Anti-Cancer Drugs 1998, 9, pp. 93–99

Research paper

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

Heather J Muenchen and Surinder K Aggarwal
Department of Zoology, Michigan State University, East Lansing, MI 48824-1115, USA.
Tel: (+1) 517 353-2253; Fax: (+1) 517 432-2789.

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.

Correspondence to SK Aggarwal

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 $2–4 \times 10^6$ 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. 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

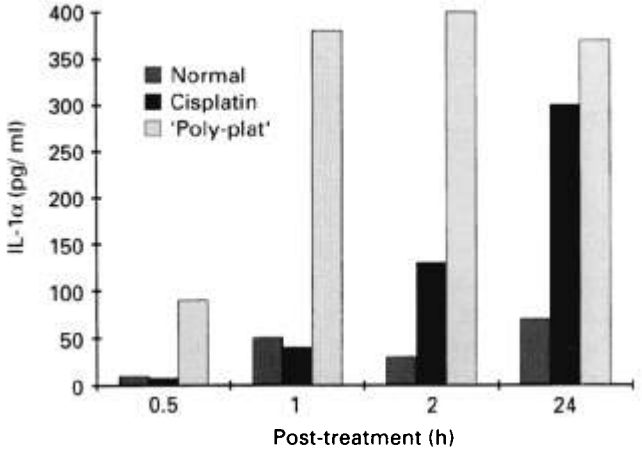

Figure 1. Bar graph showing IL-1α in the supernatants of cultured murine peritoneal macrophages treated with either 'poly-plat' or cisplatin (5 μg/ml) after 30 min, and 1, 2 and 24 h post-treatment. Note a sudden increase after 1 h of 'poly-plat' treatment compared to cisplatin. This increase stays at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

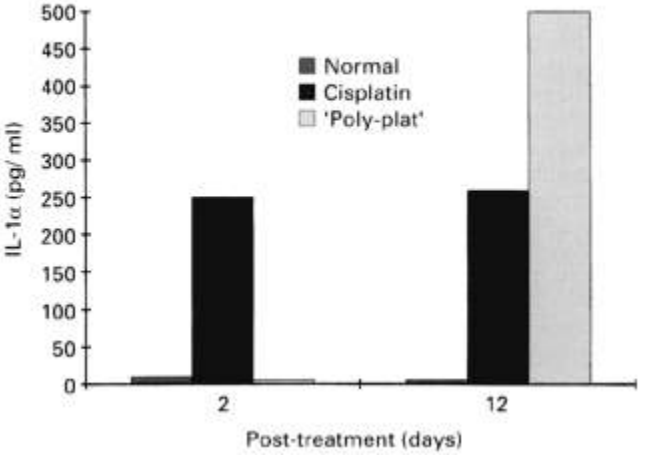

Figure 2. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages from mice treated with either 'poly-plat' or cisplatin (5 μg/ml) after 2 and 12 days. Note the large increase in IL-1α at 12 days post-injection with 'poly-plat'-treated mice. This increase is more than twice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

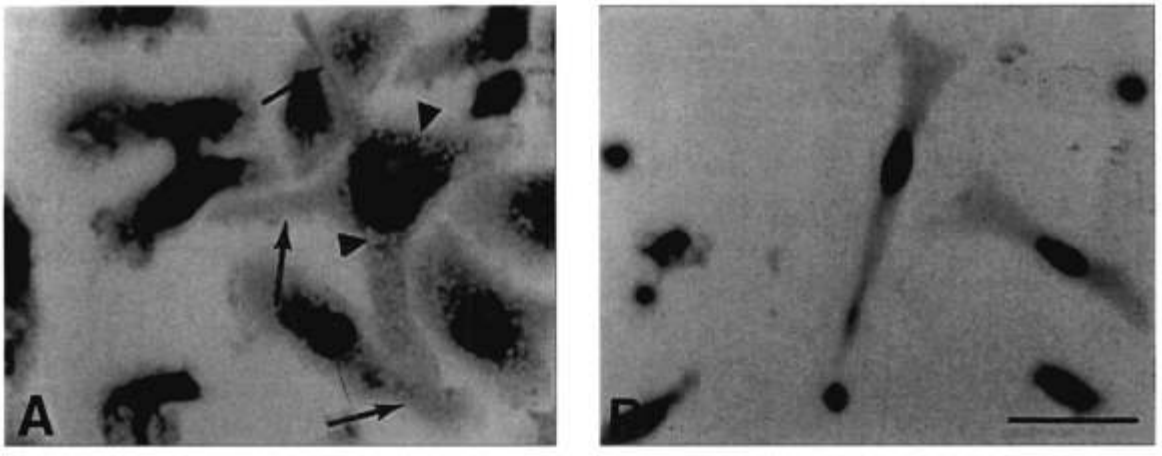

Figure 3. Light micrographs showing the murine peritoneal macrophages of mice treated with 'poly-plat' (A) and cisplatin (B) after 2 days. Note the very large nuclei, numerous lysosomes (arrowheads) and long cytoplasmic extensions (arrows) radiating from the macrophages of 'poly-plat'-treated mice. There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

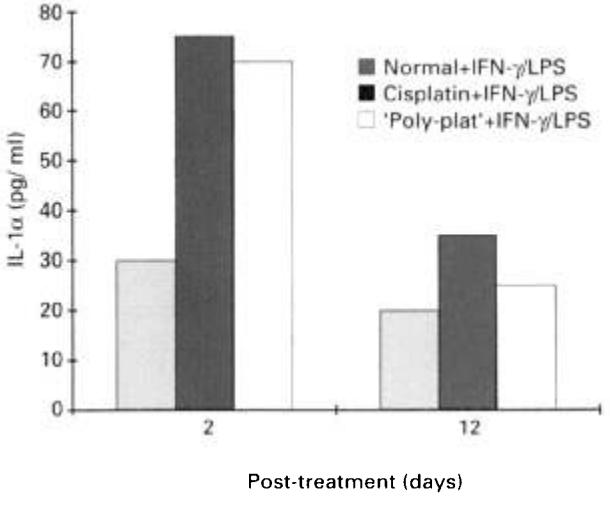

Figure 4. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages from mice treated with either 'poly-plat' or cisplatin (5 μg/ml) plus IFN-γ/LPS after 2 and 12 days. Note the initial increase of IL-1α after 2 days but a subsequent decrease after 12 days of 'poly-plat' and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

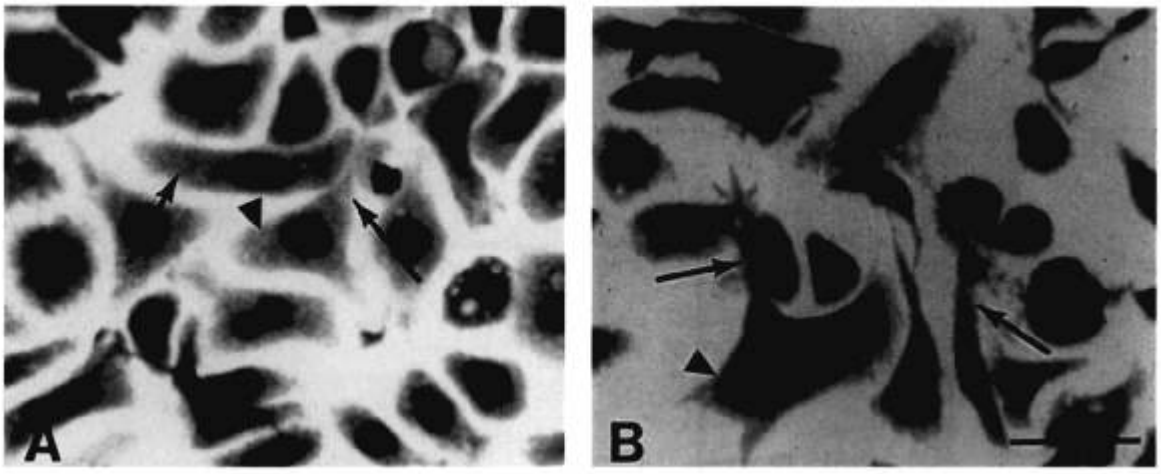

Figure 5. Light microphages showing the murine peritoneal macrophages of mice treated (2 and 12 days) with 'poly-plat' (A) and cisplatin (B) plus IFN-γ/LPS after 2 days. Note the large nuclei, numerous lysosomes and long cytoplasmic extensions (arrows) radiating from the macrophages of both 'poly-plat'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

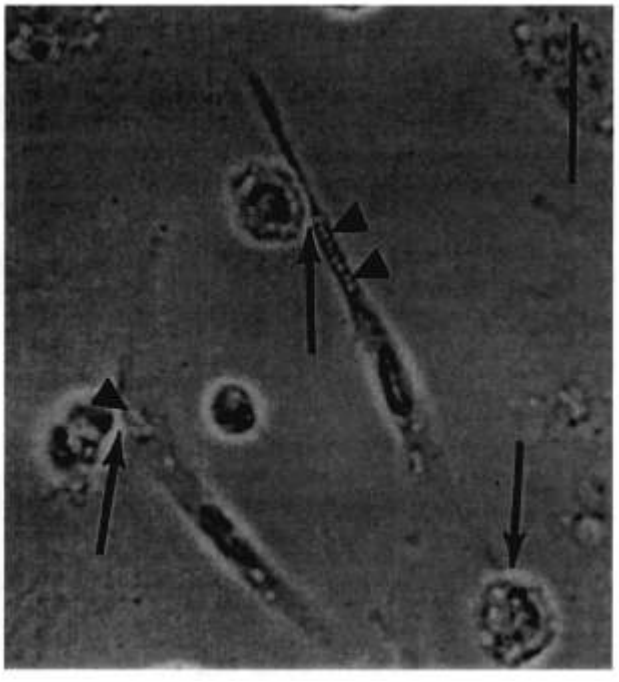

Figure 6. Light macrophages showing 'poly-plat'-treated murine peritoneal macrophages co-cultured with S180 cells for only 2 h. Note the cytoplasmic extensions in contact with tumor cells (arrows) and an increase in the lysosomal activity only after drug treatments. Untreated macrophages never establish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

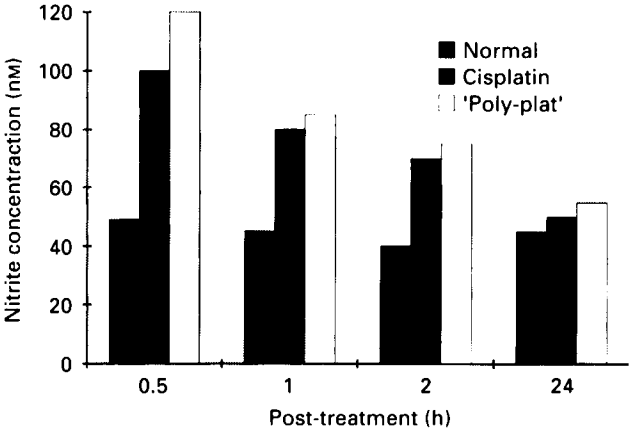

Figure 7. Bar graph demonstrating nitrite levels in the supernatants of macrophages cultures after treatment with 'poly-plat' or cisplatin at various time intervals. Note the intial increase in the nitrite levels at 30 min, but dropping to normal levels after 24 h.

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.

References

1. Aggarwal B, Totpal K. Mechanisms of regulation of cell growth by cytokines of the immune system. In: Pasquier C, Olivier R, Auclair C, eds. *Oxidative stress, cell activation and viral infection*. Basel: Birkhauser Verlag 1994: 155.
2. Palma J, Aggarwal S. Cisplatin and carboplatin mediated activation of murine peritoneal macrophages *in vitro*: production of interleukin-1α and tumor necrosis factor-α. *Anti-Cancer Drugs* 1995; **6:** 1.
3. Sodhi A, Singh S. Release of cytolytic factor(s) by murine macrophages *in vitro* on treatment with cisplatin. *Int J Immunopharmacol* 1986; **8:** 701.
4. Leh F, Wolf W. Platinum complexes: a new class of anti-neoplastic agents. *J Pharmacol Sci* 1976; **65:** 315.
5. Rosenburg B, VanCamp L. The successful regression of large solid sarcoma 180 tumors by platinum compounds. *Cancer Res* 1970; **30:** 1799.
6. Durant J. Cisplatin: a clinical overview. In: Prestayko A, Crooke S, Carter S, eds. *Cisplatin: current status and new developments*. New York: Academic Press 1980: 317.
7. Zwelling L, Kohn K. Mechanism of action of *cis*-dichlorodiammine platinum (II). *Cancer Treat Rep* 1979; **63:** 1439.
8. Bahadur A, Sarna S, Sodhi A. Enhanced cell mediated immunity in mice after cisplatin treatment. *Polish J Pharmac Pharmacy* 1984; **36:** 441.
9. Singh S, Sodhi A. Interaction between cisplatin treated macrophages and Dalton's lymphoma cells *in vitro*. *Exp Cell Biol* 1989; **56:** 1.
10. Palma J, Aggarwal S, Jiwa A. Murine macrophage activation after cisplatin or carboplatin treatment. *Anti-Cancer Drugs* 1992; **3:** 665.
11. Muenchen H, Aggarwal S, Misra H, Andrulis P. Enhanced immunostimulation by novel platinum anticancer agents. *Anti-Cancer Drugs* 1997; **8:** 323.
12. Fiebig H, Dress M, Ruhnau T, Misra H, Andrulis P, Hendriks H. GB-21, a novel platinum complex with antitumor activity in human renal and mammary xenografts. *Proc Am Ass Cancer Res* 1996; **37:** 297.
13. Kiernan J. *Histological & histochemical methods*. Oxford: Pergamon Press, 1990.
14. Ding A, Nathan C, Stuehr D. Release of reactive nitrogen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidences for independent production. *J Immunol* 1988; **141:** 2407.
15. Hsu S, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABS) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29:** 577.
16. Adams D, Hamilton T. Activation of macrophages for tumor cell kill: effector mechanisms and regulation. In: Heppner GH, Fulton AM, eds. *Macrophages and cancer*. Boca Raton, FL: CRC Press 1988: 27.
17. Stewart C, Stevenson A, Hibbs J. Effector mechanisms for macrophage-induced cytostasis and cytolysis of tumor cells. In: Heppner GH, Fulton AM, eds. *Macrophages and cancer*. Boca Raton, FL: CRC Press 1988: 39.
18. Sodhi A, Singh S. Interaction between cisplatin treated macrophages and Dalton's lymphoma cells *in vitro*. *Exp Cell Biol* 1989; **56:** 1.
19. Palma JP, Aggarwal SK. Cisplatin and carboplatin mediated release of cytolytic factors in murine peritoneal macrophages *in vitro*. *Anti-Cancer Drugs* 1994; **5:** 615.
20. Lachman L, *et al.* Natural and recombinant interleukin-1β is cytocidal for human melanoma cells. *J Immunol* 1986; **136:** 3098.
21. Okubo A, Sone S, Tanaka M, *et al.* Membrane associated interleukin-1α as a mediator of tumor cell killing by human blood monocytes fixed with paraformaldehyde. *Cancer Res* 1989; **49:** 256.
22. Gery I, Waksman B. Potentiation of cultured mouse thymocyte response by factors released by peripheral leukocytes. *J Immunology* 1986; **107:** 1778.

23. Matsubara T, Ziff M. Increased superoxide anion release from human endothelial cells in response to cytokines. *J Immunol* 1986; **137:** 3295.
24. Kharazmi A, Neilson H, Bendtzen K. Recombinant interleukin-1α and β prime human monocytes superoxide but have no effect on chemotaxis and oxidative burst response of neutrophils. *Immunobiology* 1988; **177:** 32.
25. Arouma O, Halliwell B, Dizdaroglu M. Iron ion dependent modification of bases in DNA by superoxide radical generating system hypoxanthine oxidase. *J Biol Chem* 1989; **264:** 13024.
26. Richter C, Kass G. Oxidative stress in mitochondria: its relationship to cellular calcium homeostasis, cell death, proliferation and differentiation. *Chem-Biol Interact* 1991; **77:** 1.
27. Chong Y, Heppner G, Paul L, *et al.* Macrophage mediated induction of DNA and breaks in target cells. *Cancer Res* 1989; **49:** 6652.
28. Lowenstein C, Synder S. Nitric oxide, a novel biologic messenger. *Cell* 1992; **70:** 705.
29. Moncada S, Palmer R, Higgs E. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; **43:** 109.
30. Esumi H, Tannenbaum S. Seminar on nitric oxide synthase and carcinogenesis. *Cancer Res* 1994; **54:** 297.
31. Hibbs J, Taintor R, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deaminase and amino nitrogen oxidation to nitrite. *Science* 1984; **235:** 473.
32. Stuer D, Nathan C. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 1989; **169:** 1543.
33. Krahenbuhl J, Remington J. The role of activated macrophages in specific and nonspecific cytostasis of tumor cells. *J Immunol* 1974; **113:** 507.
34. Granger D, Lehninger A. Site of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J Cell Biol* 1982; **95:** 527.
35. Drapier J, Hibbs J. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfar enzymes in the macrophage effector cells. *J Immunol* 1988; **140:** 2829.

(Received 23 September 1997; accepted 3 October 1997)