Modulation of Murine Natural Killer Cells by a Granulocytosis-inducing Tumor

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ABSTRACT

The nonmetastatic neutrophilia-inducing murine mammary carcinoma CE1460 has been shown previously to have profound effects on hemopoiesis and lymphopoesis. In this report we examined the effects of progressive growth of CE1460 on natural killer (NK) cell activity both in the bone marrow, the site of primary NK cell production, and in a peripheral site, the spleen. (BALB/c × CE)F1 mice were injected subcutaneously with trypan blue-labeled cells from in vivo passaged CE1460 or from B66. The BALB/c mammary carcinoma that does not induce neutrophilia. 3 days posttumor implantation, NK activity in bone marrow cells or spleen cells was greatly enhanced compared to normal controls. In B66 tumor-bearing mice, NK activity returned to normal by Day 7 and remained there through Day 14. In contrast, however, NK activity in CE1460 tumor-bearing mice decreased to only 10–20% of normal by Day 14. Excision of the tumor on Day 14, when WBC counts were three times normal, was followed by a rapid return of the WBC count to the normal range. NK activity in bone marrow and in spleen cells recovered somewhat but was still significantly suppressed 7 days after tumor excision. Limiting dilution analysis revealed a 3–5-fold decrease in frequency of NK precursors in bone marrow cells of mice bearing CE1460 for 7 or 14 days. The dramatic changes in NK activity observed in these experiments may reflect perturbation in production as well as an initial activation and subsequent suppression of mature NK cells.

INTRODUCTION

Multiple effects of tumor growth on immune function and hemopoiesis have been documented with numerous human and experimental tumors. One of the most informative experimental models has been the neutrophilia-inducing murine mammary adenocarcinoma CE1460, first described by Delmonte et al. in 1966 (1). The growth of this tumor is associated with multiple changes in bone marrow hemopoiesis which include increased production of neutrophils (2), their precursors and progenitors (3, 4), increased osteoclasts and bone resorption (5), decreased erythropoiesis (6), decreased primary production of B-lymphocytes (7), and thymic atrophy (8). Significantly, the tumor is nonmetastatic and appears to exert its multiple effects by the production of factors that profoundly affect hemopoiesis.

Although the exact nature of these factors is still under investigation, the selective effects of CE1460 on particular cell types made it of interest to us to test whether CE1460 influenced another bone marrow-derived cell population, NK cells. This question is of particular interest since several studies have indicated regulatory interactions between NK cells and granulocytes. For example, neutrophils can inhibit NK cell function (9) and NK cells can inhibit granulocyte-macrophage colony forming units (10). Thus the activities of these two cell types seem intimately intertwined.

As a first step in analyzing the interactions of NK cells and neutrophils in the CE1460 model system, we have assessed the effects of that tumor on NK cell activity in the bone marrow, the primary site of NK cell production (11) and in a peripheral site, the spleen. The responses to CE1460 were compared with those to a mammary carcinoma, B66, that does not induce neutrophilia. The effects of each of the tumors on the frequency of NK precursors in the bone marrow was estimated using a limiting dilution assay.

MATERIALS AND METHODS

Mice. (BALB/c × CE/J)F1 mice were bred in our vivarium and maintained on pelleted lab chow and acidified water ad libitum. Parental female BALB/c and male CE/J mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice of both sexes were used at approximately 8 to 10 weeks of age for these experiments.

Tumors. CE1460 was maintained in vivo in (BALB/c × CE)F1 mice as described previously (3) by s.c. passage at 2-week intervals. The BALB/c mammary carcinoma 66 (a gift from Dr. Gloria Heppner, Michigan Cancer Foundation) grew more slowly and was maintained in vivo by passage at 3–4-week intervals.

For transplantation into mice, in vivo grown tumors were minced and enzymatically digested using 0.025% trypsin and 0.18% versene solution to form a single cell suspension. Mice were injected s.c. in the right flank with 3 × 106 CE1460 cells or 5 × 105 B66 cells in RPMI 1640 medium without serum. Mice were checked for the presence of palpable tumor; the size of measurable tumors is reported as the mean of two perpendicular diameters ± SD. Control mice were un.injected or injected with an aliquot of the same medium used to suspend the tumor cells.

In one series of experiments, progressively growing CE1460 tumors were surgically resected under sodium pentobarbital anesthesia on Day 14 and NK activity determined at various times thereafter. Controls for these experiments were normal (nontumor bearing) mice that were sham operated.

Cellularity and Cell Preparation. At intervals after transplantation of the tumor, mice were bled from the retro-orbital sinus to assess the WBC count (2). Smears were made and stained with Wright-Giemsa. In mice with progressively growing CE1460 tumor, marked neutrophilia was invariably observed. Since the purpose of the experiment was to determine the effect of neutrophilia on NK activity, those mice with the highest WBC count were used for each day’s experiment. Significant neutrophilia was not observed in mice with B66 tumor (12).

BMC and SC suspensions were prepared as described previously for testing in the 51Cr release assay (13). In brief, SC were teased from the splenic capsule into RPMI 1640 medium containing 5% FCS. Erythrocytes were removed by hypotonic lysis; the remaining cells were washed and counted. BMC were flushed from femurs with ice cold medium. The marrow plugs were dispersed by passage through 23 and 25 gauge needles, and the number of viable nucleated cells counted. This technic yields approximately ½ as many BMC/femur as the grinding technic used in previous studies with this tumor (3, 4). For cytotoxic depletion of NK cells, BMC or SC at 2 × 107/ml were treated with 1:100 dilution of anti-ASGM1 (Wako) for 30 min at 4°C, followed by newborn rabbit serum (Pel-Freeze) as a source of C. Control cells were treated with C only. All treated cells were washed 3× with medium prior to use in the assays.

51Cr Release Assay. Log phase growth YAC-1 cells were labeled with 51Cr by addition of 0.25 mCi Na251CrO4 (New England Nuclear) to 4–10 × 106 cells. After incubation for 45 min at 37°C, the cells were centrifuged through a 2-ml layer of FCS, resuspended in RPMI 1640 with 5% FCS and held on ice for 45 min or longer before use in the assay. The target cells were then washed, counted, and diluted to 105 YAC-1/ml.
The effector SC or BMC were suspended in the same medium and plated at three concentrations to give E:T ratios of 100, 50, and 25:1, in triplicate in 96-well V-bottom plates. The effectors were plated in 100 μl/well, followed by the addition of 100 μl of target cells/well. The plates were centrifuged at approximately 500 rpm for 4 min, then placed on a rocker in a 37°C 5% CO₂ incubator for 4 h. At the end of the incubation, 100 μl was removed per well and counted in a Gamma scintillation counter. The results were calculated by:

\[
\% \text{ Kill} = 100 \times \frac{\text{Experimental cpm} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}}
\]

where spontaneous release is cpm from YAC-1 incubated in media in the absence of effector cells and maximal release is cpm from YAC-1 incubated in Triton-X. LU were calculated by linear regression analysis of the E:T ratios as described previously (13). One LU represents the number of effector cells required to lyse 10% (by BMC) or 30% (by SC) of the target cells.

Limiting Dilution Assay. BMC were plated in replicates of 24 wells at concentrations ranging from 5 × 10³/well to 0.7 × 10²/well in round-bottom wells in 0.2 ml RPMI 1640 media containing 5% FCS, 5 × 10⁻⁴ m 2-mercaptoethanol and 20% supernatant from concanavalin A-stimulated BALB/c spleen cells as a source of interleukin 2 (14). The cells were incubated at 37°C, 5% CO₂ for 7 days. Cultures were fed on Day 3 or 4 by removal of 100 μl of media and replacement with fresh media. On Day 7, 5 × 10³ ¹⁵Cr labeled YAC-1 cells were added/well and a 4-h ¹⁵Cr release assay carried out. Positive wells were those in which ¹⁵Cr release was >3 SD higher than the mean spontaneous release. The frequency of NK precursors was estimated according to Taswell (15) using a computer program kindly provided by Dr. Edward Lattime (Sloan-Kettering Institute).

RESULTS

Effect of Progressive Growth of CE1460 on NK Activity. 8-10-week-old (BALB/c × CE)F₁ mice were injected s.c. in the flank with 3 × 10⁶ CE1460 cells. On Days 3, 7, 14, NK activity was tested in BMC and SC from the tumor-bearing mice and from age- and sex-matched uninjected controls. Peripheral WBC counts, cellularity per femur or spleen, and tumor size were also determined as described in "Materials and Methods." These data are summarized in Table 1.

Growth of CE1460 and its effects on cell numbers were similar to those reported previously (2, 3). Tumor was not yet measurable on Day 3 but then grew progressively to a mean diameter of 0.8 ± 0.13 on Day 7 and 1.7 ± 0.2 on Day 14. Peripheral white cell counts were depressed on Day 3, slightly above normal on Day 7 and greater than three times normal by Day 14. As shown previously, >90% of WBC from CE1460 tumor-bearing mice were neutrophils on Day 14.

Despite the tumor-induced neutrophilia, the number of cells obtained per femur or spleen did not change markedly during the 2-week course of the experiment (Table 1). As shown elsewhere (2, 4, 16), the increase in neutrophil production is not only due to an increase in the percentage of neutrophils in the marrow but also is due to expansion of hemopoietically active marrow into previously inactive sites.

NK activity in BMC was increased dramatically on Day 3 to levels usually seen only in peripheral sites such as the spleen. By Day 7, NK activity in BMC had returned to the normal range and by Day 14 it was less than 60% of normal. A similar pattern of increase and subsequent decrease was seen in SC. However, the relative increase on Day 3 was not as great.

In order to quantitate the NK activity, we calculated lytic units. Because the activity of normal BMC is markedly less than that of SC, LU were calculated for 10% lysis by BMC and 30% lysis by SC (i.e., values that fall within the normal range of cytotoxicity at the effector:target ratios used for each effector cell population).

As shown in Fig. 1A, LU/10⁶ BMC were increased greater than 10-fold on Day 3 whereas the increase in LU/10⁶ SC was approximately 3.5-fold. On Day 7 NK activity was returning to the normal range (1.5 and 1.9 × control in BMC and SC, respectively) and by Day 14 NK activity had fallen to 25% or less of normal. Since a pronounced granulocytosis was occurring during this time, it was important to determine whether the total NK activity per femur or spleen was actually changing or whether NK cells were just being diluted out by granulocytes. When the data on lytic activity per 10⁶ cells were expressed as total LU per femur or spleen, a similar pattern was observed (Fig. 1B). LU/10⁶femur or spleen were nearly 12-fold on Day 3, were 1.5 × normal on Day 7 and 18% of normal on Day 14. The increase in LU/spleen at Day 3 was somewhat greater than the increase/10⁶ SC due to increases in splenic cellularity.

Because of the dramatic increase in NK activity observed 3 days after transplantation of CE1460, we investigated the kinetics of this response. NK activity in BMC was less than 50% of control on Day 1, was 2.5 × control on Day 2 and peaked on Day 3, followed by a return to the normal range (data not shown). Although there were some fluctuations in number of cells per femur, these were not large enough to account for changes in NK activity by either dilution or concentration. In the spleen, there was a marked increase in cellularity on Day 1 (94% increase) and an apparent decrease in NK activity. NK activity on a per cell basis peaked on Days 3 and 4, then fell toward the normal range.

Early Lytic Cells Are ASGM⁺. To test whether the cytotoxic...
cells detected early after CE1460 tumor transplantation were, in fact, NK cells, we treated BMC and SC with anti-ASGM1 and C (+) or C only (−) as detailed in “Materials and Methods” prior to use as effector cells in the Cr release assay.

Recovery of NK Activity after Tumor Excision. Subcutaneous CE 1460 tumors were allowed to grow for 2 weeks and were then surgically excised. Normal mice were sham operated to control for effects of anesthesia and surgery. NK activity and cellularity were then monitored on Days 1, 3, and 7 postop (Table 3). At 1 day after tumor removal, peripheral WBC counts were still markedly elevated in mice that had carried the CE 1460 tumor. Cellularity was normal in the femurs but reduced to only 26% of normal in the spleen. There was no significant NK activity detectable in either BMC or SC.

By Day 3 posttumor excision, the WBC counts had fallen to the normal range. The number of cells per femur was significantly greater than in normal mice. Splenic cellularity had returned to normal. NK activity was normal in BMC and still subnormal in SC, although greatly increased from Day 1 postop.

By Day 7 posttumor excision, cell numbers in the peripheral blood, femur and spleen were normal. However, NK activity was still low in the spleen and had fallen again in BMC. In the sham-operated mice, NK activity also was depressed on Day 7, suggesting a delayed effect of the anesthesia and/or surgery on marrow NK cells.

There were relatively few other changes in the sham-operated mice. WBC counts were normal throughout the 7 days as were the number of BMC/femur. Splenic cellularity was normal one day after surgery, was doubled on Day 3 and subnormal on Day 7. NK activity in SC was normal throughout the 7 days.

Effects of B66 on NK Activity. Like CE1460, the BALB/c mammary carcinoma B66 grows progressively in (BALB/c × CE)F1 mice. However, B66 does not induce neutrophilia or hypercalcemia (12). When 5 x 105 B66 cells were transplanted into the F1 mice, an increase in NK activity similar in magnitude to that induced by CE1460 was observed on Day 3 (Table 4). After the initial spike, however, NK activity returned to the normal range by Day 7 in B66 tumor-bearing mice and remained there on Days 10 and 14. Thus, the presence of a progressively growing mammary tumor was not in itself sufficient to depress NK activity. It should be noted that the B66 tumor grows more slowly than CE1460, and although more B66 than CE1460 cells were injected initially, the total tumor burden on Day 14 was less in the B66 than in the CE1460 tumor-bearing mice. The relationship between the lack of NK depression by B66 and its slower growth and/or the lack of neutrophilia remains to be explored.

Limiting Dilution Analysis of NK Precursors in CE1460 Tumor-bearing Mice. The studies described above focused on NK activity of mature NK cells. We questioned whether the CE1460 tumor would also affect NK precursor cells. Using a limiting dilution analysis (15, 17) we estimated the frequency of cells able to give rise to NK activity in BMC of normal and tumor-bearing mice (Table 5). In control (BALB/c × CE)F1 mice, 1/1293 BMC produced detectable NK lytic activity in the 7-day assay. By Day 3 after tumor transplantation, the frequency fell to 1/3350 in mice with CE1460 and to 1/4946 in B66 tumor-bearing mice. In mice bearing the CE1460 tumor the NK precursor frequency fell to 1/6550 on Day 7 and recovered somewhat to 1/3651 on Day 14. A higher, but still depressed, frequency (1/2840) was observed in B66 tumor-bearing mice on Day 14.

DISCUSSION

The data presented here demonstrate that growth of the neutrophilia-inducing mammary carcinoma CE1460 induces a biphasic change in NK activity. There is an early increase in NK activity that peaks at Day 3 in BMC and Days 3–4 in SC,
Group B and those mice were tested on Days 1, 3, or 7 following tumor excision. Group C were normal (non-tumor-bearing) mice that were sham-operated and tested in parallel with the tumor-excised mice. Group D includes the values for normal, nonoperated mice reported in Table 1.

Different from controls in tumor bearers. Significantly different from control. The number of nucleated cells per femur or spleen were not significantly different from controls in tumor bearers.

Table 4 Effects of B66 mammary carcinoma* on cellularity and NK activity of bone marrow and spleen

<table>
<thead>
<tr>
<th>Day (n)</th>
<th>WBC x 10^8/ml</th>
<th>No. cells/10^6 ± S.E.</th>
<th>% lysis of YAC-1 by*</th>
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<tbody>
<tr>
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<tr>
<td>0 (8)</td>
<td>10.2 ± 1.5</td>
<td>14.6 ± 4.2</td>
<td>72.1 ± 13.9</td>
</tr>
<tr>
<td>3 (5)</td>
<td>7.2 ± 1.5</td>
<td>17.7 ± 1.7</td>
<td>82.5 ± 4.2</td>
</tr>
<tr>
<td>7 (2)</td>
<td>8.7 ± 0.6</td>
<td>11.8 ± 2.4</td>
<td>70.9 ± 12.0</td>
</tr>
<tr>
<td>10 (2)</td>
<td>8.9 ± 1.0</td>
<td>14.4 ± 0.8</td>
<td>54.6 ± 2.3</td>
</tr>
<tr>
<td>14 (2)</td>
<td>9.4 ± 3.2</td>
<td>15.5 ± 2.9</td>
<td>79.9 ± 8.0</td>
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</table>

*B/C x CE)*, mice were injected s.c. in the right flank with 5 x 10^8 B66 cells on Day 0 and tested for NK activity on Days 3, 7, 10, and 14. *Number of white blood cells/ml ± SD. None of the experimental values were significantly different from control.

Table 5 Estimated frequency of NK precursors in BMC of control and tumor-bearing (B/C x CE)*, mice

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<th>Day</th>
<th>No. (of individual mice)</th>
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<tr>
<td>3</td>
<td>1/4946 (1/4087-1/6262)</td>
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<td></td>
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<tr>
<td>14</td>
<td>3/2840 (1/2473-1/3335)</td>
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Followed by a highly significant decline in NK activity by Day 14 of tumor growth. At that time there is a pronounced neutropenia.

In the early phase, after transplantation of 3 x 10^7 trypsinized CE1460 cells, the increase in NK activity can be seen both on a per cell basis as well as per femur or spleen. Total lytic units per femur were increased nearly 12-fold on Day 3 while LU/spleen were 4.6 x controls. We also found significantly increased NK activity in (B/C x CE)/JF, mice injected with a non-neutrophilia-inducing mammary carcinoma, B66.

Similar increases in NK activity have been observed in B6L/c mice after transplantation of 10^7 tumor cells from any of a variety of viral or chemically induced tumors (18), of an methylcholanthrene-induced sarcoma or its ascites fluid (19), or of 10^6 Ehrlich ascites cells (20). In rats, a peak of NK activity was observed 3 days after transplantation of 3 x 10^7 W/FuG-1 cells (21). Moreover, this early spike in NK activity has also been observed in mice injected with allogeneic lymphoid cells (22) or syngeneic thymocytes (18).

However, not all tumors or normal cell types induce the early (Day 3) rise in NK activity. In studies with B6L/c recipients, Herberman et al. found that neither syngeneic nor xenogeneic erythrocytes, syngeneic spleen cells nor human or rat tumor cells induced the Day 3 spike in activity (18). We have recently found that cloned in vitro grown lines of the CE1460 and B66 tumors do not induce the Day 3 rise in NK activity. The rapid rise in total NK lytic activity in both BMC and SC after implantation of CE1460 or B66 could indicate that the major effect is due to NK activation rather than production (proliferation) of new NK cells. Although the mechanism of NK stimulation is not known, the kinetics are compatible with secretion, either by the recipient's own immune system and/or by passenger leukocytes in the transplanted tissue, of NK-stimulatory lymphokines such as interferon and interleukin 2. However, our previous studies with the immunostimulator OK-432 demonstrated that NK production in the bone marrow can be increased within 1 to 2 days (13). Moreover, Biron et al. found proliferation of peripheral (spenic) NK cells after infection with lymphocytic choriomeningitis virus (23). Thus, the increases in NK activity we observed in the present study may be due both to activation and to proliferation of both primary (bone marrow) and secondary (spenic) NK cells. These possibilities remain to be resolved.

The effector cells in BMC and SC 3–6 days after transplantation of CE1460 were sensitive to treatment with anti-ASGM1 and C. This fact coupled with the observation of high lytic activity in a 4-h ^51Cr release assay strongly suggests that the effector cells are activated NK cells rather than cytotoxic macrophages. Early effector cells had only low levels of lytic activity to EL-4 targets (less than 10% lysis at 100:1). This activity also was eliminated by treatment with anti-ASGM1 and C, further supporting that the lytic cells are activated NK cells.

The second phase of response to CE1460 consisted of a gradual decline in activity to significantly depressed levels by Day 14. At that time, peripheral WBC counts were greater than

*P < 0.001 compared to Day 1 post-sham-operation (Group C).
*NS, not significantly different from post-sham-operation control on this day.
*P < 0.025 compared to post-sham-operation control on this day.

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*P < 0.001 compared to Day 1 post-sham-operation (Group C).
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*P < 0.025 compared to post-sham-operation control on this day.
3 × normal. Although tumor-induced depression in NK activity has been reported in a number of human and experimental studies (24–28), it is not a necessary correlate of tumor growth. Flannery and Brooks (29) found an increase in total NK activity in the spleens of rats bearing large spontaneous mammary carcinomas and in this study we found normal NK activity in mice 2 weeks after transplantation of the B66 carcinoma.

A feature that distinguishes the CE1460 tumor is the marked neutrophilia that accompanies tumor growth. Peripheral white cell counts reach levels three to four times normal and there is increased production of neutrophils in the expanded marrow compartment (2). The presence of high numbers of neutrophils may be sufficient to decrease NK activity. Several laboratories have reported dose-dependent inhibition of NK activity by granulocytes (9, 30, 31), which appeared to be mediated by oxygen metabolites in one study (31) but not in another (9). To produce inhibition of NK activity, granulocytes or their extracts must be present at the time of effector/target interaction. In our experiments there is a decrease in NK activity as the peripheral WBC count goes up during tumor growth and an increase in NK activity after tumor excision as the neutrophil count drops to normal. The correlation was not absolute, however, and it should be noted that 7 days after tumor excision when the WBC count had returned to normal NK activity in the spleen was less than 50% of control. This low level of peripheral NK activity may reflect the suppression of NK precursors we observed in the bone marrow of CE1460 tumor-bearing mice. The frequency of NK precursors was decreased in both CE1460 and B66 tumor-bearers 3 days after tumor transplantation, and remained depressed although the frequencies were lower in the CE1460-bearing mice. Since the total marrow volume is increased in the CE1460 tumor-bearing mice, the total number of NK precursors may actually be unchanged or increased. Whether or not this is the case remains to be tested.

These studies have shown that the neutrophilia-inducing tumor CE1460 can profoundly depress NK activity. The effects may be both on the mature effector cells as well as their bone marrow precursors. Further in vitro analyses will be required to determine whether high levels of granulocyte-stimulating growth factors by themselves are able to affect the maturation of NK precursors, or whether the effects we have observed are due to other causes.

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