Mammary Tumor Immune Enhancement in Mice by Local Hyperemia

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ABSTRACT

The mammary carcinoma MC2 causes a strong immune response in syngeneic, female C3H/He mice, and growing s.c. implants will regress spontaneously in about 20% of untreated hosts. Hyperemia is part of the local immune reaction against MC2 implants. An accelerated local hyperemic reaction at MC2 implants could be created in normal mice by the i.p. injection of blood or plasma from MC2 hosts at an early (Day 17) stage of immunization. In these mice given injections, MC2 growth was enhanced, and the incidence of spontaneous regressions was reduced. Local hyperemia caused by heat also promoted the growth of MC2 implants. In contrast, injections of blood or plasma from MC2 hosts at an advanced (Day 35) stage of immunization reduced the growth of MC2 implants and increased the incidence of their spontaneous regression. It is concluded that increased blood supply to a tumor in the absence of adequate systemic immunity favors tumor growth, and that this represents a new, additional mechanism in the immune enhancement phenomenon.

INTRODUCTION

Some important areas of investigation in tumor immunology are relatively underemphasized. As an example, the role of humoral immunity against neoplasms has received relatively little attention vis-à-vis the study of cellular effector mechanisms. One reason for this uneven research emphasis derives from serum enhancement and blocking antibody theories, which generated concern that B-cell activation and antibody production could promote neoplastic growth. The demonstration, that soluble, free tumor antigen rather than antibodies is the significant factor blocking the expression of immunity (1, 2) and that antibodies may be particularly effective against the dissemination of cancer (3–5), has done much to revitalize interest in positive aspects of humoral anticancer mechanisms. But, immune mechanisms and relationships are rarely simple. The immune responses that are essential to our survival can also be inadequate, improper, or adverse. Antibody-mediated tumor enhancement (6) and lymphocyte-mediated stimulation of tumor growth (7) are examples of incompletely understood adverse immune effects.

The purpose of this investigation was to discover why injections of plasma from immunized mice may either enhance or impede the growth of implants of the syngeneic mammary carcinoma MC2.

MATERIALS AND METHODS

Mice. All the animals used in these experiments were 8- to 10-wk-old inbred female C3H/He mice, raised and kept in an infection-controlled environment.

Received 1/28/85; revised 4/16/85, 6/28/85; accepted 10/2/85.

1 Supported by National Cancer Institute Grant CA-29660 and by a grant from Concern Foundation.

Tumors. The mammary carcinoma MC2 developed spontaneously in a multiparous C3H/He mouse and has been serially transplanted in syngeneic female mice. The sixth transplantation generation is stored in liquid N2, and the tumor was used in the seventh to 12th transplant generations. This immunogenic tumor has the characteristic of being rejected, after reaching a size of as much as 10 mm (average, 6.5 mm), by about 20% of untreated, unimmunized syngeneic female hosts. An MC2 implant may also, in occasional mice, show a period of growth, followed by a static period of 3 to 8 wk (dormancy?), which may end in reversion or renewed growth.

Mammary carcinoma 21-6 was the sixth spontaneous tumor removed from C3H/He Mouse 21. It had been shown in repeated tests to be immunogenic in transplantation assays; i.e., the C3H/He hosts cured of tumor 21-6 were resistant to the growth of reimplants of the same tumor (8). In these experiments, it was used in the third to sixth transplant generations. Tumors MC2 and 21-6 have been shown to be immunologically distinct in syngeneic mice (9).

Tumor Implantation. Tumor tissue was removed from untreated, freshly killed or live mice under the short-acting methoxyflurane anesthetic Penthrane (Abbott Laboratories, North Chicago, IL). The soft, translucent, viable tumor tissue was separated from the necrotic tumor and the surrounding host stroma by careful microdissection. The proportion of viable tissue was determined by the examination of hematoxylin-eosin-stained cryostat sections and was usually found to be greater than 80%. Two 1-mm³ pieces of living tumor tissue were implanted s.c. by trocar in the right flank to initiate new growth and to immunize test animals. Test implants for short-term histological studies were single 3-mm³ pieces of tumor placed whole in the loose s.c. connective tissue of the left flank with the point of fine scissors. The 5-mm incision in the skin was closed with a woundclip.

Tumor Measurements. The incidence of tumors at the s.c. implantation sites was checked at 7-day intervals, and the tumor size was measured with calipers. By measuring two bisecting diameters of each tumor and using only the lesser diameter to express tumor size, the possible error of measuring the added diameters of more than one focus of growth along the trocar path of implantation was avoided. Mice with progressively growing tumors were killed before they became cachectic. The mean tumor sizes 1 wk before any of the mice were killed (34 to 36 days after tumor implantation) are presented. The reported incidences exclude any tumor that eventually regressed during a 12-wk period of observation.

Mitotic Index. Two MC2 pieces were implanted, 1 cm apart, into the left flank of each mouse. One of the implants was removed for mitotic indexing on the sixth day after implantation. The mice were given one i.p. injection of 0.025 mg (1 mg/kg) of Vinristine sulfate (courtesy of Eli Lilly Co., Indianapolis, IN) 1, 2, or 4 h before the implants were removed for formalin fixation and histological sectioning. The 2-h interval was found to be optimal and was used in the present study. For each of ten randomly selected tumors per group (Table 1), 500 cells on each of three sections were counted, using a micrometer grid. The two implants were left in the mice in order to compare the mitotic indexes with the progressive growth of the tumors.

Blood Flow. The functional state of blood vessels in and around tumors was compared in actively and passively immunized mice. The mice received an injection of 0.1 ml of 0.02% resorcin-crystral violet in phosphate-buffered saline (10) via the left ventricle. The tumors with the overlying skin and adherent s.c. tissue were removed from anesthetized mice for histological examination 5 min after the injection. Quantitative
The tumor incidences in the following groups are significantly different (P < 0.05): Group 1 versus Groups 2 to 7; Group 2 versus Groups 4 to 7; Group 3 versus Groups 4 to 7. The mean tumor sizes in the following groups are significantly different (P < 0.05): Group 1 versus Groups 2 to 7; Group 2 versus Groups 4 to 7; Group 3 versus Groups 4 to 7.

### Table 1

**Transfer of MC2 host blood and plasma to MC2 implant recipients**

The tumor incidences in the following groups are significantly different (P < 0.05): Group 1 versus Groups 2 to 7; Group 2 versus Groups 4 to 7; Group 3 versus Groups 4 to 7. The mean tumor sizes in the following groups are significantly different (P < 0.05): Group 1 versus Groups 2 to 7; Group 2 versus Groups 4 to 7; Group 3 versus Groups 4 to 7.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment†</th>
<th>Mitotic index</th>
<th>Incidence</th>
<th>Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Immunized</td>
<td>3.1 ± 0.2</td>
<td>15/52 (47)</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>MC2 host blood</td>
<td>4.4 ± 0.5</td>
<td>25/55 (100)</td>
<td>7.8 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>MC2 host plasma</td>
<td>5.4 ± 0.6</td>
<td>18/20 (90)</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>21-6 host blood</td>
<td>4.8 ± 0.3</td>
<td>10/12 (83)</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>Normal blood</td>
<td>4.8 ± 0.2</td>
<td>20/25 (80)</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>6</td>
<td>Normal plasma</td>
<td>4.8 ± 0.3</td>
<td>9/12 (75)</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>Untreated</td>
<td>4.8 ± 0.4</td>
<td>39/49 (80)</td>
<td>6.1 ± 0.6</td>
</tr>
</tbody>
</table>

† Numbers are the results of five separate experiments. Group 3 was included in the experiments, and Groups 4 and 5 were included in two of the experiments. Each mouse given an injection received 1 ml of heparinized blood from Day 17 MC2 hosts 18 h before receiving s.c. MC2 test implants. An implant was removed after 6 days to determine the mitotic index. The second implant remained to determine incidence and mean size.

### RESULTS

**Immune Enhancement.** Table 1 shows the results of an experiment that compared the growth of MC2 implanted into the left flank of mice in seven groups: Group 1, mice at a moderate level of concomitant immunity by already carrying a 17-day-old MC2 implant in the right flank; Group 2, mice given injections i.p. with 1 ml of heparinized blood from Day 17 MC2 hosts 18 h before receiving the left flank implant; Group 3, like Group 2, but given MC2 host plasma; Group 4, like Group 2, but given tumor 21-6 host blood; Group 5, like Group 2, but given normal blood; Group 6, like Group 2, but given normal plasma; and Group 7, untreated mice.

The results show that the injection of whole blood or plasma from Day 17 MC2 hosts resulted in enhanced tumor growth. The injection of blood from hosts of the immunologically unrelated mammary carcinoma 21-6, or of blood or plasma from normal mice, had no effect on the recipients. In the Day 17 MC2 hosts (Group 1), the growth of second MC2 implants was inhibited, and rejections and regressions were more frequent than in untreated mice.

Figs. 1 and 2 compare the tumor-stroma interface of 6-day MC2 implants from Groups 1 and 2 (Table 1). Fig. 1 shows a tumor from Group 1 which is separated from an area of active hyperemia by a stromal layer containing numerous lymphocytes and macrophages. The MC2 implant has an area of central necrosis and a peripheral layer of live cells that has moderate mitotic activity and shows little tendency to invade the stroma. Fig. 2 presents a very different situation. The tumor implant from Group 2 is invading the host tissue, growing through the cutaneous muscle layer (panniculus carnosus) toward an area of active hyperemia. Mitotic figures are frequent.

**Local Hyperemia.** To further study the relationship of passive and active immunization to local hyperemia, the histology of the vascular reaction at s.c. implants of MC2 was surveyed in the following groups of mice: Group 1, mice given injections i.p. with 1 ml of heparinized normal mouse blood 18 h before implantation; Groups 2 to 5, mice already carrying 1- to 7-day-old MC2 implants; and Group 6, mice given injections i.p. with 1 ml of heparinized blood from Day 17 MC2 hosts.

The results presented in Table 2 show the following. (a) The development of active hyperemia at the MC2 implant in mice given normal blood was related to the development of systemic immunity. Systemic immunity has been shown to be demonstrable 1 wk after MC2 implantation (9). (b) In tumor hosts, the development of hyperemia at the MC2 test-implant was shortened by a period close to the length of the immunizing MC2 exposure. (c) The injection of whole blood from MC2 hosts caused a rapid development of hyperemia at the MC2 implant.

To determine the effect of physically induced local hyperemia on tumor growth, the growth of MC2 was compared in heat-treated and in normal s.c. sites on the same animal. Table 3 shows the combined results of two identical tests. The results

### Table 2

**Hyperemia at MC2 implants in actively and passively immunized mice**

<table>
<thead>
<tr>
<th>Day test implant removed</th>
<th>Normal blood</th>
<th>Immunizing MC2 implanted</th>
<th>MC2 host blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Day 2</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 4</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3

**MC2 growth in heat-treated skin**

A 10-mm circle of skin in the left flank was heated to produce an implantation site with a first degree burn. The normal implantation site was in the right flank of the same mouse.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated</td>
<td>17/18 (94)</td>
<td>10.1 ± 1.0</td>
</tr>
<tr>
<td>Normal</td>
<td>13/18 (72)</td>
<td>6.4 ± 0.8</td>
</tr>
</tbody>
</table>

† Numbers in parentheses, percentage.

‡ Mean ± SE. The two groups are significantly different (P < 0.05).
show that the repeated application of heat sufficient to cause a first degree burn also prepared the conditions for improved tumor growth.

**Passive Immunity.** In other studies of the local immune reaction against MC2 implants (11), *in vitro* lytic activity against MC2 cells developed in the serum of MC2 hosts only after at least 1 mo of immunization, and most predictably in mice with regressor or dormant tumors. In this experiment, blood and plasma from mice that had been cured of 35-day s.c. implants were tested for their ability to transfer passive immunity to MC2 implant recipients.

The results presented in Table 4 show that mice cured of 35-day MC2 implants had become fully resistant to a second MC2 implant. Whole blood, and to a slightly lesser degree, plasma, from cured MC2 hosts transferred a significant degree of resistance. The transfer of blood from mice cured of 35-day implants of tumor 21-6 and the transfer of normal blood and plasma were without effect on the growth of MC2 implants.

**DISCUSSION**

The phenomenon of immune enhancement of tumor growth has, since it was first described by Casey in 1932 (12) and investigated by Snell in 1954 (13), remained incompletely understood (6) (review by Winn), despite intensive study by a number of investigators. Central inhibition by specific antibody (6), immune suppression by regulatory antibodies (14), and immune suppression by regulatory T-suppressor cells (15-17) are some of the mechanisms that have been proposed.

This paper presents novel findings which suggest that factors in the plasma (and whole blood) of tumor hosts with incompletely developed concomitant immunity can cause active local hyperemia and the enhanced growth of tumors implanted into normal plasma recipients. This condition was only part of the early phase of a normal immune response, and factors in the blood of mice with fully developed concomitant immunity provided enhanced protection to normal recipients. Increased blood supply to a tumor implanted into a nonimmune host would favor tumor growth and is an additional mechanism not previously considered in explanations of the immune enhancement phenomenon. But in an adequately immunized host, on the other hand, hyperemia is an important factor in the defensive reaction. The conditions for demonstrating immune enhancement can therefore only exist in animals without adequate systemic immunity, and enhancement has, in fact, never been observed in preimmunized recipients (6).

The demonstration (Table 3) that tumor growth was enhanced where hyperemia had been induced by heat only shows that this condition in a simple form will also promote tumor growth. The demonstration supports the speculation that hyperemia may be a factor in immune tumor enhancement, but without suggesting any mechanistic relationship between two different methods of demonstration.

Accordingly, it may be suggested that the enhanced tumor growth in Group 2 (Table 1) was related to the active hyperemia shown in Fig. 2. The restrained tumor growth in Group 1 (Table 1) was related to the strong cellular infiltration in Fig. 1, which may have been facilitated by the local hyperemia around the implant.

**REFERENCES**


Fig. 1. Restrained growth of MC2, 6 days after implantation into a mouse carrying a 17-day-old implant. A narrow band of live tumor cells separates the necrotic center (right) and the stroma infiltrated by lymphocytes and macrophages. Bar, 100 μm. H & E, x 100.

Fig. 2. Rapidly growing (from right to left) MC2, 6 days after implantation into mice given injections of whole blood from MC2 hosts. Bar, 100 μm. H & E, x 100.
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