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Murine macrophage activation after cisplatin or carboplatin treatment

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Murine peritoneal macrophages when treated *in vitro* with cisplatin (9 μg/ml) or carboplatin (50 μg/ml) for 2 h are stimulated to form cytoplasmic extensions seeking out tumor cells and establishing cytoplasmic connections; however, no contact is observed with normal cells (fibroblasts and hepatocytes). In addition, cisplatin and carboplatin treatment leads to an increase in the number of lysosomes and their transfer to the tumor cells resulting in lysis (as studied by confocal microscopy). Although calcium seems to be involved in the signalling of macrophage activation, cytosolic calcium does not seem to influence this activation.

Key words: Carboplatin, cisplatin, macrophage activation.

Introduction

Cisplatin, a broad spectrum anticancer drug,[1] is currently on the market for the treatment of testicular and ovarian cancers.[2–4] It is now being tested for the treatment of head, neck and lung cancers.[5–7] Although it is an effective anticancer drug, it has severe toxic side effects, of which gastrointestinal and nephrotoxicity are the dose limiting factors.[8]

In vivo, cisplatin is known to cause hypocalcemia and hypomagnesemia.[9–11] Various calcium channel blockers, e.g. nifedipine, cause an increase in induced cytotoxicity in cisplatin resistant cell lines.[12] Cisplatin has been shown to inhibit cell division in tumor cells by depolymerization of actin-like filaments[13] known to be under the influence of calcium.[14] It seems evident that calcium plays a part in the mechanism of action of cisplatin; however, this is not yet very well understood.

Cisplatin has recently been shown to activate murine peritoneal macrophages *in vitro*.[15] These activated macrophages selectively and efficiently seek out tumor cells through the formation of cytoplasmic extensions and lysosomal transfer to these target cells causing cell death. The role of the immune system in the regression of tumors has been widely accepted[16–19] and various activating agents such as *Corynebacterium parvum*, calcium ionophore A23187 and lymphocyte mediators have been used to study macrophage activation.[20–25] In addition to the inhibition of DNA synthesis through interstrand or intrastrand crosslinking,[26–29] inhibition of transport enzymes[30] or inhibition of cytokinesis through depolymerization of microfilaments of the tumor cells,[31–33] activation of macrophages by cisplatin may be one of the major mechanisms of action for tumor regression.

Carboplatin, a second generation analog of cisplatin, has been shown to be less toxic but just as effective an anticancer agent, utilizing much higher dosages compared with cisplatin.[34] The prospect of a drug which is capable of enhancing the immune system with less severe toxic side effects is very appealing, thus this investigation of carboplatin as a macrophage activator. In order to understand the mechanism of action of cisplatin and carboplatin, the role of calcium in macrophage activation was evaluated.

Materials and methods

Cell cultures

Swiss Webster mice (Charles River Laboratories, MA) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection

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of 5 ml chilled minimal essential medium (MEM; Gibco, NY) without serum containing penicillin G sodium (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 into modified 35 mm petri dishes made by boring a hole (10 mm) in the middle and gluing a clean coverglass with silicon glue (Dow, OH) underneath. After 2 h of incubation at 37°C, cultures were washed vigorously to remove non-adherent cells, leaving only macrophages. These cultures were washed three times and incubated in fresh culture medium with 10% heat inactivated fetal calf serum (Gibco) for 3–5 days in a 5% CO_2 incubator resulting in a well spread macrophage monolayer at a density of $2–4 \times 10^6$ cells/ml. Sarcoma-180 ascites (S180; CCRFS-180II; American Type Culture Collection, MD) were maintained by serial transplantation in Swiss Webster mice. Cells were obtained by sacrificing mice whenever needed. Such cells were washed with Hank's balanced salt solution (HBSS; Gibco). After centrifugation (1000 *g*, 5 min) cells were resuspended in MEM at a concentration of 3×10^5 cells/ml of the medium. These cells served as target cells for macrophages. Normal hepatocytes were obtained by mincing a small piece of the liver through a fine wire mesh, 105 × 105 μm in size (Tetko, Inc., IL), and mouse 3T3 fibroblasts (American Type Culture Collection) were maintained in culture for a short time as target cells for the macrophages. An effector:target cell ratio of 1:10 was maintained in all experiments.

Cell viability

The viability of various cells after co-incubation with macrophages (both treated and untreated) was monitored by the trypan blue exclusion test.[35]

Macrophage-tumor cell interaction studies

To study macrophage tumor cell interaction, macrophage monolayers were treated with either cisplatin (9 μg/ml) or carboplatin (50 μg/ml) (gifts from Johnson Matthey Research Laboratories and the Chemistry Branch of the National Cancer Institute) for 2 h at 37°C. The medium was then replaced by normal medium and target cells were added. These cultures of macrophages and target cells were co-incubated for 10, 20 and 30 min, and 1, 2, 24 and 48 h. S180 cells and normal cells (hepatocytes or fibroblasts) were also treated with cisplatin (9 μg/ml) or carboplatin (50 μg/ml) for 2 h at 37°C and then presented to the untreated macrophages for varying time intervals as stated above. Coverslips seeded with macrophages and target cells were fixed with 1.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) at room temperature for 30 min. Samples were dehydrated in a graded series of ethanol, critical point dried, sputter coated with gold (thickness ~200 Å; vacuum $\sim 1 \times 10^{-1}$ Torr) using the Emscope sputter coater and viewed under the JEOL JSM 35CF scanning electron microscope operated at 15 kV. Morphological criteria used for the defining lymphocyte-mediated death of a target cell have already been established.[36,37]

Lysosomal studies

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.[7] After careful washing, these cultures were exposed to unlabelled tumor cells. As controls, tumor cells were incubated for 1 h in medium from which labelled macrophages were cultured for 1 h. These macrophage–tumor cell cultures were examined under the ACAS 570 Interactive Laser Cytometer (Meridian Instruments, MI) with confocal optics after 5, 10, 30 and 60 min intervals. Results were analyzed using the ACAS 570 Workstation.

Cytotoxicity assay

Macrophage mediated cytotoxicity was assessed by measuring the release of radioactivity as described previously.[38] Target cells (S180) were incubated in medium with 1 μCi/ml [^{3}H]thymidine (ICN Biomedicals, CA). After 24 h of incubation at 37°C, cells were washed three times with HBSS, and co-incubated with untreated and treated macrophages for 24 h in 96-well plates. Aliquots of the supernatants were taken from these cultures and counted for radioactivity using a Packard Scintillation Counter. Percent cytotoxicity was calculated using the following formula:

$$\text{percent cytotoxicity} = 100 \times \frac{\text{c.p.m. of labelled target cells cultured with macrophages} - \text{spontaneous c.p.m.}}{\text{c.p.m. of labelled cells incubated in 0.1 M NaOH} - \text{spontaneous c.p.m.}}$$

Spontaneous release was less than 10% of total release (cells incubated in NaOH).

Calcium studies

Macrophage monolayers were washed three times with HBSS for 5 min at room temperature. Fluo-3AM[39] and pluronic acid F-127[40,41] (Molecular Probes, OR) were added at a final concentration of 4 μM each for 45 min at 37°C. This was achieved by mixing 1:1 fluo-3AM (0.002 g/ml DMSO) and pluronic acid F-127 (0.0758 g/ml dH_2O), then adding 8 μl of this stock solution to 1 ml of HBSS. After loading, cells were washed thoroughly and examined under the ACAS 470 Interactive Laser Cytometer (Meridian Instruments, MI) in arbitrary units and percent differences were computed. Such cells already loaded with the fluo-3AM and pluronic acid were exposed to cisplatin (9 μg/ml) or carboplatin (50 μg/ml). This calcium indicator is non-fluorescent until hydrolyzed by non-specific esterases within the cell.[42] The excitation wavelength used was 488 nm and the emission wavelength after cytosolic calcium binding was 515 nm.[43] Fluorescent measurements were made before and after drug treatments (preferably on the same cells) for up to 2 h at 5 min intervals. A 10% neutral density filter was used to prevent excessive photobleaching which may occur during repeated and lengthy experiments. A discontinuous strategy was applied to lengthy experiments deterring the effects of any photobleaching. A 100× oil immersion lens was used for all experiments. All data was analyzed using the ACAS 570 Workstation. Unlabelled macrophages were analyzed to check for autofluorescence.

Membrane bound calcium was investigated by fixing macrophages (both control and treated macrophages) in 1% glutaraldehyde and 1% potassium pyroantimonate for 4 h at room temperature. Controls included fixing macrophages in 1% glutaraldehyde with EGTA (2.5 mM) 1 h before being treated with 1% potassium pyroantimonate. All cells were osmicated in 1% osmium tetroxide for 1 h before being processed for electron microscopy. Thin sections (700 Å) stained with uranyl acetate, lead citrate were viewed under the Philips CM-10 transmission electron microscope operated at 80 kV. Calcium pyroantimonate granules associated with the membrane were counted and subjected to statistical analysis using the Student's *t*-test (two-tailed).[44]

To evaluate the effect(s) of calcium channel blockers and calmodulin antagonist on macrophage extension formation, macrophage monolayers were treated with cisplatin (9 μg/ml), carboplatin (50 μg/ml), verapamil (10^{-5} M), nifedipine (10^{-5} M), chlorpromazine (10^{-5} M), cisplatin plus verapamil, nifedipine or chlorpromazine, or carboplatin plus verapamil, nifedipine or chlorpromazine for 2 h. Cells were washed three times and transferred to normal medium. Control cultures received the vehicle only. S180 cells were added after the various treatments. Cells were co-incubated for 4 days while monitoring the macrophages for cytoplasmic extensions and target cell cytolysis at regular intervals of 10 min up to 6 h and then 8 h intervals for up to 4 days. Cisplatin or carboplatin treated macrophages (2 h) were left in medium containing verapamil for up to 4 days while monitoring extension formation. In order to study the effect of low or high calcium in the incubation medium, cisplatin or carboplatin treated macrophages (2 h) were incubated in medium containing either EDTA at a concentration of 2.5 mM or $CaCl_2$ at a concentration of 4 mM and monitored for extension formation through 4 days. In addition, $CaCl_2$ and verapamil treated macrophages (with or without cisplatin or carboplatin treatment) were co-incubated with S180 cells for 0 and 30 min, and 1, 2, 6, 12, 24 and 48 h intervals, and processed for scanning electron microscopy. These macrophages were also stained with acridine orange for lysosomes and examined under the ACAS 570 as described earlier.

Results

Macrophage–target cell interaction

Normal murine peritoneal macrophages (Figure 1A), when treated with cisplatin (9 μg/ml) or carboplatin (50 μg/ml) for 2 h at 37°C in culture, developed cytoplasmic extensions within 10 min (Figure 1B). Untreated macrophages, when cultured with tumor cells, did not show any evidence of extensions or contact formation for up to 24 h (Figure 1C). Carboplatin treated macrophages with long extensions developed contacts and cytoplasmic continuity with the tumor cells in the form of bridges within 30 min of co-incubation (Figure 1D). Often a macrophage developed contact with many tumor cells. Within 1–2 h of co-incubation, cytoplasmic extensions were observed to shorten pulling the tumor cells towards the macrophages

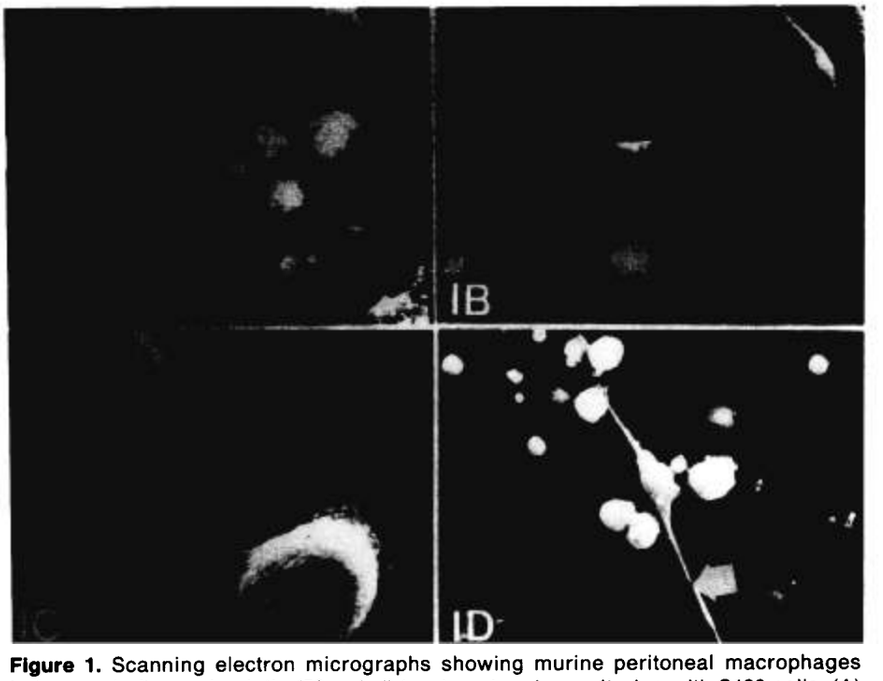

Figure 1. Scanning electron micrographs showing murine peritoneal macrophages before and after carboplatin (50 μg/ml) treatment and co-culturing with S180 cells. (A) Normal macrophages showing no cytoplasmic extensions. (B) Macrophages after carboplatin treatment (2 h). Note the long cytoplasmic extensions (arrow). (C) Untreated macrophage (arrow) and S180 cell after 24 h of co-incubation. Note the absence of contact between the two cells. (D) Carboplatin treated macrophage co-incubated with S180 cells for 30 min. Note the presence of long cytoplasmic extensions (arrow) in contact with S180 cells. Final mag. ×700 (A, B, D); ×1300 (C).

(Figure 2A). After 6–48 h of co-incubation, lysis of tumor cells occurred with eventual phagocytosis (Figure 2B). Cisplatin or carboplatin treated macrophages seemed to discriminate between normal cells (hepatocytes and fibroblasts) and tumor cells, as no extensions or contacts were established with the hepatocytes or fibroblasts (Figure 3A and B). However, the treated macrophages developed close contact with the tumor cells (Figure 3C) and excluding normal cells (hepatocytes) (Figure 3D). Untreated macrophages, when co-cultured with tumor cells that had been exposed to either cisplatin (9 μg/ml) or carboplatin (50 μg/ml) for 2 h, developed extensions. Within 1–2 h these macrophage extensions established contact with the tumor cells in a similar manner to the macrophages that had been directly stimulated by cisplatin or carboplatin. When exposed to

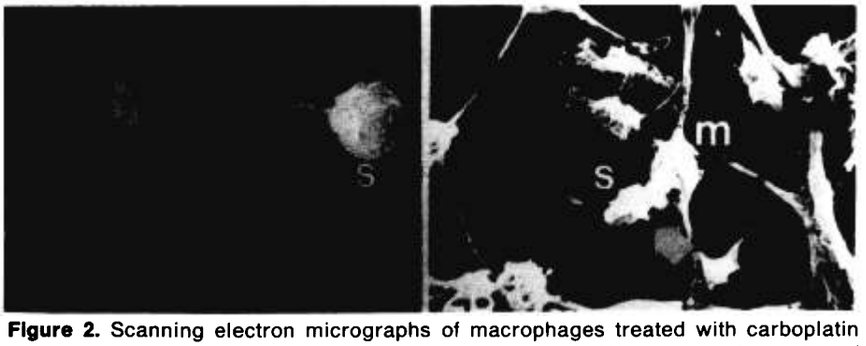

Figure 2. Scanning electron micrographs of macrophages treated with carboplatin (50 μg/ml) (m) for 2 h and co-incubated with S180 cell (s). (A) Note the shortening of the cytoplasmic extensions after 1 h of co-incubation. (B) Lysis of S180 cell (arrow) after 6 h of co-incubation. Final mag. ×1300 (A); ×700 (B).

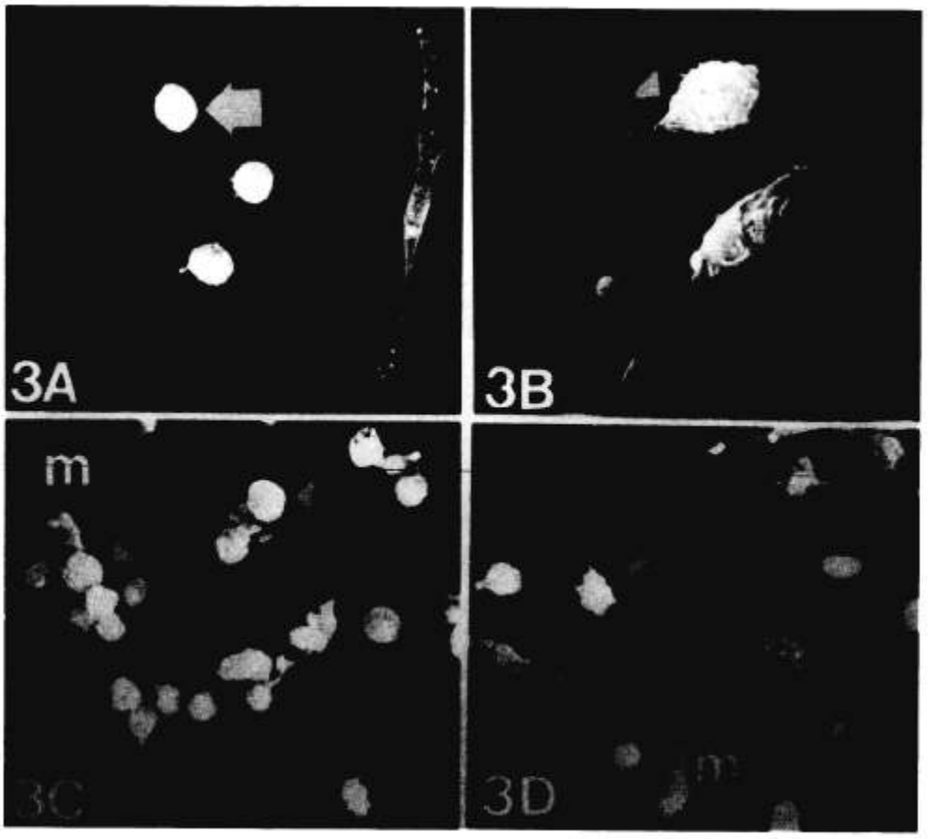

Figure 3. Scanning electron micrographs of macrophages treated with carboplatin (50 μg/ml) for 2 h and co-incubated for 24 h with: (A) normal hepatocytes (arrow); (B) fibroblasts (arrowhead). Note the absence of contact formation. (C) Note the association of tumor cells (arrowheads) and the treated macrophages (m) in comparison with (D) macrophages (m) incubated with normal hepatocytes (arrows) showing no contact formation. Final mag. ×720 (A); ×1300 (B); ×480 (C, D).

hepatocytes or fibroblasts and tumor cells simultaneously, cisplatin or carboplatin treated macrophages established contact only with the tumor cells.

Lysosomal studies

Based on fluorescence measurements after acridine orange labelling, we observed an average increase of about 59% in the number of lysosomes in the macrophages after 2 h of cisplatin treatment while there was an average increase of 83% after 2 h of carboplatin treatment (Figure 4).

There was a gradual transfer of lysosomes from the macrophages into the tumor cells over a 60 min co-incubation period (Figure 5A–H). Before the co-incubation S180 cells had very few fluorescently labelled lysosomes. However, this number increased over a 60 min period. There was no leakage of acridine orange from the labelled macrophages as incubating unlabelled tumor cells in culture medium from acridine orange labelled macrophage cultures of at least 1 h did not show any uptake of acridine orange. Thus, any increase in fluorescence (lysosomes) of the tumor cells could only have come through a direct transfer from the macrophages. When macrophages were co-cultured with S180 cells a close interaction between the macrophages and the tumor cells was observed through their apposing plasma membranes (Figure 6).

Lysis of tumor cells mediated by treated macrophages was further studied by the radioactive release assay (Table 1). When cisplatin or carboplatin treated macrophages were incubated with [^{3}H]thymidine labelled tumor cells, lysis of the tumor cells was above 50% over a period of 24 h. Interestingly, when macrophages were incubated in high calcium (4 mM) for up to 4 days, lysis did not differ significantly from controls.

Figure 4. Fluorescent images taken from the ACAS 570 of macrophages labelled with acridine orange (5 μg/ml) showing cytoplasmic fluorescence (green) and lysosomal fluorescence (red) in the untreated (A and C) and carboplatin treated (2 h) macrophages (B and D). Note the increase in lyso-somal fluorescence after carboplatin treatment (D). Bar = 5 μm.

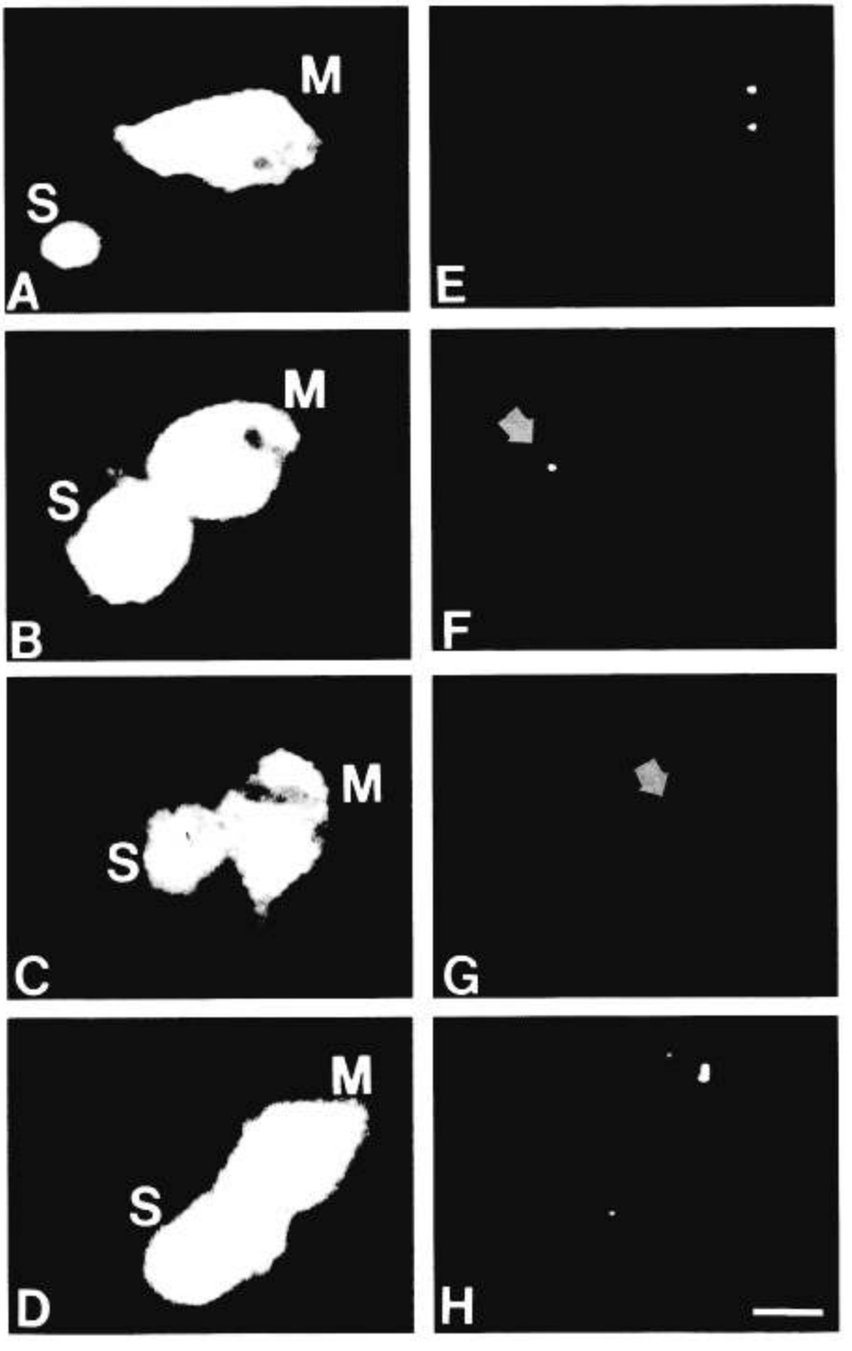

Figure 5. Fluorescent images taken from the ACAS 570 of macrophages (m) treated with carboplatin (50 μg/ml) for 2 h and labelled with acridine orange (5 μg/ml) after co-incubation with S180 cells (s) for 5, 30, 60 and 120 min. Panels (A)–(D) represent images as seen with the green detector (cytoplasmic fluorescence) and corresponding images (E–H) as seen with the red detector (lysosomal fluorescence). Note the absence of red fluorescence in the S180 cell at 5 min of co-incubation (E). There is a gradual increase in the number of lysosomes in the S180 cells after 30 (F), 60 (G) and 120 (H) min of co-incubation. Arrows point to the lysosomes probably in the process of transfer from the macrophages to the tumor cells. Bar = 5 μm.

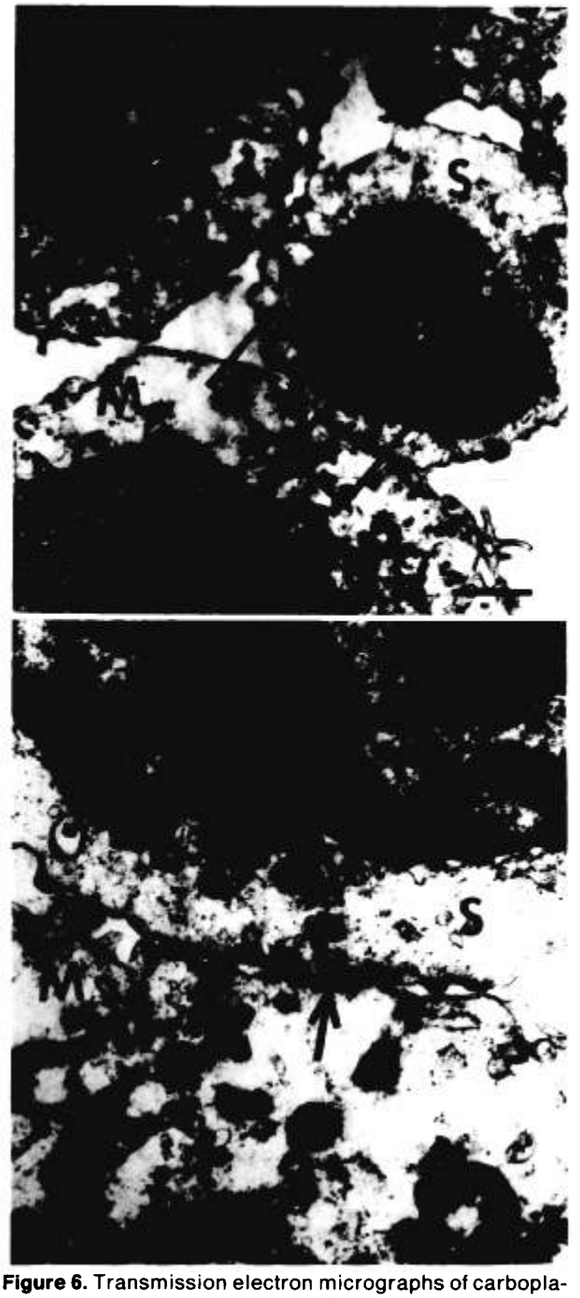

Figure 6. Transmission electron micrographs of carboplatin treated macrophage (m) and S180 cell (s) interaction. (A) Note the close association between the two cells at low magnification. (B) Higher magnification of enclosed area shown in (A). Note that the plasma membrane of the two cells are closely apposed (arrows). Bar = 1.5 μm (A); 1 μm (B).

However, cisplatin or carboplatin treatment of such cells induced immediate lysis. When macrophages were incubated in EDTA (2.5 mM), lysis of tumor cells did not differ from controls even when exposed to cisplatin or carboplatin.

Calcium studies

Using fluo-3AM as the probe, we observed a sudden increase in the intracellular calcium levels within 5 min of exposure to carboplatin by the macrophages (Figure 7). This increase gradually returned to near baseline levels after 60 min and remained so for up to 120 min when observations ceased. However, cisplatin treated macrophages demonstrated a decline in intracellular calcium levels, reaching about 20% of the normal levels after 60 min with a steady increasing trend thereafter. These intracellular calcium levels were only 25% of the normal levels by 120 min when observations ceased. Untreated macrophages did not show any significant fluctuations in intracellular calcium levels. Loss of fluorescence due to photobleaching was less than 10% and autofluorescence was not observed.

Using the potassium pyroantimonate technique to measure membrane bound calcium under the electron microscope we observed a decrease in macrophage membrane bound calcium after cisplatin (9 μg/ml) or carboplatin (50 μg/ml) treatment of 2 h compared with untreated macrophages (Figure 8). Calcium pyroantimonate granule counts on the membranes of normal macrophages were 14.8 ± 2.8 per cell versus 3.7 ± 1.64 and 4.8 ± 1.93 for cisplatin and carboplatin treated cells, respectively. This was found to be statistically significant compared with controls ($p < 0.05$). No membrane bound calcium antimonate granules were observed in macrophages incubated in EGTA (2.5 mM). The typical morphology of normal macrophages is depicted in Figure 8(A). The mitochondria appear dense; normal Golgi and rough surfaced endoplasmic reticulum were seen. However, after cisplatin treatment mitochondria appeared swollen, and the endoplasmic reticulum and Golgi membranes were bloated with prominent vesiculation from the outer nuclear envelope (Figure 8B). Similarly, carboplatin induced swelling of the endoplasmic reticulum and the vesiculation from the outer nuclear membrane; however, the mitochondria did not show marked swelling (Figure 8C).

When cultured in normal culture medium, normal macrophages did not show any significant extension formations for up to 4 days (Figure 9A). When treated with cisplatin (9 μg/ml) or carboplatin (50 μg/ml) for 2 h and placed in normal medium macrophages developed prominent extensions (Figure 9B). Macrophages treated with verapamil for 2 h and placed in normal medium demonstrated no

Table 1. Macrophage mediated S180 tumor cell lysis using [^{3}H]thymidine release assay

Treatment (24 h)	Normal medium	Cisplatin (9 μg/ml)	Carboplatin (50 μg/ml)
Normal medium	13.04 ± 2.29	53.8 ± 0.78*	57.6 ± 4.98*
Calcium chloride (4 mM)	11.54 ± 0.66	51.3 ± 0.35*	53.6 ± 0.96*
EDTA (2.5 mM)	13.83 ± 4.4	9.2 ± 0.56	10.2 ± 1.3

Numbers depict percentage Cytotoxicity ± SD. Results are averages of experiments done in triplicate. The asterisks represents data which differ significantly from controls as calculated by the Student's *t*-test (two-tailed) with $p < 0.01$.

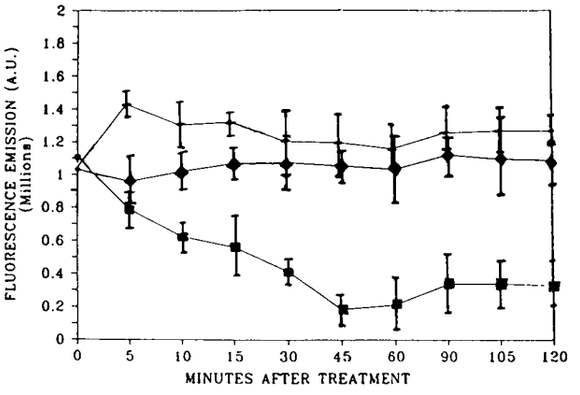

Figure 7. Graph showing the effects of cisplatin (9 μg/ml) and carboplatin (50 μg/ml) treatment on intracellular levels of calcium in macrophages as measured with the dye fluo-3AM (4 μM) using the ACAS 470 Interactive Laser Cytometer. Results are the mean of 12 cells per treatment expressed as fluorescence intensities in arbitrary units. There is an abrupt 40% increase after carboplatin treatment (+) within 5 min, reaching normal levels after 60 min. In cisplatin treated cells (■) there is a steady decline through 60 min after treatment with an upward trend thereafter. Normal cells did not show any significant changes (◆).

extensions for up to 2 days. However, such cells demonstrated extensive extension formation soon after 4 days (Figure 9C). Macrophages treated with cisplatin or carboplatin plus verapamil (2 h) also did not demonstrate extension formation for up to 2 days, but did develop extensions after 2 days (Figure 9D). Results after verapamil treatment could also be duplicated with other calcium channel blockers, such as nifedipine or the calmodulin antagonist chlorpromazine, at similar concentrations. Cisplatin or carboplatin treated (2 h) macrophages, when left in MEM plus EDTA (a calcium chelator at a concentration of 2.5 mM), showed that extension formation was completely inhibited for up to 4 days when the observations ceased. When macrophages were exposed to medium containing $CaCl_2$ (4 mM) these did develop extensions but only after 2 days of exposure. When $CaCl_2$ and verapamil treated macrophages were stained with acridine orange for lysosomes, no apparent increase in the lysosomes were seen, though extension formation was observed. However, when these macrophages were treated with either cisplatin or carboplatin, an increase in the lysosomes and their transfer to S180 cells was observed. Interestingly, EDTA treated macrophages, even when treated with cisplatin or carboplatin, did not show any lysosomal increase and transfer to S180 cells.

Discussion

Macrophages have been shown to mediate cell cytotoxic mechanisms in the destruction of tumor cells.[16,19–25,45] Macrophages are most often first activated with a biological response modifier or activator. Cisplatin is able to activate peritoneal macrophages[15] *in vitro* and cause lysis of the tumor cells. Carboplatin, a second generation analog of cisplatin, also has this capability to activate macrophages *in vitro*. This was demonstrated by contact formations established with tumor cells in much less time than when untreated macrophages were co-incubated with tumor cells. Extensions develop after cisplatin or carboplatin treatment even in the absence of tumor cells, which suggests that their presence is not a prerequisite for the development of such extensions. However, the attraction of activated macrophages seems to be specific for tumor cells only, as no contact formation was seen when normal cells (hepatocytes or fibroblasts) were co-incubated with untreated or treated macrophages. Discrimination between non-neoplastic and neoplastic cells is typical of activated macrophages.[13,15,23,45]

In the study of macrophage activation, it has been suggested that lysosomes take part in the

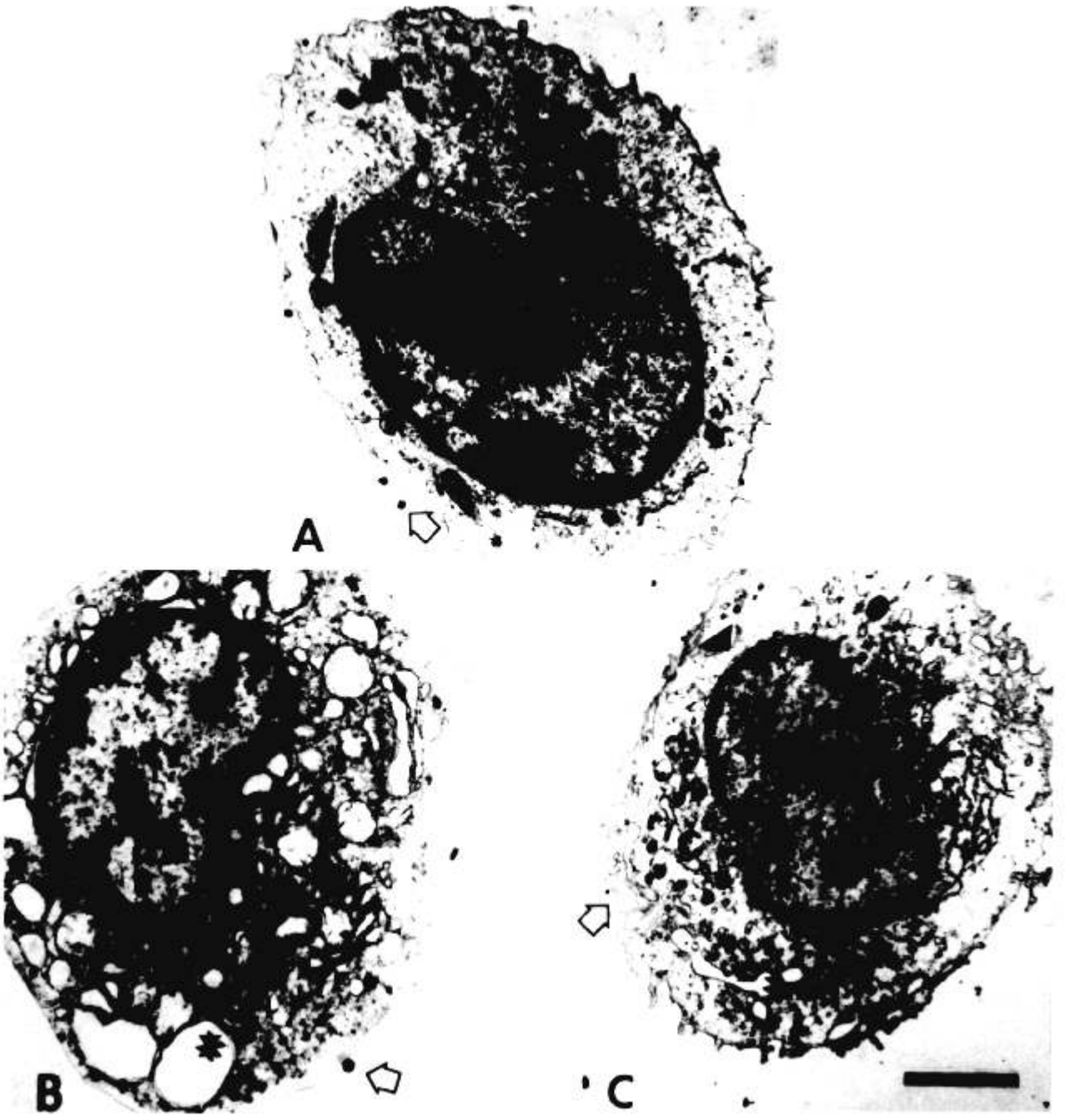

Figure 8. Electron micrographs showing the distribution pattern of calcium pyroantimonate granules on the plasma membrane of (A) untreated macrophage, (B) cisplatin treated (9 μg/ml) macrophage after 2 h and (C) carboplatin treated (50 μg/ml) macrophage after 2 h. Note the decreased number of granules (arrows) in treated macrophages as compared with the controls. The bloating of the mitochondria (>), the swelling of the nuclear membrane (▶) and endoplasmic reticulum (*) are prominent in the cisplatin treated macrophage. Some of the mitochondria, although swollen, the morphology of the nuclear membrane and the endoplasmic reticulum seem closer to normal after carboplatin. Bar = 2 μm.

process of tumor cell death.[46,47] In cisplatin activated macrophages, an increase in the number and their transfer to the tumor cells via the extensions have been documented.[15] Tumor cell lysis has been proven to be due to these transferred lysosomes.[15] Carboplatin also induces a similar increase of lysosomes in the macrophages that are transferred to the tumor cells through the extensions after contact has been established. Activation of macrophages seems to be a multi-step

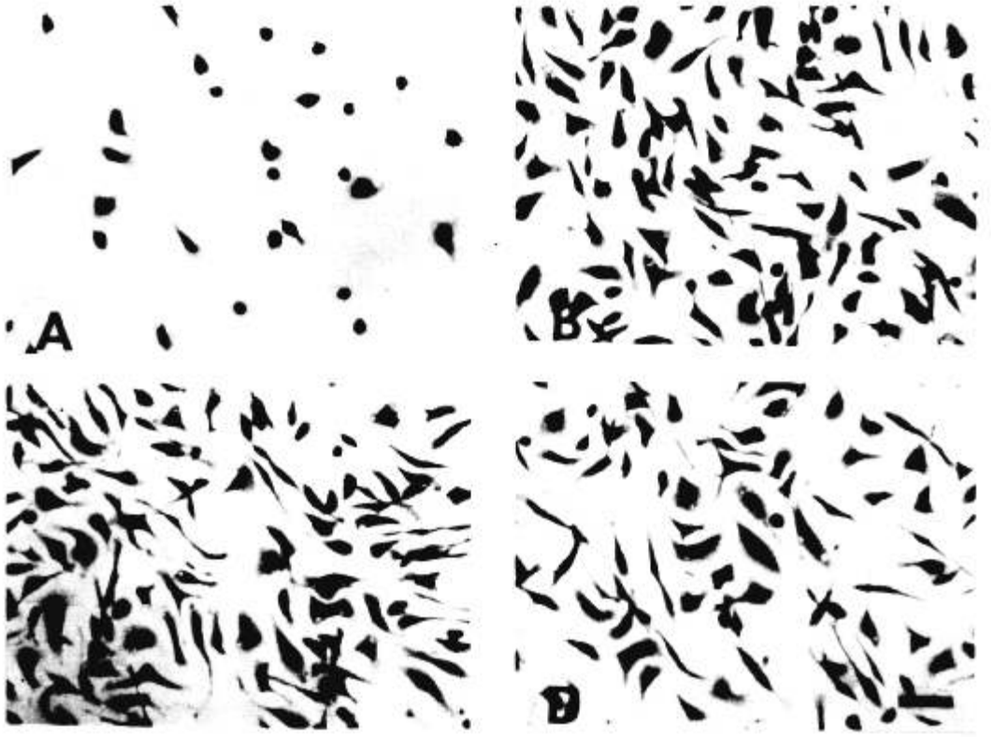

Figure 9. Light micrographs showing macrophages at 4 days in normal medium after (A) no treatment, (B) cisplatin (9 μg/ml) for 2 h, (C) verapamil (10^{-5} M) for 2 h and (D) cisplatin (9 μg/ml) plus verapamil (10^{-5} M) for 2 h. Note the extension formation after cisplatin, verapamil or cisplatin plus verapamil treatments. Bar = 30 μm.

process. There is the development of cytoplasmic extensions, an increase in the lysosomes, recognition of tumor cells leading to contact formation, transfer of the lysosomes from the macrophage to the tumor cells through these extensions and, finally, tumor cell lysis leading to tumor cell death.

Calcium has been demonstrated to have an important role in macrophage activation.[15,48] The use of calcium channel blockers or calmodulin antagonists inhibits extension formation in cisplatin or carboplatin treated macrophages for 2 days; however, extensions do develop soon after. $CaCl_2$ (4 mM) by itself is able to induce extension formation in the macrophages while 2.5 mM EDTA inhibits such extension formations even when used with cisplatin or carboplatin. These results seem to indicate a role for calcium in macrophage activation, specifically in extension formation. There does not seem to be any relationship between intracellular calcium and extension formation as both drugs induce extension formation in macrophages and diagonally opposite changes in the intracellular calcium levels. Macrophages incubated in $CaCl_2$ or EDTA seem to suggest that extension formation may be specifically regulated by calcium. When $CaCl_2$ incubated macrophages are co-cultured with tumor cells, no contact formation occurs nor can any lysosomal increase or their transfer to the tumor cells be demonstrated. EDTA inhibits all extension formations. Further, $CaCl_2$ incubated macrophages, when treated with either cisplatin or carboplatin, not only establish contact formation with the tumor cells, but also cause an increase in the lysosomes and their transfer to the tumor cells. EDTA inhibits all extension formation, lysosomal increase and transfer in the cisplatin or carboplatin treated macrophages. Calcium therefore seems only to be regulating the process of extension formation. It may be possible that calcium acts as a priming signal for macrophage activation[49,50] by cisplatin or carboplatin. Both cisplatin and carboplatin induce depletion of membrane bound calcium in macrophages to a different degree; however, both induce activation of the macrophages and their involvement in the process is not clear.

Conclusion

Cisplatin and carboplatin induce murine macrophage activation resulting in the expedition of tumor cell recognition through the development of cytoplasmic extensions which only establish contact with tumor cells. An increase in lysosomal content in the activated macrophages and the eventual transfer of these lysosomes to tumor cells via the extensions seem to be responsible for tumor cell death. Although calcium is involved in the formation of extensions, our studies seem to demonstrate that intracellular calcium may not play a major role in the macrophage activation process. It is possible that calcium acts as a priming signal and that actin may be affected. Our study suggests the enhancement of the immune system through the activation of macrophages as one of the probable mechanism(s) of action of the antitumor agents cisplatin and carboplatin.

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