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IMMUNOSTIMULATION BY NOVEL PLATINUM ANTICANCER AGENTS

presented by

Heather Jean Muenchen

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Ph.D. degree in Zoology

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Major professor

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IMMUNOSTIMULATION BY NOVEL PLATINUM ANTICANCER AGENTS

By

Heather Jean Muenchen

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ABSTRACT

IMMUNOSTIMULATION BY NOVEL PLATINUM ANTICANCER AGENTS

By

Heather Jean Muenchen

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP] is an antineoplastic drug with demonstrated activity against mainly ovarian and testicular cancers. Although cisplatin is an effective chemotherapeutic drug, it has very severe toxic side effects, of which gastrointestinal and nephrotoxicity are the dose limiting factors. Poly-[(trans-1,2-diaminocyclohexane) platinum]-carboxyamylose ("poly-plat"), 5-sulfosalicylato-trans-(1,2-diaminocyclohexane) platinum (SSP), and 4-hydroxy-α-sulfonylphenylacetato (trans-1,2-diaminocyclohexane) platinum (II) (SAP) are analogs of cisplatin with higher efficacy and potency, while eliciting less toxicity. Normal murine peritoneal macrophages, when treated with "poly-plat" and SSP (5 μg/ml) for 2 h at 37°C in culture, developed extension formations within 2 h. These cytoplasmic extensions radiate from the cell body in all directions. While SAP (5 μg/ml) treated macrophages, not showing any extension formation, instead assumed a discoid shape similar to that of the normal macrophages. Cisplatin (5 μg/ml) treated macrophages also showed similar extension formation but only after 24 h of treatment.

When these drug treated macrophages are co-incubated with S180 tumor cells they immediately establish contact with several target cells and form cytoplasmic continuity through which lysosomes are transported into the tumor cells causing their

lysis. CDDP treated cells also form cytoplasmic extensions that are fewer in number and establish contact with fewer tumor cells when compared to "poly-plat" treated macrophages. Normal macrophages do not form cytoplasmic extensions and, when co-incubated with S180 cells, fail to show any interaction.

Based on fluorescence measurements after acridine orange labeling, we observed a 500-fold increase in the number of lysosomes in the macrophages after only 2 h of "poly-plat" treatment compared to normal cells. SSP and SAP both demonstrated a 100-fold increase in the number of lysosomes. Comparatively, cisplatin treatment demonstrated only a 50-fold increase in the lysosomes. Significant increases in TNF-α levels were observed in the supernatants from SSP (5 μg/ml) treated macrophages at all times tested, with the most significant increase occurring at 2 h post-treatment (3250 pg/ml).

"Poly-plat" and SAP (5 μg/ml) demonstrated very little TNF-α activity, barely reaching 200 pg/ml at 24 h post-treatment. Cisplatin demonstrated the usual enhanced release of TNF-α at various time intervals, reaching a peak value at 2 h post-treatment (3000 pg/ml). Compared to cisplatin treatments there was an increase in IL-1α levels in the supernatants of macrophages treated with either "poly-plat", SSP, or SAP (5 μg/ml) for up to 24 h of testing.

Dedicated to my son Jadan.

ACKNOWLEDGMENTS

I wish to express my deepest thanks to Dr. Surinder K. Aggarwal, my advisor and mentor, from whom I have learned much in the areas of science and non science. His guidance, support, and patience has been greatly appreciated. I also wish to thank Drs. Will Kopachik, Thomas Corner, and Ashir Kumar for having served as my committee members.

Lastly, I would like to acknowledge my family and friends whose support has enabled me to reach my goals.

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CHAPTER 1: INTRODUCTION

INTRODUCTION

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP], a broad spectrum anticancer drug, is proven to be effective in the treatment of testicular, ovarian, prostate, bladder, head and neck, and lung cancers.[1] DNA denaturation is one of the accepted methods of its mechanism of action through its intrastrand and interstrand cross-links interfering with DNA replication and transcription. Another mechanism of action is through the activation of the immune system.[2] The activation process includes the generation of extracellular products including interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α).[3] CDDP has been shown to activate murine peritoneal macrophages *in vitro*.[4] These activated macrophages seek out tumor cells through the formation of cytoplasmic extensions and lysosomal transfer to these target cells causing cell death.[5] Although cisplatin is an effective anticancer drug, it has very severe toxic side effects, of which gastrointestinal and nephrotoxicity are the dose limiting factors.[6]

Taxol has a unique mechanism of antitumor activity in that it binds to a protein, tubulin, thus inhibiting cell division.[7] CDDP plus taxol in combination has become increasingly prevalent in clinical treatment.[7] Combination therapy is capable of producing very high response rates and is seemingly less toxic than either of the two drugs when administered alone.[7] Although, the mechanism of action for the cytotoxic interactions between CDDP and taxol have not been determined.

Poly-[(trans-1,2-diaminocyclohexane) platinum]-carboxyamylose ("poly-plat"), 5-sulfosalicylato-trans-(1,2-diaminocyclohexane) platinum (SSP), and 4-hydroxy-α-

sulfonylphenylacetato (trans-1,2-diaminocyclohexane) platinum (II) (SAP) are second generation analogs of cisplatin with higher efficacy and potency, while eliciting less toxicity.[8] This is particularly true of "poly-plat" which contains 1/5 the platinum of cisplatin.[8] The possibility of drugs enhancing the immune system with less toxicity is very encouraging.

"Poly-plat" is curative in a range of solid tumors including renal, breast, ovarian, plasma cell myeloma, and adenocarcinoma.[8] It has been shown to be up to 15X as active as equimolar amounts of cisplatin, showing inhibition in many tumors where cisplatin is ineffective. [8] SSP is curative of many cisplatin resistant tumors including M5 ovarian and several plasma cell myelomas. SSP also elicits no nephrotoxicity and is less toxic than cisplatin even at LD_{10} dose (180 mg/kg).[8] SAP, the most potent of the platinums, exhibits a wide spectrum of anti-tumor activity. It has facile reproducible synthesis and minimum acute toxicity at any effective dose from 1.85 mg to 15.0 mg/kg. We have here explored the effects of cisplatin plus taxol, "poly-plat", SSP, and SAP on the macrophages so as to understand their mechanism/s of action in enhancing the immune system.

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CHAPTER 2: ACTIVATION OF MURINE PERITONEAL MACROPHAGES AFTER CISPLATIN AND TAXOL COMBINATION

Anti-Cancer Drugs 199?, 8, pp. ?84–?89

Activation of murine peritoneal macrophages after cisplatin and taxol combination

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Cisplatin and paclitaxel are potent antineoplastic agents. Their distinctly different mechanisms of action have prompted laboratory and clinical research into their use in combination therapies. Murine peritoneal macrophages treated with cisplatin and paclitaxel in combination elicit an increase in their number of lysosomes. Drug–treated macrophages, when co-incubated with sarcoma 180 cells, establish cytoplasmic contact and transfer lysosomes into tumor cells causing tumor cell lysis. In addition, analysis of tissue culture supernatants show increased levels of interleukin-1α and tumor necrosis factor-α. Our study shows that cisplatin and paclitaxel in combination enhance elements of the immune system with greater efficacy and potency than when used alone.

Key words: Cisplatin, interleukin-1α, lysosomes, *in vitro*, macrophages, taxol, tumor necrosis factor-α.

Introduction

The remarkable clinical efficacy of paclitaxel (taxol) has resulted in numerous observations of partial and complete remission of advanced ovarian cancer in women.[1] Recently, reports of the efficacy of the drug in breast, lung and prostate cancer have aroused great interest in the antitumor compound.[1] Taxol has a unique mechanism of antitumor activity in that it binds to a protein, tubulin, thus inhibiting cell division.[2]

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP], a heavy metal platinum coordination complex, is proven to be effective in the treatment of testicular, ovarian, prostate, bladder, head and neck, and lung cancers.[3] DNA denaturation is one of the accepted methods of its mechanism of action through its intrastrand and interstrand cross-links interfering with DNA replication and transcription.[4] Another mechanism of action is through the activation of the immune system. Activated macrophages have been found to effectively destroy tumor cells by cytotoxic mechanisms.[5,6] The activation process includes the generation of extracellular products including interleukin (IL)-1α and tumor necrosis factor (TNF)-α.

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Cisplatin plus taxol in combination has become increasingly prevalent in clinical treatment.[8] Combination therapy is capable of producing very high response rates and is seemingly less toxic than either of the two drugs when administered alone.[8] Although, the mechanism of action for the cytotoxic interactions between cisplatin and taxol have not been determined. We have here explored the effects of cisplatin plus taxol on macrophages so as to understand their mechanism of action in the immune system enhancement and its efficacy in fighting cancer.

Materials and methods

Cell cultures

Swiss Webster mice (Charles River, Location, MA) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (MEM; Gibco, Grand Island, NY) without serum containing 1% antibiotic-antimycotic [penicillin G (10 000 U/ml), streptomycin sulfate (10 000 μg/ml) and amphotericin B (25 μg/ml); Gibco] into the peritoneal cavity. After gently massaging the abdominal wall, cells were aspirated and seeded onto 18 mm^2 glass coverslips, placed in 35 mm Petri dishes, at 2–4 × 10^6 cells/ml and incubated for 2 h at 37°C. Coverslips were washed vigorously to remove non-adherent cells. Cell cultures were incubated in normal medium (minimal essential media and 10% heat-inactivated fetal calf serum) at 37°C in a 5% CO_2 incubator. Sarcoma 180 ascites (S180; CCRFS-180II; American Type Culture Collection, Rockville, MD) were maintained in culture using normal medium. Cells were washed with Hank's balanced salt solution (HBSS; Gibco) and centrifuged at 1000 *g* for 5 min for use in experiments. These cells served as target cells for macrophages and were added to cultures at 3 × 10^5 cells/ml concentration.

Treatments

Cisplatin and taxol were prepared in 0.85% NaCl in 5 and 10 μg/ml concentrations, respectively. Macrophages were treated with the drugs for 2 h. The drug(s)-containing medium was replaced by normal medium, and supernatant (500 μl) was collected at 0.5, 1, 2 and 24 h for cytolytic factor/s analysis. Untreated cells in normal medium served as controls.

IL-1α assay

IL-1α was assayed using an ELISA kit (Genzyme, Cambridge, MA). The method used the multiple antibody sandwich principle,[9] where monoclonal anti-murine IL-1α was used to bind murine IL-1α present in the supernatant. A biotinylated polyclonal antibody binding the IL-1α was added and unbound material was washed out. Peroxidase-conjugated avidin was used to bind these biotin tagged complexes. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with IL-1α (15–405 pg/ml) provided in the kits and linear regression analysis was performed.

TNF-α assay

TNF-α released from supernatants of the macrophages was assayed using a specific analysis kit (Genzyme). Again, the multiple antibody sandwich principle[9] was utilized with a murine monoclonal antibody specific for murine TNF-α in the samples. A horseradish peroxidase-conjugated anti-murine TNF-α antibody was used to bind the multiple epitopes on TNF-α. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with TNF-α (35–2240 pg/ml) provided in the kits and linear regression analysis was performed.

Lysosomal assay

The quantitation of lysosomes before and after various treatments was achieved by exposing macrophage cultures to fresh medium containing acridine orange (5 μg/ml) for 30 min at 37°C in the dark.[10] After careful washing, macrophages were examined under a Zeiss 10 laser scanning confocal microscope and visual counts made.

Macrophage–tumor cell interaction studies

To study macrophage-tumor cell interaction, macrophage monolayers were treated with either cisplatin (5 μg/ml), taxol (10 μg/ml) or cisplatin (5 μg/ml) plus taxol (10 μg/ml) for 2 h at 37°C in a 5% CO_2 incubator. The medium was then replaced by normal medium and the S180 tumor cells were added. Macrophages and tumor cells were co-incubated for 2 h. Coverslips seeded with macrophages and tumor cells were fixed with 1.5% glutaraldehyde on 0.05 M phosphate buffer (pH 7.2) at room temperature for 10 min. Macrophage-tumor cell interaction was viewed using phase contrast microscopy.

Results

IL-1α release

Compared to normal there was a gradual increase in IL-1α levels in the supernatants of macrophages treated with cisplatin (5 μg/ml) plus taxol (10 μg/ml) (Figure 1). There was a gradual increase in macrophages treated with cisplatin alone. However, taxol-treated macrophages demonstrated a large increase (400 pg/ml) in IL-1α after 30 min post-treatment. These levels gradually decreased until 2 h post-treatment. After 24 h again there was an increase reaching approximately 275 pg/ml.

TNF-α release

Compared to normal, a combination treatment of the two drugs cisplatin (5 μg/ml) plus taxol (10 μg/ml) demonstrated increased levels of TNF-α after only 30 min post-treatment. A cyclical release was observed when TNF-α was viewed at 1, 2 and 24 h post-treatment. This cyclical release pattern was also true for both cisplatin and taxol when used alone, but the levels of TNF-α were not as high as after the combination treatment (Figure 2).

Macrophage activation

Murine peritoneal macrophages demonstrated extension formations after 2 h post-treatment with cisplatin alone (5 μg/ml) (Figure 3A). However, taxol (10 μg/ml) and cisplatin (5 μg/ml) plus taxol (10 μg/ml) treated macrophages did not show any extension formation, for up to 24 h, but instead assumed a discoid shape similar to that of the normal macrophages (Figure 3B–D).

Lysosomal studies

Based on fluorescence measurements after acridine orange labeling, a 100-fold increase in the number of lysosomes in the macrophages was observed only after 2 h of cisplatin (5 μg/ml) plus taxol (10 μg/ml) treatment (Figure 4A) as compared to untreated cells (Figure 4B). Both cisplatin and taxol alone demonstrated only a 50-fold increase in the number of lysosomes (Figure 4C and D).

Macrophage-tumor cell interaction studies

Macrophages co-cultured with tumor cells (S180) establish cell-cell contact within 30 min and demonstrated a transfer of their lysosomes into the tumor cells through cytoplasmic continuity assumed after co-incubation. Cisplatin-treated macrophages have been shown to transfer their lysosomes down the cytoplasmic extensions (Figure 5A). As stated earlier taxol and cisplatin plus taxol treated macrophages assume a discoid shape without cytoplasmic extensions. This, however, did not effect the ability of macrophages to establish contact with the tumor cells and transfer their lysosomes (Figure 5B and C). These tumor cells eventually undergo lysis. Untreated macrophages never established contact with tumor cells (Figure 5D).

Discussion

The antineoplastic activity that taxol has demonstrated in advanced ovarian cancer and other neoplasms in which the platinum analogs are among the most active agents has been the impetus for the development of taxol plus cisplatin combination regimens.[8] Taxol and cisplatin are the two most effective agents discovered to date for treating advanced-stage cancers.[11] Learning how best to combine these agents is the focus of preclinical and clinical studies conducted at a number of institutions. The overt effects of the anticancer drugs cisplatin and taxol appear to be DNA modification and microtubule stabilization, respectively, yet the mechanisms by which these drugs elicit tumor cell

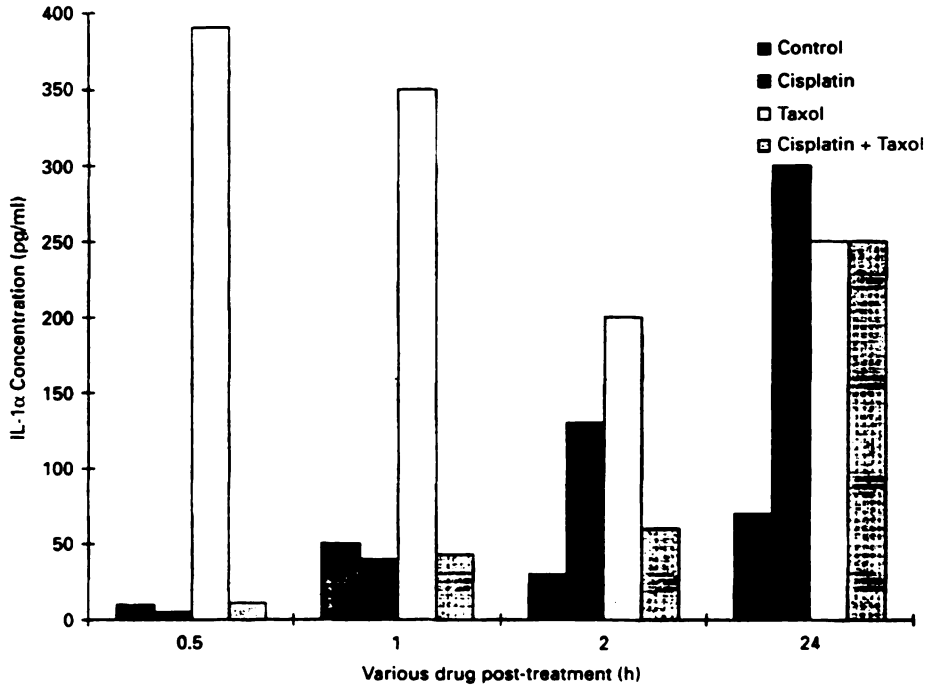

Figure 1. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages treated for 2 h with either cisplatin (5 μg/ml), taxol (10 μg/ml) or cisplatin plus taxol after 30 min, 1, 2 and 24 h post-treatment. Note the large increase at 30 min post-treatment with taxol. Both cisplatin and cisplatin plus taxol show a gradual increase in the levels of IL-1α, reaching a maximum level at 24 h post-treatment.

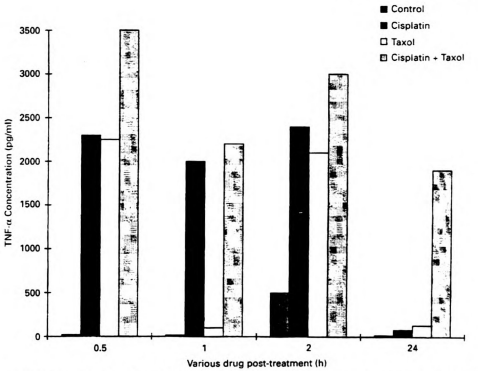

Figure 2. Bar graph showing TNF-α release in the supernatant of murine peritoneal macrophages treated for 2 h with either cisplatin (5 μg/ml), taxol (10 μg/ml) or cisplatin plus taxol after 30 min, 1, 2 and 24 h post-treatment. Note the maximum increase in TNF-α for cisplatin plus taxol at 2 h post-treatment. The release of TNF-α appears to be cyclical in all drug treatments with cisplatin plus taxol showing the highest activity.

Figure 3. Light micrographs showing macrophages after 24 h of treatment with cisplatin (A), taxol (B), cisplatin plus taxol (C) and normal (D). Note the extension formation after cisplatin treatment (arrows). Taxol and cisplatin plus taxol treated cells show mostly a discoid shape similar to that of normal macrophages. Bar = 0.5 mm.

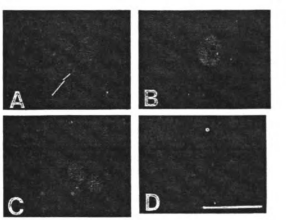

Figure 4. Fluorescent images taken from the Zeiss 10 laser scanning confocal microscope of macrophages labeled with acridine orange (5 μg/ml) showing lysosomal fluorescence in cisplatin plus taxol (A), normal (B), cisplatin (C) and taxol (D). Note the large increase in lysosomal fluorescence after cisplatin plus taxol treatment (arrows). Bar = 10 μm.

death are not well understood.[12] Both *in vitro* and *in vivo* studies conclude that taxol interacts synergistically with cisplatin in a manner that is highly schedule dependent.[12]

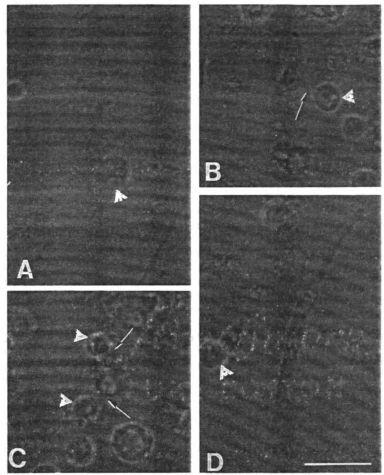

Figure 5. Phase contrast images taken from the Zeiss 10 laser scanning confocal microscope of macrophages co-incubated with S180 tumor cells (arrowheads) after 2 h of treatment with cisplatin (A), taxol (B), cisplatin plus taxol (C) and normal (D). Note that taxol and cisplatin plus taxol treated macrophages do not show long cytoplasmic extensions so very characteristic of cisplatin. However, treated cells are still able to establish contact with tumor cells and destroy them through lysosomal transfer (arrows). Bar = 10 μm.

Cisplatin stimulates immune responses by activating monocyte-macrophages and other cells of the immune system.[13] We have demonstrated here three mechanisms by which the immune system is enhanced by cisplatin and taxol combination compared to cisplatin or taxol treatments: (i) release of cytolytic factors IL-1α and TNF-α, (ii) increase in the macrophage lysosomes, and (iii) cell-cell recognition through contact between activated macrophages and tumor cells.

IL-1α was first described as a lymphocyte activating factor because of its ability to stimulate T cells.[14] Its release by activated macrophages and its cytotoxicity to tumor cells suggests IL-1α as a potent mediator in tumor cell killing by macrophages.[15] Our study demonstrates taxol as having the greatest increase in IL-1α after 30 min, while decreasing by 24 h, but yet stays higher than the untreated macrophages. Both cisplatin and cisplatin plus taxol in combination showed a gradual increase in IL-1α reaching a maximum level after 24 h post-treatment.

Increased levels of TNF-α have also been observed in activated macrophages. TNF-α is known to mediate a variety of functions which include host defense mechanisms and growth.[15] Recent studies have shown increased levels of TNF-α after cisplatin treatment. It is apparent that TNF-α is another important mediator of

tumor cell killing by macrophages. Cisplatin plus taxol combination demonstrated the greatest increase in TNF-α, reaching a maximum level after only 30 min. All three treatments seemed to cause a cyclical release of TNF-α.

Tumor cell death through macrophage activation includes the production of lysosomes.[16,17] Previous studies have shown cisplatin-treated macrophages to increase their number of lysosomes and transfer them via cytoplasmic extensions into tumor cells.[18] This transfer results in eventual lysis of tumor cells.[19] Cisplatin plus taxol combination showed the greatest increase in the level of cytoplasmic lysosomes with a 100-fold increase over that of untreated macrophages. Both cisplatin and taxol independently showed approximately a 50-fold increase in lysosomes.

Lysosomes are only released when cytoplasmic continuity between the macrophage and tumor cell has been established. Past studies have shown that cisplatin activates macrophages to form cytoplasmic extensions which make contact with tumor cells.[18] Through these extensions lysosomes are transferred and cell death occurs. Although taxol and cisplatin plus taxol combination did not activate macrophages to form cytoplasmic extensions they still established contact and formed a cytoplasmic continuity with the tumor cells. Through this cytoplasmic continuity there was an apparent transfer of lysosomes from macrophage to tumor cell and eventual cell lysis.

Conclusion

Cisplatin plus taxol combination stimulates various cytolytic factors of the immune system better than when these drugs are used independently. The macrophage activation includes production of cytoplasmic lysosomes, macrophage–tumor cell contact, and release of cytolytic factors IL-1α and TNF-α. Past *in vitro* and *in vivo* studies have shown that when these two drugs are used in combination they are more effective and less toxic than when they are used separately. Our results support activation of the immune system as an additional mechanism of action of this combination therapy. We also propose, based on our observations, that cisplatin plus taxol combination activates various cytolytic factors of the immune system better than cisplatin or taxol independently.

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CHAPTER 3: ENHANCED IMMUNOSTIMULATION BY NOVEL PLATINUM ANTICANCER AGENTS

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Enhanced immunostimulation by novel platinum anticancer agents

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'Poly-plat', SSP and SAP are second generation analogs of cisplatin which have been shown to activate murine peritoneal macrophages *in vivo* and *in vitro*. Murine peritoneal macrophages treated with 'poly-plat', SSP or SAP (5 μg/mg) for 2 h are stimulated to form cytoplasmic extensions. Drug-treated macrophages also elicit an increase in the number of lysosomes. In addition, analysis of tissue culture supernatants shows increased levels of interleukin-1α and tumor necrosis factor-α. These results show that 'poly-plat', SSP and SAP enhance the immune system with greater efficacy and potency than cisplatin.

Key words: Cisplatin, 'poly-plat', interleukin-1α, *in vitro*, lysosomes, macrophages, SAP, SSP, tumor necrosis factor-α.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP], a broad spectrum anticancer drug,[1] is proven to be effective in the treatment of testicular, ovarian, prostate, bladder, head and neck, and lung cancers.[2] Activated macrophages have been found to effectively destroy target cells by cytotoxic mechanisms.[3,4] The activation process includes the generation of extracellular products including interleukin (IL)-1α and tumor necrosis factor (TNF)-α.[5] Although cisplatin is an effective anticancer drug, it has very severe toxic side effects, of which gastrointestinal and nephrotoxicity are the dose-limiting factors.[6]

Poly-[(*trans*-1,2-diaminocyclohexane) platinum]-carboxyamylose ('poly-plat'), 5-sulfosalicylato-*trans*-(1,2-diaminocyclohexane) platinum (SSP) and 4-hydroxy-α-sulfonylphenylacetato (*trans*-1,2-diaminocyclohexane) platinum (II) (SAP) are second generation analogs of cisplatin with higher efficacy and potency, while eliciting less toxicity. This is particularly true of 'poly-plat' which contains one-fifth the platinum of cisplatin.[7] The possibility of drugs enhancing the immune system with less toxicity is very encouraging. We have here explored the effects of 'poly-plat', SSP and SAP on the macrophages so as to understand their mechanism(s) of action in enhancing the immune system.

Correspondence to SK Aggarwal

Materials and methods

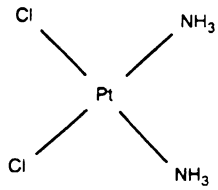
Cell cultures

Swiss webster mice (Charles River, Portage, MI) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (MEM; Gibco, Grand Island, NY) without serum containing 1% antibiotic–antimycotic [penicillin G (10 000 U/ml), streptomycin sulfate (10 000 μg/ml) and amphotericin B (25 μg/ml)] into the peritoneal cavity. After gently massaging the abdominal wall, cells were aspirated and seeded onto 18 mm^2 glass coverslips, placed in 35 mm Petri dishes at $2–4 \times 10^6$ cells/ml and incubated for 2 h at 37°C after which cells were washed vigorously to remove non-adherent cells. Cell cultures were incubated in normal medium (minimal essential media and 10% heat-inactivated fetal calf serum) at 37°C in a 5% CO_2 incubator.

Treatments

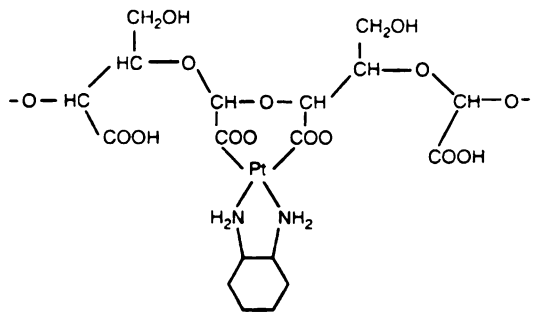
'Poly-plat' was prepared in 0.85% NaCl while SSP and SAP (Figure 1) were dissolved in 0.85% NaCl and 0.1% $NaCO_3$ in 5 μg/ml concentrations. Macrophages were treated with the drugs for 2 h. The drug(s)-containing medium was replaced by normal medium and supernatant (500 μl) was collected at 0.5, 1, 2 and 24 h for cytolytic factor(s) analysis. In addition, macrophages were also treated with cisplatin [5 μg/ml dissolved in physiological saline with 3 μl/ml of dimethylsulfoxide (DMSO; Sigma, St Louis, MO)]. Untreated cells in normal medium served as controls.

Cisplatin



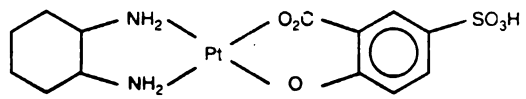
Cis-diamminedichloroplatinum(II)

'Poly-plat'



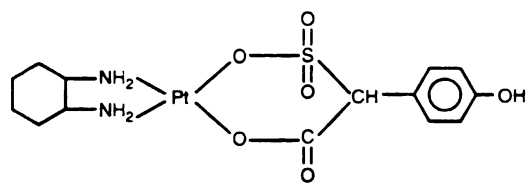
Poly-[(*trans*-1,2-diaminocyclohexane)platinum]-carboxyamylose

SSP



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

SAP



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

Figure 1. Structure of compounds studied.

IL-1α assay

IL-1α was assayed using ELISA kits (Genzyme, Cambridge, MA). The method used the multiple antibody sandwich principle,[8] where monoclonal anti-murine IL-1α was used to bind murine IL-1α present in the supernatant. A biotinylated polyclonal antibody binding the IL-1α was added and unbound material was washed out. Peroxidase-conjugated avidin was used to bind these biotin tagged complexes. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with IL-1α (15–405 pg/ml) provided in the kits and linear regression analysis was performed.[9]

TNF-α assay

TNF-α released from supernatants of the macrophages was assayed using specific analysis kits (Genzyme). Again, the multiple antibody sandwich principle was utilized with a murine monoclonal antibody specific for murine TNF-α in the samples.[8] A horseradish peroxidase-conjugated anti-murine TNF-α antibody was used to bind the multiple epitopes on TNF-α. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with TNF-α (35–2240 pg/ml) provided in the kits and linear regression analysis was performed.[10]

Lysosomal assay

The quantitation of lysosomes before and after various treatments was achieved by exposing macrophage cultures to fresh medium containing acridine orange (5 μg/ml) for 30 min at 37°C in the dark.[11] After careful washing macrophages were examined under a Zeiss 10 laser scanning confocal microscope.

Results

IL-1α release

Compared to cisplatin treatments there was an increase in IL-1α levels in the supernatants of macrophages treated with either 'poly-plat', SSP or SAP (5 μg/ml) for up to 24 h of testing (Figure 2). The greatest increases were seen 2 h post-treament (400–500 pg/ml) with a subsequent decrease from there on. IL-1α levels demonstrated a consistent increase after cisplatin treament, reaching a maximum after 24 h. Although, IL-1α levels after 'poly-plat', SSP or SAP treament demonstrated a decline after a peak at 2 h, these levels were still equal to or above those after cisplatin treatment.

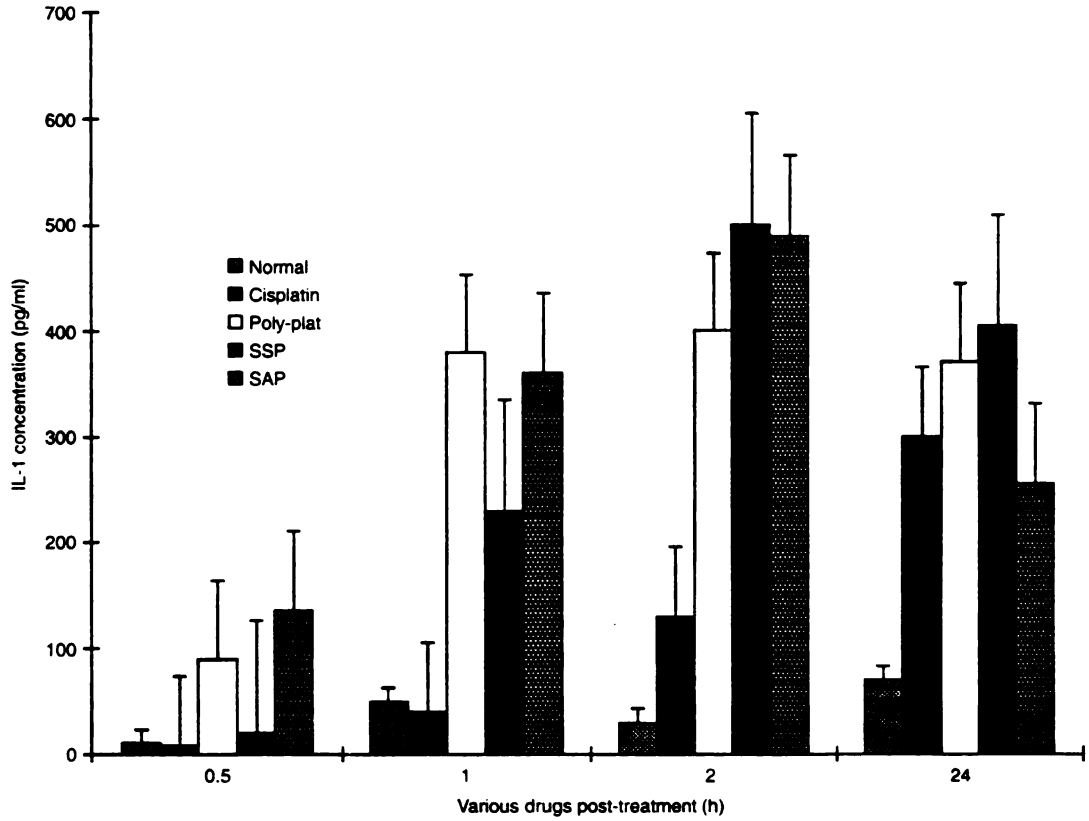


Figure 2. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages treated with either cisplatin, 'poly-plat', SSP, or SAP (5 μg/ml) after 30 min, and 1, 2 and 24 h post-treatment. Note the large increase at 2 h post-treatment in 'poly-plat', SSP and SAP. This is three times the amount of IL-1α released by cisplatin-treated macrophages.

TNF-α release

TNF-α increases were observed in SSP (5 μg/ml) at all times tested, with the most significant increase occurring at 2 h post-treatment (3250 pg/ml) (Figure 3). This level dramatically decreased at 24 h post-treatment (565 pg/ml). 'Poly-plat' and SAP (5 μg/ml) demonstrated very little TNF-α activity, barely reaching 200 pg/ml at 24 h post-treatment. Cisplatin demonstrated the usual enhanced release of TNF-α at various time intervals, reaching a peak value at 2 h post-treatment (300 pg/ml).

Macrophage activation

Murine peritoneal macrophages demonstrated extension formations after 2 h of treatment with 'poly-plat' and SSP (5 μg/ml) (Figure 4A and B). These cytoplasmic extensions radiate from the cell body in all directions. While SAP (5 μl/ml)-treated macrophages did not show any extension formation, but instead assumed a discoid shape similar to that of the normal macrophages (Figure 4C and D). Cisplatin (5 μg/ml)-treated macrophages also showed similar extension formation but only after 24 h of treatment.

Lysosomal studies

Based on fluorescence measurements after acridine orange labeling, a 500-fold increase in the number of lysosomes in the macrophages after only 2 h of 'poly-plat' treatment (Figure 5A) was observed compared to normal cells (Figure 5B). The lysosomes were plentiful in the cytoplasm of the macrophages and in the drug-induced cytoplasmic extensions

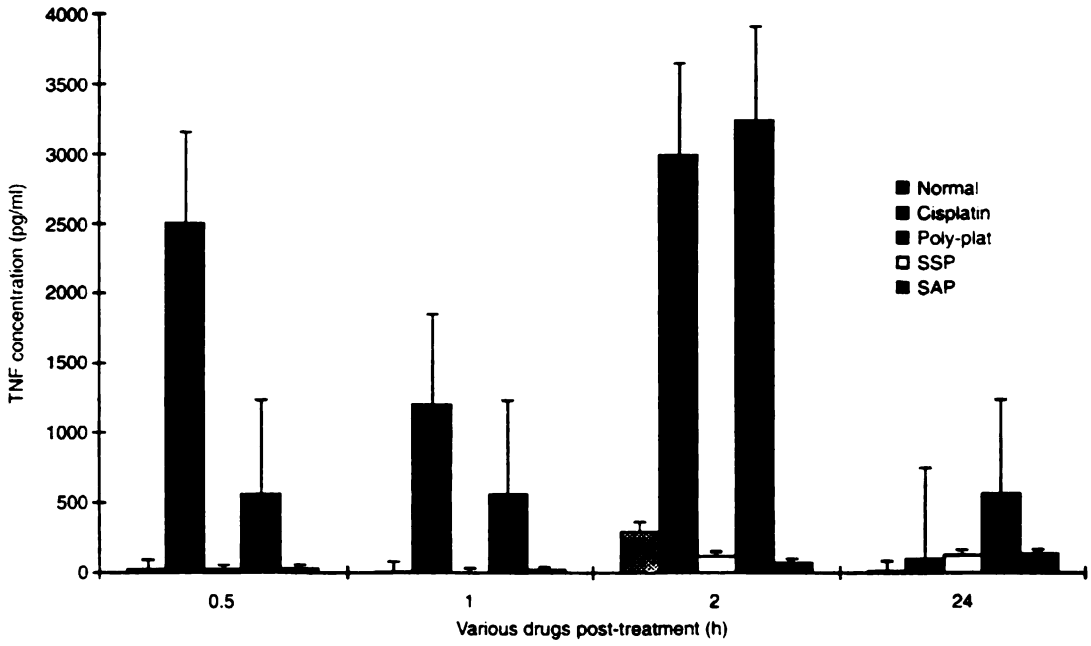


Figure 3. Bar graph showing TNF-α release in the supernatant of murine peritoneal macrophages treated with either cisplatin, 'poly-plat', SSP or SAP (5 μg/ml) after 30 min, and 1, 2 and 24 h post-treatment. Note the maximum increase in TNF-α for cisplatin and SSP 2 h post-treatment. Although, 'poly-plat' and SAP did show an increased level of TNF-α they were not significant compared to cisplatin or SSP treatments.

radiating from the cell body. SSP and SAP both demonstrated a 100-fold increase in the number of lysosomes. Comparatively, cisplatin treatment demonstrated only a 50-fold increase in the lysosomes (Figure 5C), both in the cell body and the cytoplasmic extensions.

Discussion

The anti-tumor agent cisplatin has been shown to enhance the immune system.[12] Cisplatin activates the macrophages by inducing an increase in their cytoplasmic lysosomes and promoting extension formations that establish contact with the tumor cells.[13] These macrophages lyse tumor cells through the transfer of lysosomes via the cytoplasmic extensions.[13] Cytolytic factors such as H_2O_2, superoxide anion, IL-1α, TNF-α, lysozyme and βNH have been demonstrated to be released from these treated macrophages.[5,14] Our study, using the second generation analogs of cisplatin, has shown that 'poly-plat', SSP and SAP elicit much more IL-1α, TNF-α, macrophage activation, as depicted by extension formations, and lysosomal increase compared to cisplatin.

Il-1α release by activated macrophages and its cytotoxicity to tumor target cells proves it to be potent mediator in tumor cell killing by macrophages.[14] Its release *in vitro* occurs in a cyclic manner showing its greatest increase at 2 h post-treatment after 'poly-plat', SSP and SAP. IL-1α can destroy tumor cells through several different pathways, i.e. production of superoxide, nitric oxide and hydrogen peroxide,[15,16] resulting in lipid peroxidation,[17] mitochondrial membrane depolarization and calcium mobilization, reduction in ATP synthesis.[18] and DNA base alterations.[19] Compared to cisplatin treatment, where the IL-1α release reaches a maximum at 24 h, these new drugs achieved it only after 60 min, reaching a maximum at 2 h. If the levels of IL-1α are any indication of the cytotoxicity then the efficiency of these drugs need to be tested in the *in vivo* system.

TNF-α also plays a regulatory role in inflammation and immunological response to tumors.[20] TNF-α activates production of nitric oxide which induces iron loss, and inhibits DNA synthesis, mitochondrial respiration and the citric acid cycle.[21,22] It is clear that TNF-α is an important mediator of tumor cell death by macrophages. Of the three analogs tested, only SSP demonstrated a rise in TNF-α production.

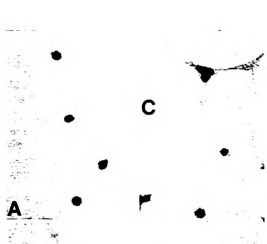


Figure 4. Light micrographs showing macrophages at 24 h in normal medium (A), and after 2 h of 'poly-plat' (B), SSP (C) and SAP (D). Note the extension formation after 'poly-plat' and SSP treatment(s). Only SAP assumes a discoid shape similar to that of untreated macrophages. Original magnification: ×1600. Bar = 0.5 mm.

Possibly the main thrust of SSP action is through increased release of TNF-α and IL-1α early enough compared to cisplatin.

Lysosomes take part in tumor cell death through macrophage activation.[23,24] Cisplatin-treated macrophages show an increase in their number of lysosomes and have been shown to be transferred to tumor cells through cytoplasmic extensions.[15] This results in the lysis of tumor cells.[25] A greater increase in lysosomes is seen in 'poly-plat'-, SSP- and SAP-treated macrophages when compared to cisplatin-treated macrophages. If lysosomal activity is any indication of cytotoxicity then 'poly-plat' may have greater efficacy in tumor cell death.

'Poly-plat' is curative in a range of solid tumors including renal, breast, ovarian, plasma cell myeloma and adenocarcinoma.[7] It has been shown to be up to 15 times as active as equimolar amounts of cisplatin, showing inhibition in many tumors where cisplatin is ineffective.[7] Recently, results have shown 'poly-plat' to have higher potency than cisplatin at the same level *in vitro* and *in vivo*.[7] This is due to 'poly-plat' having a molecular weight which is more than 10-fold higher than cisplatin. 'Poly-plat', which contains only one-fifth the platinum of cisplatin, has also been shown to be less toxic.

SSP is curative of many cisplatin resistant tumors including M5 ovarian and several plasma cell myelomas. SSP also elicits no nephrotoxicity and is less toxic than cisplatin even at LD_{10} dose (180 mg/kg). *In vitro*, SSP treatment resulted in the greatest increase in IL-1α and TNF-α release.

From our *in vitro* studies it seems that SAP, unlike cisplatin, 'poly-plat' or SSP, does not function through the activation of the immune system. However, in *in vivo* studies, it has been shown to be most potent in the regression of X5563 plasma cell myeloma, M-5 ovarian carcinoma, 6C3HED lymphosarcoma, C3H mammary adenocarcinoma, B16 melanoma and DLD-2 human colon tumor xenografts. Compared to cisplatin's 9% inhibition of the DLD-2 human colon tumor xenografts, SAP has demonstrated a 99% inhibition. Whether its mechanism of action is through increased macrophage lysosomal activity or more effective DNA cross-linking remains to be explored.

Conclusion

'Poly-plat', SSP and SAP induce murine macrophage activation via the production of cytoplasmic extensions, lysosomes, and cytolytic factors IL-1α and

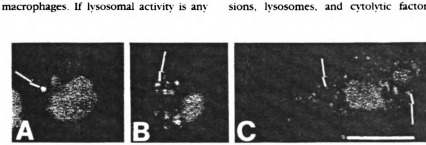


Figure 5. Fluorescent images taken from the Zeiss 10 laser scanning confocal microscope of macrophages labeled with acridine orange (5 μg/ml) showing lysosomal fluorescence in untreated (A), cisplatin (B) and 'poly-plat' (C). Note the large increase in lysosomal fluorescence after 'poly-plat' treatment. Original magnification: ×16000. Bar = 10 μm.

TNF-α. Previous investigations have shown these drugs to be more effective than cisplastin *in vitro* and *in vivo* while eliciting less toxicity. Our studies support the activation of the immune system via the mediation of cytolytic factors and lysosomes as possible mechanisms of action of these drugs. Based on our observations we propose that 'poly-plat', SSP and SAP activate various cytolytic factors of the immune system better, compared to cisplatin.

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CHAPTER 4: IMMUNE SYSTEM ACTIVATION BY CISPLATIN AND ITS ANALOG "POLY-PLAT": AN *IN VITRO* AND *IN VIVO* STUDY

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Research paper

Immune system activation by cisplatin and its analog 'poly-plat': an *in vitro* and *in vivo* study

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Swiss Webster mice treated with bolus injections of 'poly-plat' (10 mg/kg) show increased macrophage activation after 2 and 12 days when isolated in cultures. Such macrophages demonstrate an increase in the number of lysosomes and cytoplasmic extension formation with enhanced cytokine (interleukin-1α) activity. In addition, peripheral blood smears demonstrated an increase in lymphocytes and monocytes compared to cisplatin-treated animals. These results show that 'poly-plat' activates the immune system more effectively than cisplatin both *in vitro* and *in vivo*. [© 1998 Rapid Science Ltd.]

Key words: Cisplatin, interleukin-1α, *in vitro*, *in vivo*, macrophages, nitric oxide, 'poly-plat'.

Introduction

Regulation of normal and abnormal cell growth is the primary function of the immune system.[1] Many different immune system cells such as macrophages, lymphocytes and monocytes secrete various cytokines when activated by viruses, parasites, antigens or tumor cells.[2] Macrophages are now being considered as one of the most potent components of the immune system actively involved in tumor regression and tumor cell lysis. Macrophages have the capacity to destroy tumor cells not only by direct contact and phagocytic activity but also by extracellular release of cytolytic factors that are capable of killing tumor cells.[3]

Cisplatin [*cis*-dichlorodiammine-platinum(II)] is a potent antitumor compound which has been successfully used against a number of animal[4,5] and human tumors.[6] Although the primary target for cisplatin is the inhibition of DNA synthesis,[7] it has also shown development of specific cellular immune responses in tumor-bearing mice.[8] Cisplatin has been shown to activate murine peritoneal macrophages *in vitro*.[9] These activated macrophages seek out tumor cells through the formation of cytoplasmic extensions and lysosomal transfer to these target cells causing cell death.[10] Cisplatin-treated macrophages have also been induced to release various cytolytic factors including interleukin (IL)-1α and tumor necrosis factor (TNF)-α which have potent antitumor activities.[2] A combination therapy, using cisplatin and immunostimulants, has demonstrated a synergistic enhancement in its antitumor activity.

Poly-[*trans*-1,2-diaminocyclohexane)platinum]-carboxyamylose ('poly-plat') is a second generation analog of cisplatin with higher efficacy and potency, while exhibiting less toxicity.[11,12] 'Poly-plat' is equipotent to cisplatin with only one-fifth the platinum content.[12] The prospect of a new drug which is capable of enhancing the immune system with less severe side effects is very promising. Here we have further explored the effects of 'poly-plat' *in vitro* and *in vivo* on the macrophage as an effector cell and its interaction with tumor target cell(s). Although its exact mechanism of action is not known, it has been shown to enhance the immune system both *in vitro* and *in vivo* more effectively than cisplatin.[11]

Materials and methods

Cell cultures

Swiss Webster mice (Charles River, Wilmington, MA) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (MEM; Gibco, New York, NY) without serum containing 1% antibiotic-antimy-

The authors wish to thank Andrulis Pharmaceuticals (Beltsville, MD) for their generous gift of 'poly-plat' and cisplatin used in these experiments.

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cotic (Gibco) [penicillin G (10 000 U/ml), streptomycin sulfate (10 000 μg/ml) and amphotericin B (25 μg/ml)] into the peritoneal cavity. After gently massaging the abdominal wall, cells were aspirated and seeded onto 18 mm^2 glass coverslips. These were placed in 35 mm Petri dishes at 2-4 × 10^6 cells/ml and incubated for 2 h at 37°C after which cells were washed vigorously to remove non-adherent cells. Cell cultures were incubated in normal medium (minimal essential media and 10% heat-inactivated fetal calf serum) at 37°C in a 5% CO_2 incubator. Sarcoma 180 ascites (S180; CCRFS-180II; ATCC, Rockville, MD) served as target cells for macrophages. Normal hepatocytes, obtained by mincing a small piece of liver through a fine wire mesh, 105 × 105 μm in size (Tetko, IL), also served as target cells for the macrophages. An effector:target cell ratio of 1:10 was maintained in all experiments.

Treatments *in vitro*

'Poly-plat' (Andrulis Pharmaceuticals, Beltsville, MD) was prepared in 0.85% NaCl in 5 μg/ml concentrations. Macrophages were treated with the drugs for 2 h. The drug(s) containing medium was replaced by normal medium and supernatant (500 μl) was collected at 0.5, 1, 2, and 24 h for cytolytic factor(s) analysis. In addition, macrophages were also treated with cisplatin (5 μg/ml) dissolved in 0.85% NaCl with 3 μl/ml of dimethyl sulfoxide (DMSO; Sigma, St Louis, MO). Untreated cells in normal medium served as controls. Interferon (IFN)-γ/lipopolysaccharide (LPS) (Signal Transduction Laboratories, Lexington, KY) was simultaneously added to some cultures at 0.8 μg/2 μl. Coverslips were fixed and stained to view the changes in structural morphology in terms of extension formation and lysosomal changes.

Treatments *in vivo*

Mice (20 g) were injected (i.p.) with 'poly-plat' (10 mg/kg) or cisplatin (10 mg/kg) with or without subsequent injections of IFN-γ/LPS (0.8 μg/2 μl). Normal mice were given i.p. injections of 0.85% NaCl to serve as controls. Mice were sacrificed after 2 and 12 days, and peritoneal macrophages were isolated.

IL-1α assay

IL-1α was assayed using ELISA kits (Genzyme, Cambridge, MA). The method used the multiple antibody sandwich principle, where monoclonal anti-murine IL-1α was used to bind murine IL-1α present in the supernatant. A biotinylated polyclonal antibody binding the IL-1α was added and unbound material was washed out. Peroxidase-conjugated avidin was used to bind these biotin-tagged complexes. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with IL-1α (15-405 pg/ml) provided in the kits and linear regression analysis was performed.

Macrophage tumor cell interaction studies *in vitro*

Variously drug-treated macrophages were co-incubated with S180 cells for 30 min, and 1, 2 and 24 h. Coverslips were fixed and stained to view any interactions between the effector and the S180 target cells.

Peripheral blood smears

Peripheral blood smears were stained using Giemsa's stain[13] and cell counts were made of the lymphocytes, including monocytes.

Nitrite assay for estimation of NO production

The concentration of stable nitrite, end product from NO oxidation by effector macrophages, was determined by the method of Ding *et al.*[14] using Griess reagent. Briefly, 100 μl of supernatant from untreated and treated macrophages collected at various times was mixed with equal volume of Griess reagent (1% sulfanilamide, 5% phosphoric acid, 0.1% naphthylethylenediamine dihydrochloride; Sigma). The mixtures were incubated for 10 min at room temperature and the absorbance read at 540 nm. Standard curves were generated using 1 nM to 220 μM $NaNO_2$ and nitrite concentrations were determined using linear regression analysis.

Inducible NO synthase (iNOS) staining

Macrophage monolayers were stained for iNOS which catalyzes the oxidation of L-arginine to citrulline and NO using the avidin-biotin-peroxidase complex method.[15] iNOS was confirmed by the Vectastain Elite ABC Kit (Vector, Burlingame, CA).

Results

IL-1α release

There was observed a many fold increase in IL-1α levels in the supernatants of macrophages *in vitro* after 'poly-plat' (10 mg/kg) treatment for various time intervals (Figure 1) compared to cisplatin treatment. Similarly, macrophages isolated from 'poly-plat'-treated mice demonstrated a significantly higher level of IL-1α (500 pg/ml) than cisplatin treatment (250 pg/ml), but only after 12 days of treatment (Figure 2). After 2 days of 'poly-plat' treatments the levels of the IL-1α were close to normal. However, after 2 days of 'poly-plat' treatment there was an increase in the formation of cytoplasmic extensions and the lysosomes (Figure 3A) compared to cisplatin treatment (Figure 3B).

When 'poly-plat'- or cisplatin-treated mice were injected with IFN-α/LPS (0.8 μg/2 μl) there was a significant decrease in the levels of IL-1α for both the drug treatments (Figure 4). However, there was a significant induction of cytoplasmic extensions and the number of lysosomes (Figures 5A and B).

Macrophage–tumor cell interaction studies *in vitro*

Normal murine peritoneal macrophages, when treated with 'poly-plat', show cytoplasmic extensions which radiate from the cell body after only 2 h post-treatment. When these drug-treated macrophages are co-incubated with S180 tumor cells they immediately establish contact with several target cells and form cytoplasmic continuity through which lysosomes are transported into the tumor cells causing their lysis (Figure 6). Cisplatin-treated cells also form cytoplasmic extensions that are fewer in number and establish contact with fewer tumor cells compared to 'poly-plat'-treated macrophages. Normal macrophages do not form cytoplasmic extensions and, when co-incubated with S180 cells, fail to show any interaction.

Peripheral blood smears

Mice treated with 'poly-plat' demonstrated a 10-fold increase in the number of lymphocytes and monocytes both after 2 and 12 days post-treatment. Comparatively, cisplatin-treated mice demonstrated a significant decrease.

Nitrite assay for estimation of NO production

Treatment of macrophages with 'poly-plat' or cisplatin demonstrated an increase in the nitrite levels, as early as 30 min, that gradually dropped down to normal after 24 h of treatment (Figure 7). No distinct pattern was seen when comparing the relative increase in NO

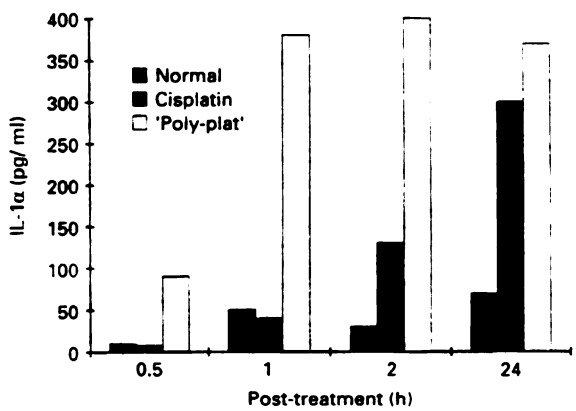

Figure 1. Bar graph showing IL-1α in the supernatants of cultured murine peritoneal macrophages treated with either 'poly-plat' or cisplatin (5 μg/ml) after 30 min, and 1, 2 and 24 h post-treatment. Note a sudden increase after 1 h of 'poly-plat' treatment compared to cisplatin. This increase stays at the same level through 24 h post-treatment. Cisplatin treatment induces a gradual increase through 24 h but is still less than that of 'poly-plat'.

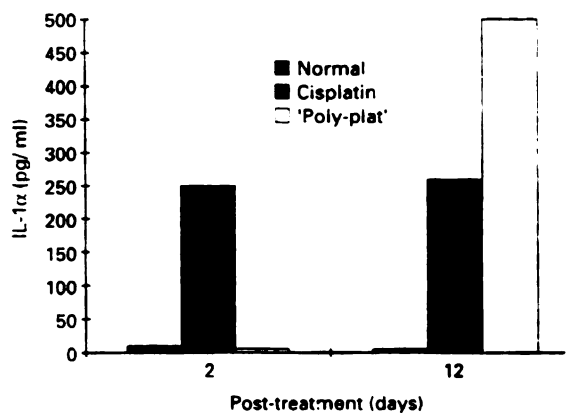

Figure 2. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages from mice treated with either 'poly-plat' or cisplatin (5 μg/ml) after 2 and 12 days. Note the large increase in IL-1α at 12 days post-injection with 'poly-plat'-treated mice. This increase is more than twice that of cisplatin-treated mice.

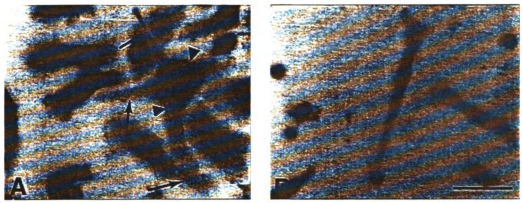

Figure 3. Light micrographs showing the murine peritoneal macrophages of mice treated with 'poly-plat' (A) and cisplatin (B) after 2 days. Note the very large nuclei, numerous lysosomes (arrowheads) and long cytoplasmic extensions (arrows) radiating from the macrophages of 'poly-plat'-treated mice. There is very little of this activation of the macrophages from cisplatin-treated mice. Bar=25 μm.

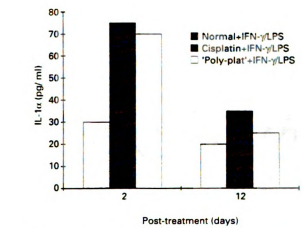

Figure 4. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages from mice treated with either 'poly-plat' or cisplatin (5 μg/ml) plus IFN-γ/LPS after 2 and 12 days. Note the initial increase of IL-1α after 2 days but a subsequent decrease after 12 days of 'poly-plat' and cisplatin treatment reaching close to normal levels.

levels between 'poly-plat'- and cisplatin-treated macrophages at the various times tested.

iNOS staining

When macrophages were treated with 'poly-plat' or cisplatin, a 10-fold increase in the expression of iNOS was visible after only 24 h post-treatment, while none was seen in untreated macrophages. A 20-fold increase in the expression of iNOS was observed in macrophages treated with 'poly-plat' or cisplatin plus IFN-γ/LPS.

Discussion

Macrophages have been implicated in the destruction of tumor cells either by direct cell–cell contact or cell cytotoxic mechanisms via the release of extracellular mediators.[16,17] 'Poly-plat' and cisplatin have both been demonstrated to be such enhancers of the immune system *in vivo* and *in vitro*.[9–11,18,19] These chemotherapeutic agents have been shown to activate murine peritoneal macrophages in a multiset fashion, increasing their ability to recognize tumor cells, establish cell–cell contact, transfer lysosomes and induce tumor cell lysis.[10,11]

Activated macrophages are known to release many cytolytic factors, including IL-1α.[20,21] IL-1α was first known to be a lymphocyte activating factor because of its ability to stimulate T-cells.[22] IL-1α can destroy tumor cells via the production of superoxide, NO and hydrogen peroxide[23,24] resulting in lipid peroxidation,[25] mitochondrial membrane depolarization and calcium mobilization, reduction in ATP synthesis,[26] and DNA base alterations.[27] In the present study we have observed not only an increase in IL-1α but also an increase in monocytes and lymphocytes, in the peripheral blood.

NO is a noxious free radical reactive nitrogen intermediate (RNI) gas found to play a major role in various biological processes.[28] It has been established as a messenger molecule regulating immune

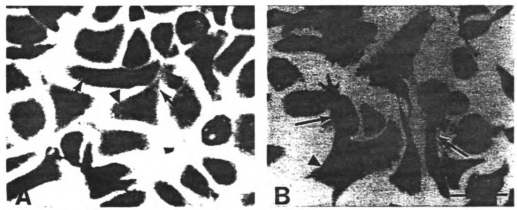

Figure 5. Light microphages showing the murine peritoneal macrophages of mice treated (2 and 12 days) with 'poly-plat' (A) and cisplatin (B) plus IFN-γ/LPS after 2 days. Note the large nuclei, numerous lysosomes and long cytoplasmic extensions (arrows) radiating from the macrophages of both 'poly-plat' and cisplatin treated mice. Bar=25 μm.

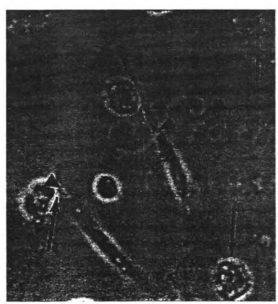

Figure 6. Light macrophages showing 'poly-plat'-treated murine peritoneal macrophages co-cultured with S180 cells for only 2 h. Note the cytoplasmic extensions in contact with tumor cells (arrows) and an increase in the lysosomal activity only after drug treatments. Untreated macrophages never establish contact with tumor cells nor show any increase in the number of lysosomes. Bar=25 μm.

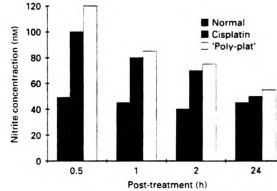

Figure 7. Bar graph demonstrating nitrite levels in the supernatants of macrophages cultures after treatment with 'poly-plat' or cisplatin at various time intervals. Note the intial increase in the nitrite levels at 30 min, but dropping to normal levels after 24 h.

functions, blood vessel dilation as well as neurotransmission.

NO is formed through the stepwise oxidation of the guanidino-nitrogen terminal atom of L-arginine to NO and L-citrulline catalyzed by NOS.[29] NO is further oxidized to form nitrite. NO exists in several forms. The constitutive form is found in endothelial cells and neurons of the central nervous system while the inducible form (iNOS) is present in macrophages, leukocytes and vascular smooth muscle cells.

iNOS is induced by a variety of factors including endotoxins (e.g. LPS) and cytokines (IL-1α, IFN-γ and TNF-α).[30] Results show that 'poly-plat' and cisplatin both influence the expression of iNOS and the observed increase in nitrite levels in the medium. It is not surprising to find an increase in NO levels after 'poly-plat' and cisplatin treatment of the macrophages and mice, since both drugs have also been demonstrated to induce IL-1α release in the medium.

NO released by primed macrophages mediates its cytotoxic effects through loss of iron,[31,32] inhibition of DNA synthesis,[32,33] mitochondrial respiration[32,34] and the citric acid cycle.[35] These have all been attributed to the inactivation of ribonucleotide reductase and the inhibition of iron-sulfur proteins NADH:ubiquinone oxidoreductase, NADH:succinate oxidoreductase and mitochondrial aconitase. In addition, the reactive oxygen species generated by macrophages could combine with NO to form substances that are more potent than NO itself.[28] NO combined with superoxides could yield peroxynitrite that decomposes to the hydroxide free radical and NO_2 free radical. Our results demonstrate an immediate increase in nitrite concentration, in 'poly-plat'- and cisplatin-treated macrophages, after 30 min in culture. These processes may attribute to the cytotoxic ability of 'poly-plat'- or cisplatin-primed macrophages inducing the destruction of tumor cells.

Conclusion

The results of this study strongly suggest the enhancement of the immune system by the anticancer agent 'poly-plat'. In addition to inducing direct cell-cell contact of the macrophages with the tumor cells, there is a release of various cytolytic factors (IL-1α and NO) from such macrophages. Release of these factors, shown to inhibit tumor cell proliferation, is evidence for the role they play in 'poly-plat'-mediated tumor cell toxicity. The major mechanism of action of 'poly-plat' is still unknown. However, our studies support the hypothesis that the enhancement of the immune system is an important mechanism of action of 'poly-plat'. Based on these findings, we propose that 'poly-plat', macrophage-mediated cell cytotoxicity, involves various cytolytic factors including IL-1α and NO. The activation of macrophages, a multi-step process, leads to target cell destruction through lysis.

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CHAPTER 5: ENHANCED IMMUNE SYSTEM ACTIVATION AFTER TREATMENT WITH NOVEL ANTINEOPLASTIC AGENTS

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Enhanced Immune System Activation after Treatment with Novel Antineoplastic Platinum Agents

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Abstract. *"Poly-plat", SSP, and SAP are second generation analogs of cisplatin (CDDP) with higher efficacy and potency. In order to understand the mechanism of action of these compounds, isolated murine peritoneal macrophages were treated with "poly-plat", SSP, or SAP (5 μg/ml) for 2 h. Treated macrophages demonstrated an increase in the number of lysosomes, but only "poly-plat" and SSP treated macrophages were stimulated to form the cytoplasmic extensions so very characteristic of cisplatin after 2 h and 24 h post-treatment. SAP showed cytoplasmic extensions only after 24 h post-treatment, and demonstrated a back to the normal discoid form when viewed at 24 h post-treatment. When drug treated macrophages were co-incubated with S180 tumor cells, cytoplasmic extensions of the macrophages developed contacts, and cytoplasmic continuity with the tumor cells, and a subsequent transfer of lysosomes from macrophage to tumor cell was observed after only 2 h of co-incubation. After 24 h of co-incubation, lysis of S180 cells was acheived. Analysis of the tissue culture supernatants collected from "poly-plat", SSP, and SAP treated macrophages demonstrated the enhanced activity of interleukin-1α of over 400 pg/ml after 2 h post-treatment, compared to only 300 pg/ml with cisplatin 24 h post-treatment. However, only SSP demonstrated an increase in TNF-α activity (2000 pg/ml) after 2 h post-treatment, which is comparable to that of cisplatin. Based on our observations we propose that "poly-plat", SSP, and SAP activate various cytolytic factors of the immune system better, than cisplatin.*

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP] is an antineoplastic drug with demonstrated activity against ovarian and testicular cancers(1). DNA denaturation is one of the accepted methods of its mechanism of action through its intrastrand and interstrand cross-links interfering with DNA replication and transcription(2). Cisplatin has also been shown to enhance specific cellular immune responses in tumor bearing mice(3), through activation of murine peritoneal macrophages *in vitro* and *in vivo*(4,5). These activated macrophages seek out tumor cells through the formation of cytoplasmic extensions and lysosomal transfer to the target cells causing their death(6). Activated macrophages have been found to effectively destroy target cells by cytotoxic mechanisms(7,8). The activation process includes the generation of extracellular products including interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and nitric oxide (NO)(5,9). Although cisplatin is an effective chemotherapeutic drug, it has very severe toxic side effects, of which gastrointestinal and nephrotoxicity are the dose limiting factors(10).

Poly-[(*trans*-1,2-diaminocyclohexane) platinum]-carboxyamylose ("poly-plat"), 5- sulfosalicylato-*trans*-(1,2-diaminocyclohexane) platinum (SSP), and 4-hydroxy-α-sulfonylphenylacetato (*trans*-1,2-diaminocyclohexane) platinum (II) (SAP) are analogs of cisplatin with higher efficacy and potency. This is especially true of "poly-plat" which contains 1/5 the platinum of cisplatin(11). The prospect of new drugs which are capable of enhancing the immune system with less severe side effects is very appealing, and thus this investigation of "poly-plat", SSP, and SAP as macrophage activators was undertaken.

Materials and Methods

Cell Cultures. Swiss webster mice (Charles River Laboratories, MA) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (MEM; Gibco, NY) without serum containing 1% antibiotic-antimycotic [penicillin G (10 000 U/ml), streptomycin sulfate (10 000 μg/ml), and amphotericin B (25 μg/ml)] into the peritoneal cavity. After gently massaging the abdominal wall, cells were aspirated and seeded onto 18 mm^2 glass coverslips, placed in 35 mm petri dishes, at 2-4 × 10^6 cells/ml and incubated for 2 h at 37°C. Cells were washed vigorously to remove

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non-adherent cells. Cell cultures were inubated in normal medium (minimal essential media and 10% heat inactivated fetal calf serum) at 37°C in a 5% CO_2 incubator. Sarcoma 180 ascites (S180; CCRFS-180II; American Type Culture Collection, MD) served as target cells for macrophages. Normal hepatocytes, obtained by mincing a small piece of liver through a fine wire mesh, 105 X 105 µm in size (Tetko, Inc., IL), also served as target cells for the macrophages. An effector target cell ratio of 1:10 was maintained in all experiments.

Treatments. "Poly-plat" was prepared in 0.85% NaCl while, SSP and SAP (Figure 1) were dissolved in 0.85% NaCl and 0.1% $NaCO_3$ at 5 µg/ml. Macrophages were treated with the drugs for 2 h. The drug containing medium was replaced by normal medium and supernatant (500 µl) was collected at 0.5, 1, 2, and 24 h for cytolytic factor analysis. In addition, macrophages were also treated with cisplatin [5 µg/ml dissolved in physiological saline with 3 µl/ml of dimethylsulfoxide (DMSO; Sigma, St. Louis, MO)]. Untreated cells in normal medium served as controls.

Lysosomal assay. The quantitation of lysosomes before and after various treatments was achieved by exposing macrophage cultures to fresh medium containing acridine orange (5 µg/ml) for 30 min at 37°C in the dark(12). After careful washing macrophages were examined under Zeiss 10 Laser Scanning Confocal Microscope.

TNF-α assay. TNF-α released from supernatants of the macrophages was assayed using specific analysis kits (Genzyme; Cambridge, MA). Again, the multiple antibody sandwich principle was utilized with a murine monoclonal antibody specific for murine TNF-α in the samples(13). An HRP conjugated anti-murine TNF-α antibody was used to bind the multiple epitopes on TNF-α. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with TNF-α (35-2240 pg/ml) provided in the kits, and linear regression analysis was performed.

IL-α assay. IL-1α was assayed using ELISA kits (Genzyme; Cambridge, MA). The method used the multiple antibody sandwich principle(13), where monoclonal anti-murine IL-1α was used to bind murine IL-1α present in the supernatant. A biotinylated polyclonal antibody binding the IL-1α was added and unbound matenal was washed out. Peroxidase-conjugated avidin was used to bind these biotin tagged complexes. A substrate solution was then added, resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450nm. Standard curves were generated with IL-1α (15-405 pg/ml) provided in the kits and linear regression analysis was performed.

Inducible nitric oxide synthase (iNOS) staining. Macrophage monolayers were stained for the inducible enzyme which catalyzes the oxidation of L-arginine to citrulline and NO using avidin-biotin-peroxidase complex method(14). iNOS was confirmed by VECTASTAIN *elite* ABC Kit (Vector Laboratories, Inc., Bullingame, CA).

Nitrite assay for estimation of nitric oxide production. The concentration of stable nitrite, the end of nitric oxide generation by effector macrophages, was determined by the method of Ding *et al*(15) based on Greiss reaction. Briefly, 100 µl of supernatant from untreated and treated macrophages collected at various times were mixed with equal volume of Greiss reagent (1% sulfanilamide, 5% phosphoric acid, 0.1% naphthylethylenediamine dihydrochloride; Sigma, MO). The mixtures were incubated for 10 min at room temperature and the absorbance read at 540 nm. Standard curves were generated using 1 nM-220 µM $NaNO_2$ and nitrite concentrations were determined using linear regression analysis.

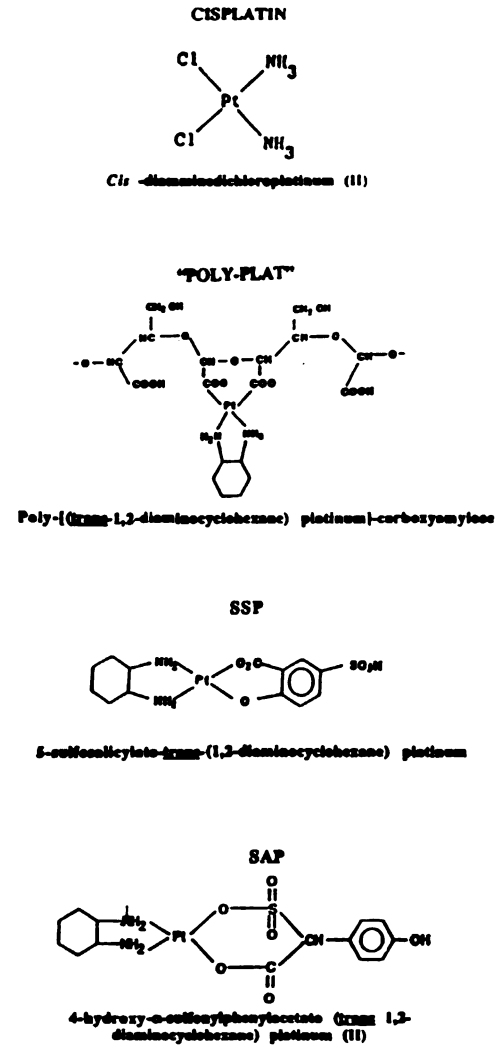

Figure 1. *Structures of various platinum coordination complexes studied.*

Results

Macrophage activation. Normal murine peritoneal macrophages, when treated with "poly-plat" and SSP (5 µg/ml) for 2 h at 37°C in culture, developed extension formations within 2 h (Figure 2A and 2B). These cytoplasmic extensions radiated from the cell body in all directions. While SAP (5 µg/ml) treated macrophages, did not show any extension formation, instead assumed a discoid shape similar to that of the normal macrophages (Figure 2C and 2D). Cisplatin (5 µg/ml) treated macrophages also showed similar extension formation but only after 24 h of treatment.

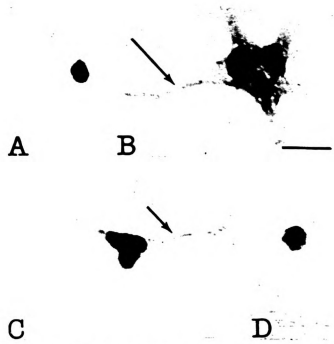

Figure 2. *Light micrographs showing macrophages at 24 h in normal medium (A); and after 2 h of "poly-plat" (B); SSP (C); and SAP (D). Note the extension formation (arrows) after "poly-plat" and SSP treatments, while SAP treated macrophages are similar in appearance to those of untreated macrophages. X1600, Bar=10μm*

Macrophage tumor cell interaction studies. When the drug treated macrophages were co-incubated with S 180 tumor cells they immediately established contact with several target cells and formed cytoplasmic continuity through which lysosomes were transported into the tumor cells causing their lysis (Figure 3). CDDP treated cells also developed cytoplasmic extensions that were fewer in number and established contact with fewer tumor cells when compared to "poly-plat" treated macrophages. Normal macrophages did not form cytoplasmic extensions and, when co-incubated with S180 cells, failed to show any interaction.

Lysosomal studies. Based on fluorescence measurements after acridine orange labeling, we observed a 500 fold increase in the number of lysosomes in the macrophages after only 2 h of "poly-plat" treatment (Figure 4A) compared to normal cells (Figure 4B). The lysosomes were plentiful in the cytoplasm of the macrophages and in the drug-induced cytoplasmic extensions radiating from the cell body. SSP and SAP both demonstrated a 100 fold increase in the number of lysosomes. Comparatively, cisplatin treatment demonstrated only a 50

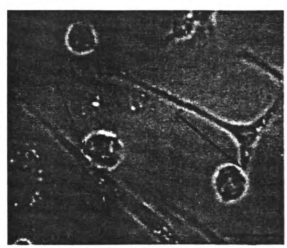

Figure 3. *Light micrograph showing "poly-plat" treated murine peritoneal macrophages co-cultured with S180 cells for only 2 h. Note the cytoplasmic extensions in contact with tumor cells (arrows) and an increased number of lysosomes. X1600, Bar=10μm*

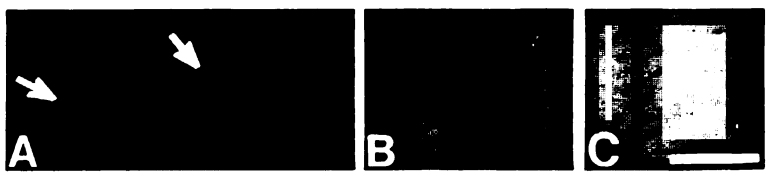

Figure 4. *Fluorescent images of macrophages labeled with acridine orange (5 μg ml) showing lysosomal fluorescence after "poly-plat" (A), normal (B), and cisplatin (C) treatments. Note the large increase in lysosomal fluorescence (arrows) after "poly-plat" treatment. X1600. Bar=10um*

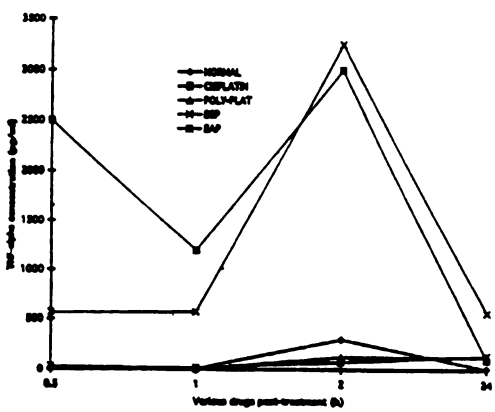

Figure 5. *Graph showing TNF-α release in the supernatant of murine peritoneal macrophages treated with either cisplatin, "poly-plat", SSP, or SAP (5 μg/ml) after 30 min, 2 and 24 h post-treatment. Note the maximum increase in TNF-α for cisplatin and SSP 2 h post-treatment. Though, "poly-plat" and SAP did show an increased level of TNF-α they were not significant compared to cisplatin or SSP treatments.*

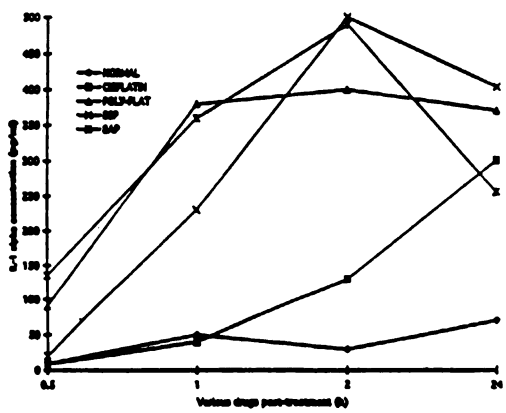

Figure 6. *Graph showing IL-1α release in the supernatants of murine peritoneal macrophages treated with either cisplatin, "poly-plat", SSP, or SAP (5ug ml) after 30 min, 2 and 24 h post-treatment. Note the large increase at 2 h post-treatment in "poly-plat", SSP, and SAP. This is three times the amount of IL-1α released by cisplatin treated macrophages.*

fold increase in the lysosomes (Figure 4C), both in the cell body and the cytoplasmic extensions.

TNF-α release. Significant increases in TNF-α levels were observed in the supernatants from SSP (5 μg/ml) treated macrophages at all times tested, with the most significant increase occurring at 2 h post-treatment (3250 pg/ml) (Figure 5). This level dramatically decreased at 24 h post-treatment (565 pg/ml). "Poly-plat" and SAP (5 μg/ml) demonstrated very little TNF-α activity, barely reaching 200 pg/ml at 24 h post-treatment. Cisplatin demonstrated the usual enhanced release of TNF-α at various time intervals, reaching a peak value at 2 h post-treatment (3000 pg/ml).

IL-1α release. Compared to cisplatin treatments there was an increase in IL-1α levels in the supernatants of macrophages treated with either "poly-plat", SSP, or SAP (5 μg/ml) for up to 24 h of testing (Figure 6). The greatest increases were seen 2 h post-treatment (400-500 pg/ml) with a subsequent decrease from there on. IL-1α levels demonstrated a consistent increase after cisplatin treatment, reaching a maximum after 24 h. However, IL-1α levels, after "poly-plat", SSP, or SAP treatment, demonstrated a decline after a peak at 2 h post-treatment but these levels were still equal to or above those after cisplatin treatment.

Inducible nitric oxide synthase (iNOS) staining. When macrophages were treated with "poly-plat", SSP, or SAP expression of iNOS was visible while none was detected in untreated macrophages. Only moderate levels of iNOS were visible in cisplatin treated macrophages.

Nitrite assay for estimation of nitric oxide production. Treatment of macrophages with "poly-plat", SSP, or SAP demonstrated increases in NO levels detected in cell culture supernatants at various times. The increase was seen as early as 30 min after treatment and persisted up to 24 h post-treatment. Again, cisplatin only demonstrated a moderate level of NO production.

Discussion

Cisplatin is able to activate peritoneal macrophages(4) *in vitro* and cause lysis of tumor cells(6). Cisplatin activates the macrophages by inducing an increase in their cytoplasmic lysosomes and promoting extension formations that establish contact with the tumor cells(6). These macrophages lyse tumor cells through the transfer of lysosomes via the cytoplasmic extensions(6). "Poly-plat", SSP, and SAP, analogs of cisplatin, also have the capability to activate macrophages *in vitro*. Our study has shown that "poly-plat", SSP and SAP elicit much more macrophage activation, as depicted by extension formations, lysosomal increases, TNF-α, IL-1α, iNOS, and NO compared to cisplatin.

Lysosomes take part in tumor cell death through macrophage activation(16,17). A greater increase in lysosomes is seen in "poly-plat", SSP and SAP treated macrophages when compared to cisplatin treated macrophages. If lysosomal activity is any indication of cytotoxicity the "poly-plat" may have greater efficacy in tumor cell death.

TNF-α also plays a regulatory role in inflammation and immunological response to tumors(18). TNF-α activates production of nitric oxide whieh induces iron loss, and inhibits DNA synthesis, mitochondrial respiration and the citric acid cycle(19,20). Of the three analogs tested only SSP demonstrated a rise in the TNP-α production. Possibly the main thrust of SSP action is through increased release of TNF-α and IL-1α early, compared to cisplatin.

IL-1α release by activated macrophages and its cytotoxicity to tumor target cells proves it as a potent mediator in tumor cell killing by macrophages(21). Its release *in vitro* occurs in a cyclic manner showing its greatest increase at 2 h post-treatment with "poly-plat", SSP and SAP. Compared to cisplatin treatment, where the IL-1α release reaches a maximum at 24 h, these new drugs achieved it after only 60 min, reaching a maximum at 2 h. If the level of IL-1α is any indication of cytotoxicity then "poly-plat" appears to be more efficient.

The iNOS is induced by a variety of factors including endotoxins (*e.g.* LPS) and cytokines (IL-1α, IFN-γ and TNF-α)(19). Results show that "poly-plat", SSP, and SAP all demonstrated enhanced levels of iNOS. NO released by primed macrophages mediates its cytotoxic effects through loss of iron(22,23), inhibition of DNA synthesis(23,24), mitochondrial respiration(23,25) and the citric acid cycle(26). In addition, the reactive oxygen species generated by macrophages could combine with NO to form substances that are more potent than NO itself(27). NO combined with superoxides could yield peroxynitrite that decomposes to hydroxide free radical and NO_2 free radical. Our results demonstrate an immediate increase in nitrite concentration, in "poly-plat", SSP, and SAP treated macrophages, after 30 min in culture. These processes may be attributed to the cytotoxic ability of "poly-plat", SSP or SAP primed macrophages inducing the destruction of tumor cells.

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CHAPTER 7: CONCLUSIONS

CONCLUSIONS

CDDP plus taxol combination stimulates various cytolytic factors of the immune system better than when these drugs are used independently. The macrophage activation includes production of cytoplasmic lysosomes, macrophage/tumor cell contact, and release of cytolytic factors IL-1α and TNF-α. Past *in vitro* and *in vivo* studies have shown that when these two drugs are used in combination they are more effective and less toxic than when they are used separately. Our results support activation of the immune system as an additional mechanism of action of this combination therapy. We also propose, based on our observations, that CDDP plus taxol combination activates various cytolytic factors of the immune system better than CDDP or taxol independently.

"Poly-plat", SSP, and SAP induce murine macrophage activation via the production of cytoplasmic extensions, lysosomes, and cytolitic factors IL-1α, IL-2 and TNF-α. Previous investigations have shown these drugs to be more effective than cisplatin *in vitro* and *in vivo* while eliciting less toxicity. Our studies support the activation of the immune system via the mediation of cytolytic factors and lysosomes as possible mechanisms of action of these drugs. Based on our observations we propose that "poly-plat", SSP, and SAP activate various cytolytic factors of the immune system bett er, compared to cisplatin.