Local Antitumor Activity of a Primary and an Anamnestic Response to a Syngeneic Guinea Pig Hepatoma

Robert C. Bast, Jr., Berton Zbar, and Herbert J. Rapp

SUMMARY—After intradermal (id) injection, the line-10 hepatoma grew progressively in nonimmune guinea pigs, whereas the line-1 hepatoma grew for approximately 2 weeks, developed central necrosis, ulcerated, and regressed. Growth of the line-10 hepatoma was suppressed when line-10 hepatoma cells were mixed with antigenically distinct line-1 hepatoma cells before id injection into syngeneic strain-2 guinea pigs. Mixture of line-10 with irradiated line-1 or viable strain-2 embryo cells did not inhibit tumor growth. Preimmunization of recipients to line-1 cells abrogated the suppression of tumor growth from mixtures of line-1 and line-10. — J Natl Cancer Inst 55: 989-994, 1975.

Established tumors regressed at sites of primary infection with BCG (1) Corynebacteria sp. (2), Listeria monocytogenes (3), and vaccinia (4). Local inhibition of tumor growth has been associated with an anamnestic response to purified protein derivative of tuberculin (PPD) (5) and to compounds that can induce contact hypersensitivity (5, 6). The administration of immunotherapeutic agents at distant sites did not affect tumor growth, and it has been postulated that tumor cells are killed, at least in part, as "bystanders" at sites of local inflammation. Support for this theory has been gathered from in vitro studies in which lymphocytes and macrophages, stimulated by nontumorous antigens or by mitogens, inhibited the growth of normal and neoplastic cells (7-14).

The relevance of in vitro studies has been challenged by repeated failure to inhibit syngeneic tumor growth in vivo at sites of a primary or anamnestic response to allogeneic murine carcinomas and sarcomas (15-18). By contrast, studies of guinea pigs and mice (19, 20) demonstrated that syngeneic tumor cells can be killed at the site of an anamnestic response to a second antigenically distinct syngeneic tumor line. A recent report suggests that a primary response to xenogeneic rat tumor cells may suppress murine tumor growth (21). Use of a strongly immunogenic guinea pig hepatoma has permitted comparison of the antitumor activity of a primary and an anamnestic response to tumor-associated transplantation antigen(s) in syngeneic recipients. In this system we found that tumor cells can be killed as bystanders at the site of a primary response to an antigenically distinct syngeneic tumor, but that an anamnestic response fails to suppress bystander growth.

MATERIALS AND METHODS

Animals.—Male Sewall-Wright strain-2 guinea pigs (450-500 g) were obtained from the Laboratory Aids Branch, Division of Research Services, NIH, and from the Animal Breeding Colony, Frederick Cancer Research Center, Frederick, Maryland. Guinea pigs were grouped 6 per cage and fed Wayne guinea pig chow daily and kale three times a week. Tap water was provided ad libitum.

Tumors.—Hepatomas were induced in strain-2 guinea pigs by the oral administration of diethylnitrosamine (22). Ascites variants of two antigenically distinct tumor lines were maintained by serial ip passage in weanling strain-2 males (22, 23). Line-1 (transplant generations 92-103), a moderately antigenic tumor, grew progressively after im, ip, or iv inoculation, but regressed after intradermal (id) injection. Line-10 (transplant generations 8-18), a weakly antigenic tumor, grew progressively after id injection with 10⁶ cells, metastasized to regional lymph nodes; and killed recipients within 60-90 days.

Preparation of tumor cell suspensions.—Ascites containing tumor cells were removed from donor animals. Cells were sedimented at 200 x g for 5 minutes, washed twice in medium 199 (Microbiological Associates, Bethesda, Md.), and resuspended in the same medium. A portion of each tumor cell suspension was diluted 1:20 in 0.13% trypan blue, and viable cells were enumerated in a hemocytometer.

Irradiation.—In one experiment, line-1 tumor cells were exposed to 12,000 R of X-irradiation as described in (24).

Embryo cells.—Torsos of strain-2 embryos (∼3.0 cm in crown-rump length) were rinsed three times with calcium-magnesium-free Dulbecco's phosphate-buffered saline (PBS). Embryos were cut into 1- to 2-mm pieces. Tissue was digested for 15 minutes with 0.25% pronase and 40 μg/ml DNase in PBS. Digestion was repeated three times: The first batch of cells was discarded and the last two were pooled. Dispersed embryo cells were filtered through two layers of sterile gauze, sedimented at 200 x g for 10 minutes at 4° C, washed once in medium 199, and resuspended in the same medium. Cells were enumerated in a hemocytometer after dilution of a sample with 0.13% trypan blue.

Immunization of animals with line-1 tumor cells.—Washed line-1 hepatoma cells were adjusted to 3 x 10⁵/ml in medium 199, and 0.1-ml samples of the tumor cell suspension were injected id into the right flanks of the animals. Injection of tumor cells was repeated weekly for 3 weeks. After an additional 2 weeks, animals were rechallenged with 3 x 10⁶ line-1 tumor cells id. All line-1-immune guinea pigs used in these studies developed induration and erythema (at least 7.5 mm in diameter) at the injection site within 24 hours after tumor cell inoculation.

Injection of mixtures containing tumor cells.—Cells were adjusted to appropriate concentrations in medium 199. Mixtures of cells were prepared immediately before id injection; cell mixtures in a volume of 0.1 ml were injected into the left flanks of the guinea pigs. In some experiments, line-10 tumor cells were injected on the left flank and line-1 tumor cells on the right flank.

Evaluation of tumor growth.—Tumors and regional lymph nodes were observed weekly for at least 90 days.

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The incidence of tumor suppression in different groups was compared statistically by the Fisher exact test. Difference in tumor size was evaluated with the Student's t-test and difference in survival with the Mann-Whitney U-test.

RESULTS

Suppression of Tumor Growth in Nonimmune Guinea Pigs Receiving Mixtures of Line-1 and Line-10 Cells

For evaluation of the antitumor activity of a primary immune response to the line-1 hepatoma, the animals received an id injection of $10^6$ line-10 tumor cells mixed with $3 \times 10^4$ to $3 \times 10^6$ line-1 tumor cells (table 1, text-fig. 1A, B, C, D). Tumor growth was completely suppressed in 4 of 5 guinea pigs given $3 \times 10^6$ line-1 tumor cells and in 2 of 4 receiving $3 \times 10^5$ line-1 cells. In an additional animal in each group, tumor growth was inhibited locally, but these guinea pigs died from progressive lymph node metastases. Smaller numbers of line-1 cells failed to affect line-10 tumor growth, either locally or within regional lymph nodes.

After the id injection of $3 \times 10^6$ or $3 \times 10^5$ line-1 cells without line-10 cells, tumor nodules reached a diameter of 10–15 mm within 10–14 days, developed central necrosis, ulcerated, and then regressed completely within 4–5 weeks. In mixtures containing comparable numbers of line-1 with $10^6$ line-10 cells, the pattern of growth and regression was similar (text-fig. 1). A dose of $3 \times 10^5$ line-1

Text-Figure 1.—A: Intradermal growth of $10^6$ line-10 tumor cells alone $\triangle$—$\triangle$, or mixed with $3 \times 10^7$ $\square$—$\square$, $3 \times 10^6$ $\bigcirc$—$\bigcirc$, and $3 \times 10^4$ (open ovals) line-1 tumor cells in nonimmune guinea pigs. Curves terminate at the death of the first animal in each group. Growth of line-10 was inhibited by $30 \times 10^6$ line-1 cells on days 18, 21 ($P<0.005$), 28, 43, and 47 ($P<0.001$). Line-10 growth was also inhibited by $3 \times 10^6$ line-1 cells from day 18 to 47 ($P<0.001$). B: Intranodal growth (same symbols). Growth of line-10 was inhibited on day 21 ($P<0.025$) and on day 28, 43, and 47 ($P<0.001$). C: Intradermal growth of different numbers of line-1 tumor cells (same symbols). D: Intranodal growth (same symbol).
TABLE 1.—Incidence of progressive growth of line-10 hepatoma in nonimmune guinea pigs inoculated at the same site with mixtures containing line-1 and line-10 hepatoma cells.a

<table>
<thead>
<tr>
<th>Number of tumor cells</th>
<th>Intradermal growth of line-10 tumor cells</th>
<th>Intranodal growth of line-10 tumor cells</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line-1</td>
<td>Intranodal Site line-10 tumor cells</td>
<td>line-10 tumor cells</td>
<td></td>
</tr>
<tr>
<td>3 X 10^7</td>
<td>10^4</td>
<td>0/5 b</td>
<td>&gt;105 d</td>
</tr>
<tr>
<td>3 X 10^6</td>
<td>10^8</td>
<td>1/4</td>
<td>&gt;110 d</td>
</tr>
<tr>
<td>3 X 10^5</td>
<td>10^8</td>
<td>5/5</td>
<td>5/5 66</td>
</tr>
<tr>
<td>3 X 10^4</td>
<td>10^8</td>
<td>4/5</td>
<td>5/5 75</td>
</tr>
<tr>
<td>0</td>
<td>10^4</td>
<td>4/4</td>
<td>4/4 66</td>
</tr>
</tbody>
</table>

a Guinea pigs received id injections containing a mixture of line-1 and line-10 hepatoma cells. After id injection, line-1 cells grew and regressed; after id injection, line-10 cells grew progressively. Measurements of intradermal and intranodal tumor growth indicate the growth of line-10 tumor and are expressed as the number of animals with progressive tumor growth/number inoculated.

b Incidence differs from line-10 controls at P = 0.008.

c Incidence differs from line-10 controls at P = 0.04.

TABLE 2.—Incidence of progressive growth of line-10 hepatoma in nonimmune and line-1-immune guinea pigs inoculated with mixtures containing line-1 and line-10 hepatoma cells.a

<table>
<thead>
<tr>
<th>Immunized to line-1 cells</th>
<th>Number of tumor cells</th>
<th>Site</th>
<th>Intradermal growth of line-10 tumor cells</th>
<th>Intranodal growth of line-10 tumor cells</th>
<th>Median survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Line-1</td>
<td>Line-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 X 10^6</td>
<td>10^4</td>
<td>Same</td>
<td>5/5</td>
<td>125^a</td>
</tr>
<tr>
<td></td>
<td>3 X 10^5</td>
<td>10^8</td>
<td>Separate</td>
<td>5/5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10^6</td>
<td>Same</td>
<td>5/5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>3 X 10^5</td>
<td>10^8</td>
<td>Separate</td>
<td>5/5</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>3 X 10^4</td>
<td>10^6</td>
<td>Separate</td>
<td>5/5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10^8</td>
<td>Same</td>
<td>5/5</td>
<td>62</td>
</tr>
</tbody>
</table>

a Guinea pigs received id injections containing tumor cells. When tumor cells were injected at the same site, mixtures of line-1 and line-10 tumor cells were injected id into the left flank. Line-10 was injected id into the left flank and line-1 into the right flank when tumor cells were injected at separate sites. Measurements of intradermal and intranodal tumor growth indicate the growth of line-10 cells and are expressed as the number of guinea pigs with progressive tumor growth/number inoculated.

b Survival significantly prolonged compared to all other groups at P = 0.018.

TEXT-FIGURE 2.—A: Intradermal growth of 10^6 line-10 tumor cells, alone △—△, or mixed with 3 X 10^4 line-1 ○—○, and with 3 X 10^5 strain-2 embryo cells □——□. Line-10 growth was inhibited by 3 X 10^6 line-1 cells on day 23 (P<0.025), 31 (P<0.005), 36 (P<0.001), 45 (P<0.001), and 52 (P<0.01). Growth of line-10 was also inhibited by strain-2 embryo cells on day 8 (P<0.005), 14 (P<0.025), 23 (P<0.025), and 31 (P<0.01). Differences on days 36-52 were not statistically significant. B: Intranodal growth (same symbols). Line-10 growth was inhibited by 3 X 10^6 line-1 cells on day 45 (P<0.025) and 52 (P<0.05). Growth of line-10 was inhibited by embryo cells on day 45 (P<0.025) and 52 (P<0.05).
TABLE 3.—Incidence of progressive growth of line-10 hepatoma in nonimmune and line-1-immune guinea pigs inoculated with mixtures containing line-10 and different numbers of line-1 tumor cells

<table>
<thead>
<tr>
<th>Immunized to line-1</th>
<th>Number of tumor cells</th>
<th>Intradermal growth</th>
<th>Intranodal growth</th>
<th>Median survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line-1</td>
<td>Line-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>8.10^7</td>
<td>10^6</td>
<td>0/5b</td>
<td>134^d</td>
</tr>
<tr>
<td>−</td>
<td>8.10^6</td>
<td>10^6</td>
<td>3/5</td>
<td>84^e</td>
</tr>
<tr>
<td>+</td>
<td>8.10^7</td>
<td>10^6</td>
<td>4/4</td>
<td>62</td>
</tr>
<tr>
<td>+</td>
<td>8.10^6</td>
<td>10^6</td>
<td>5/5</td>
<td>61</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>10^6</td>
<td>5/5</td>
<td>55</td>
</tr>
<tr>
<td>+</td>
<td>10 x 8.10^6</td>
<td>10^6</td>
<td>5/5</td>
<td>59</td>
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<tr>
<td>+</td>
<td>8.10^6</td>
<td>10^6</td>
<td>5/5</td>
<td>68</td>
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<tr>
<td>+</td>
<td>0</td>
<td>10^6</td>
<td>5/5</td>
<td>67</td>
</tr>
<tr>
<td>+</td>
<td>8.10^6</td>
<td>10^6</td>
<td>5/5</td>
<td>74</td>
</tr>
</tbody>
</table>

* Strain-2 male guinea pigs received id injections containing line-1 and line-10 tumor cells. Measurements of intradermal and intranodal tumor growth represent the growth of line-10 cells and are expressed as number of guinea pigs with progressive tumor growth at the injection site/number tested.

1 Incidence differs from controls receiving line-10 cells alone at P = 0.008.
2 Survival differs from line-10 control group at P = 0.014.
3 Survival differs from line-10 control group at P = 0.015.
4 Intratumoral injections of 3 x 10^6 line-1 cells were given twice a week for 5 weeks.

TEXT-FIGURE 3.—A: Intradermal growth of 10^6 line-10 tumor cells in line-1-immune ▲ ▲ ▲ ▲ ▲ and nonimmune □ □ □ □ □ guinea pigs; intradermal growth of 10^6 line-10 mixed with 3 x 10^6 line-1 cells in line-1-immune ○ ○ ○ ○ ○ and nonimmune □ ○ ○ ○ ○ recipients. Growth of line-10 was inhibited by line-1 in nonimmune recipients on days 21, 28, 35, 42, and 50 (P < 0.001). Growth of line-10 was not inhibited by line-1 in immune recipients. Growth of line-10 in line-1-immune recipients was less than that in nonimmune recipients on days 4 (P < 0.025) and 7 (P < 0.05), but did not differ at other intervals. B: Intranodal growth (same symbols). Growth of line-10 was inhibited by line-1 in nonimmune recipients on days 35, 42, and 50 (P < 0.001). Growth of line-10 was not inhibited by line-1 in immune recipients.

Nonimmune and Line-1-Immune Guinea Pigs

For comparison of the antitumor activity of a primary and anamnestic response to line-1, nonimmune and line-1-immune guinea pigs received id injections of mixtures of 3 x 10^6 line-1 and 10^6 line-10 tumor cells. Controls were given line-10 tumor cells alone or line-1 and line-10 tumor cells at separate sites. In nonimmune animals, id injection at the same site of 3 x 10^6 line-1 and 10^6 line-10 cells completely suppressed tumor growth in 2 of 5 recipients, inhibited local tumor growth in 5 of 5, and significantly prolonged survival relative to all other experimental and control groups. By contrast, no inhibition of line-10 growth was observed when the same mixture was injected into line-1-immune recipients (table 2, text-fig. 3A, B). Increasing the dose of line-1 cells to 3 x 10^6 failed to influence line-10 growth in line-1-immune recipients (table 3, text-fig. 4A, B). Multiple injections of line-1 were similarly ineffective in suppressing line-10 tumor growth in line-1-immune recipients (table 3).

Systemic Tumor-Specific Immunity in Guinea Pigs Receiving Mixtures of Line-1 and Line-10 Cells

Animals surviving the id injection of line-1 and line-10 were rechallenged with 10^6 line-10 tumor cells. Three of 5 that had received 3 x 10^6 line-1 cells and 4 of 4 that had received 3 x 10^6 line-1 cells resisted line-10 challenge. All nonimmune controls succumbed to an id injection of 10^6 line-10 cells.

DISCUSSION

The line-1 hepatoma is a moderately antigenic tumor that will grow in the skin of a syngeneic recipient for approximately 2 weeks and regress, leaving the animal...
specifically immune to rechallenge. Line-10, by contrast, is a weakly immunogenic hepatoma that will grow progressively after id injection, metastasize to regional lymph nodes, and kill the recipient. In this report, line-10 tumor growth was suppressed when line-1 cells were mixed with line-10 cells before id injection into nonimmune guinea pigs. Injection of line-1 cells on the contralateral flank did not affect line-10 growth. Contact with viable tumor cells was required. Admixture with irradiated line-1 cells or viable strain-2 embryo cells failed to affect line-10 growth.

Line-10 growth was not suppressed when a mixture of line-1 and line-10 cells was injected into guinea pigs immunized to line-1 tumor. Earlier reports of bystander effects in vivo have described the local antitumor activity of an anamnestic response to syngeneic tumors in mice (20) and guinea pigs (19). Growth of another guinea pig tumor, line-7, was suppressed when a mixture of line-1 and line-7 cells was injected into an animal immunized to line-1 tumor. Significantly less inhibition of line-7 growth occurred when a mixture of line-1 and line-7 cells was injected into a nonimmune recipient. The interpretation of these experiments was complicated by the presence of multiple tumor transplants in the same animal and by an observation period limited to 2 weeks. Rejection of line-1 tumor after id injection into a nonimmune guinea pig is a gradual process requiring more than a month for complete resolution. In our present study, a longer observation period and the injection of a single tumor mixture probably favored the demonstration of the antitumor activity of a primary response to line-1 cells. When the intensity of delayed cutaneous reactivity in a hyperimmune recipient is considered, however, it is not readily apparent why the anamnestic response should exert less bystander activity. In experiments with mycobacterial antigens, *chronicity* of inflammation has been a prerequisite for complete tumor suppression. Injection of living BCG into the skin of a guinea pig leads to the formation of a dermal papule that develops central necrosis, ulcerates, and heals in 4–6 weeks. Reactions produced by living BCG completely inhibit line-10 tumor growth. Injection of PPD into animals immunized to BCG leads to intense delayed cutaneous hypersensitivity reactions persisting for 48 hours. Reactions elicited by PPD failed to suppress tumor growth completely (25).

Nonspecific inhibition of syngeneic tumor growth was observed at reaction sites to some allogeneic murine lymphomas (16, 17). In allogeneic reactions to the EL 4 lymphoma, inhibition of bystander growth was not encountered if recipients were preimmunized with EL 4 tumor cells (17). Tanaka and Sasaki (21) reported a similar phenomenon in mice in which syngeneic fibrosarcoma growth could be suppressed at the site of a primary response to a xenogeneic rat tumor. Preimmunization with the rat tumor abrogated the local antitumor effect. The major effect of the anamnestic response in syngeneic, allogeneic, and xenogeneic systems may be to limit the duration of antigen-evoked inflammation.

In each reaction suppressing line-10 growth, the mixture of line-1 and line-10 tumor cells grew into a palpable nodule that developed central necrosis, ulcerated, and healed with residual scarring. Irradiated line-1 cells and a few viable line-1 cells (<3x10^6) failed to produce ulcerated nodules in nonimmune recipients and to inhibit line-10 growth. Brisk delayed cutaneous reactions followed the injection of 3x10^6 or 3x10^7 line-1 cells in line-1-immune hosts, but tumor nodules failed to grow, ulcerate, and regress.

Regression of id-injected line-1 transplants in nonimmune guinea pigs is probably mediated by an immune response. The administration of rabbit antiserum to guinea pig lymphocytes prevents the rejection of line-1 tumor. Intact animals that have rejected line-1 cells are

Hagerich JA, Wepsic T: Unpublished observations.
specifically immune to rechallenge. Line-1 immunity can be transferred with peritoneal exudate cells, and once transferred, adoptive immunity can be abrogated specifically by intracardiac injection of the same tumor line (26, 27). Judged from the ulceration observed after id injection of line-1 with or without line-10 cells, the inflammatory response associated with line-1 rejection can destroy normal epithelial cells, and tumor cells may also be killed as bystanders. Suppression of line-10 tumor growth could also result from augmented anti-line-10 immunity. The immune response to line-1 cells might exert an adjuvant effect. In contrast is the observation that 2 of 5 animals surviving the inoculation of 3 X 10⁷ line-1 and 10⁶ line-10 were not immune to rechallenge with line-10 tumor cells.

Line-1 and line-10 cells share an embryonic antigen detectable by reaction in vitro with appropriate xenonantisera (28). This is of particular interest considering the slight, but significant, inhibition of line-10 tumor growth when line-10 cells were mixed with strain-2 embryo cells before id injection (text-fig. 2). Against the possibility that a shared embryonic antigen functions as a tumor-specific transplantation antigen in vivo is the fact that uniform resistance to rechallenge with line-10 was not observed in animals surviving injection of mixtures containing line-1 and line-10 cells. Of greater significance were observations that line-10 cells grow at about a similar rate in line-1-immune and nonimmune recipients (text-figs. 3, 4), and that line-1 tumor grows, ulcerates, and regresses at a similar rate in line-10-immune and nonimmune guinea pigs.⁴

The mechanism of bystander killing is not known. Stimulated lymphocytes, activated macrophages, and vascular damage may all contribute to the destruction of tumor cells (I). We suggested that the rejection of syngeneic tumor grafts occurs in a series of steps (19, 25). Initially, sensitized lymphocytes recognize distinctive tumor antigens. Subsequently, macrophages are recruited that can kill tumor cells "nonspecifically." The demonstration of macrophage-mediated tumor killing and inhibition of tumor growth in vitro has supported this hypothesis (11–14). Hibbs found id injection of trypan blue (an inhibitor of macrophage function in vitro) inhibited primary and anamnestic responses to allogeneic and syngeneic tumor grafts in vivo (29, 30). It was more difficult to abrogate anamnestic responses than primary graft rejection. Although line-10 growth at sites of line-1 rejection in line-1-immune animals was not inhibited, it is possible that smaller numbers of line-10 cells would have been suppressed. Considered in this way, both primary and anamnestic responses are associated with the destruction of bystanders but larger numbers of bystanders are killed during a primary response.

REFERENCES

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⁴ Bast RC Jr, Zbar B: Unpublished observations.