

Local Immunological Defense of the Tumor-bearing Host in Abdominal Surgery with Special Reference to Peritoneal Macrophage-Mediated Cytostasis

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ABSTRACT : There were reported some kinds of anticancer drugs which have immunopotentiating effect. In this study, we demonstrated the i.p injection of CDDP which enhance the macrophage-mediated cytostatic effect in mice. Flow cytometry has been used to study the influence of CDDP-activated macrophages on the cell cycle of Meth-A cells, and the biochemical changes of the CDDP-activated macrophages were analysed. Macrophages harvested four days later from mice treated with i.p injection of CDDP 0.3 ~ 4 mg/kg showed a dose-dependent effect to both EL-4 and Mrth-A cells. The activation was mainly seen in the plastic adhesive cells and was diminished by treating with carrageenan. In vivo Winn assay, CDDP-activated macrophages showed a significant inhibitory effect to the proliferation of Meth-A cells for at least 15 days.

The survival time was elongated when the tumor-bearing mice were treated with i.p injection of CDDP. However, the elongative effect was diminished by the pretreatment of carrageenan. On the other hand, Flow cytometry analysis using Propidium Iodide staining was designed to study the mechanism of the tumor cell inhibitory effect by CDDP and CDDP-activated macrophages. Changes of cell elements such as RNA, protein, and mitochondria were evaluated by A.O, FITC and Rhodamin 123 staining technique respectively.

CDDP does enhance the antitumor effect of the peritoneal macrophages of mice. Using the Flow cytometry analysis we have been able to demonstrate that in contrast to CDDP which accumulate the Meth-A cells in the G₂M phase of the cell cycle, CDDP-activated macrophages appeared to have a different influence more restricted to the G₁/S block of the cycle.

CDDP-activated macrophages resembled to OK-432-activated macrophages, showing an increase of RNA, protein synthesis and an enhancement of the mitochondria membrane potential.

INTRODUCTION

Macrophage inflammatory responses which resulted in tumor lysis or proliferative inhibition seems to be one of the central importances,¹⁾ especially in cases involving solid tumors.

Tumors heavily infiltrated with macrophages are less likely to metastasize and show a good prognosis.^{2) 3) 4) 5)}

On the other hand, patients and animals with neoplasms, generally defective monocyte/macrophage function has been demonstrated.^{6) 7) 8) 9)} Whether these functions are defective before the

onset of neoplasia or become defective as a result of the tumor formation is not known. However, it is reasonable to use some regimens that can destroy the tumor cells and stimulate the host's natural immune processes in regards of the cancer therapy.

Anticancer chemotherapy was widely used in the bed-side and clinic for cancer patients as a post-operative adjuvant therapy or in the cases of progressive pleural/peritoneal carcinomatosa. However, the influence to the host's immunologic effector cells was not yet well known.

In general, chemotherapeutic agents have traditionally been thought to cause depression of immune responsiveness,^{10) 11) 12) 13)} but it has become clear that some kinds of chemotherapeutic agents e.g Cyclophosphamide,¹⁴⁾ Adriamycin,¹⁴⁾¹⁵⁾¹⁶⁾ Mitomycin C,¹⁷⁾ and Bleomycin¹⁸⁾ act to the host immune effector cells as an immunopotentiating factor.

Lichtenstein¹⁹⁾ demonstrated the enhancement of natural killer cytolysis of peritoneal and spleen cells of the mice by CDDP, Kleinerman et al.²⁰⁾ reported the enhancement of cytotoxicity of human peripheral blood monocyte by CDDP. In this study, in respect to cytostatic effect, we do the further investigation in vitro and in vivo on mice to evaluate the influence of CDDP on peritoneal macrophages. Also we analysed the kinetics of the target cell cycle and the biochemical changes of the effector cell by using the Flow cytometry. CDDP activates macrophages and inhibits the growth of tumor cells, suggesting that the drug may exert an effect similar to Biological Response Modifier (BRM) in addition to its known direct toxicity, and may have potential clinical usefulness in the design of immunotherapeutic trials for certain types of cancer.

MATERIALS AND METHODS

1) Animals, tumor cells, chemicals and reagents

C57BL/6 and BALB/C mice were obtained from Chales River Breed Laboratory, Japan. All animals were male, eight to 12 weeks old and weighed approximately 25 to 30 g. EL-4, a T-cell lymphoma of the C57BL/6 mouse and Meth-A, a fibrosarcoma of BALB/C mouse were maintained in RPMI 1640 containing 10%

FCS when used as target cells in vitro cytostasis assay and were maintained in an ascitic form in mice to be used for in vivo Winn assay.

Cis-Diammindichloroplastinum (II)(CDDP) was obtained from Nippon Kayaku Co, Ltd., Japan. Its solubility is 500 $\mu\text{g}/\text{ml}$. To administer to animals, the drugs was diluted with sterile 0.9% NaCl solution at the desired concentrations and 0.5 ml was injected i.p into each mouse.

Carrageenan type IV was obtained from Sigma Chemical Co. Thioglycolate medium was obtained from Difco Laboratories, Detroit, MI. ³H-TdR (specific activity 6.7 Ci/m mol) was obtained from New England Nuclear, Boston, MA.

2) Preparation of murine macrophages

C57BL/6 mice were given i.p injection of various doses of CDDP, and saline to controls. Peritoneal exudate cells (PEC) were harvested four days later by peritoneal lavage with ice-cold RPMI. PEC were washed two times with PBS and suspended in RPMI 1640 supplemented with 10% FCS containing penicillin(100 U/ml), streptomycin (0.05 mg/ml).

After determination by viable and differential cell counts, macrophages were adjusted to $1 \times 10^6/\text{ml}$ and 0.1 ml (1×10^5) were dispensed into the flat-bottomed wells of a 96-well culture plate (Corning). After incubation at 37 °C for 90 minutes, nonadherent PEC were removed by repeated washing with PBS, and finally approximately 45% to 55% of the initially added cells remained in cultured wells. The majority of these cells (>90%) morphologically resembled macrophages. The compound at the CDDP dose of 4 mg/kg or less causes no apparent toxicity, since >95% harvested PEC were viable and about 50% of those cells were capable of adhering to plastic plate.

3) Treatment of effector cells with carrageenan

For in vivo studies, all mice were treated with CDDP 4 mg/kg on Day-4 and some mice were given i.p injection of 1 mg carrageenan (1 mg/ml) on Day-3 and -1, respectively. PEC were harvested on Day-0, and tested in vitro for cytostasis with an E : T ratio of 10 : 1.

4) Assay for macrophage-mediated tumor cell cytostasis

The exponential phase EL-4 and Meth-A tu-

mor cells cultured in RPMI 1640 containing 10% FCS were washed twice with PBS, and resuspended in fresh RPMI 1640 containing 10% FCS culture medium. 1×10^4 cells in 0.1 ml cultured medium were added to culture wells containing macrophages (1×10^5 in 0.1 ml) or to wells containing 0.1 ml of medium only for control.

Unless otherwise noted, culture plate were incubated at 37°C in a 5% CO₂ and water-saturated atmosphere for 48 hours. Cells in each wells were pulsed with 0.5 μ Ci ³H-TdR for the final four hours and harvested with a semiautomatic multiple cell harvester (LABO SCIENCE CO LTD) to glass fiber. The amount of ³H-TdR incorporated was determined in a liquid scintillation counter (Aloka, LIQUID SCINTILLATION SYSTEM Model LSC-903). The mean cpm were obtained from triplicate cultures, and the results were presented as percentage cytostasis which was calculated by the following formula.

$$\% \text{ Cytostasis} = \left(1 - \frac{b-c}{a}\right)$$

Where "a" is cpm of cultures containing target cells only, "b" is cpm of cultures containing target cells and macrophages, and "c" is cpm of cultures containing macrophages only.

The results % cytostasis are representative of three separate experiments.

5) In vivo effect of carrageenan on CDDP treated tumor bearing mice

Group (A)-(D), each group consisting of 5 C57BL/6 male mice 10 weeks old were treated with i.p injection of EL-4 tumor cells, CDDP, and carrageenan as follows. Group (A): EL-4 tumor cells only, Group (B): EL-4 tumor cells + carrageenan, Group (C): EL-4 tumor cells + CDDP 4 mg/kg, Group (D): EL-4 tumor cells + CDDP 4 mg/kg + carrageenan.

Briefly, On Days -3 and -1 (B), (D) Groups were given i.p injection of carrageenan 1 mg per mouse, On Day 0 all groups were treated with i.p injection of EL-4 tumor cells (2×10^6 in 1 ml) which were maintained in ascitic form in C57BL/6 mice and on Day +1 (C)(D) Groups were given i.p injection of CDDP 4 mg/kg. The effect of carrageenan was expressed by the survival time (rate).

6) In vivo tumor neutralization assay (Winn assay)

Each group consist of 5 to 6 BALB/C σ^7 mice irradiated with 400 rads one day before inoculation. The effector cell suspension 0.1 ml (macrophage 5×10^7 /ml) was mixed with an equal volume of tumor cell suspension (Meth-A 1×10^7 /ml) and 0.2 ml of the cell mixture was inoculated s.c into the back of syngeneic BALB/C mouse. The results were evaluated by measuring the long axis \times short axis of the tumors. The group division was as follows: (A) inoculated with Meth-A cells only. (B) inoculated with the mixture of Meth-A cells and thioglycolate treated macrophages. (C) inoculated with Meth-A cells and CDDP treated macrophages. (D) inoculated with the mixture of Meth-A cells and macrophages treated with CDDP and carrageenan.

7) Flow cytometry

a) Propidium Iodide staining : For the analysis of the cell cycle distribution of Meth-A cells, after exposed to CDDP (0.5 μ g/ml) for 48 hours, or cocultured with macrophages, the cells were fixed with 70% alcohol for at least overnight. Alcohol-fixed cell suspensions were rinsed and treated with RNase 1 mg/ml (Sigma, RNase type 1-A) at 37°C for 20 min. Rinsed again and stained with P.I solution (50 μ g/ml) for two to three hours at 4°C.

b) Acridine Orange staining : For the analysis of RNA contdnt of macrophages, control PEC or PEC treated with various doses of CDDP and OK-432 (1 KE/mouse) were purified by the adherence method. Adherence peritoneal cells (APC) were fixed in ethanol : acetone (1 : 1 v/v) mixture for at least overnight. Fixed cells were then stained by the two-step A.O staining method as described by Darzynkiwicz.²¹⁾ Briefly fixed single cell suspensions were adjusted to 1×10^6 /ml. Aliquots of 0.2 ml of cell suspensions were mixed with 0.4 ml of ice-cold acid detergent (0.01% Triton, 0.08 N HCl, 0.15 N NaCl, 10^{-4} EDTA-Na) for 30 sec. The cells were then stained by addition of 1.2 ml of A.O solution (concentration 10 μ g/ml, diluted by phosphatecitrate buffer PH 6.0). After 5 minutes of interation the cells were measured in FACS with an argon laser (488nm). The red (>600 nm) fluorescence, and light scatter per cell were obtained for 5000 cells from each saple.

c) Fluorescein isothiocyanate (FITC) staining²²⁾: For analysis of protein content. 70% alcohol-fixed macrophages were stained with FITC 50 $\mu\text{g}/\text{ml}$ (diluted by 0.5M NaHCO_3 sodium bicarbonate PH 8.0) for 30 min at 4°C, and then measured in FACS with an excitation length of 488nm and absorption length of 530nm.

d) Supervital Rhodamine123 staining of mitochondria: Each sample of viable macrophages were stained with Rh123 10 $\mu\text{g}/\text{ml}$ (dye dilutions were made in RPMI medium containing 10% FCS) for 20 min at 37°C as described by Darzynkiewicz.²³⁾

e) Phagocytic activity of macrophages: Macrophages ($2 \times 10^5/\text{ml}$) were incubated with fluorescent latex beads ($1 \times 10^7/\text{ml}$, DIA.1 = 1.73 μ Polyscience, Inc, Warrington) for 90 min at 37°C, then the excess beads were discarded, rinsed and the macrophages were harvested by repeated pipetting with 0.2% EDTA + 0.02% trypsin solution. Centrifugation and the pellets were resuspended with RPMI medium, and measured in FACS by counting 5000 cells.

$$\% \text{ Phagocytosis} = \frac{\text{cells phagocyte at least one bead}}{\text{total cell counted}}$$

RESULT

1) Induction of tumor-cytostatic macrophages in vivo

Macrophages prepared from C57BL/6 mice treated with doses of CDDP ranging from 0.3 to 4 mg/kg showed a dose-response cytostatic effect to both syngeneic EL-4 cells and allogeneic Meth-A cells. (Fig. 1) Although the magnitude of responses of allogeneic cells (% cytostasis 52 ± 12 to 61.3 ± 9) was not so significant as syngeneic cells do (% cytostasis 78 ± 15 to 83 ± 13). A dose of CDDP lesser than 0.3 mg/kg did not show any activation effect and the dose higher than 4 mg/kg showed the adverse toxic effect to macrophages. Therefore, we considered the 4 mg/kg was the optimal dose to activate the macrophages, and in the following experiments mice were treated with a dose of 4 mg/kg. Thioglycolate was reported as having no effect on macrophages activation, but in this study the thioglycolate treated macrophages showed somewhat higher cytostatic effect than untreated macrophages does. Macrophages from

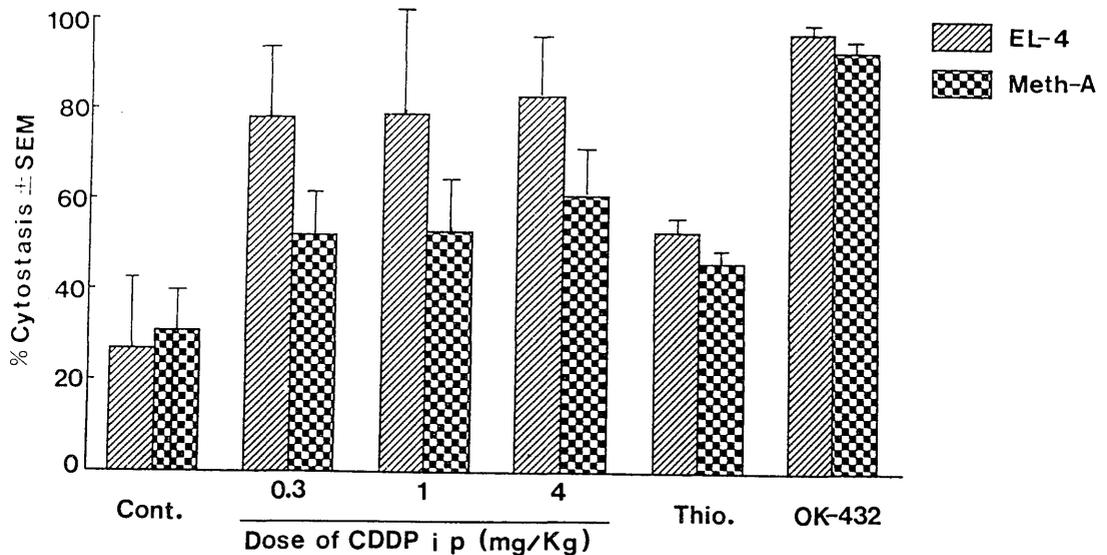


Fig. 1. Dose-response effect of CDDP on the activation of macrophage as compared to the effect of OK-432 and thioglycolate. C57/BL6 mice were treated with various doses of CDDP, 3% thioglycolate 1 ml/mouse and OK-432 1 K.E./mouse. Macrophages were prepared 4 days later for testing tumor cytostasis at an E : T ratio of 10 : 1.

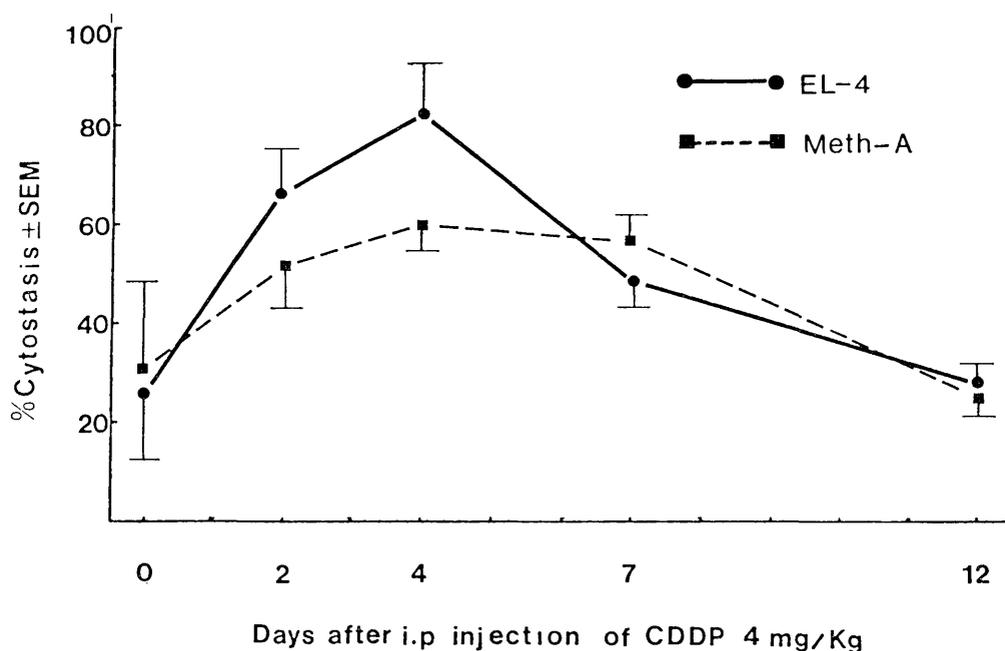


Fig. 2. Persistence of activated macrophages following a single dose of CDDP. Macrophages were prepared by the peritoneal lavage at various time intervals after treatment with CDDP 4 mg/kg, and tested for cytotaxis at an E : T ratio of 10 : 1 for 48 hrs.

mice treated with immunostimulant agent OK-432 showed a significant cytotostatic effect both to EL-4 and Meth-A cells.

2) Persistence of activated macrophages

Fig. 2 demonstrated the time course of macrophages activated by CDDP. After a single bolus of CDDP 4 mg/kg macrophages were prepared at various time intervals, cytotostatic effect was elevated as early as two days, appeared to be the maximal activated state four days later, and declined to the control state by 12 days.

3) Inactivation of CDDP-activated macrophages with carrageenan

Effector cells which caused the cytotostatic effect being investigated resembled to macrophages morphologically and in the ability to adhere to the plastic plate. In an attempt to further identify the cell type involved in the cytotostatic reaction, PEC were treated with carrageenan which was known to abrogate macrophage activity. As shown in Tab. 1 mice treated with carrageenan on Day-3 and Day-1 twice, significantly abrogate the cytotostatic effect of the PECs. These findings strongly sug-

Table 1. Effect of carrageenan on CDDP-treated macrophage

| Target cell | Treatment of mice ^a | % cytotaxis | P ^c |
|-------------|--------------------------------|------------------------|----------------|
| EL-4 | CDDP | 83.2±13.0 ^b | p<0.05 |
| | CDDP+ carrageenan | 35.5± 6.4 | |
| Meth-A | CDDP | 61.3± 9.2 | N. S |
| | CDDP+ carrageenan | 46.5±60.3 | |

^aAll mice were treated with CDDP 4 mg/kg on Day -4 and some mice were given i. p injection of 1 mg carrageenan/mouse on Day -3 and -1, macrophages were harvested on Day 0, and tested for cytotaxis with an E : T ratio of 10 : 1.

^bMean ± S. E

^cDetermined by comparison between mice given injection with and without carrageenan.

gest that CDDP acted mainly upon macrophages rather than other population of the PECs.

4) Effect of culture supernatants of activated macrophages

Macrophages prepared from mice treated with CDDP for four days, were cultured in vitro for two days, supernatants were harvested, filtrat-

ed through a 0.45 μm Millipore filter and mixed to EL-4 tumor cell cultures at various dilutions. The inhibition of tumor cells was determined two days later by ^3H -TdR incorporation. Fig. 3 shows that these supernatants inhibited the growth of tumor cells in a concentration-dependant fashion.

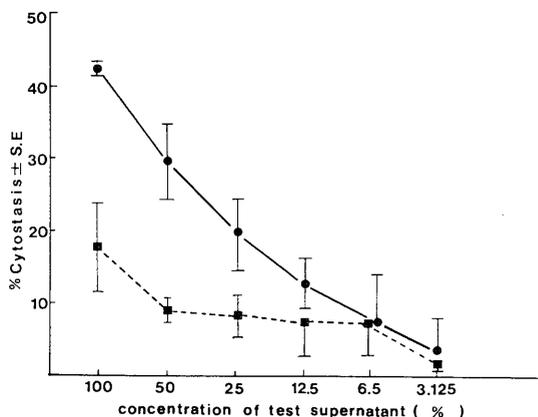


Fig. 3. Release of a cytostatic factor (s) by CDDP-activated macrophages. Macrophages prepared from C57BL/6 mice treated with i.p. injection of CDDP 4 mg/kg and saline for control were cultured 2 days. Supernatants were harvested and tested for cytotostasis of EL-4 cells at various concentrations. Concentration of supernatant are given on a logarithmic scale as percent (V/V) of the medium.

5) In vivo influence of carrageenan on the anti-tumor effect of CDDP

Compared to tumor cells transplantation only group, (all mice died within 28 days) mice received CDDP anticancer drug showed a significant therapeutic effect (survival rate of 50% at the time of 50 days). On the other hand, the therapeutic effect of CDDP was diminished when mice were pretreated with carrageenan on days -3 and -1 twice. (Fig. 4)

6) Augmentation of the in vivo tumor neutralization activity of macrophages treated with CDDP (Fig. 5)

Effector cells treated with CDDP significantly inhibited the tumor growth within 15 days ($P < 0.01 \sim P < 0.1$). However, the inhibitory ef-

fect was almost abrogated by treating with carrageenan suggesting that CDDP-activated macrophages may be the main effector cell to inhibit the tumor growth.

7) Cell cycle specificity of Meth-A cells exposure to CDDP or cocultured with CDDP-activated macrophages

Fig. 6 shows the direct cytostatic effect of CDDP by concentrations ranging from 0.005 to 15.625 $\mu\text{g}/\text{ml}$. The concentration of 0.625 $\mu\text{g}/\text{ml}$ shows cytostatic effect similar to the effect caused by CDDP-activated macrophages ($61.3 \pm 9.2\%$). Therefore, in the following Flow cytometry analysis, concentration of 0.5 $\mu\text{g}/\text{ml}$ was used in the direct CDDP cytostatic effect test.

Flow cytometry analysis using P.I staining for DNA of Meth-A cells (Fig. 7) demonstrated that in contrast to CDDP exposure (D) which accumulated Meth-A cells in G_2/M phase, CDDP and OK-432 activated macrophages appeared to block in the G_1/S phase of the cycle. The percentage fraction of the cell cycle was analyzed by the dot plot method (Tab. 2).

8) Promotion of functional phagocytosis and changes of cellular contents of macrophages treated with CDDP and OK-432

Fig. 8 demonstrated the phagocytosis of fluorescent beads by macrophages (upper) and cellular contents of protein, RNA, and mitochondria contents were analysed by the relative peak channel numbers. Both functional phagocytotic function and quantitative content changes were in dose-dependent fashion.

DISCUSSION

Cisplatin (Cis-dichlorodiamminoplatinum (II), CDDP) is a coordination of the platinum, its antitumor activity was first proved by ROSENBERG *et al.*²⁴⁾ in 1969, and is a potent chemotherapeutic drug for the treatment of a variety of human tumors.

The mechanism by which CDDP kills tumor cells is thought to be derived from direct intercalating platinum into the double helix strain of the cellular DNA, and resulted in the inhibition of cell division.²⁵⁾

The in vitro 48 hours continuous exposure test showed that effective cytostatic concentra-

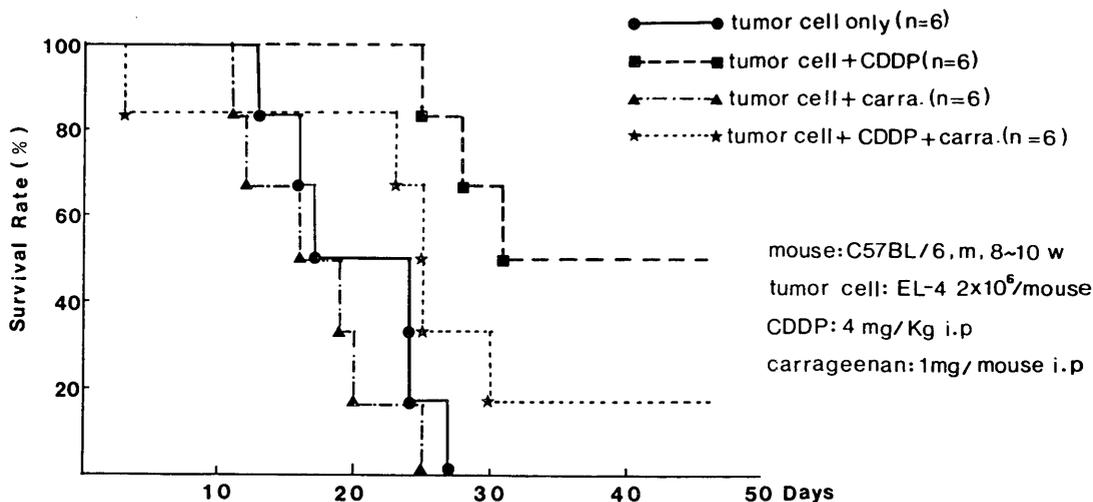


Fig. 4. In vivo influence of carrageenan on the anticancer effect of CDDP. All C57BL/6 mice received i.p injection of EL-4 cells on Day 0, some mice were treated with carrageenan on Days-3 and -1, and another mice were treated with CDDP on Day 1. The influence was evaluated by the survival rate.

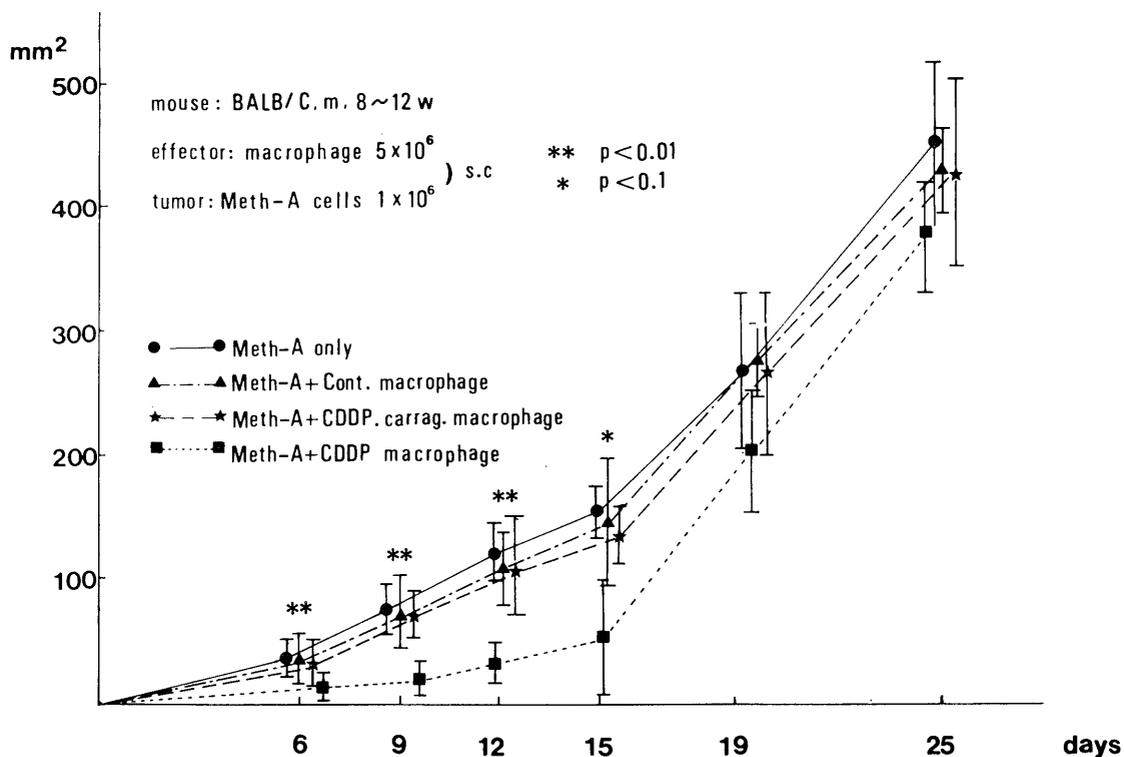


Fig. 5. Tumor neutralization assay (Winn assay) by macrophages. The effector cell suspension 0.1 ml was mixed with an equal volume of tumor cell suspension and 0.2 ml of the cell mixture was inoculated s. c into syngeneic BALB/C mouse irradiated 400 rads 5 days before the inoculation. Tumor size were evaluated by the product of major axis \times minor axis.

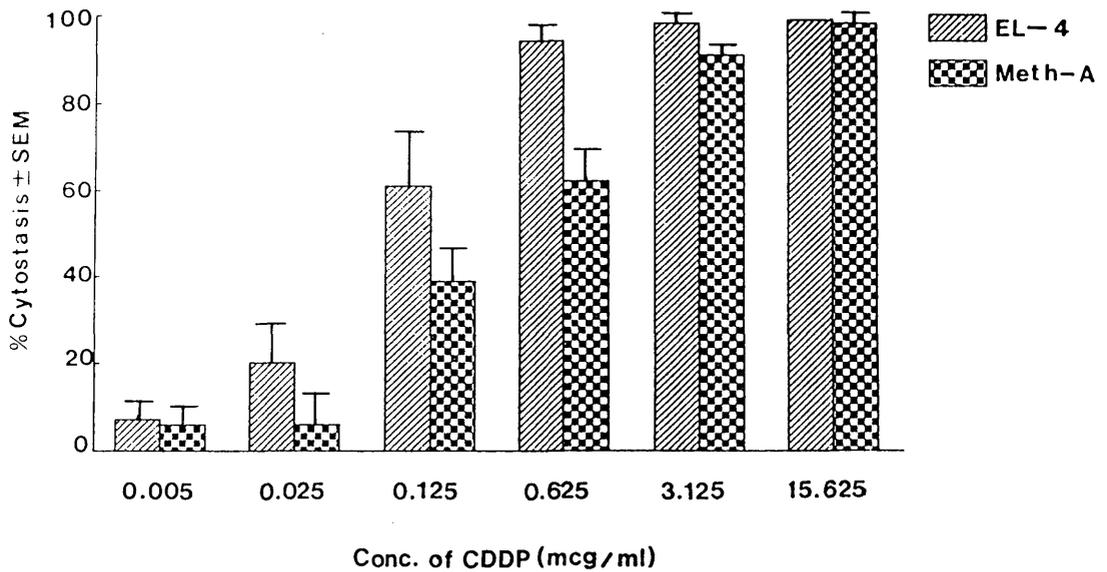


Fig. 6. Direct cytostatic effect of CDDP. Tumor cells were exposed contineously to CDDP for 48 hrs and then tested for 3H-Tdr incorporation.

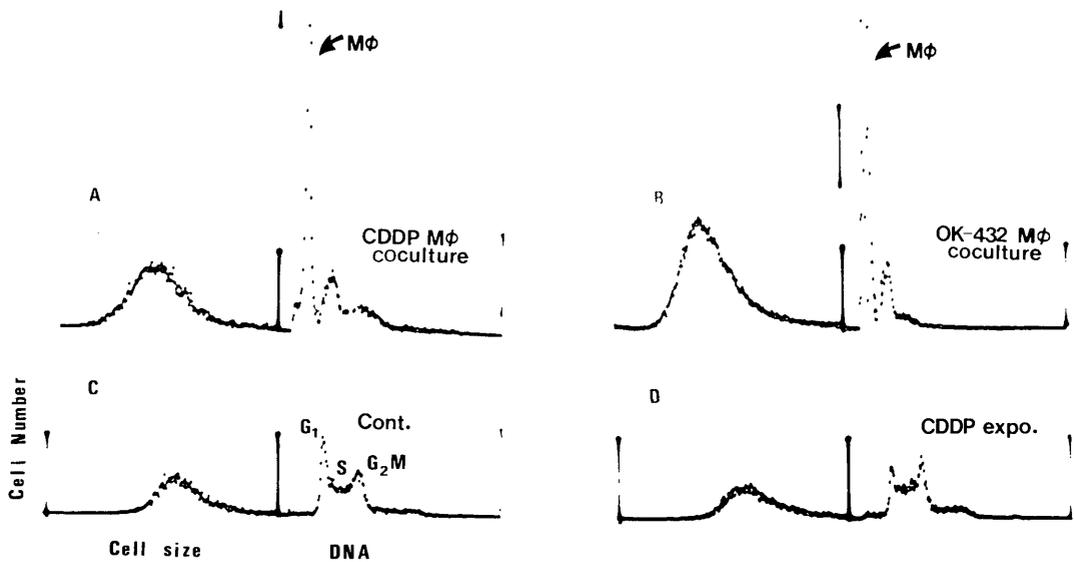


Fig. 7. DNA histograms of Meth-A cells (A) cocultured with CDDP-activated macrophages, (B) cocultured with OK-432 activated macrophages, (C) control, (D) exposed to CDDP. for 48 hrs.

Table 2. FACS-sorted phase fraction of the Meth-A cells incubated with CDDP direct and CDDP- activated macrophages

| | fraction of the cell cycle (%) | | | | | |
|--------------------------------|--------------------------------|------------------|------|-----|------------------|-----|
| | G ₁ | | S | | G ₂ M | |
| Control | 35.3 | 2.3 ^d | 30.7 | 1.7 | 26.3 | 0.6 |
| CDDP direct ^a | 25.3 | 0.6 | 37.3 | 1.2 | 37.3 | 0.6 |
| CDDP-macrophage ^b | 59.0 | 1.0 | 20.3 | 2.1 | 20.7 | 1.2 |
| OK-432 macrophage ^c | 63.2 | 3.2 | 21.3 | 1.5 | 15.5 | 1.7 |

^a 5×10^5 Meth-A cells were incubated with CDDP 0.5 $\mu\text{g}/\text{ml}$ for 48 hrs.

^b mice were treated i.p with CDDP (4 mg/kg), macrophages were prepared 4 days later and cocultured with Meth-A target cells at an E : T ratio of 10 : 1 for 48 hrs.

^c mice were treated with OK-432 1 KE/mouse 4 days.

^d mean S.E

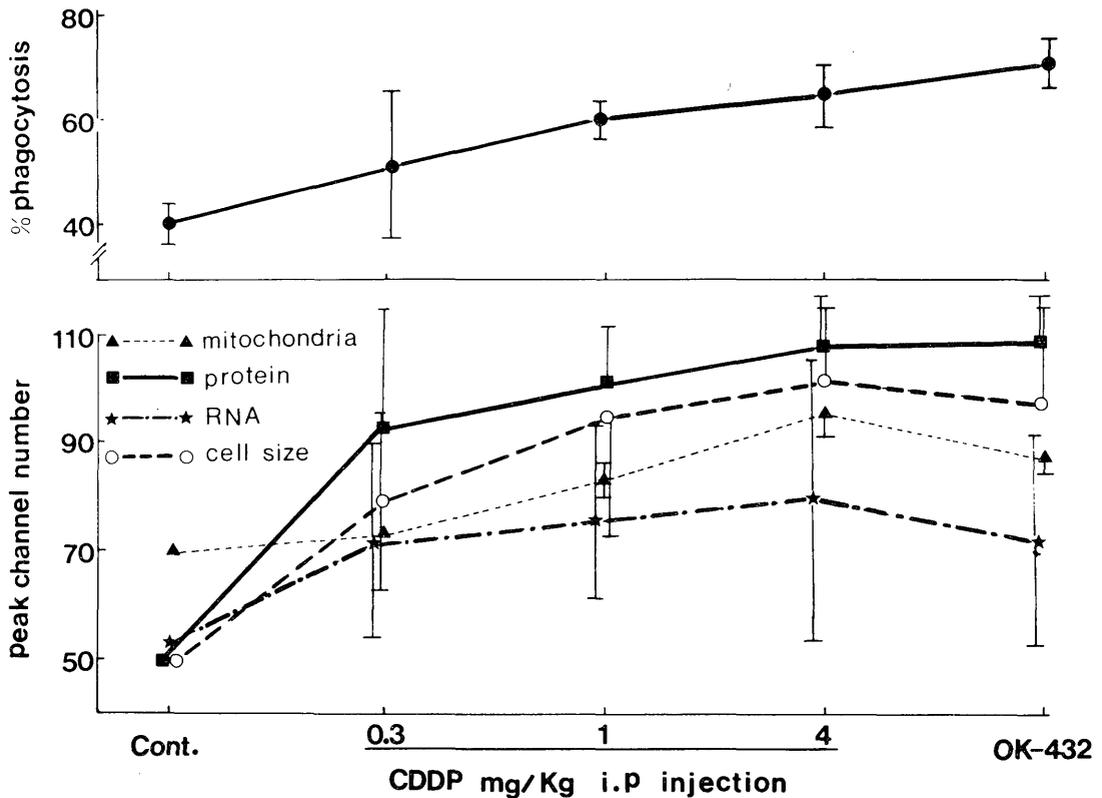


Fig. 8. Functional phagocytosis (upper) and Cellular contents (lower) changes of CDDP-activated macrophages as compared to that of OK-432 activated macrophages.

tion was at least 3000 ng/ml (Fig. 5). However other reports demonstrated that a large percentage of CDDP is excreted within two

hours of administration and thus the amount of CDDP retained in the body is low compared to that used in vitro studies.²⁶⁾ Furthermore,

within three hours of administration about 90% of the CDDP administered is protein bound. Evidence suggests that only free-CDDP is active, therefore the concentration of active drug in the circulation approaches only 100 to 280 ng/ml two hours after an i.v bolus.²⁷⁾ This is far lower than our in vitro studies. Thus, beside the direct cytotoxic effect of CDDP other mechanisms are possible.

One of these possibilities, we thought, was the host immuno-effector cell mediated anti-tumor effect. The defense mechanism of animals consists of several types of immuno-competent cells. LICHTENSTEIN *et al.*¹⁹⁾ reported the enhancement of NK cytolytic activity of the peritoneal and spleen cells by treatment of i.p injection of CDDP. However, in the present experiment, macrophages apparently were the primary target of CDDP as regard to cytostatic effect, since we investigated the peritoneal adhesive cells which morphologically resemble to macrophages and the effect was significantly abolished by treatment with carrageenan (Tab. 1, Fig. 4, 5.) which is known to abrogate macrophages activity.²⁸⁾

The antitumor effect of macrophages exposed to CDDP in vivo could be explained by several mechanisms.

First, the drug may potentiate antitumor immunity through the elimination of any immunosuppressor cell activity that appears in the tumor bearing host.^{29) 30)} In this experiment model the CDDP-activated macrophages tested in vitro was prepared from normal mice, therefore this hypothesis is unlikely to be possible.

Another mechanism could be an indirect activation of macrophages by CDDP through the release of macrophage-activating factor, a lymphokine by peritoneal lymphocytes.³¹⁾ However, other investigators²⁰⁾ have demonstrated that unlike the case of X-irradiation, treatment of lymphocyte with CDDP could not enhance the cytotoxic effect of monocyte.

A third mechanism of the toxic effect on tumor cells could be a transfer of CDDP from macrophages to tumors.^{15) 16)} This hypothesis was unlikely to be true, as was shown in Flow cytometry analysis of the target cell cycle (Fig. 7). CDDP act to the G₂M part different from CDDP-activated macrophage

which block G₁/S phase of the cell cycle.

This last mechanism: CDDP could directly activate macrophages. This hypothesis is supported by the facts that the behavior of CDDP-activated macrophages resemble to that of OK-432-activated macrophages which showed the enhancement of functional phagocytotic activity, enlargement of cell size, and the increase of cell elements, such as protein, RNA, and mitochondria.

The mechanism by which macrophages were activated with CDDP is not yet established. However, inhibition of macrophage's DNA synthesis by some macrophage-activating agents were reported. TALMADGE *et al.*³²⁾ demonstrated that M5076 cell line which in vitro displays many of the functional characteristics of an activated macrophages, when exposed to the macrophage activating agents, e.g LPS, TPA irreversibly induced cessation of cellular division. NAGANO *et al.*³³⁾ reported the inhibition of DNA synthesis in Guinea pig's peritoneal exudate macrophages, by treatment with immunoadjuvants such as MDP and LPS. These observations enable us to suggest that the inhibition of DNA synthesis (by CDDP) may be something related to the activation of macrophages, but this possibility awaits confirmation.

Macrophages from mice treated with CDDP, doses ranged from 0.3~4 mg/kg, showed a dose-dependent cytostatic effect to both EL-4 and Meth-A cells, the dose was almost coincident to that used in the clinical patients. On the other hand, the time interval required to activate macrophages was reported to be 24 hours for Adriamycin.¹⁵⁾ In the case of CDDP i.p injection, the exudate cells appeared in early phase is prominently neutrophil and lymphocyte, and it is reasonable to explain that it takes times to activate the macrophages (Fig. 2).

Macrophages activated by Bisanthrene persists for four weeks,³⁴⁾ While CDDP-activated macrophages showed relatively short persistence, four days to reach the peak and declined to base line by 12 days (Fig. 2). This fact was well coincident to the Winn assay (Fig. 5) which the CDDP-activated macrophages showed a significant antitumor effect within 15 days.

It was reported that the physical cell contact is necessary to induce the inhibition of tumor

cell proliferation,³⁵⁾ however, other reporters described that the inhibition was due to the soluble factors released by macrophages.^{36) 37)} The difference may be due to the agents which activate the macrophages. In this study, we demonstrated that after 48 hours incubation the supernatant of CDDP-activated macrophages was active for the inhibition of tumor cell proliferation, suggesting that cell-cell contact was unnecessary for cytostasis.

From the results described we conclude that in vivo, macrophages are activated by CDDP during a four to seven days duration to enlarge the cell size and change the cell elements, ultimately resulted in the resealing of soluble anti-tumor factors.

For the further investigation, Flow cytometry techniques were employed to analyse the changes of effector cells and kinetics of target cells. PROSSER *et al.*³⁸⁾ have reported that using radiolabelled uridine and leucine incorporation method in the two murine macrophages cell lines of PU5-1.8 and J774.1, when treated with BCG, RNA and protein synthesis is pronounced in spite of decreased DNA synthesis. HAMILTON *et al.*³⁹⁾ demonstrated the enhanced synthesis of a series of at least six polypeptides of 85, 80, 75, 65, 57 and 38 KD by treatment of murine peritoneal macrophages with LPS. In the present study, Flow cytometry analysis using A.O and FITC staining methods we showed the increase of RNA and protein contents of the CDDP or OK-432 activated macrophages (Fig. 8). In contrast to the dose-dependent increase of protein the RNA increase was dose-independent. Supervital staining of mitochondria was also analysed. The data we obtained was well coincide with that described by DARZYNKIEWICZ²³⁾ who showed an increase of mitochondrial uptake of Rh123 during lymphocyte stimulation by PHA.

Enhancement phagocytotic activity was another functional change accompanied by activated macrophages.¹⁷⁾ By means of latex beads phagocytosis analysed by Flow cytometry.⁴⁰⁾ CDDP dose-dependent effect to functional phagocytotic activity was shown (Fig. 8). Suggesting not only the qualitative cell elements but also the functional activity changes occurred during the activating of macrophages by CDDP.

Finally, Flow cytometry using quantitative P.I staining for DNA can distinguish target (Meth-A cell) from effector cells, because the DNA contents were different (P.I staining failed to distinguish the macrophages from EL-4 cells). Using this type of analysis, we have been able to demonstrate that in contrast to CDDP exposure, which resulted in the G₂M accumulation of the Meth-A cells, CDDP-activated macrophages like OK-432 macrophages to have a different influence which block the cells in the G₁/S phase. This difference of inhibitory mechanism suggest that the contamination of CDDP or transport of CDDP by macrophage to tumor cells were unlikely to be responsible for the tumor cell inhibition. HASKILL *et al.*¹⁶⁾ have reported the accumulation of target cells at G₂M fraction by ADR, they conclude that drug retention may be the prominent effector mechanism involved in the ADR-activated macrophages phenomenon.

The immunopotentiating effects of anticancer drugs has been reported, although mechanisms were different. In this study, we confirmed the enhancing effect of CDDP on macrophages. This is a characteristics of the possibility for the direct intracavity admission of chemotherapy for patients with abdominal or pleural malignant effusions which frequently contain significant numbers of macrophages.

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