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IL-6 in malignant pleural effusions and its augmentation by intrapleural instillation of IL-2

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SUMMARY

The levels and activities of endogenous IL-6 in malignant pleural effusions due to lung cancer before and during daily intrapleural instillations of recombinant IL-2 were examined by enzyme immunoassay and bioassay using an IL-6-dependent murine hybridoma cell line MH60.BSF2. Before therapy, malignant pleural effusions contained various levels of IL-6. Daily intrapleural instillation of recombinant IL-2 for treatment of malignant pleurisy resulted in significant augmentation of the levels and activities of IL-6 in the pleural effusions. On fractionation of the pleural effusion by chromatography, one major peak of material with a mol. wt of 24 kD showed IL-6 activity. In contrast, no significant level of tumour necrosis factor-alpha or IL-1β was detectable in pleural effusions before or during local IL-2 therapy. These data suggest that IL-2 is an important regulatory factor of secondary IL-6 production.

Keywords IL-6 IL-2 malignant pleurisy lung cancer

INTRODUCTION

IL-6 was originally identified as a factor in the culture supernatant of mitogen- or antigen-stimulated peripheral blood mononuclear cells that induced immunoglobulin production in Epstein–Barr virus (EBV) transformed B cell line or in *Staphylococcus aureus* Cowan 1-stimulated normal B cells [1]. IL-6 is known to be produced by various host cells such as monocyte/macrophages [2], T cells [3] and fibroblasts [4]. At least six forms of differentially modified IL-6 phosphoglycoproteins in the size range of 23–30 kD are secreted by induced fibroblasts and monocytes [5]. IL-6 is now considered to be a cytokine that plays a central role in the host defence mechanism by regulating immune responses, haematopoiesis and acute-phase reactions [6]. Moreover, abnormal expression of IL-6 has been reported to be related to the pathogenesis of several diseases including mesangial proliferative glomerulonephritis [7], rheumatoid arthritis [8] and several haematopoietic neoplasias, such as myeloma/plasmacytoma [8] and Castleman's disease [9].

Malignant pleurisy, seen frequently in patients with lung cancer, is of particular immunological interest, because pleural exudate cells and humoral factors produced by these effector cells are expected to play pleiotropic roles against cancer cells in the pleural cavity. For example, cancer cells in pleural effusions may produce IL-6 *in situ*, because several non-hematopoietic tumour cells and cell lines including bladder carcinoma [10], uterine carcinoma [11], renal cell carcinoma [12], glioblastoma [13], and ovarian carcinoma [14] are reported to produce IL-6. However, multiple forms of IL-6 in the size range of 23–30 kD are detected in human body fluids in local infected compartment [15]. There is, however, little information about the IL-6 level and its molecular form in malignant pleural effusions.

IL-2 is capable of inducing non-specific killer cells [16], and is widely used in immunotherapy of malignant diseases [17]. It is important to determine whether IL-2 can stimulate the host to induce secondary cytokine productions *in vivo*, which may contribute to the defence system of the host. *In vivo* IL-2 infusion was recently found to induce the secondary productions of macrophage colony-stimulating factor (M-CSF) [18], eosinophil CSF [19], interferon-gamma (INF-γ) and tumour necrosis factor-alpha (TNF-α) [20]. Nothing is yet known about the alterations of endogenous IL-6 in malignant pleural effusions during IL-2 therapy.

In the present study we demonstrated various levels of IL-6 in malignant pleural effusions. We also found that intrapleural instillation of IL-2 for treatment of malignant pleurisy augmented the local levels of IL-6 in the size of 24 kD.

MATERIALS AND METHODS

Subjects

We studied 34 patients with primary lung cancer with malignant pleural effusions (19 with adenocarcinoma, five with squamous cell carcinoma, five with small cell carcinoma and five with large cell carcinoma) before conventional anti-cancer therapy. They

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were all in-patients in Tokushima University Hospital and ranged in age from 42 to 78 years. After obtaining written informed consent, seven patients (five with adenocarcinoma and two with squamous cell carcinoma) received local daily IL-2 therapy to control malignant pleural effusions, and the six patients with a clinical response were examined in the present study. The study was approved by the Ethical Committee of Tokushima University Hospital, Tokushima.

Reagents

Fetal bovine serum (FBS) was purchased from M.A. Bioproducts (Walkerville, MD). Human recombinant IL-2 (rIL-2) was kindly provided by Takeda Pharmaceutical Co. (Osaka, Japan), and had a specific activity of $3 \cdot 5 \times 10^4$ U/mg as assayed on IL-2-dependent murine NKC3 cells [21]. Human rIL-6 (lot 735-3; specific activity 6×10^6 U/mg of protein) was a gift from Ajinomoto Co. (Kawasaki, Japan). Human natural TNF-α (specific activity $4 \cdot 0 \times 10^5$ JRU/mg of protein) was a gift from Hayashibara Institute (Okayama, Japan). Human rIL-1β [22] was kindly supplied by Dr Y. Hirai (Otsuka Pharmaceutical Co., Tokushima, Japan). None of these materials contained endotoxins (sensitivity limit 0·1 ng/ml), as judged by *Limulus* amebocyte assay (Seikagaku Kogyo Co. Tokyo, Japan).

Harvesting of supernatants of pleural effusions

Samples of pleural effusions collected by drainage were centrifuged at 400 ***g*** for 15 min at 4°C. Cell-free supernatants were filtered through membranes of 0·22 μm pore size and stored at −70°C until use.

Molecular sieve chromatography

The molecular weight of IL-6 in the pleural fluid was determined by high-performance liquid chromatography (HPLC). Briefly, the supernatant of pleural fluid was concentrated approximately 10-fold at 4°C on an immersible CX-10 ultrafilter (Millipore) with a mol. wt exclusion limit of 10 000 and dialysed against 50 mM phosphate buffer containing 120 mM NaCl (pH 7·0). The concentrated sample was applied to a TSK-GEL G3000 SW column (67·5 × 2·15 cm; Tosoh Co., Tokyo, Japan) in phosphate buffer (pH 7·0) and material was eluted with 50 mM phosphate buffer (pH 7·0) containing 30 mM NaCl at a flow rate of 1·4 ml/min. Fifty fractions were collected.

Enzyme immunoassays (EIAs) of human IL-6, IL-1β and TNF-α

The EIAs of human IL-6, IL-1β and TNF-α (sensitivity limits 0·1 ng/ml, 20 pg/ml and 20 pg/ml, respectively) were performed essentially as described previously [22,23]. Briefly, microtitration plates (Nunc, Naperville, IL) were coated with anti-IL-6, anti-IL-1β or anti-TNF-α MoAb (10 μg/ml) in 100 μl/well of PBS, pH 7·4. After overnight incubation at 4°C, the wells were blocked with 0·1% bovine serum albumin (BSA) in PBS for at least 1 h at room temperature and washed three times with PBS containing 0·05% Tween 20 (Tween-PBS). All subsequent washings were performed with this buffer. Volumes of 200 μl of test samples in PBS containing 0·1% BSA were added to duplicate wells. The plates were incubated at 37°C for 24 h and then washed three times, and 100 μl of mouse anti-IL-6 or rabbit anti-IL-1β or anti-TNF-α antibody (diluted 1:1000 with PBS containing 0·2% BSA) were added to each well. The plates were then incubated for 2 h at 37°C, washed three times, supplemented with 100 μl of peroxidase-labelled rabbit anti-mouse IgG+IgA+IgM (H+L) (for IL-6) or goat anti-rabbit IgG (H+L) (for IL-1β and TNF-α) (diluted 1:10000 with PBS containing 0·1% BSA; Zymed Laboratories, San Francisco, CA), and incubated at room temperature for 2 h. Finally the plates were washed five times, 100 μl of enzyme substrate (1 mg/ml OPD in 0·1 M sodium citrate buffer, pH 5·0) were added to each well and the plates were incubated at room temperature for 5 min. The reaction was stopped by adding 100 μl of H_2SO_4 to each well, and the absorbance at 492 nm was determined, using a Titertek Multiskan.

Bioassay of IL-6

Il-6 activity was determined by a colourimetric assay [24] using an IL-6-dependent murine hybridoma cell line, MH60.BSF2 [25]. Briefly, hybridoma cells were incubated at a concentration of 1×10^4 cells/well in 96-well Microtest III plates (Falcon, Oxnard, CA) in RPMI 1640 supplemented with 1% FBS with various dilutions of test samples or standard rIL-6 in duplicate. After 48 h of culture, the cells were treated with 3-(4,5-dimethylthiazol-2-yl)2,5,-diphenyl-tetrazolium bromide (Sigma Chemical Co., St Louis, MO) and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium formazan was measured at 550 nm with an automatic microplate spectrophotometer. Standard concentrations of rIL-6 were run in each experiment to obtain a dose–response curve.

Intrapleural administration of IL-2

Recently we showed that mononuclear cells in pleural effusions generated lymphokine-activated killer (LAK) activity when cultured *in vitro* with rIL-2 at concentrations of 0·1–1·0 U/ml, and that daily intrapleural administration of IL-2 (1000 U/day) was effective for inducing LAK activity in lymphocytes in pleural effusions in association with rapid disappearance of cancer cells from the effusions [26]. Therefore, we injected rIL-2 at a daily dose of 1000 U/person for 14 days into the pleural cavity through a Silicone-coated, 500 mm long, 7 French catheter (Hanako Medical Co., Tokyo, Japan).

RESULTS

Detection of IL-6 in malignant pleural effusions

The levels in malignant pleural effusions due to lung cancer were measured by EIA. As shown in Fig. 1, nine out of 19 specimens from patients with adenocarcinoma, one out of five from those with squamous cell carcinoma, two out of five from those with large cell carcinoma but none of five from those with small cell carcinoma showed detectable levels of IL-6. The mean (±s.d.) value of IL-6 in these 12 patients was 2·10±0·60 ng/ml (range 0·37–8·38). The bioactivity of IL-6 in malignant pleural effusions of the 12 patients who were not receiving conventional anti-cancer therapy was measured using IL-6-dependent murine hybridoma cell line MH60.BSF2 cells. As shown in Table 1, significant biological activity of IL-6 was detected in all these pleural effusions.

Phenotypic characterization of cells in malignant pleural effusions of patients before and during IL-2 therapy

Cells in the malignant pleural effusions of 18 patients before therapy consisted of 52·1±5·9% (mean±s.e.m.) lymphocytes, 32·9±6·0% macrophages and 12·7±4·6% others (including cancer cells). Phenotypic analysis showed that four patients

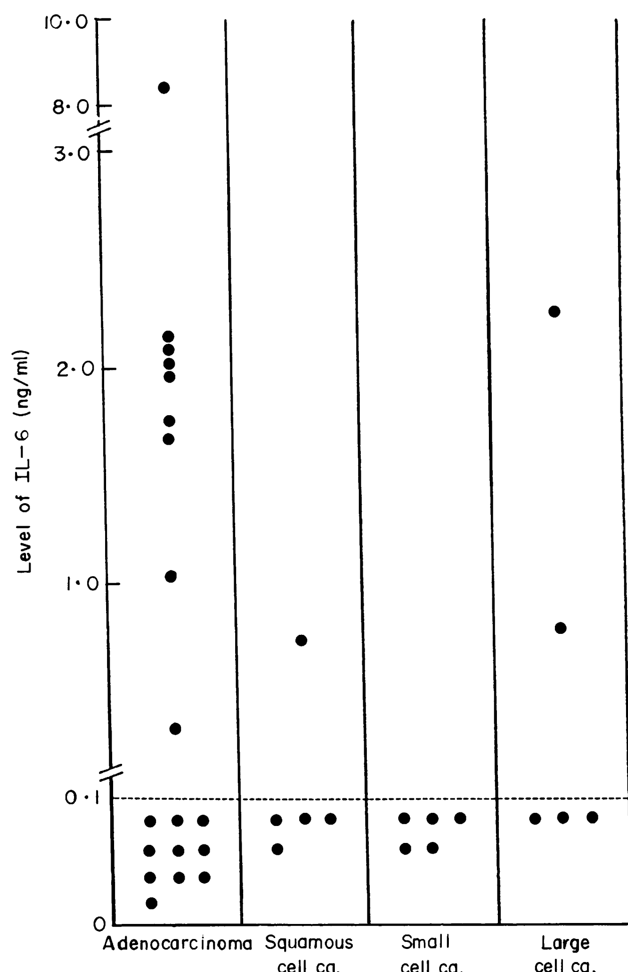

Fig. 1. Levels of IL-6 in malignant pleural effusions due to lung cancer; ca, carcinoma.

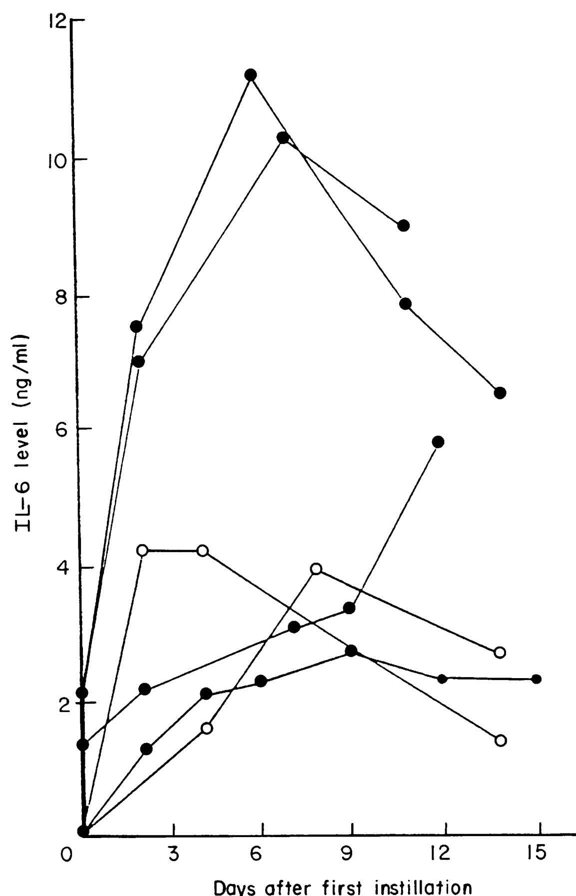

Fig. 2. Levels of IL-6 in malignant pleural effusions of patients treated by intrapleural instillation of rIL-2 (1000 U/ml) for 14 days. Four patients with adenocarcinoma of the lung (closed circles) and two patients with squamous cell carcinoma of the lung (open circles) were treated by intrapleural instillation of rIL-2 (1000 U/ml), and pleural effusion fluid before and at various times during local IL-2 therapy was harvested for EIA of IL-6.

Table 1. Level and bioactivity of IL-6 in malignant pleural effusions of patients without conventional anti-cancer therapy

Case no.	Cell type	IL-6 level (ng/ml)	IL-6 bioactivity (U/ml)
1	Adenocarcinoma	2·06	2040
2	Adenocarcinoma	2·10	3915
3	Adenocarcinoma	1·67	1550
4	Adenocarcinoma	8·38	12000
5	Adenocarcinoma	1·75	3625
6	Adenocarcinoma	1·94	4059
7	Adenocarcinoma	2·07	3524
8	Adenocarcinoma	0·37	955
9	Adenocarcinoma	1·05	3237
10	Squamous cell ca.	0·72	1865
11	Large cell ca.	0·80	2030
12	Large cell ca.	2·27	1105

The levels of IL-6 in malignant pleural effusions were measured by enzyme immunoassay. The activities of IL-6 in malignant pleural effusions were measured by bioassay using an IL-6-dependent murine hybridoma cell line MH60.BSF2. Ca, carcinoma.

before therapy had ranges of 66.5–84.5% $CD3^+$ cells (mean 79·1), 55·4–73·3% $CD4^+$ cells (mean 64·0) and 5·1–33·3% $CD8^+$ cells (mean 15·3). During daily local injections of IL-2 for 14 days, the lymphocytes in effusions of three of these patients consisted of 82·8–83·7% $CD3^+$ cells (mean 83·4), 73·9–77·0% $CD4^+$ cells (mean 75·4) and 8·6–16·5% $CD8^+$ cells (mean 12·4) on day 7, and of 42·4–86·4% $CD3^+$ cells (mean 69·3), 33·8–66·4% $CD4^+$ cells (mean 52·7) and 14·6–28·7% $CD8^+$ cells (mean 22·7) on day 14.

In vivo *induction of IL-6 production by IL-2*

For determination of whether intrapleural instillation of IL-2 influences the amount of IL-6 in malignant pleural effusions, seven patients (five with adenocarcinoma and two with squamous cell carcinoma) with malignant pleural effusions received daily local instillations of human rIL-2 (1000 U/day) for 14 days. Six of these patients had a clinical response to local IL-2 therapy, as judged by disappearance of cancer cells and rapid decrease in the volume of the effusion after IL-2 instillation. Samples of the pleural effusions were obtained before and at various times during local IL-2 therapy. Results from six patients with a clinical response to the IL-2 therapy are shown in

Fig. 2. The amounts of IL-6 in the pleural effusions increased significantly during local IL-2 therapy. One patient who failed to respond to the local IL-2 therapy showed no increase in the amount of IL-6 in the effusions (data not shown). The bioactivities of IL-6 in the effusions obtained before and during local IL-2 therapy were measured in two representative patients. The results in Table 2 show that local IL-2 administration caused significant increase in IL-6 bioactivity as well as in the amount of IL-6 in the effusions. The levels of other monokines (TNF-α and IL-1β) of the two representative patients with a clinical response are also shown in Table 2. No appreciable amount of TNF or IL-1β was detected before or during local IL-2 therapy. Moreover, no significant amount of TNF-α or IL-1β was detected in other patients treated by local IL-2 therapy (data not shown). In one patient examined (case 3), serum IL-6 was not detectable before or during local IL-2 therapy (data not shown).

Table 2. Levels of IL-6, TNF-α and IL-1β and bioactivities of IL-6 in malignant pleural effusions of patients with adenocarcinoma of the lung before and at various times during daily intrapleural instillation of IL-2 (1000 U/day).

Case no.	Days of therapy	L-6 level (ng/ml)	IL-6 bioactivity (U/ml)	TNF-α level (pg/ml)	IL-1β level (pg/ml)
1	0	2·06	2040	<20	<20
	2	7·48	24 750	<20	<20
	6	11·58	62 500	<20	<20
	11	7·84	27 000	<20	<20
	14	6·57	19 500	<20	<20
2	0	2·10	3915	<20	<20
	2	7·50	31 500	<20	<20
	7	10·91	52 250	<20	<20
	11	9·17	27 250	<20	<20

Patients with adenocarcinoma of the lung were treated by daily intrapleural instillation of IL-2 (1000 U/day) for 14 days. The levels of IL-6, TNF-α and IL-1β in malignant pleural effusions were measured by EIA. The activities of IL-6 in malignant pleural effusions were measured by bioassay using an IL-6-dependent murine hybridoma cell line MH60.BSF2.

Chromatographic pattern of IL-6 activity in malignant pleural effusion

A sample of pleural fluid obtained from case 3 on day 10 of IL-2 treatment was subjected to HPLC on a TSK-GEL G3000 SW column. As shown in Fig. 3, IL-6 measured by EIA and IL-6 bioactivity was observed in fractions corresponding to a mol. wt of 24 kD.

DISCUSSION

We found that malignant pleural effusion due to lung cancer contained IL-6, and that daily intrapleural instillation of rIL-2 resulted in significant increase in biologically active IL-6 in these pleural effusions.

There is accumulating evidence that IL-6 is detectable in the serum in a wide variety of pathological states, such as sepsis [15] and after thermal injury [27], in the cerebrospinal fluid of cases of bacterial meningitis [27] and in the synovial fluid of cases of rheumatoid arthritis [28]. These findings suggest that elevated IL-6 production is a characteristic feature of infectious and/or inflammatory reactions. Nevertheless, we found that IL-6 in pleural effusion was detectable only in one out of five cases with tuberculous pleurisy, who had 2·17 ng/ml (data not shown).

An important issue would be whether endogenous IL-6 production is related to cancer progression. Errori *et al.* [29] and

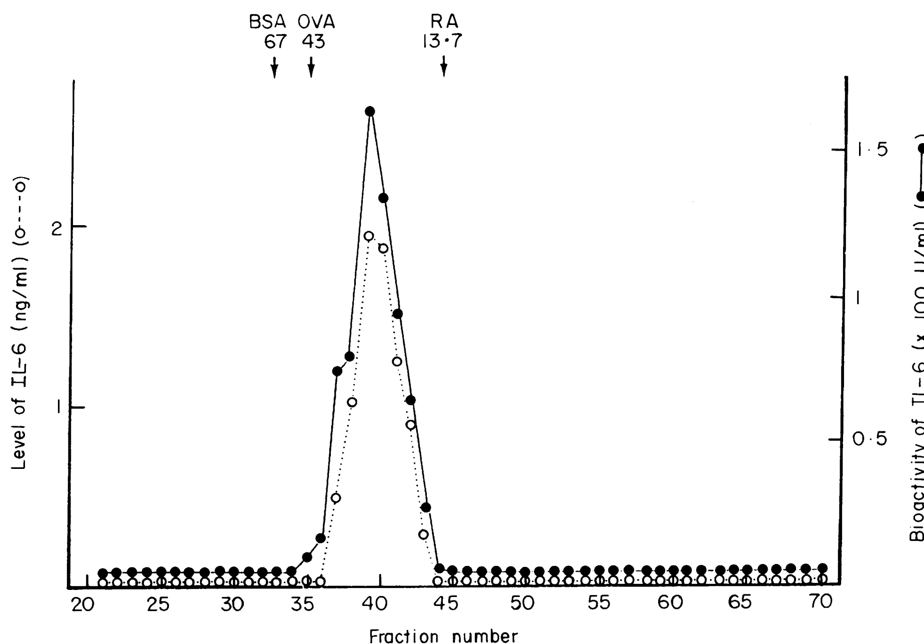

Fig. 3. Gel permeation chromatography. The pleural effusion fluid obtained from patient 3 on day 10 of treatment of IL-2 was subjected to HPLC on a TSK-GEL G3000 SW column. The following molecular weight markers were used: bovine serum albumin (BSA, 66 kD), ovalbumin (OVA, 43 kD) and ribonuclease A (RA, 13·7 kD).

Watson *et al.* [4] reported that peritoneal fluids of patients with ovarian cancer had detectable IL-6 levels. In the current study we also found various levels of IL-6 in pleural effusions of lung cancer patients. Interestingly, IL-6 level was high in pleural effusions of patients with adenocarcinoma and large cell carcinoma, but low in those of patients with small and squamous cell carcinomas (Fig. 1).

IL-6 is known to be produced by monocyte/macrophages, fibroblasts and endothelial cells. Recently, several non-haematopoietic tumour cells and cell lines were also found to produce IL-6 [10–13]. Although lymphocytes in malignant effusions before and 7 days after the daily IL-2 injections consisted largely of $CD4^+$ cells, in a preliminary study we found that pleural effusion mononuclear cells obtained from three cases before therapy did not produce detectable amount of IL-6, irrespective of IL-2 stimulation *in vitro* (data not shown). At present, we could not identify the cells capable of producing IL-6 in patients with lung cancer, but the present study clearly demonstrated that various levels of IL-6 were detectable in malignant pleural effusions due to lung cancer. Probably the other host cells and/or lung cancer cells themselves secrete IL-6, influencing defence of the host against cancer.

We found that during treatment by daily injections of IL-2, the IL-6 levels in pleural effusions were markedly increased by day 2, reached maximum levels on about day 7, and then gradually decreased by day 14. This pattern was also confirmed by bioassays using an IL-6-dependent cell line, MH60.BSF2 (Table 2). IL-6/IFN-β2 is a family of phosphoglycoproteins ranging in size from 19 to 30 kD [5], but in the present study on chromatographical fractionation of the pleural effusion of case 3 obtained on day 9 of daily infusions of IL-2, only one major peak with a mol. wt of 24 kD had IL-6 bioactivity.

The mechanism by which IL-2 causes secondary production of IL-6 *in vivo* is not yet clear. Jablons *et al.* [30] reported that i.v. administration of high doses of rTNF-α to cancer patients led to rapid increases in the circulating levels of TNF-α and IL-6 and that TNF-α was produced earlier than IL-6. Our previous study showed that IL-2 induced *in vivo* production of M-CSF [18], which is responsible for macrophage maturation. These findings suggest that production of IL-6 may be triggered secondary by released TNF-α. However, this was not the case in our study, because two cases examined did not show detectable levels of IL-1β and TNF-α in the effusions before or during local injections of IL-2, except for a small amount of TNF-α on day 7 of IL-2 treatment in one case. The recent finding that tumour cells themselves produce IL-6 also indicates the possibility that a response of the lung cancer cells to IL-2 may have contributed to the observed increase of IL-6. All our patients with a clinical response to IL-2 showed that the lung cancer cells in the effusions disappeared within 5 days [26]. Thus, it is unlikely that *in vivo* induction of IL-6 on IL-2 infusion was produced by the lung cancer cells in the effusions. However, T cells and B cells in the synovial tissues of rheumatoid arthritis patients were found to produce IL-6 constitutively [28]. Our previous investigations showed that approximately 80% of lymphocytes in malignant pleural effusions before therapy consisted of $CD3^+$ cells, and that intrapleural injections of IL-2 resulted in increase in number of T lymphocytes [26], suggesting that IL-2-activated T lymphocytes may contribute to the augmented levels of IL-6 after IL-2 infusions. Because macrophages and fibroblasts also produce IL-6 [5], IL-2 may stimulate various host cells in the pleural effusions to produce IL-6 either directly or indirectly through production of some cytokine(s) other than IL-1β and TNF-α.

In vivo IL-2-activated killer induction of lymphocytes in malignant pleural effusions was previously found to be associated with subsequent disappearance of cancer cells from the effusion within 1 week [26]. IL-6 was recently found to promote differentiation of cytotoxic T cells [31]. Moreover, in murine tumour systems, IL-6 induced *in vivo* anti-tumour activities of the host [32], suggesting a possible contribution of IL-6 to development of an anti-tumour effector mechanism. Thus, the cellular mechanism(s) working *in vivo* during IL-2-mediated cancer regression may involve a complex interaction of many cytokines.

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