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Intrapleural administration of tumour necrosis factor-alpha (TNFα) in patients with mesothelioma: cytokine patterns and acute-phase protein response

T. C. Stam[*], A. J. G. Swaak[†‡], W. H. J. Kruit[*], G. Stoter[*] and A. M. M. Eggermont[*]

[*]University Hospital Rotterdam, Daniel den Hoed Cancer Centre, Rotterdam, [†]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, Amsterdam, and [‡]Department of Rheumatology, Zuiderziekenhuis, Rotterdam, the Netherlands

Abstract

Background Tumour necrosis factor-alpha (TNFα) has been found to be very effective in the isolated limb perfusion setting for advanced extremity tumours. In a phase I study of intrapleural administration of TNFα 5 patients were followed for inflammatory response patterns.

Patients and methods Malignant mesothelioma patients were treated with repeated intrapleural administration of 0·1–0·2 mg recombinant TNFα. Samples of serum and pleural fluid were taken at different time-points before and after TNFα-administration. Levels of TNFα, interleukin-6 (IL-6), interleukin-8 (IL-8), C-reactive protein (CRP) and secretory phospholipase A_2 ($sPLA_2$) were measured using enzyme-linked immunosorbent assays (ELISAs). Alpha 1-acid glycoprotein (α1-AG) was measured by nephelometry.

Results In pleural fluid TNFα and IL-8 reached peak levels, up to 50–700 ng mL^{-1} and 6–60 ng mL^{-1}, respectively, 24 h after administration of TNFα. IL-6 (peak levels up to 250 ng mL^{-1}) and $sPLA_2$ peaked after 48 h. A slower and less dramatic pattern was observed for the levels of CRP and α1-AG. In serum no detectable levels of TNFα and no IL-8 were observed, whereas serum levels of IL-6, $sPLA_2$ and CRP showed a clear increase after intrapleural administration of TNFα. Cytokines and acute-phase proteins showed the same pattern during subsequent cycles even up to 12 cycles. Tumour regression was not observed.

Conclusions In the setting of a phase I study of repetitive intrapleural administration of TNFα in mesothelioma patients, we studied the characteristics of the inflammatory response. Intrapleural administration was followed by a clear inflammatory response locoregionally. In spite of TNFα peak levels as high as 700 ng mL^{-1} systemic levels were never detectable. The secondary cytokine response led to very high intrapleural IL-6 and IL-8 levels. Systemically IL-8 levels were never detectable whereas high IL-6 levels were induced systemically initially, with a decreased response to each intrapleural TNFα administration over time. The acute-phase response in contrast remained remarkably constant throughout the course of repeated intrapleural administrations of TNFα. Intrapleural administration of TNFα is well tolerated but associated with inconsistent and rather moderate impact on production of pleural fluid. This can be achieved by other simpler and cheaper treatment, thus we see no justification for further studies.

Keywords Acute-phase response, cytokine response, intrapleural immunotherapy, mesothelioma, TNFα.
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Department of Surgical Oncology (T. C. Stam, A. M. M. Eggermont) and Medical Oncology (W. H. J. Kruit, G. Stoter), University Hospital Rotterdam, Daniel den Hoed Cancer Centre, Rotterdam; Department of Autoimmune Diseases, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, Amsterdam (A. J. G. Swaak); and Department of Rheumatology, Zuiderziekenhuis, Rotterdam (A. J. G. Swaak), the Netherlands.

Correspondence to: A. M. M. Eggermont, MD, PhD, Department of Surgical Oncology, Daniel den Hoed Cancer Centre, PO Box 5201, 3008 AE Rotterdam, the Netherlands. Tel.: +31 10 4391911; fax: +31 10 4391011; e-mail: eggermont@chih.azr nl.

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Introduction

Malignant mesothelioma is a tumour associated with exposure to asbestos [1]. Recent studies have indicated that the incidence of malignant mesothelioma with its long time between exposure and presentation of the disease will continue to rise in the future [2].

Malignant mesothelioma is a notoriously refractory tumour to all current treatments. Neither surgery [3] nor radiotherapy [4,5] results in an increased survival. The median survival of mesothelioma is about 10 months [4,6,7]. Therefore various alternative approaches have been tested [8]. Pleural mesothelioma tends to stay locoregionally throughout most of its natural course [9]. This biological behaviour makes this disease amenable to locoregional administration of cytostatic agents or biologic agents such as interferon-gamma (IFNγ) [10], interferon-alpha [11], IL-2 [12–14]. With intrapleural administration of IFNγ or IL-2 antitumour responses have been recorded and it was speculated that TNFα might be implicated in these antitumour effects. Thus we decided to explore intrapleural administration of TNFα, a cytokine that had failed systemic administration because of excessive toxicity [15,16]. Therefore the clinical use of TNFα is restricted to locoregional application. It is already very successfully used in the treatment of irresectable sarcoma and melanoma [17,18].

There are very few reports on intracavitary TNFα in humans. TNF has been administered intraperitoneally in patients with advanced peritoneal carcinomatosis [19,20]. Regional toxicity (abdominal pain) instead of systemic toxicity was the dose-limiting factor in this setting. Intrapleural administration was studied in patients with malignant pleural effusion by Karck *et al.* [21]. Treatment with up to 200 $\mu g/m^2$ weekly, led in 3 out of 6 patients to disappearance of effusion. Because of this preclinical and clinical data, we decided to perform a phase I study in patients with stage I-IIA malignant pleural mesothelioma with rTNFα to study the clinical effect on pleural mesothelioma and evaluate the inflammatory response after this mode of administration.

We have previously shown [22] that leakage of TNFα during isolated limb perfusion (ILP) caused an acute-phase response. This was demonstrated by an increase of IL-6 directly after ILP until 2 days thereafter, followed by increase of CRP, α1-AG and α1-antitrypsin after 1 day, and decrease of negative acute-phase proteins albumin and transferrin during ILP till 6 h after ILP.

In the literature it is questioned whether the acute-phase protein response (APR) could be down regulated by a repeated stimulus [23]. Clinical studies have shown that in a number of chronic inflammatory diseases the APR is less than would be expected for the activity of inflammation [24–26]. In our patients a repeated stimulus is mimicked: at regular time intervals, 2 weeks, TNFα is administered. This enables us to investigate whether *repeated* administration of TNFα is still able to provoke an acute-phase response.

Patients and methods

Patients

Five patients with pleural mesothelioma in a phase I study were, respectively, sampled for evaluation of biologic response patterns. Demographical and clinical characteristics are summarised in Table 1. Staging and diagnosis of mesothelioma was based on computerised tomographic (CT) scan of the chest, thoracoscopic findings and histological examination of biopsy samples. According to Butchart's staging system [27] stage I is defined as tumour confined within the capsule of the parietal pleura, i.e. involving only ipsilateral pleura, lung, diaphragm and external surface of the pericardium within pleural reflection. Stage IIA is defined as mesothelioma invading chest wall or mediastinal tissues with or without lymphnode involvement ipsilaterally inside the chest.

Eligibility criteria required histologically confirmed pleural mesothelioma stage I-IIA, sufficient pleural effusion to insert an intrapleural catheter, no signs of loculation on the CT scan, no prior chemo-, radio- or immunotherapy, age < 76 years, Karnofsky performance status ≥ 80%, no cardiovascular disease, a white blood cell count $\geq 4{\cdot}0 \times 10^9 l^{-1}$, platelets $\geq 100 \times 10^9 l^{-1}$, serum bilirubin and creatinine levels within the institution's normal range, no active infection, no use of corticosteroids and obtained informed consent. The protocol was approved by the hospital's ethical committee.

Table 1 Patient characteristics and treatment response

Patient no.	Age	Sex	Stage	Treatment after 6x	Response after 12×	Response
1	63	M	I	1×0·1 mg	–	–
2	65	M	I	12×0·1 mg	SD	PD
3	63	M	IIA	18×0·2 mg	SD	SD-PD
4	65	M	I	6×0·1 mg	PD	–
5	57	M	I	6×0·1 mg	PD	–

Treatment schedule

About 2 weeks before the first administration of TNFα a Port-a-cath system was surgically inserted under general anaesthesia. The correct intrapleural position was examined radiographically and a technetium-99 m colloid scan was made to evaluate the distribution of pleural fluid throughout the pleural cavity.

Recombinant human TNFα (Boehringer Ingelheim, Germany) was administered as an intrapleural infusion, repeated every 14 days. Four patients were treated with a dose of 0·1 mg, one patient with 0·2 mg.

Tumour response evaluation

Response was evaluated after every 6 cycles using CT scan of the chest. Tumour response and toxicity were assessed according to the criteria of the World Health Organisation (1979) [28]. In case of measurable disease, complete response (CR) was defined as the disappearance of all known disease for at least 4 weeks; partial response (PR) as a decrease of > 50% in tumour size for at least 4 weeks; stable disease (SD) as a decrease of < 50% in tumour size. Progressive disease (PD) was defined as an increase of > 25% in the diameter of any lesion or the appearance of a new lesion.

Immunomonitoring and cytokine- and acute-phase protein assays

Both serum samples and pleural fluid were collected at 4 time-points during each cycle: 24 h before administration of TNFα, 24 h and 48 h after administration and at day 8. All samples were cryopreserved until testing.

Levels of TNFα, IL-6, IL-8, CRP and $sPLA_2$ were measured using enzyme-linked immunosorbent assays (ELISA). Used antibodies were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, the Netherlands).

For measuring TNFα, as described previously [29], flat-bottomed microtitre plates (Nunc, Kamstrup, Denmark) were coated overnight with purified monoclonal antibody (MAb) against TNFα (CLB-TNF/7). After washing serial dilutions of TNF-containing samples were added. Bound TNFα was detected by biotinylated sheep anti-TNFα. The detection limit of the assay was $5\,pg\,mL^{-1}$. Healthy controls were below $5\,pg\,mL^{-1}$.

The IL-6 specific ELISA was described previously [30]. A coat of CLB-IL-6/16 was applied overnight and bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. Lower detection limit was $1\,pg\,mL^{-1}$ and normal healthy control subjects were below $10\,pg\,mL^{-1}$.

For IL-8 [31] a coat of CLB-IL-8/1 MAb was applied overnight and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8. The lower detection limit of this assay was $8\,pg\,mL^{-1}$. Normal values were below $20\,pg\,mL^{-1}$.

CRP levels were measured by a sandwich ELISA using polyclonal rabbit antihuman CRP Abs as catching Abs and biotinylated MAb anti-CRP (CLB anti-CRP-2) as a detecting Ab [32]. Results were referred to a standard (Behringwerke AG, Marburg, Germany) and expressed in $mg\,L^{-1}$. Detection limit was $10\,ng\,L^{-1}$.

The ELISA used for measuring $sPLA_2$ was described before [33]. Two different monoclonal antibodies against human $sPLA_2$ were used as coating and catching antibodies, respectively. The lower limit of detection was $0{\cdot}1\,ng\,mL^{-1}$. Normal healthy volunteers were below $5\,ng\,mL^{-1}$.

Levels of α1-acid glycoprotein were measured by means of a nephelometric assay. Antisera were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, the Netherlands). Normal values were $400–900\,mg\,L^{-1}$.

Results

Cytokine and acute-phase protein levels in pleural fluid

Peak-levels (median with range) of cytokines and acute-phase proteins of all patients are represented in Table 2. In Figure 1 measurements of the first 6 cycles of patient 2 are depicted, the results of the other patients were comparable. After TNF-administration the concentration of TNFα increased in 24 h to levels in the range of $50–100\,ng\,mL^{-1}$. After 48 h still some TNFα was measurable ($150–500\,pg\,mL^{-1}$). No TNFα was measurable after 8 and 14 days. Before the first administration of TNFα IL-6 was increased in all patients, values varying from 1200 to $80\,000\,pg\,mL^{-1}$. It rose sharply to $100\,ng\,mL^{-1}$ after 24 h and $250\,ng\,mL^{-1}$ after 48 h. After 8 days it declined to $30–80\,ng\,mL^{-1}$, after 14 days levels were in the range of $15–33\,ng\,mL^{-1}$. IL-8 started at $180–260\,pg\,mL^{-1}$. It increased sharply during in the first 24 h to $2–12\,ng\,mL^{-1}$, then it decreased to $0{\cdot}7–1{\cdot}0\,ng\,mL^{-1}$ at 48 h and about $200\,pg\,mL^{-1}$ after 8 days. After 14 days levels were around $150\,pg\,mL^{-1}$. Levels of CRP increased during the first cycle from $3\,mg\,L^{-1}$ at start to $60\,mg\,L^{-1}$ after 8 days. Thereafter values remained constant around $60\,mg\,L^{-1}$. $sPLA_2$ increased during 48 h from around $5\,ng\,mL^{-1}$ ($3–9\,ng\,mL^{-1}$ after 24 h) to $20–30\,ng\,mL^{-1}$. It decreased slowly over the following 2 weeks. Levels of α1-AG remained constant around $1300\,mg\,L^{-1}$ after a slow rise from $700\,mg\,L^{-1}$. The production of cytokines demonstrated the same pattern even after cycle 12 in patient 2.

Serum-cytokine and acute-phase protein levels

In serum (Fig. 2, Table 2) no TNFα and no IL-8 was measurable in any patient. IL-6 could not be detected in 2 patients but rose sharply in the other 3, 24 h after

Table 2 Peak values (median, range) of measured cytokines and acute-phase proteins in 5 patients

Cytokine/ Acute-phase		Serum			Pleural fluid		
Protein	Pat	Time	Median	Range	Time	Median	Range
TNFα pg/mL	1*	no peak	<5		24 h	30000	
	2	no peak	<5		24 h	51500	48000–118000
	3	no peak	<5		24 h	678000	190000–1755000
	4	no peak	<5		ND		
	5	no peak	<5		24 h	2156000	218000–4395000
IL-6 pg mL^{-1}	1	24 h	160		day 8	179000	
	2	24 h	173	121–279	3×48 h,3×24 h	248000	187500–284000
	3	no peak	<1		no peak		18000–142000
	4	no peak	<1		ND		
	5†	24 h	113	91–135	24 h	286000	148500–393000
IL-8 pg mL^{-1}	1	no peak	<8		24 h	6300	
	2	no peak	<8		24 h	6000	2200–12400
	3	no peak	<8		24 h	64400	51000–71000
	4	no peak	<8		ND		
	5	no peak	<8		24 h	186500	3500–261000
CRP mg l^{-1}	1	48 h	499		day 14	83	
	2	48 h	501	385–618	no peak		3–69
	3	no peak		70–223	no peak		31–81
	4	48 h	276	216–446	ND		
	5	48 h (2×24 h)	409	327–875	day 8	88	71–198
$SPLA_2$ ng/mL	1	48 h	67		48 h	23	
	2	48 h	131	59–142	48 h	25	16–31
	3	48 h	11	9–19	no peak		0·1–7
	4	48 h	47	28–78	ND		
	5	48 h (3×24 h)	87	65–174	48 h	21	17–64
α1-AG mg l^{-1}	1	day 8	2420		ND		
	2	3xd8,3×48 h	2350	2090–2530	no peak		740–1560
	3	no peak		1800–2610	ND		
	4	48 h	2440	2320–2630	ND		
	5	no peak		2100–2540	no peak		740–1620

*Only 1 cycle.
†IL-6 was measurable only during the first two cycles, thereafter it was under detection limit ND: not measured.

TNF-administration; values were in the range of 125–275 pg mL^{-1}. At 48 h it was still measurable in the first 4 cycles. In the course of treatment IL-6 levels gradually decreased. Before the first administration of TNFα, CRP-levels in serum were increased in all patients (31–300 mg L^{-1}). CRP-levels increased slowly from around 100 mg L^{-1} after 24 h to 500 mg L^{-1} after 48 h. After 8 days CRP was still measurable: values in the range of 100 mg L^{-1}. After 14 days values were around 40 mg L^{-1}. $sPLA_2$ was increased after 24 and 48 h to about 30 ng mL^{-1} and 130 ng mL^{-1}, respectively. After a few cycles $sPLA_2$ was also measurable after 8 days: about 7 ng mL^{-1}. α1-AG was raised before administration of TNFα (1200–2000 mg L^{-1}), it increased after 24 h after administration to about 2500 mg L^{-1} at day 8. After 14 days it was around 2000 mg L^{-1}.

Toxicity and tumour response

Five male patients were treated with intrapleural infusion of TNFα. Stage I patients were treated with 0·1 mg and one patient with stage IIA mesothelioma was treated with 0·2 mg. One patient received only one dose of TNFα: Because of excessive pleural effusion, for which the patient needed drainage of pleural fluid, complicated by a haematothorax, further intrapleural TNF-administration could not be pursued. The other 4 patients were evaluable for toxicity and tumour response as they received a minimum of 6 doses up to 18 doses. Overall tolerance was quite good. All patients developed flu-like symptoms and mild fever (38–39·3 °C) during 1–2 days. Two patients had a short episode of nausea and vomiting. No grade III-IV toxicity according to the WHO-criteria [28] was observed. No hypotension or increase in heart rate was observed. Furthermore, intrapleural TNFα had no effect on number of leukocytes and thrombocytes, kreatinin and liver functions.

None of the patients had a tumour response, only one patient showed stable disease during 36 weeks. Of note, once started with treatment none of the patients needed drainage of pleural fluid any more. In 2 patients dyspnea diminished obviously during treatment. Concerning the other 2 patients: in one patient dyspnea did not change and one patient did not have complaints of breathlessness.

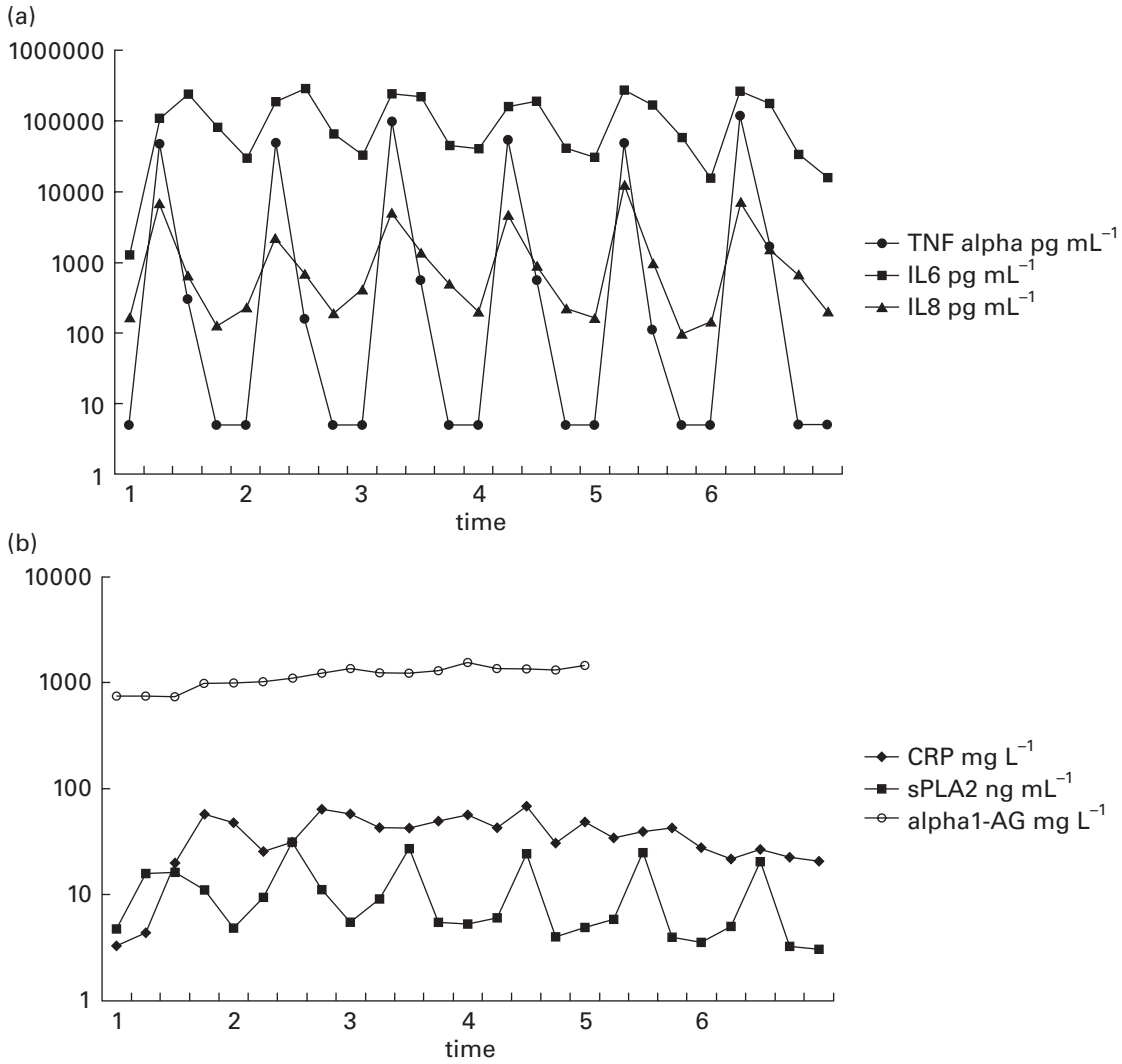

Figure 1 Cytokine (a) and acute-phase protein (b) profile in pleural fluid during the first 6 cycles of intrapleural TNF-administration in patient 2. Time-points: 24 h before administration of TNFα, 24 h after TNF-administration, 48 h after, and 8 days after. Day 14 is 24 h before the next gift of TNFα.

Discussion

In the present study the biologic effects of intrapleural administration of TNFα in patients with pleural mesothelioma was studied. The results of the cytokine measurements before administration of TNFα showed signs of an ongoing inflammatory response. This was also observed by others [34,35]. Before administration of TNFα intrapleural levels of IL-6, IL-8, CRP, $sPLA_2$ and α1-AG were elevated. In serum we found increased levels of CRP and α1-AG. Our measurements correspond to the observations of Nakano *et al.* and Monti *et al.* The high intrapleural IL-6 levels, before the first administration of TNFα, appeared to be caused by production by the mesothelioma-cells. IL-6 has been shown to be produced by mesothelioma-cells *in vitro* [36,37]. IL-6, produced in the pleural cavity, leaking to the systemic circulation, is thought to be the responsible cytokine for the induction of the acute-phase response. The mechanism of the acute-phase response has been investigated and described earlier, both *in vitro* [38,39] and *in vivo* [26,40,41].

Intrapleural administration of TNFα resulted in clear IL-6, CRP, $sPLA_2$ and α1-AG patterns in serum, with the noticeable exception for TNFα and IL-8, which never reached detectable levels systemically. This may be explained by soluble receptors for TNFα and IL-8. With respect to the IL-6 response in serum a significant increase with a peak after 48 h was observed after each TNF-administration. However the response diminished gradually in the course of time, most illustrative visible in patient 2 in which no detectable IL-6 levels were observed when TNFα had been administered more than 9 times. This points to an 'exhaustion' phenomenon or a gradual build-up of soluble IL-6 receptor levels.

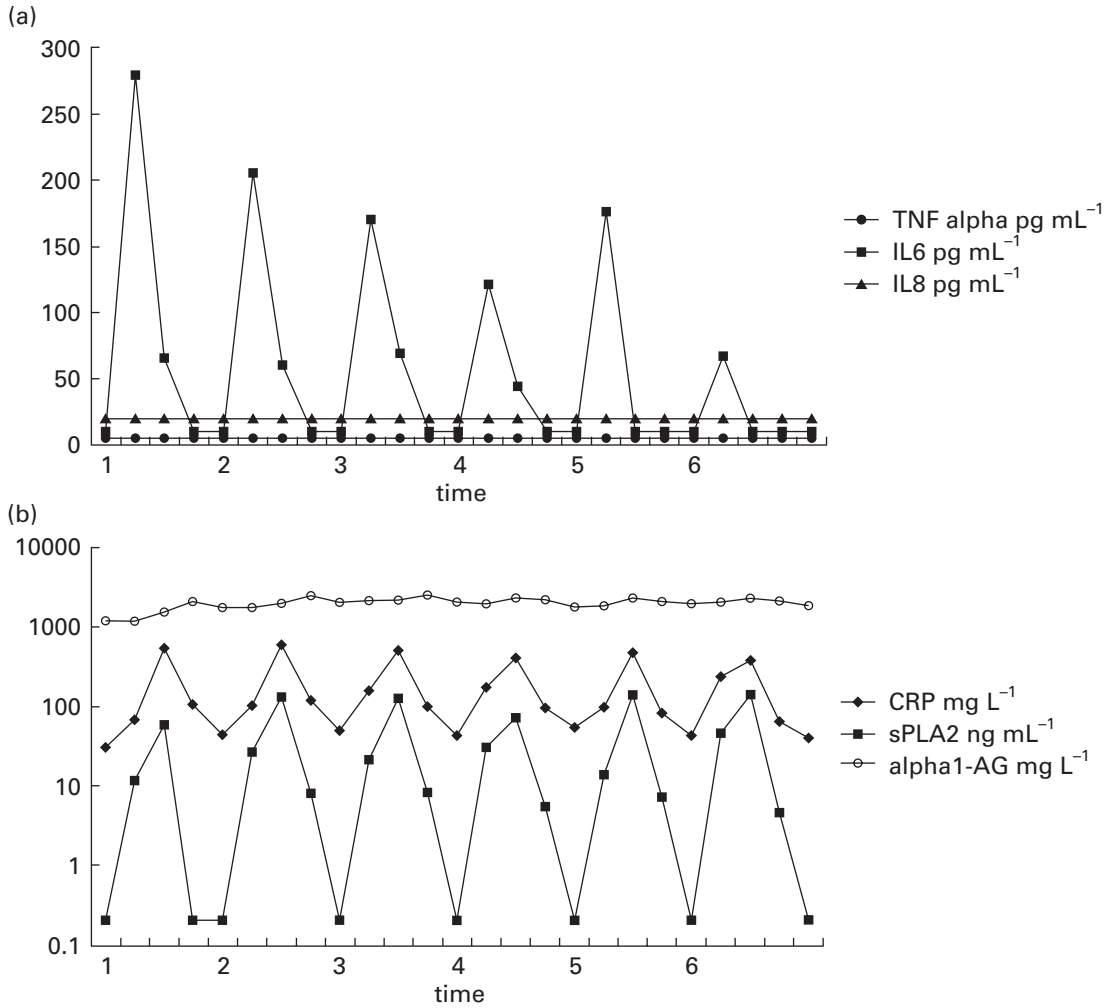

Figure 2 Cytokine (a) and acute-phase protein (b) profile in serum during the first 6 cycles of intrapleural administration of TNFα in patient 2. Time-points: 24 h before administration of TNFα, 24 h after TNF-administration, 48 h after, and 8 days after. Day 14 is 24 h before the next gift of TNFα.

However, the acute-phase response was not diminished. This may be explained by a direct stimulation of the acute-phase response by TNFα and IL-1 [26,40]. TNFα and IL-1 are only able to stimulate the positive acute-phase proteins. So in our study (we only determined positive acute-phase proteins) we could not distinguish which cytokine had the most impact on the stimulation of the APR: IL-6 or TNFα/IL-1. In contrast with the studies mentioned above [24–26], we could not find a down-regulation of the APR, even though the acute-phase response was already activated before the first TNF-administration. Administration of TNFα did increase the levels of the acute-phase proteins; this indicates that the APR was not maximally stimulated before the first TNF-administration. A possible explanation for the fact that we did not find a down-regulation of the APR could be the relatively long treatment free period of 2 weeks between the consecutive administrations of TNFα.

TNFα had no antitumour effect in these 4 mesothelioma-patients, but seemed to diminish pleural effusion. Reduction of pleural fluid was also described before [21,42]. The mechanism why TNF reduces pleural fluid is not quite clear. Most likely, fluid production by mesothelioma cells is reduced. Enhanced resorption of pleural fluid is less likely as this mechanism would be associated with higher systemic levels of TNFα and other cytokines, which we did not observe.

In conclusion, intrapleural administration of TNFα was followed by a clear inflammatory response locoregionally. In spite of TNFα peak levels as high as 700 ng mL^{-1} systemic levels were never detectable. The secondary cytokine response led to very high intrapleural IL-6 and IL-8 levels. Systemically IL-8 levels were never detectable whereas high IL-6 levels were induced systemically initially, with a decreased response to each intrapleural TNFα administration over time. The acute-phase response in contrast remained remarkably constant throughout the course of repetitive intrapleural administrations of TNFα. Intrapleural administration of TNFα is well tolerated but associated with a rather moderate impact on production of

pleural fluid. As this effect can be achieved by other simpler and cheaper treatment we see no justification for further studies.

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