Immunological and Pathological Manifestations of Murine Sarcoma Virus (Moloney) Infections

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SUMMARY

Mice were immunized against Moloney sarcoma virus (MSV) challenge by the serial inoculation of increasing doses of live MSV preparations. Only 15% of vaccinated mice developed tumors after challenge with a dose of MSV sufficient to cause tumors in 100% of control mice. This resistance was not accompanied by detectable levels of MSV-neutralizing antibody. Adult mice susceptible to MSV tumor induction displayed antibody levels following tumor regression that were sufficient to neutralize greater than $10^{2}$ median effective doses of MSV. Antibody titers were independent of initial MSV dose but were directly related to tumor duration.

The pathogenesis of MSV-induced tumors in weanling and adult mice was characterized by spontaneous regression of tumors (rhabdomyosarcomas) followed by a relapse phase. Onset of relapse was correlated with architectural changes in the mesenchymal region surrounding splenic follicles. This was followed by the appearance of metatstatic lesions in various organs and subsequent death.

INTRODUCTION

The rapidly growing tumors induced in mice by the MSV consist predominantly of fibrosarcomatous and larger, elongated myoblast cells. The shape of these cells, presence of cross-striations, and staining properties identify the tumor as a rhabdomyosarcoma (10).

A direct relationship has been established between initiating dose of MSV and tumor incidence, while an inverse relationship exists between virus dose and time to palpable tumor. Newborn mice inoculated with MSV die with rapidly progressing tumors, whereas weanling or adult mice display a significant incidence of tumor regression. Concomitants of this phenomenon are demonstrable levels of MSV-neutralizing antibody, the development of resistance to transplantation of syngeneic Moloney sarcoma cells, and infiltration of the tumor region by both leukocytes and fibrocytes (4—6, 13).

The studies reported here were undertaken to determine the relationship of MSV dose to the tumor regression and relapse phenomena as well as to the histopathological manifestations of this syndrome. Factors influencing the production of MSV-neutralizing antibody were also investigated.

MATERIALS AND METHODS

Virus. MSV lots SVRP-106 and SVRP-106S, prepared in BALB/c and NIH Swiss mice, respectively, were used. The cell-free MSV extracts were prepared to a final concentration of 1 ml of extract, equivalent to 1 g of tumor tissue, by the method of Moloney (8).

Mice. The inbred strain of BALB/c and the random-bred strain of NIH Swiss (General Purpose, Texas colony) were obtained from the Animal Production Section of the National Cancer Institute. Mice referred to as “adults” were 7 to 10 weeks old. All mice of a given experiment were of the same sex, although no difference in host response due to sex was noted.

Virus Titrations. Mice were inoculated with 0.1 ml of the appropriate MSV dilution in the left inguinal region via the s.c.-i.m. route (10). Tumor size was arbitrarily scored as 1+ to 4+ “tumor grade” according to the following system: 1+, tumor that is palpable and less than 5 mm in diameter; 2+, tumor that is clearly visible and approximately 5 to 6 mm in diameter; 3+, tumor that is approximately 7 to 10 mm in diameter; 4+, tumor that involves the entire inguinal region and is greater than 10 mm in diameter.

Virus titers were expressed as 50% end points for palpable tumor (ED₅₀) by the method of Reed and Muench (12) or in terms of in vitro FFU (7, 9).

Pathogenesis of MSV. Groups of 40 male weanling BALB/c mice/dilution (10⁶ through 10⁻³) were inoculated s.c.-i.m. in the inguinal region with 0.1 ml of MSV stock SVRP-106. All mice were observed daily and palpated for tumors through Day 126. Four mice/dilution group were sacrificed on Days 1, 4, 11, 21, and 126 after inoculation. Body weight, spleen weight to the nearest mg, and hematocrit value were determined for each mouse. All organs were examined for gross pathology, and the following organs were removed for histopathological examination: spleen, thymus, liver, lung, heart,
kidney, and normal muscle or tumor at the site of inoculation. Other tissues that displayed evidence of gross pathology were also removed for histological examination. The excised tissues were fixed in Zenker-formal solution and stained with hematoxylin and eosin. Hematocrits were determined by the microhematocrit technique, with centrifugation of heparinized blood samples at 12,000 X g for 5 min.

**Inoculation Schedule for Vaccination of Mice.** MSV lot SVRP-106, of known infectivity, was appropriately diluted so that initial inoculation of mice would begin with a dose that was 1000 times less than the dose causing tumors in mice and 100 times less than the dose causing focus production in vitro. Groups of 21 adult Swiss mice were inoculated s.c.-i.m. in the inguinal region with 0.1 ml of MSV dilutions at 2-week intervals according to the following scheme.

<table>
<thead>
<tr>
<th>Group</th>
<th>Log MSV dilutions (in order of inoculation/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-6, -5, -4</td>
</tr>
<tr>
<td>B</td>
<td>-6, -5, -4, -3</td>
</tr>
<tr>
<td>C</td>
<td>-6, -5, -4, -3, -3</td>
</tr>
</tbody>
</table>

Control mice were serially inoculated at 2-week intervals with 10^-5, 10^-4, 10^-3, and 10^-1, dilutions of normal muscle extract, prepared by the same methods used to obtain MSV from muscle tumor. Mice that received only 1 inoculation of MSV (10^-9, 10^-7, 10^-5, 10^-4, 10^-3, or 10^-2) and noninoculated mice served as additional controls. All mice were examined daily for palpable tumors. Four weeks after the final injection, tumor-bearing mice within each inoculation group were separated from those mice with no detectable tumors. All mice were bled via the axillary vessels, and sera of mice from each subgroup (positive or negative for tumors) were pooled.

**Assay for Anti-MSV-neutralizing Antibody.** All sera were diluted 4-fold in Hanks' balanced salt solution and inactivated at 56° for 30 min before testing. Equal volumes of sera and MSV, undiluted and diluted 100-fold, were incubated at 25° for 60 min. Control mixtures of diluent and MSV (10^-9, 10^-1, 10^-2, 10^-3, and 10^-4) were mixed and incubated in the same manner. Groups of 15 newborn BALB/c mice were inoculated s.c.-i.m. with 0.05 ml of each mixture. Mice were palpated for tumor for 40 days, and ED50 values were calculated for both test and control groups. The anti-MSV activity of a given serum pool was quantitatively determined by subtracting the log ED50 of the test group from the log ED50 of the control group. The value obtained was expressed as the log "neutralization index" of the serum tested.

**RESULTS**

**Quantitative Dose-Response Studies.** Ten weanling BALB/c mice/dilution group were inoculated s.c.-i.m. in the inguinal region with 0.1 ml of MSV lot SVRP-106. Rankit analysis (2) of latent period (time in days) to palpable tumor revealed an inverse relationship between MSV input dose and the Y50 value for palpable tumors. Spontaneous tumor regression was observed in approximately 90% of mice with tumors (Table 1). The dynamics of tumor growth and regression in response to 4 MSV doses are presented graphically in Chart 1, where it is shown that the rates of tumor induction and regression are essentially the same. Tumors remained at maximum size for only 2 to 4 days, regardless of the initial MSV dose. The relationship of MSV dose to the regression of tumors and subsequent relapse (to be described) is presented in Table 1. Onset of relapse was observed 2 to 3 months after complete tumor regression. In the present studies, the percentage of animals in relapse, which usually resulted in death, was independent of the dose of MSV inoculum.

**Pathogenesis of MSV Tumor Induction, Regression, and Relapse in Weanling Mice.** Gross examination of weanling BALB/c mice inoculated s.c.-i.m. in the left inguinal region with 0.1 ml of MSV (10^-9 through 10^-3 dilutions) revealed that tumors grew rapidly at the site of injection, eventually involving the entire left inguinal area and reaching a maximum size of 10 to 15 mm in diameter. Histologically, the induced neoplasm could be characterized as a rhabdomyosarcoma. Several peri cardial tumors with areas of white cell infiltration were observed in mice sacrificed 7, 11, and 21 days after MSV inoculation. Hepatic tumors were also observed during this period as round, white nodules containing undifferentiated tumor cells that were predominantly fusiform. The histology

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**Table 1. Relationship of MSV dose to response in weanling mice: tumor induction, regression, and relapse**

<table>
<thead>
<tr>
<th>MSV dilution</th>
<th>% mice with tumors</th>
<th>Time to 50% tumors: Y50 (days)</th>
<th>% regression (of mice with tumors)</th>
<th>Tumor duration (days)</th>
<th>% relapse (of mice with regressed tumors)</th>
<th>Time to onset of relapse from complete tumor regression (days)</th>
<th>% mortality (of mice inoculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-9</td>
<td>100</td>
<td>3.5</td>
<td>100</td>
<td>15.0</td>
<td>40</td>
<td>85</td>
<td>40</td>
</tr>
<tr>
<td>10^-1</td>
<td>100</td>
<td>4.6</td>
<td>90</td>
<td>21.4</td>
<td>56</td>
<td>98</td>
<td>50</td>
</tr>
<tr>
<td>10^-2</td>
<td>100</td>
<td>7.3</td>
<td>90</td>
<td>14.3</td>
<td>45</td>
<td>101</td>
<td>40</td>
</tr>
<tr>
<td>10^-3</td>
<td>90</td>
<td>12.2</td>
<td>78</td>
<td>15.2</td>
<td>43</td>
<td>62</td>
<td>30</td>
</tr>
</tbody>
</table>

Groups of 10 BALB/c mice were inoculated s.c.-i.m. in the inguinal region with 0.1 ml of the appropriate MSV dilution. The ED50 of the MSV stock used (SVRP-106) was 2.8 X 10^7/ml. The experiment was terminated at Day 159 after inoculation.
of these pericardial and hepatic tumors resembled that of the rhabdomyosarcomas described above. One hemorrhagic tumor was observed at the site of inoculation 21 days postinfection and was composed of undifferentiated cells with numerous mitotic figures.

Histological examination of spleens during the tumor induction and regression phases revealed a slight loss of splenic architecture with "tumor-like" cells present in some cases. Slight increases in spleen weight paralleled tumor development and regression, whereas body weights and hematocrit values remained normal. Regression was accompanied by a localized increase in the number of neutrophils, monocytes, and lymphocytes in the tumors, followed by heavy infiltration of fibroblasts and fibrocytes and the occurrence of a generalized fibrosis reaction.

The onset of the relapse phase, which ultimately led to death, was characterized by a pronounced growth of a new cell type in the mesenchymal region surrounding splenic follicles. These cells were refractory to staining and contained many mitotic figures, but they did not resemble the spindle-shaped tumor cells seen in other organs. Multinucleated giant cells and spindle-shaped sarcoma cells were observed in the spleen only during late relapse phase. A direct relationship between MSV dose and spleen weight was noted in mice sacrificed 18 weeks after inoculation; that is, groups of mice inoculated with MSV diluted $10^0$, $10^{-1}$, and $10^{-3}$, had average spleen weights of 179, 146, 138, and 109 mg, respectively. The average spleen weight of control mice was 87 mg.

Many livers examined during relapse phase displayed a typical leukemoid reaction with areas of vacuolated cells and necrosis. Liver tumors (Fig. 1) characterized as myosarcomas, as well as neoplasms of the pancreas (Fig. 2), myocardium (Fig. 3), lung, muscularis of the stomach, and tumors at the site of inoculation, were observed during this phase of the syndrome. Throughout tumor regression and relapse, however, no relationship was evident between initiating virus dose and any particular disease manifestation. No leukemic or lymphomatous tissues were observed throughout the investigation.

Relationship of MSV Infecting Dose to Antibody Production. These experiments were designed to study the effect of virus dose of subsequent antibody production in mice. Groups of 14 adult NIH Swiss mice/virus dilution were inoculated s.c.-i.m. in the inguinal region with 0.1 ml of MSV lot SVRP-1065. Mice were bled 45 days after inoculation, which corresponds to approximately 3 weeks after tumor regression in susceptible mice. Table 2 shows that sera from all groups of mice that did not develop tumors contained no detectable MSV-neutralizing antibody (Table 2). Latent period to tumor, as well as the time to complete tumor regression, were inverse functions of the initiating virus dose.

Chart 2 summarizes the results of an experiment in which a...
Table 3

Response of adult Swiss mice to serial inoculations of MSV

Mice were serially inoculated s.c.-i.m. with 0.1 ml of MSV dilutions in the order indicated. Mice were held 4 weeks after their last inoculation, and sera were assayed for anti-MSV activity. Control mice received a single inoculation of the MSV dilution indicated at the beginning of the experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Log MSV dose</th>
<th>Tu/Ta % tumors</th>
<th>Anti-MSV log neutralization indexb</th>
<th>Latent period to tumorc (days)</th>
<th>Tumor duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>-6</td>
<td>0/14 0</td>
<td>&gt;60.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-5</td>
<td>0/14 0</td>
<td>&gt;60.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-4</td>
<td>2/14 14</td>
<td>14.8</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-3</td>
<td>7/14 50</td>
<td>10.4</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-2</td>
<td>9/14 64</td>
<td>10.0</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1</td>
<td>14/14 100</td>
<td>6.7</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>14/14 100</td>
<td>4.8</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>A</td>
<td>-6, -5, -4</td>
<td>0/61 0</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-6, -5, -4, -3</td>
<td>8/41 20</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-6, -5, -4, -3, -1</td>
<td>3/20 15</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>B</td>
<td>-6, -5, -4, -3</td>
<td>8/8 100</td>
<td>1.5</td>
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<tr>
<td></td>
<td>C</td>
<td>-6, -5, -4, -3, -1</td>
<td>3/3 100</td>
<td>2.1</td>
<td>4.5</td>
</tr>
</tbody>
</table>

a No. of mice with tumors/no. of mice inoculated.
b See “Materials and Methods.”

c Time in days to palpable tumor.

Direct relationship was shown to exist between tumor duration (days) and log10 titer of MSV-neutralizing antibody. One hundred eighty-eight adult NIH Swiss mice were inoculated with 0.1 ml of MSV at doses 10^6 to 10^-5 in 10-fold increments s.c.-i.m. in the inguinal region. Mice were palpated for tumors daily. Three weeks after complete tumor regression, mice were sacrificed, and their sera were assayed for MSV-neutralizing antibody as described above. Each point in Chart 2 represents titers of pooled sera of 7 to 14 mice. Tumor duration and the consequent antibody titer were both independent of the dose of initial MSV inoculum.

Immunization of Mice against MSV. Mice were immunized against MSV challenge by the serial inoculation of live MSV preparations in increasing doses as described in “Materials and Methods.” The results of this experiment are summarized in Table 3. It is apparent that all 61 mice of Group A were completely protected after receiving 2 inoculations of MSV in doses beyond that which would cause tumors; the challenge dose of MSV that was used (10^-8) caused tumors in 14% of control mice. The 1st inoculum of Group A consisted of a dose of MSV equivalent to 0.01 in vitro FFU and 0.001 tumor ED50 unit for adult mice. The 2nd, or “booster,” inoculum consisted of 0.1 FU or 0.01 tumor ED50 unit of MSV.

Only 20% of the mice in Group B, which previously received inoculations of MSV diluted 10^6 to 10^-4 at 2-week intervals, developed tumors after challenge with MSV diluted 10^-3. By contrast, 50% of control mice that received a single inoculation of virus diluted 10^-3 developed tumors. The most striking prophylactic effect obtained with staggered MSV inoculations was seen in Group C, where only 15% of the immunized mice developed tumors after challenge with a dose of MSV (10^-1) sufficient to cause tumors in 100% of the control mice.

All sera from mice that were resistant to MSV challenge (Groups A, B, and C) were negative when tested for MSV-neutralizing antibody. Only the sera of mice that were susceptible to MSV tumor induction and had tumors which subsequently regressed contained antibodies that would neutralize MSV (Tables 2 and 3).

DISCUSSION

The studies reported here indicate that adult mice can be successfully protected against MSV challenge by the serial inoculation of live MSV preparations in doses less than those that would cause tumors. The critical factor in these experiments appeared to be the accurate titrating of the MSV "vac-
crease in the immune response of the host to the initial tumor and its subsequent regression could conceivably permit this splenic mesenchymal region. An insidious and progressive death of an antigenic variant of MSV previously harbored in the body in resistant mice in these studies (Tables 2 and 3) exhibited a similar latent period to tumor formation and tumor duration as did control mice receiving only 1 inoculation of MSV.

These studies also indicate that, although time to tumor formation is inversely related to virus dosage, other parameters of MSV infections (such as tumor duration, antibody response, and time to the onset of the relapse phase) are independent of the initial dose of virus that was used. The histopathological manifestations through all stages of the MSV-induced syndrome were similarly independent of the infecting dose that was used.

The pathogenesis of MSV infections of weanling mice differed in several aspects from that previously reported for newborns (5, 10, 11). Weanling mice exhibited only slight spleen weight increases and no changes in hematocrit values, whereas striking changes in these parameters were evident in mice infected with MSV shortly after birth (10). The relapse phase observed here was of particular interest, in that it was initiated by architectural changes in the mesenchymal region surrounding splenic follicles. Only subsequently did typical sarcomatous metastatic lesions appear. These observations suggest that these metastatic lesions may have been induced by MSV or an antigenic variant of MSV previously harbored in the splenic mesenchymal region. An insidious and progressive decrease in the immune response of the host to the initial tumor and its subsequent regression could conceivably permit this latent agent to express its virulence. Further studies involving extraction of virus from metastatic areas and characterization of its antigenicity are therefore suggested.

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wick, N. J.) and Dr. Kalman Perk (Hebrew University, Rehovoth, Israel) for their advice and assistance in the preparation of this manuscript and Dr. Gerald Spahn (Microbiological Associates, Walkersville, Md.) for the assay of serum samples for MSV-neutralizing activity.

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