Detection of Early and Delayed Antitumor Effects following Curative Adoptive Chemoimmunotherapy of Established Leukemia

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ABSTRACT

Advanced disseminated leukemia can be successfully eradicated by treatment with a combination of noncurative nonlethal chemotherapy plus adoptively transferred immune cells. The time course of tumor elimination following such therapy was examined by bioassay for tumor of peripheral blood and spleen cells from treated mice. Curative treatment with adoptive chemoimmunotherapy did not immediately eliminate all leukemia. Bioassay of cells from treated mice, rather, demonstrated that, following the initial tumor lysis mediated by the chemotherapy and immune cells, a period of tumor regrowth lasting several weeks preceded ultimate tumor eradication. This transient tumor regrowth detectable by bioassay never became clinically evident in the treated mice. However, immunosuppression of mice two weeks after treatment with adoptive chemoimmunotherapy resulted in recurrence of lethal tumor. The results suggest that tumor elimination following curative adoptive chemoimmunotherapy is biphasic and that the efficacy of therapy may be subject to positive and/or negative influences over a prolonged time period.

INTRODUCTION

Advanced established tumors are generally insensitive to immunotherapy alone, reflecting in part consequences of a large tumor burden. However, advanced syngeneic murine tumors have been eradicated by ACIT, noncurative nonlethal cytotoxic chemotherapy followed by adoptively transferred syngeneic cells containing T-cells immune to the relevant tumor antigens (8, 16). Simplistically, tumor eradication following ACIT might reflect destruction of most of the tumor by drug followed by rapid killing of the residual tumor cells by infused cytotoxic donor cells. However, since irradiation of immune effector cells which had been secondarily sensitized to syngeneic tumor in vitro did not abolish cytolytic activity when tested in a 4-hr in vitro assay but rendered the cells ineffective in vivo in ACIT (12), proliferation of the lymphoid cells in the host following cell transfer is presumably necessary for therapeutic efficacy.

The present studies were initiated to elucidate the reasons underlying this requirement for cell proliferation during curative ACIT by examining the time course of tumor elimination. The results revealed that curative chemoimmunotherapy did not immediately eliminate all leukemia; initial tumor lysis was incomplete, and a period of subclinical tumor regrowth lasting several weeks preceded ultimate tumor eradication. Moreover, immunosuppression of mice after cell transfer resulted in recurrence of lethal leukemia.

MATERIALS AND METHODS

Mice. Twelve- to 16-week-old C57BL/6 and BALB/c mice were obtained from Simonsen Laboratory (Gilroy, Calif.).

Tumors. FBL-3 and RBL-5 in C57BL/6 and LSTRA in BALB/c are, respectively, Friend, Rauscher, and Moloney virus-induced tumors maintained by i.p. transplantation (>300 generations) in adult syngeneic mice. These tumors express tumor-associated antigens which cross-react with each other and other Friend, Moloney, or Rauscher virus-induced tumors (9, 15).

ATS. ATS (Lot 3-9225) was purchased from Microbiological Associates (Bethesda, Md.). Sera from other production lots identically prepared have been shown to suppress in vitro and in vivo T-cell responses (1). Inoculation of 0.25 ml ATS i.p. induces no apparent morbidity or mortality in adult C57BL/6 mice.

In Vivo Immunization with FBL-3. C57BL/6 mice given inoculations of 10^6 FBL-3 s.c. develop a spontaneously regressing tumor and become resistant to subsequent challenge with otherwise lethal doses of FBL-3 inoculated i.p. (6). Therefore, mice were given inoculations of 10^6 FBL-3 s.c. 3 times at 2-week intervals, and spleen cells were obtained 14 to 21 days after the last immunization. This sensitization regimen has been shown to generate cells which are effective in ACIT (7).

In Vivo ACIT. The assay, previously described (6), consists of treating C57BL/6 mice bearing advanced established FBL-3 with a combination of nonlethal, noncurative chemotherapy and adoptively transferred immune cells. C57BL/6 mice given inoculations of 2 x 10^7 FBL-3 i.p. on Day 0 were treated on Day 5 with CY, 180 mg/kg, followed in 6 hr, to permit drug catabolism and excretion, by adoptively transferred immune cells inoculated i.p. Mice receiving no therapy or therapy on Day 5 with normal or immune cells alone died between Days 14 and 20. Treatment on Day 5 with CY alone prolonged median survival time to approximately Day 35 but cured no mice; treatment with CY plus nonimmune cells or cells immune to unrelated antigens was no more effective than was CY alone, and treatment with CY plus cells immune to FBL-3 further prolonged survival and, with a sufficient cell dose, cured mice (7).

Experiments examining in vivo suppression with ATS of the therapeutic efficacy of immune cells in ACIT were performed more than 6 months after the ACIT experiments described for bioassay studies. During this time, our FBL-3 tumor had become more virulent so that control untreated mice had a shorter

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mean survival time, and therapy with CY or CY plus the same dose of immune cells had a somewhat diminished therapeutic efficacy.

**Bioassay for Tumor.** Nucleated cells (10^6) for testing were obtained from the peripheral blood or spleen of healthy-appearing primary hosts previously inoculated with tumor i.p. and treated with ACIT. Peripheral blood and spleen cells were prepared as previously described (7). The cells were bioassayed for FBL-3 by inoculation i.p. into immunosuppressed C57BL/6 secondary hosts, which were observed for mortality from lethal tumor. The secondary hosts were immunosuppressed by pretreatment with CY, 180 mg/kg, 24 hr prior to inoculation of the bioassay sample, since this increased the lethality of FBL-3 (6) and thus enhanced the sensitivity of tumor detection.

**In Vitro Sensitization.** The culture conditions for in vitro sensitization have been described in detail (12). Briefly, C57BL/6 spleen cells which had been primed in vivo 4 weeks earlier with s.c. FBL-3 were secondarily sensitized in vitro by 5-day mixed leukocyte:tumor culture in 5% fetal calf serum with irradiated FBL-3 at a responder:stimulator ratio of 20:1 or primarily sensitized in vitro to allogeneic cells by 5-day mixed leukocyte culture with irradiated BALB/c spleen cells at a responder:stimulator ratio of 10:1.

Following in vitro sensitization, cells were tested for cytolytic activity in a 4-hr CRA as previously described (12). Cytotoxicity to allogeneic H-2^d_2^ antigens was determined by lysis of the BALB/c tumor LSTRA. Cytotoxicity generated by secondary in vitro sensitization to syngeneic FBL-3 was determined by lysis of RBL-5, another Friend, Moloney, or Rauscher virus-induced tumor of C57BL/6 origin which shares tumor-associated antigens with FBL-3 (9, 15) but which is a preferable target in the chromium release assay due to lower spontaneous release and increased sensitivity to lysis.

**RESULTS**

**Elimination of Tumor by ACIT as Determined by Bioassay.** The in vivo therapeutic efficacy of spleen cells from mice immunized with FBL-3 3 times was examined in ACIT of established FBL-3 leukemia. The cumulative results from 3 experiments are presented in Chart 1. C57BL/6 mice were given inoculation i.p. on Day 0 with 2 x 10^7 FBL-3, a syngeneic Friend leukemia. Untreated mice all died by Day 19. Treatment on Day 5 with CY prolonged median survival to Day 35 but cured no mice. Normal syngeneic spleen cells infused 6 hr after CY had no additional effect on survival. However, treatment with CY plus 5 x 10^7 immune spleen cells caused 24 of 25 mice to be tumor free at Day 150. Of note, 5 x 10^7 immune cells inoculated on Day 5 without prior chemotherapy had no effect on survival, with all mice dying by Day 19 (survival curve not shown).

Mice dying from tumor progression exhibited ascites, splenomegaly, and lymphadenopathy 2 to 7 days prior to death. The presence of disseminated tumor, which presumably exists prior to clinically apparent tumor, and the time course of tumor elimination following curative ACIT were examined by bioassaying peripheral blood and spleen cells from healthy-appearing C57BL/6 mice at various time periods following the treatment regimens shown in Chart 1. Test cells (10^6) were bioassayed for FBL-3 tumor by inoculation i.p. into C57BL/6 secondary hosts which had been immunosuppressed by pretreatment with CY so as to enhance tumor outgrowth. The potential sensitivity of this bioassay was determined by inoculating graded doses of FBL-3 into these immunosuppressed secondary hosts. FBL-3 (10^6) killed 50% of the mice, and all mice receiving 10^6 or more FBL-3 died of progressive tumor. A dose of 10^4 FBL-3 would represent only 1% contamination of the test inoculum.

Groups of mice identical with each of the treatment groups described by the survival curves in Chart 1 were prepared concurrently in each of the 3 experiments and, rather than being observed for end results in ACIT, were sacrificed at various times for bioassay. The cumulative results from 3 separate bioassay experiments for each treatment group are presented in Charts 2 and 3. The time required for tumor dissemination to occur was examined by bioassaying peripheral blood and spleen cells from untreated primary hosts which had been given inoculation with 2 x 10^7 FBL-3 i.p. on Day 0.

**Chart 1.** ACIT of FBL-3. C57BL/6 mice were given inoculation on Day 0 with 2 x 10^7 FBL-3 i.p. On Day 5, mice remained untreated, were treated with only CY (180 mg/kg), or were treated with CY followed in 6 hr by 5 x 10^7 normal C57 spleen cells or 5 x 10^7 immune C57 spleen cells i.p. Immune cells were obtained from mice immunized 3 times with s.c. FBL-3. The cumulative results of 3 experiments are presented with fractions representing the number of mice surviving per total mice in group.

**Chart 2.** Bioassay to determine the time required for tumor to disseminate in untreated mice. Primary hosts received 2 x 10^7 FBL-3 on Day 0 and remained untreated. On the specified days, the primary hosts were sacrificed, and 1 x 10^8 nucleated peripheral blood or spleen cells were bioassayed by transfer i.p. into secondary hosts which had been immunosuppressed by pretreatment with CY, 180 mg/kg. Since separate bioassay of peripheral blood and spleen cells produced similar results, the data were pooled. Each bar represents the percentage of survival of 32 secondary hosts which received test cells for bioassay from 32 primary hosts.
treated but observed rather than sacrificed for bioassay studies) never developed clinically evident tumor and were apparently cured of leukemia at Day 150 (Chart 1). Bioassays performed with cells from Days 40 and 50 revealed rapid disappearance of detectable tumor, implying that the subclinical tumor recurrence was transient. Thus, following an early but incomplete tumoricidal effect of CY and immune cells, there is subclinical tumor regrowth followed by apparent eventual tumor eradication.

**Immunosuppression with ATS.** The bioassay results from mice treated with CY plus immune cells suggested that tumor eradication requires a late antitumor effect. If this delayed tumor rejection is immunologically mediated, then immunosuppression of mice long after potentially curative ACIT might result in progressive tumor growth. ATS was selected for study as a potential immunosuppressant since low doses of ATS inoculated in vivo have been shown to preferentially eliminate memory T-cells rather than uncommitted cells (1). The selectivity and potency of our ATS were initially assessed by immunosuppression of in vitro primary and secondary responses. C57BL/6 mice, which had been primed in vivo with s.c. FBL-3 4 weeks earlier, were given inoculation i.p. with ATS in low dose, 0.04 ml, or high dose, 0.25 ml. Two days later, the mice were sacrificed, and the spleen cells were tested for generation of specific cytotoxicity following an in vitro primary allogeneic response to BALB/c cells or an in vitro secondary response to syngeneic FBL-3 (Table 1). Low-dose ATS suppressed the secondary response more than the primary response. High-dose ATS, although a somewhat more potent immunosuppressant, demonstrated no preferential suppression of the secondary response. Of note, although both doses partially abrogated the secondary response to syngeneic tumor, neither dose was sufficiently potent to eliminate all FBL-3-primed cells.

The effect of ATS, when given after adoptively transferred immune cells, on the outcome of ACIT was examined. The cumulative results of 3 experiments are presented in Chart 4. Eighty percent of mice treated on Day 5 with CY plus immune cells were tumor free at Day 100. Groups of mice previously treated on Day 5 with CY plus immune cells received a single i.p. injection of either 0.04 ml or 0.25 ml ATS on Days 15, 17, or 19. Mice receiving either a low or a high dose of ATS had a significantly increased rate of tumor recurrence and mortality ($p < 0.01$) with 50% of the mice dying from progressive tumor. Thus, ATS immunosuppression 10 to 14 days after treatment with CY and immune cells was capable of interfering significantly with tumor eradication.

**DISCUSSION**

These studies have provided some insights into the requirements for tumor eradication following ACIT. Bioassays performed with cells from untreated mice on Day 5 revealed that tumor had disseminated by the time therapy was initiated. Treatment with chemotherapy alone had a predictable immediate antitumor effect but was followed by an eventually lethal tumor recurrence. Curative therapy with CY plus immune cells demonstrated an unexpected biphasic pattern for tumor elimination characterized by early but incomplete tumor destruction, subsequent tumor regrowth, and a late antitumor effect leading to tumor eradication.

The experimental results imply that immune cells participate...
Biphasic Tumor Elimination following Chemoimmunotherapy

Table 1
ATS-pretreated spleen cells exhibit suppressed in vitro responses∗

<table>
<thead>
<tr>
<th>Group</th>
<th>In vivo pretreatment</th>
<th>In vitro stimulus</th>
<th>Primary response to allogeneic target</th>
<th>Secondary response to syngeneic tumor target</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>(FBL)</td>
<td>12 (68)</td>
<td>63 (12)</td>
</tr>
<tr>
<td>II</td>
<td>ATS (0.04 ml)</td>
<td>(FBL)</td>
<td>2 (52)</td>
<td>30 (7)</td>
</tr>
<tr>
<td>III</td>
<td>ATS (0.25 ml)</td>
<td>(FBL)</td>
<td>3 (8)</td>
<td>16 (3)</td>
</tr>
</tbody>
</table>

∗ Spleen cells were obtained from C57BL/6 mice which had been primed 4 weeks previously with s.c. FBL-3. Data presented are the means of 3 experiments.

† FBL-primed mice were given inoculations i.p. with the denoted dose of ATS 2 days prior to sacrifice and removal of the spleen cells for in vitro culture.

‡ Spleen cells were cultured for 5 days with irradiated syngeneic FBL-3 or irradiated allogeneic BALB/c spleen cells.

§ The primary response to alloantigens generated during 5-day culture was determined by lysis of the BALB/c target, LSTRA, in a 4-hr chromium release assay. Cells were tested at an effector:target ratio of 20:1.

<table>
<thead>
<tr>
<th></th>
<th>Primary response to alloantigens</th>
</tr>
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<tbody>
<tr>
<td>Lysis (%)</td>
<td>Suppression (%)</td>
</tr>
<tr>
<td>I</td>
<td>None (FBL)</td>
</tr>
<tr>
<td>II</td>
<td>ATS (0.04 ml)</td>
</tr>
<tr>
<td>III</td>
<td>ATS (0.25 ml)</td>
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</table>

Chart 4. Effect of in vivo immunosuppression with ATS on the outcome of ACIT of FBL-3. C57BL/6 mice received tumor on Day 0 and were treated on Day 5 as previously described. Groups of mice treated on Day 5 with CY plus immune cells were given inoculation on Days 15, 17, or 19 with either 0.04 ml or 0.025 ml ATS i.p. Since survival was similar independent of day of ATS administration, the cumulative results for each dose of ATS are presented. Fractions represent mice surviving per total mice in group.

In both phases of tumor elimination, a tumoricidal contribution by cytotoxic and precytotoxic cells among the adoptively transferred immune cells would be expected in the early phase of tumor elimination and is consistent with the observed delayed detection by bioassay of disseminated tumor following treatment with CY plus immune cells as compared to treatment with CY alone. Furthermore, previous studies have demonstrated that, following chemotherapy on Day 5, postponement of adoptive immunotherapy for 5 days until Day 10 resulted in diminution of the therapeutic efficacy, suggesting that the efficacy of immune cells depends in part on a contribution to tumor elimination in the period shortly following adoptive transfer (7).

Following the initial antitumor effect of CY and immune cells, a period of transient subclinical tumor growth occurred. Presumably, this reflected growth of residual FBL-3 tumor cells rather than a newly induced tumor. The early onset of detectable tumor growth following ACIT as well as the resistance of the C57BL/6 strain to virally induced leukemogenesis (14) support this presumption. Additionally, studies have suggested that the FBL-3 tumor line does not produce infectious Friend virus (4, 11). Although this analysis cannot definitively resolve between these 2 alternatives, mechanisms which permit transient growth of a new tumor induced by the same etiologic agent which induced the recently rejected tumor may be fundamentally similar to and equally important as mechanisms permitting transient growth of residual tumor.

Eventual rejection of the subclinical tumor recurrence apparently also required participation of immune cells. Immunosuppression with ATS 10 to 14 days after therapy with CY and immune cells increased the rate of recurrence of lethal tumor. Identical reductions in survival were observed following inoculation of low- or high-dose ATS. Both doses of ATS also produced relatively similar suppression of the in vitro secondary response by memory cells as compared to more disparate suppression of the in vitro primary responses. Thus, the similar effects on survival with both doses of ATS may reflect, in part, quantitatively similar elimination of sensitized cells. The persistence of immune effector cells long after ACIT which might be susceptible to ATS has been previously demonstrated; following curative ACIT, mice were resistant to challenge with FBL-3, and their spleen cells were effective in ACIT of other tumor-bearing mice (7). The fact that reduction in survival was only partial following in vivo immunosuppression with ATS could result from incomplete removal of primed effector cells, suboptimal timing of the immunosuppression, and/or participation of an additional mechanism in the eventual tumor rejection.

Thus, immune cells transferred in ACIT appear capable of mediating early and late antitumor effects against FBL-3. The mechanisms which prohibit complete tumor eradication during the early phase but permit eventual tumor rejection are not yet defined, but they presumably reflect alterations in the tumor or in the function of the immune system. Immune modulation of Friend leukemia cell surface antigens has been reported (5) and might render the tumor temporarily resistant to immune
attack or necessitate generation of immune cells with a new specificity. Alternatively, immunoregulatory phenomena such as short-lived suppressor cells of host or donor origin (2, 3, 10) or release of blocking factors (13) may transiently limit effector cell generation and/or function following the initial tumor lysis produced by CY and immune cells.

The results suggest that tumor elimination following ACIT is biphasic with early but incomplete tumor destruction followed by tumor growth and a late antitumor effect leading to tumor eradication. The efficacy of adoptive immunotherapy may be subject to positive and/or negative influences over this prolonged time period, and the outcome of protocols for treatment of antigenic tumors may vary greatly depending on the cycling and scheduling of the modalities utilized. Further analysis of the mechanisms controlling tumor elimination should help define strategies for immunotherapy which utilize this time span to preferentially facilitate and not interfere with tumor eradication.

REFERENCES

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