Suppression of Tumor Cell Growth in Vitro by a Bone Marrow Factor

John E. Dittmer, Se-Kyung Oh, Laurence Corwin, and Michael Bennett

ABSTRACT

A supernatant factor derived from monodispersed bone marrow cells was tested for its ability to inhibit the growth of tumor cell lines and freshly dispersed hematopoetic cells in vitro. The supernatant fluid from bone marrow cells was capable of inhibiting the mitogenic response of rat thymocytes to concanavalin A. It also was capable of inhibiting growth of HeLa cells, Sarcoma 180, EL-4, and BALB/c K3T3 tumor cell lines as measured by thymidine incorporation. The factor did not inhibit the growth of normal thymus cells, marrow cells, or WI-38-SV40, and F-46 tumor cell lines. From data derived from 51Cr release assays, the factor appears to be selectively cytoreductive.

Bone marrow supernatant factor is stable to heat (100°C for 10 min) and trypsin digestion, but is sensitive to carboxypeptidase B digestion. Molecular weight estimation by gel filtration chromatography on a Sephadex G-75 column indicates its apparent molecular weight to be less than 12,000. Bone marrow supernatant factor appears to function across species and strain barriers.

INTRODUCTION

A number of investigators have described factors from lymphoid or other tissues which are capable of inhibiting proliferative responses of lymphocytes (3–6, 8, 10, 14–16). Some of the same and other factors inhibit the proliferation of tumor cells (10). We describe here the findings that the supernatant fluid of washed bone marrow cells contains one or more factors capable of inhibiting the growth of tumor cells in vitro and the proliferation of mitogen-stimulated or mitogen-transformed cells in vitro, but normal marrow and thymus cells were unaffected in vitro. The BMSF4 appears to function equally well against human, rat, and mouse tumor cells.

MATERIALS AND METHODS

Animals. Mice were purchased from The Jackson Laboratory, Bar Harbor, ME, or were bred in this laboratory. Rats were purchased from Microbiological Associates, Walkersville, MD.

Preparation of Cell Suspensions and Supernatant Fluids. Rat bone marrow cells were flushed from the long bones of rats with RPMI 1640 medium (Flow Laboratories, McLean, VA). Thymus cells were obtained by mincing the organ in a small amount of media and later aspirating the tissue in larger volumes of media with a 3-ml syringe. All cell suspensions were harvested and filtered through 200-mesh/inch stainless steel gauze (Buffalo Wire Works, Buffalo, NY) to remove particulate matter. The volume of media to harvest cells from each type of organ was adjusted so as to obtain approximately 5 x 10^6 cells/ml. Usually 25 x 10^6 cells were harvested in 5 ml of media and centrifuged at 2400 rpm (900 x g), at room temperature for 8 min. BMSF so obtained was either used for experiments on that day or stored at -20°C until used. The suppressive activity of the supernatant fluids remains relatively constant for at least 2 months under these conditions.

Gel Filtration Chromatography on Sephadex G-75 Column. BMSF obtained above was concentrated through Amicon UM-05 membrane to 5 to 10 ml volume and was chromatographed on a Sephadex G-75 column (2.5 x 90 cm; LKB Instruments, Gaithersburg, MD) which was equilibrated with PBS containing 100 units penicillin/ml and 100 µg streptomycin/ml (Flow Laboratories). Individual peaks were dialyzed and lyophilized before being assayed. Molecular weight standards used to calibrate the column were blue dextran 2000, human serum IgG, human serum albumin, ovalbumin, chymotrypsinogen A. RNAse, and dinitrophenyl glycine. The flow rate was 20 ml/hr.

Enzymatic Digestion of BMSF. Twice-crystallized salt-free trypsin ( Worthington Biochemical Corp., Freehold, NJ; specific activity, 180 to 220 units/mg) was used at 1% (w/w) concentration in PBS for 1 hr at 37°C, and digestion was stopped by the addition of 1% (w/v) soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). Approximately 0.5% (w/v) of carboxypeptidase B, treated with disopropyl fluorophosphate (Sigma; specific activity, 180 units/mg protein) was used to digest the crude bone marrow factors in PBS at 37°C for 1 hr. Enzyme control and enzyme-treated sample were heat inactivated at 100°C for 10 min. Crude bone marrow suppressor factor was stable under these conditions.

Tumor Cells. Mouse EL-4 lymphoma cells, Sarcoma 180 cells, BALB/c 3T3 cells, and BALB/c K3T3 cells (previously transformed with Kirsten sarcoma virus), rat LW-12 leukemia cells, human HeLa cervical carcinoma cells, human diploid WI-38 cells, WI-38 cells previously transformed with SV40, and F-46 Friend virus-induced tumor cells derived from DBA/2 mouse were maintained in vitro. The F-46 cells were not shedding Friend virus complex, but do express viral envelope antigens.

Labeling of Tumor Cells with 51Cr. Approximately 10^6 tumor cells were suspended in 1 ml of RPMI 1640 medium enriched with 10% fetal calf serum (Flow Laboratories). They were labeled with 100 µCi 51Cr (sodium [51Cr]dichromate; New England Nuclear, Boston, MA) for 1 hr at 37°C. Cells were washed 3 times with medium and used in the chromium release assay. Approximately 5 x 10^6 tumor cells/well were incubated in Microtiter II test plates with the bone marrow supernatant for 18 hr at 37°C in a 5% CO2 environment. Cells were spun down at 800 x g, for 10 min, and 100 µl of culture supernatant was assayed for released 51Cr with a Beckman gamma counter. The percentage of cytotoxicity was calculated by

\[
\% \text{ of specific cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

Mitogenic Stimulation. Thymocytes were cultured in wells of Microtest II (Falcon Plastics, Division of Bioquest, Oxnard, CA) plates in triplicate in a total volume of 200 µl/well. Each well contained 10^5 rat thymus cells, no mitogen, or 0.5 µg Con A (Calbiochem-Behring, La Jolla, CA) and 0 to 100 µl of BMSF. The plates were cultured for 72 hr at 37°C

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3 Present address: Department of Pathology, University of Texas Health Science Center, 5323 Harry Hines Boulevard, Dallas, TX 75235.
4 The abbreviations used are: BMSF, bone marrow supernatant factor; PBS, phosphate-buffered saline (0.01 M sodium phosphate buffer, pH 7.4, plus 0.14 µ sodium chloride solution); BMC, bone marrow cells; Con A, concanavalin A.
5 Received June 24, 1983; accepted November 21, 1983.
6 R. A. Steeves, personal communication.
in a humidified atmosphere containing 5% CO₂. To each well 0.5 μCi (methyl-[3H]thymidine (New England Nuclear) in 25 μl medium was added 18 hr prior to harvesting the culture by Titertek Cell Harvester System (Flow Laboratories). Incorporation of [3H]thymidine into DNA, as detected by standard liquid scintillation counting procedures, was the measure of cell proliferation.

Growth of Cells in Petri Dishes. To obtain an independent assessment of the effect of the supernatant fluids on cell proliferation without Con A or [3H]thymidine incorporation, fibroblastic cells were seeded into 60-mm diameter plastic Petri dishes in duplicate cultures. In a total of 2 ml of Dulbecco’s minimal essential medium containing 10% heat-inactivated fetal calf serum, 3.1 to 8.1 x 10⁶ cells and 0.1 or 0.2 ml of supernatant fluid were added. The cells were cultured for 1 to 5 days and then were harvested from the Petri dishes by trypsinization (0.5% trypsin, 5 min at 26°C). The cells were enumerated with a Coulter electronic particle counter.

RESULTS

Effects of Bone Marrow Cell Supernatant Fluids on the Mitogenic Response of Thymocytes to Con A. Bone marrow cells from rats, mice, or dogs were suspended in medium and centrifuged, and the supernatant fluids were assayed for their ability to affect the ability of LEW rat thymocytes to respond to Con A. The supernatant fluid of normal adult rat and dog marrow was suppressive, and the degree of suppression was dose related (Table 1; Chart 1). The factor appears to be active across species.

Differential Sensitivity of Tumor or Mitogen-transformed and Normal Cells to the Suppressive Effect of the Supernatant Fluids. The inhibition of [3H]thymidine incorporation into thymocytes stimulated with Con A by the supernatant fluids could have been due to (a) an antiproliferative effect, (b) a nucleosidase enzyme (11), (c) “cold” thymidine (20), or (d) prevention of “activation” of thymus-dependent (T) lymphocytes (1). To distinguish among these various possibilities, we tested the ability of supernatant fluids to suppress the [3H]thymidine incorporation into Con A blasts, neoplastic, and unstimulated normal cells. The supernatant fluid from rat marrow cells was able to suppress [3H]thymidine incorporation into human-derived HeLa, mouse Sarcoma 180, and EL-4 cells, as well as Con A blasts (Table 2). However, thymocytes or marrow cells taken directly from rats or mice were not significantly affected by the supernatant fluids; therefore, it appears that the supernatant fluids discriminate between normal and tumor or mitogen-transformed cells, in most instances. However, the Friend erythropoietic tumor cells, F-46, were actually stimulated by the supernatant fluid. Cold thymidine added to the cultures inhibited [3H]thymidine incorporation in normal thymus or marrow cells, but the rat BMC supernatant failed to suppress these cells.

Effects of Marrow Supernatant Fluids on the Growth of Fibroblastic Cells. To obtain an independent assessment of the

| Experiment | Source of supernatant | Target cell | [3H]Thymidine incorporation (cpm) | % of suppression
|-------------|-----------------------|-------------|----------------------------------|------------------|
| 1 Con A blasts | Rat BMC | 1,831 | 1,247 | 32%
| 2 Sarcoma 180 | Rat BMC | 52,257 | 32,034 | 39%
| Normal TC | Rat BMC | 17,732 | 16,095 | 9.2%
| Normal BMC | Rat BMC | 45,926 | 46,910 | -1.2%
| Normal BMC | Thymidine (10 μg) | 45,926 | 635 | 99%
| 3 EL-4 | Rat BMC | 36,167 | 18,409 | 49%
| Sarcoma 180 | Rat BMC | 130,496 | 57,620 | 56%
| HeLa | Rat BMC | 43,948 | 9,135 | 79%
| Con A blasts | Rat BMC | 3,258 | 1,289 | 60%
| F-46 | Rat BMC | 16,091 | 49,778 | -209%
| 4 EL-4 | L-929 | 15,633 | 5,773 | 63%
| Normal TC | Rat BMC | 9,041 | 8,484 | -6%
| Normal TC | L-929 | 9,041 | 9,671 | -7%

* Con A blasts, mouse spleen cells (10⁶) incubated with 0.5 μg Con A for 48 hr; normal TC or BMC were taken directly from LEW rats (Experiments 2, 5, and 6) or C57BL x DBA/2 F₁ mice (Experiment 4). Blasts or tumor cells (5 x 10⁶) or normal TC or BMC (1 x 10⁶) were incubated overnight (18 hr) with [3H]thymidine supernatant fluid (50 μl/well).

Table 1

| Experiment | Source of BMSF | [3H]Thymidine incorporation (cpm) | % of suppression
|-------------|----------------|----------------------------------|------------------|
| 1 None | No mitogen | 173 | 70,609 | 100%
| Rat BMC | Con A | 64 | 85 | 100%
| 2 None | No mitogen | 186 | 46,355 | 50%
| Rat BMC | Con A | 162 | 22,988 | 50%
| Dog BMC | Con A | 154 | 3,245 | 99%
| 3 None | No mitogen | 381 | 92,983 | -10
| Mouse BMC | Con A | 403 | 102,700 | -10

* S.E. were less than 10% of the mean values in almost every instance.

- Mean values of experimentals significantly different from controls, at p < 0.05.
- BMC, 50 μl supernatant per well.
antiproliferative effects of the supernatant fluids, "target cells" were grown in Petri dishes, and growth was measured by enumerating cells at different time intervals after initiation of the cultures. Both dog and rat BMC supernatant fluids suppressed the growth of aneuploid BALB/c 3T3 and BALB/c K3T3 fibroblastic cells (Table 3). However, diploid WI-38 cells and SV40-transformed WI-38 cells were not significantly affected by rat BMC supernatant fluid. The WI-38-SV40 and the F-46 cells are the only 2 tumors tested in vitro which were resistant to the antiproliferative effects of the supernatant fluids.

Cytotoxicity of BMSF as Evidenced by 51Cr Release Assay. The ability of BMSF to inhibit the tumor in vitro appears to correlate with its ability to kill cells, as seen in a 51Cr release assay (Table 4). Forty-eight % of the EL-4 cells were lysed when 100 μl of BMSF were added to 5 x 10^4 EL-4 cells/well in Microtiter II test plates.

Preliminary Biochemical Characterization of Rat BMSF. The rat BMSF was tested for the ability to suppress proliferation of tumor cells in vitro after various treatments. The factor(s) in BMSF was stable on storage at 4° for 1 week and after boiling at 100° for 10 min. Suppressor factors found in BMSF were resistant to trypsin treatment. Carboxypeptidase B, however, removed the suppressive activity of BMSF (Table 5). Molecular weight estimation by gel filtration chromatography on a Sephadex G-75 column (Chart 2) indicates that Fraction V (M, <12,000) was the most active (Table 6).

<table>
<thead>
<tr>
<th>Table 3</th>
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<td>Effect of marrow supernatant fluids on growth of fibroblastic cells in Petri dishes</td>
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<tr>
<td>Experiment</td>
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<td>3</td>
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<td>WI-38-SV-40</td>
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Table 4 Percentage of cytotoxicity of EL-4 cells by BMSF

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<th>BMSF (μl)</th>
<th>% of cytotoxicity</th>
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<tr>
<td>100</td>
<td>48.9</td>
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<tr>
<td>50</td>
<td>30.4</td>
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<td>25</td>
<td>22.0</td>
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<td>10</td>
<td>13.9</td>
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DISCUSSION

The data presented here indicate that bone marrow cells of rats and dogs contain an antiproliferative factor that is present in the supernatant of washed cells. The factor(s) suppress mitogen-transformed blasts and most tumor cells tested in vitro. The factor(s) did not suppress the proliferation of normal thymus or marrow cells, or Friend erythroleukemia cells in vitro. Thus, the factor does not appear to affect "normal" cells or erythropoietic tumor cells but is active against many neoplastic cells. The tumor cells not affected by the factor (WI-38-SV40, F-46) are
diploid cells, although F-46 cells do have transloca
tional chromosomal abnormalities. 6

Trivial causes of decreased [%H]thymidine incorporation, e.g.,
nucleosidase activity (11) or thymidine itself (20), cannot explain the
 differential suppression of cells grown in Petri dishes (Table 3).

When several doses of Con A were tested in one experiment
involving thymus cells, the rat BMSF suppressed even at su-
praoptimal concentrations (data not shown). This indicates that
the factor does not act by interfering with binding of the mitogen.
The 51Cr release assay indicates that the factor is cytotoxic for
transformed cells (Table 4).

How this factor might distinguish between normal "resting"
cells and mitogen-stimulated thymocytes or tumor cells is not
clear. It is, however, conceivable that the factor(s) has the
potential for suppressing growth of any cell type but cannot
enter normal cells as easily as it can enter tumor cells. This
would imply that the ability of tumor cells to take up the factor
may be greater than that of normal cells (19). It should be
emphasized that a number of cell surface and biochemical
changes considered relatively specific for tumor cells have also
been observed in mitogen-transformed cells (21). Note that 10^6
"resting" thymus cells or BMC incorporated about the same
amount of [%H]thymidine as did 5 x 10^6 tumor cells (Table 2).

The BMSF was present in all washes of nondispersed bone
marrow cells. The concentration in each assay was not stan-
ardized, so that activity varied somewhat between experiments.
This probably accounts for variation in activity, especially be-
 tween BMSF isolated from dog and rat.

The factor apparently is not interferon because it differs from
interferon in several significant characteristics. Interferon is labile
at pH 7 and 57° (7), and BMSF is stable at pH 7 and 100°.
Interferon acts to inhibit viral replication (17) and cellular proli-
feration (18), and BMSF appears to be cytotoxic. The BMSF
reported here failed to inhibit Friend virus-infected cells, and
interferon is effective against Friend virus leukemia (22). In addi-
tion, interferon is inactivated by proteolytic enzymes (9), but
the BMSF is unaffected by these same enzymes.

The suppressive activity of bone marrow cell suspension
description here could be similar to the factor of rat spleen cells
(12, 14) or the splenic extract (10). Puzas et al. (13) reported that
chick bone marrow cells contain tissue-specific endogenous
inhibitors of bone cell proliferation, and Duwe and Singhal (2)
also reported a soluble factor which inhibits antibody in vivo and
in vitro, without affecting DNA synthesis of the bone marrow
cells. The bone marrow suppressor factor described by Puzas
et al. (13) shares some of the biochemical properties of our
suppressor factor, i.e., similar thermal stability, resistance to
trypsin and similar molecular weight range of less than 12,000.

However, our suppressor factor inhibits mitogenic responses of
mouse and rat splenocytes.

Experiments are currently under way to determine the origin
of the bone marrow suppressive factor and to determine whether
it is active in vivo.

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