Characterization of Macrophage Chemotaxins in Tumor Cell Cultures and Comparison with Lymphocyte-derived Chemotactic Factors

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SUMMARY

Culture fluids from five murine sarcomas were chemotactic for syngeneic peritoneal macrophages in vitro. Peritoneal macrophages from mice infected with Mycobacterium bovis, strain Bacillus Calmette-Guérin, were more responsive to the chemotactic factor in tumor cultures than were normal macrophages. Peritoneal granulocytes, however, did not significantly respond to this factor. The level of chemotactic activity in tumor cultures paralleled cell growth for all five tumors; maximal levels occurred during log growth. Culture medium alone or fluids from proliferating spleen cell cultures stimulated with mitogens did not have detectable chemotactic activity. Chromatography of the tumor culture fluids resulted in a single peak of chemotactic activity in the 15,000-molecular weight range on Sephadex G-100 and at about 7.5 mmho/cm specific conductance on diethylaminoethyl cellulose. By both biological and physicochemical characteristics, the chemotactic activity in tumor culture fluids was different from mouse lymphocyte-derived chemotactic factor.

INTRODUCTION

Accumulation of leukocytes at inflammatory foci is part of the host defense against noxious agents. One of the mechanisms that leads to localization is elaboration of molecules chemotactic for leukocytes that diffuse out from the site of release. Leukocytes then migrate up the concentration gradient to form the inflammatory focus (18). Neutrophils, basophils, eosinophils, and mononuclear phagocytes respond to chemotactic stimuli (18). Factors that preferentially attract a particular type of leukocyte have been described (16, 18); these different chemotactic factors may determine the cellular composition of inflammatory foci.

The inflammatory response at the site of neoplastic growth is characterized by a mononuclear phagocyte infiltrate. Indeed, with some rat sarcomas, macrophages may comprise up to 50% of the total cells (5). In this report, we describe a possible mechanism for macrophage accumulation in tumors. Several murine tumors elaborate a chemotactic factor in vitro that attracts syngeneic peritoneal macrophages. This tumor-derived chemotactic activity was different from lymphocyte-derived chemotactic factor in both biological and physicochemical properties.

MATERIALS AND METHODS

Tumor Cells. Tumors were obtained through the courtesy of Dr. Gerald L. Bartlett, Pennsylvania State University, Hershey, Pa. Tumor cell lines, designated 1023, 1037, 1038, and 1063, were induced by pellets of 1% 3-methylcholanthrene in paraffin implanted in the s.c. tissue of C3H/HeNcrl male mice (1). Tumors were maintained by serial trocar passage of minced tumor into immunodepressed C3H/HeN recipients (1). Split-thickness skin grafts from C3H/HeNcrl donors survived without contraction on normal C3H/HeN mice for more than 100 days (1). The 10th to 16th transplant generation of Tumor 1023, 8th to 12th generation of Tumor 1037, 50th to 54th generation of Tumor 1038, and 9th to 14th generation of Tumor 1063 were used during these experiments.

For cell culture, tumors were excised and minced. Tumor fragments were digested with 0.25% Pronase (Calbiochem, San Diego, Calif.) in 0.02 M phosphate-buffered saline, pH 7.2, with DNase, 40 µg/ml (Calbiochem). Cells obtained by enzymatic digestion were washed twice in Eagle’s minimal essential medium with antibiotics (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% (v/v) heat-inactivated FCS1 (Grand Island Biological Co.) and placed into culture in this medium. Cells were passaged in vitro by trypsinization of confluent monolayers. Tumor cell cultures used in these experiments were in the 5th to 10th passage. Cell lines for all 4 tumors were free of fibroblast and macrophage contamination by morphological criteria on Wright-stained smears prepared by cytocentrifugation (Cytospin; Shandon-Southern Instruments, Camberley, Surrey, England). Culture fluids were harvested at intervals after passage (2 × 10⁶ viable tumor cells per 6 ml medium in 25-sq cm flasks) and centrifuged at 300 × g for 15 min at 4°. Supernatant fluids were placed into sterile polypropylene tubes, stored at 4°, and assayed for chemotactic activity in the i5,000-molecular weight range on Sephadex G-100 and at about 7.5 mmho/cm specific conductance on diethylaminoethyl cellulose. By both biological and physicochemical characteristics, the chemotactic activity in tumor culture fluids was different from mouse lymphocyte-derived chemotactic factor.

1 The abbreviations used are: FCS, fetal calf serum; PC, peritoneal cells; EAMS, endotoxin-activated mouse serum; BCG, Bacillus Calmette-Guérin; LDCF, lymphocyte-derived chemotactic factor.

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becco’s modified Eagle’s medium with antibiotics (Grand Island Biological Co.). This medium contained 2 g NaHCO₃ per liter and 10% (v/v) heat-inactivated FCS. Peritoneal fluid was withdrawn through the anterior abdominal wall with a 19-gauge needle. A sample of peritoneal fluids pooled from several mice was removed for Wright stain differential and total cell counts; the remainder was centrifuged at 250 x g for 10 min at 4°C. Washed PC were resuspended in medium to 2 x i0⁶ macrophages per ml.

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Dilutions of tumor culture supernatants, tissue culture medium, and control or syngeneic mouse serum treated with bacterial endotoxins [EAMS, which contains C5a, a chemotactically active cleavage product of C5 (2, 17)], were preheated to 37°C and added to the lower wells of blind-well chemotactic chambers (Neuroprobe Corp., Bethesda, Md.). Polycarbonate membrane filters with 5-μm pores (Nucleopore Corp., Pleasanton, Calif.) were placed over the lower wells. The upper well inset was screwed into place filled with 0.2 ml of PC suspension. Chambers were incubated at 37°C in 5% CO₂ in moist air for 4 hr. After incubation, PC in the upper well were removed with a cotton swab. The filters were removed, air dried, and stained (9). Migrated macrophages in 20 oil immersion fields (field diameter, 170 μm) were counted. Chemotactic response was expressed as mean total macrophages per 20 oil fields ± S.E. for triplicate filters.

Chromatography of Culture Supernatants. Fifteen-ml volumes of culture fluids were shell-frozen in 50-ml polypropylene tubes, lyophilized, and reconstituted with 1.5 ml water within 1 hr of application to chromatography columns. Samples were applied to columns of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.) prepared as described previously (10). Eluting buffer was 0.02 M phosphate-buffered saline, pH 7.2, with cytochrome C, 0.2 mg/ml (Sigma Chemical Co., St. Louis, Mo.) added to prevent possible adsorptive loss of low concentrations of protein (10). Flow of buffer was controlled with a Pharmacia P3 pump (Pharmacia); equal fraction volumes were collected at timed intervals with an LKB fraction collector (LKB Instruments, Inc., Rockville, Md.). After measurement of absorbance at 280 nm, eluted fractions and dilutions of starting samples were assayed in duplicate for chemotactic activity. Columns were calibrated by elution of human serum containing chymotrypsinogen, 10 mg/ml (Sigma) and cytochrome c, 10 mg/ml.

For ion-exchange chromatography, DEAE-cellulose was washed with NaOH and HCl, equilibrated with 0.005 M phosphate starting buffer, pH 7.9, divided into aliquots, and autoclaved (10). One-g amounts of DEAE-cellulose in a 1% suspension were packed in 0.9-cm-diameter columns at a pressure of 1 kg/sq cm. Fifteen-ml samples of culture fluids concentrated as described above were equilibrated by dialysis with starting buffer and applied to DEAE-cellulose columns. Cellophane dialysis tubing was soaked in 0.1 M HCl for over 8 hr and washed in water overnight before use. Protein was eluted from DEAE-cellulose with a linear NaCl gradient in a volume of 16 ml. Composition of the starting gradient solution was 0.1 M NaCl in starting buffer; limit solution was 0.4 M NaCl in water. Ten-drop fractions were collected and measured for conductivity, absorbance at 280 nm, and chemotactic activity.

RESULTS

Chemotactic Stimulus in Tumor Culture Fluids for Peritoneal Macrophages. Supernatant fluids from cultures of methylcholanthrene-induced fibrosarcoma cells (Tumor 1023) were chemotactic for syngeneic mouse peritoneal macrophages in vitro (Table 1). Peritoneal macrophages from Mycobacterium bovis, strain BCG-infected mice (BCG-infected macrophages) were more responsive to the tumor-derived chemotactic activity than were an equal number of macrophages from uninfected control mice (12). The chemotactic response of peritoneal granulocytes from BCG-infected mice to the tumor-derived chemotactic activity was not significantly different from the granulocyte response to medium. These same granulocytes, however, showed a vigorous chemotactic response to complement-derived factors present in EAMS. Thus, syngeneic tumor cells in vitro elaborate factors that are chemotactic for...

Table 1

Response to chemotactic factors in tumor culture fluids

| Tumor 1023 was cultured at 2 x 10⁶ viable cells per ml Eagle’s minimal essential medium with 10% FCS in 25-sq cm plastic flasks. Culture fluids were harvested at intervals and centrifuged, and one-third dilutions of supernatants in medium were assayed for chemotactic activity. PC from mice infected 1 to 3 weeks previously with M. bovis, strain BCG (TMC No. 1029; Trudeau Mycobacterial Collection, Saranac Lake, N. Y.) and from uninfected controls were used for responding cells. A 1/100 dilution of EAMS in medium, which is chemotactic for both macrophages and granulocytes, was used as positive control. Chemotactic response was expressed as total cells per 20 oil fields, mean ± S.E., for triplicate filters. |

<table>
<thead>
<tr>
<th>Chemotactic response (migrated cells/20 oil fields) to tumor supernatants harvested at</th>
<th>Responding cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Control mice Macrophages</td>
<td>0 ± 0*</td>
</tr>
<tr>
<td>BCG-infected mice Macrophages</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>110 ± 26</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
normal and BCG-activated macrophages in vitro. These same chemotactic stimuli, however, had little effect on granulocytes.

**Correlation of Tumor Cell Growth and Appearance of Chemotactic Activity in Vitro.** We have previously reported that the elaboration of chemotactic activity in supernatants of mitogen or antigen-stimulated spleen cell cultures was not related to cell proliferation as measured by thymidine incorporation (11). Indeed, concentrations of the T-lymphocyte mitogens, phytohemagglutinin, or concanavalin A, which induced maximal cell proliferation, did not induce detectable levels of chemotactic activity; maximal levels of chemotactic activity were induced by higher mitogen concentrations, which had no proliferative activity. The relationship between cell proliferation and appearance of chemotactic activity in culture supernatants was examined for Tumors 1023 and 1063 (Chart 1). For both tumor cell lines, chemotactic activity in the culture fluids paralleled tumor cell growth. Thus, in contrast to the chemotactic lymphokine, tumor-derived chemotactic activity directly correlated with tumor cell proliferation in vitro. Maximal elaboration of the tumor-derived chemotactic activity occurred during log growth in culture. This correlation of tumor-derived chemotactic activity with log growth has been observed for 4 methylcholanthrene-induced tumors (Tumors 1023, 1037, 1038, and 1063) and with a fibrosarcoma derived from a cloned embryo cell line which underwent spontaneous neoplastic transformation in vitro (Tumor 7943) (14). The optimal cell density for production of tumor-derived chemotactic activity was about 0.10 × 10⁶ cells/sq cm. Optimal production of the chemotactic lymphokine occurred at cell densities of about 1.3 × 10⁶ cells/sq cm (20 ml of 5 × 10⁶ cells/ml in 75-sq cm flask (11)). In contrast to the relatively high levels of macrophage chemotactic activity in tumor culture fluids, the levels present in fluids from syngeneic embryo cell cultures were minimal (Table 2). Supernatants of mass embryo cultures from C3H/HeN mice had about one-fifth the macrophage chemotactic activity of supernatants from comparable numbers of tumor cells.

**Physicochemical Characterization of Tumor-derived Chemotactic Activity.** The in vitro conditions that were opti-

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**Table 2**

*Macrophase chemotactic activity in culture fluids of cell lines*

<table>
<thead>
<tr>
<th>Tumor cell lines</th>
<th>After 48 hr in culture</th>
<th>After 96 hr in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells/flask (x 10⁴)</td>
<td>Normal macrophages</td>
</tr>
<tr>
<td>1023</td>
<td>0.7 ± 0.3</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>1063</td>
<td>1.0 ± 0.3</td>
<td>28 ± 8</td>
</tr>
<tr>
<td>1038</td>
<td>1.3 ± 0.8</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>C3H embryo</td>
<td>0.8 ± 0.2</td>
<td>9 ± 3</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
for production of LDCF were very different from that necessary for elaboration of tumor-derived chemotactic activity. This difference suggested that the lymphocyte and tumor cell-derived factors might also have different physicochemical properties. LDCF was shown to have a major peak of chemotactic activity that eluted in the 40,000-molecular weight region from Sephadex G-100 (10) (Chart 2). This major peak of chemotactic activity was detected in a wide variety of in vitro conditions: with different culture medium serum supplements (human or fetal calf), with both mitogenic and nonmitogenic stimuli, and with either rapidly proliferating lymphocyte cultures or cultures with undetectable cell proliferation. Likewise, LDCF produced under a variety of in vitro conditions had a characteristic elution pattern from DEAE-cellulose (Chart 2). The major peak of chemotactic activity was detected in a specific conductance region of about 15 mmho/cm which corresponded to about 0.25 M NaCl (10).

The Sephadex G-100 and DEAE-cellulose chromatography of tumor-derived chemotactic activity was very different from the chemotactic lymphokine (Chart 3). The chemotactic activity present in Tumor 1023 culture supernatants eluted from Sephadex G-100 in the 15,000-molecular weight region. A major peak of chemotactic activity in this molecular weight region was also detected in supernatants from Tumor 1038 and 1063 cultures (Chart 3). Chemotactic activity in Tumor 1023 culture supernatants was eluted from DEAE-cellulose in a specific conductance region of about 7.5 mmho/cm, which corresponded to about 0.12 M NaCl. A similar major peak was detected in Tumor 1063 culture fluids. Thus, the tumor-derived chemotactic activity can be distinguished from the chemotactic lymphokine by both

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**Chart 2. Chromatography of lymphocyte-derived chemotactic factor.** Gel filtration and ion-exchange chromatography of LDCF. Spleen cells from BCG-infected mice were cultured with or without (PPD) 100 μg/ml PPD for 72 hr (10, 14). Fifteen-mI aliquots of supernatant culture fluids were shell-frozen, lyophilized, and reconstituted with 1.5 ml water. Samples were applied to a Sephadex G-100 column (A) or dialyzed against 0.005 M buffer, pH 7.9, and applied to a DEAE-cellulose column (B). Fractions were eluted from Sephadex G-100 with phosphate-buffered saline with cytochrome c, 0.2 mg/ml, at 3 ml/hr with timed collections of 20 min/fraction, or from DEAE-cellulose with a linear NaCl gradient. ---, absorbance of 280 nm of diluted fractions (one-fourth for Sephadex G-100, one-tenth for DEAE-cellulose); ---, specific conductance of undiluted DEAE-cellulose fractions; ---, chemotactic activity; values for duplicate filter.

**Chart 3. Chromatography of tumor cell chemotactic factor.** Gel filtration and ion-exchange chromatography of tumor culture fluids. Tumor 1023 supernatants (15 ml) were shell-frozen, lyophilized, and reconstituted with 1.5 ml water. Chromatograph procedures and chart labeling were as for Chart 2.
molecular weight and by behavior on ion-exchange chromatography.

DISCUSSION

We found that several different murine sarcomas elaborate factors in vitro that are chemotactic for syngeneic peritoneal macrophages. Activated macrophages from BCG-infected mice were more responsive to this tumor-derived stimulus than were normal peritoneal macrophages. Chemotactic hyperresponsiveness of activated macrophages also occurs to chemotactic lymphokines (LDCF), and complement-derived components present in EAMS (12). Unlike LDCF or EAMS, however, the tumor-derived chemotactic activity was only minimally effective for peritoneal granulocytes. The preferential attraction of macrophages over granulocytes has also been described for other chemotactic stimuli, notably chemotaxins from mycobacteria or cornybacillia (16, 18).

Whether the tumor-derived chemotactic activity is secreted by the cells in culture, shed from cell surface membranes, or occurs through interaction with serum components, is unknown. The appearance of tumor-derived chemotactic activity correlated with periods of rapid cell proliferation in vitro for 5 tumor cell lines. No chemotactic activity was detected in medium with serum cultured for an identical time-span, nor was chemotactic activity detected in rapidly proliferating spleen cell cultures, even though the spleen cells were cultured in the same medium at up to 10 times the density of the tumor cells. Moreover, the chemotactic lymphokine produced by mitogen or specific antigen-stimulated spleen cells over a wide range of in vitro conditions had remarkably consistent physicochemical properties (10). The chromatographic patterns of LDCF on Sephadex G-100 and DEAE-cellulose were very different from the patterns of tumor-derived chemotactic activity. LDCF showed a major peak of chemotactic activity in the 40,000-molecular weight region on Sephadex G-100 and about 15 mmho/cm specific conductance from DEAE-cellulose. In contrast, the tumor-derived activity eluted in the 15,000-molecular weight region and at about 7.5 mmho/cm specific conductance.

The in vivo role of the tumor-derived chemotactic activity and, in fact, whether the activity is limited to tumor cells, are not known. In addition to neoplastic sites, macrophage infiltration is prominent in other rapidly proliferating tissues such as granulation tissue. Chemotactic factors released by tumors and/or rapidly proliferating cells may provide an endogenous mechanism for macrophage accumulation. Accumulation of macrophages at tumor sites, mediated directly by tumor cell factors or by host resistance (lymphocyte or complement-derived stimuli), may not necessarily indicate an effective antitumor response. Although macrophages have been shown to be cytotoxic for tumor cells in vitro and in vivo in several animal species (3, 6, 8, 15), they also exert trophic effects on other cells such as fibroblasts (S. Wahl, personal communication), granulocytes (19), or B- and T-lymphocytes (7, 13, 19). Thus, a macrophage chemotaxin released by tumor cells could provide a mechanism for self-destruction or a necessary prerequisite for cell proliferation.

REFERENCES

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