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T-2 Toxin Affects Proliferation of Three Different Neoplastic Cell Lines

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The antiproliferative effect of T-2 toxin (T-2) towards mouse melanoma B16 cells, human myelogenous leukemia K562 cells, and human cervix carcinoma, HeLa cells, was studied. For the first four days of T-2 presence B16 cell survival was decreased in dose dependent fashion. However, cell survival after eleven days T-2 action may be dual: some stimulation of cell growth that was direct function of the number of seeded cells per well was observed and cell survival (for the highest number of seeded cells) six times greater than control, was noticed at 20 nM T-2 toxin concentration. A smaller cell growth stimulation (cell survival more than 3 times higher than control) was observed with a lower cell number seeded per well. Nevertheless, by eleventh day concentrations of T-2 higher than 35 nM completely inhibited B16 cell proliferation. The same trend was noticed for T-2 action towards K562 cells. Treatment of HeLa cells with various T-2 concentrations led to a marked inhibition of cell survival that was more pronounced at the end of 44th or 72nd hour, than after the 20th hour of agent's action. ICs50 values obtained in the present work, suggest that B16 cells were the most sensitive to T-2 antiproliferative action, while HeLa cells were the most resistant. When PBMC were cultured with HeLa cells the antagonism against various T-2 concentrations was observed; cell survival determined after 44, or 72 hours of cells incubation, was less decreased compared to cultures treated with T-2, or with PBMC only. In addition, it was shown that T-2 and *cis*-DDP had an antagonist effect on HeLa cells survival.

Key Words: Trichothecenes, T-2 toxin, HeLa cells, K562 cells, B16 cells, Human PBMC, MTT test

T-2 toxin, a trichothecene mycotoxin, is a secondary metabolite of several species of Fusarium fungi. Naturally occurring (mostly T-2) mycotoxicosis, affecting both man and animals after intakes of mouldy cereal grains, has been reported in literature worldwide (1-8). Toxin T-2 represents a type A trichothecene. Its chemical structure is shown in Fig. 1.

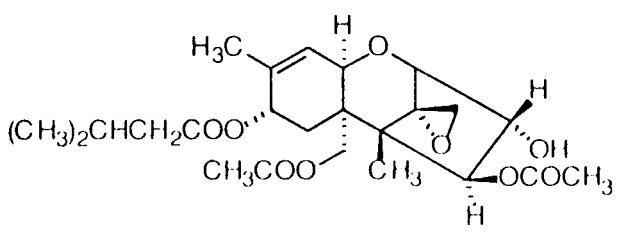

Fig. 1 - Chemical structure of T-2 toxin.

Many toxic substances in low concentrations exert various biological actions. Thus, T-2 toxin superinduced IL-2 mRNA in human tonsil lymphocytes and IL-2 production in murine and rat splenocytes (9,10). This agent could either inhibit or superinduce both IL secretion and mRNA levels in murine CD4+ T-cells (11). It was reported that preinoculation of mice with T-2 significantly enhanced resistance to Listeria monocytogenes (12). The same was observed for Escherichia coli and Staphylococcus aureus (13). Single dose and 7-day treatment with a T-2 toxin reduced the virulence of both S. hyicus or M. avium. It was concluded that the contamination of food with trichothecenes may alter the normal pattern of immunoglobulin production (14).

It was also shown that this toxin most strongly acted on rapidly dividing cells, both *in vitro* (10, 15, 16) and *in vivo* (17-19). Investigation of T-2 action on the proliferation of some human neoplastic cell lines

showed that this compound could suppress the growth of human leukaemia cell lines K562 and an EBV-transformed human lymphoid B-cell line MIN-GLI cells (20), of malignant melanoma SK-Mel/27 cells, or hepatocellular carcinoma Hep G2 cells (21), and of cervical cancer HeLa cells (22).

The aims of this work were (a): to determine the pattern of T-2 action on growth of various neoplastic cells, and to check the antiproliferative effect of T-2 in the presence of peripheral blood mononuclear cells (PBMC) or known antitumor drug *cis*-DDP.

Materials and Methods

Culture conditions for production, extraction and purification of T-2 toxin. Stock culture of F. sporotrichoides KF-38/1 was maintained as sterile water suspension. Subcultivated isolate in potato-sucrose agar for 1 week at 28°C was used for inoculum. Erlenmeyer flasks (750 ml) containing 375 ml of medium (sucrose, 50 g; yeast extract, 1 g; peptone-1 1 g; and water 1 litre, pH 5.6) (23) inoculated with spores of fungal isolate were incubated for 5 days at 28°C on horizontal excentric shaker. Fungal cultures were filtered and crude toxin was prepared from mixed culture filtrates by ethyl acetate extraction. Obtained extracts were concentrated on rotatory vacuum evaporator, dissolved in methanol+water (1+1 v/v), and the clean-up procedure as well as silica gel column chromatography were performed according to Romer et al. (24). Final T-2 toxin quantification was performed by means of gas chromatography with electron-capture detector (GC/ECD).

Chemicals. Stock solution of T-2 was made in 96% ethanol at a concentration of 3.22 mM and afterwards diluted by nutrient medium to the different final concentrations needed (in the range between 8.5-540 nM). *cis*-Diamminedichloroplatinum(II), *cis*-DDP was obtained from Bristol-Meyers-Squib (Munich, Germany). Stock *cis*-DDP solution in physiological saline (0.5 mg/ml) was diluted with nutritient medium before use to final concentrations between 1.05-16.8 μM. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemicals (St. Luis, MO, U.S.A.). MTT was dissolved, 5 mg/ml in phosphate buffer saline pH 7.2, and filtered through milipore filter, 0.22 μm, before use. RPMI 1640 cell culture medium and fetal bovine serum, (FBS) were products of Gibco (Paisley, Scotland, U.K.).

Preparation of peripheral blood mononuclear cells (PBMC). PBMC were separated from whole heparinised blood of healthy volunteers (age range 20-50 years) by Lymphoprep™ gradient centrifugation. Interface cells, washed three times with Haemaccel® aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{++}, 145 mM Cl and 35 g/L gelatine polymers, pH=7.4, were counted and resuspended in nutritient medium.

Cell culture. Mouse melanoma B16 cells, and human cervix carcinoma HeLa cells were maintained as a monolayer culture, while human myelogenous leukemia, K562 cells were grown as a suspension culture, in the same nutritient medium (RPMI 1640 medium supplemented with l-glutamine (3 mmol/L), streptomycin, and garamycin (100 μg/mL, each), 10% heat inactivated foetal bovine serum, FBS and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution). The cells were grown at 37°C in 5% CO_2 and humidified air atmosphere by twice weekly subculture.

Treatment of mouse melanoma B16 cells. Three various numbers of cells were seeded, in triplicate (1250; 625 and 312 cells per well), into three different 96-well microtiter plates and twenty hours later, five different concentrations of T-2 toxin were added to the wells to final concentrations of 8.5 nM; 17 nM; 34 nM; 68 nM and 136 nM, except to the control wells where a nutritient medium only was added to the cells. All analyses were done in triplicate. Nutritient medium with corresponding concentrations of T-2, but void of cells was used as blanc, in triplicate.

Treatment of K562 cells. Three different numbers of K562 cells were seeded per well, in triplicate, (1250; 635, and 312 cells per well) into 96-well microtiter plates. Three different plates were seeded and four hours later cells were treated with various concentrations of T-2 in the same way as B16 cells.

Treatment of HeLa cells. HeLa cells, human cervix carcinoma cells, were seeded in nutritient medium into 96-well microtiter plates, 2000 cells per well. After 20 hours, to one series of wells human PBMC were introduced keeping effector to the target cell ratio of 25:1 or 50:1. Then, simultaneously, six different concentrations of T-2 toxin (to final concentrations of 17 nM; 34 nM; 68 nM; 136 nM; 272 nM and 544 nM) were added to the group of wells with or without PBMC, but not in corresponding control wells where only nutritient medium was added. In samples used to study the com-

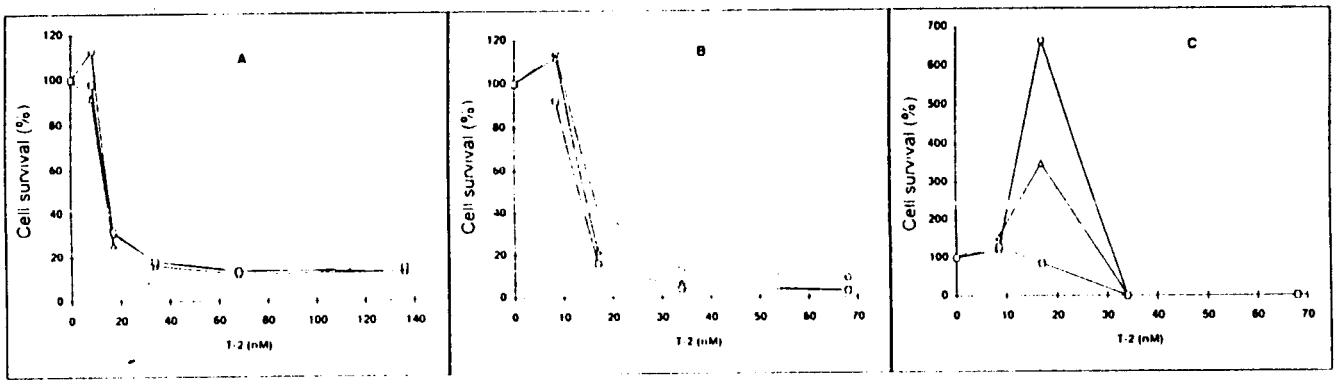

Fig. 2 - The effect of different concentrations of T-2 toxin on the survival (%) of B16 cells, at fourth (A), fifth (B) and eleventh (C) day of action. (○) 1250; (△) 625 and (□) 312 seeded cells per well. Data are the mean of triplicate. SD were less than 10%.

bined action of *cis*-DDP and T-2, twenty hours after the cell seeding, the agents were simultaneously added to the wells. All analyses were done in triplicate. Nutrient medium with or without all corresponding addition(s), but without target cells, was used as blanc, in triplicate.

Determination of B16 and K562 cell survival. Cell survival was determined by MTT test according to Mosmann's method (25) modified by Ohno and Abe (26), four, five, or eleven days after the drug addition. Briefly, 20 µL of MTT solution (5 mg/ml PBS) were added to each well. Samples were incubated for further four hours at 37°C in 5% CO_2 and humidified air atmosphere. Then, 100 µL of 10% SDS in 0.01M HCl were added to the wells. Optical density (OD) at 570 nm was red the next day. To get cell survival (%), optical density at 570 nm of a sample with cells grown in the presence of various concentration of T-2 (OD), was divided with control optical density ODc, (the OD of cells grown only in nutritient medium) x100. (OD of blanc was always subtracted from OD of a corresponding sample with target cells). Concentration IC50 was defined as the concentration of a drug required to inhibit cell survival by 50%, compared with vehicle-treated control.

Determination of HeLa cell survival. Cell survival was determined by MTT test 20, 44, 72 and 96 hours upon addition of the drug, or 20, 44 and 72 hours upon addition of PBMC, or of PBMC and the drug. In this way the effects of T-2, or of PBMC, as well as of their combination were compared, with the proliferation of the cells grown only in nutritient medium, by the same procedure used for determination of B16, or K562 cell survival. A sample with effector cells with or without the corresponding T-2 concentration was used as blanc in cases where the effect of PBMC on HeLa cell survival was examined (27). The survival of cells exposed to the combined action of *cis*-DDP and T-2 was determined after 48 and 72 hours by the same assay.

Results

The effect of T-2 toxin on B16 cell survival, as the function of the seeded cell number per well, and the function of the time of incubation, is shown on Fig 2. An antiproliferative effect of T-2 on cell survival is found as an inverse function of the seeded cell number at the beginning of the experiment. At the end of the eleventh day of T-2 action it was found that T-2 was capable of inducing a dual effect on cell survival. Some stimulation of cell growth was observed; at 20 nM of toxin concentration cell survival was six times greater (for the highest number of seeded cells) than control. In the same circumstances the smaller cell growth stimulation (cell survival 3.5 times higher than control) was observed at lower cell number seeded per well. This growth stimulation of B16 cells by 20 nM T-2 is accompanied by the formation of colonies with a greater number of non melanin-producing cells in monolayer (approx. 64) in relation to the same cells in control (approx. 16). There were more melanin-producing cells at the top layer in control than in samples with T-2. Nevertheless, concentration of T-2 toxin greater than 35 nM completely inhibited cell survival. IC50 for B16 cells, for different duration of T-2 action are shown in Table I.

The survival of human K562 cells grown in the presence of various concentrations of T-2 are shown in Fig. 3, as a function of the time of cell incubation with

the toxin. Different cell number seeded per well had various sensitivity to T-2 toxin action, and an antiproliferative T-2 effect on cell survival was observed for the first 72 hours. On the eleventh day a dual effect of T-2 was observed again: some growth stimulation, in relation to the control, could be seen at 20 nM of T-2 (cell survival was 2.4 times higher than control for the 1250 seeded cells per well). This growth stimulation induced by 20 nM T-2 for the same number of seeded cells is smaller than that determined for B16 cells. The possible explanation may be that the number of B16 cells was higher in the moment of T-2 addition (20 hrs after cells seeding, while to K562 cells toxin was added only four hours after cells seeding). Concentration of T-2 toxin greater than 35 nM completely inhibited cell growth. The IC50 for K562 cells, for different days of T-2 action, are shown in Table I.

Data on relative survival of HeLa cells grown alone, in the presence of various T-2 toxin concentrations or in culture with human PBMC, at a different effector to target ratios (0; 25:1; and 50:1) in the presence of various concentrations of T-2, determined at the end of the first, second and third day of agents and/or PBMC action, are presented on Fig. 4. Antiproliferative T-2 action on HeLa cells concentration dependent could be seen. IC50 was not the function of the time of cell incubation with T-2 for the agents action between 44 and 72 hours but an inverse function of the number of cells seeded per well at the beginning of the experiment (see Table I). Some complex relation between T-2 and PBMC action on HeLa cells in the concentration range 40-150 nM of T-2 could be observed along with an antagonistic PBMC action on T-2 inhibition of cell growth. It could be also noticed that suppression of HeLa cell survival induced by the function of some PBMC classes is lower in the presence of T-2.

Optical densities, that are directly proportional to the number of live, MTT treated, target HeLa cells - grown alone or in the coculture with PBMC at various E:T ratios, in the presence or in the absence of 34 nM of T-2 - are shown in Fig. 5 as a function of the time of the drug exposure. It could be seen that the presence of PBMC partially protected HeLa cells from the antipro-

Table I - IC50 obtained for the T-2 action on B16, K562 and HeLa cells for different period of agents action (days) and various cell number seeded per well.

Time of T-2 action	B16 IC50 (nM)	B16 No cell/well	K562 IC 50(nM)	K562 No cell/well	HeLA IC50 (nM)	HeLA No cell/well
1 day	ND*	ND	ND	/	> 540	10000
2 days	ND	ND	ND	/	56	2000
3 days	ND	ND	ND	ND	70 55 55	2500 1250 625
4 days	15 14 14	1250 625 312	27.5 26	1250 625	225 85 55	2500 1250 625
5 days	16 14.5 13	1250 625 312	21 16	1250 625	ND	ND
11 days	32.5 31.5 23	1250 625 612	30 16 7.5	1250 625 312	ND	ND

* *ND: non determined*

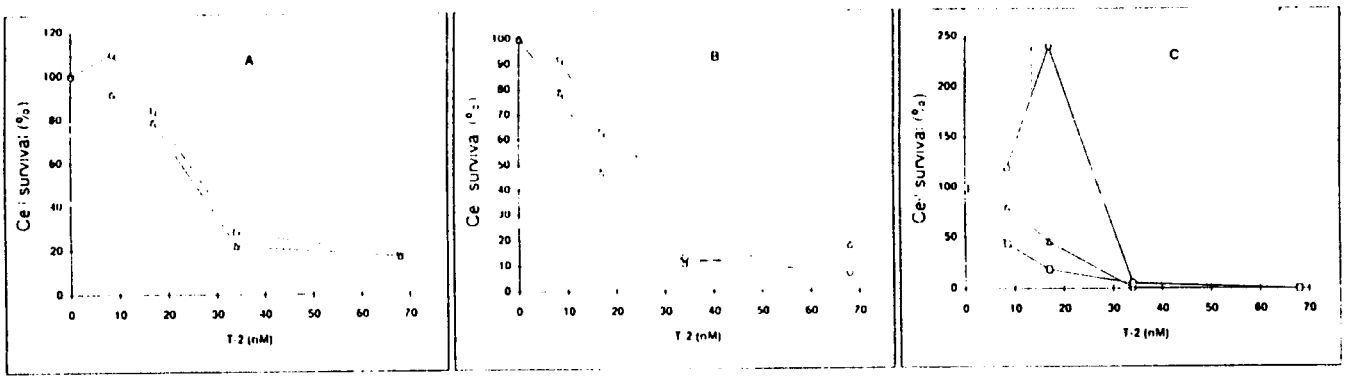

Fig. 3 - The effect of different concentrations of T-2 toxin on the survival (%) of K562 cells, at the end of fourth (A), fifth (B) and eleventh (C) day of action. (○) 1250; (△) 625 and (□) 312 seeded cells per well. Data are the mean of triplicate. SD were less than 10%.

liferative T-2 action. The effect of *cis*-DDP and T-2 toxin on HeLa cell survival is shown in Fig. 6. The antagonistic action of two drugs was observed, as the combination index, CI (28), was higher than 1.

Discussion

Although the toxic action of T-2 has been known since a long time ago (1-4), many recent reports analyse the antitumor action, especially when the agent is applied at low concentrations (20-22). T-2 toxin is a powerful inhibitor of DNA and protein synthesis in eukaryotic cells (29, 30), which is accomplished by its firm attachment to ribosomes (31-32).

Our results clearly show that low concentrations of T-2 have a dual effect on the examined cell proliferation. Extremely low T-2 levels, but defined in the range 10-25 nM, caused some stimulation of B16 and K562 cell growth. As this stimulation is proportional to the number of seeded cells per well at the beginning of the experiment, it could be assumed that some autocrine growth factors were induced and released from target cells during the T-2 action. Stimulation of target cell growth could be the consequence of this autocrine growth factor effect. This finding is in accordance with already published reports that T-2 (as well as the other known protein synthesis inhibitor, cycloheximide) could superinduce autocrine, or paracrine growth factors for various (mostly of immune origin) cell types

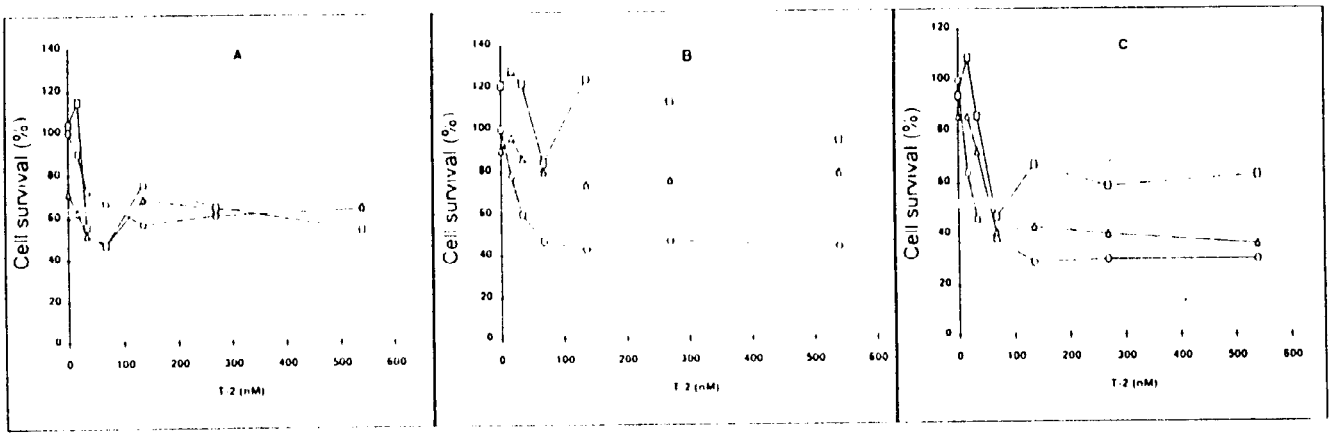

Fig. 4 - Survival (%) of HeLa cells, grown alone, or co-cultured with human PBMC is given as the function of T-2 toxin concentration, at different effector to target ratios, at 20th (A), 44th (B) and 72nd hour (C) of agents' action. In all samples 2000 of HeLa cells were seeded per well. (○) HeLa cells; (△) HeLa cells co-cultured with human PBMC E:T=25:1; (□) HeLa cells co-cultured with human PBMC E:T=50:1. E:T is given in relation to the number of seeded cells at the beginning of the experiment, 20 hours before the PBMC addition. Data are the mean of triplicate. SD were less than 15%.

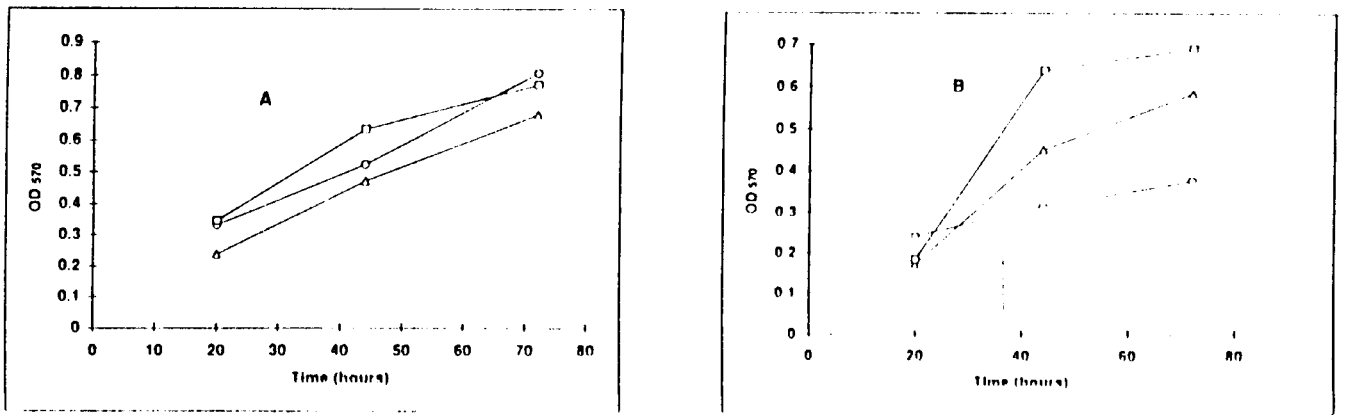

Fig. 5 - Optical density at 570 nm (OD570) of MTT assayed HeLa cells grown alone (○), or co-cultured with human PBMC at E:T ratio 25:1 (△) or 50:1 (□); at the end of 20; 44 and 72 hours of the setup of mixed cell culture (A). OD570 of HeLa cells grown alone (○) or co-cultured with human PBMC at E:T ratio 25:1 (△) or 50:1 (□); in the presence of 34 nM T-2 at the end of 20; 44 and 72 hour of the set up of mixed cell culture (B). Data are the mean of triplicate. SD were less than 10%.

(11, 33-35). The observed superinduction of some proteins was explained to be due to increased gene transcription (via inhibited synthesis of a negative protein regulator) and mRNA stability as postulated by Shaw et al. (36-38). Contrary to our findings, the effect of T-2 on the stimulation of cell growth was not reported for B16 or K526 cells (20), probably because short time of agent's action.

Higher, but still low, concentrations of T-2 toxin completely blocked cell growth stimulation, and an antiproliferative effect, already reported for K562 (20) or HeLa cells (22), was observed. The T-2 antiproliferative effect on B16 cells has not been published yet, although, it was observed earlier on human melanoma SK-MEL/27 cells (21).

Human PBMC exert a cytotoxic effect when incubated with malignant cells due to the known cytotoxic action of some of its subpopulations. In our experiment, the cytotoxic PBMC effect was weak and not proportional to the number of effector cells. Moreover, PBMC acted antagonistically to T-2 toxin, or vice versa, on HeLa cell survival. In the presence of PBMC and T-2, HeLa cell survival was higher than in the samples were only PBMC, or the corresponding concentration of T-2, were with target cells. It was unusual to see a gap in target cell survival observed at T-2 concentration between 40 and 140 nM pronounced only in HeLa cells co-cultured with PBMC. This finding could be explained by earlier published data showing that T-2 could superinduce *de novo* synthesis and release of IL-2 in CD4+

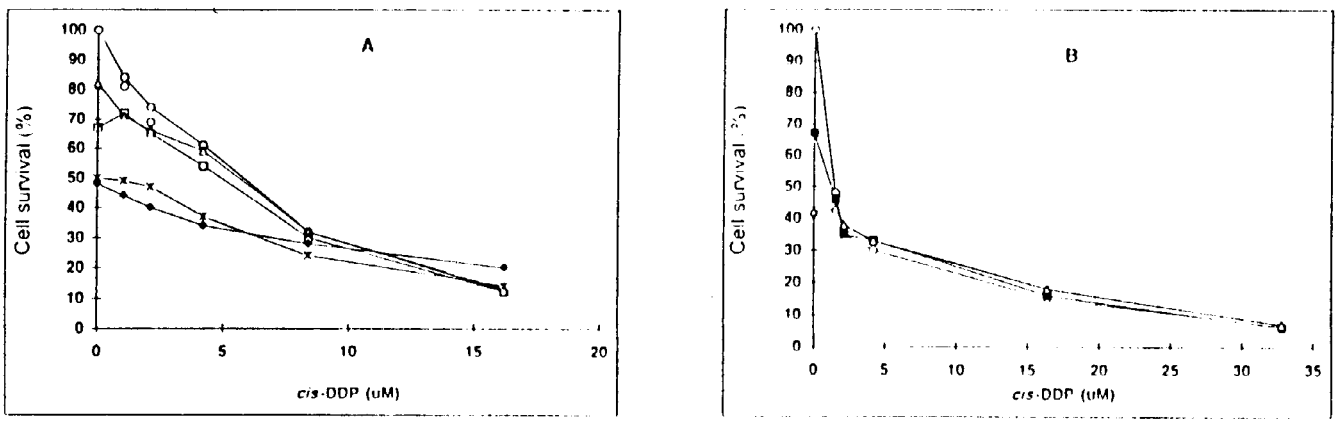

Fig. 6 - Survival of HeLa cells grown in the presence of T-2 and various cis-DDP concentrations for 48 (A) and 72 hours (B). (○) T-2 0 nM; (△) T-2 7.5 nM; (□) T-2 16 nM; (×) T-2 32 nM; (■) T-2 64 nM. Data are the mean of triplicate. SD were less than 10%.

murine T cells (11). It is known that IL-2 could enhance the cytotoxic effect of cells with NK function (39). If, by analogy, the same could happen in system investigated with the same human cells used in this experiment, the cytotoxic function of PBMC in the presence of T-2 would be stronger. However, HeLa cell survival was, even in these circumstances, higher than in the presence of PBMC only, suggesting that *in vitro*, the haematotoxic T-2 effect, observed and reported earlier (20, 40-42), could be expressed on the effector cells used in this experiment, too. Data obtained in this work suggest that, although it was proved that T-2 exerted its antiproliferative effect in low concentrations, its action on neoplastic cells could be very complex and experiments in vitro could hardly predict its final effect *in vivo*.

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