Activation of Suppressor Cells by Syngeneic Tumor Transplants in Mice

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ABSTRACT

Spleen cells from mice with syngeneic tumor transplants are hyporesponsive in mixed-lymphocyte culture. The hyporesponsiveness is due to the activation of suppressor cells. Spleen from tumor-bearing mice, treated with mitomycin and added to normal mixed lymphocyte culture (with responding cells syngeneic to the added cells), inhibits proliferation of the responding cells. Suppressor activity in the spleen cells can be detected as early as 5 days after s.c. transplantation of P-815 mastocytoma into DBA/2 mice. Tumor cells placed in cell-impermeable i.p. diffusion chambers can also activate splenic suppressor cells.

Suppressor cells can be activated in syngeneic mice by (DBA/2) P-815 cells, by (C3H) L25-cells, or by recent (C57BL/6) methylcholanthrene-induced tumors. The latter tumors retain their ability to activate suppressor cells after passing in syngeneic mice. Only one tumor, induced with methylcholanthrene in DBA/2 mice, failed to activate suppressor cells.

Suppressor activity in the spleen cells from mice with 20-day s.c. tumor transplants is not reduced after removal of glass-adherent cells. Suppressor activity is significantly decreased after removal of thymus-derived cells with anti-0 treatment and complement.

INTRODUCTION

Previously (2), we demonstrated that spleen from tumor-allosensitized mice is hyporesponsive in MLC. The hyporesponsiveness seems to be due to the presence of suppressor cells because tumor-allosensitized spleen cells inhibit the proliferation of normal spleen cells in MLC (1). The suppressor cell in tumor-allosensitized spleen appears to be a T-cell that can be separated from the cytotoxic T-cell (1).

In the present study we demonstrate the activation of suppressor cells in mice with syngeneic tumor transplants. The nature of the suppressor cell is also investigated.

MATERIALS AND METHODS

Mice, Tumor, and Sensitization. C57BL/6 (H-2^b), DBA/2 (H-2^a), C3H/He (H-2^k), and BALB/c (H-2^k) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, as 7-week-old mice and used at 9 weeks of age. Mice for passaging of tumors were obtained through the courtesy of Dr. William Bradner, Bristol Laboratories, Syracuse, N. Y. DBA/2 mastocytoma P-815 and C3H tumor L25 were maintained as ascites tumors by weekly serial passage of 5 x 10^6 cells i.p. in DBA/2 and C3H/He mice, respectively. The L25 was obtained through the courtesy of Dr. Monte Meltzer, National Cancer Institute, Bethesda, Md. Primary tumors were induced by giving mice injections of 0.1 mg MC in 0.1 ml corn oil i.m.

Solid tumors were implanted s.c. into syngeneic recipients in the axillary region with a No. 13 trocar. Ascites tumors were transplanted by injection of 10 x 10^6 cells/0.1 ml s.c. in the shoulder region or by i.p. implantation of tumor-containing, cell-impermeable diffusion chambers. The sterile diffusion chambers consisted of 4-mm-thick slices of plastic tubing (internal diameter, 8 mm) sealed on each side with a Millipore GS filter (pore size, 0.22 mu). The chambers were filled with 10 x 10^6 tumor cells/0.1 ml via a small hole on the side of the chamber, which was sealed with a hot needle before the chambers were implanted via a midventral incision. Control mice carried empty chambers.

Cell Suspension. Spleen was removed aseptically and prepared as a single-cell suspension by pressing through a 50 mesh stainless steel screen. RBC were lysed by hypotonic shock (12). Cells were counted in 0.5% acetic acid and checked for viability with trypan blue.

MLC. Cultures were maintained in RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Grand Island Biological Co.), 5 x 10^-8 M mercaptoethanol, 5% fetal calf serum (Grand Island Biological Co.), 10 /ug streptomycin per 100 ml, and 10,000 units penicillin per 100 ml (we will refer to this medium as RPMI). Microcultures were incubated in a total volume of 0.2 ml in flat-bottom microtiter plates (Linbro Chemical Co., New Haven, Conn.) at 37° and 5% CO_2 in air.

Responding cells were washed 3 times with PBS and suspended in RPMI. Stimulating spleen cells were treated with mitomycin C (kindly provided by Dr. W. Bradner, Bristol Laboratories, Syracuse, N. Y.) in PBS at 50 /ug/100 x 10^6 cells/2 ml (1) for 20 min in a 37° water bath, washed 3 times with PBS, and suspended in RPMI. Cultures consisted of 3 x 10^5 responding spleen cells and 3 x 10^6 mitomycin-treated stimulating spleen cells.

Spleen cells to be added to the MLC were pretreated with 50 /ug mitomycin in a total volume of 2 ml PBS for 20 min at 37°, washed 3 times, diluted to the appropriate concentration, and added to the MLC. The total volume of MLC was kept constant at 0.2 ml. After 72 hr, 1 /uCi [3H]thymidine (Amersham/Searle, Arlington Heights, Ill.) was added and, at 86 hr, the cultures were harvested on glass fiber strips with an automatic cell harvester (Otto Hiller Co., Madison,
Hyporesponsiveness and suppressor activity in spleen cells from DBA/2 mice carrying s.c. P-815 mastocytoma transplants for different mitomycin-treated sensitized spleen.

B. F. Argyris

The data in Table 1, Experiment 1, illustrate that spleen cells from DBA/2 mice carrying 20-day s.c. syngeneic P-815 tumor transplants are hyporesponsive when cultured with BALB/c stimulating spleen cells. To test whether this hyporesponsiveness is due to suppressor cells, we added 5 × 10^4 mitomycin-treated spleen cells from DBA/2 mice carrying s.c. transplants of P-815 mastocytoma to normal MLC. The data in Table 1, Experiment 2, indicate that suppressor cells are present in the spleen of DBA/2 mice carrying s.c. transplants of P-815 mastocytoma for 5 through 25 days. These data suggest that the P-815 can also activate suppressor cells in syngeneic mice.

To test whether a soluble factor produced by the tumor cells is responsible for the activation of suppressor cells, we sensitized DBA/2 mice with P-815 tumor cells placed i.p. in cell-impermeable diffusion chambers. The data in Table 2, Experiment 1, indicate that spleen cells from DBA/2 mice carrying P-815-filled i.p. diffusion chambers are hyporesponsive in MLC. Spleen cells from mice carrying empty chambers appear unaffected. The rise in stimulation index in this group reflects a slightly lower background proliferation in the AAm group; this illustrates the importance of expressing the results as percentage of response, which does take into account the background proliferation. In our opinion it offers a more reliable criterion of MLC reactivity. The hyporesponsiveness in Experiment 1 appears to be due to the presence of splenic suppressor cells since the addition of sensitized spleen cells to normal MLC suppresses proliferation of normal spleen cells (Experiment 2). These results suggest that the P-815 can activate suppressor cells via a soluble factor.

To test whether the activation of suppressor cells is characteristic for the P-815 mastocytoma, we used a different tumor and strain combination. The L25 is an ascites tumor, strain-specific for C3H mice. We found that C3H mice carrying 15- or 20-day s.c. transplants of the L25 tumor are hyporesponsive in MLC, and the hyporesponsiveness appears due to the presence of splenic suppressor cells (data not shown). The data in Table 3 illustrate that the L25 also can activate suppressor cells via a soluble factor. The presence of L25 in cell-impermeable i.p. diffusion chambers causes a hyporesponsiveness in MLC, which appears due to the presence of suppressor cells. These data suggest that activation of suppressor cells is not limited to the P-815 mastocytoma.

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Responding spleen (DBA/2)</th>
<th>Addition (5 × 10^5) (DBA/2)</th>
<th>Days post-tumor</th>
<th>[^3H]Thymidine incorporation (cpm)</th>
<th>SI a</th>
<th>% of response</th>
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<tr>
<td>1</td>
<td>NS °</td>
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<td>20</td>
<td>AA_m 18,495 ± 942; AB_m 89,654 ± 2,504</td>
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<td>SS</td>
<td>None</td>
<td>20</td>
<td>AA_m 13,115 ± 1,498; AB_m 34,137 ± 2,727</td>
<td>2.6</td>
<td>30</td>
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<tr>
<td>2</td>
<td>NS °</td>
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<td>0</td>
<td>AA_m 17,142 ± 441; AB_m 44,030 ± 2,767</td>
<td>2.6</td>
<td>100</td>
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<tr>
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<td>SS</td>
<td>5</td>
<td>15</td>
<td>AA_m 15,845 ± 1,161; AB_m 18,985 ± 510</td>
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<td>7</td>
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<tr>
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<td>SS</td>
<td>10</td>
<td>15</td>
<td>AA_m 19,933 ± 595; AB_m 20,697 ± 1,745</td>
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<td>10</td>
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<tr>
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<td>SS</td>
<td>20</td>
<td>20</td>
<td>AA_m 20,697 ± 1,745; AB_m 16,750 ± 1,355</td>
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<td>0</td>
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</tbody>
</table>

° SI, stimulation index; NS, nonsensitized spleen; SS, sensitized spleen; NS_m, mitomycin-treated nonsensitized spleen; SS_m, mitomycin-treated sensitized spleen.

° Cultures incubated with BALB/c stimulating spleen cells.

° Mean ± S.E.
Suppressor Cells in Syngeneic Tumor-bearing Mice

Table 2

Hyporesponsiveness and suppressor activity in spleen cells from DBA/2 mice carrying P-815 tumor i.p. diffusion chambers for 20 days

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Responding spleen (DBA/2)</th>
<th>Addition (5 x 10^6) (DBA/2)</th>
<th>[3H]Thymidine incorporation (cpm)</th>
<th>AA_m</th>
<th>AB_m</th>
<th>SI^a</th>
<th>% of response</th>
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<tr>
<td>1</td>
<td>NS</td>
<td>None</td>
<td>8,609 ± 799^c</td>
<td>76,446 ± 2,882</td>
<td>8.9</td>
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<tr>
<td></td>
<td>NS-EC</td>
<td>None</td>
<td>5,217 ± 365</td>
<td>67,887 ± 471</td>
<td>13.0</td>
<td>92</td>
<td></td>
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<tr>
<td></td>
<td>SS</td>
<td>None</td>
<td>8,419 ± 143</td>
<td>11,029 ± 373</td>
<td>1.3</td>
<td>4</td>
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<tr>
<td>2</td>
<td>NS</td>
<td>None</td>
<td>21,435 ± 1,372</td>
<td>92,685 ± 3,970</td>
<td>4.3</td>
<td>100</td>
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<td>NS</td>
<td>NS_m</td>
<td>12,522 ± 761</td>
<td>53,343 ± 3,307</td>
<td>4.3</td>
<td>100</td>
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<tr>
<td></td>
<td>NS</td>
<td>SS_m</td>
<td>18,698 ± 943</td>
<td>18,698 ± 943</td>
<td>1.5</td>
<td>15</td>
<td></td>
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</table>

^a SI, stimulation index; NS, nonsensitized spleen; NS-EC, nonsensitized spleen from mice with empty diffusion chambers; SS, spleen from mice sensitized with tumor-filled diffusion chambers; NS_m, mitomycin-treated nonsensitized spleen; SS_m, mitomycin-treated sensitized spleen.
^c Mean ± S.E.

Table 3

MLC hyporesponsiveness and suppressor activity of spleen cells from C3H mice implanted with L25-containing i.p. diffusion chambers for 15 days

<table>
<thead>
<tr>
<th>Responding spleen (C3H)</th>
<th>Addition (2.5 x 10^6) (C3H)</th>
<th>[3H]Thymidine incorporation (cpm)</th>
<th>AA_m</th>
<th>AB_m</th>
<th>SI^a</th>
<th>% of response</th>
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<tr>
<td>NS</td>
<td>None</td>
<td>19,142 ± 1,529^c</td>
<td>88,379 ± 89</td>
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<tr>
<td>SS</td>
<td>None</td>
<td>18,288 ± 820</td>
<td>7,829 ± 773</td>
<td>0.4</td>
<td>0</td>
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</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>8,319 ± 261</td>
<td>23,631 ± 564</td>
<td>3.2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>SS</td>
<td>16,258 ± 916</td>
<td>16,258 ± 916</td>
<td>2.0</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

^a SI, stimulation index; NS, nonsensitized spleen from mice with empty chambers; SS, sensitized spleen from mice with tumor-filled chambers.
^c Mean ± S.E.

Both P-815 and L25 are long-established tumors. To test the effect of recent tumors, we induced tumors in C57BL/6 mice with MC and tested their ability to activate suppressor cells. The data are recorded in Tables 4 and 5. The data in Table 4 indicate that spleen cells from the C57BL/6 mice in which the tumors were originally induced (T_A) are hyporesponsive (Experiment 1) and have suppressor activity (Experiment 3). The data from Table 4, Experiments 2 and 4, suggest that MC-induced tumors on repeated transplantation do not lose their ability to activate suppressor activity. Three other MC-induced C57BL/6 tumors were tested and found to be capable of activating suppressor cells in syngeneic mice (data not shown). The only MC-induced DBA/2 tumor that we tested, on the other hand, was incapable of activating suppressor cells (data not shown). These results indicate that recently induced tumors may, although not necessarily will, activate suppressor cells in syngeneic mice.

Our next experiments were designed to determine the nature of the suppressor cell(s) in mice with syngeneic tumor transplants. For removal of adherent cells, spleen cells from DBA/2 mice with s.c. transplants of P-815 mastocytoma or from C57BL/6 mice with MC-induced tumor were adsorbed on glass. The data in Table 5 indicate that, in both groups of mice, removal of glass-adherent cells does not lead to a loss of suppressor activity.

For removal of θ-positive T-cells, spleen cells of the previously described groups of mice were treated with AβS and complement. The AβS belonged to the same batch that was previously described and shown to eliminate MLC reactivity (2) and concanavalin A-sensitive T-cells, although it did not affect lipopolysaccharide-sensitive bone marrow-derived cells (3). Spleen cells from DBA/2 or C57BL/6 mice, with syngeneic tumor transplants, treated with AβS and complement demonstrate a considerable reduction in suppressor activity (Table 6). Loss of suppressor activity is not complete, however, as indicated by only a partial restoration of MLC reactivity.

DISCUSSION

Previously, we have shown that tumor allografts can activate splenic suppressor T-cells (1, 2). These suppressor cells could be separated from the cytotoxic T-cells by a variety of methods. With allogeneic tumor grafts, one does not know whether the observed effect is due to the allogeneic tumor antigens or tumor-specific transplantation antigens present on the surface of the tumor cells. Other studies (14, 15, 21) have shown that nonmalignant allografts can activate suppressor cells. In the present studies we demonstrate that syngeneic tumor transplants also activate splenic suppressor cells. The suppressor cells were assayed by their ability to inhibit the response of syngeneic spleen cells in MLC. The suppressor cells will also inhibit the in vitro generation of cytotoxic cells (unpublished observations).

The suppressor cell in our studies is not removed by glass adsorption and is sensitive to AβS; therefore it is most probably a T-cell. This is in agreement with the findings of others (5, 6, 9–11, 16–18), but it disagrees with the observations of several laboratories (7, 8, 19) that find that the suppressor cell is a macrophage. Conceivably, both T-cell and macrophage have suppressor activity, but they are activated at different stages of tumor growth.
We have shown in this study that tumor cells inside cell-impermeable diffusion chambers can also activate suppressor cells, indicating that a soluble factor is involved. The nature of this factor is unknown at the present time.

A number of investigators have reported the presence of suppressor cells in tumor-bearing animals. Nordlund and Gershon (11) found that the dose of tumor cells determines the activity of regulatory (suppressor) T-cells. Nelson et al. (10) have described a factor produced by splenic T-cells from tumor-bearing mice, which suppresses cell-mediated cytotoxicity (4). Small and Trainin (16) have reported that the spleen of tumor-bearing mice contains a subpopulation of cells that can enhance in vivo tumor growth. Both Manor et al. (9) and Takei et al. (17, 18) found that spleen cells from mice with early tumor transplants have no detectable suppressor activity but that splenic suppressor cells develop during the later stages of tumor growth. This does not agree with the data of Fujimoto et al. (5, 6) who found that suppressor cells develop 24 hr after tumor transplantation. Our results indicate that in DBA/2 mice the s.c. transplants of P-815 mastocytoma splenic suppressor cells are present 5 days after tumor transplantation; however, we have not tested for their presence at earlier stages.

In conclusion, our studies have shown that the suppressor cell can be activated by a variety of syngeneic tumors in a variety of mouse strains across a cell-impermeable membrane. Suppressor cells can be activated by both ascites and solid tumors. Both long-established and newly induced tumors can be active. In all cases tested we have found that the suppressor cell primarily is a T-cell.
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