Eradication of a Disseminated Syngeneic Mouse Lymphoma by Systemic
Adoptive Transfer of Immune Lymphocytes and Its Dependence upon a
Host Component(s)

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ABSTRACT

We have studied the in vivo effects of carrageenan and trypan blue on the adoptive immunotherapy of an established local and disseminated syngeneic mouse FBL-3 lymphoma. Mice receiving 500 rads total-body irradiation before injection of FBL-3 tumor into the footpad were treated 4 to 5 days later when a palpable local tumor and disseminated metastases were present. Injection of in vivo immune lymphocytes i.v. caused complete regression of footpad tumor and cured 96% of all mice (>60 days mean survival; p < 0.0005). Carrageenan or trypan blue treatment of the tumor-bearing host abrogated the therapeutic effect of adoptively transferred cells. Cure rates were significantly reduced to 27% (p < 0.004) and 0% (p < 0.0001) and mean survival times to 40.2 days (p < 0.0005) and 15.2 (p < 0.005) days for mice treated with carrageenan and trypan blue, respectively, in addition to immune cells. In vivo treatment of the immune spleen cell donors with carrageenan or trypan blue had no significant effect on the ability of those splenocytes to mediate cure when adoptively transferred cells. Cure rates were significantly reduced to 27% (p < 0.004) and 0% (p < 0.0001) and mean survival times to 40.2 days (p < 0.0005) and 15.2 (p < 0.005) days for mice treated with carrageenan and trypan blue, respectively, in addition to immune cells. In vivo treatment of the immune spleen cell donors with carrageenan or trypan blue had no significant effect on the ability of those splenocytes to mediate cure when adoptively transferred into tumor-bearing hosts, indicating that the inhibitory activity of these agents cannot be attributed to direct toxicity to immune lymphoid cells. These results demonstrate that a recipient component(s), possibly macrophages, sensitive to carrageenan and to trypan blue but relatively resistant to radiation (500 rads), plays a vital role in the cure of tumor-bearing mice that receive the adoptive transfer of immune splenocytes.

INTRODUCTION

The adoptive transfer of fresh, sensitized syngeneic T-lymphocytes can mediate the regression of established transplantable tumors in the mouse, rat, and guinea pig (2, 6, 9, 18, 27, 45, 52, 54). The precise in vivo mechanism(s) by which these immune cells mediate immunological injury to tumors upon adoptive transfer is not clear, but based upon the expression of known T-lymphocyte subset surface markers, one is able to speculate on the potential processes set in motion in vivo. For example, helper T-cells (expressing the Lyt-1 antigen in the mouse or W3/25 antigen in the rat) have been implicated in playing the central role in the rejection of a chemically induced sarcoma (3), of virally induced sarcomas (19, 34, 43), and of a leukemia/lymphoma (24), presumably by eliciting a DTH2 reaction or by serving as amplifier cells for the induction of cytolytic T-lymphocytes (3, 24, 43). Further, long-lived immune cells expressing predominantly the Thy-1+, Lyt-1+2− phenotype have been shown to specifically localize in tumors upon i.v. transfer (39). In some tumor models in mice, T-cells expressing the Lyt-1+2+ phenotype have also been shown to actively participate in the rejection process (46, 51), and it is possible that, during the course of the response in vivo, these Lyt-1+2+ cells mature into Lyt-1+2− cells (50) and/or serve as amplifier cells (56). In contrast, other laboratories have reported on the in vivo antitumor efficacy of cytotoxic lymphocytes expressing the Lyt-1−2+ phenotype. This subset of T-cells was shown by Leclerc and Cantor (33) to be capable of homing to and eliminating a small, localized MBL tumor growing s.c. Dailey et al. (10) described a long-lived Lyt-1−2+ cytolytic clone that prolonged survival of tumor-bearing mice, particularly when injected at the tumor site. More recently, the adoptive transfer of a Lyt-1−2+, glioma-specific cytotoxic T-lymphocyte clone has been shown to be effective for treating a chemically induced, syngeneic murine malignant glioma (59). The direct tumor-killing activity and the tumor-specific production of immune interferon by this clone as detected in vitro are purported to be the potential antitumor mechanisms operational in vivo. Lyt 1−, 2+ cells have also been documented as the T-cell subset responsible for elimination of allogeneic tumors (16, 17).

Considerable attention has been directed toward studies of the ability of adoptively transferred donor lymphocytes to recruit host effector components as one mechanism of tumor eradication in vivo. In particular, non-T-, noncommitted accessory cells from bone marrow, spleen, and peritoneal cells have been shown to augment the tumor-neutralizing capacity of specifically immune lymphocytes when admixed with tumor cells in the Winn test in lethally irradiated recipient mice (49, 53) and rats (1). Shu et al. (52) have shown that agents of known toxicity to monocytes-macrophages (trypan blue and carrageenan) can abrogate transferred immunity in treated guinea pigs.

We are investigating the mechanism(s) by which fresh (or cultured), sensitized T-cells act in vivo during the course of events leading to immune rejection of murine tumors. Our efforts have focused on the adoptive immunotherapy of Meth-A sarcoma (44, 46) and of the FBL-3 lymphoma (14, 15, 44, 46). With regard to the latter, results from our laboratory have indicated that the i.v. infusion of both Lyt-1+ and Lyt-2+ immune cells is required to cure mice bearing the FBL-3 tumor (46). In this paper, we describe the influence of carrageenan and trypan blue treatment on the immune response set in motion by adoptively transferred, in vivo-sensitized syngeneic T-lymphocytes that would ultimately result in the complete regression of FBL-3 tumor. We report that a recipient component(s), possibly macrophages, sensitive to these agents but relatively resistant to radiation (500 rads) is essential to the expression of adoptive immunity and cure of tumor-bearing mice.

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2 The abbreviations used are: DTH, delayed-type hypersensitivity; B6, C57BL/6 mouse; FBL-3, a Friend virus-induced lymphoma; HBSS, Hank’s balanced salt solution; MST, mean survival time.

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MATERIALS AND METHODS

Mice. Female B6 mice were 12 to 16 weeks old when used in these experiments. They were obtained from the Animal Production Colonies of the NIH, Bethesda, MD.

Tumor. FBL-3, a leukemia syngeneic to B6 mice, was originally a gift of Dr. C. C. Ting (National Cancer Institute, NIH). The tumor is highly immunogenic, with viral surface antigens that cross-react with other tumors induced by Friend, Moloney, and Rauscher viruses (23). A large number of vials of tumor from a single passage generation were cryopreserved. After thawing from storage at -70°C, tumor was injected i.p. in B6 mice and was passaged serially in ascites form. Tumor was not used after the third passage. FBL-3 tumor will grow and regress in approximately 70% of normal B6 mice if injected i.m. or s.c. although it grows progressively at these sites in irradiated mice.

Immunization. B6 mice were immunized with one i.m. injection of 10^7 live FBL-3 cells suspended in 0.05 ml sterile HBSS. Tumors grew for more than 3 weeks and then completely regressed in about 70% of mice. All mice used as immune spleen donors had no gross evidence of tumor at the time of spleen harvest.

In Vivo Assay of Adoptive Immunotherapy. Mice were given 500 rads whole-body irradiation (106Cs), and 2 to 4 hr later, 10^7 live FBL-3 tumor cells were injected into the right hind footpad in 0.05 ml of sterile HBSS. By Day 4 or 5, tumors were readily palpable, and the footpad diameter measured between 2.5 and 3 mm; the tumor was also disseminated in the regional lymph nodes and peripheral circulation (14). On the day of treatment (Day 4 or 5), mice were randomly assigned to groups and given injections i.v. with immune spleen cells in 1 ml of HBSS. We have shown previously that immune cells, but not normal cells, are effective in curing mice of both local and disseminated metastases after one i.v. adoptive transfer (14, 46). Each mouse was ear tagged and monitored for survival. Mice surviving to Day 60 were considered cured, since tumor recurrence is very rare after Day 60.

Immune Spleen Cell Suspension. Spleens were aseptically removed from FBL-3 immune donors, pooled, and crushed gently with the blunt end of a 10-ml syringe plunger in HBSS with 1% fetal calf serum. The cell suspension was washed 3 times in HBSS by centrifugation at 500 x g for 5 min. The splenocytes were then adjusted to a concentration of 10^9/ml for injection into the tail veins of recipient mice; erythrocytes were not removed before injection.

Preparation of Carrageenan and Trypan Blue. Carrageenan (type II; Sigma Chemical Co., St. Louis, MO) was dissolved in 0.85% NaCl solution to a concentration of 1 mg/ml using the double-boiler technique. The stock solution was sterilized by filtration, stored at 4°C, and brought to approximately 37°C for i.p. injections. Trypan blue (Direct Blue 14; Sigma) was dissolved in deionized water and dialyzed for 7 days with 10,000 molecular-weight salts and impurities (25). The solution was then concentrated by lyophilization. The stock solution was adjusted to 4 mg/ml in 0.85% NaCl solution, autoclaved, and stored at 4°C. In the experiments, tumor-bearing recipients of immune cells or immune mice serving as spleen cell donors received either 0.5 ml of carrageenan (0.5 mg) or of trypan blue (2 mg) per injection; the schedules of injections are described in the charts and tables.

Statistical Methods. In all experiments, the significance of differences in survival times between groups was determined by a Wilcoxon rank test (22). No mice were excluded from the statistical evaluations.

RESULTS

Effect of Carrageenan Treatment on the Expression of Adoptive Immunity. B6 mice, bearing palpable FBL-3 footpad tumors and with disseminated disease 5 days after tumor injection, were infused with 10^8 fresh immune spleen cells. As shown by the survival data in Table 1 and Chart 1, mice receiving no treatment all died of progressive tumors within 21 days of tumor injection (total MST of 3 experiments, 21.1 days). In contrast, 95% of mice receiving 10^6 immune lymphocytes underwent complete regression of tumor and were permanently cured of disease (total MST of 3 experiments, 68.5 days; p < 0.00005). To investigate the effect of carrageenan treatment of the tumor-bearing hosts on this successful adoptive therapy, the drug was administered i.p. at a dose of 0.5 mg/injection to each animal starting 1 day before adoptive transfer. A total of 7 injections was given over a 2-week period. Chart 1 and Table 1 show the survival rates of individual mice in 2 combined experiments. In groups of mice that did not receive immune lymphocytes, survival rates in untreated and carrageenan-treated animals were relatively similar, and all mice succumbed to progressively growing tumor. Carrageenan treatment alone slightly increased the total MST when compared to those mice receiving no treatment (26.1 days versus 21.1 days; p < 0.025). Carrageenan treatment of tumor-bearing hosts markedly reduced the capacity of transferred immune splenocytes to cure mice of disseminated tumor. Although the total MST for these mice was extended when compared to the untreated group (40.2 days versus 21.1 days; p = 0.005), only 4 of 15 (27%) were cured of disease; the remaining mice succumbed to progressively growing footpad tumors and disseminated disease. This is in marked contrast to mice receiving 10^6 immune splenocytes without carrageenan treatment (total MST, 68.5 days; p < 0.0005) in which 18 of 19 (95%) were cured as evidenced by complete tumor regression.

Effect of Trypan Blue Treatment on the Expression of Adoptive Immunity. We used a similar experimental protocol to study the effect of trypan blue treatment on the successful adoptive immunotherapy of mice with established FBL-3 tumor. Trypan blue was administered i.p. to recipient mice over a 2 week period at a dose of 2 mg/injection. The first 2 injections were given on Day 4 after tumor inoculation, 8 hr apart and just prior to the i.v. infusion of 10^6 fresh immune splenocytes. Chart 2 and Table 2 show the survival data. The 3 groups receiving no treatment, trypan blue alone, and trypan blue plus 10^6 immune cells had MSTs of 16.5, 16.4, and 15.2 days, respectively. These survival times were not significantly different from one another, and none of the mice in these groups was cured of disease. As was found with carrageenan, trypan blue treatment of the tumor-bearing recipients also effectively abrogated the antitumor capacity of adoptively transferred, immune splenocytes (MST of 15.2 days; p < 0.005; none of 5 mice cured). The administration of 10^6 immune cells to mice not receiving trypan blue cured all 5 treated mice (MST, >60 days; p < .001).

Lack of Effect of in Vivo Exposure of Immune Spleen Cells to Carrageenan and Trypan Blue on Subsequent Adoptive Transfer. It was necessary to determine whether the observed inhibitory effects of carrageenan and trypan blue on the adoptive immunotherapy of FBL-3 lymphoma were due to a direct toxic effect on the transferred immune lymphocytes. FBL-3 immune, B6 mice that were to serve as spleen cell donors were treated with either carrageenan or trypan blue in regimens similar to those used for treating the tumor-bearing recipients of the adoptive transfer. As observed previously (20, 52), treatment of mice with 7 injections of carrageenan or trypan blue over a 2-week period led to a slight, but noticeable, splenomegaly. Adoptive
## Table 1

**Effect of carrageenan treatment of the recipient or of the immune spleen donor on the expression of adoptive immunity to disseminated FBL-3 tumor**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Total MST</th>
<th>No. of cures/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No treatment</td>
<td>18, 21, 21, 21 (20.4)</td>
<td>14, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19 (18.3)</td>
<td>17, 18, 18, 21, 28, 30, 30, 30, 30, 30 (24.0)</td>
<td>21.1 (20)</td>
<td>0/20</td>
</tr>
<tr>
<td>3. 10&lt;sup&gt;8&lt;/sup&gt; immune splenocytes&lt;sup&gt;i&lt;/sup&gt;</td>
<td>&gt;70, &gt;70, &gt;70, &gt;70, 70 (70.0)</td>
<td>41, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70 (66.4)</td>
<td>&gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70 (70.0)</td>
<td>68.5 (19)</td>
<td>18/19</td>
</tr>
<tr>
<td>4. 10&lt;sup&gt;8&lt;/sup&gt; immune splenocytes + carrageenan&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18, 18, 35, 35, 35, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70 (43.9)</td>
<td>11, 14, 32, 35, 41, 49, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70 (36.0)</td>
<td>40.2 (15)</td>
<td>4/15</td>
<td></td>
</tr>
<tr>
<td>5. 10&lt;sup&gt;8&lt;/sup&gt; immune splenocytes from carrageenan- treated mice&lt;sup&gt;i&lt;/sup&gt;</td>
<td>&gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70 (70)</td>
<td>&gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70, &gt;70 (70)</td>
<td>70.0 (8)</td>
<td>8/8</td>
<td></td>
</tr>
</tbody>
</table>

*These values are MST (median survival time) in days.*

Group 4 vs. 1 2 3 5

| 1 | NS<sup>n</sup> | NS | NS | <0.005 |
| 2 | <0.05 | NS | <0.001 |
| 3 | <0.001 | <0.001 | <0.001 | <0.001 |
| 5 | <0.0005 | <0.0005 | <0.0005 | <0.0005 |

Group 1 vs.

| 2 | <0.01 | NS | <0.01 |
| 3 | <0.01 | <0.001 | <0.001 | <0.001 |
| 5 | <0.01 | <0.001 | <0.001 |

<sup>a</sup> FBL-3 cells (10<sup>7</sup>) injected intrafootpad after 500 rads of total-body irradiation on Day 0.

<sup>b</sup> Numbers in parentheses of Columns 2 to 4, MST.

<sup>c</sup> Numbers in parentheses of Column 5, total number of mice.

<sup>d</sup> Carrageenan (0.5 mg) injected i.p. on Days 4, 5, 7, 9, 11, 13, and 15.

<sup>e</sup> Fresh immune splenocytes (10<sup>7</sup>) from mice that were immunized with 10<sup>7</sup> i.m. FBL-3 and underwent complete tumor regression were injected i.v. on Day 5.

<sup>f</sup> Survival of >70 days counted as 70 in determining MST.

<sup>g</sup> Fresh immune splenocytes and carrageenan both given as described above.

<sup>h</sup> Donors of fresh immune splenocytes were given 0.5 mg carrageenan i.p. on Days 12, 10, 8, 6, 4, 2, and 1 before spleens were harvested on Day 0.

<sup>i</sup> NS, not significant.

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Transfer of immune splenocytes obtained from these animals retained the capacity to cure 100% of mice with disseminated FBL-3 tumor, with no significant difference from that of untreated immune splenocyte donors (Tables 1 and 2; Charts 1 and 2). These results indicate that the inhibitory effect of trypan blue and carrageenan on adoptive immunotherapy in our FBL-3 tumor model is not one of direct toxicity to donor immune lymphocytes but is, rather, an effect on a component of recipient origin.
interleukin-1 (Refs. 13 and 40). These cells are activated by interferons or other substances (Refs. 5 and 13), and the capacity to transfer antitumor immunity. Survival was checked daily.

**DISCUSSION**

It has been postulated that adoptively transferred immune lymphocytes bearing the Lyt-1+2+ phenotype mediate the in vivo rejection of skin allografts and transplantable tumors by either eliciting a DTH response (3, 11, 19, 31, 34–36) or amplifying cytolytic Lyt-1+2+ T-cell activity (16, 38). Both reactions are thought to occur through soluble mediators released by the transferred lymphocytes. Although the evidence that these processes are responsible for rejection in vivo is clearly indirect, it is hypothesized that the host contributes, in some capacity, an integral component(s) to the rejection response induced by the transfer of sensitized Lyt-1+2 donor lymphocytes. Host macrophages appear to be one such candidate in the immune response to tumors because of their direct tumoricidal activity (e.g., once activated by interferons or other substances [Refs. 5 and 13], tumor antigen-presenting capacity [Refs. 13 and 42], and secretary products [e.g., interleukin-1; Refs. 13 and 40]).

As an initial attempt to elucidate some of the complex cellular interactions that lead to the regression of established syngeneic tumors, our current study was undertaken using agents (trypan blue and carrageenan) of known, relatively selective, toxicity to macrophages. In the experiments reported in this paper, we have demonstrated that carrageenan and trypan blue treatment of mice with established FBL-3 lymphoma abrogates the curative capacity of adoptively transferred immune splenocytes. Findings of a similar nature have been reported in the adoptive immunotherapy of a syngeneic tumor in the guinea pig (52). In a skin allograft model, carrageenan has been shown to eliminate the capacity of specifically sensitized cells to cause accelerated graft rejection upon i.v. transfer (29). In light of these experiments, it is interesting to note that carrageenan has been shown to be a potent inhibitor of DTH responses in vivo (47, 48).

Although trypan blue is not ingested by viable lymphocytes, neutrophils, basophils, or eosinophils (41), and carrageenan does not appear to be cytotoxic for lymphocytes either structurally or functionally (4), except at high doses (30), it was nevertheless important to rule out any possibility that these agents were directly toxic to the adoptively transferred immune splenocytes. Thus, we showed that treatment of the immune cell donors with trypan blue and carrageenan did not adversely affect the curative capacity of their splenocytes. Further, we have found that both agents have no toxic effect on lymphokine-activated killer cell precursors when administered in vivo to normal mice.

Despite the fact that little is known regarding the in vivo effects of carrageenan and trypan blue, much information exists from in vitro studies demonstrating that these agents exhibit a potent cytotoxic effect on macrophages (8). The nonspecific cytotoxicity in vitro induced by Bacillus Calmette-Guérin (25), Corynebacterium parvum, or pyran (37) is abrogated by trypan blue. Although trypan blue and carrageenan have been shown to prevent tumor-specific rejection or to potentiate the growth of a transplantable tumor (26, 28, 32), caution must be exercised when interpreting the observed effects, as these agents have multiple activities unrelated to macrophage functions (55, 57). For example, carrageenan has been documented to inhibit the complement system (7, 12) and blood coagulation (58), as well as to affect lymphocyte circulation (21). However, with regard to

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**Table 2**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Survival times</th>
<th>MST (days)</th>
<th>No. of cures/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>12, 13, 19, 22</td>
<td>16.5</td>
<td>0/4</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>10, 14, 17, 19, 22</td>
<td>16.4</td>
<td>0/5</td>
</tr>
<tr>
<td>10^8 immune splenocytes + trypan blue</td>
<td>&gt;60, &gt;60, &gt;60, &gt;60, &gt;60</td>
<td>60.0</td>
<td>5/5</td>
</tr>
<tr>
<td>4. 10^8 immune splenocytes + trypan blue</td>
<td>14, 15, 17, 22, 22</td>
<td>15.2</td>
<td>0/5</td>
</tr>
<tr>
<td>5. 10^8 immune splenocytes from trypan blue-treated mice^d</td>
<td>&gt;60, &gt;60, &gt;60, &gt;60</td>
<td>60.0</td>
<td>4/4</td>
</tr>
</tbody>
</table>

^d FBL-3 cells (10^7) injected intraperitoneal after 500 rads daily of total-body irradiation on Day 0.

^p values: Group 1 versus 2, p = not significant; Group 1 versus 3, p < 0.01; Group 1 versus 4, p = not significant; Group 1 versus 5, p < 0.01; Group 4 versus 1, p = not significant; Group 4 versus 2, p = not significant; Group 4 versus 3, p < 0.005; Group 4 versus 5, p < 0.01.

^b Two mg trypan blue injected i.p. on Days 4 (2 injections), 5, 7, 9, 11, and 13.

^c Fresh immune splenocytes (10^7) from mice that were immunized with 10^7 i.m.

^e FBL-3 and underwent complete tumor regression were injected i.v. on Day 4.

^f Survival of >60 days counted as 60 in determining MST.

^g Fresh immune splenocytes and trypan blue both given as described above.

^h Donors of fresh immune splenocytes were given 2 mg trypan blue i.p. on Days 15, 13, 11, 9, 5, 3, and 1 (2 injections) before spleens were harvested on Day 0.

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the latter effect, interference with the transportation of i.v.-transferred immune cells to the tumor challenge sites in the guinea pig has not been apparent (52). Moreover, we have found that carrageenan administration does not affect the capacity of lymphokine-activated killer cells to cause the regression of established pulmonary sarcoma metastases upon i.v. transfer. At present, we cannot explain the slight prolongation in survival of tumor-bearing hosts treated with carrageenan alone.

The experiments reported here demonstrate an important role for a host component(s) in our model of the adoptive immunotherapy of a disseminated FBL-3 lymphoma. It appears that the major effect of carrageenan and trypan blue to abrogate the therapeutic effect of transferred cells occurs by an alteration of host macrophage participation in the rejection process.

REFERENCES


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