Paracrine Rather Than Autocrine Regulation of Myeloma-Cell Growth and Differentiation by Interleukin-6

By Bernard Klein, Xue-Guang Zhang, Michel Jourdan, Jean Content, Frédéric Houssiau, Lucien Aarden, Marc Piechaczyk, and Regis Bataille

To explore the mechanisms involved in the pathogenesis of human multiple myeloma (MM), we investigated the potential role of interleukin-6 (IL-6), a B-cell differentiation factor in humans, and a growth factor for rat/mouse heterohybridomas and murine plasmacytomas. Using a heterohybridoma assay, we found that two well-documented human myeloma cell lines, RPMI 8226 and U266, did not secrete IL-6 and did not express RNA messengers for IL-6. Neutralizing antibodies to IL-6 did not inhibit their proliferation, and recombinant IL-6 did not stimulate it. Taken together, these data show that IL-6 is not the autocrine growth factor of these human myeloma cell lines. A high production of IL-6 was found in the bone marrows of patients with fulminating MM, compared with patients with inactive or slightly active MM, or to healthy donors. This IL-6 production was assigned to adherent cells of the bone-marrow environment but not to myeloma cells. A spontaneous proliferation of myeloma cells freshly isolated from patients was observed in short-term cultures. Recombinant IL-6 was able to amplify it two- to threefold. The spontaneous proliferation of the myeloma cells was inhibited by anti-IL-6 antibodies and induced by recombinant IL-6. After 2 to 3 weeks of culture, the myeloma-cell proliferation progressively declined and no IL-6-dependent myeloma cell lines could be obtained despite repeated additions of fresh IL-6 and costimulation with other cytokines such as tumor necrosis factor (TNF)β, or IL-1β. These data demonstrated a paracrine but not autocrine regulation of the growth and differentiation of myeloma cells by IL-6.

SEVERAL CYTOKINES (B-cell growth factors, BCGFs; and B-cell differentiation factors, BCDFs) have recently been found to be involved in the proliferation and differentiation of human B cells.1 Of these cytokines, B-cell stimulatory factor 2 (BSF-2) was shown to induce the final maturation of B cells into immunoglobulin-secreting cells (ie, plasma cells) and might be involved in the pathogenesis of autoimmune diseases.1,2 A cDNA of this cytokine, produced by a human HTLV1-infected T-cell clone (ie, TCL-Nal), was recently cloned, and the deduced sequence was shown to be identical to that of interferon-beta 2 (IFN-β).3,4 IFN-β was originally described as a by-product of interferon-beta 1, produced by poly(I)- poly(C)-induced human fibroblasts.5,6 IFN-β is also produced by human fibroblasts and osteoblasts when stimulated by interleukin-1 (IL-1) or tumor necrosis factor (TNF).7,8 By bacterial lipopolysaccharide,9 or by diacylglycerols and calcium ionophore.10 It has little or no antiviral activity11,12 and is a growth factor for hybridomas and mouse plasmacytomas.13,14 (reviewed in reference 20). BSF-2/IFN-β2 was also shown to be identical to hybridoma growth factor (HGF), whose cDNA was cloned from human monocytomas.15 It also induces HLA class-I antigens on fibroblasts,16 the production of acute-phase proteins by hepatocytes,17 the production of IL-2 by murine T cells,18 the proliferation of human and murine T cells,19-21 the differentiation of cytotoxic T cells,22 the proliferation and differentiation of murine B cells in synergy with IL-1,23 and the proliferation and differentiation of murine hematopoietic precursors.24-26 Thus, the cytokines termed BSF-2, IFN-β2, and HGF are one and the same cytokine, and because of its pleiotropic effects, the name IL-6 is now generally used. A murine equivalent of human IL-6 has been purified and partially sequenced (ie, interleukin HP1, hybridoma-plasmacytoma growth factor, plasmacytoma growth factor).1,3,8,27 A cDNA was recently cloned and was shown to share significant homology with the human IL-6 gene.3,28 Murine IL-6 is necessary for in vitro survival and proliferation of murine plasmacytomas, which usually grow only in vivo; it is probably involved in the establishment and maintenance in vivo of peritoneal pristane-induced BALB/c plasmacytomas.32-35 As for human IL-6, murine IL-6 is mainly produced by macrophages, fibroblasts, and T-cell lines.36-38

The existence of a specific factor (IL-6) for the proliferation of murine plasmacytomas prompted us to investigate the possibility that its human equivalent might be involved in multiple myeloma (MM). Several clues support this hypothesis: (a) conditioned media from adherent spleen cells of BALB/c mice primed by intraperitoneally injected pristane or mineral oil, and containing high levels of murine IL-6 have been previously reported to support the growth of human myeloma stem cells39-42; and (b) specific receptors for IL-6 were recently found on lymphoblastoid cell lines, but the highest levels were found on U26641-42 and RPMI 8226 (J. Content, unpublished results, March 1988), two well-documented human myeloma cell lines (HMCLs), suggesting that these cell lines could be directly sensitive to this factor. In contrast to recently proposed theories by Kawano et al,43 our present results favor a paracrine rather than an autocrine regulation of myeloma-cell growth and differentiation by IL-6.

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MATERIALS AND METHODS

Patients

We studied the production of IL-6 by bone-marrow cells from 29 patients with either MM (n = 26) or plasma cell leukemia (PCL, n = 3) after obtaining informed consent. The diagnostic criteria were those of the Southwest Oncology Group of the United States.44 Of the myelomas, 20 were of IgG, 8 of IgA, and one Bence Jones. Sixty percent of the patients had the kappa light chain subtype. The mean age was 64 years (range, 48 to 78) and the sex ratio of M:F was 2:6. Eighteen patients were studied during fulminating disease, characterized by extensive lytic bone lesions, recurrent hypercalcemia, death within 6 months, and PCL for three patients. The other 11 patients were studied during an inactive or slightly active phase of their disease, as determined by criteria previously reported.45 IL-6 production by both peripheral-blood and bone-marrow cells of one patient with PCL was also studied. Following a remission phase induced by chemotherapy, this patient had a fulminating relapse with extramedullary proliferation, hypercalcemia, and death within a month. In the terminal phase of the disease, 90% of the cells in the peripheral-blood and bone-marrow samples were malignant plasma cells (>98%).

The presenting features were those of almost entirely of malignant plasma cells (>98%). Solitary plasmacytoma originating from bone marrow were malignant plasma cells. The tumor, obtained by surgical resection, was composed of malignant plasma cells (<98%). Finally, bone marrow from seven healthy donors (HDs) was studied after obtaining informed consent.

We also studied the presence of IL-6 in normal sera and in the sera of 99 patients with MM, including patients with active or inactive disease. The diagnostic criteria were those of the Southwest Oncology Group.44 The presenting features were those of a standard MM population.45

Cultures of Bone Marrow and HMCLs

Short-term culture supernatants of bone-marrow cells and HMCLs. Bone-marrow samples from normal individuals and from patients with MM were prepared as previously described.46 A plasmacytoma obtained by surgical resection was gently teased with extramedullary proliferation, hypercalcemia, and death within a month. In the terminal phase of the disease, 90% of the cells in the peripheral-blood and bone-marrow samples were malignant plasma cells (<98%). Solitary plasmacytoma originating in bone marrow was included in the study. The tumor, obtained by surgical resection, was composed of malignant plasma cells (<98%). Finally, bone marrow from seven healthy donors (HDs) was studied after obtaining informed consent.

We also studied the presence of IL-6 in normal sera and in the sera of 99 patients with MM, including patients with active or inactive disease. The diagnostic criteria were those of the Southwest Oncology Group. The presenting features were those of a standard MM population.

Bone-marrow cells were isolated by centrifugation of the heparinized peripheral blood over Ficoll-Hypaque gradients. Supernatants were produced as follows: 10^6 cells/mL, 24 hours at 37°C in PRMI 1640 supplemented with 2 mmol/L L-glutamine, 5 x 10^{-2} mol/L 2-mercaptoethanol (2-ME), and with or without 1% fetal calf serum (FCS). Supernatants were filtered (0.22 μm) and stored at −20°C until use.

Culture supernatants from two HMCLs (ie, RPMI 8226, and U266) were also prepared (5 x 10^5 cells/mL, cultured for one or three days in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 5 x 10^{-3} mol/L 2-ME, and 5% FCS). The RPMI 8226 was purchased from Flow Laboratories (Irvine, Scotland). The U266 cell line was a gift from Dr Nilsson (Uppsala, Sweden). Their main features have been previously published47 and were regularly checked in our laboratory. RPMI 8226 produced only light chains and had numerous typical chromosome markers. U266 expressed high levels of cytoplasmic IgE2 immunoglobulins.

Supernatants from long-term cultures of adherent bone-marrow cells and fibroblast cell lines. Monolayers of adherent cells were obtained from bone marrows of six different patients and three HDs after 4 to 6 weeks of culture in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 5 x 10^{-3} 2-ME, and 5% FCS. At confluence, adherent cells were washed with fresh medium and cultured for 24 hours and 48 hours in RPMI 1640 with 5% FCS. The supernatants were filtered (0.22 μm) and stored at −20°C until use.

Cytokine Assays

IL-6 bioassay. The presence of IL-6 in the culture supernatants of the HMCLs, of the bone-marrow adherent cell cultures, and in the sera of patients with MM was evaluated using the IL-6-dependent hybridoma cell line B9 as previously described in detail.48 One of the known cytokines, except IL-6, induced proliferation of this cell line (B. Klein, unpublished results, February 1988). Briefly, 5 x 10^3 B9 cells were cultured in 96-well flat-bottomed culture dishes in 200 μL of RPMI 1640 culture medium with 5% FCS, and serial dilutions of the sample. Three days later, 0.5 μCi of (3H)TdThd was added to each culture well, incubated for eight hours, and (3H)TdThd incorporation was determined as previously described.49 rIL-6 was used as an internal standard in all assays. One unit of IL-6 was defined as the amount inducing half-maximal proliferation and amounted to approximately 1 pg of rIL-6.

IL-6 in the culture supernatants of bone marrows was assayed using another specific and highly sensitive bioassay.50 Briefly, 2 x 10^8 IL-6-dependent hybridoma cells (mouse/mouse hybrid TT1D1) were incubated in 200 μL of culture medium in the presence of serial dilutions of supernatants. After four days, the number of cells was evaluated by colorimetric determination of hexosaminidase levels. One unit of IL-6 was defined as the amount required to obtain half-maximal growth of the cells and amounted to approximately 1 pg of IL-6. A selected spleen-cell concanavalin A (ConA) supernatant containing approximately 1,000 U/mL IL-6 was used as an internal standard in all IL-6 assays.

IL-1 assays. IL-1 activity was tested using a standard lymphocyte activating factor (LAF) bioassay, as previously described.51 The control IL-1 was recombinant IL-1 kindly supplied by Biogen Laboratories (Geneva). One unit of IL-1 was defined as the amount inducing a half-maximal proliferative response of the thymocytes. IL-1β was also measured using a standard radioimmunoassay purchased from Cistron Laboratories (Pine Brook, NJ).

TNF bioassay. TNF activity was tested using a modified cytotoxic assay against the L929 fibroblast cell line.52 Briefly, L929 cells were cultured for 24 hours in 96-well flat-bottomed microtiter plates at a density of 4 x 10^3 cells per well in 100 μL of RPMI 1640 culture medium supplemented with 5% FCS. Then 100 μL of serial dilutions of the sample were added to quadraplicate culture wells together with 20 μL of a solution of 10 μg/mL actinomycin D (Sigma, St Louis). Twenty-four hours later, the supernatants were removed and 100 μL of crystal blue (Merck, Darmstadt, WG; 5% crystal blue in 1:4 mixture of methanol:water) was added and left for ten minutes. The wells were then gently washed five times with tap water and the absorbance was determined at 540 nm using an automatic multichromatographic photometer (Flow Laboratories, Irvine, Scotland). The TNF activity of a sample, expressed in units per milliliter, was defined as the reciprocal of the dilution that halved the absorbance in the culture wells. We have found that the sensitivity of the assay is to within 1 U/mL. Recombinant IL-1 is not cytotoxic in this bioassay53 (unpublished data).

Activation of Malignant Plasma Cells in Vitro

Activation of malignant plasma cells freshly isolated from bone marrow. Bone-marrow samples from ten patients with MM and peripheral-blood cells from three patients with PCL were cultured at 10^6 cells per milliliter in Iscove medium supplemented with 5 x 10^{-3} mol/L 2-ME and 5% FCS, in the absence or presence of rIL-6 at various concentrations (100, 500, or 1,000 U/mL). At culture days 0, 3, 7 and 15, the percentages of malignant plasma cells were determined by intracytoplasmic immunofluorescence using anti-kappa or anti-lambda light chain antibodies directly coupled to fluorescein (Kallestad, Austin, TX). The percentages of plasma cells in the S-phase were determined using an anti-bromodeoxyuri-
dine monoclonal antibody (ie, Bu-I, a generous gift from N. Gonchoroff, Mayo Clinic, Rochester, MN) and a rhodamine-labeled goat anti-mouse Ig (Cappel Laboratories, Malvern, PA) in a double fluorescence technique described elsewhere.28,29

Stimulation of HMCLs. The effect of rIL-6 on the proliferation of two HMCLs (RPMI 8226, U266) was also evaluated using cultures with low cell densities to increase the requirement of the tumor cells for exogenous growth factor, as described elsewhere.26 Cell lines were cultured in RPMI 1640 supplemented with 2-mmol/L L-glutamine, 5 \times 10^{-5} \text{mol/L 2-ME}, and 5% FCS.

Sources of IL-6 and Anti-IL-6 Antiserum

Two sources of rIL-6 were used. One rIL-6 (provided by J. Content) was produced in virtually pure form by injection of IL-6 mRNA into Xenopus laevis oocytes, as described elsewhere.19 Supernatants of oocytes not injected with IL-6 mRNA contained no IL-6 activity and were used as controls in each experiment using this source of IL-6. IL-6 from the second source (provided by L. Aarden) was purified rIL-6 produced in Escherichia coli by molecular cloning and expression of complementary DNA derived from human monocyte-poly(A+) RNA, as described elsewhere.31 Monocyte-derived IL-6 (usually termed HGF)49 was obtained by culturing 10^6 peripheral-blood mononuclear cells from healthy individuals for 24 hours in RPMI 1640 culture medium and 5% FCS. The rabbit anti-IL-6 antibodies were provided by J. Content. They were obtained by repeated immunization of rabbits with IL-6 and neutralized IL-6 (40,000 U/mL).

Northern Blot Analysis

Total cellular RNA was extracted by the guanidium/hot phenol method described by Maniatis et al27 and analyzed by electrophoresis of 20 μg of RNA through 1% agarose-formaldehyde gels followed by Northern blot transfer to geneScreenPlus nylon membrane (DuPont, Boston) as described by Thomas.28 The hybridization with 32P-labeled plasmid DNA and the autoradiographic procedure were reported elsewhere.29 The human IL-6 cDNA and the full-length rat glyceraldehyde-3-phosphate dehydrogenase pRGAPDH13 cDNA were described elsewhere.50,51

RESULTS

Patients With Fulminant Multiple Myeloma Secreted Large Amounts of IL-6

As shown in Fig 1, spontaneous secretion of IL-6 was found in the culture supernatants of bone-marrow cells from healthy donors and patients with MM. Eighteen patients with fulminating disease (extensive lytic bone lesions with major fractures, recurrent hypercalcemia, PCL in three cases, and death within 6 months) had spontaneous IL-6 levels (833 ± 945 U/mL) significantly higher than those of 11 patients with inactive or slightly active disease (119 ± 81 U/mL) and those of healthy donors (147.2 ± 71 U/mL). Twelve of the 18 patients (67%) with fulminating disease had IL-6 levels >362 U/mL, ie, three standard deviations above the mean value observed in patients with inactive/slightly active disease. Within this group of patients with fulminating MM, the levels of IL-6 secretion in the bone-marrow cultures were not correlated with the proportions of plasma cells present in these cultures. A low but significant spontaneous secretion of IL-6 was observed in the bone marrows of healthy donors (147.2 ± 71 U/mL), while the IL-6 levels of patients with inactive/slightly active MM remained within the normal range. IL-6 was completely undetectable (threshold of detection: <10 U/mL) in one normal bone marrow, two bone marrows from patients with inactive disease, and two from patients with fulminant disease (Fig 1). Thus, bone marrows from patients with inactive/slightly active myeloma could be distinguished from those of patients with fulminating disease on the basis of IL-6 levels. As described in the Materials and Methods section, the same supernatants from bone-marrow cultures were also tested for IL-1 activity (LAF bioassay), IL-1β activity (radioimmunoassay), and TNF activity (L929 cytolysis bioassay). The results in normal bone-marrow samples were: LAF = 0.7 ± 1.2 U/mL, IL-1β = 0.37 ± 0.23 ng/mL, TNF = 1.7 ± 1.9 U/mL; in bone marrow from patients with inactive/slightly active disease: LAF = 2.8 ± 4.2 U/mL, IL-1β = 0.58 ± 0.15 ng/mL, TNF = 1.9 ± 1.9 U/mL; and in bone marrow from patients with fulminating disease: LAF = 3.5 ± 7.8 U/mL, IL-1β = 0.91 ± 0.5 ng/mL, TNF = 2.5 ± 3.4 U/mL. Thus, neither LAF, IL-1β, nor TNF activity significantly discriminated between any categories of bone marrow, as opposed to IL-6.
Table 1. Production of IL-6 by Cells of the Bone-Marrow Environment But Not Myeloma Cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Non-T Cells*</th>
<th>HLA-DR Negative Non-T Cells*</th>
<th>Adherent Non-T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,600 (60%)</td>
<td>0 (95%)</td>
<td>2,300</td>
</tr>
<tr>
<td>2</td>
<td>874 (83%)</td>
<td>4 (96%)</td>
<td>649</td>
</tr>
<tr>
<td>3</td>
<td>1,381 (56%)</td>
<td>18 (93%)</td>
<td>1,679</td>
</tr>
</tbody>
</table>

*In parentheses, percentage of myeloma cells in the cell population.

IL-6 Was Secreted by Cells of the Bone-Marrow Environment, But Not by the Myeloma Cells

As bone marrow from patients with fulminating disease had the largest percentages of myeloma cells (data not shown), we considered the possibility that IL-6 was secreted by malignant plasma cells. Several findings supported the idea that IL-6 was secreted mainly by cells of the bone-marrow environment rather than by the myeloma cells: (1) As shown in Table 1, the separation of the bone-marrow cells into adherent and nonadherent cells clearly indicated that IL-6 was secreted by the bone-marrow adherent cells, and not by the nonadherent cell population containing the myeloma cells. (2) Virtually pure populations of freshly isolated myeloma cells secreted very small amounts of IL-6, if any (Table 2). In one case (patient A), this population was obtained from a bone-marrow plasmacytoma removed by surgical resection and contained >98% plasma cells. In the other case (patient B), it was obtained from the massive invasion of the peripheral blood with 50,000 myeloma cells/μL. The low production of IL-6 (20 U/mL) could not be further increased by IL-1β, TNFβ, or PMA, which are known to be potent IL-6 inducers in fibroblasts (Table 2).

Interestingly, the bone-marrow cells of patient B, comprising 90% myeloma cells like the peripheral blood, secreted large amounts of IL-6 (3,200 U/mL), indicating the importance of the bone-marrow environment for IL-6 production (Table 2). (3) Human myeloma cell lines with a plasmacytic phenotype (RPMI 8226, U266) did not secrete IL-6 (data not shown), and did not express any IL-6 mRNA (Fig 2). (4) Monolayers of bone-marrow adherent cells, established from six patients with MM, secreted high IL-6 levels after 4 to 6 weeks of culture without any stimulation (Table 3). This was not specific of myeloma patients since monolayers from HDs, established in the same conditions, also secreted significant IL-6 levels (Table 3).

Low Levels of IL-6 in Sera From Patients With MM

Since IL-6 was found in the sera or the biological fluids of patients with autoimmune diseases, 99 sera from patients with MM were assayed for its presence. Low, but significant levels of IL-6 were found in these sera (range, 0 to 150 U/mL).

IL-6-Induced Myeloma-Cell Proliferation

Since spontaneous high IL-6 levels were detected in patients with fulminating disease and since IL-6 is a paracrine growth factor for murine plasmacytomas, we wanted to determine whether it was a growth factor for myeloma cells. Myeloma cells were isolated from the bone marrow of ten patients with active MM and from the peripheral blood of three patients with PCL. The percentages of myeloma cells in the S-phase were determined specifically using an anti-S-phase antibody. Myeloma cells did not proliferate or proliferated very little in vivo (Table 4). After seven days of culture, spontaneous proliferation was found in eight of 13 patients, and the percentages of the myeloma cells in the S-phase ranged from 3% to 10% of total myeloma cells. rIL-6 (optimal concentration, 1,000 U/mL) doubled this proliferation (Table 4). In addition, spontaneous proliferation was abolished by neutralizing antibody to IL-6 (Table 5). rIL-6 reversed the inhibitory action of the anti-IL-6 antibody and induced proliferation of myeloma cells (Table 5). After 15 days, exogenous rIL-6 did not enhance myeloma-cell prolif-

Table 2. Production of IL-6 by Peripheral-Blood or Bone-Marrow Myeloma Cells

<table>
<thead>
<tr>
<th>Patients</th>
<th>IL-6 (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Stimulation</td>
</tr>
<tr>
<td>Patient A</td>
<td>0</td>
</tr>
<tr>
<td>Peripheral blood cells (&gt;98% myeloma cells)</td>
<td>20</td>
</tr>
<tr>
<td>Bone-marrow cells (90% myeloma cells)</td>
<td>3,200</td>
</tr>
</tbody>
</table>

Cells were cultured for two days at 10^6 cells/mL in RPMI 1640 and 5% FCS. In some groups, recombinant IL-1β, or TNFβ, or PMA was added at the initiation of cultures. Patient A had bone-marrow plasmacytoma, and a sample was obtained from a surgically resected tumor. Patient B had plasma-cell leukemia, and peripheral blood and bone marrow were taken at the same time.
INTERLEUKIN-6

GAPDH

Fig 2. Absence of IL-6 mRNA in human myeloma cell lines. Total cellular RNAs (20 μg) were fractionated by electrophoresis through a 1% agarose gel containing 1 M formaldehyde and transferred onto a nylon membrane. Hybridizations were successively carried out with the nick-translated IL-6 and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. The GAPDH probe was used as an internal control to assay for mRNA integrity. The monocytes were peripheral-blood monocytes obtained after 24-hour adherence on plastic culture dishes.

ration anymore and the cultures progressively declined (data not shown). Higher rIL-6 concentrations (>1,000 U/mL) or addition of IL-1β or TNFα did not further increase proliferation, and no myeloma cell lines were obtained by continuous addition of rIL-6. In five of 13 patients, no spontaneous proliferation of myeloma cells was found (Table 4), nor was it induced by rIL-6 alone or in combination with IL-1β, TNFα, IL-2, IFN-γ, or PMA (Table 4 and unpublished results). This difference between responsive and unresponsive patients did not correspond to a difference in the stage of the disease (Table 4).

On six separate occasions, rIL-6 did not increase the proliferation of the RPMI 8226 HMCL plated at low cell density in order to increase its dependence on exogenous factors (Fig 3). The proliferation of the U266 HMCL was slightly increased in two of five separate experiments (Fig 3). In addition, anti–IL-6 antibody did not at all inhibit the proliferation of these HMCLs when used at a concentration inhibiting 500 U of IL-6 (data not shown).

DISCUSSION

Cooperation between malignant plasma cells and cytokine-producing accessory cells appears to be a major mechanism in the genesis of murine plasmacytomas. After having been injected intraperitoneally with mineral oil, susceptible mice develop inflammatory granuloma in the peritoneal cavity associated with large local IL-6 production. Then polyclonal plasma cells colonize the inflammatory granuloma, and finally a monoclonal plasmacytoma with characteristic translocations emerges.6 It is of major interest to note that murine malignant plasma cells do not produce IL-6. On the other hand, they are absolutely dependent on the continuous addition of exogenous IL-6 for proliferating in vitro.32-35 For these reasons, we considered the possibility that IL-6 was involved in the pathogenesis of human MM.

Large amounts of IL-6 were secreted by bone-marrow environment, but not by myeloma cells in patients with fulminating MM. Several observations supported our conclusion: (1) In the bone marrow of patients with MM, IL-6 was secreted by adherent cells (mainly monocytes and stromal cells), but not by HLA-DR-negative nonadherent cells, consisting mainly of myeloma cells. (2) Neither pure fresh myeloma cells or true myeloma cell lines (RPMI 8226 and U266)47,48 secreted any detectable IL-6, or contained any IL-6 mRNA. (3) Long-term cultures of adherent stromal cells from the bone marrow of patients with MM spontaneously secreted large amounts of IL-6. This was not specific of patients with MM since long-term cultures of bone-marrow adherent cells of HDs also spontaneously secreted IL-6. Thus, a similar situation occurs in human MM and murine plasmacytomas, namely that IL-6 is secreted in large amounts by the tumor environment, but not by malignant plasma cells. Kawano et al recently reported that in patients with MM, bone-marrow cells, enriched in myeloma cells, secreted IL-6 (ie, 0.4 U to 6 U/10^6 cells/mL); they concluded that the myeloma cells were secreting IL-6 themselves.49 However, as the amount of IL-6 corresponding to 1 unit was not indicated in the abovementioned paper, and as IL-6 secretion in total bone-marrow cells was not compared with that in purified myeloma cells, this work was initially difficult to evaluate. A following paper by the same investigators indicated that the heterohybridoma MH60 used in these studies for testing IL-6 was as sensitive as the heterohybridomas we used, one unit corresponding to 5 pg of IL-6.60 Thus, we can conclude that Kawano et al published results similar to ours, namely that bone-marrow cells enriched in

Table 3. IL-6 Production in Long-Term Cultures of Bone-Marrow Adherent Cells From Patients With Active MM

<table>
<thead>
<tr>
<th>Adherent Cells From Patients With Active MM</th>
<th>IL-6 (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloma patients</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>550</td>
</tr>
<tr>
<td>2</td>
<td>620</td>
</tr>
<tr>
<td>3</td>
<td>5,120</td>
</tr>
<tr>
<td>4</td>
<td>1,610</td>
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<tr>
<td>5</td>
<td>2,800</td>
</tr>
<tr>
<td>6</td>
<td>1,700</td>
</tr>
<tr>
<td>Healthy donors</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>540</td>
</tr>
<tr>
<td>2</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>1,140</td>
</tr>
</tbody>
</table>

Bone-marrow adherent cells were cultured for 4 to 6 weeks. No malignant plasma cells were detected in the final cultures of myeloma bone marrow. Fresh medium (RPMI 1640 and 5% FCS) was added to the confluent cultures, and after further culturing for two days, the supernatants were assayed for IL-6 using the IL-6-dependent B9 cell line.
Table 4. Effect of rIL-6 on the Proliferation in Vitro of Myeloma Cells Freshly Isolated From Bone Marrow

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>IL-6 in Culture Medium (U/mL)</th>
<th>Day 0 of Culture</th>
<th>Day 7 of Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myeloma Cells in Culture (%)</td>
<td>Myeloma Cells in the S-Phase (%)</td>
<td>Myeloma Cells Culture (%)</td>
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<tr>
<td>1</td>
<td>1,000</td>
<td>14</td>
<td>8</td>
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<td></td>
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<td>72</td>
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<tr>
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<td>2,000</td>
<td>90</td>
<td>92</td>
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<td>7</td>
<td>1,000</td>
<td>95</td>
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<td></td>
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<td></td>
<td>95</td>
</tr>
<tr>
<td>8</td>
<td>10,000</td>
<td>95</td>
<td>93</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cultures with spontaneous proliferation on day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>9‡§</td>
</tr>
<tr>
<td>10‡§</td>
</tr>
<tr>
<td>11‡§</td>
</tr>
<tr>
<td>12‡§</td>
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<tr>
<td>13‡§</td>
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<tr>
<td>1,000</td>
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<td>1,000</td>
</tr>
<tr>
<td>1,000</td>
</tr>
<tr>
<td>1,000</td>
</tr>
</tbody>
</table>

*Not very active disease. †IL-6 produced in E. coli. ‡Very active disease. §Plasma-cell leukemia. §§IL-6 produced in oocytes.

Myeloma cells secreted only very small amounts of IL-6 compared with the total bone-marrow cells. This low secretion by cell populations enriched in myeloma cells could easily be the result of a minor contamination by residual monocytes, since heterohybridoma bioassays are sensitive enough to detect the IL-6 secreted by a single monocyte. Supporting the conclusion that myeloma cells do not secrete IL-6, we found that the myeloma cell lines RPMI 8226 and U266 secreted no IL-6 and contained no IL-6 mRNA. On the contrary, Kawano et al reported the presence of IL-6 mRNA in the U266 cell line, without precisely determining if it secreted IL-6 or not. This may be explained by their use of a dot-blot analysis, which did not allow them to exclude a nonspecific hybridization, compared with the northern blot analysis we used. This may also be explained by the probable existence of different clones of the U266 cell line, which was established 20 years ago, and carried on in many laboratories. The U266 cell line we used had a typical phenotype of plasma cells and produced IgEX immunoglobulins.

IL-6 induced a transient proliferation of myeloma cells. We studied this point by direct determination of the proliferating myeloma cells using an anti–S-phase monoclonal anti-
IL-6 IN HUMAN MULTIPLE MYELOMA

What is the role of IL-6 in human MM? The inability to obtain IL-6-dependent myeloma cell lines raises the question of the phenotype of the myeloma stem cell and the exact role of IL-6 in human MM. The only argument in favor of a plasmacytic tumor stem cell in MM is the existence of a small number of plasmacytic HMCLs, such as RPMI 8226 and U266. However, these plasmacytic HMCLs are not at all representative of the large majority of patients. They have been obtained only from a very small proportion of patients with fulminant disease and extramedullary proliferation, and from the extramedullary sites, not from bone marrow. In this small proportion of patients, additional oncogenic events may occur, allowing the plasma-cell compartment to metastasize and generate HMCLs in vitro. We have previously shown that these plasmacytic HMCLs produce an autocrine growth factor and a B-cell growth factor, the two factors being copurified in a peak of 70 kilodalton (Kd) apparent molecular weight. This autocrine growth factor is not IL-1α, IL-1β, IL-2, IL-4, TNFα, TNFβ, IFNα, IFNγ, and has biological properties close to those of BCGF 1156 (B. Klein, unpublished results, May 1987). Interestingly, the present results indicate that IL-6 is not the autocrine growth factor of these plasmacytic HMCLs. In the large majority of patients with active MM, no plasmacytic HMCLs can be obtained from the bone marrow, despite high amounts of

Table 5. Inhibition of Myeloma-Cell Proliferation by Neutralizing Antibodies to IL-6

<table>
<thead>
<tr>
<th>Antibodies to IL-6</th>
<th>Patient 1</th>
<th>85% Myeloma Cells</th>
<th>Myeloma Cells in the S-Phase (%)</th>
<th>IL-6* (U/mL)</th>
<th>Myeloma Cells in the S-Phase (%)</th>
<th>IL-6* (U/mL)</th>
<th>Patient 2</th>
<th>91% Myeloma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulation</td>
<td>10</td>
<td>530</td>
<td>8</td>
<td>1,270</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IL-6†</td>
<td>1.5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IL-6 plus rIL-6 (3,000 U/mL)</td>
<td>8</td>
<td>160</td>
<td>6</td>
<td>624</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rIL-6 (3,000 U/mL)</td>
<td>25</td>
<td>4,129</td>
<td>10</td>
<td>3,601</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bone-marrow cells were enriched in myeloma cells by removing adherent cells and T cells. The percentage of myeloma cells in the S-phase as well as the IL-6 levels in the cultures were determined on day 3 of culture. In some groups, recombinant IL-6 (3,000 U/mL) was added at the initiation of cultures.

*The IL-6 levels in the culture supernatants were determined using the B9 cell line.
†The anti-IL-6 antibodies were added at the initiation of cultures at a concentration inhibiting 2,000 units of IL-6.

body. Spontaneous proliferation of myeloma cells was found in samples from eight of 13 patients after seven days of culture. It was abolished by neutralizing antibodies to IL-6 and reinduced by exogenous rIL-6. This clearly indicates that the spontaneous proliferation of myeloma cells was mediated by endogenous IL-6 secreted by the bone-marrow environment. rIL-6 (optimal dose, 1,000 U/mL) further increased this spontaneous proliferation, with 10% to 25% of the myeloma cells in the S-phase on day 7 of culture. If the S-phase is estimated to last for one third of the cell cycle, our results indicate that 30% to 75% of the myeloma cells were cycling in response to IL-6. This is unexpected for myeloma cells, which are usually considered to be nonproliferative. Over long periods of time, the cultures progressively declined despite repeated additions of fresh IL-6 or costimulation with other cytokines such as TNFα, IL-1β, IL-2, or IFN-γ. We have not yet been able to obtain an IL-6-dependent myeloma-cell line. It has recently been reported that IL-6 induced the proliferation of bone-marrow cells enriched in myeloma cells without the establishment of IL-6-dependent myeloma cell lines.64 In this report, the proliferation was assayed using tritiated thymidine incorporation. We rejected this proliferation assay because IL-6 is a growth factor for many cell lineages and it is impossible to determine which of these cells are really proliferating. The double immunofluorescence method used in the present study, combining an anti-S-phase monoclonal antibody and anti-immunoglobulin light chain antibodies, clearly demonstrated that IL-6 is a growth factor for myeloma cells, without the possibility of expanding them in vitro. IL-6 may also have this property for normal plasma cells. Indeed, plasma cells generated in cultures of pokeweed-mitogen stimulated B cells are proliferating, and IL-6 is essential for the generation of plasma cells in such cultures. In addition, we have found that polyclonal plasma cells from reactive (ie, inflammatory) bone marrow proliferate spontaneously, and that this proliferation is increased in response to IL-6 (X.G. Zhang, unpublished results, July 1987).

EFFECT OF IL-6 ON THE PROLIFERATION OF HUMAN MYELOMA CELL LINES

Fig. 3. Effect of IL-6 on the proliferation of human myeloma cell lines. Human cell lines were cultured at low cell densities to increase their requirement for exogenous growth factors. rIL-6 was added at different concentrations at the initiation of the cultures, and proliferation was assayed after two days of culture using [3H]thymidine incorporation. The IL-6-dependent B9 cell line was used as a control for the activity of rIL-6 (●—●). Results are means ± SDs of the [3H]thymidine incorporation determined in six replicate culture wells. RPMI 8226 (●—●): U266 (△—△).
IL-6 produced constitutively. Several reports even suggest that the putative myeloma stem cell could be an immature B cell. B cells and pre-B cells bearing the idiotypic determinants of the myeloma monoclonal protein have been found. More recently, pre-B/B cells showing a double phenotype of immature B cells and plasma cells have been found in the bone marrow of patients with MM. The pre-B/B cells were able to generate pre-B/B cell lines not infected with the Epstein Barr virus and having patient-specific cytogenetic markers. All these observations suggest that the myeloma stem cell could be an immature B cell. This stem cell would proliferate but would also continuously differentiate, generating malignant plasma cells with a limited proliferative capacity. Since IL-6 is a potent B-cell differentiation factor, it might be essential for inducing the continuous differentiation of these tumor stem cells into myeloma cells and for inducing a transient proliferation of the myeloma cells. After longer culturing, the stem-cell compartment may be dried up by continuous differentiation, resulting in a decrease in the number of proliferating myeloma cells and the gradual culture death. Taking into account all these arguments and the present data, the recent proposal that IL-6 is an autocrine growth factor for myeloma cells, allowing the emergence of the tumoral clone, appears to be unwarranted.

Acknowledgment

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