In Vitro Tumoricidal Activity of Resting and Glucan-Activated Kupffer Cells

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Kupffer cells compose 80–90% of fixed tissue macrophages and have been suggested to play an important role in hepatic antitumor resistance. In the present study, the ability of resting and activated Kupffer cells to lyse syngeneic mammary adenocarcinoma BW10232 cells was evaluated. Activated Kupffer cells were isolated from C57Bl/6J mice following single of multiple intravenous (IV) injections of glucan (0.45 mg/mouse), a potent macrophage-activating agent. Mice receiving 5% (w/v) dextrose served as control. Resting Kupffer cells induced significant (P < .05) 4% and 12% specific lysis of adenocarcinoma cells at target:effector ratios of 1:10 and 1:50, respectively. Kupffer-cell-mediated tumoricidal activity was depressed on day 1 following a single IV injection of glucan. By day 3 postglucan, the antitumor activity of Kupffer cells returned to control levels and was enhanced on days 5 and 10. Following multiple IV injections of glucan on days −5, −3, and −1, Kupffer-cell-mediated cytoxicity was elevated on days 1 and 4. These observations demonstrate that 1) resting Kupffer cells are significantly cytotoxic to adenocarcinoma cells at T:E ratios of 1:10 and 1:50 and 2) following a transient inhibition of Kupffer-cell-mediated tumoricidal activity, glucan was effective in significantly enhancing the antitumor activity of Kupffer cells.

Key words: tumor cells, macrophages, immunomodulator, liver

INTRODUCTION

Kupffer cells compose the largest population of fixed tissue macrophages in the body [1,5]. The location of Kupffer cells, along the hepatic sinusoids, is ideal for interaction with exogenously or endogenously derived toxins, microorganisms, and tumor cells entering the liver via the hepatic portal and/or arterial circulations. In regard to hepatic antitumor activity, recent advances in Kupffer cell isolation techniques have made the evaluation of Kupffer-cell-mediated tumoricidal activity possible. Initial studies have demonstrated the cytolytic/cytostatic effect of Kupffer cells to a variety of allogeneic and syngeneic tumor cells in vitro [5,12,17]. These observations have led to the recognition of Kupffer cells as an important component of hepatic antitumor activity.

Administration of glucan, a beta-1,3-linked polyglucose immunomodulator, to mice bearing subcutaneous implants of reticulum cell sarcoma M5076 will result in a marked decrease in hepatic metastases and enhancement of long-term survival [20]. The enhancement of hepatic antitumor activity by glucan coincided with increased Kupffer-cell-mediated in vitro tumor cytotoxicity. These observations further suggested the importance of Kupffer cells in combating neoplastic invasion of the liver.

In the present study, the antitumor activity of Kupffer cells was characterized by investigating the in vitro tumoricidal activity of resting and glucan-activated Kupffer cells. Specifically, this study was designed to evaluate 1) the cytotoxic activity of resting Kupffer cells to adenocarcinoma BW10232, 2) the ability of isolated Kupffer cells to lyse adenocarcinoma following a single intravenous (IV) injection of glucan, and 3) the antitumor activity of Kupffer cells following multiple IV injections of glucan. Additionally, studies were undertaken to compare the in vitro tumoricidal activity of Kupffer cells to resting and glucan-activated peritoneal macrophages and splenic macrophages.

MATERIALS AND METHODS

Mice

C57Bl/6J (14–18 g) mice, purchased from Jackson Laboratory (Bar Harbor, ME), were maintained in plastic cages and fed Purina Laboratory Chow and water ad

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libitum. All experimental procedures were undertaken with strict adherence to the *Guiding Principles in the Care and Use of Animals* as approved by the Council of The American Physiological Society.

**Glucan**

Glucan was prepared by previously outlined methods [20] and was endotoxin-free as determined by the *Limulus* lysate procedure.

**Tumor Cells**

Adenocarcinoma BW10232 cells, originally purchased from Jackson Laboratory (Bar Harbor, ME), were isolated from C57BI/6J mice bearing subcutaneous implants of the tumor cell line. Adenocarcinoma cells were maintained in vitro by cultivating the cells in tissue culture flasks (Corning 25100) containing RPMI-1640 media supplemented with 7.5% fetal bovine serum, penicillin-streptomycin (50 μg/ml), gentamicin sulfate (50 μg/ml), and amphotericin B (2.5 μg/ml).

**Experimental Design**

In studies designed to evaluate the effect of a single injection of glucan on Kupffer-cell-mediated tumoricidal activity, C57BI/6J mice received 0.45 mg of glucan IV on day 0. Control animals received 5% (w/v) dextrose in water (D5W) in the same regimen. Kupffer cells, splenic macrophages, and peritoneal macrophages were isolated from control and glucan-treated animals on days 1, 3, 5, 10 and 14 posttreatment.

Kupffer cells antitumor activity following multiple glucan injections was determined by administering glucan IV (0.45 mg) on days −5, −3, and −1. Control animals received isovolumetric D5W in the same regimen. Kupffer cells and peritoneal macrophages were isolated on days 1, 4, and 8 posttreatment.

**Isolation of Kupffer Cells**

The livers of C57BI/6J mice were perfused via the hepatic portal vein with 10 ml of Hank’s balanced salt solution (HBSS) followed by 10 ml of 0.125% (w/v) collagenase type IV (Sigma Chemical Company, St. Louis, MO) in HBSS. The liver was aseptically excised, placed in a plastic culture dish containing collagenase-HBSS solution, and minced with a sterile razor blade. The resulting suspension was transferred to a sterile 50-ml beaker containing 10 ml of 0.125% collagenase-HBSS and incubated for 40 min at 37°C in a Dubnoff oscillating metabolic incubator. The preparation was then transferred to a 50-ml centrifuge tube and centrifuged (10g for 10 min) to sediment connective tissue debris and hepatocytes. The resulting supernatant, containing primarily nonparenchymal cells, was recovered, washed (3×) with HBSS, and resuspended to a volume of 5 ml with HBSS. The nonparenchymal cell suspension was combined with 7 ml of 30% (w/v) metrizamide (Accurate Chemical And Scientific, Westbury, NY) in HBSS and overlaid with 0.5 ml of HBSS. The resulting suspension was centrifuged (1,500g for 20 min) and the Kupffer cell band, which developed at the HBSS-metrizamide interface, was retrieved and washed (3×) with media. The recovered cells were cultured in Corning 96-well microtiter plates for 2 hr and were washed (3×) with media to remove nonadherent cells. Media was replenished over the adherent cell population at concentrations of 2 × 10⁵ or 1 × 10⁶ cells/well. Kupffer cells were allowed to incubate overnight (12–16 hr) prior to initiating cytototoxic assays. The adherent cell population was determined to be > 85% macrophages by peroxidase staining [10].

**Isolation of Splenic Macrophages**

The spleens of C57BI/6J mice were aseptically excised and minced with 25-gauge needles. The resulting cell suspension was passed through a 22-mesh steel screen to remove connective tissue debris and was centrifuged (100g for 10 min). Erythrocytes were removed by hypotonic lysis; the preparation was centrifuged (100g for 10 min); and the resulting splenocyte preparation was resuspended in media. One hundred μl of splenocyte suspension was pipetted into microtiter wells (Corning 25860), incubated for 2 hr, and washed with media to remove nonadherent cells. Media was replenished over the adherent cell population at concentrations of 1 × 10⁶ or 2 × 10⁵ cells/well. The adherent cell population was determined to be > 80% macrophages by peroxidase staining [10].

**Isolation of Peritoneal Macrophages**

Peritoneal macrophages were isolated by lavaging the peritoneal cavity with 10 ml of media. The cells were washed (2×), resuspended, in media and incubated (37°C, 5% CO₂) in 96-well microtiter plates for 2 hr. The cultures were washed to remove nonadherent cells and media was replenished over the adherent cell population at a concentration of 2 × 10⁵ or 1 × 10⁶ cells/well. Macrophages were incubated for 12–16 hr prior to initiating cytototoxic assays. The adherent cell population was determined to be > 95% macrophages by peroxidase staining [10].

**Macrophage Purification and Depletion**

Hepatic and splenic macrophages were further purified through the employement of monoclonal anti-thy 1. Isolated splenocytes or hepatic nonparenchymal cells were pipetted into microtiter wells containing 10 μl of monoclonal anti-thy 1 (Accurate Chemical Co., Clone N1MR-1) and incubated (4°C) for 30 min. The plates were centrifuged (100g for 10 min), the supernatant was discarded, and 200 μl of 100% fetal bovine serum was added to each
well. The plates were incubated (37°C, 5% CO₂) for an additional 2 hr and washed (3 ×) to remove nonadherent cells and 200 µl of media was replenished over the adherent cell population (1 × 10⁶ cells/well).

In macrophage depletion studies, splenocytes and hepatic nonparenchymal cells (2 × 10⁷/well) were aliquoted into Falcon 24-well tissue culture plates and incubated for 2 hr. The nonadherent cells were recovered and transferred to tissue culture wells containing 100 µl of monoclonal antimacrophage supernatant (Accurate Chemical and Scientific, clone M170.15), incubated for 30 min (4°C), centrifuged (100g for 10 min), and resuspended in 100% fetal bovine serum. The resulting cultures were incubated for 1 hr (37°C, 5% CO₂); the nonadherent cell fractions were removed, washed (3 ×), and pipetted into microtiter wells (1 × 10⁶ cells/well).

Cytotoxicity Assay

Adenocarcinoma cells were incubated for 72 hr in media supplemented with 10 µCi/ml of ³H-thymidine (New England Nuclear, Boston, MA, 6.7 Ci/mmol). The cells were detached from the culture flask surface by gentle washing, harvested, and washed (3 ×) with media to remove unincorporated ³H-thymidine. The cells were then placed in Corning microtiter wells (2 × 10⁶/well) containing macrophages at target to effector ratios (T:E) of 1:10 or 1:50. An additional 100µl of media was added to each well to bring the total volume to 300 µl. The resulting cultures were incubated (37°C, 5% CO₂) for 72 hr and centrifuged (100g for 10 min) and 100 µl of the culture supernatants was transferred to scintillation vials for determination of ³H-thymidine release. Maximum ³H-thymidine release was determined by freeze/thawing (3 ×) 300 ul (2 × 10⁶ cells) of radiolabeled adenocarcinoma cell suspension. Cytotoxicity was expressed as % specific lysis, which was calculated as follows:

\[
\text{% Specific lysis} = \frac{\text{Max release (cpm) - media control (cpm)}}{\text{Experimental (cpm) - media control (cpm)}} \times 100
\]

Statistics

All data were evaluated by one-way or two-way analysis of variance. Comparison between groups was performed employing Student’s t-test or Newman-Keuls’ multiple range test. A probability of P < .05 was considered significant.

RESULTS

Comparative Evaluation of the Cytotoxic Activity of Resting Kupffer Cells, Splenic Macrophages, and Peritoneal Macrophages

Kupffer cells, splenic macrophages, and peritoneal macrophages were significantly cytotoxic to adenocarcinoma cells at a T:E ratio of 1:10 as indicated by 4, 8, and 9% specific lysis of adenocarcinoma cells, respectively (Table 1). At a T:E ratio of 1:50, Kupffer cells, splenic macrophages, and peritoneal macrophages induced 12, 10, and 29% specific lysis of adenocarcinoma cells, respectively. In addition, significant increases in tumoricidal activity of 48 and 93% were observed for Kupffer cells and peritoneal macrophages, respectively, at a T:E ratio of 1:50 when compared to the 1:10 ratio (Table 1). Splenic macrophages did not induce a signifi-

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**TABLE 1. The Comparative Tumoridal Activity of Resting Kupffer Cells, Peritoneal Macrophages, and Splenic Macrophages**

<table>
<thead>
<tr>
<th>Group</th>
<th>T:E ratio</th>
<th>³H-thymidine release (cpm)</th>
<th>% specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum release</td>
<td>—</td>
<td>4,291 ± 378</td>
<td>100</td>
</tr>
<tr>
<td>Media control</td>
<td>—</td>
<td>467 ± 33</td>
<td>0</td>
</tr>
<tr>
<td>Kupffer cells 10:1</td>
<td>10:1</td>
<td>621 ± 33b</td>
<td>4.0 ± 0.3b</td>
</tr>
<tr>
<td>Kupffer cells 50:1</td>
<td>50:1</td>
<td>916 ± 103bc</td>
<td>11.7 ± 1.4bc</td>
</tr>
<tr>
<td>Splenic macrophages 10:1</td>
<td>10:1</td>
<td>768 ± 109b</td>
<td>7.9 ± 1.3b</td>
</tr>
<tr>
<td>Splenic macrophages 50:1</td>
<td>50:1</td>
<td>860 ± 58b</td>
<td>10.3 ± 0.6b</td>
</tr>
<tr>
<td>Peritoneal macrophages 10:1</td>
<td>10:1</td>
<td>807 ± 69b</td>
<td>8.9 ± 0.8b</td>
</tr>
<tr>
<td>Peritoneal macrophages 50:1</td>
<td>50:1</td>
<td>1,560 ± 83bc</td>
<td>28.6 ± 1.4bc</td>
</tr>
</tbody>
</table>

*Isolated Kupffer cells, peritoneal macrophages, and splenic macrophages were incubated with ³H-thymidine-labeled adenocarcinoma cells (2 × 10⁴/well) for 72 hr at target:effector (T:E) ratios of 1:10 and 1:50; ³H-thymidine release was determined and % specific lysis calculated as an index of cytotoxic activity. Values are expressed as mean ± SE. N = 23-69/group. F = 21.27.

Statistical comparisons performed employing media control as reference, P < .05.

Statistical comparisons performed employing macrophages at T:E ratio of 1:10 as reference, P < .05.
significant increase in specific lysis of adenocarcinoma cells at the 1:50 ratio when compared to the 1:10 ratio. Based on the level of macrophage-mediated antitumor activity observed at the 1:50 ratio, this T:E ratio was employed in the evaluation of Kupffer cell, splenic macrophage, and peritoneal macrophage-mediated cytotoxic activity following glucan administration.

Enhancement of Kupffer Cell, Splenic Macrophage, and Peritoneal Macrophage-Mediated Tumoricidal Activity Following a Single IV Injection of Glucan

On day 1 following glucan administration, the antitumor activity of Kupffer cells was significantly depressed compared to control (Table 2). Kupffer-cell-mediated antitumor activity was not significantly altered on day 3 following a single injection of glucan. Glucan significantly enhanced Kupffer-cell-mediated tumoricidal activity on days 5 and 10. By day 14, no significant difference in the antitumor activity of Kupffer cells was observed in glucan-treated mice when compared to control (Table 2).

On day 1 postglucan, the cytotoxic activity of peritoneal macrophages was significantly depressed (Table 3). By day 3, glucan-treated peritoneal macrophages exhibited a significant increase in tumoricidal activity which was maintained on days 5 and 10. Peritoneal macrophage-mediated antitumor activity was not significantly altered 14 days following a single glucan injection (Table 3).

Splenic macrophage-mediated cytotoxicity to adenocarcinoma cells was not significantly altered on days 1 and 3 postglucan (Table 3). On days 5 and 10 postglucan, the tumoricidal activity of splenic macrophages was increased. The antitumor activity of splenic macrophages was not significantly different from control on day 14 postglucan (Table 3).

Enhancement of Kupffer Cell and Peritoneal Macrophage Antitumor Activity by Multiple Glucan Injections

Glucan administration on days −5, −3, and −1 significantly enhanced Kupffer-cell-mediated antitumor activity on days 1 and 4 (Table 4). The antitumor activity of Kupffer cells was not significantly altered on day 8 postglucan.

Glucan, administered on days −5, −3, and −1, significantly enhanced the tumoricidal activity of peritoneal macrophages on days 1, 4, and 8 (Table 4).

Evaluation of Effector Cells Mediating the Antitumor Activity of Glucan

In confirmation of previous experiments, glucan significantly enhanced splenic macrophage-mediated cytotoxicity to adenocarcinoma cells on day 10 following a single IV injection (Table 5). Removal of T-lymphocytes from the adherent splenocyte population by treatment with monoclonal anti-Thy 1 did not significantly alter the cytotoxicity of control or glucan-treated splenic macrophages to adenocarcinoma cells. Nonadherent splenocytes, rendered free of macrophages by treatment with anti-macrophage monoclonals, exhibited a significant decrease in cytotoxicity to adenocarcinoma cells compared to splenic macrophage-enriched preparations. The macrophage-depleted splenocyte preparations did not exhibit significant tumoricidal activity (Table 5).

A single IV injection of glucan on day 0 significantly enhanced the antitumor activity of Kupffer cells on day 10 (Table 5). Glucan-activated Kupffer cells which were further purified by treatment with anti-Thy 1 monoclonals were also significantly cytotoxic to adenocarcinoma cells. In addition, a significant decrease in antitumor activity was observed in the Kupffer cell group purified with anti-Thy 1 compared to Kupffer cells purified by adherence. Macrophage-depleted nonadherent hepatic nonparenchymal cells obtained from glucan-treated mice were not significantly cytotoxic to adenocarcinoma cells (Table 5).

DISCUSSION

The present study demonstrates the ability of Kupffer cells, isolated from normal mice, to lyse adenocarcinoma cells in vitro at T:E ratios of 1:10 and 1:50. These observations support previous findings [12,16,17] which indicate that Kupffer cells normally reside in a partially
activated state as indicated by their cytotoxic/cytostatic effect on tumor cells in vitro. Nolan and colleagues have suggested that Kupffer cell activation occurs due to interaction of Kupffer cells with endotoxin and other macrophage-activating stimuli entering the liver via the hepatic portal circulation [3,15]. However, the tumoricidal activity of Kupffer cells does not appear to be maximally stimulated under normal conditions since the administration of glucan, a potent macrophage-activating agent, will further enhance Kupffer-cell-mediated antitumor activity. This observation is supported by studies which have demonstrated that macrophage-activating agents such as lymphokines, endotoxins, or muramyl tripeptide will stimulate Kupffer-cell-mediated tumoricidal activity [6,7,21,22].

Glucan, a potent macrophage-activating and tumor-inhibitory agent, is effective in the therapy of a variety of syngeneic experimental tumors [2,8,9,20]. Glucan has also been shown to induce tumor regression following intraslesional administration in human malignancy

### Table 3. Enhancement of Peritoneal and Splenic Macrophage-Mediated Tumoricidal Activity Following a Single IV Injection of Glucan

<table>
<thead>
<tr>
<th>Days postglucan</th>
<th>Peritoneal macrophages</th>
<th>Splenic macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D5W control</td>
<td>Glucan</td>
</tr>
<tr>
<td>Media control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>17.7 ± 1.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.4 ± 1.3&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>31.8 ± 6.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>55.1 ± 1.1&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>16.3 ± 2.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>39.2 ± 5.1&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>24.0 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38.7 ± 1.1&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>20.4 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23.2 ± 2.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Glucan (0.45 mg) was injected IV on day 0. Peritoneal and splenic macrophages were isolated on days 1, 3, 5, 10, and 14. Isolated peritoneal and splenic macrophages were incubated with radionabeled adenocarcinoma cells (2 × 10<sup>6</sup>) for 72 hr at a T:E ratio of 1:50; 3<sup>H</sup>-thymidine release was determined and % specific lysis calculated. Values are expressed as mean ± SE. N = 6-17/group.

<sup>d</sup>F = 43.27.
<sup>e</sup>F = 12.23.
<sup>d</sup>Statistical comparisons performed employing media control as reference, P < .05.
<sup>e</sup>Statistical comparisons performed employing D5W control as reference, P < .05.

### Table 4. Enhanced Tumoricidal Activity of Kupffer Cells and Peritoneal Macrophages Following Multiple IV Injections of Glucan

<table>
<thead>
<tr>
<th>Days postglucan</th>
<th>Kupffer cells</th>
<th>Peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D5W control</td>
<td>Glucan</td>
</tr>
<tr>
<td>Media control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>12.4 ± 1.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.7 ± 1.1&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>6.4 ± 0.4</td>
<td>15.7 ± 2.0&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>20.8 ± 2.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.3 ± 3.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Glucan (0.45 mg) was injected on days -5, -3, and -1 prior to harvesting Kupffer cells and peritoneal macrophages on day 1, 4, or 8. Isolated hepatic and peritoneal macrophages were incubated with adenocarcinoma cells (2 × 10<sup>6</sup>) for 72 hr at a target:effector ratio of 1:5; 3<sup>H</sup>-thymidine release was determined and % specific lysis calculated as an index of cytotoxic activity. Values are expressed as mean ± SE. N = 8/group.

<sup>d</sup>F = 8.26.
<sup>e</sup>F = 27.43.
<sup>d</sup>Statistical comparisons performed employing media control as reference, P < .05.
<sup>e</sup>Statistical comparisons performed employing D5W control as reference, P < .05.
The mechanisms mediating the antitumor effect of glucan have not been fully delineated. However, evidence indicates that macrophages play an important role in glucan's antineoplastic activity [4,8].

Browder and colleagues [2] have shown that IV glucan therapy will significantly enhance long-term survival and reduce hepatic tumor cell burden in rats receiving an intraportal challenge of $1 \times 10^6$ leukemic cells. Additional studies performed by Williams and co-workers [20] have shown glucan to be effective in altering the outcome of experimental hepatic metastatic disease. Glucan administration decreased metastatic burden and primary tumor weight resulting in increased long-term survival. Enhanced Kupffer-cell-mediated antitumor activity was correlated with increased hepatic tumor resistance, suggesting the importance of Kupffer cells in modulating the antimitastatic effect of glucan. The present study extends these initial observations by demonstrating the ability of glucan to enhance Kupffer cell antitumor activity for up to 10 d following single or multiple intravenous injections.

In order to further characterize the effector cells mediating the enhanced antitumor activity in glucan-treated mice, macrophage purificaton and depletion studies were performed. These studies were designed to determine whether cytotoxic effector cells such as natural killer (NK) cells [11] and cytotoxic T-lymphocytes [14] were involved in glucan-enhanced cytotoxicity to adenocarcinoma cells. Cytotoxic effector cells in spleen and liver preparations were evaluated since 15–20% of the adherent cell populations from these two sources were determined not to be macrophages by peroxidase staining. The present study indicates that NK cells and cytotoxic T-lymphocytes do not appear to be important in glucan-enhanced splenic macrophage-mediated cytotoxicity to adenocarcinoma cells since macrophage-depleted nonadherent splenocytes were not significantly cytotoxic to adenocarcinoma cells in a 72-hr cytotoxicity assay. In addition, removal of T-lymphocytes from the adherent splenocyte population by treatment with monoclonal anti-Thy 1 did not significantly alter the cytotoxic activity of the adherent splenocyte preparations. This observation is supported by the findings of Cook and co-workers [4], which showed that glucan was an effective antitumor agent in congenitally athymic, T-cell-deficient mice. However, adherent T-lymphocytes and/or NK cells may partially mediate the cytotoxicity observed in adherent hepatic nonparenchymal cell preparations since removal of lymphocytes from this adherent cell population by treatment with anti-Thy 1 monoclonals resulted in a significant decrease in cytotoxic activity. These studies support the hypothesis that Kupffer cells play an important role in hepatic antitumor surveillance. In addition, Kupffer-cell-mediated tumoricidal activity

TABLE 5. Evaluation of Effector Cells Mediating the Antitumor Activity of Glucan*

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody</th>
<th>% specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D5W control</td>
</tr>
<tr>
<td>Media control</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Splenic macrophages$^b$</td>
<td></td>
<td>18.1 ± 1.6$^d$</td>
</tr>
<tr>
<td>Splenic macrophages$^b$</td>
<td>anti-Thy 1</td>
<td>21.0 ± 2.2$^d$</td>
</tr>
<tr>
<td>Non-adherent$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splenocytes</td>
<td>antimacrophage</td>
<td>2.9 ± 0.3$^f$</td>
</tr>
<tr>
<td>Kupffer cells$^c$</td>
<td></td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>Kupffer cells$^s$</td>
<td>anti-Thy 1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Hepatic non-parenchymal cells$^c$</td>
<td>antimacrophage</td>
<td>0</td>
</tr>
</tbody>
</table>

*Kupffer cells and splenic macrophages were isolated on day 10 following IV administration of glucan (0.45 mg) or D5W on day 0. Macrophages were purified by adherence and/or treatment with anti-Thy 1. Nonadherent splenocytes and hepatic nonparenchymal cells were rendered free of macrophages by treatment with antimacrophage monoclonals. Effector cells were incubated with $^3$H-thymidine-labeled adenocarcinoma cells ($2 \times 10^7$/well) at a T:E ratio of 1:50 for 72 hr; $^3$H-thymidine release was determined and % specific lysis calculated. Values are expressed as mean ± SE. N = 8–12/group.

$^b$Statistical comparisons performed employing media control as reference, $P < .05$.

$^c$Statistical comparisons performed employing D5W control as reference, $P < .05$.

$^d$Statistical comparisons performed employing nonantibody-treated macrophages as reference, $P < .05$. 

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Browder and colleagues [2] have shown that IV glucan therapy will significantly enhance long-term survival and reduce hepatic tumor cell burden in rats receiving an intraportal challenge of $1 \times 10^6$ leukemic cells. Additional studies performed by Williams and co-workers [20] have shown glucan to be effective in altering the outcome of experimental hepatic metastatic disease. Glucan administration decreased metastatic burden and primary tumor weight resulting in increased long-term survival. Enhanced Kupffer-cell-mediated antitumor activity was correlated with increased hepatic tumor resistance, suggesting the importance of Kupffer cells in modulating the antimitastatic effect of glucan. The present study extends these initial observations by demonstrating the ability of glucan to enhance Kupffer cell antitumor activity for up to 10 d following single or multiple intravenous injections.

In order to further characterize the effector cells mediating the enhanced antitumor activity in glucan-treated mice, macrophage purification and depletion studies were performed. These studies were designed to determine whether cytotoxic effector cells such as natural killer (NK) cells [11] and cytotoxic T-lymphocytes [14] were involved in glucan-enhanced cytotoxicity to adenocarcinoma cells. Cytotoxic effector cells in spleen and liver preparations were evaluated since 15–20% of the adherent cell populations from these two sources were determined not to be macrophages by peroxidase staining. The present study indicates that NK cells and cytotoxic T-lymphocytes do not appear to be important in glucan-enhanced splenic macrophage-mediated cytotoxicity to adenocarcinoma cells since macrophage-depleted nonadherent splenocytes were not significantly cytotoxic to adenocarcinoma cells in a 72-hr cytotoxicity assay. In addition, removal of T-lymphocytes from the adherent splenocyte population by treatment with monoclonal anti-Thy 1 did not significantly alter the cytotoxic activity of the adherent splenocyte preparations. This observation is supported by the findings of Cook and co-workers [4], which showed that glucan was an effective antitumor agent in congenitally athymic, T-cell-deficient mice. However, adherent T-lymphocytes and/or NK cells may partially mediate the cytotoxicity observed in adherent hepatic nonparenchymal cell preparations since removal of lymphocytes from this adherent cell population by treatment with anti-Thy 1 monoclonals resulted in a significant decrease in cytotoxic activity. These studies support the hypothesis that Kupffer cells play an important role in hepatic antitumor surveillance. In addition, Kupffer-cell-mediated tumoricidal activity
can be enhanced in situ up to 10 d following IV administration of the macrophage-activating agent glucan. Pharmacologic enhancement of Kupffer-cell-mediated antitumor activity, which previous studies have associated with reduction in hepatic metastases [18--20], suggests the possible value of employing Kupffer-cell-activating biologic response modifiers in the therapy of hepatic neoplasia. Based on the relative ineffectiveness and, in many cases, severe toxicity of currently employed therapeutic modalities in the treatment of hepatic metastases, the administration of immunomodulating agents, either alone or in combination with other antineoplastic agents, may prove to be a beneficial new approach to the therapy of secondary hepatic neoplasia. In addition, immunotherapy may also be beneficial when employed in a prophylactic regimen prior to surgical resection of malignant tissue since the potential for tumor cell seeding of portal blood and subsequent hepatic metastases is enhanced following surgical manipulation. Preoperative Kupffer cell activation may prevent the establishment of malignant lesions in the liver and therefore may be of potential benefit in these patients.

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