Evidence that tumor antigens enhance tumor growth in vivo by interacting with a radiosensitive (suppressor?) cell population
(tumor immunity/blocking factors/Winn assays/immunological enhancement)

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ABSTRACT The growth of a small number of cells from each of two chemically induced BALB/c sarcomas was enhanced when x-irradiated (15,000 rads) cells of the same sarcoma were mixed with the tumor inoculum. This enhancement did not occur if the recipients had been given a total body x-irradiation of 450 rads. Tumor neutralization (Winn) tests showed that tumor cells irradiated in vitro enhanced tumor growth only in the presence of radiosensitive cells present in the spleens of both nonimmune and tumor-bearing mice. On the basis of these findings we postulate that tumor antigen blocks effective tumor immunity by a mechanism that involves a suppressor cell population.

Even though lymphocytes from tumor-bearing animals often can kill cells from the respective tumors in vitro, neoplasms that possess tumor-specific transplantation antigens (TSTA) are usually not rejected by their hosts in vivo. Rather, for tumors carrying TSTA to be rejected, the animals must be first immunized against the tumor and the cell dose used for subsequent challenge must not be greater than 1–3 orders of magnitude above the minimal dose needed for outgrowth in unimmunized controls (1).

Several mechanisms which contribute to the escape of antigenic tumors from immunological control have been described (2–6). Specific blocking factors (SBF), which inhibit ("block") the in vitro destruction of tumor cells by immune T lymphocytes, represent one of the more extensively immunologically specific escape mechanisms (7, 8). Tumor antigens (7–8), antibodies (7), antigen–antibody complexes (3, 10), and probably also host-cell derived immunosuppressive factors, different from antibodies (11), can act as SBF. Suppressor cells that can turn off the immune response to tumor antigens in a specific or nonspecific way represent another escape mechanism that has attracted great attention lately (5, 12–15).

Tumor antigen can both enhance tumor growth (9, 16, 17) and abolish delayed hypersensitivity to itself in vivo (17). To achieve this, the antigen can be inoculated in the form of a soluble extract or as intact tumor cells that have been rendered incapable of dividing by a heavy dose of x-irradiation (in this paper, referred to as HR cells). One interpretation of these findings is that the antigen inhibits the effector cells directly or prevents their activation. This interpretation is supported by the evidence that free antigen can inhibit the cytotoxic effect of sensititized T lymphocytes in vitro (8, 18, 19). However, certain other findings indicate that antigen will impair anti-tumor immunity by interacting with a suppressor cell population rather than directly with the effector cells (18). Furthermore, two sets of recent data suggest that this interaction results in the production of specific immunosuppressive substances.

First, a Thy-1-positive lymphocyte population present in the spleens of tumor-bearing mice has been shown to synthesize SBF in vitro (20). Thy-1-positive lymphocytes from nonimmune (control) mice could also make SBF in vitro, provided that a T-cell deprived spleen cell population from mice carrying the respective tumor (and itself unable to form SBF) was added (20). This latter observation indicates that SBF can be produced by T lymphocytes from nonimmune donors; operationally, these lymphocytes can be referred to as "suppressor lymphocytes." Second, SBF isolated from the serum of mice with 3-methylcholanthrene-induced sarcomas as glycoproteins with a molecular weight of 56,000 were found to bind to both the respective tumor cells and to "unblocking" antibodies obtained from the serum of tumor-immunized mice; the same un-blocking antibodies could not specifically bind to the respective tumor antigens (11). These SBF were therefore not likely to be TSTA but instead were probably specific immunosuppressive factors similar to those seen in certain nontumor systems (21, 22).

This paper describes experiments performed on three transplanted lines of 3-methylcholanthrene-induced BALB/c sarcomas to study whether tumor antigen can enhance tumor growth in vivo by interacting with host (suppressor) cells different from the actual effector cells.

MATERIALS AND METHODS

Mice and Tumors. BALB/c mice were bred by brother/sister mating in our laboratory and were regularly checked for their ability to accept intrasternal skin grafts; 8- to 12-week-old females were used for all experiments.

Two sarcomas were used (1420 and 1425); these had been induced in female BALB/c mice by intramuscular inoculation of 0.1 mg of 3-methylcholanthrene dissolved in triocetanil. The tumors were maintained by serial transplantation for 8–15 passages in syngeneic females. In experiments using graded cell doses for tumor challenge, tumors were trypsinized and cell suspensions were prepared in Waymouth's medium (GIBCO) without fetal calf serum. In all experiments, except those presented in Table 1, mice were inoculated subcutaneously on both sides of the back, providing two tumor "sites" per mouse.

All mice were individually ear tagged, and the treatment of the mice was unknown to the person scoring the animals. The mice were examined twice weekly for tumor development; two perpendicular tumor diameters were measured. Mean tumor diameters (±SEM) for all "sites" per group were calculated, with negative sites being counted as 0. Statistical significance was estimated by Student's t test.

Immunization of Mice. Mice were inoculated subcuta-

Abbreviations: TSTA, tumor-specific transplantation antigens; SBF, specific blocking factor(s); HR cells, tumor cells x-irradiated with 15,000 rads in vitro.
### Table 1. Effect of HR cells on growth of sarcoma 1420 and 1425 in preimmunized and in untreated syngeneic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment*</th>
<th>Type of tumor cells inoculated†</th>
<th>Tumor diameter (mean ± SEM), mm‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No x-ray (10⁶/mouse)</td>
<td>HR (10⁶/mouse)</td>
</tr>
<tr>
<td>1</td>
<td>Imm. to 1420</td>
<td>1420</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1420, other side</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1425, other side</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1420, same side</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1425, same side</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Imm. to 1425</td>
<td>1425</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1420, other side</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>1425, other side</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>1420, same side</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>1425, same side</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>11</td>
<td>Nonimmune</td>
<td>1420</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>1420, other side</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>1425, other side</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>1420, same side</td>
<td>1.8 ± 0.2c</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>1425, same side</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>16</td>
<td>Nonimmune</td>
<td>1425</td>
<td>None</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>1420, other side</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>1425, other side</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>1420, same side</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>1425, same side</td>
<td>1.2 ± 0.2c</td>
</tr>
</tbody>
</table>

* Immune mice were injected subcutaneously 10 days before challenge with 10⁶ HR tumor cells.
† All mice were given test challenge subcutaneously on the right side of the back. HR cells were either admixed with the cells used for challenge ("same side") or were given on the contralateral side ("other side").
‡ Means of two perpendicular tumor diameters at 2–5 weeks after challenge with tumor cells. Each group consisted of 10 mice. For differences between tumor diameters in experimental groups and groups without HR cells ("none"); * P < 0.05; ** P < 0.01; † P < 0.001.

The mixtures were incubated at room temperature for 30 min and then inoculated subcutaneously into each flank. Controls were always included to make certain that spleen cells from tumor-bearing mice did not contain cells that could grow out to palpable tumors (they never did).

### RESULTS

#### Effect of Admixed HR Tumor Cells on Tumor Growth
Using tumors 1420 and 1425, we first studied whether the admixture of HR cells in the inoculum influenced the ability of a small dose (10⁶) of cells from the same or a different tumor to grow in syngeneic mice. The recipient mice were either untreated or had been preimmunized against the respective tumors. They were inoculated subcutaneously on the right side.

Table 1 shows that added HR cells significantly enhanced the growth of the challenge cells in both untreated and preimmunized syngeneic hosts, as compared to groups in which only the tumor cells were inoculated. This enhancement was seen only when HR and tumor cells were inoculated together and not when the HR cells were given on the contralateral side.

1420 HR cells enhanced sarcoma 1420 and not 1425, and 1425 HR cells enhanced sarcoma 1425. Although 1425 HR cells also gave some enhancement of sarcoma 1420, this enhancement was not statistically significant. The same pattern of specificity was observed when the data were expressed as fractions of all inoculated tumor sites having tumor at 2, 3, and 4 weeks after challenge rather than as mean tumor diameters (data not shown).

#### Effect of X-Irradiation of Recipient Mice on Tumor En-
hancement by HR Cells. The enhancing effect of added HR cells was studied further by performing an experiment similar to that described in Table 1 but including groups in which the preimmunized mice were given 450 rads of total body x-irradiation the day before challenge. As seen before in nonirradiated, preimmunized mice, HR cells enhanced tumor growth. However, HR cells did not enhance tumor growth in immunized mice that had been preirradiated (Table 2).

Effect of Spleen Cells from Normal or Tumor-Bearing Mice on Tumor Growth in Preirradiated Recipients. Because whole-body x-irradiation abolished the ability of HR cells to enhance tumor growth, we reasoned that a radiosensitive (suppressor?) cell population was responsible for tumor enhancement. Therefore, neutralization (Winn) tests were performed to determine whether spleen cells could enhance the growth of tumor 1425 in sublethally irradiated mice. Spleen cells from both normal and tumor-bearing hosts were tested and were either left untreated or preirradiated with 450 rads. To provide cells reacting to the TSTA of tumor 1425, spleen cells from mice that had been preimmunized against 1425 were added to the mixtures inoculated.

As shown in Table 3, tumor growth was inhibited in mice receiving tumor cells that had been mixed with immune lymphocytes (group 2), compared to mice inoculated with tumor cells and nonimmune ("normal") spleen cells (group 5) or tumor cells alone (group 1).

The growth of tumor in mice receiving mixtures of tumor cells and spleen cells from either normal or tumor 1425-bearing animals was identical to the growth in mice receiving only tumor cells. Furthermore, preirradiation of the normal or tumor-bearers' spleen cells prior to mixing with tumor cells also had no effect on growth of the tumor cells (data not shown).

When spleen cells from mice bearing tumor 1425 (group 3) or from nonimmune (normal) mice (group 5) were added to the immune cells, tumor growth was greater than that seen with immune cells alone, although this difference was not statistically significant. However, if the tumor-bearer or normal spleen cells were given 450 rads (groups 4 and 6), this tendency of enhanced growth was completely eliminated. Concordant data were obtained in both of two subsequent similar experiments.

Combined Effect of HR Cells and Spleen Cells on Tumor Growth in Preirradiated Hosts. The experiments described up to this point indicated that HR cells can enhance tumor growth (Table 1) and that a radiosensitive lymphoid cell population can facilitate tumor growth in the presence of a systemic immune response to TSTA (Table 2). They suggested, furthermore, that a radiosensitive cell population, represented in the spleens of normal or tumor-bearing mice, could facilitate tumor growth in the presence of a local immune response to tumor (Table 3). We speculated that the reason why spleen cells were not more effective in enhancing tumor growth than shown in Table 3 may have been that the amount of antigen released from the inoculated tumor cells was insufficient for a spleen cell suppressor function to be fully established. Three experiments were performed to investigate this possibility, and the data were concordant. Table 4 presents one of these experiments. Sublethally irradiated mice were inoculated with 105 1425 tumor cells together with 5 X 106 immune ("anti-1425") spleen cells, 1420 or 1425 HR cells (as a source of tumor antigen provided in addition to that probably released from the challenge cells), and spleen cells from nonimmune BALB/c mice. Groups were included in which no HR cells were added, as well as groups

Table 3. Effect of added spleen cells from normal or tumor-bearing mice on growth of sarcoma 1425 in irradiated mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of spleen cells*</th>
<th>Tumor diameter (mean ± SEM), mm†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At 8 days</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>Anti-1425</td>
<td>0±</td>
</tr>
<tr>
<td>3</td>
<td>Anti-1425 + 1425 tu.</td>
<td>0.5 ± 0.1a</td>
</tr>
<tr>
<td>4</td>
<td>Anti-1425 + 1425 tu. (450 rads)</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>Anti-1425 + norm.</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Anti-1425 + norm. (450 rads)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Anti-1425 spleen cells (immune cells) were from mice immunised with 1425 HR cells 10 days previously; 1425 tu. spleen cells were from mice carrying 1425 tumor transplants with a mean diameter of ~10 mm; normal spleen cells were from nonimmune mice. In some groups, the spleen cells were irradiated with 450 rads before they were mixed with the tumor cells and inoculated. None means only tumor cells given.

†Group 1 included 10 mice; the other groups included 5 mice each; all mice were inoculated on both sides (two "sites" per mouse). For differences between tumor diameters in groups receiving nonirradiated and preirradiated spleen cells: a, P < 0.05; c, P < 0.001.
in which the normal spleen cells were either deleted or pre-
treated with 450 rads in vitro.

Normal spleen cells without added HR cells (group 2) gave
slight enhancement of tumor growth compared to growth of
tumors in sites where only immune cells were present (group
1). This enhancement was statistically significant but was only
observed early (10 and 13 days) and was similar to the results
in Table 3. 1425 HR cells enhanced tumor growth but this en-
hancement occurred only when nonirradiated normal spleen
cells were included in the cell mixtures inoculated (group 5).
Furthermore, the addition of 1420 HR cells (a noncrossreacting
tumor) to the inoculum of tumor cells, immune cells, and spleen
cells had no effect on tumor growth.

Finally, a preliminary experiment was performed to test
whether the spleen cells that act in concert with HR cells in
enhancing tumor growth are sensitive, in the presence of com-
plement, to treatment with a previously described anti-T
cell serum (23) and complement. The data showed that the
spleen cells were sensitive to this serum (in the presence of
complement). Subsequent experiments using mouse anti-
Thy/sera have given similar results (data not shown).

DISCUSSION

We demonstrate that cells from chemically induced BALB/c
sarcomas 1420 and 1425 grew better when inoculated into mice
together with heavily x-irradiated (HR) cells from the same
sarcoma than when given alone; the HR cells were known to
possess TSTA because they could induce transplantation re-
sistance to the respective tumors. Tumor enhancement was seen
both in mice preimmunized to cells of the respective sarcomas
and in untreated animals.

The enhancement seen with tumor 1425 was highly specific;
only a weak statistically nonsignificant enhancement was ob-
served when cells from sarcoma 1420 were inoculated together
with HR cells from tumor 1425. The latter finding may reflect
some immunological crossexreaction between the two sarcomas
(unpublished data). The fact that sublethal irradiation of the
mice totally abolished the enhancing effect of the HR cells
(Table 2) argues against its being simply due to a "feeder" effect
of the added HR cells (24). The HR cells enhanced tumor
growth only when mixed with the test challenge, indicating that
the dose of antigen inoculated (5 x 10^6 nondividing cells) was
too small to achieve a systemic effect. Our findings agree with
published reports that antigen can enhance tumor growth (8,
9, 16, 17), and they may explain why the tumor transplantation
resistance detected in preimmunized hosts is generally over-
come when the mice are challenged with large inocula.

The remainder of our experiments concerned the question of
whether the observed enhancing effect of HR cells was due to
a direct inactivation of the effector cells or was more indirect
(e.g., involving a suppressor cell mechanism).

Suppressor cells are generally sensitive to a sublethal dose of
X-irradiation (25), whereas the ability of preimmunized mice
to reject antigenic tumor cells is known to be resistant to such
radiation (1). Experiments performed to test whether HR cells
could enhance tumor growth in sublethally x-irradiated hosts
preimmunized to the tumor showed that the enhancing effect of
antigen was indeed abolished by radiation. That led us to test
whether spleen cells from either nonimmune or tumor-bearing
mice could facilitate tumor growth in sublethally irradiated
mice when mixed with spleen cells from mice immune to the
respective tumor. A radiosensitive population of spleen cells was
found to partially abrogate the ability of an immune lympho-
cyte population to inhibit tumor growth. No difference was seen
in this respect between spleen cells from nonimmune mice and
from animals bearing the respective tumor; these data are at
variance with those of Fujimoto et al. (26), probably because of
differences between the experimental systems studied.

We speculated that the effect seen with added tumor-bearer
or normal spleen cells was only partial because the amount of
tumor antigen present was insufficient to fully activate sup-
pressor cells. We therefore determined whether both 1425 HR
cells and nonimmune spleen cells were needed for substantially
enhancing the growth of tumors 1425 in sublethally irradiated
mice; spleen cells from mice immunized to 1425 were added as
the source of tumor immunity. Significant enhancement was
seen only when the 1425 tumor cells used for challenge were
mixed with both 1425 HR cells and nonirradiated spleen cells.
The spleen cells responsible for this effect were sensitive to
treatment with an anti-T serum and complement, indicating that
they were T lymphocytes. However, a much better charac-
terization of the surface markers of the suppressor cells is
needed.

We suggest the following model, which is similar to one
proposed by Gershon et al. (12), as an explanation of our find-
ings. Tumor antigen in the form of HR cells, which will induce

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Table 4. Combined effect of HR and spleen cells on growth of sarcoma 1425 in irradiated syngeneic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Test inoculum*</th>
<th>Tumor diameter (mean ± SEM), mm†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 x 10^6 HR cells</td>
<td>5 x 10^6 norm BALB/c spleen cells</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>No x-ray</td>
</tr>
<tr>
<td>3</td>
<td>Given 450 rads</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>1425</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>1420</td>
<td>No x-ray</td>
</tr>
<tr>
<td>6</td>
<td>1420</td>
<td>Given 450 rads</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>No x-ray</td>
</tr>
<tr>
<td>9</td>
<td>Given 450 rads</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

* Each group consisted of 10 mice, inoculated on both sides of the back. In all groups, the tumor mixtures inoculated included 5 x 10^6 cells from
the spleen of mice immunized against sarcoma 1425 and 10^6 sarcoma 1425 cells.
† For differences between tumor diameters in corresponding groups receiving spleen cells compared to groups not receiving spleen cells (but
the same type of HR cells); * P < 0.05; † P < 0.001. Differences between tumor diameters in groups 2 and 5 are significant at P < 0.001 for
each of the four time points; tumor diameters in group 8 do not differ significantly from those in group 2.
transplantation immunity under certain conditions, can enhance tumor growth under other conditions, acting alone or together with antibodies in the form of antigen–antibody complexes. This enhancement operates via an indirect mechanism which involves a T lymphocyte population present in the spleen (and probably also elsewhere) and which is sensitive to sublethal doses of x-irradiation. This lymphocyte population performs a suppressor-cell function. As indicated by published findings (7, 8, 18, 19), tumor antigen may also inhibit the effect (or "prekiller") cells directly, but we postulate that such inhibition occurs primarily when the antigen is present in a dose sufficiently large to compete with tumor cells for the receptors on the immune lymphocytes. Furthermore, we postulate that lymphocytes represented in normal spleens are, in the presence of tumor antigen, easily recruited into performing the suppressor function; this would explain why nonimmune lymphocytes were as efficient as tumor-bearers' lymphocytes in enhancing tumor growth. Our data are similar to findings on the in vitro formation of SBF by mouse spleen cultures (20, 27), as summarized in the introduction. They suggest that the suppressor cell effects observed in this study might be related to SBF production and/or action.

Further experiments should clarify whether this model provides a major mechanism whereby antigenic tumors can escape from immunological destruction and to what extent the operationally defined suppressor cells inhibit effector cell activity by producing soluble SBF, including the 56,000-dalton immunosuppressive molecule recently identified (11) and antigen–antibody complexes (10). If, indeed, effector cell activity is primarily inhibited via a suppressor cell mechanism, rather than by tumor antigens directly, there may be a good possibility for therapeutic intervention. However, one must also consider alternative explanations of the findings observed, including, among others, the modification by spleen cells of antigens present on HR tumor cells into a form capable of inhibiting immune effector cells (from a form that is not) and the formation of some lymphokines by spleen cells exposed to tumor antigens "immunostimulatory" (28) to the tumor and thereby counterbalancing tumor inhibition by immune lymphocytes.

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