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Mouse Anti-human Interleukin-6 Receptor Monoclonal Antibody Inhibits Proliferation of Fresh Human Myeloma Cells *in vitro*

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Interleukin-6 (IL-6) is a major growth factor in multiple myeloma. We investigated the effect of mouse anti-human IL-6 receptor monoclonal antibody (anti-IL-6R mAb) on the *in vitro* proliferation of freshly isolated myeloma cells from 21 patients to evaluate the therapeutic potential. The addition of anti-IL-6R mAb inhibited more than 30% of the spontaneous proliferation of myeloma cells in 9 of 21 cases in a dose- (0.1 to 20 μg/ml) and time-dependent manner. The inhibitory effects of anti-IL-6R mAb did not differ significantly from that of anti-IL-6 mAb, and were correlated with the extent of the response of myeloma cells to IL-6. Flow cytometric analysis showed that all myeloma cells expressed IL-6R, whose intensity was not correlated with either the extent of response of myeloma cells to IL-6 or the inhibitory effects of anti-IL-6R mAb on proliferation of myeloma cells. Although our study showed heterogeneity in the proliferative responses of myeloma cells to IL-6 and anti-IL-6R mAb, these observations suggest the possibility of using anti-IL-6R mAbs for treating some patients with multiple myeloma whose growth depends on IL-6.

Key words: Multiple myeloma — IL-6 — Anti-IL-6 receptor antibody — Anti-IL-6 antibody

IL-6,[5] which is identical with BSF-2 and with mouse hybridoma-plasmacytoma growth factor,[1,2] promotes the growth of human myeloma cells via autocrine and paracrine mechanisms.[3–6] Its serum level in multiple myeloma reflects disease activity.[7] These observations prompted us to evaluate the therapeutic effects of anti-IL-6 mAb, previously shown to inhibit the growth of myeloma cells *in vivo*.[8]

IL-6 mediates its functions through two membrane proteins, a ligand-binding molecule (IL-6R) with a molecular weight of 80 kDa,[9] and a non-ligand-binding signal transducer (gp130).[10] Human myeloma cells and cell lines express IL-6R[3,11] and IL-6R mRNA.[12] PM1, a mouse mAb against 80 kDa IL-6R, inhibits the binding of IL-6 to the receptor and blocks the IL-6-dependent growth of myeloma cell lines *in vitro*[13,14] and *in vivo*.[15] Recently, reshaped human PM1 antibodies were designed as therapeutic agents for administration to human patients in repeated doses.[16] To evaluate the therapeutic potential of anti-IL-6R antagonist, we investigated the effect of a mouse anti-human IL-6R mAb, PM1, on the growth of freshly isolated and prepared myeloma cells in comparison with that of anti-IL-6 mAb *in vitro*.

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[5] Abbreviations: IL-6, interleukin-6; IL-6R, the specific receptor for IL-6; mAb, monoclonal antibody; SI, stimulation index; TdR, thymidine.

MATERIALS AND METHODS

Patients and preparation of cells Twenty-one patients with multiple myeloma, including 3 with plasma cell leukemia, were included in this study. Multiple myeloma was classified according to the clinical staging system proposed by Durie and Salmon[17] (Table I). Samples were obtained for study after informed consent had been obtained. Bone marrow samples were obtained from patients with advanced myeloma at the time of pretreatment or relapse, and contained >50% of myeloma cells. Specimens consisted of portions of surgically resected plasmacytomas, ascites fluid and pericardial effusion which showed a massive infiltration of myeloma cells. These specimens were subjected to Ficoll-Hypaque density centrifugation, and the adherent cells were depleted by adherence on plastic petri dishes. Nonadherent cells were subsequently subjected to T cell depletion by rosetting with sheep red blood cells treated with 2-S-aminoethylisothiouronium bromide (Sigma, St. Louis, MO) as previously described.[18] Bone marrow samples were purified on discontinuous Percoll gradients and by depletion of monocytes and myeloid cells using antimyelomonocytic mAb Leu M1 (CD15) (Becton Dickinson, Mountain View, CA) plus rabbit complement (Hoechst Behering, Germany) as previously described.[3,19] This method afforded a purified cell fraction that consisted of >95% myeloma cells.[19]

Table I. Patient Characteristics and Expression of IL-6 Receptor

No.	Diagnosis	Isotype	Stage at sampling	Disease status	Sample	IL-6R expression by flow cytometry (%)
1	MM	IgG/λ	IIA	relapse	ascites	6.1
2	MM	IgA/κ	IIIA	relapse	plasmacytoma	ND
3	MM	BJ/κ	IIIB	relapse	plasmacytoma	4.4
4	PCL	IgE/κ	IIIA	at diagnosis	BM	48.2
5	PCL	IgG/κ	IIIA	at diagnosis	BM	95.8
6	MM	BJ/λ	IIIB	at diagnosis	BM	33.7
7	MM	IgA/λ	IIIA	terminal	PE	ND
8	MM	IgG/κ	IIIA	at diagnosis	BM	3.8
9	MM	IgG/κ	IIIB	at diagnosis	BM	20.7
10	MM	IgA/κ	IIIB	at diagnosis	BM	50.0
11	MM	IgA/λ	IIIA	at diagnosis	BM	19.5
12	MM	BJ/κ	IIIB	terminal	PE	7.7
13	MM	BJ/κ	IIIB	at diagnosis	BM	11.1
14	MM	IgG/λ	IIA	relapse	plasmacytoma	6.0
15	MM	IgG/κ	IIIB	at diagnosis	BM	63.2
16	MM	IgG/κ	IIIA	at diagnosis	BM	51.0
17	PCL	IgA/λ	IIIA	at diagnosis	BM	15.7
18	MM	IgG/κ	IIIA	relapse	BM	44.7
19	MM	IgA/λ	IIIA	at diagnosis	BM	89.9
20	MM	IgG/κ	IIA	at diagnosis	BM	46.2
21	MM	IgA/λ	IIIA	at diagnosis	BM	9.9

PCL, plasma cell leukemia; BM, bone marrow; PE, pericardial effusion; ND, not done.

Cell culture Freshly isolated myeloma cells (>95%) were cultured in triplicate at 10^5/well in RPMI1640 medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT) in 96-well, U-bottomed culture plates. Cells were incubated at 37°C under a humidified atmosphere of 5% CO_2 in air for 72 h with normal mouse IgG, a mouse anti-human IL-6R mAb (10 μg/ml), or with a mouse anti-human IL-6 mAb (10 μg/ml) in the presence or absence of recombinant human IL-6 (100 U/ml). Recombinant human IL-6 (specific activity 6 U/ng) was generously provided by Ajinomoto Company (Tokyo). Prior to harvesting, cells were pulsed with 1 μCi of ^{3}H-TdR (Amersham, UK) for 12 h. The radioactivity incorporated was counted in a liquid scintillation counter.

Mouse mAbs A mouse anti-human IL-6R mAb (IgG_1 class: PM1) and a mouse anti-IL6 mAb (IgG_1 class: MH166) were used in this study. PM1 and MH166 have been described elsewhere.[13-15] These mAbs were prepared by Chugai Pharmaceutical Company, Shizuoka.

IL-6R expression by flow cytometry A mouse anti-human IL-6R mAb, MT18, which does not compete with IL-6 for binding to IL-6R,[13] was used for detecting IL-6R by an indirect immunofluorescence method as previously described.[20] In brief, cells were washed three times and suspended in phosphate-buffered saline. After blocking with normal human IgG (Green Cross Company, Tokyo) for 30 min at 4°C, cells were incubated with MT18 for 3 h at 4°C. Nonspecific staining was excluded using normal mouse IgG (Becton Dickinson). After being washed twice, cells were stained with fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse IgG (Coulter, Hialeah, FL) for 30 min at 4°C, washed twice and resuspended in phosphate-buffered saline. Cells were analyzed by flow cytometry (Epics Profile, Coulter).

Statistical methods Data are reported as the mean ± SD of triplicate cultures. The *t* test was used to evaluate the statistical significance of differences among anti-IL-6R mAbs and anti-IL-6 mAb and for correlation analysis. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Inhibitory effect of anti-IL-6R mAbs on proliferation of myeloma cells The inhibitory effects of anti-IL-6R mAb on proliferation of freshly isolated myeloma cells are shown in Table II and Table III. Without exogenous IL-6, the mean percentage of inhibition of anti-IL-6R mAb and anti-IL-6 mAb was 25.5±22.2 and 22.4±22.5% (mean±SD), respectively. In the presence of exogenous IL-6 (100 U/ml), the values were 19.6±19.0 and 20.0±18.0%, respectively. There was no significant difference in the inhibitory effects between the anti-IL-6R

Table II. Effects of mAbs on Spontaneous Proliferation of Myeloma Cells

Case No.	^{3}H-TdR Incorporation				
	Control Ig	Anti-IL-6R		Anti-IL-6	
	cpm	cpm	% inhibition	cpm	% inhibition
1	2114±298	556±165**	73.7	445±270**	78.9
2	19487±1021	15800±481**	18.7	15608±2730	19.7
3	7721±2330	6876±2923	10.9	7025±452	9.0
4	430±35	477±5	0	418±183	2.8
5	8814±694	5671±295**	35.7	6504±368**	26.2
6	2154±185	1711±201*	20.6	2229±359	0
7[a]	1720±595	1183±424	31.2	ND	
8	4031±1509	3428±1193	15.0	2923±1002	27.5
9	1341±248	989±323	26.2	1437±239	0
10	3688±217	1039±173**	71.8	1465±141**	60.2
11	12446±716	11994±895	3.6	10652±10652	14.4
12	13245±708	13927±762	0	16259±2146	0
13	7535±976	8080±764	0	6946±523	7.8
14	2824±1049	1894±562	32.9	1246±1257	55.9
15	5535±1462	3542±463	36.0	3490±737	36.9
16	2426±462	2182±47	10.1	2023±330	16.6
17	5612±697	5679±286	0	4975±661	11.4
18	6230±1238	4864±568	21.9	ND	
19	4344±383	2868±523*	36.0	2923±163**	32.3
20	4661±1643	1874±1431	59.8	5645±1952	0
21	2352±969	1596±164	32.1	1725±595	26.7
Mean±SD			25.5±22.2[b]		22.4±22.5[b]

Results are expressed as the mean cpm and % inhibition of triplicate cultures. ND: not done.
a) 40,000/well. *b*) No significant difference of % inhibition among groups.
* $P<0.05$, ** $P<0.01$, statistically significant difference of % inhibition.

mAb and the anti-IL-6 mAb. Anti-IL-6R mAb inhibited more than 30% of the proliferation in 9 of the 21 cases, and the mean inhibitory effect in these cases was 45.5%; statistical significance was reached in 6 of 21 cases (Table II) and in 5 of 21 cases (Table III). The proliferation of myeloma cells was markedly inhibited by 73.7% in case 1 and by 71.8% in case 10. Discrepancies between the effect of the anti-IL-6 mAb and that of the anti-IL-6R mAb were observed in case 20 (Table II) and in cases 16 and 21 (Table III). However, the difference in these cases was not statistically significant. In the presence of exogenous IL-6, SI by IL-6 ranged from 0.95 to 2.24 (mean: 1.30).

The effect of anti-IL-6R mAb on proliferation was dose- (0.1 to 20 μg/ml) (Fig. 1) and time-dependent (Fig. 2) when myeloma cells were used in case 1, in whom a significant IL-6-dependent growth was observed.

In evaluating the relationship between the inhibitory effects of anti-IL-6R mAb and the extent of response to IL-6, we observed a positive correlation between them (Fig. 3A).

IL-6R expression by flow cytometry All the myeloma cells examined from 19 patients expressed IL-6R as evaluated by flow cytometric analysis (Table I). The expression of IL-6R ranged from 3.8 to 95.8% (mean: 33.0%). Representative flow cytometric patterns appear in Fig. 4. The intensity of IL-6R expression was not correlated with either the extent of the response to IL-6 or the inhibitory effect of anti-IL-6R mAb on the proliferation of myeloma cells (Fig. 3B, C).

DISCUSSION

Several studies have demonstrated that IL-6 stimulates the growth of human myeloma cells.[3-6] It was initially reported to be an autocrine growth factor for myeloma cells based on the observations that freshly isolated myeloma cells constitutively express IL-6 mRNA, produce IL-6, bear IL-6R and proliferate *in vitro* in the presence of exogenous IL-6.[3] Other investigators have found that IL-6 is a paracrine growth factor.[4] Regardless of the mechanism, the serum level of IL-6 reflects disease activity in multiple myeloma.[7] Therapeutic trials using mouse anti-IL-6 mAb,[8] mouse anti-IL-6R mAbs,[13-15] IL-6 antisense oligonucleotides[21,22] and IL-6-*Pseudomonas* exotoxin[23] have reportedly shown an inhibition of growth of myeloma cells.

IL-6 mediates its functions through two membrane proteins, a ligand-binding molecule (IL-6R) with a molecular mass of 80 kDa,[9] and a non-ligand-binding signal

Table III. Effects of mAbs on IL-6-dependent Proliferation of Myelona Cells

Case No.	^{3}H-TdR Incorporation					
	Control Ig + IL-6		anti-IL-6R + IL-6		anti-IL-6 + IL-6	
	cpm	SI	cpm	% inhibition	cpm	% inhibition
1	4731±553	2.24	1753±247**	62.9	1305±633**	72.4
2	22409±3615	1.15	20833±226	7.0	21405±3735	4.5
3	8448±853	1.09	7923±2444	6.2	8154±458	3.5
4	408±59	0.95	510±19	0	392±46	3.9
5	11553±1431	1.31	ND		ND	
6	2452±359	1.14	2113±166	13.9	2363±139	0
7[a]	2171±129	1.26	ND		ND	
8	5823±2208	1.44	5131±1774	11.9	5517±1653	5.3
9	2526±598	1.88	1544±273	38.9	1829±648	27.5
10	4889±546	1.32	2516±898**	48.5	3622±496*	25.9
11	17978±5041	1.44	13488±2236	24.9	13169±2174	26.7
12	19583±1137	1.48	14350±1308**	26.7	16070±560**	17.9
13	7829±330	1.04	8328±1202	0	7025±1167	10.3
14	3895±777	1.38	2004±667*	48.5	2598±1116	33.3
15	6171±789	1.11	4365±344*	29.3	3871±408*	37.3
16	2815±390	1.16	2667±516	5.3	2083±349	26.0
17	6708±142	1.19	7662±1264	0	6477±1844	3.3
18	6283±1737	1.01	7690±898	0	ND	
19	4452±1155	1.02	3481±730	21.8	3602±229	19.6
20	6722±1198	1.44	5239±1513	22.1	6183±1599	8.0
21	2858±1093	1.21	2742±1076	4.1	1895±350	33.7
Mean±SD		1.30±0.30		19.6±19.0[b]		20.0±18.0[b]

Results are expressed as the mean cpm and % inhibition of triplicate cultures. SI, Stimulation index. ND, not done.
a) 40,000/well. *b*) no significant difference of % inhibition among groups.
$*P<0.05$, $**P<0.01$, statistically significant difference of % inhibition.

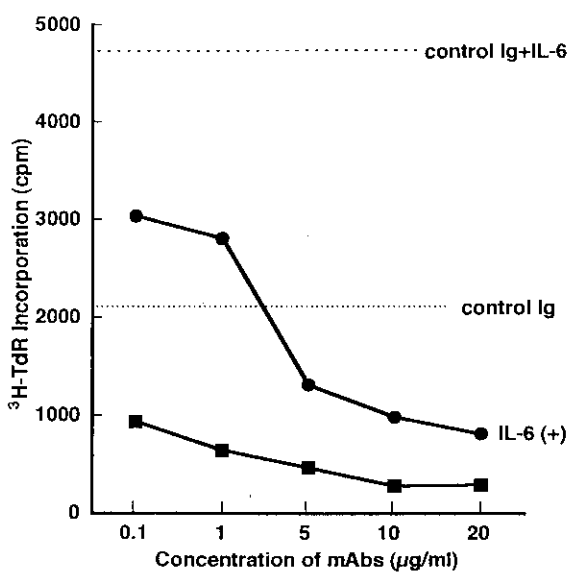

Fig. 1. Dose-dependent effect of anti-IL-6R mAbs on spontaneous and IL-6-dependent proliferation of myeloma cells. Myeloma cells from case 1 were cultured for 72 h in the presence of anti-IL-6R mAb (0.1 to 20 μg/ml) with (●) or without (■) recombinant human IL-6 (100 U/ml). Results are expressed as the mean cpm of triplicate cultures.

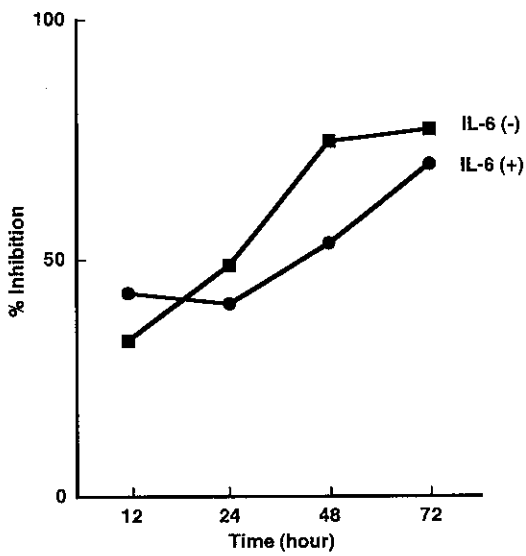

Fig. 2. Time-dependent effect of anti-IL-6R mAbs on spontaneous and IL-6-dependent proliferation of myeloma cells. Myeloma cells from case 1 were cultured in the presence of anti-IL-6R mAbs (10 μg/ml) with (●) or without (■) recombinant human IL-6 (100 U/ml) for the indicated times. Results expressed as the mean percentage inhibition of triplicate cultures.

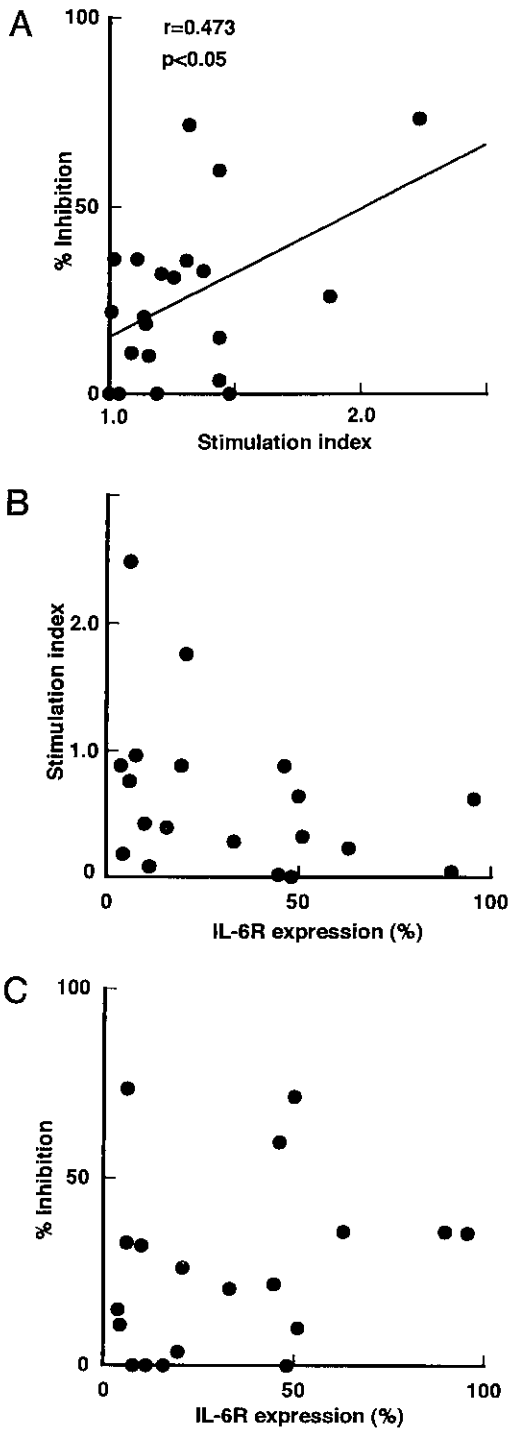

Fig. 3. Relationship between the responsiveness to IL-6 and the effect of anti-IL-6R mAb (A), between the IL-6R expression and the responsiveness to IL-6 (B), and between the IL-6R expression and the effect of IL-6R mAb (C). A positive correlation was observed between inhibitory effect of anti-IL-6R mAbs on spontaneous proliferation and the extent of response of myeloma cells to IL-6.

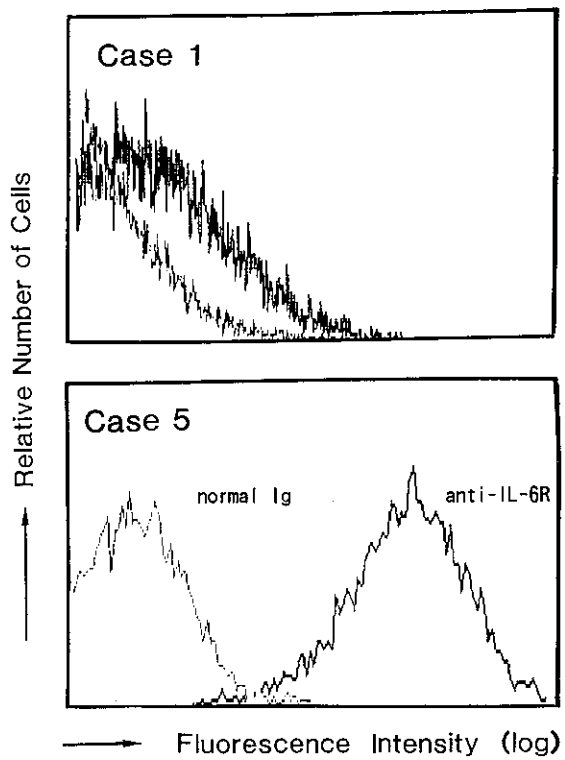

Fig. 4. IL-6R expression determined by flow cytometry. IL-6R expression was analyzed by flow cytometry after staining with MT18 mAb and FITC-anti mouse IgG as described in "Materials and Methods." Case 1 had weakly expressed IL-6R when compared with case 5 (6.1% vs. 95.8%). However, the proliferation of the myeloma cells in case 1 was strongly inhibited by anti-IL-6R mAb.

transducer (gp130).[10] Myeloma cell lines bear IL-6R amounting to over 10,000 binding sites/cell[11] and express IL-6R mRNA.[12] Recent studies demonstrated that an anti-IL-6R mAb, PM1, inhibits the proliferation of a human myeloma cell line both *in vitro*[13, 14] and *in vivo*.[15] However, the effects of anti-IL-6R mAb on freshly isolated myeloma cells have not previously been described. In the present study, we demonstrated inhibitory effects of a mouse anti-IL-6R mAb, PM1, on the proliferation of freshly isolated myeloma cells *in vitro*. Anti-IL-6R mAb inhibited the spontaneous proliferation of myeloma cells in 9 of 21 cases and the mean percentage inhibitory effect in these cases was 45.5%. Our study also suggested that the inhibition was time-dependent. So, the inhibition might have been much more significant if we had incubated the cells with the mAb for a longer period.

This inhibitory effect was correlated with the extent of the response of the myeloma cells to IL-6. In case 1, the growth of myeloma cells was significantly inhibited by

anti-IL-6R mAb. The myeloma cells obtained from this patient's ascites fluid responded to exogenous IL-6. Furthermore, we observed a high level of IL-6 (91.0 pg/ml) in the ascites fluid. These findings suggested that IL-6 promoted the growth of myeloma cells via an autocrine and/or paracrine mechanism, and that the administration of anti-IL-6R mAb may be useful in treating this case.

Nilsson *et al.* reported that freshly isolated myeloma cells respond to IL-6 in approximately 40 to 60% of the patients.[24] Zhang *et al.* reported that IL-6 was a potent growth factor in myeloma with a high labeling index, and that the strongest response was observed in patients with plasma cell leukemia.[4] In contrast, Asaoku *et al.* reported that myeloma cells in the early stage disease responded to IL-6 better than those in the advanced stage.[5] Of the 21 patients in our study, 18 had advanced stage III myeloma, including 3 cases with plasma cell leukemia. Only case 1 with stage II myeloma showed SI over 2.0. Our results are consistent with those of Asaoku *et al.* Unfortunately, we did not examine the labeling index of the myeloma cells. Furthermore, while Zhang *et al.* incubated myeloma cells for 7 days with a high titer of recombinant IL-6 (1000 U/ml), we incubated them for 72 h with IL-6 at the concentration of 1000 U/ml. Further examination of the relationship between the responsiveness to IL-6 and disease severity in myeloma is warranted.

Using flow cytometry, we demonstrated that all the myeloma cells expressed IL-6R. However, the intensity of such expression correlated neither with the extent of the response to IL-6 nor with the inhibitory effect of anti-IL-6R mAb. Kawano *el al.* also reported that the number of IL-6R on myeloma cells is not correlated with the responsiveness to exogenous IL-6.[3] One possible explanation of this discrepancy is that IL-6 downregulates the expression of both the IL-6R[25] and IL-6R gene.[26] Using radiolabeled IL-6, no specific binding was detected on an IL-6-dependent myeloma cell line. However, when IL-6 was removed, high-affinity IL-6R appeared on the cell surface.[26] We did not examine whether the intensity of IL-6R expression of myeloma cells was changed by addition of IL-6 and the mAbs during the *in vitro* culture. However, this regulation mechanism of IL-6R may explain the discrepancy observed in case 1, in whom a high level of IL-6 (91.0 pg/ml) in the ascites fluid was observed. In this case, the intensity of IL-6R expression of myeloma cells was very low but the growth was strongly inhibited by the mAb, suggesting that IL-6 had downregulated the IL-6R expression *in vivo*. Another possible explanation is the effect of soluble IL-6R on myeloma cells. Recently, soluble IL-6R which binds to IL-6 and potentiates the IL-6-dependent responses has been reported.[10] Myeloma cell lines have been reported to release the soluble IL-6R, which serves for potentiating IL-6 functions.[27] Furthermore, serum soluble IL-6R levels have been shown to be increased in patients with plasma cell disorders, suggesting that circulating soluble IL-6R may potentiate the IL-6 response of myeloma cells *in vivo*.[28] Unfortunately, we did not examine the level of soluble IL-6R in the culture supernatant or in the patient's serum. Further studies on the role of soluble IL-6R in multiple myeloma seem warranted. A third possible explanation is that signal transduction by myeloma cells may be somewhat different from that of normal plasma cells. Recently, mutations in the intracytoplasmic domain of gp130 were found in a human myeloma cell line and in tumor cells of patients with multiple myeloma, whereas no mutation was found in the cytokine receptor homologous domain of IL-6R (α-chain).[29] Therefore, the responsiveness of myeloma cells to IL-6 rather than the expression of IL-6R on myeloma cells should be examined before administering anti-IL-6R mAb treatment to patients with multiple myeloma.

The heterogeneity of multiple myeloma has been documented cytogenetically,[30] phenotypically,[21, 31, 32] and biologically.[2, 3, 33] We also demonstrated the heterogeneity of the inhibitory effect of anti-IL-6R mAbs, responsiveness to IL-6 and IL-6R expression. Clinical trials with anti-IL-6 mAb in a patient with multiple myeloma have shown a complete inhibition of the proliferation of myeloma cells *in vivo*.[9] Our study has shown that the inhibitory effect of anti-IL-6R mAb resembled that of anti-IL-6 mAb and suggests that the administration of this substance may be a new treatment strategy in some patients with multiple myeloma whose tumor growth depends on IL-6. Recently, reshaped human PM1 antibodies have been designed as therapeutic agents for administration to human patients in repeated doses,[16] and the *in vivo* effects of anti-IL-6R mAb are currently being investigated using human myeloma cells transplanted into IL-6 transgenic severe combined immunodeficiency mice.

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