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Impact of cadmium in T lymphocyte subsets and cytokine expression: differential regulation by oxidative stress and apoptosis

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Abstract Cadmium (Cd), a possible human carcinogen is a potent immunotoxicant. In rodents it causes thymic atrophy and splenomegaly, in addition to immuno-suppression and modulation of humoral and/or cellular immune response. Oxidative stress and apoptosis appear to be underlying mechanism of Cd induced thymic injury. To understand the involvement of reactive oxygen species (ROS), intracellular glutathione (GSH) and apoptosis in modulation of T-cell repertoire, we studied the effect of Cd (10, 25 and 50 μM) on primary T lymphocytes of BALB/c mice at different time intervals (6, 12 and 18 h). We observed a dose and time dependent decline in $CD4^+$/$CD8^+$ ratio (a bio-indicator of immunotoxicity) as a result of significant suppression of $CD4^+$ subsets (helper T-cells) and enhancement in $CD8^+$ cells (cytotoxic T-cells) At the same time, the $CD4^+CD8^+$ (DP) cell population was lowered while the $CD4^-CD8^-$ (DN) cells were increased. The oxidative stress and apoptotic data revealed almost similar ROS generation in both $CD4^+$ and $CD8^+$ cells, but relatively more marked GSH depletion and apoptosis in $CD4^+$ than in $CD8^+$ population. On further analysis of $CD4^+$ T-subsets, cytokine release (IL-2 and IFNγ) by Th 1 cells and IL-4 by Th 2 cells were shown to be significantly suppressed in a dose responsive manner. The highest inhibition was observed in IFNγ, then IL-2 followed by IL-4. In conclusion, our data demonstrates that T-cell apoptosis by Cd, more in $CD4^+$ than in $CD8^+$ cells appear related to higher depletion of intracellular glutathione. Th 1 cells of $CD4^+$ sub-population are more responsive to Cd than Th 2, leading to higher suppression of IL-2 and IFNγ than IL-4 and hence, the study unravels to some extend, the underlying events involved in Cd immunotoxicity.

Keywords Cadmium · T-subsets · Apoptosis · Oxidative stress · Interleukin-2 · Interleukin-4 · Gamma-Interferon

Introduction

The immunotoxicity of Cd in a variety of mammalian and non mammalian animal species is well documented (Descotes 1992). Sporadic reports on cadmium induced abnormal humoral and/or cellular responses (Dan et al. 2000) are available (Ohsawa et al. 1983; Koller 1998). In vivo, Cd causes thymic damage, modulation of proliferative rate of rat thymocytes (Morselt et al. 1988), marked loss of thymus weight, T-cell depletion and thymic atrophy (Suzuki et al. 1981; Mackova et al. 1996; Liu et al. 1999). Differential effects on rat blood lymphocyte by Cd (Lafuente et al. 2004) and altered T-cell

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phenotypic changes in mice (Dong et al. 2001) further add to the understanding of its immunotoxic potential.

Regarding the T-cell phenotypes, its development is initiated within the thymus from T-lymphocyte precursors to $CD4^-$ $CD8^-$ (double negative) immature T-cells, subsequently to $CD4^+$ $CD8^+$ (double positive) thymocytes and eventually to mature T-cells expressing either $CD4^+$ or $CD8^+$ single positive, prior to their release in the circulation (Rothenberg 1992). $CD4^+$ and $CD8^+$ are among a number of surface molecules that are active in the +ve and −ve selection of thymocytes (Conroy and Alexander 1996). Dong et al. (2001) reported differential susceptibility of various T subsets to the apoptogenic effects of Cd in the order of $CD8^+ > CD4^-$ $CD8^-$ (double negative, DN) > $CD4^+$ $CD8^+$ (double positive, DP) > $CD4^+$. In addition, a decline in DP cells and a marked lowering in $CD4^+/CD8^+$ ratio mainly due to a significant increase in $CD8^+$ subsets, was also observed. A number of other immunotoxins such as dioxins, fusarenon-X, linomide, dexamethasone and arsenic (Baumann et al. 1997; Harring et al. 1997; Lai et al. 1998; Miua et al. 1998; Gupta et al. 2003) are shown to display phenotypic alterations in T lymphocytes. Recent evidences suggest that the mechanism of Cd-induced damage includes the production of free radicals that alter mitochondrial activity and trigger apoptosis (Achanzar et al. 2000; Shen et al.2001; Harstad and Klaassen 2002).

Our earlier studies demonstrated that oxidative stress markers i.e. ROS and GSH acted as intracellular signals and preceded mitochondrial membrane depolarization and caspase-3 activation in Cd induced apoptosis in murine thymocytes and splenocytes (Pathak and Khandelwal 2006a, b, 2007).

With the knowledge that Cd induces oxidative stress accompanied by apoptosis in murine T-lymphocytes, the current study was designed to evaluate (a) the differential effects of ROS production and GSH depletion on T-subsets and the (b) influence of altered T lymphocytes on programmed cell death and cytokine (IL-2, IL-4 and IFNγ) secretion.

Such a systematic investigation will further elucidate the role of oxidative stress in Cd induced apoptosis in the regulation of thymocyte subsets and aid in better understanding of the underlying nature of immunotoxic damage by Cd on thymus.

Materials and methods

Chemicals

All the chemicals were of highest grade purity available. Cadmium chloride ($CdCl_2$), RPMI 1640, Antibiotic-antimycotic solution, Dulbecco's phosphate buffered saline (PBS), Fetal bovine serum (FBS), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and all other chemicals were purchased from Sigma Aldrich, USA. Annexin V-FITC from Biovision and 5′-Chloromethylfluorescein diacetate (CMF-DA) was from Molecular Probes. FITC-conjugated anti-CD4 monoclonal antibody, PE-conjugated anti-CD4 monoclonal antibody, PE-conjugated anti-CD8 monoclonal antibody, mouse Interleukin-2, Interleukin-4 and gamma-Interferon ELISA kits were purchased from eBiosciences.

Preparation of thymocyte suspension

Thymus was dissected from male BALB/c mice (4–6 weeks old) and single cell suspension prepared under aseptic conditions. The suspension was passed through 100 μM stainless steel mesh and suspended in complete cell culture medium (RPMI 1640 containing HEPES and 2 mM glutamine, supplemented with 10% FBS and 1% antibiotic–antimycotic solution). The cell density was adjusted to ca. 1.5×10^6 cells/ml and the viability of the freshly isolated cells was always over 95% (trypan blue exclusion test).

For the monitoring of various parameters in the present investigation, we have used different Cd conc (10, 25 and 50 μM) and the selection was based on our earlier studies (Pathak and Khandelwal 2006a). All the parameters were analyzed thrice as independent experiments.

Flow cytometry analysis

All the following assays were carried out on thymocytes treated with Cd (10, 25 and 50 μM) for different time intervals (6, 12 and 18 h) at 37°C in a CO_2 incubator. The flow cytometric analysis were done on BD-LSR flow cytometer. Cell debris, characterized by a low FSC/SSC was excluded from analysis. As dual staining was performed for all the assays, appropriate electronic compensation was adapted

among the two fluorescence channels to overcome residual spectral overlap. The data was analysed by Cell Quest software and mean fluorescence intensity was obtained by histogram statistics.

Assesssment of T-lymphocyte phenotyping

The T-lymphocyte phenotyping was conducted to analyze the effect of Cd based on CD4 and CD8 surface molecules. Positioning of quadrants on FITC/PE dot plots was performed and $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (Double positive; DP) and $CD4^-CD8^-$ (Double negative; DN) subpopulations were distinguished. Briefly, after incubation time (6, 12 and 18 h), the harvested cells were resuspended in 1 ml PBS. An aliquot of 100 µl was incubated with 5 µl FITC-conjugated anti-CD4 monoclonal antibody and 5 µl PE-conjugated anti-CD8 monoclonal antibody for 30 min in dark at room temperature, after which 400 µl PBS was added to each sample. The FITC and PE fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

Assessment of apoptotic cells

The apoptotic cell distribution was analysed by Annexin V binding in T-subsets. Positioning of quadrants on FITC/PE dot plots was performed and live $CD4^-/CD8^-$ cells (LL: Annexin V^-/PE^-), apoptotic $CD4^-/CD8^-$ cells (LR: Annexin V^+/PE^-), apoptotic $CD4^+/CD8^+$ cells (UR: Annexin V^+/PE^+) and live $CD4^+/CD8^+$ cells (UL: Annexin V^-/PE^+) were distinguished. Briefly, after the treatment period, the harvested cells were suspended in 1.0 ml PBS. An aliquot of 100 µl was incubated with 5 µl PE-conjugated anti-CD4 or 5 µl PE-conjugated anti-CD8 monoclonal antibody for 30 min in dark at room temperature. Annexin V-FITC (5 µl) was added 15 min prior to the completion of incubation time and 400 µl PBS was added to each sample. The FITC and PE fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

Reactive Oxygen Species (ROS) measurement

The generation of ROS in $CD4^+$ and $CD8^+$ T-subsets was detected by DCF fluorescence. Positioning of quadrants on DCF/PE dot plots was performed and $CD8^-DCF^-$ (LL), $CD8^-DCF^+$ (LR), $CD8^+DCF^+$ (UR) and $CD8^+$ DCF^- (UL) were distinguished. Thymocytes were incubated with Cd and DCFH-DA (100 µM final conc) was added simultaneously to the medium. The cells were harvested and suspended in 1 ml PBS. An aliquot of 100 µl was incubated with 5 µl PE-conjugated anti-CD4 or 5 µl PE-conjugated anti-CD8 monoclonal antibody for 30 min in dark at room temperature and 400 µl PBS was added to each sample. The DCF and PE fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

Glutathione (GSH) measurement

The cellular level of GSH in $CD4^+$ and $CD8^+$ T-subsets was monitored by CMF-DA. Positioning of quadrants on CMF/PE dot plots was performed and $CD8^-$ CMF^- (LL), $CD8^-$ CMF^+ (LR), $CD8^+$ CMF^+ (UR) and $CD8^+$ CMF^- (UL) were distinguished. After treatment, the cells were incubated with CMF-DA (1.0 µM final conc) for 30 min in dark at 37°C. After harvesting, the cells were suspended in 1 ml PBS. An aliquot of 100 µl was incubated with 5 µl PE-conjugated anti-CD4 or 5 µl PE-conjugated anti-CD8 monoclonal antibody for 30 min in dark at room temperature and 400 µl PBS was added to each sample. The CMF and PE fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

Effect of Cd on cytokines release

The release of Interleukin-2 (IL-2), Interleukin-4 (IL-4) and gamma-Interferon (IFNγ) in thymocytes were measured by using Mouse IL-2, IL-4 and IFNγ ELISA kits, respectively. Briefly, 1.0×10^4 cells in 200 µl of complete medium were seeded in 96-well plate with or without 2.5 µg/ml Con A and Cd (10, 25 and 50 µM) and incubated for 72 h at 37°C in a CO_2 incubator. The plate was centrifuged and supernatant was used for measuring cytokine release by ELISA. The ELISA plates were incubated overnight with 100 µl/well of capture antibody in coating buffer under cold conditions. After aspirating the coating solution, the wells were washed 3

times with 300 µl wash buffer and after removing the buffer carefully, 200 µl of blocking solution was added for 1.0 h at 15–25°C. The plates were washed and the standard or sample solution (100 µl) was added into each well in triplicate and incubated for 2.0 h at 15–25°C. After washing the plates 5 times, 100 µl of detection antibody solution was added and the plates were further incubated for 1 h at 15–25°C, followed by washing. 100 µl of Avidin-HRP was then added and incubated for 30 min and the plates were again washed 7 times. They were further incubated with 100 µl of substrate solution for 15 min and after addition of 50 µl of stop solution, the absorbance was measured at 450 nm.

Statistical analysis

Significance of mean values of different parameters between the treatment groups were analysed using Two Ways Analysis of Variance (ANOVA) considering dose and time as independent variables and each parameter saperately as dependent variable. The homogeneity of variance between the treatments was ascertained before applying ANOVA. Pair wise comparisons were done by calculating the least significant difference.

Results

T-cell phenotypic alterations

The effect of Cd on thymocyte differentiation and development was measured by cell surface marker expression using flow cytometry. The relative frequency of each subpopulation and the $CD4^+/CD8^+$ ratio was determined at 6, 12 and 18 h. Cadmium treatment altered the thymocyte surface marker expression, leading to distinct phenotypic changes which were clearly evident at 12 h and 18 h but not at 6 h, as shown in Table 1. These alterations were reflected by all the Cd conc (10, 25 and 50 µM) and increased in a dose and time dependent manner.

The percentage of $CD4^+$ cells at 50 µM conc decreased from 11.8 (control) to 4.6 ($P < 0.01$) at 12 h, which further fell to 2.0 ($P < 0.001$) at 18 h. Simultaneously, the $CD8^+$ cells increased to 23.8 ($P < 0.01$) from 6.4 (control) and at a later time to 31.0% ($P < 0.001$). The $CD4^+/CD8^+$ ratio therefore, dropped from 1.8 to 0.1. Even the lowest Cd conc (10 µM) could alter the various T subsets, displaying the $CD4^+/CD8^+$ ratio of 0.9 and 0.5 at 12 h and 18 h, respectively. The $CD4^+CD8^+$ (DP) also declined, whereas $CD4^-CD8^-$ (DN) registered a gradual dose and time related increase (Table 1).

Table 1 Effect of Cd on T lymphocyte phenotypes

Time	Group	$CD4^-CD8^-$	$CD4^+$ $CD8^+$	$CD4^+$	$CD8^+$	CD4/CD8
6 h	Con	0.76 ± 0.2	80.47 ± 5.6	12.24 ± 3.6	7.02 ± 1.6	1.74
	Cd 10	1.12 ± 0.3	81.21 ± 3.4	11.64 ± 2.4	6.84 ± 2.4	1.70
	Cd 25	0.68 ± 0.2	79.02 ± 5.3	11.02 ± 3.3	6.56 ± 1.8	1.68
	Cd 50	1.09 ± 0.3	79.32 ± 3.2	11.40 ± 4.2	6.87 ± 2.3	1.66
12 h	Con	1.26 ± 0.2	80.98 ± 3.6	11.86 ± 2.5	6.41 ± 1.3	1.85
	Cd 10	2.21 ± 0.8	75.62 ± 5.4	9.82 ± 3.4	11.25 ± 3.2	0.87
	Cd 25	11.02 ± 2.1[c, *]	58.32 ± 5.1 [c, *]	7.05 ± 2.8[c, *]	17.16 ± 3.8[c, *]	0.41
	Cd 50	18.42 ± 3.8[b, **]	46.82 ± 4.6[b, **]	4.65 ± 0.8[b, **]	23.82 ± 4.4[b, **]	0.19
18 h	Con	2.95 ± 0.8	81.66 ± 5.2	11.02 ± 2.3	5.86 ± 1.4	1.88
	Cd 10	4.61 ± 1.4	67.42 ± 2.8	8.52 ± 1.5[c, *]	16.02 ± 4.1[c, *]	0.53
	Cd 25	17.53 ± 2.3[b, **]	42.00 ± 4.3[b, **]	4.02 ± 0.9[b, **]	24.02 ± 5.2[b, **]	0.16
	Cd 50	24.42 ± 4.2[a, ***]	35.12 ± 3.6[a, ***]	2.01 ± 1.3[a, ***]	31.02 ± 6.2[a, ***]	0.06

Freshly isolated thymocytes (1.5×10^6) were treated with Cd (10–50 µM) for 6, 12 and 18 h and the cells were stained with FITC-conjugated anti-CD4 monoclonal antibody and PE-conjugated anti-CD8 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively

Each value represents mean ± S.D ($n = 3$). [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$ with respect to one dose and at different time points *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ with respect to control at one time, using two way ANOVA

ROS in T-subsets

After ascertaining T-subset alterations by Cd, we further investigated the ROS levels in $CD4^+$ and $CD8^+$ cells. The ROS levels were measured on flow cytometer using DCFH-DA fluorescent dye. The $CD4^+$ cells displaying DCF fluorescence comprised of mature $CD4^+$ (SP) as well as $CD4^+CD8^+$ (DP) cells. Maximum fluorescence in both the subsets was exhibited at 6 h by the highest Cd conc ($P < 0.001$) (Table 2) indicating marked ROS generation. In the $CD4^+$ cells, the DCF^+ fluorescence continued to decline upto 18 h ($P < 0.001$). Whereas, the $CD8^+$ cells displayed almost three fold rise at all time points ($P < 0.001$ at 6 h and 12 h, $P < 0.01$ at 18 h). This effect was dose related and was more pronounced with the two higher doses of Cd. On comparing the two T subsets at 18 h, Cd causes a higher ROS production in $CD8^+$ cells with dose and time ($P < 0.01$) than $CD4^+$ cells.

GSH in T-subsets

Another bio-indicator of oxidative stress i.e. GSH was also studied in $CD4^+$ and $CD8^+$ cells using CMF-DA (GSH sensitive fluorescence probe), on flow cytometer. A significant lowering trend in GSH was observed in both $CD4^+$ and $CD8^+$ cells at all time points (Table 3). The $CD4^+$ cells appeared more responsive to the effect of Cd exhibiting almost 10 fold ($P < 0.001$) decline in GSH at 18 h. The $CD8^+$ cells on the other hand, were less affected and the fall in GSH was 50% of that observed in $CD4^+$ cells. The highest Cd conc showed maximum changes in GSH levels. No time related significance was observed in the two subsets at various Cd conc.

The data reveals higher depletion of GSH in $CD4^+$ with dose, than in $CD8^+$ cells. The initial CMF fluorescence at 6 h was almost similar in both the subsets ($P < 0.001$).

Table 2 Effect of Cd on ROS generation in T-subsets

	6 h		12 h		18 h	
	$CD4^+DCF^+$	$CD8^+DCF^+$	$CD4^+DCF^+$	$CD8^+DCF^+$	$CD4^+DCF^+$	$CD8^+DCF^+$
Con	36.21 ± 3.6	25.21 ± 2.6	36.72 ± 3.2	26.12 ± 2.2	38.52 ± 2.4	25.62 ± 2.2
Cd 10	52.51 ± 2.8	36.42 ± 2.2	53.12 ± 4.1	32.22 ± 1.6	43.42 ± 3.6	30.61 ± 3.6
Cd 25	72.32 ± 4.2**	68.37 ± 5.4***	62.80 ± 3.8*	52.27 ± 3.2**	40.62 ± 2.5[b]	45.58 ± 1.8[c, *]
Cd 50	86.01 ± 4.8***	82.61 ± 4.2***	62.20 ± 5.8*	60.24 ± 3.4***	35.32 ± 3.6[a]	54.81 ± 2.4[b, **]

Freshly isolated thymocytes (1.5×10^6) were treated with Cd (10–50 μM) for 6, 12 and 18 h and the cells were stained with DCFH-DA (100 μM) and PE-conjugated anti-CD4 monoclonal antibody/PE-conjugated anti-CD8 monoclonal antibody. The DCF and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively

Each value represents mean ± SD ($n = 3$). [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$ with respect to one dose and at different time points *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ with respect to control at one time, using two way ANOVA

Table 3 Effect of Cd on GSH levels in T-subsets

	6 h		12 h		18 h	
	$CD4^+CMF^+$	$CD8^+CMF^+$	$CD4^+CMF^+$	$CD8^+CMF^+$	$CD4^+CMF^+$	$CD8^+CMF^+$
Con	88.83 ± 6.2	80.20 ± 5.4	87.61 ± 5.4	75.72 ± 6.2	85.83 ± 5.2	70.90 ± 4.9
Cd 10	64.42 ± 5.4	61.15 ± 3.8	60.21 ± 4.6	60.83 ± 5.4	36.58 ± 4.6**	62.30 ± 5.3
Cd 25	45.30 ± 6.2**	38.62 ± 4.1**	38.29 ± 3.8***	40.62 ± 4.5**	26.50 ± 3.2***	44.58 ± 3.2**
Cd 50	26.40 ± 4.8***	21.26 ± 2.6***	12.92 ± 3.4***	38.65 ± 3.8**	9.01 ± 2.4***	18.88 ± 3.5***

Freshly isolated thymocytes (1.5×10^6) were treated with Cd (10–50 μM) for 6, 12 and 18 h and the cells were stained with CMF-DA (1 μM) and PE-conjugated anti-CD4 monoclonal antibody/PE-conjugated anti-CD8 monoclonal antibody. The CMF and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively

Each value represents mean ± S.D ($n = 3$). *** $P < 0.001$, ** $P < 0.01$ with respect to control at one time, using two way ANOVA

The oxidative stress (ROS and GSH) results show that ROS generation was marginally higher in $CD8^+$ cells, whereas GSH depletion was more marked in $CD4^+$ cells.

After assessing the levels of ROS and GSH in both the T-subsets, the next step was to determine the extend of apoptosis in both $CD4^+$ and $CD8^+$ cells, in order to understand an association between the two.

Apoptosis in T-subsets

The $FITC^+$ cells represent apoptotic cells since Annexin binds to externalized phosphatidylserine in cells undergoing programmed cell death. A dose and time dependent increase in apoptotic population was observed in $CD4^+$ cells (Table 4). With the highest Cd conc, the FITC positive cells were gradually enhanced with time and at 18 h, the apoptotic cell number rose to 31.3% ($P < 0.001$) from 2.9% in control cells. From an initial increase of 3.9 folds at 6 h, it became 10.7 at 18 h. Although, the data on $CD8^+$ cells showed that the apoptotic cell population with 50 μM Cd, also increased with dose ($P < 0.01$) and time, from 5.1% at 6 h to 12.6 at 18 h ($P < 0.05$), it was much lower than that observed in $CD4^+$ cells.

Marked loss in $CD4^+$ T-cells, may be assigned to significant apoptosis by Cd, which appears not directly related in the case of $CD8^+$ cells. Results on oxidative stress and apoptosis signify both SP and DP population. In this study, the number of immature T-cells undergoing oxidative stress and apoptosis by Cd cannot be defined, but at the same time, the phenotypic data (Table 1) indicates substantial loss in $CD4^+CD8^+$ population and a significant rise in $CD4^-CD8^-$.

Cytokine release

To further investigate the role of $CD4^+$ towards cytokine production, we analyzed IL-2, IL-4 and IFNγ, in cells treated with Cd. A dose dependent decrease in IL-2, IL-4 and IFNγ as evident in Fig. 1, indicated progressive inhibition of cytokine production which may be related to a major decrease in $CD4^+$ sub population. Significant reduction in IL-2, IFNγ and IL-4 was observed in Con A stimulated T-cells. In unstimulated cells, only the highest Cd conc was able to suppress IL-2 and IFNγ production. With 50 μM Cd, the reduction in cytokine levels at 72 h was in the order IFNγ {5 folds ($P < 0.001$)}, then IL-2 {2.3 folds ($P < 0.001$)} followed by IL-4 {1.5 folds ($P < 0.01$)} and the cells responded in a dose dependent manner. It appears that reduction in $CD4^+$ population by Cd (Table 1) would yield lesser number of Th 1 and Th 2 cells and that inhibition of Th 1 was higher than Th 2, based on the cytokine data. The decrease in $CD4^+$ subset, an early event, was observed as early as 12 h and the cytokine levels in stimulated and unstimulated T-cells were seen at 72 h.

These results suggest that immunosuppression by Cd could be a consequence of diminished IL-2 and IFNγ production by Th 1 and IL-4 by Th 2 cells. GSH and ROS appeared to be the early targets of Cd followed by high incidence of apoptosis in $CD4^+$ cells. On the other hand, GSH depletion and ROS generation did not contribute largely in causing

Table 4 Effect of Cd on apoptosis in T-subsets

	6 h		12 h		18 h	
	$CD4^+AV^+$	$CD8^+AV^+$	$CD4^+AV^+$	$CD8^+AV^+$	$CD4^+AV^+$	$CD8^+AV^+$
Con	2.02 ± 0.8	1.12 ± 0.4	3.20 ± 0.9	1.84 ± 0.6	2.91 ± 0.6	2.12 ± 0.8
Cd 10	2.42 ± 0.5	2.24 ± 0.5	5.18 ± 1.4	3.14 ± 0.8	8.34 ± 1.4 [c, *]	5.26 ± 1.2
Cd 25	3.32 ± 1.2	4.20 ± 1.0	9.34 ± 1.8 [c, *]	4.40 ± 1.3	14.62 ± 2.3 [b, **]	8.15 ± 0.8*
Cd 50	7.80 ± 1.4*	5.12 ± 1.2	19.46 ± 3.6[c, **]	7.62 ± 1.8*	31.28 ± 3.4[a, ***]	12.56 ± 1.7 [c, **]

Freshly isolated thymocytes (1.5×10^6) were treated with Cd (10–50 μM) for 6, 12 and 18 h and the cells were stained with Annexin V-FITC and PE-conjugated anti-CD4/PE-conjugated anti-CD8 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively

Each value represents mean ± SD ($n = 3$). [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$ with respect to one dose and at different time points *** $P < 0.001$, ** $p < 0.01$, * $P < 0.05$ with respect to control at one time, using two way ANOVA

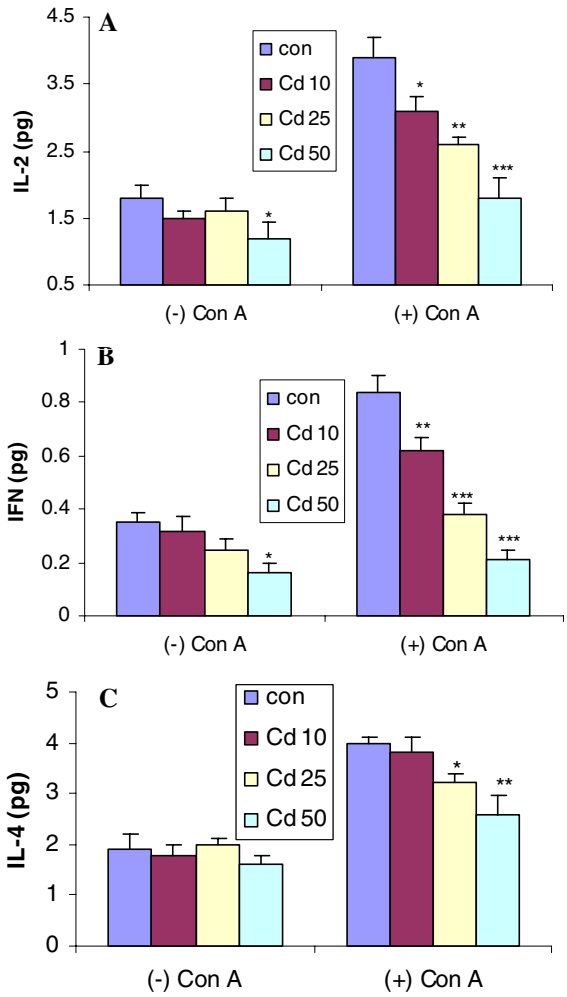

Fig. 1 Effect of Cd on cytokine levels**.** Freshly isolated thymocytes (1.0×10^4) were treated with Cd (10–50 μM), and Con A (2.5 μg/ml) for 72 h at 37°C. The cytokines (IL-2, IL-4 and IFNγ) were measured by ELISA kits. Each bar represents mean ± SD (n = 3). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ as compared to control, using one-way ANOVA

apoptosis in $CD8^+$ cells, resulting in an increase in $CD8^+$ T subsets.

As $CD4^+$ and $CD8^+$ cells also form part of $CD4^+CD8^+$ T-cell population and the surface marker would tag both the population, the latter would probably also respond to oxidative stress and apoptosis.

Discussion

Immunotoxicity by Cd is well documented and is reported to cause oxidative stress and apoptosis in immune cells including both B- and T-cells (Pathak and Khandelwal 2006a, b). The fact that, in vivo exposure to Cd leads to thymic cell depletion and atrophy (Suzuki et al. 1981; Mackova et al. 1996; Pathak and Khandelwal 2007) indicates that Cd may interfere with the T-cell selection process in the thymus and alter the T-cell maturation process. Marked increase in $CD4^-CD8^-$ cells in the present investigation is suggestive of this proposition. Thymocyte apoptosis plays a crucial role in T-cell development and differentiation through negative selection mechanisms (Kisiclow 1995) and various immunotoxins act through induction of thymocyte apoptosis via different signaling pathways (Pieters et al. 1994; Kamath et al. 1998).

Cadmium exposure significantly suppressed the proportion of $CD4^+$ cells followed by $CD4^+CD8^+$ (DP). The $CD4^-CD8^-$ (DN) and $CD8^+$ cells on the other hand, were markedly enhanced. Changes in T-subsets by Cd observed by us slightly differed from that of Dong et al. (2001) who found no significant change in the $CD4^+$ cell population. The normal distribution of these four sub-populations in mouse corroborated with their values. The nature of such changes during apoptosis is not well understood and is still controversial whether the phenotypic changes occur due to apoptosis or vice versa. Apoptosis at 6 h in both $CD4^+$ and $CD8^+$ cells followed by phenotypic alterations at 12 h, suggest that these changes may be dependent on apoptosis and there appears to be a correlation between the two. Simultaneous depletion of $CD4^+CD8^+$ cells with dose and time of Cd exposure could also be related to the direct influence of Cd on immature thymocytes, since the apoptotic data (Table 4) indicates apoptosis in total cell population i.e. SP and DP cells and the increase in $CD8^+$ subsets is indicative of factors other than apoptosis. The possibility of Cd causing downregulation of their surface markers during the final maturation (SP) state also exists. Lowering of $CD4^+/CD8^+$ ratio in blood is normally used as an index of the immunotoxic measure of a xenobiotic. Significant decline of $CD4^+/CD8^+$ ratio, in the present investigation, was related to disappearance of $CD4^+$ (helper cells) and appearance of $CD8^+$ cells (cytotoxic or suppressor cells). Alterations in these two subsets truly reflected the immunotoxicity of Cd. Such an increase in $CD8^+$ thymocyte may also be associated,

at least in part, with the carcinogenic effect of Cd (Dong et al. 2001).

Our data on oxidative stress markers (ROS and GSH) in total $CD4^+$ and $CD8^+$ cells indicated ROS generation and GSH depletion in both $CD4^+$ and $CD8^+$ cells. The former appeared to be more susceptible to oxidative stress particularly GSH, exhibiting higher apoptosis and displaying $\sim$10 fold inhibition in CMF fluorescence than $CD8^+$ cells, which was only four folds at 18 h . However, the involvement of ROS cannot be denied, since our earlier reports indicate ROS increase as early as 15 min followed by GSH in primary murine thymocytes under the influence of Cd (Pathak and Khandelwal 2006a, b). Generation of ROS and depletion of GSH, changes in mitochondrial permeability, release of cytochrome C, and activation of caspase-3 are major elements of the mitochondrial pathway of Cd induced apoptosis (Hengartner 2000; Zamzani and Kroemer 2001; Gupta 2003). Ankrust et al. (1995) have also reported decreased levels of total and reduced glutathione in $CD4^+$ lymphocytes in common variable immunodeficiency (CVI) patients characterized by persistant immune activation in vivo, decreased number of $CD4^+$ lymphocytes in peripheral blood and splenomegaly.

To understand the cascade of events eventually leading to modulation of cellular immune responses, the data on cytokine release (IL-2 and IFNγ) by Th 1 cells and (IL-4) by Th 2 cells revealed inhibition of cytokine production. In addition to IL-3 and GM-CSF, Th 1 cells of helper T-cells (CD4) produce IL-2, IFNγ and TNF β which activate Tc and macrophages to stimulate cellular immunity and inflammation. Th 2 cells secrete IL-4, IL-5, IL-6 and IL-10, which stimulate antibody production by B cells. IL-4 stimulates Th 2 activity and suppresses Th 1 activity, while IL-12 promotes Th 1 activities. Th 1 and Th 2 cytokines are antagonistic in activity. Th 1 cytokine IFNγ inhibits proliferation of Th 2 cells, while IL-2 and IFNγ also stimulate B cells to secrete IgG_{2a} and inhibit secretion of IgG_1 and IgE. IL-4 stimulates B cells to secrete IgE and IgG_1.

The Cd induced inhibition in cytokines release, could be because of lowered $CD4^+$ population resulting in decreased proportion of Th 1 and Th 2 cells or an indirect influence of the metal mediated by the endocrine system (i.e. pituitary hormones) (Lafuente et al. 2004). These cells mediate cytotoxic and local inflammatory reactions, and therefore play important roles in combating intracellular pathogens including virus bacteria and parasites. Our observations on IL-2 and IFNγ release, corroborated with the reports on chromium (Ram et al. 2002), where the authors showed significant inhibition of Con A induced cytokine production by chromium.

In conclusion, our data demonstrates that T-cell apoptosis by Cd, more in $CD4^+$ than in $CD8^+$ cells appear related to higher depletion of intracellular glutathione. Th 1 cells of $CD4^+$ sub-population are more responsive to Cd than Th 2, leading to higher suppression of IL-2 and IFNγ than IL-4 and hence, the study unravels to some extend, the underlying events involved in Cd immunotoxicity.

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