Immunotherapy and Chemotherapy of Moloney Sarcoma Virus-induced Tumors in Mice

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SUMMARY

Regression of autochthonous (primary) murine sarcoma virus (Moloney) (MSV)-induced tumors was first detectable in 13% of BALB/c mice inoculated with MSV at 3 weeks of age and increased in incidence thereafter. This correlated with the ability to resist transplanted BALB/c Moloney sarcoma (MS) cells. Treatment of young BALB/c mice bearing palpable primary tumors with spleen cells or with serum from adult BALB/c or CDF (BALB/c × DBA)F1 mice whose autochthonous tumors had regressed resulted in complete tumor regression in 34—40% of the hosts. Such immune cells, in conjunction with cyclophosphamide (CY), were also effective in eradicating palpable transplanted tumors.

Primary tumors were moderately sensitive to CY. However, CY given to young mice bearing primary tumors transiently inhibited tumor growth and doubled host survival time, whereas CY given to adult tumor-bearing mice decreased tumor growth but also depressed immunologic reactivity, thereby preventing tumor regression. This illustrates a potential danger in treating a host already responding against his tumor with a chemotherapeutic agent possessing potent immunosuppressive activity. Specific immunotherapy prevented the deleterious effect of CY. Most adult BALB/c mice bearing palpable primary tumors and inoculated with CY were cured by spleen cells or by serum from BALB/c, CDF, or (BALB/c × C57BL/6)F1 mice hyperimmunized by MSV, but not by cells from unimmunized mice or by anti-BALB/c serum. The results show that established primary MSV-induced tumors can be cured by lymphoid cells or serum from syngeneic or allogeneic donors whose autochthonous MSV-induced tumors have regressed.

INTRODUCTION

MSV rapidly induces tumors in newborn and adult mice (4). The tumors have been reported to be rhabdomyosarcomas (7, 15). Among the unique features of this tumor system is a high incidence of complete regression of autochthonous (primary) tumors (4). The possibility that regression is immunologically mediated has been suggested by the following observations: (a) MSV-induced tumors possess tumor-specific transplantation antigens (3). (b) Autochthonous tumor regression depends upon an intact immunologic responsiveness of the host. Tumors induced in normal adult mice regress, whereas those induced in newborn mice (4), or in adult mice immunologically suppressed by pretreatment with sublethal X-irradiation (4), cortisone (16), or neonatal thymectomy (12), tend to grow progressively and kill the host. (c) Autochthonous tumor regression is preceded by, accompanied by, and followed by a specific humoral and cellular immune response against the tumor and/or virion antigens (6) and is associated with a progressive infiltration of the tumor by lymphocytes (6).

This study deals with the following three problems: (a) The development of immunologic responsiveness to MSV-induced tumor antigens in BALB/c mice as a function of age; (b) The effect of a chemotherapeutic agent with both antitumor and immunosuppressive activity on tumor-bearing hosts which are or are not reacting adequately to the tumor; (c) The eradication of established primary or transplanted MSV-induced tumors destined to grow progressively and kill the host either by serum or lymphoid cells from adult syngeneic or allogeneic donors whose autochthonous MSV-induced tumors have regressed.

MATERIALS AND METHODS

Mice. BALB/c (H-2d), CDF (BALB/c × DBA/2)F1, and CBF (BALB/c × C57BL/6)F1 mice with an H-2d/H-2b genotype were obtained from the production colonies of Texas Inbred Mice and the Charles River Breeding Laboratories.

Virus. A pool of MSV, designated RP#114, was kindly provided by Dr. J. B. Moloney. It had been extracted from BALB/c tumor tissue and had been passed in weanling BALB/c mice for 114 generations. Tumors were induced with this pool in adult BALB/c mice and were removed on Day 10 when they contained abundant MSV (6). Virus was extracted by differential ultracentrifugation (14) (1 ml of extract per gram of tumor tissue). The resultant pool, designated RP#114-S-1, was used for all experiments.

Tumor Induction. Tumors were always induced by inoculating 0.05 ml of a 101 dilution of the MSV pool i.m. into the right hind limb. Mice were palpated daily, and the tumor diameters were measured by calipers.

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1Supported by Grant No. CA 10777 from the National Cancer Institute, NIH.
2Abbreviations used are: MSV, murine sarcoma virus (Moloney); MS Moloney sarcoma (cells); CY, cyclophosphamide; HBSS, Hank's balanced salt solution.

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Transtplantable Moloney Sarcoma. A solid sarcoma, originally obtained from Dr. J. B. Moloney and designated SV122-TR-4, was used. It originated in a BALB/c mouse and has been maintained by serial transplantation in adult BALB/c mice for 72 generations. The tumor, when inoculated as $10^7$ cells or as trocar fragments, grows progressively, metastasizes to all limbs, and kills almost all normal adult BALB/c mice. It contains oncogenic MSV and is antigenic in histocompatible hosts (3, 6). Trocar fragments or tumor cell suspensions, prepared as previously described (6), were always inoculated s.c.

Drug. CY was dissolved in distilled water, and the appropriate concentrations injected i.p. in a volume of 0.01 ml per gram body weight.

Preparation of Spleen Cell Suspensions. Spleens were cut into small fragments and pressed through a stainless steel mesh. The cells were then washed 3 times in HBSS, centrifuged once at 400 rpm (30 X g) for 5 minutes, and twice at 2000 rpm (700 X g) for 5 minutes. The concentration of trypan blue-unstained nucleated cells was determined and adjusted with HBSS. The cells were injected i.p. in a volume of 0.05 ml in young recipients or 0.1-0.15 ml in adults.

Sera. Normal or immune donors of spleens were exsanguinated at the time of sacrifice and splenectomy, and their sera used the same day. For immunotherapy, sera were injected i.p. in a volume of 0.1 ml per mouse. An anti-BALB/c serum was obtained by injecting adult C57BL/6 mice with $1 \times 10^7$ BALB/c spleen cells i.p. at weekly intervals and bleeding them 2 weeks after the fifth injection. The serum was stored at $-70^\circ$C until testing.

Preparation of Antibody against Donor $\gamma$-Globulin. Sera from BALB/c mice immunized against DBA or C57BL/6 $\gamma$-globulin by the method of Lieberman and Dray (13) were tested against serially diluted serum from DBA or C57BL/6 mice by end-point precipitation in a double diffusion system in an agarose gel, as previously described (8). The center well contained BALB/c anti-DBA or anti-C57BL/6 serum. DBA, C57BL/6, CDF, or CBF sera could always be diluted 1:32 or 1:64 and still yield precipitin lines with the specific anti-globulin. The presence of DBA or C57BL/6 $\gamma$-globulin in sera of BALB/c mice injected with CDF or CBF lymphoid cells was similarly tested. The results expressed as the reciprocal of the highest dilution of the test serum which produced a precipitin line with the undiluted BALB/c anti-DBA or anti-C57BL/6 serum.

RESULTS

Ontogeny of Immunologic Responsiveness to MSV-induced Tumor Antigens. BALB/c mice of various ages were inoculated with MSV, or with $10^5$ or $10^6$ viable syngeneic MS cells. The incidence of autochthonous tumor regression and resistance to transplanted MS cells, as a function of host age at inoculation of MSV or MS cells, is presented in Chart 1. Both were first detectable in mice challenged with MSV or MS cells at 3 weeks of age and increased progressively with age. The results suggested the appropriate hosts for immunotherapeutic studies.

Effect of Sublethal CY on MSV-induced Tumors Destined to Kill the Host. Two-week-old BALB/c mice, bearing palpable tumors induced by inoculation of MSV 6 days earlier, were treated once with CY (115 mg/kg). The results are presented in Table 1. CY transiently inhibited tumor growth and doubled host survival time. No regressions occurred with or without CY. Similarly, adult BALB/c mice were inoculated with trocar fragments of the transplantable MS and with CY (224 mg/kg) 24 hours later. Table 1 shows that CY inhibited the outgrowth of MS fragments. No cures were observed. The results suggested that both primary and transplanted MS cells were sensitive to the antitumor effect of CY.

Effect of CY on Regression of Primary Tumors in Adults. Although it is known that adult mice immunologically suppressed before MSV inoculation develop progressively growing tumors and die (4), the effect of immune suppression after the tumor is already palpable has not been studied. CY, a drug which is immunosuppressive even when administered after the host has been exposed to antigen (2), was deemed appropriate for study. Thus, the experiments involved the treatment of a host, who is reacting adequately to his tumor, with a drug which can kill tumor cells but which can concurrently depress the host's immune response.

Adult BALB/c mice were treated once with CY (in doses which killed no normal adult mice) either prior to MSV inoculation or after they had developed palpable tumors. Control mice received MSV but no CY. The results, presented in Table 2, show that mice treated with CY 47 days prior to MSV inoculation developed tumors which, like tumors induced in untreated adult mice, ultimately regressed. However, mice treated a week or a day before MSV inoculation, or at any time after the tumors were already palpable, ultimately died with progressively growing tumors. Cumulatively, 85% of untreated control mice exhibited complete tumor regression, whereas 92% of tumor-bearing mice treated with CY ultimately died with tumor.

The growth curves of tumors treated with CY (224 mg/kg) are depicted in Chart 2. Mice treated with CY one day before

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chart1.png}
\caption{Autochthonous tumor regression (---) and resistance to syngeneic transplanted Moloney sarcoma cells (---) in BALB/c mice, as a function of their age at inoculation of Moloney sarcoma virus or Moloney sarcoma cells. Higher doses of Moloney sarcoma cells; e.g., $10^7$ grew and killed all mice of all ages.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{ll}
\hline
Age of inoculation (weeks) & % Survivors \\
\hline
<1 & 100 \\
1-2 & 92 \\
2-3 & 85 \\
3-4 & 65 \\
4-5 & 47 \\
5-6 & 35 \\
6-7 & 20 \\
7-8 & 10 \\
8-12 & 0 \\
\hline
\end{tabular}
\caption{Effect of CY on MSV-induced Tumors Destined to Kill the Host.}
\end{table}
MSV developed progressively growing tumors and died. However, mice given CY when tumors were already palpable (7 days after MSV inoculation) exhibited inhibition of tumor growth for 2–3 weeks, followed by progressive tumor growth and death of the host by Day 40 after MSV inoculation.

Immunotherapy. The preceding studies indicated the choice of hosts for immunotherapeutic approaches to the treatment of established primary or transplanted MSV-induced tumors. Progressively growing fatal tumors could be induced by inoculating adult mice with trocar fragments of transplantable MS, young mice with MSV, or adult mice with MSV plus CY. Since regression of autochthonous MSV-induced tumors renders mice immune to MSV-induced tumor antigens (6, 10), specific immunization of donors of serum or lymphoid cells could be achieved by inoculating them with MSV and waiting for regression of the resultant tumors.

In all experiments, tumor-bearing BALB/c hosts were treated with lymphoid cells or serum from syngeneic or hemisyngeneic mice. Furthermore, the donors used produced γ-globulin antigenically distinguishable from that of the host. Therefore, the persistence of donor γ-globulin in host sera could be used as a marker for the persistence of donor lymphoid cells in the host.

Chemoimmunotherapy of Transplanted Tumors. Since lymphoid cells from adult mice whose autochthonous MSV-induced tumors had regressed have been shown to neutralize
transplanted MS cells in vitro (5), and since MSV-induced tumors are somewhat sensitive to the antitumor effect of CY, an attempt was made to treat mice bearing palpable transplanted tumors with CY plus specifically immunized F₁ lymphoid cells.

Adult BALB/c mice were inoculated with trocar fragments of syngeneic MS cells. All mice which exhibited palpable local tumors 8 mm in diameter on Day 14 were inoculated with CY (224 mg/kg). Four hours later, they received 1 X 10⁸ spleen cells from normal adult CDF mice or from adult CDF mice whose autochthonous MSV-induced tumors had regressed one month before. The results are depicted in Chart 3. All untreated mice died with tumor, as did 10/10 mice treated only with immune cells (not charted). CY retarded tumor growth and prolonged host survival. Treatment of tumor-bearing mice with CY and normal CDF spleen cells did not prolong host survival beyond that observed with CY alone. By contrast, treatment with CY plus immune CDF spleen cells resulted in further prolongation of survival in 4/10 mice and complete cures in 6/10 tumor-bearing mice. Thus, palpable transplanted tumors were eradicated by a combination of chemotherapy and lymphoid cells from mice specifically sensitized against MSV-induced tumor antigens.

Syngeneic Immunotherapy of Primary Tumors in Young Mice. BALB/c mice, aged 3 weeks or less, were inoculated with MSV 8 weeks before use, developed tumors which regressed 3 weeks later, were reinoculated with MSV 2 weeks after regression (and failed to develop tumors), and were sacrificed as donors 3 weeks later. Control tumor-bearing mice of the same age and weight were treated with spleen cells or serum from normal adult CDF mice or remained untreated.

Chart 4 shows that 15 of 24 tumor-bearing mice treated with hyperimmunized spleen cells or serum were completely cured, in contrast with only 1 out of 22 mice treated with normal cells or serum. Once again, although tumor regression occurred after treatment with immune cells or serum, regression after treatment with immune serum was associated with gross retardation of tumor growth, whereas regression after treatment with immune cells was not.

Tumor-bearing mice which received immune CDF spleen cells were bled 6 and 14 days later, and their sera tested for the presence of DBA γ-globulin. At 6 days, 6/8 mice whose tumors ultimately regressed were positive (at 1:4), and 2 were negative. By contrast, 4/5 mice which ultimately died with tumor were negative, and one died with tumor before bleeding. Thus, the therapeutic effect of donor spleen cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumors regressed</th>
<th>No. of tumors treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3/71</td>
<td>3/75</td>
</tr>
<tr>
<td>Normal serum</td>
<td>3/75</td>
<td>4/83</td>
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<tr>
<td>Normal spleen</td>
<td>29/78</td>
<td>27/90</td>
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<tr>
<td>Immune spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum from immune spleen donors</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Syngeneic immunotherapy of primary murine sarcoma virus (Moloney)-induced tumors in young BALB/c mice. Young mice bearing palpable tumors were injected with spleen cells once or with serum on 3 consecutive days.

"Immune" donors were adult mice whose autochthonous tumors had regressed.
Immunotherapy and Chemotherapy of MSV Tumors

Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumor-bearing mice treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1/28</td>
</tr>
<tr>
<td>Normal BALB/c spleen</td>
<td>2/29</td>
</tr>
<tr>
<td>Normal CBF spleen</td>
<td>0/8</td>
</tr>
<tr>
<td>Normal CDF spleen</td>
<td>0/8</td>
</tr>
<tr>
<td>Normal CDF serum</td>
<td>0/8</td>
</tr>
<tr>
<td>C57BL/6 anti-BALB/c serum</td>
<td>1/8</td>
</tr>
<tr>
<td>Immune BALB/c spleen</td>
<td>20/24</td>
</tr>
<tr>
<td>Immune CBF spleen</td>
<td>10/12</td>
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<tr>
<td>Immune CDF spleen</td>
<td>8/13</td>
</tr>
<tr>
<td>Serum from immune CDF spleen donors</td>
<td>6/9</td>
</tr>
</tbody>
</table>

Immunotherapy of primary MSV-induced tumors in cyclophosphamide (CY-treated adult BALB/c mice. MSV, murine sarcoma virus (Moloney).

aMice bearing palpable tumors were injected with CY (180 mg/kg) and four hours later, with spleen cells once or with serum for four consecutive days.

b"Immune" donors had exhibited autochthonous tumor regression and had been reinoculated twice with MSV.

appeared to be correlated with their persistence in the host. All mice were negative by Day 14.

Immunotherapy of Primary MSV-induced Tumors in CY-treated Adult Mice. Since CY administered to tumor-bearing adult mice temporarily inhibited tumor growth but prevented regression, an attempt was made to save the mice by treatment with large numbers of lymphoid cells or serum from specifically hyperimmunized donors. Adult BALB/c mice bearing palpable primary MSV-induced tumors (6–8 mm in diameter) were inoculated with CY (180 mg/kg). Four hours later they received $2.5 \times 10^8$ spleen cells from BALB/c, CDF, or CBF mice which had been inoculated with MSV 13 weeks before use, which had developed tumors that regressed, which had been reinoculated with MSV 3 and 7 weeks after regression (no tumors developed), and which had been sacrificed as donors 2 weeks later. One group of tumor-bearing CY-treated mice was treated with serum from the immune CDF donors (0.1 ml/day x 4). Control mice received only CY, CY plus spleen cells from normal adult mice of the 3 strains, CY plus normal CDF serum, or CY plus C57BL/6-anti-BALB/c serum.

The results are presented in Table 4. Almost all tumor-bearing mice treated with CY alone died with tumor. Treatment of such CY-treated mice with normal spleen cells or serum or with anti-BALB/c serum had no significant effect on tumor growth, and almost all mice died with tumor. By contrast, 76% of tumor-bearing CY-treated mice given hyperimmune spleen cells or serum were cured. Chart 5 depicts the growth curves of tumors treated with CY plus normal or hyperimmune syngeneic spleen cells in one experiment. The latter treatment resulted in rapid disappearance of the tumors. Tumors treated with hyperimmune hemisyngeneic spleen cells, or with hyperimmune serum yielded similar growth curves.

All survivors treated with nonsyngeneic spleen cells were bled 20 and 36 days after treatment, and their sera tested for the presence of donor-type γ-globulin, as a marker for the persistence of donor lymphoid cells in the host. The results, presented in Chart 6, do not permit firm conclusions. As expected, CDF cells which, like the BALB/c host, are H-2d, persisted longer than did CBF cells (H-2d/H-2b). Mice "cured" by immune CDF cells tended to have higher titers of DBA γ-globulin for a longer time than did mice given the same cells but not "cured" of tumor. However, this correlation was not seen with the transient chimerism induced by immune CBF cells. Mice given normal CDF or CBF spleen cells also had donor γ-globulin in their sera at 20 days but died with tumor before the second bleeding.
Regression of established primary murine tumors is unique to the Moloney sarcoma system. The possibility that regression represents an immunologic rejection of antigenic tumor cells by the autochthonous host has been suggested by: (a) the presence of tumor-specific transplantation antigens on MSV-induced tumors (3); (b) the dependence of autochthonous tumor regression upon an intact immunologic competence of the host (4, 16); (c) the progressive infiltration of regressing tumors by lymphocytes (6); and (d) the association of autochthonous tumor regression with a humoral and cellular immune response to MSV-induced tumor antigens (6). The specific immune response is demonstrable by a variety of tests. Mice with regressing autochthonous tumors resist challenge with transplanted syngeneic MS cells and produce antibody detectable by virus-neutralization and immunofluorescence tests (6). Furthermore, their lymphoid cells or sera are cytotoxic to MS cells by the colony-inhibition test (10). The results presented in this paper further substantiate the view that regression is indeed immunologically mediated.

Studies on ontogeny of immunologic responsiveness to MSV-induced tumor antigens in BALB/c mice suggest that effective responsiveness, as manifested by regression of autochthonous tumors or rejection of transplanted tumors, first occurs at 3 weeks of age. However, even younger mice bearing progressively growing tumors are known to respond to some extent, since their lymph node cells or sera are cytotoxic to MS cells by the colony-inhibition test (10). Thus, the tumors grow even in the presence of an immune response against them.

Palpable primary tumors induced in young mice, and destined to kill the host within one week after becoming palpable, were successfully treated with spleen cells from syngeneic adult mice whose autochthonous MSV-induced tumors had regressed. The therapeutic effect was not associated with gross inhibition of tumor growth, suggesting that the lymphoid cells may require time to be effective. Serum from the immune spleen donors also caused regression but was associated with gross retardation of tumor growth. This is compatible with a direct cytotoxic mechanism of action and is consistent with the reported ability of specific antiserum to inhibit the outgrowth of transplanted MS cells (12). A greater frequency of cures was obtained by treating smaller, though still palpable, tumors with larger numbers of spleen cells or more injections of serum from adult CDF mice hyperimmunized with MSV.

Specifically immune spleen cells, in conjunction with chemotherapy, were also effective against a transplantable MS. Sixty percent of BALB/c mice bearing palpable transplanted tumors were cured by a combination of sublethal CY and spleen cells from CDF mice whose autochthonous MSV-induced tumors had regressed. The results are comparable to those obtained in treating a Moloney leukemia by a similar approach (9). CY was given so as to decrease the number of tumor cells and concurrently depress the host’s response to the F1 lymphoid cells. The dose of CY employed permits long-term persistence of CDF cells in the BALB/c host (8). Finally, CY has a very short biologic half-life in the mouse and cannot injure spleen cells injected 4 hours later (11).

CY inhibited the growth of primary as well as transplanted MSV-induced tumors. However, whereas CY was beneficial to mice bearing autochthonous tumors destined to kill the host, it was ultimately deleterious to adult mice bearing autochthonous tumors destined to regress. Although CY given to adults with palpable primary tumors temporarily inhibited tumor growth, it also depressed host reactivity against the tumor and prevented tumor regression. Presumably, the latter result represents an interaction between the antitumor and the immunosuppressive effects of the drug. The results illustrate a potential danger in treating a host already responding against his tumor with a chemotherapeutic agent possessing potent immunosuppressive activity.

The deleterious effect of CY on adult mice bearing palpable primary tumors could be reversed by spleen cells or serum from donors specifically sensitized to MSV-induced tumor antigens. Seventy-six percent of BALB/c mice bearing palpable primary tumors and inoculated with CY were cured with spleen cells from adult BALB/c, CDF, or CBF mice hyperimmunized by MSV but not with cells from normal adult mice of the 3 strains. Similarly, serum from hyperimmune donors was curative, whereas normal serum or anti-BALB/c serum was not effective.

In all immunotherapy experiments, the persistence of non-syngeneic donor lymphoid cells in the host was monitored by
the persistence of donor γ-globulin in host sera. Chimerism of variable degrees was documented in young mice and in CY-treated adults. Some of the data, as well as data reported previously (9), suggest a correlation between persistence of donor lymphoid cells and their therapeutic effectiveness. The question, however, still requires further study.

Although the cures observed in the immunotherapy experiments are attributed to the lymphoid cells or serum administered, the possibility of a host contribution must also be considered. The function of the donor cells or serum may be only to retard tumor growth until the young or CY-treated adult host develops or regains immunologic competence or to decrease the total load of antigen to a level which the host can handle. The effect of specific antiserum, namely, tumor growth inhibition followed by complete regression, is consistent with this view. However, the effect of immune cells, namely, ultimate regression without rapid tumor inhibition, is more difficult to explain within this framework.

The remote possibility also exists that the therapeutic effect is totally mediated by the host, either via immunization by additional antigen present in donor cells or serum or by a transfer of immunologic information, as postulated by Alexander (1). The former mechanism is considered unlikely in the immunologically hyporesponsive host with tumors which normally grow very rapidly and kill in one week. Furthermore, treatment of young tumor-bearing mice with additional antigen, i.e., MSV, had no curative effect. The possibility of information transfer via immune cells cannot be ruled out and is being studied. Such a mechanism, however, does not satisfactorily explain the striking effect of specific antisera.

Finally, it is assumed that immunotherapy involves the killing of the primary tumor cells. However, the tumors are known to contain and release oncogenic MSV, which decreases in concentration as the tumors regress (6). The contribution of potential reinfection and reinduction of tumor at the original site to the growth of the primary tumor is not known. Therefore, the possibility that the therapeutic effect of lymphoid cells or serum is somehow mediated via an effect on reinfection or reinduction cannot be evaluated.

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