Augmentation of Neutrophilic Granulocyte Progenitors in the Bone Marrow of Mice With Tumor-Induced Neutrophilia: Cytochemical Study of In Vitro Colonies

By Minako Y. Lee and Joan L. Lottsfeldt

Transplantation of CE mammary carcinoma into mice has been shown to produce marked neutrophilia. Previous studies in vivo indicated a significant increase in marrow neutrophil production in these mice, but regulatory mechanisms of this neutrophilia have not been well understood. In order to obtain information about neutrophil production mechanisms at the progenitor cell level, the profile of marrow granulocyte-macrophage progenitors in mice with neutrophilia induced by this tumor was quantitatively analyzed by cytochemical staining of in vitro colonies to distinguish colonies of neutrophils (N-colony), macrophages (M-colony), and mixed cells (NM-colony). Cell cycle kinetics of progenitors were studied by in vivo administration of cytocidal drugs. The absolute number of N-colonies in a femur increased significantly and reached three times normal three to four weeks after tumor implantation. The number of NM-colonies also increased significantly by the fourth week, but the number of M-colonies was unchanged. The number of N-colonies in a femur related directly to the degree of neutrophilia. The increased number of N-colonies from the marrow of tumor-bearing mice was not attributed to a different time course of colony growth nor to a different sensitivity to CSA; instead, a significantly larger fraction of neutrophilic progenitors from the tumor-bearing mice were in active cell cycle than were those of normal mice. The day 14 tumor-bearing mouse serum demonstrated N-colony stimulating activity while the sera of normal mice and day 7 tumor-bearing mice were inhibitory for in vitro colony growth. These studies demonstrated an increase in the numbers and turnover rate of marrow neutrophil progenitors in CE tumor-induced neutrophilia, suggesting that this tumor stimulates proliferation of these progenitors in vivo.

STUDIES OF progenitor cells using in vitro colony formation have been applied widely in past years to investigate the mechanisms of granulopoiesis. Recent advances in the understanding of in vitro colony formation have revealed a wide range of heterogeneity in colony-forming cells (CFC) or colony-forming units in culture (CFU-c) concerning their proliferation and differentiation potentials as well as their responsiveness to stimulating factors. In addition, stimulating factors themselves are also heterogeneous regarding their origins and specific biologic activities.

Murine models of tumor-induced neutrophilia have been used by various investigators to study the mechanisms of neutrophil production by analysis of CFU-c. In particular, the murine models with tumors that demonstrate colony-stimulating activity (CSA) in vitro have attracted investigators to study the role of CSA in granulopoiesis in vivo. However, most of these studies revealed no increases in the femoral marrow CFU-c in spite of severe neutrophilia in tumor-bearing animals; consequently, the mechanisms of neutrophil stimulation in the marrow have remained unclear.

We have demonstrated previously by in vivo quantitative and kinetic measurements that the neutrophilia of mice bearing a transplantable CE mammary carcinoma was due to a marked increase in the neutrophil production rate from the marrow, suggesting the stimulation of this cell line in tumor-bearing animals. In order to understand the regulatory mechanisms of neutrophilic granulocyte production at the progenitor cell level, we have examined the profile of colony formation in this mouse model by applying cytochemical enzyme staining to colonies grown in vitro.

MATERIALS AND METHODS

Mice

Female BALB/c and male CE mice were obtained from the Jackson Laboratories (Bar Harbor, Me) and (BALB/c x CE)F1 hybrid mice were bred in the Vivarium at the University of Washington. All experiments were performed on (Balb/c x CE)F1 hybrid mice of both sexes between the ages of 12 and 16 weeks. Mice were killed in groups of four to six at various time intervals after transplantation of the tumor. Age- and sex-matched mice injected with Hank's balanced salt solution were used as controls.

Tumor Cells

CE mammary carcinoma was used to induce neutrophilia in the mice. The CE mammary carcinoma cell line, which has been maintained in our laboratory by a series of subcutaneous transplantsations in host mice (CE or [BALB/c x CE]F1 mice) was used. The transplantation of 3.5 to 5.0 x 10⁴ viable tumor cells, inoculated...
subcutaneously into the flanks of mice as described before \(^1\) invari-
ably produced significant neutrophilia two to three weeks after
tumor transplantation.

**Preparation of Cell Suspensions**

Two femora from each mouse were isolated and marrow cell
suspensions were prepared by grinding the whole femora using a
mortal and pestle as described previously. \(^{14}\) Spleen cells were
prepared by gently squeezing and teasing the organ with 18-gauge
needles in Hank’s balanced salt solution. The number of total
nucleated cells for spleen and bone marrow was counted using a
hemocytometer.

**Assays of Granulocyte-Macrophage
Colony-Forming Cells**

Approximately 10\(^3\) nucleated bone marrow cells or 5 \(\times\) 10\(^3\) spleen
cells were added to each milliliter of supplemented Medium 199
(M.A. Bioproducts, Walkersville, Mass) containing 0.3\% Bacto agar
(Difco Laboratories, Detroit), 20\% FCS (Sterile Systems, Logan,
Utah) and 5\% post-endotoxin mouse serum (see below) as a source of
CSA. \(^1\) Three cultures were prepared for each sample in 35 \(\times\) 10-mm
plastic Petri dishes and incubated at 37 °C in a humidified atmo-
sphere with 5\% CO\(_2\). Colonies grown by day 8 of incubation were
studied by routine scoring. In some experiments, colonies were
scored on days 4, 8, and 12 of culture.

**Cytochemical Staining of Colonies
and Colony Counts**

Staining of colonies in the agar gel was performed by a modifica-
tion of the methods described by Kubota et al, \(^9\) and the cytochem-
ical demonstration of the specific esterase of the primary granules of
neutrophilic granulocytes was performed by a modification of a
method described previously. \(^{11,11}\) Briefly, the entire agar gel was
transferred gently from the Petri dish onto a 50 \(\times\) 75-mm glass slide
and allowed to dry under two
layers of filter paper (Whatman, No. 1). After complete drying, the top dry filter paper was removed, and
colonies in agar gel were fixed by flooding the agar and the
remaining filter paper with 10\% (vol/vol) neutral buffered formalin
for ten minutes at room temperature. After removing the last filter
paper, the slides were washed gently with tap water and allowed to
dry. The slides were incubated in a reagent mixture containing
naphthol ASD chloroacetate (Sigma Chemical Co, St Louis) with
hexazotized basic fuchsin in 0.1 mol/L Michaelis buffer at 37 °C for
45 minutes in a water bath and counterstained with toluidine blue as
described previously. \(^{17}\) With this staining, neutrophilic granulocytes
in a colony demonstrated strongly positive cytoplasmic red staining,
while monocytes or macrophages remained unstained.

Stained colonies were examined under an inverted microscope
using a \(\times\) 10 objective lens. Colonies were defined as groups of 50 or
more cells, and each stained colony was classified as a neutrophilic
colony (N-colony) if more than 90\% of the cells in the colony were
neutrophilic granulocytes and a macrophage colony (M-colony) if
more than 90\% of the cells in the colony were macrophages. Colonies
composed of a mixture of neutrophilic granulocytes and macro-
phages were classified as neutrophil-macrophage colonies (NM-
colony). Progenitors of these colonies were defined as CFU-N,
CFU-M, and CFU-NM, respectively. All colonies appearing in an
agar plate were scored and classified into these three categories in
triplate plates per sample.

**Preparation of Post-endotoxin Serum**

Post-endotoxin stimulated mouse serum was prepared according to
the method described. \(^{18}\) Approximately 80 (BALB/c \(\times\) CE)F1
hybrid mice were injected intraperitoneally with 50 \(\mu\)g of *Salmon-
ella typhosa* lipopolysaccharide (Difco Laboratories, Detroit). Two
hours later, blood was collected by cardiac puncture under ether
anesthesia and pooled. The resulting serum was separated by
centrifugation at 1800 rpm, filtered with 0.45 \(\mu\)m millipore filters
(Miller-HA, Millipore Corp, Bedford, Mass) and kept at \(-20 °C
until use as a source of CSA.

**Collection of Mouse Serum**

Blood was collected by cardiac puncture under ether anesthesia
from groups of mice bearing tumors for various time periods. Serum
was separated, filtered, and stored as described for post-endotoxin
serum.

**Cytocidal Drug Treatment In Vivo**

In vivo cytocidal experiments were performed using hydroxyurea \(^9\)
and cytosine arabinoside. \(^9\) Hydroxyurea (Sigma Chemical Co, St
Louis) solutions were prepared in normal saline immediately before
use and 1 mg/g body weight was given intraperitoneally to a group
of mice. Equal volumes of normal saline were given intraperitoneally
to control mice. Two hours later the mice were killed, femoral
marrow cells were removed by grinding the whole femur as described
above, and the bone marrow cells were pooled from two mice in each
hydroxyurea-treated or control group. Subsequently, bone marrow
cells were cultured in vitro for N-, NM-, and M-colony formation as
above, but using six culture dishes per sample. Cytosine-1-\(\beta\)-d-arabinofuranoside (Sigma Chemical Co) was dissolved in
sterile saline immediately before use, and a dose of 0.6 mg/g body
weight was given intraperitoneally to a group of mice. Equal volumes
of saline were given to a control group. Two hours later, the mice
were killed and bone marrow cells were processed in the same
manner as described for experiments with hydroxyurea.

**Blood Cell Counts**

Blood leukocyte counts, differential counts, and hematocrits were
determined from the first drop of blood obtained from the lateral
orbital sinuses. The concentration of leukocytes was determined with
a Coulter counter. Differential counts of two hundred cells were
done on Wright-Giemsa stained smears.

**RESULTS**

**Tumor Growth and Blood Pictures**

In spite of numerous passages of the tumor through host mice (CE or [BALB/c \(\times\) CE]F1) during the past several years, the biologic behavior of this mammary
carcinoma appeared unchanged, and the transplantation of the tumor invariably produced remarkable
neutrophilia in host mice as the tumor grew. The white
blood cell counts of 14-day tumor-bearing mice aver-
aged 37,800 \(\pm\) 5,700/\(\mu\)L, and the counts of 21-day
tumor-bearing mice averaged 96,400 \(\pm\) 31,200/\(\mu\)L.
The control white blood cell counts were 7,200 \(\pm\)
1,800/\(\mu\)L. Over 85\% of blood leukocytes were neutro-
phils in tumor-bearing mice. Hematocrit values
dropped from 53.2 \(\pm\) 2.5\% to 30.2 \(\pm\) 5.7\% by the third
week after tumor transplantation. These blood pictures
as well as marked granulocytic hyperplasia of the
marrow and extramedullary hemopoiesis in the spleen
of tumor-bearing mice were essentially the same as
described in detail previously. \(^5,12,13\)
Colony Morphology

Figure 1 illustrates the appearance of stained colonies. Colonies containing neutrophilic granulocytes are recognized easily by the bright red staining of primary granules of neutrophilic granulocytes. Colonies composed of many immature granulocytes were more densely aggregated and demonstrated a stronger esterase reaction, because the primary granules are more abundant in promyelocytes and myelocytes than the later stages of granulocytes.17

Colony Enumeration

Changes in bone marrow cellularity, colony numbers, and colony types before and after transplantation of the neutrophilia-inducing tumor are summarized in Table 1. In normal mice, 20.7 ± 3.6% of colonies were of macrophages. The remaining 43.8 ± 2.3% were a mixture of neutrophils and macrophages. The number of nucleated cells per femur decreased slightly with tumor growth, as others have also observed in mice with tumor-induced neutrophilia.5,6 The number of in vitro colony-forming cells per 10⁷ nucleated cells increased after day 14 (P < .005), and there was a significant increase in the number of colony-forming cells per femur (P < .005) after 21 days of tumor growth.

Colony Type Evaluation

The proportion of N-colonies increased significantly from 20.6 ± 2.4% of control value to 34.3 ± 1.9% at 14 days (P < .005) and to 43.3 ± 3.9% at 28 days (P < .005) after tumor transplantation; the proportion of colonies composed of macrophages decreased with tumor growth. As shown in Table 1, the absolute

Fig 1. Granulocyte and macrophage colonies grown in agar and stained for chloroacetate esterase reaction of the primary granules of the neutrophilic granulocyte. The enzyme reaction is demonstrated as bright red color in the cytoplasm of neutrophilic granulocytes. Magnification ×64. (A) A tight neutrophilic granulocyte colony (N-colony); (B) a loose neutrophilic granulocyte colony (N-colony); (C) a colony composed of a mixture of macrophages and neutrophils, (NM-colony); (D) a macrophage colony (M-colony).
number of N-colonies of femoral bone marrow increased significantly \( (P < .005) \) after 14 days; by day 28 the absolute number of NM-colonies also increased \( (P < .005) \), while the absolute number of M-colonies did not show significant changes with tumor growth. The tumor's effect on differentiation of progenitors thus appeared to be primarily on the neutrophilic cell line. There was a direct relationship between the number of N-colonies per femur and the blood neutrophil counts of mice (Fig. 2), suggesting these N-colonies were derived from progenitors that are likely responding in vivo to a neutrophilic stimulator in tumor-bearing mice.

The spleens of tumor-bearing mice were quite variable in size and cellularity. Spleens were smaller than normal at 14 days, then enlarged in the third and fourth week with marked extramedullary hemopoiesis and an increment in all types of colonies. The proportion of N-colonies to NM- and M-colonies at the later weeks was higher than that of normal \( (P < .05) \) (Table 2).

**Time Course of Colony Growth**

The proportion of granulocytic and macrophage colonies has been shown to vary after different times in culture.\(^{21}\) To determine whether or not the increase in the proportion of neutrophilic colonies in tumor-bearing animals was due to different kinetics of colony growth, we examined colonies on days 4, 8, and 12 of culture. Bone marrow cells were pooled from three normal mice (WBC: 7097 ± 504/μL) and three 14-day tumor-bearing mice (WBC: 71,200 ± 6,900/μL). As shown in Figure 3, the number of neutrophilic colonies from tumor-bearing mice increased as early as day 4 of culture and remained higher than that of normal mice throughout the culture period. In comparison with this, the number of macrophage colonies was similar in normal and tumor-bearing mice and increased with the culture period in both groups of animals.

Further observations of these N-colonies revealed some qualitative changes at various culture days. Neutrophilic colonies developed from normal mouse bone marrow at day 4 of culture were small (50 to 80 cells), demonstrated a bright red esterase reaction, and approximately 73% of the colonies were tight and compact. Day 4 colonies from tumor-bearing mice were similar in appearance to those from normal mice, and about the same proportion were as tight and compact as N-colonies of normal mice. Day 8 colonies were larger and less tightly packed; 15.1 ± 1.4% of N-colonies from normal bone marrow were tight and compact, while 9.7 ± 5.3% of those from tumor-bearing mice belonged to the tight-and-compact category. By day 12 of culture, all colonies from normal and tumor-bearing mice were loose and dispersed, cell outlines were indistinct and showed less esterase reaction than day 4 colonies, suggesting that the neutrophils matured and disintegrated in culture.

**Response of Progenitors to CSA**

The response of progenitors to varying doses of CSA was compared between the bone marrow of normal and tumor-bearing mice. Bone marrow cells were pooled from three normal animals and three 14-day tumor-bearing mice. As shown in Figure 4, without added CSA in culture, there was no spontaneous colony

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**Table 1. The Number and Differential Types of In Vitro Colonies Grown from the Femoral Bone Marrow of Mice Following Transplantation of the CE Mammary Carcinoma**

<table>
<thead>
<tr>
<th>Days After Tumor Transplant</th>
<th>Number of Mice Studied</th>
<th>Number of Cells per Femur (× 10⁶)</th>
<th>Number of Colonies per 10⁶ Marrow Cells</th>
<th>Number of Colonies per Femur (× 10²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total CFU-c</td>
<td>N-colony</td>
<td>NM-colony</td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>37.9 ± 3.9</td>
<td>143.7 ± 19.9</td>
<td>27.8 ± 6.2</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>36.8 ± 4.0</td>
<td>167.8 ± 11.7</td>
<td>35.6 ± 2.8</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>29.2 ± 3.7</td>
<td>186.8 ± 35.6</td>
<td>59.4 ± 18.4</td>
</tr>
<tr>
<td>21</td>
<td>10</td>
<td>27.3 ± 5.1</td>
<td>295.9 ± 106.7</td>
<td>94.6 ± 23.7</td>
</tr>
<tr>
<td>28</td>
<td>4</td>
<td>31.2 ± 2.0</td>
<td>279.3 ± 18.4</td>
<td>121.4 ± 17.8</td>
</tr>
</tbody>
</table>

Values are given as the mean ± 1 SD.
\*P < 0.005 when compared with day 0. by t test.
\*P < 0.025 when compared with day 0. by t test.
\*P < 0.05 when compared with day 0. by t test.

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![Fig 2. The number of N-colonies per femur related to the blood neutrophil counts. Neutrophilic colony data were grouped according to the degree of neutrophilia of the mouse from which colonies were analyzed. Each dot represents one animal. Dotted lines indicate mean colony counts of the group.](from www.bloodjournal.org by guest on September 14, 2017. For personal use only.)
growth from the marrow of either normal or tumor-bearing mice. At a minimum dose of CSA, twice as many total colonies and N-colonies developed per 10^5 cells from the marrow of tumor-bearing mice compared with the marrow of normal mice. With increasing doses of CSA, both total colonies and N-colonies increased, but the slope of lines appeared similar in the two groups of mice, indicating that the CSA sensitivity of progenitors of tumor-bearing mice is not different from that of normal mice.

Cell Cycle Kinetics of Progenitors

An increased number of cells in a progenitor cell compartment can be achieved by their increased rate of reproduction within the compartment, enhanced entrance of more primitive stem cells into the compartment, or delayed release of cells from the compartment. We conducted in vivo hydroxyurea cytocidal experiments to examine cell cycle kinetics of progenitors. The cytocidal rate of progenitors from the bone marrow of normal mice, two-week tumor-bearing mice, and three-week tumor-bearing mice treated with hydroxyurea or saline is summarized in Table 3. There was a significant \( P < .025 \) increase in the cytocidal rates of total CFU-c and neutrophilic progenitors of marrow cells of 21-day tumor-bearing mice when it was compared with that of normal marrow. The tumor's effect on cell cycle kinetics appeared limited to neutrophilic progenitors, since only an insignificant increase was observed in macrophage colony formation.

Comparable results were also obtained by cytosine arabinoside treatment in vivo. The cytocidal rate of neutrophilic progenitors of 14-day tumor bearers with high neutrophil counts (58,000 to 60,000/μL) was 48.9%, that of 14-day tumor bearers with medium high neutrophil counts (38,000 to 40,000/μL) was 28.7%, and that of normal mice was 9.3 ± 2.4% (mean ± 1SEM). The killing rate of macrophage progenitors was not different between the groups of mice. These results indicated that a larger proportion of granulocyte progenitors of the bone marrow of tumor-bearing mice were in active cell cycle than progenitors of normal mouse marrow, and the increased rate of

![Fig 3. Differential types of colonies from bone marrow of a normal mouse (left) and a two-week tumor-bearing mouse (right) harvested at three different culture periods. The means ± 1 SD of data obtained from two experiments are shown. □, N-colony; □, NM-colony; □, M-colony.](image)

![Fig 4. The response of progenitors to varying doses of CSA in vitro. Bone marrow cells were pooled from three normal mice and three 14-day tumor-bearing mice and cultured with varying concentrations of post-endotoxin serum (as CSA). Closed circles represent total CFU-C of normal mouse marrow; closed triangles represent N-colony of normal mouse marrow; open circles represent total CFU-C of tumor-bearing mouse marrow; open triangles represent N-colony of tumor-bearing mouse marrow. The means ± 1 SD of data collected from three experiments are plotted.](image)

Table 2. The Number and Differential Types of In Vitro Colonies Grown from the Spleen of Mice Bearing the CE Mammary Carcinoma

<table>
<thead>
<tr>
<th>Days After Tumor Transplant</th>
<th>Number of Mice</th>
<th>Number of Cells per Spleen ( \times 10^6 ) per 10^5 Cells per Spleen</th>
<th>Number of Colonies</th>
<th>Colony Differentials (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N-colony</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>15.6 ± 7.1</td>
<td>29.5 ± 10.9</td>
<td>22.9 ± 8.1</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>25.4 ± 9.5</td>
<td>37.4 ± 4.3*</td>
<td>44.3 ± 4.9</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>21.3 ± 6.5</td>
<td>37.8 ± 1.1</td>
<td>44.5 ± 0.8</td>
</tr>
</tbody>
</table>

Values are given as the mean ± 1 SD.  
* \( P < .05 \) when compared with day 0 by \( t \) test.  
† \( P < .005 \) when compared with day 0 by \( t \) test.
reproduction within the compartment probably was attributed to the increased number of neutrophilic progenitors.

**Colony Stimulator in Serum**

To further our investigation of the mechanisms of increased neutrophil production in tumor-bearing animals, the sera of tumor-bearing mice were evaluated on days 2, 7, and 14 after tumor implantation for colony-stimulating activity. As shown in Figure 5, the serum from day 2 tumor-bearing mice showed some colony-stimulating activity, but the majority of colonies grown with this serum were macrophages. The serum of day 7 tumor-bearing mice as well as the serum of normal mice (data not shown) did not stimulate any colony growth. Interestingly, however, the serum of day 14 tumor-bearing mice stimulated colony growth, and the majority of colonies grown with this serum were neutrophilic granulocyte colonies.

A full dose-response curve of the stimulator in day 14 serum is shown in Figure 6. With increasing doses of this serum, not only did the number of N-colonies increase, but the N-colonies that were stimulated contained a greater number of cells. Since both normal and day 7 sera did not stimulate any colony growth, we examined the inhibitory activity of these sera. Test sera were added to culture medium containing normal marrow cells and a usual dose (5%) of postendotoxin serum as CSA. As shown in Figure 7, both sera inhibited the growth of colonies at a concentration over 5%. At this concentration, day 2 serum was inhibitory mostly to the growth of N-colonies but not M-colonies. In contrast, day 14 tumor mouse serum did not show any inhibitory activity (Fig 8). These findings indicated the presence of an inhibitor of colony growth in the serum of normal mice of this strain and in the serum of mice with early stages of tumor growth, while there appeared to be a neutrophilic colony stimulator in the serum of mice bearing the tumor for two weeks.

**DISCUSSION**

A number of investigators have described tumor-associated leukemoid reactions in humans and animals. Marked neutrophilia in CE mice bearing a transplantable mammary carcinoma (CE 1460 adenocarcinoma) was first described by Delmonte et al., and production of a possible granulopoietic factor from the tumor was suggested. Despite the fact that this

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**Table 3. Cytocidal Rate of Marrow Progenitors by In Vivo Hydroxyurea Treatment of Mice Bearing the CE Mammary Carcinoma**

<table>
<thead>
<tr>
<th>Days After Tumor Transplant</th>
<th>Cytocidal Rate (%)*</th>
<th>Total CFU-c</th>
<th>CFU-N</th>
<th>CFU-NM</th>
<th>CFU-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.2 ± 5.3</td>
<td>14.2 ± 7.9</td>
<td>13.8 ± 6.9</td>
<td>23.5 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>15.5 ± 6.1</td>
<td>15.5 ± 6.0</td>
<td>15.4 ± 6.9</td>
<td>15.8 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>32.3 ± 1.2†</td>
<td>37.6 ± 1.0†</td>
<td>24.5 ± 4.8</td>
<td>33.9 ± 4.6‡</td>
<td></td>
</tr>
</tbody>
</table>

*Values are given as the mean ± 1 SEM obtained from at least four experiments in each time point. In each experiment, pooled marrow cells from two of the saline-treated and two of the hydroxyurea-treated mice were processed in parallel. The age, sex, body weight, tumor age and white blood cell counts of both groups of mice were matched in each experiment. Colony growth was expressed as colony numbers per femur wall = calculated according to the formula: Rate = (saline treated - hydroxyurea treated)/(saline treated) × 100.

†P < 0.025 when compared with day 0 by t test.
‡P < 0.10 when compared with day 0 by t test.
mouse model of tumor-induced neutrophilia has been known for years and investigated by several researchers,\textsuperscript{5,12} the in vivo mechanism for this neutrophilia has not been fully clarified.

In an attempt to improve our understanding of the mechanism in tumor-induced neutrophilia, we have previously characterized the kinetics of neutrophil production in this animal model. Such studies showed that there was a marked increase in marrow neutrophil production as a result of expanded hemopoietic tissue and a shortened marrow neutrophil transit time,\textsuperscript{13} indicating in vivo stimulation of marrow neutrophil production. We undertook the present study to acquire information on the process of neutrophil production at the progenitor cell level. We applied methods to identify colonies of the neutrophilic cell line to improve specificity of in vitro colony analysis in neutrophilia.

We demonstrated that as the tumor grew and neutrophilia developed in the mice, there was a progressive increase in neutrophilic progenitors in the bone marrow. There was a direct relationship between the number of marrow neutrophil progenitors and blood neutrophil counts. A larger portion of neutrophilic progenitors of tumor-bearing mice with neutrophilia and further substantiate our previous studies, which indicated that the primary mechanism of CE tumor-induced neutrophilia was on bone marrow neutrophil production\textsuperscript{13} and not accumulation of neutrophils in the blood as suggested by others.\textsuperscript{5}

Our findings are in contrast to those observed by other investigators who studied bone marrow colonies of mice with various tumor-induced neutrophilia and did not find an increment of colony-forming cells in the bone marrow\textsuperscript{5,7,11} but found primarily accumulations of stem cells and progenitor cells in the spleens of tumor-bearing mice.\textsuperscript{6,7,11} Although it is conceivable that different mediators were involved in other cases of tumor-induced neutrophilia, one of the explanations for this difference could be the type of stimulator used to stimulate in vitro colony growth. Others used L cell-derived CSA, which is now known to stimulate primarily macrophage colony growth.\textsuperscript{4} We used post-endotoxin-stimulated serum prepared from syngeneic mice to stimulate colony growth in vitro, since this type of CSA has been shown to stimulate progenitors of the granulocyte-macrophage series and produce more granulocytic colonies than the L cell-derived stimulator.\textsuperscript{4} Therefore, it is believed that we examined a different progenitor cell subpopulation, one that includes neutrophilic granulocyte progenitors as well as macrophage progenitors, which were studied in earlier reports.

Some transplantable tumors that cause neutrophilia in mice have been shown to produce colony-stimulating activity in vitro.\textsuperscript{7-11} Whether or not the production of such activity from the tumor in vitro has any relevance to neutrophilia of tumor-bearing mice has been controversial.\textsuperscript{5,24} In the CE tumor-bearing mouse model, earlier investigators believed in a granulocytosis-promoting activity of the CE tumor.\textsuperscript{23} In subsequent studies by others, this tumor did not appear to produce
CSA in vitro, and the plasma of CE tumor-bearing mice barely demonstrated any CSA by conventional assays. Although specific identification of in vitro colonies enabled us to detect the presence of neutrophilic colony stimulating and inhibitory activities in the serum of CE tumor-bearing mice in this study, the presence of inhibitors in the serum of our mice could well mask the stimulatory activities by such bioassays. We need to investigate further the biologic properties and the origin of these serum activities to determine their physiologic significance. It is possible that neutrophilia of CE tumor-bearing mice may be mediated through a different granulopoietic factor or factors than CSA. We have shown recently that the CE tumor-induced neutrophilia was associated with an excessive osteoclastic bone resorption. Whether or not such bone changes have any relationship to the neutrophilia of mice has to be elucidated by further studies. Specific identification of in vitro colonies as we applied here will allow us to further dissect the mechanisms of neutrophil production in the early stage of cell development.

We conclude from these studies that neutrophilic granulocyte progenitors are markedly increased in numbers in the bone marrow of mice with CE tumor-induced neutrophilia. A greater fraction of these progenitors are in active cell cycle than those of normal mice, suggesting this tumor stimulates neutrophilic progenitor cells in host mice. The increase in numbers and turnover rate of neutrophilic progenitors may well contribute to the marked neutrophilia of mice, as does the expanded hemopoietic tissue we described previously. The physiologic significance of N-colony stimulating activity found in the serum of 14-day tumor-bearing mice needs to be examined by further studies.

REFERENCES

Augmentation of neutrophilic granulocyte progenitors in the bone marrow of mice with tumor-induced neutrophilia: cytochemical study of in vitro colonies

MY Lee and JL Lottsfeldt