Production of Cytokines by Bone Marrow Cells Obtained From Patients With Multiple Myeloma

By Alan Lichtenstein, James Berenson, Dean Norman, Mei-Ping Chang, and Anne Carlile

Previous work with continuously cultured multiple myeloma lines suggested that cytokine production by tumor cells may mediate some of the medical complications of this disease. To further investigate this issue, we assayed freshly obtained bone marrow (BM) cells from myeloma patients for the in vitro production of cytokines and the presence of cytokine RNA. Production of cytokine protein was assessed by bioassays with the aid of specific neutralizing anticytokine antibodies. These assays detected interleukin-1 (IL-1) and tumor necrosis factor (TNF) secretion by myeloma BM cells, which was significantly greater than secretion from similarly processed BM cells of control individuals. In contrast, lymphotoxin and interleukin-2 (IL-2) production could not be detected. The levels of IL-1 and TNF produced in vitro peaked at 24 hours of culture and correlated with stage and the presence (or absence) of extensive osteolytic bone disease. Northern blot analysis demonstrated the presence of IL-1 beta and TNF RNA in uncultured myeloma BM cells but no detectable IL-1 alpha or lymphotoxin RNA. In addition, the amount of cytokine RNA correlated with protein production, being significantly greater in patients’ BM cells than in control marrow. These data suggest a role for IL-1 beta and/or TNF in the pathophysiology of multiple myeloma and argue against a role for lymphotoxin or IL-2.

MATERIALS AND METHODS

Patients and Controls

Sixteen patients with multiple myeloma as defined by the Myeloma Task Force were studied. All patients underwent BM examination, serum protein electrophoresis, full skeletal surveys, and were staged according to the Durie-Salmon classification. Four patients were diagnosed as having monoclonal gammopathy of undetermined significance (MGUS). They were asymptomatic with normal BM examinations and skeletal surveys. Ten age- and sex-matched patients with non-neoplastic disease had BM examinations for investigation of anemia. They form the control population. The extent of bone disease of myeloma patients was described as extensive or minimal. Extensive disease was defined as skeletal surveys demonstrating either multiple lytic lesions (more than three) or the combination of diffuse osteoporosis and two or more pathologic fractures. Minimal bone disease was defined as either osteoporosis alone, or three or fewer lytic lesions, or normal skeletal surveys. The percent plasma cell infiltration and macrophage presence was determined by differential counts of Wright’s-Giemsa-stained BM smears with examination of at least 300 cells.

Isolation of Bone Marrow Cells

Bone marrow samples were aspirated after informed consent was obtained in accordance with the Human Subjects Review Boards of the West Los Angeles VA and UCLA Medical Center. After injection through needles of decreasing gauge, the cells were further separated by Ficoll-Hypaque (FH) density centrifugation.

Culture of BM Cells

BM cells were cultured in 10 x 75 culture tubes at a concentration of 10^6/mL and a volume of 1 mL/tube. Cells were cultured in RPMI media supplemented with 10% fetal calf serum (FCS, Reheis, Phoenix), 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin-streptomycin. The media and serum were specifically selected for low endotoxin levels, and endotoxin contamination was not detected by the Limulus assay (less than 0.125 eu/mL). The cells were cultured for 24, 48, or 72 hours at 37°C in a humidified atmosphere of 5% CO_2. After culture, the tubes were centrifuged and the supernatants were immediately harvested, pooled, sterile filtered, and frozen at -70°C until testing. All samples were assayed within 8 months of cell culture, and 90% of samples were assayed within 6 months.

Interleukin-1 Assay

The thymocyte coproliferation assay was used as described by Mizel et al. Assays were performed in flat-bottom Microtest II
96-well titer plates. Each well contained 10^4 thymocytes obtained from 6- to 8-week-old C3H/HeJ mice in 100 μL of RPMI media with 10% FCS and 100 μL of sample supernatant. Phyoehagglutinin (PHA) (Wellcome, Research Triangle Park, NC) was added at 0.5 μg/well. The plates were incubated at 37°C in 5% CO2 for 72 hours and pulsed at 64 hours with 3H-thymidine (New England Nuclear, Boston), 0.4 μCi/well in 10 μL of media. Cells were harvested with an automatic harvester and counted in a Beckman scintillation counter (model LS-350). Activity units were calculated by probit analysis adapted from the method of Gillis et al., which was initially developed for quantification of IL-2. In probit analysis, activities of serial-diluted patients' samples were compared with a control (model LS-350). Activity units were calculated up to 500 U/mL and had no effect on the activity of thymocytes. Even tenfold higher concentrations of the antibodies maintained their specificity for their relative cytokines.

**Assay for IL-2**

IL-2 was assayed using the murine IL-2-sensitive cell line CTLL2 as previously described.

**Northern Blot Analysis**

Analysis was performed on uncultured cells. Total RNA was extracted by homogenizing cells in guanidine thiocyanate and by ultracentrifugation through a cesium chloride gradient. Twenty-five micrograms of total RNA was electrophoresed in a 1.2% agarose gel in the presence of formaldehyde and transferred to nylon filters. The recombinant probes for the human IL-1 alpha, IL-1 beta, and lymphotoxin genes had been previously described. They were oligolabeled with [32P]deoxycytidine triphosphate. Hybridization, washing, and autoradiography were carried out as previously described. The RNA was first hybridized with an alpha probe to ensure equivalent RNA was loaded in each lane. The specificity of the probes used was confirmed by several methods. First, the probes were all oligolabeled inserts and we obtained the appropriately sized restriction fragments when we cut out insert DNA. Second, hybridization of these probes on filters with RNAs from human bone marrow and peripheral blood mononuclear cell fractions showed appropriately sized RNA fragments as previously reported. Third, DNA digested with several different restriction enzymes gave the appropriately sized human germline bands when these probes were used in Southern blot analysis of human granulocyte DNA. In addition, phorbol myristate acetate (PMA) stimulated HL-60 cells showed marked expression of TNF and lymphotoxin genes. The L929 assay does not detect IL-2, IL-6, or Interleukin-6 protein. The specific activity of the lymphotoxin was 2 x 10^5 U/mg protein.

**Statistics**

The t-test was used to determine P values.

**RESULTS**

**Patients and Controls**

Sixteen patients (14 males, two females) with myeloma were studied. The percentage of plasma cells in their BM ranged from 10% to 60%. The percentage of monocyte/macrophage cells was less than 5% in all cases. The remaining BM cells were composed of normal myeloid and erythroid elements. At diagnosis, two patients were stage I, five were stage II, and eight were stage III. One other patient was diagnosed as smoldering myeloma at presentation and, under observation, transformed into stage II myeloma. All
BM specimens were obtained prior to any initiation of therapy. At diagnosis, five patients had extensive bone disease, with all demonstrating multiple lytic lesions (three or more lesions in all five patients), osteoporosis (all five patients), and pathologic fractures (two of five patients). The other 11 patients had inactive bone disease with either completely normal skeletal surveys (two patients), only osteoporosis (six patients), or the presence of only one lytic lesion (three patients).

Four patients with MGUS were also studied. They had normal skeletal surveys, BM plasmacytosis less than 5%, and serum M-proteins less than 3 g/dL. They have been followed 1 to 3 years without significant change in their M-protein level.

Ten control patients were studied. All were hospitalized and under investigation for nonmalignant hematologic disorders. Their mean age (59 years) and sex (nine males; one female) were not significantly different from that of the myeloma patients (mean age 65 years).

**Bioassays**

**IL-1.** Nucleated BM cells were first isolated by density centrifugation. By differential counts on Wright-Giemsa-stained cytosmears, the isolated BM cells contained percentages of cell types comparable with preseparated cells. Thus the percentage of plasma cells in cultured marrow ranged from 15% to 70%, and the macrophage percentage was still below 5%. IL-1 levels in BM supernatants were determined by the thymocyte coproliferation assay. As shown (Fig 1), IL-1 levels in 24-hour supernatants from myeloma patients with bone disease (n = 5) were significantly greater (P less than 0.05) than from patients without bone disease (n = 11) and from controls (n = 10). The differences between the three groups were not as clear when 48- and 72-hour supernatants were tested. Four patients with MGUS had IL-1 levels (145 ± 55 U/mL) in 24-hour supernatants that were midway between the controls and myeloma patients without extensive bone disease. Maximal IL-1 activity in supernatants from myeloma patients' BM cells was always present at 24 hours, with decreasing levels at 48 and 72 hours. These kinetics are possibly due to a use of the produced cytokine by actively metabolizing cells. In contrast, MGUS and control supernatant activity appeared to plateau after 24 hours. IL-1 activity was not detected in serum from any patient.

The specificity of these assays was determined with anti-IL-1 antibodies. In each patient anti-IL-1 antibodies decreased supernatant-induced thymocyte coproliferation by over 95%. Although the anti-IL-1 antibody we used cannot distinguish between IL-1 alpha and IL-1 beta, Northern blot analysis suggested the only species we detected in the bioassay was IL-1 beta (see below).

The IL-1 produced in vitro did not correlate with the percent of plasma cells in the marrow specimens. For example, marrow cells from two patients with severe bone disease that contained 26% and 17% plasma cells produced 420 and 340 U/mL of IL-1 activity at 24 hours. In contrast, cells from one patient, containing 47% plasma cells, only produced 110 U/mL. Also, IL-1 levels showed no correlation with the percent of marrow macrophages. However, the levels did correlate roughly with Durie-Salmon stage at diagnosis. This was not surprising, as the degree of bone disease roughly parallels the tumor burden in individual patients, and IL-1 levels were correlated with the presence or absence of extensive bone disease.

We were able to repeatedly study IL-1 production from BM cells of one patient during the evolution of her disease. This individual was followed for approximately 2 years with the diagnosis of smoldering myeloma. During this time she had no symptoms of myeloma and negative skeletal surveys. IL-1 production by BM cells was 80 to 100 U/mL. Over a 6-month period she then developed overt myeloma with decreased hematocrit and a pathologic fracture. A repeat assay demonstrated her BM cells (containing a comparable percent of plasma cells) now produced 180 U/mL of IL-1 activity.

**Tumor necrosis factor/lymphotoxin.** BM supernatants were unable, by themselves, to achieve cytolysis of L929 targets as previously reported. However, in the presence of actinomycin-D, cytotoxic activity was present with kinetics similar to that of IL-1 production (Fig 2). The myeloma patients as a group were clearly associated with higher levels

![Fig 1. IL-1 activity in supernatants obtained at one, two, or three days. Data presented as mean values ± SD for each group of patients. Patients with "bone disease" had extensive osteolysis (see "Materials and Methods"), and those with "no bone disease" had minimally active bone disease (see Materials and Methods). Activity at day 1 for patients with extensive bone disease was significantly (P < .06) greater than for patients with minimal bone disease, patients with benign monoclonal gammopathy (BMG or MGUS), or controls. At day 2, it was only significantly greater than with patients with BMG or controls, and at day 3, there were no significant differences.](www.bloodjournal.org)
of activity v control individuals. There was also a significant difference between the values from patients with and without bone disease. Activity of supernatants harvested from MGUS patients' BM cells was, once again, midway between controls and myeloma patients. Cytotoxic activity was not detected in any serum samples from patients.

Anti-TNF and antilymphotoxin antibodies were used to distinguish which lymphokine was responsible for L929 cytotoxicity. As shown in Table 1, these antibodies specifically neutralize the effect of either recombinant TNF or purified lymphotoxin in the L929 assay. In these experiments we used 400 neutralizing units of antilymphotoxin antibody and 2,500 neutralizing units of anti-TNF antibody. In preliminary experiments not shown, even tenfold higher concentrations of these antibodies maintained their specificity. The table also shows the effects of the antibodies on cytotoxic activity mediated by supernatants from BM cells of three patients with extensive bone disease and three without bone disease. The anti-TNF antibodies completely abrogated activity in all cases, while antilymphotoxin antibodies had no effect. Identical data (complete abrogation with anti-TNF antibodies and no effect with antilymphotoxin antibodies) was obtained with supernatant-induced L929 cytotoxic activity from cells of the other myeloma and MGUS patients as well as the control patients.

Production of TNF in vitro did not correlate with the degree of plasma cell infiltration of the marrow or percent of macrophages but roughly correlated with Durie-Salmon stage at diagnosis.

IL-2. IL-2 was not detected in any BM supernatants at 24, 48, or 72 hours by its bioassay.

Northern blot analysis. Northern blot analysis was performed on freshly obtained uncultured BM cells. We obtained sufficient RNA to probe for TNF and IL-1 beta in eight patients with myeloma (three with extensive bone disease) and five normal controls. All eight specimens from myeloma patients contained the 16S RNA IL-1 beta product. In contrast, RNA for IL-1 alpha was not detected in any sample. Of the five control specimens, only two showed a faint band corresponding to IL-1 beta RNA. Since these individuals had some minimal IL-1 activity in the bioassay (Table 2) and also had undetectable IL-1 alpha RNA on Northern analyses, it is possible an IL-1 beta band may have appeared in the other normal controls if we allowed a longer film exposure.

Northern blot analysis also confirmed the results of the TNF/lymphotoxin bioassay. Uncultured BM cells from all eight myeloma patients contained 18S TNF RNA, but none had detectable lymphotoxin RNA. Three of the five controls had very small amounts of TNF transcripts detected.

Table 2 shows the relationship between the Northern data assayed on uncultured BM cells and the protein production data assayed in supernatants from the same specimens after 24 hours of culture. As shown, there was a clear distinction

Table 1. Inhibition of Supernatant-Induced L929 Cytotoxicity by Anti-TNF Antibody

<table>
<thead>
<tr>
<th>Sample</th>
<th>No Antibody</th>
<th>Anti-LT Antibody</th>
<th>Anti-TNF Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant TNF 50 ng/mL</td>
<td>750</td>
<td>775</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Purified LT 5 ng/mL</td>
<td>100</td>
<td>&lt;5</td>
<td>120</td>
</tr>
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<td>Patients with bone disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant A</td>
<td>410</td>
<td>440</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Supernatant B</td>
<td>180</td>
<td>170</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Supernatant C</td>
<td>250</td>
<td>250</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Patients without bone disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant A</td>
<td>65</td>
<td>75</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Supernatant B</td>
<td>55</td>
<td>60</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Supernatant C</td>
<td>85</td>
<td>80</td>
<td>&lt;5</td>
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L929 cytotoxicity assay performed with six different supernatants in the presence or absence of antibodies to either LT (lymphotoxin) or TNF. The specificity of the antibodies had been previously documented (see Materials and Methods).
Table 2. Correlation of Bioassay Data to Northern Blot Analysis

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>IL-1 Bioassay U/mL</th>
<th>IL-1 Beta RNA</th>
<th>TNF Bioassay U/mL</th>
<th>TNF RNA</th>
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<tr>
<td>Myeloma patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>420</td>
<td>++++</td>
<td>600</td>
<td>++++</td>
</tr>
<tr>
<td>2*</td>
<td>266</td>
<td>+++</td>
<td>250</td>
<td>+++</td>
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<tr>
<td>3*</td>
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<td>1</td>
<td>73</td>
<td>±</td>
<td>15</td>
<td>±</td>
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<tr>
<td>5</td>
<td>35</td>
<td>–</td>
<td>5</td>
<td>–</td>
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</table>

Bioassay results on supernatants obtained after 24 hours of BM cell culture. Relative RNA expression on uncultured BM cells arbitrarily defined as:
- ± = RNA was barely detectable;
- + = 1 x;
- ++ = 2 to 5 x the amount present in the 1 x lane;
- +++ = 6 to 10 x the amount present in the 1 x lane;
- ++++ = over 10 x the amount present in the 1 x lane.

*Myeloma patients with extensive bone disease.

between the high levels of IL-1 beta and TNF RNA in the myeloma patients' BM cells and the minimal amounts in the normal controls. There is also a rough correlation between the levels of protein secreted in the bioassay and the cytokine RNA for individual myeloma patients. As examples, Figs 3 and 4 demonstrate the difference in amount of cytokine transcripts between three myeloma patients and three normal controls.

DISCUSSION

Appropriate bioassays were used to assess in vitro production of cytokines from BM cells of myeloma patients.

CONTROL

| 1 | 2 | 3 |

MYELOMA

| 1 | 2 | 3 |

![IL-1 Beta Image]

IL-1 BETA

Fig 3. Twenty-five micrograms of total cellular RNA were loaded in each lane, electrophoresed on denaturing formaldehyde agarose gels, transferred to nylon filters, and hybridized with the IL-1 beta cDNA probe. Molecular size of the IL-1 beta mRNA transcript is shown on the right. The examples consist of control patients 1, 2, and 3 and myeloma patients 1, 2, and 3 from Table 2.

Although these bioassays may be more sensitive than immunoreactive assays,23 they lack specificity, especially since many of the cytokines share some biological properties. Specificity was obtained with the use of specific neutralizing antibodies. They clearly demonstrated an increased production of IL-1 and TNF by BM cells from patients with myeloma and nondetectable lymphotoxin and IL-2 production. Northern blot analysis strongly suggested that the IL-1 species detected was IL-1 beta rather than IL-1 alpha. It also supported the TNF/lymphotoxin bioassay results, as TNF mRNA was present in all samples studied while lymphotoxin mRNA was not detected. Since BM cells from myeloma
patients also produce IL-6, we cannot rule out a possible synergy between this cytokine (or another unidentified cytokine) and IL-1 beta or TNF in their respective bioassays. This is particularly true with the thymocyte proliferation assay where IL-6 clearly synergizes with IL-1. Although others have indicated a possible effect of TNF in the thymocyte assay, with the mitogen concentration used in our assay, we have previously documented TNF has no effect. In addition, although IL-2 can be detected in our thymocyte assay, the more sensitive CTLL 2 assay was negative in all cases. It is thus unlikely that IL-2 caused the positive results in the thymocyte assay. The fact that anti-IL-1 antibodies abrogated thymocyte proliferation further confirms the presence of IL-1 in these supernatants.

We did not detect any increased levels of IL-1 or TNF in the serum of our patients. This raises the concern that the cytokine levels detected in bioassays may not reflect the in vivo situation but result from induction during in vitro culture. The RNA data, however, support the notion that the cytokines are actually produced in vivo. Northern blot analyses were performed on freshly obtained uncultured marrow and correlated with the bioassay in two ways: (1) the specificity of the detected cytokines (ie, presence of TNF RNA and protein production and absence of lymphotoxin RNA and protein), and (2) the quantitative differences in protein and RNA levels between myeloma patients and normal controls (Table 2 and Figs 3 and 4).

There was a rough correlation between levels of protein production for the two cytokines in individual patients (Table 2). The samples that contained large amounts of TNF usually also demonstrated high IL-1 activity. Similarly, the levels of gene transcription were also correlated in individual patients (Table 2). This raises the question of whether the IL-1 beta and TNF genes are transcribed constitutively in myeloma BM in vivo or whether the levels of mRNA and protein production result from continued induction by these or other cytokines. For example, TNF production within the marrow might account for IL-1 gene transcription and protein production, since it is a potent inducer of IL-1 in vitro. Even the BM cells of our control patients produced detectable, albeit minimal, levels of thymocyte proliferation and L929 cytotoxicity, that was proven to be specific for IL-1 and TNF with the use of specific antibodies. In addition, IL-1 and TNF mRNA have been found in lymphoid tissues of normal individuals. It could thus be argued that the balanced action of these cytokines maintains homeostasis in different tissues of normal individuals and that the detected overproduction in myeloma BM reflects secondary activation of regulatory mechanisms.

This also raises the question of the cell of origin of IL-1 and TNF overproduction. Although it is possible that myeloma plasma cells express the genes and secrete these cytokines, an equally tenable possibility is that BM macrophages are the producer cells. Monocytes and macrophages are considered to be the main cellular source of both IL-1 and TNF, and an activated state of such cells could account for a higher level of cytokine activity. There is some precedence for this in that cells of the monocyte/macrophage lineage are activated in myeloma, and this activation may play a role in the detected hypogammaglobulinemia of these patients. We have not been able to sufficiently separate our marrow specimens into macrophage-depleted or -enriched (and plasma cell-depleted) populations to accurately answer this question. In situ hybridization with cytokine gene probes may allow a resolution of this issue. Garrett et al studied five cultured myeloma cell lines, one of which was established from a patient with active bone disease. These myeloma cells expressed both lymphotoxin and TNF mRNA but not IL-1 RNA. Of the three cytokines, only production of lymphotoxin was detected in cell culture.
Furthermore, the osteoclast activating-factor activity of the myeloma cell supernatants was neutralized by antilymphotoxin antibodies. These data suggested to the authors that production of lymphotoxin is related to bone destruction in patients with myeloma. Although we have not assayed bone resorption in vitro, our results argue against this notion. Lymphotoxin production and mRNA could not be detected in 16 consecutive freshly obtained BM specimens from myeloma patients, five of whom had extensive osteolytic bone disease. On the other hand, the production of IL-1 and TNF correlated with the extent of bone disease and may play a role in its development. Alternatively, since TNF and IL-1 levels also correlated with stage of disease, cytokine overproduction may simply reflect tumor burden or disease activity and not necessarily be related to the presence of bone disease.

In preliminary studies with BM supernatants from five patients with marrow malignancies not associated with bone resorption (one case of chronic lymphocytic leukemia [CLL], one hairy cell leukemia, one case of Waldenström's macroglobulinemia, and two cases of non-Hodgkin's lymphoma), we have not detected significantly increased levels of IL-1 or TNF. However, other investigators have detected IL-1 production by CLL and acute lymphoblastic leukemia cells. Thus overproduction of IL-1 by malignant BM cells may not be specific for accompanying osteolytic bone lesions. On the other hand, a study by Iwato supported the notion that, at least in some myeloma patients, IL-1 beta overproduction may mediate bone disease. IL-1 alpha or beta have also been implicated in osteolytic bone disease accompanying adult T-cell leukemia, squamous cell carcinoma of the head and neck, and ideopathic osteoporosis. Finally, other cytokines that we have not investigated, like transforming growth factor, can resorb bone and may be important.

The inconsistency between the current study and that of Garrett may simply be due to the differences between freshly obtained myeloma marrow and continuously cultured lines. Studies by Klein et al and Kronke et al on cultured myeloma lines reported results similar to Garrett's study. The former could not detect production of IL-1 from three continuously cultured myeloma lines, and the latter detected lymphotoxin and TNF mRNA in several lymphohemopoietic lines, but only lymphotoxin was secreted.

In summary, BM cells from patients with multiple myeloma produce IL-1 and TNF at significantly greater levels than similarly prepared cells from control individuals. In contrast, lymphotoxin and IL-2 were not produced by these cells. Work is in progress to determine whether any of the medical complications of myeloma, such as osteolytic bone disease and nonparaprotein hypomunoglobulinemia, may be due to overproduction of these cytokines.

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Production of cytokines by bone marrow cells obtained from patients with multiple myeloma

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