Enhanced Activity of Mouse Peritoneal Cells after Aclacinomycin Administration

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ABSTRACT

We have investigated the activation of mouse peritoneal macrophages by injection of aclacinomycin (ACM). Macrophages from ACM-treated mice have an increased phagocytic activity as measured by Candida ingestion. The microbicidal activity indirectly evaluated by chemiluminescence and superoxide determination in response to stimulation with zymosan and 40-phorbol-12-myristate-13a-acetate is also greater in the cells from treated mice. Direct measurement of the cytostatic function, and of in vitro and in vivo cytotoxicity shows comparable significant increases against L1210 or P815 target cells. The enhanced antitumoral activity could not be attributed to the residual presence of ACM in the peritoneal cells since no drug was detected by high-performance liquid chromatography and since their freeze-thaw lysates incubated with P81S cells did not modify the growth of tumor cells as measured by [3H]-thymidine incorporation. We also checked that the presence of ACM did not influence the intensity of the chemiluminescence. In all tests performed, only i.p. ACM administration could stimulate the peritoneal cells.

Since the doses of ACM inducing an increase in macrophage activity are effective on the survival of tumor-bearing mice, the participation of this mechanism in tumor control might be suggested.

INTRODUCTION

In the treatment of neoplastic disease by oncostatic or cytotoxic drugs, serious side effects often occur. Among these are opportunist infections and secondary malignancies. It might be assumed therefore that drugs which have an acceptable therapeutic effect without compromising host immune defenses would be of particular interest (1).

Moreover, evidence has accumulated indicating that some drugs were able to stimulate immune responses including antitumor mechanisms (2–6). We have previously reported that after administration of ACM2 immune response to sheep red blood cells is enhanced (7) due to the elimination of suppressor blood cells (8).

In the present study, we examined the modification of peritoneal cell activities observed after ACM injection. We studied the phagocytic activity and the production of free-oxygen radicals which are major mechanisms in infection control. Their cytostatic and cytotoxic activities were studied as well. A good concordance between the various tests performed allowed us to conclude that, after i.p. administration of ACM, the peritoneal cell population shows greater phagocytic and in vitro antitumor activities. The later phenomenon correlates with the observation that in vivo-enhanced destruction of i.p.-injected tumoral cells is also more intense in treated mice.

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2 The abbreviations used are: ACM, aclacinomycin; PC, peritoneal cells; PMA, 45-phorbol-12-myristate-13a-acetate; dThd, thymidine; cpm, counts per minute; NK, natural killer; E:T, effector-target cell ratio; HPLC, high-performance liquid chromatography; LD50, dose level of 50% lethality.

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 (Villejuif, France). They were used within 2 weeks after delivery.

Drug. Aclacinomycin, a cytotoxic antibiotic isolated from cultures of Streptomyces galilaeus, was kindly supplied by Laboratories Roger Bel- lon (Neully/Sene, France). For use, the drug was dissolved in pyrogen-free saline at a concentration of 1 mg/ml. This solution, stored at ~20°C, was used within 8 days.

Preparation of PCs. B6 mice were sacrificed by cervical dislocation and peritoneal cells were obtained by washing with 6 ml of cold Hanks’ balanced salt solution without phenol red (pH 7.2). Smears were prepared by cytocentrifugation and colored with Giemsa stain for differential counts. Living cells were determined by trypan blue exclusion.

Some experiments were done with purified adherent cells. In these experiments, the percentage of macrophages in PC was estimated on Giemsa-stained smears before and after plastic adherence. Suspensions, adjusted to the appropriate concentration, were distributed into 96-well tissue culture plates (NUNC) (0.2 ml/well) for cytotoxicity testing, or put in plastic cuvettes (Clinicon) for chemiluminescence assay (0.5 ml/tube). The cells were allowed to adhere for 2 h at 37°C in a 5% CO2 atmosphere. The medium and the nonadherent cells were removed by aspiration and the adherent cells were washed twice with RPMI 1640 for cytotoxicity or phenol-red-free Hanks’ balanced salt solution for chemiluminescence. Approximately 80% of the adherent cells were found to be macrophages.

Culture Reagents. RPMI 1640 tissue culture medium (GIBCO) was supplemented with 10% fetal calf serum (GIBCO), containing 16 mg/ml endotoxin, lot 10F7651S, 1% nonessential aminoacids (GIBCO), 1% sodium pyruvate (GIBCO), and antibiotics (RPMI 1640 supplemented).

Tumor Target Cells. The murine L1210 leukemic tumor cells and mastocytoma P815 were maintained by weekly in vivo passages of 2 × 106 cells in syngeneic mice. Radiolabeled tumor target cells were prepared by incubating 5 × 106 tumor cells in 0.1 ml with 0.1 mCi 51Cr-labeled sodium chromate (Amersham) for 60 min at 37°C. The cell suspension was washed three times with RPMI 1640 tissue culture medium and diluted to 2 × 106 cells/ml in RPMI 1640-supplemented medium.

Phagocytic Assay. A total of 106 PC were allowed to adhere to glass coverslips for 30 min at 37°C in a humidified atmosphere containing 5% CO2. The slides were carefully washed with Hanks’ balanced salt solution at 37°C to remove nonadherent cells and covered with 0.6 ml of fresh serum from normal or ACM-treated mice containing Candida albicans at a concentration of 2 × 105 elements per ml. This organism was isolated from cultures of a leukemic patient’s blood, and maintained on Sabouraud’s medium (Institut Pasteur Production, Paris). The slides were incubated at 37°C for 30 min to allow ingestion of Candida by the adherent cells. The coverslips were rinsed with tissue culture medium to remove noningested Candida, air dried, stained with Giemsa, and mounted inverted on microscope slides. Each preparation was read independently by two observers under a phase contrast microscope at high magnification (> 800).

Three hundred cells were examined to calculate the percentage of cells having phagocytosed at least one Candida. The number of Candida phagocytosed per macrophage was also measured. A phagocytic index was calculated as the average number of Candida per macrophage, i.e., the total number of ingested Candida divided by the number of macrophages (9). The values presented in this work are the mean and standard deviation of five mice individually assayed. Each test was performed twice with similar results.
Measurement of PC Chemiluminescence. PC were suspended at a concentration of 10^6/ml in phenol red-free Hanks' solution. Chemiluminescence measurements were performed in an LKB 1250 luminometer at 37°C in a light-tight chamber with 1 ml of PC suspension. Twenty μl of luminol (Sigma Chemical Co., St. Louis, MO) were added at a final concentration of 5 x 10^-4 M. When background light emission became constant, either 100 μl of a suspension of opsonized Zymosan (15 mg/ml) (Sigma) or PMA (10 ng/ml) (Sigma) was added, and photomultiplier output was recorded for 60 min (3).

Figs. 1–4 are drawn with the average values ± standard deviation obtained from groups of 10–14 individually assayed mice. Each in vitro test (Fig. 5) was performed in triplicate and repeated three times with similar results.

Assay for O2 Production. O2 generation was assayed by reduction of ferricytochrome C (10). 10^6 PC, suspended in phenol-red-free Hanks' balanced salt solution, were placed in 35-mm diameter Petri dishes, to which 100 μl of 1 mg/ml ferricytochrome C solution (type III; Sigma) was added to obtain a 80-μM final concentration. The production of O2 was stimulated by addition of luminol (1.5 mg/ml final concentration) or PMA (10 ng/ml final concentration), and the PC were incubated for 90 min at 37°C in 5% CO2. After incubation, the cell-free supernatants were transferred to ice-cooled tubes, centrifuged at 2000 x g, and their absorbances were read at 550 nm against a blank of ferricytochrome C incubated for 90 min at 37°C in the absence of cells. The amount of O2 produced was calculated using a differential absorption coefficient:

$$\Delta A_{254} = 2.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$$

(A)

Absorption measurements were performed in a Cary 220 spectrophotometer (Varian). The results were expressed as nmol O2/10^6 PC/90 min. The specificity of cytochrome C reduction was verified by inhibition with 300 units/ml of superoxide dismutase (type I; 3000 units/mg protein, Sigma). The values presented are the mean ± standard deviation of data from five mice individually measured and the experiment was performed three times with similar results.

Growth Inhibition Assay. The assay was performed as previously described (11). A total of 2 x 10^4 tumoral cells were incubated as quadruplicate samples, either alone or in the presence of effector PC, at different ratios in flat-bottom, 96-well plastic microtiter plates (NUNC) with 0.2 ml RPMI 1640 supplemented culture medium in a 5% CO2 incubator at 37°C for 48 h. DNA synthesis of tumor cells was measured by adding 1 μCi of [3H]Thd (Amersham) to each well 8 h before the cells were harvested. The test was carried out at 48 h by harvesting the cells on a multiple-suction filtration apparatus (Dynatech 2000) and [3H]Thd incorporation was determined in an LKB Rack β liquid scintillation counter. The percentage of growth inhibition of P815 cells due to the presence of macrophages was calculated by comparison with P815 cells cultivated alone. The PC were obtained from six mice per group and each experiment was performed three times with similar results.

Cytotoxicity Assay. PC at various concentrations in 0.2 ml of supplemented RPMI 1640 medium were incubated with 2 x 10^4 51Cr-labeled target P815 or L1210 cells for 4 or 18 h at 37°C in humidified atmosphere with 5% CO2 in flat-bottomed 96-well plastic microtiter plates (NUNC). After centrifugation, 0.1 ml of supernatant was counted for radioactivity for 1 min in a 7 counter (LKB 1272; test cpm). Spontaneous release (spontaneous cpm) was determined by incubating 51Cr-labeled target cells alone, and total labeling (total cpm) determined by counting of a 0.1-ml aliquot of target cell suspension. The following formula was used to compute the percentage of cytotoxicity:

$$\text{Test cpm} - \text{spontaneous cpm} \over \text{total cpm} - \text{spontaneous cpm} \times 100$$

(B)

Results

Spontaneous release by target cells did not exceed 10% for 4-h assays or 25% for 18-h assays. All E:T cell ratios were carried out in triplicate and each experiment was performed three times (12).

Evaluation of In Vivo Cytotoxicity. The method of Porteous and Munro (13) was used. B6D2F1 mice received 10^5 L1210 or P815 cells i.p., and, 5 days later, four injections of 1 μCi [32P]UdR. On day 7, the tumoral cells were harvested in Hanks' balanced salt solution containing heparin, centrifuged at 200 x g at 4°C, resuspended, counted with trypsin blue (viability 97%), and adjusted to a concentration of 2 x 10^7 living cells per ml. B6D2F1 mice were injected i.p. with 10^7 labeled cells in 0.5 ml.

Uptake of [32P]UdR into the thyroid was prevented by including 0.1% potassium iodine in the drinking water, starting 2 days before the injection of the labeled cells. The animal bedding was changed daily to reduce ingestion of saw dust contaminated by radioactive urine.

For the measurement of the remaining radioactivity, mice were introduced into a tube of appropriate size and counted in toto in a γ counter (Packard) for 1 min. The results are expressed as the percentage of the injected radioactive ten mice were used per group.

HPLC Sample Preparation and ACM Determination. The drug was extracted from the medium or the cell lysate by mini chromatography on C-18 Sep-Pack cartridges (Waters Association) according to Robert (14).

The last eluate was evaporated to dryness at 37°C and reconstituted in 50 μl of mobile phase.

ACM was measured by HPLC as described by Andrews et al. (15) with fluorescence detection. The quantitation was made by the external standard method and the sensitivity limit was 65 pmol in the 20μl-injected sample (S/N = 3).

Statistics. The values were compared using Student's t test, except for the in vivo cytotoxicity for which the nonparametric Mann-Whitney test was used.

Results

Cellular Composition of the Peritoneal Cell Population after ACM Injection. Mice were injected i.p. with 4 mg/kg of ACM. This dose was chosen because it caused the highest increase in the plaque-forming cell response to sheep red blood cells and in the lifespan of L1210 leukemia-bearing mice (7, 16). No difference was observed on day 4 after injection between the cellular composition of the peritoneal washings between control (lymphocytes 55.4 ± 3.6%, macrophages 37.1 ± 3.0%, neutrophils 3.6 ± 2.1%, and mast cells 4.2 ± 3.3%) and treated mice (lymphocytes, 63.3 ± 5.4%; macrophages, 30.3 ± 4.2%; neutrophils, 5.1 ± 1.4%; and mast cells, 2.1 ± 1.7%)

Phagocytic Activity of Peritoneal Macrophages after ACM Injection. The phagocytic activity of peritoneal macrophages was measured by Candida ingestion. Table 1 shows the values obtained with cells harvested from normal or ACM-injected mice (4 mg/kg i.p. on day 4). The percentage of cells having ingested at least one Candida was significantly greater among the population from ACM-injected mice than controls. The phagocytic index was also greater, reflecting the higher ingestion of Candida per cell after ACM treatment.

To evaluate the possible effect of the oncostatic drug persisting in the blood 4 days after injection, normal cells were harvested from groups of 10-14 individually assayed mice. Each experiment was performed twice with similar results.

Table 1

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>% of cells having ingested at least one Candida</th>
<th>Phagocytic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal macrophages in normal serum</td>
<td>66 ± 5*</td>
<td>1.56 ± 0.12</td>
</tr>
<tr>
<td>ACM macrophages in ACM serum</td>
<td>87 ± 8</td>
<td>2.34 ± 0.29</td>
</tr>
<tr>
<td>Normal macrophages in normal serum</td>
<td>71 ± 3</td>
<td>1.48 ± 0.06</td>
</tr>
<tr>
<td>ACM macrophages in ACM serum</td>
<td>89 ± 2</td>
<td>2.23 ± 0.13</td>
</tr>
</tbody>
</table>

* Values, mean ± SD of five mice.
incubated in serum of ACM-injected mice, or ACM-treated cells in normal serum. The enhanced phagocytic activity of the peritoneal macrophages did not depend upon the serum in which they were incubated because this enhancement is observed even when the treated cells were incubated in normal serum but not when normal cells were incubated in ACM serum (Table 1).

Chemiluminescence Response of PC after ACM Injection. The PC from individual untreated mice or mice given 4 mg/kg ACM i.p. 4 days earlier were stimulated with zymosan or PMA and their chemiluminescence response measured as a function of the time of incubation. Zymosan appeared to be a better stimulant than PMA. In both cases, the intensity of light, expressed in mV, emitted by PC from ACM-injected mice was twice as high as that of cells from untreated mice (Fig. 1).

The intensity of the chemiluminescence response was dependent upon the ACM dose. As shown in Fig. 2, an enhancement of PC chemiluminescence in response to zymosan was already observed after the i.p. injection of 2 mg/kg ACM on day —4. This stimulating effect reached its maximal value after injection of 4 mg/kg and decreased slightly when the dose was increased to 8 mg/kg.

The route of administration of the drug was a critical parameter since no enhancement of the chemiluminescent response was observed when the optimal ACM dose (4 mg/kg) was injected i.v. or i.m. (Fig. 3). As a positive control for this experiment, ACM was injected i.p., the chemiluminescence was identical to the one represented in Fig. 2 (data not shown).

In order to ascertain the role of macrophages in light emission, PC suspensions were adjusted to a concentration of $3 \times 10^5$ macrophages in each suspension and the chemiluminescent response to zymosan was measured before and after purification of PC by adherence to plastic. As shown in Fig. 4, chemiluminescence enhancement was unequivocally observed with plastic-adherent PC from mice given 4 mg/kg ACM i.p. 1 or 4 days before cell harvest. However, the chemiluminescence emitted by the adherent population was slightly less intense both in control and in ACM-treated groups than in the whole unfractionated PC population. This comes likely from the elimination of neutrophils which are more potent light emitters than macrophages to phagocytic stimulus (17). After each test, the adherent cells were detached with EDTA at 4°C. Over 77% of the cells had the morphology of macrophages.

Since Adriamycin has been reported to increase the chemiluminescence in Ehrlich tumor cells (18), we investigated whether the presence of residual ACM could not directly modify the intensity of PC chemiluminescence. For this purpose, the chemiluminescent response of $10^6$ normal PC to zymosan was measured either in the presence of $10^{-3}$ M ACM or after preincubation of the cells for 4 h with $10^{-3}$ M ACM, followed by three washings. As shown in Fig. 5, the curves of chemiluminescence emission by ACM-treated PC in vitro were strictly
MACROPHAGE ACTIVITY AFTER ACLACINOMYCIN ADMINISTRATION

Fig. 5. Chemiluminescence of normal PC treated in vitro with ACM. A, PC (10^6) were incubated with 5 x 10^{-3} M ACM for 4 h and then washed before measurement of their chemiluminescent response to opsonized zymosan. B, PC (10^6) were incubated with 5 x 10^{-3} M ACM during the measurement of their chemiluminescent response to opsonized zymosan. C, chemiluminescent response of untreated PC to opsonized zymosan. Each test was performed in triplicate and repeated three times with similar results.

Table 2 Production of superoxide by peritoneal cells of mice previously injected with aclacinomycin

<table>
<thead>
<tr>
<th>Treatment of the mice</th>
<th>Cells in vitro stimulated by</th>
<th>nmol of O_2^- per 10^6 peritoneal cells per 90 min</th>
<th>P&lt;br&gt;value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL*</td>
<td>PMA</td>
<td>4.9 ± 2.7‡</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>ACM</td>
<td>PMA</td>
<td>52.0 ± 9.7‡</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>NIL</td>
<td>zymosan</td>
<td>12.3 ± 4.1‡</td>
<td></td>
</tr>
<tr>
<td>ACM</td>
<td>zymosan</td>
<td>68.7 ± 16.3‡</td>
<td></td>
</tr>
</tbody>
</table>

* NIL, no treatment.
‡ Values, mean ± SD of five mice.

Fig. 6. Cytostatic activity of PC on P815 tumor cells after ACM administration (4 mg/kg on day —4). PC-to-tumor cell ratios (E:T) of 40:1 to 10:1 were used and [3H]thymidine incorporation by tumor cells was measured after a 48-h incubation, at 40:1 E:T (P < 0.01).

Results presented in Table 2 show that, regardless of whether the stimulus was zymosan or PMA, PC from mice given 4 mg/kg ACM i.p. on day —4 liberated higher amounts of O_2^- (P < 0.001) than normal PC.

In Vitro Assays of PC Antitumor Activity. PC were examined for their in vitro cytostatic activity on P815 tumor cells. Fig. 6 shows that PC from mice given 4 mg/kg ACM i.p. on day —4 more efficiently inhibited tumor cell growth, as estimated by [3H]thymidine incorporation after 48 h of contact, than PC from control mice at all E:T ratios used (P < 0.01 at 40:1 E:T).

Cytotoxic activity was measured on 51Cr-labeled P815 tumor cells incubated for 18 h with PC at different E:T ratios. The results, expressed as the percentage of chromium-51 released into the culture supernatants, are presented in Table 3. A noticeable augmentation of the cytotoxicity was observed at the 40:1 E:T ratio when the drug was injected i.p. at the dose of 4 mg/kg on day —4. No effect was observed when the drug was injected i.v. or i.m.

The influence of the ACM dose when administered i.p. on day —4 on the cytotoxic and cytostatic activities of PC was investigated using an E:T ratio of 40:1. As shown in Fig. 7, a strong inhibition of tumor cell growth was already observed with PC from mice given 2 mg/kg ACM. Increasing the dose to 4 or 8 mg/kg only resulted in a slight augmentation in the cytostatic activity. In contrast, the cytotoxicity assay, PC from mice given 2 mg/kg ACM did not behave differently from normal PC. However, a marked increase in the cytotoxic activity was observed after injection of 4 mg/kg ACM and still more with 8 mg/kg.

Characterization of the Effector Cells of PC Cytotoxicity after ACM Injection. It is now well established that two types of nonspecific killer cells, namely macrophages and NK-cells, can be detected in activated PC (21, 22). Therefore, experiments were performed to identify the cytotoxic cell, the activity of which is increased by ACM injected i.p. at the dose of 4 mg/kg on day —4. NK-cells have been distinguished from cytotoxic macrophages by their failure to adhere to plastic, their inability to kill P815 tumor cells, and by their ability for being selectively detected in a 4-h chromium-51 release assay (22).

Results presented in Table 4 show for the first time that ACM injection did not modify either PC cellular composition or the adherence property of the cell population. Unfractionated PC from ACM-treated mice displayed an increased cytotoxic activity when measured in a 18-h chromium-51 release assay, as compared to normal unfractionated PC. This enhancement was observed whether macrophage-sensitive, NK-cell-unsensitive (P815), or macrophage- and NK-cell-sensitive (L1210) target cells were used. In both normal and ACM-treated groups, the cytotoxic activity was recovered in the plastic adherent cell population in which over 75% of the cells had macrophage morphology. The increment in cytotoxicity resulting from

Table 3 In vitro cytotoxicity of peritoneal cells measured after an 18-h incubation with P815 51Cr-labeled cells. Influence of the route of administration of aclacinomycin

<table>
<thead>
<tr>
<th>Route</th>
<th>% Chromium-51 release effector-to-target ratios (mean ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>40:1</td>
</tr>
<tr>
<td>i.m.</td>
<td>8.7 ± 1.3</td>
</tr>
<tr>
<td>i.v.</td>
<td>9.2 ± 0.9</td>
</tr>
<tr>
<td>i.p.</td>
<td>28.1 ± 1.5</td>
</tr>
<tr>
<td>Controls</td>
<td>10.9 ± 1.2</td>
</tr>
</tbody>
</table>

Fig. 7. Cytostatic (left) and cytotoxic (right) activity of PC after ACM administration. Influence of the dose of ACM injected on day —4. Macrophages to P815 tumor cell ratios was 40:1. Cytostaticity was evaluated by [3H]thymidine incorporation by tumor cells after 48 h of incubation and cytotoxicity was evaluated by chromium-51 release by prelabeled tumor cells after 18 h of incubation.
ACM administration was still observed in the plastic-adherent cell population with values of cytotoxicity close to that obtained with the whole population. In contrast, the residual cytotoxicity recovered in the plastic nonadherent population (more than 85% lymphocytes) was not significantly different in control and ACM-treated groups after a 18 h incubation with both target cells.

NK-cell activity was investigated by measuring PC cytotoxicity against L1210 tumor cells in a 4-h chromium-51 release assay. This activity was mainly recovered in the plastic nonadherent cell population but did not exceed 7% at a 40:1 E:T ratio. This confirms that normal PC are not a major source of cytotoxic activity against LI210 tumor cells in vitro. These data strongly suggest that macrophages, but not NK-cells, are the effectors of PC cytotoxicity and that their activity can be strongly increased by ACM injection.

**In Vivo Cytotoxic Assay.** In the above experiments, PC stimulated by ACM showed an enhanced cytotoxic activity in vitro for tumor cells. In order to determine whether this phenomenon could have an in vivo counterpart, we compared control and ACM-treated mice (4 mg/kg on day -4) for their capacity to eliminate [125I]UdR-labeled L1210 or P815 tumor cells injected i.p. on day 0. The rate of whole body radioactivity decay has been shown to be directly correlated with the tumor cell destruction potential of the host (13).

As shown in Table 5, the percentage of remaining radioactivity was significantly lower in mice given ACM by the i.p. route, as compared to normal mice, on days 2 and 3 after LI210 tumor cell inoculation and on days 1, 2, and 3 after P815 tumor cell injection. In contrast, no difference in radiolabeled tumor cell elimination was observed when ACM was injected i.v. or i.m.

Possible Influence of Residual ACM in PC Cytotoxicity. Two experiments were performed in order to ascertain whether residual ACM in PC might be directly responsible for their in vitro antitumor activity. PC lysates, obtained by disrupting $8 \times 10^6$ cells either from ACM-treated or control mice by three cycles of freeze thawing, were added to $2 \times 10^4$ P815 tumor cells for 48 h, at which time [3H]dThd incorporation was measured. In parallel, ACM was added to tumor cell cultures at concentrations varying from $10^{-5}$ to $10^{-12}$ M in order to directly quantitate the cytostatic potential of the drug.

In another experiment, a direct dosage of ACM present in PC lysates was performed by HPLC. In three different assays, ACM was not detectable in $5 \times 10^5$ PC from mice injected i.p. with 4 mg/kg on days -1, -4, or -8, or with 8 mg/kg on day -4, that is, less than 150 pmol were present. When the cells were harvested only 4 h after i.p. injection of 4 mg/kg ACM, 20% of injected ACM was recovered, and probably also ACM metabolites, as shown by the presence of additional peaks (data not shown). These results argue against the direct involvement of residual ACM in the potent antitumor activity of PC from ACM-treated mice.

**Kinetics of the Stimulation of PC Activities after ACM Injection.** All the experiments reported above were performed 4 days after ACM injection. As we also wanted to determine the kinetics of stimulation of PC activities after ACM i.p. injection at the dose of 4 mg/kg, additional experiments were performed. Fig. 9A shows the modifications observed in the chemiluminescence response of PC to zymosan, as measured 1–12 days after drug injection. Each point represents the mean value of the peak of light emission by PC from 10 mice individually assayed. The maximum of chemiluminescence stimulation was observed

<table>
<thead>
<tr>
<th>MACROPHAGE ACTIVITY AFTER ALCALINOMYCIN ADMINISTRATION</th>
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<tr>
<td>Peritoneal cells were fractionated by plastic adherence for 2 h at 37° C at 5% CO2. Chromium-51 labeled L1210 or P815 cells were incubated for 4 or 18 h at 37° C in a CO2 incubator and the Chromium-51 release was measured. Each experimental group was compared with the corresponding control group by Student’s t test.</td>
</tr>
</tbody>
</table>

### Table 4 Cytotoxic activity of peritoneal cells from ACM-injected mice

<table>
<thead>
<tr>
<th>Cellular composition (%)</th>
<th>Chromium-51 release effector-to-target ratio 40:1 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1210 at 4 h</td>
</tr>
<tr>
<td>Control</td>
<td>Macrophages</td>
</tr>
<tr>
<td></td>
<td>84</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>77</td>
</tr>
<tr>
<td>Adherent cells</td>
<td>44</td>
</tr>
<tr>
<td>Nonadherent cells</td>
<td>77</td>
</tr>
<tr>
<td>ACM (4 mg/kg day -4)</td>
<td>37</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>14</td>
</tr>
<tr>
<td>Adherent cells</td>
<td>76</td>
</tr>
<tr>
<td>Nonadherent cells</td>
<td>64</td>
</tr>
</tbody>
</table>

* NS, not significant.
MACROPHAGE ACTIVITY AFTER ACLACINOMYCIN ADMINISTRATION

![Graph: Growth inhibition of P815 tumor cells by increasing doses of ACM and by peritoneal cell lysates from mice injected with various doses of ACM.]

We observed an increase in the chemiluminescence of PC in response to opsonized zymosan as a phagocytic stimulus or to PMA as a membrane disturbing agent (10). The fact that plastic-adherent cells, 75% of which have a macrophage morphology, emitted about 80% of the chemiluminescence of the whole population of PC strongly suggests that the macrophages are responsible for light emission and are stimulated by the drug. The enhancement of chemiluminescence response was already maximal 24 h after ACM injection and then declined slowly, being undetectable on day 12. Four mg/kg of ACM seems to be the optimal dose and the i.p. route is the only one to be effective.

The stimulation of an oxidative burst which is considered as the origin of chemiluminescence (19) was confirmed by the direct measurement of \( O_2^- \) reduction by PC harvested 4 days after ACM injection (4 mg/kg i.p.). The \( O_2^- \) is the first radical formed during the respiratory burst of phagocytic cells and is the precursor of bactericidal hydrogen peroxide (20). Taken together, these data strongly suggest that both phagocytic and bactericidal activities of macrophages can be rapidly and durably enhanced after i.p. administration of ACM. It is important to stress that infections cause serious problems in cancer patients, who are chronically immunodepressed either by the tumor or by the therapy (24–26). These observations are in agreement with results obtained with Adriamycin. Five days after administration of this drug, the phagocytosis of sheep red blood cells coated with anti-sheep red blood cell antibodies is increased (27, 28). However, it has been reported that the capacity of human polymorphonuclear cells to phagocytose Escherichia coli in vitro is diminished by a 15-h preincubation with Adriamycin (29). On the other hand, tumor cells are sensitive to oxygen free radicals liberated by phagocytic cells, but only in the antibody-dependent cell-mediated cytotoxicity test tumor cells have they been shown to be the appropriate stimulus able to generate the activation of the respiratory burst (30, 31). Our work refers only to the increased formation of oxygen radicals as an indirect evidence of the microbicidal activity of macrophages.

In vitro antitumor activity of PC from ACM-treated mice is significantly increased and the optimal cytostatic and cytotoxic activities are observed in cells harvested 4 days after injection of 8 mg/kg ACM. A 2 mg/kg dose stimulates cytostatic but not cytotoxic activity and the kinetics of stimulation of PC is comparable in both tests, the maximum activity being observed 24 h after ACM injection (4 mg/kg i.p.). This stimulation persists for 4 days, but 8 days after injection, the response is back to normal values.

The PC of i.p. ACM-injected mice 4 days before cell harvest, whether unfractionated or purified by plastic adherence (75% macrophages), are more cytotoxic after an 18-h incubation than untreated cells towards P815 (NK resistant) or L1210 cells (NK sensitive) and the values of cytotoxicity obtained with plastic-adherent cells are close to the values of the unfractionated population. These data tend to prove that the activation affects mainly the macrophages as it has been reported for other anthracyclines (12, 32, 33). The fact that plastic nonadherent PC in both populations showed the same cytotoxic activity against L1210 cells after 4 h of incubation suggests that the NK-cells were not stimulated by ACM.

The in vitro cytotoxic activity of PC, measured against P815 or L1210 cells on day 4 after ACM injection, has also been correlated with an enhanced in vivo tumor cell destruction.

It is worth noting that, in all the tests performed, the increases in the activity of the PC cells were observed only when ACM

**DISCUSSION**

The results presented here demonstrate that ACM, when injected i.p. to mice at doses proven to be efficient in the treatment of murine tumors and to enhance the response to sheep red blood cells in mice (4 mg/kg) (7, 16), strongly increases a variety of macrophage activities.

Phagocytosis of *Candida* organisms by peritoneal macrophages is enhanced on day 4 after ACM injection. In addition, we also indirectly considered the microbicidal activity of PC after ACM injection, which is generally accepted to be mediated by the participation of \( O_2^- \) radicals released during phagocytosis (23).
was injected i.p. We can then suggest that the drug does not reach a sufficiently high peritoneal concentration when it is administered i.v. or i.m. at doses that do not cause generalized toxicity. A similar finding has been observed after i.v. injection of Adriamycin (33, 34). This observation might be of great interest in the treatment of tumors developing in the peritoneal cavity such as ovarian carcinoma where the i.p. route can be used as a treatment modality (35). Additional data should be provided, however, because of the discrepancy of some of the results reported: PC from mice injected s.c. with Adriamycin inhibit better than normal cells the growth of MBL-2 lymphoblastoid leukemia cells (36).

It is important to consider whether the presence of residual drug could modify or not the intensity of the responses measured. ACM has, like all anthracyclines, oxidative properties and is considered to be responsible to their heart cytotoxicity (37). This drug induces the formation of superoxide ions in cells by electron exchange between reduced nucleotides and the anthracycline quinone (38). Our results raise the question as to the role of the drug remaining in the macrophages that could have affected the results of the tests, either because it would increase the chemiluminescence, or directly destroy the tumor cells.

Our results suggest that this is not likely because values of chemiluminescence were normal when the PC were incubated for 4 h with ACM or when ACM was added during the test. It cannot be a direct antitumoral activity of ACM remaining in PC because the amounts determined by high-performance liquid chromatography were lower than that needed to produce an antitumoral activity, and because the lysis of treated cells was not toxic to P815 tumor cells (Fig. 8). It has been claimed that ACM is metabolized rapidly and is no more present after a 4-h incubation in vitro with Friend’s leukemia cells (39). In our conditions, we were able to detect 20% of injected ACM 4 h after i.p. injection (data not shown). This discrepancy can be accounted for by the method used (in vitro versus in vivo) or by differences in cell metabolism (Friend leukemia cells versus PC). On the other hand, there is a difference between the metabolism of Adriamycin and ACM in mice: 24 h after the i.p. injection of 10 mg/kg Adriamycin, Salazar and Cohen (12) reported that the drug is still detectable in the PC, and that the whole cells or their lysates are cytotoxic for P815 cells. However, the activation of PC persists for 5–7 days while the drug is no longer detectable into the cells.

Finally, our observations do not exclude the fact that in different experimental conditions higher doses of Adriamycin and daunorubicin (half or quarter of LD₅₀) can reduce the suppressor activity induced by the injection of higher doses of antigens (40). Ishizuuka et al. (41) and ourselves (8) have also shown that lower doses of ACM (a quarter of LD₅₀) eliminate suppressor cells. Both effects, on macrophages and on suppressor cells, might be observed when appropriate protocols are used, indicating a similar effect of anthracyclines on the immune system in spite of their biological and biochemical differences.

The interest of the above results as a whole comes from the fact that the ACM potentiating activity in macrophages is not toxic to P815 tumor cells (Fig. 8). It has been claimed that lower doses of ACM (a quarter of LDS₀) eliminate antigens (40). Ishizuuka et al. (41) and ourselves (8) have also shown that lower doses of ACM (a quarter of LD₅₀) eliminate suppressor cells. Both effects, on macrophages and on suppressor cells, might be observed when appropriate protocols are used, indicating a similar effect of anthracyclines on the immune system in spite of their biological and biochemical differences.

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MACROPHAGE ACTIVITY AFTER ACLACINOMYCIN ADMINISTRATION

Enhanced Activity of Mouse Peritoneal Cells after Aclacinomycin Administration

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