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Phenotypic alterations and cytokine expression by cadmium in murine lymphocytes: An in vitro and *in vivo* study

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Introduction

Among the heavy metals (Pb, Hg, Cd), Cd is a metal of concern since it is a Grade I human carcinogen and a potent immunotoxicant . It has a complex toxic mechanism because of its long biological half-life and multi-organ toxicity. Cadmium causes damage both to humoral immune response and cell mediated immunity (Descotes, 1992; Dan *et al.*, 2000). A number of *in vivo* experiments demonstrate B and T cell alterations (Cifone *et al.*, 1989; Lefuente *et al.*, 2004; Pathak and Khandelwal, 2007), marked loss of thymus weight, T cell depletion and thymic atrophy (Borgman *et al.*, 1986; Mackova *et al.*, 1996; Liu *et al.*, 1999). The underlying mechanism for the causation of immune responses by Cd are not well understood, but in part relate to oxidative stress and lymphocyte apoptosis. We have earlier reported that oxidative stress markers such as ROS and GSH act as intracellular signals for the downstream cascade of apoptotic events in murine lymphocytes. Further, Cd induced apoptosis appears to suppress the mitogenic stimulated cell proliferative response, as well.

To have a better understanding as to how Cd acts on the lymphoid cells and affect its function, we designed two experiments, i) An *in vitro* study, where splenocytes and thymocytes were exposed to varying Cd concs and the other (ii) *in vivo* study, where BALB/c mice were injected a single i.p. dose of Cd. In these two experiments, we ascertained the influence of Cd on splenic (CD3 and CD19) and thymic ($CD4^+$ and $CD8^+$) cell population and the release of cytokines (gamma interferon {IFN-γ} and interleukin-2 {IL-2}), a function of $CD4^+$ cells.

Interestingly, we observed almost similar effects of Cd. Cadmium significantly lowered the $CD4^+/CD8^+$ ratio and inhibited cytokine release. These findings were dose related *in vitro* and time dependent *in vivo*.

Materials and Methods

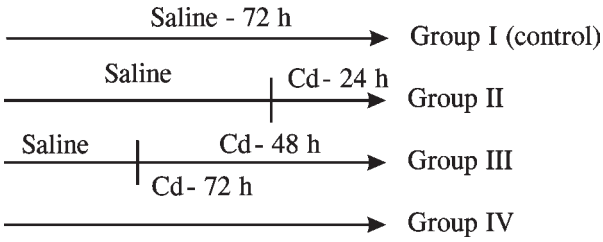
All the chemicals were of highest grade purity available. Cadmium chloride ($CdCl_2$), RPMI 1640, Antibiotic-antimycotic solution, Dulbecco's phosphate buffered saline (PBS), Fetal bovine serum (FBS), and all other chemicals were purchased from Sigma Aldrich, USA. FITC-conjugated anti-CD3 monoclonal antibody, FITC-conjugated anti-CD4 monoclonal antibody, PE-conjugated anti-CD19 monoclonal antibody, PE-conjugated anti-CD8 monoclonal antibody and mouse Interleukin-2 and gamma-Interferon ELISA kits where from eBioscience.

The BALB/c mice were maintained in IITR animal house under standard conditions. They were fed with standard rodent pellet and water *ad libitum*. Our animal house and breeding facility are registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and CPCSEA guidelines were followed (IAEC approval obtained).

For *in vivo* study four to six weeks old male mice were divided in four groups of six animals each. Grouping chart is as below:

Saline (0.1 ml/20 g/day, i.p.), Cd as $CdCl_2$ (1.8 mg/kg, i.p.).

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The animals were sacrificed at one time point, i.e. 72 h. Thymus and spleen were excised and single cell suspension were prepared under aseptic conditions. The suspensions were filtered through a stainless steel mesh and centrifuged at 200 x g for 10 min at 4°C. Thymocytes were resuspended in RPMI medium and splenocytes were suspended in 5.0 ml hypotonic erythrocyte lysing solution (2.42 g Tris and 7.56 g NH_4Cl in 1.0 l deionised water, pH adjusted to 7.2). After 5 min incubation followed by centrifugation, the cells were resuspended in complete medium and the cell density was adjusted to ca. 1.5 x 10^6 cells / ml . For *in vitro* study, freshly isolated thymocytes and splenocytes were treated with different Cd concs (10, 25 and 50 μM) for 18 h. The selection of dose and time is as previously described (Pathak and Khandelwal, 2006a and 2007a).

The T-cell phenotypes were analyzed based on their 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. The splenic population was assessed, based on CD3 (T cells) and CD19 (B cells) surface molecules. Briefly, the cells were suspended 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 thymocytes. In the case of splenocytes, 5 μl FITC-conjugated anti-CD3 monoclonal antibody and 5 μl PE-conjugated anti-CD19 monoclonal antibody were added separately for 30 min in dark at room temperature. After incubation 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, on flow cytometer and 10,000 events were acquired. The flow cytometric analysis was done on BD-LSR flow cytometer. Cell debris, characterized by a low FSC/SSC was excluded from analysis and the data was analysed by Cell Quest software .

The release of Interleukin-2 (IL-2) and gamma-Interferon (IFN-γ) in thymocytes and splenocytes were measured by using Mouse IL-2 and IFN-γ ELISA kits respectively. Briefly, 1.0x10^4 cells in 200ml of complete medium were seeded in 96-well plate with or without one or more reagents (2.5 μg/ml Con A, Cd) 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 / sample solution (100 μl) were added into each well and incubated for 2.0 h at 15 - 25°C. After washing the plates 5 times, 100 μl of detection antibody solution was added to each well and the plates were further incubated for 1.0 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. The plates were further incubated with 100 μl of substrate solution for 15 min, 50 μl of stop solution was added to each well and the absorbance measured at 450 nm.

Significance of mean of different parameters between the treatment groups was analysed using one way analysis of variance (ANOVA) after ascertaining the homogeneity of variance between the treatments. Pair wise comparisons were done by calculating the least significant difference.

Results and Discussion

Our previous reports demonstrated that Cd induced apoptosis at 6 h in primary murine lymphocytes *in vitro* and *in vivo*, it was at 24 h in spleen and much later i.e. 48 h in thymus. We were the first to show a close link of oxidative stress and apoptotic pathway *in vivo* and this communication

is an addition to further understanding of immunotoxic potential of Cd. Here, we studied the thymic and splenic cell population and immune function by Cd. A dose dependent decline in $CD4^+$/$CD8^+$ ratio *in vitro* (Fig. 1) and a similar time related response *in vivo* (Fig. 2) was evident . The lowering in the $CD4^+/CD8^+$ ratio of ~ 0.17 by 25 μm Cd was comparable to the 72 h data of *in vivo* study. A profound increase in the $CD8^+$ cells and a simultaneous loss in $CD4^+$ cells appeared responsible for the decline in $CD4^+/CD8^+$ ratio. 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). Apoptosis at 6 h in both $CD4^+$ and $CD8^+$ cells followed by phenotypic alterations at 12 h, suggest that the latter may be dependent on apoptosis and suggest a correlation between the two (Pathak and

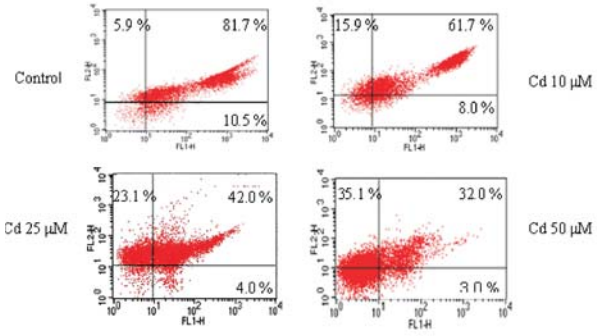


Fig.1: T-cell phenotypic alterations by Cd in vitro. Freshly isolated murine thymocytes were incubated with Cd (10-50 μM) for 18 h at 37°C. The cells were stained with FITC-conjugated anti-CD4 monoclonal antibody and PE-conjugated anti-CD8 monoclonal antibody . The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plots representing one of the three independent experiments. LL : $CD4^-$ $CD8^-$ cells ($FITC^-/PE^-$), LR : $CD4^+$cells ($FITC^+/PE^-$), UR : $CD4^+CD8^+$ cells ($FITC^+/PE^+$) and UL : $CD8^+$ cells ($FITC^-/PE^+$).

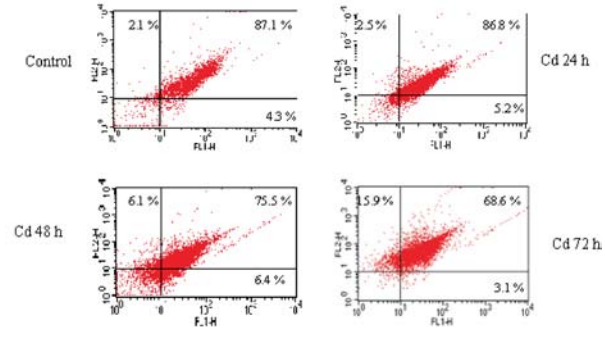


Fig.2: T-cell phenotypic alterations by Cd *in vivo*. The thymocytes isolated from mice treated with Cd (1.8 mg/kg) ip for 24, 48 and 72 h were stained with FITC-conjugated anti-CD4 monoclonal antibody and PE-conjugated anti-CD8 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plots representing one of the three independent experiments. LL:$CD4^-$ $CD8^-$ cells ($FITC^-/PE^-$), LR: $CD4^+$cells ($FITC^+/PE^-$), UR:$CD4^+$ $CD8^+$ cells ($FITC^+/PE^+$) and UL:$CD8^+$ cells ($FITC^-/PE^+$).

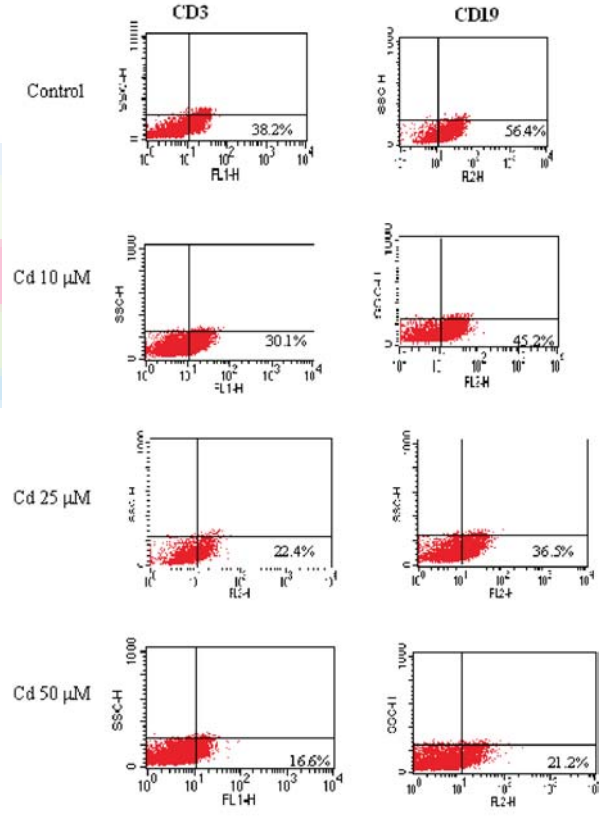


Fig.3: Alterations in splenic population by Cd *in vitro*. Freshly isolated murine splenocytes were incubated with Cd (10-50 μM) for 18 h at 37°C. The cells were stained with FITC-conjugated anti-CD3 monoclonal antibody and PE-conjugated anti-CD19 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plots representing one of the three independent experiments.

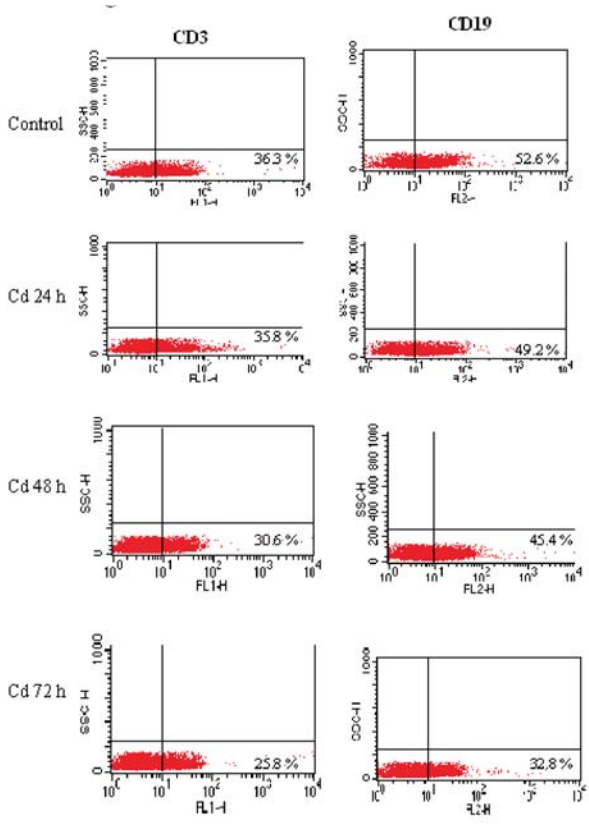


Fig.4: Alterations in splenic population by Cd *in vivo*. The splenocytes isolated from mice treated with Cd (1.8 mg/kg) ip for 24, 48 and 72 h were stained with FITC-conjugated anti-CD3 monoclonal antibody and PE-conjugated anti-CD19 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plots representing one of the three independent experiments.

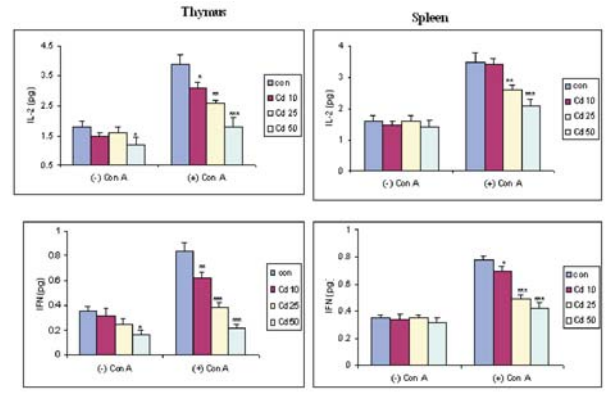


Fig.5: Effect of Cd on cytokine levels *in vitro*. Freshly isolated thymocytes and splenocytes ($1x10^4$) were treated with Cd (10-50 μM), and Con A (2.5μg /ml) for 72 h at 37°C. The cytokines (IL-2 and IFNγ) were measured by ELISA kits. Each bar represents mean$\pm$ S.D (n = 3). $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ as compared to control, using one-way ANOVA.

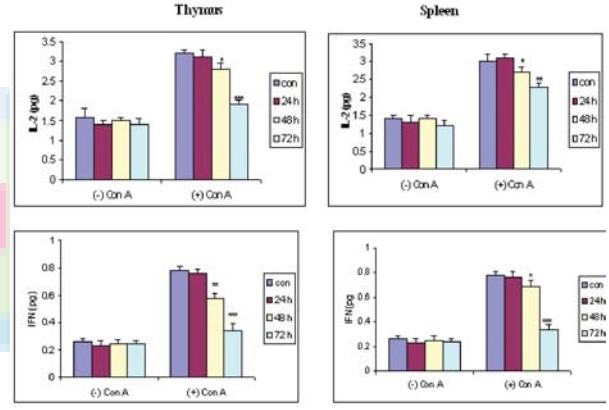


Fig.6: Effect of Cd on cytokine levels *in vivo*. The thymocytes and splenocytes isolated from mice treated with Cd (1.8 mg/kg) ip for 24, 48 and 72 h were incubated with and Con A (2.5μg /ml) for 72 h at 37°C. The cytokines (IL-2 and IFNγ) were measured by ELISA kits. Each bar represents mean$\pm$S.D (n=3). $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ as compared to control, using one-way ANOVA.

Khandelwal, 2008). An increase in $CD8^+$ thymocyte may also be associated, at least in part, with the carcinogenic effect of Cd (Dong *et al.* 2001).

Similar phenotypic alterations have been reported with other immunotoxicants such as dioxin and arsenic trioxide (Lai *et al.*, 1998; Gupta *et al.*, 2003). Modulation of splenic cell population by Cd is shown in Fig. 3 and 4. The T and B cells were substantially lowered as a result of metal's toxic response. Cadmium when added *in vitro*, exhibited a dose dependent lowering not earlier than 18 h (6 h data not shown). Around 2.5 fold decreased in CD3 and CD19 was observed with the highest Cd conc and ~ 1.6 fold with the median dose.

The *in vivo* affect of Cd on splenic cell population (Fig. 4) shows almost similar pattern of T and B cell decrease. Around 1.5 fold loss in the two cell population, correspond to *in vitro* data of 25 μM Cd. The *in vitro* apoptosis at 6 h (Pathak and Khandelwal, 2006a,b) and *in vivo* at 24 h (Pathak and Khandelwal, 2007) preceding phenotypic alterations, strongly suggest the apoptotic influence on modulation of B and T cell population. Thymocytes undergoing apoptosis upregulate a number of cell surface markers

(Kishimoto *et al.*, 1995). We have earlier reported potentiation of oxidative stress followed by mitochondrial caspase dependent apoptotic pathway in both *in vitro* and *in vivo*.

Another important immune function indicator is to evaluate the effect of a compound on the secretion of cytokines. IL-2 and IFN-γ release was seen in Con A stimulated splenic cells. The data in Fig. 5 and 6, demonstrated significant inhibition by Cd, the effect being dose related *in vitro* and time dependent *in vivo*. Cytokine data of the median dose of Cd corroborates with altered T- phenotypes (lowered $CD4^+$ cells) and is comparable to 72 h data of Cd *in vivo*.

The above results further elucidate the immunotoxic potential of Cd and dose effect relationship can be established. Since *in vitro* results with 25 μm Cd are similar to the 72 h effect of 1.8 mg Cd dose *in vivo*, detailed *in vitro* studies may be an alternate to animal models for delineation of Cd induced immunotoxicity.

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