Alterations in Murine Host Defense Functions by Adriamycin or Liposome-encapsulated Adriamycin*1

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ABSTRACT

Peritoneal exudate cells (PEC) from C57BL/6 mice were collected on different days following an i.p. injection of Adriamycin (10 mg/kg) as free drug (ADM) or encapsulated in liposomal containers of ADM. Macrophages harvested from mice at various times (Days 4–14) after either drug treatment were responsive to in vitro lipopolysaccharide induction of tumoricidal activity, maximum response being seen on Day 7. In addition, 18 days after treatment, significant macrophage tumoricidal activity was observed only in the ADM/Lip-treated group. When supernatants from cultures of PEC obtained 7 days after treatment were assayed for interleukin 1 following lipopolysaccharide stimulation, activity was found with both ADM- and ADM/Lip-treated cells. Without lipopolysaccharide stimulation, only PEC from ADM-treated mice elaborated factor(s) with interleukin 1-like activity. Both ADM and ADM/Lip induced significant PEC-natural killer (PEC-NK) activity by Day 4, while the ADM/Lip treatment sustained PEC-NK activity more effectively than free drug at later time points (7 or 11 days posttreatment). Drug-induced PEC-NK activity (Day 7) was (a) ablated by treatment in vitro with anti-asialo GM1 antibody and complement, and (b) associated with a population of PEC nonadherent to plastic. A transient suppression of splenic NK activity was seen 4 days following either ADM or ADM/Lip administration with recovery to control level by Day 7.

These data demonstrate that following ADM or ADM/Lip administration some of the changes necessary for macrophage tumoricidal activation must have occurred in vivo. Liposome encapsulation of ADM extended the duration of ADM-induced augmentation of certain host defenses.

INTRODUCTION

Nonspecifically activated murine or human immune effector cells can recognize and selectively destroy tumor cells in vitro. Pharmacological agents of bacterial or other origins are currently being investigated for promotion of this activity. In studies of drug-induced modulation of host defenses, cells of the monocyte-macrophage lineage are frequently identified as important mediators of immunopharmacological effects (1, 2). Other studies indicate that activated macrophages may circumvent the problem of tumor heterogeneity and that their action may not lead to the development of resistant tumor cells (3).

Increased phagocytic activity and enrichment of immature macrophages in the spleens of ADM4 (5 mg/kg, i.v.)-treated mice has been observed (4). In studies of tumor growth inhibition by ADM within the peritoneal cavity, macrophages were suggested as mediators of optimal drug activity (5, 6). These and other studies (7–12) indicated that macrophages exposed to ADM in vitro or in vivo may inhibit tumor growth as a result of either sequestration and subsequent release of drug by the macrophage or through direct macrophage-mediated tumoricidal activity. Salazar and Cohen (9) reported that ADM (10 mg/kg, i.p.) elicited a population of peritoneal exudate cells that were cytotoxic to tumor cells in the absence of measurable drug. It should also be mentioned that in vitro, macrophage-mediated destruction of tumor cells has been reported to be independent of target cell resistance to ADM (7).

The entrapment of cytokines or bacterial cell wall derivatives in liposomes potentiates the induction of cell-mediated tumoricidal activity in vitro and in vivo (11, 13). These results were ascribed to increased incorporation of the agent into macrophages and phagocytic components of the reticuloendothelial system. The encapsulation of ADM in liposomes increases the therapeutic index of the drug in some animal tumor model systems (14–16). The basis of this effect seems to be a reduction in toxicity, with the antitumor activity of the drug being retained or augmented (15). The mechanism(s) responsible for these effects is not known at present but could include altered and/or augmented antitumor host defense systems as well as altered drug pharmacokinetics and disposition. The purpose of this study was to compare the ability of free ADM with ADM encapsulated in a standardized liposome preparation (17) to induce alterations of some host defense mechanisms.

MATERIALS AND METHODS

Animals. Female C57BL/6Cr or C3H/HeJ mice, 6-wk-old, were obtained from the Goodwin Institute for Cancer Research, Plantation, FL, and Jackson Laboratory, Bar Harbor, ME, respectively.

Agents Used. ADM was obtained from Adria Laboratories, Columbus, OH. The drug either was used free or was encapsulated in phosphatidylglycerol, phosphatidylcholine, cholesterol, α-tocopherol (1:4:4:0.1 molar ratio) liposomes which were extruded through 0.2-μm nucleopore filters, as previously described (17). A dose of 10 mg/kg of free or encapsulated ADM was administered i.p. to C57BL/6N mice. ADM concentration in liposomes was determined after chloroform/methanol extraction by fluorescence at excitation 468 nm, emission 585 nm. The ADM concentration was determined from standard curves. Between 50 and 60% of the initial starting concentration of ADM was entrapped in liposomes in different preparations resulting in 12–18 μg of ADM/μmol of lipid. An equivalent quantity of liposomes (~300 μmol/kg) alone was injected i.p. in Lip controls. All injections were done using the same vol/wt ratio. Other agents used were: LPS, E. coli strain O111: B4 (Difco Laboratories, Detroit, MI); IL-2, lot 81-1232 (Collaborative Research, Inc., Waltham, MA); anti-asialo GM1 antiserum (Wako Chemicals, Dallas, TX); Low-Tox-M rabbit complement (Accurate Chemical & Scientific Co., Westbury, NY); phytohemagglutinin (Wellcome Laboratories, Beckenham, England); 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY).

Media. RPMI 1640 was supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 25 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer (Research Organics, Cleveland, OH), and 50 μg/ml gentamycin (Gibco Labs., Grand Island, NY). This medium was used to maintain cell lines and for experimental cultures and assays. The endotoxin levels in the fetal calf serum were in the range of 0.005–0.033 ng/ml.

Macrophages and NK Cytotoxicity Assays. YAC-1 (a lymphoma of A/Sn origin) and P815 (a mastocytoma of DBA/2 origin) were main-

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tained as continuous cultures. For cytolytic assays, tumor cells (5 \times 10^6/0.1 ml) were incubated with 0.1 mCi of Na_2^51CrO_4 (specific activity, 250–500 \muCi/\mug chromium) (Amersham, Arlington Heights, IL) for 30–45 min and washed 3 times and viable cells were counted. Radiolabeled target cells (10^4/well) were added to plated effector cells. All incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

PEC were obtained from C57BL/6 mice following sacrifice by cervical dislocation. The peritoneal cavity was lavaged with 7 ml of ice cold Hanks’ balanced saline solution (pH 7.4; Gibco, Chagrin Falls, OH) using a 10-ml syringe and 22-gauge needle. PEC from at least six mice were pooled, centrifuged at 300 \times g for 5 min, and resuspended in culture media. Spleens were aseptically removed on the day of sacrifice and single cell suspensions prepared by passage through coarse (50-mesh) and fine (200-mesh) stainless steel gauze, as described previously (18). Viable cells, by trypan blue exclusion, were counted on a hemacytometer in all assays. In general, PEC and spleen cell populations prepared as indicated were found to be \geq 90% and 95% viable, respectively. The total numbers of cells recovered from normal control mice ranged between 2 and 5 \times 10^8 per mouse for PEC and between 4 and 6 \times 10^6 per mouse for spleen cells.

For cytotoxic macrophage assays, 0.1-ml aliquots of PEC suspensions (1.25 \times 10^6 cells/ml) were dispensed into the wells of flat-bottomed, 96-well flat-bottomed, 96-well microwell plates (Linbro; Flow Labs, McLean, VA). The cells were allowed to adhere during a 24-h incubation and then the plates were washed 3 times with warm media to remove nonadherent PEC. These adherent PEC monolayers (>90% macrophages by morphology and nonspecific esterase [Sigma Diagnostic Kit; Sigma Chemical Co., St. Louis, MO]) were incubated for 18 h with or without LPS in a final volume of 0.2 ml. The macrophage monolayers were then washed and 10^6 radiolabeled P815 cells were added to each well in a final volume of 0.2 ml. After an 18-h incubation the plates were centrifuged at 300 \times g for 5 min. A 0.1-ml volume of supernatant was removed from each well and 51Cr release was determined in a gamma counter (Model 1197; Searle Analytic Inc., Des Plaines, IL). The possibility that the nonadherent cells could develop lytic activity was also evaluated, where appropriate, by adding P815 target cells to wells which had not been washed; the level of lytic activity observed was not significantly different from that of washed cells and therefore the data are not presented. For cytotoxic NK assays, peritoneal exudate cells or spleen cells, at various concentrations, were plated in round-bottomed microwell plates (Linbro; Flow Labs, McLean, VA) in a volume of 0.1 ml and 10^6 radiolabeled YAC-1 cells were added in 0.1 ml. After the addition of YAC-1 target cells the plates were incubated for 4 h, followed by centrifugation and quantifying of 0.1 ml of supernatant as described previously (18). Viable cells, by trypan blue exclusion, were counted on a hemacytometer in all assays. In general, PEC and spleen cell populations prepared as indicated were found to be \geq 90% and 95% viable, respectively. The total numbers of cells recovered from normal control mice ranged between 2 and 5 \times 10^8 per mouse for PEC and between 4 and 6 \times 10^6 per mouse for spleen cells.

In all assays of lytic activity the percentage of specific 51Cr release was determined using the following formula:

\[
\% \text{ of specific release} = \frac{\text{Experimental release} - \text{SR}}{\text{MR} - \text{SR}} \times 100
\]

The spontaneous release (SR) was determined by incubating 51Cr-labeled target cells alone for 4 h in NK assays or with nonactivated, adherent resident PEC for 18 h in tumoricidal macrophage assays. Spontaneous release was 6–10% in 4-h assays and 18–24% in 18-h assays. Maximal releasable (MR) label was determined by adding 0.1 ml of 1% Triton X-100 to 10^6 labeled target cells in 0.1 ml.

Anti-Asialo GM1 Antbody + Complement Treatment. Anti-asialo GM1 antibody was added directly to PEC in the microwells at a final dilution of 1:20. After 30 min on ice the cultured effector cells were spun down and supernatant was removed and resuspended. Low-toxicity rabbit complement was added (final dilution, 1:20) and the cultures were incubated for 45 min. After 3 washings, radiolabeled YAC-1 target cells were added for the 4-h NK assay.

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Anti-Asialo GM1 Antibody + Complement Treatment. Anti-asialo GM1 antibody was added directly to PEC in the microwells at a final dilution of 1:20. After 30 min on ice the cultured effector cells were spun down and supernatant was removed and resuspended. Low-toxicity rabbit complement was added (final dilution, 1:20) and the cultures were incubated for 45 min. After 3 washings, radiolabeled YAC-1 target cells were added for the 4-h NK assay.

Assay for ADM Equivalents in PEC. ADM was extracted from 10^6 PEC/sample by sonication in 36.5% HCl/absolute ethanol (1:11) in an ice bath for 5 min. This sonicated lyase was centrifuged (12,150 \times g) for 20 min at 4–6°C. The supernatant was measured for fluorescence at 668 nm excitation and 585 nm emission using a Perkin-Elmer (Model 650-10S) spectrofluorometer.

Assay for IL-1 Activity. A standard thymocyte comitogenic assay for IL-1 (19) was used to test 18-h adherent PEC culture supernatants for IL-1-like activity. Thymocytes (5 \times 10^4) from C3H/HeJ mice were added in 0.1 ml/well to flat-bottomed, 96-well plates. An equal volume of adherent PEC culture supernatant was added for a final volume of 0.2 ml. To facilitate a proliferative response, the media were supplemented with phytohemagglutinin (0.8 \mug/ml) and 2-mercaptoethanol (2.5 \times 10^3 \muM). Cultures were incubated for 72 h. Four hours before harvesting, the cultures were pulsed with [3H]thymidine (New England Nuclear; 2 Ci/mm; 1 \muCi/50 \mul/well).

Cells were harvested on glass fiber filters with an Otto Hiller cell harvester. The radioactivity was measured by scintillation counting using a Beckman counter (Model LS 7000). Thymidine uptake by the thymocytes was expressed as mean cpm ± SD.

Assay for IL-2 Activity. This procedure was described in a previous report (20). Briefly, using a long-term IL-2-dependent cytolytic T-cell line (CTCL-1), 10^5 cells in 0.1 ml were added to 0.1-ml volumes of conditioned media from adherent PEC cultures. The cells were incubated for 24 h and pulsed with [3H]thymidine (1 \muCi/well) 4 h before harvest. Results are expressed as the arithmetic mean of cpm dThd uptake ± SD. A semipurified rat IL-2 preparation was used as a positive control.

Data Presentation and Statistical Analysis. The data presented are from triplicate cultures ± standard deviation. All experiments have been repeated at least 3 times, and one representative experiment is presented unless otherwise stated. The Student’s t test was used to evaluate the statistical significance of differences between experimental values where indicated.

RESULTS

Kinetics of Drug-induced Effects on Macrophage Cytotoxicity. An ex vivo experimental system was used to analyze the role free or encapsulated ADM could have on macrophage tumoricidal activity. Different groups of mice were injected i.p. on various days prior to harvesting peritoneal exudate cells. The experimental groups used in all experiments were (a) untreated control mice, (b) control mice injected with liposomes, (c) mice injected with ADM (10 mg/kg), and (d) mice injected with ADM/Lip (10 mg/kg). Peritoneal macrophages adherent to plastic were cultured with or without LPS for 18 h before cytotoxic activity was measured in a standard 18-h chromium release assay. An earlier report had demonstrated that ADM induces tumoricidal peritoneal macrophages in a similar system (9). Therefore, in initial experiments the possibility that drug encapsulation would alter the duration and/or magnitude of such a response was evaluated on various days following treatment (Fig. 1). In all treatment groups, only background activity (0–8% specific 51Cr release) was observed with adherent PEC cultures without LPS addition. LPS-stimulated cytosis of P815 cells by control or Lip-control PEC was at background levels throughout (data not shown) while drug-treated groups had elevated activity on Days 4 through 14. Only LPS (1.0 \mug/ml)-stimulated PEC from the ADM/Lip-treated mice displayed significant macrophage tumoricidal activity 18 days after treatment (P < 0.05). This was a consistent finding observed in a total of 3 separate experiments. Macrophage activation at lower LPS concentrations (10^{-1}–10^{-2} \mug/ml) was greatest on Day 7 regardless of the mode of ADM administration.

Kinetics of Drug-induced Effects on PEC-NK Activity. To ascertain if under similar conditions the effect of liposome
encapsulated ADM was different from that of free drug on a second nonspecific effector mechanism, PEC-NK activity was assayed. Freshly harvested peritoneal exudate cells were mixed with radiolabeled YAC-1 lymphoma cells (10⁴) in a 4-h lytic assay (Fig. 2). Resident PEC displayed minimal NK activity as did PEC from liposome-treated control mice. In contrast, similar high levels of PEC-NK activity were attained 4 days after ADM or ADM/Lip treatment (Fig. 2). The ADM/Lip treatment was more effective than that with free drug in sustaining PEC-NK activity on Days 7, 11, and 14 (P < 0.01). Both diminished thereafter and no differences between the two drug treatments were seen on Day 18 (Fig. 2) or on Days 21 or 27 (data not shown). The NK activity in both drug-treated groups, however, remained significantly (P < 0.01) above control levels on all days tested.

IL-1-like Activity of PEC Culture Supernatant. Recent studies have proposed many different biological activities for IL-1. Enhancement of the tumoricidal activity of macrophages and NK cells are two such activities (21). Furthermore, Cohen et al. reported the production of an IL-1-like activity by PEC from ADM-treated mice (22). Supernatants of 18-h cultures of adherent peritoneal macrophages (Day 7), therefore, were analyzed in the standard thymocyte proliferation assay for the detection of IL-1 activity (Table 1). Since IL-2 is also active in this assay system, supernatants were simultaneously tested for IL-2 activity using an IL-2 dependent CTCL-1 line (20). All supernatants with significant activity in the thymocyte proliferation assay for IL-1 were inactive in the IL-2 assay. Control and Lip-control PEC supernatants had detectable IL-1-like activity only following stimulation of the cells with the highest tested LPS concentration (1000 ng/ml). In cultures without LPS stimulation only ADM PEC released factor(s) with IL-1 activity into cell-free supernatants (P < 0.05). Supernatants from both ADM and ADM/Lip PEC had IL-1-like activity following stimulation with LPS at concentrations ranging from 0.001 to 1000 ng/ml (P < 0.01). While the elaboration of IL-1-like activity from ADM/Lip PEC was dependent on the concentration of LPS, no significant increase in this activity from ADM PEC was observed with LPS exposure.

PEC-ADM Content. As an indication of ADM retention by cells in the peritoneal cavity, PEC lysates were assayed for ADM (fluorescence equivalents; Table 2). Slight fluorescence was detectable 4 and 7 days after free drug administration. Increased levels of fluorescence were observed in ADM/Lip PEC on Days 4 and 7 but by Day 11 although some fluorescence was still detected, the level was not statistically significantly greater than that of the control PEC.
Table 1 IL-1 and IL-2 activities in supernatants from 18-h cultures of adherent PEC ± LPS

<table>
<thead>
<tr>
<th>LPS (ng/ml)</th>
<th>Control</th>
<th>Lip</th>
<th>ADM</th>
<th>ADM/Lip</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.226 ± 405</td>
<td>1.930 ± 185</td>
<td>9.142 ± 1.179&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.942 ± 534&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,000</td>
<td>4.821 ± 1.096&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.120 ± 1.520</td>
<td>10.598 ± 831&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.827 ± 279&lt;sup&gt;d&lt;/sup&gt; &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>2.653 ± 452</td>
<td>2.492 ± 933</td>
<td>10.446 ± 226&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.140 ± 711&lt;sup&gt;d&lt;/sup&gt; &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1</td>
<td>2.262 ± 319</td>
<td>2.218 ± 567</td>
<td>11.588 ± 953&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.553 ± 483&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.001</td>
<td>2.240 ± 582</td>
<td>1.961 ± 138</td>
<td>9.693 ± 230&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.720 ± 599&lt;sup&gt;d&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Cytotoxicity assay: Peritoneal exudate cells (5 x 10<sup>5</sup>) were cultured with IC<sub>f</sub> radiolabeled YAC-1 cells and treated with anti-asialo GM<sub>1</sub> antibody and complement. Percentage of specific <sup>51</sup>Cr release was determined after 4 h. The medium was removed and complement in fresh medium (final dilution, 1/20) was added for 45 min at 37°C. The medium was removed and complement in fresh medium (final dilution, 1/20) was added for 45 min at 37°C. The medium was removed and complement in fresh medium (final dilution, 1/20) was added for 45 min at 37°C. The medium was removed and complement in fresh medium (final dilution, 1/20) was added for 45 min at 37°C.

Table 2 ADM equivalents in PEC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip</td>
<td>0.2 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>ADM</td>
<td>4.2 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>ADM/Lip</td>
<td>14.4 ± 6.2</td>
<td>3.5 ± 1.5</td>
<td>2.1 ± 2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of 3 experiments, ADM equivalents (ng/10<sup>6</sup> cells) ± SD.

Table 3 Elimination of Day 7 PEC natural killer activity by anti-asialo GM<sub>1</sub> antibody and complement

<table>
<thead>
<tr>
<th>PEC population</th>
<th>Untreated&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Anti-asialo GM&lt;sub&gt;1&lt;/sub&gt; antibody + complement&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment no. 1</td>
<td>2.5 ± 1.0</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>Control</td>
<td>4.8 ± 0.4</td>
<td>0.8 ± 0.9</td>
</tr>
<tr>
<td>Lip-control</td>
<td>16.9 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>ADM/Lip</td>
<td>37.1 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Experiment no. 2</td>
<td>0.7 ± 2.0</td>
<td>0.7 ± 2.0</td>
</tr>
<tr>
<td>Lip-control</td>
<td>2.1 ± 2.1</td>
<td>0.2 ± 0.8</td>
</tr>
<tr>
<td>ADM/Lip</td>
<td>3.49 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 ± 0.8</td>
</tr>
</tbody>
</table>

<sup>b</sup> Peritoneal exudate cells were cultured as in <sup>a</sup> either without the addition of antibody and complement (untreated), or with the addition of only one of the two (data not shown since it was not significantly different).

<sup>c</sup> Anti-asialo GM<sub>1</sub> antisera (final dilution, 1/20) was added to PEC for 30 min at 4°C. The medium was removed and complement in fresh medium (final dilution, 1/20) was added for 45 min at 37°C. After 3 washes, radiolabeled YAC-1 target cells were added for the 4-h NK assay.

<sup>d</sup> Significantly different from control PEC activity (P < 0.001).

<sup>e</sup> ADM/Lip PEC activity significantly greater than ADM PEC activity.

Characterization of NK Peritoneal Effector Cells from Day 7 Treated Mice. In order to verify that the effector cells in the PEC population that were active against YAC-1 tumor cells had NK properties, PEC were treated with anti-asialo GM<sub>1</sub> antisera and complement (Table 3) in vitro prior to the 4-h cytotoxicity assay. Complement or antibody alone controls did not alter the responses (data not shown). The anti-YAC-1 activities of ADM PEC and ADM/Lip PEC were abrogated by in vitro asialo GM<sub>1</sub> antibody plus complement treatment. Since asialo GM<sub>1</sub> antigen can be expressed on certain subsets of macrophages (23), a population depleted of macrophage by plastic adherence was examined. The nonadherent cell population was recounted and assayed in parallel with corresponding whole PEC populations (Fig. 3). Consistent with the properties of NK cells the PEC-NK activity of the nonadherent effector cells was similar to that of the whole effector cell populations with the overall pattern of activities being the same. In summary, drug-induced PEC-NK activities had characteristics ascribed to noninduced natural killer cells.

Spleenic NK Activity. The i.p. administration of ADM (10 mg/kg) to C57BL mice had been previously shown to result in a decrease in NK activity in the spleen (24). Fresh spleen cells were added to <sup>51</sup>Cr-labeled YAC-1 cells at different effector:target cell ratios of 12.5:1 (●), 25:1 (▲), and 50:1 (□). * significantly different from control (P < 0.01), 0, ADM/Lip significantly different from ADM (P < 0.01).

Fig. 3. NK activity, 7 days after treatment, of whole PEC in comparison to that of nonadenheter PEC. Activity of whole PEC or those nonadherent to plastic after 2 h was assessed in a 4-h chromium release assay. Results shown were obtained at effector:target cell ratios of 12.5:1 (●), 25:1 (▲), and 50:1 (□). * significantly different from control (P < 0.01). 0, ADM/Lip significantly different from ADM (P < 0.01).

DISCUSSION

Previous studies have implicated macrophages in the antitumor effectiveness of ADM (5, 6). Studies with allogeneic and syngeneic tumor models have offered support to the postulate that ADM can activate macrophages in vivo (5, 6, 9, 11, 12, 25). Investigations performed with macrophages exposed to drug in vitro have demonstrated their resistance to the direct cytotoxicity of ADM and their ability to store and subsequently release drug at levels sufficient to exert tumor cytoidal activity (7, 8, 26). These findings suggest that the storage and release of drug by macrophages in ADM-treated animals may be therapeutically advantageous at tumor sites with macrophage infil-
tation. Such redistribution of drug by the reticuloendothelial system does not preclude the concurrent induction of augmented host responses for which there is considerable experimental evidence (4, 9, 22, 24, 25, 27, 28).

Liposomes as a drug delivery system may offer some therapeutic advantages in the delivery of cytotoxic agents to tumors (14). Thus when free and encapsulated ADM are compared on different schedules, such as a single i.v. injection, 5 daily i.v. injections, or 5 biweekly i.v. injections, ADM/Lip is approximately one-third as toxic as free ADM (29). The maximal tolerated single i.v. push doses of free ADM and ADM/Lip are approximately 10 mg/kg and 30 mg/kg, respectively. α-Tocopherol was included in the liposome preparations in order to reduce peroxidation of liposomal lipids and the possible generation of toxic free radicals by ADM. Whereas α-tocopherol in liposomes has been shown to reduce lipid peroxidation (30) its effects on ADM entrapped in liposomes have not been investigated. ADM/Lip containing α-tocopherol has been used previously in therapeutic (15, 16) and toxicity studies (29).

In experimental models, the route of administration (i.v., i.p., p.o.) and the location of the tumor are critical considerations in designing effective treatment protocols using liposomes. For example, ara-C entrapped in liposomes can increase the therapeutic index against i.p. inoculated L1210 by single i.p. or i.v. injections compared with an i.p. multiple dose schedule of free ara-C or i.v. infused ara-C (31). When ADM/Lip is administered i.p. the liposomes leave the peritoneal cavity via the lymphatics and are retained in lymph nodes (32). Since in metastatic ovarian cancer these regional lymphatics often become occluded with tumor, this is a situation in which liposomes could also improve drug delivery to critical sites. A similar approach has been used in chemotherapeutic studies in murine liver metastatic models. ADM/Lip by the i.v. route was more effective than free ADM against liver metastasis of a reticulum cell sarcoma (15) and two colon carcinoma cell lines (16). Furthermore, liposome uptake by the reticuloendothelial system has been shown to facilitate delivery of biological response modifiers to effector cells in the lungs, liver, spleen, and lymph nodes (13).

A previous report (9) indicated that administration of free ADM (10 mg/kg, i.p.) to mice induced activation of peritoneal macrophages to a tumoricidal state. In this study macrophages from mice 7 days after ADM treatment apparently did not require in vitro activation and were cytolytic for tumor cells in the absence of detectable drug. In contrast to these reported findings, in the study reported herein it was found that while macrophage activation was also augmented 7 days following administration of ADM it was dependent upon the addition of relatively low concentrations of LPS (0.5–1.0 μg/ml) to the culture media. This difference may be due to differences in the strains of mice (C3Heb/FeJ versus C57BL/6) or fetal calf serum content of such components as endotoxin. Macrophages from mice treated with ADM therefore correspond to “primed macrophages” (i.e., previously exposed to a priming signal such as lymphokine and/or γ-interferon) and require only a triggering signal such as LPS (33) for activation to occur. Weinberg et al. (34), using populations of cloned macrophages, observed that differentiation of macrophages toward the tumoricidal state parallels their responsiveness to LPS. In their report, in vivo Baccillus Calmette-Guérin primed macrophages were also induced to be tumoricidal by relatively low LPS concentrations in vitro (0.5–1.0 μg/ml). Thus, the macrophage from the ADM-treated mice, regardless of mode of drug administration, apparently had undergone regulatory signaling in vivo resulting in a primed state similar to that induced by BCG.

Increases (2- to 3-fold) in the number of cells harvested on Day 7 from the peritoneal cavity of ADM- (but not ADM/Lip)-treated mice were seen in this study. Such ADM-induced increased cell recoveries have been observed in previous studies (9, 24) and are consistent with a drug-induced local inflammation. Similar to Salazar and Cohen’s reported cytology findings (9) there was no marked change in the percentage of the total PEC population which were morphologically identifiable as macrophages (data not shown). It is not clear, however, whether the increased numbers of cells, including macrophages, in the peritoneal cavity resulted from proliferation in situ or migration from other sites. Nevertheless, ADM-induced changes in macrophage function in this local environment would appear to be a combined result of inflammation and effects on regulatory signaling.

The elaboration of an IL-1-like soluble factor and the augmentation of NK activity observed with PEC from mice 7 days after ADM treatment are functional capacities commonly associated with macrophage activation (33). In a recent study using human peripheral blood monocytes as effector cells, monocyte-derived IL-1 did not induce cytotoxic macrophages (35). Human IL-1 was found, however, to maintain the responsiveness of monocytes for cytocidal activation by LPS or lymphokine (35) and it may be playing a similar role in the ADM-induced effects studied here. In comparison to PEC from mice treated with ADM 7 days earlier, those of mice treated with ADM/Lip had similar tumoricidal macrophage activity and greater NK activity, but their ability to elaborate IL-1-like soluble factors was dependent upon in vitro LPS stimulation. This difference between the induction of functional activity of macrophages following administration of ADM or ADM/Lip may be similar to the recently reported in vitro finding that agents such as LPS, γ-interferon, and muramyl tripeptide-phosphatidylethanolamine in the free form stimulate IL-1 release but are ineffective when encapsulated in liposomes (36).

The results of the kinetic experiments showed that the magnitude of optimal PEC macrophage or NK cytolytic activity obtained after ADM administration was similar whether the drug was given in free or encapsulated form. The administration of ADM/Lip, however, extended the duration of the drug-induced priming of peritoneal macrophages up to 18 days following treatment. While the levels of peritoneal NK from ADM-treated mice were augmented on all days compared to
control, they were even greater following ADM/Lip treatment on Day 7, 11, or 14. This extension in the duration or magnitude of augmented PEC macrophage and NK activities, respectively, may be due to a persistence of intra- and/or extracellular drug in the peritoneal environment following ADM/Lip treatment, but significant levels of ADM-associated fluorescence were not detectable in extracts of cells obtained from animals 11 days posttreatment. Tumor cell cytolyis by rerelease of ADM from macrophages in the in vitro assays has been the subject of some debate (7, 9, 10), however, it is unlikely to have occurred at time points as late as those (14 or 18 days) studied herein. Furthermore, cytolytic activity was not observed with macrophages from drug-pretreated mice, on any of the days tested, without LPS stimulation. Even if one were to postulate that LPS stimulation elicits ADM release from macrophages, this should not have been a factor since the assay procedure included washing between LPS stimulation and addition of radiolabeled P815 target cells. Finally, the fact that PEC cytolytic activity was lower on Day 4 than for Day 7 also argues against direct drug transfer as a mechanism and suggests that the effects at later time points (Days 7, 11, 14, 18) are due to earlier drug interactions with host defenses.

The depressed splenic NK activity observed following ADM or ADM/Lip treatment may represent an immunomodulation by activated macrophages in the spleen. Removal of nylon wool adherent spleen cells from the effector cell population has shown to reverse this suppression (24). While the mechanism of this macrophage-mediated suppression has not been completely verified, it may involve cyclooxygenase metabolites such as prostaglandin E2 which have been found to be elevated in spleen cells from ADM-treated mice (20). In this study, the observed suppression of splenic NK was transient in both ADM- and ADM/Lip-treated mice. The effect of ADM on the tumoricidal activation of splenic macrophages is currently under investigation.

Increases in the survival of mice treated with ADM prior to tumor challenge have been reported (25). In addition, the administration of ADM combined with the macrophage activator maleic anhydride divinyl ether (37) or the lymphokine IL-2 (38) increased the survival of tumor-bearing mice. Although immunomodulation by ADM was not directly considered in these latter studies, these findings support the possibility that the ADM-induced augmentation of tumoricidal macrophage and natural killer activities observed in vitro have therapeutic relevance in vivo. The addition of biological response modifiers to a drug protocol such as that studied herein might be facilitated by the less stringent time dependency of macrophage activation and enhancement of NK activity with effector cells from ADM/Lip-pretreated mice versus ADM-pretreated mice.

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REFERENCES


Alterations in Murine Host Defense Functions by Adriamycin or Liposome-encapsulated Adriamycin

Kenneth Mace, Eric Mayhew, Enrico Mihich, et al.