Potentiation of Immunity to Murine Leukemia (LSTRA) by Bacillus Calmette-Guérin

Gerald L. Bartlett, John W. Kreider, and Dallas M. Purnell

Abstract

LSTRA murine leukemia injected s.c. or intradermally was uniformly lethal for BALB/c x DBA/2 F1 mice at doses of 10^3 cells or greater. Mice that survived after injection of near-threshold tumor cell doses usually were not immune to the tumor. Few mice were cured by excision of the injection site tumor ≥6 days after injection, but those that were cured developed tumor immunity. Irradiation of the tumor cells with 8000 rads reliably prevented growth. Injection of 10^6 LSTRA cells exposed to 8000 rads (Lx) immunized few animals to LSTRA challenge while 10^7 Lx protected most of the mice against challenge with 10^6 or 10^7 tumor cells. LSTRA-induced immunity was effective against a tumor line induced by the murine sarcoma virus (Moloney) but not against a spontaneous mammary carcinoma or an antigenic, chemically-induced sarcoma. Lymphoid cells from immune mice were effective in the local passive transfer (Wlhn) test. The low level of immunity induced by 10^6 Lx was significantly potentiated by admixture of 5 x 10^3 or 5 x 10^4 colony-forming units Bacillus Calmette-Guérin in the tumor cell vaccine. Bacillus Calmette-Guérin given alone did not protect mice against LSTRA, and admixture of a higher dose of Bacillus Calmette-Guérin (5 x 10^6 colony-forming units) did not alter the weak immunity induced by the 10^6 Lx. Thus, potentiation of tumor immunity by an immune stimulant may occur with a relatively low optimum dose; higher doses may evoke lesser effects.

Introduction

A major objective of immunotherapy is the destruction of malignant cells that have spread beyond the primary site and the regional lymph nodes. Frequently, such disease is undetectable or inaccessible for injection. Therefore, it is critically important to induce a potent, systemic tumor immunity in tumor-bearing individuals. We have been studying a promising model for such treatment with a tumor-specific vaccine. The advantages of LSTRA are that it is by nature a disseminated disease, it is responsive to chemotherapy, experiments are relatively short term, and animals are inexpensive and available in adequate supply. The behavior of this model is similar to but not identical with the line 10 vaccine system. This article describes some of the basic features of the tumor and of its interaction with a standard immune stimulant, BCG. In all of these experiments, the efficacy of the presumed immunizing treatments was assessed by tumor cell challenge following immunization.

Materials and Methods

Animals. Male mice of the BALB/c x DBA/2 F1, (hereafter called CD2FI) hybrid strain were obtained from Charles River Breeding Laboratories Wilmington, Mass., through the auspices of the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute.

Tumors. The transplantable murine leukemia, LSTRA, was obtained from Dr. John Pearson, National Cancer Institute. The tumor was originally induced in a BALB/c mouse by the Moloney strain of murine leukemia virus. The recommended passage procedure was i.p. injection of a 1:1000 dilution of the tumorous ascites fluid at weekly intervals (hereafter termed "blind" passage). That procedure commonly yielded ascites preparations that were grossly bloody and that occasionally were contaminated with coliform bacteria. Subsequently, we adopted a quantitative passage procedure to decrease the probability of contamination. At each passage, tumorous ascites fluid was harvested quantitatively (usually about 1.5 ml fluid) under aseptic conditions into 400 units of heparin (Riker Labs, Inc., Northridge, Calif.; 0.9% benzyl alcohol as pre-
servative). A portion of the fluid was cultured in thioglycolate broth medium, and the cells were counted in a hemacytometer. For documentation of morphology, cells in the ascites fluid were centrifuged onto microscope slides, fixed in methanol, and stained with May-Grunwald Giemsa. Injection i.p. of $10^6$ cells yielded a harvest in 7 days of approximately 1 to 3 x $10^8$ tumor cells and approximately equal numbers of erythrocytes ($10^8$ cells resulted in a comparable harvest in 6 days). With this procedure bacterial contamination was rare. Contaminated lines were discarded and replaced with sterile, frozen tumor stock. In comparative tests (some of which are reported below), tumor from the 2 passage procedures behaved similarly, except that injection of cells from blind passage into immunosuppressed mice led to early, nontumor death. We presumed that the immunologically deficient mice were unable to cope with the bacterial contaminants that frequently accompanied the blind passage. Following s.c. or i.d. tumor cell inoculation, tumor growth was evaluated primarily by the time of death due to tumor. The following criteria were used to attribute death to lethal tumor growth: spleen weight, >200 mg; presence of palpable injection site tumor; or presence of heavy leukemic infiltrate of liver or lung in hematoxylin eosin-stained sections. Differences in the frequency of survival ("cures") were evaluated for statistical significance by a 2 x 2 contingency table method for small numbers (19).

Tumor MSC-1, a cell culture line of a murine sarcoma virus-induced tumor (BALB/c origin), was obtained from Dr. Howard T. Holden, National Cancer Institute, and was maintained in cell culture. Inocula of $10^6$ cells in immunocompetent CD2F1 mice grew temporarily and regressed completely. The CaD2 mammary adenocarcinoma received from The Jackson Laboratory (Bar Harbor, Maine) arose spontaneously in a DBA/2 female mouse. It was maintained by serial s.c. transplantation of tumor cell suspensions in female CD2F1 mice. Sarcoma ASC-37 was induced in a BALB/c male mouse by s.c. implantation of a pellet containing 5% 7,12-dimethylbenz(α)anthracene in paraffin. It was used in its second to fourth transplant generations.

Cell suspensions of the solid tumors were prepared by digestion with pronase (2.5 mg/ml; Calbiochem, La Jolla, Calif.) and DNase (200 Kunitz units/ml; Sigma Chemical Co., St. Louis, Mo., or Calbiochem) in modified Eagle's basal medium (Flow Laboratories, Rockville, Md.) with CaCl2 (11.1 mg/liter). Cells were counted in a hemacytometer with exclusion of trypan blue as the criterion of viability. Excision of tumors was performed under anesthesia with a single indentation. The nuclear material stained a uniform, granular deep purple and frequently contained one or more large, pale nucleoli with peripheral chromatin condensation. The cytoplasm stained a bright blue. The most consistent and prominent cytological characteristics of the tumor cells were the presence of numerous clear cytoplasmic vacuoles and a poorly demarcated, pale area in the nuclear concavity. The tumor cells were nonphagocytic when incubated with carbon particles and were negative when stained for acid phosphatase (12).

**Tumorigenicity and Immunogenicity of Small Inocula of LSTRA Cells.** At periodic intervals we assessed the tumorigenicity of LSTRA by injection of graded doses or single low doses of tumor cells into normal CD2F1 mice (Table 1). The tumorigenicity was quite consistent. The incidence of progressive growth was slightly higher from a given dose injected s.c. than from the same dose injected i.d. For i.d. injections, $10^3$ cells usually yielded 100% lethal tumor growth while $10^2$ cells resulted in 50 to 80% tumor incidence.

For determination of whether exposure to subthreshold numbers of tumor cells could immunize mice to LSTRA, the survivors of tumorigenicity tests were challenged with $10^5$ LSTRA cells 42 days after the initial inoculum. Only 10% of survivors of the challenge yielded tumors.

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Route of injection</th>
<th>Initial inoculum</th>
<th>Challenge inoculum ($10^6$ cells) in survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial inoculum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chal-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inoc-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>um (10^6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>survi-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>i.d.</td>
<td>1/10</td>
<td>8/10</td>
</tr>
<tr>
<td>2</td>
<td>i.d.</td>
<td>5/10</td>
<td>10/10</td>
</tr>
<tr>
<td>3</td>
<td>i.d.</td>
<td>7/12</td>
<td>9/10</td>
</tr>
<tr>
<td>4</td>
<td>i.d.</td>
<td>8/10</td>
<td>10/10</td>
</tr>
<tr>
<td>4a</td>
<td>i.d.</td>
<td>7/10</td>
<td>10/10</td>
</tr>
<tr>
<td>1</td>
<td>s.c.</td>
<td>5/15</td>
<td>15/15</td>
</tr>
<tr>
<td>2</td>
<td>s.c.</td>
<td>7/10</td>
<td>15/15</td>
</tr>
</tbody>
</table>

**Results**

**Cytological Characteristics of LSTRA.** Cytocentrifuge preparations or smears of cells obtained from ascites passage and stained by the May-Grunwald Giemsa method were examined by light microscopy. The suspensions consisted of a mixture of small and large poorly differentiated lymphocytes. The nuclei were round to oval, occasionally with a single indentation. The nuclear material stained a uniform, granular deep purple and frequently contained one or more large, pale nucleoli with peripheral chromatin condensation. The cytoplasm stained a bright blue. The most consistent and prominent cytological characteristics of the tumor cells were the presence of numerous clear cytoplasmic vacuoles and a poorly demarcated, pale area in the nuclear concavity. The tumor cells were nonphagocytic when incubated with carbon particles and were negative when stained for acid phosphatase (12).

**Immune Stimulant.** Phipps strain BCG was obtained as a frozen suspension culture (TMC 1029; Trudeau Institute, Saranac Lake, N. Y.) and was maintained at −70° until used. The stock vials were thawed rapidly to 37°. Middlebrook 7H9 broth medium with ADC enrichment (Baltimore Biological Laboratory), at the time of use.

**Radiation.** Materials to be irradiated were exposed to γ-radiation ($^{60}$Co) in a Gammacell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada). The dose rate was approximately 90 rads/sec.

**Immunogenicity and Resistance of Small Inocula of LSTRA Cells.** At periodic intervals we assessed the tumorigenicity of LSTRA by injection of graded doses or single low doses of tumor cells into normal CD2F1 mice (Table 1). The tumorigenicity was quite consistent. The incidence of progressive growth was slightly higher from a given dose injected s.c. than from the same dose injected i.d. For i.d. injections, $10^3$ cells usually yielded 100% lethal tumor growth while $10^2$ cells resulted in 50 to 80% tumor incidence.

For determination of whether exposure to subthreshold numbers of tumor cells could immunize mice to LSTRA, the survivors of tumorigenicity tests were challenged with $10^5$ LSTRA cells 42 days after the initial inoculum. Only 10% of survivors of the challenge yielded tumors.
were done to determine the radiation dose required to prevent growth of LSTRA from large inocula. Tumor from the initial survivors rejected the threshold dose of tumor cells. In this system injection of subthreshold numbers of tumor cells was not a reliable means of inducing tumor rejection immunity.

Rate of Tumor Dissemination. Peripheral leukocyte and differential counts were performed daily in a group of 4 mice that received 10⁵ LSTRA cells i.d. and in 4 others that were not given injections. Leukocyte counts in normal mice ranged from 10 to 22 × 10⁶ cells/ml, with occasional 1-day fluctuations above that range. The range was greater in the LSTRA-injected mice (10 to 30 × 10⁶ cells/ml), again with occasional brief fluctuations. Only one of the tumor-bearing mice had a clear, significant leukocytosis, and that developed only 2 days before death due to the tumor (44 and 68 × 10⁶ cells/ml on the last 2 days of life). Identifiable circulating tumor cells were rare in smears from all the tumor-bearing mice. Despite the lack of an identifiable leukemic phase, the mice given LSTRA injections died 9 to 12 days after tumor cell injection with local i.d. tumor nodules, hepatosplenomegaly, and diffuse perivascular tumor cell infiltrates in most tissues. We concluded that quantitative and qualitative examination of peripheral blood was not a useful criterion for assessing course of the disease.

We determined the time required for LSTRA to disseminate after i.d. injection of the tumor cells. Mice received 10⁴ LSTRA cells i.d., and at various times thereafter the injection sites were excised. Two such experiments were done. Tumors were generally not palpable prior to Day 7. Excision of the injection site before Day 6 cured most of the animals, but by Day 7 or 8 local surgery was usually not curative. We concluded that dissemination of LSTRA beyond the cutaneous injection site required approximately 1 week.

A traditional means of inducing tumor immunity has been to permit temporary tumor growth followed by curative excision or ligation of the tumor before it spread beyond the primary injection site (20, 38). Therefore, we tested tumor rejection immunity in the mice that survived following excision of the LSTRA implants. Four to 6 weeks after initial tumor cell injections, mice were challenged with either 10³ or 10⁴ LSTRA cells i.d., contralateral to the primary inoculum site. Mice that had carried the initial tumor for 1, 2, or 3 days were not immune to challenge, while approximately one-half of the mice cured by surgery on Day 5 showed some immunity. Those mice that survived after 7 or 8 days of temporary tumor growth were all immune to challenge, but they represented only a small remnant of the initial cohort. We concluded that mice could be immunized to LSTRA by temporary tumor growth and excision but that the method was impractical due to tumor dissemination before development of immunity.

Tumorigenicity and Immunogenicity of Irradiated LSTRA Cells. In our initial experiments with this model (4), we found that a dose of 2000 rads was sufficient to prevent growth of inocula containing 10⁴ LSTRA cells. We later found that larger inocula (10⁵ cells) exposed to that dose of radiation frequently were tumorigenic. Three experiments were done to determine the radiation dose required to prevent growth of LSTRA from large inocula. Tumor from blind passage was used in 2 experiments, and cells from quantitative passage were used in one. The results were essentially identical so the data have been pooled in Table 2. Irradiation with 8000 rads was required to reliably prevent growth.

Mice that survived after inoculation of 10⁷ irradiated cells were tested for tumor immunity by challenge with 10⁴ LSTRA cells i.d. in the left flank (Table 2). Greater than 90% of the mice rejected the challenge inoculum. We concluded that injection of a large number of lethally irradiated LSTRA cells was an effective means of inducing immunity to LSTRA challenge.

Quantitative immunogenicity of Lx and Adjuvant Effect of BCG on Immunization by Lx. Earlier experiments (6) had shown that appropriate mixtures of viable (nonirradiated) LSTRA cells and BCG could induce immunity to LSTRA cell challenge. The results in Table 2 indicated that BCG was not required for immunization. The next group of experiments defined the quantitative aspects of immunization with Lx and determined whether BCG functioned as an adjuvant for LSTRA immunity.

In the first experiment control mice were unimmunized or were given i.d. injections of 5 × 10⁶ CFU BCG. Other groups were immunized (i.d.; right flank) with 10⁴, 10⁵, or 10⁶ Lx given alone, at the same time as BCG in the left flank, or admixed with BCG. Four weeks after immunization the groups were divided into subgroups and challenged with 10³, 10⁴, or 10⁵ viable LSTRA cells (i.d.; ventral skin). Mice that survived greater than 6 weeks after challenge were considered immune. None of the control mice (untreated or BCG injected) survived. Pretreatment with 10⁶ Lx was ineffective against all challenge doses, and 10⁶ Lx protected a few animals against only the low challenge dose (4 of 7 survivors versus 0 of 7 in the untreated controls; p < 0.035). Immunization with 10⁷ Lx protected most mice against all 3 challenge doses. Immunity in mice treated with both Lx and BCG, whether given as separate injections or as mixtures, was not significantly different from immunity induced by the comparable dose of Lx alone. We concluded that 10⁶ Lx was a subimmunogenic dose, that 10⁷ Lx produced barely detectable immunity, and that 10⁸ Lx was immunogenic.

<table>
<thead>
<tr>
<th>Radiation dose (rads)</th>
<th>No. of mice surviving/no. of mice given injections of irradiated cells</th>
<th>No. of mice surviving/no. of mice challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/26</td>
<td>0/25</td>
</tr>
<tr>
<td>4000</td>
<td>1/26b</td>
<td>1/1</td>
</tr>
<tr>
<td>6000</td>
<td>18/26</td>
<td>16/18</td>
</tr>
<tr>
<td>8000</td>
<td>26/26</td>
<td>23/26</td>
</tr>
</tbody>
</table>

a Untreated control mice (no initial injection).

b Animals in this group survived significantly longer than did mice receiving unirradiated cells.
against challenge with $10^6$ viable cells (100 times the lethal dose for normal mice). Under the conditions of this experiment, BCG did not function as an adjuvant or as a systemic immune stimulant for LSTRA immunity.

To extend the search for adjuvant effects of BCG, we evaluated a range of BCG doses and tested immunity with a higher tumor cell challenge dose. Some mice were not treated; other groups were immunized with $10^6$ or $10^7$ Lx either alone or admixed with 1 of 3 doses of BCG ($5 \times 10^3$, $5 \times 10^4$, or $5 \times 10^5$ CFU). Twenty-eight days after immunization the groups were subdivided and were challenged with $10^4$, $10^5$, or $10^6$ LSTRA cells. The experiment was performed twice (13-month interval) with 7 mice per subgroup the first time and 10 mice per subgroup the second time. The results were quite similar (except that the level of Lx-induced immunity was somewhat higher the second time), so the data have been pooled for graphic presentation (Chart 1).

Again, unimmunized mice or mice immunized with BCG alone were unable to reject the challenge inocula. Immunization with $10^6$ Lx (Chart 1a, open bars) protected more than one-half of the mice challenged with $10^4$ cells but did not protect a significant proportion of mice against the higher challenge doses. Addition of BCG to the $10^6$ Lx had no significant effect on immunity to $10^4$ cell challenge. The proportion of mice immune to the larger challenge inocula was significantly improved by admixture of $5 \times 10^5$ or $5 \times 10^6$ CFU BCG with the $10^6$ Lx but not by addition of $5 \times 10^5$ CFU BCG. The highest BCG dose was significantly less effective than was either of the lower doses when challenge was with $10^6$ LSTRA cells ($p < 0.02$), and it was significantly less effective than the lowest dose tested ($p = 0.04$) when mice were challenged with $10^6$ cells. The higher tumor cell dose ($10^7$ Lx; Chart 1b) protected the majority of mice against challenge with $10^4$ or $10^6$ cells, and that effect was not altered by admixture with any of the 3 doses of BCG. Fewer (53%) of the mice immunized with $10^7$ Lx rejected a challenge of $10^4$ viable cells. That proportion was improved by admixture with $5 \times 10^5$ CFU BCG. These experiments confirmed the previous conclusions concerning the relative immunogenicity of different doses of Lx. Two subsequent pretreatment experiments have included mice that were immunized with $10^6$ Lx with or without admixture of $5 \times 10^5$ BCG and challenged 28 days later with either $10^4$ or $10^6$ LSTRA cells. The data for the replicate groups of the 4 experiments are presented in Table 3 to illustrate the reproducibility of the adjuvant effect of the BCG. Preimmunization with BCG alone has never had any effect on growth of a subsequent inoculum of LSTRA. We conclude that BCG may function as an adjuvant for LSTRA immunity, evidenced primarily by improved resistance to the higher challenge doses. The adjuvant effect was most apparent when the dose of specific immunogen (Lx) was suboptimal; under that condition the effective dose was $5 \times 10^3$ to $5 \times 10^5$ BCG, and the higher dose was totally ineffective.

Specificity of Resistance to LSTRA Challenge. The specificity of LSTRA immunity was tested initially in CD2F$_1$ mice that had rejected LSTRA plus BCG mixtures and that rejected a subsequent challenge of $10^6$ LSTRA cells (“LSTRA immune”); controls were normal, non-age-matched CD2F$_1$ mice. In the first test, groups of 8 mice were challenged with the indicated dose of LSTRA cells i.d. in the opposite flank.

<table>
<thead>
<tr>
<th>No. of mice that survived/no. of mice challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenge (10$^6$ LSTRA)</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>Experiment</td>
</tr>
<tr>
<td>1$^a$</td>
</tr>
<tr>
<td>2$^a$</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

$^a$ Data in the first 2 lines were included in Chart 1.
challenged with $10^4$ LSTRA or $10^4$ MSC-1 cells. LSTRA killed all the controls and was rejected by all the immune mice. MSC-1 grew to $\geq 3$ mm in all the controls (mean maximum diameter, $4.4 \pm 0.4$ mm) prior to regression, while it never reached 3-mm diameter in any of the immune mice (mean maximum diameter, $0.2 \pm 0.2$ mm). In the second test, groups of 10 control or LSTRA-immune mice were challenged with $10^4$ LSTRA or with $5 \times 10^4$ CaD2 cells. LSTRA grew progressively in 9 of 10 controls, but it did not grow in immune mice. CaD2 killed all mice of both the normal and immune groups with no difference in tumor growth rate or day of death.

In the next 2 experiments, groups of mice were unimmunized (controls) or were immunized with $5 \times 10^4$ CFU BCG only, with $10^7$ Lx only, with Lx plus BCG, or with Sarcoma ASC-37 (by temporary tumor growth and excision). One day prior to challenge, all mice were irradiated with 450 rads. In the first experiment, one-half of the mice were challenged with $10^4$ LSTRA cells, and the other one-half received $10^4$ cells of ASC-37 (Table 4). Mice pretreated with BCG alone or with ASC-37 were not immune to LSTRA challenge, while mice treated with either Lx or Lx plus BCG were strongly immune. The challenge dose of ASC-37 was at the threshold for growth, since the tumor incidence was only 90% in controls and the tumors appeared late. All mice immunized with ASC-37 rejected the challenge inoculum of that tumor. Pretreatment with BCG, Lx, or Lx plus BCG caused rejection of ASC-37 in 40 to 44% of the mice. Although those results were not significantly different from the controls, they suggested a weak, nonspecific inhibition of the threshold dose of ASC-37 by those pretreatments. Thus, ASC-37 did not immunize against LSTRA, but the specificity of the effects of BCG, Lx, or Lx plus BCG was not clear. In the second experiment (Table 4), groups of mice received similar pretreatments and were challenged with $10^4$, $10^6$, or $10^8$ cells of ASC-37. Again, a dose of $10^4$ cells was near the threshold for growth (70% tumor incidence in the controls), but the tumors grew slightly faster than in the previous experiment. There was no evidence of nonspecific inhibition of growth in mice pretreated with BCG, Lx, or BCG plus Lx. Immunization with ASC-37 protected most of the mice against challenge with up to $10^4$ ASC-37 cells. We concluded that the resistance to LSTRA observed in this study was a specific tumor rejection immunity since it could not be induced by BCG alone nor by another antigenic tumor alone, and mice immune to LSTRA were not immune to unrelated tumors; the immunity was directed at antigens that were shared by LSTRA and the MSC-1 tumor line.

Local Passive Transfer of Immunity to LSTRA. Mice that had been immunized with LSTRA plus BCG mixtures and that had survived LSTRA cell challenges were pooled from several experiments. They were rechallenged and boosted with $10^4$ LSTRA cells i.d. Four days later those mice and untreated mice of a similar age, were exsanguinated by cardiac puncture, serum was prepared, and the spleens and lymph nodes were collected. The lymphoid cell suspensions were mixed with LSTRA cells [test of Winn (43)] in various ratios (10:1, 100:1, 1000:1), and other LSTRA cells were suspended in the sera (dilutions of 1:2 or 1:20). The mixtures were incubated at 37° for 30 min and then were injected i.d. into normal CD2F1 mice, $10^4$ LSTRA cells/mouse. The results showed that lymphocytes from immune donors in a ratio of 1000:1 suppressed growth of the LSTRA cells (6 of 10 survivors versus 0 of 10 in controls; $p = 0.005$). Lower ratios of the immune lymphocytes and all ratios of the normal lymphocytes were not effective, nor was the serum from immune or normal donors at either concentration used. We concluded that the resistance to LSTRA that we have observed was mediated in part by lymphoid cells. This experiment does not exclude antibody involvement in the resistance.

**DISCUSSION**

This report is the initial stage in the development of LSTRA as a model for studying vaccine immunotherapy of cancer. The use of a long transplanted tumor in such an application will be facilitated by a thorough awareness of the biological background of the tumor as well as by an analysis of its current characteristics. Despite the rather frequent use of LSTRA in recent years, we found that information in the literature concerning its origin and its early biological and immunological behavior was fragmentary. In the light of our findings, we have reviewed published information concerning the etiological, pathological, antigenic, and growth characteristics of LSTRA. This summary provides some evidence concerning relative stability of those characteristics in the tumor line and includes features that are essential to the proper interpretation of our data.

In 1960, Moloney (33) described the pathogenesis of a large number of primary leukemias induced by the Moloney strain of murine leukemia virus and reported the cellular transplantation of leukemia from mice bearing primary leukemia into syngeneic recipients. Subsequent articles involved an unnamed, transplantable, Moloney virus-induced leukemia of BALB/c origin, which may have been LSTRA (15, 25). However, the first report in which LSTRA was identified by that name was published in 1964 (21), and by then the tumor had already undergone 107 in vivo transplantations. We have not been able to locate a specific description of the origin and pathogenesis of LSTRA or of

**Table 4**

<table>
<thead>
<tr>
<th>Pretreatment†</th>
<th>First experiment</th>
<th>Second experiment ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^4$ LSTRA</td>
<td>$10^4$ ASC-37</td>
</tr>
<tr>
<td>Unre,cted</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>controls</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>$5 \times 10^4$ BCG</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>$10^7$ Lx</td>
<td>7/10</td>
<td>0/10</td>
</tr>
<tr>
<td>$10^8$ Lx</td>
<td>9/10</td>
<td>0/10</td>
</tr>
<tr>
<td>$10^8$ Lx + $5 \times 10^4$ BCG</td>
<td>9/10</td>
<td>0/10</td>
</tr>
<tr>
<td>$10^6$ ASC-37, excision</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

† ASC-37 was excised on Day 14 (first experiment) or on Day 21 (second experiment). Mice in all groups were exposed to 450 rads 6 days after excisions and were challenged 1 day later.

‡ All mice in the second experiment were challenged with ASC-37.

* p < 0.005, compared to untreated controls; for all other groups, p > 0.05, compared to untreated controls.

DECEMBER 1978

4405
its conversion to the ascites passage form.

Moloney (33) and Dunn et al. (16) described the primary Moloney virus-induced tumors as lymphocytic leukemias, and 3 papers with LSTRA have described the induced tumors as lymphocytic, lymphatic, or lymphoid (9, 22, 36), but details of the cytology of LSTRA have not been published. In 3 recent reports, LSTRA cells lacked surface immunoglobulin, Thy-1.2 antigen, and complement receptors; were nonphagocytic; and possessed monocyte Fc receptors (for immunoglobulin-erythrocyte complexes), Ia8 antigens, and H-2 antigens (14, 41, 42). Those features are more suggestive of a monocytic than of a lymphocytic origin. In our experience, the cells were morphologically lymphocytic although quite undifferentiated. They were nonphagocytic and lacked histochemically demonstrable acid phosphatase.

A review of articles with LSTRA revealed some changes in its growth characteristics associated with prolonged transplantation. In early articles (107–171 transplant generations), the threshold dose was 1.0 to 1.5 x 10^4 cells in BALB/c or CD2F1 mice with a median survival time of approximately 20 days (9, 21). Later, at approximately 300 transplant generations, 10^5 cells were 100% lethal in CD2F1 mice with a median survival time of 16 days (23), but 5 x 10^6 cells were required for 100% takes in BALB/c x C57BL/6F1 mice (18). We found that 10^6 cells were uniformly lethal in CD2F1 mice with a median survival of 14 days.

The LSTRA tumor line has been called either a leukemia (e.g., 21, 36), a lymphoma (e.g., 17) or both (31, 32). Pearson et al. (36) published evidence that LSTRA could be detected in the peripheral blood by bioassay (injection of blood into susceptible hosts) beginning about 5 days after s.c. injection of 10^6 cells, and Fefer (17) stated that dissemination occurred about 3 days after injection of 10^6 cells. We found that there was not a consistent leukemic phase to the disease following i.d. injection of tumor cells. Dissemination beyond the injection site occurred about one week after i.d. injection of 10^6 tumor cells, and the pattern of tissue infiltrates indicated that the tumor had spread by the hematogenous route.

Information is scarce concerning virus production by LSTRA. Injection of 8 x 10^8 irradiated (10,000 rads) LSTRA cells s.c. into newborn BALB/c mice caused splenomegaly in all 8 recipients. That was taken as presumptive evidence for the release of infectious Moloney virus from the irradiated tumor cells (32). In contrast, when LSTRA cells were grown in vitro, they did not release any detectable murine leukemia virus into the culture supernatant (14).

In addition to the cell surface markers described previously, LSTRA expresses various tumor-associated antigens that can be detected on the cell membrane by serological techniques. Antiserum to the murine sarcoma virus (Moloney) react with LSTRA cells (18), as do sera from mice immunized with allogeneic tumors induced by Friend, Moloney, or Rauscher viruses (24). LSTRA cells also reacted with an antiserum induced in C57BL/6 mice by hyperimmunization with a syngeneic, methylcholanthrene-induced tumor; that antiserum also reacted with several other syngeneic, methylcholanthrene-induced tumors. Additional data in that report were considered consistent with the presence of common, embryonic antigens on the cross-reacting tumors (11). Nord and Weissman (34) demonstrated approximately 5 x 10^6 Moloney antigen-specific sites per LSTRA cell using a radiolabeled xenogeneic (rat) antiserum.

Several investigators have tested the sensitivity of LSTRA cells to tumor rejection immunity induced by various means. Immunization of adult BALB/c mice with viable Moloney leukemia virus resulted in immunity for only a very brief period, while formalin-inactivated virus produced no protection at all (21). In contrast, immunization with murine sarcoma virus produced strong immunity to challenge (18, 31). Irradiated tumor cells (either LSTRA or allogeneic Moloney virus-induced tumors) were weakly immunogenic in BALB/c mice (21, 32) but seemed to be more immunogenic in CD2F1 mice (30). Allografts of viable Moloney virus-induced tumors were either weakly immunogenic [a CD2F1, tumor (21)] or strongly immunogenic [a C57BL/6 tumor (32)]. Likewise, the immunogenicity of Rauscher virus-induced tumors was variable (9, 30).

LSTRA cells are immunogen under certain conditions. As mentioned in the previous paragraph, irradiated LSTRA cells can induce immunity to LSTRA, and that resistance extends to other Moloney virus-induced tumors (both syngeneic and allogeneic) but was ineffective against a Rauscher virus-induced tumor (21, 32). Growth of LSTRA in vivo under chronic exposure to certain chemotherapeutic drugs led to the development of LSTRA sublines that were more immunogenic than was the parental tumor (13, 30). A significant proportion (40%) of mice cured of LSTRA by chemotherapy or by chemotherapy plus BCG were resistant to challenge with 2500 LSTRA cells (35). Likewise, some mice that survived after injection of mixtures of LSTRA cells and BCG were immune to LSTRA cell challenge; the probability of being immune was critically dependent on the doses of tumor cells and BCG in the immunizing inoculum (6). LSTRA cells did not stimulate syngeneic lymphocytes in an in vitro mixed lymphocyte-tumor interaction (10).

Several different methods have been used to immunize animals against tumor challenge. In the initial such report, Gross (26) found that when sarcoma cells were injected i.d. into mice, tumors regressed more frequently than if they were injected s.c., and the survivors were immune. He also found that near-threshold inocula of the L2C leukemia of strain 2 guinea pigs, although tumorigenic when given s.c., frequently regressed after i.d. injections, and again the survivors were immune to challenge (27, 28). In our study of LSTRA, doses as low as 10^5 cells were tumorigenic in most animals whether injected s.c. or i.d. Tumor growth was never evident in the remainder of the mice, and they were not immune to challenge. Another common approach to the induction of tumor immunity has been to permit tumors to grow temporarily and then to cure the animals by ligation or by surgery (20, 38). That is feasible with many tumors because they tend to remain localized at the injection site. LSTRA disseminated rather early so that the duration of temporary growth was limited to 5 to 7 days, and even then not all the animals were cured by surgery. The mice that were cured after 6 days of tumor growth were immune to challenge. The lack of immunity following earlier excision could be due to inadequate antigen dose or to inadequate duration of exposure or both. Thus, the viable, nonirradiated tumor cells were immunogenic, but the method of
temporary tumor growth was quite inefficient due to the rapid dissemination of the tumor from the injection site. Numerous investigators have used irradiated tumor cells to elicit tumor immunity (1, 39), but that method is not always successful even with tumors that are immunogenic by other procedures (1, 2, 7). Irradiated LSTRA cells were immunogenic in other studies (21, 32), and our results confirmed those observations. When a large number of irradiated cells (10³) was used, strong immunity was obtained. Some of the immunized mice could reject 1000 times the minimal tumorigenic dose. Lower immunizing doses produced infrequent and weak immunity. The number of cells required for immunization may explain the difficulty of immunizing by the 2 previous methods (sub-threshold inocula or temporary tumor growth).

Another method of immunization has been to mix viable tumor cells with an immune stimulant. That approach was quite effective for some tumors that were nonimmunogenic by other procedures (7), but induction of tumor immunity was not essential for suppression of tumor in a mixed inoculum (8). We have previously reported that injection of appropriate mixtures of LSTRA cells and BCG immunized mice against subsequent challenge with LSTRA cells (6). In that study we did not test the specificity or the transferability of the induced resistance. We have now found that mice immunized by LSTRA-BCG mixtures could reject LSTRA cells and could accelerate the rejection of a murine sarcoma virus tumor line that shares Moloney virus-related antigens (18), but they were not immune to etiologically unrelated tumors. Likewise, mice immune to another tumor or immune to BCG were not immune to LSTRA. The resistance was transferable in the Winn test by lymphoid cells. Thus the resistance induced by LSTRA-BCG mixtures can be considered a valid example of specific tumor rejection immunity.

In our previous report (6) we did not determine whether the admixed BCG was serving as a true adjuvant (i.e., augmenting the level of immunity induced by a given dose of immunogen) or if it simply provided a means of preventing growth of the immunizing tumor cells, comparable to irradiation. These results show that viable tumor cells or irradiated tumor cells were immunogenic, without the influence of BCG. However, under appropriate conditions (sub-immunogenic dose of irradiated tumor cells and low BCG dose), BCG exerted a significant adjuvant effect on the immunogenicity of Lx.

Low doses of adjuvants were superior to higher doses in at least 2 other tumor immunity systems. Scott found that mixtures of Corynebacterium parvum and irradiated P-815 mastocytoma cells induced stronger immunity than did the irradiated tumor cells alone (40). The lowest dose of C. parvum (3.5 μg) was slightly but not significantly better than the highest dose tested (175 μg). Piessens et al. (37) showed that challenge inocula of a rat mammary tumor grew more slowly in rats that had survived after injection of tumor cells mixed with a low dose of BCG (6 x 10⁴ CFU) than in untreated rats or in rats treated with mixtures containing more BCG (6 x 10⁸ CFU), although the groups were small (3 and 5 rats) the data were statistically significant. We have reported here that the highest dose of BCG we tested was significantly less effective than were the lower doses when used as an adjuvant with the Lx vaccine. The inverse dose-response relationship was similar to that which we have previously observed with nonirradiated LSTRA cells (6), although the absolute dosages were different. The conclusion is obvious and worth emphasis. When immune stimulants are used as adjuvants to augment immunity to tumor antigens, the optimal dose may be relatively low and higher doses may be significantly less effective or ineffective.

In summary, nontumorigenic LSTRA cells can induce specific tumor rejection immunity, and that resistance can be significantly potentiated by an immune stimulant. Those observations commend the use of this host-tumor system in studies of active, systemic, specific immunotherapy.

**ACKNOWLEDGMENTS**

We thank Dean C. Katsilas and Alfred J. Hockley, III, for expert technical assistance.

**REFERENCES**

G. L. Bartlett et al.


Potentiation of Immunity to Murine Leukemia (LSTRA) by Bacillus Calmette-Guérin

Gerald L. Bartlett, John W. Kreider and Dallas M. Purnell


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/38/12/4401

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.