**In Vitro and in Vivo Effect of Adriamycin Therapy on Monocyte Activation by Liposome-encapsulated Immunomodulators**

Melissa M. Hudson, John S. Snyder, Norman Jaffe, and Eugenie S. Kleinerman

Departments of Cell Biology [M. M. H., J. S. S., E. S. K.] and Pediatrics [M. M. H., N. J., E. S. K.], The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

The purpose of these studies was to determine the effect of Adriamycin (ADR) on the ability of liposome-encapsulated immunomodulators to activate human blood monocytes to the tumoricidal state. We undertook these experiments because we envisioned using encapsulated activators in addition to chemotherapy to destroy pulmonary micrometastases in patients with osteosarcoma (OS). Prior to the initiation of such therapy, it was important to determine whether chemotherapy interferes with monocyte function. First, human peripheral blood monocytes were isolated from normal donors and preincubated with ADR (0.5-500 ng/ml) for 1 h and then washed prior to the addition of free or liposome-encapsulated activators. After 18-24 h incubation, the activating agents were washed off and [3H]IdUrd-labeled A375 melanoma cells were added. Lysis of radiolabeled tumor cells was quantified 72 h later. Monocytes were also incubated with ADR for 24 h in the presence of free or liposome-encapsulated activators and their cytotoxicity quantified. ADR had no effect on the ability of either free or liposome-encapsulated agents to activate monocyte tumoricidal function.

We also studied the in vivo effect of ADR therapy on monocyte function in nine patients with OS. At the time of diagnosis and 1 month after ADR therapy (75 mg/m²) patient monocytes could be activated to the tumoricidal state by liposome-encapsulated agents at levels equal to or greater than pretherapy levels. Monocytes isolated from four patients with OS 1 day after ADR therapy and then activated by liposome-encapsulated agents also demonstrated tumoricidal activity. These studies indicated that the monocytes isolated from osteosarcoma patients treated with ADR can be activated in vitro to kill tumor cells and that additional therapy with liposome-encapsulated immunomodulators may be combined with ADR in the treatment of metastatic pulmonary OS.

INTRODUCTION

Adjuvant chemotherapy after surgical resection of primary osteosarcoma has improved survival rates from 20% to 2 years to approximately 60% at 5 years (1-3). Unfortunately, despite the use of effective adjuvant chemotherapy, about 40% of patients with OS still develop pulmonary metastases. In our experience approximately 30% of the total patient population develop their pulmonary metastases while receiving adjuvant chemotherapy suggesting residual tumor cell clones proliferate. To improve these results, we hope to use monocytes activated by liposome-encapsulated immunomodulators in vivo as an additional adjuvant therapy for micrometastatic pulmonary disease in patients with OS.

Human monocytes can be activated to the tumoricidal state following incubation with a variety of soluble and liposome-encapsulated agents (4). Furthermore, these activated monocytes selectively lyse malignant cells, not normal cells (5). Eighty to ninety % of liposomes injected i.v. into mice are taken up in the liver, spleen, and peripheral blood monocytes; 8-10% localize in the pulmonary microvasculature without evidence of local or systemic toxicity (6, 7). Liposome-encapsulated materials can thus be directed to pulmonary macrophages to augment the host's defense against pulmonary metastases. Indeed, it has been demonstrated that multiple i.v. injections of liposome-encapsulated agents activate murine pulmonary macrophages, leading in turn to eradication of pulmonary micrometastases (8, 9).

We believe it is important to combine this liposome treatment with other established treatment modalities early in therapy in an attempt to salvage the significant patient population who develop metastases during therapy. However, if chemotherapy interferes with monocyte function, the proposed therapy would offer no advantage for increased tumor kill. Because Adriamycin is a mainstay of many chemotherapeutic regimens used to treat OS, we determined whether ADR treatment would interfere with liposome therapy's potential. To that end, we evaluated the effect of ADR on monocyte activation by liposome-encapsulated agents using an in vitro tumor cell cytotoxicity assay. We also evaluated the in vivo effect of ADR on monocyte function in children with OS.

MATERIALS AND METHODS

Reagents. RPMI 1640 medium, HBSS without Ca²⁺ or Mg²⁺, FBS, and minimal essential medium with Earle's balanced salt solution (CMEM) were purchased from M. A. Bioproducts, Walkersville, MD. rIFN-γ was supplied by Genentech, Inc., South San Francisco, CA. Recombinant interleukin-1β was purchased from Cistron (Pine Brook, NJ). MDP was the gift of Ciba-Geigy Ltd., Basel, Switzerland. Human AB serum was purchased from Sigma, St. Louis, MO.

Drugs. Adriamycin (NSC 123127) was obtained from the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment, National Cancer Institute. It was suspended in sterile distilled water at 1 mg/ml and then serially diluted in RPMI 1640 medium with 5% human AB serum. The drug was used within 60 min following suspension.

Separation of Monocytes. Mononuclear leukocytes were collected from the peripheral blood of normal donors by separation on Ficoll-Hypaque gradients (1300 x g for 10 min) (lymphocyte separation medium; Litton Bionetics; Kensington, MD) and washed twice in HBSS (50 x g for 15 min) to remove platelets. Mononuclear leukocytes were further purified by being layered onto performed continuous Percoll gradients in 50-ml polycarbonate tubes and spun in swing-out buckets (275 x g for 50 min). Upon centrifugation, cell populations separated into two distinct bands in the Percoll gradient on the basis of their relative densities. The upper band contains monocytes (75-90%) as determined by nonspecific esterase staining and morphological examination, while the lower band is primarily composed of lymphocytes...
cells were added as described below. After 18 to 24 h incubation the culture wells were washed twice with HBSS to remove activating agents and [125I]IdUrd-radiolabeled target medium or ADR for 24 h in the presence of free or encapsulated activators. Monocytes were also incubated with 5% human AB serum. Monocytes were preincubated with medium or ADR (0.5-500 ng/ml) for 1 h and then washed twice with HBSS prior to the addition of free or liposome-encapsulated activators. Free activators included RPMI 1640 with 5% human AB serum with LPS (1 μg/ml) or IFN-γ (10^4 U/ml) plus MDP (100 ng/ml). The liposome-encapsulated activator used was MTP-PE, which is a lipophilic derivative of MDP (see “Preparation of Liposomes”). Liposome preparations were also suspended in RPMI 1640 with 5% human AB serum. Monocytes were also incubated with medium or ADR for 24 h in the presence of free or encapsulated activators. After 18 to 24 h incubation the culture wells were washed twice with HBSS to remove activating agents and [3H]dThd-radiolabeled target cells were added as described below. Cells cultured from a tumor line derived from human melanoma, was provided by Dr. Raymond Ruddon (National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD). Monolayer cultures were maintained on plastic in CMEM supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, twofold-concentrated vitamin solution, and L-glutamine (M. A. Bioproducts) at 37°C in an incubator providing a humidified atmosphere containing 5% CO2 in air.

The murine helper T-cell clone D10.G4.1 was kindly provided by Dr. C. Janeway. The cell line was called in interleukin-2-supplemented Clic’s medium and passed in C57B1/6 spleen cells every 3 weeks. C57B1/6 spleen cells cultures were both the alloantigen H-2K recognized by the D10 clone and the feeder effect. Assays of Monocyte-mediated Cytotoxicity. Cytotoxicity was assessed by lysis of radiolabeled A375 tumor cells in a 72-h assay. Target cells in exponential growth phase were incubated for 24 h in medium containing [3H]dThd (0.3 μCi/ml; specific activity, 200 mCi/μmol; New England Nuclear, Boston, MA). The cells were washed twice to remove unincorporated radiolabel and harvested by trypsinization with 0.25% trypsin (Difco) and 0.02% EDTA for 1 min and washed again. The labeled cells were resuspended in CMEM supplemented with 5% FBS, and 10^5 target cells were plated into the culture wells to provide a 1:20 target-to-effector cell ratio. Radiolabeled tumor cells were also plated alone as an additional control group. The culture wells were washed after 24 h to remove nonadherent tumor cells, refed with fresh medium, and then incubated for an additional 48 h.

Cell-to-cell contact between effector and target cells is required for killing to occur in this adherent cell assay. Washing the culture wells after 24 h removed the potential error introduced by cells that did not adhere but were not necessarily killed in the 3-day assay. At 72 h after the addition of the target cells, the assay was terminated by washing culture wells two times with HBSS. The adherent viable cells were lysed with 0.1 ml of 0.5 N NaOH, and the radioactivity in the lysate was measured in a gamma counter. The cytotoxic activity of the monocyte was calculated as follows.

\[
\text{% spontaneous cytotoxicity} = \left( \frac{\text{cpm of target cells alone}}{100} \right) - \left( \frac{\text{cpm of target cells with control monocytes}}{\text{cpm of target cells alone}} \right)
\]

\[
\text{% generated cytotoxicity} = \left( \frac{\text{cpm of target cells with control monocytes}}{100} \right) - \left( \frac{\text{cpm of target cells with activated monocytes}}{\text{cpm of target cells with control monocytes}} \right)
\]

Statistical Analysis. Experimental results were analyzed for their statistical significance by Student's t test.

Preparation of ADR-stimulated Supernatants for IL-1 Assays. To determine if ADR stimulates monocyte production of soluble IL-1, peripheral blood monocytes obtained from normal donors were separated and cultured as described above. Monocytes (1 x 10^6) were added to each well of a 24-well flat-bottomed plate (Falcon, Oxford, CA) and allowed to adhere for 1.5 to 2 h at 37°C. Nonadherent cells were then removed by washing three times with HBSS. ADR (500 ng/ml) or LPS (1 μg/ml) or LPS (1 μg/ml) (positive control) was added to the culture wells. A second group of monocytes was treated with LPS (1 μg/ml) in the presence of ADR to determine if ADR had any effect on the ability of LPS to stimulate monocyte-mediated IL-1 production. Supernatants were collected separately 24 h later, spun at 225 x g for 5 min to remove residual cells and cellular debris and stored at —20°C. Monocyte cultures were then refed with CMEM supplemented with 5% FBS and supernatants were again collected at 48 h, spun and frozen.

For preparation of intracellular IL-1, the monocyte monolayers were washed thoroughly, fed with fresh medium, frozen at —20°C and thawed. This freeze-thawing process was repeated three times and the preparations were centrifuged and the supernatants harvested.

ADR was removed from the supernatants by extensive dialysis against distilled water (Spectrograde no. 1, M, 600-8000; Spectrum, Los Angeles, CA) in the event that ADR reduced nonspecific toxicity to the D10.G4.1 cell line. To assure that ADR would not interfere with secreted IL-1 and could be successfully dialyzed from the supernatant, the positive control supernatant (LPS-stimulated) was divided into two fractions, and ADR (500 ng/ml) was added to one fraction. Both fractions were then dialyzed as described above prior to use in the IL-1 assay.

D10.G4.1 Assay. Intracellular and extracellular IL-1 activities were measured by using the D10.G4.1 murine T-cell line as previously described (10). Following exposure to IL-1 and concanavalin A, these T-cells will proliferate and their proliferation can be quantified by using [3H]Tdr incorporation. Briefly, D10.G4.1 cells were cultured in bulk quantities for 14 to 16 days following exposure to H-2K^b antigen-presenting spleen cells (C57B1/6) and frozen at 2.5 million cells/aliquot in medium containing 20% FBS and 8% dimethyl sulfoxide. On the day of the assay, cells were quickly thawed, washed twice with medium, and seeded at 10,000 cells/well in medium containing 2.5 μg/ml concanavalin A (Sigma, St. Louis, MO) together with serial dilutions of collectible test and positive control supernatants in 96-well microtiter plates. After 48 h incubation, 0.2 μCi of [3H]Tdr was added to each well. Cells were harvested in a semiautomatic cell harvester (Skatron Inc., Sterling, VA). The amount of [3H]Tdr incorporation was quantified by using a scintillation counter.

Preparation of Liposomes. Lyophilized liposomes with MIP-PE were supplied by Ciba-Geigy, Basel, Switzerland. MLVs were prepared from the freeze-dried liposome preparations by adding 2.5 ml of phosphate buffered saline without Ca^2+ or Mg^2+ to the vials containing 1-palmitoyl-2-oleoyl-phosphatidylcholine and dioleoyl-phosphatidylserine (7:3 molar ratio, 250 mg of total lipids) with or without 1 mg of MTP-PE. Prior to use in assays all vial contents were again vortexed for an additional 30 min. The MLVs were reproducible and uniform with respect to particle size distribution (mean size, 2.3 μm) as determined by image analysis as described previously (11). Serial dilutions of the MLV ranging from 25 to 100 nmol phospholipid/well were made using RPMI 1640 medium supplemented with 5% human AB serum. Hereafter MLVs containing MTP-PE will be referred to as test liposomes and MLVs without MTP-PE will be referred to as control liposomes.

Phagocytosis of Liposomes. Peripheral blood monocytes were isolated from normal donors as described above and adjusted to 10^6 monocytes/ml in RPMI 1640 medium with 5% human AB serum. Monocytes were plated on a 12-mm diameter round sterile glass coverslip in a 24-well tissue culture dish. After 1–1.5 h, nonadherent cells were removed by washing with HBSS three times. Liposomes were added directly to the monolayers (1000 nmol/well in 10 μl) and incubated.
bated for 30 min before the addition of medium alone or medium containing ADR (500 ng/ml). The monocyte cultures were then placed in 37°C incubator providing a humidified atmosphere containing 5% CO₂.

Monocyte samples were removed at 16–18 h and fixed with 2% paraformaldehyde and 3% glutaraldehyde for 10 min. The samples were stained for 5 min with 2–3 ml of Nile Red (1 μg/ml), washed once with phosphate buffered saline, and examined by fluorescence microscopy using 488/520 excitation/emission filters. Serial frozen sections were cut from other monocyte cultures and postfixed with 0.1 M cacodylate buffer for 1 h and then processed for transmission electron microscopy as previously described (12).

Patients. Our patient population consisted of children referred to the pediatric osteosarcoma service at M. D. Anderson Hospital and Tumor Institute. Age range at diagnosis was 9–16 years. Seven patients were female and four were male. The tumor-bearing bone was the femur in six, tibia in three, humerus in one, and scapula in one: patients were free of metastatic tumor at the initiation of therapy as determined by radiographic studies of the tumor-bearing bone, skeletal survey, chest radiographs, CT scan of lungs, and radionuclide bone scan.

Heparinized blood (10–20 ml) was drawn from nine of the above patients prior to the initiation of therapy and approximately 1 month after i.v. ADR therapy (75 mg/m²). Peripheral blood mononuclear leukocytes were isolated on Ficoll-Hypaque gradients as described above, washed twice in HBSS, and then resuspended in RPMI 1640 medium supplemented with 5% human AB serum. Monocytes (10⁶) were added to each well of a Terasaki monoclonal clusters plate (Costar, Cambridge, MA) and allowed to adhere for 1.5 to 2 h at 37°C. Nonadherent cells were removed by washing the wells three times with HBSS. Monocytes were then incubated at 37°C for 24 h in medium alone, medium containing soluble activators [rIFN-γ (10⁴ U/ml) plus MDP (100 ng/ml) or LPS (1 μg/ml)] or medium containing either control or test liposomes. The monocyte cultures were then washed two times with medium prior to the addition of [³²P]UdR-labeled A375 melanoma tumor cells. Tumor cells were added to give a target-to-effector ratio of 1:20, and monocyte-mediated cytotoxicity was assayed as described above.

A second group of four patients who were clinically free of disease had blood samples drawn immediately prior to ADR therapy and 1 day after ADR therapy. Peripheral blood monocytes were then isolated and the cytotoxicity assay was performed to assess their ability to respond to liposome-encapsulated activators.

RESULTS

Effect of ADR on Spontaneous Monocyte-mediated Cytotoxicity. The effect of ADR on spontaneous monocyte-mediated cytotoxicity was assessed by incubating monocytes from normal donors with serial concentrations of the drug for 1 h. The monocyte cultures were then washed and replaced with medium for 24 h at which time [³²P]UdR-labeled target cells were added. Cytotoxicity was determined after 72 h of cocultivation. Monocytes exposed to ADR for either 1 or 24 h compared with that of control monocytes exposed to medium alone showed no significant difference in percentage of spontaneous monocyte-mediated cytotoxicity (Table 1). These experiments demonstrate that ADR by itself did not directly activate the immune effector cell to become tumoricidal. We have previously demonstrated that ADR enhances the cytotoxic function of monocytes against red blood cells (13). This 24-h red blood cell assay however differs from the 72-h tumor cytotoxicity assay described in this study in that the red blood cells are killed intracellularly and lysis is mediated by oxygen radicals (14). By contrast, tumor cell killing described here occurs extracellularly (15) and is not mediated by oxygen radicals (16).

Effect of ADR on the Activation of Monocyte-mediated Cytotoxicity by Free Activators. The effect of ADR on activation of monocyte-mediated cytotoxicity by free activators was assessed by preincubating peripheral blood monocytes from normal donors with ADR (0.5–500 ng/ml) for 1 h. Culture wells were then washed two times prior to the addition of soluble LPS (1 μg/ml). The second group of monocytes was incubated with ADR for 24 h in the presence of soluble LPS (1 μg/ml), then washed prior to the addition of [³²P]UdR-labeled A375 melanoma cells. Cytotoxicity was determined 72 h later. The free activator LPS (1 μg/ml) produced 38% monocyte-mediated cytotoxic activity (Fig. 1). Monocytes incubated in the presence of ADR for 1 h and then exposed to LPS could still be rendered tumoricidal to similar levels (42–54% cytotoxicity). Likewise, monocytes incubated with LPS in the presence of ADR for 24 h continued to demonstrate monocyte-mediated cytotoxicity against A375 melanoma cells (Fig. 1). Variability within this assay can be as much as 10–15%; therefore, the only significant decrease in tumor killing was noted at the 50 ng/ml ADR dosage (P < 0.02). The reason cytotoxicity decreased at this dose only, a decrease seen in two of three experiments, is unclear.

Effect of ADR on Interleukin-1 Production by Monocytes. Recent studies have suggested that ADR stimulated the production of IL-1 by various cells (17, 18). Since monocyte-mediated tumoricidal activity has recently been shown to be closely associated with monocyte-mediated IL-1 production (19–21), we also assayed the ability of ADR to induce monocyte-mediated IL-1 production. Monocyte cultures were incubated in the presence of ADR (500 ng/ml) for 24 h, the cultured supernatants were harvested, dialyzed to remove ADR, and then assayed for the presence of IL-1. Medium-treated monocytes do not release IL-1 under these conditions. Monocyte cultures incubated with LPS (1 μg/ml) then dialyzed served as a positive control for IL-1 production. Another group of monocytes was treated with LPS (1 μg/ml) in the presence of ADR to assess if the drug interfered with monocyte-mediated IL-1 production stimulated by LPS. All supernatants collected from monocyte cultures 24 and 48 h after treatment with ADR failed to show IL-1 activity, while positive control supernatants col-

Table 1  Effect of ADR on spontaneous monocyte-mediated cytotoxicity

<table>
<thead>
<tr>
<th>Treatment of monocytes</th>
<th>Residual adherent radioactivity (cpm ± SD)</th>
<th>% killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells alone (no monocytes)</td>
<td>4286 ± 354</td>
<td>0</td>
</tr>
<tr>
<td>Medium²</td>
<td>3490 ± 187</td>
<td>0</td>
</tr>
<tr>
<td>ADR (0.5 ng/ml x 1 h)³</td>
<td>3894 ± 40</td>
<td>0</td>
</tr>
<tr>
<td>ADR (5.0 ng/ml x 1 h)³</td>
<td>4200 ± 196</td>
<td>0</td>
</tr>
<tr>
<td>ADR (50 ng/ml x 1 h)³</td>
<td>3511 ± 103</td>
<td>0</td>
</tr>
<tr>
<td>ADR (500 ng/ml x 1 h)³</td>
<td>3718 ± 118</td>
<td>0</td>
</tr>
<tr>
<td>ADR (0.5 ng/ml x 24 h)³</td>
<td>3411 ± 269</td>
<td>0</td>
</tr>
<tr>
<td>ADR (5.0 ng/ml x 24 h)³</td>
<td>3772 ± 339</td>
<td>0</td>
</tr>
<tr>
<td>ADR (50 ng/ml x 24 h)³</td>
<td>3390 ± 241</td>
<td>3</td>
</tr>
<tr>
<td>ADR (500 ng/ml x 24 h)³</td>
<td>3941 ± 493</td>
<td>0</td>
</tr>
<tr>
<td>rIFN-γ + MDP³</td>
<td>1840 ± 105</td>
<td>52</td>
</tr>
</tbody>
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cytes were treated in the presence or absence of ADR (0.5-500 ng/ml) to assess the effect of ADR on the ability of liposome-encapsulated agents to activate monocytes. The second group of monocytes was incubated with ADR for 24 h in the presence of soluble LPS. After 18-24 h of incubation, activating agents were washed off and radiolabeled tumor cells were added. Lysis of radiolabeled A375 melanoma tumor cells was quantified 72 h later. Above data are from one representative experiment of 2. In a third experiment, no decrease in cytotoxic function was observed at 50 ng/ml.

Effect of ADR on Phagocytosis of Liposomes. Liposome uptake is necessary for monocyte activation. Therefore we investigated the uptake of fluorescently labeled liposomes by monocytes in vitro. Fig. 3 shows a transmission electron micrograph of normal donor monocytes incubated with test liposomes in the presence or absence of ADR. Monocytes incubated with test liposomes in the presence of ADR showed no difference in numbers of liposomes inside the cells compared with control monocytes.

Effect of ADR on the Activation of Monocyte-mediated Cytotoxicity by Liposome-encapsulated Activators. To assess the effect of ADR on the ability of liposome-encapsulated agents to activate monocyte-mediated tumoricidal function, monocytes were treated in the presence or absence of ADR (0.5-500 ng/ml) with different concentrations of control or test liposomes ranging from 25 to 100 nmol phospholipid/2 x 10^6 cells. As an additional control, monocytes were also treated with free LPS (1 µg/ml) or rIFN-γ plus MDP. Monocytes incubated with free LPS demonstrated 40 ± 6% tumoricidal activity. The incubation of monocytes with test liposomes led to the generation of 39 ± 3% cytotoxicity. Monocytes incubated in the presence of ADR for 24 h during the activation process with test liposomes likewise showed cytotoxic activity of 31 ± 9% (Fig. 4). The Student's t test shows no significant difference among these mean values. We conclude that in vitro ADR had no effect on the ability of the liposome-encapsulated immunomodulator MTP-PE to activate tumoricidal function in normal donor monocytes. Pretreatment of monocytes with ADR for 1 h also failed to inhibit the ability of liposome-encapsulated agents to activate tumoricidal function (data not shown). Additional experiments in which tumor cell targets were cocultured with both liposome-activated monocytes and suboptimal doses of ADR revealed no synergistic or additive effects between monocyte cytotoxic activity and ADR (data not shown).

Effect of ADR Therapy on Patient Monocyte-mediated Cytotoxicity by Liposome-encapsulated Activators. To determine if ADR therapy in vivo affects monocyte function in patients with OS, peripheral blood monocytes were isolated separately from nine patients with OS at the time of referral, prior to the initiation of any therapy, and 1 month after ADR therapy (75 mg/m²). These monocytes were then incubated with control or test liposomes (25-100 nmol/2 x 10^6 cells). Monocytes were also treated with free activators (LPS, 1 µg/ml, or rIFN-γ + MDP) as an additional control to rule out any inherent monocyte defect. Fig. 5 shows the results of these studies. The peripheral blood monocytes from each patient isolated 1 month after ADR therapy could be activated in vitro by test liposomes to kill tumor cells. In fact, the activated cytotoxic function was increased in seven of nine patients. Student's t test showed this increase to be of statistical significance (P < 0.02). Similar results were obtained with the peripheral blood monocytes from these patients 2 and 3 weeks after ADR therapy (data not shown). Thus, ADR appeared to have no adverse effect on the ability of test liposomes to activate monocytes in vitro. There...
ADRIAMYCIN THERAPY ON MONOCYTE ACTIVATION

Fig. 3. Phagocytosis of liposomes by human blood monocytes. Transmission electron photomicrographs of human blood monocytes after 24 h incubation with test liposomes containing MTP-PE in the presence (A) and absence (B) of ADR. L, intracellular liposome; bar, 2 μm.

Fig. 4. Effect of ADR on the activation of monocyte-mediated cytotoxicity by liposome-encapsulated activators. Human peripheral blood monocytes were isolated from normal donors and incubated with ADR (0.5 to 500 ng/ml) for 24 h in the presence of free rIFN-γ (10⁴ U/ml) + MDP (100 ng/ml) or liposome-encapsulated MTP-PE. After 18–24 h incubation, the activating agents were washed off and [³²P]dUrd-labeled A375 melanoma tumor cells were added. Lysis of radiolabeled target cells was quantified 72 h later. Representative experiment of 10.

The monocytes from one patient could not be activated by liposome-encapsulated agents at the time of diagnosis (Fig. 5), and yet these monocytes were stimulated to kill tumor cells by soluble agents (Fig. 6). The failure of liposome-encapsulated agents to activate tumoricidal function may be secondary to the inability of this patient's monocytes to phagocytose the liposomes, although no data supporting this hypothesis are available. However if liposomal uptake does not occur universally in this patient population, liposome therapy may not be effec-
ADRIAMYCIN THERAPY ON MONOCYTE ACTIVATION

Fig. 6. Tumorilytic activity of activated monocytes from one OS patient. Ten ml of heparinized blood was drawn from one patient (see Fig. 4) prior to the initiation of therapy and 1 month after ADR therapy (75 mg/m²). Peripheral blood monocytes were isolated and incubated with free activators [LPS (1 µg/ml) and rIFN-γ (10⁴ U/ml) plus MDP (100 ng/ml)] and test MLVs containing MTP-PE. After 18-24 h incubation the activating agents were washed off and [125I]IdUrd-labeled tumor cells were added. Lysis of radiolabeled cells was quantified 72 h later.

Table 2 Effect of in vivo ADR therapy on monocyte activation

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>% Generated cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free activators*</td>
</tr>
<tr>
<td></td>
<td>Pre-ADR  Post-ADR</td>
</tr>
<tr>
<td>1</td>
<td>55 ± 2  67 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>83 ± 3  83 ± 14</td>
</tr>
<tr>
<td>3</td>
<td>81 ± 6  56 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>56 ± 7  66 ± 2</td>
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</tbody>
</table>

* Free activators consisted of LPS (1 µg/ml) or rIFN-γ (10⁴ U/ml) plus MDP (100 ng/ml).

DISCUSSION

Our results demonstrate that ADR has no effect on the ability of liposome-encapsulated immunomodulators to activate human blood monocytes. Normal monocytes preincubated with ADR exhibited tumorcidal activity after exposure to liposome-encapsulated activators (Fig. 4). Likewise, monocytes isolated from patients with OS who had received ADR 1 day to 1 month previously continued to demonstrate in vitro activation of monocyte-mediated cytotoxicity by liposome-encapsulated activators (Fig. 5). We have also demonstrated that ADR had no
effect on the activation of monocytes by free immunomodulators (Fig. 1) and did not alter monocyte IL-1 secretion (Fig. 2) or monocyte phagocytosis (Fig. 3). These data indicate that ADR probably will not interfere with monocyte activation by liposome-encapsulated agents or subsequent monocyte-mediated tumor cell lysis. Thus, liposome-encapsulated agents may be effectively combined with ADR therapy.

Metastatic pulmonary disease in patients with OS is a significant problem. Fifty to eighty % of patients with OS are considered to have microscopic pulmonary tumor at diagnosis. Eighty % of patients who undergo surgical resection of tumor-bearing bone as their only therapy develop pulmonary metastases 6 to 9 months after diagnosis (1). Combined surgical and chemotherapeutic treatments have resulted in survival rates of 60% at 5 years (1-3). However, a substantial number of patients (40%) still are not responsive to conventional chemotherapy and eventually develop metastases. Even more significant is the 30% of patients with OS who develop pulmonary metastases while receiving adjuvant chemotherapy.

Activation of monocyte-mediated cytotoxicity by liposome-encapsulated activators is a particularly attractive therapy for metastatic pulmonary disease in patients with OS because it is a highly effective way to deliver immunomodulators to macrophages in the pulmonary vasculature (6, 22). Once activated, it is our hope that these pulmonary macrophages will eradicate residual tumor cells. Such cells can be more distal to the blood vessels or in the center of the tumor and thus not be exposed to an adequate concentration of drug. It is unlikely that liposome-encapsulated immunomodulators could serve as a single-therapeutic modality. The ratio of monocyte to tumor cells is critical for successful tumor eradication (22). If the number of tumor cells is too high, the number of macrophages in the body may be inadequate to handle the tumor burden alone. We, therefore, see liposome-encapsulated monocyte-activating agents being used as an addition to existing chemotherapeutic and surgical regimens in an adjuvant setting. We feel it is important to include this therapy early in the course of treatment of OS since only microscopic disease exists in these patients after removal of the primary tumor, thus the tumor burden (i.e., tumor to monocyte ratio) is favorable for effective destruction by activated monocytes.

However, if chemotherapy even transiently depresses monocyte function, the effectiveness of liposome therapy would be decreased. Because of this possibility, it was important to determine the effect of ADR on the activation of monocyte-mediated cytotoxicity by liposome-encapsulated activators. ADR was chosen because it is frequently used in chemotherapeutic protocols for OS. We assayed the ability of patients' monocytes to be activated by liposome-encapsulated agents at various times after ADR therapy to determine the timeframe of chemotherapeutic effects. This information is vital to proposing treatment schedules that would include liposome therapy. Our data indicate that monocytes isolated from osteosarcoma patients treated with ADR can be activated in vitro to kill tumor cells and that ADR does not suppress this monocyte function. Furthermore, IL-1 secretion by monocytes and the phagocytic properties of monocytes were also not altered by ADR. There was a suggestion of enhanced tumoricidal activity in some patients' monocytes activated by liposomes after exposure to ADR. The mechanism for this enhanced activity is unclear at this time and could not be explained by transfer of ADR to the tumor cells or increased IL-1 secretion. Whether the slight increase in intracellular IL-1 observed (Fig. 2) can explain the enhanced killing is only speculative at this time and needs further investigation. We have previously shown that agents that stimulate monocytes to secrete oxygen radicals and peroxides do not stimulate tumor cell killing as measured in this assay and have further demonstrated that tumor cell killing is not mediated via oxygen radicals (16). Therefore, it is unlikely that the enhanced tumoricidal activity can be explained by ADR's capacity to generate free radicals and peroxides.

Our demonstration of enhanced monocyte activation following in vivo ADR therapy adds to the numerous reports that ADR stimulates host immunity. Riccardi et al. (23) demonstrated that ADR was able to induce resistance in lethally irradiated mice against transplanted lymphoma cells. This ADR-induced antitumor response was present as late as 15–30 days following drug treatment, showed no genetic restriction, and was not due to direct antitumor action by the drug. Furthermore, antimacrophage agents such as silica or carrageenan abrogated the response suggesting that ADR induced the activation of macrophage cytotoxic activity. Santoni et al. (24) found that a single i.p. injection of ADR resulted in increase of the cytolytic activity by peritoneal exudate cells felt to be related to enhanced NK activity. Tomazic et al. (25) demonstrated that ADR added directly to murine spleen cell cultures enhanced the development of cytotoxic activity against allogeneic tumor cells. The augmented response was related to effects on the nonadherent spleen cell fraction suggesting that T-effector cells played a role in enhanced cytotoxic response. In other murine studies Orsoni et al. (26) demonstrated that spleen cell populations obtained from mice treated with ADR showed enhanced cytotoxic activity when cocultivated with allogenic tumor cells compared to spleen cells from untreated animals. Further work has shown that the augmented response is secondary to ADR-modified cell populations in both monocyte-macrophage precursors as well as T-regulatory cells (27, 28). Similarly, Arinta et al. (29) reported twofold higher cell-mediated cytotoxicity of peripheral blood mononuclear cells obtained from patients after administration of ADR. ADR treatment was associated with an increase in the percentage of T-suppressor cells and significantly higher levels of Interleukin-2 production by peripheral blood mononuclear cells which was felt to be responsible for ADR augmentation of cytotoxic response. Our studies support the hypothesis that ADR's effect on the host's immune system (in addition to its direct antitumor effect) may play an important role in tumor cell eradication.

Biological response modification as an adjuvant therapy is currently being investigated in many treatment regimens for a variety of tumors. The combination of standard chemotherapeutic agents, such as ADR, capable of enhancing natural host immunity, with these biological agents may further contribute to improved disease free survival. Our results indicate that ADR may enhance the ability of liposome-encapsulated monocyte activators to stimulate tumoricidal function. This data is encouraging because it suggests that ADR and liposome therapy may be combined. Based on the Goldie and Goldman hypothesis for drug resistance (30) it is essential to employ as many effective forms of therapy as early as possible in the course of adjuvant treatment. While residual tumor cells may not be ADR-resistant, repeated exposure to ADR could render a resistant population. However if activated macrophages can destroy these small numbers of remaining cells, repeated treatment with ADR becomes unnecessary. Of interest is the finding that activated mouse macrophages can kill ADR-resistant as well as ADR-sensitive tumor cells (31). This data suggests that the monokines released by activated macrophages can kill tumor cells regardless of their drug sensitivity.
Recent reports in the literature demonstrate the limitations of current chemotherapeutic regimens in improving the 60% metastasis-free survival achievable in osteosarcoma patients (32). The conclusion of this study is that novel forms of therapy in addition to current chemotherapy are needed if we are to succeed in our quest for improving these percentages. We, therefore, propose that biological therapy aimed at activating monocyte effector cells may be an effective addition to ADR in the adjuvant treatment of metastatic pulmonary OS as well as other adjuvant settings where pulmonary micrometastases are suspected.

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