**Effect of Immune Status on the Development of Artificially Induced Metastases in Different Anatomical Locations**

Jan Vaage, Kuang Chen, and Suzanne Merrick

*Section of Experimental Radiotherapy, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025*

**SUMMARY**

Using a syngeneic methylcholanthrene-induced mouse sarcoma of recent origin and with demonstrated immunogenicity, we studied the effect of the immune status of the host on tumor growth in four different anatomical locations. For simulation of metastatic spread, tumor cells were selectively “seeded” in the lungs via the tail vein and in the liver via hepatic portal vein injection. Tumor growth was also studied in s.c. and i.p. injection sites.

Host resistance following sensitization was expressed most strongly in the liver and lungs and less strongly in the s.c. and i.p. locations. Sensitization by living tumor implants surgically removed after a period of growth left the hosts more resistant than the mice with sensitizing implants that had been killed by local irradiation. Immunotherapy consisting of s.c. injection of killed tumor tissue after challenge in addition to presensitization resulted in stronger resistance than presensitization alone.

The incidence of tumor growth was higher in sublethally irradiated mice than in untreated, normal control mice. This indicates that even the presumably weak first phase of the developing immune response initiated in control mice by the challenge implant was important in controlling the development of tumor growth.

**INTRODUCTION**

The malignancy of solid tumors in clinical cancer is determined by the tendency of the tumor to invade and metastasize. In laboratory rodents, induced and spontaneous carcinomas are frequently found to metastasize, while sarcomas induced experimentally with polycyclic hydrocarbons or plastic disc implants grow progressively at the site of induction or implantation and rarely develop metastases. Therefore, attempts to study host factors involved in the control of metastatic growth of sarcomas in mice must involve injection of tumor cells to simulate the natural spread from a primary tumor to distant sites.

The role of tumor antigenicity and immune resistance among the factors that influence the ability of disseminated tumor cells to establish metastatic tumor growth has not been investigated as extensively as has the immunology of tumors growing in the local implantation site. Kim (8) found that, among several methylcholanthrene-induced mammary carcinomas in rats, the ability to metastasize was seen among weakly antigenic and nonantigenic tumors, and highly antigenic tumors did not metastasize. Deodhar and Crile (3) found that primary allotransplants of Sarcoma 180, which very rarely gave rise to metastases in intact hosts, metastasized in nearly all recipients that had been immunologically suppressed with antilymphocyte serum. Vaage and Weiss (14) found that mice responded to tumor-specific immunotherapy after cell suspensions of autochthonous mammary carcinomas had been injected i.v. or i.p. to simulate metastatic extension of tumor growth.

The present investigation has attempted to determine whether antitumor immunity is equally effective in any anatomical site or is expressed more strongly in certain organs or tissues. If this is the case, as the present data indicate, then local or regional immunological differences, as well as local or regional anatomical and physiological differences, are factors that determine where metastatic tumors may develop.

**MATERIALS AND METHODS**

**Mice.** All the animals used in these experiments were 12-week-old male mice of inbred strain C3Hf/Bu from the specific-pathogen-free breeding colony maintained by the Section of Experimental Radiotherapy of The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston. The animals were kept in rooms maintained at 72°F and 50% humidity, 5 mice/cage. The cages, which contained feed and bedding, were sterilized for 4 min at 276°F in a high-vacuum autoclave. The drinking water was also sterilized and adjusted to pH 2.5 with HCl. The cages were covered with Isocaps sterilized with ethylene oxide.

**Tumor.** A fibrosarcoma induced in a female C3H mouse by methylcholanthrene (12) was kept in liquid nitrogen and reintroduced into syngeneic mice. It was used in these experiments in the 3rd and 4th transplant generations for sensitizing implantation and for preparation of tumor cell suspensions.

**Surgical Procedures.** Complete removal of tumors implanted s.c. was performed under Nembutal anesthesia. A circular incision in the skin was made around the edge of the tumor, and the tumor was removed by blunt dissection. The incision was closed with wound clips.

**Portal vein injections were made through a ventral midline**
incision with the use of a glass needle drawn from 750-μ glass tubing attached to a 1-ml syringe by a length of fine polyethylene tubing.

Irradiation Procedures. Animals received whole-body irradiation on a 250-kVP, 30-μA Maxtron X-ray machine with a 0.5-mm Cu and 1-mm Al filter. The dose rate was 90 rads/min at 50 cm from the source, calibrated with a Victoreen chamber. Five mice were irradiated at 1 time in a rotating, compartmentalized box. The total dose to the midplane of the mice was 400 rads.

The mice with sensitizing tumor implants killed by irradiation were given Nembutal anesthesia and treated under hypoxic conditions on a parallel opposing dual-source 137Cs machine with a field size of 3 cm in diameter. The tumor and the skin were clamped for 15 min to cut off the blood supply before irradiation. The dose rate was 1052 rads/min and the total dose to the tumor was 8000 rads in a single treatment. A circular clamp 3 cm in diameter excluded the body of the tumor host from the field of exposure during irradiation. Scatter irradiation was 3% 0.5 cm from the edge of the field and 0.3% at 1 cm. The scatter irradiation had been shown in previous tests to have no effect on the immune status of the mice (J. Vaage, unpublished data).

Tumor cell suspensions used for booster vaccines were killed by exposure to 10,000 rads on the 137Cs machine.

Tumor Implantation. Implantation s.c. of 1- x 1-mm pieces of living tumor tissue was used to initiate tumor growth for immunization. An incision was made in the skin of the flank, and a tumor piece was placed under the skin by means of a trocar. The incision was closed with a wound clip.

Challenge implantation of presensitized and control mice was by injection of viable (trypan-blue-negative) tumor cells suspended in TC Medium 199 (Difco, Detroit, Mich.) and consisting predominantly of single cells and a few clumps of up to 10 cells each. The mechanical preparation of single-cell suspensions from tumor tissue has been described elsewhere (13).

Statistical Analysis. The effect of treatment is described in terms of differences in tumor incidence following challenge or in terms of differences in amount of tumor mass following challenge. For comparison of tumor incidence, the χ² test was used when the number of mice in a single test group was 20 or more; the Fisher test (11) was used when the number was less than 20.

Differences between groups were considered significant only when the p value of comparison was 0.05 or less.

RESULTS

Table 1 represents data from 2 similar experiments, which followed the same procedure to test the reproducibility of the results. Since the results of the 2 tests were nearly identical, the data have been combined. In each test, 12-week-old male C3H mice were distributed randomly to 4 groups, which received different immunological treatment: (a) presensitization with booster injections of killed tumor cells; (b) presensitization only; (c) no treatment; and (d) depression by sublethal whole-body irradiation. Each group was further divided into 4 subgroups, which were challenged via different routes of injection: (a) hepatic portal vein; (b) tail vein; (c) peritoneal cavity; and (d) s.c. tissue of the flank. The i.v. and i.p. injections contained 5 × 10⁶ viable tumor cells suspended in TC medium 199 (Difco); the s.c. injections contained 2 × 10⁵ cells. These doses had been shown in previous tests to be suitable for the demonstration of specific host resistance (12).

Following injection of tumor cells via the portal vein, tumor growth was found only in the liver, with the exception that, in 12 of 71 mice given injections by this route, tumor growth was also found in the peritoneal cavity. This probably occurred because some of the cell suspension was spilled during the process of injection. Following injection of tumor cells via the tail vein, tumor growth was found mainly in the lungs. In the 32 mice of 75 given injections via the tail vein that developed tumors, tumor growth was found only in the lungs in 19 mice; in the lungs and kidneys in 10 mice; in the lungs, kidneys, and heart in 1 mouse; and in extrapulmonary areas (the liver and adrenal gland) only in 2 mice. When injected i.p., the tumor cells grew attached to the peritoneum, and in only 1 of 73 animals given injections via this route was tumor growth found outside the peritoneal cavity (in the lungs). When injected s.c. tumor cells grew only at the injection site.

In each of the 2 experiments, the presensitized mice had been given implants s.c. of 1- x 1-mm pieces of living tumor tissue to initiate tumor growth for immunization. After a period of growth, 19 days in the 1st experiment and 21 days in the 2nd, when the sensitizing implants measured about 10 x 10 mm in diameter, the implants were either removed by resection or killed by high-dose local irradiation to compare the effects on host resistance of cure by surgical or irradiation therapy. The sensitizing implants were resected or irradiated about 24 hr before the challenge injections. The immunologically depressed group had no prior exposure to the tumor and received whole-body irradiation about 24 hr before challenge.

The booster injections consisted of 0.05 ml of irradiation-killed tumor cell suspension in 0.9% NaCl solution equivalent to 0.01 g of tumor, wet weight, and were given s.c. 7 and 14 days after challenge. The 2 experiments were terminated 14 and 19 days after challenge, respectively, for the animals challenged i.v. and i.p. and after 20 and 28 days, respectively, for the animals challenged s.c. Frequent palpations to detect the development of i.p. growth and measurements of s.c. growth decided the termination times in each experiment. Each animal was killed by cervical dislocation. The s.c. implants were measured with calipers, and a careful search was made for the extent and location of “metastatic” tumor growth.

The sarcomatous nature of metastatic visceral tumor growth was determined by histological examination of randomly chosen samples.

To arrive at a common denominator describing the amount of tumor tissue growing s.c. where measurements were easily made and in the viscera where accurate measurements were not practical, we gave values from 0 to 3 according to the number and size of growth found at autopsy. The values were determined at blind readings and were derived according to the following grading system. 0, no tumor growth found by gross
Table 1

Influence of immune status on the number and size of developing tumor implants

Sensitizing implants were irradiated or removed surgically on Day 18, and the mice were challenged on Day 19. Mice received whole-body irradiation 1 day before challenge.

<table>
<thead>
<tr>
<th>Immune status</th>
<th>Route of challenge</th>
<th>Radiotherapy</th>
<th>Surgery</th>
<th>Combined</th>
<th>Radiotherapy</th>
<th>Surgery</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presensitized + booster&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Portal vein</td>
<td>0/11</td>
<td>0/11</td>
<td>0/20 (0%)</td>
<td>0/11</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Tail vein</td>
<td>0/11</td>
<td>1/10</td>
<td>1/21 (5%)</td>
<td>0/11</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>3/10</td>
<td>4/11</td>
<td>7/21 (33%)</td>
<td>1.60</td>
<td>2.00</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>6/12</td>
<td>1/9</td>
<td>7/21 (33%)</td>
<td>2.20</td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>Sum and average</td>
<td>9/44</td>
<td>6/39</td>
<td>15/83 (18%)</td>
<td>2.00</td>
<td>1.70</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presensitized</td>
<td>Portal vein</td>
<td>1/9</td>
<td>0/10</td>
<td>1/19 (5%)</td>
<td>2.00</td>
<td>0/20</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>Tail vein</td>
<td>2/13</td>
<td>0/11</td>
<td>2/24 (8%)</td>
<td>2.50</td>
<td>0/25</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>4/11</td>
<td>7/10</td>
<td>11/21 (52%)</td>
<td>2.00</td>
<td>1.60</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>6/10</td>
<td>1/9</td>
<td>7/19 (37%)</td>
<td>2.00</td>
<td>1.00</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>Sum and average</td>
<td>13/43</td>
<td>8/40</td>
<td>21/83 (25%)</td>
<td>2.30</td>
<td>1.50</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total of the above</td>
<td>Portal vein</td>
<td>22/87&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14/79&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36/166 (22%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.00</td>
<td>1.60</td>
<td>1.90</td>
</tr>
<tr>
<td>Control</td>
<td>Tail vein</td>
<td>8/16</td>
<td>14/15</td>
<td>8/16 (50%)</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>14/15</td>
<td>14/15</td>
<td>14/15 (93%)</td>
<td>2.60</td>
<td>2.60</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>10/14</td>
<td>10/14</td>
<td>10/14 (71%)</td>
<td>2.10</td>
<td>2.10</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td>Sum and average</td>
<td>46/60&lt;sup&gt;e&lt;/sup&gt;</td>
<td>77%&lt;sup&gt;e&lt;/sup&gt;</td>
<td>46/60 (77%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.20</td>
<td>2.20</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed</td>
<td>Portal vein</td>
<td>12/16</td>
<td>12/16</td>
<td>12/16 (75%)</td>
<td>2.30</td>
<td>2.30</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td>Tail vein</td>
<td>15/15</td>
<td>15/15</td>
<td>15/15 (100%)</td>
<td>2.60</td>
<td>2.60</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>15/16</td>
<td>15/16</td>
<td>15/16 (94%)</td>
<td>2.80</td>
<td>2.80</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>14/15</td>
<td>14/15</td>
<td>14/15 (93%)</td>
<td>2.60</td>
<td>2.60</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>Sum and average</td>
<td>56/62&lt;sup&gt;e&lt;/sup&gt;</td>
<td>90%&lt;sup&gt;e&lt;/sup&gt;</td>
<td>56/62 (90%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.60</td>
<td>2.60</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values from 0 to 3 expressing the total amount of tumor tissue found in the various anatomical locations at autopsy (see text). Average values for groups and subgroups were calculated by dividing the sum of the values by the no. of tumor-positive mice.

<sup>b</sup> Booster injection of killed tumor cells on Days 7 and 14 after challenge.

<sup>c</sup> 15/83 vs. 21/83 (0.05 < p < 0.1).

<sup>d</sup> 22/87 vs. 14/79 (0.05 < p < 0.1).

<sup>e</sup> 36/166 vs. 46/60 + 56/62 (p < 0.001).

<sup>f</sup> 46/60 vs. 56/62 (0.01 < p < 0.05).

examination or by histological examination of visceral organs from randomly chosen samples. 1, 1 to 5 tumors from 1 to 3 mm in diameter and/or 1 tumor of not more than 5 mm in diameter found in visceral organs and in the peritoneal cavity; s.c. tumors 3 to 7 mm in diameter. 2, more than 5 tumors of from 1 to 3 mm in diameter and/or up to 5 tumors of not more than 5 mm in diameter found in visceral organs in the peritoneal cavity; s.c. tumors 8 to 14 mm in diameter. 3, multiple tumors larger than 5 mm in diameter found in visceral organs or in the peritoneal cavity (Solitary large tumors were never seen; they were invariably part of massive tumor growth); s.c. tumors larger than 14 mm in diameter. The results of this experiment are presented in Table 1.

The data show that mice cured of their sensitizing tumor implants had acquired a high degree of resistance to challenge, and the cumulative values indicate that booster injections with killed tumor cells enhanced the resistance. The additive effect of booster injections is on the borderline of statistical significance, yet although weak the effect was repeatable in 2 experiments.

The data further show that the mice cured by surgery were, on the average, more resistant than the mice cured by radiotherapy. This is expressed in the incidence of tumor growth, as well as in the tumor growth values. These values are also on the borderline of statistical significance, but they are in agreement with the results of a separate investigation of the effects of residual tumor tissue on host resistance (J. Vaage, unpublished data).

Because tumor growth rates vary greatly between individual tumors and hosts, the tumor growth values for those presensitized subgroups in which few of the mice developed tumors tend to become meaningless by themselves, but the average values for the major groups may still be regarded as an indication of differences in levels of host resistance to tumor growth.

The mice in the untreated control groups were less susceptible to challenge than the mice immunologically depressed by sublethal irradiation. This may be interpreted to mean that the immune response initiated by the challenge injection of tumor cells was prompt enough and strong enough to cause the rejection of some tumor implants and influence the growth of others.
Host resistance was particularly well expressed in the organs reached by portal and tail vein injections. In the case of the liver, the low incidence of tumor growth in presensitized mice may have been in part due to host factors other than immune resistance. This was indicated in the presumably unresponsive sublethally irradiated mice, where the incidence of tumor growth was lower in the livers than in other sites.

The difference in the level of resistance between the differently treated groups is most unequivocally expressed when the numbers of mice that developed tumors are compared, but it is also evident in the average tumor growth values calculated for the tumor-bearing mice.

**DISCUSSION**

The factors that determine where tumor cells disseminated via lymph and blood become established and grow remain a subject of speculation. It is not possible to predict the extent and location of secondary tumors in individual cases of clinical cancer, nor can it be explained why certain tissues, such as muscle, show relatively few metastases. The published information on this subject was reviewed by Fisher and Fisher (4) in 1967, and they concluded that the location of metastatic tumor growth is determined by a combination of factors. These include intrinsic characteristics of the tumor cells, mechanical factors affecting the blood flow through an organ, and the biological properties of various tissues and organs. More recently, Hofer et al. (7) used radioactively labeled tumor cells to trace their distribution following injection. They found that the ability to migrate is a characteristic of a given tumor cell population and that the distribution of disseminated tumor cells is determined by factors in the organs reached by the circulating tumor cells. In sensitized mice, the differences in the level of antitumor immune resistance compared, but it is also evident in the average tumor growth values calculated for the tumor-bearing mice.

Previous work (3, 8, 14) has indicated that immunological factors influence the ability of disseminated tumor cells to establish metastatic growth. This investigation supports the previous findings and shows that in the mouse there are differences in the level of antitumor immune resistance between different anatomical locations. In sensitized mice, the organs reached by portal vein and tail vein injections were almost totally resistant to a challenge dose of tumor cells that caused growths in a high percentage of the unsensitized control animals. The liver appears to be more resistant to "metastases" than the lungs, although the liver seems to retain more of the injected tumor cells. This is indicated by the observation that the liver was the only organ with tumor growth after portal vein injection, whereas the tail vein injections were followed by tumor growth in the kidneys, the liver, and the adrenal gland, as well as in the 1st filtering organ, the lungs. The occurrence of tumor growth in the peritoneal cavity after portal vein injection is considered to have been caused by a technical error associated with the difficulty of injecting into the portal vein without accidentally seeding the peritoneal cavity. The data indicate that the s.c. site is more resistant to tumor growth than the peritoneal cavity in all groups except the group of sublethally irradiated mice. The difference in resistance between the 2 sites becomes more significant when the difference in challenge doses, $5 \times 10^6$ cells injected i.p. against $2 \times 10^5$ cells injected s.c. is taken into consideration. The relative readiness with which tumors become established in the peritoneal cavity is not what one might expect considering the high immunological activity of peritoneal cells in cytotoxicity tests (1, 5, 6) and in adoptive transfer of immunity (9). This activity may be partially offset by the favorable growth conditions in the peritoneal cavity.

The lower values for tumor incidence and tumor growth in the untreated control group (77%) compared with the immunologically depressed group (90%) indicate that the primary immune response that follows the challenge injection of tumor cells into the untreated control mice was prompt and strong enough to cause the rejection of the injected tumor cells in a significant number of the mice. This lends support to the immunological surveillance concept (2), which states that host resistance factors may arrest the development of antigenic tumors soon enough after their appearance that an unknown proportion of them regress before being noticeable.

Surgical extirpation of a sensitizing tumor implant left the host more resistant than the animal with a tumor left in situ after it was killed by irradiation. This seems paradoxical in view of the fact that booster injections of additional killed tumor cells enhanced the immune response of both surgically cured and irradiation-cured mice. This observation may show how very critical the time and quantity of the antigenic stimulus is to the expression of host resistance. The presence of a large mass of recently killed tumor tissue in a sensitized animal at the time of challenge may serve specifically to desensitize or depress resistance at a critical moment, whereas a much smaller amount of killed tumor tissue injected 1 and 2 weeks after challenge may boost the immune resistance. Much more information is needed in this area and may be derived from animal models before specific immunotherapy is ready for testing in clinical trials.

**ACKNOWLEDGMENTS**

We extend our appreciation to Dr. Herman D. Suit for his support and interest in this work.

**REFERENCES**

3. Deodhar, S. D., and Crile, G., Jr. Enhancement of Metastases by


Effect of Immune Status on the Development of Artificially Induced Metastases in Different Anatomical Locations

Jan Vaage, Kuang Chen and Suzanne Merrick