Circulating Tumor Antigens versus Immune Serum Factors in Depressed Concomitant Immunity

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SUMMARY

Injections of serum, presumed to contain soluble fibrosarcoma antigens but no homologous antibody, inhibited the recovery of antitumor transplantation immunity in mice recently cured of a large s.c. fibrosarcoma implant. Recovery from the specific immune impairment associated with a large antigenic tumor normally follows soon after complete excision of the tumor. The additional injection of serum from immunized animals averted the inhibition of recovery by antigen-containing serum.

INTRODUCTION

The question of whether concomitant immunity exists in a tumor host essentially asks whether or not an animal or a patient bearing a tumor is still able to resist the growth of more cells of the same tumor in the form of natural metastatic spread or in the form of experimental tumor inocula. In other terms, will an antigenic tumor mass neutralize, by one mechanism or another, immune resistance factors to an extent that may leave the host with impaired or abrogated resistance to secondary incursions by the neoplastic cells?

The existence of concomitant immunity has been observed recently by several investigators (5, 9, 14, 18, 21, 25, 33, 34, 36, 37, 39). A previous publication (33) reviewed most of the relevant literature published prior to 1971.

The question of what factors may impair the effectiveness or the strength of concomitant immunity has received several possible and not mutually exclusive answers. One explanation proposes that an antigenic tumor mass may act like a sponge (20). By antigenic attraction and binding, the specific resistance factors may be absorbed as soon as they are formed and can therefore not be found systemically in detectable amounts until the antigenic mass has been removed. This explanation is not supported by experimental evidence from transfer studies with sensitized lymphoid cells labeled with tritium (19, 32) in which it was shown that sensitized cells were not specifically attracted to antigenic deposits. However, specific and preferential localization of antitumor antibodies has been observed (3, 12, 24). Another possible cause for the impairment of concomitant immunity was observed by Alexander et al. (1), who found that lymphoid blast cells appeared to be retained in lymph nodes draining an antigenic tumor and were not released into the circulation until the tumor had been removed. Specific and local retention of antibodies other than at the tumor mass has not been documented. A 3rd explanation that has received wide support from in vivo investigations in allogeneic systems (17) and from in vitro investigations in syngeneic and autochthonous systems (15, 16, 26) postulates that immune serum factors may enhance tumor growth by blocking or otherwise interfering with cell-mediated antitumor immune protection. A 4th explanation, which is supported by the present report and by previous reports (33, 34, 37), proposes that circulating tumor antigen in excess may be a significant ingredient in the impairment of host immune resistance factors. The presence of tumor-specific antigens in tumor-host serum has been observed and measured by Gold and Freedman (11) and by Thomson and associates (30, 31). Observations by Hellström et al. (14), Sjögren et al. (26), Brawn (6), Currie and Basham (7), Currie and Gage (8), and Baldwin et al. (2) showed that sera that could be presumed to contain specific antigen could impair in vitro lymphocyte cytotoxic activity. The specific and dose-dependent depression of in vivo host resistance by tumor in situ or by injected tumor tissue has been reported in previous publications (33, 34, 37).

This study has investigated in vivo the effect of serum presumed to contain soluble tumor antigen and of serum presumed to contain tumor-specific antibodies on the recovery of depressed concomitant host resistance to tumor growth.

MATERIALS AND METHODS

Animals. All experimental animals were 12-week-old female C3Hf/He mice from the defined-flora,2 pathogen-free breeding colony maintained by the Department of Radiation Medicine at the Massachusetts General Hospital, Boston, Mass., and from a similar colony of the same inbred C3Hf/He line maintained by the Department of Cancer Therapy Development at the Pondville Hospital.3 Both male and female C3Hf/He mice were used as serum donors.

Tumors. The fibrosarcoma had been induced in a female C3Hf/He mouse by methylcholanthrene and kept in liquid nitrogen. It was reintroduced into syngeneic mice to be used in these experiments in the 3rd to 6th transplant generations for

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2The mice carry only the following enteric bacteria: Clostridium sp., Bacteroides sp., Peptostreptococcus sp., Bacillus sp.

3This work was started at the Massachusetts General Hospital and completed at the Pondville Hospital.

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sensitizing implantations and for preparation of tumor-cell suspensions. The mammary carcinoma had developed spontaneously in a multiparous C3H/He mouse. It had also been kept in liquid nitrogen and was used in this study in the 3rd and 4th transplant generations.

Tumor Implantation. Tumor tissue was removed from freshly killed or live anesthetized donor animals, and skin and necrotic tissue were removed before the tissue was placed in tissue culture medium in a sterile Petri dish kept on a bed of crushed ice.

Implantation of two 1 cu-mm pieces of living tumor tissue s.c. was used to initiate antitumor immunity. An incision was made in the skin of the right flank, and the pieces of tumor tissue were placed under the skin by means of a trocar. The incision was closed with a wound clip. In all of the experiments reported here, s.c. sensitization procedures were done on the right side of the animals, and s.c. challenge implantations were done on the left side. Challenge implantation of presensitized and control mice was by injection of viable (trypan blue-negative) tumor cells suspended in TC Medium 199 (Difco Laboratories, Inc., 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 of tumor tissue with the use of 110 mesh polyester cloth (PE 162 screen cloth, from TET/Kressilk, Elmsford, N. Y.) has been described in a previous publication (37).

Surgical Procedures. Removal of tumors implanted s.c. was done under pentobarbital anesthesia. A circular incision was made in the skin around the edge of the tumor, and the tumor was removed by blunt dissection. A cautery set equipped with pencil handle and platinum eye tip (National Statham Inc., Elmhurst, N. Y.) was used to close bleeders. The incision was closed with wound clips.

Statistical Analyses. The effect of treatment is described in terms of differences in tumor incidence and in tumor size following challenge.

For comparison of tumor growth, Student's t test was used to evaluate differences between 2 groups of mice. The test for linear trend in proportions (28) was used for evaluation of differences between more than 2 groups.

Differences between groups were considered significant only when p was 0.05 or smaller.

RESULTS

The Effect of Serum, Presumed to Contain Tumor Antigen, on the Resistance of the Host to Tumor Challenge. In the 1st experiment, male C3Hf mice that were to serve as serum donors were first given 400 R of whole-body X-radiation. Beginning on the day of the radiation exposure, the mice were given 2 daily administrations of 50 mg inocula of radiation-killed (5000 R) fibrosarcoma cells suspended in 0.5 ml of 0.85% NaCl solution injected both i.p. and s.c. Other mice were similarly given injections of 50-mg doses of killed mammary carcinoma cells. The injections were given for 6 consecutive days. The last injections were given in the morning, and the mice were bled out by cardiac puncture in the afternoon. The pooled serum was stored at -11°F.

The experimental mice had been prepared in the following manner. Twenty-six days before the day of challenge, the mice received 1 s.c. sensitizing implant of a 1 cu-mm piece of living fibrosarcoma in the right flank. On the 26th day of growth, when the tumors were, on the average, 15 x 15 mm, the tumors were removed surgically. Previous studies (33, 34, 36, 37) have shown that, with a tumor that large, the host would be in a state of depressed concomitant immunity and that immune recovery would follow soon after tumor excision. The surgically cured mice were divided into 4 groups of 10 mice each, and their resistance level was tested on the day following surgery by 2 simultaneous challenge inocula of 10^6 suspended living fibrosarcoma cells injected s.c. at the left shoulder and at the left hip. Groups of unsensitized, untreated control mice were challenged with cell suspensions containing 3.3 x 10^5, 1 X 10^6, or 3.3 x 10^4 living cells per inoculum to confirm that the challenge dose of 10^6 cells for the experimental animals constituted neither an excessive nor an insufficient dose. All mice in 1 experiment were challenged at the same time with tumor cells from the same cell suspension.

The mice in experimental Group 1 received no treatment other than surgery. The mice in Group 2 were given s.c. injections of 5 mg of radiation-killed fibrosarcoma cells in the right flank on the day of challenge and for 4 consecutive days thereafter. Previous studies (33, 34, 36, 37) have shown that this procedure will prevent the recovery from specific immune impairment that normally follows the excision of a large antigenic tumor. The mice in Group 3 were given i.p. injections of 0.5 ml of serum from donors treated with killed fibrosarcoma cells on the day of challenge and for 4 consecutive days thereafter. The mice in Group 4 were given i.p. injections of 0.5 ml of serum from donors treated with killed mammary carcinoma cells on the day of challenge and for 4 consecutive days thereafter.

The incidence of tumors at the challenge injection sites was checked at weekly intervals and the size of the tumors was measured with calipers and recorded from the time they became palpable. By measuring 2 bisecting diameters of each tumor and using only the lesser of the 2 diameters to indicate tumor size, the possible error of measuring the added diameters of more than 1 focus of growth arising along the needle path of implantation was avoided and a better indication of tumor growth was achieved. Each experiment was terminated when more than 1 mouse in any group became cachectic because of progressive tumor growth. The mean values per group at the last recording of tumor sizes are presented in the tables. This procedure was used for each experiment in this investigation.

Table 1 presents data from 2 similar experiments that followed the same procedures to test the reproducibility of the results. Since the results of the 2 experiments were similar, the data have been combined. Statistical evaluations of the differences in tumor development following challenge show that resistance was strong in the mice treated with tumor resection only (Group 1 versus Group 6) and that the resistance was impaired by the injection of killed fibrosarcoma cells (Group 2) or by the injection of serum from immunodepressed and fibrosarcoma-treated mice (Group 3) but not by the injection of serum from immunodepressed mice treated with an antigenically unrelated mammary carcinoma (Group 4).
Antigen and Antibody in Depressed Tumor Immunity

Table 1
The effect of injection of killed FS<sup>a</sup> cells, or serum from immunodepressed, FS-treated mice, on the resistance of the host to FS challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of cells injected</th>
<th>No. of tumors&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Av. tumor size (mm)&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Surgery only</td>
<td>$1 \times 10^3$</td>
<td>8/40</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>Surgery + killed FS cells</td>
<td>$1 \times 10^5$</td>
<td>18/40</td>
<td>4.95</td>
</tr>
<tr>
<td>3</td>
<td>Surgery + FS serum</td>
<td>$1 \times 10^4$</td>
<td>15/40</td>
<td>4.13</td>
</tr>
<tr>
<td>4</td>
<td>Surgery + MC serum</td>
<td>$1 \times 10^4$</td>
<td>6/40</td>
<td>1.50</td>
</tr>
<tr>
<td>5</td>
<td>Unsensitized</td>
<td>$3.3 \times 10^5$</td>
<td>40/40</td>
<td>12.60</td>
</tr>
<tr>
<td>6</td>
<td>Unsensitized</td>
<td>$1 \times 10^4$</td>
<td>31/40</td>
<td>10.08</td>
</tr>
<tr>
<td>7</td>
<td>Unsensitized</td>
<td>$3.3 \times 10^4$</td>
<td>29/40</td>
<td>8.70</td>
</tr>
</tbody>
</table>

<sup>a</sup>FS, fibrosarcoma; MC, mammary carcinoma.

<sup>b</sup>The sensitized tumors were implanted s.c. in the right flank and removed after 26 days of growth at an average size of about 15 x 15 mm. Treatment consisted of (a) surgical removal of the tumors, (b) surgery plus injections of suspensions of killed FS cells, or (c) surgery plus injections of serum from radiated mice treated with suspensions of killed FS or MC cells (see text).

<sup>c</sup>All mice were challenged with s.c. injections of tumor cells at the left shoulder and hip. Group 6 differs significantly from each of Groups 1 to 4. Group 1 (or Group 4) versus Group 2 (or Group 3), $p < 0.05$; Group 1 (or Group 4) versus Group 2 (or Group 3) versus Group 6, $p < 0.001$.

<sup>d</sup>Only the differences in average tumor size have been evaluated statistically.

<sup>e</sup>The average value for each group was derived by dividing the sum of the tumor size by the total number of s.c. challenge implantations (2/mouse) in each group.

4). These results thus confirm and expand previously published observations of specific depression of immune protection by residual or injected tumor tissue (33, 34, 37) by indicating that circulating tumor antigens may interfere with the expression of the antitumor resistance in sensitized mice.

The Effect of Immune Serum versus the Effect of Serum Presumed to Contain Tumor Antigen, on the Resistance of the Host to Tumor Challenge. The next experiment was designed to determine any possible effects on host resistance if serum, presumed to contain specific antibodies but no homologous antigen, were to be administered along with serum presumed to contain tumor antigen but no homologous antibodies and known to impair resistance in sensitized mice.

As in the previously described experiment, male C3Hf serum donors were given whole-body X-radiation followed by a series of injections of killed fibrosarcoma cells before the serum was collected.

The immune serum used in this experiment was taken from female C3Hf mice first used in other experiments either as controls or as experimental animals. The mice all carried a progressively growing s.c. fibrosarcoma or mammary carcinoma implant. Any additional exposure had for these mice been limited to injections of killed tumor tissue. The tumors were surgically removed and 1 week later the mice were given s.c. booster injections of 5 mg of killed fibrosarcoma or mammary carcinoma cells in 0.2 ml of 0.85% NaCl solution. One week later the mice were bled out by cardiac puncture. The antiserum for each tumor was pooled and stored at $-11^\circ$.

The experimental protocol followed the general procedures of the previously described experiment. The experimental mice in groups of 10 were prepared with sensitizing s.c. fibrosarcoma implants followed by surgical removal at an average size of about 15 x 15 mm. On the day following surgery, the mice were challenged and the treatment was started. The mice in experimental Group 1 received no treatment other than surgery. The mice in Group 2 were given i.p. injections of 0.5 ml of serum from radiated donors treated with killed fibrosarcoma cells on the day of challenge and for 4 consecutive days thereafter. The mice in Group 3 were given i.p. injections of both 0.5 ml of serum from the radiated donors and 0.5 ml of fibrosarcoma antiserum on the day of challenge and for 4 consecutive days thereafter. The mice in Group 4 were treated like the mice in Group 3 but were given mammary carcinoma antiserum in place of the fibrosarcoma antiserum. The i.p. injections of antiserum preceded the i.p. injections of serum from radiated donors each time.

The incidence and size of tumors at the challenge injection sites were checked and recorded as in the previously described experiment.

Table 2 presents the combined results of 2 separate but similar experiments. Statistical evaluation of the differences in tumor development following challenge shows that resistance was strong in the mice treated with tumor resection only (Group 1 versus Group 6) and that the resistance was impaired by the injection of serum from immunodepressed and fibrosarcoma-treated mice (Group 2). This impairment was averted, partially but significantly, by the additional injection of fibrosarcoma antiserum (Group 3) but not by the additional injection of mammary carcinoma antiserum (Group 4).

DISCUSSION

The question posed in this study, of whether the serum of a tumor host may contain some factors that may impair and
The effect of injection of serum from immunodepressed, FS-treated mice and serum from immunocompetent, FS-treated mice on the resistance to FS challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of cells injected</th>
<th>No. of tumors</th>
<th>Av. tumor size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Surgery only</td>
<td>$1 \times 10^3$</td>
<td>12/40</td>
<td>2.78</td>
</tr>
<tr>
<td>2</td>
<td>Surgery + FS serum</td>
<td>$1 \times 10^3$</td>
<td>22/40</td>
<td>5.53</td>
</tr>
<tr>
<td>3</td>
<td>Surgery + FS serum + FS antiserum</td>
<td>$1 \times 10^3$</td>
<td>16/40</td>
<td>3.65</td>
</tr>
<tr>
<td>4</td>
<td>Surgery + FS serum + MC antiserum</td>
<td>$1 \times 10^3$</td>
<td>25/40</td>
<td>5.75</td>
</tr>
<tr>
<td>5</td>
<td>Unsensitized</td>
<td>$3.3 \times 10^3$</td>
<td>40/40</td>
<td>12.68</td>
</tr>
<tr>
<td>6</td>
<td>Unsensitized</td>
<td>$1 \times 10^3$</td>
<td>40/40</td>
<td>11.53</td>
</tr>
<tr>
<td>7</td>
<td>Unsensitized</td>
<td>$3.3 \times 10^3$</td>
<td>35/40</td>
<td>9.08</td>
</tr>
</tbody>
</table>

* a FS, fibrosarcoma; MC, mammary carcinoma.
* b The sensitizing tumors were implanted s.c. in the right flank and removed after 26 days of growth at an average size of about 15 x 15 mm. Treatment consisted of (a) surgical removal of tumors, (b) surgery plus injections of serum from radiated mice treated with suspensions of killed FS cells, (c) same treatment as b plus injections of serum from FS-immune mice, (d) same treatment as b plus injections of serum from MC-immune mice (see text).
* c All mice were challenged with s.c. injections of tumor cells at the left shoulder and hip. Group 6 differs significantly from each of Groups 1 to 4. Group 1 (or Group 3) versus Group 2 (or Group 4), p < 0.05; Group 1 (or Group 3) versus Group 2 (or Group 4) versus Group 6, p < 0.001.
* d Same as Table 1.
* e Same as Table 1.

The results of this investigation showed that serum factors, presumed but not proven to be antibodies, may also act in immune protection by neutralizing excessive free tumor antigens to maintain or to promote the recovery of protective immunity.

The results of the present in vivo tests do not exclude the possibility that growth-enhancing factors other than tumor antigens could have been present in the enhancing serum and do not permit any conclusion that may be at variance with the well-documented in vitro phenomenon of serum factors that can block cell-mediated destruction of neoplastic target cells.

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The positive role of antibodies in host resistance to solid tumor growth has been observed by investigators using s.c. (4, 22), i.m. (10, 13, 23), or i.v. (38) tumor implantation sites, under conditions where the protective effect could be assumed to be due to antibody-mediated or cell-assisted cytotoxic effects. The results of this investigation showed that immune serum factors, presumed but not proven to be antibodies, may also act in immune protection by neutralizing excessive free tumor antigen to maintain or to promote the recovery of protective immunity.

The results of the present in vivo tests do not exclude the possibility that growth-enhancing factors other than tumor antigens could have been present in the enhancing serum and do not permit any conclusion that may be at variance with the well-documented in vitro phenomenon of serum factors that can block cell-mediated destruction of neoplastic target cells.
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