Biologic Effects of Anti–Interleukin-6 Murine Monoclonal Antibody in Advanced Multiple Myeloma

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In patients with advanced multiple myeloma (MM) there is an excess of production of interleukin-6 (IL-6) in vivo, and elevated serum levels are associated with plasmablastic proliferative activity and short survival. These data prompted us to perform a clinical trial with a murine anti–IL-6 monoclonal antibody (MoAb) to neutralize the excess of this putatively deleterious factor in these patients. Ten MM patients with extramedullary involvement frequently were treated with anti–IL-6 MoAb. The MoAb was administered intravenously to 9 patients; 1 patient with malignant pleural effusion received intrapleural therapy. Of the 3 patients who succumbed to progressive MM after less than 1 week of treatment (including the only 1 treated locally), 2 with evaluable data exhibited marked inhibition of plasmablastic proliferation. Among the 7 patients remaining more homogeneous receiving the anti–IL-6 MoAb for more than 1 week, 3 had objective antiproliferative effect marked by a significant reduction of the myeloma cell labelling index within the bone marrow. One of these 3 patients achieved a 30% regression of tumor mass. However, none of the patients studied achieved remission or improved outcome as judged by standard clinical criteria. Of major interest, objective antiproliferative effects were associated with complete inhibition of C-reactive protein (CRP) synthesis and low daily IL-6 production in vivo. On the other hand, the lack of effect in 4 patients was associated with a higher IL-6 production and inability of the MoAb to neutralize it. Anti–IL-6 was also associated with resolution of low-grade fever in all the patients and with worsening thrombocytopenia and mild neutropenia. The generation of human antibodies to Fc fragment of the murine anti–IL-6 MoAb observed in 1 patient was associated with dramatic progression. These data show that anti–IL-6 MoAb can suppress the proliferation of myeloma cells and underscore the biologic role of IL-6 for myeloma growth in vivo. Furthermore, suppression of CRP and worsening of neutropenia/thrombocytopenia both indicate that IL-6 is critically involved in acute-phase responses and granulopoiesis/thrombopoiesis.
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INTERLEUKIN-6 (IL-6), a pleiotropic cytokine produced by a range of cells, plays a central role in both host defense mechanisms and acute-phase responses.[1] Pioneering studies have shown that this cytokine controlled the proliferation[2-5] but did not induce the differentiation[6,7] of immature myeloma cells in vitro. First, anti–IL-6 MoAb blocked the spontaneous proliferation of freshly explanted myeloma cell in short-term cultures and addition of exogenous IL-6 further increased it.[3-5] Second, myeloma cell lines whose proliferation is dependent on addition of exogenous IL-6 have been obtained from every patient with extramedullary proliferation.[8] All studies agreed that large amounts of IL-6 are produced by the tumoral microenvironment in response to stimulating myeloma cells.[3,9,10] Several studies have reported an autocrine production of IL-6 by the myeloma cells themselves.[2,11] However, this production was only very minor (1/1,000) as compared with that of the tumoral microenvironment. For these reasons, these studies could not exclude a minor contamination by environmental cells or more simply a weak activation of IL-6 gene in myeloma cells by exogenous IL-6, as has been previously reported.[12] The involvement of this cytokine in vivo is supported by the relationship of increased IL-6 serum levels to in vivo tumor cell kinetics, disease severity, and survival of patients with multiple myeloma (MM).[13,14] These data led us to develop a clinical trial to define the effects of a murine anti–IL-6 monoclonal antibody (MoAb) in patients with advanced MM (mainly plasma cell leukemia). The rationale of this project was also supported by data obtained in mice showing that (1) IL-6 is a major paracrine plasmacytoma growth factor and that (2) anti–IL-6 and anti–IL-6 receptor MoAbs significantly inhibit the in vivo expansion of plasmacytomas and improve the survival of inoculated mice.[15]

PATIENTS, MATERIALS, AND METHODS

Patients

Nine patients with advanced and progressive MM, mainly primary and secondary plasma cell leukemia (PCL) (n = 6), refractory to standard chemotherapy and with a life expectancy less than 1 month were treated with anti–IL-6 MoAb. The clinical course of a tenth patient treated similarly and previously reported has been included in the current study, considering new data on IL-6 production in vivo.[16] Written informed consent was obtained from all the patients. Selected pretreatment characteristics of the 10 patients are summarized in Tables 1 and 2. All but 1 patient were treated with intravenous (IV) B-E8 anti–IL-6 MoAb, usually at a standard dose of 20 mg/day for at least 4 days (up to 68 days). One patient (no. 3) received B-E8 intrapleurally for a malignant pleural effusion at a daily dose of 20 mg for 3 days. Response to treatment was evaluated using standard criteria, including performance status, body temperature, weight, and improvement of symptoms such as anemia, hypercalcemia, occurrence of infections, and regression of monoclonal component and myeloma cell mass.

Materials and Methods

Infection of anti–IL-6 MoAb. Injection of anti–IL-6 MoAb was performed in vivo as previously described.[16] Nine of the 10 patients

Table 1. Pretreatment and Treatment Characteristics of Patients

	Case No.									
	1*	2	3*	4	5	6†	7*	8*	9	10
Sex/Age (yr)	M 59	F 51	F 57	M 44	F 54	M 61	F 45	F 56	M 59	M 65
Clinical status	MM with secondary PCL	Fulminating MM with EMI	Refractory primary PCL (pleural effusion)	Refractory MM	Refractory MM with progressive bone disease and hypercalcemia gram-negative sepsis on day 12	Refractory primary PCL	MM with secondary PCL	MM with secondary PCL	MM with secondary PCL	Fulminating MM with EMI
Daily doses	AK 2 mg d0, 5 mg d1, 10 mg d2, 30 mg d3, 10 mg d4	GK 10 mg d0 → d3	GL 20 mg d0 → d2 (locally)	AK 40 mg d0 → d1 10 mg d2 → d23 20 mg d24 → d39 40 mg d40 → d43	GK 10 mg d0 → d14 40 mg d15 → d25 80 mg d26 → d29 40 mg d30 → d44	GK †	GL 20 mg d0 → d2 10 mg d3 → d10 20 mg d11 → d16	AK 20 mg 30 → d10	AK 20 mg d0 → d11 10 mg d12 → d16	AK 20 mg d0 → d12
Treatment duration (d)	5	4	3 local treatment	44	45	68	17	11	17	13

Abbreviation: EMI, extramedullary involvement, mainly epidural involvement—primary and secondary PCL (all had EMI other than blood invasion).
* Successful obtaining of an IL-6–dependent human myeloma cell line.
† See Klein et al[16] for more details.

received B-E8 and 1 received mab-8 MoAb, which were administered according to a schedule outlined in Table 1. Doses of anti–IL-6 MoAb were selected to provide serum concentrations similar to those known to inhibit myeloma cell proliferaion in vitro and were adjusted according to patients' clinical status.

Anti–IL-6 MoAb. Anti–IL-6 MoAb (B-E8 and mab-8, both IgGκ) were prepared by J. Wijdenes (Innotherapie, Besançon, France) and L. Aarden (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands), respectively, by immunizing mice with rIL-6[17,18] These MoAb were purified and submitted to stringent analyses required before use, as previously described.[16] The purified MoAb were sterile and free of pyrogenic activity.

Collection of peripheral blood cells and serum samples. Peripheral blood or bone marrow samples for all studies, including pharmacokinetic studies, were collected at 7 AM, 1 hour before mab-8 or B-E8 injections. Peripheral blood serum and plasma were stored at −20°C until use. Peripheral blood, pleural effusion, or bone marrow mononuclear cells were obtained by centrifugation of heparinized samples over Ficoll-Hypaque gradients.

Proliferation and culture assays of myeloma cells. The percentages of myeloma cells were determined by cytoplasmic immunofluorescence using anti-κ or anti-λ light chain antibodies directly coupled to fluorescein (Kallestadt, Austin, TX). The percentages of plasma cells in S phase (ie, the labeling index) were determined using an antibromodeoxyuridine MoAb (Immunotech, Marseilles, France) and a rhodamine-labeled goat antimouse Ig (Jackson Laboratories, West Grove, PA) in a double fluorescence technique described elsewhere.[3] Cells were cultured at 10^6 cells/mL in RPMI 1640 medium supplemented with 5×10^{-5} mol/L 2ME and 5% fetal calf serum for 5 days, hence referred to as culture medium. In some groups, 10 μg/mL of anti–IL-6 MoAb (mab-8 or B-E8) or of control purified murine serum IgG (Sigma Chemicals, St Louis, MO) was added at initiation of the cultures. To reverse the effect of anti–IL-6 MoAb, the MoAb was incubated with human rIL-6 (100 ng/mL) for 2 hours before being added to cultures. On day 5, the percentages of myeloma cells and myeloma cells in S phase were determined as described above.

Evaluation of circulating anti–IL-6 MoAb levels. Levels of circulating anti–IL-6 MoAb were determined by enzyme-linked immunosorbent assay (ELISA). Polystyrene plates (Immuno I; Nunc, Kamstrup, Denmark) were coated overnight with purified goat IgG against mouse Ig (10 μg/mL in phosphate-buffered saline [PBS]; Tago Inc, Burlingame, CA) at room temperature. Plates were saturated with 1% bovine milk proteins (BMP) in PBS for 1 hour. After five washes in PBS containing 0.05% Tween 20 (Sigma Chemicals), 100 μL of the patient plasma diluted in PBS-BMP-Tween (1% BMP in PBS containing 0.05% Tween 20) was added for 1 hour at room temperature. Plates were washed five times with PBS-BMP-Tween before a solution containing peroxidase-conjugated goat antimouse IgG (Jackson Laboratories) was added for 1 hour and then washed again five more times. Reactivity was determined by the intensity of the enzymatic reaction to substrate O-phenylene diamine (for 20 minutes) measured by absorbance at 492 nm (Titertek Multiskan Mc; Flow, Irvine, UK) after the addition of 50 μL 2 N H_2SO_4. Mouse Ig concentrations were calculated using a reference curve obtained with serial dilution of mab-8 or B-E8 MoAb.

Detection of human antibodies to anti–IL-6 MoAb. The presence of human antibodies to anti–IL-6 MoAb was also checked by ELISA. Plates (Immuno I; Nunc) were coated with either 10 μg/mL (in PBS overnight at room temperature) of mab-8, B-E8, or a control MoAb of the same isotype (CD37, BL14) as IgG1. To ensure that the plates were coated with the same amount of each MoAb a peroxidase-conjugated goat antimouse IgG (Jackson Laboratories) was used. Plates were saturated with PBS-BMP, and patient's serum samples (diluted 1/50 in PBS-BMP) were added for 1 hour at room

Table 2. Results of Anti–IL-6 Therapy in 7 Patients Receiving More Than 1 Week of Treatment

Pretreatment Features	Responsive to Anti–IL-6 MoAb			Unresponsive to Anti–IL-6 MoAb			
Patients treated more than 1 week	4	5	6	7	8	9	10
Ig type, light chain subtype	AK	GK	GK	Gλ	AK	AK	AK
Types of tumors	MM	MM	Primary PCL	Sec. PCL	Sec. PCL	Sec. PCL	MM
Presenting fever (correction with MoAb)	–	–	+ (yes)	–	–	+ (yes)	+ (yes)
Severe subsequent infection	–	+	–	–	+ (death)	–	–
Hypercalcemia (correction with MoAb)	–	+ (no)	+ (partial control)	+ (no)	+ (no)	+ (no)	+ (no)
Platelet counts ($10^3/\mu L$) (% maximum suppression)	148 (45%)	272 (86%)	184 (56%)	88 (52%)	57 (37%)	17 (40%)	122 (35%)
Neutrophil counts/μL (% maximum suppression)	1,845 (80%)	2,800 (78%)	3,000 (65%)	4,300 (95%)*	3,784 (stable)	2,500 (70%)	2,700 (stable)
CRP serum levels† (mg/L) (% maximum suppression)	5 (100%)	21 (100%) Undetectable (<1 mg)	90 (100%)	46 (96%)	90 (75%)	60 (88%)	123 (60%)
Daily in vivo IL-6 production (μg)	Not done	1.7	4	>46	>165	>47	>341
Overall changes of myeloma Ig	↗	↗	↘ –30%	↗	↗	↗	↗
Documented antiproliferative effects on myeloma cells	Yes	Yes	Yes	No	No	No	No
Survival (d)	93	45	128	17	11	17	13

*Due to toxic chemotherapy.

† Pretreatment values—CRP has been monitored as shown in the Fig 2. Platelet and neutrophil counts were monitored every day until death (patients no. 7, 8, 9, and 10), then every other day, then twice a week.

temperature. Human Ig were detected by incubating plates with peroxidase-conjugated goat antihuman IgG or IgM (Jackson Laboratories). Reactivity was determined as above.

IL-6 bioassay and source of IL-6. Biologic activity of IL-6 was evaluated using the B9 hybridoma bioassay as previously described.[3] B9 hybridoma was a generous gift of L. Aarden (Amsterdam, The Netherlands).

Evaluation of IL-6 production in vivo. The overall daily production of IL-6 was evaluated according to a methodology previously described by ourselves.[19,20] It is based on the observation that the anti–IL-6 MoAb induces IL-6 to circulate in the form of stable monomeric immune complexes and protect it from rapid clearance in vivo. In patients with partial inhibition of CRP production, the consumption of IL-6 by cells was not completely inhibited and the true in vivo production was compulsorily greater than our evaluation. The ability of neutralizing IL-6 by the anti–IL-6 MoAb was estimated fro the abaques we previously published.[20]

RESULTS

Anti–IL-6 MoAb Serum Concentrations and Immunization

In all patients receiving systemic therapy, serum anti–IL-6 MoAb (approximately 1 μg/mL) was detectable 24 hours after the first IV injection and increased progressively to an average value of 5 μg/mL on days 2 to 4 and to 5 to 17 μg/mL on days 7 and 8. No B-E8 MoAb was detected in the serum of the patient receiving intrapleural injections during the first 2 days. Concentrations of 6 to 17 μg/mL were sustained in 5 patients (no. 4, 5, 7, 8, and 10) and declined to

undetectable levels in 1 patient (no. 9). Human antibodies to murine B-E8 MoAb were observed in 2 patients (no. 8 and 9) on days 9 to 11 resulting in rapid clearance of circulating anti–IL-6 MoAb despite continued drug administration in one patient (Fig 1). No anti–B-E8 or anti–mab-8 activity was found in the other patients' serum. For patient no. 6, the data have been detailed elsewhere.[16]

Effects on General Clinical and Biologic Symptoms

The results are outlined in Table 2. There were no life-threatening side effects.

Clinical effects. Four patients (no. 1, 6, 9, and 10) with low-grade fever ranging from 37°C to 38°C, without evidence of overt infection showed a normalization in body temperature within 2 days of treatment with anti–IL-6 MoAb. This was frequently associated with disappearance of fatigue and a slight but significant improvement of the performance status and pain.

Biologic effects. Except patient no. 2 (shortest treatment) and 3 (local treatment), all the patients developed further worsening of thrombocytopenia (35% to 86%; median, 40%). One patient with marked thrombocytopenia (no. 9, 17,000/μL) before therapy required platelet transfusion. Six of these 8 patients also showed neutrophil reduction (65% to 95%), including 1 due to toxic antibiotherapy (patient no. 7). Three patients (no. 2, 5, and 8) developed bacterial infections, which were lethal in patients no. 2 and 8. The
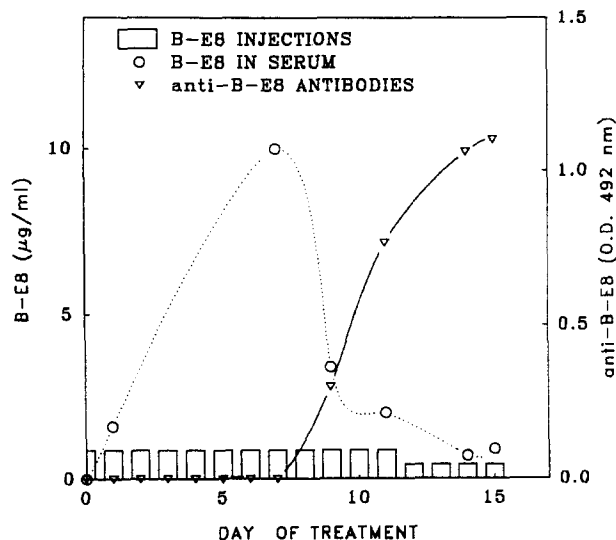
Fig 1. Clearance of B-E8 MoAb after anti–B-E8 immunization in patient no. 9.

Table 3. Inhibition of Myeloma Cell Growth (Labeling Index) by the Anti–IL-6 MoAb In Vitro

Patient No.	Sample Origin	Controls	With Anti–IL-6 MoAb	With Anti–IL-6 MoAb and IL-6
1	Blood	30	0.7	17
2	Bone marrow	21	0	21
3	Bone marrow	18	0.6	8
	Peripheral blood	16	1	12
	Pleural effusion	16	0.5	8
6	Bone marrow	8	0.3	10
7	Bone marrow	1	0.5	1.5
8	Peripheral blood	1.5	0	1.5

Values are the percentage of myeloma cells in S phase. See Patients, Materials, and Methods.

neutropenia was not different in these patients from that of the others.

Because C-reactive protein (CRP) production by freshly explanted human hepatocytes is dependent on IL-6 in vitro,[21] CRP serum levels were determined before and during intravenous anti–IL-6 MoAb. Before treatment, CRP serum levels were elevated in all the patients, ranging from 5 to 160 mg/L (median, 60 mg/L; normal values, <1 mg/L by nephelometry). A significant reduction in CRP of at least 50% was observed in all the patients receiving anti–IL-6 therapy. Sustained suppression to undetectable levels (<1 mg) was observed in 3 patients (no. 4, 5, and 6) receiving more than 1 week of therapy (Fig 2). In the other 4 cases (no. 7, 8, 9, and 10), CRP serum levels were reduced by 60% to 96% but remained above normal levels (Fig 2 and Table 2). It is noteworthy that the maximum CRP serum level reduction correlates with the maximum and absolute platelet counts reduction (Table 2).
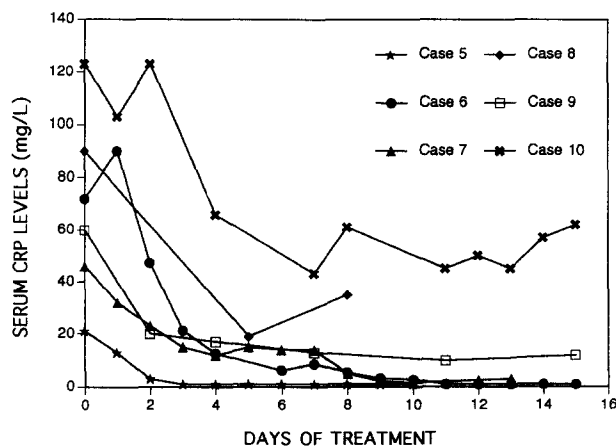
The daily production of IL-6 has been estimated as previously described by ourselves.[20] Data are not evaluable in patients no. 1 and 2 (too short a treatment) and not available in patient no. 4. For the others, the daily IL-6 production ranged from 1.7 to more than 341 μg/d. Of note is that CRP was totally inhibited in patients with the lowest IL-6 production (no. 5 and 6, Table 2).

Anti-proliferative Effects of Anti–IL-6 MoAb

In vitro studies. Mononuclear cells from bone marrow (no. 2, 3, 5, and 10), blood (no. 1, 3, 8, and 9), and pleural effusion (no. 3) samples were cultured as previously described in Patients, Materials, and Methods with anti–IL-6 MoAb or anti–IL-6 MoAb and IL-6; mab-8 was used in patient no. 1 and B-E8 in the other cases. In all patients exhibiting S phase values greater than 1% in vivo, complete inhibition o myeloma cell proliferation was observed (Table 3) as previously published by ourselves in many patients with MM.[3,4,6] Similar results were previously published for patient no. 6.[16] In agreement with these results, IL-6–dependent cell lines were established in 4 patients (no. 1, 3, 7, and 8).

In vivo studies. Concomitantly with in vitro investigations, in vivo cytokinetic studies were performed in patient no. 3 while receiving anti–IL-6 MoAb intrapleurally for a malignant pleural effusion and in patients no. 1, 4, 5 and 6.

Patient no. 3 with primary PCL exhibited primary treatment drug resistance with subsequent development of a malignant pleural effusion. Upon local administration of 20 mg of B-E8 anti–IL-6 MoAb on 3 successive days, a 80% decrease of the concentration of tumor cells and of their proliferative compartment (from 16% to 2.5% myeloma cells in S phase) was observed (Fig 3A). No diffusion of the anti–IL-6 MoAb was found outside the pleural fluid. In this case, the local concentration of MoAb was 20 μg/mL on day 1 of treatment, increasing up to 60 μg/mL on day 3. This finding was in agreement with a diffusion volume of 1 L. In these conditions, this in vivo model was very close to an in vitro situation. The dramatic decrease of myeloma cell proliferation and of myeloma cell counts during treatment was
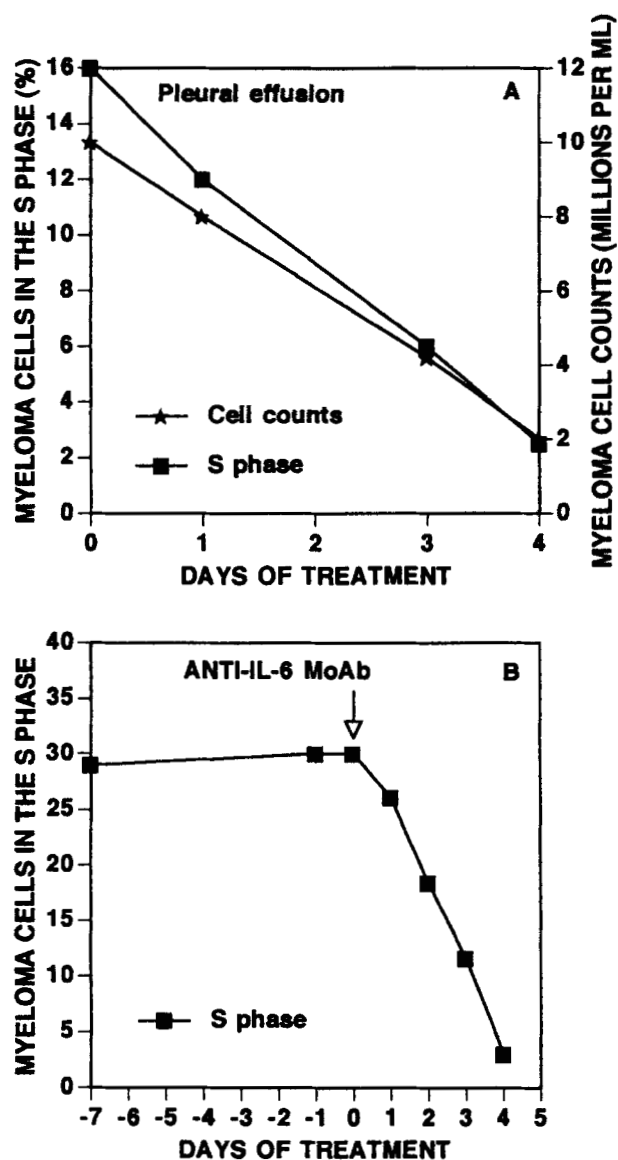

Fig 2. Significant reduction of serum CRP levels in 6 patients with a more than 1 week of treatment with the B-E8 anti–IL-6 MoAb.

Fig 3. (A) Significant reduction in both myeloma cell counts and myeloma cells in S phase in patient no. 3 during B-E8 anti–IL-6 therapy. (B) Significant reduction in the percentages of myeloma cells in S phase in patient no. 1 during mab-8 anti–IL-6 therapy.

cell proliferation decreased markedly in vivo, reaching a low S-phase fraction of 3% after 4 days (Fig 3B). The absolute increase in circulating myeloma cells to 55,000/μL throughout the 4 days of treatment with anti–IL-6 may have resulted from sustained tumor cell proliferation, albeit at a markedly reduced level of 3% versus 30% at the onset of therapy.

Patient no. 2 died quickly and was not evaluable for antiproliferative effects of anti–IL-6 MoAb. In patients no. 4, 5, and 6, a transient but significant reduction of myeloma cell labeling index was obtained within the bone marrow. In patient no. 4, a reduction from 19% to 3% of the myeloma bone marrow labeling index was observed, with a subsequent increase to 14%. In patients no. 5 and 6, a reduction from 1.5% and 4.5%, respectively, to 0% was noted. Disease progressed in the other 4 patients and no antiproliferative effect was documented.

Antitumoral Effects of Anti–IL-6 MoAb

None of the patients treated had improved outcome or achieved remission as defined by standard clinical response criteria for MM. The observed effects after anti–IL-6 administration in this poor-risk patient population must therefore be categorized as biologic effects. Indeed, although tumor cell proliferation was inhibited in peripheral blood in 1 patient (no. 1) and in pleural effusion in another (no. 3), both died within 1 week due to their terminal disease stage. A third patient (no. 2) had central nervous system involvement and died on day 5. The remaining patients (no. 4 through 10) were treated for 13 to 68 days (median, 17 days). As emphasized in the previous section, a clear-cut objective antiproliferative effect was observed on malignant cells in vivo in 3 patients (no. 4, 5, and 6). Among them, 1 patient (no. 6) achieved 30% tumor cytoreduction for 2 months, but relapse occurred after stopping anti–IL-6 MoAb. Although patients no. 4 and 5 had significant (but transient) reduction in their marrow plasma cell labeling index, disease progressed. Furthermore, fast disease progression was observed in 4 other patients (no. 7, 8, 9, and 10). CRP synthesis was transiently inhibited in patient no. 9, in association with reduction of overall white blood cells and plasmablasts (86% inhibition) between days 3 and 6. However, a progression occurred on day 7 in conjunction with the emergence of human antibodies to Fc fragments of murine B-E8 MoAb that was no longer detectable. Of major interest (and as outlined in Table 2) a clear correlation was found between in vivo daily IL-6 production, inhibition of CRP synthesis, and antiproliferative effects. Such effects were only observed in patients no. 4, 5, and 6, presenting the lowest IL-6 production. In these 3 patients, the B-E8 MoAb was able to inhibit CRP. In the 4 other patients presenting the highest production of IL-6, the B-E8 MoAb was unable to completely inhibit CRP synthesis and thus IL-6. These results are summarized in Table 2.

DISCUSSION

In vitro studies performed in our patients receiving anti–IL-6 MoAb in vivo simply and clearly show that, among the various putative myeloma cell growth factors, IL-6 plays

therefore in agreement with the inhibition of myeloma cell proliferation by the anti–IL-6 MoAb that was observed in short-term cultures in vitro in this patient (Table 3).

Patient no. 1 developed secondary PCL, with 11,200 myeloma cells/mL in peripheral blood and an S phase fraction of 30%. In our experience and that of others, such proliferative activity in peripheral blood was quite unusual. Considering this high proliferative activity and that B-E8 MoAb was administered intravenously, thus in the close contact of tumor cells, the situation was very similar to that of patient no. 3 and that of in vitro studies. Upon administration of gradually increasing doses of mab-8, in vivo drug levels increased progressively to 7.7 μg/mL on day 4 and myeloma

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