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Production of Interleukin-1 by Bone Marrow Myeloma Cells

By Federico Cozzolino, Maria Torcia, Donatella Aldinucci, Anna Rubartelli, Anna Miliani, Alan R. Shaw, Peter M. Lansdorp, and Renato Di Guglielmo

Plasma cells isolated from bone marrow (BM) aspirates of 12 patients with multiple myeloma (MM) and nine patients with monoclonal gammopathy of undetermined significance (MGUS) were analyzed for production of cytokines with bone-resorbing activity, such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and lymphotoxin (LT). Culture supernatants of plasma cells from MM, but not from MGUS or normal donor, invariably contained high amounts of IL-1-β and lower amounts of IL-1-α. With a single exception, TNF/LT biologic activity was not detected in the same supernatants. IL-6 was present in two of five supernatants tested. Normal B lymphocytes released both IL-1 and TNF/LT activities for four days after activation in vitro; however, production of these cytokines ceased at the final stage of plasma cell. Unexpectedly, the mRNA extracted from MM plasma cells hybridized with TNF- and LT-specific, as well as IL-1-specific probes, although the culture supernatants did not contain detectable TNF/LT biologic activity. When tested in the fetal rat long bone assay, MM plasma cell supernatants displayed a strong osteoclast-activating factor (OAF) activity, which was greatly reduced but not completely abolished by neutralizing anti-IL-1 antibodies. Anti-TNF or anti-LT antibodies were ineffective in the same test. We conclude that the IL-1 released in vivo by malignant plasma cells has a major role in pathogenesis of lytic bone lesions of human MM.
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LYTIC BONE LESIONS are a prominent feature of multiple myeloma (MM). Since the original description by Mundy et al,[1] ample evidence has accumulated that bone resorption is related to the action of factor(s) produced by the malignant cells, termed osteoclast-activating factor (OAF). The biochemical characterization of OAF revealed that several different proteins can mediate this biologic effect.[2] Among these are interleukin-1 (IL-1)-α, IL-1-β, tumor necrosis factor (TNF), and lymphotoxin (LT).[3-6] Further studies showed that these factors interact with osteoblasts, which are ultimately responsible for metabolic activation of osteoclasts.[7,8]

In this study, we investigated production of the above factors by freshly isolated bone marrow (BM) plasma cells from patients with monoclonal gammopathy (MG) or normal subjects and by plasma cells generated in cultures of peripheral blood lymphocytes (PBLs). We show that malignant plasma cells invariably produce relevant amounts of IL-1 and, in a single case, TNF as well. Although MM plasma cells expressed the genes for both TNF and LT in the cases studied, their biologic activities were never detected in culture supernatants. We therefore conclude that IL-1 is the predominant factor responsible for the lytic bone lesions of MM.

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MATERIALS AND METHODS

Patients. Twenty-one patients with MG were studied, 12 with MM and nine with MG of undetermined significance (MGUS). Diagnosis of MM was established according to conventional criteria,[9] and the patients were selected for the presence of lytic bone lesions. Patients with MGUS were selected according to the stability of their hematologic and humoral parameters for >5 years. BM specimens were obtained, after informed consent, from needle biopsies performed for periodic controls.

Reagents. Purified human recombinant (hr) IL-1-α and IL-1-β were obtained as described.[10,11] Both had a specific activity of 1.3×10^7 half-maximal units per milligram in the thymocyte costimulation assay. Purified hrTNF, hrLT, hrIFN-γ, specific activity 9.6×10^6 and 8.9×10^7 units per milligram in the L929 cytotoxicity assay, and 2.1×10^7 NIH reference units per milligram, respectively, were the kind gift of Biogen S.A., Geneva, Switzerland. Neutralizing antisera specific for IL-1-α, IL-1-β, and TNF were obtained in rabbits, using the recombinant proteins as immunogen. They contained 10^7, 0.5×10^6, and 2×10^7 neutralizing units per milliliter, respectively. The absence of cross-reactivity of anti-IL-1-α and anti-IL-1-β antisera was assessed in the LAF assay, in which both failed to affect thymocyte proliferation in response to IL-1-β and -α, up to concentrations of 1:50. The mouse monoclonal antibody (MoAb) LTX-9 to human LT, 10^5 neutralizing units per milligram, was provided by Dr G. Adolf, Ernst-Boehringer-Institut für Arzneimittelforschung, Vienna.

The DNA probes specific for IL-1-α and IL-1-β consisted of *Eco* RI fragments from pSPhIL1α.2 and pSPhIL1β.2, recognizing the coding regions of IL-1-α and IL-1-β, respectively. The probes specific for TNF and LT, provided by Dr E. Kawashima, Biogen S.A., have been described elsewhere.[12]

Isolation and culture of cells. BM samples were diluted 1:1 with Hanks' balanced salt solution (HBSS) and mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Milan) density-gradient centrifugation. Cells at the interface were resuspended in RPMI 1640 culture medium (Flow Laboratories, Milan) supplemented with 2 mmol/L L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin (hereafter referred to as complete medium, CM), and 10% heat-inactivated fetal calf serum (FCS, Flow). Normal BM samples were obtained from patients undergoing cardiac surgery and treated similarly. Plasma cells were purified from the above suspensions by a direct rosetting procedure with ox RBCs coated with the MoAb

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purified from ascites fluid of mice injected with the OK T10 hybridoma clone (ATCC, American Type Culture Collection, Rockville, MD) or with the A10 hybridoma clone (gift of Dr F. Malavasi, University of Turin, Italy), as described previously.[13] Alternatively, cells expressing the PCA-1 were separated by the indirect rosetting procedure as described previously.[14] Cells (2×10^7) were resuspended in 400 μL 1:10 dilution of PCA-1 MoAb (Coulter-Kontron, Milan) in HBSS and kept at 4°C for 30 minutes. After two washes with cold HBSS, cells were incubated with a 1% suspension of ox RBCs coated with $F(ab')_2$ fragments of affinity-purified goat anti-mouse Igs at 37°C for one hour. Rosetting cells were then separated by Ficoll-Hypaque density gradients, and RBCs were lysed by incubating at 4°C for ten minutes with a 0.83% NH_4Cl solution. Aliquots of cells obtained by either procedure were cytocentrifuged and examined for the percentage of plasma cells by May-Grünwald-Giemsa staining. When necessary, the cell suspension was further purified by a one-step complement-mediated cytotoxicity as described previously.[12] Cells (1×10^7) were incubated at 4°C in 1 mL CM-FCS containing a mixture of purified OK T11 MoAb (ATCC), 1:20 final dilution, B1 MoAb (Coulter-Kontron), 1:40 final dilution, and MY 4 MoAb (Coulter-Kontron), 1:20 final dilution. After incubation on ice for 30 minutes, nontoxic rabbit complement (Pel-Freeze, Società Italiana Chimici, Milan), 1:2 final dilution, was added, and the suspension was kept at 37°C for one hour. Dead cells were removed by centrifugation on Ficoll-Hypaque gradients. The purity of the cell suspension was checked again by cytoplasmic immunofluorescence analysis using FITC-conjugated rabbit anti-human Ig antiserum. Contaminating cells were represented mainly by erythroblasts, with few lymphoid cells. Cell suspensions containing $<90\%$ viable plasma cells were discarded.

Normal BPBLs were purified from buffy coats by an indirect rosetting procedure using the B1 MoAb. Small tonsil B lymphocytes were obtained by depletion of T cells by double rosetting with neuraminidase-treated sheep erythrocytes and Percoll (Pharmacia) density-gradient fractionation as described previously.[13] The cells were cultured at 1×10^6/mL in CM-FCS for seven days with killed *Staphylococcus aureus* of the I Cowan strain (donated by Dr S. Romagnani, University of Florence). At the end of the incubation period, cells were harvested and centrifuged over Ficoll-Hypaque gradients to remove cell debris. Plasma cells were isolated by the procedures described above.

BM MNCs and purified plasma cells were cultured for 48 hours at 37°C in a humidified atmosphere with 5% CO_2 with or without hr interferon-γ (hrIFN-γ), 2,000 U/mL, phytohemagglutinin (PHA, Wellcome, Pomezia, Italy), 1 μg/mL, or lipolysaccharide from *E coli* (Sigma, St Louis), 10 μg/mL; supernatants were harvested and tested for IL-1, TNF/LT, and OAF activities. In some experiments, cells were incubated for four hours in CM-FCS with cycloheximide (cyc, Sigma), 1 μg/mL, then washed six times and cultured as above. For the kinetic analysis of cytokine production, normal B PBLs were cultured in 24-well plates at 1×10^6 cells/mL. Supernatants were harvested daily and replaced with fresh CM-FCS; aliquots of cells were cytocentrifuged, fixed, and analyzed for intracytoplasmic Ig by immunofluorescence as above. The RPMI 8226 myeloma cell line was obtained from ATCC (Cat. no. 155-CCL) and cultured in CM-FCS.

IL-1 assay. IL-1 activity in culture supernatants was determined as described previously.[15] C3H/HeJ mouse thymocytes (1.5×10^6) were cultured in triplicate for 72 hours in 96-well plates, with 1 μg/mL PHA alone, or PHA plus serial dilutions of the supernatants to be assayed. In some experiments, anti–IL-1-α or anti–IL-1-β antibodies, or both, were added at a final dilution of 1:500. The cells were pulsed in the last eight hours with ^{3}H-thymidine (^{3}H-TdR, Amersham, Amity-PG, Milan) 0.5 μCi/well, harvested, and counted in a liquid scintillation counter. One unit of IL-1 was defined as the amount of supernatant capable of doubling the ^{3}H-TdR incorporation by stimulated thymocytes.

IL-6 assay. The assay was performed as described previously.[16] Five thousand B13.29 cells per well were cultured for 48 hours in flat-bottom microtiter plates in 200 μL Iscove's modified Dulbecco's medium (IMDM) supplemented with 50 μmol/L 2-mercaptoethanol, 5% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin with or without twofold dilutions of the samples to be tested or of a standard preparation containing 4,000 U/mL. Cells were pulsed for the last six hours with ^{3}H-TdR 0.2 μCi/well, and the uptake was determined by liquid scintillation counting. One U/mL is the concentration that yields half-maximal ^{3}H-TdR incorporation in the assay.

TNF/LT assay. The assay was performed as described by Colotta et al.[17] WEHI 164 sarcoma cells (a gift from Dr A. Mantovani, Istituto di Ricerche Farmacologiche "Mario Negri," Milan), 0.5 to 1.0×10^6/mL, were incubated in CM-FCS at 37°C with 1 μg/mL actinomycin D (Sigma) for three hours, washed extensively, resuspended in CM-FCS, and labeled with ^{51}Cr, as described previously[15]; 5×10^3 cells/well were incubated for six hours in triplicate with or without serial dilutions of test supernatants or with serial dilutions of hrTNF or hrLT to determine a standard curve. At the end of the incubation period, aliquots of cells were lysed in 0.1 N HCl to determine maximal lysis, 80 μL were harvested from each well, and radioactivity was detected in a liquid scintillation counter. Specific lysis was determined according to the following formula: Percentage of specific lysis $= [(\text{Exp} - \text{Sp})/(\text{Max} - \text{Sp})] \times 100$, where Exp is radioactivity release induced by test supernatants, Sp is radioactivity spontaneously released by labeled cells, and Max is radioactivity released by HCl-treated cells. Distinction between the two cytokines was obtained with monospecific antibodies.

OAF assay. The fetal rat long bone assay was performed as described by Dewhirst et al.[4] Pregnant rats were injected with 200 μCi ^{45}Ca (NEN, Dupont Italia, Florence) on the 18th day of gestation. Bone shafts of radii and ulnae were microdissected from 19-day fetuses and cultured in 0.5 mL BGJ_b medium (GIBCO, Mascia Brunelli, Milan) containing 1 mg/mL bovine serum albumin (BSA, Sigma) for one day to reduce exchangeable ^{45}Ca. One bone (radius or ulna) was then incubated in medium containing a diluted test supernatant, and the controlateral bone was cultured in medium alone as control. In some experiments, anti–IL-1-α and IL-1-β, or anti-TNF antibodies, at a final dilution of 1:100, or anti-LT antibodies at a final dilution of 1 μg/mL, were added. Five pairs of bones were used for each dilution. Bones were cultured for two days, media were replaced, and the cultures were continued for three more days. The percentage of ^{45}Ca released from a bone into the medium during the entire culture was determined by measuring the radioactivity in medium 1, medium 2, and the trichloroacetic acid (TCA)-solubilized bone by a liquid scintillation counter. OAF-induced resorption was expressed as the difference between treated and control percentages of ^{45}Ca released during the culture. Maximal resorption was determined by using 200 ng/mL parathyroid hormone. For each group of five pairs, the mean and SE were calculated. Significance was determined by a two-tailed Student's *t* test.

RNA isolation and blot hybridization. The procedures used were essentially those described by Maniatis et al.[18] Cells were lysed in 4 mol/L guanidinium isothiocyanate, layered over 5.7 mol/L cesium chloride, and centrifuged at 100,000 g at 20°C for 18 hours. The RNA pellets were recovered and precipitated with ethanol. Aliquots of 2.0, 1.0, and 0.5 μg RNA (measured by spectrophotometry and ethidium bromide staining of test minigels) were slot-blotted to a synthetic nylon transfer membrane (Gene Screen Plus, Dupont Italia) using $10\times$ standard saline citrate (SSC) ($1 \times$ SSC = 150

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mmol/L NaCl, 15 mmol/L citrate, pH 7.0). Membranes were prehybridized at 60°C in 1% sodium dodecyl sulfate (SDS), 1 mol/L NaCl, 10% dextran sulfate, then hybridized in the same solution to which 100 μg/mL salmon sperm DNA and 1×10^6 cpm/mL ^{32}P-labeled probe were added. Membranes were washed in 2 × SSC for ten minutes, 2 × SSC/1% SDS for one hour at 60°C, and 0.1 × SSC for one hour at room temperature. After drying, membranes were exposed to x-ray film with intensifying screens. DNA probes were labeled to a specific activity of $\approx 10^9$ cpm/μg with hexanucleotide primers and ^{32}PdCTP, as described previously.[19]

RESULTS

Production of cytokines by plasma cells from MG patients. Plasma cells were isolated from BM specimens of 12 patients with MM and nine patients with MGUS and tested for production of factors with IL-1 and TNF/LT activity. Cells were cultured for 48 hours with or without activation stimuli such as LPS, PHA, and hrIFN-γ. Table 1 shows that a clear-cut difference exists between the two types of plasma cell populations. All MM plasma cell culture supernatants contained large amounts of IL-1 activity, not increased by stimulation in culture. In contrast, supernatants of plasma cells from MGUS patients consistently failed to display IL-1 activity, in either unstimulated or stimulated cultures. When tested against WEHI 164 cells, neither MM nor MGUS plasma cell supernatants from unstimulated or stimulated cultures showed a significant cytotoxic activity, a finding which suggests that TNF or LT was not released by the malignant cells. The only exception was MM case 9, in which plasma cells spontaneously released TNF/LT activity in the supernatants (Table 1). This activity was entirely neutralized by monospecific anti-TNF antibodies (data not shown). To rule out that the presence of inhibitors of TNF or LT could interfere with the biologic assay, known amounts of both cytokines were added to some supernatants which were subsequently reassayed. The same amounts of TNF and LT added were detected within the supernatants (Table 1).

IL-1 can be produced by a variety of cell types, including monocytes/macrophages, granulocytes, and possibly immature myeloid cell precursors.[20] To ascertain whether the IL-1 detected in the above supernatants was actually produced by plasma cells and not by other contaminating cell types, unfractionated MM BM samples and purified plasma cell suspensions thereof were cultured in parallel and supernatants were assayed for IL-1 activity as described above. Table 2 shows that in the single cases purified plasma cells invariably released higher amounts of IL-1 in comparison to unfractionated BM cells. Coculture experiments with graded numbers of autologous PB monocytes or granulocytes yielded results consistent with the above data, since the highest IL-1 activity was detected within the supernatants of purified plasma cell suspensions (Table 2).

Table 1. Cytokine Production by Plasma Cells From MG Patients

	IL-1 Activity (U/mL)		TNF/LT Activity Specific Cytotoxicity (%)		TNF/LT Activity Specific Cytotoxicity (%) Cultured With*	
Supernatants	Unstimulated	Stimulated†	Unstimulated	Stimulated†	hrTNF	hrLT
From MM plasma cells						
1	212	239	2.2	4.4	36.5	38.5
2	195	183	3.1	4.8	32.3	37.4
3	318	345	1.0	1.8	34.2	35.0
4	158	185	0.7	1.5	35.6	35.3
5	244	233	2.1	3.4	31.4	32.8
6	133	121	3.2	4.5	37.1	38.1
7	118	147	1.5	2.3	ND	ND
8	85	89	1.4	2.8	ND	ND
9	189	197	11.7	13.7	ND	ND
10	225	215	2.0	2.1	ND	ND
11	157	171	4.8	6.1	ND	ND
12	284	275	0.6	1.3	35.8	36.7
From MGUS plasma cells						
1	<10	<10	0.8	1.5	36.9	37.2
2	<10	<10	1.5	2.4	35.8	36.4
3	<10	<10	1.9	3.2	37.8	36.5
4	<10	<10	2.1	4.1	ND	ND
5	<10	<10	3.1	3.8	ND	ND
6	<10	<10	2.1	2.7	ND	ND
7	<10	<10	1.1	1.8	ND	ND
8	<10	<10	0.5	1.2	ND	ND
9	<10	<10	0.8	1.4	ND	ND

Plasma cells 1×10^6 isolated by rosetting techniques were cultured for 48 hours with or without stimulants; the supernatants were recovered and tested for IL-1 activity in the thymocyte costimulation assay using hrIL-1β as standard and for TNF/LT activity in the WEHI 164 cell cytotoxicity assay using hrTNF or hrLT as standard. Results are the mean of triplicate determinations. SD was consistently <10%.

*The same LPS-stimulated supernatants were assayed with or without 5 U/mL hrTNF or hrLT. These amounts of hrTNF and hrLT in the standard curves yielded 35.8% ± 4.2% and 36.9% ± 3.3% specific cytotoxicity, respectively.

†Cells were cultured with LPS, 10 μg/mL, PHA, 1 μg/mL, or hrIFN-γ, 2,000 U/mL.

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Table 2. IL-1 Production by Unfractionated BM Cells or Purified Plasma Cells From MM Patients

Supernatants	IL-1 Production (U/mL) by Cells From Patient*			
	7	10	11	12
From BM MNC	72	180	85	213
From purified plasma cells	128	230	157	284
From purified plasma cells + autologous monocytes				
1:0.5	96	178	131	224
1:1	75	135	95	171
1:2	45	74	51	84
From purified plasma cells + autologous granulocytes				
1:0.5	110	191	175	234
1:1	81	141	124	180
1:2	48	68	81	92

BM mononuclear cells (MNCs), obtained through F-H centrifugation, were cultured for 48 hours in parallel with purified plasma cells and purified plasma cells contaminated with autologous monocytes or granulocytes at the indicated ratios. The final cell number in each culture was 1×10^6. Supernatants were recovered and tested for IL-1 activity.

*Percentage of plasma cells in MNC suspensions was as follows: patient 7, 51%; patient 10, 73%; patient 11, 48%; and patient 12, 78%.

To rule out that the IL-1 detected in culture supernatants could have been taken up by plasma cells in vivo and subsequently released in vitro, purified MM plasma cells were pulsed for six hours with cyc, washed, and cultured for 48 hours more. Table 3 shows that culture supernatants of cyc-pretreated plasma cells showed markedly reduced IL-1 activity. The same supernatants, however, did not affect the thymocyte proliferative response induced by addition of exogenous IL-1 (data not shown). This finding rules out that some cyc had been carried over in the biologic assay.

To characterize the molecules produced by the malignant cells further, IL-1 activity was assayed with polyclonal antibodies monospecific for IL-1-α and IL-1-β. Figure 1 shows that addition of anti–IL-1-β caused a >80% decrease in the thymocyte proliferation induced by IL-1-rich supernatants, whereas anti–IL-1-α antibodies caused only a modest decrease. Addition of both antibodies, however, failed to abolish completely the IL-1 activity of the supernatants. Since IL-6 [interferon-β_2 (IFN-β_2), B-cell stimulatory factor-2, hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor] is active in the thymocyte comitogenic assay,[21] culture supernatants of purified plasma cells from five cases of MM were analyzed for the presence of this cytokine. Table 4 shows that significant amounts of IL-6 in two cases, and no cytokine at all in the remaining three, were detected. Thus, a minor part of IL-1 activity in some supernatants of unstimulated cells could be related to an effect of IL-6.

Table 3. IL-1 Activity in Supernatants From Cycloheximide-Treated MM Plasma Cells

Supernatants	IL-1 Production (U/mL) by Plasma Cells From Patient			
	1	3	2	4
From unstimulated cells	215	320	180	198
From LPS-stimulated cells	241	345	190	201
From unstimulated cyc-treated cells*	48	37	22	25
From LPS-stimulated cyc-treated cells*	47	35	25	24

*MM plasma cells were incubated with cyc 1 µg/mL for six hours, washed extensively, and cultured at 1×10^6/mL for 48 hours with or without LPS 10 µg/mL. Supernatants were recovered and tested for IL-1 activity. In the same assay, controls with exogenous IL-1 indicated that no residual cyc was present.

Production of cytokines by normal plasma cells. Normal B lymphocytes and Epstein-Barr virus (EBV)-transformed lymphoblastoid B-cell lines produce both IL-1 and LT, either spontaneously or following in vitro activation.[22-24] Whether these functions are maintained during B-cell differentiation is not known, however. To clarify this issue, different cultures of tonsil or B PBLs were stimulated with SAC, and the supernatants were recovered at various intervals and tested for IL-1 and TNF/LT activities. The time-course analysis confirmed that resting B cells did not produce detectable amounts of cytokines, and that significant IL-1 and TNF/LT activities were present in the supernatants of B cells stimulated for 24, 48, and 72 hours (Fig 2). Experiments performed in the presence of neutralizing antibodies showed that ≈70% of cytotoxic activity against WEHI 164 cells was neutralized by anti-LT antibodies (data not

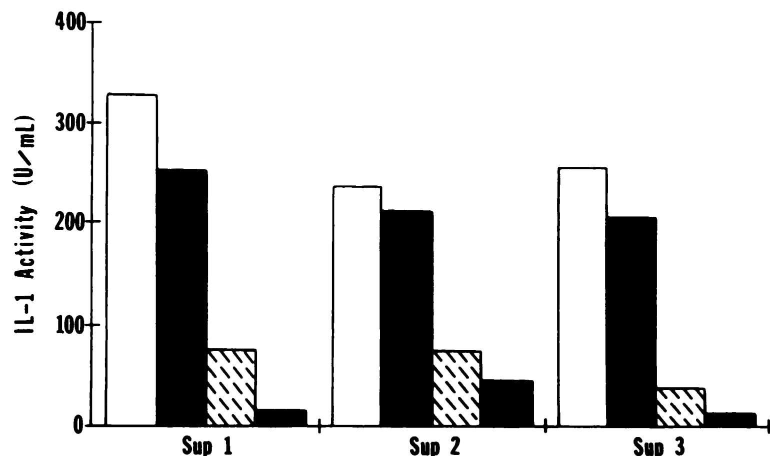

Fig 1. Effect of anti–IL-1-α and anti–IL-1-β antibodies on IL-1 activity of MM plasma cell supernatants. MM cell supernatants were tested untreated (□) and treated with anti–IL-1-α (▨), anti-IL-1-β (▧), or both (■), at a final dilution of 1:500. Results of three of seven experiments.

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Table 4. IL-6 Production by Plasma Cells From MM Patients

Supernatants of MM Plasma Cells From Case	IL-6 Activity (U/mL)
3	3,000
7	500
9	20
11	3
12	10

Plasma cells from MM patients were cultured for 48 hours at 1 × 10^6/mL. Supernatants were harvested and tested for IL-6 activity in the B13.29 hybridoma cell proliferation assay. A standard preparation containing 4,000 U/mL IL-6 was used as positive control.

shown). Then the activities gradually decreased, and the supernatants of cells cultured ≥120 hours were virtually devoid of both IL-1 and TNF/LT. As expected, the percentage of cIg^+ cells in the same cultures gradually increased from 1% to 5% on day 3 to 25% to 30% on day 8 (Fig 2). Thus, differentiating B cells appear to lose progressively their ability to produce cytokines. To assess more directly the function of plasma cells, $OKT10^+$ cells from stimulated suspensions at day 8 were isolated and cultured for 48 hours more, with or without various stimuli. In addition, since B lymphocytes obtained from different tissues have been suggested to differ in their functional properties,[25] plasma cells from BM specimens of cardiac surgery patients were isolated and tested for their ability to release cytokines. Again, the culture supernatants were devoid of both IL-1 and TNF/LT activities (data not shown), confirming that production of these molecules is a property of activated B cells.

OAF activity in plasma cell culture supernatants. To investigate whether and, perhaps more important, the extent to which the plasma cell-derived IL-1 could account for the lytic bone lesions of MM, the OAF activity of purified plasma cell supernatants from five cases of MM, two cases of MGUS, and two normal donors were tested in a fetal rat long bone assay, with or without neutralizing anti–IL-1, anti-TNF, or anti-LT antibodies. All MM plasma cell supernatants induced a strong ^{45}Ca release by cultured rat calvariae (Fig 3). More important, the addition of anti–IL-1 antibodies caused reduction in the bone-resorbing activity, which never reached the background levels, however. OAF activity in the presence of LT- and TNF-specific antibodies was not significantly affected (Fig 3). This finding is consistent with the absence of TNF/LT activity in plasma cell supernatants. Expectedly, supernatants from purified MGUS or normal donor plasma cells did not contain OAF activity (data not shown).

Cytokine production by the RPMI 8226 myeloma cell line. RPMI 8226 is a continuous cell line derived from the PB of a patient with MM.[26] This cell line could represent a useful model for comparison with freshly isolated MM plasma cells. We tested the culture supernatants of the RPMI 8226 cell line for IL-1 and TNF/LT activities. Table 5 shows that a high TNF/LT activity was detected and that it could be blocked completely by anti-LT antibodies. On the contrary, no IL-1 activity was formed in the same supernatants. These results are therefore in contrast with those obtained from analysis of MM plasma cell supernatants.

Cytokine-specific mRNA production by RPMI 8226 cells and MM plasma cells. To determine whether the genes encoding for TNF, LT, or IL-1 were expressed in MM cells and RPMI 8226 cells, total cellular mRNA from cells of MM case 10 and from RPMI 8226 cells was analyzed by slot-blot hybridization with DNA probes specific for IL-1-α, IL-1-β, TNF, and LT. Unexpectedly, the cells from the MM case contained mRNA specific for TNF and LT, in addition to that specific for IL-1-α and IL-1-β (Fig 4). This result was confirmed by the mRNA analysis of two additional MM cases (cases 4 and 7, not shown). The mRNA from RPMI 8226 cells hybridized with LT- and TNF-specific, but not with IL-1-α– or IL-1-β–specific, DNA probes, as expected (Fig 4).

DISCUSSION

Cytokines are a group of proteins endowed with powerful, pleiotropic biologic properties. Although no definite evidence for a critical role played by such proteins in the mechanisms of disease has yet been provided,[27] several studies have suggested that inappropriate or excessive production of some cytokines may be harmful.[28-30] Therefore, a fine control of their biologic activities by specific or nonspecific inhibitors is

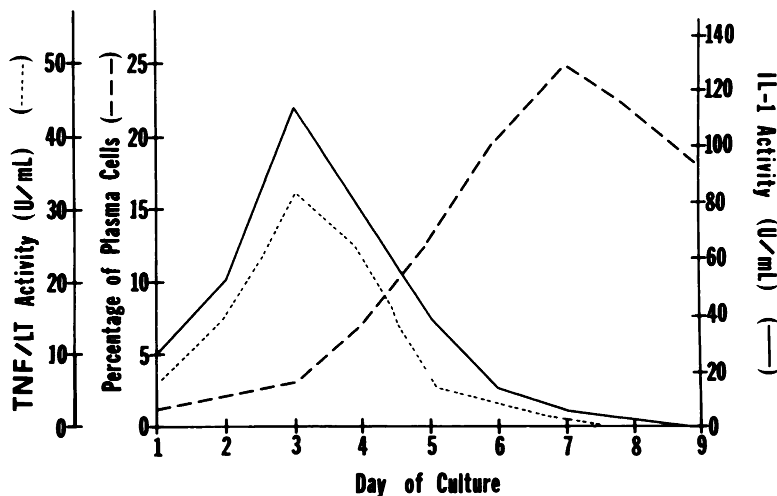

Fig 2. Time-course analysis of cytokine production by normal tonsil B lymphocytes. B lymphocytes were cultured at 1 × 10^6/mL in CM-FCS with SAC (1:2,500 final dilution). Supernatants were harvested daily and tested for IL-1 activity in the thymocyte costimulation assay and for TNF/LT activity in the WEHI 164 cytotoxicity assay.

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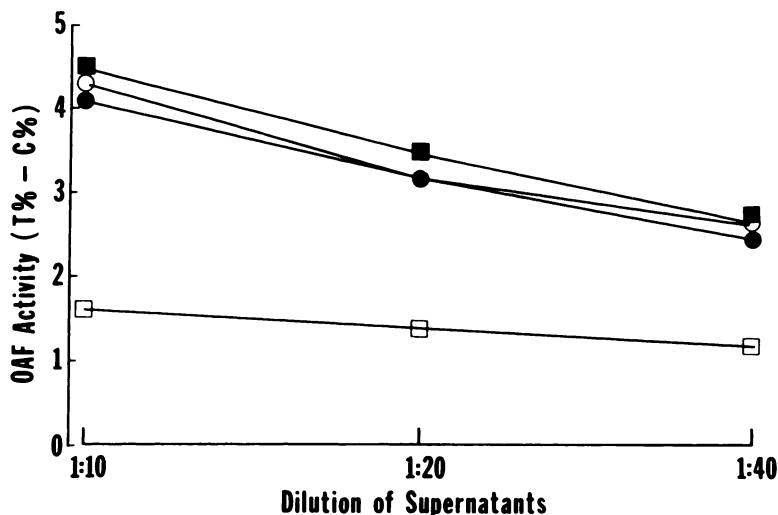

Fig 3. Effect of anticytokine antibodies on OAF activity of MM cell supernatants. MM cell supernatants, untreated (■) or treated with anti–IL-1-α and anti–IL-1-β (□), with anti-LT (O), or with anti-TNF (●), were tested for OAF activity in the fetal rat long bone assay. Results of one of five representative experiments.

a biologically effective mechanism which could be eventually used as therapeutic tool.

This study provides evidence that malignant plasma cells from BM of MM patients produce IL-1 in vitro. The purity of the suspensions tested, confirmed also by lack of effect of stimulants on cytokine release, together with the coculture experiments with autologous normal cells, ruled out that cell types other than myeloma plasma cells are responsible for IL-1 production. IL-1 was actively synthesized in vitro by MM plasma cells, in high amounts as compared with the amount released by normal stimulated monocytic cells. Production of IL-1 was constitutive, and stimulation of the cells in vitro did not produce further increase in IL-1 release. Finally, MM cell supernatants showed a strong OAF activity in organ cultures, which was affected by monospecific anti–IL-1 antibodies. In contrast, plasma cells from MGUS patients, normal marrow samples, or from stimulated blood or tonsil B cell cultures did not release detectable amounts of IL-1, even after stimulation. These results indicate that MM plasma cells and MGUS plasma cells display distinctive differences for some biologic features. The latter point could be useful for differential diagnosis between MGUS and low-mass MM. Low amounts of IL-6 were spontaneously secreted by plasma cells from some cases of MM. They could account for the minor residual IL-1 activity detected in the presence of neutralizing antibodies.

Table 5. Cytokine Activity in RPMI 8226 Cell Supernatants

Stimulus	IL-1 Activity (U/mL)	TNF/LT Activity (Specific Cytotoxicity %) Supernatants Alone	Anti-LT Antibodies
Nil	<10	30	2.1
PHA	<10	75	3.2
LPS	<10	71	2.8
SAC	<10	75	3.2

Cells 1 × 10^6/mL were cultured for 48 hours with or without stimulants. The supernatants were recovered and tested for IL-1 activity in the thymocyte costimulation assay and for TNF/LT activity in the WEHI 164 cell cytotoxicity assay with or without anti-LT antibodies (1 μg/mL final dilution). Results are the mean of triplicate determinations. SD was consistently <10%.

With a single exception, TNF/LT activity was never detectable in plasma cell culture supernatants. The presence of inhibitors of TNF or LT was reasonably excluded by the evidence that exogenous factors added to supernatants as internal standards were entirely measured in the assay. The WEHI 164 cell cytotoxicity assay is, in our experience, capable of detecting as little as 50 pg/mL TNF/LT. Thus, the possibility that lesser quantities were actually produced cannot be dismissed. Consistent with this hypothesis is the presence of TNF- and LT-specific mRNA in MM plasma cells. An alternative explanation for the discrepancy between the presence of mRNA and the absence of detectable TNF/LT activity could be posttranscriptional events, affecting translation, secretion, or both, as has been observed in other systems.[31-36]

MM plasma cells produced mRNA for both the main

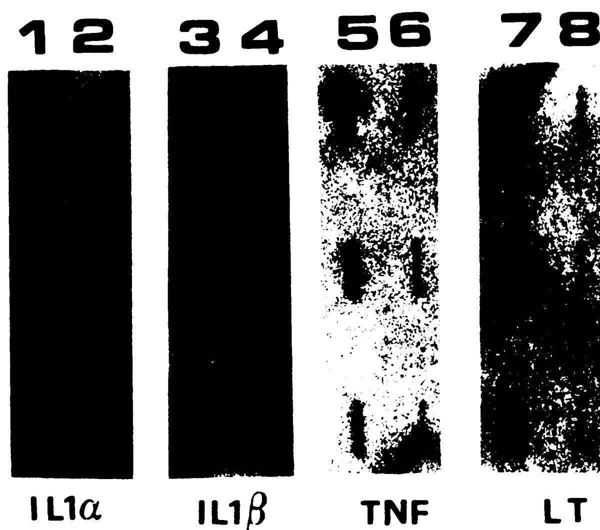

Fig 4. Slot-blot hybridization of mRNA extracted from MM plasma cells (lanes 2, 4, 6, and 8) and from RPMI 8226 cells (lanes 1, 3, 5, and 7) with DNA probes specific for IL-1-α (lanes 1 and 2), IL-1-β (lanes 3 and 4), TNF (lanes 5 and 6), and LT (lanes 7 and 8). The amount of mRNA loaded onto each lane was checked by test minigel.

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molecular species of IL-1, α and β, and their culture supernatants contained both proteins. More than 80% of the IL-1 activity within the supernatants was accounted for by IL-1-β, which is about ten times more active than IL-1-α in inducing osteoclast activation.[5] The strong OAF activity exerted by these supernatants in organ cultures was therefore expected, as was the marked reduction in the presence of anti–IL-1 antibodies. However, OAF activity was not completely abolished. Comparable results have been obtained in models using continuous myeloma cell lines.[37] Since the concentrations of the antibodies were in great excess and always capable of neutralizing the thymocyte costimulatory activity of recombinant IL-1, we suggest that another factor or factors could be responsible for the minor residual OAF activity. The inefficacy of neutralizing antibodies against LT or TNF in the OAF assay, together with the absence of detectable cytotoxic activity against WEHI 164 cells in most MM cell supernatants, suggests that these cytokines are not responsible for the residual bone-resorbing activity. We therefore conclude that IL-1 is the major mediator of bone resorption induced by BM MM cells.

Our observations concerning freshly isolated MM plasma cells are in contrast with those of Garrett et al, who reported recently that several continuous cell lines obtained from MM patients produce abundant LT and do not release detectable amounts of IL-1.[37] We could confirm the above findings at both the mRNA and protein levels, using the RPMI 8226 cell line. Nevertheless, BM cells, freshly isolated from marrow aspirates of MM patients, show inverse functional properties. We believe that in vitro established cell lines do not fully reproduce the behavior of malignant cells, which is best assessed by studying malignant cells freshly obtained ex vivo. Indeed, myeloma cell lines perhaps arise from and hence are representative of the stem cell population,[38,39] which accounts for a minor proportion of the malignant clone.[40] Intraclonal maturation is a well-characterized phenomenon that occurs in almost every neoplasia. Use of different lymphokine programs, as a new functional property, may be acquired together with differentiation.

We do not now know which function, if any, IL-1 subserves in the overall biology of the malignant cell. However, the observation that IL-1 and LT are produced by normal B lymphocytes during the combined processes of proliferation and differentiation, their synthesis being switched off at the final step of plasma cells, indicates that expression of these proteins is finely regulated in normal cells. That the malignant cells from MM patients and the myeloma cell lines maintain IL-1 and LT production, respectively, may suggest that these cytokines are involved in their abnormal proliferative and/or maturational activity. Recently, IL-6 was reported to be an autocrine growth factor for human myeloma cells,[41] which express a high-affinity receptor molecule on their surface. This finding could explain the low IL-6 activity detected in our culture supernatants. Moreover, Kehrl et al showed that LT induces proliferation of SAC-stimulated B lymphocytes,[42] and Scala et al reported that IL-1 is an autocrine growth factor for an EBV-infected lymphoblastoid B cell line.[43] Because IL-1 is a major promoter of IL-6 expression in several cell types,[44,45] both cytokines may participate in a more complex autocrine loop. In this connection, precise identification of the cytokines produced by the malignant cells is of importance for a better understanding of myeloma cell biology.

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