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IMMUNE SYSTEM ACTIVATION AS A MECHANISM OF TUMOR INHIBITION FOLLOWING NOVEL PLATINUM DRUG ADMINISTRATION

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IMMUNE SYSTEM ACTIVATION AS A MECHANISM OF TUMOR INHIBITION FOLLOWING NOVEL PLATINUM DRUG ADMINISTRATION

By

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ABSTRACT

IMMUNE SYSTEM ACTIVATION AS A MECHANISM OF TUMOR INHIBITION FOLLOWING NOVEL PLATINUM DRUG ADMINISTRATION

By

Dale J. Telgenhoff

An effective method of destroying tumors in the body is through the activation of the immune system. Activated macrophages seek out tumor cells, make contact with cytoplasmic extensions, and transfer lysosomes to the tumor cell. Activated macrophages also release various cytokines (IL-1, IL-2, TNFα) which further activate the immune system and cause tumor necrosis. Cisplatin is a potent anti-neoplastic agent which works via three mechanisms: DNA crosslinking, inhibition of the mitotic spindle, and macrophage activation. Other drugs have been developed which are more potent at macrophage activation and much less toxic. These drugs, in particular Poly-plat, are much larger in their molecular structure compared to cisplatin, with numerous branching side chains radiating from a central platinum atom. In equal doses poly-plat has less platinum than cisplatin and is more effective with less toxicities.

In vivo treatment, using platinum antineoplastic agents results in an increase in macrophage activity, including an increase in extensions, cytolytic factors (cytokines), and numbers. The increase in the number of macrophages is a result of monocyte sequestration and differentiation. Activation of macrophages enhances the organism's ability to seek out and lyse tumor cells. Unfortunately, direct application of platinum compounds to the organism causes stress to the animal due to the drugs, of which gastrointestinal and nephrotoxicity are the dose limiting factors. By isolating leukocytes from an animal, treating them with activating drugs, then returning them to the animal we

saw a sequestration and activation response similar to that of treating the animal directly with the drug. Further, we did not see the toxicity caused by the antineoplastic agents since the drug itself was not being introduced. A decrease in the harsh side effects is beneficial to the patient, since they will not have to endure the pain and malaise associated with chemotherapy.

Dedicated to my wife Kelly and my daughter Gwen, for enduring.

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I wish to thank my advisor, Dr. Surinder Aggarwal, for his guidance and support throughout my years at Michigan State University. His patience and dedication encouraged my development as a researcher and as an instructor. I would also like to thank Drs. R. Neal Band, Will Kopachik, and Ashir Kumar for having served as committee members. Their ideas and review of manuscripts has been critical in the development of this dissertation.

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KEY TO ABBREVIATIONS

CDDP	Cisplatin
CdK	Cyclin Dependent Kinase
DAB	Diaminobenzidine
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbant Assay
FBS	Fetal Bovine Serum
G1	Growth Phase 1
G2	Growth Phase 2
HS	Horse Serum
IL-1	Interleukin-1
IL-2	Interleukin-2
KOH	Potassium Hydroxide
LPS	Lipopolysaccharide
MEM	Minimal Essential Medium
PBS	Phosphate Buffered Saline
NaCl	Sodium Chloride
NF-κB	Nuclear Factor κ B
PCNA	Proliferating Cell Nuclear Antigen
PMA	Phorbol Myristate Acetate
Poly-plat	(Poly-[(*trans*-l,2-diaminocyclohexane) platinum] carboxyamylose)
RPMI	RPMI Medium
S	Interphase

S.D.	Standard Deviation
SAP	(4-Hydroxy-α-sulfonylphenylacetato [*trans* 1,2 diaminocyclohexane] platinum II)
SDS	Sodium Dodecyl Sulfate
SSP	(5-sulfosalicylato-*trans*-[1,2-diaminocyclohexane] platinum)
TEM	Transmission Electron Microscopy
TNFα	Tumor Necrosis Factor α
TUNEL	TdT Mediated dUTP Nick-end Labeling
v/v	Volume/Volume
w/v	Weight/Volume
x g	Gravity

INTRODUCTION

Cisplatin (*cis*-Diamminedichloroplatinum II) is an inorganic water soluble platinum containing complex which has seen wide use as an anti-cancer agent (Figure 1) (Goodman et al. 1996). Since its development in 1965, it has been used to treat testicular, head, neck, bladder, and ovarian cancer with varying degrees of success (Abrams and Murrer 1993), (Bosl et al. 1980). Cisplatin's major mechanisms of action include inhibition of DNA synthesis by interstrand and intrastrand crosslinking, inhibition of cytokinesis, induction of apoptosis (Nehme et al. 1997), (Ferreira et al. 2000), and macrophage activation (Abrams and Murrer 1993), (Fichtinger-Schepman et al. 1986). Cisplatin appears to enter the cell by diffusion, where the chloride ions are lost by hydrolysis leaving two active ligand sites which are able to bind DNA forming interstrand and intrastrand crosslinks (Figure 2), which ultimately arrest the cell cycle at G1, S, or G2 (Fichtinger-Schepman et al. 1986) (Sundaralingam et al. 1985) (Gonzalez et al. 2001). These platinum-DNA adducts also alert DNA damage sensors (ATM, DNA-PKcs, and ATR), which phosphorylate p53, ultimately resulting in the activation of the caspase cascade and apoptosis (Porter et al. 1997).

Although cisplatin has been shown to be effective in treating various types of cancers (Abrams and Murrer 1993) drug resistance is a major problem, especially with repeated doses of the drug (Welters et al. 1998); (Beale et al. 2000). Factors affecting resistance include an increase in drug efflux, a decrease in drug influx, increases in glutathione levels, DNA repair, and drug tolerance (Henkels and Turchi 1999), (Beale et al. 2000), (Nishimura et al. 1996). Cisplatin is administered intravenously and has an initial half-life in the plasma of 25 to 50 minutes (Goodman et al. 1996).

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Cisplatin activates the immune system by inducing macrophages to form cytoplasmic extensions which seek out and phagocytose tumor cells (Palma and Aggarwal 1994). Activated macrophages also release various cytokines (IL-1, TNFα), which further activate the immune system and cause tumor necrosis (Basu et al. 1991; Muenchen et al. 1997). The activation of macrophages is probably accomplished through the release of nuclear factor kappa B (NF-κB) from its sequestration in the cytoplasm by IκB (Maldonado et al. 1997). NF-κB is a protein which, when released from the IκB complex, migrates to the nucleus and is responsible for the transcription of inflammatory cytokines (Brach et al. 1993), (Stacey et al. 1996).

One of the inflammatory cytokines also produced by activated macrophages is interleukin-2 (IL-2) (Burkitt and Aggarwal 2000). IL-2 is primarily secreted by activated T lymphocytes, but has been shown to be released in great quantities by fibroblasts and macrophages following treatment with platinum anti-cancer agents (Burkitt and Aggarwal 2000), (Telgenhoff and Aggarwal 2000). IL-2 activates cells with IL-2 receptors (T and B lymphocytes, natural killer cells, and macrophages) to proliferate (Sharon 1998) and differentiate into cells with increased immune system activity (Collins and Oldham 1995), (Ding et al. 1988). IL-2 has also been shown to induce monocyte differentiation into macrophages through activation of the macrophage colony stimulating factor gene (Brach et al. 1993), (Zhu et al. 1993). The use of IL-2 as a treatment for cancer has been shown to induce regression of metastatic cancers in selected patients that had failed on other chemotherapies (Rosenberg et al. 1988).

Cisplatin has been shown to activate Kupffer cells by increasing their number and cytoplasmic extensions (Telgenhoff and Aggarwal 1998). The Kupffer cells are the

resident macrophages in the liver, comprising the largest population of fixed tissue macrophages in the body (Xu et al. 1984). They are generally stellate in appearance, and reside in the sinusoids, interdigitating their filipodia in the endothelial lining (Wisse 1974). Kupffer cells constitute 15% of the total liver cell population in number but only 2.5% of the overall protein content (Kuiper et al. 1994). During episodes of hepatic stress, however, they have been shown to greatly increase in size and number (Toge et al. 1981), (Wisse 1974). Although self-replication has been observed with chronic hepatic stimulation, population increases during acute stimulation are generally attributed to monocyte differentiation (Bouwens and Wisse 1982),(van der Rhee et al. 1979a). Due to the increase in both size and number, the Kupffer cell population is better equipped to deal with hepatic insult and return the body to a state of homeostasis (McCuskey and McCuskey 1990), (Shi et al. 1996).

Other drugs have been developed which are more potent at macrophage activation and much less toxic (Fiebig et al. 1996), (Drees et al. 1995). Three novel second generation platinum containing antineoplastic agents have been developed; SAP (4-Hydroxy-α-sulfonylphenylacetato [*trans* 1,2-diaminocyclohexane] platinum II), SSP (5-sulfosalicylato-*trans*-[1,2-diaminocyclohexane] platinum), and Poly-plat (Poly-[(*trans*-1,2-diaminocyclohexane) platinum]-carboxyamylose) (Andrulis Pharmaceuticals, Bethesda, MD) (Figure 3) which have been shown in initial studies to be more effective in the treatment of cancer and less toxic than cisplatin (Muenchen et al. 1997). These drugs, in particular Poly-plat, are much larger than cisplatin, with numerous branching side chains radiating from a central platinum atom. In equal doses Poly-plat has less platinum than cisplatin, which is a possible reason for the decrease in

toxicity. Poly-plat has been shown to activate macrophages to produce inflammatory cytokines in greater quantities than cisplatin (Muenchen and Aggarwal 1998).

Typically macrophages have been activated by treating the animal with the drug (*in vivo*). *In vivo* treatment of macrophages with platinum antineoplastic agents results in an increase in macrophage activity, including an increase in extensions, cytolytic factors (cytokines), and numbers (Telgenhoff and Aggarwal 1998), (Bahadur et al. 1984), (Gupta and Sodhi 1987). The increase in the number of macrophages present is a result of monocyte sequestration and differentiation (van der Rhee et al. 1979b), (Tanner et al. 1982). Activation of macrophages enhances the organisms ability to seek out and lyse tumor cells (Adams and Hamilton 1988), (Bucana et al. 1976).

The objectives of the following research were to examine how platinum compounds affect an organism *in vitro* and *in vivo*, to understand the mechanisms of action of less toxic platinum compounds, to determine the ability of these drugs to enhance the immune response, and to use the stimulation of the immune system to treat tumor burdened animals. The first group of experiments involved examining the ability of cisplatin to induce changes in Kupffer cell morphology, with the aim of understanding the effectiveness of this compound at stimulating immune cells. Next we compared the activation of Kupffer cells by SAP, SSP, and Poly-plat to that seen following cisplatin treatment. We then examined the effects of treating both tumor cells and macrophages directly with Poly-plat. Based on the data from these experiments we hypothesized that immune cells treated with Poly-plat were better equipped to scavenge tumor cells. To show this we activated immune cells *in vitro* and used them to treat tumor burdened animals.

The following thesis builds the premise for cancer treatment which entails activating immune cells outside of the body and then returning them to the tumor burdened patient. In this way the patient never encounters the toxic effects of the drug. While adoptive transfer is not a novel concept (Wang et al. 1986), (Herscowitz et al. 1981), it has not been performed with platinum agents with known immunomodulatory effects. It is our hope the techniques developed here will be pursued to a greater degree in cancer immunotherapy.

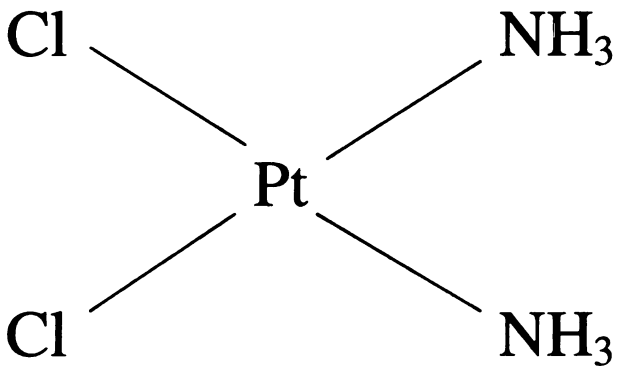


Figure 1: Structure of *cis*-diamminedichloroplatinum II (cisplatin)

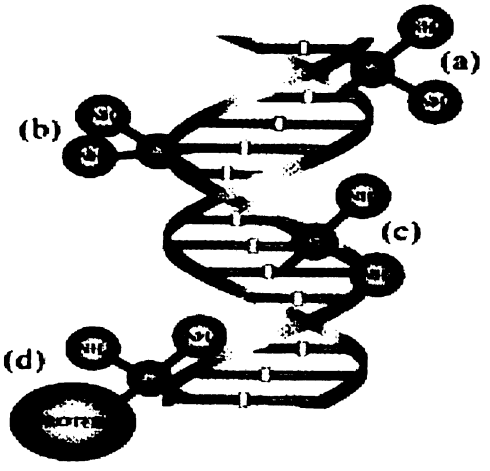
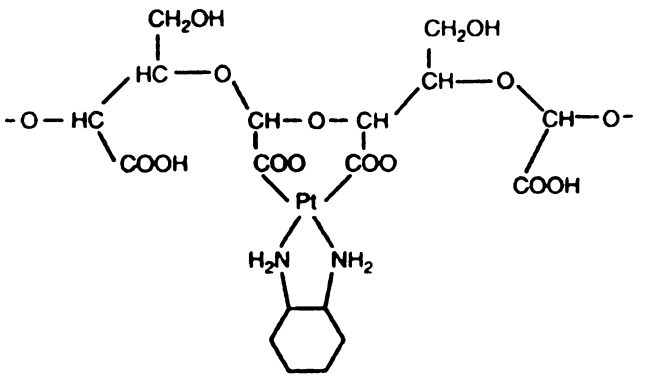


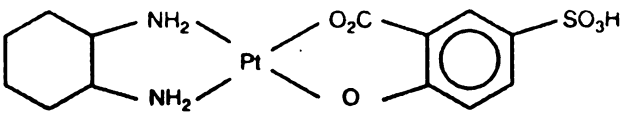
Figure 2: Main adducts formed in the interaction of cisplatin with DNA. (a), interstrand cross-link. (b), 1,2-intrastrand cross-link. (c), 1,3-intrastrand cross-link, (d), protein-DNA cross-link. (Gonzalez et al. 2001)

'Poly-plat'



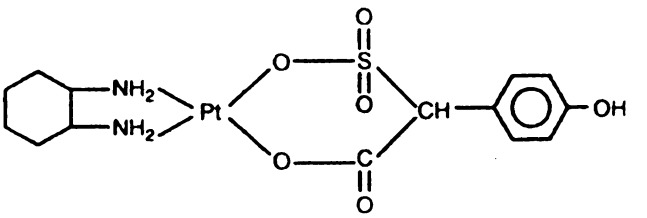
Poly-[(*trans*-1,2-diaminocyclohexane)platinum]-carboxyamylose

SSP



5-sulfosalicylato-*trans*-(1,2-diaminocyclohexane)platinum

SAP



4-hydroxy-α-sulfonylphenylacetato(*trans*
1,2-diaminocyclohexane)platinum(II)

Figure 3: Structure of novel anti-neoplastic agents SAP, SSP, and Poly-plat (Muenchen et al. 1997)

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CHAPTER 1: HISTOCHEMICAL AND MORPHOLOGICAL IDENTIFICATION OF KUPFFER CELLS ACTIVATED BY CISPLATIN

SUMMARY

Cisplatin is a potent anti-cancer agent which has been shown to activate Kupffer cells. These activated macrophages demonstrate an increase in cytoplasmic extensions, lysosomes, and peroxisomes elevating their anti-tumor activity. Wistar rats were treated with cisplatin (9 mg/kg) and sections of liver were excised for light and electron microscopic (TEM) analysis at 1, 6, 15, and 30 days post treatment. Non-specific esterase staining was used to differentiate Kupffer cells using light microscopy, while morphologic criteria were used for TEM analysis. Liver sections taken 6 days post cisplatin treatment showed the greatest number of activated macrophages, with the highest degree of activation.

INTRODUCTION

In inflammatory reactions the body utilizes a number of different mechanisms to restore homeostasis. In the case of organ inflammation the lymphoid system induces monocytes to enter the infected organ and differentiate into macrophages (Cohn 1978), (Ogawa et al. 1978), (van der Rhee et al. 1979a). These macrophages, while maintaining many of their monocyte characteristics, undergo a number of changes to make them more efficient scavengers of invading agents (Karnovsky and Lazdins 1978), (Ogawa et al. 1978), (Toge et al. 1981). An important change which occurs is an increase in the number of peroxisomes (van der Rhee et al. 1979b), which increase tumor cytotoxicity (Singh and Sodhi 1988).

The Kupffer cells are the resident macrophages in the liver, comprising the largest population of fixed tissue macrophages in the body (Xu et al. 1984). They are generally stellate in appearance, and reside in the sinusoids, interdigitating their filipodia in the endothelial lining (Wisse 1974a). Kupffer cells constitute 15% of the total liver cell population in number but only 2.5% of the overall protein content (Kuiper et al. 1994). During episodes of hepatic stress, however, they have been shown to greatly increase in size and number (Toge et al. 1981), (Wisse 1974a). Although self-replication has been observed with chronic hepatic stimulation, population increases during acute stimulation are generally attributed to monocyte differentiation (Bouwens and Wisse 1982),(van der Rhee et al. 1979a). Due to the increase in both size and number, the Kupffer cell population is better equipped to deal with hepatic insult and return the body to a state of homeostasis (McCuskey and McCuskey 1990), (Shi et al. 1996).

Cisplatin is a broad spectrum anti-cancer agent. Since its development in 1965, it has been used to treat testicular, head, neck, bladder, and ovarian cancer with varying degrees of success (Abrams and Murrer 1993), (Bosl et al. 1980). Cisplatin's major mechanisms of action include inhibition of DNA synthesis by interstrand and intrastrand crosslinking, inhibition of cytokinesis, and macrophage activation (Abrams and Murrer 1993), (Palma et al. 1992). Cisplatin activates the immune system by inducing macrophages to form cytoplasmic extensions which seek out and phagocytose tumor cells (Palma and Aggarwal 1994). More recently, cisplatin has been shown to activate Kupffer cells by increasing their number and cytoplasmic extensions (Telgenhoff and Aggarwal 1998).

In the present study electron microscopic analysis was utilized to examine the cytoplasmic extensions in greater detail. Morphological characteristics were used to determine Kupffer cell number and size, and these data were compared with light microscopic studies in order to obtain a better understating of cisplatin's effect on the Kupffer cell population.

MATERIALS AND METHODS

Animals. Six male Wistar rats (Charles Rivers Laboratories, Wilmington MA) weighing 160-180 grams were kept on a twelve hour light, twelve hour dark cycle with free access to feed and water. All rats were weighed two days prior to the first injection and each day thereafter until they were sacrificed on day eight for light microscopic studies. The rats received an intraperitoneal injection of cisplatin (1.8 mg/kg with .85% (w/v) normal saline as the vehicle of injection) for five consecutive days beginning on day three and continuing through day seven. Controls received only the normal saline.

Tissue Preparation. On day eight the rats were euthanized using carbon dioxide, followed by decapitation using the guillotine. The liver was excised and cut into small blocks (1 cm^3) and mounted on a cryostub in OCT mounting media at -20° C (Miles Scientific, Elkhart, IN). The frozen tissue was sectioned at -20° C using a slow fluid motion. Two sections (7-10 μm) of each treatment were collected on glass coverslips. The sections were then air dried. They were immersed in a fixative consisting of 1% (v/v) glutaraldehyde, 5% (w/v) sucrose in 0.5M cacodylate buffer, pH 7.2, for 30-60 seconds at 4° C. Following fixation, the coverslips were washed in sodium cacodylate buffer.

Esterase Staining. A diazonium staining method was employed with Napthol AS-LC-Acetate (Sigma, USA) as the substrate at a pH of 7.1. The sections were immersed in the enzyme incubation medium for 40 minutes at 37° C. Following incubation, the sections were washed briefly in distilled water, then mounted onto glass slides using glycerin jelly.

The sections were examined for non-specific esterase staining using a Zeiss III photomicroscope. The population of macrophages in each of the sections were quantitatively rated based on the number of stained macrophages present within the tissue. These cells were counted per unit area using the same magnification with each section. Cell morphology was also examined by focusing through the 10 μm section.

Electron Microscopy. For electron microscopy studies nine male Wistar rats were treated as described but were followed through a thirty day post-treatment period. On days 8, 13, 22, and 37 the cisplatin treated rats (days 8 and 37 for the control) were injected with Equithesin and opened via abdominal incision. A 22 gauge catheter attached to a 0.85% (w/v) normal saline (room temperature, 70 cm) perfusion apparatus was inserted into the left ventricle. A bag containing 2% (v/v) paraformaldehyde in phosphate buffer (room temperature, 70 cm) was attached to the apparatus via a distal port. The perfusion was started and the right atrium was cut to allow drainage. After 90 seconds the saline line was closed and the paraformaldehyde line was opened. After 15 minutes of perfusion fixation the liver was excised and transferred to a petri dish. The periportal region was excised and transferred to another dish containing 2% (v/v) glutaraldehyde in PBS (pH 7.3). The liver was diced into 1 mm segments and washed in cold PBS for peroxidase staining, using the DAB method. The tissues were then post-fixed in 1% (w/v) OsO_4 in PBS (pH 7.3) for 60 minutes at 4° C, followed by three washings in 0.1 M sodium acetate. The tissues were then washed in a graded acetone series and embedded in araldite.

The sectioning was performed on the LKB Ultramicrotome and collected on copper mesh grids, which were then stained with uranyl acetate and lead citrate. The tissues were viewed on the Phillips CM-10 TEM.

RESULTS

Light Microscopy. The esterase stained control sections showed distinct macrophage staining within the hepatic sinusoid (Figure 1a). The cells appeared discoid in shape, without cytoplasmic extensions radiating from the cell body. Sections observed from the cisplatin treated animals (Figure 1b) showed a ten fold increase in the macrophage population, compared to the controls (Table 1). Also, the degree of cytoplasmic extensions radiating from the cell body were greatly increased (Figure 1b, insert). In transmission electron microscopic studies, normal Kupffer cells are visualized spanning the sinusoidal lumen with extensions interdigitating into the Space of Disse (Figure 2). Identification of the Kupffer cells was based on peroxidase staining using the DAB method, which created electron dense deposits in the Kupffer cells, enabling differentiation from other sinusoidal cell types. In cases where the DAB did not penetrate thoroughly, morphologic characteristics unique to Kupffer cells were used for identification. These included location in the sinusoid, nuclear shape, vermiform bodies, and annulate lamellae. The organelles visible in the cytoplasm include mitochondria, golgi apparatus, lysosomes, peroxisomes, and endoplasmic reticulum.

Electron Microscopy. Morphologically, the cisplatin treated liver showed an increase in the size and number of Kupffer cell extensions compared to the control (Figure 3). The cytoplasmic to nuclear ratio was twice as much as in the control tissue. Also noted was a three-fold increase in organelles in the treated cells over the controls (Table 1). This included an increase in mitochondria, lysosomes, peroxisomes, and various other vacuoles. Extensions, which increased one day post cisplatin treatment (Figure 4a) were more prevalent in Kupffer cells at day six (Figure 4b), and decreased in

at days fifteen (Figure 4c) and thirty (Figure 4d). Other signs of increased activation such as increase in Kupffer cell numbers, mitochondria, electron lucent and dense organelles, and vermiform bodies were much more prevalent in day six tissues over any other time. Also seen in tissue six days post treatment was cell to cell interaction between the Kupffer cells and natural killer (pit) cells (Figure 5). This type of interaction was not seen in any other treatment mode.

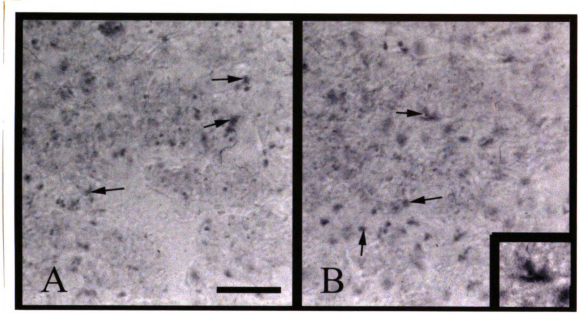


Figure 1 Light micrograph showing esterase staining within the hepatic sinusoid of the control tissue (A) and the cisplatin treated tissue (B). Note the increase in Kupffer cells (arrows) and increase in extensions (insert, 2x) following cisplatin treatment. (Bar = 50 μm).

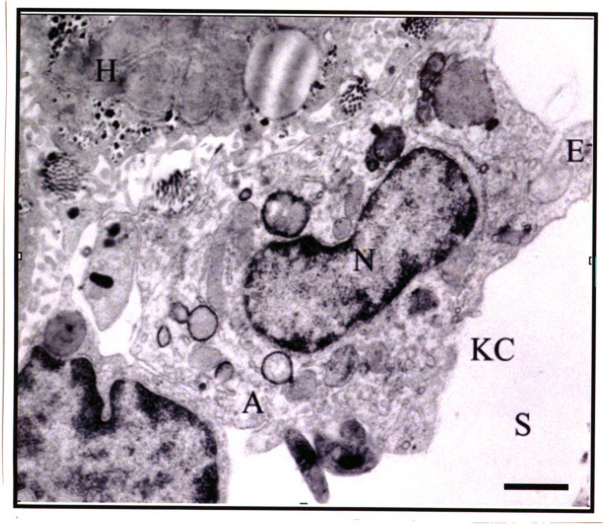


Figure 2 Normal Kupffer cell (KC) in the sinusoidal lumen (S). Very few extensions (E) are exhibited. *N*, nucleus; *A*, annulate lamellae; *H*, hepatocyte (Bar = 1.0 μm).

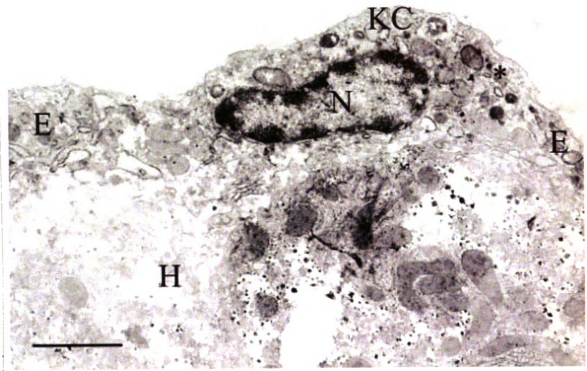


Figure 3 Cisplatin activated Kupffer cell (KC) along the endothelial lining. Extensions (E) can be seen branching out from the center. An increase in lysosomes and peroxisomes (*) is also noted. *N*, nucleus; *H*, hepatocyte (Bar = 3.0 μm).

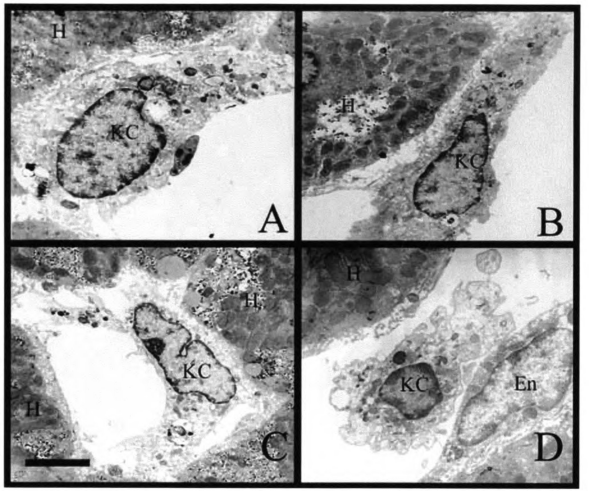


Figure 4 After one day post-cisplatin treatment (A) the Kupffer cells on average exhibited one or more extensions. Six days post-cisplatin treatment (B) showed the highest increase in numbers, extensions, and cytoplasmic organelles. These signs of activation were decreased by day fifteen (C), and returned to normal by day thirty (D). *KC*, Kupffer cell; *H*, hepatocytes; *En*, endothelial cell.
(Bar: A = 1.5 μm, B = 1.9 μm, C = 2.6 μm, and D = 1.5 μm)

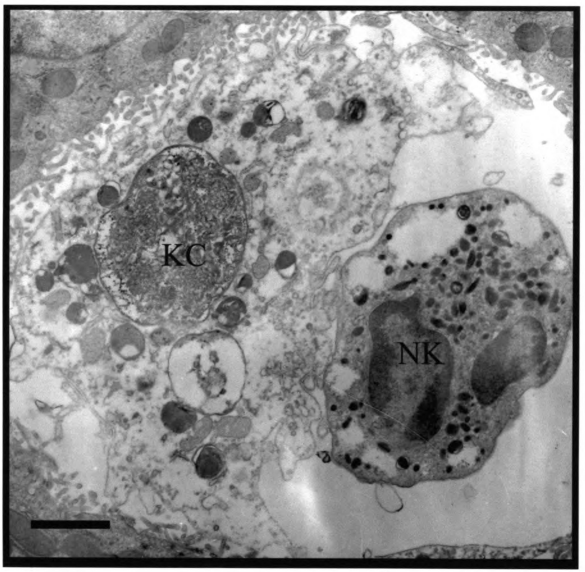
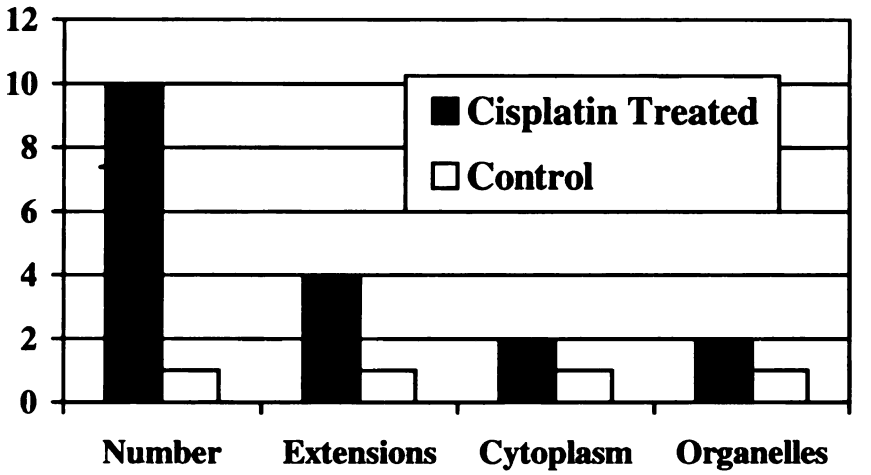


Figure 5 Hepatic sinusoid six days post-cisplatin treatment showing interaction between activated Kupffer cell (KC) and natural killer cell (NK) (Bar = 1.5 μm).



Treatment	Average Number of Cells per Viewing Area	Average Number of Extensions	Cytoplasm to Nuclear Ratio	Number of Organelles
Normal Saline	2.2 ± 0.4	0.7 ± 0.5	2.2:1	13 ± 2.5
Cisplatin (9 mg/kg)	19.8 ± 4.0	3.2 ± 1.1	4.1:1	27 ± 6.8

Figure 6 Graph showing the proportional changes in the liver Kupffer cells following cisplatin treatment. The number of Kupffer cells increased nearly ten-fold (based on esterase staining). Other changes include increases in extension formation, cytoplasm, and cellular organelles (based on TEM images). Actual changes are recorded in table below graph and are averages based on 50 areas viewed per treatment.

DISCUSSION

The staining pattern observed in light microscopy was the result of the non-specific esterases found in the tissue. The mammalian liver has been shown to exhibit one of the highest concentrations of these esterases compared to other organs, thus making esterase staining a valuable tool for the observation of the various cell types (Smedsrod et al. 1985). Specifically, cells of monocyte lineage can be identified due to the abundance of histochemically detectable esterases within the cytoplasm (ten Hagen et al. 1996). These cells are differentiated from the positively staining hepatocytes due to their location in the sinusoid. Difficulty arises when trying to quantify the number of Kupffer cells using this method in non-perfused tissue, as circulating monocytes will also stain positive (van der Rhee et al. 1977). Peroxidase staining can be used to differentiate Kupffer cells from monocytes, but hepatocytes do stain, making difficulties in counting (Bouwens et al. 1986), (Wisse 1974b). Antibodies against Kupffer cells and in-situ hybridization techniques are also effective ways to visualize and quantify these cells, but these techniques vary in reproducibility and can be very expensive (Bodenheimer et al. 1988), (ten Hagen et al. 1996).

Electron microscopic visualization is the most effective method of differentiation due to the unique morphological qualities of the Kupffer cells. Their location, size, and overall appearance are fairly constant in both scanning and transmission electron microscopy (Toge et al. 1981), (Wisse 1977). More specifically, they contain organelles found exclusively in Kupffer cells; bristle coated micropinocytic vesicles and vermiform bodies (Wisse 1974b). Using these morphologic characteristics we were able to determine the Kupffer cell population did not increase as dramatically as previously

described (Johnson and Aggarwal 1996). In fact, in tissues one day post-treatment there is no noticeable increase in Kupffer cells numbers compared to the control. The previous estimation was due to positive esterase staining in circulating monocytes, contributing to the overall number. These monocytes migrated to the liver, but had not differentiated into macrophages (Tanner et al. 1982).

It is not until six days post-treatment that we see an overall increase in the number of Kupffer cells. On average, each section of liver revealed a three-fold increase in Kupffer cells over the control. Self proliferation of Kupffer cells is impossible due to the inhibition of cell division in cisplatin treated tissue (Rosenberg et al. 1965), therefore the increase in numbers are solely the result of monocyte differentiation. These Kupffer cells are more active in phagocytosing invader cells, due to their increase in cytoplasmic extensions (Toge et al. 1981). These extensions have been shown to seek out and either phagocytose invader cells, or present them to natural killer cells for destruction (Malter et al. 1986).

Macrophage activation is a rapid event, usually occurring within 1-2 days after initial administration of the activating agent (Cohen et al. 1986), (Drysdale et al. 1983), (Malter et al. 1986). Monocytic differentiation into macrophages, however, takes a longer period of time (van der Rhee et al. 1979a). This is in part due to the need to migrate to the area of differentiation, then develop the attributes of the specific tissue macrophage. For cisplatin treated tissues, we have shown this differentiation occurs between one and six days post-treatment. After the six day peak, the size and number of Kupffer cells begins to return to pre-treatment levels, and by day thirty return to normal.

CONCLUSION

Kupffer cells are activated by the anti-cancer agent Cisplatin. Activation entails two events, each working independently. These events are macrophage activation and monocyte recruitment. Macrophage activation occurs rapidly, with noticeable changes in shape and function occurring within one day post treatment (Toge et al. 1981). Monocyte recruitment is a more lengthy process (van der Rhee et al. 1979a). Monocyte migration occurs within one day post-treatment, but differentiation takes up to five days post-treatment before the optimal number of Kupffer cells have been produced. The liver, in an effort to restore homeostasis, regulates the number of Kupffer cells, reducing them by day fifteen and returning them to their pre-treatment numbers by day 30. The exact mechanism of this regulation is unknown.

Due to the increase in numbers and cytoplasmic extensions, the Kupffer cell population is better equipped to deal with hepatic insult. Activated Kupffer cells, efficient in phagocytosing invader cells, are effective scavengers of invading organisms in the bloodstream due to their location in the sinusoid (Malter et al. 1986), (Ohnishi et al. 1982). An increased understanding of the mechanisms controlling Kupffer cell size and number can lead to more effective treatment of liver distress.

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CHAPTER 2: KUPFFER CELL ACTIVATION AFTER TREATMENT WITH CISPLATIN AND ITS SECOND GENERATION NOVEL ANALOGS SAP, SSP AND POLY-PLAT

SUMMARY

SAP (4-Hydroxy-α-sulfonylphenylacetato [*trans* 1,2-diaminocyclohexane] platinum II), SSP (5-sulfosalicylato-*trans*-[l,2-diaminocyclohexane] platinum), and Poly-plat (Poly-[(*trans*-l,2-diaminocyclohexane) platinum]-carboxyamylose) are second generation analogs of cisplatin (CDDP) with higher efficacy than cisplatin. *In vitro* studies using these compounds have shown activation of peritoneal macrophages in terms of various cytokines secreted. The present study was undertaken to examine the effects of these new compounds on Kupffer cell activation and their role in detoxification.

Wistar rats (100-120g) were given intraperitoneal injections of either cisplatin (9 mg/kg), SAP (10 mg/kg), SSP (10 mg/kg), or Poly-plat (10 mg/kg) in 0.9% (w/v) normal saline over 5 days. Controls received equal amounts of 0.9% normal saline. On day 6 the animals were sacrificed and the livers fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). Liver tissues were post-fixed in 1% (w/v) osmium tetroxide, dehydrated, and embedded for electron microscopy. Thick sections (1 mm) were placed in KOH for 15 minutes to remove araldite, and stained with hemotoxylin and eosin for light microscopy.

Light microscopy studies revealed an increase in the non-parenchymal cells in the treated tissues compared to the normal. Also evident under light microscopy was an increase in extension formation. The most obvious increases occurred with all three analogs with respect to cisplatin. Transmission electron microscopy disclosed increases in size, extensions, and cytoplasmic contents in treated tissues compared to the control. Extension formation was greatest in the Poly-plat treated tissues, showing more

extensions per Kupffer cell on average than any other treatment. There was also a corresponding increase in cytoplasm, lysosomes, and mitochondria beyond other treatments.

These results show SAP, SSP, and Poly-plat exhibit an increased ability to activate the immune system compared to the widely used antineoplastic drug cisplatin. Of the three compounds, Poly-plat appears to be the most promising analog for immune system activation.

INTRODUCTION

An effective method of destroying tumors in the body is through the activation of the immune system (Bahadur et al. 1984), (Zacharchuk et al. 1983). Activated macrophages seek out tumor cells, make contact with cytoplasmic extensions, and transfer lysosomes to the tumor cell (Palma and Aggarwal 1995). Cisplatin is a potent anti-neoplastic agent which works via macrophage activation (Abrams and Murrer 1993). Other drugs have been developed which are more potent at macrophage activation and much less toxic (Fiebig et al. 1996), (Drees et al. 1995). Three novel second generation platinum containing antineoplastic agents have been developed; SAP (4-Hydroxy-α-sulfonylphenylacetato [*trans* 1,2-diaminocyclohexane] platinum II), SSP (5-sulfosalicylato-*trans*-[l,2-diaminocyclohexane] platinum), and Poly-plat (Poly-[(*trans*-l,2-diaminocyclohexane) platinum]-carboxyamylose) (Andrulis Pharmaceuticals, Bethesda, MD) which have been shown in initial studies to be more effective in the treatment of cancer and less toxic than cisplatin (Muenchen et al. 1997). These drugs, in particular Poly-plat, are much larger than cisplatin, with numerous branching side chains radiating from a central platinum atom.

The present study was undertaken to examine the effects of these new compounds on Kupffer cell activation in the liver as well as non-specific esterase activity in the hepatocytes. Circulating monocyte levels were also examined to correlate increases in resident macrophages with monocyte levels.

MATERIALS AND METHODS

Animals. Wistar rats (125-135 grams) were kept on a twelve hour light, twelve hour dark cycle with free access to feed and water. All rats were weighed two days prior to the first injection and each day thereafter until they were sacrificed. Three rats each were given intraperitoneal injections of either cisplatin (9 mg/kg), SAP (10 mg/kg), SSP (10 mg/kg). or Poly-plat (10 mg/kg) in 0.9% (w/v) normal saline over 5 days. Controls received equal amounts of 0.9% normal saline. Tail vein blood draws were performed each day throughout the experiment. 22 gauge needles were inserted into the tail veins of the rats and 0.5 ml was withdrawn. Blood was smeared onto slides and air-dried. Monocyte levels were determined morphologically by Giemsa staining of air-dried blood smear slides. The number of monocytes per viewing area were counted and averaged for 25 areas per treatment.

Tissue Preparation. On day 6 the animals were anesthetized with carbon dioxide and the animal was opened via abdominal incision. The livers were removed and either fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for TEM analysis or were placed in OCT compound and frozen using dry ice and acetone for light microscopy. Tissue fixed in glutaraldehyde was post-stained in 1% (w/v) osmium tetroxide, dehydrated through a graded acetone series, and embedded in araldite for transmission electron microscopic observation. Sections were cut on the ultramicrotome, stained with uranyl acetate and lead citrate, and examined on the Phillips CM-10 TEM. Frozen tissue was cut at 10 μm thickness on the cryotome and stained for the demonstration of non-specific esterase using the azo dye method [3]. Stained sections were viewed and staining intensity determined on the Zeiss laser scanning microscope.

RESULTS

Weight Changes. Animals given only the vehicle of injection continued to gain weight throughout the course of the experiment, with an average weight gain of 35 grams. Animals treated with platinum compounds gained weight initially, but reached a plateau three days after treatment. On average these animals only gained 20 grams. Cisplatin treated rats gained weight initially, but after day three steadily lost weight, for an average weight gain of only 5 grams (Table 1).

Circulating Monocyte Levels. Monocyte levels following treatment with cisplatin or Poly-plat decreased following treatment with the platinum agents, possibly due to sequestration of the monocytes into resident macrophages (Figure 1). In the Poly-plat treated rats this decrease was followed by a sharp increase indicating an immune response to the decrease. Cisplatin treatment caused a more lengthy suppression of circulating monocyte levels. Monocyte levels in SAP and SSP treated animals were similar to the control (not shown).

Gross Morphology. Prior to euthanasia animal behavior was monitored and recorded. Animals receiving cisplatin appeared listless after the third day of injection, which continued until the end of the experiment. Cisplatin treated animals ate very little food, and had loose, infrequent stool. All other animals treated appeared normal, with food intake similar to the untreated rats. After euthanasia the internal organs were removed and examined (Figure 2). The rats treated with cisplatin had bloated stomachs, filled with undigested food. The intestines had a slippery feel, with a slime-like coating not seen in other rats. The kidneys of the cisplatin treated rats were slightly smaller than untreated rats, and had a grainy appearance on the surface. All other platinum

compounds had a morphology similar to the untreated rats.

Morphology. In the normal liver Kupffer cells were round and exhibited few extensions (Figure 3a). Cisplatin treatment demonstrated an increase in Kupffer cell extensions (Figure 3b). Extension formation following SAP and SSP treatment was similar to CDDP (Not Shown). Poly-plat treatment, however, revealed an even more pronounced activation with a greater increase in extension formation (Figure 3c). Quantification of Kupffer cells was performed using non-specific esterase staining. The normal liver exhibited a moderate amount of esterase staining, and was used as a standard to compare the drug treated against (Figure 4a). The CDDP treated liver had an increase in esterase staining in the sinusoids, and a significant decrease in the hepatocytes (Figure 4b). SAP treatment showed decreased staining in the sinusoids and increased staining in the hepatocytes (Figure 4c). SSP treatment had decreases in staining in both the sinusoids and the hepatocytes (Figure 4d). Poly-plat treatment demonstrated a significantly higher degree of staining in the sinusoids than CDDP, and a moderate amount of staining in the hepatocytes, comparable to normal liver (Figure 4e).

Table

Weight Changes Following Platinum Drug Administration

Days of Treatment

Treatment	1	2*	3*	4*	5*	6*	7	8
Control	135	139	143	148	155	162	164	170
Cisplatin	130	139	143	150	146	142	140	135
SAP	132	137	142	145	146	149	152	154
SSP	132	138	140	141	143	141	148	150
Poly-plat	131	136	146	150	152	154	153	155

Table 1: Changes in weight of rats following platinum drug administration. The values listed are the averages of three rats per treatment group. Asterisks denote days of treatment.

Figure

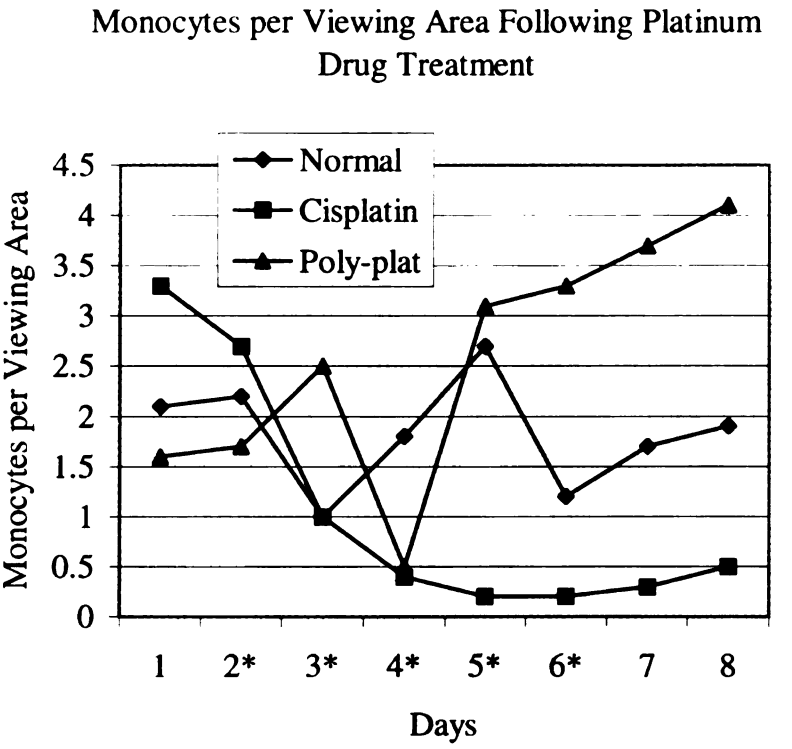

Figure 1: Circulating monocyte levels following treatment with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml). Note the decrease in monocytes following treatment with the platinum agents, followed by a sharp increase indicating an immune response to the decrease. Cisplatin treatment caused a more lengthy suppression of circulating monocyte levels.

Figure

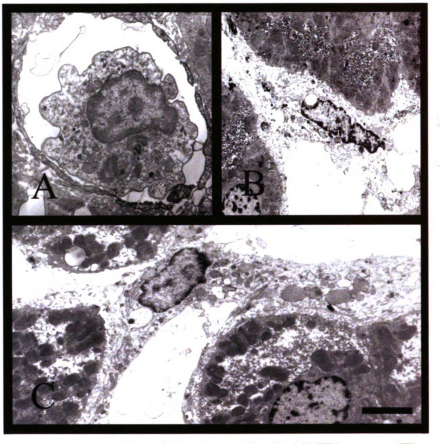

Figure 3: Kupffer cells from control (A), cisplatin (B), and Poly-plat (C) treated rat liver. Note the large extensions evident in the Poly-plat treated liver. Bar = 2 μm.

Figur

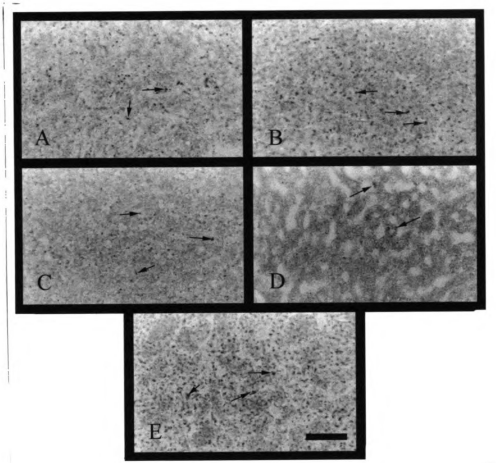

Figure 4: Light micrographs showing esterase staining in the liver of rats which received no treatment (A) or treatment with cisplatin (B), SAP (C), SSP (D), or Poly-plat (E). Black arrows denote Kupffer cells. Note the increase in the number of Kupffer cells in the cisplatin and Poly-plat treated tissues. The SAP and SSP treatments were decreased below normal. The hepatic esterase (background staining) was decreased following cisplatin and SSP treatment. Hepatic esterase was normal in SAP and Poly-plat treated tissues. Bar = 50 μm.

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Table

Summary of The Effects of Platinum Agents

Treatment	Extension Formation	Sinusoidal Esterase	Hepatic Esterase
Normal	None	+ +	+ +
Cisplatin	Increased	+ + +	+
SAP	Increased	+ ½	+ + +
SSP	Increased	+	+
Poly-plat	Large Increase	+ + + +	+ +

Table 2. Kupffer cell extension formation and non-specific esterase staining in treated and untreated rat liver. Staining intensity was measured visually and an average score was obtained from 10 slides per treatment using these ratings: (-) None (negative control), (+) Low, (+ +) Moderate, (+++) High, (++++) Very High. Data collected for extension formation from 50 TEM images per treatment group. Sinusoidal and hepatic esterase data collected from 25 light microscopy images per treatment group.

DISCUSSION

Stimulation of the immune system is a major function of many antineoplastic agents. Cisplatin stimulates the immune system through macrophage activation (Palma et al. 1992), making the macrophages more efficient in phagocytosing tumor cells. The severe toxic side effects evident in cisplatin treatment seem to be due to the disruption in sulphydryl containing mitochondrial dehydrogenase enzymes. The disruption of these enzymes prevents calcium uptake thereby reducing calcium levels in the cell to cytotoxic levels (Aggarwal 1993). SAP, SSP, and Poly-plat show no such disruption, and as a result have been shown to be much less toxic.

Due to low toxicity SAP, SSP, and Poly-plat would appear to be alternatives to CDDP. SAP and SSP both activate macrophages, however there is no significant increase in Kupffer cell numbers after SAP and SSP treatment, as shown by sinusoidal esterase staining. Poly-plat not only increases Kupffer cell activation, but also increases the number of Kupffer cells. Non-specific esterases catalyze the breakdown of ester bonds, which is a major mechanism of detoxification in the liver (Williams 1985). SAP, SSP, and Poly-plat all contain ester bonds, and a decrease in hepatocellular esterase would result in increased circulating concentrations of unhydrolyzed drugs. It is unknown whether these drugs are active in their natural or biotransformed state. If ester hydrolysis is required for drug activation, then the decreased levels of esterase found in SSP treated liver may result in a reduced pharmacological effect. Conversely, the normal levels of esterase found in Poly-plat treated hepatocytes may explain the increased activation seen in Kupffer cells after Poly-plat administration. It is unclear why Kupffer cell numbers are low in SAP treated livers, yet hepatic cell esterase levels are higher than

found in the normal tissue. These results show that Poly-plat is more effective than cisplatin, SAP, or SSP in macrophage activation, with less toxic side effects compared to cisplatin.

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CHAPTER 3: EFFECTS OF POLY-PLAT ON TUMOR CELLS *IN VITRO*

SUMMARY

The action of poly-[(trans-1,2-diaminocyclohexane)platinum]-carboxyamylose (Poly-plat) on tumor cells was examined using human fibrosarcoma (HT1080) and Walker rat carcinoma (WRC-256) in culture. These cells were treated with Poly-plat (10 μg/ml) or cis-dichlorodiammine platinum (cisplatin) (10 μg/ml) for 2-5 days. Apoptosis assays (morphology and TdT-mediated dUTP nick-end labeling) were used to determine cytotoxic effects of drugs on tumor cells. Cell protein extracts were collected and analyzed for the cell cycle regulatory proteins p27 (an inhibitor of cell cycle progression) and Proliferating Cell Nuclear Antigen (a marker of cell proliferation) via Western blot analysis. The results demonstrate the ability of Poly-plat to arrest the growth of tumor cells but not induce apoptosis in these cell lines.

INTRODUCTION

Cisplatin, a potent anti-neoplastic agent, works via inhibition of DNA replication (Fichtinger-Schepman et al. 1986), inhibition of the cytokinesis (Abrams and Murrer 1993), induction of apoptosis (Nehme et al. 1997); (Ferreira et al. 2000), and macrophage activation (Palma et al. 1992). Cisplatin exerts its cytotoxicity via the formation of interstrand and intrastrand crosslinks, which ultimately arrests the cell cycle at G1, S, or G2 (Fichtinger-Schepman et al. 1986) (Sundaralingam et al. 1985). These platinum-DNA adducts also alert DNA damage sensors (ATM, DNA-PKcs, and ATR), which phosphorylate p53, ultimately resulting in the activation of the caspase cascade and apoptosis (Porter et al. 1997). Although cisplatin has been shown to be effective in treating various types of cancers (Abrams and Murrer 1993) drug resistance is a major problem, especially with repeated doses of the drug (Welters et al. 1998); (Beale et al. 2000). Factors affecting resistance include an increase in drug efflux, a decrease in drug influx, increases in glutathione levels, DNA repair, and drug tolerance (Henkels and Turchi 1999); (Beale et al. 2000) (Nishimura et al. 1996).

The interactions between cisplatin and DNA are believed to be the main cause of apoptosis in cisplatin mediated cell death (Gong et al. 1999) (Henkels and Turchi 1999). Other platinum based drugs have been developed which are more potent at macrophage activation and are less toxic (Fiebig et al. 1996) (Drees et al. 1995). Poly-plat (Poly-[(*trans*-1,2-diaminocyclohexane) platinum]-carboxyamylose) is one such compound (Fiebig et al. 1996). Poly-plat has been shown to activate macrophages to produce inflammatory cytokines in greater quantities than cisplatin (Muenchen and Aggarwal

1998). Poly-plat is less toxic than cisplatin in equal doses of the drugs (Muenchen et al. 1997).

Two cell cycle regulatory proteins, Proliferating Cell Nuclear Antigen (PCNA) and p27, have important roles in the cell cycle. PCNA is required for DNA replication during the S-phase of the cell cycle (Kurki et al. 1986). Increased levels of PCNA in cell cultures indicate an increase in proliferation of the cells (Giordano et al. 1991). p27 is a protein belonging to the family of CDK inhibitors, which inhibit progression through the cell cycle (Luo et al. 1995), (Sgambato et al. 2000). An increase in p27 levels in rapidly proliferating cell types following treatment with antineoplastic agents is a good marker for cell cycle inhibition (Oshita et al. 2000). Specifically, p27 binds to Cdk4/cyclin D complexes resulting in cell cycle arrest at G_1, the growth phase prior to DNA replication.

MATERIALS AND METHODS

Cell Cultures. The HT1080 human fibrosarcoma cells were a gift from the Michigan State University Carcinogenesis Laboratory (East Lansing, MI). WRC-256 Walker rat carcinoma cells were purchased from American Type Cell Culture (Rockville, MD). Cell lines were kept in MEM supplemented with 10% (v/v) horse serum and 1% (w/v) antibiotic [penicillin G (10,000 U/ml) and streptomycin sulfate (10,000 µg/ml)] and stored in T25 flasks at 37° C in a 5% CO_2 incubator.

Treatments. Cisplatin and Poly-plat were dissolved in 0.1 ml dimethyl formamide, followed by the addition of MEM for a final concentration of 1 mg/ml. Drug solutions were prepared fresh daily. Twelve flasks of HT1080 and WRC-256 cells were used per treatment. Cells were treated with cisplatin (10 µg/ml), Poly-plat (10 µg/ml), or drug solvent alone in the T25 flasks for five days. Three flasks per treatment were removed and the supernatant was poured off. Cultures were rinsed with MEM and 1 ml trypsin/EDTA (0.25g trypsin/0.1g EDTA in 0.22% (w/v) NaCl) was added for five minutes to remove adherent cells. Horse serum (5 ml) was added to each flask to inhibit trypsin and the entire solution was poured into a 50 ml conical polypropylene tube. Cells were centrifuged at 1000 *g* for five minutes, washed once with MEM and resuspended in fresh medium. Cell counts were performed using an American Optical hemacytometer via dye exclusion with 0.2% (w/v) trypan blue. This was repeated 1, 2, and 5 days after treatment.

Apoptosis Assay. HT1080 cells were seeded onto 18 mm^2 glass coverslips at 1 X 10^6 cells/ml and placed in 35 mm plastic Petri dishes. Cells were incubated for 4 hours at 37° C in a 5% CO_2 incubator. Fresh MEM containing 10% horse serum (5 ml) was

added to the Petri dishes. HT1080 cells were then treated with cisplatin (10 μg/ml), Poly-plat (10 μg/ml), or drug solvent for two hours. The cells were rinsed with MEM and cultured in normal medium. Coverslips were removed from culture after 48 hours, fixed in 2% (v/v) glutaraldehyde in phosphate buffered saline (PBS), and stained by the *In situ* cell death detection kit, using double strand DNA breaks by the TdT-mediated dUTP nick-end labeling (TUNEL) reaction (Sanders and Wride 1996). In addition to the TUNEL assay coverslips were fixed in 2% glutaraldehyde in PBS and stained with Harris hematoxylin (Kiernan 1999). All coverslips were dehydrated in graded ethanol, cleared in xylene, and mounted on glass slides with Permount mounting medium. Slides were viewed on the Zeiss LSM 210 confocal microscope and in transmitted light. TUNEL slides were also viewed in fluorescence with an excitation wavelength of 488 nm and emission of >520 nm. The experiments described above were prepared in triplicate.

PCNA and p27 Analysis. Cultures of HT1080 cells were grown to 75% confluence in twelve T75 flasks. Six flasks were treated with Poly-plat (10 μg/ml, prepared as listed above), the remaining six with the vehicle only. After two hours the treatment was removed and the flasks were rinsed with PBS. Fresh medium was added to the flasks and they were returned to the incubator. Three flasks from each treatment group were removed at 24 and 48 hours post treatment and the medium was aspirated. Cell lysing solution was added to each flask (300 mM urea and 2% (w/v) SDS in PBS) and the flasks were scraped with plastic scrapers. The solution was pipetted into screw cap 2 ml tubes and sonicated for 1 minute with a tip sonicator. The tubes were then placed in a boiling water bath for five minutes. The extracts were then frozen at -20° C until all extracts were collected.

Protein Separation. The protein extracts were removed from the freezer and thawed at room temperature. Three 10 μl aliquots were taken from each tube and the protein concentration for each sample was determined from an average of the three using the BCA protein assay and the spectrophotometer. 15 μg of protein from each sample was mixed with loading buffer [glycerol: β-mercaptoethanol: bromphenol blue (1 mg/ml in water) in a 3:1:0.5 ratio, to total 30 μl] and loaded on a 10-20% tricine gel. Four identical gels were prepared. A marker protein ladder was added in lane 1. The gels were run at 55 mA for 4 hours, until the dye front approached the bottom. The gels were then electroblotted onto a nitrocellulose membrane for 90 minutes at 100 volts. The blots were stained with ponceau S for 2 minutes, rinsed briefly in PBS, and photographed for transfer of protein. The protein marker ladders were marked with ink and the blots were rinsed in PBS to remove the ponceau S. The blots were then blocked in blocking buffer (7.5% (w/v) milk and 0.1% (v/v) Tween-20 in PBS) for one hour at room temperature.

Antibodies. Mouse anti-p27 and mouse anti-PCNA antibodies were prepared separately in 1:500 dilutions in blocking buffer. The four blots were placed in plastic bags and two were filled with mouse anti-p27 antibody solution, two with mouse anti-PCNA antibody solution. The bags were sealed and placed at 4° C overnight. Following day the blots were rinsed with PBS three times for ten minutes each. The blots were again placed in bags and sealed with goat anti-mouse HRP conjugated secondary antibody (1:1000 dilution in blocking buffer) for 2 hours at room temperature. The blots were rinsed three times with PBS for ten minutes each.

Visualization of Protein Bands. The blots were placed on parafilm and excess PBS was removed. ECL working detection reagent (Amersham) was applied in the dark

and the blots were covered with parafilm for five minutes. Excess ECL reagent was removed and the blots were placed between sheets of plastic and taken to the dark room. The blots were exposed to film for times ranging from 5-30 seconds. The film was developed and scanned with a Microtek scanner. Banding intensity was determined using Image Pro Plus imaging software.

RESULTS

Cell Growth Inhibition. As measured by trypan blue exclusion untreated WRC-256 cells increased in numbers from 2.1 X $10^5 \pm 0.3$ to 3.5 X $10^5 \pm 0.2$ after 24 hours and 6.8 X $10^5 \pm 1.1$ after 48 hours (Figure 1). WRC-256 cells treated with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml) were inhibited to a cell growth rate nearly one half that of the normal cells. Untreated HT1080 cells also showed a rapid growth rate, from 2.0 X $10^5 \pm 0.3$ at the beginning of the experiment to 1.2 X $10^6 \pm 1.1$ after five days. HT1080 cells treated with Poly-plat (10 μg/ml) showed growth inhibition at 24 and 48 hours (Figure 2). However after five days of treatment the HT1080 cells returned to a normal growth rate. Between 24 and 48 hours no growth was seen in the Poly-plat treated HT1080 cell cultures.

Apoptosis Assay. HT1080 cells stained with hematoxylin after 48 hours in culture and examined by transmission microscopy appear large and show multiple mitotic figures (Figure 3 A-C; Table 1). Apoptosis can be visualized in untreated HT1080 cells after 48 hours in culture by nuclear fragmentation (Figure 3 D-F) at a rate of 1.1% (Table 1), or 2.1% using the TUNEL assay (Figure 3 G, H). The TUNEL assay detected cells with morphological signs of apoptosis (DNA fragmentation) and cells which appeared normal histologically (cells with DNA strand breaks but no morphological aspects of apoptosis). No mitotic figures were seen in the cisplatin and Poly-plat treated cells after 48 hours. The Poly-plat treated cells had an apoptotic rate similar to the untreated cells. Only the cisplatin treated cells had a marked increase in apoptosis at 48 hours after treatment.

PCNA and p27. PCNA and p27 were examined for their role in progression through the cell cycle. The nitrocellulose blots were photographed after Ponceau S staining to show equivalence of protein loading in the gels (Figure 4). PCNA levels after 24 hours of Poly-plat treatment were roughly equal to that of the untreated cells. By 48 hours the PCNA levels had nearly tripled (Figures 5 and 6). p27 levels were higher than the control at 24 hours after Poly-plat treatment and continued to increase after 48 hours (Figures 5 and 7).

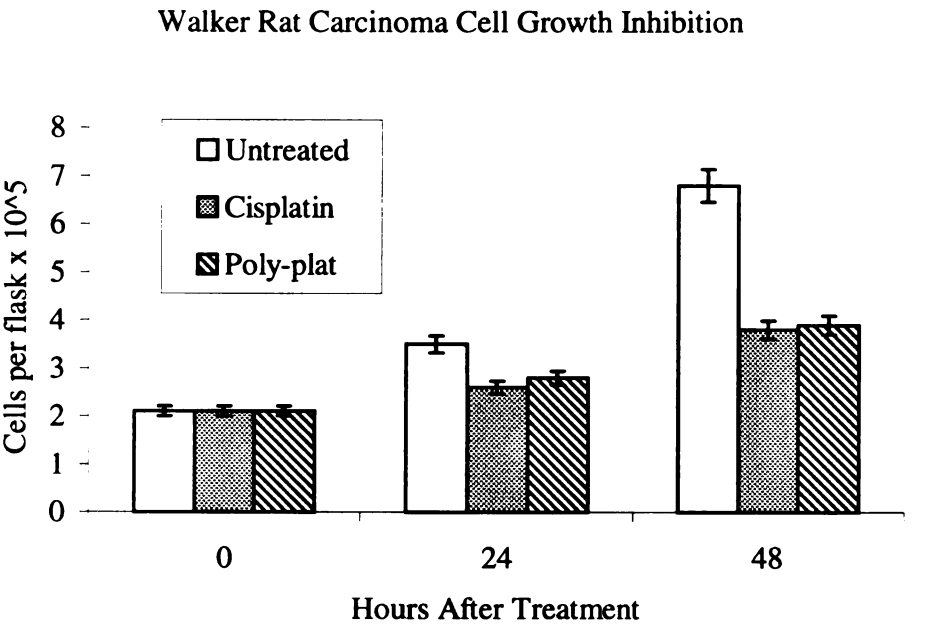

Figure 1: Graph showing the effects cisplatin (10 μg/ml) or Poly-plat (10 μg/ml) treatment on WRC-256 cell growth. Note the inhibition of cell growth in cisplatin and Poly-plat treated cultures after 24 and 48 hours. Cells numbers were measured by trypan blue dye exclusion and standard deviation calculated from triplicate runs of experiment.

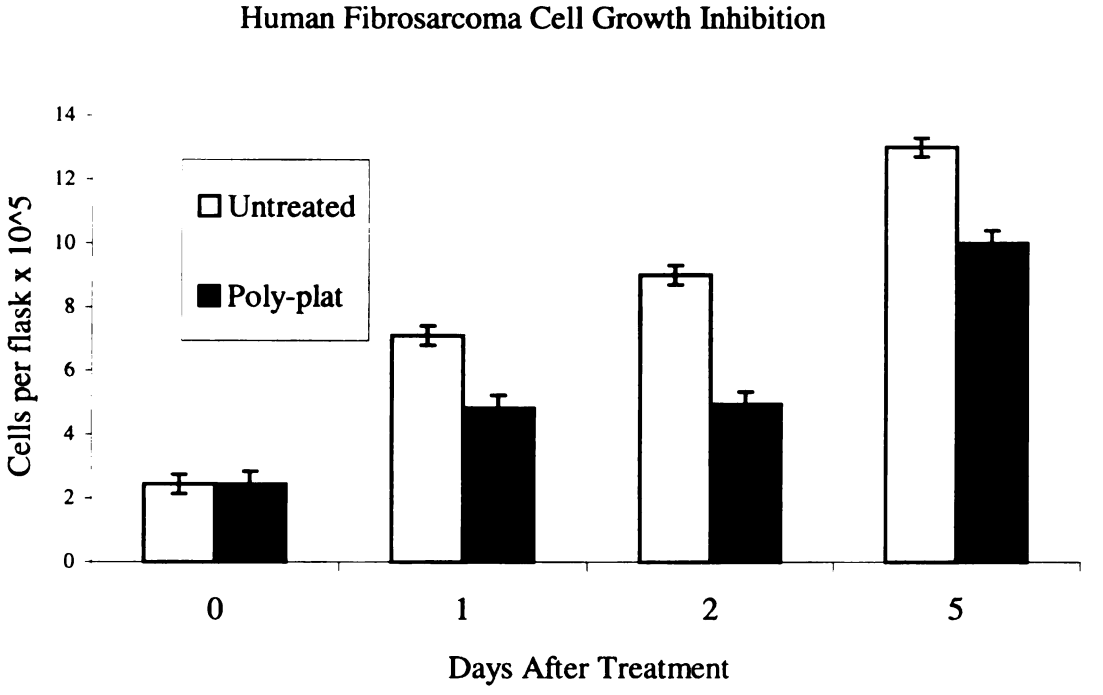

Figure 2: Graph showing the effects Poly-plat (10 μg/ml) treatment on HT1080 cell growth. Note the inhibition of cell growth in Poly-plat treated cultures after 1 and 2 days. Cell growth returns to normal by day 5. Cells numbers were measured by trypan blue dye exclusion and standard deviation calculated from triplicate runs of experiment.

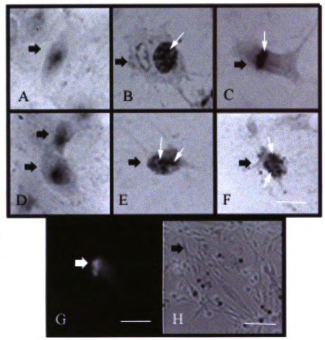

Figure 3: Light micrographs showing HT1080 cells (large arrows) after 48 hours. Untreated cells stained with hematoxylin shown not undergoing mitosis [A], in prophase (determined by condensation of the nucleus, white arrow) [B], and metaphase (determined by chromosomes lined up at the equator, white arrow) [C]. Neither cisplatin (10 μg/ml) nor Poly-plat (10 μg/ml) treated cells showed any mitotic activity after 48 hours of treatment. Untreated cells stained with hematoxylin also showed very little apoptotic activity [D]. Apoptosis was increased slightly in cells treated with Poly-plat (10 μg/ml) after 48 hours [E] and can be seen with hematoxylin staining as fragmentation of the nucleus into smaller membrane bound vesicles (white arrows). An increase in HT1080 cells undergoing apoptosis is seen after cisplatin treatment (10 μg/ml) after 48 hours [F] (fragmented nucleus, white arrows). The TUNEL assay was also performed and apoptotic cells were seen either via fluorescence [G; untreated HT1080 cell undergoing apoptosis after 48 hours] or transmission [H; Poly-plat treated cells (10 μg/ml) after 48 hours treatment]. Bar = 20 μm (A-G); 80 μm (H).

HT1080 Cells Following Treatment

Treatment	Mitotic[1] Figures	Apoptosis[1] (Hematoxylin)	Apoptosis[2] (TUNEL)	Viable Cells[3] (X 10^5)
Untreated	2.0 ± 0.2	1.1 ± 0.3	2.7 ± 1.8	6.8 ± 0.5
Cisplatin	0	18 ± 2.0	26 ± 2.7	3.2 ± 0.8
Poly-plat	0	3 ± 1.1	4 ± 1.3	4.6 ± 0.6

Table 1: Table of human fibrosarcoma (HT1080) cells after treatment with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml).

[1] Visualized in transmitted light from hematoxylin stained slides.

[2] Apoptosis TUNEL assay examined in fluorescence using 488 nm excitation and > 520 nm emission.

[3] Viable cells counts x 10^5 (± S.D.) calculated via hemacytometer using trypan blue dye exclusion. Beginning cell count was 2.1 X 10^5 for all treatments.

* Values for mitotic figures and apoptosis given in percent ± S.D. of 250 total cells counted per treatment.

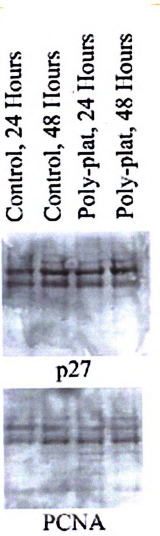

Figure 4: Blots stained using Ponceau S staining solution to visualize protein bands on nitrocellulose membrane. Note the protein bands in each lane are similar in size and position indicating equal loading of protein extracts.

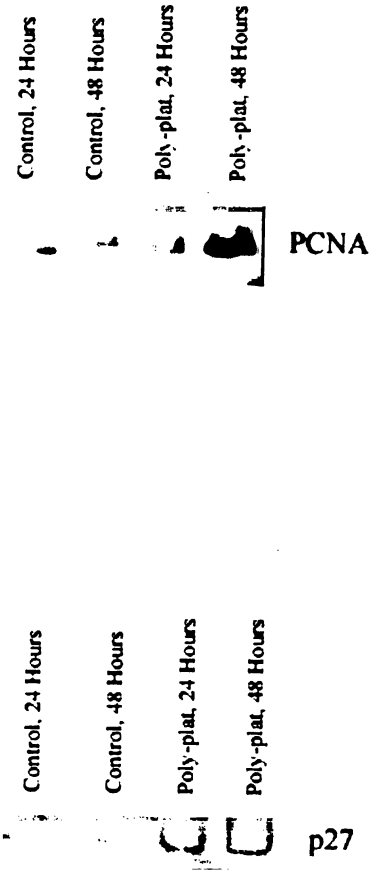

Figure 5: PCNA and p27 levels in HT1080 cells at 24 and 48 hours after treatment with Poly-plat. Note the large increase in PCNA levels in the Poly-plat treated cells after 48 hours, indicating an increase in proliferation. p27 levels are elevated at 24 hours after Poly-plat treatment, but remain constant over the following 24 hours.

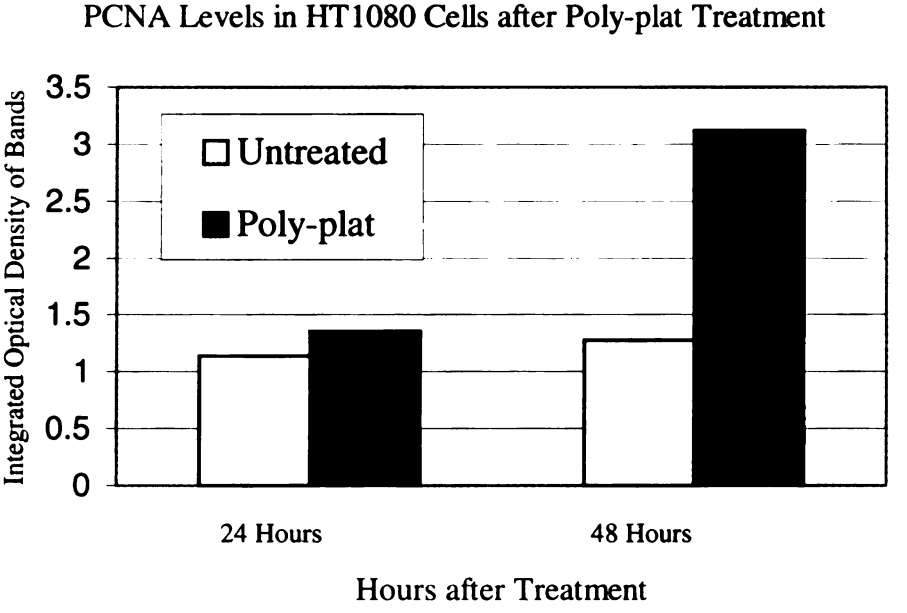

Figure 6: PCNA levels in HT1080 cells following Poly-plat treatment. Levels determined via three Western Blots of protein extracts of treated cells, and analysis of band optical density performed using Image Pro Plus, taking an average of the three blots.

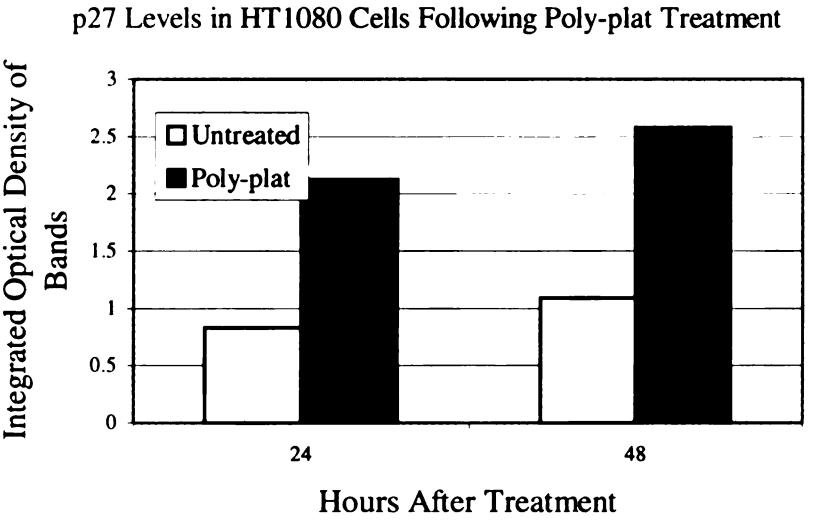

Figure 7: p27 levels in HT1080 cells following Poly-plat treatment. Levels determined via three Western Blots of protein extracts of treated cells, and analysis of band optical density performed using Image Pro Plus, taking an average of the three blots.

DISCUSSION

The safe and effective use of chemotherapy in clinical practice requires a thorough understanding of the drug's actions as well as its associated toxicity. Cisplatin has been used to treat testicular, ovarian, head, neck, and bladder cancers with a high degree of success (Palma and Aggarwal 1995). It is believed to enter the tumor cell by diffusion and disrupt the DNA double helix by interstrand and intrastrand crosslinking (Roberts and Pascoe 1972) (Goodman et al. 1996). The major toxicity caused by cisplatin is impairment of renal tubular function (Walker and Gale 1981). Irreversible kidney damage can occur at higher doses or repeated courses (Goodman et al. 1996). In addition to other toxicity, marked nausea and vomiting occur in almost all patients (Abrams and Murrer 1993); (Goodman et al. 1996). For some, this can be a dose-limiting factor.

Other less toxic platinum analogs have been examined for their ability to stimulate the immune response (Telgenhoff and Aggarwal 1998). Of these, Poly-plat appears to be the most effective at immune cell activation (Muenchen et al. 1997). In this study we examined Poly-plat's effectiveness at inhibiting tumor cell growth. Both WRC-256 and HT1080 cells were inhibited by Poly-plat treatment. This inhibition was shown to be nearly equal to that of treating with cisplatin using the WRC-256 cells. HT1080 cell growth was inhibited between days one and two, however the growth rate returned to normal by day five. This data coupled with the lack of apoptosis in these cells when treated with Poly-plat alone could indicate that these cells are arrested in the cell cycle until repair mechanisms can be activated.

Further evidence of cell cycle arrest was apparent from the Western blot data. Levels of p27 were elevated in Poly-plat treated cells 24 and 48 hours after treatment. An elevation of p27 has been shown to indicate cell cycle arrest in tumor cells following platinum drug treatment (Oshita et al. 2000). This data correlates well with the data from figure 2, which shows a growth stasis between 24 and 48 hours after Poly-plat treatment. PCNA levels were near normal levels 24 hours after Poly-plat treatment. A large increase in PCNA levels 48 hours after treatment indicates a push for the cell to continue through the cycle. PCNA is present during all phases of the cell cycle, increasing in late G_1 and reaching a peak in S phase (Kurki et al. 1986). Increased levels of this protein following Poly-plat treatment may indicate the cells are recovering from the insult and are upregulating proteins required for cell cycle progression. Growth resumes and approaches near normal levels five days after treatment, presumably due to cell damage repair mechanisms in the tumor cell.

From these studies it can be assumed that Poly-plat alone *in vitro* is ineffective at inducing apoptosis in tumor cells. Further it has been shown that tumor cells treated with Poly-plat recover from treatment within five days. Since Poly-plat has been shown to be effective against certain tumors *in vivo*, it is possible that Poly-plat's effectiveness lies not in it's ability to kill tumor cells, but rather in it's ability to slow tumor cell growth until an effective immune resonse can be mounted. It may also be seen that the main effect of Poly-plat is the induction of immune system activation.

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CHAPTER 4: IMMUNE CELL ACTIVATION AND TUMOR CELL INTERACTION FOLLOWING POLY-PLAT TREATMENT

SUMMARY

Rat peritoneal macrophages and mouse fibroblasts were treated with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml) for 2 hours and allowed to grow in normal medium for 48 hours. Supernatants from treated cells were collected at 0, 4, 18, 24, and 48 hours and IL-2 levels were examined using the enzyme linked immunosorbant assay (ELISA) technique. Optimal dosing of Poly-plat was determined via IL-2 production from macrophages treated with varying doses. Cultures of peritoneal macrophages were treated with cisplatin (10 μg/ml) or Poly-plat (5 μg/ml) for two hours and allowed to grow in normal medium for 24 hours. Cytotoxicity of these supernatants was examined using HT1080 and WRC-256 cells. Apoptosis assays were used to determine cytotoxic effects of macrophage supernatants on tumor cells. Macrophage movement following treatment with Poly-plat was also examined. These results demonstrate the ability of Poly-plat to activate macrophages to induce apoptosis in these cell lines.

INTRODUCTION

Activated macrophages seek out tumor cells, make contact through cytoplasmic extensions, and transfer lysosomes to the tumor cell (Palma and Aggarwal 1995), (Muenchen and Aggarwal 1998). Activated macrophages also release various cytokines (IL-1, TNFα), which further activate the immune system and cause tumor necrosis (Basu et al. 1991), (Muenchen et al. 1997). One of the inflammatory cytokines also produced by activated macrophages is interleukin-2 (IL-2) (Burkitt and Aggarwal 2000).

IL-2 is primarily secreted by activated T lymphocytes, but has been shown to be released in great quantities by fibroblasts and macrophages following treatment with platinum anti-cancer agents (Burkitt and Aggarwal 2000), (Telgenhoff and Aggarwal 2000). IL-2 activates cells with IL-2 receptors (T and B lymphocytes, natural killer cells, and macrophages) to proliferate (Sharon 1998) and differentiate into cells with increased immune system activity (Collins and Oldham 1995), (Ding et al. 1988). IL-2 has also been shown to induce monocyte differentiation into macrophages through activation of the macrophage colony stimulating factor gene (Brach et al. 1993), (Zhu et al. 1993). The use of IL-2 as a treatment for cancer has been shown to induce regression of metastatic cancers in selected patients that had failed on other chemotherapies (Rosenberg et al. 1988).

Poly-plat has been shown to activate macrophages to produce inflammatory cytokines in greater quantities than cisplatin (Muenchen and Aggarwal 1998). The purpose of this study was to examine the effects of Poly-plat on activating macrophages and other cell types to release IL-2, and to determine the optimal dose for Poly-plat based on IL-2 production. The ability of Poly-plat treated macrophages to induce apoptosis in

tumor cells was also examined, as well as the ability of macrophages to seek out tumor cells *in vitro*.

MATERIALS AND METHODS

Cell Cultures. Mouse 3T3 fibroblasts were obtained from Dr. Peter Davidson (Michigan State University) and cultured in Minimal Essential Medium (MEM) with 10% (v/v) Fetal Bovine Serum (FBS). Rat peritoneal macrophages were isolated from methoxyflurane anesthetized 8 week old male Wistar rats (Charles River, MA) via peritoneal injection of 10 ml chilled RPMI Medium (Gibco, NY) following institutional guidelines (Palma et al. 1992). After gently massaging the abdominal wall, cells were aspirated and placed in conical centrifuge tubes and centrifuged at 1000x *g* for five minutes. The supernatant was removed and the cells were resuspended in RPMI with 10% (v/v) horse serum (Atlanta Biologicals, GA). A cell count was performed via hemacytometer using the trypan blue dye exclusion method (Sigma, MO) and 3 x 10^5 cells were placed in each T25 flask and incubated at 37° C for 2 hours, after which cells were washed with RPMI to remove non-adherent cells and fresh RPMI with 10% (v/v) horse serum was added. The HT1080 human fibrosarcoma cells were a gift from the Michigan State University Carcinogenesis Laboratory (East Lansing, MI). Macrophage and HT1080 cell lines were kept in RPMI supplemented with 10% (v/v) horse serum and 1% (w/v) antibiotic [penicillin G (10,000 U/ml) and streptomycin sulfate (10,000 µg/ml)] and stored in T25 flasks at 37° C in a 5% CO_2 incubator.

Treatments. Cisplatin and Poly-plat were dissolved in 0.1 ml dimethyl formamide, followed by the addition of MEM for a final concentration of 1 mg/ml. Drug solutions were prepared fresh daily. Six flasks of 3T3 cells and macrophages were treated with either cisplatin (10 µg/ml), Poly-plat (10 µg/ml), or the drug vehicle alone for 2 hours at 37° C. The cells were rinsed in PBS and fresh MEM with 10% (v/v) fetal

bovine serum was added to the 3T3 cells, RPMI with 10% (v/v) horse serum was added to the macrophages. Supernatants were obtained from each culture at 0, 4, 18, 24, and 48 hours. ELISA kits (Pharmingen, CA) were used to analyze IL-2 levels. The method utilized the multiple antibody sandwich principle, where a monoclonal antibody to rat IL-2 was used to capture rat IL-2 present in samples. A biotinylated polyclonal antibody binding the captured IL-2 was added and unbound material was washed out. Peroxidase conjugated avidin was used to bind these biotin tagged complexes, resulting in a color change. Standard curves were generated with IL-2 (0-200 pg/ml) provided in the kits. The reaction was stopped by acidification and absorbance was read at 450 nm using an Umax kinetic spectrophotometer microplate reader (Molecular Devices, CA). 3T3 cells were trypsinized and counted via hemocytometer at 24 and 48 hours. Duplicate macrophage and 3T3 cultures were grown on coverslips and treated as described above. Coverslips were fixed in buffered formalin and viewed using phase contrast optics on the Zeiss 210 Laser Scanning microscope.

Optimal Dose Determination. In order to determine the optimal dose for induction of IL-2 release from macrophages activated with Poly-plat macrophages were obtained as previously described and cultured in 60 mm culture plates in RPMI with 10% (v/v) horse serum. Plates were treated with either control medium, cisplatin (2, 5, 10, 25, 50 or 100 μg/ml), or Poly-plat (2, 5, 10, 25, 50, or 100 μg/ml); two plates for each treatment dose. Plates were treated for 2 hours at 37° C. The media was aspirated, the plates were rinsed with PBS, and fresh RPMI with 10% (v/v) horse serum was added. The plates were returned to the incubator and 500 μl of the medium was removed from each plate at 0, 4, 24, and 48 hours after treatment and replaced with 500 μl of normal

media. The supernatants were frozen at –20° C until all were collected. The ELISA IL-2 assay was performed on the supernatants as previously described.

Apoptosis Assay. HT1080 cells were seeded onto 18 mm^2 glass coverslips at 1 x 10^6 cells/ml and placed in 35 mm plastic Petri dishes. Cells were incubated for 4 hours at 37° C in a 5% CO_2 incubator. Fresh RPMI containing 10% (v/v) horse serum (5 ml) was added to the Petri dishes. Macrophages (1 x 10^5) were obtained as previously described and co-cultured with half of the HT1080 cell cultures for an additional 2 hours. Macrophages were also cultured alone in normal medium. All three culture types (HT1080, macrophages, and HT1080 + macrophages) were then treated with cisplatin (10 μg/ml), Poly-plat (5 μg/ml), or drug solvent for two hours. The cells were rinsed with RPMI and cultured in RPMI with 10% (v/v) horse serum. HT1080 and HT1080 + macrophages coverslips were removed from culture after 48 hours, fixed in 2% (v/v) glutaraldehyde in PBS, and stained by the In situ cell death detection kit (Boehringer-Mannheim, CA), using double strand DNA breaks by the TdT-mediated dUTP nick-end labeling (TUNEL) reaction (Sanders and Wride 1996). The supernatants from the treated macrophages were removed after 24 hours and placed in Petri dishes containing untreated HT1080 cells (1 x 10^6). The cells were incubated at 37° C for an additional 48 hours. Coverslips were removed, fixed, and stained using the TUNEL reaction. In addition to the TUNEL assay coverslips from all three cultures (HT1080, HT1080 + macrophages, and HT1080 + macrophage supernatant) were fixed in 2% (v/v) glutaraldehyde in PBS and stained with Harris hematoxylin. All coverslips were dehydrated in graded ethanol, cleared in xylene, and mounted on glass slides with Permount mounting medium. Slides

were viewed on the Zeiss LSM 210 confocal microscope and in transmitted light. The experiments described above were prepared in triplicate.

Macrophage Movement. Macrophages and HT1080 cells obtained as previously described. 1x 10^4 Macrophages were suspended in 1 ml of RPMI with 10% (v/v) horse serum and placed as a single droplet on one end of a glass slide. 1 x 10^4 HT1080 cells were also suspended in 1 ml of RPMI with 10% (v/v) horse serum and placed as a single droplet on the opposite end of the slide, so that neither droplet was touching. The slides were placed in an incubator for six hours at 37° C with 5% CO_2. The slides were then rinsed to remove non-adherent cells and placed in Petri dishes containing RPMI with 10% (v/v) horse serum. Twelve slides were prepared in this way and were divided into four groups of three slides each. The groups were treated with either phorbol myristate acetate (PMA, 10 ng/ml, dissolved in DMSO and prepared in RPMI with 10% (v/v) horse serum), lipopolysaccarhide (LPS, 10 μg/ml prepared in RPMI with 10% (v/v) horse serum), Poly-plat (5 μg/ml, prepared as previously described), or normal medium plus DMSO (3 μl/ml) as a control. These slides remained in culture for 48 hours in the incubator. The slides were then rinsed in PBS and fixed with 2% (v/v) glutaraldehyde in PBS. Peroxidase staining was performed to visualize macrophages using the DAB method (Kiernan 1999), and slides were counterstained with 1% (w/v) mentanil yellow. The slides were dehydrated in a graded series of ethanol, cleared in xylene, and coverslips were applied with Permount mounting medium. Slides were viewed on the Zeiss 210 laser scanning microscope.

RESULTS

IL-2 Release. There was an increase in IL-2 levels detected in the supernatants of the treated macrophages with Poly-plat (10 μg/ml) and cisplatin (10 μg/ml) for the various times tested (Figure 1). The levels of IL-2 released in the cisplatin treated macrophages was above normal after 12 hours, and remained high even after 48 hours. The Poly-plat treated macrophages demonstrated a very high level of IL-2 throughout the duration of the experiment compared to the normal or cisplatin treated cultures. IL-2 release from Poly-plat treated fibroblasts was also much higher than cisplatin or the untreated (Figure 2).

Cell Morphology. Untreated macrophages appear discoid in shape (Figure 3a), gradually becoming longer and flattened after 48 hours (Figure 3b). Cisplatin or Poly-plat treated macrophages show a large increase in extensions and cytoplasmic content after 48 hours (Figures 3c and 3d). The extensions formed in Poly-plat treated macrophages were generally longer and more in number (Figure 3e). In addition, the rate of fibroblast growth appeared increased in Poly-plat treated cultures possibly due to the elevated release of IL-2 (Figure 4). Fibroblasts experienced growth inhibition after cisplatin treatment (Table 1). The was no significant increase in fibroblast growth in the Poly-plat treated cultures compared to the control. The appearance of increased growth (Figure 4) could be due possibly to the increase in size of the fibroblasts.

Dose Determination. Prior to conducting further experiments, a dose response curve was generated for cisplatin and Poly-plat with the intention of discovering the optimal dose for IL-2 production in cultured macrophages. For the cisplatin treated macrophages the optimal dose ranged between 5 and 10 μg/ml, with the greatest response

at 48 hours in the cells treated with 10 μg/ml (Figure 5). In the Poly-plat treated macrophages the largest response was at 5 μg/ml, at the 24 hour mark (Figure 6). Average IL-2 levels declined after this point.

Apoptosis Assay. As reported in chapter 3, apoptosis can be visualized in untreated HT1080 cells after 48 hours in culture by nuclear fragmentation at a percent apoptotic of 1.1% (Table 2), or 2.1% using the TUNEL assay. No mitotic figures were seen in the Cisplatin and Poly-Plat treated cells after 48 hours. The Poly-plat treated cells had an apoptotic index similar to the untreated cells. Only the Cisplatin treated cells had a marked increase in apoptosis at 48 hours after treatment. HT1080 cells which were cultured with macrophages and left untreated had an apoptosis percent increase twice that of HT1080 cells alone after 48 hours (Table 3). Cells co-cultured and treated with Cisplatin showed no appreciable difference in the rate of apoptosis. HT1080 cells co-cultured with macrophages and treated with Poly-plat had a large increase in the apoptotic rate, nearly nine times that of treating the cells with Poly-plat alone. The addition of untreated macrophage supernatant to the HT1080 cells had no effect on cell division or apoptosis (Table 4). The addition of Cisplatin treated macrophage supernatant to the HT1080 cells increased the apoptotic rate nearly six times (in both assay systems), but when Poly-plat treated macrophage supernatant was added the apoptotic rate for the HT1080 cells was even greater than that of the cells treated with the cisplatin treated macrophage supernatant.

Macrophage Movement Assay. To examine the extent of movement macrophages were co-cultured with HT1080 cells at opposite ends of a glass slide. Both cell populations were treated with agents known to induce macrophage activation (PMA,

LPS) and Poly-plat. HT1080 cells (Figure 7a) and macrophages (Figure 7b) cultured at opposite ends of a glass slide have a tendency to stay separated when left untreated for 48 hours. When treated with macrophage activating agents LPS (Figure 7c) and PMA (Figure 7d) the macrophages are found in conjunction with the tumor cells. Poly-plat treatment (Figure 7e) yields a response similar to that seen with PMA. Individual macrophages (Figure 7f) are also much more spread out following Poly-plat treatment.

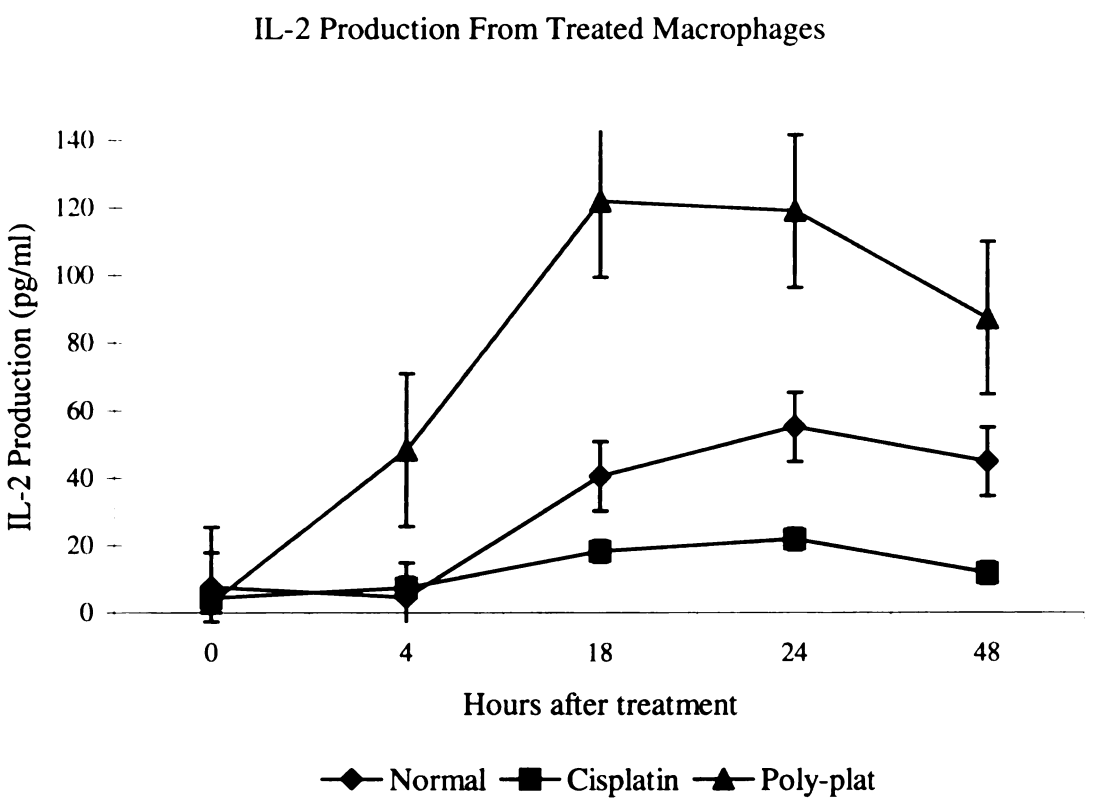

Figure 1: Graph showing IL-2 release in the supernatant of macrophages treated with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml) at various times. Note the increase in IL-2 production in Poly-plat treated macrophages. Standard deviations were calculated from triplicate analysis of supernatants.

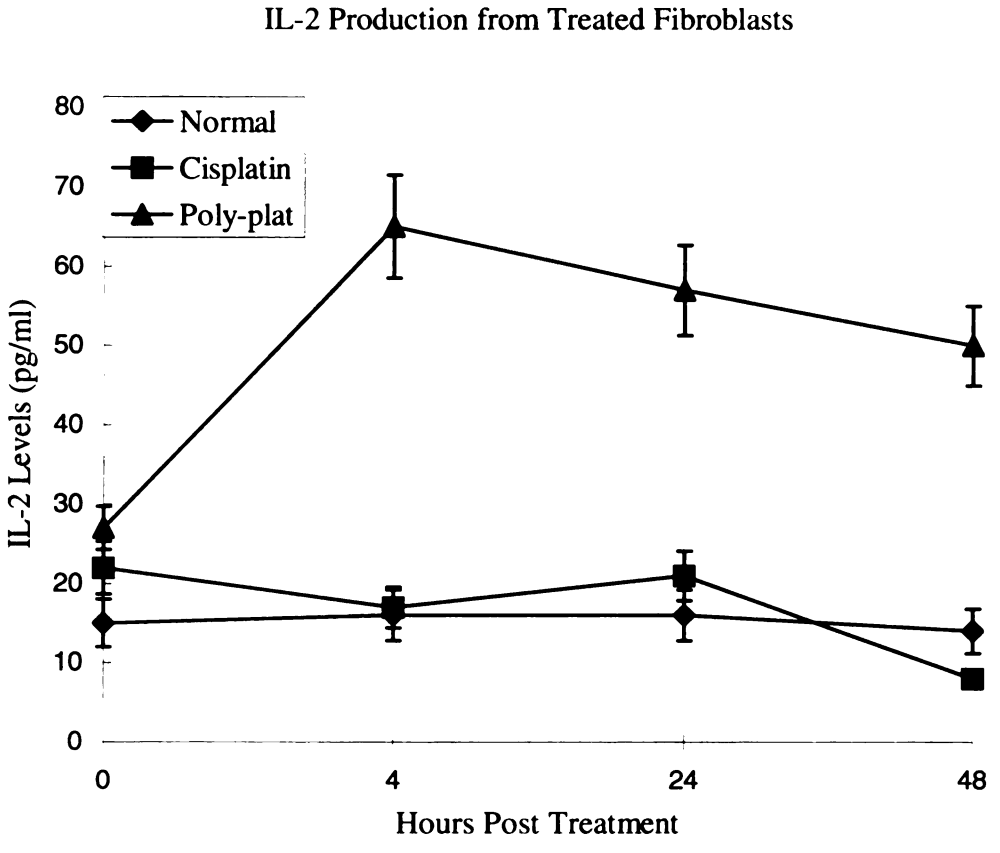

Figure 2: Mouse 3T3 IL-2 assay showing IL-2 production in cultured mouse fibroblasts treated with cisplatin (10 mg/L), Poly-plat (10 mg/L), or normal saline. Poly-plat was the only treatment which resulted in an increase in IL-2 production. Standard deviations were calculated from triplicate analysis of supernatants.

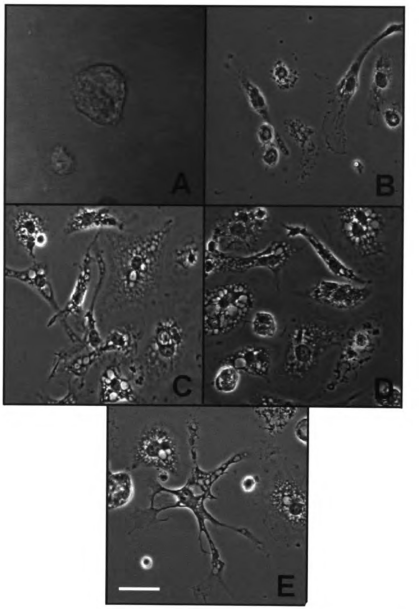

Figure 3: Light micrographs showing macrophages at 0 hours (A) and 48 hours (B) in normal medium, and 48 hours after cisplatin (C) and Poly-plat (D) treatments. Note the increase in cytoplasmic extensions and lysosomes formation following cisplatin and Poly-plat treatment. Poly-plat macrophages in general show a greater degree of extension formation (E). Bar = 5 μm (A), 2.5 μm (B-E).

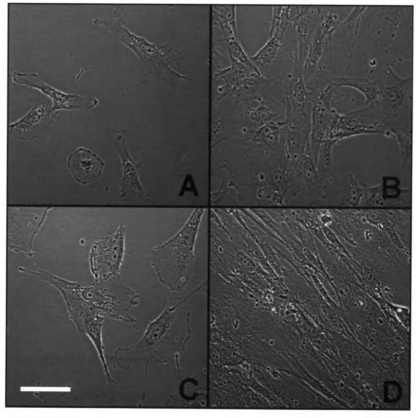

Figure 4: Light micrographs showing mouse 3T3 cells at 0 hours (A) and 48 hours (B) in normal medium, and 48 hours after cisplatin (C) and Poly-plat (D) treatments. Bar = 2.5 μm.

Mouse 3T3 Fibroblast Cell Counts Following Treatment with Platinum Compounds

Treatment	0 Hours	24 Hours	48 Hours
Untreated	8.1×10^4	1.23×10^5	2.02×10^5
Cisplatin	--	9.50×10^4	4.80×10^4
Poly-plat	--	1.12×10^5	2.32×10^5

Table1: Changes in mouse 3T3 fibroblast cell count following treatment with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml). Cisplatin caused a reduction in overall cell count, while Poly-plat increased the number of cells slightly above the untreated count. Counts are averages of three experiments.

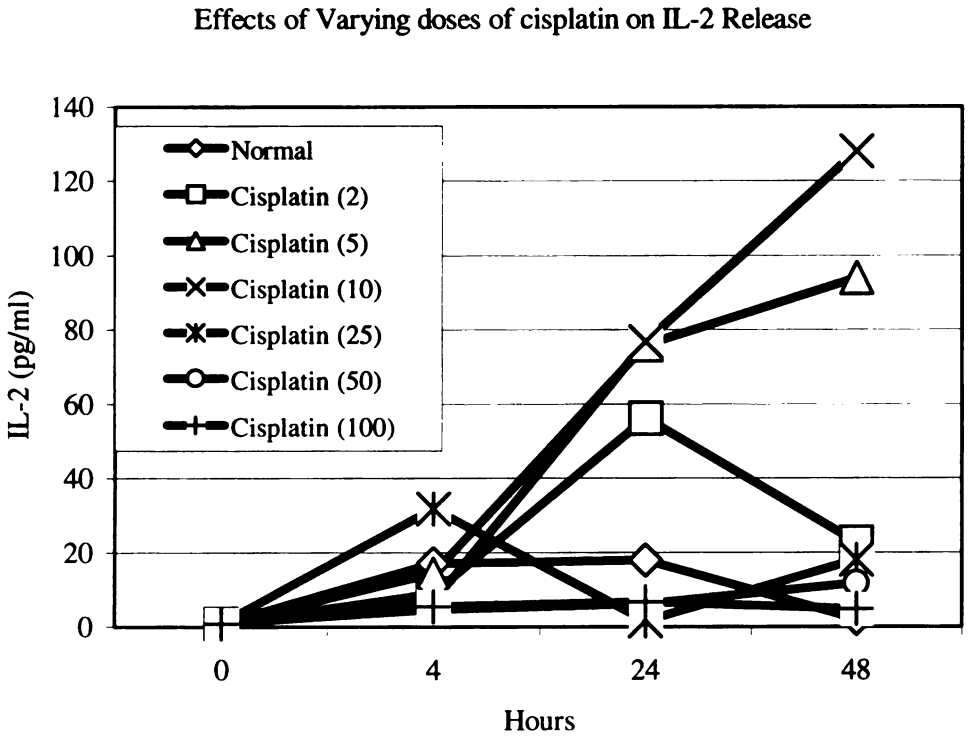

Figure 5: Dose optimization for IL-2 release from cisplatin treated macrophages. Doses shown are in μg/ml. Only doses lower than 10 μg/ml showed any increase in IL-2 levels, with the maximal release at 10 μg/ml.

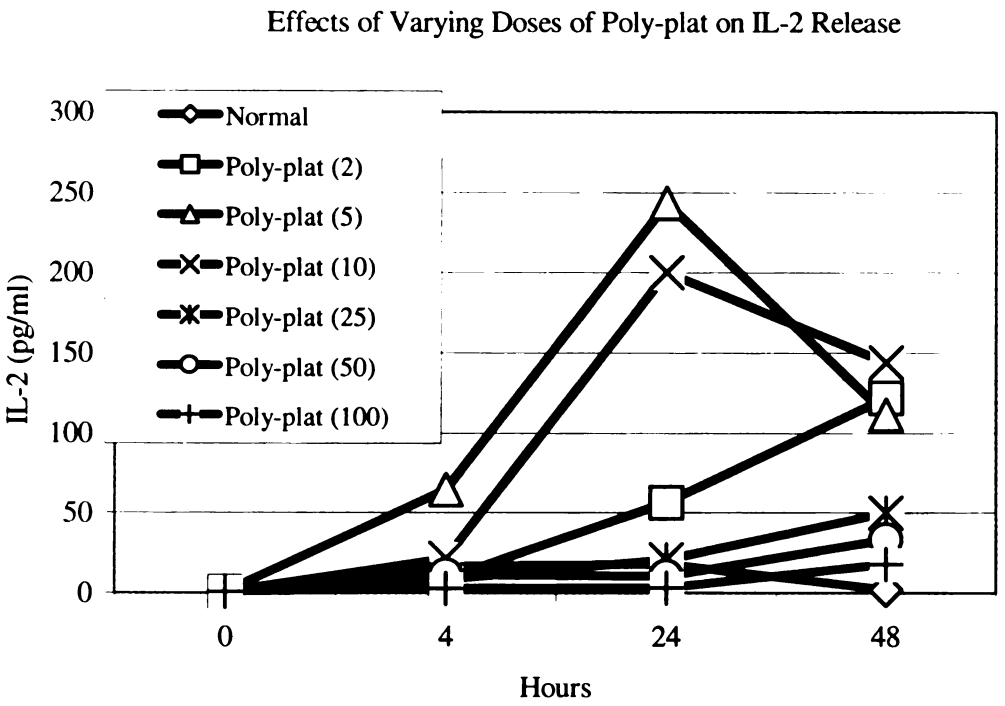

Figure 6: Dose optimization for IL-2 release from Poly-plat treated macrophages. Doses shown are in μg/ml. The maximal response was seen in the cells treated with 5 μg/ml after 24 hours in culture.

HT1080 Cells Following Treatment

Treatment	Mitotic[1] Figures	Apoptosis[1] (Hematoxylin)	Apoptosis[2] (TUNEL)	Viable Cells[3] (X 10^5)
Untreated	2.0 ± 0.2	1.1 ± 0.3	2.7 ± 1.8	6.8 ± 0.5
Cisplatin	0	18 ± 2.0	26 ± 2.7	3.2 ± 0.8
Poly-plat	0	3 ± 1.1	4 ± 1.3	4.6 ± 0.6

Table 2: Table of human fibrosarcoma (HT1080) cells after treatment with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml).
[1] Visualized in transmitted light from hematoxylin stained slides.
[2] Apoptosis TUNEL assay examined in fluorescence using 488 nm excitation and > 520 nm emission.
[3] Viable cells counts x 10^5 (± S.D.) calculated via hemacytometer using trypan blue dye exclusion. Beginning cell count was 2.1 X 10^5 for all treatments.
* Values for mitotic figures and apoptosis given in percent ± S.D. of 250 total cells counted per treatment.

HT1080 cells co-cultured with rat macrophages

Treatment	Mitotic[1] Figures	Apoptosis[1] (Hematoxylin)	Apoptosis[2] (TUNEL)	Viable Cells[3] (X 10^5)
Untreated	1.3 ± 0.2	1.9 ± 0.2	4.6 ± 1.0	2.0 ± 0.5
Cisplatin	0	22 ± 2.8	28 ± 9.2	0.3 ± 0.8
Poly-plat	0	18 ± 1.6	35 ± 3.3	0.8 ± 0.6

Table 3. Table of human fibrosarcoma (HT1080) cells co-cultured with macrophages after treatment with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml).

[1] Apoptosis visualized in transmitted light from hematoxylin stained slides.

[2] Apoptosis TUNEL assay examined in fluorescence using 488 nm excitation and > 520 nm emission.

[3] Viable cells counts x 10^5 (± S.D.) calculated via hemacytometer using trypan blue dye exclusion. Beginning cell count was 2.1 X 10^5 for all treatments.

Values for mitotic figures and apoptosis given in percent ± S.D. of 250 total cells counted per treatment.

HT1080 cells treated with macrophage supernatant

Treatment	Mitotic Figures	Apoptosis (Hematoxylin)	Apoptosis (TUNEL)	Viable Cells (X 10)
Untreated Macrophage supernatant	2.2 ± 0.4	2.1 ± 0.3	2.4 ± 0.8	2.4 ± 1
Cisplatin Macrophage supernatant	1.8 ± 0.3	12 ± 3	18 ± 3.3	1.3 ± 0.6
Poly-plat Macrophage supernatant	2.7 ± 0.4	19 ± 4	21 ± 4.5	1.0 ± 0.6

Table 4. Table of human fibrosarcoma (HT1080) cells exposed to macrophage supernatant from macrophages treated with cisplatin (10 μg/ml) or Poly-plat (10 μg/ml).

[1] Visualized in transmitted light from hematoxylin stained slides.

[2] Apoptosis TUNEL assay examined in fluorescence using 488 nm excitation and > 520 nm emission.

[3] Viable cells counts x 10^5 (± S.D.) calculated via hemacytometer using trypan blue dye exclusion. Beginning cell count was 2.1 X 10^5 for all treatments.

Values for mitotic figures and apoptosis given in percent ± S.D. of 250 total cells counted per treatment.

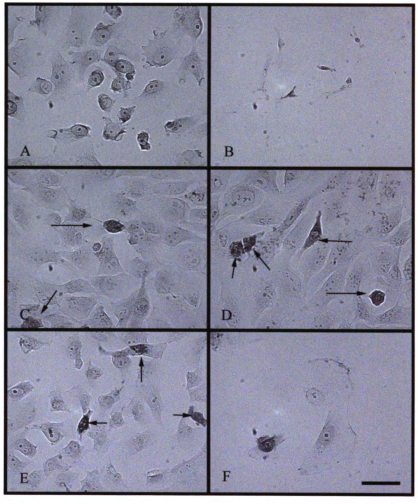

Figure 7: HT1080 cells (A) and macrophages (B) cultured at opposite ends of a glass slide have a tendency to stay separated when left untreated for 48 hours. When treated with macrophage activating agents LPS (C) and PMA (D) the macrophages are found in conjunction with the tumor cells. Poly-plat treatment (E) yields a response similar to that seen with PMA. Individual macrophages (F) are also much more spread out following Poly-plat treatment. (Arrows indicate macrophages stained for peroxidase). Bar = 5 μm (A, C-F); 10 μm (B).

DISCUSSION

IL-2 is an inflammatory cytokine, typically associated with T lymphocytes, inducing clonal expansion and producing tumor infiltrating lymphocytes which play a pivotal role in tumor regression. Macrophages treated with cisplatin have been shown to seek out and lyse tumor cells, and release cytokines such as TNF-α and IL-1α (Palma et al. 1992). This study reveals another mechanism whereby activated macrophages are immune system stimulators through the release of high levels of IL-2. Poly-plat treated macrophages release even greater quantities of IL-2 than cisplatin, and reveal an increase in morphologic characteristics of activation. Non-immune cells are also stimulated to release IL-2, as is seen by the 3T3 cells. These cells rapidly divide in culture, but this division is inhibited following cisplatin treatment. Poly-plat treatment however, does not inhibit fibroblast division, and may actually increase cell division possibly through the high amounts of IL-2 released. Therefore it may be that Poly-plat's antitumor effects rely not on its ability to stop cell division, but rather on its enhancement of immune cells to seek out and lyse the tumor cells. Further experimentation is required to determine the effect of blocking IL-2 in the supernatant with IL-2 antibodies.

Poly-plat is a large compound, with a molecular weight of 707. Compared to cisplatin, which has a molecular weight of 132, in equal doses its molarity is nearly one-seventh. This could account for the decreased toxicity of Poly-plat. For consistency in past experiments the dose of Poly-plat has been kept the same as that of cisplatin, but in order for Poly-plat to be effective its dose-response relationship needed to be determined. Since the goal of the current research is to find more effective ways of enhancing the immune system function, and since increased IL-2 levels have been shown to correlate

with macrophage activation, we chose IL-2 as a marker for dose response (Burkitt and Aggarwal 2000), (Basu et al. 1991). Although 10 μg/ml of Poly-plat has been shown to be effective in the past at inducing IL-2 release, our studies show the maximal release of IL-2 to be from Poly-plat at a dose of 5 μg/ml. This dose is used through the remainder of the experimentation with Poly-plat.

In this study we examined Poly-plat's effectiveness at inhibiting tumor cell growth through the activation of the immune response. Poly-plat alone is unable to induce apoptosis in HT1080 cells. However, when co-cultured with macrophages there is a tremendous increase in the apoptotic rate for these cells. This is even true for HT1080 cells which were not co-cultured with macrophages, but which were treated with macrophage supernatant from macrophages which had been treated with Poly-plat. This increase in apoptosis may be caused by various cytokines (IL-1α and TNF-α) which have been shown in previous studies to increase dramatically in macrophages following treatment with Poly-plat (Muenchen and Aggarwal 1998). Macrophages in this study have also been shown to seek out tumor cells when treated with Poly-plat, moving across an entire glass slide. Morphologically the macrophages appear different, but in addition the tumor cells must be releasing some factor following treatment which allows the macrophage to find them. Similar chemotactic responses have been seen *in vivo* by immune cells (Springer and Anderson 1986),(Fumarulo et al. 1980).

The development of a tumor is caused by an aberrant cell that escapes detection by the immune system (Sharon 1998). By activating immune cells with these drugs we hope to empower immune cells to seek out and destroy tumor cells. This study has shown growth inhibition in Poly-plat treated tumor cells. These tumor cells are able to

recover from treatment with Poly-plat, either through repair mechanisms or loss of drug stability. The addition of immune cells, as are found in vivo, also causes these cells to go apoptotic. The two mechanisms (cell growth inhibition and apoptosis) may be the cause of Poly-plat's effectiveness in treating animals burdened with tumors.

CONCLUSION

The ability of cisplatin to effectively treat tumors relies in part on its ability to bind to the DNA and to activate the immune system. Poly-plat activates immune system cells to a greater extent than cisplatin, and has fewer toxic side effects. Activation of immune cells has been shown through an increase in cell size and content, an increase in IL-2 production, and an increase in chemotactic movement. Although it does not appear to cause apoptosis or inhibit cell division, its immunomodulatory effects need to be explored to gain a better understanding of its role in the immune system. Previous studies have shown its effectiveness at treating various cancers *in vivo*. To further explore its potential as an antineoplastic agent its effects will need to be explored in animals with a depleted or deficient immune response. Through an increased understanding of how the immune system is modulated by chemicals such as Poly-plat we can gain insight into more efficient ways to harness its power.

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CHAPTER 5: MACROPHAGE ACTIVATION AS AN ANTI-TUMOR MECHANISM FOLLOWING ADMINISTRATION OF PLATINUM ANTINEOPLASTIC AGENTS

SUMMARY

Activated macrophages seek out tumor cells, make contact with cytoplasmic extensions, and transfer lysosomes to the tumor cell. Activated macrophages also release various cytokines (IL-1, IL-2, TNFα) which further activate the immune system and cause tumor necrosis. Typically macrophages have been activated by treating the animal with the immune stimulating drug (*in vivo*). Unfortunately, direct application of platinum compounds, such as cisplatin or Poly-plat, to the organism causes stress due to the toxicity of the drugs. The goal of the following research was to examine the effects of cisplatin and Poly-plat on activating the immune cells *in vitro* and returning them to another animal in order to examine the degree of immunostimulation. These results were compared to treating the animal directly with cisplatin or Poly-plat. It was found that the blood cells from the donor animals were activated by the drugs and were able to cause an immune response in the recipient animal, which was seen in the increase of serum interleukin-2 levels and resident macrophages (Kupffer cells). Although the response was not as great as that of treating the animal with the drugs directly, it was much less toxic to the animal as evidenced by histochemical staining for the enzymes succinate dehydrogenase and alkaline phosphatase. This treatment method was then applied to tumor burdened animals and resulted in tumor regression nearly equal to that of treating with the platinum drugs.

INTRODUCTION

An effective method of destroying tumors in the body is through the activation of the immune system (Bahadur et al. 1984), (Zacharchuk et al. 1983). Activated macrophages seek out tumor cells, make contact with cytoplasmic extensions, and transfer lysosomes to the tumor cell (Palma and Aggarwal 1995), (Muenchen and Aggarwal 1998). Activated macrophages also release various cytokines (IL-1, IL-2, TNFα) which further activate the immune system and cause tumor necrosis (Muenchen et al. 1997), (Basu et al. 1991). Cisplatin is a potent anti-neoplastic agent, which works via three mechanisms: DNA crosslinking, inhibition of the mitotic spindle, and macrophage activation (Abrams and Murrer 1993), (Palma et al. 1992). Other drugs have been developed which are more potent at macrophage activation and much less toxic (Fiebig et al. 1996), (Drees et al. 1995). These drugs, in particular Poly-plat (Poly-[(*trans*-1,2-diaminocyclohexane) platinum]-carboxyamylose) (Andrulis Pharmaceuticals, Bethesda, MD), are much larger than cisplatin, with numerous branching side chains radiating from a central platinum atom. In equal doses Poly-plat has less platinum than cisplatin, which is a possible reason for the decrease in toxicity.

Typically macrophages have been activated by treating the animal with the drug (*in vivo*). *In vivo* treatment of macrophages with platinum antineoplastic agents results in an increase in macrophage activity, including an increase in extensions, cytolytic factors (cytokines), and numbers (Telgenhoff and Aggarwal 1998), (Bahadur et al. 1984), (Gupta and Sodhi 1987). The increase in the number of macrophages present is a result of monocyte sequestration and differentiation (van der Rhee et al. 1979), (Tanner et al. 1982). Activation of macrophages enhances the organisms ability to seek out and lyse

tumor cells (Adams and Hamilton 1988), (Bucana et al. 1976). Unfortunately, direct application of platinum compounds to the organism causes stress due to the toxicity of the drugs, of which gastrointestinal and nephrotoxicity are the dose limiting factors (Walker and Gale 1981), (Wang and Aggarwal 1997).

By isolating white blood cells from an organism, treating them with macrophage activating drugs, then returning them to the animal we should see a sequestration and activation response similar to that of treating the animal directly with the drug. Further, we will not see the toxicity caused by the antineoplastic agents since the drug itself is not being introduced. A decrease in the harsh side effects is beneficial to the patient, since they will not have to endure the pain and malaise associated with chemotherapy.

MATERIALS AND METHODS

Blood Cells. White blood cells were isolated from nine adult male Sprague-Dawley rats via cardiac puncture. The rats were first anesthetized with methoxyflourane and kept unconscious using a methoxyflourane mask. The chest and abdomen were sterilized with betadine and an 18-gauge needle was inserted into the heart. The blood obtained was placed in tubes containing EDTA to prevent coagulation, and the white blood cells were separated by centrifugation on a Ficoll gradient. The isolated leukocytes (which were composed of granulocytes and mononuclear leukocytes) from each rat were placed in three separate sterile T-25 flasks and treated with either cisplatin (10 μg/ml), Poly-plat (5 μg/ml), or normal saline for two hours. The drugs were removed and the cultures were rinsed with phosphate buffered saline. Supernatants were collected at 0, 4, 18, 24, and 48 hours, and IL-2 levels were measured using ELISA kits. In addition, duplicate plates of cells were treated as above, rinsed to remove the drug, and remained undisturbed in culture for 48 hours. After this time the cultures were treated with 10% (v/v) trypsin for 5 minutes to remove adherent cells. The cells were then collected in normal saline, stained with tryphan blue, and their viable numbers were determined via hemocytometer using the dye exclusion method.

Immune Cell Transplant. Fifteen 10-week-old healthy untreated Spague-Dawley rats were used as blood donors. Each day for five consecutive days three rats were placed in methoxyflourane chambers for two minutes. Upon removal, they were placed face up and their chest was scrubbed with betadine. An 18-gauge needle was then inserted and 8-10 ml of blood was withdrawn via cardiac puncture, after which the rats were euthanized. The blood was then placed in sodium heparin tubes. The leukocytes were separated by

centrifugation for 10 minutes at 2000x g. The white cells were then placed in sterile tubes and each was treated with either normal saline, cisplatin (10 μg/ml), or Poly-plat (5 μg/ml). The tubes were then placed on a rotator for two hours to prevent cell adhesion. The tubes were then centrifuged for 10 minutes at 4000x g. The supernatant was removed and the cells were rinsed in phosphate buffered saline to remove any of the drug. They were again centrifuged; the supernatant discarded, and rinsed a second time. The cells from each treatment group were counted via hemacytometer and separated into three equal portions in normal saline. The cells were then injected into the peritoneal cavity of nine 10-week-old Sprague-Dawley rats that came from the same breeding line as the donor rats. This treatment regimen was repeated each day for five consecutive days. Six additional rats from the same breeding line were treated directly with either cisplatin (10 mg/kg), Poly-plat (5 mg/kg), or normal saline via intraperitoneal injection over a five day period. On the seventh day the rats were euthanized with methoxyflourane. A cardiac puncture was performed on each rat to collect blood, which was placed in serum separator tubes for serum IL-2 analysis. The IL-2 analysis was performed three times using an ELISA kit. The liver and kidney of each rat was removed and flash frozen in OCT compound for enzyme analysis.

Enzyme Analysis. Frozen sections from each rat were cut at 10 μm on a cryotome and air-dried on coverslips. Triplicate sections from each animal were stained for either peroxidase, succinate dehydrogenase, or alkaline phosphatase activity using histochemical methods (Kiernan 1999). Following staining the coverslips were dehydrated through a graded series of ethanol, cleared in xylene, and mounted on

microscope slides using Permount mounting medium. Enzyme levels were examined on the Zeiss 210 laser scanning microscope.

Tumor Cells. Ten T-75 flasks of Walker rat carcinoma (WRC) cells were kept in MEM supplemented with 10% (v/v) horse serum and 1% (w/v) antibiotic [penicillin G (10,000 U/ml) and streptomycin sulfate (10,000 µg/ml)] at 37° C in a 5% CO_2 incubator. The cells were given fresh medium daily until they reached 90% confluency. The cells were then rinsed with phosphate buffered saline and removed exposed to 3 ml of trypsin EDTA solution for five minutes at room temperature. Flasks were tapped to dislodge adherent cells and 10 ml of horse serum was added. The cells were collected into 50 ml centrifuge tubes and centrifuged at 4000 rpm to pellet the cells for 5 minutes. The supernatant was removed and the cells were rinsed with phosphate buffered saline and resuspended. The centrifuge and wash was repeated twice more.

Tumor Burdened Animals. Twenty-four 10-week-old healthy untreated Spague-Dawley rats were given injections using an 18 gauge needle of 1 x 10^6 WRC cells from cultures described above. These injections were given subcutaneously in the right hip region, which had been shaved and scrubbed with betadine prior to the injection. These injections were repeated for two additional days. After five days only nine rats developed measurable tumors in the region. The animals which did not develop tumors were euthanized.

Tumor Treatments. Treatments began when all tumor burdened animals developed tumors larger than 1 cm as measured by linear calipers. Three rats were treated with either normal saline, cisplatin (10 mg/kg), or Poly-plat (5 mg/kg) over a five day period. The remaining six rats were divided into groups of two and treated for five

consecutive days with leukocytes isolated from donor rats and treated with phosphate buffered saline, cisplatin, or Poly-plat as previously described. The treated leukocytes were injected subcutaneously at the site of the tumor using an 18 gauge needle. Measurements of tumor growth were performed daily throughout the treatments and for six days after treatment. At day eleven all animals were euthanized using Methoxyflourane. Tissues were collected and frozen in OCT mounting media at -20° C.

RESULTS

Blood Cells. The treated cells increased in number, presumably through release of IL-2. IL-2 levels were increased in the Poly-plat treated cell supernatants (Figure 1). Cisplatin IL-2 levels decreased below the levels of the untreated cells. The cells were initially plated at 2.2 x 10^5 cells. After 24 hours the untreated cells remained at 2.2 x 10^5. The cells treated with cisplatin dropped to 1.86 x 10^5 cells, and Poly-plat treated cells also dropped to 1.98 x 10^5. After 48 hours in culture the untreated cell count was 2.8 x 10^5. The cisplatin count plummeted to 0.9 x 10^5. The Poly-plat count showed the most dramatic increase in cell numbers, rising to 3.6 x 10^5 (Table 1).

Serum IL-2. In order to determine potential benefits of transferring immune cells, serum IL-2 levels from animals given immune cells treated *in vitro* was examined. The animals which were treated with normal saline had the lowest IL-2 serum levels (29.1 pg/ml), followed closely by the animals which were treated with saline treated blood (29.5 pg/ml). Those receiving cisplatin and Poly-plat directly had the highest levels of serum IL-2 (51.2 and 67.8 pg/ml, respectively). The animals which received blood that had been treated with cisplatin (35.0 pg/ml) or Poly-plat (54.5 pg/ml) did not have levels as high as the direct treatment, but higher than the untreated (Table 2).

Enzyme Assays. Each of the animals that received donor blood had enzyme levels of SDH and AP that were similar to that of the control animals (Figure 2). The cisplatin treated rats had enzyme levels which were significantly lower than the control, indicating a high degree of toxicity. The Poly-plat treated rats also showed a decrease in the two enzymes, though not to the extent of the cisplatin treated. Resident macrophages in the liver (Kupffer cells) show a high degree of peroxidase staining, and therefore this

enzyme was used as a marker to examine macrophage numbers. Both cisplatin and Poly-plat treated rats showed an increase in Kupffer cell numbers over that of the control (2.5 and 3 times as many) (Figure 3). The animals treated with white blood cells that had been exposed to these drugs showed on average twice as many Kupffer cells as either the normal or the rats treated with blood alone.

Tumor Regression. Of the nine rats which developed tumors only two of the animals showed no signs of regression, the untreated control and the rat receiving leukocytes which were not treated (Figure 4). All other treatments caused a regression in tumor size as measured by linear calipers. On day eleven the tumors were measured and the rats were euthanized with methoxyfluorane. Upon examination of the internal organs it was discovered that tumor metastasis had occurred to the lung, spleen, and kidney in the control animal. Macrophage metastasize was also seen in the rat treated with Poly-plat in the lung and the spleen. No other metastasis was seen in any of the other treatment groups.

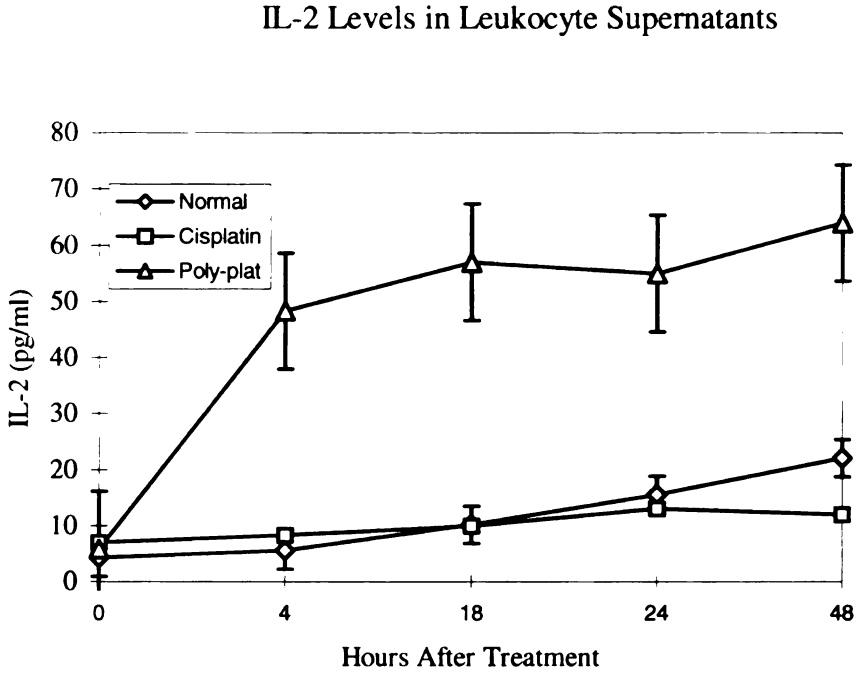

Figure 1: Graph showing IL-2 levels in treated leukocytes following treatment with cisplatin (10 μg/ml), Poly-plat (5 μg/ml), or normal saline *in vitro*. Note the increase in IL-2 levels following Poly-plat treatment. [Average of three repetitions ± S.D.]

Leukocyte Counts Via Hemacytometer
Following Treatment

Treatment	0 Hours	24 Hours	48 Hours
Control	2.2×10^5	2.2×10^5	2.8×10^5
Cisplatin	2.2×10^5	1.86×10^5	0.9×10^5
Poly-plat	2.2×10^5	1.98×10^5	3.6×10^5

Table 1: Table showing Leukocyte numbers from three rats per treatment group following treatment with cisplatin (10 μg/ml), Poly-plat (5 μg/ml) or normal saline. Counts of viable cells performed via hemacytometer and averages of ten counts given as total cell count per flask.

IL-2 Levels in Serum of Treated Rats

	IL-2 (pg/ml)
Control (2)	29.1 +/- 2.7
Cisplatin (2)	51.2 +/- 9.8
Poly-plat (2)	67.8 +/- 5.5
Untreated Leukocytes (3)	29.5 +/- 5.7
Cisplatin Treated Leukocytes (3)	35.0 +/- 8.2
Poly-plat Treated Leukocytes (3)	54.5 +/- 8.1

Table 2: IL-2 levels in animals which received either normal saline (2 rats), cisplatin (10 μg/ml, 2 rats), Poly-plat (5 μg/ml, 2 rats), Leukocytes from donor animals (3 rats), leukocytes from donor animals treated with cisplatin (10 μg/ml, 3 rats), or leukocytes from donor animals treated with Poly-plat (10 μg/ml, 3 rats). The IL-2 levels from the animals treated directly with the platinum drugs was the highest, however the leukocytes which were treated with the platinum drugs elicited a release of IL-2 much greater than the control as well.

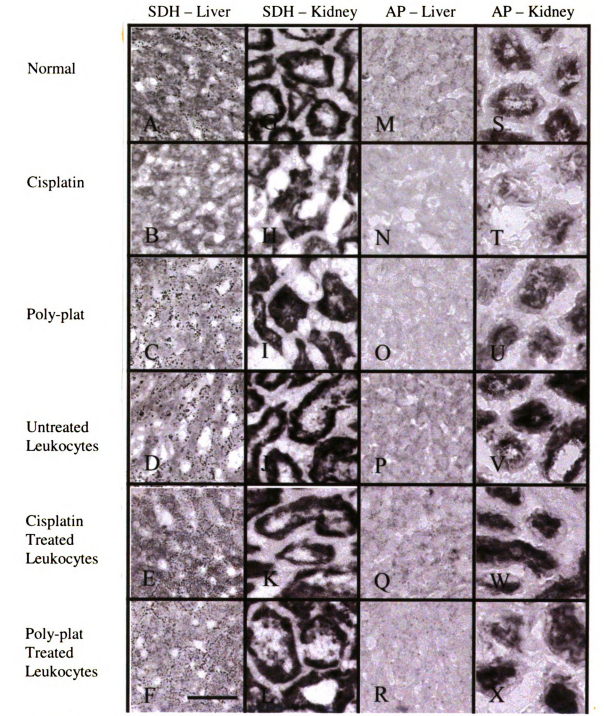

Figure 2: Enzyme histochemistry for succinate dehydrogenase (SDH) and alkaline phosphatase (AP) of liver and kidney from control animals (A, G, M, S) or animals treated with cisplatin (B, H, N, T), Poly-plat (C, I, O, U), untreated leukocytes (D, J, P, V), leukocytes treated with cisplatin (E, K, Q, W), or leukocytes treated with Poly-plat (F, L, R, X). The enzyme levels in the cisplatin treated tissues showed the greatest decrease. Bar = 50 μm.

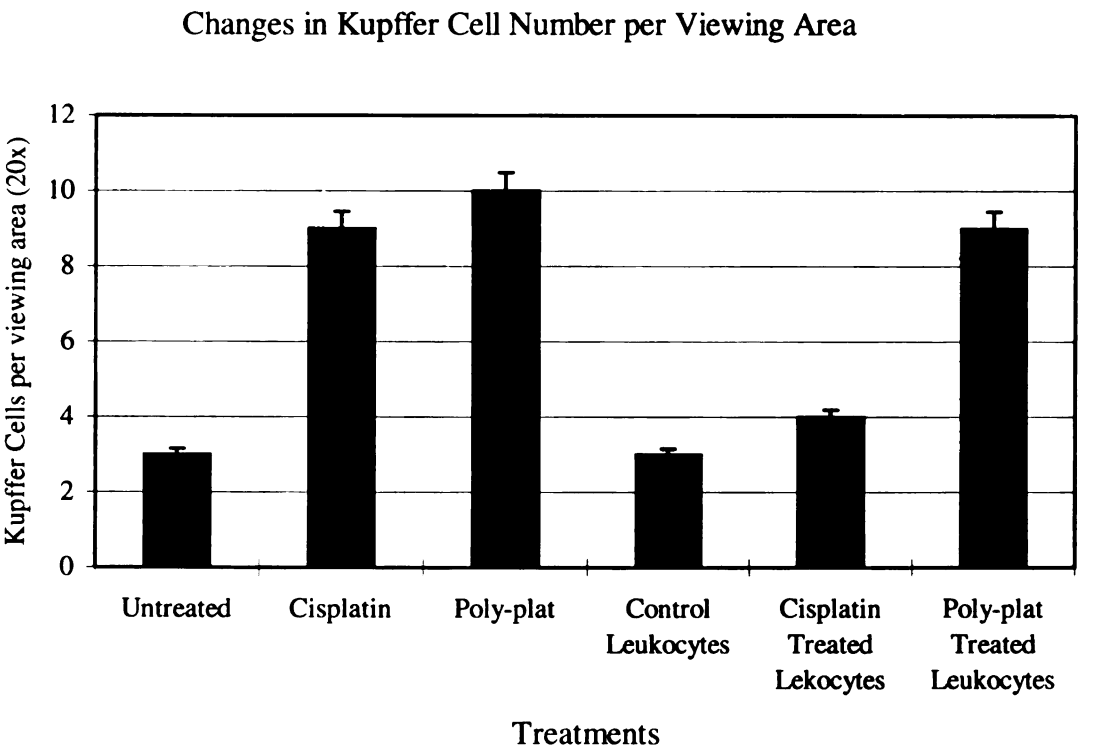

Figure 3: Graph showing changes in Kupffer cell number as seen via peroxidase staining following various treatments. Averages and standard deviations were obtained from viewing twenty areas in the sections from each rat, for a total of 40 (untreated, cisplatin, Poly-plat) or 60 (leukocyte treatments) data sets. Note the increase in Kupffer cells from treating rats with leukocytes exposed to Poly-plat (5 μg/ml) is nearly equal to that of treating directly with cisplatin (10 μg/ml) or Poly-plat (5 μg/ml).

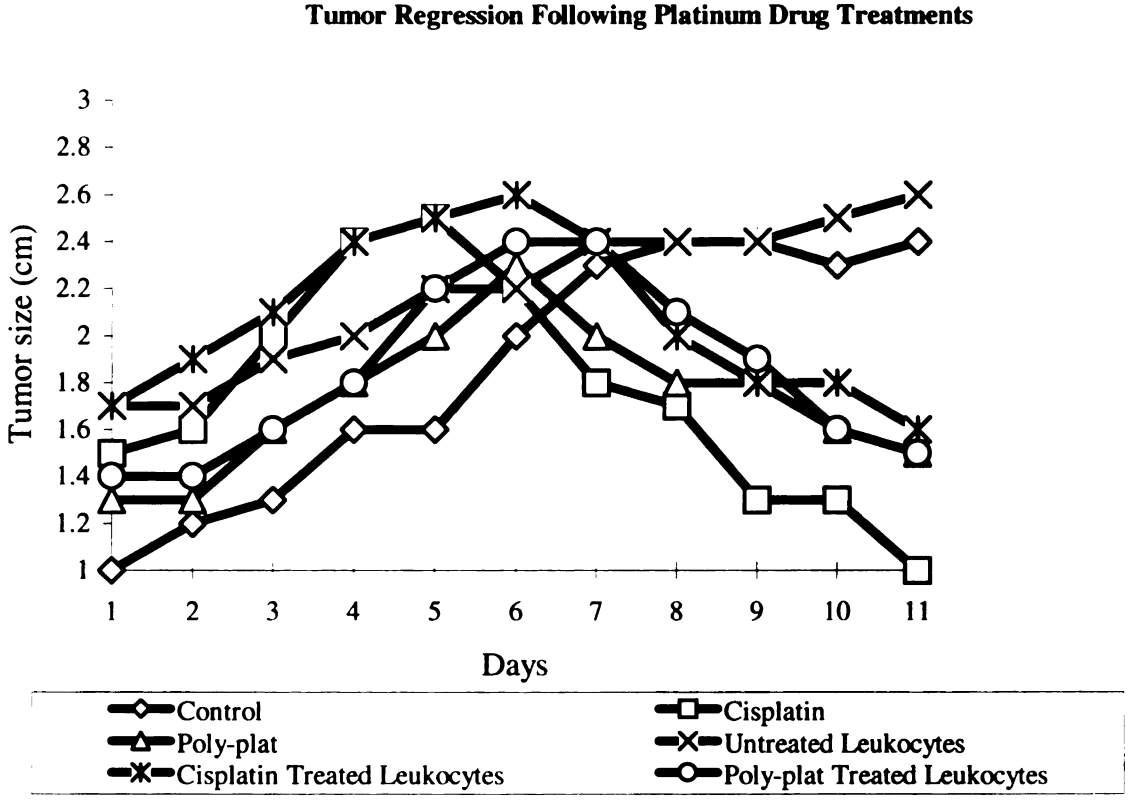

Figure 4: Tumor regression of tumors induced in the right hip of rats via injection of WRC cells. Data shown from rats which developed tumors $\geq$ 1 cm in diameter. Three rats were treated with either normal saline, cisplatin (10 mg/kg), or Poly-plat (5 mg/kg) on days 1-5. The remaining six rats were divided into groups of two and treated for five consecutive days with leukocytes isolated from donor rats and treated with either normal saline, cisplatin (10 μg/ml), or Poly-plat (5 μg/ml). Only two groups showed no regression of tumors with treatment, the control and the untreated leukocyte treated animal.

DISCUSSION

The safe and effective use of chemotherapy in clinical practice requires a thorough understanding of the drugs actions as well as its associated toxicity. Cisplatin has been used to treat testicular, ovarian, head, neck, and bladder cancers with a high degree of success (Palma and Aggarwal 1995). It is believed to enter the tumor cell by diffusion and disrupt the DNA double helix by interstrand and intrastrand crosslinking (Roberts and Pascoe 1972). The major toxicity caused by cisplatin is impairment of renal tubular function. Irreversible kidney damage can occur at higher doses or repeated courses (Goodman et al. 1996). In addition to other toxicity, marked nausea and vomiting occur in almost all patients (Abrams and Murrer 1993), (Goodman et al. 1996). For some, this can be a dose-limiting factor. The goal of our research has been to find ways to deliver this and other highly effective drugs without the associated side effects.

One concept we have been studying is the ability of cisplatin to activate the immune system. We have seen cisplatin's effects on immune cells and believe this to be an important part of it effectiveness (Telgenhoff and Aggarwal 1998). We have also examined other less toxic platinum analogs for their ability to stimulate the immune response. Of these, Poly-plat appears to be the most effective at immune cell activation (Muenchen et al. 1997). The development of a tumor is caused by an aberrant cell that escapes detection by the immune system. By activating immune cells with these drugs we hope to empower immune cells to seek out and destroy tumor cells selectively without the associated toxicity of directly treating the animal with the drug.

Only in recent times have we seen the rise of a new type of therapy for treating cancers, which in some instances has shown to be as effective as surgery, radiation, and

chemotherapy. This new therapy, referred to as biological therapy, is defined as cancer treatments that act primarily through natural host defense mechanisms or by the administration of natural mammalian substances (Rosenberg 1991). Adoptive immunotherapy, which is the transfer of immune cells that have antitumor activity to a tumor-bearing host, is a type of biological therapy. Dr. Steven Rosenberg at the National Institutes of Health has been a leader in adoptive immunotherapy. His initial studies involved using lymphocytes that had been exposed to the tumor either *in vitro* or *in vivo*. These cells were grown in culture and returned to the tumor-bearing host (Rosenberg 1991). He also used IL-2 to induce the clonal expansion of T lymphocytes (Lotze et al. 1990). IL-2 is an inflammatory cytokine, typically associated with T lymphocytes, inducing clonal expansion and producing tumor infiltrating lymphocytes which play a pivotal role in tumor regression (Lotze et al. 1990). Rosenberg's most recent work in the field has been with combination adoptive immunotherapy. He has used IL-2, which does show antitumor activity by itself, in conjunction with alpha interferon and tumor infiltrating lymphocytes (Rosenberg et al. 1988). The difference in our study in comparison to Dr. Rosenberg's is that we use immune cells that have not been exposed to a specific tumor. The immune cells in our studies are activated by a platinum compound that results in a more generalized activation. This generalized activation has been shown to be effective in various tumor models, including Walker rat carcinoma, human fibrosarcoma, murine sarcoma 180, and human ovarian teratocarcinoma cells (Palma et al. 1992) (Burkitt and Aggarwal 2000).

Macrophages treated with cisplatin have been shown to seek out and lyse tumor cells, and release cytokines such as TNF-α and IL-1α (Palma et al. 1992). This study

reveals another mechanism whereby activated macrophages are immune system stimulators through the release of high levels of IL-2. Poly-plat treated macrophages release even greater quantities of IL-2 than cisplatin, and show an increase in morphologic characteristics of activation. Through this generalized form of activation we have seen immune cells returned to the animal and create an immune response which, while not on the same level as treating with the drugs directly, shows promise due to the increase in circulating levels of IL-2 and the lack of toxicity.

The Walker rat carcinoma is an aggressive tumor, which has been shown to readily metastasize to the lung and other organs (Bellamy and Hinsull 1978). On treating the tumor burdened animals with activated immune cells we saw a regression of the localized tumor and no metastasis. Animals which were not treated showed a steady growth in the size of the tumor, and metastasis occurred in two of the animals. It is unclear why the Poly-plat treated animal developed secondary tumors while the initial tumor regressed. In previous studies, Poly-plat was shown to slow tumor cell growth without inducing apoptosis (unpublished data). Poly-plat may be arresting the tumor cells replicative cycle, but is ineffective at preventing metastasis. A larger treatment group will need to be utilized to determine Poly-plat's effectiveness.

In determining the effectiveness of treating leukocytes *in vitro* and using those to cause tumor cell regression we have developed a system of adoptive immunotherapy which avoids the use of cultured macrophages (which are difficult to obtain in large numbers) and that could be applied clinically with minimal discomfort or risk to the patient. Further studies are required to determine the effectiveness of this treatment

method on spontaneous tumors, which are generally more difficult to treat than transplanted tumors (Rockwell and Moulder 1990).

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CONCLUSION

The safe and effective use of chemotherapy in clinical practice requires a thorough understanding of the drugs action's as well as their associated toxicities. The goal of our research has been to find ways to deliver highly effective drugs without the associated side effects. One concept we have been studying is the ability of platinum based compounds to activate the immune system. We have seen cisplatin's effects on immune cells and believe this to be an important part of it effectiveness (Telgenhoff and Aggarwal 1998). Stimulation of the immune system is a major function of many antineoplastic agents. Cisplatin stimulates the immune system through macrophage activation (Palma et al. 1992), making the macrophages more efficient in phagocytosing tumor cells. The effects of chemotherapeutic agents on modulating host immune defenses have been identified in many studies. Matheson *et al.* demonstrated that low doses of methotrexate and 5-fluorodeoxyuridine augmented natural killer cell activity (Matheson et al. 1983). The activation of macrophages to destroy tumor cells was seen in rats treated with mitomycin C (Ogura et al. 1982) and in mice treated with adriamycin and cyclophosphamide (Stoychkov et al. 1979). Macrophage activation is considered to be an important part of the organisms response to chemotherapy (Ohanian et al. 1980). Macrophage activation occurs rapidly, with noticeable changes in shape and function occurring within one day post treatment (Toge et al. 1981). Monocyte recruitment, an event which increases the number of available macrophages to counter insult, is a more lengthy process (van der Rhee et al. 1979).

The severe toxic side effects evident in cisplatin treatment seem to be due to the disruption in sulphydryl containing mitochondrial dehydrogenase enzymes (Aggarwal

1993). The disruption of these enzymes prevents calcium uptake thereby reducing calcium levels in the cell to cytotoxic levels. SAP, SSP, and Poly-plat are novel platinum agents which show no such disruption, and as a result have been shown to be much less toxic (Muenchen and Aggarwal 1998), (Telgenhoff and Aggarwal 1998). Due to low toxicity SAP, SSP, and Poly-plat would appear to be alternatives to CDDP. SAP and SSP both activate macrophages, however, there is no significant increase in Kupffer cell numbers after SAP and SSP treatment, as shown by sinusoidal esterase staining. Poly-plat not only increases Kupffer cell activation, but also increases the number of Kupffer cells present. In this research we examined Poly-plat's effectiveness at inhibiting tumor cell growth. Both WRC-256 and HT1080 cells were inhibited by Poly-plat treatment. This data coupled with the lack of apoptosis in these cells when treated with Poly-plat alone could indicate that these cells are arrested in the cell cycle until repair mechanisms can be activated. Poly-plat activates immune system cells to a greater extent than cisplatin, and has fewer toxic side effects. Activation of immune cells has been shown through an increase in cell size and content, an increase in IL-2 production, and an increase in chemotactic movement. Poly-plat treated macrophages release even greater quantities of IL-2 than cisplatin, and show an increase in morphologic characteristics of activation.

Chemotherapy is a widely used treatment for cancer, but the mechanisms of drug action are still being discovered. While early studies (Rosenberg and VanCamp 1970), (Roberts and Pascoe 1972) focused on chemotherapeutic agent's abilities to crosslink DNA and prevent cell cycle progression, more recent experiments were designed to examine the role of inducing apoptosis in tumor cells (Jiang et al. 1999), (Nehme et al.

1997). One of the main problems with this type of research is that drugs exert their effects differently depending on the cell type used, and may even react differently to the same type of tumor in different subjects. In one study cisplatin mediated apoptosis by down-regulating inhibitory proteins of the Fas/FasL signalling pathway in sarcoma cells (Kinoshita et al. 2000), in another study lung cancer cells exposed to cisplatin underwent apoptosis without utilizing the Fas/FasL pathway (Ferreira et al. 2000). Cells from the same lineage can also become resistant to cisplatin and fail to undergo apoptosis either due to a decrease in cisplatin uptake or through a change in DNA repair ability (Blommaert et al. 1998), (Fink et al. 1997).

Based on the studies conducted with cisplatin, our goal was to examine the effects of Poly-plat on inducing apoptosis. From these studies it can be assumed that Poly-plat alone *in vitro* is ineffective at inducing apoptosis in tumor cells. Further it has been shown that tumor cells treated with Poly-plat recover from treatment within five days. Since Poly-plat has been shown to be effective against certain tumors *in vivo*, it is possible that Poly-plat's effectiveness lies not in it's ability to kill tumor cells, but rather in it's ability to slow tumor cell growth until an effective immune resonse can be mounted. Further evidence of cell cycle arrest following Poly-plat treatment was apparent from the levels of p27 and PCNA. p27 was elevated in Poly-plat treated cells 24 and 48 hours after treatment. An elevation of p27 has been shown to indicate cell cycle arrest in tumor cells following platinum drug treatment (Oshita et al. 2000). A large increase in PCNA levels 48 hours after treatment indicates a push for the cell to continue through the cycle. Increased levels of this protein following Poly-plat treatment

may indicate the cells are recovering from the insult and are upregulating proteins required for cell cycle progression.

Adoptive immunotherapy, which is the transfer of immune cells that have antitumor activity to a tumor-bearing host, is a type of biological therapy (Rosenberg 1990). In most studies involving immunotherapy the cytotoxic T-cell is the effector of choice (Basu et al. 1991), (Rosenberg et al. 1988), (Wang and Rosenberg 1996). Several studies have examined the effects of various agents on inducing macrophages to kill tumor cells (Han et al. 1999), (Johnson et al. 1986), (Ogura et al. 1982). Very few studies treated macrophages with an immune stimulating drug and returned the macrophages to tumor burdened animals with the effect of alleviating the tumor (Wang et al. 1986), (Di Luzio et al. 1976). The possible reason for the lack of interest in this method is the difficulty in obtaining large populations of macrophages for treatment in a clinical setting. In addition, macrophages do not survive well in culture, and rarely proliferate due to already being terminally differentiated cells (Cohn 1978), (Herscowitz et al. 1981).

In our studies macrophages were activated by cisplatin or Poly-plat which resulted in a generalized form of activation. Activation of immune cells was shown through an increase in cell size and content, an increase in IL-2 production, and an increase in chemotactic movement. Poly-plat treated macrophages released even greater quantities of IL-2 than cisplatin, and showed an increase in morphologic characteristics of activation. This generalized activation was shown to be effective in various tumor models, including Walker rat carcinoma, human fibrosarcoma, murine sarcoma 180, and human ovarian teratocarcinoma cells (Palma et al. 1992) (Burkitt and Aggarwal 2000).

In order to develop an adoptive immunotherapy system utilizing Poly-plat clinically we had to use immune cells which were readily obtainable and which would result in an immune response similar to that seen with macrophages *in vitro*. We chose to use peripheral circulating leukocytes for the adoptive transfer technique. Leukocyte populations contain lymphocytes which have been shown in previous studies to be good effectors of tumor cell kill (Cole et al. 1994) (Rosenberg et al. 1988), and monocytes which differentiate into macrophages when activated (Kleinerman et al. 1980), (Sutton and Weiss 1966).

On treating the tumor burdened animals with leukocytes activated with cisplatin and Poly-plat we saw a regression of the localized tumor and no metastasis. Animals which were not treated showed a steady growth in the size of the tumor, and metastasis occurred in two of the animals. In addition, we saw none of the associated toxicities of treating the animal directly with the drugs. Due to the ease of obtaining large numbers of leukocytes from an organism this treatment method can easily be applied in the clinical setting. Leukocytes can even be obtained from the tumor burdened individual (for non leukemia or lymphoma cancers), treated, and returned to the same individuals without cross reactivity. The treatment could be repeated many times with little to no side effects for the patient. A decrease in harmful side effects is not only easier for the patient to tolerate, but also decreases damage to the kidney and liver. The immune system can be a powerful tool in combating disease, but occasionally cancer cells evade detection. Through an increased immune response following adoptive immunotherapy it may be possible to boost the systems awareness to the tumor and mount a greater, more efficient response.

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