Biochemical Pharmacology, Vol. 58, pp. 85–93, 1999.


ELSEVIER

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00082-9

# Specific Dual Effect of Cycloheximide on B Lymphocyte Apoptosis: Involvement of CPP32/Caspase-3

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**ABSTRACT.** Cycloheximide (CHX) is known to stimulate or to prevent apoptosis, according to the cell type used. We found that CHX, in a dose-dependent way, exerted the two opposite effects in B lymphocytes. $CHX^{high}$ (2.5 μg/mL) inhibited protein synthesis (>90%) and greatly increased B cell apoptosis but failed to prevent apoptosis induction by dexamethasone (DEX) or dibutyryl-cAMP (dbcAMP), which is in opposition with CHX activity in thymocytes. On the contrary, $CHX^{low}$ (0.05 μg/mL), modestly inhibited protein synthesis (<15%) and reduced spontaneous as well as drug-induced apoptosis and further augmented the protection conferred by interleukin-4 or lipopolysaccharide. To examine the role of caspases in CHX effects, we used the broad spectrum peptide caspase inhibitor Z-VAD-fmk: it totally abrogated spontaneous as well as drug- and $CHX^{high}$-induced cell death. Apoptosis was associated with CPP32/caspase-3 activation, since cleavage of CPP32/caspase-3 and caspase-3 activity were increased by DEX, dbcAMP as well as by $CHX^{high}$ treatment. Meanwhile, caspase-3 activity was reduced by $CHX^{low}$ treatment. Therefore, CHX exerts opposite effects on B lymphocyte apoptosis which are associated with a dual action on caspase-3 activation. BIOCHEM PHARMACOL **58**;1:85–93, 1999.


**KEY WORDS.** apoptosis; B lymphocytes; caspase; cycloheximide; dexamethasone; IL-4

Apoptosis, or physiological cell death, is essential for the normal development and homeostasis of the immune system [1–4]. This active process, characterized by distinct morphological and biochemical features, is believed to require the expression of specific death genes [5–7]. Apoptosis of B cells must be tightly controlled to allow appropriate B lymphocyte differentiation and prevent the occurrence of malignancy or autoimmunity [8]. B lymphocytes have been reported to undergo rapid apoptosis when removed from their natural environment, the process being greatly increased by the protein synthesis inhibitor CHX† [9]. Although macromolecular syntheses were initially considered as essential for active cell death [10, 11], it now appears that, according to the experimental cell system used, the presence of synthesis inhibitors can lead to either prevention [12] or stimulation of apoptosis [13, 14]. CHX, a translation inhibitor which can induce expression of specific genes and stabilization of specific mRNA [15, 16], has been widely employed, leading to contradictory conclusions [17]. Indeed, the prevention of cell death by CHX has been attributed to interference with an active process through inhibition of the synthesis of killer gene products [6] as well as to an overexpression of several life gene products, including Bcl-2 and molecules involved in mitochondrial function [18]. Meanwhile, the effect of CHX on the induction of apoptosis has led to the speculation that the apoptotic machinery could be constitutively expressed in a latent form and restrained by labile life proteins or antiapoptotic factors [9, 19].

It now appears that activation of one or several cysteine proteases of the caspase family constitutes an essential and common event in the effector stage of the apoptotic process [20–22]. In particular, CPP32/caspase-3-like proteases appear to be critical in apoptosis of mammalian cells [23, 24]. Caspase activation can be induced by a wide variety of death signals, including Fas activation [25], growth factor deprivation [26], or drugs [27]. Caspase activation, and ensuing apoptosis, can be totally blocked by peptide antagonists, designed to mimic known sequences of caspase substrates [28]. We previously reported that apoptosis was greatly increased by the protein synthesis inhibitor CHX in a B cell hybridoma [29] or in B lymphocytes [30]. In the present study, we further investigated the mechanism whereby CHX affected B lymphocyte apoptosis. In order to investigate the involvement of caspases, activation of ICE-like and caspase-3-like proteases were examined and the effect of the peptide caspase inhibitor Z-VAD-fmk was analyzed. We found that CHX exerted a dose-dependent

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† *Abbreviations:* CHX, cycloheximide; dbcAMP, dibutyryl-cAMP; DEX, dexamethasone; DAPI, 6-diamidino-2-phenylindol; IL-4, interleukin-4; and LPS, lipopolysaccharide.

Received 13 July 1998; accepted 12 January 1999.

dual action, since high doses of CHX increased apoptosis, while low doses reduced constitutive as well as drug-induced apoptosis and potentiated the protection afforded by IL-4. This dual activity was linked to caspase activation. Thus, we demonstrated that CPP32/caspase-3 was implicated in B cell apoptosis and that caspase activity was greatly increased by high doses of CHX in parallel with apoptosis induction, both effects being blocked by the peptide caspase inhibitor Z-VAD-fmk. Meanwhile, low concentrations of CHX reduced both caspase activation and the apoptotic process.

## MATERIALS AND METHODS

### *Animals and Materials*

Female C57BL/6 mice were purchased at 6–8 weeks of age (Iffa Credo). Recombinant mouse IL-4 was obtained from Genzyme. Polyclonal antibody directed against CPP32 was purchased from UBI. Z-VAD-fmk was purchased from BACHEM Biochimie. The caspase substrates YVAD-pNA and DEVD-pNA were purchased from Alexis Biochemicals. The culture medium used throughout was RPMI 1640 (Biomedia) supplemented with 25 mM HEPES, 2 mM L-glutamine, standard antibiotics, 50 μM 2-mercaptoethanol and 8% heat-inactivated fetal bovine serum (Biomedia). Actinomycin D, CHX, dbcAMP, DEX, DAPI, ethidium bromide, and LPS (*Salmonella enteritidis*) were purchased from Sigma.

### *Cell Preparation and Culture*

Thymocytes were obtained from mice 6–8 weeks old. B lymphocytes were purified as previously described [30]. Briefly, splenocytes ($3–4 \times 10^7$/mL in RPMI medium) were treated with a cocktail of anti-T cell monoclonal antibodies at 4° for 60 min followed by incubation with low-tox complement at 37° for 60 min (Cedarlane). After centrifugation on a discontinuous Percoll gradient, cells at the 65 to 70% interface were identified as B lymphocytes by anti-immunoglobulin M FITC (fluorescein isothiocyanate) staining (more than 98% Ig-positive cells). Cells ($5 \times 10^5$ cells/mL) were cultured in 24-well plates (Nunclon) in 6% $CO_2$ at 37°. For cytometric analysis, cells were fixed in cold 70% ethanol and kept at −20° until analysis.

### *Analysis of Apoptosis and Cell Cycle by Flow Cytometry*

Fixed cells ($5 \times 10^5$ cells/mL) were washed twice with HBSS (Hanks' Balanced Salt Solution) and then stained with DAPI (2.5 μg/mL) at 37° for 30 min. DNA content was quantified by flow cytometric analysis performed on a PARTEC CA II flow cytometer (Chemunex) equipped with a 100 W mercury lamp (type HBO). Fluorescence at 455 nm was recorded as a function of DNA content. Each histogram was generated until the analysis reached at least 15,000 cells. The percentage of apoptosis was determined from the sub-G1 events.

### *Analysis of DNA Fragmentation in Agarose Gels*

DNA from $5 \times 10^5$ B lymphocytes was extracted as previously described [31]. Briefly, after cell lysis, DNA was extracted and precipitated with 70% ethanol at −20° overnight. Electrophoresis was performed in 1% agarose gel containing 1 μg/mL ethidium bromide. ϕX174RF/Hae III digest was used as DNA size marker.

### *Determination of [$^3$H]Thymidine Incorporation*

B lymphocytes ($10^5$ cells/well) were cultured in 96-well microtiter plates (0.2 mL final vol.) in the presence of various stimuli. Proliferation was assessed on day 3 by pulsing cultures with 0.5 μCi of tritiated thymidine ([$^3$H]TdR, Amersham) for 6 hr before harvesting. [$^3$H]TdR uptake was expressed as mean cpm for triplicate cultures.

### *Assay for Caspase Activity*

After 8 hr of culture in the presence or absence of drugs, as indicated, thymocytes or B lymphocytes ($5 \times 10^6$) were washed and lysed in Triton X-100 buffer (0.5% Triton X-100, 2 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride) for 20 min on ice. Cell lysates were added with 0.5 mL of ICE buffer (100 mM HEPES-KOH pH 7.5, 10% sucrose, 10 mM dithiothreitol, and 0.1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate)) containing 100 μM DEVD-pNA or YVAD-pNA caspase substrates, and then incubated for 8 hr at 37°. Enzyme-catalyzed release of *p*-nitroaniline was monitored at 400 nm.

### *Assay for CPP32 Cleavage*

CPP32 cleavage was assessed by Western blotting of whole cell extracts from B lymphocytes using a polyclonal anti-CPP32 antibody (UBI). Briefly, $5 \times 10^6$ cells were collected and washed once in PBS, resuspended in sample buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.1% BBP, 10% 2-mercaptoethanol) and boiled for 5 min. Samples corresponding to 30 μg protein were run on 15% SDS-PAGE gels (mini-PROTEAN II, BioRad). Proteins were transferred to polyvinylidene difluoride membranes (Imobilon P, Millipore), which were then blocked in TBS (Tris-buffered saline) containing 0.05% Tween-20 and 1% gelatin. After an overnight incubation at 4° with anti-CPP32 antibody (2 μg/mL), CPP32 and cleavage product were detected by using the ECL method (Amersham) according to the manufacturer's instructions.

### *Statistical Analysis*

Results are expressed as the arithmetic mean ± SD for three experiments. Mean values were compared by using the Student's *t*-test and the level of significance is indicated in figures and tables; a *P* value of 0.05 was considered as

significant, a value of 0.01 as very significant, and a value of 0.001 as highly significant.

## RESULTS

### *Differential Effects of CHX on the Induction of Apoptosis in Thymocytes and B Lymphocytes*

In order to determine whether inhibition of protein synthesis affected spontaneous or drug-induced apoptosis, mouse thymocytes and spleen B cells were cultured in the absence or presence of 10 μg/mL CHX, a concentration shown to be effective at totally inhibiting protein synthesis. Apoptosis was characterized by morphological examination and DNA fragmentation, and quantified by flow cytometric analysis after DAPI staining of cell nuclei. Figure 1 shows that, in agreement with previous reports, CHX did not significantly increase (48.7 ± 5.2% vs 41.9 ± 6.6% in controls, $P > 0.1$) the spontaneous apoptosis occurring after 24 hr of culture in thymocytes, but that it almost totally inhibited the proapoptotic activity of the glucocorticoid analog, DEX, and of the cAMP-increasing agent, dbcAMP ($P < 0.01$). The response of B lymphocytes to CHX was quite different, since CHX increased spontaneous apoptosis (72.1 ± 6.5% vs 47.9 ± 5.2%, $P < 0.01$) while failing to protect cells against DEX- (89.2 ± 3.4% vs 92.1 ± 4.0% without CHX, $P > 0.2$) and dbcAMP-induced apoptosis (86.2 ± 2.5% vs 83.9 ± 8.2% without CHX, $P > 0.8$), which remained at high levels.

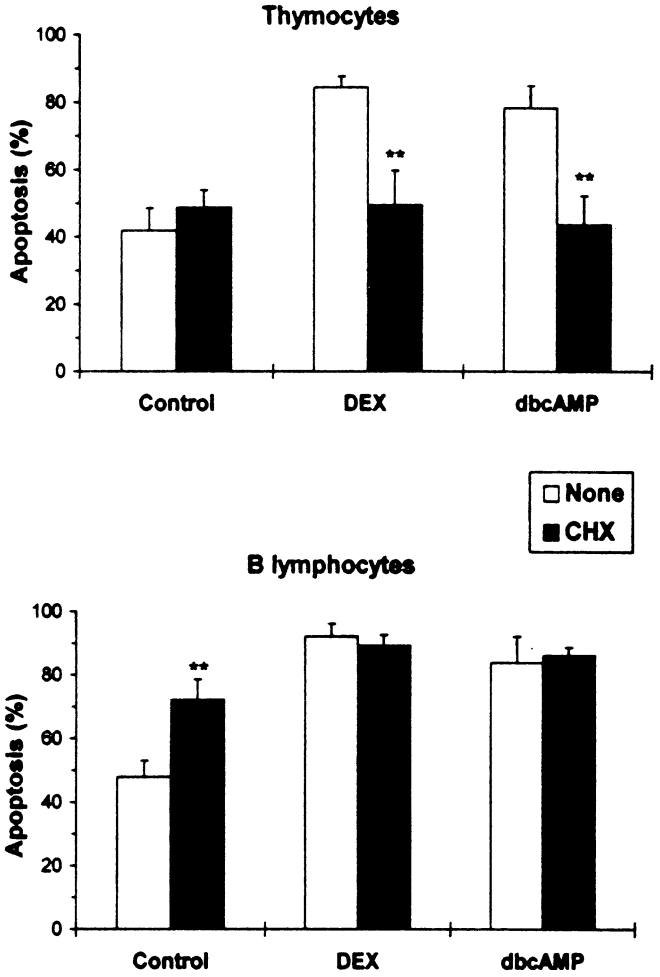


**FIG. 1. Effect of cycloheximide on thymocyte and B lymphocyte apoptosis. Cells ($5 \times 10^5$ cells/mL) were incubated for 24 hr in the absence or presence of CHX (10 μg/mL) with or without dexamethasone (DEX, 5 nM) or dbcAMP (100 μg/mL) as indicated. The percentage of apoptosis was determined by flow cytometry (cells containing less than 2n DNA). Results are expressed as the means of three different experiments, with error bars indicating standard deviation. **, $P < 0.01$ compared with control. Thymocyte apoptosis observed in the absence versus presence of CHX: control 41.9 ± 6.6% vs 48.7 ± 5.2%; DEX 84.3 ± 3.2% vs 49.4 ± 10.3%; dbcAMP 78.2 ± 6.5% vs 43.5 ± 8.6%. B lymphocyte apoptosis observed in the absence versus presence of CHX: control 47.9 ± 5.2% vs 72.1 ± 6.5; DEX 92.1 ± 4.0% vs 89.2 ± 3.4%; dbcAMP 83.9 ± 8.2% vs 86.2 ± 2.5%.**

### *Dose-Dependent Dual Effect of CHX on B Lymphocyte Apoptosis*

Since B lymphocytes appeared to be highly sensitive to apoptosis induction by CHX, a dose–response study was performed which revealed opposite effects of the protein synthesis inhibitor. The percentage of apoptotic cells was reduced at 0.05 and 0.1 μg/mL ($P < 0.01$), with an optimal protection observed for 0.05 μg/mL ($CHX^{low}$) with a decrease of $\Delta = -13.6 \pm 2.1\%$ ($P < 0.01$), and strongly enhanced at higher concentrations (1 μg/mL and above, $P < 0.01$), with a reproductive and optimal activity at 2.5 μg/mL ($\Delta = +21.2 \pm 3.5\%$, $P < 0.01$), routinely used in the following experiments (Fig. 2A). The dose-dependent opposite effects of CHX on B cell apoptosis were further confirmed by laddering of DNA in gel electrophoresis (Fig. 2B). Moreover, we observed that two other inhibitors of protein synthesis, emetine and anisomycin, led to similar dual effects (data not shown). We next investigated whether an inhibitor of transcription also exerted a dual effect on B cell apoptosis. Actinomycin D dose dependently (5–100 ng/mL) increased apoptosis in B lymphocytes (Fig. 2C), but low concentrations (0.01–1 ng/mL) failed to protect against cell death (data not shown). These results suggest that the protection conferred by low doses of CHX took place downstream from the transcription step.

### *Effect of CHX on Apoptosis, Protein Synthesis, and Proliferation*

In an attempt to better define the mechanism whereby $CHX^{low}$ induced protection against apoptosis, we compared its activity to that of IL-4, a potent rescuing cytokine mimicking T cell help activity [9, 32, 33]. As shown in Fig. 3A, the protection conferred by IL-4 (50 U/mL) was triggered very rapidly and did not require the constant presence of the cytokine, since a 1-hr pulse was sufficient for an optimal protection (more than 30% reduction at 24 hr, $P < 0.001$). In contrast, an 1- or 4-hr pulse with

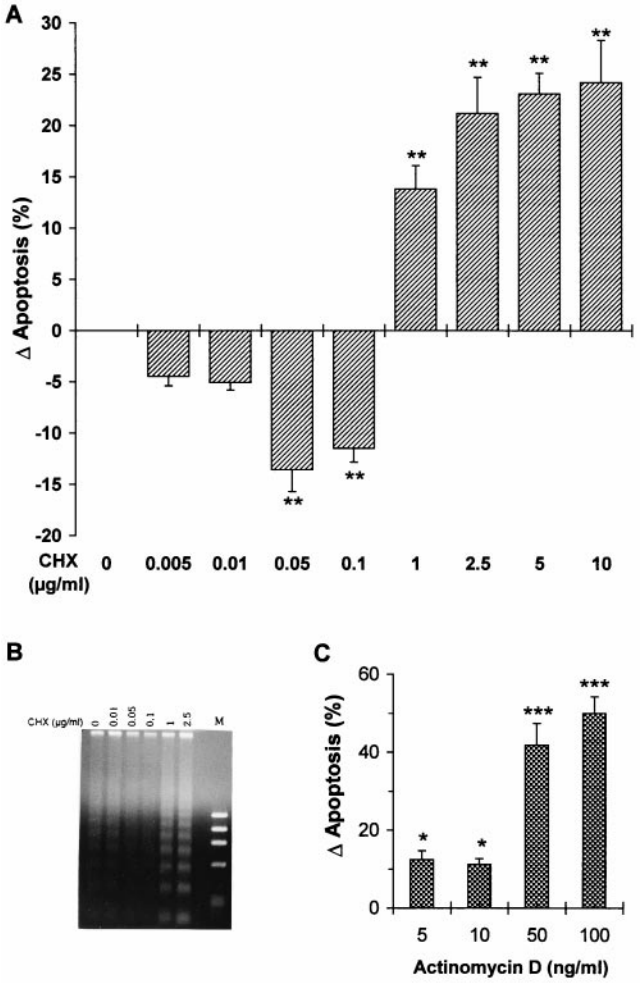


FIG. 2. Dose–response of CHX and actinomycin D on B lymphocyte apoptosis. B lymphocytes purified from mouse spleen cells ($5 \times 10^5$ cells/mL) were incubated for 24 hr with increasing concentrations of CHX (A) or actinomycin D (C) as indicated. Results are expressed as the means of three different experiments, with error bars indicating standard deviation of the difference in percentage of apoptosis between cells cultured in the presence or absence of inhibitor (Δ). *, $P < 0.05$; **, $P < 0.01$ compared with untreated cells. (A) Values obtained in the presence of increasing doses of CHX (Δ with control at 47.9 ± 5.2%): 0.005 μg/mL −4.5 ± 0.9%; 0.01 μg/mL −5.1 ± 0.7%; 0.05 μg/mL −13.6 ± 2.1%; 0.1 μg/mL −11.5 ± 1.3%; 1 μg/mL 13.8 ± 2.3%; 2.5 μg/mL 21.2 ± 3.5%; 5 μg/mL 23.1 ± 2.0%; 10 μg/mL 24.2 ± 4.1%. (B) Agarose gel electrophoresis of DNA extracted from cells cultured in the absence or presence of increasing doses of CHX. ϕX174RF/Hae III digest was used as DNA size marker (M). (C) Values obtained in the presence of increasing concentrations of actinomycin D (Δ with control at 47.9 ± 5.2%): 5 ng/mL 12.4 ± 2.3%; 10 ng/mL 11.2 ± 1.5%; 50 ng/mL 41.8 ± 5.6%; 100 ng/mL 50.0 ± 4.2%.

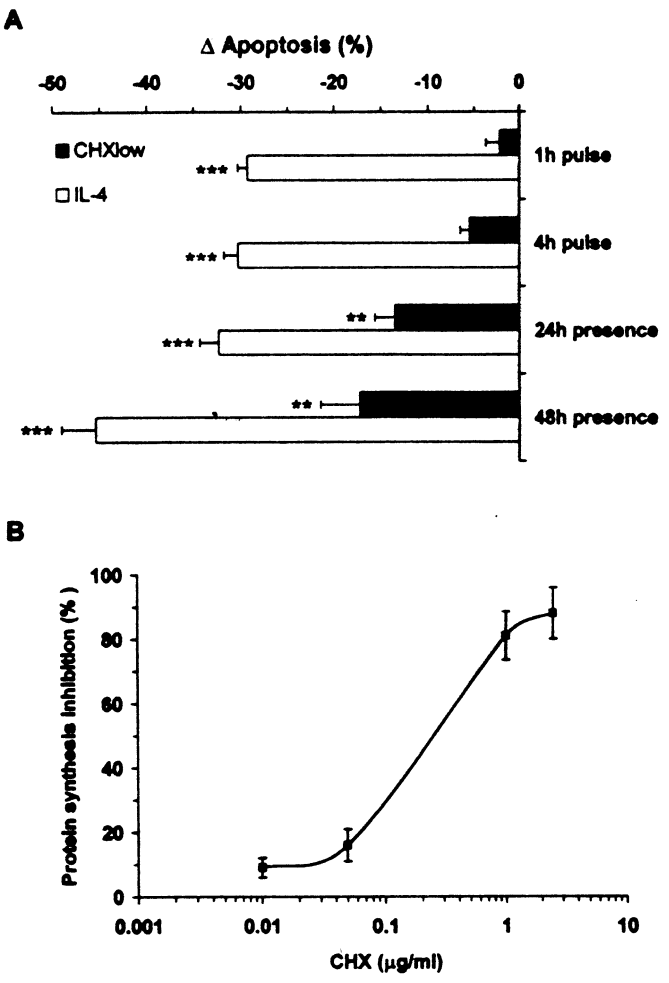


FIG. 3. Effect of CHX on apoptosis and protein synthesis in B lymphocytes. (A) B cells were pretreated for 1 and 4 hr with or without $CHX^{low}$ (0.05 μg/mL) or IL-4 (50 U/mL), washed and then cultured for 24 hr, or cells were cultured for 24 and 48 hr in the presence of $CHX^{low}$ or IL-4 as indicated. Results are expressed as the means of three different experiments, with error bars indicating standard deviation of the difference between treated and untreated cells (Δ). **, $P < 0.01$; ***, $P < 0.001$ compared with untreated cells. Values obtained in presence of $CHX^{low}$ (Δ with control of 47.9 ± 5.2% at 24 hr and of 67.7 ± 6.3% at 48 hr): 1-hr pulse −2.2 ± 1.5%; 4-hr pulse −5.5 ± 1.0%; 24-hr presence −13.6 ± 2.1%; 48-hr presence −17.3 ± 4.2. Values obtained in the presence of IL-4 (Δ with control of 47.9 ± 5.2% at 24 hr and of 67.7 ± 6.3% at 48 hr): 1-hr pulse −29.3 ± 1.0%; 4-hr pulse −30.3 ± 1.5%; 24-hr presence −32.4 ± 2.0%; 48-hr presence −45.5 ± 3.6. (B) Dose–effect of CHX on inhibition of *de novo* protein synthesis as measured by inhibition of $^{14}C$-labeled aminoacid uptake after an overnight incubation. Results are expressed as mean % inhibition of triplicate samples, with error bars indicating standard deviation in CHX-treated versus untreated cells.

$CHX^{low}$ did not significantly rescue cells from apoptosis ($P > 0.2$). Nevertheless, when $CHX^{low}$ was left throughout the two-day culture, it led to a long-lasting protection ($P < 0.01$). This suggested that $CHX^{low}$ acted through a constant repression of apoptosis by inhibiting the synthesis of a factor involved in the apoptotic cascade. The effect of CHX on *de novo* protein synthesis was thus investigated. Figure 3B indicates that, after an overnight incubation, CHX induced a dose-dependent and sustained decrease in overall protein synthesis. The proapoptotic high doses inhibited protein synthesis by approximately 90%, whereas the low protective dose of CHX induced less than 15% inhibition.

We then examined whether $CHX^{low}$ affected spontane-

**TABLE 1. Effect of $CHX^{low}$, $CHX^{high}$, and LPS on cell cycle distribution and proliferation**

| Treatment | Cell distribution | | | | Proliferation (cpm) |
|---|---|---|---|---|---|
| | Apoptosis (%) | G1 (%) | S (%) | G2/M (%) | |
| Control | 67.7 ± 6.3 | 23.9 ± 3.1 | 3.9 ± 0.9 | 1.6 ± 0.5 | 2920 ± 637 |
| $CHX^{low}$ | 48.8 ± 5.2 | 44.9 ± 3.2 | 4.0 ± 0.8 | 2.3 ± 0.2 | 893 ± 211 |
| $CHX^{high}$ | 92.9 ± 3.6 | NS* | NS* | NS* | 78 ± 17 |
| LPS (5 μg/mL) | 42.3 ± 4.2 | 41.6 ± 2.0 | 12.6 ± 1.2 | 3.6 ± 0.8 | 87731 ± 2941 |
| LPS + $CHX^{low}$ | 31.9 ± 2.3 | 57.7 ± 2.9 | 6.9 ± 1.0 | 3.4 ± 0.6 | 20491 ± 297 |
| LPS + $CHX^{high}$ | 85.2 ± 3.9 | 10.6 ± 1.6 | 3.3 ± 0.5 | 0.9 ± 0.3 | 161 ± 24 |

B lymphocytes were cultured in the presence or absence of drugs as indicated. They were recovered after 48 hr for apoptosis and cell cycle analysis after DAPI staining, or after 72 hr for proliferation assay by [$^3$H]thymidine incorporation. Values show the means ± SD of three different experiments.

* NS, not significant.

ous or mitogen-induced cell growth. As shown in Table 1, $CHX^{high}$, in agreement with its proapoptotic activity, totally inhibited spontaneous and LPS-induced cell proliferation as determined by thymidine incorporation and cell cycle analysis. The low dose of CHX also reduced cell proliferation and increased the frequency of cells in the G1 compartment. Table 1 also shows that, consistent with previous findings [34], LPS (5 μg/mL) reduced apoptosis (42.3 ± 4.2% vs 67.7 ± 6.3% in controls at day 2) and that $CHX^{low}$ further augmented the repression of cell death (to 31.9 ± 2.3%); at the same time, however, it inhibited LPS-induced cell proliferation (to less than 25%) and cell cycle progression by inhibiting progression of cells into the S and G2/M phases.

### *Inhibition of Drug-Induced Apoptosis by $CHX^{low}$*

To investigate whether $CHX^{low}$ was also effective in protecting B cells against drug-induced apoptosis, cells were cultured for 24 hr in the absence or presence of DEX (5 nM) or dbcAMP (100 μM) with or without $CHX^{low}$ and IL-4, used alone or in combination. As shown in Fig. 4, $CHX^{low}$ partially protected B cells against both spontaneous and drug-induced apoptosis. IL-4 provided a better protection and its activity was further increased by $CHX^{low}$. Thus, in the presence of IL-4 and $CHX^{low}$, the frequency of apoptotic cells was reduced from 48% to 10% in controls, and from 92% to 37%, and 84% to 21% in the presence of DEX and dbcAMP, respectively. Drug effect on apoptosis was thus totally abrogated.

### *Dose-Dependent Dual Activity of CHX on Caspase Activation*

In order to investigate whether caspase activation was involved in the opposite effects of CHX on B cell apoptosis, we employed a broad-range caspase inhibitor and measured caspase activity in cytoplasmic extracts. As shown in Fig. 5A, Z-VAD-fmk totally inhibited spontaneous as well as DEX-, dbcAMP- and $CHX^{high}$-induced apoptosis, indicating that B lymphocyte apoptosis was mediated through caspase activation. To characterize the type of caspase involved in B lymphocyte apoptosis, the presence of ICE/caspase-1-like or CPP32/caspase-3-like activity in cytoplasmic extracts was investigated by measuring the cleavage of their respective chromogenic peptide substrates, YVAD-pNA or DEVD-pNA. Apoptotic cells were totally devoid of caspase-1-like activity (data not shown). In contrast, Fig. 5B shows that the cleavage of the caspase-3 DEVD-pNA substrate was increased by DEX (0.689 ± 0.052 vs 0.426 ± 0.030, $P < 0.001$), dbcAMP (0.585 ± 0.075 vs 0.426 ± 0.030, $P < 0.01$), and $CHX^{high}$ (0.582 ± 0.035 vs 0.426 ± 0.030, $P < 0.01$). In every case, DEVDase activity was abrogated by the peptide inhibitor Z-VAD-fmk. This indicated that CPP32/caspase-3 activation was implicated in

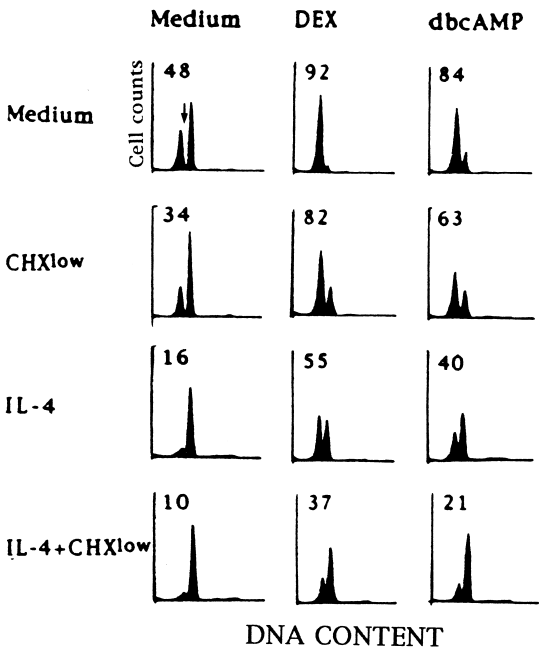


**FIG. 4. Protection of B lymphocytes against spontaneous and drug-induced apoptosis. B cells ($5 \times 10^5$ cells/mL) were cultured for 24 hr in medium alone or with DEX (5 nM) or dbcAMP (100 μM) in the absence or presence of $CHX^{low}$ (0.05 μg/mL) or IL-4 (50 U/mL) used alone or in combination. Apoptosis was determined by flow cytometry as the frequency of the subdiploid cell population, to the left of the arrow, whose percentage is presented on the left of the histograms. At least 15,000 cells were analyzed for a constant scale on the ordinate axis. Results are those of a typical experiment.**

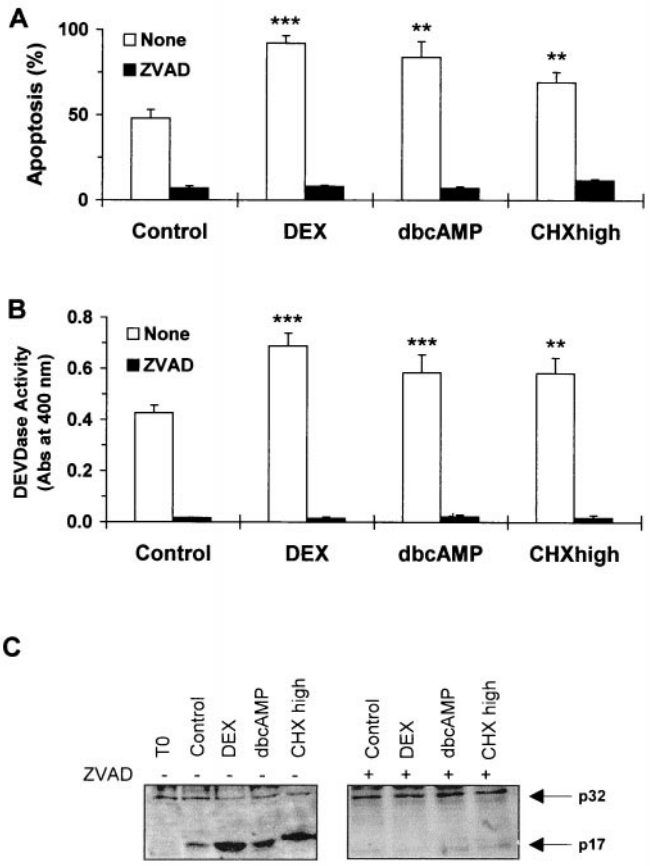


**FIG. 5. Involvement of CPP32/caspase-3-like activity in B lymphocyte apoptosis. B cells were cultured in medium (control) or in the presence of DEX or dbcAMP with or without caspase inhibitor Z-VAD-fmk (50 μM) as indicated. (A) Apoptosis was quantified by cytometric analysis after 24 hr of culture. Data shown represent the mean percentage of apoptosis of three different experiments with error bars indicating standard deviation. **, $P < 0.01$; ***, $P < 0.001$ compared with control cells. Apoptosis observed in the absence versus presence of Z-VAD-fmk: control 47.9 ± 5.2% vs 7.0 ± 1.2%; DEX 92.1 ± 4.0% vs 8.1 ± 0.5%; dbcAMP 83.9 ± 8.2% vs 6.9 ± 1.0%; $CHX^{high}$ 69.0 ± 6.5% vs 11.6 ± 0.9%. (B) Caspase activity was measured after a culture of 8 hr by hydrolysis of the specific DEVD-pNA substrate. Results expressed as mean absorbance units at 400 nm of three different experiments, with error bars indicating standard deviation. **, $P < 0.01$; ***, $P < 0.001$ compared with control cells. DEVDase activity measured in the absence versus presence of Z-VAD-fmk (absorbance units): control 0.426 ± 0.030 vs 0.016 ± 0.002; DEX 0.689 ± 0.050 vs 0.015 ± 0.005; dbcAMP 0.585 ± 0.070 vs 0.021 ± 0.008; $CHX^{high}$ 0.582 ± 0.060 vs 0.018 ± 0.009. (C) Western blot analysis of cleavage of CPP32/caspase-3 in untreated or treated cells was performed as described in Materials and Methods. Lane 1 represents extract from B lymphocytes at the initiation of cultures (T0), and lanes 2–5 extracts obtained from cells cultured for 8 hr in the absence or presence of DEX, dbAMPc, or $CHX^{high}$, with or without Z-VAD-fmk, as indicated.**

the apoptosis of normal primary B lymphocytes. The implication of CPP32/caspase-3 was demonstrated further by Western blot analysis. Figure 5C shows that the 32 kD band (p32) corresponding to CPP32/caspase-3 was present in B cells at the initiation of cultures (T0) and decreased in cells undergoing apoptosis. Thus, the low mw band (p17) corresponding to the active cleavage product of CPP32 appeared in cultured B cells (control) and was increased by DEX, dbcAMP, or $CHX^{high}$ treatment. As previously observed for DEVDase activity, Z-VAD-fmk greatly inhibited CPP32/caspase-3 cleavage and the 32 kD band remained at a high level.

The differential effect of CHX on thymocyte and B cell apoptosis was further demonstrated by measuring DEVDase activity. Table 2 shows that, in parallel with data on thymocyte apoptosis (Fig. 1), $CHX^{high}$, by itself, did not significantly increase DEVDase activity (0.424 ± 0.035 vs 0.394 ± 0.043, $P > 0.2$). In addition, $CHX^{high}$-induced inhibition of apoptosis was accompanied by a reduction in DEX- or dbcAMP-induced caspase activation (0.265 ± 0.082 vs 0.800 ± 0.069, $P < 0.001$ and 0.212 ± 0.069 vs 0.490 ± 0.035, $P < 0.001$, respectively). In contrast, in B lymphocytes, $CHX^{high}$, which increased apoptosis and failed to antagonize drug-induced apoptosis (Fig. 1), did increase DEVDase activity (0.582 ± 0.035 vs 0.426 ± 0.030, $P < 0.01$), but did not affect the DEX- (0.677 ± 0.068 vs 0.689 ± 0.052, $P > 0.8$) and dbcAMP-induced increase in DEVDase activity (0.575 ± 0.023 vs 0.585 ± 0.075, $P > 0.8$). Moreover, the dose-dependent opposite effects of CHX on B cell apoptosis were reflected on DEVDase activity (Table 2), since the low protective dose of CHX (0.05 μg/mL), as with IL-4, reduced DEVDase activity ($P < 0.05$ and $P < 0.01$, respectively), in parallel with its effect on apoptosis. $CHX^{low}$, which potentiated the protective activity of IL-4 (Fig. 4), also potentiated the reduction in DEVDase activity afforded by IL-4 in DEX- (0.404 ± 0.056 vs 0.494 ± 0.025, $P < 0.05$) and dbcAMP-treated cells (0.320 ± 0.065 vs 0.437 ± 0.042, $P < 0.05$).

## DISCUSSION

This study shows that CPP32/caspase-3-like protease was activated during apoptosis of B lymphocytes and that, according to the dose used, CHX increased or decreased caspase activation, reflecting the opposite effects of this inhibitor of protein synthesis on B cell apoptosis. It was initially stated that B lymphocyte apoptosis does not require *de novo* protein synthesis since spontaneous apoptosis was increased by CHX [9, 35]. This favored the hypothesis of the pre-existence of the death machinery which must be restrained by the continuous synthesis of short-lived protective protein(s), including members of the Bcl-2 family [9]. Accordingly, we observed that CHX stimulated B cell apoptosis at a concentration (2.5 μg/mL = $CHX^{high}$) that inhibited global protein synthesis. Interestingly, at that concentration, CHX, which is known to antagonize drug-induced apoptosis [10], effectively inhibited DEX- and dbcAMP-induced apoptosis in thymocytes, but failed to affect drug-induced apoptosis in B lymphocytes. Unexpectedly, lowering the concentration of CHX led to an opposite

**TABLE 2. Caspase-3-like activity in thymocytes and B lymphocytes**

| Cell | Caspase activity in absorbance units at 400 nm (mean ± SD) | | |
|---|---|---|---|
| | Control | DEX 5 nM | dbcAMP 100 μM |
| Thymocytes | | | |
| None | 0.394 ± 0.043 | 0.800 ± 0.069 | 0.490 ± 0.035 |
| $CHX^{high}$ | 0.424 ± 0.035 | 0.265 ± 0.082* | 0.212 ± 0.069* |
| B Lymphocytes | | | |
| None | 0.426 ± 0.030 | 0.689 ± 0.052 | 0.585 ± 0.075 |
| $CHX^{high}$ | 0.582 ± 0.035‡ | 0.677 ± 0.068 | 0.575 ± 0.023 |
| $CHX^{low}$ | 0.363 ± 0.025† | 0.581 ± 0.043§ | 0.457 ± 0.036** |
| IL-4 50 U/mL | 0.318 ± 0.038‡ | 0.494 ± 0.025‖ | 0.437 ± 0.042** |
| $CHX^{low}$ + IL-4 | 0.280 ± 0.034‡ | 0.404 ± 0.056¶ | 0.320 ± 0.065†† |

Thymocytes or B lymphocytes were cultured for 8 hr in the presence or absence of drugs as indicated. Caspase activity was determined by DEVD-pNA hydrolysis after 8 hr at 37°. For thymocytes, values show the means ± SD of three different experiments: *, $P < 0.01$ between CHX-treated and -untreated cells for control, DEX, and dbcAMP treatments. For B lymphocytes, values show the means ± SD of three different experiments: †, $P < 0.05$, ‡, $P < 0.01$ compared with medium alone (control/none); §, $P < 0.05$, ‖, $P < 0.01$, ¶, $P < 0.001$ compared with DEX-treated cells (DEX/None); **, $P < 0.05$, ††, $P < 0.01$ compared with dbcAMP-treated cells (dbcAMP/None).

effect, $CHX^{low}$ (0.05 μg/mL) being able to reduce spontaneous as well as drug-induced apoptosis. Moreover, $CHX^{low}$ potentiated the protection conferred by IL-4, leading to total inhibition of drug-induced apoptosis. The prevention of apoptosis by $CHX^{low}$ described here was observed neither in thymocytes nor in macrophages, the latter being sensitive only to CHX-induced apoptosis ([36] and data not shown). The dual activity of CHX thus appeared to be specific to B lymphocytes, which is in agreement with previous findings suggesting that the signaling pathways leading to apoptosis in B lymphocytes differ from those occurring in other cells [37]. In addition, the dual effect of CHX does not appear to be due to an intrinsic property of this drug, since it was also observed with the two other protein synthesis inhibitors, anisomycin and emetin.

LPS is a polyclonal B lymphocyte activator which strongly stimulates proliferation and differentiation. It was reported that LPS partly protects immature [34] and mature [38] B cells against spontaneous or drug-induced apoptosis. Here, we observed that $CHX^{low}$ increased the rescuing activity of both LPS and IL-4; nevertheless, $CHX^{low}$ greatly inhibited LPS-induced proliferation and cell cycle progression. Therefore, it appeared that the antiapoptotic activity of $CHX^{low}$ was associated with an antiproliferative effect. Similarly, IL-4 protected B lymphocytes against apoptosis in parallel with an inhibition of cell proliferation ([39, 40] and data not shown). Thus, these results suggest that, in contrast with LPS, $CHX^{low}$ provided a pure survival signal. This is in agreement with a previous report showing that CHX (0.1 μg/mL) was able to partially protect splenic B cells from whole anti-immunoglobulin-induced apoptosis but failed to restore thymidine incorporation and cell cycle re-entry [41].

Initially, the protection against apoptosis conferred by CHX was attributed to an inhibition of gene death expression [6], but it now appears that it is rather associated with an overexpression of life gene products [18]. It is noteworthy that a high concentration of CHX led to total inhibition of protein synthesis and could thus induce apoptosis by an unspecific metabolic stress as proposed for other treatments [42, 43], whereas we hypothesize that the low dose of CHX leading to less than 15% inhibition of protein synthesis was not perceived as a vital insult and could exert its protective effect by a specific repression of some pro-apoptotic factors. The long-lasting $CHX^{low}$-induced protection described here occurred after a lag time and required the constant presence of the drug. This suggested that $CHX^{low}$ could act by repressing the activation of factors, with a high turnover, involved in the executive phase of the apoptotic process. Thus, we explored the possibility that $CHX^{low}$ could interfere with the proteolytic cascade which ultimately leads to activation of a cysteine protease of the ICE/caspase family, which is crucial for the execution phase of apoptosis [44].

We first observed that Z-VAD-fmk, an irreversible and wide spectrum inhibitor of caspase activity, totally inhibited spontaneous and drug-induced apoptosis in B lymphocytes, including $CHX^{high}$-induced apoptosis. This showed that the apoptotic pathway in B lymphocytes, as in most cells, implicated caspase activation. We then observed that CPP32/caspase-3-like protease was cleaved during apoptosis and that caspase-3-like activity was increased in apoptotic B cells. The presence of this member of the caspase family had previously been detected following the triggering of apoptosis in Ramos BL cells [45], in B-chronic leukemia cells [46], and in irradiated human B lymphocytes [47], supporting the importance of CPP32/caspase-3-like proteases in the B cell lineage. Few investigators have examined whether CHX directly affected caspase activation. It has been reported that prevention by CHX of butyrate-induced apoptosis in Jurkat cells [48] and of $K^+$/serum deprivation-induced apoptosis in neurons [49] was accompanied by an inhibition of CPP32 activation and that induction of apoptosis by CHX in HL-60 promyelocytic leukemia cells was inhibited by caspase inhibitors [50]. We demonstrated that caspase-3-like activity was similarly increased by DEX or dbcAMP treatment in thymocytes and B lymphocytes and that, interestingly, $CHX^{high}$, which antagonized drug-induced apoptosis in thymocytes, also reduced drug-induced caspase activity. Meanwhile, in B

lymphocytes, $CHX^{high}$ affected neither apoptosis nor the increase in caspase activity induced by DEX and dbcAMP although, by itself, it stimulated both phenomena. On the other hand, $CHX^{low}$ reduced, in parallel, spontaneous or drug-induced cell death and caspase activity. In addition, the intrinsic protective activity of IL-4 was increased by $CHX^{low}$ and was associated with a reduction in caspase activation. These results show a strong relationship between induction of apoptosis and activation of CPP32/caspase-3 and demonstrate that the opposite effects of CHX on apoptosis in B lymphocytes were reflected in caspase activation.

Since activation of CPP32/caspase-3 is a distal step in the apoptotic cascade [51, 52], it cannot be stated that CHX directly acted on caspase activation. The regulation of CPP32/caspase-3-like activity by cycloheximide may just represent a secondary event of its effect on an earlier step. It is becoming evident that mitochondrial alterations constitute early and essential steps in the apoptotic cascade, leading to activation of executive caspases (reviewed in [53]). Therefore, the dose-dependent effect of CHX on mitochondrial potential ($\Delta\Psi m$) and redox status is under investigation to define earlier steps in the dual effect of CHX described here. Moreover, an analysis of protein neosynthesis during CHX-induced stimulation or repression of apoptosis should allow an identification of essential proteins implicated in the choice between survival or apoptosis.

In summary, our findings point to CPP32/caspase-3 as a crucial component in the death machinery in primary B lymphocytes and provide new data concerning the mechanism whereby inhibition of protein synthesis affects apoptosis.

*This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS ERS 0571), Paris-Sud University, and the Association pour la Recherche sur le Cancer (ARC), and a fellowship from ARC (C. L.). We thank N. Esquirol for excellent technical assistance.*

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