Therapy of Spontaneous Lung Metastasis of Murine Renal Adenocarcinoma by Systemic Administration of Liposomes Containing the Macrophage Activator CGP 31362

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ABSTRACT

Current therapies for renal cell carcinoma have been limited by the unresponsiveness of metastatic disease to conventional treatments. Although the use of biological response modifiers as adjuvant therapy has generally not been successful against disseminated disease, in situ activation of macrophages to a tumoricidal state by liposome-encapsulated immunomodulators has been shown to eradicate metastatic cancer in murine tumor models. We, therefore, designed experiments to evaluate the ability of a new macrophage activator, CGP 31362, a synthetic bacterial cell wall analogue, to cause regression of spontaneous lung metastases in mice whose primary renal adenocarcinoma was removed by nephrectomy. Delivery of the CGP 31362 to the lungs was accomplished by its encapsulation in multilamellar phospholipid liposomes (MLV-CGP 31362). Therapy with repeated i.v. injections of MLV-CGP 31362 significantly reduced the number of lung metastases in nephrectomized mice. Therapeutic efficacy of MLV-CGP 31362 was influenced by the encapsulation ratio of CGP 31362 to total phospholipid, the dose of injected liposomes, and the frequency of administration. Optimal therapy was achieved by combining the use of i.v. MLV-CGP 31362 with the s.c. injection of recombinant murine γ interferon. Administration of MLV-CGP 31362 prior to removal of the primary tumor and continuing postoperatively was superior to postoperative therapy alone. Several lines of evidence indicate that in situ activation of macrophages was responsible for the therapeutic effects of MLV-CGP 31362: (a) macrophages harvested from the lungs of treated mice had significant tumoricidal activity against cultured renal carcinoma cells, (b) activated macrophages, as defined by the MRP-14 marker, were present in lung tumor nodules of treated mice but not untreated mice, and (c) the in situ activation of alveolar macrophages was consistent with the in vivo deposition of 60% of radiolabeled MLV-CGP 31362 liposomes in the lungs following i.v. injection. The results reported here represent the first in vivo evaluation of MLV-CGP 31362 and offer additional evidence that macrophage activation can be a potent treatment for metastatic disease when used in combination with therapies that reduce tumor burden.

INTRODUCTION

Twenty-five thousand new cases of RCC3 were diagnosed in the United States in 1989. One third of these patients presented with clinical metastatic disease (1). In addition, up to 50% of the patients with apparently localized carcinomas subsequently develop metastatic disease. Although the primary tumor can usually be removed surgically, modalities aimed at eradicating metastatic disease have not been as successful. Most deaths in patients with renal cancer are caused by the growth of metastases that are resistant to conventional therapy because metastases are heterogeneous with respect to a variety of biological properties, including sensitivity to chemotherapeutic agents, karyotype, growth rate, and antigenicity (2–4). Neoplastic cells are genetically unstable, so the resistant subpopulations appear before therapy and also continue to evolve during therapy (5, 6).

Chemotherapy, the traditional form of therapy for metastatic disease, is nonspecific and associated with high toxicity. Vinblastine, the nitrosoureas, and hydroxyurea have elicited minor responses in RCC, but complete responses are rare. Therapy with biological response modifiers has been attempted as a means of augmenting host antitumor immunity. Some success against advanced murine renal carcinomas has been achieved using IL-2-stimulated lymphocytes, alone or in combination with Adriamycin (7–9). Unfortunately, subsequent clinical trials with these agents have not been as successful (10–14).

During the past decade, many studies have shown that macrophages can be activated to a tumoricidal state by lymphokines (15–24) and interaction with microorganisms or their products (25, 26), especially when these materials are encapsulated within phospholipid liposomes (26–29). Once in the circulation, liposomes are rapidly engulfed by free and fixed reticuloendothelial cells (primarily of the liver, lungs, and lymph nodes) (30–33).

Recent studies have shown that encapsulation of a synthetic analogue of the outer wall of Gram-negative bacteria, MTP-PE, into liposomes can activate macrophages in situ and induce regression of spontaneous lung metastases of murine melanoma (34–37) and canine osteosarcoma (38) and can activate tumoricidal properties in blood monocytes of cancer patients (26). Moreover, the results of phase I clinical trials indicate that, at the minimum dose sufficient to produce desirable biological effects, these liposomes are well tolerated (39). Renal cancer may be a good candidate for therapy based upon the in situ activation of macrophages, because it is resistant to most chemotherapeutic strategies, and there have been reports of successful immunological intervention in this disease (5–9).

A new macrophage activator, CGP 31362, is a synthetic lipoprotein analogue of a Gram-negative bacteria cell wall. It is a potent activator of macrophages and produces superior tumoricidal activity, compared to that observed for MTP-PE (40, 41). MLV-CGP 31362 is able to activate macrophages at a lower liposome concentration than that required by MLV-MTP-PE and, unlike MLV-MTP-PE (20, 26, 40), optimal tumoricidal activity produced by MLV-CGP 31362 does not require the presence of γ interferon (40). Moreover, macrophages activated with MLV-CGP 31362 release a wider range of diffusible mediators than those treated with MLV-MTP-PE.
THERAPY OF LUNG METASTASIS BY MLV-CGP 31362

(40). These properties of MLV-CGP 31362 have recommended its use for in situ activation of macrophages to enhance host resistance against cancer metastasis. We, therefore, determined whether multiple i.v. administrations of MLV-CGP 31362 could inhibit the growth of spontaneous lung metastases in the murine RENCA tumor model.

MATERIALS AND METHODS

Animals. Specific pathogen-free BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were maintained in facilities approved by The American Association for Accreditation of Laboratory Animal Care, in accordance with United States Department of Agriculture, Department of Health and Human Services, and NIH regulations and standards.

Tumor Cell Culture. RENCA cells from a spontaneous renal adenocarcinoma syngeneic to BALB/c mice (42) were grown as monolayer cultures in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, and non-essential amino acids. The complete medium was free of endotoxin, as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 in air. All cell cultures were free of Mycoplasma, reovirus type 3, pneumonia virus of mice, K virus, encephalitis virus, lymphocyte choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

Reagents. CGP 31362 is a synthetic analogue of a fragment of a lipoprotein from the outer wall of Gram-negative bacteria. Its molecular weight is 1184. This immunomodulator and the encapsulating phospholipid (synthetic phosphatidylcholine and phosphatidylserine, 7:3 molar ratio), as a dry lyophilisate, were the gifts of Ciba-Geigy, Ltd. (Basel, Switzerland). The CGP 31362:phospholipid ratios used were 1:125, 1:250, and 1:1000 (mg:mg). Multilamellar liposomes were prepared from the dry lyophilisate at room temperature by hydrating the vial of lyophilized phospholipids and CGP 31362 in HBSS for 1 min at room temperature, followed by vigorous shaking for 6 min using a Vortex shaker. Control liposome preparations contained the same amount of phospholipid but no CGP 31362. Treatments consisted of i.v. injections of the indicated amount of MLV suspended in 0.2 ml of Ca2+/Mg2+-free HBSS.

rIFN-γ (specific activity, 17 × 10^6 IU/mg) was the gift of Genentech, Inc. (South San Francisco, CA). rIFN-γ was administered s.c., at a dose of 50,000 units/injection, in 0.2 ml Ca2+/Mg2+-free HBSS containing 0.1% albumin.

Development of Spontaneous Lung Metastasis Model. The RENCA-BALB/c model of spontaneous metastasis was patterned after the original studies described by Salup et al. (7–9). Cultured RENCA cells (50% confluent) were given fresh medium 24 h before harvest. The cells were then rinsed in Ca2+/Mg2+-free HBSS and overlaid for 2 min with a 0.25% trypsin/0.02% EDTA solution. The flask was tapped, and the cells were pipetted to produce a single-cell suspension. The cells were then washed in HBSS, and their viability was ascertained by trypan blue dye exclusion. Suspensions with >95% viability were used for in vivo studies. The cell suspension was kept at 4°C.

BALB/c mice were anesthetized by i.p. injection of 0.15 ml sodium pentobarbital (10 mg/ml), washed in alcohol, and exsanguinated by severing of the renal arteries and veins with a pair of vascular clips. Nephrectomy of the injected kidney was performed 10 min after injection. The kidneys were removed and weighed. Each kidney was weighed and placed in Bouin’s fixative for 24 h prior to fixation in 10% buffered formalin. The lung colonies were counted using a dissecting microscope.

Survival Experiment. The effect of MLV-CGP 31362 on the overall survival of BALB/c mice with spontaneous RENCA lung metastasis was determined by i.v. therapy with 5 μmol of MLV-CGP 31362 beginning on day 7 after tumor cell injection, i.e., 3 days before nephrectomy. The treatment was continued postoperatively twice weekly for 3 weeks. Some animals received MLV-CGP 31362 postoperatively, while others received HBSS or MLV-HBSS. Because rIFN-γ has been shown to produce synergistic activation of macrophages with muramyl dipeptide (44, 45), we administered both MLV-CGP 31362 and rIFN-γ to determine whether this combination also acted synergistically in inhibiting spontaneous lung metastasis of RENCA. Five μmol of MLV-CGP 31362 were administered once, twice, or 3 times per week, alone or with a s.c. injection of 50,000 units rIFN-γ twice per week.

Throughout these experiments, all mice were examined daily until control mice became moribund, at which time all mice were killed and necropsied. Statistical analysis of the results was performed by the Mann-Whitney Test. All experiments were carried out at least twice.

Experiments were designed using optimal parameters described for the use of MLV-MTP-PE (36–38), and no toxicity of MLV-CGP 31362 was observed, as defined by the behavior of the mice, weight loss, or hair texture, using any of the indicated doses or preparations. We first determined the optimal dose of active CGP 31362 and the optimal ratio of CGP 31362 to phospholipid MLV. In this series of experiments, mice were treated twice weekly for 3 weeks (total of six treatments) with either HBSS, control preparation of MLV containing HBSS (MLV-HBSS) or MLV-CGP 31362. Four different amounts of CGP 31362 (0.125, 0.25, 0.50, and 1 mg) were encapsulated within 250 mg of MLV. The dose of MLV was 5 μmol/injection.

The next series of experiments were designed to determine whether the dose of MLV with CGP 31362 influenced the outcome of therapy. Mice were given injections, twice weekly for 3 weeks, of 1.25, 2.5, 5, or 10 μmol of MLV (0.5 mg CGP 31362/250 mg lipid). In the next experiment, we determined the influence of treatment schedule on the outcome of therapy. Thus, mice were treated with 5 μmol of MLV-CGP 31362 (0.5 mg in 250 mg lipid) once, twice, or 3 times per week for 3 weeks. Following this study, we examined the effect of preoperative therapy on the inhibition of lung metastasis. Mice were treated with MLV-CGP 31362 3 days before nephrectomy, and therapy continued postoperatively twice per week for 3 weeks (six treatments). Some animals received MLV-CGP 31362 postoperatively, while others received HBSS or MLV-HBSS. Because rIFN-γ has been shown to produce synergistic activation of macrophages with muramyl dipeptide (44, 45), we administered both MLV-CGP 31362 and rIFN-γ to determine whether this combination also acted synergistically in inhibiting spontaneous lung metastasis of RENCA. Five μmol of MLV-CGP 31362 were administered once, twice, or 3 times per week, alone or with a s.c. injection of 50,000 units rIFN-γ twice per week.

Assay of Macrophage-mediated Tumor Cell Cytotoxicity. Macrophage-mediated cytotoxicity was assessed by a radioative label-release assay, as previously described in detail (47). Target RENCA cells in exponential growth phase were incubated for 24 h in medium supplemented with [3H]thymidine (≥2000 Ci/mmol; Dupont, Boston, MA). The target cells were rinsed 4 times with warm HBSS to remove
unincorporated label, harvested by a brief trypsinization, and resuspended in medium. The cells were plated (1 × 10⁶/well) to yield a macrophage:target ratio of 10:1. Radiolabeled target cells were plated into wells containing no macrophages as an additional control. After 72 h, the cultures were washed twice with HBSS, and the remaining adherent viable target cells were lysed with 0.1 ml of 0.1 M NaOH. The lysate was measured for radioactivity, and the percentage of macrophage-mediated cytotoxicity was calculated from:

\[
\text{Percentage of cytolysis} = \frac{[A - B]}{A} \times 100
\]

where \(A\) is the cpm in cultures of target cells and AM from untreated mice and \(B\) is the cpm in cultures of target cells and AM from mice of the treated groups.

Immunohistological Staining for Macrophages. Fresh lungs from nephrectomized, tumor-bearing mice were harvested 24 h following the second treatment (day 15) and were snap-frozen in liquid nitrogen. Frozen sections were fixed for 10 min in cold acetone, washed 3 times in PBS, treated with 1% Triton X-100 for 5 min, and rinsed 3 times in PBS. Nonspecific binding sites were blocked by incubation of the samples in PBS containing 1% normal goat serum and 1% bovine serum albumin, for 20 min. The samples were then incubated with primary polyclonal antibodies specific for macrophages (rat anti-mouse (F4/80) (48) or activated macrophages [rabbit anti-mouse MRP14 (49, 50), directed against a calcium-binding protein found in activated macrophages], for 18 h in a 4°C humidified chamber, followed by three rinses in PBS and incubation for 5 min in PBS/normal goat serum/bovine serum albumin (both antibodies supplied by Ciba-Geigy, Ltd.). The samples were incubated with second antibody (Auroprobe goat anti-rat or goat anti-rabbit, gold-labeled; Amersham, Arlington Heights, IL) for 1 h, washed 3 times in PBS, fixed with 2% glutaraldehyde in PBS for 10 min, and washed 4 times in distilled water. The samples were treated with 50 µl of Silver Intense for 5–7 min, and stained macrophages were visualized under bright field microscopy.

Image Analysis. The area of representative tumor nodules and the number of macrophages present in the given tumor areas were measured using an IBAS analyzer (Carl Zeiss Instruments, Thornwood, NY).

In Vivo Distribution of MLV-CGP 31362. The RSC of BALB/c mice were injected i.v. with 10⁵–10⁶ RENCA cells. Nephrectomy was done 10 days later. Twenty h after nephrectomy, the mice were given injections of 5 µmol MLV-CGP 31362 that contained 131I-phenylpropionyl-phosphatidylethanolamine as a trace marker (51). Groups of five mice were killed at 5, 15, 30, 60, 120, and 180 min, and radioactivity in the lungs, liver, kidneys, and spleens (~200,000 cpm/mouse) was determined.

RESULTS

Optimization of MLV-CGP 31362 Administration. Initial experiments demonstrated inhibition of the number of spontaneous lung metastases in nephrectomized mice following repeated injections of MLV-CGP 31362. Photographs of representative lungs are shown in Fig. 1. Whereas lungs from MLV-HBSS-treated animals had generally >150 RENCA nodules (Fig. 1A), lungs of mice given injections of MLV-CGP 31362 had less than 30 nodules (Fig. 1B). The cross-sections of lungs shown in Fig. 2 dramatically illustrate the differences in the number of lung nodules between mice treated with MLV-HBSS (Fig. 2A) and those treated with MLV-CGP 31362 (Fig. 2B).

Dose of CGP 31362 and Lipid Content of MLV-CGP 31362. Nephrectomized mice with established lung metastases were treated twice per week for 3 weeks (six treatments) with either saline (HBSS), control liposomes containing HBSS (MLV-HBSS), or 5 µmol/injection of the CGP 31362 (0.125, 0.25, 0.50, or 1.0 mg) incorporated within 250-mg phospholipid liposomes (MLV-CGP 31362). The results of this experiment are shown in Table 1. There was no difference in the median number of lung metastases of mice treated with either HBSS or MLV-HBSS. All MLV containing CGP 31362 significantly decreased the number of lung metastases, compared with the controls. Treatment of mice with MLV-CGP 31362 (0.5 mg/250 mg lipid) produced the greatest reduction in the median number of lung metastases, as well as a significant decrease in the number of animals with regional metastasis. For this reason, all further experiments were performed with MLV containing 0.5 mg CGP 31362/250 mg lipid.

Mice were treated with different amounts of MLV-CGP 31362, and the results are shown in Table 2. Mice treated with HBSS or MLV-HBSS (5 µmol/injection) had no significant differences in the number of lung metastases. All animals treated with MLV-CGP 31362 had a significant reduction in the number of lung metastases, compared to control mice, with the maximum inhibition occurring at 5 µmol. Therapy with this dose resulted in a significant reduction in the median number of lung metastases, compared with all other amounts of liposomes.

Schedule of Liposome Treatment. The effects of scheduling therapy on the efficacy of MLV-CGP 31362 in inhibiting spontaneous lung metastasis are summarized in Table 3. Again, there was no difference between HBSS and MLV-HBSS in inhibiting lung metastasis. The importance of the appropriate schedule is
Granulocyte-macrophage colony-stimulating factor (GM-CSF) therapy with MLV-CGP 31362 significantly reduced the incidence of lung metastases. These results also demonstrate that systemic therapy either twice per week or 3 times per week (for a total of 3 weeks) was equally effective in reducing the median number of lung metastases following once per week therapy. The treatment dose was 5 μmol.

Table 1 Inhibition of spontaneous lung metastasis of murine renal adenocarcinoma: effect of varying the CGP 31362/phospholipid ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of lung metastases</th>
<th>Regional recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HBSS</td>
<td>99</td>
<td>17–&gt;150</td>
</tr>
<tr>
<td>2</td>
<td>MLV-HBSS</td>
<td>114&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15–&gt;150</td>
</tr>
<tr>
<td>3</td>
<td>MLV-CGP 31362 (0.125)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3–&gt;150</td>
</tr>
<tr>
<td>4</td>
<td>MLV-CGP 31362 (0.250)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9–&gt;150</td>
</tr>
<tr>
<td>5</td>
<td>MLV-CGP 31362 (0.50)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1–&gt;57</td>
</tr>
<tr>
<td>6</td>
<td>MLV-CGP 31362 (1.0)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5–&gt;150</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not significant versus group 1.
<sup>b</sup>mg active material/250 mg total lipid (phosphatidylcholine:phosphatidylserine, 7:3 molar ratio). All mice were given i.v. injections twice weekly for 3 weeks. The treatment dose was 5 μmol.
<sup>c</sup>P < 0.05 versus groups 1 and 2.

Table 2 Inhibition of spontaneous lung metastasis of murine renal adenocarcinoma: effects of MLV dose

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of lung metastases</th>
<th>Regional recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>67</td>
<td>36–&gt;150</td>
</tr>
<tr>
<td>MLV-HBSS</td>
<td>64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31–&gt;150</td>
</tr>
<tr>
<td>MLV-CGP 31362 (5 μmol)</td>
<td>34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17–62</td>
</tr>
<tr>
<td>MLV-CGP 31362 (10 μmol)</td>
<td>46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8–56</td>
</tr>
<tr>
<td>MLV-CGP 31362 (25 μmol)</td>
<td>37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8–76</td>
</tr>
<tr>
<td>MLV-CGP 31362 (1.25 μmol)</td>
<td>41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6–71</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not significant versus HBSS.
<sup>b</sup>P < 0.008 versus HBSS or MLV-HBSS.
<sup>c</sup>P < 0.04 versus 1.25, 2, or 10 μmol therapy.

Table 3 Effect of schedule of injection or addition of rIFN-γ on the inhibition of spontaneous lung metastasis by MLV-CGP 31362

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of lung metastases</th>
<th>Regional recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>9</td>
<td>63</td>
</tr>
<tr>
<td>MLV-HBSS</td>
<td>9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>IFN-γ (s.c.)</td>
<td>6</td>
<td>43&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-HBSS + IFN-γ</td>
<td>9</td>
<td>31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-CGP 31362, 1/wk</td>
<td>9</td>
<td>49&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-CGP 31362, 2/wk</td>
<td>9</td>
<td>24&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-CGP 31362, 3/wk</td>
<td>10</td>
<td>22&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-CGP 31362, 1/wk, + IFN-γ</td>
<td>10</td>
<td>15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-CGP 31362, 2/wk, + IFN-γ</td>
<td>10</td>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MLV-CGP 31362, 3/wk, + IFN-γ</td>
<td>10</td>
<td>15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not significant versus HBSS.
<sup>b</sup>P < 0.01 versus HBSS only.
<sup>c</sup>P < 0.05 versus HBSS only.

Fig. 3. Percentage of survivors as a function of time after injection of 10⁶ RENCA cells into the left renal subcapsule of BALB/c mice. All mice were nephrectomized on day 11 and randomized to treatment groups: HBSS only, MLV-HBSS (MLy), rIFN-γ (50,000 units/injection s.c.) (IFN-γ), MLV-HBSS plus rIFN-γ (MLy + IFN-γ), MLV-CGP 31362 (5 μmol/injection) (31362), or MLV-CGP 31362 plus rIFN-γ (31362 + IFN-γ). All mice received treatments twice weekly for 3 weeks.

Table 4 Comparison of pre- and postoperative treatment of spontaneous lung metastasis with MLV-CGP 31362

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>No. of lung metastases</th>
<th>Regional recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS at each time</td>
<td>38</td>
<td>14–48</td>
</tr>
<tr>
<td>Postoperative only</td>
<td>31362</td>
<td>17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>31362</td>
<td>14–29</td>
<td></td>
</tr>
<tr>
<td>Preoperative + postoperative</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0–10</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.015 versus postoperative.
<sup>b</sup>P < 0.001 versus HBSS only.
<sup>c</sup>P < 0.01 versus HBSS only.
<sup>d</sup>P < 0.001 versus HBSS only.
<sup>e</sup>P < 0.015 versus postoperative group.
Evidence for in Situ Activation of Alveolar Macrophages Following Therapy with MLV-CGP 31362. The antitumor effects of therapy with MLV-CGP 31362 could be demonstrated in two ways. First, alveolar macrophages that were harvested from the lungs of tumor-bearing nephrectomized mice 24 h after the second treatment with MLV-CGP 31362 were tumoricidal for cultured RENCA cells (44 ± 10% cytolysis), whereas alveolar macrophages from untreated tumor-bearing animals demonstrated no cytotoxicity (−7 ± 2%). The kinetics and duration of macrophage-mediated cytotoxicity in situ are the subject of a separate report (56). Second, although macrophages could be identified around the periphery of a lung nodule from both treated and untreated mice using the F4/80 polyclonal antibody (Fig. 4, A and B), only the mice treated with MLV-CGP 31362 had activated macrophages within the lung metastases, as identified by staining with the MRP-14 polyclonal antibody (Fig. 4, C and D). Quantitation of MRP-14-positive cells within these early metastatic lesions by image analysis revealed a 4-fold increase of activated macrophages in the tumor of treated mice (Table 5).

In Vivo Localization of MLV-CGP 31362. The 125I-PE-labeled liposomes containing CGP 31362 were mainly deposited in the lungs (60%), liver (35%), and spleen (19%) within 5 min after i.v. injection (Fig. 5). The percentage of label decreased in the lungs and liver over 3 h, to about 20%, and the amount found in the spleen remained relatively constant. Less than 2% of the labeled liposomes were detected in the kidneys.

Table 5 Presence of activated macrophages in lung metastases

<table>
<thead>
<tr>
<th>Treatment of mice</th>
<th>No. of MRP-14 positive-staining cells</th>
<th>Area/nodule*</th>
<th>No. of cells/area</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV-CGP 31362</td>
<td>&gt;100</td>
<td>4.10 ± 05</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>&gt;100</td>
<td>3.58 ± 05</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>&gt;100</td>
<td>2.20 ± 05</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>3.78 ± 05</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>1.91 ± 05</td>
<td>35</td>
</tr>
<tr>
<td>MLV-HBSS</td>
<td>36</td>
<td>3.71 ± 05</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>2.66 ± 05</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6.51 ± 04</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.89 ± 05</td>
<td>10</td>
</tr>
</tbody>
</table>

* Average area/nodule, ×10^2 μm².

DISCUSSION

The present studies were motivated by the previous demonstration that repeated i.v. injections of liposomes containing immunomodulators, such as muramyl tripeptide phosphatidylethanolamine, into mice with established lung and lymph node metastases eradicated metastatic disease (34, 36, 37). The therapy was not restricted to one tumor type, because success has been reported for animal models of melanoma, fibrosarcoma, osteosarcoma, and skin and cecal tumors (16, 34, 36–38, 52–55). The results presented here represent the first in vivo evaluation of liposome CGP 31362 and provide the initial experience with this form of macrophage-mediated therapy against metastatic renal adenocarcinoma.

Fig. 4. Sections of lung tumor nodules that were processed for visualization of macrophages by F4/80 antibody (A and B) or activated macrophages by MRP14 antibody (C and D). Lung nodules are from mice treated with either MLV-HBSS (A and C) or MLV-CGP 31362 (B and D).
The RENCA model in BALB/c mice has been previously used as a model for human renal cell carcinomas. Death of mice occurs as a result of lung metastases (7–9). Our experience with this tumor line is similar. This tumor demonstrates progressive local growth, with metastasis to the lymph nodes, lungs, and, rarely, other sites. The orthotopic transplantation of this tumor results in tumor growth within its natural environment, and the tumor burden can be reduced by performing a nephrectomy, without compromising the renal function of the host. Because this model is reliable, we have used it to assess a novel approach to biological therapy of RCC. We have characterized some of the parameters necessary to obtain therapeutic results against spontaneous lung metastases of RCC. The results provide evidence that activated macrophages may infiltrate lung metastases subsequent to systemic administration of MLV-CGP 31362. Twice per week injections with 5 μmol total lipid that contained 8 μg of the active CGP 31362 were optimal for this animal model of RCC. However, we noted that the therapeutic effects of MLV-CGP 31362 were less than optimal when 16 μg of active material were delivered by either 5 μmol of liposomes (Table 1) or 10 μmol (Table 2). This result may indicate that optimal tumoricidal activation of lung macrophages (or subsequent in situ events) is dependent on the amount of activator delivered. This result parallels observations made on the in vitro activation of AM of BALB/c mice with MLV-CGP 31362, which emphasize the importance of the ratio of active material to the total amount of phospholipids presented to macrophages (56).

The use of rIFN-γ in addition to MLV-CGP 31362 enhanced the therapeutic efficacy of the latter. The presence of rIFN-γ has been shown to be required for the optimal activation of alveolar macrophages (44, 45). The activation of macrophages to a tumoricidal state has been shown to require at least two activation stimuli, resulting in a defined sequence of events (57, 58). rIFN-γ probably enables macrophages to respond to a second signal, which then triggers the macrophages to lyse tumor cell targets. However, in vitro studies indicate that MLV-CGP 31362 produces maximal tumoricidal activity of macrophages in the absence of rIFN-γ (41); hence, the improvement of in vivo therapy by the addition of rIFN-γ may be related to augmentation of host response.

The most significant inhibition of RENCA lung metastasis was observed when therapy included a single i.v. treatment with MLV-CGP 31362 prior to nephrectomy. It is likely that this enhanced inhibition was due to macrophage activation at a time when tumor burden within the lung was low. Alternatively, the activation of macrophages prior to nephrectomy may reduce the immunosuppressive effects of surgery. Several lines of evidence suggest that the inhibition of growth of lung metastases in this model is mediated by macrophage killing of tumor cells.

First, activation of macrophages to the tumoricidal state against RENCA cells was demonstrated. Second, activated macrophages, as defined by the MRP-14 marker (49, 50), were identified within lung tumor nodules taken from treated mice early in the course of their disease, whereas the nodules of untreated mice did not contain activated macrophages. Third, activation of alveolar macrophages is consistent with the measured deposition of >60% of MLV-CGP 31362 liposomes being found in the lungs immediately after i.v. injection. Fourth, MLV-CGP 31362 has no direct cytotoxic effects on cultured RENCA cells (data not shown). While the therapy employed in this report was effective in inhibiting spontaneous lung metastases, it was not curative. Earlier studies showed that surviving tumor cells in the lungs of mice treated with macrophage activators were as sensitive to macrophage-mediated killing as cultured parental cells (54). This suggests that tumor burden may limit the effectiveness of this therapy and strengthens the rationale for early immune stimulation, such as with preoperative therapy. Future studies using this strategy will also utilize other animal tumor models that are responsive to this form of therapy (34, 52–55).

In order to develop therapeutic strategies against disseminated cancer, it is important to incorporate a modality that may be able to circumvent the problem of tumor cell heterogeneity (2–5). Macrophage activation can serve as a potent therapeutic approach in this regard. In light of recent studies that have demonstrated the immunosuppressive properties of the RENCA tumor (59, 60) and the known ability of IL-2 to augment chemotherapy in this tumor model (8, 9), it would be of interest to evaluate combination therapy using macrophage activators plus IL-2. Such work is in progress.

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