JASEM ISSN 1119-8362

Full-text Available Online at
http:// www.bioline.org.br/ja

J. Appl. Sci. Environ. Mgt. 2004
Vol. 8 (2) 63 - 66

Cell cycle analysis of interleuklin-6 Stimulated B9 hybridoma cell line in the Presence of cadmium

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ABSTRACT: Interleukin-6 is a multifunctional Cytokine. *In–vitro*, interleukin-6 (IL-6) stimulated murine hybridoma B9 cell proliferation is well established. Cadmium inhibition of this response to IL-6 has been previously reported. Cell cycle analysis of IL-6 stimulated B9 cells in the presence or absence of cadmium (Cd) was performed in this study to examine the mechanism of Cd inhibition of B9 cell line proliferation. DNA stained cells were analyzed using electronically programmable flow cytometer. The results show a requirement for IL-6 signals in Go/G1 phase activation and transition out of S-phase of the cell cycle. Cd induced blockage of cells in the S-phase of the cell cycle was observed when IL-6 stimulated cells were treated with 10µM $CdC1_2$. Increasing the concentration of IL-6 from 3 to 100 pg/ml reversed the Cd-blockage of cells in the S-phase of the cell cycle. The ability of IL-6 (at high concentration) to reverse Cd-induced blockage of cells in the S-phase of the cell cycle, suggests that IL-6 signal (s) required for transition of cells out of S-phase is a potential target for Cd. @JASEM

The immunosuppressive properties of heavy metals have being assessed by in-vivo tests in rodents. From the results of such study it is clear that immunosuppression induced by toxic metals may carry a risk of increased susceptibility to infectious agents (Lawrence (1981); Bouley et al., (1982)). How these events occur at the molecular level is unknown. A number of studies have reported the involvement of cytokines in metal mediated immunomodulation. In one of such studies, Cadmium, lead, Copper and Zinc (each at 10 or 100 µM) inhibited antigen induced Interleukin-2 production by D.11.10 cell line (Smith and Lawrence 1988). Kowolenko and his colleagues (1989) have demonstrated that lead acetate (50 or 100 µM) inhibited in-vitro responses of these cells to CSF-1. Non-cytotoxic concentrations of cadmium have been reported to inhibit IL-6 stimulated B9 hybridoma cell proliferation (Orupabo et al., 1992). The mechanism by which Cd inhibits this IL-6 response is not known.

Cell proliferation occurs in phases. A quiescent cell, which is not growing, is said to be in the resting (Go) state. If cell division is triggered, the cells first enters the gap (G1) phase of the cell cycle during which there is an increase in both RNA and protein synthesis. When the cells start to make new DNA they transit into DNA synthetic (S) phase of the cell cycle. During this phase, the DNA content of the cells increase until they have doubled, after which the cells enter the second gap (G2) phase. Finally the cells enter the mitosis (M) phase and either divides returning to G1 phase if cell division is to be sustained, or to the stationary Go- phase. Cellular DNA content in the G2/M phases is double the DNA content of Go/G1 phases. Cells in the S-phase will have a DNA content lying between the extreme of Go/G1 and G2/M phases (Gary and Coffino 1979). The aim of this study was to identify the mechanism by which Cd inhibits IL-6 stimulated B9 cell proliferation by analysis of cells in the different phases of the cell cycle.

MATERIALS AND METHODS

The IL-6 dependent B9 hybridoma cell line established by Dr. L. A. Aarden, was provided by Dr. S. Hopkin (Stafford, U. K). Cells were cultured in "RPMI 1640" medium supplemented with 5% heat inactivated foetal calf serum, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 µM glutamine and 5 x 10^{-5} M 2-mecaptoethanol was used as basal medium. The cell line was maintained at between 5 and 50 x 10^4/ml with recombinant human IL-6 at 3 pg/ml. Cell cultures were incubated in a humidified incubator at 5% CO_2 and 37^o C.

Cell cycles analysis of B9 cells was performed using a cell staining technique described by Barlogie et al., (1976). This technique involves use of mithramycin, a DNA-specific florescent dye that complexes with native DNA and ethidium bromide, which is an intercalating agent that preferentially binds to double stranded DNA. Combination of both dyes in a cell staining technique produces a fluorescent intensity proportional to the amount of DNA present.

Flow cytometry is based on a flow system in which fluorescent stained cells are transported in a liquid medium one by one, through intense light that excites the dye. The resulting fluorescence that is measured and recorded is proportional to the amount of DNA to which the dye is bound. Ten thousand nuclei per sample are counted on an EPICS V flow cytometer. For excitation a coherent innova-90 5W enhanced argon ion laser was used at 100 mW and 488 nm wavelength. Cell cycle analysis was performed using PARA 1 (Coulter Electronic Software). PARA 1 fits mathematically defined distributions to experimental data and derives the area under each section of the histogram. Gaussian distribution is assumed for

Go/G1 and G2/.M phases while the S-phase is derived by subtraction of Go/G1 plus G2/M form the total nuclei count. The G2/M and S-phases are combined to determine the proliferative index.

RESULTS

The incubation time chosen for this study was based on the kinetics of B9 cell line proliferation. Increase in cell number was observed after 24 hours in the presence of IL-6 (figure 1). Cells were stimulated with either 3 or 100 pg/ml IL-6 required for half maximal or maximal cell proliferation respectively, in presence or absence of 10µM $CdCl_2$ and incubated for 6 or 24 hours in culture before cell cycle analysis (Table 1).

TABLE 1. Cell cycle analysis of B9 cell line response to IL-6 and Cd.

Culture condition	Culture time (hr)	Cell cycle phase Go : S : M %			Proliferative index (s+m)
Blank	0	70	22	8	30
	6	82	16	4	20
Blank		81	10	9	19
IL-6 (3pg/ml)		81	16	4	20
IL-6 (3pg/ml) + Cd 10µM		82	6	12	18
IL-6 (100 pg/ml)		84	11	4	15
IL-6 (100 pg/ml) + Cd 10µM					
Blank		60	18	22	40
IL-6 (3pg/ml)	24	36	24	40	64
IL-6 (3pg/ml) + Cd 10µM		44	42	14	56
IL-6 (100 pg/ml)		23	28	49	77
IL-6 (100 pg/ml) + Cd 10µM		34	27	39	66

The cell cycle data shows 70% of cells reside in the Go/G1-phase and 30% in the proliferative (S + M) phase of the cell cycle before (0 hour) initiation of culture.

Cells harvested 6 hours after initiation of culture shows that about 80% of the cells reside in Go/G1-phase with proliferative index of about 20% in the absence or presence of IL-6. The observable difference is in the distribution of cells between S-phase and M-phase of the cell cycle. In the presence of IL-6 there is transition of cells from S-phase to M-phase when compared with un-stimulated cell population. This suggest that IL-6 is involved in the activation of cells from S-phase to M-phase of the cell cycle within the first 6 hours in culture. The distribution pattern in the presence of 10µM $CdCl_2$ shows that cadmium inhibits IL-6 activation of cells out of S-phase of the cell cycle.

Cells harvested after 24 hours in culture shows the population of cells committed to divide as 64% and 77% respectively for half maximal and maximal IL-6 stimulation compared with 40% in the un-stimulated cell population. This observation support the proliferative role of IL-6. Analysis of IL-6 (3pg/ml) stimulated B9 cells in the presence of Cd shows 42% of cells in S-phase and 14% in the M-phase compared with 24% and 40% respectively in the absence of Cd. In the presence of high IL-6 (100 pg/ml) concentration, 27% of Cd treated cells were in the S-phase and 39% in the M-phase compared with 27% and 39% respectively in the absence of Cd. The results shows that cd induced blockage of cells in S-phase of the cell cycle was reversed with high concentration of IL-6 (100pg/ml) in the 24 hour cell population. The results are expressed as the cumulative percentage of cells in Go, S and M phases of the cell cycle. Each data point represents the average of duplicate analysis, and representative of three separate experiments.

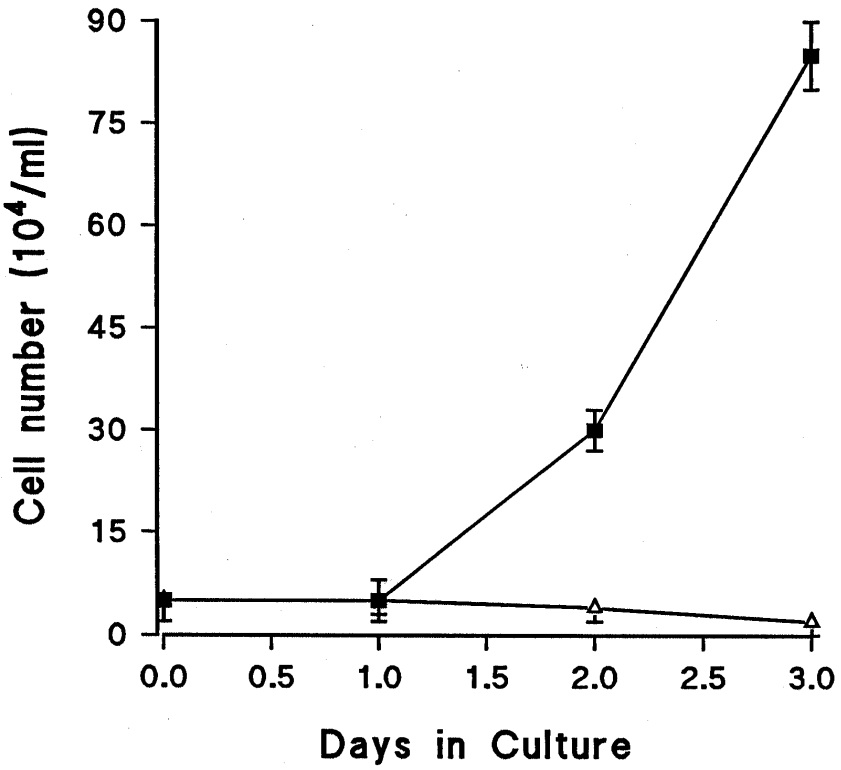

Fig 1. Time response of B9 cells to IL-6. B9 cells cultured for various time intervals with (■) or without (Δ) IL-6. The results are plotted as means ± 95% confidence intervals (n=6).

DISCUSSION

DNA distribution and cell cycle analysis of IL-6 stimulated B9-cells in the presence or absence of Cd was examined to identify the mechanism of Cd inhibition of B9 cell proliferation. The result shows that the proportion of cells that transverse out of Go-phase of cell cycle increased with increased IL-6 concentration. Addition of cadmium to the culture medium reduced the proportion of cells that transverse out of S-phase to the mitotic (M) phase. Increasing the concentration of IL-6 from 3 to 100 pg/ml reversed the Cd- induced blockage of cells in the s-phase of the cell cycles.

Cadmium is known to compete with calcium for binding sites on a variety of calcium-binding proteins or displace calcium at transport sites (Prased, 1985). Previous studies have demonstrated a direct calcium involvement in the initiation of DNA synthesis in many cell types (Whitfield et al., 1980). It is therefore possible that Cd blockage of cells in the S-phase of the cell cycle observed in this study, may be mediated by direct effect on calcium mediated cell functions.

Acknowledgement: This work was sponsored by the Association of Commonwealth Universities

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