

6-Mercaptopurine Decreases the Bcl-2/Bax Ratio and Induces Apoptosis in Activated Splenic B Lymphocytes

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SUMMARY

6-Mercaptopurine and related purine antimetabolites are used in the treatment of several B cell disorders. These drugs inhibited the proliferation of mature splenic B cells after being triggered with polyclonal mitogens. In addition to the antiproliferative effects, 6-mercaptopurine, 2-mercaptopurine, and aminoguanidine evoked a rapid apoptotic cell death in activated B cells that started at 6 hr after drug treatment and therefore preceded DNA synthesis. Incubation of activated B lymphocytes with 6-mercaptopurine blocked the low but sustained nitric oxide release observed in these cells that contrib-

utes to the prevention of apoptotic cell death; the addition of chemical nitric oxide donors significantly antagonized the apoptosis elicited by these drugs. The inhibition of nitric oxide synthesis elicited by mercaptopurines correlated with a decrease in the release of nitric oxide-derived species to the culture medium and in the intracellular levels of cGMP. The ratio between the amounts of Bcl-2 and Bax, two proteins involved in the control of apoptosis in mature B cells, markedly decreased as result of mercaptopurine treatment.

The introduction of MP into the treatment of certain forms of lymphoblastic leukemia was a major success in the development of therapeutic protocols based on the use of antimetabolites of nucleic acid synthesis (1, 2). Indeed, 6-MP remains an important drug in the maintenance therapy of acute lymphoblastic leukemia (1–4). 6-MP has also been used in immunosuppressive therapy and in the treatment of steroid-unresponsiveness inflammatory bowel disease (3, 5). Many studies have been devoted to the establishment of the mechanism of action of these drugs and to the characterization of the metabolic pathways involved in nucleic acid antimetabolite fate as well as in the monitoring of the plasma levels of these substances (5). The main enzyme activity involved in the depletion of circulating 6-MP levels is thiopurine methyltransferase, which promotes *S*-methylation of 6-MP as the first step in the degradative pathway. This enzyme exhibits a high degree of genetic polymorphism in humans (6), and the individual variations in this activity provide a plausible explanation for the diversity in the efficacy of 6-MP treatment. For this reason, related drug derivatives have been envisaged as alternative or complementary candidates in antimetabolite therapy (e.g., 6-mercaptopurine, several chloropurines, and azathioprine) (7, 8). Because these drugs exhibit structural analogy with physiological

purines, they inhibit the synthesis and interconversion of purine nucleotides (1, 9). These analogues incorporate into both RNA and DNA and therefore may affect several cell functions (9). However, evidence is accumulating that suggests that the number of cellular function targets affected by purine antimetabolites is larger than previously supposed. For example, 2-chlorodeoxyadenosine induces a rapid apoptotic death and potentiates cell-mediated cytotoxicity by natural killer cells in various lymphocytic disorders (10, 11).

Most of the studies of MPs have been done using lymphoma cell lines; however, little is known about how these drugs affect normal lymphocyte cell function and why they exhibit a high degree of specificity for the treatment of certain B cell disorders. It is of interest to know, therefore, whether MPs influence the physiological process of B cell triggering. In the absence of stimuli, isolated mature B cells initiate a rapid process that leads to apoptotic cell death, whereas cell activation rescues the cells from apoptosis and initiates a series of events that promote cell growth and differentiation (12, 13). Current research stresses the importance of the maintenance of high levels of the Bcl-2 proto-oncogene in the survival of mature B lymphocytes (14). However, although overexpression of Bcl-2 seems to protect cytokine-deprived cells (i.e., T lymphoblasts in the absence of interleukin-2), it seems that Bcl-2 inhibits the apoptosis of cells that fail to receive survival signals (15). Bcl-2 was identified in human lymphomas exhibiting a translocation be-

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ABBREVIATIONS: MP, mercaptopurine; ISO, dinitrate isosorbide; LPS, lipopolysaccharide; NOS, nitric oxide synthase; NO, nitric oxide; SIN-I, 3-morpholinolysynonimine; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

tween chromosomes 14 and 18 (16) and has been involved in the prolonged survival of several B cell lines (e.g., acute lymphoblastic leukemia, non-Hodkin's lymphomas, and other lymphoid disorders involving B and T lymphocytes) (16, 17). Although the signals relevant to the control of the Bcl-2 levels remain unknown, the survival function of Bcl-2 depends on the extent of binding to proteins such as Bax and Bcl-x_s that seem to antagonize its activity (18). In addition to this, different data suggest that the generation of low but sustained levels of NO is an additional condition that improves B cell survival (19, 20). Considering these results, we investigated the ability of 6-MP to induce a rapid apoptosis in B cells as part of the pharmacological effect of this drug. In addition to this, we tested the hypothesis of decreased NO synthesis as a likely candidate to trigger B cell death after treatment with 6-MP and related drugs. Our results show that in activated B cells, MPs promote a rapid apoptotic cell death, a fall in NOS activity, and a decrease in the Bcl-2/Bax ratio.

Materials and Methods

Chemicals. [³H]Thymidine was obtained from New England Nuclear Research Products (Boston, MA). Purine and related metabolites and biochemicals were from Sigma Chemical (St. Louis, MO). LPS was from Difco (Detroit, MI). Materials and chemicals for electrophoresis were from BioRad (Richmond, CA). The ELISA cell-death kit was from Boehringer-Mannheim Biochemica (Mannheim, Germany). Other chemicals and biochemicals were from Boehringer-Mannheim or Merck (Darmstadt, Germany). Antibodies were from Santa Cruz Biochemicals (Santa Cruz, CA). A fusion protein of murine CD40-human Cγ1 was used to immunize Lewis rats from which hybridomas were produced (a generous gift from Dr. J. Andersson, Basel Institute of Immunology, Basel, Switzerland). An anti-CD40 mAb (FGK 45.5) corresponding to an IgG, κ, was purified on a protein G/Sepharose column, and the optimal concentration for proliferation was 5–10 μg/ml.

Mice. Eight- to 10-week-old inbred BALB/c mice were used throughout the experiments and were from our colony.

Preparation of lymphocytes. Spleens from four or five animals were removed under sterile conditions, and the B cell-enriched suspension was obtained after treatment with anti-Thy-1 antibody plus rabbit C (20). Resting B lymphocytes were prepared by centrifugation on a discontinuous Percoll gradient, and cells sedimenting at $\rho > 1.072$ (mg/ml) were used. To remove macrophages, the lymphocyte suspension was treated with anti-Mac-1 antibody plus rabbit C (21). After three washes in RPMI 1640, the cells were resuspended in RPMI 1640 supplemented with 10% of batch-tested nonstimulatory fetal calf serum, 2 mM glutamine, antibiotics, and 50 μM β-mercaptoethanol (culture medium). The B cell population purity was determined by immunofluorescence on an EPIC profile cytofluorometer and resulted in the expression of >97% Ig⁺ and <1% of Thy-1.2⁺ and Mac-1⁺, respectively. B220⁺ cells were purified by flow cytometry sorting.

Analysis of DNA fragmentation. Internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis, by detection of mononucleosomes and oligonucleosomes in the cytosol (ELISA cell death kit; Boehringer-Mannheim), and by flow cytometry as follows. The cell suspension (1–2 × 10⁶ cells) was centrifuged at 200 × *g* at 4° for 10 min, and the pellet was washed twice with ice-cold PBS. After resuspension in 250 μl of PBS, an equal volume of 20 mM EDTA, 0.5% Triton X-100, and 5 mM Tris-HCl, pH 8.0, was added, and the incubation was continued for 15 min at 4°. Nuclei were removed by centrifugation at 500 × *g* for 10 min, and the resulting supernatant was centrifuged at 30,000 × *g* for 15 min (soluble fraction). The fragmented DNA present in the soluble fraction was precipitated

with 70% ethanol plus 2 mM MgSO₄, and aliquots were treated for 1 hr at 55° with 0.3 mg/ml proteinase K. After two extractions with phenol/chloroform, the DNA was analyzed in a 2% agarose gel and stained with 0.5 μg/ml of ethidium bromide (20). The DNA present in the soluble fraction was also analyzed using an ELISA cell death kit in which the histone-associated DNA fragments were detected using a sandwich ELISA with anti-histone and anti-DNA-peroxidase antibodies. The relative degree of apoptosis was quantitatively determined by measuring the peroxidase activity at 405 nm and calculating the ratio between the enzyme activity of a sample incubated for a given period of time and the corresponding value at time 0 hr after activation (enrichment factor). Alternatively, B lymphocytes were labeled *in vivo* in the presence of 0.005% propidium iodide (20, 22, 23). In this way, apoptotic cells exhibited transient fluctuations in the membrane permeability, which allowed a characteristic staining that was distinct from that of necrotic cells. Cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France) equipped with a 5-W argon laser, and the forward scatter was plotted against the propidium iodide fluorescence. Data were calculated using the density plot function, and the percentage of apoptotic cells (quadrants R3 plus R4) was determined. Viable or necrotic cells are represented in the R1 and R2 quadrants, respectively. These cell populations were confirmed by DNA analysis after cell sorting (20).

[³H]Thymidine incorporation. Proliferation was determined by culturing 5 × 10⁴ cells/well onto 96-well plates. [³H]Thymidine (1 μCi) was added for the last 15-hr period of culture. The thymidine incorporation was measured by scintillation counting after retention over GF/C glass-fiber filters under acidic conditions.

Determination of NO. NO release was determined spectrophotometrically on the basis of the accumulation of nitrite in the medium (phenol red free) according to the method of Misko *et al.* (24). The culture medium (0.1–0.5 ml) was centrifuged, and the nitrite present in the supernatant was determined by the addition of 50 μl of 100 μM 2,3-diaminonaphthalene (dissolved in 0.6 M HCl). After incubation for 10 min at room temperature, the tubes were filled with 50 μl of 1 N NaOH, and the fluorescence was measured at 450 nm after excitation at 365 nm. A standard curve was constructed with NaNO₂.

Determination of cGMP. B220⁺ B lymphocytes (10⁶ cells) were challenged with 0.5 mM isobutyl-1-methylxanthine at the time of ligand addition to favor the accumulation of this cyclic nucleotide. The cells were collected by centrifugation, and after washing with PBS, the cell pellet was resuspended in 0.2 ml of an ice-cold mixture of ethanol/water (2:1 v/v). After thorough mixing and centrifugation in an Eppendorf centrifuge, samples were speed vacuum-dried, and cGMP was measured using a specific binding kit according to the recommendation of the supplier (Amersham International, Buckinghamshire, UK).

Detection of Bcl-2 and Bax. B cells (5 × 10⁶) were washed twice and after two cycles of freezing and thawing were homogenized in PBS. Aliquots of 15 μg of protein were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amounts of Bcl-2 and Bax were determined by Western blotting and immunodetection using the ECL technique. The mAb hamster anti-mouse Bcl-2 3F11 (kindly donated by Dr. G. Nuñez, Ann Arbor, MI) was generated by immunization of hamsters with purified recombinant Bcl-2 protein expressed in *Escherichia coli* and recognizes a 23-kDa protein corresponding to the truncated bcl-2 gene product in lysates from bacteria transformed with *bcl-2* cDNA. Anti-Bax antibody was obtained from Santa Cruz Biochemicals. Alternatively, Bcl-2 levels were measured *in situ* by flow cytometry as follows. After fixation for 20 min with one volume of 4% formaldehyde at room temperature, the cells were labeled with biotinylated anti-B220 and then were permeabilized with 0.5% saponin in PBS. After extensive washing with PBS, the cell pellet was resuspended with PBS containing 0.5% bovine serum albumin and incubated for 45 min with anti-Bcl-2 antibody, followed by rabbit anti-hamster IgG-fluorescein isothiocya-

nate antibody. Quantitative analysis was performed on a flow cytometer using additional staining with B220 to confirm the homogeneity of the cell population (12, 20).

RNA extraction and analysis. Total RNA was extracted from the same number of cells for each experiment ($3-4 \times 10^6$ cells) according to the guanidinium thiocyanate method (25). RNA was size-separated by electrophoresis in a 0.9% agarose gel containing 2% formaldehyde and 3-(*n*-morpholino)propanesulfonic acid buffering system (20) and transferred to Nytran membranes (NY 13-N; Schleicher & Schuell, Keene, NH). The level of *bcl-2* mRNA was determined using a *Hind*III fragment from the *bcl-2* cDNA as probe (15) that was labeled with [α - 32 P]dCTP using the Random Primed labeling kit (Boehringer-Mannheim). The hybridized membrane was exposed to X-ray film (Kodak X-OMAT). Quantification of the films was performed by laser densitometry (Molecular Dynamics, Sunnyvale, CA) using the hybridization with an 18S ribosomal probe for normalization.

Protein determination. Protein was measured using the Bio-Rad protein assay reagent.

Statistical analysis. Values are mean \pm standard error of three or four experiments performed in duplicate. Statistical significance was estimated with Student's *t* test for unpaired observations, with *p* < 0.05 considered significant. In studies of individual RNA or protein immunodetection samples, linear correlations between increasing amounts of input RNA or protein and signal intensity were observed (correlation coefficient was >0.9).

Results

MPs inhibit B cell proliferation. B cell activation by polyclonal mitogens such as LPS promoted proliferation of *ex vivo* cultured lymphocytes, and the maximal thymidine incorporation was observed 72 hr after triggering. When cells were incubated with 6-MP, 2-MP, or a related antimetabolite such as 2-aminopurine or aminoguanidine, a clear inhibition of thymidine incorporation was observed (Fig. 1A). This inhibition was dose dependent, and the half-maximal effect was obtained at concentrations of 0.8, 1.0, 1.5, and 2.5 μ M for 6-MP, 2-MP, 2-aminopurine, and aminoguanidine, respectively. The inhibition of proliferation elicited by these antimetabolites assayed at a concentration of 50 μ M cannot be attributed to a delay in the cell cycle because failure in thymidine incorporation resulted during a 108-hr observation period (not shown). 6-MP also inhibited the proliferation induced by anti-CD40 mAb or LPS plus anti-CD40 mAb (Fig. 1B).

6-MP induces programmed cell death of activated B cells. Treatment of activated B cells with MPs produced morphological changes associated with those described for programmed cell death (i.e., chromatin condensation). On analysis, purified resting B cells ($\rho > 1.072$) exhibited at 8 hr of culture the characteristic DNA laddering pattern on agarose gel electrophoresis (Fig. 2). Treatment with 6-MP of nonactivated B cells did not affect the intensity of the DNA laddering. Cells activated with LPS, anti-CD40 mAb, or LPS plus anti-CD40 mAb exhibited a reduced laddering pattern; however, treatment with 6-MP markedly produced an intense DNA degradation (Fig. 2, + lanes). The extent of the DNA laddering bands on 6-MP incubation was more intense in activated cells than in the nonstimulated counterparts, suggesting an enhanced sensitivity to the action of the drug after B cell triggering. The apoptotic effect of 6-MP was observed at times that preceded the initiation of the S phase of the division cell cycle. To obtain more quantitative data on

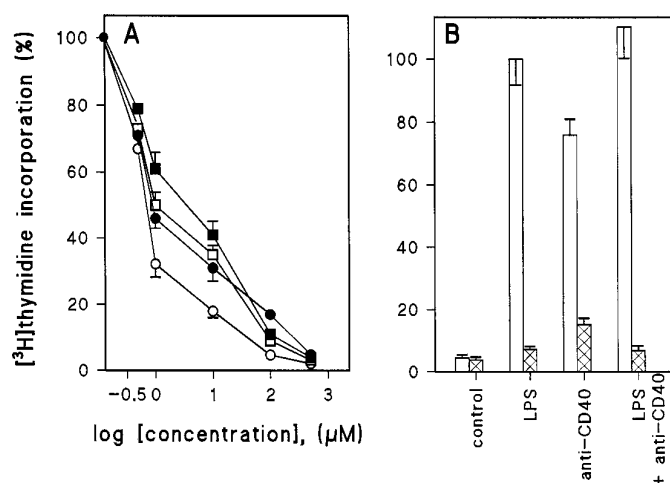


Fig. 1. Effect of MP and related antimetabolite analogues on B cell proliferation. A, Cultures of B lymphocytes (5×10^4 cells) were stimulated for 72 hr with 2 μ g/ml LPS and different concentrations of 6-MP (○), 2-MP (●), 2-aminopurine (□), and aminoguanidine (■). The cells were pulsed during the final 15 hr of culture with 1 μ Ci of [3 H]thymidine, and the radioactivity incorporated into the trichloroacetic acid-insoluble pellet was determined. B, [3 H]Thymidine incorporation of cells incubated with 2 μ g/ml LPS, 10 μ g/ml anti-CD40 mAb, or both in the absence (open bars) or presence (cross-hatched bars) of 50 μ M 6-MP. Total radioactivity incorporated in LPS-stimulated cells was $110,711 \pm 12,107$ dpm. Results are mean \pm standard error of four experiments [error bars are given only for 1 and 10 μ M antimetabolite concentration (A) or mean \pm standard error of three experiments assayed in duplicate (B)].

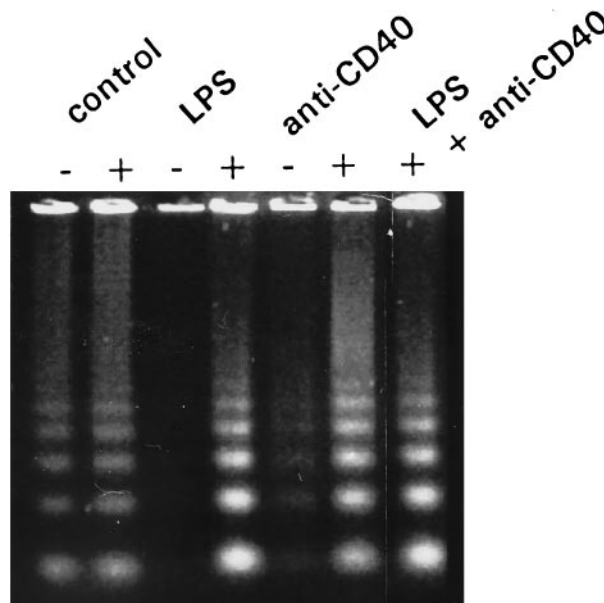


Fig. 2. 6-MP promotes apoptosis in activated B cells. Purified small B lymphocytes (1×10^6) were incubated for 8 hr in the absence (–) or presence (+) of 20 μ M 6-MP and the indicated mitogens (2 μ g/ml LPS and 10 μ g/ml anti-CD40 mAb). After this incubation period, the cells were centrifuged and homogenized; after isolation, the DNA was analyzed in a 1% agarose gel. Results show a representative experiment of four.

the DNA fragmentation, the enrichment in nucleosomal moieties in the cytosol was followed using a two-antibody sandwich ELISA that detected the presence of DNA-histone complexes. As shown in Fig. 3, the enhancement in DNA degradation after 6-MP challenge of cells stimulated with

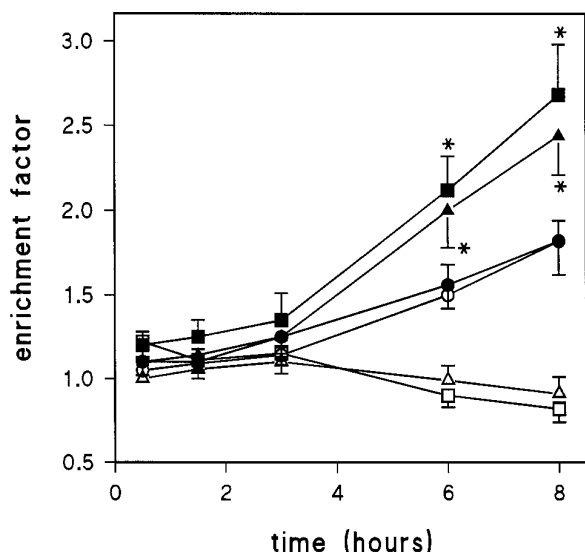


Fig. 3. Time course of DNA fragmentation in activated B cells treated with 6-MP. Purified small B lymphocytes were stimulated in the absence (*open symbols*) or presence (*filled symbols*) of 20 μM 6-MP without mitogen (\circ and \bullet) or with 2 $\mu\text{g}/\text{ml}$ LPS (\square and \blacksquare) or 10 $\mu\text{g}/\text{ml}$ anti-CD40 mAb (\triangle and \blacktriangle). At the indicated times, the presence of mononucleosomes and oligonucleosomes in the cytosol was measured using a sandwich ELISA with anti-histone and anti-DNA-peroxidase antibodies. Results are mean \pm standard error of three experiments assayed in duplicate. *, $p < 0.001$, statistically different from the corresponding condition in the absence of 6-MP.

LPS (2 $\mu\text{g}/\text{ml}$) or anti-CD40 mAb (10 $\mu\text{g}/\text{ml}$) was statistically significant after 6 hr of culture, whereas in nonactivated cells, the effect of 6-MP on apoptosis was minimal over the period of time analyzed.

LPS-dependent NO production in B cells is inhibited by MPs. Results from several groups suggest that a low release of NO inhibits apoptosis in mature B cells through a mechanism that involves sustained levels of Bcl-2 (19, 20). For this reason, we investigated whether 6-MP treatment influences NO synthesis in B lymphocytes. Using the model of LPS triggering of B cells, a low but sustained nitrite accumulation was observed after a lag period of ~ 2 hr, and a 4-fold increase was exhibited at 4 hr (Fig. 4A). This NO synthesis was detected using highly purified B lymphocytes (flow cytometrically sorted B220⁺ cells). The presence of 6-

and 2-MP in the incubation medium at concentrations in the range that produce $>90\%$ inhibition of proliferation (50 μM) abolished the accumulation of nitrite in the culture medium (Fig. 4A). This blockage in NO synthesis occurred during the initial 4 hr of culture. To ensure that the nitrite measured in the culture medium effectively corresponded to a NOS-dependent activity, LPS-stimulated cells were incubated with *N*^G-methyl-L-arginine, an inhibitor of NOS, and a drastic decrease in nitrite synthesis was observed (Fig. 4A). One of the targets of NO is the stimulation of soluble guanylate cyclase. As Fig. 4B shows, LPS promoted an increase in the intracellular concentration of cGMP, a process that was completely blocked when cells were treated with 6-MP, and therefore confirmed the synthesis of NO after LPS stimulation.

The apoptosis induced by MPs is partially blocked by NO donors. To investigate whether the blockage of NO synthesis exerted by MP in LPS-activated B cells might participate in the development of apoptosis elicited by MPs, B lymphocytes were challenged with substances that pharmacologically release NO once incorporated into the cell, therefore bypassing the step of NOS activation (19, 20). In this way, LPS-activated B lymphocytes were incubated in the presence of 6-MP and SIN-I, a NO donor, and apoptosis was followed by flow cell cytometry after *in vivo* staining of the cells with propidium iodide. As shown in Fig. 5 and Table 1, the percentage of LPS-activated cells displaying apoptosis (quadrants R3 plus R4) significantly decreased after treatment with SIN-I (Fig. 5f), indicating that this early effect of MP was easily bypassed through signals triggered by NO release. When the effect of NO donors was assayed in resting nonactivated cells, the extent of protection was similar regardless of the treatment with 6-MP, which suggests that this drug does not promote apoptosis in resting cells (Table 1). Similar results were obtained when apoptosis was followed by nucleosomal enrichment of the cytosol (Table 1). Furthermore, to determine whether this rescue of B cells from apoptosis was able to restore the proliferative capacity of cells incubated in the presence of 6-MP, B cells were stimulated with LPS, and the response to NO donors was determined. As shown in Fig. 6A, only a partial recovery of proliferation was obtained when cells were treated with SIN-I or ISO. The optimal effect was dependent on the con-

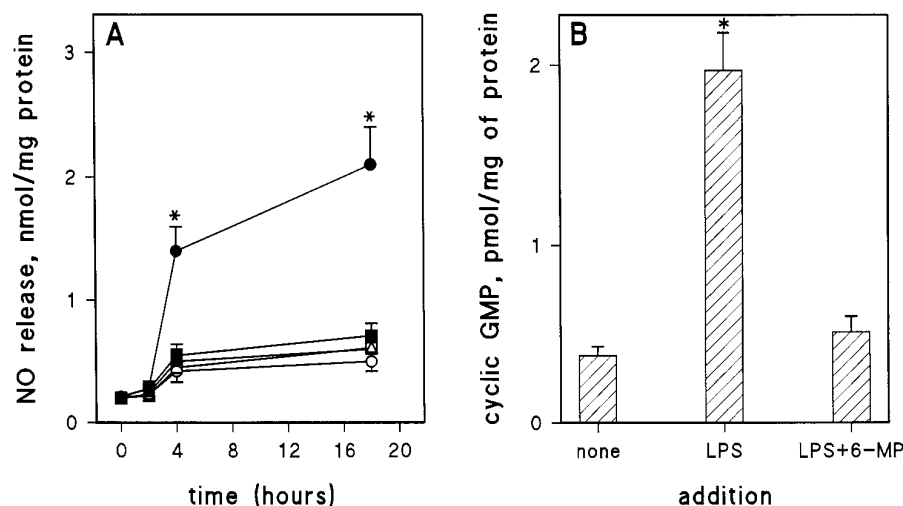


Fig. 4. Time course of NO release and cGMP concentration in cultured B lymphocytes. Small B cells were purified by flow cytometry sorting (B220⁺) and cultured in 24-well dishes (5×10^5 cells/well) in phenol red-free RPMI 1640 medium containing 50 μM β -mercaptoethanol, 10% fetal calf serum, and 1 mM arginine. Cells were incubated in the absence (\circ) or presence (\bullet) of 2 $\mu\text{g}/\text{ml}$ LPS supplemented with 50 μM 6-MP (\square), 50 μM 2-MP (\blacksquare), or 100 μM *N*^G-methyl-L-arginine (\triangle). A, Total NO production was assayed as nitrite accumulation in the medium. B, cGMP concentration was measured after 4 hr of incubation with 2 $\mu\text{g}/\text{ml}$ LPS in the absence or presence of 50 μM 6-MP. Results are mean \pm standard error of four cell preparations. *, $p < 0.001$, significantly different from the corresponding controls without MP.

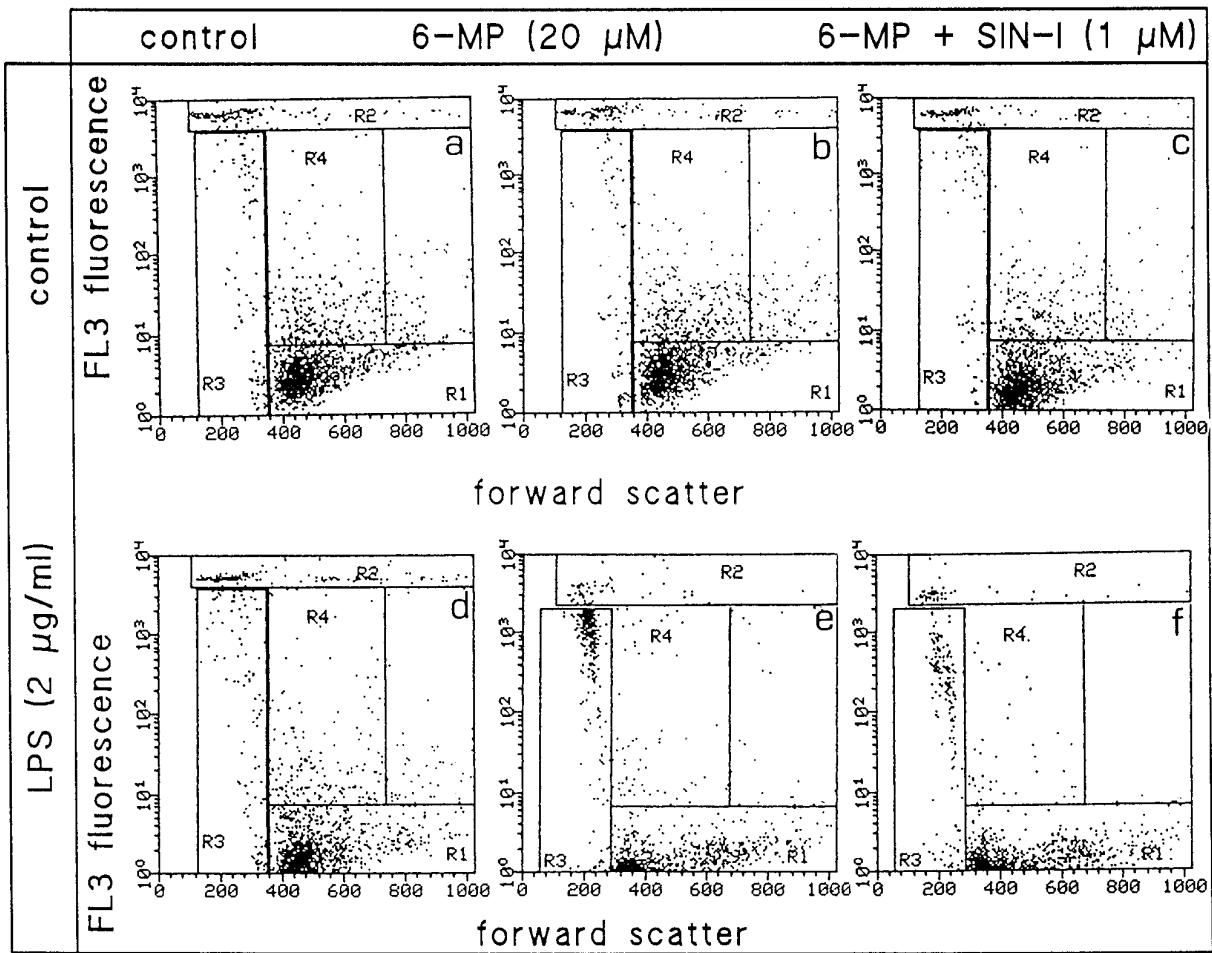


Fig. 5. NO donors prevent the apoptosis induced by MPs in B cells. B cells were incubated *in vivo* with propidium iodide in the absence (a–c) or presence (d–f) of 2 µg/ml LPS and 20 µM 6-MP (b and e) or 20 µM 6-MP and 1 µM SIN-I (c and f). After 6 hr of incubation, the cells were analyzed by flow cell cytometry to determine the percentage of apoptotic cells (R3 plus R4 quadrants), which is summarized in Table 1. The nature of intact, necrotic, and apoptotic cells (quadrants R1, R2, and R3/R4, respectively) was confirmed by the analysis of DNA integrity in agarose gels. Results show a representative experiment of three.

TABLE 1
NO donors protect against 6-MP-induced apoptosis of activated B cells

Small B cells were incubated for 6 hr *in vivo* with propidium iodide and the indicated stimuli. Apoptosis was determined by measuring the percentage of cell in quadrants R3 + R4 after analysis by flow cell cytometry (see text) or after the release of nucleosomal moieties to the cytosol (see Fig. 3 enrichment factor). Values are mean ± standard error of three experiments.

Addition	6-MP (20 µM)	Cells in quadrants R3 + R4	Enrichment
		%	
None	–	17 ± 2	1.6 ± 0.2
None	+	19 ± 3	1.6 ± 0.3
None + SIN-I (1 µM)	–	12 ± 1	1.2 ± 0.1
None + SIN-I (1 µM)	+	13 ± 2	0.9 ± 0.1
LPS (2 µg/ml)	–	10 ± 1	1.0 ± 0.1
LPS (2 µg/ml)	+	23 ± 3	2.1 ± 0.3
LPS (2 µg/ml) + SIN-I (1 µM)	–	11 ± 1	1.0 ± 0.1
LPS (2 µg/ml) + SIN-I (1 µM)	+	14 ± 2	1.0 ± 0.2

centration of 6-MP used, and the average range of concentration of NO donors that promoted a maximal thymidine incorporation was 0.1–1 µM. These results indicate that rescue of B cells by SIN-I or ISO was dependent on the dose of antimetabolite used and suggest that in addition to the block-

age of NO synthesis, other signaling pathways were also affected by these drugs, although they displayed a lower sensitivity to MPs.

To further analyze the temporal pattern of effects elicited by 6-MP on cell growth, an experiment was carried out in which LPS-stimulated cells were incubated with SIN-I or ISO and 6-MP was added at different times through the culture. As shown in Fig. 6B, the maximal inhibition was obtained when 6-MP was added during the first 24 hr of culture, a period of time during which the protection by NO donors (especially SIN-I) was maximal. However, as progression in the cell cycle occurred, the inhibitory effect of 6-MP, as well as the protection by SIN-I, markedly decreased, suggesting the existence of multiple steps affected by 6-MP treatment.

MPs decrease the Bcl-2/Bax ratio in stimulated B cells. One of the main mechanisms involved in the induction of apoptotic death in mature B cells is a decrease in the levels of Bcl-2 or, alternatively, an increase in the levels of Bax (17, 18). To address whether Bcl-2 and Bax levels correlate with the apoptosis that occurs after treatment of activated B cells with 6-MP, the amount of these proteins was quantified in total cell extracts. When LPS-activated cells were incubated

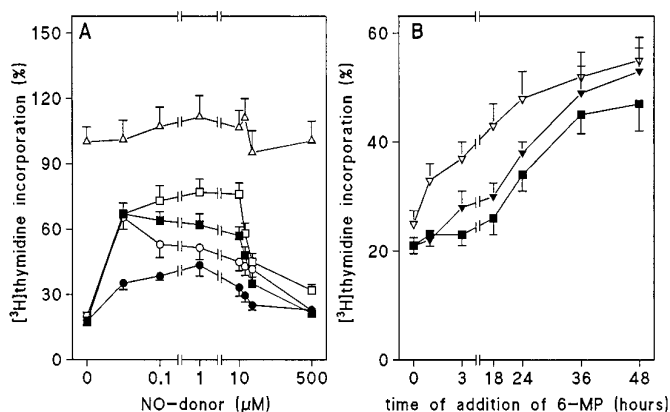


Fig. 6. Effect of NO donors on proliferation of B cells treated with 6-MP. A, LPS-activated B cells were incubated with the indicated concentrations of the NO donors SIN-1 (open symbols) or ISO (filled symbols) in the absence (▽) or presence of 50 μM (○ and ●) or 10 μM (□ and ■) 6-MP, and [³H]thymidine incorporation was measured after 72 hr of culture. B, Alternatively, LPS-stimulated cells incubated in the absence (■) or in the presence of 1 μM SIN-1 (○) or 1 μM ISO (●) were challenged at the indicated times after starting the culture with 50 μM 6-MP, and [³H]thymidine incorporation was measured after a total incubation period of 72 hr. The radioactivity incorporated in cells stimulated with LPS was 93,110 ± 8,325 dpm. Results are mean ± standard error of three experiments assayed in triplicate.

with 6-MP, only the levels of Bcl-2 decreased after 8 hr of culture (Fig. 7), whereas an important increase in Bax was observed after 6 hr. These results agree with the observation of apoptosis after 6 hr of incubation with 6-MP (Fig. 3); therefore, the decrease in the Bcl-2/Bax ratio might participate in the apoptosis observed after 6-MP incubation.

The mRNA levels of *bcl-2* in LPS-activated B cells decreased after 8 hr of incubation with 6-MP (44% of the corresponding level in the absence of 6-MP; Fig. 8, A and C). The protein levels of Bcl-2 were measured *in situ* as previously described (15). As shown in Fig. 8B, the decrease of Bcl-2 was delayed with respect to the *bcl-2* mRNA content. However, after 18 hr in culture, the amount of Bcl-2 was 44% lower than that in LPS-activated cells cultured in the absence of

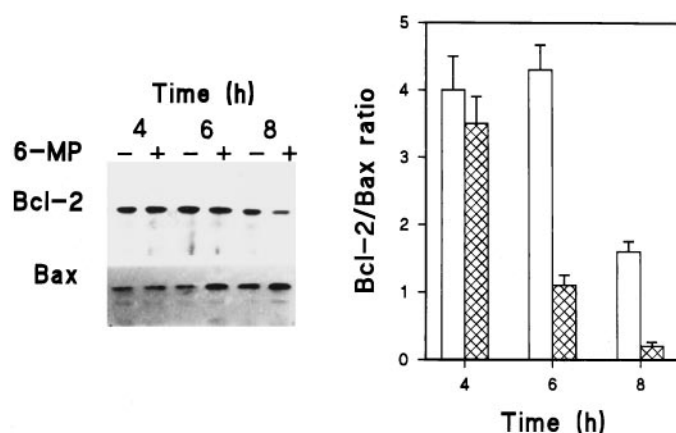


Fig. 7. Bcl-2 and Bax levels in LPS-activated B lymphocytes treated with 6-MP. B cells were incubated with 2 μg/ml LPS in the absence (–) or presence (+) of 20 μM 6-MP for the indicated periods of time. After homogenization, aliquots of total cell extracts were analyzed by Western blotting using anti-Bcl-2 and anti-Bax antibodies. Right, ratio between band intensities corresponding to cells treated in the absence (open bars) or presence (cross-hatched bars) of 6-MP of three experiments (mean ± standard error).

6-MP. These results suggest that 6-MP inhibits the transcription of *bcl-2*, although the effect at the protein level seems to be less effective, probably because of the high half-life of this protein.

Discussion

Inhibition of *de novo* pathway of purine biosynthesis is a well established protocol to achieve immunosuppression and to block proliferation of various types of lymphocyte lines (1, 2, 26). 6-MP and related purine analogues have proved to be effective in the therapy of childhood acute lymphoblastic leukemia, and the pharmacology of these drugs is well established (26, 27). However, despite this wide clinical use of purine antimetabolites, less is known regarding the cell-specific effects of these drugs on naive or stimulated normal B lymphocytes and even on B cell lymphomas. For this reason, we focused our study on the characterization of early effects elicited after drug treatment. In the current study, we used purified splenic B cells as a model system to study the effect of purine antimetabolites on mature small B lymphocytes ($p > 1.072$). Stimulation of B cells was carried out using polyclonal activators such as LPS, CD40 ligation, or a combination to elicit a wide proliferative response when cells were cultured *ex vivo*.

According to our results, at doses in the low micromolar range, 6-MP and related metabolites drastically inhibited the mitogen-induced proliferation of B cells, a result that should be expected in view of the pharmacological properties of these molecules (28, 29). However, the interesting observation was that most of the cells not only failed to enter the S phase of the cell cycle but also died as a result of apoptotic cell death, suggesting that in addition to DNA synthesis inhibition, these drugs interfere with the signaling pathway triggered by LPS that rescues B cells from the characteristic apoptosis of nonactivated mature cells (12, 28–30). The induction of apoptosis by MPs required cell activation, and the process was observed after 6 hr of treatment. Therefore, it might be concluded that the antiproliferative effects of these drugs are due, at least in part, to programmed cell death rather than to failure in DNA synthesis. This rapid apoptosis induction was confirmed by the observation of a DNA internucleosomal laddering in agarose gels, by immunodetection of nucleosomes in the cytosol, and by the appearance of a characteristic shift in the population distribution when cells stained *in vivo* with propidium iodide were analyzed by cytofluorometry. Regarding apoptosis induction by antitumor agents, it is now accepted that the apoptotic susceptibility of a lymphoma depends on the activation state and on the interaction with specific cytokines or costimulatory cells. For example, 5-azacytidine is very effective in HL-60 cells at the G1 phase (31); cyclosporine inhibits the apoptosis induced by 2-chloro-adenosine in mouse thymocytes (32); and antigen/receptor engagement abrogates the Fas-dependent apoptosis in B cells (33). Also, the cell specificity of antimetabolite drugs is reinforced by the observation that some of them (e.g., 6-MP) are poor cytostatic and antiproliferative drugs for several neuroblastoma cell lines (34).

In B cells, several lines of evidence suggest that one of the mechanisms by which apoptosis occurs is through a decrease in the Bcl-2 levels (13, 14). Alternately, *bcl-2* translocation into the immunoglobulin loci yields to a high level of expres-

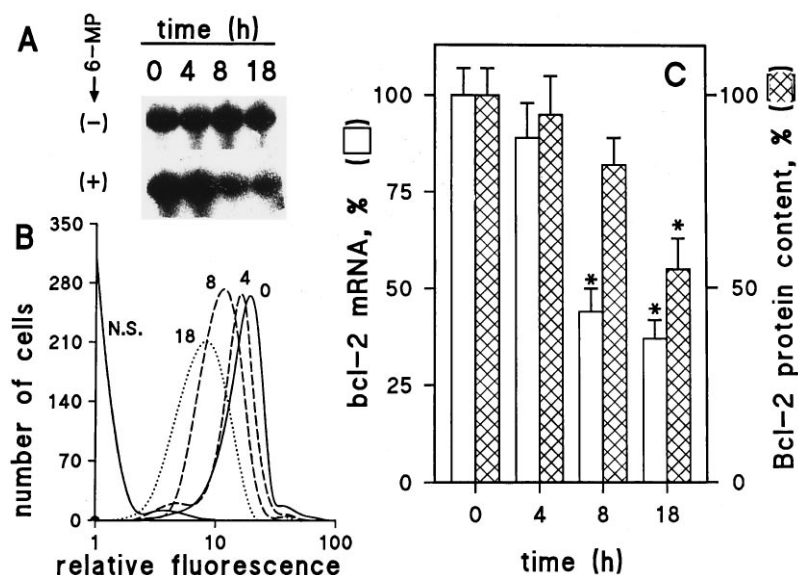


Fig. 8. Effect of MP on *bcl-2* levels. A and B, cells were incubated for the indicated periods of time with 2 μ g/ml LPS in the absence (–) or presence (+) of 20 μ M 6-MP, and *bcl-2* mRNA levels were measured using a specific probe. The corresponding Bcl-2 protein levels were measured by flow cytometry in cells incubated with 2 μ g/ml LPS and 20 μ M 6-MP. The label of each curve corresponds to the time of incubation. N.S., curve obtained using an unspecific anti-hamster antibody (B). The quantitative analysis corresponding to the ratio of the mRNA levels in the presence and absence of 6-MP (after normalization for the 18S ribosomal RNA content) was expressed as percentage with respect to 0 time (C, open bars). The protein levels of Bcl-2 corresponding to three experiments as described in B are given (C, cross-hatched bars). *, $p < 0.001$ compared with values at 0 hr.

sion of this gene and is responsible for ~80% of follicular lymphomas in humans (35). However, Bcl-2 function is modulated through the interaction with other members of this protein family, such as Bax, that act in a dominant negative fashion (18). Taking into account this background of information, we investigated whether 6-MP treatment promoted changes in Bcl-2 and Bax levels and therefore could provide a mechanism by which to understand the antimetabolite triggering of apoptotic cell death. Our results show that although the Bcl-2 levels exhibited a late and moderate decrease compared with the kinetics of apoptosis, Bax levels increased more rapidly, resulting in important changes in the Bcl-2/Bax ratio after 6 hr of treatment. The possibility of the participation of other members of the Bcl-2 family (e.g., Bcl-x, Bcl-x_s, and Bak) in the regulation of this apoptotic death remains to be established. To our knowledge, the apoptotic effects of MPs have been unrecognized, although apoptotic death of various lymphocyte populations induced after treatment with adenosine antimetabolites (i.e., 2-chloro-2'-deoxyadenosine and 2- β -D-arabinosyladenosine) was previously reported (11).

Our results also show that MPs, through a mechanism not yet identified, inhibit NOS activity in stimulated B cells and therefore prevent endogenous NO synthesis. In this regard, various groups have stressed the relevance of the synthesis of NO to prevent apoptosis in resting B cells (19, 20). Indeed, the presence of low levels of functional NOS activity in activated B cells is an original observation of this work. This observation could be interpreted in the sense that NO plays a role in the process of B cell activation (12, 30). There are various conditions under which NO modulates the apoptosis of cells from the immune system: in polymorphonuclear leukocytes, NO inhibits free radical generation, protecting against cytotoxicity (36), and in human B lymphocytes infected with the Epstein-Barr virus, NO modulates the transition from the replicative to the lytic phase of the virus (19). However, activation of B cells with LPS or anti-CD40 antibodies are only two experimental models of polyclonal B cell triggering, and therefore further work is required to ensure that NOS activity is present in physiologically activated B lymphocytes. Indeed, under more physiological conditions, it

is possible that NO can originate from neighboring cells (i.e., dendritic cells) that modulate B cell development through cognate interactions (37).

In conclusion, our results show that MP treatment of activated B cells blocks NO synthesis and induces programmed cell death, a process that can be partially reversed by the addition of exogenous NO donors. Also, the effect of MPs on Bcl-2, Bax, and related genes that modulate B cell death and survival may contribute to a understanding of the pharmacological efficacy of this drug for the treatment of B cell disorders. Following the results of this work, the use of clinically characterized B cell lymphomas can provide additional clues to unravel the intracellular pathways affected in the course of antimetabolite treatment.

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