxin are the major soluble intracellular antioxidants implicated in the regulation of apoptosis and necrosis, and it has been postulated that an excessive depletion of GSH and thioredoxin predisposes cells to necrotic cell death [43]. This may explain why ebselen and some of its derivatives have inhibitory effects on human cancer cell growth at micromolar concentrations.

Oxidative stress induced by HN2 also produces lipid peroxidation in membranes, which in turn causes hydroxide phospholipids [22, 44]. Lipid hydroperoxides can induce apoptosis by their capacity to increase cytoplasmic calcium [45]. It was reported that preincubation of human plasma with micromolar concentrations of ebselen strongly reduces the level of hydroperoxide phospholipids [46]. Recently, Ramakrishnan has demonstrated that radiation-induced peroxide generation was blocked by ebselen [26]. Ebselen is able to abolish the detelerious effects of hydroperoxides by reducing them to the corresponding alcohols [10]. In many cases, ebselen's cytoprotective effect is due to its hydroperoxide-reducing capacity. Taken together, these results indicate that lipid peroxidation and oxygen radicals may have a pivotal role in the induction of apoptosis by HN2 and that ebselen is a potent inhibitor of apoptosis induced by the latter.

The involvement of ROS as apoptotic signaling molecules in cell death induced by alkylating agents has been suggested by some authors (for review, see [47]). The apoptotic process triggered by HN2 was associated with an oxidative stress and up-regulation of mitochondrial Bcl-2 protein [7], and we show here that intracellular depletion of GSH and ROS production is involved in cell death induced by this alkylating agent. Moreover, glutathione, a non-protein antioxidant present in millimolar concentrations in mammalian cells, is implicated in maintaining the cellular oxidation–reduction balance and has been shown to rescue cells from a wide variety of exogenous insults [47]. Reduced GSH represents the intracellular defense against free radicals and is involved in the elimination of reactive peroxides and hydroperoxides [48]. We show that ebselen at 10 μM did not significantly enhance the cellular GSH pools during the 72-hr preincubation period, whereas it did prevent the

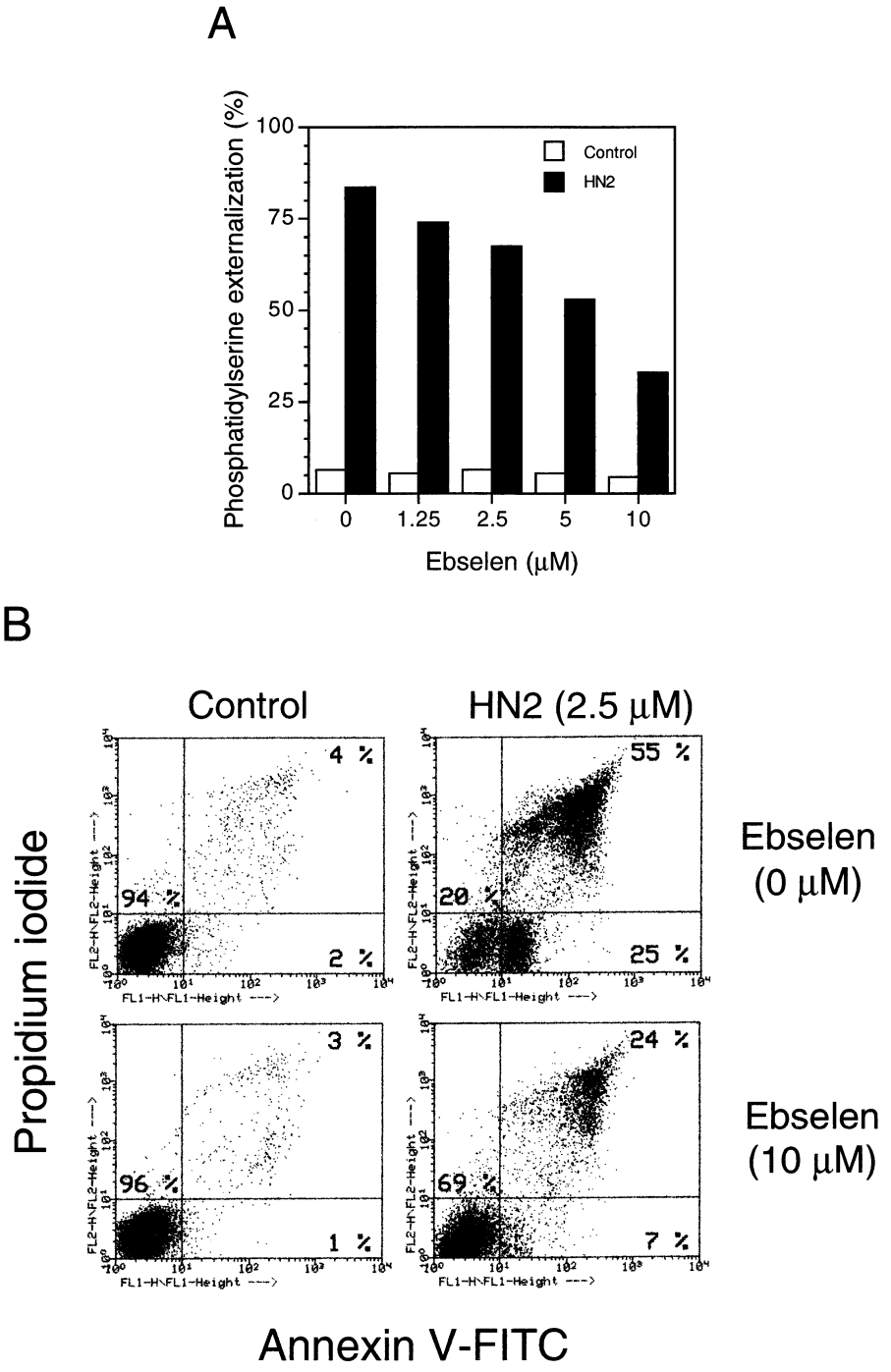

FIG. 7. Inhibition of phosphatidylserine externalization by ebselen in HN2-treated MOLT-4 cells. (A) Cells were preincubated with different concentrations of ebselen for 72 hr, washed, and treated with 2.5 μM HN2. The cells were collected 24 hr thereafter and stained with annexin V–FITC. Phosphatidylserine externalization was quantified by flow cytometry analysis. (B) Dot–plot representation of the double staining PI versus annexin V–FITC as determined 24 hr after HN2 treatment. The cells were labeled and analyzed by flow cytometry as described in Materials and Methods. Results are representative of 3 independent experiments. (This experiment was repeated three times with similar results.) FL1-H (band pass filter, 530/30 nm) and FL2-H (band pass filter, 585/42 nm) are the relative intensities of logarithmic fluorescence determined in channels 1 and 2, respectively.

decrease in GSH content and protect MOLT-4 cells from apoptosis triggered by HN2. Nevertheless, intracellular ebselen can generate a storage pool of ebselen–GSH adducts [49] which in turn, by reacting directly with HN2 or indirectly with HN2-induced peroxides, may exert a protective action. In addition to the formation of ebselen–GSH, ebselen could react with other extracellular protein thiols such as serum albumin, generating a selenol intermediate which may play an important role in the protective effect [50]. The mechanisms of the protective effect of ebselen are unknown. These selenol intermediates, through a chemical reaction with GSH and other thiols, may also be able to react as nucleophiles with HN2. These reactions may lead to the inactivation of HN2. However, it seems unlikely that this mechanism accounts for the entirety of ebselen's protective action. Nevertheless, it must be noted that ebselen-treated MOLT-4 cells were washed twice before the addition of fresh culture medium containing the alkylating agent. Moreover, similar results were obtained using a fetal bovine serum-free culture medium (data not shown).

Recently, alterations in mitochondrial functions have been found to play a key role in the effector phase of

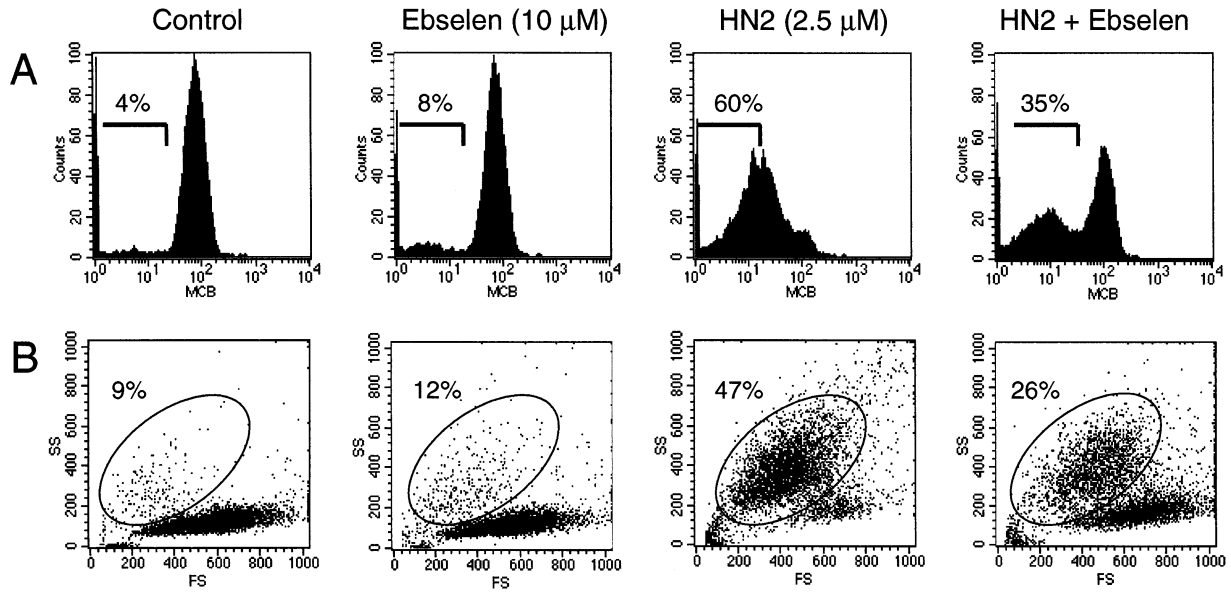

FIG. 8. Prevention of intracellular GSH content by ebselen in HN2-treated MOLT-4 cells. Cells were preincubated with a single concentration of ebselen for 72 hr, washed, and treated with HN2 for 24 hr (see Materials and Methods). (A) Ebselen inhibited depletion of cellular GSH pools as shown by flow cytometry histograms from MCB staining. (B) Apoptosis is also inhibited by ebselen as detected by dot–plots of side (SS) and forward scatter (FS) using flow cytometry. This experiment was repeated in triplicate with similar results.

apoptosis induced by different agents [51, 52]. These alterations include the disruption of mitochondrial transmembrane potential, the opening of permeability transition pores, and the generation of ROS. In addition, the release of cytochrome *c* mitochondrial protein induces the formation of the apoptosome complex (procaspase-9, cytochrome *c*, apoptotic protease-activating factor, and dATP), which becomes activated under certain conditions and in turn is able to activate the cytoplasmic caspase cascade [53, 54]. Caspases are expressed in cells as inactive zymogens (pro-

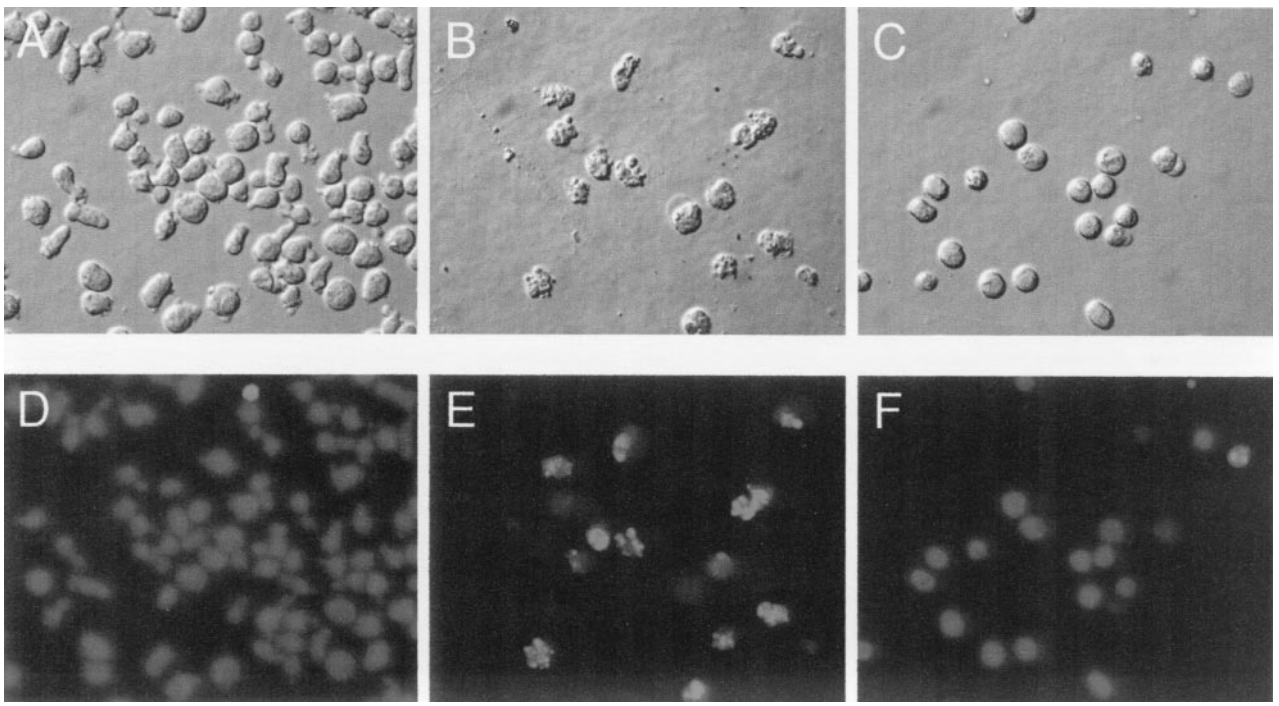

FIG. 9. Microscopic morphology of MOLT-4 cells dying from apoptosis in the presence of HN2. (A and D) MOLT-4 cells pretreated with ebselen alone (10 μM), (B and E) in the presence of HN2 alone (2.5 μM), or (C and E) in the presence of HN2 plus ebselen, and then observed under phase-contrast microscope (A, B, and C), and fluorescent microscope after double staining with MCB/PI (D, E, and F). Note the typical apoptotic change after HN2 addition and the protective effect of ebselen on apoptosis induced by HN2. Also, GSH depletion and cell permeability are prevented by ebselen-pretreated MOLT-4 cells.

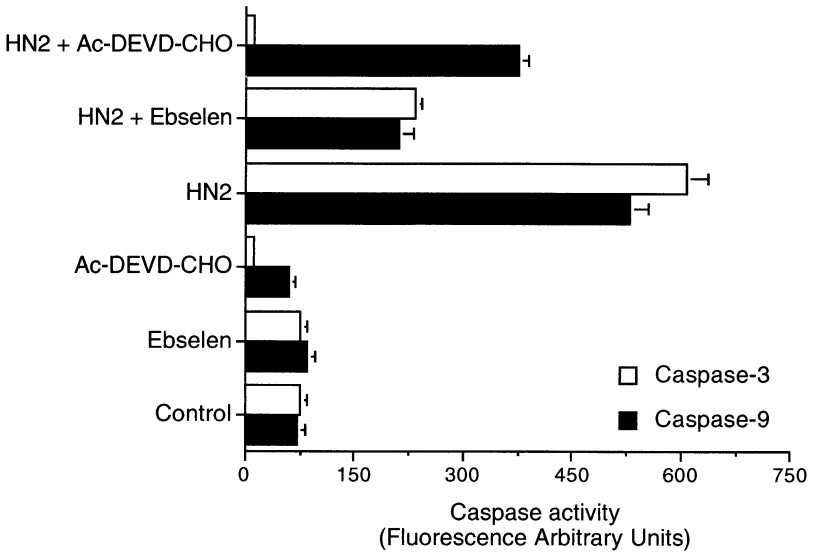

FIG. 10. Activation of caspases during apoptosis induced by HN2. MOLT-4 cells were preincubated with or without 10 μM of ebselen for 72 hr and then washed before the addition of HN2 (2.5 μM) for 24 hr. MOLT-4 cells were pretreated with a single concentration (100 μM) of Ac-DEVD-CHO 1 hr prior to HN2 addition. Whole cell lysates prepared at 24 hr after HN2 treatment were assayed for caspase activity determination as described in the Materials and Methods section. Caspase-3-like or caspase-9-like proteolytic activity was examined by monitoring cleavage and release of AMC from the fluorogenic substrates Ac-DEVD-AMC or Ac-LEHD-AMC, respectively. Activities are represented as the intensity of fluorescence of amion-4-methylcoumarin. Data are representative of three experiments.

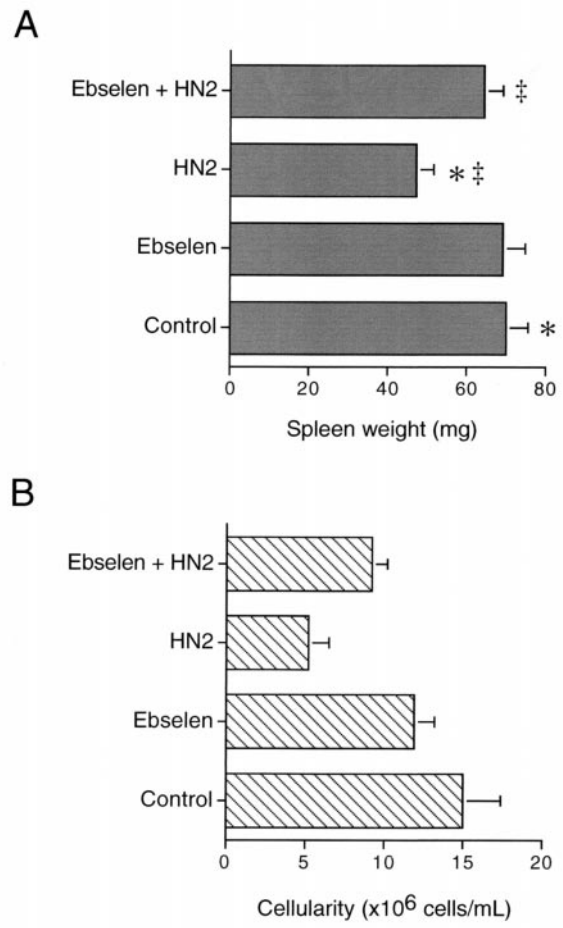

FIG. 11. Influence of ebselen on the spleen weight and lymphocyte cellularity of HN2-treated BALB/c mice. (A) The effect of single ebselen (4 mg/kg) injection on the reduction in spleen weight and (B) lymphocyte cellularity 24 hr prior to a dose of HN2 (6 mg/kg). Spleen were removed 24 hr after HN2 injection. Differences between spleen weight of HN2-treated mice versus control were statistically significant at: *, $P < 0.03$; and the protective effect of ebselen against spleen weight loss was significant at: ‡, $P < 0.05$ (Mann–Whitney test). Values are means of quadruplicate determinations.

caspases) and converted during the apoptotic process into their active forms by proteolytic cleavage. Therefore, our results indicate that caspase-9 (as an activator of caspases) is activated in HN2-induced apoptosis. Our data describe the capacity of ebselen pretreatment to reduce HN2-induced mitochondrial oxygen radical generation in MOLT-4 cells, suggesting that this antioxidant could also intercept signal apoptotic pathways, possibly by preventing intracellular GSH depletion, mitochondrial membrane disruption, and caspase-9 proteolytic activation. Similar results were obtained with overexpression of mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx) in M15 cells in which PHGPx has been shown to prevent apoptosis induced by different neoplastic drugs but unable to inhibit cell death induced by Fas-specific antibodies [55]. This suggested that mitochondrial PHGPx selectively suppressed release of cytochrome *c*, caspase-3 activation, and apoptosis via the mitochondrial death pathway, in which production of hydroperoxide is a major apoptotic factor.

In conclusion, pretreatment of normal or transformed lymphocytes with ebselen prevented all the biochemical changes known to be associated with apoptosis, thereby conferring resistance on the cytotoxic action of HN2. When injected *in vivo*, ebselen was also protective against spleen weight and cell loss. Whatever the precise mechanism by which ebselen prevents spleen weight loss from HN2-induced cytotoxic damage, it appears as one of the most attractive antioxidants in its category and may find utility as a cytoprotective compound against the cytotoxicity of nitrogen mustard in both clinical and non-clinical situations.

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