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Kyunghee Burkitt

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S. K. Agrawal

Major professor

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IMMUNE SYSTEM ACTIVATION BY CISPLATIN AND "POLY-PLAT"

By

Kyunghee Burkitt

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ABSTRACT

IMMUNE SYSTEM ACTIVATION BY CISPLATIN AND "POLY-PLAT"

By

Kyunghee Burkitt

Murine peritoneal macrophages, human fibroblasts and human ovarian teratocarcinoma cells were treated with cisplatin (10 μg/ml) and "poly-plat" (10 μg/ml) for 2h and cultured for 2-96h. After "poly-plat" treatment, macrophages developed cytoplasmic extensions much faster and also secrete higher levels of interleukin-2 (IL-2), compared to cisplatin. "Poly-plat" treated human fibroblasts also showed significant release of IL-2 (138 pg/ml), compared to cisplatin treated (64 pg/ml) or normal controls (38 pg/ml) after 8h of treatment. However, human ovarian teratocarcinoma cells didn't show significant increase of IL-2 levels after "poly-plat" treatment except only after 2h and 24h of treatment. Human fibroblasts demonstrated a 53% increase in cell numbers after "poly-plat" treatment, compared to 37% after cisplatin. However, after "poly-plat" treatment, human ovarian carcinoma cells showed an initial decrease (15%) in cell numbers and then an increase (23%) up to 24h post-treatment. Based on our studies, we propose that "poly-plat" is more effective in activation of the macrophages and human fibroblasts in terms of various cytokines.

To my family

ACKNOWLEDGEMENTS

The last four years at MSU have brought me many challenges and beautiful memories. First of all, I would like to say a special thank to Dr. S.K. Aggarwal for his guidance and support. I had so many difficulties the last couple of years. At times, I almost gave up. But, I learned three valuable things, independence, confidence and above all, modesty. There were always people around me who supported me during difficult situation. Dr. Tanaka, Dr. Ross, and Judy Smith deserve my thanks for their special care and direction. I also can't forget about the loving and endless support from my family in Korea. I thank my father for his financial support and critical guidance, and my mother for her warm and cheerful mental support and my brother for his deep love, and finally my best friend kyung-min for her support. There is also another best friend and my loving husband, Kevin G. Burkitt, who is a my best critic and also counselor at all times as he will be in the future. I really thank him for his devotion through my studies.

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INTRODUCTION

Cisplatin [*cis*-dichlorodiammine-platinum(II)], a broad spectrum anticancer drug, presently is being used for the treatment of testicular and ovarian cancers (1, 2). One of the major proposed mechanisms of this anti-tumor activity is interaction with DNA that results in the inhibition of DNA replication and transcription (3).

Recent studies have demonstrated that immune system activation might be another mechanism of anti-tumor activity of this drug (4). There are many immune system cells such as macrophages, lymphocytes and monocytes, of which macrophages are one of the most important immune system cells because of their phagocytic activity and also the release of various cytokines (5).

Cisplatin has been shown to activate murine peritoneal macrophages *in vitro* and *in vivo* (4, 6). Activated macrophages mediate tumor lysis by lysosomal transfer to tumor cells and through the release of various cytokines such as IL-1α and TNF-α and interleukin-2 (4, 5).

Interleukin-2 (IL-2) was originally described as "T cell growth factor" (7). IL-2 differentiates T cells as well as stimulates NK (natural killer) cells and LAK (lymphocyte activated killer) cells (8-11). Therapeutic application of IL-2 injection has been involved in the treatment of neoplastic conditions (12), infectious diseases such as hepatitis B (13) and malignancies associated with AIDS (14). Recent studies have demonstrated that transfected human fibroblast with the IL-2 gene are stimulated to induce high levels of IL-2 which increases the population of $CD3^+$, $CD56^+$ lymphocytes to fight tumor cells (15). IL-2 appears to have an important role not only in activating immune responses,

but also in gene therapy which has been used for the treatment of various cancers (16-18).

Poly-[trans-1, 2-diaminocyclohexane platinum]-carboxyamylase (poly-plat), a second generation analog of cisplatin, contains only one fifth the amount of platinum compared to cisplatin and has been shown to be less toxic and more effective as an anticancer agent (19). Recent studies show that "poly-plat" is more effective in activating macrophages than cisplatin by inducing higher levels of cytokines such as IL-1α and TNF-α (20, 21).

In the present study we describe the results of treating various cell lines from normal (Human fibroblasts, Murine peritoneal macrophages) and tumor cells (Human ovarian teratocarcinoma cells) after "poly-plat" treatment, and compare those after cisplatin treatments.

MATERIALS AND METHODS

Cell culture

1. Macrophages: Swiss webster mice were anesthetized using CO_2 and killed by cervical dislocation. Peritoneal macrophages were isolated by injecting 5ml of a chilled Dulbecco's modified eagle medium (DMEM;Gibco, NY) into the peritoneal cavity. After gently massaging the abdomen wall, the peritoneal fluid was aspirated. Almost equal amounts of cells (2×10^6 cells/ml) were seeded onto glass coverslips and then were placed in 35mm Petri dishes and incubated for 2h at 37°C. After 2h of incubation, the coverslips were washed with phosphate buffer solution (PBS; pH 7.2) to remove non-adherent cells, leaving the attached macrophages. Cells were then incubated in fresh DMEM with 10% fetal bovine serum for further experiment.

2. Human fibroblasts and human ovarian teratocarcinoma: Human fibroblasts and ovarian teratocarcinoma cells were obtained from Dr. C.C. Chang of Michigan State University. Cell cultures were incubated in a DMEM with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. When cells were confluent in culture flasks, cells were then trypsinized and subcultured into plastic culture dishes with an equal number of cells in each culture dish .

Drug treatment

Plated cells were treated with cisplatin (10 μg/ml) and "poly-plat" (10 μg/ml) prepared in 0.85% NaCl for 2h at 37°C in a 5% CO_2 incubator. After drug treatment, the drug containing medium was replaced by normal DMEM with 10% fetal bovine serum.

Cell counting

Cells in various cultures (human fibroblasts and human ovarian teratocarcinoma cells) were counted before and after drug treatment. Subsequently, counts were maintained after 8, 16, 24, 36, 48, 72 and 96h of treatment. To count cells at each time interval, cells were washed with PBS and trypsinized with 50% trypsin-EDTA diluted with PBS. Cell suspension (200μl) was mixed with 500μl of trypan blue (0.4%) and 300μl of HBBS. Cells were counted in the 16 square leukocyte chamber to determine the cells/ml. Cells/ml were calculated using the following formula: average counted cells per square (n=9) × dilution factor × 10^4. Cells/ml were multiplied by the original volume of fluid (10ml) in the culture dish to get the number of total cells.

Slide preparation

In order to visualize changes in cell population (human fibroblasts and human ovarian teratocarcinoma cells) after any drug treatment, same number of cells were plated onto 18mm glass coverslips. After 2h incubation on the coverslips, cells were washed with a phosphate buffer solution (PBS) to remove non-adherent cells. Coverslips were then transferred into the plastic culture dish with normal medium and

drugs were added for 2h. Coverslips were then removed from the culture dish, washed, and put on normal medium. Sample coverslips were removed from these cultures at intervals of 8, 16, 24, and 48h. These were fixed in 2% (0.02g/ml) glutaraldehyde in 0.05M phosphate buffer (pH 7.4) for 2 minutes and then rinsed in distilled water. Methylene blue was used to help visualize cellular details.

IL-2 Assay

The drug containing medium was replaced by normal medium and aliquots of the supernatant were collected at 0.5, 1, 2, 4, 8, 16, 24, 72, 96h post-treatment from various cell cultures. An enzyme-linked immunosorbent assay (ELISA) kit (Pharmagen, San Diego, CA) was used for IL-2 assay. Monoclonal anti-mouse IL-2 was added to each well of microplate, and then the antibody was incubated overnight at 4°C. After incubation, the antibody was aspirated from each well and washed with 0.05% PBT (PBS with 0.05% Tween-20) to remove non-adherent materials. IL-2 standards (200,100, 50, 25, 12.5, 6.3 and 3.1 pg/ml) and samples (cell culture supernatants) were added to each well and incubated for 2h at room temperature. All samples were then washed with 0.05% PBT and working detector (Detection Ab + Avidin-horseradish peroxidase conjugate) was added to bind the primary and secondary antibody complex. A substrate solution (Tetramethylbenzidine and Hydrogen Peroxide) was added to give color change. The reaction was stopped by adding stop solution, 2 N H_2SO_4. Absorbance was read at 450nm. Standard curves were generated with IL-2 (3.1-200pg/ml) provided in the kit and linear regression analysis was performed twice.

RESULTS

Morphological changes after drug treatment on murine peritoneal macrophages

Morphological changes of cisplatin and "poly-plat" treated murine peritoneal macrophages were observed at 2, 16, 24h post-treatment (Fig 1-3).

No extensions were observed in the normal (Fig 1A) or the cisplatin treated cultures after 2h post-treatment (Fig 1B). However, short extension formations were observed after "poly-plat" treatment (Fig 1C). Similarly, no extension formation was observed even at 16h post-treatment in either the normal (Fig 2A) or the cisplatin (Fig 2B) treated macrophages. However, pseudopod formation was dramatically increased after "poly-plat" treated macrophages (Fig 2C).

Both normal and cisplatin treated macrophages demonstrated pseudopod formation only after 24h post-treatment. About 20-30% of the total population of non-treated macrophages formed short extensions (Fig 3A) and cisplatin treated macrophages showed between short and medium developed extensions on 50-60% of total cell population (Fig 3B). "Poly-plat" treated macrophages demonstrated cytoplasmic extension in 70-80% of the cells. These extensions were long and slender (Fig 3C).

Changes in cell population in drug treated human fibroblasts

Numbers of normal and drug treated human fibroblasts were counted before treatment with cisplatin or "poly-plat" and immediately after treatments. Subsequently cell counts were made at 8, 16, 24, 36 and 48h post-treatment (Table1). "Poly-plat"

treated fibroblasts showed a greater increase in cell numbers compared to cisplatin treated fibroblasts. An initial increase of about 15% was observed immediately after "poly-plat" treatment, at the same time, normal cells and cisplatin treated fibroblasts showed only 1% and 11% increase respectively. There were continuous increases in cell numbers of cisplatin and "poly-plat" treated fibroblasts up to 24h post- treatment when numbers started to decrease while the normal cells continued to multiply.

To visualize any changes in cell population, human fibroblasts were observed using light microscopy immediately after drug treatments and also after 8 and 16 of cisplatin or "poly-plat" treatment (Fig 4-6). To show the increase in the number of cells observed at each time interval, we used percentages (based on Table 1) to describe changes in cell numbers. Immediately after "poly-plat" treatment, there was observed a 15% increase in the fibroblastic cells (Fig 4C), while cisplatin treated fibroblasts demonstrated an 11% increase (Fig 4B). Normal fibroblasts demonstrated a minimal increase of no more than 1% (Fig 4A).

A greater increase in cell numbers was observed after 8h of treatments (Fig 5). "Poly-plat" treated cells appeared very compact (Fig 5C), about a 23% increase in cell numbers from normal fibroblasts (Fig 5A). However, cisplatin treated cells (Fig 5B) showed only a 15% increase from normal cells. After 16h of treatments, cisplatin (Fig 6B) and "poly-plat" (Fig 6C) treated cells showed an 8% and 15% increase, respectively, compared to the normal cells (Fig 6A).

Changes in cell population on drug treated human ovarian teratocarcinoma cells

Normal cells and drug treated human ovarian teratocarcinoma cells were counted before and after 2h of drug treatments. Subsequently, cell counts were made at 8, 16, 24, 36, 48, 72 and 96h post- treatment (Table 2). Normal cells were maintained in cultures without any treatments for comparison at each time interval. Cisplatin treated ovarian carcinoma cells showed a continuous decrease in cell numbers at each of the time intervals. However, there was an initial decrease of about 15% in cell numbers after 2h of "poly-plat" treatment. "Poly-plat" treated cells then showed a gradual increase to about 23% from the initial decrease up to 24h post-treatment. After 24h of "poly-plat" treatment, however, there was a gradual decrease in cell numbers reaching close to those after 96h of cisplatin treatment.

To visualize any changes in cell population, human ovarian teratocarcinoma cells were observed using light microscopy before any treatments and also after 24 and 48h of treatments with cisplatin or "poly-plat" (Fig 7-9). To show the increase in the number of cells observed at each time interval, we used percentages (based on Table 2) to see changes in cell numbers on the glass slide. After 24h of treatment, non-treated tumor cells (Fig 7B) showed twice the number of cells (141% increase) compared to those before treatment (Fig 7A) while 59% of the cells were destroyed after cisplatin treatment (Fig 8A). However, cell numbers increased to about 5% after 24h of "poly-plat" treatment (Fig 8B) from those before treatment (Fig 7A). After 48h of treatment, "poly-plat" showed about a 19% decrease in cell numbers (Fig 9B) from 24h post-treatment whereas those of cisplatin treatment stayed almost the same (Fig 9A).

IL-2 release

1. Macrophages: There was an increase in the IL-2 level detected in the supernatants of the macrophages treated with either cisplatin (10 μg/ml) or "poly-plat" (10 μg/ml) for the various times tested (Fig 10). Significant differences in the IL-2 level between cisplatin and "poly-plat" treatment were observed after 4h of treatment. The greatest increase (166pg/ml) in the IL-2 level of "poly-plat" treatment was observed after 16h of treatment. The IL-2 level of both cisplatin and "poly-plat" decreased to 81pg/ml and 125pg/ml after 24h of treatment.

2. Human fibroblasts: No significant differences in the IL-2 level of normal, cisplatin and "poly-plat" treated fibroblasts were observed until 2h post-treatment (Fig 11). After 4h of treatment, "poly-plat" treated cells released IL-2 (52pg/ml) at the rate of about 9 times that of untreated fibroblasts. The IL-2 level reached a maximum (138 pg/ml) after 8h of "poly-plat" treatment. However, the IL-2 level of cisplatin treated cells showed only half (64 pg/ml) of the IL-2 level compared to "poly-plat" treated cells. The IL-2 level of both cisplatin and 'poly-plat' treated cells decreased to 25 and 31 pg/ml, respectively, after 16h of treatment.

3. Human ovarian teratocarcinoma: IL-2 levels of up to 82 pg/ml were observed after 0.5h of "poly-plat" treatment compared to normal (43 pg/ml) and after cisplatin treatment (58 pg/ml). This level increased up to 104 pg/ml after 2h of "poly-plat" treatment. Interestingly, normal cells also demonstrated an increase in the IL-2 levels (97 pg/ml) after 1h in normal medium without any treatment. After 2h of "poly-plat"

treatment, IL-2 levels decreased (37 pg/ml) until 16h post-treatment (Fig 12). There was again an increase in the IL-2 levels after 24h of "poly-plat" (104 pg/ml) and after cisplatin treatment (97 pg/ml). However, this increase did not remain constant. After 48h of treatment, it was lower than its peak at 24h post-treatment (see Fig 12).

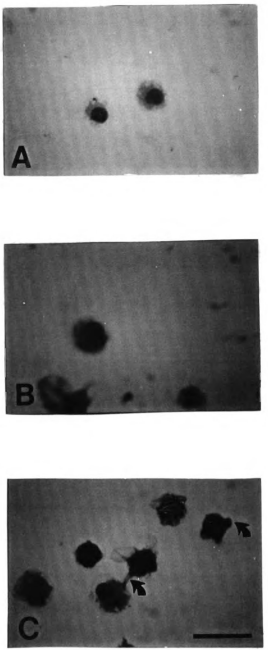

Fig1. Light micrographs showing murine peritoneal macrophages at 2h in normal medium after (A) no treatment, (B) cisplatin (10 μg/ml) and (C) "poly-plat" (10 μg/ml) treatment for 2h. Note the short cytoplasmic extension formation (arrows) after "poly-plat" treatment, while normal and cisplatin treated macrophages show no extension formation. Final magnification: ×2000 (A, B, C), Bar = 7.5 μm

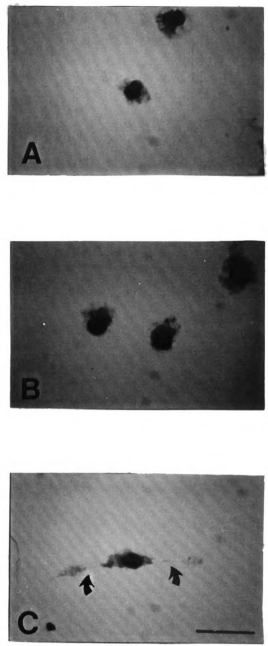

Fig 2. Light micrographs showing murine peritoneal macrophages at 16 h in normal medium after (A) no treatment, (B) cisplatin (10 μg/ml) for 2h, (C) "poly-plat" (10 μg/ml) for 2h. Note the cytoplasmic extensions (arrows) were observed after "poly-plat" treatment as bidirectional in their formation, while no cytoplasmic extensions were observed in normal or cisplatin treated macrophages. Final magnification: ×2000 (A, B, C), Bar = 7.5 μm

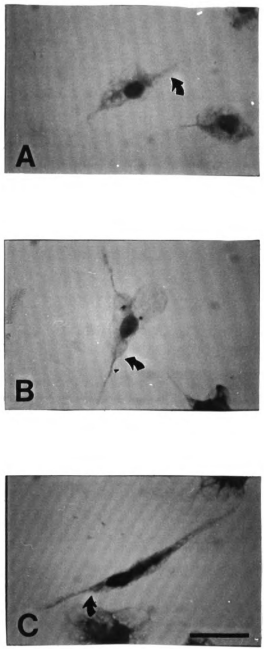

Fig 3. Light micrographs showing macrophages at 24h in normal medium after (A) no treatment, (B) cisplatin (10 μg/ml) and (C) "poly-plat" (10 μg/ml) treatment for 2h. Note the cytoplasmic extensions (arrows) after normal, cisplatin and "poly-plat" treatment. "Poly-plat" treated macrophages have a more developed extension formation compared to normal and cisplatin treated macrophages. Final magnification: ×2000 (A, B, C), Bar = 7.5 μm

TABLE 1. Number of human fibroblasts before and after drug treatments

Treatment	Cell numbers						
	before treatment	after treatment (hr)					
		0*	8	16	24	36	48
Control	0.88×10^6	0.89×10^6	1.02×10^6	1.10×10^6	1.47×10^6	1.58×10^6	2.13×10^6
CDDP	------	0.98×10^6	1.18×10^6	1.19×10^6	1.22×10^6	1.19×10^6	1.13×10^6
Poly-plat	------	1.01×10^6	1.25×10^6	1.27×10^6	1.35×10^6	1.25×10^6	1.16×10^6

* Specifies the number just after 2h of drug treatment, when the cells were placed in a normal medium without any drug. Results are averages of experiments done in triplicate.

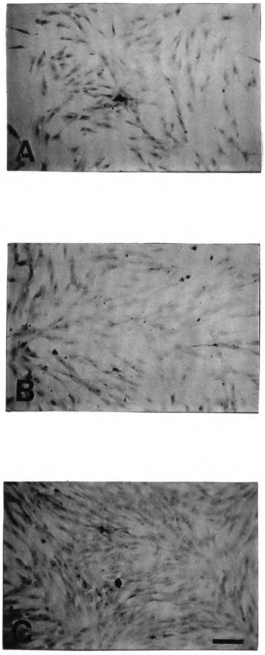

Fig 4. Light micrographs of human fibroblasts showing changes in the number of cells immediately after (A) no treatment, (B) cisplatin (10 μg/ml) and (C) "poly-plat" (10 μg/ml) treatment for 2h. Note the increase in cell numbers were observed immediately after cisplatin (0.98×10^6 cells/ml) and "poly-plat" (1.01×10^6 cells/ml) treatment. The cell numbers are much higher after "poly-plat", compared to cisplatin.
Final magnification: ×200 (A, B, C), Bar = 37.5 μm

Fig 5. Light micrographs showing human fibroblasts at 8h in normal medium after (A) no treatment, (B) cisplatin (10 μg/ml) and (C) "poly-plat" (10 μg/ml) treatment for 2h. Note the large increase (1.25×10^6 cells/ml) in cell numbers after 8h of "poly-plat" treated fibroblasts. Final magnification: ×200 (A, B, C), Bar = 37.5 μm

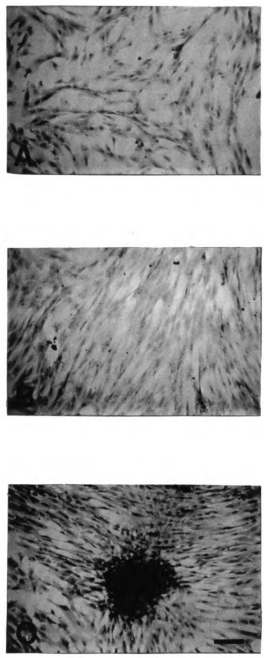

Fig 6. Light micrographs showing human fibroblasts at 16h in normal medium after (A) no treatment, (B) cisplatin (10 μg/ml) treatment for 2h, (C) "poly-plat" (10 μg/ml) treatment for 2h. Note the clump formation in "poly-plat" treated fibroblasts probably due to a large increase in cell number (1.27×10^6 cells/ml). Final magnification: ×200 (A, B, C), Bar = 37.5 μm

TABLE 2. Number of human ovarian teratocarcinoma cells before and after drug treatments

Treatment	Cell number								
	before treatment	after treatment (hr)							
		0*	8	16	24	36	48	72	96
Control	4.1×10^6	4.2×10^6	7×10^6	8.9×10^6	9.9×10^6	10.4×10^6	12×10^6	16.5×10	22.1×10^6
CDDP	------	4×10^6	3.9×10^6	2.6×10^6	1.7×10^6	0.5×10^6	0.41×10^6	0.4×10^6	0.4×10^6
Poly-plat	------	3.5×10^6	3.8×10^6	3.9×10^6	4.3×10^6	3.5×10^6	2.9×10^6	2.3×10^6	0.5×10^6

* Specifies the number just after 2h of drug treatment, when the cells were placed in a normal medium without any drug. Results are average of experiments done in triplicate.

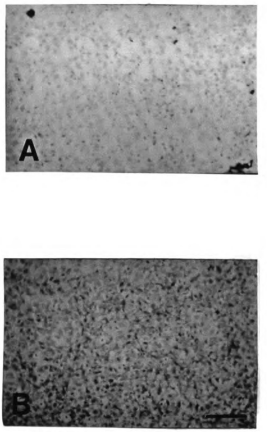

Fig 7. Light micrographs showing human teratocarcinoma cells in normal medium immediately before any drug treatment (Fig A: 4.1×10^6 cells/ml) and after 24h of incubation in normal medium without any drug treatment (Fig B: 9.9×10^6 cells/ml). Note the increase in cell numbers after 24h incubation. Final magnification: ×200, Bar = 37.5 μm

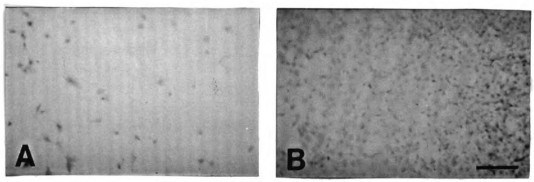

Fig 8. Light micrographs showing human teratocarcinoma cells at 24h in normal medium after (A) cisplatin (10 μg/ml) and (B) "poly-plat" (10 μg/ml) treatment for 2h. Number of cells greatly decreased (1.7×10^6 cells/ml) after cisplatin treatment, however, there was an increase in cell number (4.3×10^6 cells/ml) after "poly-plat" treatment. Final magnification: ×200 (A, B), Bar = 37.5 μm

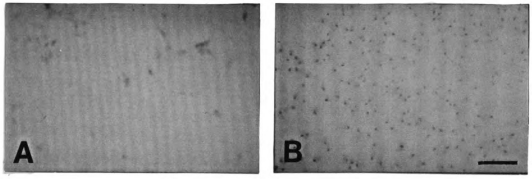

Fig 9. Light micrographs showing human teratocarcinoma cells at 48h in normal medium after (A) cisplatin (10 μg/ml) and (B) "poly-plat" (10 μg/ml) treatment for 2h. Note the continuous decrease (0.41×10^6 cells/ml) in cell numbers with cisplatin treatment while there was less of decrease in cell numbers (2.9×10^6 cells/ml) with "poly-plat" treatment. Final magnification: ×200 (A, B), Bar = 37.5μm

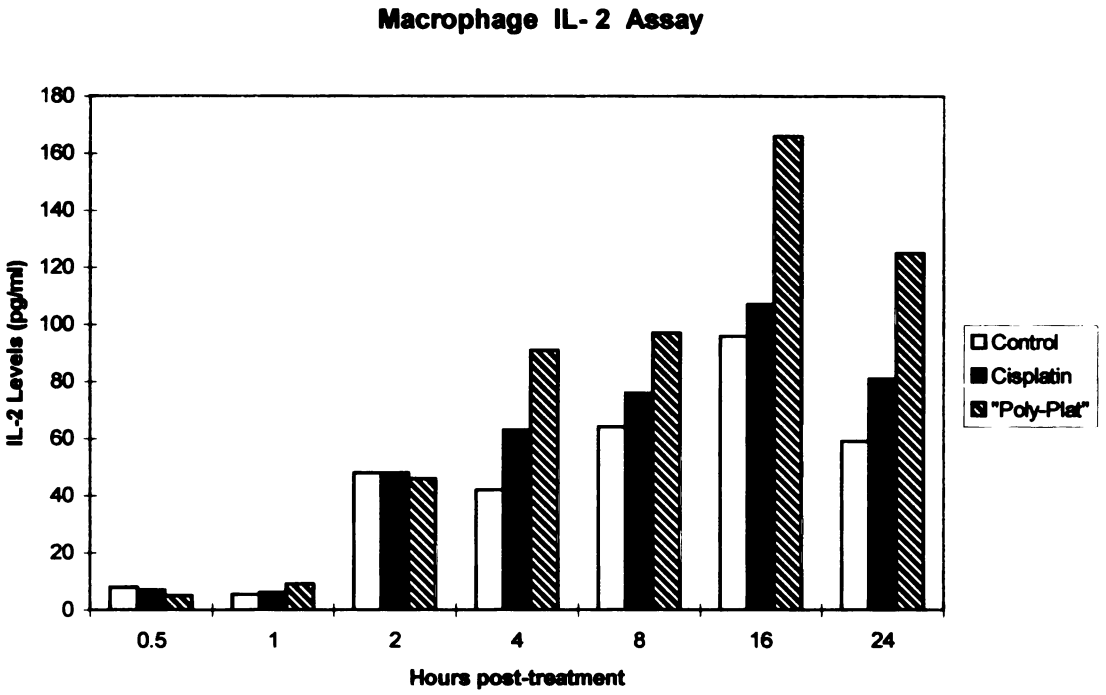

Fig 10. Bar graph* showing IL-2 release in the supernatants of macrophages treated with either cisplatin (10 μg/ml) or "poly-plat" (10 μg/ml) at various time intervals. Note the gradual increase in IL-2 for "poly-plat" treatment up to 16h post-treatment with the highest level at 16h of "poly-plat" treatment. Cisplatin treatment induced a gradual increase through 16h but is still less than that of "poly-plat" treatment.

* Each bar represents the mean from 2 sets of readings.

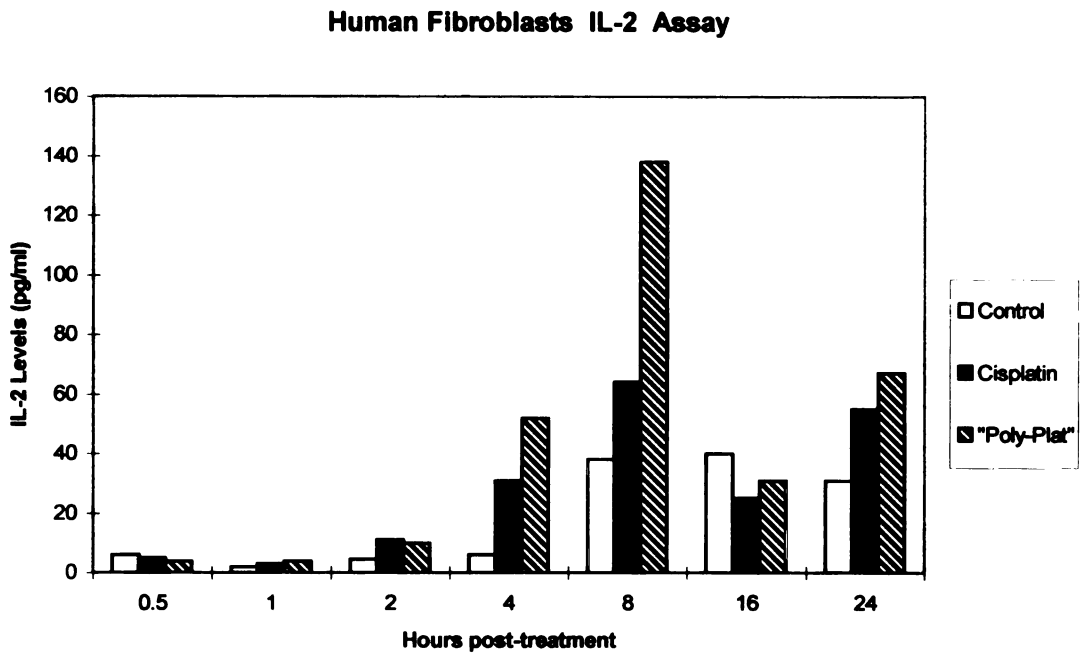

Figure 11. Bar graph* showing IL-2 release in the supernatants of human fibroblasts treated either with cisplatin (10 μg/ml) or "poly-plat" (10 μg/ml) at various time intervals. Note the sudden increase (138 pg/ml) after 8h of "poly-plat" treatment compared to cisplatin. After 16h of "poly-plat" treatment, Il-2 levels (31 pg/ml) dramatically decreased.

* Each bar represents the mean from 2 sets of readings.

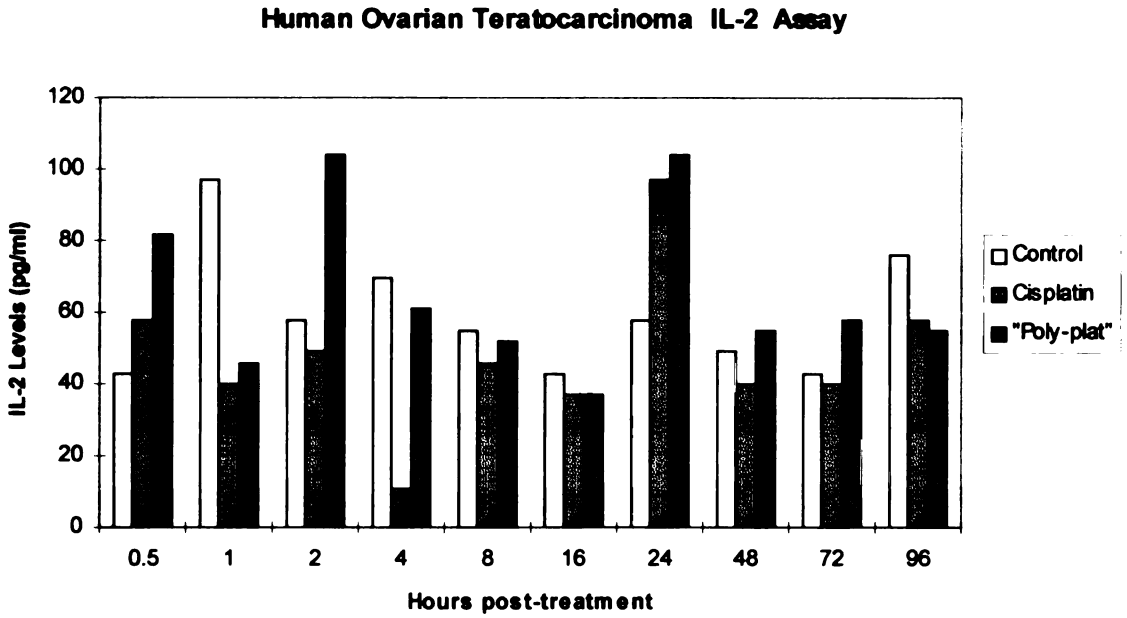

Figure 12. Bar graph* showing IL-2 release in the supernatants of human ovarian teratocarcinoma cells treated with either cisplatin (10 μg/ml) or "poly-plat" (10μg/ml) at various time intervals. Highest IL-2 level (104 pg/ml) was observed after 2h and 24h of "poly-plat" treatment. Cisplatin also induced IL-2 releases reaching a peak after 24h of treatment.

* Each bar represents the mean from 2 sets of readings.

DISCUSSION

Cisplatin, a broad spectrum anticancer drug, can activate murine peritoneal macrophages *in vitro* and *in vivo* (4, 6). Activated macrophages seek out tumor cells through cytoplasmic extensions and lysosomal transfer to the tumor cells causing tumor cell death (4). In order to increase the efficacy, second generation analogs of cisplatin have been reported. "Poly-plat", one such analog of cisplatin, has been shown to activate macrophages better than cisplatin by releasing significant levels of cytokines like IL-1α and TNF-1α (20, 21). IL-1 and TNF-1α have been known to be involved in direct antitumor activity and increase the expression of MHC (major histocompatibility complex) molecules on the cell surface (22, 23) .

In the study of macrophage activation, it has been demonstrated that macrophages activated by "poly-plat" increase cytoplasmic extension formation and also increase the release of cytokine, interleukin-1α, better than cisplatin (20). Other studies have shown that "poly-plat" generates 10 times the number of lysosomes compared to cisplatin, which are transferred to tumor cells through the cytocrine process (19).

In the present study, we have observed cytoplasmic extension formation within 2h after "poly-plat" treatment while cisplatin treatment induces only after 24h. It was observed that there was a greater release of IL-2 from macrophages after "poly-plat" treatment compared to cisplatin. Studies have demonstrated that the ability of IL-2 to destroy tumor cells is based on various effects on immune response to tumor cells through activation of cytotoxic lymphocytes and increased surface antigen or receptors on tumor cells involved in tumor cell recognition and lysis (24, 25). Since IL-2

stimulates the immune system (7, 26), investigators have used IL-2 gene expression vectors which have been transfected into fibroblasts in order to stimulate IL-2 release. Released IL-2 from the fibroblasts stimulate an increase in cell population of CD3+ CD56+ lymphocytes which are involved in tumor cell lysis(15).

"Poly-plat" can stimulate not only macrophages, but also non-immune system cells like human fibroblasts. Studies on cell population of fibroblasts show that a 53% increase in cell number was observed after 24h of "poly-plat" treatment, while there was only a 38% increase after cisplatin treatment. An increase in cell population of fibroblasts seems to be followed by maximum release of IL-2 (138 pg/ml). Recent studies have demonstrated that T cells are stimulated to express high affinity IL-2 receptor and secrete IL-2 in the presence of an antigen (27). The binding of IL-2 to IL-2 receptor signals the T cells to release IL-2 which in turn induces T cell proliferation (27). Since IL-2 receptors have been detected on human fibroblasts (28), it is possible that our observation of cell proliferation on human fibroblasts might be due to IL-2 interaction with IL-2 receptor.

Unlike murine macrophages or human fibroblasts, "poly-plat" treated human ovarian teratocarcinoma cells didn't show significant release of IL-2. "Poly-plat" treatment increased the release of IL-2 to a maximum level (104 pg/ml) after 2h of treatment, decreasing until 16h followed by another peak level after 24h. Correspondingly, tumor cell population decreased by 15% after 2h of "poly-plat" treatment with a slight increase (23%) subsequently through 24h. However, a significant decrease (88%) was seen until 96h post-treatment. It has been suggested that IL-2 interaction with IL-2 receptor results in cell proliferation of human breast

carcinoma cell line through autocrine regulation on the cells (29). Similar situations probably apply to tumor cells in our study.

IL-2 receptors consist of three structural domains (α, β, γ). When IL-2 binds IL-2 receptors, serine-rich region in the β chain seems to be involved in two signal transduction pathways: activating tyrosine kinase of src family and induction of c-myc (30). C-myc is the early response gene which is induced within 15 minutes of growth factor treatment. When growth factor is present, concentration of Myc protein is elevated and then reaches to a new steady value which triggers cells to escape G_o phase and to proliferate (31). Based on these findings, we suggest that proliferation of human fibroblasts and human ovarian tumor cells in our study might be due to the increased expression of c-myc followed by binding of IL-2 to IL-2 receptors.

Cells that are treated with anticancer agents activate an injury response that eventually induces cell death by apoptosis (32-36). After 24h of "poly-plat" treatment, an almost 3 times faster rate of tumor cell death was observed than with cisplatin treatment. One of the possible mechanisms in increasing cell death might be the involvement of nitric oxide (37, 38). Nitric oxide is generated in mammalian cells by the conversion of L-arginine to L-citrulline. Since nitric oxide released from activated macrophages has been demonstrated to be involved in killing tumor cells (37, 38), it is possible that nitric oxide might be released from "poly-plat" treated tumor cells (ovarian teratocarcinoma cells). Higher production of nitric oxide might have occurred after "poly-plat" treatment as compared to cisplatin treatment, thereby causing increased tumor cell death. Changes in the level of nitric oxide has not been studied after "poly-plat" treatment on ovarian cancer cells. Studies on the mechanisms which control

positive and negative regulation on the tumor cells needs to be investigated. Further studies of the level of interleukin-2 receptor and nitric oxide on the different tumor cells may be required to answer this question.

CONCLUSION

This study focused on treating various cell cultures (macrophages, human fibroblasts and human ovarian carcinoma cells) with cisplatin and "poly-plat" to see the difference in level of cytokine (IL-2) and also in cell numbers before and after treatments. "Poly-plat" treated macrophages stimulated cytoplasmic extensions more efficiently than cisplatin and also released significant levels of cytokine, IL-2. We have also found that "poly-plat" stimulates cell proliferation (human fibroblasts) followed by increased level of IL-2.

Based on these findings, we suggest that "poly-plat" is more effective in macrophages activation and also inducing higher levels of IL-2 from different cell cultures, compared to cisplatin.

REFERENCES

1 Bosl G, Lange P, Franley E, et al: Vinblastine, bleomycin and cis-diamminedichloroplatinum in the treatment of ovarian and testicular cancer. Am J med *68*: 492-496, 1980.

2 Williams S, Einhorn L: Cisplatinum in the treatment of testicular and other cancers. Adv Intern Med *27*: 531-545, 1982.

3 Heiger-Bernays W, Essigman J, Lippard S: Effect of the antitumor drug cisdiamminedichloropaltinum [II] and related paltinum complexes on eukaryotic DNA replication. Biochemistry *29*: 8461-8466, 1990.

4 Palma J, Aggarwal S, Jiwa A: Murine macrophages activation after cisplatin or carboplatin treatment. Anticancer drugs *3*:665-675, 1992.

5 Palma J, Aggarwal S: Cisplatin and carboplatin mediated activation of murine peritoneal macrophages in vitro; production of interleukin -1 alpha and tumor necrosis factor-alpha. Anti-Cancer Drugs *6*: 311-316, 1995.

6 Sodhi A, Singh S: Interaction between cisplatin treated macrophages and Dalton's lymphoma cells in vitro. Exp Cell Bio *56*: 1-11, 1989.

7 Smith K: T cell growth factor. Immunol Rev *51*: 337-357, 1980.

8 Ortaldo J, Mason A, Gerald J, Henderson LE, Farrar W, Hopkins R, Herberman R, Rabin H: Effects of natural and recombinant IL-2 on relgulation of IFN-γ, production and natural killer activity: lack of involvement of the TAC antigen for these immunoregulatory effects. J Immunol *133*: 779-783, 1984.

9 Trinchieri G, Matsumoto-Kobayashi M, Clark SC, Seehra J, London L, Perussia : Response of resting human peripheral blood natural killer cells to interleukin-2. J Exp Med *160*:1147-1169, 1984.

10 Lotzova E, Savary C, Herberman RB: Induction of NK cell activity against fresh human leukemia in culture with interleukin-2. J Immuol *138*: 2718-2727, 1987.

11 Rosenstein M, Yron I, Kaufmann Y, Rosenberg SA: Lymphokine activated killer cells: Lysis of fresh syngeneic NK-resistant murine tumor cells by lymphocytes cultured in interleukin-2. Cancer Res *44*: 1946-1953, 1984.

12 Kolitz JE, Mertelsmann R: The immunotherapy of human cancer with interleukin 2: present status and future directions. Cancer Invest *9*:529-542, 1991.

13 Weinberg A, Basham TY, Merigan TC: Regulation of guinea-pig immune functions by interleukin 2: critical role of natural killer activity in acute SV-2 genital infection. J Immunol *137*: 3310-3317, 1986.

14 Berstein ZP, Porter MM, Gould M, et al: Prolonged administration of low-dose interleukin-2 in human immunodeficiency virus-associated malignancy results in selective expansion of innate immune effectors without significant clinical toxicity. Blood *86*: 3287-3294,1995.

15 Engel B, Laws HJ, Buttlies B, Kahn T, Gobel U, Burdach S: Induction of a CD3+/CD56+ lymphocyte population following gene therapy with transgenic IL-2 secreting fibroblasts in a child with peripheral neuroectodermal malignancy. Medical and pediatric oncology *31*: 56-60, 1998.

16 Flemming CL, Patel PM, Box G, et al: Sarcoma cells engineered to secrete IFN-gamma or IL-2 acquire sensitization to immune cell killing via different mechanisms. Cytokine *9*: 328-332, 1997.

17 Sobol RE, Fakhrai H, Shawler D, et al: Interleukin-2 gene therapy in a patient with glioblastoma. Gene Ther *2*: 164-167, 1995.

18 Tohmatsu A, Okino T, Stabach P, Padula SJ, Ergin MT, Mukherji B: Analysis of cytolytic effector cell response in vitro against autologous human tumor cells genetically altered to synthesize interleukin-2. Immunol Lett *35*: 51-57, 1993.

19 Muenchen H, Aggarwal S, Misra H, Andrulis P: Enhanced immunostimulation by novel platinum anticancer agents. Anticancer Drugs *8*: 323-328, 1997.

20 Muenchen H, Aggarwal S: Immune system activation by cisplatin and its analog "poly-plat": an *in vitro* and *in vivo* study. Anticancer Drugs *9*: 93-99, 1998.

21 Muenchen H, Aggarwal S: Enhanced immune system activation after treatment with novel antineoplastic platinum agents. Anticancer research *18*: 2631-2636, 1998.

22 Asher A, Mule J, Rosenberg S: Recombinant human necrosis factor mediates regression of a murine sarcoma in vivo via Lyt-2^+ cells. Cancer Immunol Immunother *28*: 153-156, 1989.

23 Dejong R, Stokkers P, Lamme E, et al: Regulation of T-cell differentiation by CD2 and CD28 accessory molecules. Immunology *74*: 175-182, 1991.

24 Oppenheim JJ, Ruscetti FW, faltynek C: Cytokine. *In*: Basic and Clinical Immunology (Stites DP, Terr Al, eds). Singapore, Prentice Hall, 1991, pp 78-100.

25 Greenberg PD. Adoptive T cell therapy of tumors: Mechanisms operative in the recognition and elimination of tumor cells. Advances in Immunology. *49*: 281-335, 1991.

26 Gearing A, Thorpe R, Bird C, Spitz M: Human B cell proliferation stimulated by interleukin-2. Immunol Lett *9*: 105-108, 1985.

27 Fraser J, Irving B, Crabtree G, Weiss A: Regulation of interleukin-2 gene enhancer activity by the T-cell accessory molecule CD 28. Science *251*: 313-316, 1991

28 Plaisance S, Rubinstein E, Alileche A, et al: Expression of the interleukin-2 receptor on human fibroblasts and its biological significance. Int Immunol *4*: 739-746, 1992.

29 Katano M, Matsuo T, Morisaki T, et al: Increased proliferation of a human breast carcinoma cell line by recombinant interleukin-2. Cancer Immunology Immunotherapy *39*: 161-166, 1994.

30 Shibuya H, Yoneyama M, Ninomiya-Tsuji J, Matasumoto K, Taniguchi T: IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: Demonstration of a novel role for c-myc. Cell *70*: 57-67,1992.

31 Evan G, Littlewood T: The role of c-myc in cell growth. Curr Opin Genet Dev *3*: 44-49, 1993.

32 Dive C, Hickman JA: Drug-target interactions: only the first step in the commitment to a programmed cell death. Br J Cancer *64*: 192-196, 1991.

33 Fisher DE: Apoptosis in cancer therapy: crossing the threshold. Cell *78*: 539-542, 1994.

34 Hickman JA: Apoptosis induced by anticancer drugs. Cancer Metast Rev *11*: 121-139, 1992.

35 Dive C, Wyllie AH: Apoptosis and cancer chemotherapy. *In*: Cancer Chemotherapy (Hickman JA, Tritton TR, eds). Oxford, Blackwell, 1993, pp 21-56.

36 Hickman JA, Potten CS, Merritt AJ, Fisher TC: Apoptosis and cancer chemotherapy. *In*: The role of apoptosis in development, Tissue homeostasis and Malignancy (Dexter TM, Raff MC, Wyllie AH, eds). London, Chapman and Hall, 1994, pp 83-89.

37 Hibbs J, Taintor R, Vavrin Z: Macrophage cytotoxicity: role for L-arginine deaminase and amino nitrogen oxidation to nitrite. Science *235*: 473-476, 1987.

38 Stuehr D, Nathan C: Nitric oxide: A macrophage product responsible for cytostasis and repiratory inhibition in tumor target cells. J Exp Med *169*: 1543-1555, 1989.