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The acute phase protein response in patients receiving subcutaneous IL-6

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SUMMARY

IL-6, tumour necrosis factor-α (TNF-α) and IL-1 are thought to be the key mediators of the acute phase response although much of the evidence is based on *in vitro* studies. It is not clear to what extent each of the acute phase proteins are regulated *in vivo* by each of these cytokines. The aim of this study was to examine the effects of IL-6 treatment in eight patients with cancer on the concentrations of an extensive range of positive and negative acute phase proteins. It was part of a larger investigation to assess the value of IL-6 in the management of chemotherapy-induced thrombocytopenia. IL-6 was administered by a daily subcutaneous injection for 7 days at a dose level of 1, 3, or 10 μg/kg/day. Increases in the positive acute phase proteins, serum amyloid A, C-reactive protein, α_1-acid glycoprotein, α_1-antichymotrypsin, haptoglobin, α_1-antitrypsin, fibrinogen, complement component C3, and caeruloplasmin, were observed, with the greatest incremental changes and fastest responses being seen for C-reactive protein and serum amyloid A protein. The negative acute phase proteins transferrin, transthyretin and retinol binding protein all fell to a nadir within 48–96 h after the first IL-6 injection. Increases in complement component C4 were only found in two patients, which may be related to the increase in circulating TNF-α concentrations found only in these patients. This study has therefore shown that IL-6 is capable of causing changes in the majority of acute phase proteins *in vivo*. Although secondary induction of TNF-α was not observed in the majority of patients examined, it is still possible however that other cytokines involved in regulation of the acute phase response, such as IL-1, may have been induced and contributed to the overall response.

Keywords acute phase proteins IL-6 *in vivo* cytokine TNF

INTRODUCTION

The acute phase proteins (APP) are a family of approximately 30 plasma proteins produced in increased amounts by the liver in inflammation [1,2]. Their roles include mediating inflammation (e.g. C-reactive protein (CRP), some complement components), inhibiting proteases (e.g. α_1-antitrypsin (AAT) and α_1-antichymotrypsin (ACT)) and scavenging free radicals (e.g. haptoglobin (HPT), hemopexin (HPX) and caeruloplasmin (CPL)). The rate and magnitude of change in their plasma concentration reflects their induction by different cytokines, their molecular size, volume of distribution, and rate of catabolism both in the circulation and at the site of inflammation.

It is apparent from *in vitro* studies using hepatocyte cultures, and from correlations of serum IL-6 concentrations with APP levels in various inflammatory states *in vivo*, that IL-6 is a principal regulator for most APP genes. However the regulation of the APP is complex and the pattern of response seen is determined by the presence of several mediators. These fall into four major groups, two of which consist of cytokines acting as primary regulators and two of which consist of co-factors or modulators [3]. The IL-1 group of cytokines, IL-1α and β and the tumour necrosis factors TNF-α and β, induce the production of type 1 APP which include α_1-acid glycoprotein (AGP), serum amyloid A protein (SAA), CRP and complement component C3. The IL-6 family of cytokines (IL-6, IL-11, leukaemia inhibitory factor, oncostatin M, and ciliary neutrophilic factor) induce the synthesis of type-2 APP, fibrinogen (FB), AAT, ACT, HPT, HPX and CPL. In most cases IL-1 or TNF have no effect on the production of type 2 APP but the IL-6 group of cytokines can induce and synergistically enhance the production of type 1 APP. However this distinction is partly dependent on the species and type of hepatocyte culture as for

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example IL-1, IL-6 or TNF either failed to induce CRP production [4,5] or only the combination of IL-1 and IL-6 induced CRP production [6,7] in the human hepatocyte cell lines HepG2 and Hep3B2, whereas IL-6 alone induced CRP expression in primary human hepatocytes [8]. Studies using gene targeting to produce IL-6 deficient mice [9–11] showed only a slight reduction in LPS-induced APP production, implicating other cytokines such as TNF and IL-1 in this response. However induction of APP synthesis following a sterile injury was absent, indicating that under these circumstances IL-6 is the principal mediator of the APP response.

The concomitant decrease in concentration of the 'negative' APP which include albumin (ALB), transthyretin (TTR), transferrin (TF) and retinol-binding-protein (RBP), has been variously thought to be due to transfer to the extravascular space, or to increased catabolism. However, recent studies indicate that IL-1, TNF and IL-6 are all capable of decreasing transcription of genes for albumin [12–14] and IL-6 has been shown to decrease transcription of the TTR gene in HepG2 cells [14].

The two groups of factors which modulate cytokine-induced APP synthesis are the glucocorticoids and the growth factors such as insulin, insulin-like growth factor, hepatic growth factor, fibroblast growth factor and transforming growth factor-β [3]. Whereas glucocorticoids can directly stimulate some APP production, their principal role appears to be to synergize with the cytokines [4,8,15]. The growth factors also tend to modulate the response to the APP-inducing cytokines [15,16]. Although most of the cytokine-induced changes in APP production are thought to be mediated at the transcriptional level [2,3], post-transcriptional modulations such as alterations in stability or processing of mRNA [17–19] and altered glycosylation [20] are also important and may contribute to their regulation.

It is clear from the *in vitro* studies and murine gene targeting experiments above, that IL-6 plays a primary role in the regulation of the acute phase protein response. The recent availability of recombinant IL-6 for use in phase I/II clinical trials in cancer patients, both for its potential anti-cancer effect and as a thrombopoietic agent, has enabled its *in vivo* effects on APP to be studied in humans. Several studies have each examined two or three APP and increases in CRP, SAA, FB, C3 and HPT have been described together with a decrease in ALB, during IL-6 therapy [21–24]. In this study we describe in detail the changes observed in an extensive range of APP during IL-6 therapy (as a thrombopoietic agent) and relate these to cytokine changes.

MATERIALS AND METHODS

Patients

Eight patients (seven male and one female) who had been entered into a randomized phase I/II study of recombinant glycosylated human IL-6 (Sigosix™, Ares Serono, Geneva, Switzerland) were studied. The IL-6 was administered as part of an investigation into the clinical use of IL-6 in the management of thrombocytopenia induced by myelosuppressive chemotherapy. Six of the patients had advanced colorectal cancer and two had melanoma (Table 1). All patients were aged between 40 and 61 years of age, had a normal haematopoietic function as assessed by a full blood count, had no evidence of severe heart, lung or renal disease, had a Karnofsky performance status of greater than 60, had no evidence of active systemic infection or autoimmune disease, and had not received any form of chemotherapy within 2 months before entering the trial. Liver function tests were normal in the majority of patients with the exception of Patient 3 (markedly elevated alkaline phosphatase, ALT, bilirubin and γ-glutamyl transpeptidase), and Patients 5 and 8 (slight elevation of alkaline phosphatase only). These abnormalities were judged clinically to be due to metastatic disease and not hepatic disfunction. Computed tomography scans prior to the start of the study showed Patients 4 and 8 to have four or five small liver metastases, Patient 5 to have one large liver metastasis, and Patient 3 diffuse metastatic involvement throughout the liver with the possibility of co-existing fatty infiltration/cirrhosis.

Each patient was given a subcutaneous injection of rhIL-6 once daily for 7 days at a dose of either 1, 3 or 10 μg/kg/day, doses which in preclinical trials were well tolerated and exerted significant thrombopoietic activity. The biological activity of the IL-6 preparation was in the range of $12–14 \times 10^6$ IU/mg protein as assessed by Ares Serono using the T1165 *in vitro* bioassay [25]. Venous blood samples were taken prior to

Table 1. Details and identification of the patients described in this study and their pre-treatment IL-6 and TNF-α concentrations

Patient	Symbol	Cancer type	IL-6 dose (ug/kg/day)	Pre-treatment plasma IL-6 (pg/ml)	Pre-treatment plasma TNF-α (pg/ml)
1	□	Melanoma	1	1	16
2	○	Melanoma	1	7	14
3	△	Colorectal	1	39	40
4	☆	Colorectal	1	1	17
5	◆	Colorectal	3	4	15
6	●	Colorectal	3	13	15
7	▲	Colorectal	10	1	12
8	◇	Colorectal	10	80	25

Normal values would be expected to be $<8{\cdot}5$ pg/ml and <20 pg/ml, respectively, based on the manufacturer's quoted findings and also on our findings on a limited number of healthy volunteers.

treatment and at 2 or 3 day intervals thereafter into both EDTA containing tubes and plain tubes. Within 30 min of collection, samples were centrifuged, the serum and plasma removed and stored in multiple aliquots at −70°C until analysed.

Acute phase protein assays

All the acute phase proteins except SAA and fibrinogen were measured in serum by standard procedures using a Behringwerke Nephelometric Analyser 100 (Behringwerke AG, Germany). Antibodies, standards and controls were purchased from Behringwerke AG, Dako (Dako, Denmark), Incstar (Incstar, USA) and the Protein Reference Unit (PRU, Sheffield, UK). Fibrinogen was also assayed nephelometrically but using EDTA plasma. SAA was determined using a competitive inhibition ELISA as previously described [26]. Briefly, affinity-purified goat anti-human SAA was coated onto Immunolon 2 plates (Dynatech) and following blocking with PBS-Tween, sample or standards were added to the wells together with an alkaline-phosphatase-SAA conjugate and incubated for 1 h at room temperature. Bound conjugate was detected with para-nitrophenyl phosphate under standard conditions. Samples were standardized against an 'in-house' standard [27]. Plasma viscosity was measured by a modified Harkness method using a Coulter viscometer [28].

Cytokine assays

IL-6 and TNF-α were measured in EDTA plasma samples using immunoassays with reported sensitivities of 2 and 3 pg/ml, respectively (EASIAs, Medgenix Diagnostics, Belgium). Full details of the validation and evaluation of these assays for these samples and detailed IL-6 pharmacokinetics of these plus additional patients are described elsewhere (Banks *et al.*, submitted).

RESULTS

The symbols used and patient treatment groups are shown in Table 1.

Acute phase protein responses

The positively reacting acute phase proteins, SAA, CRP, AGP, ACT, HPT, AAT, FB, C3, C4, and CPL, together with plasma viscosity, all increased following IL-6 injection (Fig. 1a–k). In general there was a dose-response relationship between the IL-6 dose administered and the peak changes in APP concentrations. However, there was considerable overlap between the dose groups and the degree of change also depended on the initial concentrations of acute phase proteins. For example, Patient 8 had elevated concentrations of many of the APP prior to treatment and showed a less marked response to IL-6 for many proteins, although treated at the highest dose level. The greatest incremental changes and the fastest responses were seen for CRP and SAA which in the majority of patients fell to initial values by 5–10 days after the end of therapy. AAT, ACT, AGP, FB and HPT all increased outside the reference range but responded more slowly and in most patients had not returned to baseline by 20 days. C3 showed more modest increases whilst C4 only increased in two of the patients. Although most patients showed a rise in plasma viscosity with IL-6 treatment, there was no consistent relationship with dose of IL-6.

The negative acute phase proteins TF, TTR and RBP all fell to a nadir by 48–96 h after the first IL-6 injection, rising to pre-injection levels or higher by about 3 to 9 days after cessation of therapy (Fig. 2a–c).

The pattern of response in the majority of patients was comparable and a typical example of the relative changes in all positive and negative acute phase proteins measured for one patient is shown (Fig. 3a, b). An exception was Patient 1 who showed an initial fall followed by an exaggerated rebound increase in AAT, CPL, C3, and C4, accompanied by an exaggerated initial fall in the negative APP and a rebound increase. The concentrations of some of the APP (e.g. AGP, HPT, FB) were elevated in most of the patients prior to commencing treatment, despite the fact that in most of these patients their pre-treatment IL-6 and TNF-α concentrations were within the normal range (Table 1), with the most marked exceptions being Patient 8 and Patient 3.

The relationship between the APP response and IL-6 and TNF-α

The time course of the relationship of CRP to IL-6 has been studied in three patients (Fig. 4a, b) and it is clear that at all doses of IL-6, the CRP remained elevated after the IL-6 concentrations returned to normal. It is also apparent that the IL-6 concentration began to fall before the end of the treatment period. Interestingly the concentrations of CRP reached in the patients receiving the two lower IL-6 doses were comparable despite differences in plasma IL-6 concentration. No increase in TNF-α concentrations was found in three of the five patients (Patients 5, 6 and 8) in whom this cytokine was measured sequentially (Banks *et al.*, submitted), but a transient increase from 12 to 24 pg/ml by 48 h was observed in Patient 7, returning to normal concentrations 48 h later. However a sustained and gradual rise in TNF-α from 15 pg/ml pre-treatment to 36 pg/ml by day 12 was seen in Patient 1 (Banks *et al.*, submitted). Earlier time-point samples following the first IL-6 injection (hourly intervals until 6 h, then 12 and 24 h) were also examined in one patient (Patient 8), in order to rule out the possibility of an earlier transient rise in TNF-α, but again no change was detected.

DISCUSSION

A number of studies in man and non-human primates have examined the effects of IL-6 on the serum levels of various APP, though none has taken a comprehensive view, nor have the negative APP been examined in detail [21–24,29,30]. Both intravenous infusion and subcutaneous injection of IL-6 have been examined and the various studies together have shown increases in CRP, SAA, FBR, HPT, C3, AGP and CPL, together with decreases in TTR and albumin. In this study we have shown that AAT, ACT, AGP, CPL, CRP, C3, FB, HPT and SAA and the plasma viscosity all increased and the negative APP decreased following subcutaneous administration of IL-6 to patients with cancer. There was generally a dose–response relationship between the incremental change and the amount of IL-6 administered. The time course and the incremental increases were comparable to those seen in acute trauma such as surgery [31].

It is also interesting to note that there appears to be no difference in the induction of type 1 and type 2 APP, clearly

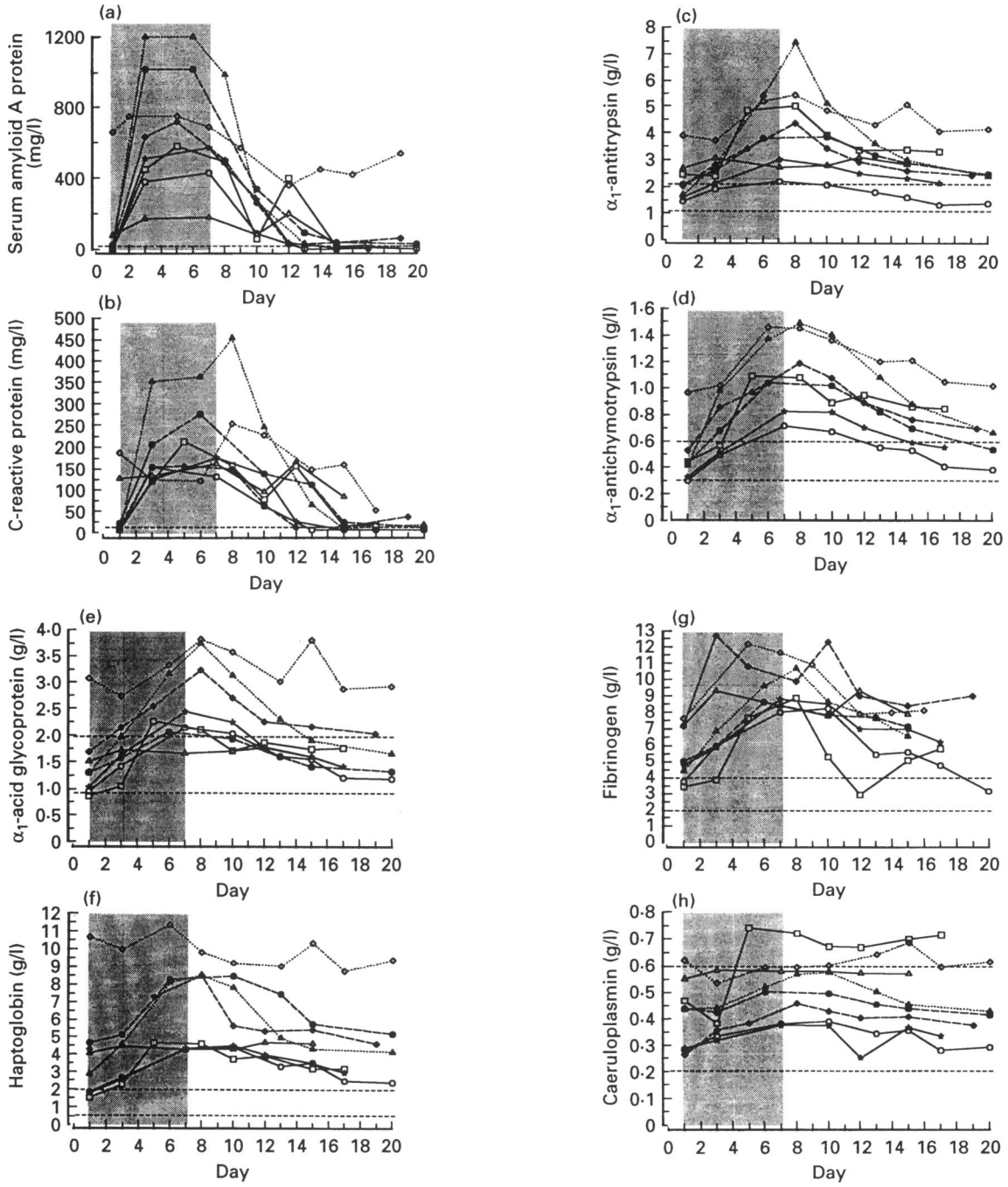
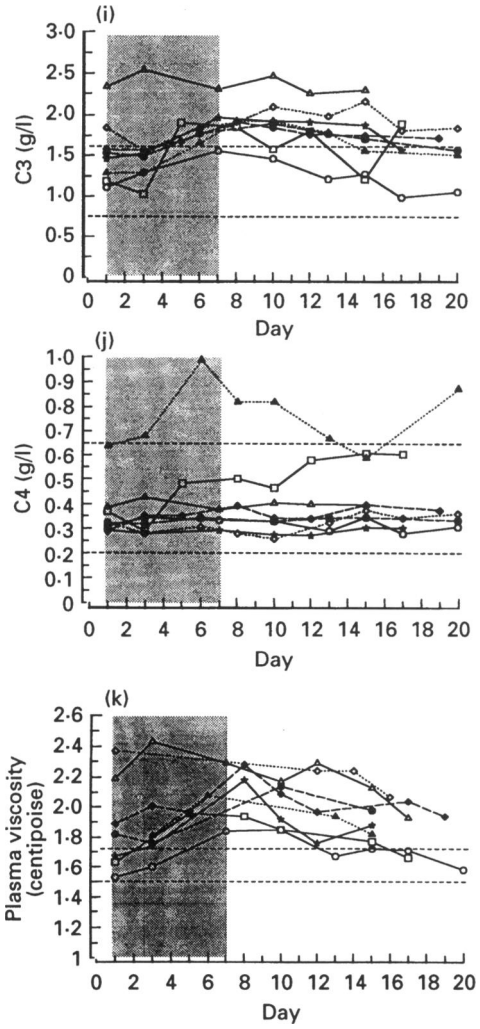


Fig. 1. Concentrations of the positive acute phase proteins (a) serum amyloid A, (b) C-reactive protein, (c) α_1-antitrypsin, (d) α_1-antichymotrypsin, (e) α_1-acid glycoprotein, (f) haptoglobin, (g) fibrinogen, (h) caeruloplasmin, (i) C3, (j) C4, and (k) plasma viscosity in patients with advanced cancer during (shaded area) and after IL-6 therapy. Symbols are as shown in Table 1. IL-6 was administered by a subcutaneous injection at the doses indicated on each of days 1 to 7 with the day 1 blood sample being taken prior to treatment. IL-6 at 1 μg/kg/day (——), IL-6 at 3 μg/kg/day (- - - -), and IL-6 at 10 μg/kg/day (· · · ·). The horizontal dotted lines represent the upper and lower limits of the reference ranges (upper limit if single line).

underlining the importance of IL-6 as an inducer of all APP genes, with the exception of C4 as discussed later. The possibility that some of the genes could be expressed as a result of secondary induction of TNF-α production by IL-6 was unlikely in three of the five patients in whom TNF-α was measured (and in an additional three patients in the study whose acute phase proteins were not measured; Banks *et al.*, submitted), where no increase was found, even in samples taken shortly after the start of treatment. This is in agreement with other studies [21,32]. However, this does not rule out the possible induction of other cytokines and their soluble receptors which may influence the acute phase response, such as IL-1



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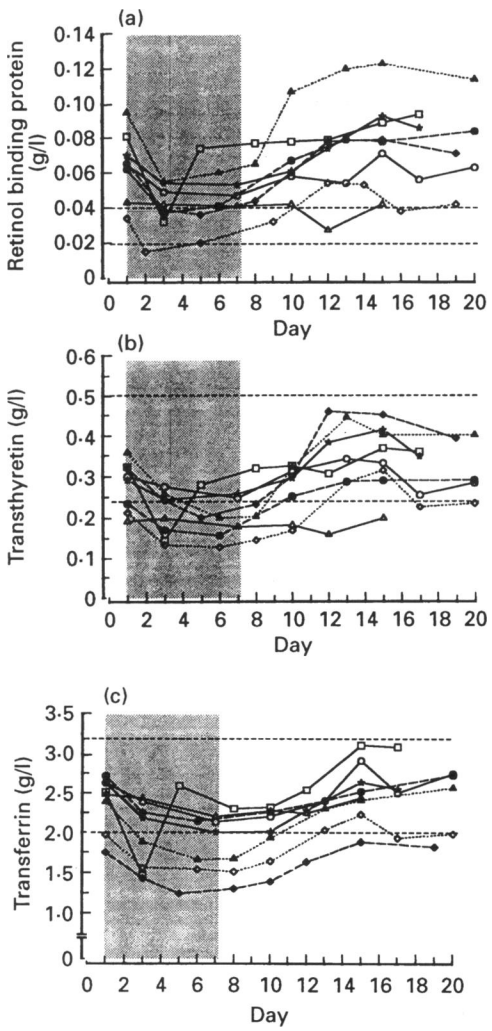

Fig. 2. Concentrations of the negative acute phase proteins (a) retinol-binding protein, (b) transthyretin, and (c) transferrin in patients with advanced cancer during (shaded area) and after IL-6 therapy. Symbols are as shown in Table 1. IL-6 was administered by a subcutaneous injection at the doses indicated on each of days 1 to 7 with the day 1 blood sample being taken prior to treatment. IL-6 at 1 μg/kg/day (——), IL-6 at 3 μg/kg/day (- - - -), and IL-6 at 10 μg/kg/day (····). The horizontal dotted lines represent the upper and lower limits of the reference ranges.

or interferon-γ, although IL-1 was not found in previous studies [21,32]. Interestingly, Patient 1 who showed a consistent increase in TNF-α, demonstrated an abnormal or exaggerated response in AAT, CPL, C3, and the negative APP. Only C3 would be expected to be influenced by TNF-α based on their division into type I and II APP.

C4 has been reported not to increase in patients receiving IL-6 [22]. However, we found an increase in C4 in two patients, both of whom were the only two patients to show any increase in circulating TNF-α concentrations during IL-6 administration, although the most abnormal TNF-α was seen in Patient 3 prior to IL-6 treatment and in whom C4 did not change. In *in vitro* studies, IL-1, IL-6 and TNF-α have been found not to induce hepatic C4 production [33–35], whereas interferon-γ induced synthesis of C4 both by hepatoma cells and by monocytes [34,36]. In contrast both interferon-γ and IL-6 induced C4 synthesis by a human intestinal epithelial cell line [37]. As administration of TNF-α has been reported to produce an increase in interferon-γ concentrations [38], this could be occurring in the two patients in whom the TNF-α concentrations increased (although modestly) following IL-6 treatment, and account for the changes in C4.

Elevated concentrations of several positive APP were found in Patients 3 and 8 prior to treatment and this was probably due to their elevated IL-6 and TNF-α concentrations at the start of the study. Increased IL-6 and TNF-α concentrations have been reported in patients with colorectal cancer, with higher levels being related to enlarged diameter and more extensive tumour, and the presence of liver metastases [39,40]. This may account for the elevated values seen in Patient 8 who had extensive liver metastases and Patient 3 who had extensive abdominal wall tumour at the time of the study. Raised IL-6 and TNF-α concentrations have also been found in patients with various liver diseases including cirrhosis [41,42] and this may contribute to the results seen in Patient 3.

Despite much discussion in the literature about the mechanism of the negative acute phase response, there is now ample

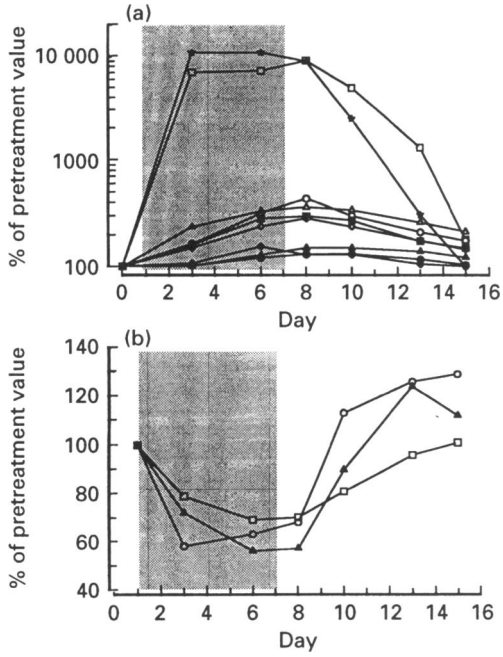

Fig. 3. Profile of the changes in (a) positive and (b) negative acute phase proteins relative to initial values in Patient 7 during (shaded area) and after 7 days treatment with IL-6 at 10 μg/kg/day. (a) □, C-reactive protein; ○, α_1-antitrypsin; ▲, α_1-antichymotrypsin; ◇, α_1-acid glycoprotein; ■, haptoglobin; ●, caeruloplasmin; ▲, C3; ◆, C4; ☆, serum amyloid A. (b) ○, retinol-binding protein; ▲, transthyretin; □, transferrin.

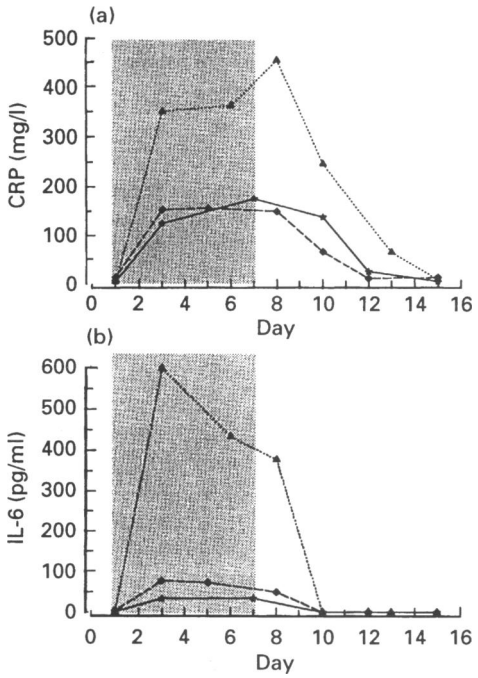

Fig. 4. Concentrations of IL-6 and C-reactive protein in one patient from each of the IL-6 dose groups during (shaded area) and after IL-6 therapy. Symbols are as shown in Table 1. IL-6 was administered by a subcutaneous injection at the doses indicated on each of days 1 to 7 with the day 1 blood sample being taken prior to treatment. IL-6 at 1 μg/kg/day (——), IL-6 at 3 μg/kg/day (- - - -), and IL-6 at 10 μg/kg/day (· · · ·).

evidence to suggest that it is due to reduced transcriptional activity of the genes involved [12–14]. As with the positive APP the degree of change in the negative APP is comparable with that seen in inflammation [31]. This hierarchy of response may reflect the biological half-life of the proteins in the circulation, as it mirrors very closely the pattern of decrease found in starvation [43]. The reason for the elevated levels of the negative APP prior to treatment in most of the patients is not clear. The rebound in TTR levels to values above baseline has not been reported in inflammation or trauma so far as we are aware. It does however suggest a positive regulatory mechanism inducing synthesis of this protein when the negative regulation of IL-6 is removed.

Interestingly, the preliminary data on IL-6 presented here indicate that the IL-6 concentrations are falling before the completion of IL-6 treatment, suggesting that IL-6 is either being metabolized at a greater rate, or that complexes are forming which may not be recognized by the IL-6 assay [44]. This is being investigated further.

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