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Ochratoxin A Induces Apoptosis in Human Lymphocytes through Down Regulation of Bcl-x_L

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Ochratoxin A (OTA) is a widespread mycotoxin contaminating feed and food. Besides its potent nephrotoxicity, OTA also affects the immune system. We demonstrate here a role for Bcl-x_L in OTA-induced apoptosis in human lymphocytes. In particular, human peripheral blood lymphocytes and the human lymphoid T cell line, Kit 225 cells, underwent apoptosis in a time- and dose-dependent manner. This apoptosis was inhibited by z-VAD.fmk, suggesting that caspases were responsible for the induction of apoptosis. Moreover, OTA triggered mitochondrial transmembrane potential ($\Delta\psi m$) loss and caspase-9 and caspase-3 activation. Interestingly, Bcl-x_L protein expression was decreased by OTA treatment, whereas Bcl-2 protein level was not affected. Down-regulation of *bcl-x_L* mRNA was not observed in cells treated with OTA. Overexpression of Bcl-x_L in Kit 225 cells protected them against mitochondrial perturbation and retarded the appearance of apoptotic cells. Taken together, our data indicate that mitochondria are a central component in OTA-induced apoptosis and that the loss of Bcl-x_L may participate in OTA-induced cell death.

Key Words: **ochratoxin A; Bcl-x_L; mitochondria; lymphocyte; apoptosis.**

Ochratoxin A (OTA) is a ubiquitous fungal metabolite known for its nephrotoxicity, carcinogenicity, genotoxicity, and immunotoxicity (Bondy and Pestka, 2000; Kuiper-Goodman and Scott, 1989; Marquardt and Frohlich, 1992). This wide spectrum of toxicity is largely due to its long half-life, which is estimated to be 35.5 days in humans (Studer-Rohr *et al.*, 2000). OTA is a nephrotoxin for all animal species studied to date, and this is also probably the case for humans. In fact, OTA has been considered as the major agent responsible for the Danish porcine nephropathy and the Balkan endemic nephropathy in humans (Kuiper-Goodman and Scott, 1989; Petkova-Bocharova and Castegnaro, 1991). OTA has been shown to induce apoptosis at nanomolar concentrations in human kidney cells (Schwerdt *et al.*, 1999) and in the renal canine epithelial cell line MDCK-C7 (Gekle *et al.*, 2000). The fact that OTA is such a potent mycotoxin relies on its ability to disturb cellular physiology in multiple ways, particularly by inhibiting the phenylalanyl-tRNA-synthetase (Baudrimont *et al.*, 1997; Creppy *et al.*, 1983a), leading to the inhibition of protein synthesis. Additionally, OTA induces lipid peroxidation in kidney microsomes (Rahimtula *et al.*, 1988) and also alters calcium homeostasis in liver microsomes from treated rats (Khan *et al.*, 1989).

OTA has been shown to be immunosuppressive *in vivo* and *in vitro* (Muller *et al.*, 1995, 1999; Stormer and Lea, 1995). Several studies in mice have shown that OTA treatment resulted in depletion of lymphoid cells and suppression of the antibody response (Creppy *et al.*, 1983b; Muller *et al.*, 1995). OTA also induced macrophage activation (Boorman *et al.*, 1984), altered natural killer cell activity (Luster *et al.*, 1987), and downregulated lymphocyte proliferation of murine and human origins (Prior and Sisodia, 1982; Stormer and Lea, 1995; Thuvander *et al.*, 1995). Concentrations as low as 5 ng OTA/kg body weight have been shown to suppress immune responses in mice (Haubeck *et al.*, 1981).

Apoptosis is an important process in a wide variety of different biological systems and also in chemical-induced cell death (Cohen, 1997). The immune system is now recognized as a target organ for many xenobiotics such as drugs and chemicals, which are able to trigger unwanted apoptosis or to alter the regulation of programmed cell death. Reducing the number of immune-competent cells after xenobiotic treatment can lead to immunosuppressive effects, resulting in an increased susceptibility to tumors or infectious diseases. Many experimental works have dealt with the influence of xenobiotics on the immune system. Glucocorticoids used as immunosuppressive and anti-inflammatory agents are known to provoke apoptosis of thymocytes and activated T cells (Perrin-Wolff *et al.*, 1995). Low doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affect thymocyte development, and the maturation of $CD4^+$ $CD8^+$ double-positive cells is skewed toward CD8 single-positive cells (Nohara *et al.*, 2000). Fungal toxins have been also reported to have immunotoxic effects (Bondy and Pestka,

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2000). The trichothecenes, notably T-2 and HT-2 toxins, provoke a rapid and strong apoptosis of human lymphoid cells (Holme *et al.*, 2003; Shifrin and Anderson, 1999).

OTA immunotoxicity has been explored to different degrees in multiple species, including rodents, poultry, and pigs (Bondy and Pestka, 2000). Among the possible mechanisms leading to immunosuppression, a decrease in the number of lymphocytes due to direct cytotoxicity or to apoptosis has been previously described with xenobiotics. Ochratoxin A has been shown to induce apoptosis in different cellular models. These observations prompted us to examine if the immunosuppressive effects of OTA could be due to lymphocyte apoptosis. Our results show that OTA induces a dose-dependent apoptosis of human peripheral blood lymphocytes through a mitochondrial pathway leading to caspase activation. In addition, we observe that OTA provokes a decrease of Bcl-x_L expression that may be a trigger for OTA-induced apoptosis.

MATERIALS AND METHODS

Cells and culture conditions. Blood was collected from donors (with an average age of 36 years) who were clearly informed about the aim of the study and gave their informed consent. Blood was sampled by venipuncture in sterile tubes containing EDTA and then diluted in PBS. Peripheral blood mononuclear cells (PBMC) were separated from blood by density gradient centrifugation with Ficoll-hypaque Plus (Eurobio, Les Ulis, France) at 2000 rpm for 30 min. PBMC were lifted from the interface and washed twice in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum, 25 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin and 1mM sodium pyruvate (RPMI complete medium). The cells were then resuspended in complete medium at 2×10^6 cells/ml at 37°C in 5% CO_2. Kit 225 cells, an IL-2-dependent human leukemic CD4+ T cell line, were cultured in RPMI complete medium (Hori *et al.*, 1987). Kit 225-bcl-x_L stable cell lines were established as follows. Exponentially growing Kit 225 cells (10^7) were washed in RPMI 1640 buffer and resuspended in 150 μl of RPMI 1640 containing 10 μg of control plasmid (pcDNA3-Neo) or pcDNA3-bcl-x_L construct myc-tagged (a kind gift from Dr Armelle Biola, INSERM U461, Châtenay-Malabry, France). After 10 min incubation on ice, cells were electroporated using a Bio-Rad Laboratories, Inc. gene pulser (Ivry-sur-Seine, France) set at 250 V and 960 μF. Cells were then maintained on ice for 10 min and resuspended in complete medium. Selection of stably transfected cells was initiated 48 h after electroporation using 800 μg/ml G418. Stably transfected cells were selected by a 2-week treatment with G418 and cloned by limiting dilution. Individual clones were analyzed by immunoblotting using the 9E10 anti-Myc antibody, and cell lines expressing different levels of protein were selected for further analysis.

Cell treatment. OTA (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, Sigma) to make a stock solution of 50 mM, from which final concentrations were prepared. Appropriate concentrations of OTA or DMSO (control) were added to cell suspension and incubated at 37°C in 5% CO_2 for indicated times. *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk; 50 μM; Bachem, Weil am Rhein, Germany), dissolved in DMSO, was added 1 h before the addition of OTA.

Measurement of apoptosis. Cells were incubated for various times with different concentrations of OTA, washed with PBS, and permeabilized with ethanol by incubation overnight at –20°C. The cells were then washed with PBS, treated with RNase (20 μg/ml) and stained with propidium iodide (PI, 50 μg/ml). Apoptosis was determined by the quantification of DNA hypodiploidy using flow cytometry. Data acquisition was performed using Cellquest® software (Becton Dickinson).

Assessment of mitochondrial transmembrane potential ($\Delta\psi_m$). To determine changes in the inner mitochondrial transmembrane potential ($\Delta\psi_m$), we used $DiOC_6(3)$ (80 nM), a fluorochrome known to incorporate into all cells driven by the $\Delta\psi_m$. PI (5 μg/ml), which enters cells with damaged plasma membrane, was also used to differentiate apoptosis from necrosis. Cells treated with OTA were labeled with $DiOC_6(3)$ at 37°C for 10 min, and then PI was added at 4°C. The cells were scored immediately by cytofluorometric analysis (Becton Dickinson). Quadrants were fixed using the untreated control. Early apoptotic cells are in the lower left quadrant, and the late apoptotic cells undergoing secondary necrosis are in the upper left quadrant (Fig. 4A).

Western blot. After different treatments, cells were lysed for 30 min in NP40 lysis buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1 mM EDTA, 10 mM NaF, and 0.5% NP-40), supplemented with inhibitor cocktail (1 mM PMSF, 1 mM sodium orthovanadate, 1 mM DTT, 1 μg/ml aprotinine, and 1 μg/ml leupeptine) and centrifuged at 15,000 rpm for 20 min to remove cell debris. After protein concentration determination using the bicinchonic acid protein assay, equal amounts of protein were loaded on a 15% SDS–PAGE. Proteins were transferred to a polyvinylidine difluoride (PVDF) membrane in a transfer apparatus containing transfer buffer (25 mM tris, 192 mM glycine, and 20% methanol). After saturation of nonspecific binding sites with 5% of dry low-fat milk in TBS Tween 20 (0.290) for 1 h, membranes were probed with the different antibodies: anti-caspase-3 (Alexis, San Diego, CA), anti-caspase-9 (Cell Signaling, OZYME, France), anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Bcl-x_L (BD Transduction Laboratories, San Diego, CA). The membranes were then probed with the corresponding secondary antibodies coupled to horseradish peroxidase for 1 h, developed with the addition of enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK), and exposed to film (Kodak, NY).

RT-PCR. Total cellular RNA was isolated from 5×10^6 cells using the Trizol® reagent (Invitrogen, Paisley, UK). The amount of RNA recovered was measured by spectrophotometry. cDNA was synthesized by reverse transcriptase from 2 μg total RNA using 4 μM oligo(dT) primer, 2 μM of dNTP (Amersham, Piscataway, NJ), 20 U of Rnasin (Promega, Madison, WI), and 2 U of AMV reverse transcriptase (Promega) in a total volume of 25 μl.

mRNA expression levels for *bcl-x_L* were determined using PCR. The oligonucleotide primer sequences were as follow: *bcl-x_L* sense, 5′-AGGATACAGCTGGAGTCAGT-3′; *bcl-x_L* antisense, 5′-ACCTGCATCTCCTTGTCTAC-3′. *β-actin* cDNA amplification was used as an internal control. The *β-actin* sense primer was 5′-GGGTCAGAAGGATTCCTATG-3′, and the antisense primer was 5′-GGTCTCAAACATGATCTGGG-3′; 5 μl of the cDNA was used for amplification by PCR. Amplification of cDNAs was performed using 50 pmol primers, 200 μM dNTP, and 1 U Taq DNA polymerase (Qbiogene, Illkirch, France) with reaction buffer. An initial 5-min denaturation was carried out. Cycles of denaturation, annealing and extension were then performed for 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C for *bcl-x_L*, and 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C for *β-actin*. PCR reactions were performed at various cycle numbers to ensure that the results obtained were within the linear range of the amplification curve. PCR reactions were then performed at 30 cycles for *bcl-x_L* and 23 cycles for *β-actin*. We used the expression ratio $(bcl\text{-}x_L/\beta\text{-}actin)_{OTA}/(bcl\text{-}x_L/\beta\text{-}actin)_{control}$ as measured by densitometry to evaluate *bcl-x_L* gene expression.

Measurement of bcl-x_L mRNA stabilization. Kit 225 cells were untreated or treated with 5 μM OTA for 14 h before the addition of 1 μg/ml actinomycin D to arrest transcription. The kinetics of *bcl-x_L* mRNA degradation in the control and OTA-treated cells were assessed using RT-PCR analysis.

Statistics. The data are presented as mean values ± SE. For statistical analysis, the dependence between response (the percentage of subG1 cells, time or doses of OTA) was assessed by linear regressions through values of slope or interactions. Comparisons between slopes were made by parallelism tests between adjusted lines or by interaction assessments. For Figure 7, ANOVA was performed because the data were in a $3 \times 2 \times 2$ factorial design. Statistical difference was achieved if $p < 0.05$.

RESULTS

Ochratoxin A Triggers Apoptosis in PBMC

In this study, we investigated whether the induction of apoptotic cell death could contribute to the effects of OTA on the immune system, using human peripheral blood mononuclear cells (PBMC) collected from healthy donors. Apoptosis is characterized by chromatin oligonucleosomal fragmentation that translates into reduced propidium iodide staining during cell cycle analysis (i.e., subG1 cells). Global analysis of the variation of subG1 cells percentages showed a statistical difference according to treatment time and doses of OTA. An overall comparison of the slopes (control, 5 μM OTA, and 10 μM OTA) in Figure 1A shows a highly significant difference ($p < 0.0001$). Detailed comparisons show a nonsignificant difference between 5 μM and 10 μM ($p = 0.11$) and significant differences between control and OTA at 5 μM ($p = 0.016$) or 10 μM ($p = 0.001$); significance is not modified by Bonferroni correction. Moreover, slopes (3.01 for 24 h and 4.02 for 48 h) for Figure 1B are significantly different ($p = 0.0007$). Thus, OTA treatment resulted in a marked increase in the percentage of subG1 cells in a time- and dose-dependent manner (Fig. 1A and Fig. 1B). The percentage of apoptotic cells incubated with 10 μM of OTA increased from 11 $\pm$ 2.3 to 35 $\pm$ 5.3% after 7 and 24 h of treatment, respectively. In addition, phytohemagglutinin-stimulated PBMC also showed a similar pattern of apoptosis induction (data not shown). Interestingly, OTA used at 1 μM was found to trigger significantly the apoptotic death of PBMC at both 24 and 48 h (12 $\pm$ 3 and 23 $\pm$ 0.5%), respectively (Fig. 1B). OTA is known to act as a chronic toxin in humans due to its long half-life (Studer-Rohr *et al.*, 2000). We therefore exposed PBMC for 24 to 120 h with OTA concentrations as low as 0.1 μM. As shown in Figure 1C, OTA at the concentration of 0.5 and 1 μM induced cell apoptosis that was strongly dependent on time according to a linear regression test. The variations of the percentage of subG1 cells are dependant on both variables "dose of OTA" and "time," with a strong interaction between the variables ($p < 0.0001$), which has for consequence a quasi-null difference along the time between doses 0 and 0.1 μM (around 0.03), and large differences between 0 and 0.5 μM (0.122) and 1 μM (0.217) as well. Thus, cells exposed to OTA at 0.1 μM for 5 days did not show signs of apoptosis.

Ochratoxin A-Induced Apoptosis Involves the Activation of Caspases

The central executioner of the apoptotic machinery is a family of proteases called caspases. To determine whether these proteases were implicated in lymphocyte apoptosis during OTA exposure, PBMC from different donors were preincubated with the pan-caspase inhibitor, z-VAD.fmk at 50 μM for 1 h, and cells were then exposed to OTA. The influence of z-VAD.fmk was assessed by interaction with dose. The slopes of the response versus dose were different with or without z-VAD.fmk ($p = 0.010$). So treatment with z-VAD.fmk significantly reduced the induction of apoptosis by OTA as de-

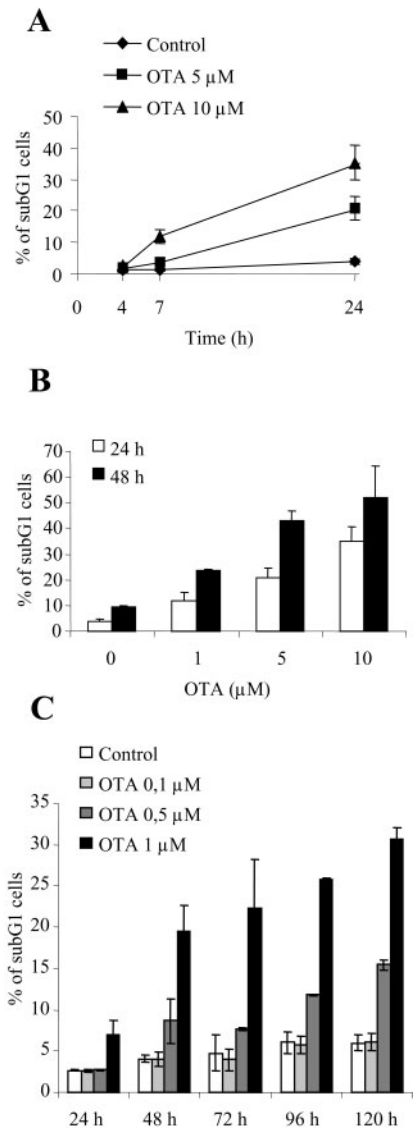

FIG. 1. OTA-induced apoptosis in human peripheral blood mononuclear cells (PBMC). PBMC were incubated with varying concentrations of OTA and labeled with propidium iodide. Cells were analyzed by flow cytometry, and subG1 cells with hypodiploid DNA corresponding to apoptotic cells were quantified. (A) OTA induces apoptosis in a time-dependent manner. Data represent the means $\pm$ SE of five independent experiments from five different donors. Control cells were incubated with DMSO 0.02%. An overall comparison of the slopes (control, 5 μM OTA, 10 μM OTA) shows highly significant difference ($p < 0.0001$). (B) The percentage of apoptotic cells increases in a dose-dependent manner. This figure represents the means $\pm$ SE of three independent experiments from three different donors. An overall comparison of the slopes (24 and 48 h) shows highly significant difference ($p < 0.001$). (C) Low doses of OTA induced apoptosis in PBMC following 5 days of exposure. The variations of the percentage of subG1 cells are dependant of both variables "dose of OTA and time," with a strong interaction between the variables ($p < 0.0001$). Data represent the means $\pm$ SE of three independent experiments from three different donors.

termined by quantification of subG1 cells, suggesting a role for caspases in this apoptotic process (Fig. 2).

Caspases are activated by cleavage at specific residues, and to confirm the presence of active caspases in the cytosol of PBMC treated with OTA, we performed immunoblot analysis of caspase-3 and -9 (Fig. 3). A decrease in the quantity of both pro-caspase-9 and -3, molecular weights of 46 and 32 KDa, respectively, was observed (lanes 2, 3, and 4), indicating caspase activation. In addition, the anti-caspase-3 antibody could detect the p17 fragment corresponding to the fully activated caspase (Han *et al.*, 1997). The presence of z-VAD.fmk partially prevented the loss of caspase-9 proform and led to accumulation of caspase-9 p35 fragment (lanes 5 and 6). z-VAD.fmk also totally abrogated the apparition of the caspase-3 p17 fragment (lanes 5 and 6). Therefore, the inhibitory effect of z-VAD.fmk on caspase processing correlates with its ability to protect cells against OTA-induced apoptosis. However, the persistence of partially processed caspase-3, p20 fragment, indicates that caspase inhibition was not absolute, which may explain the residual cell death observed in Figure 2 despite z-VAD presence. In any case, these results establish that caspase activity is required for OTA-induced PBMC apoptotic cell death.

Ochratoxin A Induces Mitochondrial Membrane Permeabilization

Caspase-9 activation and mitochondrial membrane permeabilization (MMP) are intimately linked in numerous models of apoptosis. Indeed, MMP triggers caspase-9 activation by the release of mitochondrial proteins, such as cytochrome c, participating in the formation of the apoptosome (Green and Reed, 1998; Kroemer and Reed, 2000; Wang, 2001). This membrane permeabilization is often accompanied by loss of inner mitochondrial transmembrane potential ($\Delta\psi_m$). OTA induced a time- and dose-dependent decrease of $\Delta\psi_m$, as shown in Figure 4A and Figure 4B, showing that this mycotoxin was able to induce mitochondrial dysfunctions of a type known to lead to caspase-9 activation. Interestingly, treatment of PBMC with low concentrations of OTA such as 0.5 μM with prolonged incubation time was found to induce mitochondrial perturbation. In fact, the interaction between dose and time was found highly significant ($p < 0.0001$). The slopes of the percentage of cells with low $\Delta\psi_m$ versus time for the doses 0 or 0.1 μM of OTA are very close to zero; they are positive for the dose 0.5 μM (0.150 h^{-1}) and for dose 1 μM (0.342 h^{-1}) (Fig. 4C).

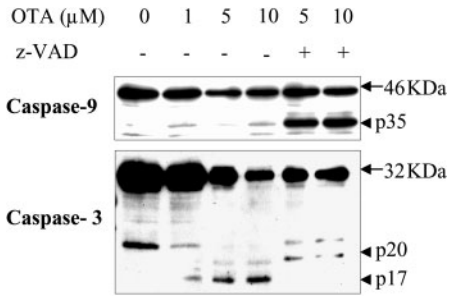

FIG. 3. OTA-induced caspase-3 and caspase-9 activation in PBMC. Cells were incubated with OTA at different concentrations for 24 h. z-VAD.fmk (50 μM) was added 1 h before OTA treatment. Total cell lysates were prepared and electrophoresed in a 15% SDS–PAGE gel and then transferred to PVDF membranes. The membranes were probed with either anti-caspase-3 or anti-caspase-9 antibodies. The results shown are representative of three independent experiments. Arrows indicate the pro-caspase form, and arrowheads indicate the cleaved fragments of pro-caspases.

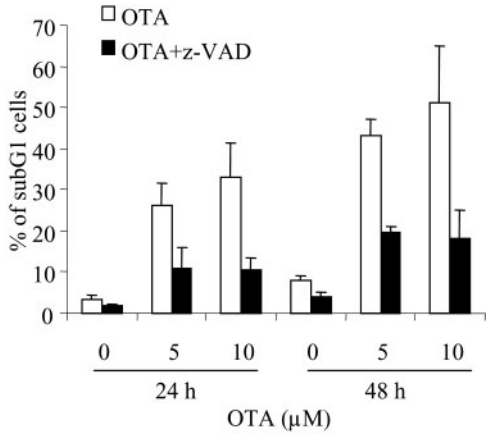

FIG. 2. OTA-induced apoptosis is inhibited by z-VAD.fmk. PBMC from different donors were preincubated with z-VAD.fmk (50 μM) for 1 h and then treated with OTA for 24 and 48 h. Apoptosis, measured by the subG1 peak, was evaluated by flow cytometry. Data represent the means ± SE of three independent experiments from three different donors. An overall comparison of the slopes of the response versus dose (with or without z-VAD.fmk) shows highly significant difference ($p < 0.01$).

OTA Triggers Apoptosis and Mitochondrial Membrane Permeabilization in Kit 225 Cells

To investigate the mechanisms of OTA-induced apoptosis in lymphoid cells, we used a human lymphoid cell line, Kit 225, which is more amenable to transfection than primary cells. We first investigated the apoptotic pathway in this cell line after OTA treatment. Figure 5A shows the dose-response for OTA-induced apoptosis in Kit 225 cells as measured by subG1 cells quantification. The percentage of subG1 cells increased with the time of exposure (24 and 48 h) and the concentration of OTA (5 and 10 μM). To confirm that induction of apoptosis in this model involved the mitochondria, cells were simultaneously stained with $DiOC_6(3)$ and PI. OTA induced an early drop in $\Delta\psi_m$ that increased with time (Fig. 5B). Caspase-9 and caspase-3 were also activated in this cell line by OTA (data not shown). These results indicate that, as is the case for PBMC, OTA-induced cell death in Kit 225 cells implicates mitochondrial perturbations, thus validating this cell line for mechanistic evaluation.

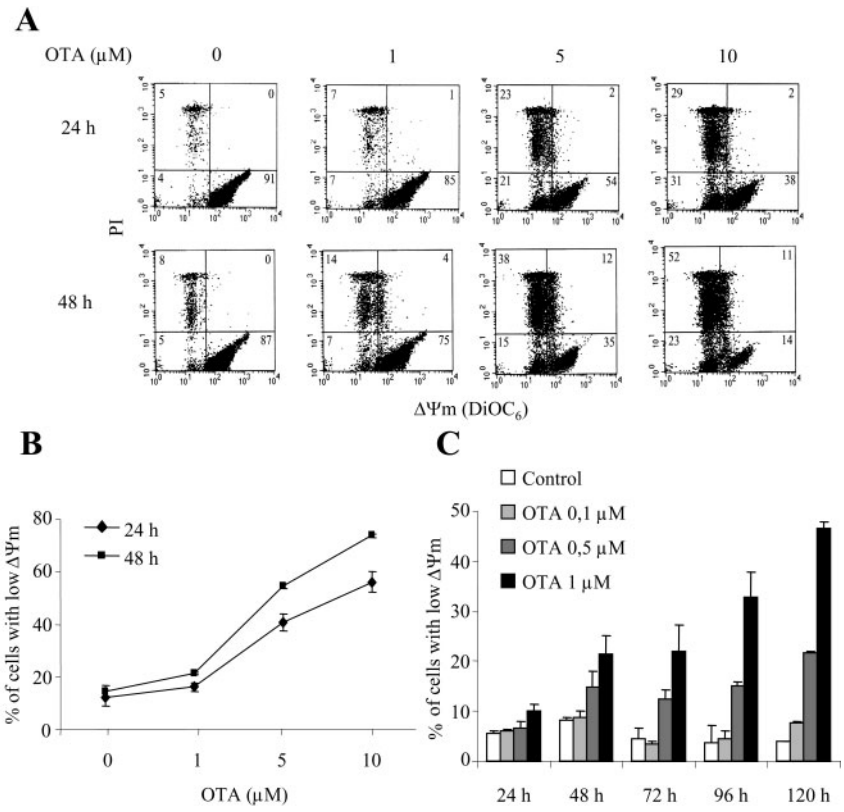

FIG. 4. OTA treatment provoked a decrease in the mitochondrial transmembrane potential ($\Delta\psi_m$) in PBMC. (A) Cells treated with OTA at different concentrations for 24 and 48 h were simultaneously stained with $DiOC_6(3)$ and propidium iodide, followed by cytofluorometric analysis. Numbers indicate the percentage of cells found in the corresponding quadrant. Early apoptotic cells are in the lower left quadrant, and the late apoptotic cells are in the upper left quadrant. Results shown were from a representative experiment. (B) Quantification of data from three different experiments. The role of the dose of OTA is significant: the slopes differ from zero ($p < 0.0001$). The slopes are also different between 24 h (4.54 μM^{-1}) and 48 h (6.04 μM^{-1}), with $p < 0.05$. Results shown correspond to the sums of the percentage found in the lower left and upper left quadrants of these different experiments. (C) Loss of $\Delta\psi_m$ in PBMC treated with low concentrations of OTA for up to 120 h. Data represent the means ± SE of three independent experiments from three different donors. The interaction between dose and time was found highly significant ($p < 0.0001$).

OTA Induces a Decrease in the Protein Level of Bcl-x_L

Pro-survival proteins such as Bcl-2 and Bcl-x_L are known to protect the cell against diverse cytotoxic signals (Chao *et al.*, 1995; Cory, 1995) that trigger the mitochondrial apoptotic pathway by preventing mitochondrial alterations and cytochrome c release. Therefore, we evaluated the Bcl-2 and Bcl-x_L protein level by Western blots in Kit 225 cells treated with OTA. Bcl-x_L protein content was decreased at 5 μM of OTA at 24 h and was nearly completely absent at 10 μM after 48 h of OTA treatment (Fig. 6). In contrast, Bcl-2 protein level was not affected by OTA treatment (Fig. 6).

To further investigate the role of Bcl-x_L decrease in OTA-induced apoptosis, Kit 225 cells were stably transfected with the *bcl-x_L* gene. As shown in Figure 7A, Bcl-x_L overexpression in Kit 225 cells protected cells from OTA-induced apoptosis, in contrast to the mock-transfected cells, as measured by the percentage of subG1 cells. Moreover, caspase-3 activation was inhibited (data not shown), and $\Delta\psi_m$ was preserved in OTA-treated cells overexpressing Bcl-x_L (Fig. 7B). Data treated by ANOVA with doses (0 and 10 μM), time (24 and 48 h) and clones (mock, Kit-Bcl-x_L clone 1, and clone 13) showed significant differences between effects of doses ($p < 0.0001$) and no significant difference between 24 and 48 h ($p = 0.34$). Differences between mock and Kit-Bcl-x_L clone 1 were highly significant ($p < 0.0001$) and significant ($p < 0.05$) between mock and Kit-Bcl-x_L clone 13. Thus, the degree of protection induced by Bcl-x_L overexpression was correlated with the level of exogenous Bcl-x_L expression as shown with the two clones tested (Fig. 7A, inset).

However, in Kit 225-bcl-x_L clones although a significant protection from OTA-induced apoptosis was present, we still observed apoptosis, suggesting that overexpression of Bcl-x_L did not fully protect the cells over time. When we evaluated the protein level of exogenous Bcl-x_L (Bcl-x_L-Myc) in OTA-treated cells, our results showed a significant decrease, demonstrating that Bcl-x_L-Myc was also lost over time (Fig. 7C).

OTA Treatment Did Not Affect bcl-x_L Gene Transcription

As a first step to unveil the cause of Bcl-x_L decrease, we asked whether Bcl-x_L was cleaved by caspases, as previously described in other models (Clem *et al.*, 1998). The failure of z-VAD.fmk to prevent endogenous Bcl-x_L decrease in Kit 225 cells treated with OTA (Fig. 8A) implies that Bcl-x_L is probably not a substrate for caspases in these cells.

We then examined whether OTA treatment decreases the expression of *bcl-x_L* mRNA. Figure 8B shows that the decrease in Bcl-x_L was not due to transcriptional down-regulation, as the expression of *bcl-x_L* mRNA was unaffected by OTA treatment at both 14 and 24 h. To determine whether the loss of Bcl-x_L was due to an alteration of mRNA stabilization, we assessed the degradation rate of *bcl-x_L* mRNA in Kit 225 cells treated with 5 μM OTA for 14 h before the addition of actinomycin D.

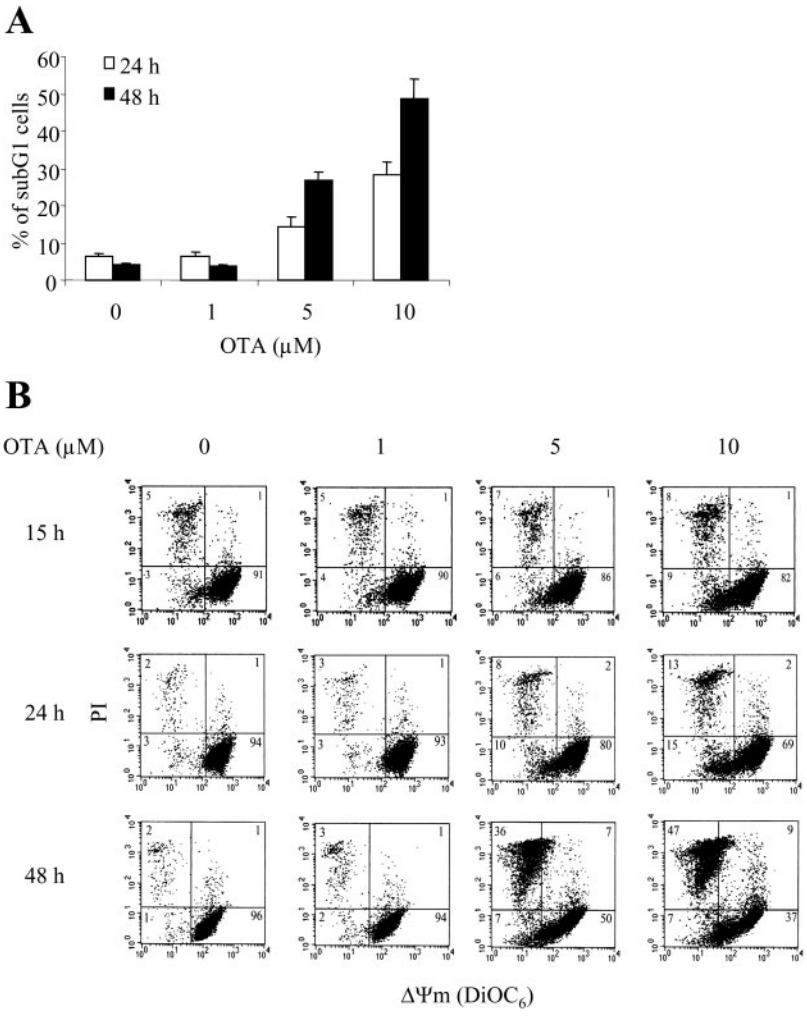

FIG. 5. OTA-induced apoptosis in the human Kit 225 lymphoid cell line. (A) Cells treated with OTA at different concentrations for 24 and 48 h were subjected to ethanol fixation and staining with propidium iodide followed by flow cytometry quantification of DNA loss. The role of the dose of OTA is significant: the slopes differ from zero ($p < 0.0001$). The slopes are also different between 24 h (2.24 μM^{-1}) and 48 h (4.70 μM^{-1}) with $p < 0.0001$. Data represent the means ± SE of five independent experiments. (B) OTA decreased the mitochondrial transmembrane potential in Kit 225 cells. After the indicated treatment, cells were stained simultaneously with $DiOC_6$ and propidium iodide followed by cytofluorometric analysis. Numbers indicate the percentage of cells found in the corresponding quadrant. Data shown were representative of three independent experiments.

The comparison of *bcl-x_L* mRNA decay (2, 3, 4, 6, and 8 h) obtained from cells treated with OTA to *bcl-x_L* mRNA degradation observed in control cells showed no difference (Fig. 8C). The aforementioned data suggest that the decrease of Bcl-x_L due to OTA was probably not occurring at the transcriptional level.

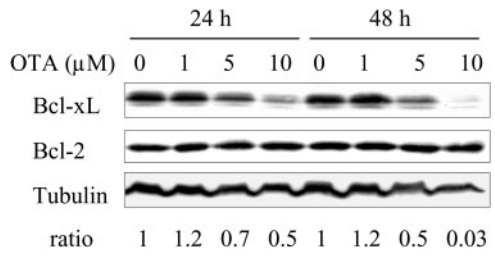

FIG. 6. OTA decreased Bcl-x_L level in Kit 225 cells. Cell extracts obtained after the indicated treatment were subjected to immunoblotting with antibodies specific for Bcl-x_L, Bcl-2, and β-tubulin as a loading control. Bcl-x_L expression was evaluated by densitometry using Image Quant® software, and the ratio $(Bcl\text{-}x_L/\beta\text{-}tubulin)_{OTA}/(Bcl\text{-}x_L/\beta\text{-}tubulin)_{control}$ was calculated.

OTA Induces Bcl-x_L Decrease in PBMC

To further corroborate the role of Bcl-x_L in OTA-induced apoptosis, we investigated whether the loss of Bcl-x_L was confined to Kit 225 cells or whether it was also found in PBMC. As observed in Kit 225 cells, we noted a significant decrease in the Bcl-x_L protein level at different concentrations such as 5 μM OTA at 24 h, whereas Bcl-2 expression was not modified, confirming the observation in a relevant model (Fig. 9).

DISCUSSION

In the present study, we report that OTA induces apoptosis in human lymphocytes, with the mitochondria playing a pivotal role in coordinating caspase activation. We also observe that OTA decreases Bcl-x_L protein level without affecting gene transcription, suggesting a mechanism that could be a trigger for apoptosis.

To elucidate the mechanism by which OTA triggers apoptosis, we used two types of cells: PBMC and Kit 225 cells. These

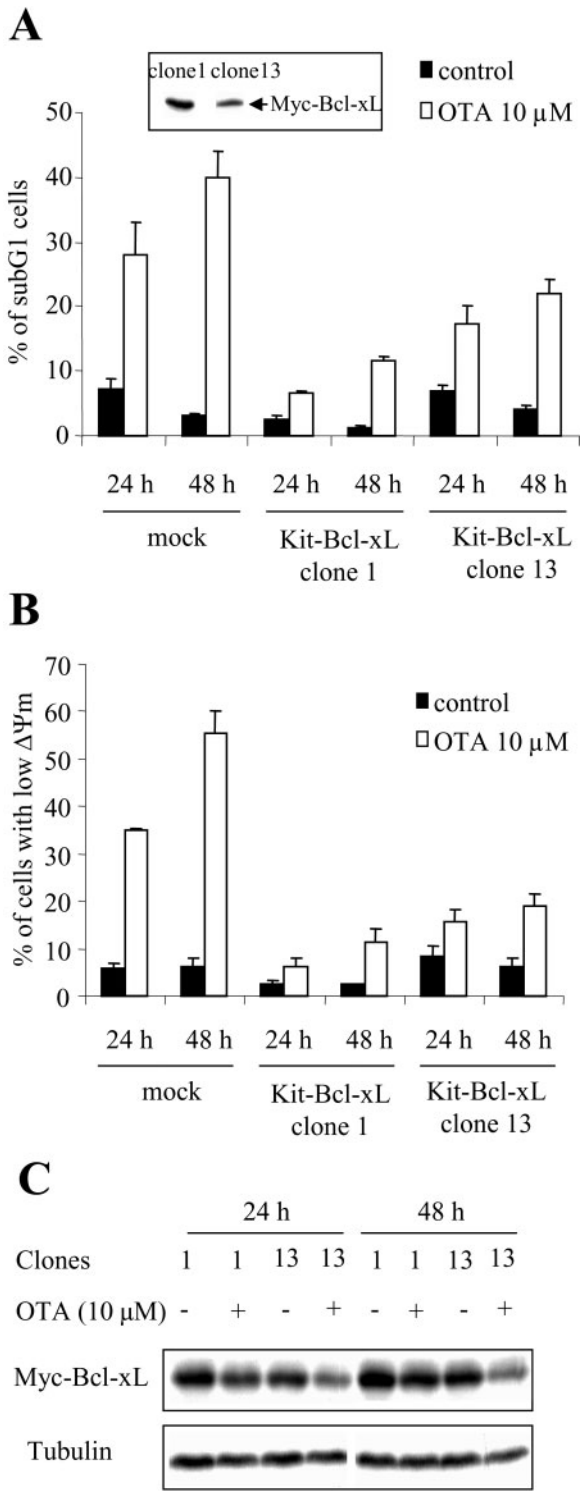

FIG. 7. Kit 225 cells overexpressing Bcl-x_L were protected from OTA-induced apoptosis. (A) Kit 225 cell lines stably transfected with the human *bcl-x_L* gene or the control plasmid (Neo) were exposed to the indicated dose of OTA during 24 and 48 h and were subjected to ethanol fixation and staining with propidium iodide followed by FACS quantification of subG1 cells. Inset represents an anti-Myc immunoblotting showing the expression level of Myc-Bcl-x_L of the two represented clones. (B) Mitochondrial membrane stabilization by Bcl-x_L prevented OTA-induced $\Delta\psi_m$ loss. Kit 225 cells stably transfected with vector only (Neo) or with the plasmid coding for human Bcl-x_L, were treated with OTA, and the frequency of cells with low $\Delta\psi_m$ was assessed. Data treated by ANOVA with doses (0 and 10 μM), time (24 and 48 h), and clones (mock, Kit-Bcl-x_L clone 1, and clone 13) show significant differences between effects of doses ($p < 0.0001$) and no significant difference between 24 and 48 h ($p = 0.34$). (C) OTA decreased exogenous Myc-Bcl-x_L level in Kit 225-bcl-x_L cells. Kit 225-bcl-x_L cells were treated for the indicated period with 10 μM OTA. Cell extracts obtained after the indicated treatment were subjected to immunoblotting with anti-Myc.

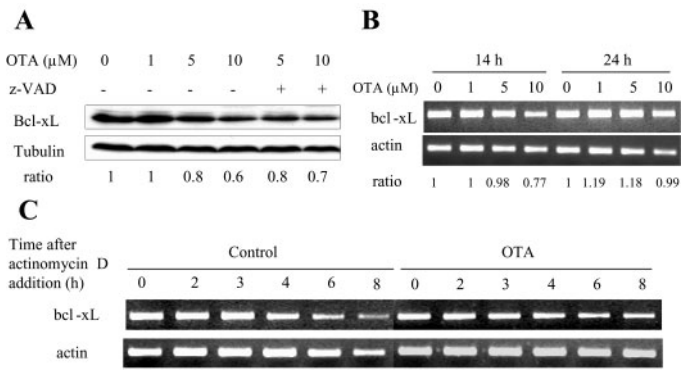

FIG. 8. OTA did not downregulate Bcl-x_L at the transcriptional level or by activated caspases. (A) z-VAD failed to inhibit the degradation of Bcl-x_L. Kit 225 cells were treated for the indicated period with OTA at different concentrations; z-VAD (50 μM) was added 1 h before OTA treatment. Cell extracts obtained after the indicated treatment were subjected to immunoblotting with anti-Bcl-x_L. Bcl-x_L expression was evaluated by densitometry using Image Quant® software, and the ratio $(Bcl\text{-}x_L/\beta\text{-}tubulin)_{OTA}/(Bcl\text{-}x_L/\beta\text{-}tubulin)_{control}$ was calculated. (B) OTA did not inhibit *bcl-xL* transcription. *bcl-x_L* and *β-actin* mRNA of Kit 225 cells treated for 14 and 24 h with OTA were evaluated by RT-PCR. Gene expression was evaluated by densitometry using Image Quant® software, and the ratio $(bcl\text{-}x_L/\beta\text{-}actin)_{OTA}/(bcl\text{-}x_L/\beta\text{-}actin)_{control}$ was calculated. One representative experiment out of three is shown. (C) OTA did not affect *bcl-x_L* mRNA stabilization. Kit 225 cells were treated with 5 μM OTA for 14 h before the addition of 1 μg/ml actinomycin D. The kinetics of *bcl-x_L* mRNA degradation was examined using RT-PCR analysis. This data was representative of three independent experiments.

cells undergo apoptosis in a time- and dose-dependent manner. In addition, low doses of OTA such as 0.5 μM triggered apoptosis in PBMC after 4 days of incubation with OTA, suggesting that chronic exposure to low doses of OTA may expose humans to inadvertent immunosuppression. Seegers *et al.* have also reported that OTA induced DNA degradation associated with apoptosis, but this observation was made in phytohemagglutinin-stimulated human lymphocytes (Seegers *et al.*, 1994).

In our models, OTA was shown to induce apoptosis via the activation of caspase-9 and caspase-3. Other examples of xenobiotics capable of activating the caspase pathway similarly to OTA have been described previously. For instance, acetaminophen was shown to induce a caspase-dependent apoptosis in PBMC by triggering the activation of caspase-3, 9, and 8 independently of Fas with mitochondria as a primary target

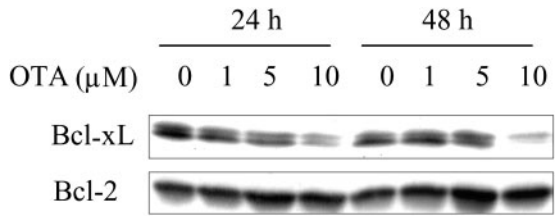

FIG. 9. OTA decreased Bcl-x_L expression in PBMC. Cell extracts obtained after the indicated treatment were subjected to immunoblotting with antibodies specific for Bcl-x_L and Bcl-2. The results shown were from a representative experiment out of three.

(Boulares *et al.*, 2002). Silica can also initiate caspase-9 and caspase-3 activation in a macrophage cell line by destabilizing mitochondrial integrity (Thibodeau *et al.*, 2003).

Mitochondria are organelles that play a central role in the apoptotic process. In fact, several pro-apoptotic signals target the mitochondria and culminate in MMP (Green and Reed, 1998; Kroemer and Reed, 2000), leading to the release of cytochrome c, formation of the apoptosome, and activation of caspase-9 and consequently caspase-3 (Li *et al.*, 1997). $\Delta\psi_m$ loss was found in cells incubated with OTA, implying that mitochondria play a role in the mechanism of OTA-induced apoptosis. These data suggested that $\Delta\psi_m$ loss after OTA treatment preceded the caspase activation and subsequently was responsible for the induction of apoptosis. Moreover, the $\Delta\psi_m$ loss triggered by OTA was not inhibited by z-VAD treatment (data not shown), suggesting that caspases are not implicated in the onset of $\Delta\psi_m$ loss in opposition with what was reported in other models (Finucane *et al.*, 1999; Ricci *et al.*, 2003).

Bcl-2 and Bcl-x_L are anti-apoptotic proteins that have been first described to potently inhibit dexamethasone or γ-irradiation-induced apoptosis and also cytokine deprivation-induced apoptosis (for a review, see Kroemer *et al.*, 1998). It has been suggested that both proteins inhibit apoptosis at the level of mitochondrial function by preventing cytochrome c efflux to the cytosol, thus inhibiting caspase-9 activation (Hu *et al.*, 1998; Vander Heiden *et al.*, 1997). Since in previous reports a decline in the expression of Bcl-2 and Bcl-x_L was thought to be responsible for the onset of apoptosis, we investigated the possible role of the anti-apoptotic proteins, Bcl-2 and Bcl-x_L, in OTA-induced apoptosis. Herein, OTA triggers the decrease of Bcl-x_L protein, while Bcl-2 level was not affected. When Bcl-x_L was overexpressed in Kit 225 cells, both OTA-induced apoptosis and $\Delta\psi_m$ loss were strongly inhibited. This effect was also found in Kit 225 cells overexpressing Bcl-2 (data not shown). Moreover, pro-apoptotic Bax, largely cytosolic in healthy cells, was found to translocate to mitochondria in Kit 225 cells treated with OTA at 24 and 48 h (data not shown). All these data suggest that OTA induced a perturbation in the equilibrium between pro and anti-apoptotic proteins of the Bcl-2 family.

Bcl-x_L has been shown to delay apoptosis induced by various stimuli (Boise *et al.*, 1993; Fang *et al.*, 1994; Gonzalez-Garcia *et al.*, 1994; Shiraiwa *et al.*, 1996) and to play a stronger protective role against apoptosis than Bcl-2 in certain circumstances (Gottschalk *et al.*, 1994). Bcl-x_L has also been reported to control apoptosis mediated by nitric oxide and reactive oxygen species (Shimizu *et al.*, 1995). In macrophages, Bcl-x_L, but not Bcl-2, was able to regulate their susceptibility against nitric oxide toxicity (Lakics *et al.*, 2000; Okada *et al.*, 1998). Recent studies have demonstrated that the down-regulation of Bcl-x_L by different treatments such as retinoic acid (Fujimura *et al.*, 2003), honokiol (Yang *et al.*, 2002) and arsenic trioxide (Hyun Park *et al.*, 2003) was correlated with apoptosis, while Bcl-2 level remained unchanged.

The rapid loss of Bcl-x_L protein after OTA treatment appeared to be unrelated to transcription inhibition, since *bcl-x_L* mRNA in Kit 225 cells was not downregulated over time compared to untreated cells. Furthermore, OTA had no effect on *bcl-x_L* mRNA stabilization compared to control cells. These results would suggest that OTA could activate a proteolytic mechanism leading to Bcl-x_L protein decrease. Indeed, in Kit 225 cells overexpressing exogenous Bcl-x_L under the control of the CMV promoter, we have also shown a decrease in exogenous Myc-tagged Bcl-x_L likely due to proteolytic activity induced by OTA treatment. The failure of z-VAD.fmk to prevent endogenous Bcl-x_L breakdown in Kit 225 cells also suggested that Bcl-x_L was not cleaved by caspases in our model and that other proteases may be involved in the proteolysis of Bcl-x_L. Recent studies, however, pointed out that noncaspase proteases such as cathepsins, calpains, and the proteasome complex can contribute to cell death and also cleave death-related substrates including caspases (Egger *et al.*, 2003; Jaattela and Tschopp, 2003). Bcl-x_L decrease has been previously documented upon treatment by xenobiotics such as retinoic acid (Fujimura *et al.*, 2003). However, in this case, retinoic acid clearly inhibits *bcl-x_L* mRNA expression by suppression of transcription. Other mycotoxins such as T-2 and HT-2 toxins have been shown to induce apoptosis in HL-60 cells due to the activation of both caspase-9 and caspase-8 and consequently caspase-3. However, Bcl-2 and Bcl-x_L levels were not altered in these models (Holme *et al.*, 2003).

OTA has been shown to inhibit both DNA and protein synthesis under certain conditions. High concentrations of OTA (above 100 μM) inhibited phenylalanyl-tRNA synthetase (Baudrimont *et al.*, 1997) and, consequently, general protein synthesis (Creppy *et al.*, 1979, 1983a). We do not believe that inhibition of protein synthesis could account for the observed decreased Bcl-x_L level after OTA treatment of human lymphocytes. Indeed, we did not observe any changes in the protein level of other proteins such as Bcl-2. Moreover, the preincubation of cells with L-phenylalanine, a competitive inhibitor of OTA for this effect, did not protect cells from OTA-induced apoptosis (data not shown). This is consistent with the results of Bruinink and Sidler who reported the inability of L-phenyl-

alanine to protect neuronal cells from toxicity due to OTA (Bruinink and Sidler, 1997).

In conclusion, OTA induces apoptosis by disrupting mitochondrial function in human T-lymphocytes. Our work demonstrates for the first time that Bcl-x_L loss due to OTA accompanies lymphocyte demise and may be the trigger for the apoptotic process. These data also indicate that OTA-induced apoptosis is dose- and time-dependent, and low concentrations of OTA provoke $\Delta\psi_m$ loss and cell death after a 4-day exposure. These observations have to be integrated with the fact that OTA has a long elimination half-life (Studer-Rohr *et al.*, 2000), and exposure to low doses upon a long period may lead to immunotoxic effects. Additional research is needed to elucidate the mechanism(s) responsible for OTA-induced apoptosis leading to Bcl-x_L decrease.

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