Association of Host Immunity with 5-Fluorouracil-initiated Cure of Plasmacytoma LPC-1 in BALB/c Mice

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ABSTRACT

Previous experiments in the chemotherapy of plasmacytoma LPC-1 in syngeneic BALB/c mice revealed a correlation between the effectiveness of the drug or drug combination and the proportion of mice subsequently immune to tumor challenge. Additional parameters were examined to clarify this observation and to determine which immune mechanism(s) were involved in drug-initiated immunity. The magnitude of the rejection response to LPC-1 in mice cured of LPC-1 was high. The 50% tumor-producing dose in cured mice was $10^{0.5}$ compared with $10^{1.5}$ in normal mice, a resistance ratio of $10^{5}$. Duration of immunity decreased with time, from 90 to 100% survivors at 4 months to <50% at 7 to 12 months, and decreased with increasing size of challenge dose. Immunity of cured mice to LPC-1 was highly specific for LPC-1 (85% survivors) and partially specific for plasmacytoma ADJ-PC-5 (29% survivors). The cured mice were not immune to two other plasmacytomas and a sarcoma, all syngeneic with BALB/c. In assays for cell-mediated cytotoxicity, peritoneal exudate but not spleen or lymph node cells were cytolytic to $^{51}$Cr-labeled LPC-1 target cells from cured mice after challenge with mitomycin C-treated LPC-1. Lymphoid cells from cured, unchallenged mice were inactive. Time studies of cell-mediated cytotoxicity using peritoneal exudate cells from LPC-1-implanted mice before, during, and after therapy with 5-fluorouracil revealed 2 major peaks of cytolysis, on Days 18 and 77. Treatment of lymphocytes with anti-Thy-1.2 plus complement destroyed all cytolytic activity. In all experiments, complement-dependent cytotoxicity in sera was absent. These observations suggest that destruction of residual tumor cells after chemotherapy may be due to specific immunological responses.

INTRODUCTION

Although drug treatment may result in "complete" remission of tumors in some patients, the inexplicable failure in others raises important questions concerning the mechanisms by which chemotherapy might be curative. All too frequently, termination of remission, as indicated by tumor recurrence, reveals the temporary nature of many remissions as well as the degree of host control over tumor growth.

Recent studies in the chemotherapy of a mouse plasmacytoma in syngeneic BALB/c mice have revealed a unique correlation between the efficacy of the various therapies (percent-age of mice in complete remission) and the proportion of each treatment group subsequently resistant to challenge with viable tumor cells (19). The data suggested that drug activity and host immunity may act conjointly to induce tumor remission and that the ensuing degree of host resistance to tumor challenge may be the consequence of previous direct action of the drug(s) on the tumor cells and of drug influence on host immune mechanisms.

In this paper, we describe the degree, duration, and specificity of the drug-initiated tumor immunity and determinations of the immune mechanisms possibly associated with this process. The findings, which appear to separate effects of therapy from effects of host on tumor growth, should further our understanding of the procession of events leading to remission, cure, and tumor immunity.

MATERIALS AND METHODS

Tumors and Hosts. PCT LPC-1, MOPC-70A, RPC-20 [described previously (17)], and ADJ-PC-5 (13) and sarcoma Meth A (11) are isogenic with BALB/c mice (Charles River Breeding Laboratory, Inc., Wilmington, Mass.). The myeloma proteins secreted by these PCT are, respectively, IgG2a, IgG1, Bence Jones, and IgG2a. Tumors were passaged i.p. by implantation of 0.1 to 0.2 ml of undiluted 7- to 10-day ascitic fluid. Tumor growth was progressive and lethal to 100% of the mice.

Therapy. Chemotherapy of LPC-1 was as described (17, 20). Briefly, young female BALB/c mice weighing 16 to 19 g were implanted i.p. with $1 \times 10^5$ LPC-1 cells. Drug treatment of randomly distributed, tumor-implanted mice was initiated 7 days later. The standard treatment protocol consisted of single daily doses administered s.c. 6 times weekly for 4 weeks to groups of 10 mice. We obtained long-term survivors by giving the mice 5-FU in 0.85% NaCl solution at 16 mg/kg/day or a combination of 5-FU for the first 2 weeks and 1,3-dimethyluracil at 31.3 mg/kg/day in 0.5% carboxymethylcellulose in 0.85% NaCl solution during the last 2 weeks. 5-FU was generously supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, and liberal

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\[2\text{1 To whom requests for reprints should be addressed, at Donald S. Walker Laboratory, Sloan-Kettering Institute for Cancer Research, 145 Boston Post Road, Rye, N. Y. 10580. Received February 4, 1980; accepted May 12, 1980.} \]
amounts of 1,3-dialbylurea were supplied by the Montsanto
Chemical Company, St. Louis, Mo. Control mice, 20/ exper-
iment, were given the drug vehicle on the same schedule. Mice
surviving and apparently tumor free 90 days after tumor im-
plantation were designated cures; death of cured mice from
tumor recurrence after this time was rare. When warranted,
mice were autopsied upon termination of experiments and
examined grossly and microscopically to verify presence or
absence of tumor.

Media. The basic medium used throughout, unless noted
otherwise, was RPMI 1640 (Microbiological Associates, Inc.,
Bethesda, Md.). When supplemented with 10% decomple-
cented calf serum (Grand Island Biological Co., Grand Island,
N. Y.) and 5 x 10^{-5} M 2-mercaptoethanol, it was designated
RPMI 1640-10 (1640-20, 20% calf serum; 1640-0, no calf
serum). Preservative-free heparin sodium, 5 units/ml, was
included when tumor cells or CTL were harvested.

Cytotoxic Lymphocyte Isolation. CTL were derived from
spleens, peritoneal exudates, and pancreatic and mesenteric
lymph nodes. Spleens were removed, cleaned of fascia, and
minced with scissors in heparinized RPMI 1640-10. The SPC
suspension was homogenized with a loosely fitting Teflon ho-
mogenizer. The homogenate was passed through a premois-
tened 200 mesh stainless steel screen and then centrifuged at
350 x g for 5 min. The pellet was suspended in Tris-ammonium
chloride (3) and quickly centrifuged at 350 x g for 5 min. The
pellet was again washed and suspended in RPMI 1640-10.

After injection of heparinized RPMI 1640-10, PEC were
aspirated from abdomens of experimental mice, pooled, and
processed as for SPC. Mesenteric and pancreatic lymph nodes
were resected, teased into suspension, and transferred to
centrifuge tubes on ice where barge debris was permitted to
settle. The supematant was aspirated, and the bymphoid cells
were resected, teased into suspension, and transferred to
2 Falcon tissue culture dishes (35 x 10 mm) which were
incubated on a rocking platform (BebcoGlass, Vineland, N. J.)
at 6 oscillations/mm. Duplicate dishes containing tumor cells
alone were included as controls for measuring spontaneous
release. Normal cells (SPC, PEC, LN) added to the labeled
target tumor cells had little or no effect on spontaneous 51Cr
release. A second set of controls, in duplicate, was used for
determining maximal 51Cr release from the 1 x 10^{5} tumor cells.
After 4.5 hr incubation, duplicate aliquots of the contents of
each dish were transferred to small test tubes, and the released
51Cr was counted in a 5- scintillation counter (Nuclear-Chicago
Corp., Des Moines, Iowa). Maximal release of 51Cr was deter-
mined by lysing the pellet with distilled water. Specific cytotox-
icity was calculated using the formula:

\[
\% \text{ of specific cytotoxicity} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximal release} - \text{cpm spontaneous}} \times 100
\]

Anti-θ Serum. θ-bearing lymphocytes were eliminated with
anti-θ serum and complement. Monoclonal anti-Thy 1.2 (ob-
tained from Dr. U. Hämmerling of our Institute) was incubated
with 5 x 10^{7} PEC for 30 min at 37° after addition of adsorbed
guinea pig complement.

RESULTS

Magnitude, Duration, and Specificity of Immunity. BALB/
c mice cured of the syngeneic plasma cell tumor LPC-1 by
cytotoxic drug therapy were found to be immune to challenge
with LPC-1 (19). We have now quantitated the magnitude,
duration, and specificity of the immunity in cured mice.

Groups of 5 cured mice were challenged i.p. with doses of
LPC-1 ranging from 10^{3} to 10^{7} cells to determine the magnitude
of immunity. Ten normal mice implanted with 10^{5} cells were
included as controls. Since results for a duplicate experiment
were similar, they were pooled. The experiments were termi-
nated at 90 days. Of 20 normal control mice, all but 3 devel-
oped tumors when given 10^{3} LPC-1 cells (15% survivorship)
(Table 1). In contrast, 40 to 100% of cured mice survived
challenges doses ranging from 10^{3} to 10^{7} cells. The TD_{50}
in cured mice was 10^{6.5} [approximated by the method of Reed
and Muench (14)], while titration in normal control mice yielded
a TD_{50} of 10^{4.8}. Thus, the resistance ratio was 10^{6} or 100,000-
fold greater in cured mice.

The duration of immunity to LPC-1 tumor was determined as
follows. Groups of cured BALB/c mice maintained for various
periods of time were challenged i.p. with 10^{3}, 10^{4}, or 10^{5} LPC-
1 cells. Age-matched, 0.85% NaCl solution-treated mice not
previously exposed to tumor were used as controls. The ex-

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* M. N. Teller and I. Smullyan, unpublished observations.
experiment was terminated 33 to 34 days after challenge, and the abdomens of the mice were examined for presence of tumor.

The data presented in Chart 1 indicate that the duration of resistance to tumor challenge is related both to the time elapsed before challenge and to the magnitude of the challenge dose. When cured mice were challenged after an interval of 4 months (from time of first inoculation of tumor), all but one (of 30) survived regardless of dose; whereas, when cured mice were challenged after 7 months or more, less than one-half survived, the proportion decreasing to zero as the dose of tumor challenge increased. At all challenge doses, the proportion surviving after the 4-month interval was significantly higher (p < 0.01) than after the 2 longer intervals combined. (No significant difference at any dose was noted between the 2 longer intervals of 7 and 12 months.) All control mice implanted with the various doses of tumor cells died with progressively growing tumor.

To determine the specificity of resistance of mice cured of LPC-1, groups were challenged with the same tumor, with other PCT, or with a sarcoma, all isogeneic with the BALB/c mouse. First, each tumor line was titrated to determine its TD_{50} in normal mice. Groups of 10 normal mice were implanted i.p. with 10-fold increments from 10^3 to 10^6 cells each of LPC-1, the other PCT (ADJ-PC-5, MOPC-70A, or RPC-20), or Meth A sarcoma. The number of tumor-free survivors was recorded 60 days later. Each titration was duplicated, the data were pooled, and the TD_{50} was calculated (14) for each tumor. The specificity of resistance of cured mice to LPC-1 was determined by implanting 2 duplicate groups of 10 cured mice, along with 5 normal control mice (10 for ADJ-PC-5), i.p. with 100 TD_{50} doses of each tumor.

It is apparent from the results that a high degree of specificity exists in mice cured of LPC-1 (Table 2). All normal control mice inoculated with 100 TD_{50} doses of each tumor died with progressive tumor growth. Survival rates for challenge were: LPC-1, 85%; ADJ-PC-5, 29%; MOPC-70A or RPC-20, 5%; and Meth A, 0%. Occasional prolonged survivals were observed (in the LPC-1 and MOPC-70A groups), with death associated with growth of solid rather than ascites tumor. Thus, in mice cured of LPC-1, a high degree of immunity was demonstrated to LPC-1 itself (p < 0.01), an intermediate degree (p < 0.05) was shown to ADJ-PC-5, and little or none was demonstrated to the other 2 PCT or the sarcoma. Notably, the myeloma protein IgG_{2a} is characteristic of both LPC-1 and ADJ-PC-5, which showed the highest degree of cross-resistance, but different myeloma proteins are associated with the other plasma cell tumors.

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Challenge cell dose</th>
<th>Survivors ( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>( 10^3 )</td>
<td>3 15</td>
</tr>
<tr>
<td>Cured</td>
<td>10</td>
<td>( 10^3 )</td>
<td>6 60^a</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>( 10^4 )</td>
<td>9 90^b</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>( 10^5 )</td>
<td>4 40</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>( 10^6 )</td>
<td>10 100^b</td>
</tr>
</tbody>
</table>

^a Mice tumor free 90 days after tumor implantation.

^b Significantly different from control group (p < 0.01).

**Table 2**

<table>
<thead>
<tr>
<th>Challenge tumor</th>
<th>Myeloma protein</th>
<th>Challenge cell dose</th>
<th>MST^b (day)</th>
<th>Range (days)</th>
<th>90-day survivors^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPC-1</td>
<td>IgG_{2a}</td>
<td>( 3.2 \times 10^3 )</td>
<td>&gt;80</td>
<td>37–79</td>
<td>17/20</td>
</tr>
<tr>
<td>ADJ-PC-5</td>
<td>IgG_{2a}</td>
<td>( 1.0 \times 10^3 )</td>
<td>21.0</td>
<td>16–37</td>
<td>6/21</td>
</tr>
<tr>
<td>MOPC-70A</td>
<td>IgG_{1}</td>
<td>( 3.2 \times 10^3 )</td>
<td>26.0</td>
<td>19–87</td>
<td>1/20</td>
</tr>
<tr>
<td>RPC-20</td>
<td>Bence Jones</td>
<td>( 1.0 \times 10^3 )</td>
<td>28.0</td>
<td>23–36</td>
<td>1/20</td>
</tr>
<tr>
<td>Meth A</td>
<td>None</td>
<td>( 3.2 \times 10^3 )</td>
<td>13.0</td>
<td>10–14</td>
<td>0/20</td>
</tr>
</tbody>
</table>

^a 100 x 50% lethal dose.

^b MST, median survival time.

^c Number of survivors/number inoculated.
Cell-mediated Immunity against LPC-1 in Cured Mice.

Since cured mice were capable of rejecting large numbers of tumor cells (Table 1), it was desirable to examine possible mechanisms involved in protecting immune mice. In a series of experiments, PEC, SPC, and LN were collected from both unchallenged and challenged cured mice. Challenge was carried out by injecting i.p. 1 × 10^7 MIT C-treated or irradiated LPC-1 cells 5 days prior to assay. L:T ratios used were 150:1 to 200:1 for LN and SPC and 25:1 to 100:1 for PEC. Table 3 shows the results of these experiments.

Neither PEC nor SPC from cured mice, which did not receive a challenge tumor dose, showed measurable CMC at L:T ratios of 20:1 to 80:1 (PEG) or 200:1 (SPC). Only PEC from mice cured of LPC-1 and challenged with LPC-1 showed significant cytotoxicity in vitro to LPC-1.

T-Cell Characteristic of CTL Population. The cytolytic activity of PEC suspensions was due to T-cells. Table 4 shows the results of a representative standard CMC assay in which treatment of PEC with anti-Thy 1.2 plus complement reduced activity of PEG suspensions was due to T-cells. Table 4 shows the results of these experiments.

Periodicity of CTL Development during and after Therapy. The resistance of cured mice to challenge with LPC-1 decreased with time (Chart 1). If the CTL response was involved in maintaining cure status, its activity would decrease chronologically in cured mice. This correlation was sought by assessing the recall capacity at various times during and after chemotherapy.

A large group of mice was implanted i.p. with 1 × 10^6 LPC-1 cells and treated with 5-FU, 16 mg/kg/day s.c., starting on Day 7, in accord with the standard protocol. A control group of 20 tumor-implanted mice was given the vehicle alone. The median survival time for the controls was 15.0 days (range, 13 to 18 days). All control mice died with progressively growing tumor. The 5-FU-treated mice were randomized into 2 groups. One group was challenged with 1 × 10^6 MIT-C-treated LPC-1 i.p. 5 days prior to harvesting of CTL; the second remained unchallenged. SPC and PEC were harvested on Days 0 (before tumor implantation), 7, during (Day 18), and after (Day 35, etc.) therapy with 5-FU (see "Materials and Methods"). One of 2 groups of mice were inoculated (challenged) additionally i.p. with 1 × 10^7 MIT-C-treated LPC-1 cells 5 days prior to assay; SPC and PEC were harvested on the days indicated and incubated with ^51Cr-labeled LPC-1 target cells for 4.5 hr. L:T ratios for SPC were 200:1 throughout; for PEC, L:T ratios were 25:1. ■, PEC of immunized mice; □, PEC of unimmunized mice; ○, SPC of immunized mice; △, SPC of unimmunized mice.

1 cells and treated with 5-FU, 16 mg/kg/day s.c., starting on Day 7, in accord with the standard protocol. A control group of 20 tumor-implanted mice was given the vehicle alone. The median survival time for the controls was 15.0 days (range, 13 to 18 days). All control mice died with progressively growing tumor. The 5-FU-treated mice were randomized into 2 groups. One group was challenged with 1 × 10^6 MIT-C-treated LPC-1 i.p. 5 days prior to harvesting of CTL; the second remained unchallenged. SPC and PEC were harvested on Days 0 (before tumor implantation), 7, during (Day 18), 18, 35 (2 days after last dose of 5-FU), 49, 63, 77, and 93 (3 days after reaching cure status).

CMC was minimal or absent in SPC and PEC from unchallenged tumor-bearing mice, with one exception occurring on Day 18 (Chart 2). Cytotoxicity of PEC from those mice was 13.1 ± 0.5%. Cytolytic activity of spleen cells from challenged mice was measurable only on Day 77 of the experiment (13.5 ± 1.4%). The greatest and most reproducible activity was present in PEC from challenged mice. Percentage of cytotoxicity was: 33.7 ± 0.6 (Day 18); 8.2 ± 0.9 (Day 35); 9.4 ± 1.1 (Day 49); 14.4 ± 0.6 (Day 63); and 40.2 ± 2.4 (Day 77). No measurable cytotoxicity was found on Days 0, 7, 33, and 90. In 3 other assays, CMC of PEC on Day 90 averaged 15 to 25%.

**DISCUSSION**

The immune response, perhaps of primary importance in the rare spontaneous cure, appears to play a significant and possibly crucial role as an adjunct to chemotherapy in the treatment of cancer (1, 5, 15, 19). In our studies with LPC-1, complete remissions of i.p. tumors were obtained by treatment with various drugs, singly and in combination (19). The most effective drugs not only yielded a higher proportion of cures but also rendered the mice comparatively more resistant to challenge with the syngeneic tumor cells. These results suggest that at least 2 factors are involved in the induction of resistance to tumor challenge: (a) the effectiveness of the drugs in killing the tumor cells; and (b) the ability of host immune mechanisms to function so that tumor immunity may be established and
remain operative despite any immunodepressive effect of the therapeutic drug and of the tumor.

Duration in tumor immunity of cured mice seems finite. As measured by viable tumor challenge, resistance to LPC-1, high at 4 months, diminished at approximately 7 months after the primary implant and was nearly gone by the 12th month (Chart 1). It cannot be determined from this experiment whether the lesser degree and duration of immunity in the older cured mice were the result of a longer elapsed time between tumor exposure or were due to their more advanced age, about 15 months when challenged. In studies of others, lower resistance to challenge was found in the aged cured mice than in the young (18), suggesting that decreased immunological reactivity of the older mice (16) may be a mitigating factor.

Table 2 shows a high degree of specificity associated with the resistance of LPC-1-cured mice. Highest resistance was demonstrated to LPC-1 itself and partial resistance to ADJ-PC-5, corroborating the results of other investigators (2, 9). Besides the possible sharing of common tumor-associated transplantation antigens, both LPC-1 and ADJ-PC-5 secrete myeloma protein IgG2a. Whether myeloma proteins play a major role as an antigen in the induction of drug-initiated tumor immunity may be clarified by comparisons with nonsecreting variants, a possibility currently under investigation.

CMC toward 3HCr-labeled LPC-1 target cells was assessed in vitro during and after therapy as a possible immune mechanism responsible for effecting cure of progressively growing LPC-1 in BALB/c mice. Selective removal of T-cells from the PEC population by anti-th serum and complement abrogated cytolytic activity, indicating a thymus origin.

In our studies, significant CTL responses were demonstrable only after tumor challenge during and after completion of therapy. This finding correlates with the results previously reported by Glaser and Herberman (7). In their studies, CTL activity was apparent only after challenge of mice in which Gross virus-induced tumor had regressed. Dislosed in our study, however, is the apparent periodicity in inducible CTL activity in mice undergoing curative therapy (Chart 2). We believe that the kinetics of developing inducible CMC activity followed by a periodicity in expressing recall capacity reflects the trigonal relationship between therapy, tumor, and host. The time study revealed several time periods when CMC activity could be readily induced in mice undergoing therapy which resulted in cures. The periodicity, we believe, represents 3 phases of immune responsiveness, reflecting perturbations in the immune system initiated during tumor growth and therapy.

The absence of significant CTL activity during the initial 7 days after tumor implantation reflects the nonspecific suppressive effects of progressive tumor growth [see review by Naor (10)]. In the first phase, the effect of therapy on tumor load was expressed as a significant CTL response to in vivo LPC-1 challenge in the treated animals 18 days after tumor implantation (11 days after therapy initiation). It may be at this particular juncture, when tumor-induced immune suppression is relieved by therapy and when drug toxicity on immune function is minimal or not apparent that success or failure of therapy-induced remission is programmed. Preliminary experiments support this conclusion in that viable tumor cells, which progressively grow and kill normal BALB/c mice, are readily aspirated from treated mice on Day 18. The treated mice from which viable tumor was obtained achieve 90-day cure status.4

This finding suggests that the residual tumor which resisted therapy was controlled by immunity of the host. The report of Mantovani et al. (8) gives additional support to this hypothesis. In their experiments, mice which remained immunosuppressed for more than 9 days after cessation of therapy died of progressive tumor growth, while animals which were immunologically competent achieved cure status.

The period of low inducible CMC activity between Days 18 and 77 (Chart 2) may reflect compromise of the immune system by cumulative or delayed drug toxicity. It appears that drug abrogation of tumor-induced immune suppression may predispose cells involved in immune responsiveness to the toxic effects of the cytoxic agent, a situation which has been shown to occur in intestinal mucosa cells from mice bearing L1210 leukemia (12). These investigators showed that the intestinal mucosa cells from mice bearing progressively growing L1210 were not subject to the toxic effects of therapy until the tumor load was significantly reduced by drug administration, thus subjecting newly proliferating intestinal cells to drug toxicity.

The results described suggest that destruction of residual tumor cells after chemotherapeutically induced regression is due to specific immunological responses. Consistent with those observations, present studies in our laboratory suggest that induction of remission in mice bearing well-established syngeneic tumors may be the result of interaction between drug therapy and host immune mechanisms. This interaction appears to implicate the effectiveness of the individual drug treatment in killing the tumor cell and the ability of host immune mechanisms to function so that immunity to the tumor may be established and maintained during and/or after therapy.

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