In Vivo Effects and Parallel in Vitro Cytotoxicity of Splenocytes Harvested from Treated or Control C57BL/6J Mice after Adjuvant Immunotherapy of Pulmonary Metastases Using Xenogeneic RNA Specific to B16 Murine Melanoma

Bosco Shang Wang, Glenn Steele, Jr., John A. Mannick, Marguerite Fallon, and Suzanne R. Onikul

Department of Surgery, Peter Bent Brigham Hospital [B. S. W., G. S., J. A. M., S. R. O.], and Sidney Farber Cancer Institute [G. S., M. F.], Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Following excision of B16 melanoma isografts from C57BL/6J mice, 80 to 100% of untreated animals develop progressive pulmonary metastases after 4 to 6 weeks and die within 100 days. Immune RNA (I-RNA) extracted from the lymphoid tissues of guinea pigs immunized with B16 melanoma or antigenically distinct syngeneic Lewis lung carcinoma was incubated in vitro with splenocytes harvested from normal C57BL/6J mice. After B16 isograft excision, animals receiving five separate i.p. injections (every other day) of 75 × 10⁶ B16 I-RNA-treated syngeneic splenocytes showed significantly improved long-term survival as summarized from three successive experiments (52%) compared to animals receiving Lewis lung carcinoma I-RNA-treated splenocytes (p < 0.0001). The effect of I-RNA treatment was RNase sensitive. Splenocytes were harvested from selected animals in each group 1 to 5 weeks after adjuvant therapy and tested for in vitro cytotoxicity against B16 murine melanoma targets. [¹²⁵]Iododeoxyuridine assays, “long-term” ⁵¹Cr assays, and microcytotoxicity assays with visual counting of remaining target cells were performed in parallel. Splenocytes harvested from B16 I-RNA-treated animals were consistently and significantly cytotoxic to B16 melanoma target cells when compared to splenocytes harvested from Lewis lung carcinoma I-RNA-treated animals. This in vitro cytotoxic effect was demonstrated from the second through the fifth week after in vivo treatment when animals in the control groups began to die from progressive pulmonary metastases.

INTRODUCTION

The demonstration of effective immunotherapy even when predicted by various in vitro assays of specifically altered immune parameters has been uniformly unsuccessful or un reproducible in animal tumor models and in humans (22). At best, immunoprevention experiments have shown that some tumor-associated antigens defined by in vitro cytotoxicity assays may function as tumor rejection antigens in vivo but only when immunization has preceded either carcinogen treatment (17, 21) or tumor isograft challenge (4, 15, 18). Naturally, these immunoprevention models are not directly analogous to humans with growing tumor or to patients who are at risk of recurrence after surgical excision of their primaries.

Xenogeneic I-RNA extracted from the lymphoid tissues of immunized animals has been reported to transfer specific immune responsiveness both in vitro and in vivo (4, 15, 23, 26). In vitro studies have demonstrated that specific I-RNA increases cytotoxicity against tumor targets even when effector cells are harvested from animals with large tumor burden (6). As is the case with other potentially attractive forms of immunotherapy, however, I-RNA has produced no unequivocal benefit when animals bearing tumor isografts received specific I-RNA therapy even when therapy is begun early after isograft inoculation (5).

Although unproven, there is theoretical advantage in the application of systemic antitumor therapy at a time when host tumor burden is minimal or when no gross tumor is evident but the risk of recurrence is high (9). Host antitumor defenses may be optimal in such a setting, and the implication of I-RNA success in preventing death from metastases after complete tumor isograft excision has been published (14, 25).

In the present investigation, we have attempted to confirm that adjuvant treatment using xenogeneic I-RNA will prevent the development of metastases leading to death after complete surgical excision of B16 murine melanoma isografts. In addition, we have asked whether the in vivo effects of immunotherapy can be monitored in vitro by 3 separate assays for CMC using B16 melanoma target cells and splenocytes sequentially harvested from selected animals in each of the in vivo treatment and control groups.

MATERIALS AND METHODS

Animals. C57BL/6J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and fed a standard pellet diet and water ad libitum.

Tumors. B16 melanoma and 3LL were obtained from The Jackson Laboratory and from Dr. A. E. Bogden, Mason Research Institute, Worcester, Mass., respectively. Tumor isografts were maintained by serial subcutaneous transplantation in adult male C57BL/6J mice. Both tumors caused death from pulmonary metastases in a high percentage of mice following complete excision of primary tumor isografts. Although syngeneic, B16 and 3LL have been shown by previously published in vivo immunotherapy protocols to be antigenically distinct (25).

Cell Cultures. Monolayer cultures of B16 melanoma were maintained in Weymouth’s medium containing 10 to 20% fetal serum. 1-ANA. I-RNA, immune RNA; CMC, cell-mediated cytotoxicity; 3LL, Lewis lung carcinoma; RPMI medium 1640, Roswell Park Memorial Institute Medium 1640; CFA, complete Freund’s adjuvant.

1 This research was supported in part by the Brigham Surgical Group, Inc., and by National Cancer Institute Grants CA 22513-02 and CA 21644.
2 Supported in part by American Cancer Society Fellowship JFCF 372-A. To whom requests for reprints should be addressed, at Department of Surgery, Peter Bent Brigham Hospital, 721 Huntington Avenue, Boston, Massachusetts 02115.
3 The abbreviations used are: I-RNA, immune RNA; CMC, cell-mediated cytotoxicity; 3LL, Lewis lung carcinoma; RPMI medium 1640, Roswell Park Memorial Institute Medium 1640; CFA, complete Freund’s adjuvant.
calf serum. Early generations of B16 stored at —70° in Weymouth's medium plus 10% dimethyl sulfoxide were periodically defrosted, passaged several times in culture medium, and used as target cells for 2 to 3 weeks in the various cytotoxicity assays to be described below.

**Effectors Cells Used in Cytotoxicity Assays.** Spleens were removed from selected sacrificed animals in treatment and control groups throughout the in vivo immunotherapy trials for use as effector cells in the in vitro cytotoxicity assays. Each spleen was teased apart in RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N.Y.) and rinsed through 40 and 80 mesh stainless steel screens. Cell suspensions were further purified by Ficoll-Hypaque gradient centrifugation (13). Effector cell populations consisted of more than 95% monocytes with greater than 96% viability as judged by trypan blue dye exclusion.

**In Vitro Assays of CMC.** Three separate in vitro assays were performed in parallel to determine the cytotoxic effect of splenocytes harvested from each of the in vivo treatment and control groups. Cultured B16 murine melanoma cells were used as targets for all assays. Individual in vitro assays were performed by separate individuals in 2 different laboratories. Results were not compared until the entire experimental set was complete.

The first in vitro assay used to monitor CMC is a modification of the technique described by Cohen et al. (3). Viable B16 melanoma cells (10⁵) were labeled with 0.25 μCi of [125I]iododeoxyuridine (specific activity, >2000 Ci/mmol; New England Nuclear, Boston, Mass.) in each well of a Falcon Microtest II culture plate at 37° in a 10% CO₂:90% water-saturated air atmosphere for 24 hr. Excess [125I]iododeoxyuridine was then aspirated, and each cell was washed once with RPMI Medium 1640. Splenocytes were suspended in complete RPMI Medium 1640 containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 100 units penicillin per ml, and 100 μg streptomycin per ml and added to the labeled B16 cells at a spleenocyte:target cell ratio of 500:1. After 48 hr incubation, splenocytes and detached target cells were removed by washing the wells with RPMI Medium 1640. Remaining adherent tumor cells were fixed with a thin film of plastic spray, and individual wells were cut out with a band saw. Residual radioactivity, reflecting remaining tumor cells in each well, was counted in a Packard Model 5110 γ-counter. The mean cpm were obtained from 3 replicate wells. To emphasize the specificity of tumor immunity mediated by I-RNA, splenocytes obtained from mice treated with 3LL I-RNA were used as the major control effector cells. Therefore, the cytotoxicity index (CI) was calculated as follows:

\[
CI = \frac{\text{cpm of tumor cells exposed to splenocytes from 3LL I-RNA-treated mice} - \text{cpm of tumor cells exposed to splenocytes from 3LL control mice}}{\text{cpm of tumor cells exposed to splenocytes from 3LL I-RNA-treated mice}} \times 100
\]

The second in vitro assay of CMC used was the "long-term" 51Cr technique described by Steele et al. (19). In this assay, 10 x 10⁵ B16 melanoma cells/0.2 ml Weymouth's medium containing 10% heat-inactivated fetal calf serum were incubated in flat-bottomed sterile 2-ml glass ampuls (Flow Laboratories, Solna, Sweden) for 2 to 4 hr at 37° in a 5% CO₂:95% air atmosphere. After target cell attachment, medium was aspirated and replaced with 0.2-ml volumes of sodium chromate (51Cr; specific activity, 56 mCi/mg). Target cells were labeled in a concentration of 0.2 mCi/ml medium. After 2 hr incubation, 51Cr solution was aspirated, and each ampul was gently washed 5 times with 0.7-ml volumes of Weymouth's medium containing 5% inactivated fetal calf serum. Mouse splenocytes harvested from each of the in vivo treatment or control groups were added in 0.2-ml volumes of Weymouth's medium at effector cell:target cell ratios of 100:1, 30:1, and 10:1. After 1 hr, 1.8 ml of Weymouth's medium containing 5% inactivated fetal calf serum were added to each ampul. After 20 to 24 hr of incubation at 37° in a 5% CO₂:95% air atmosphere, each ampul was measured for total radioactivity in a γ-counter. Ampuls were then centrifuged at 600 x g for 4 to 5 min, and 0.2 ml of supernatant was removed for measurement of released radioactivity. The remaining 1.8 ml were aspirated and discarded, and 0.7-ml volumes of Weymouth's medium were added to each ampul. After this washing medium was aspirated, the remaining radioactivity was measured in the ampuls empty except for retained adherent target cells. Cytotoxic effect was calculated in 2 ways:

\[
\frac{\text{Radioactivity released per 0.2 ml x 10}}{\text{Total radioactivity}} \times 100
\]

and

\[
\frac{\text{Remaining radioactivity after final wash}}{\text{Total radioactivity}} \times 100
\]

Each splenocyte population was tested in triplicate at the 3 effector cell:target cell ratios, and mean percentage of cytotoxicity ± S.E. was calculated.

The third in vitro assay for CMC consisted of a previously described microcytotoxicity technique with visual counting of remaining B16 target cells (after Giemsa staining) following 48 hr incubation with the various splenocyte populations (19). The effector cell:target cell ratios used were 3000:1, 2000:1, and 1000:1. Samples were tested at each effector cell:target cell ratio in at least 8 parallel wells. Splenocyte-mediated cytotoxicity was considered present if splenocytes harvested from mice sacrificed in the specific immunotherapy treatment group caused a statistically significant decrease in the number of target cells when compared to splenocytes harvested from the nonspecifically treated or control groups. Data from all of the in vitro assays were analyzed for statistical significance with Student's t test.

**Preparation of I-RNA.** The details of I-RNA preparation have been described elsewhere (26). Briefly, Hartley guinea pigs were immunized by footpad injection of a mixture of tumor cells and CFA. Two weeks later, spleens and lymph nodes of immunized guinea pigs were harvested, and RNA was extracted from these tissues by the hot phenol method. RNA was stored in ethanol at —20° until use. Only nondegraded RNA, as determined by ultracentrifugation in sucrose density gradients, was used (24). After the complete removal of ethanol, RNA was dissolved in RPMI Medium 1640 and added to a Tris-NH₄Cl-treated splenocyte suspension prepared from normal C57BL/6J mice at a concentration of 750 to 1000 μg for every 5 x 10⁵ splenocytes in 1 ml of RPMI Medium 1640. After incubation at 37° in a 10% CO₂:90% air atmosphere for 30 min, the cells were collected by centrifugation and brought to
a concentration of $75 \times 10^6$ RNA-incubated splenocytes/ml of RPMI Medium 1640.

**Experimental Design.** The *in vivo* adjuvant immunotherapy protocol and parallel *in vitro* CMC testing using splenocytes from selected animals in each experimental group are summarized in Chart 1. Sets of 8- to 10-week-old male C57BL/6J mice (45 to 60/set) were given injections of $2 \times 10^5$ B16 melanoma cells i.m. in their left hind limbs. B16 cells used for all *in vivo* experiments were harvested from s.c. isografts passed in syngeneic 8- to 10-week-old male mice. Fourteen days later, the limbs were amputated after isografts became palpable in all animals. Beginning 2 days later, each animal received 5 i.p. injections (every other day) of $75 \times 10^6$ syngeneic splenocytes that had been variably treated *in vitro* as illustrated in Chart 1. The survival rate of each experimental group of 15 animals was recorded until 100 days after the primary isograft excision. Selected survivors were sacrificed after 100 days and autopsied to prove the absence of pulmonary metastases. The significance of the differences in survival between treatment and control groups were analyzed by Fisher’s exact $x^2$ test.

Parallel *in vitro* cytotoxicity testing by 1 of 3 CMC assays was performed at weekly intervals from Day 10 through Day 38 after isograft amputation. The actual assays performed to demonstrate CMC of splenocytes harvested from sacrificed animals in each treatment and control group are detailed in Chart 1.

**RESULTS**

**In Vivo Effect of Adjuvant Therapy.** Survival of animals in treatment and control groups is summarized in Chart 2. Splenocytes incubated with specific B16 I-RNA significantly increased long-term survival to 52% ($p < 0.0001$). Selected surviving animals were sacrificed and autopsied to prove absence of pulmonary metastases. No animals surviving 100 days after amputation of the tumor isograft had gross or microscopic evidence of metastases. Treatment of B16 I-RNA with pancreatic RNase (50 $\mu$/ml) for 30 min prior to incubation with splenocytes destroyed the effectiveness of therapy.

**Parallel in Vitro CMC.** When B16 melanoma cells were exposed to splenocytes from animals sacrificed in the various treatment or control groups, clear-cut and statistically significant increases in CMC were demonstrated only in the animals who had received specific B16 I-RNA immunotherapy. Tables 1 to 3 present the details of a single parallel set of 3 CMC assays performed at Day 24 after primary isograft resection (Day 14 after completing immunotherapy). The 2 pools of splenocytes harvested from animals receiving specific immunotherapy are identified as Pool 1 and Pool 2 to enable comparison of interassay CMC produced by the same effector cell populations. There is good agreement among the 3 tests for CMC in the various experimental and control groups.

Charts 3 to 5 summarize the time course of *in vitro* CMC by each of the 3 assays. Again, there is good agreement among the techniques, and only the splenocytes from B16 I-RNA-treated animals showed consistent and significant CMC effect on B16 targets at all intervals.

**DISCUSSION**

These results confirm the effectiveness of xenogeneic I-RNA treatment and control groups is summarized in Chart 2. Splenocytes incubated with specific B16 I-RNA significantly increased long-term survival to 52% ($p < 0.0001$). Selected surviving animals were sacrificed and autopsied to prove absence of pulmonary metastases. No animals surviving 100 days after amputation of the tumor isograft had gross or microscopic evidence of metastases. Treatment of B16 I-RNA with pancreatic RNase (50 $\mu$/ml) for 30 min prior to incubation with splenocytes destroyed the effectiveness of therapy.

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Splenocytes harvested from animals receiving Effector cell-target cell ratio on remaining B16 target cells Cytotoxicity index (\%) 3LL non-tumor-specific I-RNA 500:1 321 ± 30 43\(^a\) Tumor-specific B16 I-RNA #1 500:1 173 ± 9 43\(^a\) Tumor-specific B16 I-RNA #2 500:1 170 ± 13 47\(^b\) RNase-pretreated B16 I-RNA 500:1 305 ± 25 5\(^b\)

\(^a\) Cytotoxicity index

\[^{125}\]Iododeoxyuridine with 3LL splenocytes - \[^{125}\]Iododeoxyuridine with "test" splenocytes \[^{125}\]Iododeoxyuridine with 3LL splenocytes \times 100

\(^b\) Mean ± S.E.
\(^c\) Not significant.

Table 2

In vitro cytotoxicity of splenocytes harvested from I-RNA-treated and control mice on B16 melanoma targets

Comparison of effects of splenocytes from animals receiving Effector:target ratio \(^{51}\)Cr released \(^{51}\)Cr retained Differences in effects of splenocytes by

<table>
<thead>
<tr>
<th>Comparison of effects of splenocytes from animals receiving</th>
<th>Effector:target ratio</th>
<th>(^{51})Cr released</th>
<th>(^{51})Cr retained</th>
<th>Differences in effects of splenocytes by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-specific B16 I-RNA #1 vs. 3LL I-RNA</td>
<td>100:1</td>
<td>17(^a)</td>
<td>10.5(^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>8.4(^b)</td>
<td>1.8(^b)</td>
<td></td>
</tr>
<tr>
<td>Tumor-specific B16 I-RNA #2 vs. 3LL I-RNA</td>
<td>100:1</td>
<td>8.5(^b)</td>
<td>5(^b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>8.7(^b)</td>
<td>3(^b)</td>
<td></td>
</tr>
<tr>
<td>RNase-pretreated B16 I-RNA vs. 3LL I-RNA</td>
<td>100:1</td>
<td>10.1(^a)</td>
<td>5.6(^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>-2.1(^c)</td>
<td>-1(^c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>-1(^c)</td>
<td>-1(^c)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) p < 0.05.
\(^b\) p < 0.01.
\(^c\) Not significant.

In vivo and in Vitro Effects of I-RNA Adjuvant Therapy

In vivo and in vitro effects of I-RNA adjuvant therapy have previously been reported in both transplantation and animal tumor models (1, 11, 12). Cytotoxicity and proliferation assays have shown specific and significant transfer of immune reactivity after I-RNA exposure of effector splenocytes or lymphocytes (23, 26). Increased specific cytotoxic effect of already immune splenocytes or lymphocytes harvested from animals with large volumes of tumor isograft has also been reported after in vitro exposure of the effector cells to xenogeneic I-RNA (6). The parallel in vitro CMC data documented in this investigation demonstrate, however, that in vivo xenogeneic I-RNA treatment may modify a specific host immune response measurable in vitro. All 3 CMC assays demonstrated similar specific in vitro
Table 3. Summary of serial [125I]iododeoxyuridine cytotoxicity assay data using splenocytes harvested from B16 I-RNA-treated versus RNase-exposed I-RNA-treated animals on B16 melanoma targets. Percentage of cytotoxicity is calculated as described in Table 3.

<table>
<thead>
<tr>
<th>Splenocytes harvested from animals receiving</th>
<th>Effector:target ratio</th>
<th>Surviving B16 target cells</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3LL non-tumor-specific I-RNA</td>
<td>3000:1</td>
<td>275 ± 18$^a$</td>
<td>1000:1</td>
</tr>
<tr>
<td>Tumor-specific B16 I-RNA RNA #1</td>
<td>3000:1</td>
<td>127 ± 7$^b$</td>
<td>2000:1</td>
</tr>
<tr>
<td>Tumor-specific B16 I-RNA RNA #2</td>
<td>3000:1</td>
<td>110 ± 10$^c$</td>
<td>2000:1</td>
</tr>
<tr>
<td>RNase-pretreated</td>
<td>3000:1</td>
<td>246 ± 23$^d$</td>
<td>1000:1</td>
</tr>
<tr>
<td>B16 I-RNA</td>
<td>2000:1</td>
<td>283 ± 21$^e$</td>
<td>1000:1</td>
</tr>
</tbody>
</table>

* Mean ± S.E.  
$^a$ p < 0.001.  
$^b$ Not significant.

Chart 3. Summary of serial [125I]iododeoxyuridine cytotoxicity on B16 melanoma targets using splenocytes harvested from animals sacrificed at weekly intervals after B16 I-RNA or RNase-exposed I-RNA. Cytotoxicity index is calculated as stated in Table 1, Footnote a.

Chart 4. Summary of serial long-term $^{51}$Cr data using splenocytes harvested from B16 I-RNA-treated or RNase-exposed I-RNA-treated animals on B16 melanoma targets. Cytotoxic effect is calculated as stated in Table 2.

Cytoxicity of splenocytes from I-RNA-treated animals. Significantly increased in vitro CMC was found only in the group of animals whose survival was improved by specific I-RNA therapy. We hypothesize, therefore, that the B16 I-RNA splenocyte treatment may have functioned at least in part by specifically altering host CMC in vivo.

Many investigators have reported widely varying results in attempting to correlate changes in specific in vivo and in vitro immunological parameters (10, 16, 20). Individual in vitro assay techniques present their own intrinsic objective and subjective difficulties and many monitor specific cytotoxic effects of particular subsets of effector cells with differing sensitivity (19). The 3 separate in vitro assay techniques were performed in parallel in this investigation to minimize the subjective difficulties of any single assay and also to compare variations in each in vitro result with the in vivo immunotherapy results. No attempt was made to fractionate subsets of effector cells used in the cytotoxicity assays, and it is therefore possible that the uniformity of CMC results presented here might not have been obtained with a more restricted effector cell population.

Host immune parameters other than CMC may of course have been effected by the I-RNA therapy. Several of these other immunological parameters are presently under investigation. We have obtained preliminary evidence, for instance, that natural killer activity monitored by a short-term $^{51}$Cr release assay using splenocytes harvested from I-RNA-treated versus nontreated C57BL/6J mice on reference AJ Maloney virus-induced lymphoma targets, YAC-1, is significantly decreased.

The implications of these data are 2-fold. (a) Fidler and Kripke (8) have presented evidence that B16 melanoma iso- grafts may be heterogeneous, with defined cell populations predetermined to become metastases. Our investigations have shown in vivo therapeutic effect in permanently preventing pulmonary metastases but only temporary and equivocal ther- apeutic benefit against the primary tumor isograft itself. We have, therefore, examined whether particular sublines of B16 melanoma which metastasize more frequently (7) are paradoxically more sensitive to specific CMC after xenogeneic I-RNA than are B16 sublines metastasizing less frequently. (b) Since this animal model is analogous to several human tumors (with significant risk of recurrence after clinically curative primary tumor resection), the application of adjuvant xenogeneic I-RNA therapy in a well-controlled human trial is being planned.

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