Enhanced Activity of Peritoneal Cells after Aclacinomycin Injection: Effect of Pretreatment with Superoxide Dismutase on Aclacinomycin-induced Cytological Alterations and Antitumoral Activity


ABSTRACT

Peritoneal macrophages from mice injected with aclacinomycin (ACM) (4 mg/kg, i.p.) showed increased functional activity, as assessed by increased antitumoral activity in vitro and in vivo and zymosan-triggered chemoluminescence. They also showed ultrastructural signs of activation (increased number of cytoplasmic organelles), and atypical alterations (giant vacuoles and giant lysosomes containing heterogeneous myeloid bodies, lipofuscin-like substance, cytoplasmic debris, and a fine granular material). As these atypical alterations could be due to the generation of superoxide following ACM injection, superoxide dismutase (SOD) was injected 1 h prior to ACM administration. Neither the morphological characteristics of activation, nor the enhanced metabolic and antitumoral activities induced by ACM were affected by SOD pretreatment, but the atypical alterations were inhibited in a dose-dependent manner. Heat-inactivated SOD did not prevent their appearance. The atypical alterations were not found in peritoneal macrophages from talc or lipopolysaccharide-injected mice, and were also prevented by SOD pretreatment, indicating that the alterations are due to anthracycline treatment. Finally, 185SOD was phagocytized by peritoneal macrophages in vitro and in vivo and not by L1210 tumoral cells, explaining why the atypical alterations induced by ACM were no longer seen after SOD pretreatment. The unchanged direct oncostatic activity of ACM following SOD pretreatment suggests that this combination may have some wider perhaps clinical, potential.

INTRODUCTION

ACM2 is an oncostatic of the ATC family which possesses a marked activity against a wide variety of human and murine tumors (1, 2).

We have previously reported that 4 days after i.p. injection of ACM to mice, although the drug was not detectable in peritoneal cells the activity of the peritoneal macrophages was enhanced. We observed an increase of the oxygen-free radical production as measured by chemoluminescence or by $O_2^-$-spe

On the other hand, in preliminary electron microscopy studies, we have observed that the i.p. administration of ACM to mice induced ultrastructural features of functional activation (increase of cytoplasmic organelles) in macrophages (9), and also atypical formation of giant lysosomes and giant vacuoles which may reflect drug-induced deleterious effects.

The toxic effects of the oxygen-free radicals on macrophages after ATC administration are not known. However, macrophages are able to store ATC (10-12), and on the other hand, in physiological conditions, they secrete high amounts of oxygen-free radicals in response to stimulus. However, this secretion of oxygen-free radicals during phagocytosis is controlled and does not appear to be toxic for these cells in normal conditions (13).

The superoxide anion thus plays an important role in both situations, as it is the first radical formed either in heart injury after ATC administration or in the macrophages after phagocytic stimulus (5, 6, 14). The enzyme responsible for the detoxification of superoxide in the cells is the superoxide dismutase (SOD) that transforms the $O_2^-$ into $H_2O_2$ (15).

This is why it appeared interesting to study whether treatment with SOD before ACM administration to mice could modify drug-induced morphological alterations and functional activities of peritoneal macrophages and whether SOD affects the antitumoral activity of ACM.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57Bl/6 (B6) and (C57Bl/6 × DBA/2) F1 (B6D2F1) 6-week-old male mice were obtained from the breeding center of the Institut de Recherches Scientifiques sur le Cancer (IRSC, Villejuif, France). They were used within 2 weeks of delivery.

Drugs

Aclacinomycin and Adriamycin were kindly supplied by Laboratoires Roger Bellon (Neuilly-sur-Seine, France). The drugs were dissolved (1 mg/ml) in 0.9% NaCl. The solutions were stored at $-20^\circ$C and used within 8 days. The drug doses in all experiments were 4 mg/kg i.p. for ACM and 5 mg/kg i.p. for ADM.

Superoxide dismutase was obtained from Sigma Chemical, St. Louis, MO (S-8524) and dissolved in Hanks’ balanced salt solution without phenol red (pH 7.2) at a concentration of 500 U/ml, stored at $-20^\circ$C, and used within 8 days. The doses used in all in vivo experiments (7, 25, 50, or 100 units/mouse) were injected i.p. in a standard volume of 0.2 ml 1 h before ACM, ADM, talc, or LPS injection. Talc (2 mg/mouse) or LPS (Escherichia coli 055:B5 Difco) (5 μg/mouse) were injected i.p.

Culture Reagents

RPMI 1640 tissue culture medium (GIBCO) was supplemented with 10% fetal calf serum (GIBCO, endotoxin (0.22 ng/ml), batch IFO42595), 1% nonessential amino acids (GIBCO), 1% sodium pyruvate (GIBCO), and antibiotics. This medium is referred to as RPMI-S.
Preparation of Peritoneal Cells

Mice were sacrificed by cervical dislocation and peritoneal cells obtained by washing with 6 ml of cold Hanks' balanced salt solution without phenol red (pH 7.2). Smears were prepared by cytocentrifugation and stained with Giemsa and nonspecific esterase staining for differential counts; live cells were counted by trypan blue dye exclusion.

In some experiments, the macrophages were purified by plastic adherence. Cells were identified by Giemsa and nonspecific esterase staining, counted, and adjusted to the desired concentration. The peritoneal cell suspension was placed in 96-well plastic tissue culture plates (Nunc) (0.2 ml/well) and macrophages were allowed to adhere for 2 h at 37°C in an atmosphere of 5% CO₂. The medium and nonadhering cells were aspirated off and the plates washed three times with RPMI-S. 78% of the adherent cells had the morphology and the staining properties of macrophages.

Tumor Target Cells

Murine L1210 leukemic cells and mastocytoma P815 tumor cells were maintained by weekly in vivo passages of 2 x 10⁶ cells in syngeneic mice. Radiolabeled P815 cells were prepared by incubating 5 x 10⁵ cells in 0.1 ml with 0.1 mCi ⁵¹Cr as sodium chromate (Amersham) for 60 min at 37°C. The cell suspension was washed three times with RPMI 1640 medium and diluted to 2 x 10⁴ cells/ml in RPMI-S medium.

Electron Microscopy

Peritoneal macrophages were obtained by washing the peritoneal cavity. They were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4) for 1 h at 4°C, postfixed in 1% osmium tetroxide and embedded in Epon-Embedlite. Semithin sections were stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate, and observed with a Joel 100C transmission electron microscope. In each case, a total of 200 cells from eight mice were examined by groups of 40 cells taken on five sections.

Measurement of Macrophage Chemoluminescence

For each test 10⁶ peritoneal cells were suspended in 1 ml of phenol red-free Hanks' balanced salt solution. These populations contained between 25 and 30% macrophages and 3 to 5% neutrophils. Chemoluminescence elicited with zymosan (final concentration of 5 µg/ml) with a LKB 1250 luminometer during 60 min at 37°C as has been described (16). Groups consisted of 10–15 mice each, with each mouse measured individually.

The chemoluminescence-emission curves were traced and their areas calculated. The results are reported as a percentage of the untreated control group emission on the corresponding day.

Growth Inhibition

The assay used was slightly modified from that described previously (17). Peritoneal cells from six mice were pooled and seeded in quadruplicate at various concentrations in 96-well tissue culture plates. Nonadhering cells were removed and the macrophage monolayers were overlaid with 2 x 10⁴ P815 cells in 0.2 ml of RPMI-S to give effector/target (E:T) cell ratios of 40:1, 20:1, 10:1. The cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C for 48 h. Tumor cell DNA synthesis was measured by adding 1 µCi of [³H]dThd (Amersham) to each well 8 h before harvesting the cells. The percentage of P815 growth inhibition due to the presence of macrophages was calculated by comparison with P815 cells cultured alone. Each experiment was performed three times.

In Vitro Cytotoxicity

Macrophage monolayers prepared as described above were incubated with 2 x 10⁵ ⁵¹Cr-labeled P815 target cells in 0.2 ml RPMI-S for 18 h at 37°C in humidified atmosphere with 5% CO₂. The plates were centrifuged and 0.1 ml of supernatant was removed from each well and counted in a LKB 1217 gamma-counter (test cpm). Spontaneous release was determined by incubating ⁵¹Cr-labeled P815 target cells alone (spontaneous cpm), and total labeling was determined by counting a 0.1-ml aliquot of target cells (total cpm). The percentage of cytotoxicity for each effector/target cell ratio was calculated from:

\[
\text{Test cpm - spontaneous cpm} \times 100 \\
\text{Total cpm - spontaneous cpm}
\]

Spontaneous release by target cells did not exceed 20% of total cpm. The tests were carried out in triplicate at each effector/target cell ratio, and each experiment was performed three times.

Evaluation of in Vivo Cytotoxicity

The method of Porteous and Munro (18) was used. B6D2F₁ mice were injected i.p. with 10⁶ P815 cells, and, 5 days later, given four injections of 1 µCi [¹²⁵I]dUrd. On Day 7, tumor cells were harvested in Hanks' balanced salt solution containing heparin, centrifuged at 200 x g at 4°C, resuspended, counted with trypan blue (viability 98%), and adjusted to a concentration of 2 x 10⁶ live cells per milliliter. B6D2F₁ mice were injected i.p. with 10³-labeled cells in 0.5 ml. Uptake of [¹²⁵I]dUrd into the thyroid was prevented by including 0.1% potassium iodide in the drinking water, starting 2 days before the injection of the labeled cells. The animal bedding was changed daily to reduce ingestion of sawdust contaminated with radioactive urine.

The remaining radioactivity was measured by placing the mice in an appropriate size tube and counting them in toto in a gamma-counter (Packard) for 1 min. The results are expressed as the percentage of injected radioactivity. 10 mice were used per group.

Direct in Vitro Cytotoxicity of ACM

10⁶ P815 or L1210 cells were suspended in RPMI-S and ACM was added to a final concentration of 0.1, 0.05, or 0.01 µg/ml of tumor cell suspension. SOD was added to half the tubes at a concentration of 25 or 50 U/ml. The suspensions were incubated for 2 h at 37°C in a 5% CO₂ atmosphere, the cells were washed three times with RPMI 1640, and their viability was measured by trypan blue exclusion. 10⁴ viable cells were then plated in flat-bottom 96-well plastic microtiter plates (Nunc) for 24 h. 8 h before the end of the culture 1 µCi of [³H]dThd was added to each well. The cells were then harvested and the incorporated radioactivity determined. Each experiment was performed in triplicate and repeated three times with similar results.

Survival of Mice Injected with Tumor Cells

Groups of mice were injected i.p. with 2 x 10⁶ P815 tumor cells, and 2 h later, with 4 mg/kg ACM i.p. In other groups, 100 U SOD were injected i.p., either alone or 1 h before ACM. As toxicity controls, mice (10 per group) were given ACM, SOD, or SOD-ACM. Survival was recorded. The experiment was performed twice.

Iodination of SOD

1.2 mg of SOD in 120 µl PBS was radiolabeled with 1 mCi Na[¹²⁵I] according to McConahey and Dixon (19) as modified by Marguerie et al. (20). The [¹²⁵I]SOD was diazylated overnight against PBS which was changed twice. [¹²⁵I]SOD concentration was determined according to Bradford (21) after standardization with unlabeled SOD. Specific activities ranged from 4 to 8 x 10⁸ cpm/molecule of [¹²⁵I]SOD. Incorporation of [¹²⁵I]SOD into [¹²⁵I]SOD was checked by electrophoresis on 12.5% polyacrylamide gel according to Laemmli (22) and autoradiography for 2 h at ~30°C on Kodak Ortho-G films.

[¹²⁵I]SOD Internalization

Experiments were performed with enriched populations of peritoneal macrophages harvested 4 days after i.p. injection of 1 ml thioglycolate (Institut Pasteur, Paris, France). More than 90% of the cells were macrophages. The same experiments were repeated with more physiological normal peritoneal cell, containing 30% of macrophages. Each experiment was repeated at least twice with comparable results, and carried out at least in triplicate.

In Vitro Experiments. 9 x 10⁵ L1210 leukemic cells or peritoneal
macrophages from thioglycollate-injected mice were incubated at 37°C in 120 µl of PBS containing 14.3 µg (50 units) of [125I]SOD. Controls consisted of L1210 or peritoneal cells incubated with [125I]SOD and a 10-fold excess of unlabeled SOD.

**In Vivo Experiments.** 50 units of [125I]SOD in 200 µl of PBS were injected i.p. into normal or thioglycollate-injected mice. Controls were normal or thioglycollate-treated mice injected i.p. with cytochalasin B (100 µg/mouse) (Sigma) 30 min before [125I]SOD-injection. Cytochalasin B was selected because it efficiently inhibited [125I]SOD internalization by peritoneal macrophages in our experimental conditions.

At specified times, three aliquots (40 µl) were taken from the cell suspension containing [125I]SOD and cooled to 0°C to block cell membrane movements. A 10-fold excess of unlabeled SOD was added for 15 min to displace cell surface bound [125I]SOD. The remaining bound proteins were then removed by digestion for 15 min at 0°C with an equal volume of a freshly prepared solution of pronase (0.5 mg/ml) (Boehringer, Mannheim), 50 mM EDTA (pH 7.4). The remaining internalized radioactivity was determined according to Levesque et al. (23). Aliquots of proteolysed cell suspension were transferred onto a double-layer gradient containing an upper phase of 200 µl 8% sucrose in PBS and a lower phase of 100 µl dibutyl phthalate/dimethyl phthalate mixture (10.5/1 v/v). After 2 min of centrifugation at 12,000 x g, the tips of the tubes containing the cells were cut off with a razor blade and counted in a LKB gamma counter. [125I]SOD internalization was calculated after subtraction of the background obtained either in the presence of a 10-fold excess of unlabeled SOD (in vitro experiments) or in the presence of cytochalasin B (in vivo experiments).

**Statistics**

The values were compared using Student's t test, except for the in vivo cytotoxicity and mouse survival for which the nonparametric Mann-Whitney test was used. This test was selected because it allows comparisons without taking in account the standard deviation and is also more accurate.

**RESULTS**

**Ultrastructural Studies**

**Normal Peritoneal Macrophages.** Fig. 1 shows the ultrastructural appearance of peritoneal macrophages harvested 1, 4, 8, and 12 days after injection of 0.2 ml saline. Macrophages had indented nuclei with finely distributed chromatin forming small clumps on the inner face of the nuclear membrane and variable nucleoli. Cytoplasm was abundant, contained scattered mitochondria, and displayed variable amounts of rough endoplasmic reticulum in long and short cisternae, medium-size Golgi apparatus, and numerous primary lysosomes; secondary lysosomes were rarely seen.

**ACM-treated Macrophages.** The cells from treated mice showed the large number of organelles characteristic of increased functional activity (Fig. 2), with a well-developed Golgi apparatus, an abundant rough endoplasmic reticulum in long and short cisternae, numerous mitochondria, and secondary lysosomes. There were always more organelles than in control populations. Primary lysosomes were still present. There were also, as defined by standard criteria (24, 25), other ultrastructural alterations: giant vacuoles and atypical giant lysosomes, containing heterogenous material such as myeloid bodies, lipofuscin-like substances, cytoplasmic debris and fine granular material (Figs. 2 and 3). Peritoneal macrophages presenting at least one of these alterations were considered atypical. After ACM treatment 23 ± 5% of the cells presented these features on Day 1 and 11 ± 4% on Day 4. Ultrastructural features of functional activation were less evident 8 days after injection with ACM (Fig. 4). Atypical giant lysosomes were not found in this population, but large secondary lysosomes resembling giant atypical lysosomes were observed in 5 ± 3% of the cells. The ultrastructural characteristics of ACM-treated macrophages on Day 12 were comparable to those of control populations (Table 1).

**Influence of the Route of ACM Administration.** We have previously reported that when ACM is injected i.v. or i.m. (4 mg/kg, Day —4), the peritoneal cells do not show enhanced metabolic or antitumoral activity (3). Similarly, the ultrastructure of peritoneal macrophages from i.v. or i.m. injected mice was similar to that of normal cells (data not shown).

**SOD-ACM-treated Macrophages.** Mice were given 7, 25, or 50 units of SOD i.p., and ACM (4 mg/kg i.p.) 1 h later. The peritoneal macrophages were harvested 4 days later. No giant atypical lysosome and no clear membrane-bound giant vacuoles were observed in 50-unit SOD-treated macrophages (Fig. 5). Atypical giant lysosomes and vacuoles were present in 4 ± 4% of the 25-unit SOD-treated macrophages and in 7 ± 2% of the macrophages given 7 units of SOD. In contrast, the morphological signs of increased functional activity were present in all the SOD-ACM-treated groups. SOD specificity was checked by analysis of macrophages from mice injected with 50 units of heat-inactivated SOD (100°C for 60 min) before ACM administration (4 mg/kg i.p. on Day —4). Their ultrastructure was...
EFFECT OF SOD ON ACTINOMYCYIN-ACTIVATED PERITONEAL CELLS

Since all ATC liberate free oxygen radicals and are cardiotoxic, we examined the morphological alterations induced by ADM which is reported to be more cardiotoxic than ACM (4, 26). Mice were given 5 mg/kg of ADM, a dose which enhanced the antitumoral activity of peritoneal macrophages (27). Fig. 6 shows that, after ADM injection, peritoneal macrophages contained giant atypical lysosomes and giant vacuoles in 26 ± 6% of the cells (Table 1). These alterations were strictly comparable to those observed in ACM-treated macrophages. Pretreatment with 50 units of SOD/mouse similarly inhibited their development; they were present in only 8 ± 5% of the cells, P < 0.01 (Fig. 7).

Cells harvested 24 h after i.p. injection of LPS (5 μg/mouse), a potent macrophage activator, had morphological characteristics of activation but none of the atypical alterations observed in ACM and ADM groups. Pretreatment with SOD (50 units/mouse) did not change their morphology (Fig. 8).

Peritoneal macrophages from mice injected with an inert irritant, talc (2 mg/mouse i.p.), 4 days earlier had a similar cytoplasmic organization and were devoid of atypical alterations found in ATC groups, irrespective of whether the mice were pretreated or not with SOD (Table 1).

These data indicate that the ultrastructural atypical alterations observed in our experimental conditions are due to ATC injection. Since they are inhibited by SOD they must be due to generation of O₂⁻. These observations are supported by the fact that heat-inactivated SOD had no effect and that the atypical changes are not seen in peritoneal macrophages from LPS or talc-injected mice.

Chemoluminescence

The chemoluminescence emitted by stimulated phagocytic cells gives an overall evaluation of oxygen-free radical production. The following experiment was performed to check whether SOD administration, while preventing the development of ultrastructural atypical alterations, also modified the metabolic activation of peritoneal cells.

The chemoluminescence elicited with opsonized zymosan was measured on various days after injection of ACM (4 mg/kg i.p.). Half of the mice were pretreated with SOD (25 or 50 units/mouse i.p.) 1 h before ACM injection. The chemoluminescence response of peritoneal cells from ACM-treated mice was greater than that of the control group with a peak value 24 h after ACM injection. The intensity of the chemoluminescent response dropped progressively on Days 4 and 8 after ACM injection and reached control values by Day 12. Injection of 25 or 50 units of SOD 1 h before ACM administration did not change either the intensity of chemoluminescence or its kinetics. The chemoluminescence on Day 1 and on Day 4 after administration of 50 units of SOD was comparable to that of normal cells, indicating that the enzyme does not modify the response of macrophages to zymosan (Table 2).

In Vitro Inhibition of P815 Tumor Cell Growth

Groups of mice were injected with ACM either alone or 1 h after SOD injection (50 or 25 units i.p.) and, 4 days later, their peritoneal macrophages were assayed for cytostatic activity on P815 tumor cells. ACM-treated macrophages inhibited tumor cell proliferation more efficiently than did normal macrophages at all the E/T cell ratios studied. No difference was observed between ACM and SOD-ACM treated cells. SOD injection did not prevent the enhancement of the cytostatic activity induced by ACM. The cells from SOD-treated mice had the same activity as normal cells (Fig. 9).

Fig. 3. Peritoneal macrophages from mice either 1 or 4 days after ACM injection (4 mg/kg i.p.). Note giant lysosomes (GL) containing (upper) granular electron dense material and cytoplasmic debris and (lower) myelinoid bodies and lipofuscin-like material (EM 6750X).

Fig. 4. Peritoneal macrophages from mice injected with ACM 8 days earlier (4 mg/kg i.p.). Note that secondary lysosomes (arrows) and Golgi areas (g) are less numerous than in Fig. 2. Large secondary lysosomes (LSL) resembling atypical giant lysosomes (EM 2700X).

Comparable to that of macrophages from mice injected with ACM alone; atypical alterations were seen in 10 ± 3% of the cells (Table 1).

Ultrastructure of Peritoneal Macrophages from Mice Treated with ADM, LPS, or Talc. The specificity of the observed atypical alterations was assessed by comparing the effects of various agents on the ultrastructure of peritoneal macrophages.
Table 1 Percentage of peritoneal macrophages with atypical giant lysosomes (containing myelinoid bodies, lipofuscin-like substance, cytoplasmic debris, fine granular material) and/or giant vacuoles after treatment

<table>
<thead>
<tr>
<th>Days after treatment and method of administration</th>
<th>ACM (4 mg/kg) SOD</th>
<th>ADM (5 mg/kg) SOD</th>
<th>LPS (5 µg/mouse) SOD</th>
<th>TALC (2 mg/mouse) SOD</th>
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<td>4</td>
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* Treatment 30 min after SOD-injection i.p.
* SOD pretreatment (i.p. units/mouse).
* Heat-inactivated SOD (100°C/60 min).

In each case five sections of 40 cells were examined under electron microscopy. The results are expressed as the mean ± standard deviation of the percent of altered cells. For ACM group ± SOD (on Day —4) the linear regression was calculated, the coefficient of correlation of which was 0.97. Student’s t test between ADM and SOD-ADM P < 0.01 and between ACM and ADM (on Day —4) P < 0.05.

In Vitro Cytotoxic Effect on P815 Tumor Cells

Peritoneal macrophages from mice injected with ACM or with ACM plus SOD, as above, were assayed for cytotoxic activity on 51Cr-labeled P815 tumor cells. The cytotoxicity of ACM-treated macrophages was greater than that of normal cells. There was no difference between ACM and SOD-ACM-treated cells (Table 3). SOD injection did not prevent enhancement of the cytotoxic activity induced by ACM and the injection of SOD alone did not modify the cytotoxic activity of peritoneal macrophages.

In Vivo Tumor Cell Destruction

The effect of SOD on the stimulation of peritoneal cell antitumoral activity by ACM and on the direct cytotoxicity of ACM, was assessed by injecting B6D2F1 mice with ACM alone or 1 h after SOD injection (50 units/mouse i.p.), either 4 days before or 1 h after the injection of [125I]dUrd-labeled P815 tumor cells. The whole body radioactivity of the mice was counted in toto on several days following tumor cell injection.

As shown in Table 4, the percentage losses of radioactivity were greater in mice given ACM or ACM-SOD 4 days before tumor cell inoculation than in controls. The stimulation of in vivo antitumoral activity induced by ACM administration was thus not modified by SOD pretreatment.

The radioactivity loss in mice injected with ACM or SOD-ACM 1 h after tumor cell inoculation was greater than in mice treated 4 days earlier, probably reflecting the direct cytotoxic effect of ACM at this time; SOD did not prevent the in vivo direct and indirect cytotoxic effects of ACM.

Direct in Vitro Antitumor Activity of ACM and SOD-ACM

L1210 or P815 tumor cells were incubated for 2 h with varying doses of ACM (0.1, 0.05, 0.01 µg/ml) with or without SOD (25 or 50 units/ml) to test the ability of SOD to modify...
EFFECT OF SOD ON ALCINOMYCIN-ACTIVATED PERITONEAL CELLS

SOD-ACM-injected animals. In contrast, the survival of SOD-injected mice was strictly comparable to that of control mice, indicating that SOD did not stimulate natural peritoneal cell cytotoxicity and had no antitumoral activity. Mice were also given ACM, SOD, or SOD-ACM to evaluate the toxicity of the treatment: no death was recorded up to 60 days after treatment (data not shown).

Internalization of $[^{125}I]$SOD by Peritoneal Macrophages and Peritoneal Cells

The protection of peritoneal macrophages from ACM toxicity by SOD and the sensitivity of tumoral cells in the presence of SOD were examined by following the penetration of $[^{125}I]$-labeled enzyme in peritoneal macrophages and in L1210 leukemic cells.

No free radioactivity was detected by autoradiography after electrophoresis of $[^{125}I]$SOD (Fig. 11). All the radioactivity was

Table 2 Chemoluminescence of peritoneal cells from mice injected i.p. with ACM, either alone or 1 h after SOD

Each individual curve of the chemoluminescence response induced by zymosan was drawn and its area was calculated. The results are reported as the percentage of the untreated control group (100%) on the corresponding day (mean ± SD) to 15 mice per group. ACM 4 mg/kg.

Table 3 In vitro cytotoxicity of peritoneal cells from mice injected i.p. with ACM, either alone or 1 h after SOD

Mice received i.p. on Day -4 ACM (4 mg/kg) either alone or 1 h after 50 U SOD. Their peritoneal macrophages were incubated with $[^{3}H]$dThd by the SOD-ACM-treated cells was similar to that of cells incubated with ACM alone.

Survival of Mice Injected with Tumor Cells

The survival of mice challenged with $2 \times 10^6$ P815 cells and treated with ACM or SOD-ACM on Day 0 (SOD 100 units i.p./mouse 1 h before ACM injection), confirmed the above experiments. ACM-injected mice survived longer than control mice (Fig. 10) and there was no difference between ACM and SOD-ACM-injected animals. In contrast, the survival of SOD-injected mice was strictly comparable to that of control mice, indicating that SOD did not stimulate natural peritoneal cell cytotoxicity and had no antitumoral activity. Mice were also given ACM, SOD, or SOD-ACM to evaluate the toxicity of the treatment: no death was recorded up to 60 days after treatment (data not shown).

Table 4 In vivo cytotoxicity of mice injected i.p. with ACM, either alone or 1 h after SOD

$[^{125}]$Urd-labeled P815 tumor cells for 18 h and the label release measured. Statistical analysis: Student's $t$ test: between A and control, $P < 0.01$; between B and control, $0.02 < P < 0.05$; between C and control: not significant.

Table 5 shows that incorporation of $[^{125}]$dUrd by the SOD-ACM-treated cells was similar to that of cells incubated with ACM alone.
EFFECT OF SOD ON ALCALCINOMYCIN-ACTIVATED PERITONEAL CELLS

contained in a M, 16,000 band corresponding to reduced SOD, as confirmed by Coomassie brilliant blue staining.

Peritoneal macrophages incubated in vitro with [125I]SOD showed no significant incorporation after 15 min (10 ± 5 × 10^3 molecules of SOD per cell), but significant incorporation after 30 min (47.5 ± 5.5 × 10^3 molecules per cell) and 60 min (55 ± 10 × 10^3 molecules per cell). In a similar experiment, L1210 leukemic cells internalized almost no [125I]SOD (Fig. 12).

The in vivo internalization of [125I]SOD by peritoneal macrophages 1 h after its i.p. injection was also studied. Peritoneal cells from thioglycolate-injected mice, and containing more than 90% macrophages, internalized 92.14 ± 8.73 × 10^3 molecules per cell. Peritoneal cells from normal mice containing approximately 30% macrophages internalized 30.36 ± 3.34 × 10^3 molecules per cell. These results suggest that SOD is internalized by peritoneal macrophages but not by tumor cells in our experimental conditions (Fig. 12).

DISCUSSION

Peritoneal macrophages from ACM-treated mice show two types of ultrastructural modifications: increased intracytoplasmic organelles indicating functional activation, and atypical alterations such as giant lysosomes and giant vacuoles possibly due to toxic effects of ACM. Pretreatment with SOD does not affect the morphological signs of activation but impairs the formation of atypical elements in a dose-dependent manner suggesting an uncontrolled, ACM-induced O_2^- generation in these cells.

These atypical ultrastructural alterations of macrophages are not specific to ACM or ADM since similar alterations have been induced in other experimental conditions (28) and have also been reported in human lysosomal storage disease (29) and in disorders with high phagocytic activity resulting in the ac-

Table 5: In vitro cytotoxicity of ACM is not modified by the addition of SOD

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<th>Tumor (units/ml)</th>
<th>ACM (µg/ml)</th>
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<tr>
<td></td>
<td>0.1</td>
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<tr>
<td>L1210</td>
<td>2,608 ± 271</td>
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<td></td>
<td>2,048 ± 551</td>
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<td>2,368 ± 752</td>
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P815

|                 | 694 ± 84   | 5,603 ± 108 | 13,013 ± 697  | 452,222 ± 25,378 |
|                 | 756 ± 61   | 4,567 ± 1,039| 12,278 ± 646  | 431,700 ± 14,572 |
|                 | 654 ± 148  | 4,785 ± 183  | 11,727 ± 785  | 477,500 ± 18,420 |

The in vivo internalization of [125I]SOD by peritoneal macrophages 1 h after its i.p. injection was also studied. Peritoneal cells from thioglycolate-injected mice, and containing more than 90% macrophages, internalized 92.14 ± 8.73 × 10^3 molecules per cell. Peritoneal cells from normal mice containing approximately 30% macrophages internalized 30.36 ± 3.34 × 10^3 molecules per cell. These results suggest that SOD is internalized by peritoneal macrophages but not by tumor cells in our experimental conditions (Fig. 12).

Cumulation of lipid material (30). Nonspecific stimuli however, such as LPS or tcalc do not induce the atypical ultrastructural alterations found in ACM-treated cells. ADM injection leads to the formation of the same giant lysosomes and giant vacuoles in peritoneal macrophages as those found in ACM-treated cells, suggesting that these ATC share the same mechanisms of action. The atypical alterations are more frequent in ADM-treated cells than in ACM-treated cells, which may be correlated with the higher cardiotoxicity of ADM (4, 26). The development of atypical morphological alterations induced by ATC administration can be prevented by pretreatment with SOD,
but not by heat-inactivated SOD. These findings support the hypothesis that an abnormal generation of O$_2^-$ in those cells is implicated in the formation of atypical ultrastructural features in ACM-treated macrophages.

The published reports on the morphological characteristics of heart lesions due to ATC injury which are related to the generation of free oxygen radicals, suggest that there is a modification of lipid metabolism (28–30) since they include the presence of myeloid bodies and lipid peroxidation in cardiac cells (26, 31) and these lesions can be minimized by the administration of antioxidants such as α-tocopherol or glutathione (32, 33).

We have also investigated whether the oncostatic activity of ACM is modified by SOD pretreatment, which has been shown to inhibit the formation of atypical morphological alterations. Although it has been suggested that free-oxygen radicals are in part responsible for the antitumoral activity of ATC (34), P815 and L1210 tumor cells were killed by ACM even in presence of SOD in our experimental conditions, possibly because the size of SOD molecule (M$_r$ 33,000) renders it unable to cross the plasma membrane by diffusion (35). This is supported by the fact that [125I]SOD is internalized in vitro and in vivo by peritoneal macrophages, which are known to have high pinocytotic and phagocytotic activities (13, 14) while [125I]SOD is not internalized by L1210 leukemic cells.

These data are consistent with the hypothesis of a direct detoxification of O$_2^-$ anions generated after ACM treatment by the ingested SOD in peritoneal macrophages, and provides an explanation for the continued oncostatic activity of ACM after SOD pretreatment. Yoda et al. (32) similarly found that glutathione, a potent inhibitor of O$_2^-$, does not impair the cytotoxic effect of ADM in vivo against L1210 leukemic cells and minimizes heart injury. The common feature of these two observations is the increased antioxidant capacity of the myocardial cells and the macrophages, although it results from two different mechanisms. The reduced glutathione in the plasma converts plasma cysteine to cysteine, which rapidly enters in the cells and accelerates intracellular glutathione synthesis (36) while SOD is directly internalized by macrophages.

The metabolic and antitumoral activities of peritoneal macrophages can be enhanced by administration of an ATC such as ADM or ACM (3, 10, 11, 12, 27) and SOD pretreatment does not interfere with this stimulation. This feature may be evaluable in the control of tumor cells or infections.

The mechanism by which ACM enhances the activity of peritoneal macrophages is not understood but it is necessary to consider that inflammation is the response to any injury and its characteristic depends on the intensity and the type of injury. The oxidative properties of ATC may play an important role in the ATC induced injury in the peritoneal cavity. In clinical trials, i.p. chemotherapy with ADM is limited by the onset of aseptical peritonitis (37).

It is possible that ACM is toxic for macrophages, and that their enhanced activity after ACM injection could be attributed to a direct stimulation of the remaining cells and/or to the recruitment of a new cell population to the peritoneal cavity as a consequence of inflammation.

There is an apparent contradiction in the chemoluminescent data, as SOD pretreatment does not appear to modify the chemoluminescent response of normal or ACM-treated cells while it inhibits atypical alterations apparently induced by O$_2^-$ generation. However it has been shown (38) that the secretion of free oxygen radicals by peritoneal macrophages constitutes the normal response to phagocytic stimuli as shown in Table 2 and is largely a membrane phenomenon. This may be quite different from the abnormal and uncontrolled generation of O$_2^-$ radicals within the macrophages induced after ACM treatment which lead to the atypical alterations.

This apparent lack of SOD effect on chemoluminescence may also result from the particular experimental conditions used, as we and others (38, 39) have shown that direct addition of SOD to normal or ACM-treated cells during measurement can dramatically reduce light emission. We have also observed (data not shown) a 30% inhibition of chemoluminescence when ACM-treated mice are given 50 units of SOD 60 min before cell harvest. Thus, chemoluminescence can be inhibited by SOD, but as the present tests were done 1 to 12 days after SOD injection the antioxidant enzyme had probably been metabolized or inactivated.

On the other hand, antitumoral tests with macrophages have never shown that tumor cells can elicit respiratory burst in macrophages in spite of the fact that malignant cells could be sensitive to free oxygen radicals.

The present results could be relevant in i.p. chemotherapy where ACM has considerable potential (40), where pretreatment with SOD could decrease ATC toxicity without affecting its oncolytic or immunoregulatory properties. But further studies will be needed to develop that proposition.

The present work supports the notion that the side-effects from ATC administration due to oxygen free radicals could affect different cells, and that the utilization of antioxidants may help cellular detoxification of free oxygen radicals. A better understanding of the role of oxygen-free radicals in the side-effects of ATC, and their behavior in the presence of antioxidants should lead to an increase in the efficiency of these drugs and to a decrease in their side-effects.

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Enhanced Activity of Peritoneal Cells after Aclacinomycin Injection: Effect of Pretreatment with Superoxide Dismutase on Aclacinomycin-induced Cytological Alterations and Antitumoral Activity

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