Increased Chemotaxis of Leukocytes from Mice Bearing Tumors

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Abstract

Increased chemotaxis toward activated serum was demonstrated by leukocytes from bone marrow, spleen, and peripheral blood of tumor-bearing mice as compared with those of normal mice. Increased chemotaxis was correlated with the duration of tumor growth, which, in turn, was correlated with size of tumor. Upon surgical tumor removal, chemotaxis fell to normal levels unless metastasis had occurred. In each case, the extent of chemotaxis was correlated with the volume of the metastasis. Increased chemotaxis was seen in relation to the growth of a mammary adenocarcinoma as well as that of a chemically induced fibrosarcoma. The major chemotactic in serum was shown to be complement derived, and sera from tumor-bearing and normal animals were equally effective. The presence of a tumor resulted in an increase in the percentage of polymorphonuclear cells in the bone marrow. The numbers of cells which migrated were independent of the cellular composition of the bone marrow in normal mice. In contrast, a negative correlation was found between the percentage of lymphocytes present and the number of bone marrow cells which migrated toward activated serum when the cells originated in a tumor-bearing mouse. The data suggested the increased chemotaxis was a property of the cells rather than the soluble substances in the serum.

Introduction

In contrast to the interest concerning the function of lymphocytes and macrophages in tumor-bearing animals, there is little attention has been given to the role of cells of the granulocytic series in such hosts. However, there is some evidence that granulocytes may play a significant role in protecting a host from its tumor. For example, it has been shown that polymorphonuclear cells are cytotoxic to tumor cells (3-5). In addition, it has been demonstrated that bone marrow cells, which are comprised largely of granulocytes, are active in chemotaxis (6).

Several investigators have shown that chemotaxis of leukocytes (including polymorphonuclear cells) may be measured by migration under agarose toward a known chemotactant (7-10).

Materials and Methods

Animals. Female C3HeB/FeJ mice (Jackson Laboratory), 8 to 12 wk old, were fed laboratory chow and water ad libitum.

Tumors. (a) A spontaneous mammary carcinoma arising in a female C3H mouse and carried in female C3HeB/FeJ mice was used. Tumor cells were isolated from a tumor approximately 10 mm in diameter. The tumor was minced with scissors and washed through an 80-mesh nylon screen with Medium 199 (Gibco, Grand Island, NY) containing penicillin (50 units/ml) and streptomycin (50 ¡g/ml). Viability was assessed by trypan blue dye exclusion. A tumor was produced by s.c. injection of 1 x 106 viable cells in 0.1 ml medium into the left hind leg at the base of the tibia. For metastatic studies, the primary tumor was removed by amputation of the leg at the upper end of the femur. All lung metastases visible to the naked eye were measured with a micrometer, and volume was calculated using the average of 2 radii. Mice with recurring tumor growth at the amputation site were eliminated from the study.

(b) A fibrosarcoma was produced by s.c. injection of 0.1 ml of 0.1% 3-methylcholanthrene (Fisher, Fair Lawn, NJ) in the flank. Resultant tumors were transferred by s.c. injection of 1-mm3 fragments in the axillary region.

Leukocytes. Using a 25 gauge needle, bone marrow cells were harvested by flushing femurs with Hank's modified balanced salt solution (Flow Laboratories, McLean, VA) supplemented with penicillin, streptomycin, and 2 mm L-glutamine. Spleen cells were obtained by perfusing the organ with medium. WBC were separated from blood drawn from the retroorbital venus plexus with a pipet coated with ammonium heparin (400 units/ml; Scientific Products, Mc Gaw Park, IL). Blood was transferred to heparin-coated tubes and centrifuged at 500 rpm at 4°C for 10 min. WBC were aspirated from the cell-plasma interface.

Differential cell counts were made prior to the experiment on cytospurfe (Shandon, Sewickley, PA) slide preparations stained with Giemsa or on plates stained with Wright's stain after migration. A minimum of 200 cells was counted per preparation.

Sera used as chemotactant or for inhibition of complement component C3 include the following: guinea pig serum (fresh frozen; Cordis, Miami, FL); serum from normal mice; serum from tumor-bearing mice; and goat antiserum to murine C3 or to guinea pig C3 (Cappel, Cochranville, PA). The antisera to C3 were dialyzed at 4°C overnight against three changes of phosphate-buffered saline, pH 7.2, in order to remove preservative. They were sterilized by filtration through 0.2-µm polycarbonate filters (Gelman, Ann Arbor, MI).

For studies of inhibition of C3, equal volumes of anti-C3 and the respective serum were mixed and incubated at 37°C for 30 min prior to use.

Chemotaxis measurement was adapted from the methods of John and Sieber (9) and Nelson et al. (10). Agarose (Seakem; Marine Colloids Division, FMC, Rockland, ME) was dissolved by autoclaving and held at 56°C. A gel was prepared by mixing at 45°C five parts Medium 199 containing 4% 1 M4(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 8.0, penicillin, streptomycin, and L-glutamine) with one part 5% agarose. Five ml of the mixture were placed into a 60-mm tissue culture dish (Falcon No. 3002). When solidified, the gel was cut with a template: 2.4-mm holes; 2.4 mm apart; three wells per set; six sets per plate. The resultant pattern resembled spokes of a wheel. The gel in the wells was removed with an 18 gauge needle. In 5-µl volumes, fresh frozen serum was placed in the outer well, heat-treated serum (56°C, 30 min) in the inner well, and cells (2.5 x 107/ml) in supplemented balanced salt solution containing 1% human albumin) in the center well. Complement in the fresh serum was activated by contact with the agarose (11). Each test was replicated 10 to 12 times. Plates were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO2 in air, the cells were fixed for 30 min with 3 ml absolute methanol per dish, the agarose was removed, and the cells were stained with Wright's stain. Migration was determined microscopically using a ×10 objective and ×10 eyepieces. One eyepiece contained a grid ruled in 100 squares. The number of migrating cells was counted within the width of the grid for the entire length of migration beginning at the edge of the well.

Statistics. Data were analyzed by Student's t test and, in some cases, also by multiple regression. Product-moment correlation coefficients were determined for some data. Some data were transformed to logarithms prior to analysis in order to normalize the distribution.

Results

Leukocytes from bone marrow, peripheral blood, and spleens of mice bearing a mammary adenocarcinoma were compared...
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Table 1  Chemotaxis toward activated guinea pig serum of leukocytes from bone marrow, peripheral blood, and spleen of normal mice and mice bearing a mammary adenocarcinoma

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Normal mouse</th>
<th>Tumor-bearing mouse</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>228.8 ± 25.4* (11)</td>
<td>689.2 ± 82.1 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>247.3 ± 93.9 (6)</td>
<td>618.8 ± 58.0 (6)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>49.7 ± 13.9* (11)</td>
<td>521.2 ± 51.6 (11)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Probability, the Student’s t test: normal versus tumor bearers.
* Mean ± SE of the number of migrating cells.
* Numbers in parentheses, number of experiments.
* Significance of normal spleen versus bone marrow, P < 0.001; of normal spleen versus peripheral blood, P < 0.05.

with cells from normal animals in their migration toward activated guinea pig serum (Table 1). In every case leukocytes from tumor bearers migrated in increased number. The magnitude of migrating cells from tumor-bearing mice was similar from the three cell sources. In normal mice, however, fewer spleen cells migrated than did cells from peripheral blood or bone marrow, which may have been a reflection of the presence of fewer polymorphonuclear cells in spleens of normal animals: 12%, compared to 58.5% for tumor bearers. The assay was terminated after 4 h, a time optimal for measuring migration of polymorphonuclear cells but premature for measuring mononuclear cell migration which was optimal at 24 h.

The following group of studies was performed with bone marrow cells only. To determine whether there was a correlation between degree of migration of bone marrow cells and tumor size, we measured migration weekly following tumor implantation (Table 2) and then correlated time of tumor growth with size (Fig. 1). It was found that increased numbers of bone marrow cells migrated as the tumor grew through 21 days but not thereafter. It is interesting to note that, although fewer numbers of cells migrated toward heat-inactivated than fresh guinea pig serum, numbers of cells from tumor bearers were greater than those from normal animals. The same phenomenon occurred if no chemotactant were present (chemokinesis). Numbers of cells which migrated spontaneously were similar in number to those migrating toward heat-treated guinea pig serum.

When days of tumor growth were plotted versus volume (mm³), an allometric growth curve resulted (Fig. 1). When both variables were transformed to logarithms, a straight line resulted. This pattern of growth is consistent with standard growth (12). These data demonstrate that the size of tumor in this model is correlated with length of time of tumor growth.

To determine whether the reactivity of the bone marrow cells was a result of a local reaction in the tumor-bearing leg under investigation, bone marrow from the normal contralateral leg also was tested. No consistent differences were found, and tumor bearers showed increased chemotaxis with cells obtained from either leg. This finding allowed us to study chemotaxis of bone marrow cells subsequent to, as well as at, the time of primary tumor removal by leg amputation.

Amputation of the tumor-bearing limb caused chemotaxis to revert to normal by 14 days (Fig. 2), at which time metastases were less than 1 mm³ in size. Bone marrow cells from these animals migrated in numbers similar to those of normal amputees and of normal untreated mice. However, 28 days after removal of the primary tumor in animals bearing metastases from 1 to 300 mm³ in size, a large increase in numbers of bone marrow cells which migrated was noted. The numbers of cells which migrated showed a high correlation with the volume of the metastases (r = 0.537, P < 0.005; Fig. 3).

After tumor removal by leg amputation at 21 days of tumor growth, 24 animals were followed for survival. The animals could be divided into 2 groups: those which lived at least 175 days after amputation (41.7%), and those which died between 13 and 63 days (average, 37.2) after amputation (58.3%). These percentages were quite similar to those of the above experiment in which 51.6% had visible lung metastases and 48.4% did not. Those animals with metastases were identified by increased chemotaxis at 28 days after removal of primary tumor but not at 14 days. Because the average survival was 37 days, most animals with metastases could have been identified before they died.

To determine whether this increased chemotactic response was an isolated or general phenomenon, bone marrow cells from mice bearing a fibrosarcoma were examined in comparison with those from mice bearing a mammary adenocarcinoma and those from normal animals (Table 3). In addition, we examined a variety of sera (i.e., guinea pig, normal mouse, mammary adenocarcinoma bearer, and fibrosarcoma bearer) to see if the source of chemotactant influenced the migration of the cells. It was found that bone marrow cells from fibrosarcoma-bearing mice were similar in their migration to those from mice bearing mammary adenocarcinoma and that both were significantly greater than those from normal animals. Further, the source of the chemotactant used had no effect on the numbers of cells which migrated: serum from normal or tumor-bearing mice attracted equal numbers of cells from each respective source. However, the use of sera heated (56°C, 30 min) so as to inactivate complement resulted in a dramatic decrease in the numbers of cells which migrated. Nevertheless, significantly greater numbers of cells from mice bearing either tumor migrated toward each serum than cells from normal mice.

Complement was identified as providing the primary chemotactant in the serum in that a significant depression of chemotaxis resulted when the sera were incubated with anti-C3

Table 2  Number of bone marrow cells migrating toward guinea pig serum from normal and mice bearing tumor for different periods of time

<table>
<thead>
<tr>
<th>Days of tumor growth</th>
<th>Activated</th>
<th>Heat treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal BMC*</td>
<td>Tumor BMC</td>
</tr>
<tr>
<td>1-7</td>
<td>69.7 ± 8.3 (5)</td>
<td>84.0 ± 10.8 (14)</td>
</tr>
<tr>
<td>8-14</td>
<td>68.4 ± 8.0 (6)</td>
<td>184.0 ± 27.9 (9)</td>
</tr>
<tr>
<td>15-21</td>
<td>118.7 ± 16.2 (5)</td>
<td>752.6 ± 77.0 (22)</td>
</tr>
<tr>
<td>22-28</td>
<td>79.6 ± 10.1 (7)</td>
<td>552.0 ± 128.0 (7)</td>
</tr>
<tr>
<td>&gt;28</td>
<td>66.1 ± 6.9 (5)</td>
<td>403.6 ± 76.5 (8)</td>
</tr>
</tbody>
</table>

* BMC, bone marrow cells; NS, not significant.
* Probability, the Student’s t test: normal versus tumor bearers.
* Mean ± SE of the number of migrating cells.
* Numbers in parentheses, number of experiments.

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Fig. 1. Days of tumor growth plotted versus volume (mm³). Twelve animals were averaged at each time point except 32 days, which had an n of 9. When time and volume were converted into logarithms, a straight line resulted (dotted line).

Fig. 2. Number of bone marrow cells which migrated toward activated guinea pig serum from animals bearing 21-day tumors. Measurements were made at the time of amputation of the tumor-bearing leg and 14 or 28 days later. ■, tumor-bearing leg amputated; □, normal leg amputated; ◆, normal animal; ▼, tumor-bearing leg amputated, metastasis in lung. Bars, SE.

prior to use in the experiment (Table 4).

Differential counts of the cells which migrated revealed that greater than 93% were immature polymorphonuclear cells in both tumor bearers and normal mice. To see if the composition of the bone marrow cell mixture added to the wells affected the migration, differential counts were also performed prior to the experiment (Table 5). These showed an increase of segmented neutrophils in tumor bearing mice which was mirrored by a relative decrease in the number of lymphocytes. A decrease in the numbers of eosinophils also was noted. A further analysis was performed to determine if there was a correlation of the percentage of cell type present with the number of cells which migrated from individual animals within each group. Results are indicated by the r values beside differential percentage (Table 5). No significant correlations occurred for normal animals, whereas a negative correlation was seen for percentage of lymphocytes present and number of cells which migrated for bone marrow cells from tumor bearers ($P = 0.04$). No significant correlations of number of cells which migrated and percentage of other cell types present in tumor-bearer bone marrow cells occurred.

DISCUSSION

Although it appears as if chemotaxis in this study simply corresponded to granulocyte leukocytosis, further analysis indicates something else must have been occurring. There is a possibility that the presence of the tumor resulted not only in an increased number of granulocytes in the bone marrow but actually made them more chemotactic. If increased numbers of granulocytes alone had been the determining factor, there would have been a significant correlation between the percentage of polymorphonuclear cells and the number of cells which migrated in the normal animal. Even in the tumor-bearing mice, it was not the percentage of polymorphonuclear cells which affected cell migration but the percentage of lymphocytes.

The increased chemotaxis demonstrated in this study also occurred in models of necrosis (Staphylococcus aureus and turpentine), but the degree of chemotaxis was not as great as that of cells from tumor bearers. Although tumor necrosis may have been a factor in the present study, it was not a prominent feature, because animals which subsequently developed metastases had a concomitant rise in chemotaxis activity compared to the animals which did not develop metastases. Further, there was little or no necrosis in the metastases. Our data correspond with those of others who showed that granulocytosis developed concomitantly with enhancement of metastases (13). A similar phenomenon has been described by other investigators who showed that a tumor promoter initiated centrosome splitting in a manner similar to that induced by treatment of randomly locomoting cells with a chemotactic factor (14).

Other studies of chemotaxis of cells derived from animals or patients with tumors, including metastatic breast cancer, have indicated that there is a decrease in chemotaxis (15–18). It is important to point out that those studies were carried out using monocytes or macrophages. In fact, in the in vitro studies reported, care was taken to eliminate granulocytes (16, 17). The 

3 H. V. Ratajczak, M. G. Lewis, and K. Duggal, unpublished observations.
present data do not conflict with those findings, since different cell types were being evaluated. Only in one study were both macrophages and granulocytes studied in the same model (19), and the ability of those cells to respond to an in vivo inflammatory stimulus in a tumor bearer was evaluated. Macrophage chemotaxis was less than that of normal controls, while granulocyte chemotaxis was the same as or greater than that of normals.

The major chemotactant in the serum was identified as complement derived in the present study. Not only did heat treatment reduce the chemotactic stimulus, but the chemotactic stimulus was less than that toward serum from a normal mouse. The number of bone marrow cells which migrated toward serum from a mammary adenocarcinoma-bearing mouse was less than that toward serum from a normal mouse. The magnitude of all other migration tests did not differ with respect to source of serum from mice.

### Table 3 Chemotactant properties of sera from mice bearing different tumors as chemotactants for bone marrow cells from those animals

<table>
<thead>
<tr>
<th>Source of bone marrow cells</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Mammary adenocarcinoma</td>
</tr>
<tr>
<td>Activated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>58.5 ± 10.6$\text{a}$</td>
<td>568.6 ± 96.6$\text{a}$</td>
</tr>
<tr>
<td>Mouse: normal</td>
<td>112.5 ± 19.9</td>
<td>763.6 ± 108.1</td>
</tr>
<tr>
<td>Mouse: mammary adenocarcinoma</td>
<td>155.9 ± 25.8</td>
<td>944.4 ± 33.8</td>
</tr>
<tr>
<td>Mouse: fibrosarcoma</td>
<td>166.8 ± 24.0</td>
<td>825.0 ± 126.8</td>
</tr>
</tbody>
</table>

### Table 4 Effects of preincubation of serum with antiserum to C3 on chemotaxis of bone marrow cells from normal or tumor-bearing mice

<table>
<thead>
<tr>
<th>Source of bone marrow cells</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Mammary adenocarcinoma</td>
</tr>
<tr>
<td>Mouse: normal</td>
<td>199.5 ± 0.7$\text{a}$</td>
<td>537.6 ± 25.1</td>
</tr>
<tr>
<td>Mouse: normal + anti-murine C3</td>
<td>1.5 ± 0.5</td>
<td>100.4 ± 13.3$\text{a}$</td>
</tr>
<tr>
<td>Mouse: mammary adenocarcinoma</td>
<td>190.6 ± 43.6</td>
<td>503.4 ± 36.6</td>
</tr>
<tr>
<td>Mouse: mammary adenocarcinoma + anti-murine C3</td>
<td>2.4 ± 1.3</td>
<td>45.8 ± 10.9$\text{a}$</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>139.8 ± 31.2</td>
<td>465.3 ± 40.9</td>
</tr>
<tr>
<td>Guinea pig + anti-guinea pig C3</td>
<td>60.4 ± 13.7</td>
<td>357.6 ± 30.2$\text{a}$</td>
</tr>
<tr>
<td>Anti-murine C3</td>
<td>0.9 ± 0.6</td>
<td>48.0 ± 23.7</td>
</tr>
<tr>
<td>Anti-guinea pig C3</td>
<td>1.5 ± 1.0</td>
<td>62.4 ± 29.5</td>
</tr>
</tbody>
</table>

### Table 5 Influence of cell types present on migration of bone marrow cells from normal versus tumor-bearing mice

<table>
<thead>
<tr>
<th>Source of bone marrow cells</th>
<th>Normal (n = 12)</th>
<th>Tumor bearer (n = 12)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>162.4 ± 22.3$\text{a}$</td>
<td>664.7 ± 41.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Differential**

- Blast: 8.8 ± 1.1 (r = 0.072$\text{a}$)
- Promyelocytes: 4.0 ± 0.3 (r = -0.320)
- Myelocytes: 6.3 ± 0.5 (r = -0.430)
- Metamyelocytes: 37.3 ± 0.9 (r = -0.049)
- Segmented neutrophils: 20.8 ± 0.9 (r = 0.478)
- Lymphocytes: 16.0 ± 1.3 (r = -0.371)
- Monocytes: 3.3 ± 0.4 (r = -0.031)
- Eosinophils: 3.6 ± 0.4 (r = -0.560)

**Notes:**

- Mean ± SE of the number of cells which migrated from inidividual animals within each group.
- r = correlation coefficient of percentage of cell types present with number of cells which migrated from individual animals within each group.
- Significance level of r = 0.04.

- Mean ± SE (number).
- Differential percentage ± SE. Average counts of duplicate preparations, 200 cells counted per slide.
- Significance of normal versus tumor bearers.
- P = 0.001.
- P = 0.002.
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treatment dramatically lower the effectiveness of serum in this regard, but preincubation with antiserum to C3 also greatly reduced its chemotactant properties.

Our data indicated that the change in migration of leukocytes from tumor-bearing mice was a reflection of the presence of more than one kind of tumor. Tumors caused an increase in number of polymorphonuclear cells with a complementary decrease in number of lymphocytes. Only for tumor bearers was the composition of bone marrow cells a deciding factor in the number of cells which migrated. Cells from tumor-bearing mice had increased spontaneous migration (chemokinesis) as well as migration toward heat-inactivated serum. The increased migration toward activated complement (chemotaxis) was equal if the source of complement was serum from a normal mouse or one bearing a tumor. These data suggested that the increased chemotaxis of leukocytes from tumor-bearing mice was a property of the cells rather than the soluble substances in the serum.

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