Brief Definitive Report

Mouse Plasmacytoma Growth In Vivo: Enhancement by Interleukin 6 (IL-6) and Inhibition by Antibodies Directed against IL-6 or Its Receptor

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Summary

Murine plasmacytomas show a striking dependence on interleukin 6 (IL-6) for their growth in vitro. Here, we present evidence suggesting that IL-6 also plays an essential role in the in vivo development of these tumors. This conclusion is based on the finding that the tumorigenicity of an IL-6-dependent plasmacytoma cell line was increased ~100-fold on transfection with an IL-6 expression vector, whereas it was inhibited in animals treated with monoclonal antibodies capable of blocking the binding of IL-6 to its receptor. Injection of these antibodies 1 d before tumor challenge protected >50% of the mice and retarded tumor growth in all animals. Tumors arising in antibody-treated mice retained their IL-6 dependence in vitro, suggesting that the level of protection could be improved if stronger IL-6 antagonists were available.

It is now well established that IL-6 is a critical factor for the in vitro growth and survival of mouse plasmacytomas (1, 2), and there is increasing support for the idea that it plays an important role in the in vitro proliferation of human myelomas as well (3–6). The notion that IL-6 may also be of importance for the in vivo growth of these tumors is supported by the following observations: (*a*) pristane-induced granulomas, which produce large amounts of IL-6 (1), are critical not only for induction (7) but also for early transplantation of mouse plasmacytomas (8); (*b*) overexpression of IL-6 in transgenic mice, although not sufficient to cause the development of plasma cell tumors, induces strong plasmacytosis (9); and (*c*) transfection with IL-6 cDNA was recently shown to increase the tumorigenicity of an IL-6-dependent B cell hybridoma (10). While none of these findings formally proves that IL-6 is required for the in vivo growth of plasmacytomas, they clearly call for a direct evaluation of this possibility.

In the present report, we addressed this question by examining whether the tumorigenicity of a mouse plasmacytoma could be increased by transfection with an IL-6 cDNA and, more importantly, whether it could be inhibited by administration of antibodies directed against IL-6 or against its receptor.

Materials and Methods

Plasmid Construction. A mouse IL-6 cDNA fragment of 827 bp was isolated from clone pHP1B5 (11) by digestion with EcoRI and DraI. This fragment, which contains the entire coding sequence of IL-6 but lacks ~250 bp at the 3′ end of the gene, was subcloned into the SalI site of plasmid pBMGneo (12). Northern blot analysis was performed as described (11) using ^{32}P-labeled mIL-6 cDNA.

Transfection of Plasmacytoma Cells with IL-6 cDNA. IL-6-dependent T1033C2 plasmacytoma cells were transfected by electroporation, and transfectants containing the IL-6 expression vector were selected in G418. This population, termed TB6D2, secreted IL-6 (~500 pg/10^6 cells/24 h, as measured in the 7TD1 assay), and could be cloned in the absence of exogenous IL-6. A clone designated TB6D2.M was selected for further study. A control group of cells was transfected with the same plasmid containing the IL-6 cDNA cloned in missense orientation. As expected, this population, TB6D1, remained IL-6 dependent. TB6D1 cells were cloned in the presence of IL-6, and a clone termed TB6D1.M was selected for further study. Northern blot analysis revealed the presence of IL-6 message in TB6D2 but not in TB6D1 cells.

Antibodies. mAb 6B4 is a neutralizing anti-IL-6 IgG1 antibody of rat origin (13). Antibody 15A7 is a rat IgG2b that is directed against a cell surface component of IL-6 receptor–positive cells and blocks IL-6 binding. The derivation and characteristics of this antibody will be described elsewhere (P. Coulie et al., manuscript in preparation). Control rat antibodies included Lo-DNP1 (IgG1 anti-DNP), Lo-DNP57 (IgG2b anti-DNP) (both gifts of Dr. H. Bazin, Unit of Experimental Immunology, University of Louvain), and 2B5 (IgG1 anti–mouse IgG1). Hybridomas were grown in pristane-primed BALB/c *nu/nu* mice, and the ascitic fluid was collected under sterile conditions. To avoid contamination with LPS, the ascites were mostly used without further purification. For some experiments, however, antibody 15A7 was purified by affinity chromatography on mouse anti–rat κ-Sepharose (antibody MARK1 given

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Table 1. *Increased Tumorigenicity of IL-6-Producing T1033C2 Plasmacytoma Cells*

Dose of tumor cells	Tumor incidence	
	IL-6-dependent cells (TB6D1.M)	IL-6-producing cells (TB6D2.M)
	d	*d*
10^6	7/9 (39)	9/9 (16)
3×10^5	3/9 (41)	9/9 (20)
10^5	2/9 (41)	9/9 (20)
3×10^4	0/9	9/9 (26)
10^4	0/9	8/9 (30)

BALB/c mice were injected intraperitoneally with the indicated cell doses and tumor development was followed over a 110-d period. Median survival time is indicated in parentheses and is given for tumor-bearing animals.

by Dr. H. Bazin). $F(ab')_2$ fragments were prepared from the purified antibody by overnight digestion with pepsin at 37°C in 0.1 M acetate buffer, pH 4.2. Under these conditions, digestion was complete as verified by gel filtration on a Superose-12 FPLC column.

Results and Discussion

Increased Tumorigenicity of IL-6-producing Plasmacytoma Cells. The influence of IL-6 on plasmacytoma cell growth in vivo was examined in normal BALB/c mice using T1033C2 cells transfected with an IL-6 cDNA expression construct. All mice injected intraperitoneally with 3×10^4 IL-6-producing cells (TB6D2.M) developed a tumor and died in 40 d. By contrast, in mice injected with the same number of IL-6-dependent cells, transfected with a missense construct (TB6D1.M), not a single tumor arose, even after 110 d, when the experiment was terminated. A high tumor incidence could nevertheless be obtained with TB6D1.M cells, but only after injection of 100-fold higher cell doses (Table 1). In addition, even when TB6D1.M tumors developed, their growth rate was considerably slower than that of the autonomous cells.

These differences could not be ascribed to intrinsic changes in the growth rate of the cells because doubling times measured in vitro in the presence of saturating IL-6 were of the same order for both types of transfectants (10.2 h for TB6D1.M and12 h for TB6D2.M). They were also not due to the selection of unrepresentative clones because similar results were obtained with the uncloned transfectant populations. Moreover, the increased mortality of mice injected with IL-6-sufficient plasmacytoma cells did not result from a toxic effect of IL-6 on the host because P815 mastocytoma cells transfected with the IL-6-encoding plasmid and secreting amounts of IL-6 higher than those produced by TB6D2.M (i.e., 3,000 pg/ml/24 h/10^6 cells) did not show increased tumorigenicity. It therefore appears that the increased tumorigenicity of T1033C2 cells producing IL-6 is a direct consequence of the plasmacytoma growth factor activity of the cytokine. These observations, which extend the findings reported by Tohyama et al. (10) for an IL-6-dependent B cell hybridoma, prove that the plasmacytoma growth-promoting activity displayed by IL-6 in vitro is not a culture artifact.

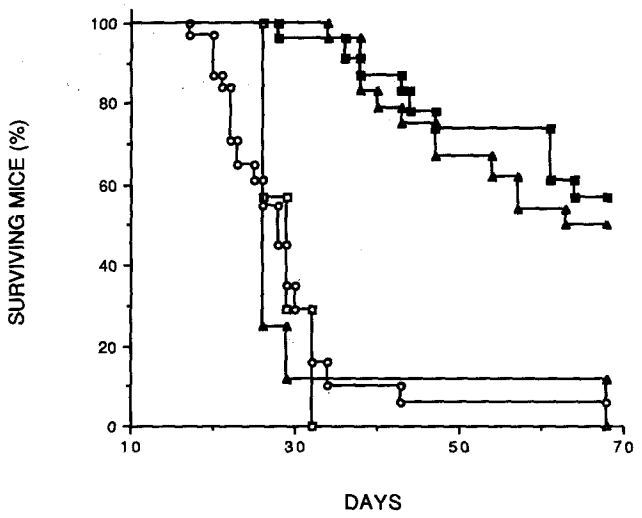

Figure 1. Inhibition of T1033C2 plasmacytoma growth in mice treated with anti-IL-6 or anti-IL-6R antibodies before tumor challenge. BALB/c mice were given a 1-ml subcutaneous injection of monoclonal rat antibodies in the back 24 h before intraperitoneal challenge with 10^6 T1033C2 cells. Antibodies, administered in the form of ascitic fluid obtained in nude mice, included control anti-DNP antibodies Lo-DNP1 (IgG1; eight mice) (△) and Lo-DNP57 (IgG2b; seven mice) (□), anti-IL-6 antibody 6B4 (IgG1; 24 mice) (▲), and anti-IL-6R antibody 15A7 (IgG2b; 23 mice) (■). Additional controls were injected with saline (31 mice) (○).

Inhibition of Plasmacytoma Growth In Vivo by Antibodies Blocking the Action of IL-6. The increased tumorigenicity of IL-6-producing T1033C2 cells does not prove that T1033C2 cells actually require IL-6 for growth in vivo. To test this hypothesis, we examined whether antibodies capable of neutralizing the plasmacytoma growth factor activity of IL-6 in vitro would inhibit tumor formation by IL-6-dependent T1033C2 cells. We used two rat mAbs: one, termed 6B4, that is directed against IL-6 (13), and one, termed 15A7, that is directed against the IL-6 receptor, in as much as it binds to IL-6R-positive cells and competes with IL-6 for binding. The antibodies were administered subcutaneously 24 h before intraperitoneal challenge with a tumorigenic dose of IL-6-dependent plasmacytoma T1033C2. Data pooled from a series of experiments are shown in Fig. 1. In mice injected with medium or with isotype-matched control ascites, tumor incidence was >95% with a median survival time of 25–29 d. By contrast, in the group of mice treated with antibody 6B4, 50% of the animals were protected, and the remainder had a median survival time of 38 d. When the animals were treated with antibody 15A7, the protection rate was ~60%, and the median survival time of tumor-bearing animals was prolonged to 40 d. This level of protection was obtained with 1–1.5 ml of ascites (equivalent to 0.5–3 mg of antibodies).

The results described above indicate that the IL-6 dependence of plasmacytomas T1033C2 is not exclusively an in vitro

Table 2. *IL-6 Dependence of T1033C2 Tumors Arising in Mice Treated with Anti-IL-6 Antibody*

Culture conditions	Tumor D	Tumor E
	10^6/culture	
No antibody	1.050	ND
Control antibody 2B5	ND	1.030
Anti-IL-6	0.087	0.010
Anti-IL-6R	ND	0.007
IL-6	7.600	29.000

Cells were recovered from two tumors that arose in BALB/c mice injected with 10^6 T1033C2 cells after treatment with anti-IL-6 antibody 6B4. Cells were cultured without added IL-6 at an initial concentration of 3×10^5 cells/ml in the presence of the indicated antibodies (10 μg/ml), and cell growth was evaluated after 4 and 5 d for tumors D and E, respectively. Additional cultures were supplemented with 5 ng/ml of rIL-6.

phenomenon. They do not, however, rule out the possibility that IL-6 acts only during a short period of time required by the cell to adapt from in vitro to in vivo conditions. To address this question, antibodies were administered 2 wk after tumor challenge, at a time when visible tumors had already developed. Under these conditions, antibody 15A7 was found to induce partial necrosis of some tumors. However, complete remissions were never observed, although the treatment resulted in a significant increase in the survival time of the mice (37 ± 1.61 d vs. 26.6 ± 2.2 d in the control group; $p = 0.022$ by Wilcoxon's test). Anti-IL-6 had no significant effect under these experimental conditions, indicating that this antibody is less potent than 15A7 as an inhibitor of T1033C2 growth in vivo. This difference could not be ascribed to opsonisation of the tumor cells by the anti-IL-6R antibody because $F(ab')_2$ fragments retained the antitumor activity of the intact antibody (data not shown). Moreover, the intact antibody failed to inhibit the growth of FLOPC21, a GM-CSF-dependent plasmacytoma, which binds 15A7 to the same extent as T1033C2.

The failure of our antibodies to block T1033C2 growth completely could be due to either to their relatively poor efficiency as IL-6 antagonists or to the emergence of IL-6-independent variants. To discriminate between these possibilities, we tested the IL-6 dependence in vitro of cells recovered from ascitic tumors arising in mice treated with anti-IL-6 antibodies. These tumors contained a substantial number of macrophage-like adherent cells secreting enough IL-6 to support some growth of the plasmacytoma cells in vitro. As shown in Table 2 for cells derived from two such tumors, this basal level of growth was enhanced ~10-fold on addition of exogenous IL-6 and inhibited 10–100-fold on addition of anti-IL-6 or anti-IL-6R antibodies. This result indicates that the development of T1033C2 tumors in mice treated with anti-IL-6 antibodies is not due primarily to the emergence of IL-6-independent variants, which in turn suggests that the antitumor protection could be improved if stronger IL-6 antagonists were available.

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