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ORIGINAL ARTICLE

Cell death and cytokine production induced by cadmium in leukocytes of cord blood and peripheral blood of adults

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Abstract: Cadmium has been shown to alter humoral and cell-mediated immune *response in vivo* and *in vitro*. The immune system of newborns is particularly vulnerable to the toxic effect of cadmium, but there is only limited knowledge concerning the influence of cadmium on cytokine production by the immune cells of newborns. Therefore, the aim of this paper was to compare the influence of cadmium on cell viability and cytokine production by cord blood cells and adult blood cells. The results of the study demonstrate that cord blood leukocytes were more sensitive than adult leukocytes to cadmium cytotoxicity, measured as the percent of cells undergoing apoptosis. Cord blood cells exhibited defects n PHA-and LPS-induced IL-2, IFN-γ and TNF-α production, compared with adult blood cells. Cadmium at 10 μM concentration enhanced IFN-γ production in cord blood cells and TNF-α production in the blood cells of adults; however, it decreased the production of IFN-α and IL-10 in both types of cells. The data show that cadmium-induced apoptosis of cord blood leukocytes, as well as enhanced production of pro-inflammatory IFN-γ, may result in deeper alterations in the immune cell functions of newborns after exposure to cadmium, than in adults.

Key words: cadmium, cord blood cells, apoptosis, cytokines

INTRODUCTION

Cadmium is a toxic 'heavy' metal of continuing occupational and environmental concern, with a wide variety of adverse effects. There are several sources of human exposure to cadmium, including employment in primary metal industries and consumption of tobacco products [1]. Cadmium is also present at low concentrations in the environment of industrial areas.

Cadmium accumulates primarily in the human liver and kidneys, where it is bound to metallothioneins (MT). In pregnant women, cadmium is present in maternal blood; it may also accumulate in the placenta during the first semester of pregnancy, and may transfer into the fetal blood [2, 3]. Measurement of cadmium concentration in the liver tissue of newborns and infants indicates that it can reach values of about 2.9 ng/1 gram of liver wet mass [4, 5]. Maternal exposure to cadmium seems to increase the risk of early delivery, which leads to a low birth weigh [6]. Moreover, several studies suggest that the immune system of the developing fetus and the newborn baby is particularly vulnerable to the toxic effect of cadmium [7,8].

Cadmium has been classified as a human carcinogen and has been shown to alter immune response, affecting humoral and cell-mediated responses, phagocytic activity of macrophages and natural killer (NK) cell functions [9, 10, 11]. Cadmium may influence the expression of genes related to inflammatory responses, especially such cytokines as IL-1, TNF-α or IL-8 [12,13,14]. Generally, it seems likely that at low concentrations cadmium is able to stimulate the immune system, while at higher concentrations inhibitory and suppressive reactions have been observed [9, 15, 16].

There are only a few papers concerning the influence of cadmium on newborn immune cell functions [7,8], therefore, it was of interest to compare the influence of different cadmium concentrations on cell viability and production of several cytokines by cord and adult blood cells.

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MATERIALS AND METHOD

Blood samples. Adult blood was obtained from 10 healthy, non-pregnant volunteer women, 23-33-years old. Cord blood samples were obtained from 10 healthy, full-term (38-42 weeks) babies after normal vaginal delivery. Blood was taken from the placental end of the cord and collected into tubes with heparin (20 U/ml of Heparinum, Polfa), following ethics committee approval. Criteria of exclusion were complicated pregnancy, pre-maturity, infections and congenital abnormalities. All samples were processed directly after arrival at the laboratory (1-2 h after collection).

Induction of cytokines. For cytokine induction, whole cord and adult blood was diluted in Eagle's Minimal Essential Medium (MEM) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin to obtain a density of leukocytes of 1×10^6 cells/ml, and the

autologous serum was added to concentration of 10%. Cell suspensions were distributed (2 ml/well) into 24-well plastic plates (Falcon, Bedford , MA, USA) and induced to produce IFN-α with Newcastle disease virus, Radom velogenic strain, 5 $TCID_{50}$/leukocyte. IL-1β, IFN-γ, TNF-α, IL-6 and IL-10 were induced with 10 μg/ml PHA (Sigma, St. Louis, MO, USA) together with 2 μg/ml LPS from E.coli 0111:B4 (Sigma,). IL-2 was induced with 50 μg/ml PHA. At the onset of cell cultures, $CdCl_2$ (Sigma) solution was added (10 μl) to some samples to obtain the final concentration in diluted blood of 1 μM – 10 μM. Cells were incubated at 37°C in 5% CO_2 in air for 24 h (IL-1β, IL-6, IFN-α, TNF-α and IL-10) or for 72 h (IFN-γ, IL-2). Culture supernatants were collected and stored at -80°C for cytokine assay.

Cytokine assays. Concentrations of cytokines were measured in culture supernatants using specific ELISA assays (Endogen Inc. Woburn, MA, USA) according to the manufacturer`s instructions. The minimal detectable level of IL-1β was <1 pg/ml, IL-2 <4 pg/ml, IL-6 <1 pg/ml, IL-10 <3 pg/ml, TNF-α <5 pg/ml, IFN-α <3 pg/ml and IFN-γ <2pg/ml. The results were expressed as pg of cytokine per 1 ml of cell culture medium (10^6 of leukocytes).

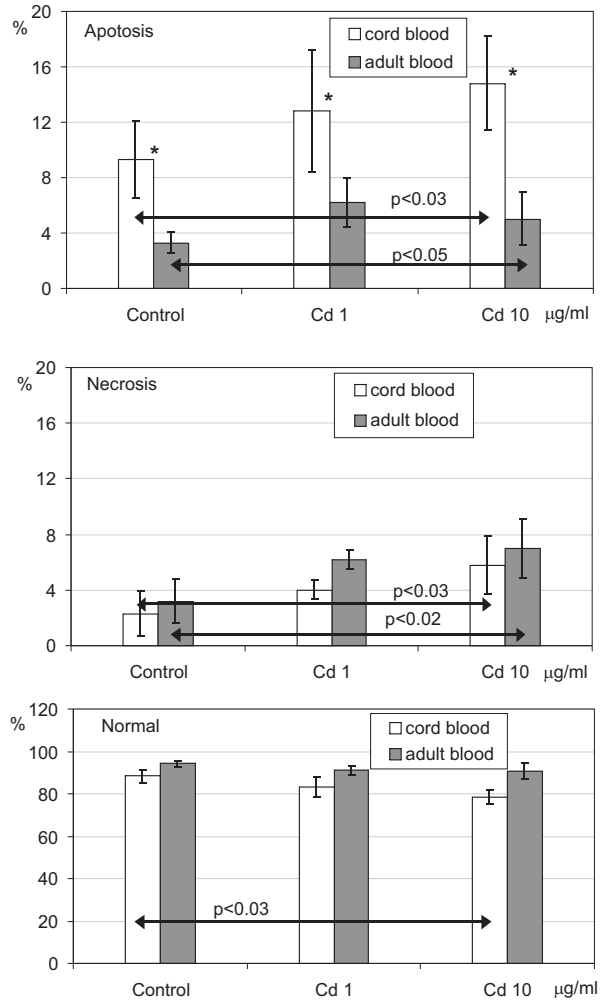
Separation of blood mononuclear cells and apoptosis measurement. Blood samples were diluted with phosphate buffered saline (PBS), layered onto Lymphoprep (1.077, Nycomed, Oslo, Norway), and centrifuged at 300×g for 20 min. Cells from the interphase were collected, washed 3 times in medium, and suspended in MEM supplemented with 10% of fetal calf serum at the density of 1×10^6 cells/ml. Cell suspensions were distributed into 24-well plastic plates and treated with 1-10 μM $CdCl_2$ (final concentration) together with mitogens (PHA 10 μg/ml and LPS 2 μg/ml) for 24 h at 37°C. After incubation with cadmium, cells were collected, centrifuged and suspended in 50 μl of MEM. A 25-μl aliquot of cells was stained by addition of 1 μl of stock solution of 100 μg/ml acridine orange, plus 100 μg/ml of ethidium bromide in PBS. After 2 min, the suspension was placed on a microscope slide and covered with a coverslip. A minimum of 500 cells were scored under the fluorescence microscope and categorized as normal, apoptotic or necrotic. The results were expressed as percent of cells from each category. 'Healthy' cells had a normal green nucleus; apoptotic cells had condensed or fragmented bright green chromatin. Late apoptotic cells with secondary necrosis displayed condensed or fragmented orange chromatin, whereas cells that had died from direct necrosis had a structurally normal orange nucleus [17]. Both apoptotic types of cells were placed in the 'apoptotic' category.

Statistics. Values were expressed as mean±S.D. Significance of differences was determined by an analysis of variance (Statistica computer package). A number of statistical tests were used: these included Mann-Whitney test for comparisons between 2 groups (control and examined), Kruskal-Wallis test, multiple comparison post-hoc Tukey`s test, and Spearman`s correlation analysis. P values <0.05 were considered to be significant.

RESULTS

Mononuclear blood cells were stained with acridine orange and ethidium bromide and categorized under fluorescence microscopy as normal, apoptotic or necrotic. Figure 1 shows that cadmium caused apoptotic and necrotic death of both types of blood cells, but the percent of apoptotic cells, as well as the sum of both apoptotic and necrotic cells after incubation with 10 μM $CdCl_2$ concentration, were about twice as high in cord blood than in adult blood cells. A concentration of 100 μM $CdCl_2$ almost exclusively caused cell necrosis (data not shown) in both types of cells.

In order to compare the influence of cadmium on cytokine production in cord blood and adult blood cells, we used whole blood cell cultures. Whole blood samples were diluted with medium to obtain the density of leukocytes of 1×10^6/ml, and induced for cytokine production as described in Materials and Methods. The level of cytokines was measured in supernatants of cell cultures. Cord blood cells not treated with cadmium (controls) produced less IL-2, IFN-γ and TNF-α than adult blood cells. The levels of IL-1β, IL-10, IFN-α and IL-6 were comparable in both cord and adult blood cells (Fig. 2 [a-g]).



* Statistically significant in comparison to adult cells at p< 0.05

Figure 1 Apoptosis and necrosis of mitogen activated (PHA+LPS) cord blood and adult blood cells after 24 h of incubation with $CdCl_2$. Cells were stained with acridine orange and ethidium bromide and categorized under fluorescence microscope as normal, apoptotic or necrotic. The results are expressed as percent of apoptotic, necrotic or normal cells± S.D.

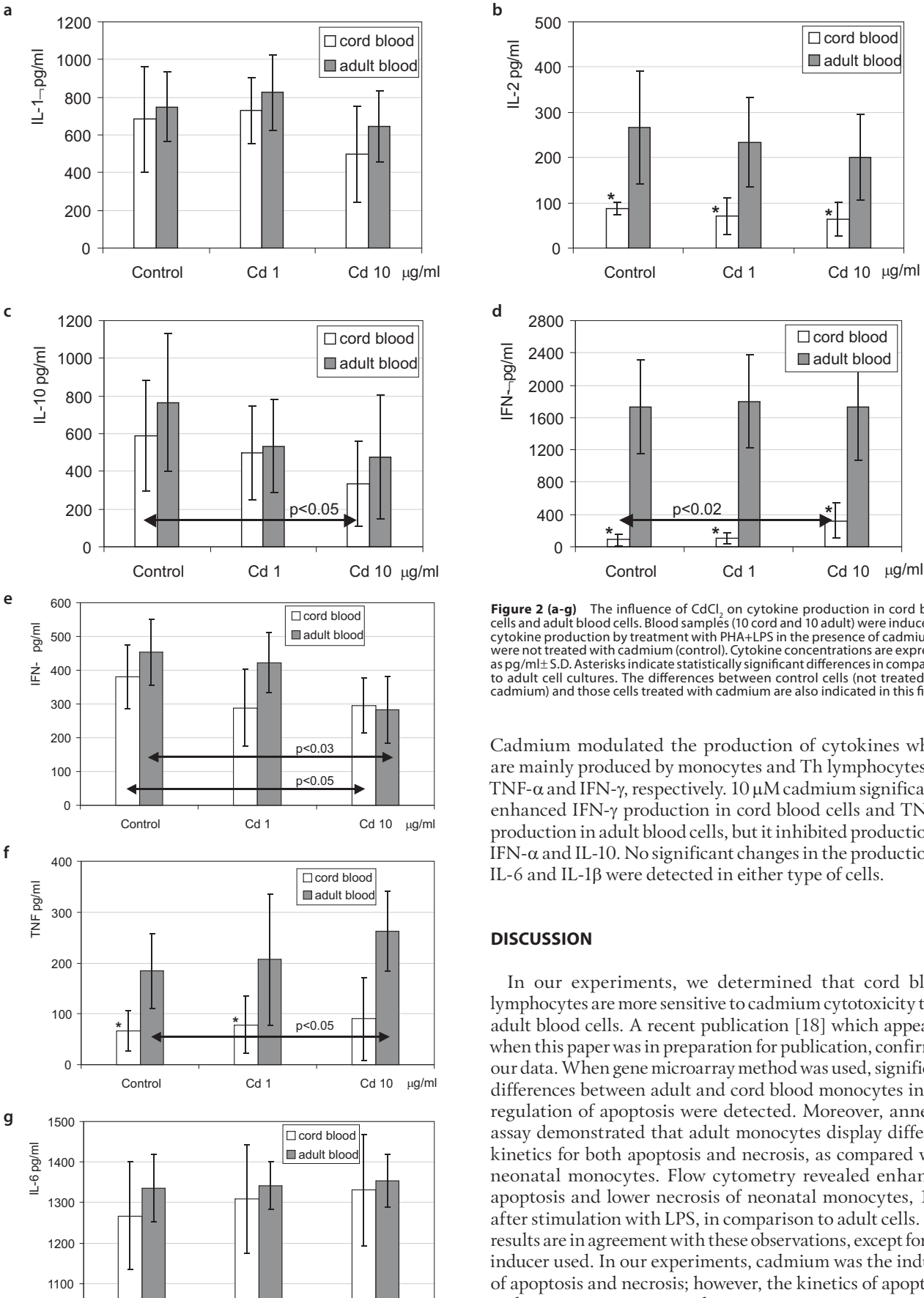


Figure 2 (a-g) The influence of $CdCl_2$ on cytokine production in cord blood cells and adult blood cells. Blood samples (10 cord and 10 adult) were induced for cytokine production by treatment with PHA+LPS in the presence of cadmium, or were not treated with cadmium (control). Cytokine concentrations are expressed as pg/ml± S.D. Asterisks indicate statistically significant differences in comparison to adult cell cultures. The differences between control cells (not treated with cadmium) and those cells treated with cadmium are also indicated in this figure.

Cadmium modulated the production of cytokines which are mainly produced by monocytes and Th lymphocytes, i.e. TNF-α and IFN-γ, respectively. 10 μM cadmium significantly enhanced IFN-γ production in cord blood cells and TNF-α production in adult blood cells, but it inhibited production of IFN-α and IL-10. No significant changes in the production of IL-6 and IL-1β were detected in either type of cells.

DISCUSSION

In our experiments, we determined that cord blood lymphocytes are more sensitive to cadmium cytotoxicity than adult blood cells. A recent publication [18] which appeared when this paper was in preparation for publication, confirmed our data. When gene microarray method was used, significant differences between adult and cord blood monocytes in the regulation of apoptosis were detected. Moreover, annexin assay demonstrated that adult monocytes display different kinetics for both apoptosis and necrosis, as compared with neonatal monocytes. Flow cytometry revealed enhanced apoptosis and lower necrosis of neonatal monocytes, 14 h after stimulation with LPS, in comparison to adult cells. Our results are in agreement with these observations, except for the inducer used. In our experiments, cadmium was the inducer of apoptosis and necrosis; however, the kinetics of apoptosis and necrosis were very similar.

Several developmental deficiencies of the neonatal-host defence system have been described, including a defect in the

activity of NK cell and the monocyte/macrophage system [19, 20]. Also, the cytokine production examined in cord blood cells was impaired. Generally, the results indicate that cord blood cells produce less IL-4, IFN-γ, TNF-α and IL-10 than adult peripheral blood cells, and amounts of IL-2 and IL-6 comparable to adults [21, 22, 23, 24].

In our experiments, we determined that cord blood cells induced with PHA+LPS produced significantly less TNF-α and IFN-γ after induction with PHA+LPS, and less IL-2 than adult cells after induction with PHA alone (50μg/ml). On the other hand, the levels of IL-1β, IL-10, IFN-α and IL-6 were comparable to those in adult blood cells.

Our results concerning TNF-α are in agreement with those of several other authors, including Sautois et al. (1997) [23], who used LPS with PHA to induce TNF-α, Serushago et al. (1996) [25], who used *Listeria monocytogenes* as inducer, Chalmers et al. (1998) [26], who used PMA with ionomycin and monensin, and Matsuda et al. (1996) [27], who used respiratory syncytial virus as the inducer. It seems likely that the defect in TNF-α production in cord blood cells is inducer-independent.

In our experiments, cord blood cells produced significantly less IL-2 than adult cells. The differences in the results indicating defective IL-2 production and those detecting normal IL-2 response in cord blood cells are rather connected with a different time of IL-2 yield than the inducer used. Han and Hodge (1999) [22] and Miles et al. (2003) [28] have shown that IL-2 was produced earlier in response to PHA in cord blood cells than in adult blood. When samples were collected after 24 h of incubation, cord blood cells produced amounts of IL-2 compared to adults, while after 72 h a significant drop in IL-2 activity was observed in cord blood, and a significant increase in IL-2 level in adult blood cell cultures. In our study, we measured IL-2 in supernatants of blood cell cultures after 72 h of incubation when the differences in its production in cord and adult blood cells were the highest, and the effect of the cadmium on cytokine production was at its strongest.

In most papers, as well as in our experiments, IFN-γ production by cells of neonates was determined to be lower, compared to adults, after induction with phorbol esters, ionomycin, enterotoxin B (SEB), PHA with LPS, or PHA alone [26, 29, 23]. However, when IL-12 or allogenic cells were used as the IFN-γ inducer, cytokine production was similar in both cord and adult blood cells [30]. Since we used PHA with LPS as IFN-γ inducer, our results are in agreement with those obtained by Sautois et al. (1997) [23].

In our study, the level of IFN-α produced in cord blood cells was comparable to that of adult cells, when non-inactivated NDV was used as inducer. This result differs from that obtained by Neustock et al. (1993) [31], who observed significantly diminished IFN-α production in cord blood cells also after UV- irradiated NDV stimulation. In accordance with Clavell and Bratt (1971) [32], we believe that such differences may be due to different mechanisms involved in IFN induction by the infectious and the UV-inactivated virus .

The results indicating normal IL-1β production in cord blood cells are in agreement with the results obtained by Sautois et al. (1997) [23], who, after stimulation with LPS (25μg/ml) together with PHA (5 μg/ml), observed that mononuclear cells isolated from cord blood produced IL-1β levels comparable to the blood cells of adults.

Low IL-10 production in cord blood cells after LPS stimulation has been described, but when allogenic cells were the inducers, the cord leukocytes produced similar amounts of this cytokine as the leukocytes of adults [33, 34].

Some observations [35, 36] suggest that the neonatal immune system is programmed against generation of Th1-immune response and low IFN-γ production, interpreted as a curtailment of early autocrine activation of T cells. In our experiment, IFN-γ was modulated by cadmium, and in cord blood cells 10 μM cadmium enhanced IFN-γ production. Until now, no such enhancement of IFN-γ production in blood cultures has been described in the literature. IFN-γ is a pleiotropic cytokine that modulates the immune function, macrophage activation, apoptosis and other cellular functions, and therefore its enhancement by cadmium may result in the development of immune system hyper-reactivity, resulting in allergic or autoimmune reactions in newborns exposed to cadmium [12, 37].

The acute phase of immune response involves cytokines, such as TNF-α assayed in the present study. The slight increase in their production after treatment of cord blood cells, and especially adult blood cells, with 10 μM cadmium concentrations (as determined in our experiments) is in agreement with the observations of other authors [10] and can partially explain the intense inflammatory cell infiltration in cases of severe kidney damage resulting from cadmium exposure [38]. Moreover, increased levels of plasma activity of TNF-α have also been noted following cadmium intoxication in rats [39], which may play a role in the development of systemic inflammatory response.

In our study, the level of cadmium-induced apoptosis of mitogen activated leukocytes was dose-dependent, and more apoptotic cells appeared after incubation with 10 μM cadmium concentration than with 1 μM concentration. Moreover, 10 μM cadmium concentration modulated production of cytokines, decreasing the release of some of them (IFN-α, IL-2 and IL-10) while enhancing the release of others (IFN-γ and TNF-α). Recent publications have revealed that cadmium modulates cytokine production via different pathways, involving such transcription factors as NF-κB in the case of cadmium induced IL-8 (Hyun et al., 2007) [14], or AP-1 transcription factor in the case of TNF-α induction (Souza et al., 2004) [40]. As the cells which are relatively resistant to the toxic effect of cadmium were characterized as T lymphocytes (De La Fuente et al., 2002) [41], it can be expected that Th-derived cytokines, such as IL-2, IFN-γ and IL-10, should be more resistant to cadmium influence than monokines such as IL-1β, IFN-α or TNF-α; however, the results of our study do not confirm this hypothesis.

SUMMARY

This study demonstrates that cord blood cells are more sensitive to cadmium cytotoxicity than adult blood cells. At the same cadmium concentration within the range of 1-10 μM, the level of apoptosis was higher in cord blood cells than in adult blood cells. Moreover, 10 μM of cadmium modulated cytokine production, significantly enhancing IFN-γ production in cord blood cells and TNF-α production in adult blood cells, while decreasing the production of IFN-α and IL-10 in both types of cells. IL-6 production was relatively resistant to the effects of cadmium. The data show that exposure of the developing human immune system to cadmium may result in deeper alterations in the immune cell functions than in adults.

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