Depletion of Lymphocyte Subpopulations in Primary and Secondary Lymphoid Organs of Mice by a Transplanted Granulocytosis-inducing Mammary Carcinoma

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ABSTRACT

Transplanted CE mammary carcinoma causes a marked increase in the production of neutrophilic granulocytes in mice associated with the expansion of hemopoietic marrow into the peripheral skeleton. Changes in lymphocyte populations in the femoral marrow, the expanding peripheral marrow, and the spleen were examined for a period of 3 weeks post-tumor transplantation using fluoresceinated antisera specific for B- and T-cells. As tumor growth and granulocytic hyperplasia progressed, B- and T-cells became reduced in femoral marrow and the spleen, but lymphocytes of undefined function, devoid of T- and B-cell surface antigens (null cells), transiently increased in femoral marrow and in the spleen. In the expanding peripheral marrow, such null cells increased and remained as the predominant cell type until granulocytic hyperplasia took place. These changes suggest shifts in the site of myelogenous lymphocyte production or in the differentiation program of lymphocytes. The thymus invariably showed marked atrophy as which, as shown in adrenalectomized animals, could not be explained entirely by tumor-induced stress. Thus, the massive granulocytopenic effects of CE mammary carcinoma are coupled with marked changes in lymphocyte populations due, most likely, to the tumor’s influence on primary lymphoid organs.

INTRODUCTION

A number of cells known to be involved in antitumor responses are derived from the bone marrow. These include NK cells as well as macrophage precursors, which may participate in antitumor responses in a variety of ways, and B-cells, which eventually secrete antibody required for antibody-dependent cell-mediated cytotoxicity. The production of these potential effector cells may be influenced by neoplasms which perturb the normal pattern of hemopoiesis. CE mammary carcinoma has been found to induce a marked neutrophilic response in the blood and the bone marrow, and mice transplanted with this tumor have (a) greatly augmented rates of neutrophil production (15), (b) an increased population of granulocyte-macrophage-committed stem cells (17), and (c) fatty marrow in their peripheral skeleton replaced by hemopoietic marrow which initially consists of lymphoid cells and is later replaced by predominant granulocytopenia (16). The objective of the present study was to gauge the effect of the granulocytosis-inducing tumor on the lymphocyte population of the bone marrow and thymus, which are considered primary lymphoid organs, and in the spleen, which is considered a secondary lymphoid organ. Following the transplantation of CE mammary carcinoma, we have quantitated changes in lymphocyte subpopulations in femoral bone marrow, in the expanding marrow of the metatarsal bones and caudal vertebral, and in the spleen. B- and T-cells were identified with fluoresceinated antisera, and changes in null cells were computed from the total lymphocyte count. In addition, we have examined the effects of CE mammary carcinoma on the thymus in order to assess the extent to which the splenic changes might be a reflection of altered lymphocytopoiesis in the 2 primary sites of lymphocyte production. To test whether or not some of the observed changes could be attributed to tumor-induced stress, we have repeated experiments in adrenalectomized mice. For comparing the stress effect of CE mammary carcinoma, 1460 MCA was used which has no obvious hemopoietic effects.

MATERIALS AND METHODS

Mice. BALB/c and CE mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). The experiments were performed on 30 female CE and 120 male and female BALB/c x CE F1 (hereafter called CCEF,) mice bred in the vivarium, University of Washington. Mice were killed at the age of 14 to 16 weeks in groups of 3 at various time intervals after transplantation of the tumor.

Tumor Cells. During the past 2 years, CE 1460 mammary adenocarcinoma (7) used in these studies has been passaged in our laboratory through CE, CCEF, and CBA/Ca strains of mice by s.c. inoculation at 2 to 3 weekly intervals. All of these mice are of the H-2b haplotype, and we were unable to grow the tumor in several other mouse strains of haplotypes other than H-2b. Dr. I. and Dr. K. E. Hellström (Fred Hutchinson Cancer Research Center, Seattle, Wash.) kindly donated 1460 MCA. Tumor cell homogenates were prepared as described previously (15), and approximately 1 x 106 viable tumor cells were injected s.c. in the flank of mice using 0.2 ml of the crude homogenate. The growth of both tumors was localized without metastases, and the CE 1460 carcinomas reached diameters of approximately 2 cm 3 to 4 weeks after inoculation.

Adrenalectomy. Bilateral adrenalectomy (18) was performed on 14-week-old male CCEF, mice under pentobarbital anesthesia, supported by preoperative injection of 0.25 mg hydrocortisone acetate (Merck Sharp & Dohme, West Point, Pa.). Postoperatively, mice received 1 ml of 0.9% NaCl solution i.p., and their drinking water was replaced by an acidified solution of 0.9% NaCl solution. Age- and sex-matched sham-operated mice received the same pre- and postoperative treatment. Ten days after the operation, tumor cells were inoculated as described. Mice were killed in groups of 4 at various time intervals, and the wet weight of the thymus and thymic cellularity was recorded.

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2 To whom requests for reprints should be addressed, at Division of Hematology, Department of Medicine RM-10, University of Washington School of Medicine, Seattle, Wash. 98195.
3 The abbreviations used are: NK, natural killer; 1460 MCA, methylcholanthrene-induced fibrosarcoma; CM, caudal-metatarsal bones.

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Blood Cell Counts. Just before the mice were killed, leukocyte concentration in orbital sinus blood was determined by Coulter Counter. Two hundred leukocytes were classified on Wright-Giemsa-stained smears.

Bone Marrow and Spleen Cell Suspension. One femur from each mouse and all segments of caudal vertebrae as well as 10 metatarsal bones from each mouse (CM) were isolated and placed in a Petri dish containing cold Hanks' balanced salt solution. Cells were always suspended in ice-cold medium consisting of Hanks' balanced salt solution, 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), and 0.1% Na$_2$PO$_4$ (referred to hereafter as "medium").

Marrow cell suspensions were prepared using a modification of the method described by Carsten et al. (3). Bones were cut into small pieces, were placed in 3 ml of medium in a small porcelain mortar, and were crushed and ground with a pestle until no intact bone pieces remained. The solution was flushed up and down with a Pasteur pipet several times to obtain a single cell suspension. The cell suspension was then layered over 3.0 ml of cold Hanks' balanced salt solution containing 35% fetal calf serum and 0.1% NaN$_3$ in a 15-ml plastic centrifuge tube. The remaining bone chips were ground similarly twice more, and a total of 8.5 ml of cell suspension was likewise layered into the same 15-ml test tube. After the tube was allowed to stand for 60 min in ice, the top 10.0 ml of the suspension were gently transferred to a second tube. This procedure allowed ground bone fragments to sediment to the bottom of the tube while marrow cells remained in the top layer. Only a negligible number of cells sedimented with the bone fragments. Spleen and thymus cell suspensions were prepared by pressing small pieces of the organ through lens paper in medium as described previously (20). The total number of nucleated cells was determined in femur and CM marrow and spleen cell suspensions using Turk solution and a hemocytometer.

Antisera. For surface staining of $B$-cells, goat anti-mouse IgM (Cappel Laboratories, Downingtown, Pa.) was used. $F(ab')_2$ fragments were obtained by pepsin digestion and were conjugated with fluorescein isothiocyanate (5). $T$-cells were identified by fluorescein-conjugated monoclonal anti-mouse Thy 1.2 (Becton, Dickinson and Co., Sunnyvale, Calif.).

Immunofluorescence. RBC were lysed osmotically in spleen and bone marrow suspensions (20), and in each instance, 2 x 10$^6$ cells in 50 $\mu$l medium were incubated on ice for 30 min with 25 liters of one of the appropriately diluted fluoresceinated antisera. Cells were then washed twice, and wet mounts were prepared from a drop of cell suspension for counting under a Leitz Ortholux II fluorescent microscope with a x100 oil immersion objective. Cells with circumferential bright green ring fluorescence were considered positive. Damaged cells were identified as cells with dull and uniform fluorescence. The remaining cell suspension was spun in the cytocentrifuge and stained with Wright-Giemsa for morphological examinations.

Cell Counts. On wet-mount preparations stained with fluorescein isothiocyanate-conjugated $T$- or $B$-cell reagents, 1000 consecutive cells were scored as fluorescence positive or negative or as damaged cells. On Wright-Giemsa-stained slides, 1000 consecutive cells were scored as lymphocytes, other hemopoietic cells, or damaged cells. The absolute number of lymphocytes and their $B$- and $T$-subclasses were calculated from the total nucleated cell counts, the differential counts on Wright-Giemsa-stained slides, and the counts obtained from the preparations stained with fluoresceinated antisera. The number of "null" or "double negative" lymphocytes was obtained by subtracting the number of $T$- and $B$-lymphocytes from the total number of lymphocytes. The results were expressed in absolute cell numbers of $T$-, $B$-, or "null" lymphocytes per femur, CM, or spleen.

RESULTS

Peripheral Blood. As illustrated in Table 1, the blood leukocyte response to the transplanted tumor was in agreement with previous findings (7, 15), and a remarkable neutrophilia was observed in both CE and CCEF, mice as the tumor progressed. The growth rate of the tumor was identical in both strains of mice. The number of blood lymphocytes remained essentially unchanged.

Marrow and Spleen Cellularity. In preliminary experiments, we tested the feasibility of studying marrow cells obtained by grinding the bones. It was found that the grinding produced approximately 20% more nucleated cells/femur than did flushing of the medullary cavity with culture medium (17). More than 92% of both femur and CM cells were viable immediately after grinding. Although in CM it was physically impossible to evaluate completeness of cell extraction from the bones, the ground marrow suspension from these small bones of tumor-bearing mice yielded adequate numbers of cells for study, and contaminating bone fragments were as few as in femoral cell suspensions.

The total number of nucleated cells in femoral marrow was unaffected in both strains by tumor transplantation (Table 2). As shown in Table 1, the splenic weight increased by about 50% at 1 week and decreased at 2 weeks after transplantation in both strains. In CE mice, the spleen remained small at 3 weeks, but in CCEF, mice, it enlarged again at 3 weeks. These weight changes were reflected also in splenic cellularity (Table 2).

Before transplantation of the tumor, the peripheral bone marrow was overwhelmingly fatty. An attempt to obtain marrow cells from CM of normal mice yielded only 6 x 10$^6$ cells when the bones of 10 animals were pooled. Therefore, quantitative cellular studies from these marrows were considered not feasible. By 1 week post-tumor transplantation, however, these peripheral marrows gave fairly consistent cell yields (Table 2). The pooled CM of a mouse with marked granulocytosis yielded almost as many marrow cells as a femur. Tumor cells were never seen in either the bone marrow or the spleen.

**Table 1**

<table>
<thead>
<tr>
<th>Wk post&lt;br&gt;tumor&lt;br&gt;transplant</th>
<th>Tumor diame&lt;br&gt;ter (mm)</th>
<th>Neutrophils (x 10$^5$/μl)</th>
<th>Lymphocytes (x 10$^7$/μl)</th>
<th>Thymus wt (mg)</th>
<th>Spleen wt (mg)</th>
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</thead>
<tbody>
<tr>
<td>Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>0</td>
<td>1.5 ± 0.9$^*$</td>
<td>6.7 ± 0.8</td>
<td>10.07 ± 1.00</td>
<td>7.10 ± 3.70</td>
</tr>
<tr>
<td>1</td>
<td>11.3 ± 1.2</td>
<td>16.8 ± 1.9</td>
<td>5.3 ± 0.9</td>
<td>1.88 ± 0.90</td>
<td>11.28 ± 10.90</td>
</tr>
<tr>
<td>2</td>
<td>17.3 ± 0.6</td>
<td>69.1 ± 13.2</td>
<td>3.0 ± 1.0</td>
<td>13.0 ± 8.9</td>
<td>55.8 ± 15.6</td>
</tr>
<tr>
<td>3</td>
<td>20.8 ± 1.3</td>
<td>71.4 ± 12.4</td>
<td>4.8 ± 2.4</td>
<td>11.5 ± 2.6</td>
<td>59.3 ± 9.0</td>
</tr>
<tr>
<td>CCEF</td>
<td>0</td>
<td>1.5 ± 0.8</td>
<td>5.6 ± 0.7</td>
<td>89.1 ± 18.0</td>
<td>80.8 ± 13.9</td>
</tr>
<tr>
<td>1</td>
<td>12.0 ± 1.3</td>
<td>9.0 ± 2.1</td>
<td>5.9 ± 0.9</td>
<td>10.64 ± 6.1</td>
<td>134.4 ± 22.8</td>
</tr>
<tr>
<td>2</td>
<td>16.8 ± 2.3</td>
<td>62.2 ± 14.0</td>
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</tr>
<tr>
<td>3</td>
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<td>135.5 ± 19.1</td>
<td>7.5 ± 1.9</td>
<td>7.6 ± 0.8</td>
<td>114.0 ± 58.2</td>
</tr>
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*$^*$ Mean ± S.D.
Granulocytopenia and Erythropoiesis. An overwhelming neutrophilic hyperplasia was observed in the femoral marrow and spleen of both strains, and by the end of the observation period, neutrophils predominated also in CM marrow. Erythropoiesis was markedly reduced in femur marrow, and erythroblasts were rarely found in CM marrow; but splenic erythropoiesis was maintained (Table 2).

Lymphocytes. The changes in the incidence of total lymphocytes and of their B- and T-subclasses in bone marrow and spleen are shown in Table 2. Chart 1 shows the effect of the tumor on the absolute numbers of B-, T-, and null or double-negative lymphocytes per organ.

The total number of lymphocytes decreased in femoral marrow following tumor inoculation, while in the CM of most mice, lymphocytes accounted for approximately 40% of cells over the 3-week period. However, the percentage of lymphocytes was also reduced in mice with the most severe neutrophilia. The total splenic lymphocyte population progressively decreased in both strains of mice (Table 2).

Concordant with our previous findings (21) in normal femoral marrow, about 50% of lymphocytes were B-cells. After tumor transplantation, their percentage and absolute number fell progressively in the femoral marrow, while in CM marrow, a considerable number of B-cells appeared as hemopoiesis expanded (Chart 1). The absolute size of the splenic B-cell population fell sharply from over $6 \times 10^8$ to less than 50% of the control value by Week 2 and remained low at 3 weeks.

T-cells accounted initially for less than 3% of femoral bone marrow lymphocytes and by 2 weeks were practically undetectable in femoral marrow in both strains. They appeared in CM marrow, but the incidence was much lower than that of B-cells, and in the spleen, their population decreased to less than 30% of control value (Chart 1).

Prior to tumor inoculation, null lymphocytes accounted for approximately 40% of the lymphocytes in femoral marrow. The absolute number of null lymphocytes in the femoral marrow of both strains of mice temporarily increased at 1 week but eventually decreased by the third week. In CM marrow, null cells were the predominant type of lymphocytes. In both strains, CM contained as many null lymphocytes as did a single normal femur (Chart 1). In the spleen, null lymphocytes temporarily increased at 2 weeks in CE mice and at 1 week in CCEF, mice, but as granulocytes increased, the number of splenic null cells declined in both strains (Chart 1).

Effect on the Thymus. The granulocytosis-inducing CE carcinoma produced pronounced thymic involution in both CE and CCEF, mice (Table 1). Two experiments were performed to test whether or not this thymic atrophy was due to a specific influence on the thymus or to nonspecific stress effects in the tumor-bearing animals. Since the hemopoietic effects of CE mammary carcinoma on CE and CCEF, mice were essentially identical as shown above and the same degree of thymic atrophy was observed in both strains (Table 1), the following experiments were performed using only CCEF, mice which were more readily available. In one experiment, 1460 MCA, which has no hemopoietic effects demonstrable in the blood, was transplanted into CCEF, hosts, and its effects on the peripheral neutrophil count and the thymus were compared with those observed in mice bearing a CE mammary carcinoma. In the other experiment, groups of mice were adrenalectomized along with sham-operated controls, and some members of each group received CE mammary carcinoma.

In contrast to the marked neutrophilia produced by the transplantation of CE mammary carcinoma, there was no such response to the 1460 MCA tumor of comparable size. The mean WBC count of 1460 MCA tumor-bearing mice remained close to base-line level [10.5 ± 1.6 (S.D.) x 10^3/μl] and 4 weeks after tumor transplantation was 11.7 ± 5.0 x 10^3/μl.
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Chart 1. Quantitative changes of marrow lymphocyte subpopulations in the femoral (F) and CM marrow and the spleen (SPL) of CE and CCEF, mice following transplantation of CE mammary carcinoma. •, B-cells; ▲, T-cells; ○, null cells. Bars, S.D.

The thymus of these mice under the presumed stress of the 1460 MCA tumor weighed 79.2 ± 15.3 mg at 4 weeks, not different from the control value (Chart 2), indicating that, unlike CE mammary carcinoma, the 1460 MCA tumor had no significant effect on granulocytosis or the thymus.

The adrenalectomy experiments revealed that the thymic atrophy seen with the CE mammary carcinoma could not be ascribed entirely to stress effects (Chart 3). In normal mice not transplanted with tumors, there was no change in thymus weight over a 16-day period, and adrenalectomized and sham-operated animals were comparable. Mice transplanted with the CE mammary carcinoma showed a significant fall in thymic weight on Day 14 in both adrenalectomized and sham-operated groups. In adrenalectomized mice by Day 16, there was an increase in thymus weight, and this was less evident in sham-operated mice. Thymic cellularity, for which the data are not shown, paralleled the changes in thymic weight in all groups of animals. Adrenalectomy did not alter the granulocytic response of tumor-bearing mice. The WBC counts of adrenalectomized tumor-bearing mice reached 47.1 ± 12.1 x 10³/µl at 14 days post-tumor transplantation, while those of sham-operated tumor-bearing mice and control mice were 41.8 ± 2.8 x 10³/µl and 9.5 ± 1.1 x 10³/µl, respectively.

DISCUSSION

A number of studies have documented the influence of a variety of tumors on hemopoiesis which was reflected in "leukemoid reactions," granulocytic hyperplasia of the bone marrow, and redistribution and quantitative changes in hematopoietic stem cells (1, 2, 4, 7, 12, 14, 17, 19, 22). A greatly augmented marrow granulocyte production to near 10 times the normal value in CE carcinoma-bearing mice has been well documented by our quantitative kinetic measurements (15). The present study was to investigate a possible impact of CE mammary carcinoma and/or the massive granulocyte proliferation on marrow lymphocyte populations. Some cells included in the marrow lymphoid population may participate in various antitumor host defense mechanisms, and some may have various stem cell functions (9, 23, 26).

The studies reported here demonstrate that transplanted CE mammary carcinoma causes a marked diminution in the lymphocyte population of bone marrow accompanied by thymic atrophy. Marrow lymphocyte loss was most pronounced in B-cells, while null cells in the marrow initially and transiently increased.

In newly developed marrow of the peripheral skeleton, the incidence of lymphocytes remained higher than in femoral marrow. Most of these cells were of the null surface phenotype, suggesting a shift in the site of marrow lymphocyte production. Splenomegaly, if present, was mainly due to extramedullary hemopoiesis, and both B- and T-cells decreased in the spleen. The decrease in lymphocytes in bone marrow, thymus, and spleen was not reflected in the peripheral blood. All these changes in response to the CE mammary carcinoma were essentially identical in the syngeneic CE strain and in CCEF, hybrid mice.
The changes observed in the spleen can be attributed at least partly to the diminished number of lymphocytes in the bone marrow and thymus. Lymphocytes are known to seed to secondary lymphoid organs from both the thymus and the marrow. Postnatally, in the normal animal, the bone marrow is the primary site for the origin of B-lymphocytes [reviewed by Rosse (23)], and practically all young B-cells in the spleen are direct immigrants from the marrow. Although the spleen can contribute significantly to B-cell production when hemopoiesis in the bone marrow is eliminated (11, 25), the spleen of animals bearing the CE mammary carcinoma had evidently not readjusted to reverse the decline in the splenic B-cell population, nor has the activated, vascularized, peripheral bone marrow assumed a major compensatory role in B-cell production. The total number of B-cells recovered from all caudal vertebrae and 10 metatarsal bones represented a mere fraction or, at most, the equivalent of the B-cell population normally present in a single femur. Thus, it is very likely that not only the splenic but the total marrow B-cell population was reduced in quantitative terms.

The accumulation of mononuclear cells in activated peripheral hemopoietic marrow was due chiefly to lymphoid cells devoid of both T- and B-cell surface antigens. As we have found previously, the CE mammary carcinoma exerts its granulocytopenic effect in the absence of the spleen (15), and bone marrow lymphocyte changes in splenectomized mice were the same as those in nonsplenectomized mice.* It is unlikely that the mononuclear cells observed in the peripheral marrow represent circulating blood lymphocytes which consist almost entirely of differentiated T- and B-cells. The largest population of null lymphocytes is found normally in hemopoietic marrow (21), where the majority of them will mature spontaneously into B-cells, while others can be induced in vitro to express T-cell membrane markers (23, 24). *"Null"* cells may include NK cells as well as a variety of stem cells or progenitor cells. That some of the lymphoid cells in activated fatty marrow have stem cell or progenitor cell potential is espoused by the observation that the granulocyte-macrophage colony-forming units content is increased in the peripheral bones of mice transplanted with CE mammary carcinoma (17). It is not known what proportion of the "null" cells observed in our studies are hematopoietic progenitors; pre-B-, pre-T-, or NK cells; or NK cell precursors.

Bone marrow T-lymphocytes declined in our tumor-bearing mice, and there was a significant fall also in the splenic population of T-cells. These changes are consistent with the thymic atrophy observed in mice bearing CE mammary carcinoma which could not be attributed to stress. These findings suggest that CE mammary carcinoma exerts an effect not only on the marrow but also on the thymus. At present, it is not possible to conclude what primary target various neoplasms influence in hemopoietic differentiation. The proliferation and differentiation of pluripotent stem cells are influenced by various regulatory factors as well as by organ-dependent microenvironment and cellular interactions (6). CE mammary carcinoma apparently promotes proliferation and differentiation of stem cells into an almost entirely granulocytic cell line. The observation that a granulocytosis-promoting factor responsible for granulocytosis (8) remains to be confirmed. Our findings raise the intriguing possibility of competition between granulocytic and lymphocytic progenitor cells in the marrow, but how such a competition is mediated awaits further experimentation. In view of initial increase in null lymphocytes, one must also consider the possibility that the differentiation program of newly produced lymphocytes may be altered under the influence of the tumor.

The marked decrease in the populations of bone marrow lymphocytes and of B-cells in general raises the question of what impairment the deletion of these cells may lead to in host defenses against the tumor. It has been shown that newly generated lymphocytes discharged from the marrow home to various neoplasms (10, 13), which apparently do not inhibit lymphocyte production. It remains to be determined what relationship such tumor-seeking marrow-derived lymphocytes may have to the null lymphocytes which can be recovered from the marrow and spleen of mice bearing CE mammary carcinoma, and whether or not these cells can contribute to antitumor responses of the host.

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