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Interleukin-6 involvement in mesothelioma pathobiology: inhibition by interferon α immunotherapy

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Abstract A role for interleukin-6 (IL-6) in malignant mesothelioma has been suggested by the clinically presenting symptoms of mesothelioma patients, which include fever, weight loss and thrombocytosis. A murine model of malignant mesothelioma was therefore used to examine the potential role of IL-6 in this cancer type and whether the effect of interferon α (IFNα) therapy on mesothelioma might be mediated, in part, by regulating IL-6 levels and/or IL-6-induced pathobiology. A panel of human and murine mesothelioma cell lines was assayed for endogenous IL-6 production in a bioassay, and for IL-6-mRNA expression. Four out of 5 human and 5 out of 15 murine mesothelioma cell lines produced moderate to high levels of bioactive IL-6 in vitro. This result was corroborated by mRNA detection. One of the representative murine cell lines, AB22, was chosen for further in vivo studies in the murine mesothelioma model. In AB22-inoculated mice detectable serum IL-6 levels were found to precede macroscopically detectable tumour growth, clinical signs (cachexia, abdominal distension, diarrhoea) and changes in the peripheral lymphoid organs (cell depletion and functional depression). Treatment with anti-IL-6 antibody curtailed the clinical symptoms ($P < 0.001$), as did treatment with recombinant human (rhu) IFNα ($P < 0.001$). Neither anti-IL-6 antibody nor rhuIFNα had a direct growth-inhibitory effect on the AB22 mesothelioma cell line in vitro, however, in vivo rhuIFNα treatment of mice inoculated with AB22 cells attenuated both IL-6 mRNA expression in the tumours and serum IL-6 levels, ameliorated the depression of lymphocyte activities, and enhanced the number of tumour-infiltrating lymphocytes and macrophages. On the basis of these results it is suggested that IL-6 mediates some of these effects, directly or indirectly, and that a combination therapy of rhuIFNα and anti-IL-6 antibody may be an improved palliative treatment for patients with malignant mesothelioma.

Key words Mesothelioma · IL-6 · Cancer cachexia IFNα Immunotherapy

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Introduction

Weight loss and fever are among the more common presenting complaints of patients with malignant mesothelioma [23]. Thrombocytosis is also a common haematological finding in this disease, which is otherwise not characterized by specific biochemical or haematological abnormalities, and hepatomegaly is noted occasionally [5, 23]. These symptoms are reminiscent of some of the reactions seen in inflammation, which are now known to be caused by endogenous cytokines. Of these interleukin-6 (IL-6), a pleiotropic cytokine produced by protean cell types [37], is known to play a role in the acute-phase protein response, fever and cachexia in inflammation [26], as well as being a regulator of thrombocytosis [12, 13]. Recently, a direct role for IL-6, produced by tumour cells and macrophages, in cancer cachexia was unambiguously established in a murine model of colon cancer [33, 34].

Mesothelioma is an aggressive, uniformly fatal serosal tumour, with a median survival time of only 8–9 months from diagnosis [28]. Mesothelioma is non-responsive to conventional therapy such as surgery and

chemo- and radiotherapy, and even palliative treatment appears to have only modest impact in the management of patients [24]. Immunotherapy with recombinant human IFNα2a (rhuIFNα2a), although only modestly successful [36], would appear to be the best treatment option currently available. However, it clearly requires improvement to make a significant positive change in prognosis for a larger proportion of patients. A prerequisite for improvement is a better understanding of the mechanisms whereby IFNα expresses its anticancer activity. It was previously shown in a murine mesothelioma model that treatment with IFNα attenuates growth factor production in the tumour milieu, in particular TGFβ, and curtails the deactivation of tumour-infiltrating lymphocytes (TIL) and macrophages (TIM) [3, 10]. However, these findings do not preclude the possibility that other mechanisms may also contribute to the antitumour effect of IFNα. Recently IL-6 production by several human mesothelioma cell lines was described [30], thus making this cytokine's involvement in the paraneoplastic syndromes in malignant mesothelioma plausible. This hypothesis was supported by findings in a murine model of mesothelioma, where cachexia and hepatocyte degeneration, and concomitant high serum IL-6 levels were found consistently with one of the mesothelioma cell lines used [2].

The aims of the present studies were to investigate the possible role of IL-6 in the pathobiology of malignant mesothelioma and, to characterize further the mechanisms involved in the antitumour effect of IFNα in mesothelioma.

Materials and methods

Cell lines and culture

The human and murine mesothelioma cell lines used in these studies have previously been described [2, 6, 20]. The cells were grown in culture Dulbecco's modified Eagle medium with 5% fetal bovine serum in (FBS) as previously described [2], and used for mouse inoculation at an in vitro passage level no greater than 16 past in vivo mouse passage. For production of mesothelioma-cell-conditioned medium, cells were seeded in 16-mm tissue-culture wells at different starting densities and allowed to grow for 48 h. Following three changes of serum-free medium at 3- to 4-h intervals, the cells were incubated with medium with 0.2% or 0.5% FBS for a further 36 h (murine cells) or 48 h (human cells). The culture supernatants were collected, clarified and stored at − 85° C until assayed for IL-6 bioactivity. The cells in the conditioning plates were trypsinized and counted.

Animals

BALB/c mice (specific-pathogen-free, female, 6–8 weeks old) were obtained from the Animal Resources Centre (Murdoch, Western Australia) and maintained under standard conditions in the University Department of Medicine animal holding area. All animal experimental procedures were approved by the UWA Animal Welfare Committee, and followed the NH&MRC Recommendations for Experiments in Animals.

Tumour challenge protocol and pathology studies

The protocol for tumour inoculation and the assessment of clinical and pathology parameters have been previously described in detail [2, 3, 10]. Briefly, single-cell suspensions of AB22 cells in pyrogen-free saline were inoculated i.p. The mice were observed twice daily by two independent observers and given a clinical score based on the following features: body weight (scores of 0–3), behaviour (scores of 0–5) and physical appearance, including abdominal distension (0–5), absence or presence of diarrhoea (0–3) and hydration state (0–1). A score of 0 indicated no detectable abnormalities and 17 the maximum for a moribund animal [2, 3, 10]. Animals were killed when clinical scores exceeded 10–11. Anaesthetized animals were bled by cardiac puncture immediately before euthanasia, and the serum was stored at − 85° C until tested. A post mortem examination was conducted on all animals and tissues collected for further processing as described [2].

Interferon treatment protocol

Two recombinant human IFNα hybrids with in vivo and in vitro functional activity in rodents were used in the present studies. Recombinant huIFNα B/D hybrid (CGP 35269), huIFNαB/D, was generously provided by Dr. H. K. Hochkeppel, Ciba-Geigy Ltd., Basel. It had a specific activity of 2.5×10^8 units/mg protein, as tested in antiviral assays on human and murine cells. Recombinant huIFNαA/D was supplied by Roche, and had a specific activity of 5×10^7 antiviral units/mg protein. Both IFNα preparations were diluted in pyrogen-free saline, with 1% normal mouse serum as carrier, immediately before the daily i.p. injections, consisting of 1×10^5 units/mouse given in a volume of 100 µl [3]. Placebo treatment consisted in diluent only, and had no effect in itself on tumour growth or clinical signs. The animals were examined twice daily throughout the experiments and given a clinical score according to the above criteria.

Antibodies and other reagents

The following mAb to murine antigens were used for analysis of surface antigen expression, as blocking Ab in cytokine bioassays or for in vivo treatment: RM2-1 (rat anti-CD2), KT3.2 (rat anti-CD3), GK1.5 (rat anti-CD4), 53.7.3 (rat anti-CD5), 53.6.72 (rat anti-CD8), I21.1.1 (rat anti-CD18, the β chain of LFA-1), M1/70 (rat anti-CD11b), B3B4 (rat anti-CD23), 7D4 (rat anti-CD25), M5/114 (rat anti-Ia), F4/80 (rat anti-macrophage), XMG1.2 (rat anti-IFNγ), 6B4 (rat anti-IL6). The origin and production of these have been described previously [2]. Secondary reagents comprised biotinylated rabbit anti-(rat Ig) and streptavidin-horseradish-peroxidase from DAKO a/s (Glostrup, Denmark), and fluorescein-isothiocyanate-(FITC)-conjugated goat anti-(rat Ig) from Tago Inc. (Burlingame, Calif.).

Recombinant cytokines for use as standards in bioassays comprised rmuIFNγ (Genzyme), rhuILβ1, rhuIL-6 and recombinant human tumour-necrosis factor α (rhuTNFα; Boehringer-Mannheim), recombinant human transforming growth factor β1 (rhuTGFβ1; Genzyme). Phytohaemagglutinin was from Wellcome (Dartford, Kent, UK), concanavalin A from Pharmacia (Uppsala, Sweden), and lipopolysaccharide and chemicals were purchased from Sigma (Castle Hill, NSW). Ficoll-Hypaque and Percoll were from Pharmacia (North Ryde, NSW), and micro blood tubes (Microtainer) were from Becton-Dickinson.

Procurement of lymphocytes and peritoneal macrophages and in vitro functional assays

Single-cell suspensions were made from lymph nodes and spleens and their proliferative responses to and cytokine induction by mitogens were assessed as previously described in detail [2]. Macrophages were retrieved from the peritoneal cavities of normal or tumour-bearing mice as previously described [2]. Following an overnight incubation the cytokine production by adherent cells (macrophage purity above 98% as assessed by non-specific esterase staining [1]) in response to lipopolysaccharide ± IFNγ was assessed as described [2]. Macrophage-mediated tumour cell growth inhibition was measured using the protocol of Ferrari et al. [8] with a 48-h co-incubation period.

Cytofluorometric analysis

Spleen and lymph node cells were analysed for cell-surface antigens with the above-listed rat mAb and secondary FITC conjugate on a FACScan flow cytometer (Becton Dickinson) as described [2].

Immunocytochemistry

Assessment of the phenotypic profiles of TIL and TIM was performed as previously described [2]. Briefly, cryosections were immunolabelled in a triple-layer protocol using CD-specific mAb and the biotin/streptavidin conjugates listed above. For each section (at least two sections/tumour) five representative 40 × objective fields were selected and *all* positively labelled cells counted. Areas with extensive necrosis were excluded as were areas compromised by artefactual (processing-related) distortion of tissue structure.

Cytokine bioassays

Stored mononuclear cell MNC- and macrophage culture supernatants and sera were assayed for cytokine bioactivities (IL-1, IL-6, IL-3/GM-CSF, TNFα, IFNγ) as previously described [2]. In particular, IL-6 was assayed for using the factor-dependent B9 cell line [9]. This cell line is reportedly also responsive to IL-11 [4]; however, blocking studies using the mAb 6B4 or goat anti-muIL-6 (British Biotechnology Products Ltd.) showed that the activity detected in our samples was indeed due to IL-6 (data not shown). The sensitivities of the various bioassays, in our hands, were as follows: IL-1, 0.5 pg/ml or 0.025 U/ml; IL-3/granulocyte/macrophage-colony-stimulating factor (GM-CSF), 0.1 U/ml; IL-6, 0.03 U/ml; IFNγ, 0.02 U/ml; TNFα, 50 pg/ml.

Analysis for IL-6 mRNA by polymerase chain reaction (PCR) and Southern blotting

Murine-IL-6-specific primers (5′-TTG CCT TCT TGG GAC TGA TGC-3′, 5′-GCA TTG GAA ATT GGG GTA GGA-3′; product size 481) were used to amplify IL-6 mRNA by reverse transcriptase/PCR (RT-PCR) using a protocol previously described in detail [10]. Briefly, total RNA extracted from cryosectioned tumours or tumour cells grown in vitro was reverse-transcribed into cDNA using random primers and avian myeloblastosis virus reverse transcriptase, and amplified by PCR using the IL-6 primers, and co-amplified β-actin primers in each tube as an internal control. Amplification was limited to 27 cycles to remain within the logarithmic phase for both genes. The resulting products were separated on a 2% agarose gel and transferred to nylon membranes. The membranes were hybridized with biotinylated IL-6 and β-actin-specific probes and the binding visualized using streptavidin-peroxidase-coupled luminol-based enhanced chemiluminescence (Amersham ECL reagent) as described [16].

Table 1 Interleukin-6 (IL-6) bioactivity in conditioned media from human and murine mesothelioma cell lines. The data shown are representative for three independent experiments

Mesothelioma cell line	Bioactivity[a] (U/10^6 cells)
Human[b]	
DeHmP3	< 1
Ju77	175
Lo68	1007
No36	381
ONe58	501
Sty51	6339
Murine[c]	
AB1	13
AB2	< 1
AB12	< 1
AB13	< 1
AB22	1587
AC16	67
AC28	< 1
AC29	4
AC31	289
AC32	< 1
AC34	< 1

[a] IL-6 production during 24 h in confluent cultures grown in DMEM (murine) or RPMI-1640 medium (human) with 0.5% serum
[b] Described in [20]
[c] Described in [6]

Statistical analysis

For the in vivo experiments the times to onset of symptoms or death were compared using the log-rank test. Mitogen responses and cytokine levels were compared using the Wilcoxon rank-sum test, with P values below 0.05 considered statistically significant.

Results

IL-6 mRNA expression and protein production by mesothelioma cells

Conditioned media from a panel of human and murine mesothelioma cell lines, maintained at 30% or 100% confluence at low serum levels, were assayed for IL-6 bioactivity. Out of 6 human mesothelioma cell lines, 5 produced IL-6 (Table 1). This result was also confirmed by enzyme-linked immunosorbent assay (not shown). Out of 11 murine mesothelioma cell lines, 5 produced detectable bioactive IL-6 (Table 1), and this result was corroborated by immunocytochemical labelling of IL-6 protein in fixed cytospin preparations (not shown). By RT-PCR, IL-6 mRNA was demonstrated in 10 of these cell lines (Fig. 1), suggesting that, in some cell lines, either IL-6 synthesis was below the sensitivity cut-off for the bioassay, or the protein was

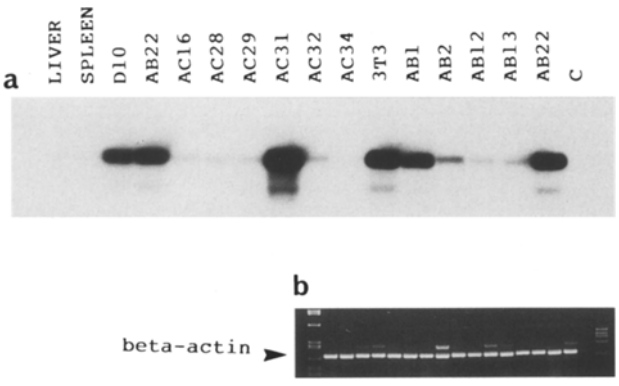

Fig. 1a, b In vitro interleukin-6 (IL-6) mRNA expression in murine mesothelioma cell lines detected by reverse transcriptase/polymerase chain reaction (PCR). Total RNA was extracted from mesothelioma cell cultures, reverse-transcribed and subjected to PCR using IL-6-specific primers and β-actin as a co-amplified internal control. The PCR products were transferred to nylon membrane and hybridised with an IL-6-specific probe

degraded by tumour cell proteases. The AB22 cell line was chosen for further in vivo studies since its IL-6 production levels were comparable to those of the human MM cell lines. Furthermore, this cell line produces no or negligible amounts of IL-1, TNFα, GM-CSF or TGFβ [2, 10], and it was reasoned that it might therefore allow for a less ambiguous picture of the role of IL-6 in mesothelioma pathobiology than would other mesothelioma lines, which produce high amounts of TGFβ in addition to IL-6 [2, 10].

IL-6 has been reported to be an autocrine/paracrine growth factor for a number of tumours [19]. However, neither exogenous IL-6 nor neutralization of the endogenous IL-6 had any apparent effect on the in vitro growth of AB22 cells (or any other mesothelioma cell line tested; data not shown).

IL-6 effects in mesothelioma pathobiology

Intraperitoneal inoculation of AB22 mesothelioma cells caused, after a lag phase determined by the number of cells inoculated, a syndrome characterized by sudden-onset cachexia, diarrhoea, a variable degree of abdominal swelling (due to solid tumour growth in the mesenterium and accumulation of haemorrhagic ascites), rough coat, dehydration and eventually, after several days, listlessness and death [2]. The cachexia was notable for its sudden onset and rapid progression, even though the animals were active and continued to eat and drink regularly until shortly before succumbing. In time-course studies it was found that clinical symptoms and macroscopically apparent tumour growth were preceded by high serum levels of IL-6 (Fig. 2a) and a progressive disappearance of cells from

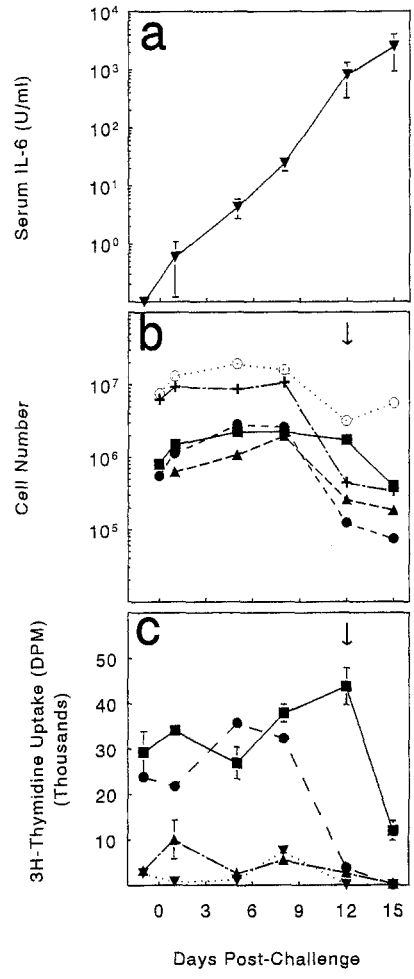

Fig. 2a–c Serum IL-6 levels (**a**), total cell numbers in lymphoid organs and the peritoneal cavity (**b**), and phytohaemagglutinin (PHA) response of spleen and peripheral lymph node cells of mice during the intraperitoneal development of AB22 tumours. Animals were challenged with 2×10^5 AB22 mesothelioma cells, and 4–6 animals were randomly sacrificed on the days indicated after challenge. The data in **b** depict mean numbers of (··· ○ ···) spleen cells, (–– + ––) lymph node cells, (—■—) peritoneal macrophages, (– – ● – –) peritoneal lymphocytes, and (––▲––) free mesothelioma cells in the peritoneal cavity/ascitic effusion, determined in 4–6 animals at each point. The SD were less 10% and are not shown for the sake of clarity. The data in **c** are representative for a range of mitogen doses tested and are the mean ± SEM of 4–6 animals at each point. –·▲·– Spleen cells without mitogen; —■— spleen cells stimulated with 4 μg/ml PHA, (···▼···) lymph node cells without mitogen, and ––●–– lymph node cells stimulated with 4 μg/ml PHA. Similar results were obtained with concanavalin A stimulation (not shown). **b, c** *arrows*, the day of onset of clinical signs of tumour-associated disease

the peripheral lymphoid organs and the peritoneal cavity (Fig. 2b). During the same period the spleen and lymph node cells showed an initial steady or slightly heightened proliferative responsiveness to phytohaemagglutinin (Fig. 2c) and concanavalin A (data not shown), followed by an almost complete abolition of responses by the time the animals were distinctly cachectic, but not yet listless (day 15 in the experiment

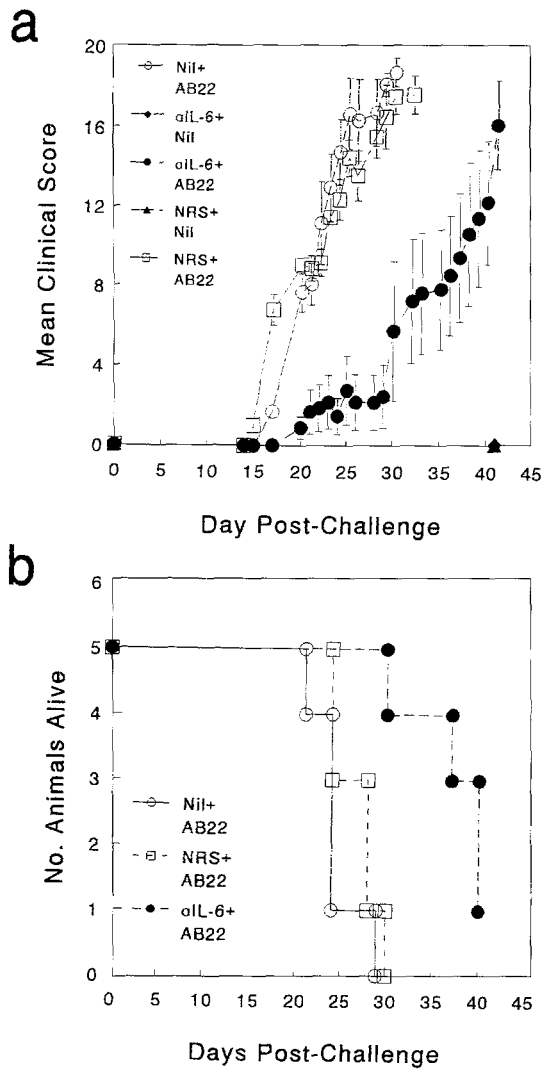

Fig. 3a, b Effect of in vivo treatment of mice with AB22 tumours with anti-IL-6 antibody (6B4; 1 mg/mouse twice weekly, commencing on the day of mesothelioma cell inoculation) on clinical performance (**a**) and survival rate (**b**). Control groups received either no treatment or normal rat serum. The animals were examined daily and given a clinical score as described in Materials and methods. –○– AB22-mesothelioma-cell-challenged mice receiving no treatment, –◆– normal mice receiving anti-IL-6 antibody, –●– AB22-cell-challenged mice treated with anti-IL-6 antibody, –▲– normal mice treated with normal rat serum, –□– AB22 mesothelioma cell challenged mice treated with normal rat serum. The differences in clinical scores between untreated and control-treated and the anti-IL-6-treated tumour-bearing animals are highly significant ($P < 0.001$)

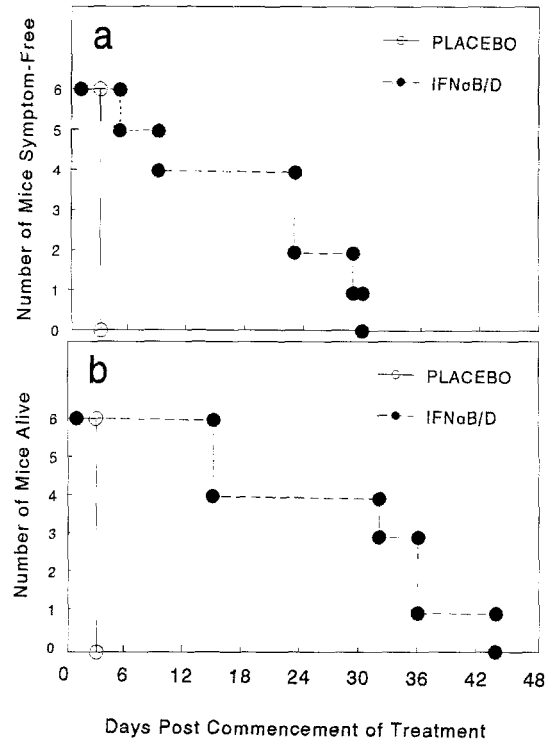

Fig. 4a, b Effect of treatment with rhuIFNαB/D on clinical performance (**a**) and survival rate (**b**) of AB22-mesothelioma-cell-challenged mice. Mice were inoculated ip. with 1×10^6 AB22(P1) mesothelioma cells. Treatment began on day 6 after challenge, and consisted in daily injection of (––●––) 1×10^5 U/mouse in 100 µl pyrogen-free saline with carrier protein or (–○–) diluent only [3], resulting in a significant improvement of both clinical performance ($P = 0.014$) and survival rate ($P < 0.001$) with interferon α (IFNα) treatment

depicted in Fig. 2c). Spontaneous and mitogen-induced IL-3/GM-CSF and IFNγ production remained low throughout the time course. Phenotypically, there was a progressive decrease in CD2 expression by both spleen and lymph node cells 12–15 days after tumour cell inoculation, and low CD3, CD5 and LFA-1 expression on lymph node cells on day 15 (data not shown).

Twice-weekly treatment of animals with rat anti-(murine IL-6) mAb (6B4; 1 mg/mouse for each inoculation), starting on the day of tumour cell challenge, dramatically attenuated the onset of clinical symptoms and the clinical course ($P < 0.001$). Control rat serum had no effect (Fig. 3). No differences in the post mortem appearance of untreated and control-treated animals were apparent, whereas anti-IL-6-treated animals had less macro- and microscopic liver damage and no or only moderate thymus atrophy and depletion of body fat depots, despite a sizeable tumour mass at the time they were terminated.

Effect of rhuIFNα on in vivo development of IL-6- producing mesotheliomas

Animals challenged i.p. with 1×10^6 AB22(P1) tumour cells and then treated daily, from day 6 after challenge, with 1×10^5 units rhuIFNαB/D had a significantly ($P = 0.014$) protracted disease course (Fig. 4a) and improved survival rate (Fig. 4b, $P < 0.001$) compared to placebo-treated animals. Although only tested in a small number of animals, serum IL-6 levels were notably lower in the IFNαB/D-treated mice (mean ± SEM = 7.3 ± 3.8 U/ml, range 0.0–13.3 U/ml, $n = 3$)

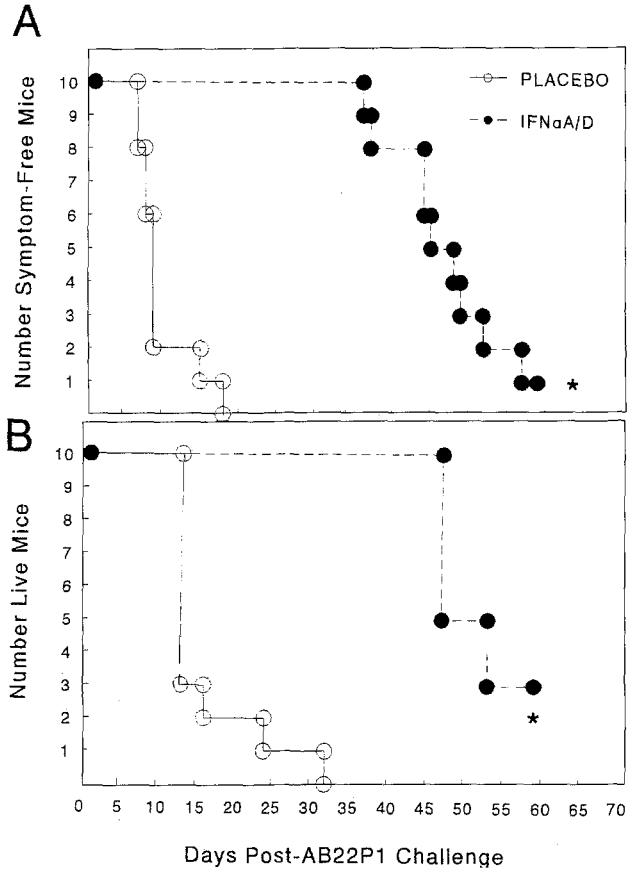

Fig. 5A, B Effect of treatment with rhuIFNαA/D on clinical performance (**A**) and survival rate (**B**) of mice (10/group) inoculated i.p. with AB22 mesothelioma cells. Mice were challenged i.p. with 2×10^5 tumour cells, and treatment began on day 4 after challenge (same treatment regime as in Fig. 4). –○– Placebo treatment, –●– rhuIFNαA/D treatment. Both clinical performance and survival rate were significantly ($P < 0.001$) improved by the IFNα treatment (*). The experiment was aborted on day 59, at which time 1 of 3 surviving animals still showed no clinical, macro- or microscopic signs of tumour development

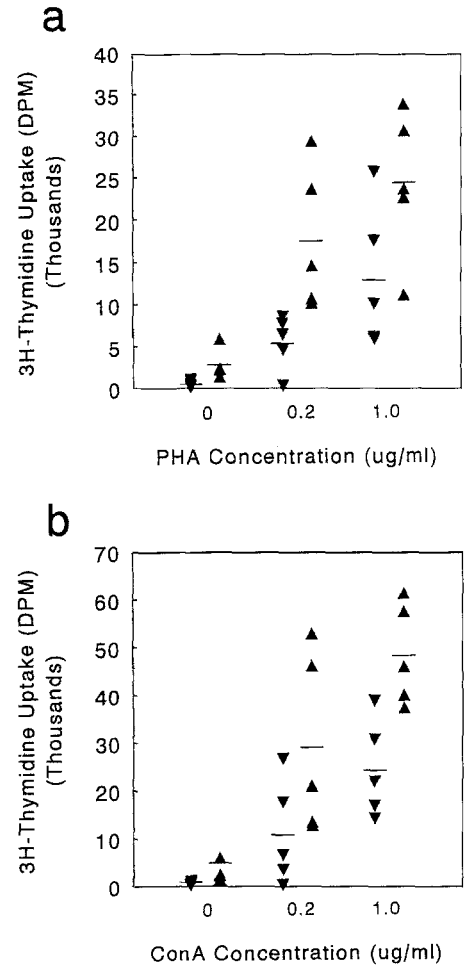

Fig. 6a, b Effect of in vivo IFNα treatment on the mitogen responsiveness on spleen cells from individual mice challenged i.p. with AB22 mesothelioma cells. Group 1 (▼): animals receiving placebo and showing severe signs of tumour development (cachexia, diarrhoea, intraperitoneal tumour mass and liver degeneration). Group 2 (△): animals receiving rhuIFNαA/D therapy and killed simultaneously with the group 1 animals (5 randomly selected animals from a group initially consisting of 15). At this time point (12 days after tumour cell challenge, 8 days after commencement of treatment) none of the treated animals had macro- or microscopic signs of tumour growth, and only low serum IL-6 levels (see Fig. 7 compared to animals in group 1)

than in the placebo-treated mice (mean ± SEM = 49.2 ± 23.6 U/ml, range 2.0–145.9 U/ml, $n = 6$). Whereas there were no apparent differences between the two groups with respect to the extent of tumour necrosis and haemorrhage, degenerative changes in the liver appeared less pronounced in the IFNα-treated mice.

An outcome similar to the one shown in Fig. 4 was obtained when the experiment was repeated with a smaller tumour cell challenge (1×10^5 cells/mouse i.p.) and daily treatment with rhuIFNαA/D (1×10^5 units mouse^{-1} day^{-1}) from day 4 after challenge until the experiment was aborted on day 59 (Fig. 5). On day 12 after challenge, when 7 out of 10 placebo-treated animals were killed because of severe cachexia, five animals in the IFNα-treated group ($n = 15$) were randomly selected and killed. No tumour growth or organ pathology was apparent macro- or microscopically in the 5 IFNα-treated animals, whereas the placebo-treated animals presented with the pathology typical of AB22 mesothelioma [2].

Peritoneal leucocytes and spleen cells were procured and assessed phenotypically and functionally for the immunological effects of IFNα treatment. Phenotypically the spleen cells from the two groups, only differed by a slightly higher frequency of $CD8^+$ cells in IFNα-treated mice compared to placebo-treated mice (mean ± SD: 11.4 ± 4.1% compared to 4.6 ± 2.6%). However, both with respect to a mitogen-induced proliferative response (Fig. 6) and cytokine production (Table 2), cells from IFNα-treated animals were notably more responsive. Peritoneal macrophages from placebo-treated animals showed a higher spontaneous

Table 2. Cytokine production by spleen cells and peritoneal macrophages from animals with intraperitoneal maligant mesothelioma receiving placebo or recombinant human interferon α (rhuIFNa) therapy (*GM*-CSF granulocyte macrophage-colony-stimulating factor, *PHA*, phytohemagglutinin, *ConA*; concanavalin A, *ND* not detected)

Cytokine	In vivo treatment	In vitro stimulation			
		Spleen cells[a]			Peritoneal macrophages[b]
		Nil	PHA	ConA	Nil
IFNγ	Placebo	0.0	0.0	21.7 ± 21.7	ND
	rhuIFNαA/D	0.4 ± 0.4	209.2* ± 103.8	58.8 ± 28.8	ND
IL-3/ GM-CSF	Placebo	0.7 ± 0.3	4.7 ± 1.1	6.4 ± 2.0	ND
	rhuIFNαA/D	1.2 ± 0.1	16.0** ± 3.4	16.4** ± 4.3	ND
IL-6	Placebo	ND	ND	ND	4976
	rhuIFNαA/D	ND	ND	ND	3140
IL-1	Placebo	ND	ND	ND	60.9
	rhuIFNαA/D	ND	ND	ND	38.4

[a] Data represent mean ± SEM of 5 animals/group for cultures of 1×10^5 cells/150 µl incubated for 48 h
[b] Values are from cultures (triplicate cultures of 2×10^5 macrophages/150 µl) of pooled cells from 5 animals/group. Incubation was for 48 h
* $P < 0.001$ as compared to the placebo group
** $P < 0.05$ as compared to the placebo group

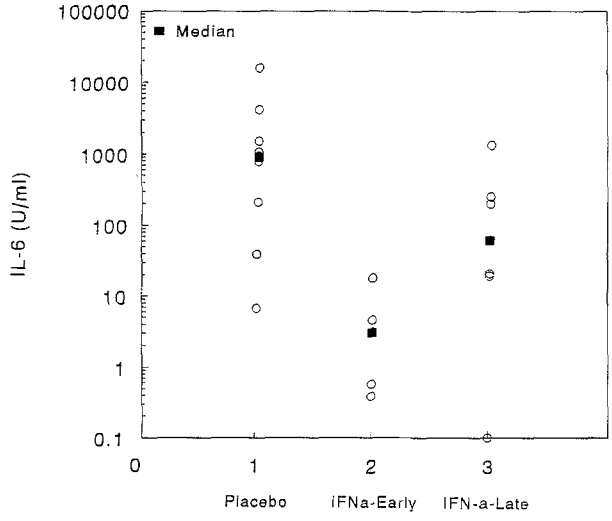

Fig. 7 Serum levels of IL-6 in animals with intraperitoneal AB22 tumours, receiving either placebo (and killed on day 12 for humane reasons), or rhuIFNαA/D treatment. From the latter group, initially consisting of 15 mice, 5 animals were killed at the time when 7 of 10 placebo-treated mice showed severe cachexia and were killed. None of the 5 rhuIFNα-treated mice had detectable mesothelioma growth. The remaining rhuIFNα-treated animals were killed when their condition deteriorated through tumour growth. The data depicted are from the same animals represented in Fig. 6

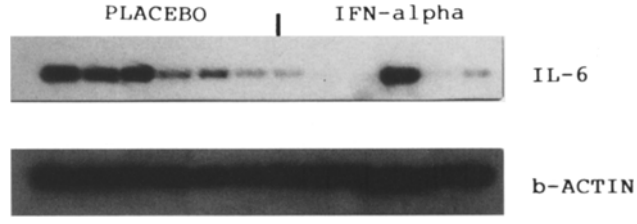

Fig. 8 Effect of in vivo rhuIFNα treatment on IL-6 mRNA expression in individual AB22 mesothelioma tumours sampled from animals in the experiment depicted in Fig. 4. Total RNA was extracted from cryopreserved tumours, and reverse-transcribed. The resulting cDNA was amplified using IL-6- and β-actin-specific primer pairs in a co-amplification PCR reaction over 27 cycles at 56° C. Gel loading was adjusted according to the β-actin product levels. Following transfer of products to nylon membrane these were probed with biotinylated β-actin and muIL-6-specific probes, and specific hybridization visualised by enhanced chemiluminescence (ECL)

release of IL-6 and IL-1 than cells from IFNα-treated mice (Table 2). However, no differences were seen with respect to their ability to inhibit tumour cell growth (autologous as well as heterologous) in vitro without or with prior lipopolysaccharide + IFNγ stimulation (in both instances 20–50% inhibition over an E:T ratio-range of 0.1–10; data not shown).

Serum levels of IL-6 were significantly lower in the IFNα-treated animals, killed prior to visible tumour growth and clinical disease, compared to placebo-treated animals ($P < 0.05$; Fig. 7). Remarkably, IFNα-treated animals succumbing to tumour growth after 35–52 days of treatment still had serum IL-6 levels lower than the placebo-treated group (Fig. 7). Furthermore, the treatment with rhuIFNα attenuated IL-6 mRNA expression in the tumours (Fig. 8), and an increase in $CD3^+$, $CD5^+$ and $CD4^+$ TIL as well as in $F4/80^+$ macrophage-like cells was noted in tumours from IFNα-treated animals (Fig. 9). TNF was not detected in serum from any animal, regardless of treatment type, and serum IL-1 levels were generally low, or

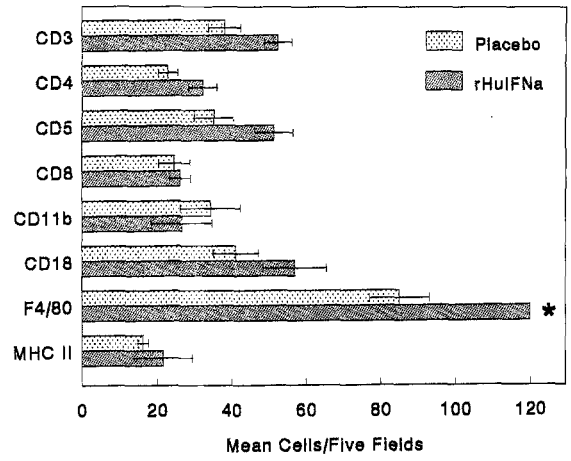

Fig. 9 Effect of in vivo rhuIFNαA/D treatment on the numbers and phenotypic profile of tumour-infiltrating lymphocytes and macrophages in AB22 mesothelioma tumours. Consecutive cryosections from tumours collected following euthanasia were immunolabelled with the CD-specific mAb listed in Materials and methods, and the total number of positively labelled cells in five representative microscope (40 × objective) fields were recorded. * The frequency and density of F4/80$^+$ cells were so high that exact counts could not be made, and the numbers were set to be at least 120 per five 40 × objective fields

undetectable, showing no relation to disease severity (data not shown).

Discussion

Cachexia is a common clinical feature in human cancer patients and is highly correlated with a negative medical outcome [32]. Although the role of TNFα, the "classical", cachexia-inducing cytokine, as a mediator of cancer cachexia has been the subject of intense study, most investigations have failed to demonstrate elevated TNFα levels in cancer patients [27]. More recently a role for IL-6, a pleiotropic cytokine with pro-inflammatory, immunoregulatory and haematopoietic effects [31], in cachexia of some cancer types has been demonstrated [11, 17, 25, 33, 34]. Our findings confirm and extend this function of IL-6 in a solid tumour of mesenchymal origin: high serum levels of IL-6 were associated with cachexia and liver damage, and these effects could be curtailed by treatment with anti-IL-6 antibody. TNFα bioactivity was not detected in body fluids of the tumour-bearing animals, and IL-1 levels were low and had no apparent relation to clinical manifestations. Furthermore, treatment with rhuIFNα curtailed IL-6 mRNA expression in the tumours and delayed tumour growth, liver damage and onset of clinical symptoms, as well as attenuating the effect of tumour growth on both peripheral lymphoid organs and TIL/TIM. A role for IL-6 in mesothelioma-associated thrombocytosis has previously been suggested [30], whereas it has not yet been implicated in the, probably more important [23], symptoms such as fever and cachexia.

Most established human mesothelioma cell lines examined so far produce IL-6 [30] (this study), as do some of the murine mesothelioma cell lines. The AB22 murine mesothelioma cell line used in the present studies, produced levels of IL-6 in vitro comparable to those produced by human mesothelioma cell lines. This was confirmed for AB22 tumours in vivo, as judged by immunocytochemical labelling for IL-6 protein (unpublished data) and expression of IL-6-specific mRNA (Fig. 8). In contrast, no or negligible levels of TGFβ, IL-1, TNFα, or GM-CSF were detected [2, 10]. This may have allowed for the unambigous demonstration of IL-6 involvement in the clinico-pathological manifestations of mesothelioma in this study. In addition, other cells, in particular macrophages and lymphocytes, could be expected to produce IL-6 during the course of the antitumour immune response [34, 37]. However, the relative importance of the various cell types in IL-6 production may vary with tumour type and site of growth. Thus, when AB22 cells are inoculated s.c., no serum IL-6 can be detected and the mice gain weight normally even when the tumours become sizeable and heavily infiltrated with TIL and TIM [2], and even though IL-6 mRNA can be detected in the tumours (unpublished data). In contrast, intraperitoneal growth of another murine mesothelioma cell line, AC29, which by itself produces bioactive IL-6 at only barely detectable levels (Table 1), results in detectable IL-6 levels in serum just before the animals succumb. This occurs acutely, probably as an indirect result of excessive levels of TNFα and TGFβ [2, 3, 10].

Although no direct role for IL-6 as a growth factor or inhibitor [18] in mesothelioma cell growth in vitro could be discerned (unpublished data), IL-6 may nevertheless be important for tumour progression by stimulating angiogenesis [22]. Indeed, the AB22 tumours are typically very well vascularised, in contrast to AC29 tumours [2], for example. It therefore remains possible that IFNα, which also does not have a direct inhibitory effect on mesothelioma growth [3], in part exhibits its inhibitory effect on the AB22 tumour by inhibiting IL-6 production by the mesothelioma cells and macrophages, and thus the angiogenic activity, thereby attenuating establishment of focal tumour growth. Whether the eventual subsidence of the antitumour effect of rhuIFNα, is due to production of rhuIFNα-neutralizing antibodies [29], or to tachyphylaxis, as often observed in cytokine therapy, or some other mechanism remains to be elucidated.

Another mechanism whereby IFNα may function is by stimulating the local and systemic antitumour immune response, directly or indirectly [7]. The cause of the disappearance of cells from the peripheral lymphoid organs and functional depression of the remaining cells around the time of clinical manifestations of mesothelioma development is unknown, although

several mechanisms, not mutually exclusive, could be envisaged, including (a) chronic exposure to tumour antigens at inappropriate levels [21, 38], (b) cytokine-induced changes in lymphocyte trafficking [28] and (c) corticosteroid- or cytokine-induced apoptosis of lymphocytes [14]. IFNα treatment appeared to ameliorate the immunological depression, but whether this is due to a direct stimulatory effect [7] or to the curtailed tumour development or to some other effect [3] is not yet known.

The implications for the human disease of the present findings could be the design of a combined therapeutic and palliative approach consisting of simultaneous treatment with rhuIFNα [36] and anti-IL-6 antibody [15, 35] or soluble IL-6 receptors, perhaps in further combination with conventional treatments such as removal of any effusion present [24].

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