

Effect of arsenic, cadmium and lead on the induction of apoptosis of normal human mononuclear cells

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(Accepted for publication 13 March 2002)

SUMMARY

The aim of this work was to investigate the effect of cadmium, lead and arsenic on the apoptosis of human immune cells. Peripheral blood mononuclear cells (MNC) were incubated with increasing concentrations of these metals and then cellular apoptosis was determined by flow cytometry and by DNA electrophoresis. We found that arsenic induced a significant level of apoptosis at 15 μM after 48 h of incubation. Cadmium had a similar effect, but at higher concentrations (65 μM). In addition, cadmium exerted a cytotoxic effect on MNC that seemed to be independent of the induction of apoptosis. In contrast, concentrations of lead as high as 500 μM were nontoxic and did not induce a significant degree of apoptosis. Additional experiments showed that arsenic at concentrations as low as 1.0 μM had a significant pro-apoptotic effect when cells were cultured in the presence of this pollutant for more than 72 h. Non-T cells were more susceptible than T lymphocytes to the effect of arsenic and cadmium. Interestingly, MNC from children chronically exposed to arsenic showed a high basal rate of apoptosis and a diminished *in vitro* sensibility to this metalloid. Our results indicate that both arsenic and cadmium are able to induce apoptosis of lymphoid cells, and suggest that this phenomenon may contribute to their immunotoxic effect *in vivo*.

Keywords immunodeficiency lymphocytes non-T cells programmed cell death toxicity

INTRODUCTION

Apoptosis or programmed cell death plays a key role in the development and homeostasis of the immune system [1,2]. Apoptosis is triggered by a variety of physiological and pathological stimuli that induce different cells to participate in its own death process [1,2]. The morphological changes that characterize this process include loss of microvilli and cell–cell junctions, cytoplasm shrinkage and condensation of nuclear chromatin [1,2]. The biochemical changes that occur during apoptotic cell death include the translocation of phosphatidylserine to the outer layer of cell membrane, the increase of intracellular Ca^{2+} and a characteristic DNA cleavage pattern [1–4].

Although the mechanisms involved in apoptosis may differ in distinct cell types, this phenomenon can be divided into three distinct phases: in the initiation phase, apoptosis is induced through cell membrane receptors (mainly those of the TNF receptor family) by growth factor withdrawal or DNA damage, among others. The second or effector phase involves mitochondrial alterations as well as different intracellular signals (Ca^{2+} ,

ceramide, AMPc, oxygen metabolites) that result in the activation of caspases, a phenomenon that is partially under the control of bcl-2 gene family members [5–7]. Finally, during degradation or third phase, the activation of catabolic enzymes originates the characteristic structural features of apoptosis [6].

Apoptosis occurs under a wide range of physiological and pathological conditions, such as normal cell turnover, withdrawal of growth factors, immune-mediated cytotoxicity, and deletion of autoreactive lymphocytes [2,8]. Although apoptosis is very important in the homeostasis of the immune system, this type of cell death can be also associated with disease. An abnormal resistance to the induction of apoptosis has been observed in different neoplastic or autoimmune conditions. In contrast, an enhanced apoptosis has been found in disorders such as AIDS, aplastic anaemia and neurodegenerative disorders [8]. In addition, a growing number of agents that induce apoptosis of lymphoid cells, such as chemotherapeutic drugs, irradiation, ethanol, mercury, p-dioxin, etc. have been described [9–12]. Abnormalities in apoptosis of immune cells can lead to immunoregulatory defects, which may result in immunodeficiency, autoimmune disease or malignancy [8].

Lead (Pb), cadmium (Cd) and arsenic (As) are extremely toxic environmental pollutants. It is well known that exposure to these metals causes damage in different tissues, including the

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immune system [13–15]. Pb is an ubiquitous toxic metal with different sources of environmental exposure [13]. The activation of cellular functions due to its calcium mimicking effect and inhibition of the activity of different proteins through its binding to sulphhydryl (SH) groups of amino acids have been described as the main mechanisms of toxicity of this metal [13,16]. There is evidence that Pb is able to affect the immune system. Both, acute and chronic exposures to lead diminish the resistance to *Listeria monocytogenes* [17]. In addition, it has been reported that lead inhibits nitric oxide (NO) production that increases TNF- α secretion, together with up-regulation of TNF- α receptor expression on activated mononuclear cells [18,19]. Furthermore, there is evidence that lead increases the number of antibody-producing cells [20], and that favours the activation of Th2 lymphocytes [21].

Cadmium is also able to mimic calcium and to bind to SH groups of proteins [14,16]. Exposure to cadmium occurs mainly via tobacco and occupational industries. Cadmium affects both the cellular and humoral immune responses and seems to have a clear carcinogenic effect in humans [14]. Its immunotoxicity also has been suggested by impaired resistance to *L. monocytogenes* as well as by the inhibition of antigen-specific T cell responses and the induction of abnormalities in antibody production [22,23].

Arsenic, a human carcinogen, is a worldwide contaminant that is found in soil, water and air. Arsenic is an uncoupler of mitochondrial oxidative phosphorylation that induces generation of reactive oxygen species [24]. Different studies have indicated that arsenic is immunotoxic [15]. Lymphocytes from individuals chronically exposed to this metalloid show a longer generation time compared to controls [25]. Another study reported that arsenic interferes with the antigen-presenting function of splenic macrophages [26]. Finally, arsenic is able to alter the response of IgM and IgG antibody-forming cells to sheep erythrocytes, and the proliferative response of lymphocytes to phytohaemagglutinin [27,28].

The mechanisms responsible for the immunotoxicity of As, Cd and Pb have not been elucidated fully. It has been reported that lead and cadmium cause the destruction of the cell membrane of human lymphocytes and monocytes [29]. In addition, cadmium and arsenic are able to induce apoptosis of hamster ovary cells and rat testicular tissue [24,30]. However, the possible effect of these metals on the apoptosis of immune cells has not been reported. The aim of this work was to assess the effect of As, Cd and Pb on the induction of programmed cell death of human peripheral blood mononuclear cells (MNC). We have found that As and Cd, but not Pb, induce apoptosis of immune cells, mainly B lymphocytes and monocytes. Interestingly, this effect of As was also detected *in vivo*, in children chronically exposed to this contaminant.

MATERIALS AND METHODS

Experimental design

Peripheral blood MNC were obtained from healthy donors. Cells from each subject were exposed for different periods of time to increasing concentrations of sodium arsenite (0–100 μ M), cadmium chloride (0–300 μ M) or lead acetate (0–500 μ M), and then apoptotic cells were quantified by different techniques. All experiments were carried out in quadruplicate. Differences among treatments were determined by the indicated tests. In addition, the preferential effect of metals on MNC subsets was studied. Finally, children with and without chronic exposure to

arsenic were also studied: MNC were obtained and the presence of apoptotic cells was assessed at 0, 12 and 36 h, with or without the addition of As.

Subjects

MNC were obtained from blood samples of 15 healthy adults. As indicated in the Results and figure legends, not all experiments were performed in every one of them. Seven children (4–6 years old) living in a town in which a gold mine is in the urban zone were also studied. These children lived near to the raw mineral breaker and were chronically exposed to arsenic, the main by-product of the gold extraction process. Their urinary levels of As ranged from 94 to 240 μ g/g of creatinine (mean = 143.9 μ g/g of creatinine). Five children who lived in a nearby town and who were not exposed to As were studied as controls. Their arsenic urinary levels ranged from 17 to 34 μ g/g of creatinine (mean = 24.8 μ g/g of creatinine). In all cases, written consent was signed by the parents before the study.

Isolation of mononuclear cells

MNC were obtained from heparinized blood by Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, USA) density gradient centrifugation. After washing with phosphate-buffered saline solution (PBS), MNC were suspended at 1×10^6 cells/ml in RPMI-1640 culture medium (Diagnostics Inc., Mequon, WI, USA), supplemented with 10% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. T and non-T cells were obtained by rosetting with sheep red blood cells (SRBC) as described [31]. Cell viability was determined by trypan blue dye exclusion and was always >95%.

Detection of apoptosis

Cellular apoptosis was detected by four different methods, as follows.

DNA content assay. This assay was performed as described [32]. Briefly, MNC (1×10^6 /ml) were incubated with metals, as indicated above, washed with PBS and resuspended in 2 ml lysis buffer (1 mg/ml sodium citrate, 40 μ g/ml RNase, 50 μ g/ml propidium iodide and Triton X-100, all reagents from Sigma). Then, cell samples were incubated at 4°C for 30 min in the dark before analysis on an EPICS Profile II flow cytometer (Coulter, Hialeah, FL, USA); a minimum of 20000 cells per sample were analysed. Apoptotic, hypodiploid cells were registered and results were expressed as the percentage of apoptotic cells.

Annexin V binding. After their exposure to metals, 1×10^6 cells were washed in PBS, resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), and stained with FITC-conjugated annexin V (Pharmingen, Becton Dickinson Co., San Diego, CA, USA). Then, cells were incubated for 15 min in the dark at room temperature, washed with binding buffer and analysed by flow cytometry. Results were expressed as the percent of annexin V-binding cells.

DNA fragmentation. Cells were treated as described above, and then were rinsed with PBS, resuspended in 1 ml of lysis buffer (Tris-Cl 10 mM pH 7.6, EDTA 10 mM pH 8, NaCl 50 mM, SDS 20% and proteinase K 100 μ g/ml), and incubated overnight at 42°C. DNA was precipitated with isopropanol and rinsed three times in 70% ethanol and dried. DNA samples were electrophoresed in a 1% agarose gel with 0.5 μ g/ml ethidium bromide at 60 mV for 90 min. DNA was visualized by ultraviolet transillumination and photographed with a Polaroid MP4 camera system.

DNA breaks fluorescence labelling. The APO-DIRECT™ staining kit (Phoenix Flow Systems, San Diego, CA, USA) was used according to the manufacturer's instructions. This end-labelling method (TUNEL) employs a terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP to detect the DNA breaks that are produced by endonucleases during apoptosis. In some experiments, TUNEL and annexin V staining were combined with labelling with different monoclonal antibodies specific for MNC subsets (CD3, CD19, CD14). Cells were analysed in a FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA) and results were expressed as the percent of positive apoptotic cells.

Arsenic quantification

Urinary As levels were determined as described previously [33]. Briefly, urine samples were digested and As content determined

by the hydride evolution technique using a Perkin-Elmer 3110 atomic absorption spectrometer. This method detects total As levels. Results were expressed as μg of As/g of urinary creatinine. Arsenic urinary levels are a reliable marker of recent arsenic exposure [15].

RESULTS

To determine whether Cd, As or Pb were able to induce apoptosis of MNC *in vitro*, cells were cultured in the presence of increasing concentrations of CdCl_2 , NaAsO_2 or $\text{Pb}(\text{COO})_2$ for 48 h, and apoptosis was then detected by propidium iodide staining and flow cytometry analysis. As shown in Fig. 1a, As induced a significant level of apoptosis of MNC at $15 \mu\text{M}$, whereas Cd had a similar effect at higher concentrations ($85\text{--}200 \mu\text{M}$, Fig. 1a).

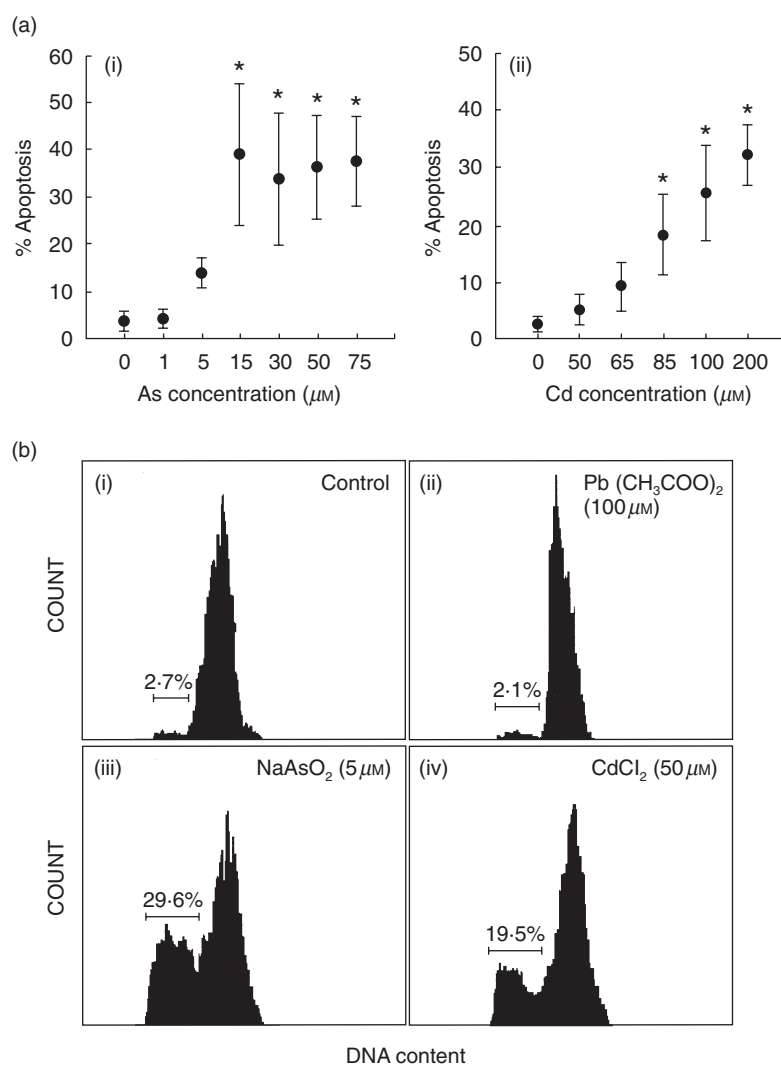


Fig. 1. *In vitro* effect of arsenic and cadmium on the apoptosis of normal human MNC. (a) Dose–response assays. Peripheral blood MNC were incubated in the presence of increasing concentrations of sodium arsenite (i) or cadmium chloride (ii) for 48 h and then the percentage of apoptotic cells was determined by propidium iodide staining and flow cytometry analysis, as described in Materials and methods. Data correspond to the arithmetic mean and s.d. of the percentage of apoptotic cells of four independent experiments. Asterisks indicate $P < 0.05$ compared to the negative control. (b) Peripheral blood MNC obtained from a healthy donor were cultured in the absence (i), or presence of $100 \mu\text{M}$ lead (ii), $5 \mu\text{M}$ arsenic (iii), or $50 \mu\text{M}$ cadmium (iv) for 48 h. Then, MNC apoptosis was determined as in (a). The percentage of hypodiploid, apoptotic cells is indicated. Results correspond to one healthy individual out of 10 studied.

However, a great individual difference was evident in the susceptibility of MNC from different donors to the effect of these substances (Fig. 1a,b), mainly for As (range 16.5–68.3% apoptosis, at 15 μM for 48 h, $n = 10$, DNA content analysis). For some MNC samples As and Cd concentrations as low as 5 and 50 μM , respectively, induced a significant level of apoptosis (Fig. 1b). In contrast, Pb did not induce apoptosis of MNC from any individual, even at very high concentrations (500 μM , Fig. 1b, and data not shown). Kinetics experiments performed incubating MNC with 15 μM As, 100 μM Cd or medium alone (control) showed that after 16 h of exposure to arsenic or cadmium, approximately 13% of MNC were apoptotic, and that this percentage increased to 22.7 and 16.2% at 24 h, respectively (Fig. 2a). Arsenic showed a higher induction of apoptosis at almost all times of incubation compared to Cd, and reached approximately 70% at 72 h of cell culture. Additional experiments showed that low doses of arsenic (1.0 μM) were also able to induce apoptosis in an important fraction of MNC when cells were incubated for longer periods of time (Fig. 2b). Interestingly, the number of viable cells in the cultures incubated in the presence of Cd (100 μM) diminished from 2×10^6 to less than 1×10^6 after 48 h of incubation (Fig. 2c). In the case of As, it was also detected an important decrease of viable cells (from 2×10^6 to 0.5×10^6), but the percentage of apoptotic cells

in these cultures was very high, approximately 50% (Fig. 2d). These results suggested that Cd exerted an additional cytotoxic effect on MNC through a mechanism different to the induction of apoptosis.

The induction of apoptosis by As and Cd was confirmed using alternative techniques for apoptosis detection. Figure 3a shows the results of an experiment in which apoptosis was detected by annexin V-FITC staining. In this case, a very high percentage of cells became annexin V-positive (29.7% and 92.8% for Cd and As, respectively) after 24 h of cell culture. In addition, the flow cytometry analysis of cells stained for DNA breaks by TdT/FITC-dUTP end-labelling confirmed that As and Cd were able to induce apoptosis (Fig. 3b). Interestingly, these techniques showed a higher sensitivity than DNA content analysis for apoptosis detection (compare Figs 1a and 3c). On the other hand, when MNC were treated with As or Cd, the characteristic pattern of internucleosomal DNA fragmentation (ladder) was detected after 24 h of incubation with both metals, whereas control cells did not show evidence of DNA damage (data not shown). As expected, when MNC were preincubated with 1 mM ZnSO₄ (an endonuclease inhibitor) for 1 h before metal addition (100 μM Cd or 15 μM As), a significant inhibition of programmed cell death was observed (data not shown).

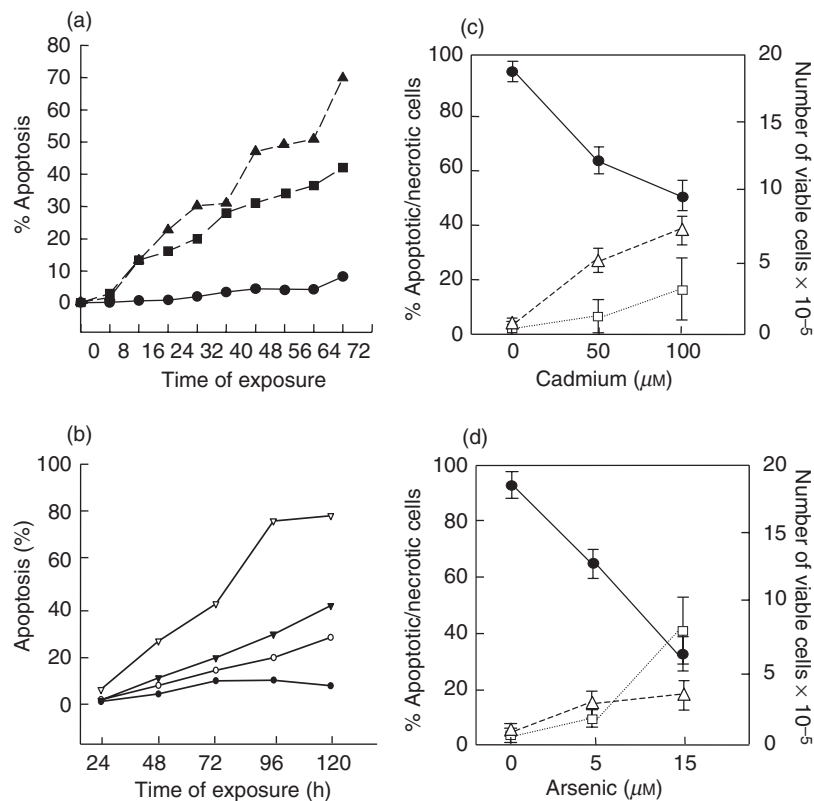


Fig. 2. *In vitro* effect of As and Cd on the apoptosis and necrosis of normal human MNC. (a, b) Kinetics analysis of the effect of As and Cd on the apoptosis of MNC. Cells were exposed to NaAsO₂, CdCl₂ or culture medium alone for the indicated periods of time and then the percentage of cells undergoing apoptosis was determined by propidium iodide staining, as described in Material and methods. Results correspond to one representative experiment out of three performed. (a) \blacktriangle , As (15 μM); \blacksquare , Cd (100 μM); \bullet , medium alone. (b) ∇ , As (5.0 μM); \blacktriangledown , As (2.5 μM); \circ , As (1.0 μM); \bullet , medium alone. (c, d) Effect of As and Cd on MNC viability. Peripheral blood MNC were incubated in the presence of the indicated concentrations of Cd (c) and As (d) for 48 h and then the number of viable and necrotic cells was determined by trypan blue staining and direct microscope counting. In cell cultures run in parallel, the percentage of apoptotic cells was determined as in (a). Results correspond to the arithmetic mean and s.d. of five independent experiments. (c, d) \bullet , Viable cells; \square , apoptotic cells; \triangle , necrotic cells.

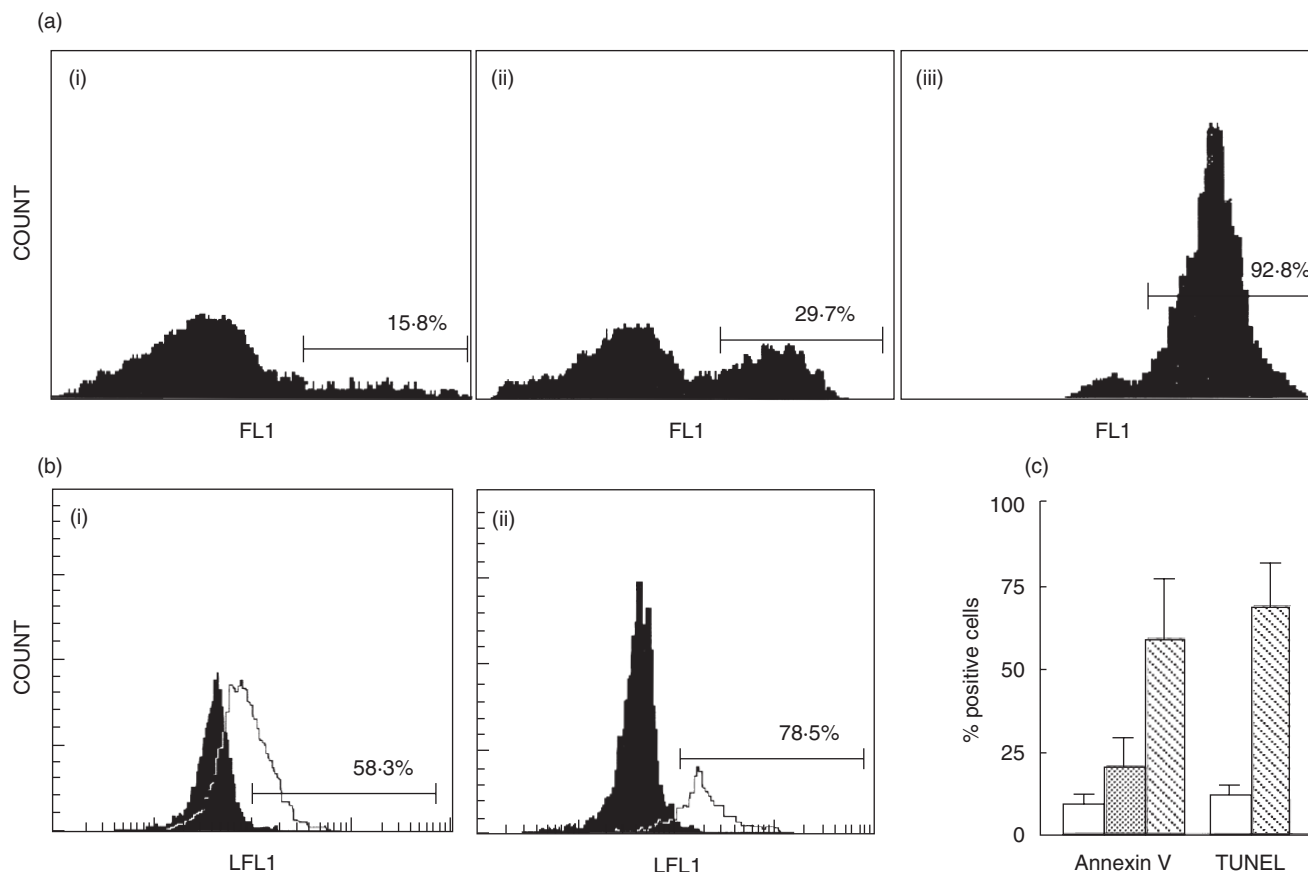


Fig. 3. Detection of apoptosis induced by As and Cd by annexin V binding and TUNEL assays. (a) MNC cultured in absence (i) or presence of 100 μM cadmium (ii) or 15 μM arsenic (iii) for 24 h were stained with the phosphatidylserine-binding protein annexin V-FITC, and analysed by flow cytometry. Results of one experiment out of five performed are shown. Numbers correspond to the percentage of positive cells. (b) Arsenic induces DNA breaks in MNC. Peripheral blood MNC cultured in the presence or not of 15 μM As for 24 h were stained for DNA breaks by TdT/FITC-dUTP end-labelling (TUNEL), as described in Material and methods. Solid histograms correspond to cells incubated in medium alone and empty histograms to cells incubated with As. Lymphocytes (i) and monocytes (ii) were analysed separately, according to their side and forward scatter characteristics. Results of one representative experiment out of four performed are shown. (c) Results of all experiments described in (a) (annexin V binding) and (b) (TUNEL) are shown. Data correspond to the arithmetic mean and s.d. of five (annexin V) and four (TUNEL) independent experiments. Values of TUNEL correspond to the percentage of apoptosis of the whole MNC population. Empty bars = medium alone; grey bar = Cd 100 μM ; hatched bars = As 15 μM .

We then investigated whether the immunotoxic effect of Cd and As was preferentially exerted on a MNC cell subset. T and non-T cells were isolated by rosetting with SRBC, and then the apoptotic effect of As and Cd on these cells was tested. As shown in Fig. 4a, non-T cells were more susceptible to the induction of apoptosis compared to T lymphocytes. This effect was more evident in cells treated with As. Double-labelling experiments using anti-CD3, anti-CD14 and anti-CD19 MoAbs plus annexin V-FITC or TUNEL staining confirmed that non-T cells had a higher susceptibility to the effect of As compared to T lymphocytes (Fig. 4b,c).

The important pro-apoptotic effect of As found *in vitro* prompted us to investigate whether this phenomenon could be observed in individuals exposed to this metalloid. Figure 5a shows that fresh isolated MNC from children chronically exposed to As, and with high urinary levels of this contaminant, exhibited a significantly enhanced basal percentage of hypodiploid cells ($P = 0.001$, Mann-Whitney *U*-test). However, a non-significant correlation between the basal percentage of apoptotic cells and

the concentration of urinary As ($r_s = 0.56$, $P > 0.05$, Fig. 5b) was found in these exposed children. Interestingly, when MNC from these children were cultured in the presence of As during 12–36 h, the percentage of apoptotic cells was significantly lower compared with cells from non-exposed children (Fig. 5c, $p = 0.037$, Mann-Whitney *U*-test).

DISCUSSION

It is well known that apoptosis plays a key role in the homeostasis of the immune system, and in recent years it has become clear that programmed cell death is also involved in many pathological conditions, either as the result of its enhancement or inhibition [8]. Thus, abnormalities in programmed cell death are involved in different immunological diseases, including AIDS [8,34,35]. In this regard, it seems evident that the uncontrolled elimination of immune cells may account for immunosuppression or immune dysregulation, depending on the lymphoid cell subset affected. Recently, the number of toxic agents with an apoptotic-inducing

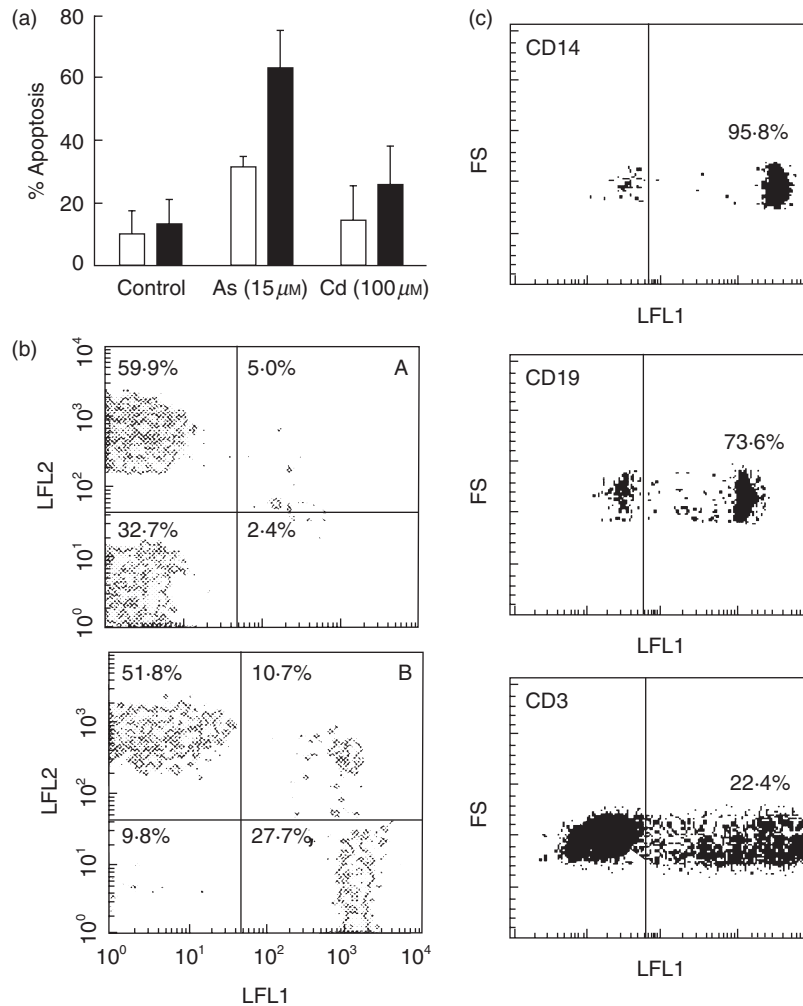


Fig. 4. Non-T cells have a high susceptibility to the induction of apoptosis by arsenic and cadmium. (a) T and non-T cells were cultured in the presence or absence of 15 μ M arsenic or 100 μ M cadmium for 24 h, and then the percentage of apoptotic cells was determined by flow cytometry after staining with propidium iodide. Results correspond to the arithmetic mean and s.d. of the percentage of apoptosis of four independent experiments. \square , T cells; \blacksquare , non-T cells. (b) Peripheral blood MNC were incubated in medium alone (A) or in the presence of 15 μ M As for 24 h (B), and then cells were double-stained for CD3 (LFL2) and annexin V (LFL1). Numbers correspond to the percentage of cells in each quadrant. Results of one representative experiment out of three performed are shown. (c) MNC incubated with 15 μ M As for 18 h were stained by TdT/FITC-dUTP end-labelling (TUNEL) and then labelled with the indicated PE-conjugated antibodies. Cells positive for each antibody were electronically gated and analysed in the histograms that are shown. Data of one representative experiment out of three performed are shown. Numbers correspond to the percentage of apoptotic cells in each MNC subset.

effect on immune cells has increased [9–12], and these substances seem to be responsible for immunodeficiency.

Metals such as arsenic, cadmium and lead are important polluting agents, and in many places around the world humans are exposed to high concentrations of these metals. It has been described widely that both the acute or chronic exposure to Cd, As or Pb are associated with damage at different levels [13–15]. However, the effects of lead, cadmium and arsenic on immune cells have received little attention. Our results demonstrate that arsenic is able to induce apoptosis of an important fraction of MNC. Although the degree of apoptosis was variable among the individuals tested, cells from all donors exhibited an important apoptotic response to As, with a significant effect at 15 μ M after 48 h of incubation. These data are in agreement with another study in which 40 μ M arsenite was able to induce apoptosis of

chinese hamster ovary cells [24]. On the other hand, using a different experimental model (rat thymocytes), it was found that exposure to low concentrations of NaAsO₂ (0.01–1.0 μ M) resulted in apoptotic changes after 6 h of incubation [36]. In this regard, we detected that when MNC were cultured for longer periods of time (≥ 3 days) very low doses of As were able to induce apoptosis of a significant proportion of MNC. Therefore, arsenic seems to be an efficient stimulus of apoptosis in different cells, including human normal lymphoid cells.

In the case of Cd, we found that higher concentrations than As were necessary to induce apoptosis of MNC. This finding is consistent with a report showing that in rats, the toxic effect of arsenic is higher compared with cadmium [37]. However, our data indicate that Cd exerts an important toxic effect on MNC that is not mediated through the induction of apoptosis. It would be

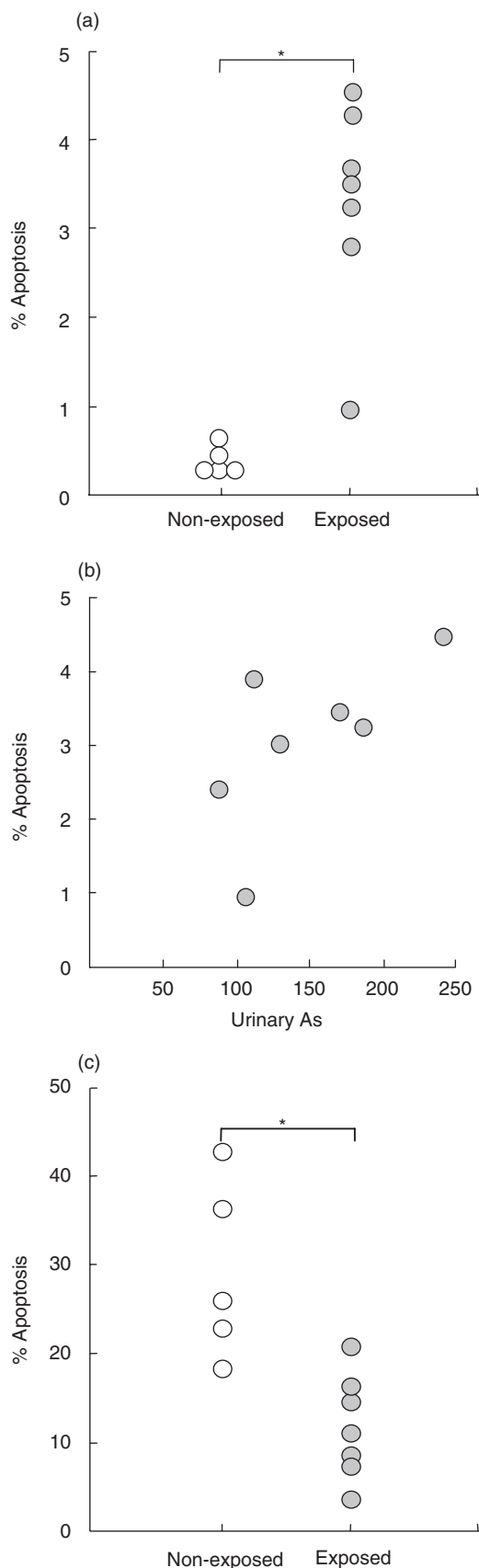


Fig. 5. Individuals exposed to arsenic show enhanced *in vivo* apoptosis of MNC and diminished sensitivity to As *in vitro*. (a) Peripheral blood MNC were isolated from seven children with chronic exposure to and high urinary levels of As (filled circles), and five healthy non-exposed matched controls (open circles). The fresh isolated cells were immediately stained with PI and analysed for hypodiploid cells by flow cytometry. Asterisk indicates $P = 0.001$. (b) Lack of significant correlation between the percent of apoptotic MNC and As urinary levels in the seven children shown in (a). r_s , Spearman correlation coefficient. $r_s = 0.56$; $P > 0.05$. (c) MNC from As-exposed and non-exposed children were incubated for 36 h in the presence of $15 \mu\text{M}$ As. Then, apoptotic cells were detected as in (a). Asterisk indicates $P = 0.037$.

interesting to elucidate the additional mechanism of toxicity of Cd on lymphoid cells. On the other hand, our results are in agreement with previous reports in which the administration of CdCl_2 to rodents induced apoptosis in liver and testicular cells [38,39]. However, other studies using isolated nuclei from mammalian cells, indicate that Cd has a dual effect, inhibiting the endonuclease activity triggered by calcium, but inducing this enzymatic activity in the absence of calcium, probably by replacing it [40,41]. It is worth mentioning that our experiments with Cd were performed in the presence of Ca^{2+} , and that under such experimental conditions we observed induction of apoptosis in all MNC samples tested.

We found that lead was unable to induce apoptosis of MNC even at very high concentrations ($500 \mu\text{M}$). This finding is in agreement with a previous study on the effect of Hg, Ag, Cu, Pb and Zn on human lymphocytes, in which a lower toxicity of lead was found compared with the other metals [29]. Nevertheless, it has been reported that lead is able to promote the apoptosis of other cells as cerebellar neurones [42], and that this phenomenon occurs at low concentrations ($1 \mu\text{M}$). Thus, although lead seems to be able to induce apoptosis, this effect is not observed in normal human MNC. It will be interesting to explore additional mechanisms of lead toxicity on lymphoid cells. On the other hand, the lack of induction of apoptosis by lead makes evident that the effect of As and Cd is specific and that the induction of apoptosis of lymphoid cells is not an indiscriminate phenomenon triggered by any metal.

The stimuli involved in the induction of apoptosis are very variable depending on cell type. Since MNC is a heterogeneous cell population, it is feasible that some of these cells are more susceptible to the toxic effect of As and Cd. In this regard, the shape and the plateau of the dose-response curves of these metals (Fig. 2) suggest that there are two cell subsets with high and low sensitivity to the induction of apoptosis by these metals. Therefore, we decided to determine whether or not the effect of arsenic and cadmium was differentially exerted on T and non-T cells. Interestingly, we found that the latter cells were more susceptible to the induction of apoptosis by both metals. Additional double-labelling experiments (CD3-CD14-CD19/annexin V or TUNEL) further indicated that As mainly exerts its pro-apoptotic effect on B lymphocytes and monocytes. A preferential toxicity on a lymphoid cell subset has also been observed in the case of ethanol, which induces mainly apoptosis of B cells compared to T cells [10]. On the other hand, we have found previously that stimulation through CD50 mainly triggers the programmed cell death of a T cell subset [43].

It is worth mentioning that the concentrations of As that induced apoptosis of MNC *in vitro* were similar to those observed *in vivo*, in individuals exposed to this pollutant (0.5–7.5 μM , see [44]). Therefore, it is feasible that the *in vitro* effect of Cd and As on MNC observed by us indeed occurs *in vivo* in the individuals with chronic exposure to these metals. In fact, the high basal level of MNC apoptosis found in children chronically exposed to and with high urinary levels of As suggest strongly that this metal exerts its pro-apoptotic effect on MNC *in vivo*. This point is supported further by the low *in vitro* sensitivity of the MNC from children exposed to As, suggesting that the susceptible cells have been partially eliminated *in vivo* in these individuals. However, we did not find a significant correlation between the urinary level of As and the percentage of basal apoptosis in the exposed children. It is possible that this phenomenon is due to the small number of children studied ($n = 7$) as well as to the individual differences in the susceptibility to the effect of As observed in this study. On the other hand, the toxic effect of Cd and As could be exerted on other immune cells such as Langerhans cells, which have a key role in the induction of immune response. In this regard, it has been reported that there is a low number of epidermal Langerhans cells in the skin neoplasias induced by As [45]. It will be interesting to assess *in vivo*, in individuals with chronic exposure to As or Cd, the presence of apoptotic cells in skin biopsies.

The preferential effect of As and Cd on B cells and monocytes could account for the immunotoxic effects of these metals on the humoral immune response, or the functions carried out by monocytes/macrophages such as NO production [18]. However, it has also been reported that both As and Cd have a deleterious effect on the T cell-mediated immune response [22,27], a phenomenon that, at first glance, is not in agreement with our results. It is feasible that the defects in the cellular immune response induced by As and Cd are related to deficiencies in antigen presentation, a function exerted mainly by B cells and mononuclear phagocytic cells, including Langerhans cells. An alternative possibility, as stated above, is that Cd and As have mechanisms of immunotoxicity additional to their pro-apoptotic effect.

Our results demonstrate, for the first time, that As and Cd, but not Pb, are able to induce apoptosis of human normal MNC. These findings are in agreement with the use of arsenic in the treatment of acute promyelocytic leukaemia [46].

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