Induction and Regression of Primary Moloney Sarcoma Virus-induced Tumors in Mice

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SUMMARY

Newborn BALB/c, C57BL/6, and (BALB/c × C57BL/6) F1 (CBF) mice were equally susceptible to tumor induction by Moloney sarcoma virus (MSV). A significant incidence of spontaneous regressions of primary tumors was observed—a phenomenon unique to this tumor system. Of tumors induced in newborn BALB/c, C57BL/6, and CBF mice, 4/151, 20/43, and 12/49, respectively, regressed. Serum from mice whose primary tumors regressed neutralized the oncogenicity of MSV. The three strains of adult mice were equally susceptible to tumor induction by MSV. X-irradiation of adult mice with 350 R one day prior to MSV inoculation did not affect susceptibility to oncogenesis, but affected the fate of the tumor. Whereas all tumors induced in unirradiated adult mice completely regressed, many tumors induced in X-irradiated mice grew progressively and killed the hosts. It is therefore suggested that in this tumor system, with its remarkably short latency period to tumor formation, the immunologic competence of the host may not be a prime factor in tumor induction, but may determine whether a primary tumor, once formed, will regress or kill the host. All the adult mice were bled 9, 21, and 50 days after MSV inoculation, and their sera tested against Moloney lymphoma cells by the indirect fluorescent antibody test. No relationship between antibody production and tumor regression was evident.

INTRODUCTION

Moloney (16) recently noted the development of sarcomas in newborn mice inoculated with a potent Moloney leukemia virus preparation. Cell-free extracts of the sarcomas induced tumors at the site of inoculation in virtually all newborn mice and in weanling BALB/c mice. Pathologic studies (17) revealed the tumors to be rhabdomyosarcomas. By electron microscopy, Dalton (5) observed virus particles, morphologically indistinguishable from the murine leukemia viruses, budding from connective tissue cells.

Transplantable MSV-induced tumors were difficult to obtain because they regressed in most normal histocompatible recipients. However, tumors transplanted into preirradiated mice usually grew progressively. The tumors were shown to be antigenic in histocompatible mice. Mice pretreated with MSV or Moloney sarcoma cells were resistant to the transplantation of histocompatible Moloney sarcoma cells. Their sera neutralized the oncogenicity of MSV. Transplantation studies, MSV neutralization tests, and serologic studies suggested antigenic similarity between Moloney sarcoma cells and lymphomas induced by FMR viruses (7).

In the course of the latter studies, a significant incidence of spontaneous regressions of primary MSV-induced tumors in newborn mice was observed. To determine the role of the host's immunologic competence in this unique phenomenon, the induction and regression of primary MSV-induced tumors was investigated in three strains of newborn mice, and in normal and X-irradiated adult mice.

MATERIALS AND METHODS

Mice

Male and female BALB/c, C57BL/6, and CBF mice, 12–16 weeks old, were used. All mice were obtained from the production colonies of Texas Inbred Mice and Batelle Memorial Institute. Pregnant females were observed daily. Mice used as "newborns" were always less than 72 hours old.

Viruses

All experiments were performed with a single pool of MSV, Lot No. SV-216-RP No. 45, a one gram equivalent tumor extract from Moloney sarcomas of BALB/c origin (7).

X-irradiation of Mice

This procedure was performed as previously described (7).

The Indirect Fluorescent Antibody Test

The technic developed by Möller (14), using suspensions of viable cells, was employed; 0.05 ml of undiluted antisera was added to a pellet containing $2 \times 10^6$ trypan blue-unstained cells. The suspension was incubated at 37°C for 20 minutes and washed 4 times in Hank's Balanced Salt Solution. The resulting pellet was incubated for 20 minutes at 37°C with 0.05 ml of fluorescein-conjugated goat-antimouse globulin (Hyland) diluted 1:5. After 4 more washings, the cells were examined under the fluorescence microscope. Samples were read blind. Cells manifesting diffuse fluorescence of the type that Möller demonstrated to be indica-
tive of dead cells were omitted from consideration. This was not a significant problem as the viability of our cell preparations was usually greater than 90%. Viable cells were classified as stained or unstained. All cells exhibiting bright green granular or sectorial fluorescence or any other staining pattern other than that characteristic of dead cells were considered positive. Since only the presence or absence of specific antibody was of interest, 5 fields (approximately 300–400 cells) per sample were scanned, and the frequency of stained viable cells was estimated. Each sample was therefore graded as follows: negative, less than 20% of cells positive; <1, 20–40% of cells positive; 1+, 40–50% of cells positive; 2+, 50–70% of cells positive; 3+, 70–90% of cells positive; and 4+, 90–100% of cells positive. Specific sera staining more than 40% of cells were considered "positive" for antibody. Each test included a negative control (normal serum) and a positive control (specific antiserum with known activity). The negative control was always examined for 120–180 viable cells, and the precise frequency of staining determined. Normal serum usually stained less than 10% of the cells.

Sera

Mice were bled from the retroorbital sinus, and the individual sera obtained stored at less than —70°C. Occasionally, sera from many mice were pooled and distributed into 0.2-ml aliquots and similarly stored.

Virus Neutralization

MSV in 10⁻³ or 10⁻⁴ dilutions was mixed with an equal volume of normal C57BL/6 serum or with undiluted specific antiserum. The mixtures were thoroughly stirred, incubated at room temperature for 90 minutes, and inoculated (0.05 ml intramuscularly) into newborn BALB/c mice. The mice were examined daily for palpable tumors.

RESULTS

Induction and Regression of Primary Tumors in Newborn Mice

Three strains of newborn mice were inoculated intramuscularly with 0.05 ml of graded dilutions of MSV. All mice were palpated daily for local tumors. The results are shown in Table 1. MSV at a 10⁻⁴ dilution induced tumors in only 2 out of 20 BALB/c newborns at 80 days. Thus, the dose-response curve was sharp. The latency period, which was remarkably short, increased with decreasing doses of MSV. The 3 strains were equally susceptible to tumor induction. However, they exhibited different frequencies of spontaneous tumor regressions. Cumulative data reveal that 3%, 47%, and 24% of primary tumors regressed in BALB/c, C57BL/6, and CBF newborns, respectively.

Immunity in Mice Whose Primary Tumors Regressed

Ten C57BL/6 and 10 CBF mice whose primary, neonatally induced tumors regressed at 4 weeks were bled at eight weeks of age. Serum from each mouse reacted with 40–100% of histocompatible Moloney lymphoma cells by the fluorescence test. The sera, when pooled, stained 80% of lymphoma cells. Table 2 shows that the pooled antisera neutralized the oncogenicity of MSV. Only 1 of 12 newborn BALB/c mice inoculated with MSV and antiserum developed tumors, in contrast to 16 of 17 mice receiving MSV and normal C57BL/6 serum.

Induction of Tumors in Normal and Irradiated Adult Mice

To determine the susceptibility of adult mice to oncogenesis by MSV, 3 strains were inoculated with 0.1 ml of graded dilutions of MSV. The effect of transient immunodepression on susceptibility to oncogenesis was also tested by inoculating MSV 24 hours after sublethal X-irradiation of the host. Six out of 44, 0/43, and 0/45 BALB/c, C57BL/6, and CBF mice, respectively, died 4–5 days after X-irradiation, before any tumors were palpable. Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Induction</th>
<th>Regression</th>
<th>Induction</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>2/29 [26]</td>
<td>0/35</td>
<td>2/27 [51]</td>
<td>4/151</td>
</tr>
</tbody>
</table>

* Induction is represented as the (No. of mice developing tumors) / (No. of mice inoculated); mean latency period to tumor detection, in days, is in brackets.

+ Regression is represented as the (No. of mice whose local tumors completely regressed) / (total No. of mice in whom tumors were induced); mean latency period to disappearance of palpable local tumors, in days, is in brackets. All mice whose tumors did not regress died with progressively growing tumors.

* CBF, (BALB/c ♀ × C57BL/6 ♂)F₁ mice.
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shows that the 3 strains of unirradiated adult mice were equally susceptible to tumor induction. Preirradiation of the host exerted no detectable effect on susceptibility to oncogenesis even with the lowest dose of MSV. Cumulative data for all strains and all doses of MSV reveal that 105/133 normal mice developed tumors, compared with 102/125 X-irradiated mice.

Regression of Primary Tumors in Adult BALB/c Mice

Table 4 shows that all tumors induced in normal BALB/c mice completely regressed. However, tumors induced in irradiated mice often grew progressively. Cumulatively, all 36 tumors induced in normal mice regressed, whereas only 9/32 tumors induced in irradiated mice regressed.

Regression of Primary Tumors in Adult CBF Mice

All tumors induced in normal CBF mice regressed, whereas tumors induced in X-irradiated mice often grew progressively. Table 5 reveals that all 28 tumors induced in normal mice with the higher doses of MSV regressed, whereas only 9/18 tumors induced in irradiated mice regressed. However, tumors induced with the lowest dose of MSV regressed in both normal and X-irradiated hosts. Thus, the difference observed between the fate of normal and X-irradiated mice with induced tumors was strongly dependent upon the dose of MSV used to induce the tumors. The greater the dose of MSV inoculated, the higher was the incidence of deaths with tumor in X-irradiated BALB/c and CBF mice.

\[
\text{TABLE 2}
\]

Neutralization of Moloney Sarcoma Virus Oncogenicity by Antiserum from C57BL/6 and (BALB/c \( \delta \times C57BL/6 \) \( \delta \)) \( F_1 \) Mice Whose Primary Neonatally Induced Tumors Regressed

<table>
<thead>
<tr>
<th>Dilution of Moloney sarcoma virus</th>
<th>( 10^{-1} )</th>
<th>( 10^{-2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>8/8 [7]^a</td>
<td>8/9 [18]</td>
</tr>
<tr>
<td>Antiserum</td>
<td>1/6 [68]</td>
<td>0/6</td>
</tr>
</tbody>
</table>

\( ^a \) (No. of mice developing tumors) / (No. of mice inoculated); mean latency period to tumor detection, in days, is in brackets. Diluted Moloney sarcoma virus was incubated with an equal volume of serum; 0.05 ml of the resultant suspension was inoculated into newborn BALB/c mice.

\[
\text{TABLE 3}
\]

Induction of Primary Tumors in Normal and X-irradiated Adult Mice Inoculated with Moloney Sarcoma Virus

<table>
<thead>
<tr>
<th>Strain</th>
<th>X-ray (R)</th>
<th>Dilution of Moloney sarcoma virus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( 10^{-1} )</td>
<td>( 10^{-2} )</td>
<td></td>
</tr>
<tr>
<td>(BALB/c ( \delta \times C57BL/6 ) ( \delta )) ( F_1 )</td>
<td>15/15 [7]</td>
<td>13/16 [11]</td>
<td>7/15 [14]</td>
</tr>
</tbody>
</table>

\( ^a \) (No. of mice developing tumors) / (No. of mice inoculated); mean latency period to tumor detection, in days, is in brackets.

One-tenth ml of diluted Moloney sarcoma virus was inoculated i.m. into normal mice or into mice preirradiated 24 hours earlier.
TABLE 5
Growth and Regression of Primary Moloney Sarcoma Virus-induced Tumors in Normal and X-irradiated Adult (BALB/c × C57BL/6 ♀) F1 Mice

<table>
<thead>
<tr>
<th>Moloney sarcoma virus dilution</th>
<th>X-ray (R)</th>
<th>No. tumors regressed/No. induced</th>
<th>Median day of regression, after Moloney sarcoma virus inoculation</th>
<th>Deaths with tumor/No. tumors induced</th>
<th>Median day of death</th>
<th>mmts*</th>
</tr>
</thead>
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<tr>
<td>10^{-1}</td>
<td>350</td>
<td>15/15</td>
<td>19</td>
<td>0/15</td>
<td>14</td>
<td>56</td>
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<td></td>
<td></td>
<td>1/15</td>
<td>23</td>
<td>13/15</td>
<td>56</td>
<td>21</td>
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<tr>
<td>10^{-2}</td>
<td>350</td>
<td>13/13</td>
<td>18</td>
<td>0/13</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8/13</td>
<td>34</td>
<td>4/13</td>
<td>63</td>
<td>15</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>350</td>
<td>7/7</td>
<td>17</td>
<td>0/7</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8/8</td>
<td>21</td>
<td>0/8</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

* mmts, mean maximal tumor size, in mm.

One additional mouse is alive with a palpable tumor on Day 100.

3–6, were bled 9, 21, and 50 days after MSV inoculation. Serum from each mouse was tested for antibody by the indirect fluorescent antibody test. Attempts to prepare a suspension of viable sarcoma cells adequate for use as target cells were unsuccessful. However, transplantation studies and serologic tests have shown that Moloney sarcoma cells and Moloney lymphoma cells (or their causative viruses) are antigenically similar (7). The antigenic similarity was supported by the following serologic observations: (a) sera from mice immunized with Moloney lymphoma cells effectively neutralized the oncogenicity of MSV; (b) sera

TABLE 6
Growth and Regression of Primary Moloney Sarcoma Virus-induced Tumors in Normal and X-irradiated Adult C57BL/6 Mice

<table>
<thead>
<tr>
<th>Moloney sarcoma virus dilution</th>
<th>X-ray (R)</th>
<th>No. tumors regressed/No. induced</th>
<th>Median day of regression, after Moloney sarcoma virus inoculation</th>
<th>Deaths with tumor/No. tumors induced</th>
<th>Median day of death</th>
<th>mmts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-1}</td>
<td>350</td>
<td>14/14</td>
<td>17</td>
<td>0/14</td>
<td>13</td>
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<td></td>
<td></td>
<td>11/11</td>
<td>32</td>
<td>0/11</td>
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<td>10^{-3}</td>
<td>350</td>
<td>8/8</td>
<td>21</td>
<td>0/8</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/6</td>
<td>22</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* mmts, mean maximal tumor size, in mm.

Two additional mice are alive with palpable tumors on Day 100.

TABLE 7
Specific Antibody Production by Normal and X-irradiated Adult Mice Inoculated with Graded Doses of Moloney Sarcoma Virus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Moloney sarcoma virus dilution</th>
<th>X-ray (R)</th>
<th>Group 1a</th>
<th>No. of mice producing antibody</th>
<th>Day 21 1 2 3</th>
<th>Day 50 1 2 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-1}</td>
<td>350</td>
<td>0/7</td>
<td>1/15</td>
<td>8/15</td>
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<tr>
<td></td>
<td>10^{-2}</td>
<td>350</td>
<td>0/12</td>
<td>0/14</td>
<td>4/14</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>350</td>
<td>0/3</td>
<td>0/7</td>
<td>0/5</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>10^{-1}</td>
<td>350</td>
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<td>9/14</td>
<td>12/14</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>350</td>
<td></td>
<td>0/13</td>
<td>12/13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>350</td>
<td></td>
<td>6/12</td>
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<td>2/3</td>
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<td>1/13</td>
<td>10/15</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>350</td>
<td>0/4</td>
<td>0/1</td>
<td>0/13</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>350</td>
<td></td>
<td>9/13</td>
<td>13/13</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* Groups: No. 1, mice dying with progressive tumor growth; No. 2, mice with tumors which ultimately regressed; No. 3, mice who were always free of tumor.

1 No. of mice producing antibody

X-ray was considered positive if it reacted with 40% or more Moloney lymphoma cells by the fluorescent test.

a CBF, (BALB/c ♀ × C57BL/6 ♂) F1 mice.

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from mice immunized with Moloney sarcoma cells reacted against histocompatible Moloney lymphoma cells by the indirect fluorescent antibody test; (c) Moloney sarcoma cells absorbed appreciable amounts of anti-Moloney lymphoma activity, as measured by the fluorescence test.

In the absence of any assay for the detection of antibody directed specifically against Moloney sarcoma, sera from mice inoculated with MSV were tested for antibody against Moloney lymphoma cells. The target cell employed was LSTRA—a Moloney virus-induced ascitic lymphoma of BALB/c origin, in its 298th transplant generation. Sera that stained 40% or more of the lymphoma cells were considered positive.

No mice of any strain produced detectable antibody 9 days after MSV inoculation. Table 7 presents the antibody production by the 3 strains of normal and X-irradiated mice 21 and 50 days after receiving graded doses of MSV. For the purpose of discussion, mice treated in any given way were subdivided into those dying with tumors (Group 1), those whose tumors regressed (Group 2), and those in whom tumors never arose (Group 3). Although tumor incidence and regression was comparable among the 3 strains of normal mice, the antibody response was different. Table 7 shows that only 1/43 normal BALB/c mice produced detectable antibody at 21 days, in contrast to 23/45 C57BL/6 and 23/46 CBF mice. Similarly, at 50 days, only 14/43 BALB/c mice produced fluorescent antibody, compared with 30/45 C57BL/6 and 41/45 CBF mice. On an individual mouse basis, there was no relationship between antibody production, tumor induction, presence or absence of tumor, size of tumor, or time to tumor regression.

X-irradiated mice of the 3 strains inoculated with MSV differed in their antibody response. At 21 days after MSV inoculation (22 days after X-irradiation), almost no mice of any strain produced detectable antibody. At 50 days, however, 29/38 C57BL/6 mice formed antibody, as contrasted with 8/43 CBF and 0/22 BALB/c mice.

**DISCUSSION**

C57BL/6 newborn mice are relatively resistant to oncogenesis by FMR, Gross, and polyoma viruses (9, 11, 13, 15, 18). However, Perk and Moloney (17) readily induced tumors by inoculating C57BL/6 neonates with MSV. The data presented confirm the equal susceptibility of BALB/c and C57BL/6 newborn mice, as well as CBF mice, to oncogenesis by graded doses of MSV.

However, the 3 strains of newborn mice exhibit marked differences in their response to the induced tumors. Whereas 3% of primary tumors induced in BALB/c newborns spontaneously regressed, 47% of tumors induced in newborn C57BL/6 mice and 24% of tumors in CBF newborns regressed. Regression of primary, neonatally induced tumors is unique to this tumor system. All C57BL/6 and CBF mice whose tumors regressed produced circulating antibody in adulthood, as demonstrated by the indirect fluorescent antibody test and MSV neutralization tests.

The relative resistance of C57BL/6 newborns to viral oncogenesis is postulated to be a function of the host response, rather than of target organ sensitivity (10). This has been well substantiated by Law (12), who demonstrated that C57BL/6 newborns, who are normally resistant to polyoma virus, could be rendered susceptible by thymectomy. C57BL/6 mice apparently develop immunologic competence early in life. This is suggested by: (a) their relative resistance to the neonatal induction of homograft tolerance (2, 19); (b) their ability to reject skin homografts within the first few days of life (3); and (c) the ability of lymphoid cells from 5-day-old C57BL/6 mice to induce a graft-versus-host reaction (1). This rapid development of immunologic competence in newborn C57BL/6 mice may indeed explain their resistance to oncogenesis by most viruses characterized by long latency periods to tumor formation. However, oncogenesis by MSV is remarkably rapid, with most tumors becoming palpable in 6-10 days. This may provide too little time for either the development of general immunologic competence, or for the elicitation of an adequate specific immune response. With time, both are attained and regression of the tumor occurs. Immunologic competence may not, therefore, be the critical factor in tumor induction in this system, but may determine whether a primary tumor will grow or regress.

The data on induction and regression of primary tumors in adult mice are consistent with the above formulation. Normal adult BALB/c, C57BL/6, and CBF mice were equally susceptible to tumor induction, in contrast to the well-known resistance of adult C57BL/6 mice to oncogenesis by other murine tumor viruses. In contrast to the results reported in the polyoma tumor system (11), depression of immunologic competence by X-irradiation did not affect host susceptibility to oncogenesis. However, whereas all tumors induced in normal mice regressed, a significant number of tumors induced in preirradiated mice—especially BALB/c and CBF mice—grew progressively. Similar results were obtained in cortisone-treated BALB/c weanling mice inoculated with MSV (D. Shachat, unpublished data). Analogous observations have been reported in the Shope papilloma system. This virus normally induces skin fibromas in normal adult rabbits. The fibromas regress in a few weeks, and the host is thereafter immune to the virus. However, inoculation of the virus into X-irradiated (4) or cortisone-treated rabbits (8) induces generalized, invasive, progressively growing tumors. Finally, primary Rous sarcomas induced in adult chickens often regress (6). However, the effect of X-irradiation on regression in that system has not been studied.

Tumor deaths in X-irradiated adult mice inoculated with MSV occurred, in order of decreasing frequency, in BALB/c, CBF, and C57BL/6 mice. X-irradiated mice of the 3 strains also differed in their antibody production. The pattern of tumor deaths and antibody production may reflect a difference in the radiosensitivity of the immune response between the 3 strains. At 50 days after MSV inoculation, most C57BL/6, a few CBF, but none of the BALB/c mice, produced detectable antibody.

However, antibody—of the type tested—does not appear to be related causally to tumor regression. This view is based largely on the following findings:

1. For any given mouse, no relationship was observed between antibody production, presence or absence of local tumor at time of bleeding, tumor size, tumor regression, or time to tumor regression.

2. Although tumors induced in normal mice of all three strains regressed at comparable times, C57BL/6 and CBF mice formed specific antibody at 21 days after MSV inoculation, whereas BALB/c mice did not.

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