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IL-1 receptor antagonist (IL-1Ra) does not inhibit the production of C-reactive protein or serum amyloid A protein by human primary hepatocytes. Differential regulation in normal and tumour cells

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SUMMARY

The synthesis of some class 1 acute-phase proteins (APP), including C-reactive protein (CRP) and serum amyloid A (SAA) protein is completely blocked by the IL-1 receptor antagonist (IL-1Ra), whereas the production of fibrinogen, a class 2 APP, is increased by IL-1Ra in hepatoma cells, but this has never been tested in human hepatocytes in primary culture. Since previous studies on the contributions of cytokine inhibitors in connective tissues diseases suggested that IL-1 and tumour necrosis factor-alpha (TNF-α) might play an important role in the regulation of CRP, we decided to examine in more detail the respective roles of IL-1β, IL-6, and TNF-α and their inhibitors in the production of APP by human primary hepatocytes *versus* the hepatoma cell line PLC/PRF/5. In the hepatoma cell line, IL-1β and/or TNF-α had synergistic effects with IL-6 on the production of CRP and SAA. In contrast, these cytokines were devoid of effect in normal hepatocytes. The production of fibrinogen was increased by IL-6 and decreased by IL-1 (and TNF-α) in both cell types. The secretion of CRP and SAA by primary hepatocytes incubated with a cytokine-rich mononuclear cell-conditioned medium was totally unaffected by IL-1Ra or anti-TNF-α antibodies. In contrast, the addition of IL-1Ra increased the production of fibrinogen by both hepatoma cells and primary hepatocytes incubated with the mononuclear cell-conditioned medium. We therefore conclude that IL-1β and TNF-α do not exert any significant effect on the synthesis of CRP and SAA by human primary hepatocytes.

Keywords IL-1 receptor antagonist C-reactive protein serum amyloid A protein acute-phase protein human hepatocytes human hepatoma cells

INTRODUCTION

Acute-phase proteins (APP) are molecules involved in the maintenance of the body's homeostasis and integrity. Although APP are synthesized in physiological conditions, their production is markedly increased during inflammatory responses. These molecules are mainly synthesized by hepatocytes. IL-6 is considered to be the major inducer of APP synthesis [1], although several other cytokines, such as IL-1 [1], tumour necrosis factor-alpha (TNF-α) [1], IL-11 [2], leukaemia inhibitory factor (LIF) [3], oncostatin M [4], ciliary neurotrophic factor [5] and, to a lesser extent, transforming growth factor-beta (TGF-β) [6] or interferon-gamma (IFN-γ) [7] have also been shown to participate in the regulation of these proteins. Previous studies on rat and human hepatoma cell lines have shown that APP can be divided into two classes according to the effects of cytokines on their regulation. Class 1 APP are induced mainly by IL-1, combinations of IL-1 and IL-6, or combinations of both cytokines plus glucocorticoids [8–11]. This class includes serum amyloid A (SAA), C-reactice protein (CRP), haptoglobin, C3 complement, and α_1-acid glycoprotein. Class 2 APP, including fibrinogen, α_1-antitrypsin, α_1-antichymotrypsin, and α_2-macroglobulin [8,9], respond mainly to IL-6, LIF, IL-11, or to combinations of these cytokines plus glucocorticoids [2,3,8,9]. Their production is not induced by IL-1 [8,9] and, in some instances such as for fibrinogen, IL-1 may down-regulate the effect of IL-6 [12]. Two recent studies have shown that the addition of IL-1Ra, one of the specific inhibitors of IL-1, completely inhibits the synthesis of CRP or SAA protein by the hepatoma cell line Hep 3B

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incubated with lipopolysaccharide (LPS)-stimulated monocyte-conditioned medium (CM) [13,14], whereas this factor increased the production of fibrinogen. These results suggested that IL-1Ra might decrease class 1 APP response *in vivo*, but have not been confirmed on normal human hepatocytes.

Only limited data are available on the production of APP by primary cultures of human hepatocytes. It has been shown that IL-6 alone could fully stimulate the synthesis of CRP and SAA protein [15] by normal hepatocytes, in contrast to the situation in hepatoma cell lines. The production of fibrinogen was down-regulated by IL-1 and TNF-α in both hepatocyte primary cultures [15] and hepatoma cells.

In two recent studies, we compared cytokine and cytokine inhibitor levels in the serum from patients with connective tissue diseases, characterized by a weak APP response with those from patients with rheumatoid arthritis or spondylarthropathies, who generally display a high APP response. We found that cytokine inhibitor levels, particularly IL-1Ra and/or TNF soluble receptors (TNF-sR), were significantly higher in the connective tissue diseases [16,17]. These findings suggest that IL-1 and TNF-α could play a significant role in the production of CRP by normal hepatocytes. We therefore investigated in more detail the respective contributions of these cytokines to the regulation of APP in primary cultures of human hepatocytes, focusing on the relative importance of IL-1β, IL-6, or TNF-α and the effects of their inhibitors in the synthesis of CRP, SAA protein, and fibrinogen. The results were compared with those obtained with the hepatoma cell line PLC/PRF/5, which is known to produce these APP.

MATERIALS AND METHODS

Reagents

Recombinant human (rh) IL-1β, rhTNF-α, rhIL-1Ra, anti-rhTNF-α, and anti-rhIL-6 antibodies were purchased from British Bio-Technology Products (Abingdon, UK). Biological activities were given by the supplier. IL-1β activity ranged from 1 to $3{\cdot}3 \times 10^8$ U/mg, IL-1Ra activity from 1 to $1{\cdot}4 \times 10^5$ U/mg, TNF-α activity from 2 to 5×10^7 U/mg. rhIL-6 was a gift from Amgen Corp. (Thousand Oaks, CA). Its activity (7×10^7 U/mg) was determined with the B9 cell proliferation assay. The effects of goat anti-rhIL-6 antibodies at concentrations from 1 to 10 μg/ml were determined by their ability to block the activity of a supernatant from LPS-stimulated monocytes in 1:20 dilution on the B9 cell proliferation assay. Rabbit anti-CRP and anti-fibrinogen antibodies were purchased from Dako (Glostrup, Denmark).

Preparation of mononuclear supernatant-conditioned medium

Mononuclear cells were purified from blood of a healthy blood donor by centrifugation on Ficoll–Paque and attachment to plastic. Adherent mononuclear cells were incubated at 4×10^6 cells/ml in RPMI 1640 (GIBCO, Life Technologies Ltd, Paisley, UK) supplemented with 2 μg/ml LPS (*Escherichia coli* 0.55 B5; Sigma, St Louis, MO), 10% fetal calf serum (FCS), 200 U/ml penicillin, 100 μg/ml streptomycin. After 5 h, the supernatant was collected, centrifuged twice, and stored in aliquots at −20°C. The levels of IL-1β and TNF-α in the supernatant were tested by commercially available enzyme-linked immunoassays (IL-1 EIA, Immunotech, Marseille, France; TNF-α EASIA, Medgenix Ltd, Fleurus, Belgium). The concentration of IL-6 was assessed in the B9 bioassay.

Human hepatocyte primary cultures

Liver explants (15–30 g) were taken from patients undergoing partial hepatectomy for benign tumours. This procedure received the approval of the institutional ethics committee of the Department of Surgery, and informed consent was obtained from the patients. The hepatocytes were isolated from normal areas by perfusion with a calcium-free medium followed by a collagenase solution. The cells were suspended in RPMI 1640 supplemented with 10% newborn calf serum, centrifuged at 40 *g* to separate hepatocytes from other cell types, washed three times, and resuspended in RPMI supplemented with 10% FCS. Cell viability, as determined by trypan blue exclusion, varied from 80% to 90% in the various experiments. More than 90% of the cells presented a typical hepatocyte morphology. Hepatocytes (75×10^3 cells/0·5 ml per well) were seeded on 48-well culture plates (Corning Glass, Corning, NY) and cultured in RPMI supplemented with 10% FCS, 10^{-7} M final concentration insulin, 200 U/ml penicillin, 100 μg/ml streptomycin. After adherence of the cells, the culture medium was replaced by fresh RPMI supplemented with 5% FCS. After 24 h, cell culture conditions were changed to RPMI supplemented with 0·2% bovine serum albumin (BSA), 10^{-8} M final concentration insulin, and 10^{-8} M final concentration dexamethasone (DXM). After 48 h of culture, the cells were stimulated and 10^{-7} M final concentration DXM was added. Supernatants were collected at 24 and 48 h, aliquoted, and kept frozen at −20°C until assessed for APP concentrations. Experiments were performed in duplicate.

PLC/PRF/5 cell line

The human hepatoma cell line PLC/PRF/5 was purchased from the American Type Culture Collection (Rockville, MD) and maintained in MEM (GIBCO) supplemented with 10% FCS and non-essential amino acids, L-glutamine (GIBCO), 200 U/ml penicillin, 100 μg/ml streptomycin, and amphotericin B. The PLC/PRF/5 cells were initially treated for 10 days with ciprofloxacine to eradicate mycoplasma contamination. The efficacy of this treatment was assessed by the 4′,6-diamine-2′-phenindole dihydrochloride (DAPI) test (Boehringer Mannheim Biochemica, Mannheim, Germany). The cells were maintained for less than 10 passages before use in the various experiments. For the induction of APP, PLC/PRF/5 were seeded in 48-well culture plates (40×10^3 cells/well) until confluence was reached. At this time, 10^{-8} M final concentration DXM was added. Twenty-four hours later, the culture medium was replaced by fresh medium containing 2·5% FCS, 10^{-7} M final concentration DXM and the various stimulating factors to be tested. The supernatants were collected after 24 and 72 h, aliquoted, and frozen at −20°C until the determination of APP concentrations. Experiments were performed in duplicate.

Human skin fibroblast stimulation

The production of IL-6 by normal human skin fibroblasts was used to test the efficacy of TNF-*a* or IL-1β, and the inhibitory effect of IL-1Ra or anti-rhTNF-α antibodies. Skin fibroblasts were kindly provided by J.-M. Dayer (Geneva, Switzerland). Cells were seeded in 48-well culture plates (10 000 cells/well) in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 200 U/ml penicillin, and 100 μg/ml streptomycin for 24 h. The medium was replaced by DEM supplemented with 1% FCS and the stimulating factors. Cells were incubated 24 h and the culture supernatants collected.

IL-6 quantification by the B9 bioassay

The biological activity of IL-6 was assessed by the B9 cell proliferation assay [18]. Briefly, supernatants in 1:100 and 1:1000 dilution in RPMI were added to B9 cells (10×10^3/well) in microtitre plates (Falcon, Lincoln Park, NJ). After 48 h incubation at 37°C, 50 μl tetrazolium benzene sulphonic acid (Boehringer Mannheim Biochemica) were added and proliferation assessed by spectrophotometry. IL-6 levels were determined as a function of the standard curve using rhIL-6. The specificity of this test has been confirmed by using specific goat anti-rhIL-6 blocking antibodies. IL-6 determinations were performed in triplicate.

ELISA for CRP

A sandwich ELISA procedure was used to measure the concentrations of CRP as previously described [19]. Briefly, 96-well microlitre plates (Nunc, Roskilde, Denmark) were coated with a rabbit anti-CRP antibody (Dako) diluted to 1:3000 in 0·05 M barbitone buffer pH 8·8 (50 μl/well). The plates were washed and 50 μl/well of culture supernatants were added at different dilutions. The standard curve was prepared with purified human serum CRP (Dako) diluted in the medium used for the cell cultures. The plates were incubated at 37°C for 2 h and washed. Peroxidase-conjugated rabbit anti-CRP antibody (Dako) was diluted 1:3000 in PBS-Tween + 10% FCS and 50 μl/well were added. The plates were incubated at 37°C for 2 h, washed, and 100 μl of substrate were added to each well. The substrate was 1,2-phenylenediamine (OPD) (Dako) diluted in phosphate/citrate buffer pH 5·0, and incubated at room temperature for 15 min; the reaction was stopped with 50 μl of 2 M sulphuric acid. The sensitivity of this assay was approximately 0·4 ng/ml, as defined by the lowest level of CRP reliably detected.

ELISA for fibrinogen

Plates were coated with a rabbit anti-fibrinogen antibody (Dako) diluted 1:800 in 0·05 M barbitone buffer. The standard curve was prepared with purified human plasma fibrinogen (Diagnostica Stago, Asnières, France) diluted in cell culture medium and ranged from 2000 to 7·81 ng/ml. A peroxidase-conjugated rabbit anti-fibrinogen antibody (Dako) was used as second antibody. The reaction with OPD was stopped after 3 min with 2 M sulphuric acid. The detection limit of the assay was 8 ng/ml, as defined by the lowest level of fibrinogen reliably detected.

ELISA for SAA

The determination of the concentration of protein SAA in the culture supernatants was performed by ELISA (Biosource, Camarillo, CA) according to the supplier's instructions. The sensitivity of this assay was approximately 9 ng/ml, as defined by the lowest level of SAA reliably detected.

Immunoprecipitation of radiolabelled CRP

For immunoprecipitation experiments, the culture medium was removed after 16 h of stimulation and the cells cultured in methionine-free MEM supplemented with ^{35}S-methionine for 8 h. The supernatants were then collected and stored at −20°C until use. Rabbit anti-CRP antibodies, the same as those we used for the ELISA, were added (1:100) to each culture supernatant and allowed to react overnight at 4°C. The mixture was then treated with staphylococcal protein A-Sepharose beads for 1 h at 4°C. After six washes, bound proteins were analysed by SDS–PAGE (4–20% gradient gel) under reducing conditions. The gels were stained and dried before autoradiography.

Statistical analysis

Comparisons between the levels of APP obtained with different stimulations were performed using the unpaired Student's *t*-test. $P<0.05$ was considered significant.

RESULTS

Kinetics of IL-6-induced CRP production

We first determined the kinetics of CRP production by hepatocytes (Fig. 1a) and PLC/PRF/5 cells (Fig. 1b) stimulated or not with rhIL-6. The cells were also cultured in the presence of DXM, in order to induce the synthesis of APP, as already shown in previous studies on the production of APP by hepatoma cells and primary hepatocytes (reviewed in [1]). The secretion of CRP by stimulated hepatocytes increased faster than that of PLC/PRF/5 cells. After 24 h of induction, the concentrations of CRP in the culture medium of IL-6-stimulated hepatocytes had clearly increased compared with those of unstimulated cells, whereas levels of CRP in the culture medium from PLC/PRF/5 cells remained low. The levels of CRP appeared significantly increased by IL-6 in PLC/PRF/5 cells at 72 h only. We therefore decided to determine the concentrations of APP in the supernatants from hepatocytes and hepatoma cells at 24 h and 72 h, respectively.

Effects of IL-6, IL-1β and TNF-α on CRP production

The hepatocyte primary cultures were incubated with the different cytokines, alone or in combination, and the concentrations of CRP were measured in the culture supernatants after 24 h. As shown in Fig. 2a, IL-6 alone, even at low concentrations (0·1 ng/ml), was able to induce the production of CRP. The response of CRP to IL-6 was dose-related. In contrast, IL-1β and TNF-α alone could not trigger any detectable elevation. Furthermore, the concentrations of CRP

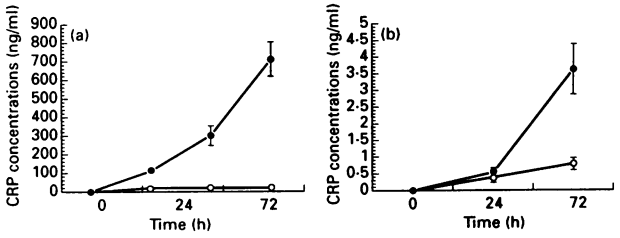

Fig. 1. Kinetics of C-reactive protein (CRP) release in the supernatants from human primary hepatocyte (a) and from PLC/PRF/5 cell line cultures (b). The supernatants of unstimulated cells (○) and cells incubated with 1 ng/ml IL-6 (●) were harvested at the indicated times and concentrations of CRP were determined in duplicate. Values are the mean ± s.d. of two stimulations.

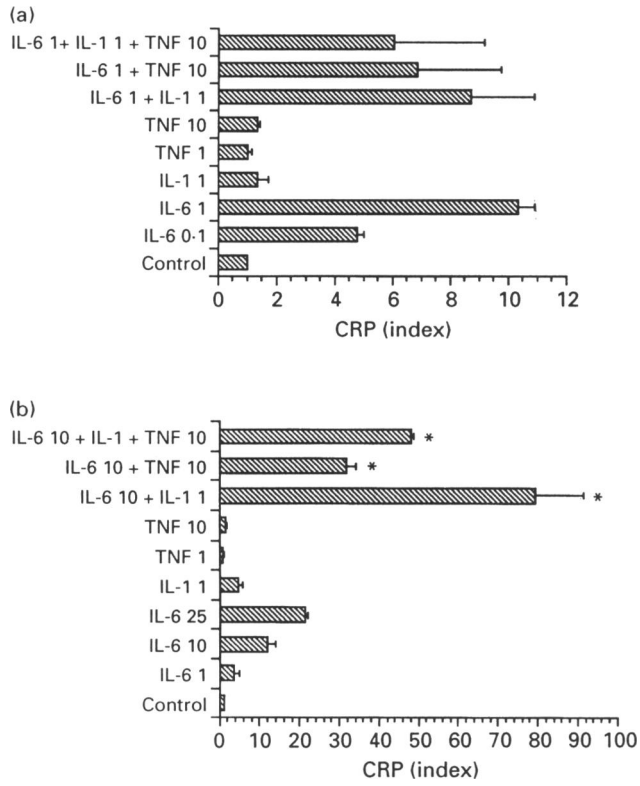

Fig. 2. C-reactive protein (CRP) production in response to IL-6, IL-1β and tumour necrosis factor-alpha (TNF-α). Human primary hepatocytes (a) and PLC/PRF/5 cells (b) were stimulated by the three cytokines alone or in combination at the indicated concentrations (ng/ml). Supernatants were then collected and their content in CRP analysed by ELISA in duplicate. The results are expressed as the ratio of CRP over the levels measured in supernatants from unstimulated cells. Each value represents the mean ± s.d. of two experiments and each experiment was performed in duplicate. * Significantly different from stimulation by IL-6 alone.

obtained with IL-6 alone were not significantly increased by adding IL-1β, TNF-α or both. Comparable results were obtained when supernatants were tested after 48 h culture (data not shown). The production of CRP by PLC/PRF/5 cells stimulated by different cytokines is shown in Fig. 2b. The production of CRP was also induced by IL-6 alone, but required much higher concentrations than for hepatocyte primary cultures. This response was also dose-related. However, the addition of IL-1β, TNF-α, or both significantly increased the effect of IL-6 alone ($P<0{\cdot}05$). The concentrations of CRP were always markedly higher in the supernatants of hepatocyte primary cultures.

Effects of IL-6, IL-1β and TNF-α on SAA production

We then examined the effect of IL-6, IL-1β, and TNF-α on the production of SAA, another type 1 APP, by normal hepatocytes. Results comparable to those on CRP were obtained; Fig. 3 shows that the SAA production was stimulated by IL-6 even at low concentrations (0·1 ng/ml). The levels of SAA obtained with IL-6 alone were not significantly increased by further addition of IL-1β, TNF-α or both. In the supernatants of PLC/PRF/5, concentrations of SAA remained very low

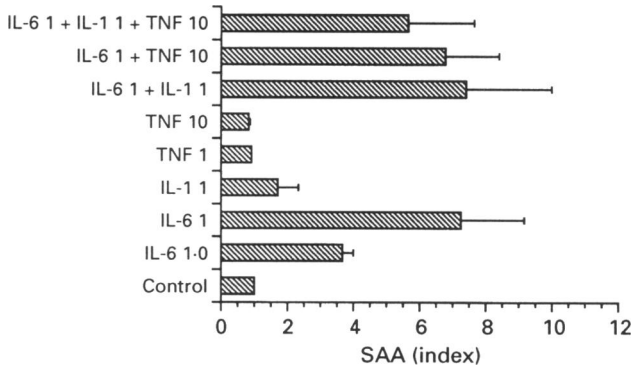

Fig. 3. Serum amyloid A (SAA) production in response to IL-6, IL-1β, and tumour necrosis factor-alpha (TNF-α). Human primary hepatocyte cultures were incubated with the three cytokines alone or in combination at the indicated concentrations (ng/ml). Supernatants were then collected and their content in SAA analysed by ELISA. The results are expressed as the ratio of SAA over the levels measured in supernatant from unstimulated cells. Each value represents the mean ± s.d. of two experiments, and each experiment was performed in duplicate.

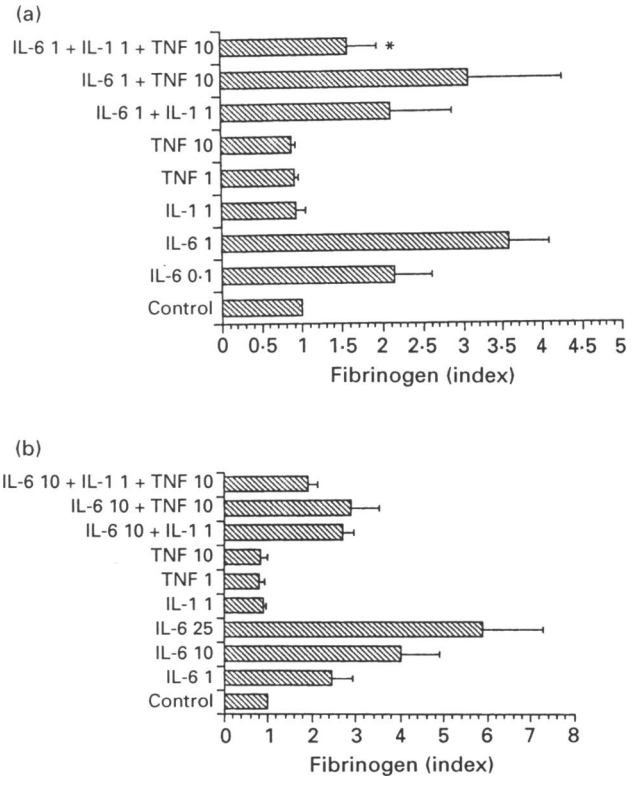

Fig. 4. Fibrinogen production in response to IL-6, IL-1β, and tumour necrosis factor-alpha (TNF-α). Human primary hepatocytes (a) and PLC/PRF/5 cells (b) were stimulated by the three cytokines alone or in combination at the indicated concentrations (ng/ml). Supernatants were then collected and their content in fibrinogen analysed by ELISA in duplicate. The results for each condition are expressed as the ratio of fibrinogen over the levels measured in supernatants from unstimulated cells. Each value represents the mean ± s.d. of two experiments, and each experiment was performed in duplicate. * Significantly different than stimulation by IL-6 alone.

Table 1. Levels of C-reactive protein (CRP), fibrinogen, and serum amyloid A (SAA) protein in the culture supernatants of conditioned medium (CM)-stimulated hepatocytes with or without addition of IL-1Ra, anti-rhTNF-α or anti-rhIL-6 antibodies

Anti-rhIL-6 (μg/ml)	0	0	0	0	0	0	1	10
IL-1Ra (ng/ml)	0	0	20	100	500	0	0	0
Anti-rhTNF-α (μg/ml)	0	0	0	0	0	1	0	0
CXM (1:20)	0	+	+	+	+	+	+	+
CRP (ng/ml)	21 ± 2·1	307 ± 3·54	313 ± 24	396 ± 42	395 ± 37	298 ± 3·54	27 ± 0·5	24 ± 9·1
Fibrinogen (μg/ml)	0·6 ± 0·1	1·4 ± 0·1	1·9 ± 0·46	2·3 ± 0·34	2·7 ± 0·1	1·5 ± 0·3	0·6 ± 0·01	0·5 ± 0·04
SAA (μg/ml)	0·62 ± 0·14	4·60 ± 0·67	4·74 ± 0·81	4·63 ± 0·80	ND	4·61 ± 0·9	0·81 ± 0·2	0·64 ± 0·17

Values represent mean ± s.d. of two stimulations analysed in duplicate by specific immunoassays.

(⩽ 90 ng/ml) even when doses of up to 25 ng/ml IL-6 were used to stimulate the cells. However, the addition of 1 ng/ml IL-1β to IL-6 increased significantly the levels of SAA to 2·5 μg/ml (not shown).

Effects of IL-6, IL-1β and TNF-α on fibrinogen production

In order to compare the results obtained with CRP and SAA (type 1 APP) with a type 2 APP, we examined the release of fibrinogen in the same supernatants. Figures 4a,b show the concentration of fibrinogen in the culture supernatants of hepatocytes or PLC/PRF/5 cells incubated with different cytokines for 24 h and 72 h, respectively. IL-6 induced the production of fibrinogen by both cells, and the response of fibrinogen to IL-6 was dose-related. Neither IL-1β nor TNF-α alone were able to increase the levels of fibrinogen. In contrast, the concentrations of fibrinogen tended to decrease when these cytokines were added to IL-6 in primary hepatocytes and hepatoma cells. When IL-1β and TNF-α were added together with IL-6, the production of fibrinogen by primary hepatocytes was significantly decreased below the levels induced by IL-6 alone ($P<0·05$). A comparable result was found in the supernatant from PLC/PRF/5 cells, but the difference between IL-6 alone and IL-6 plus IL-1β and TNF-α was not significant ($P<0·08$).

Effects of IL-1Ra, anti-TNF-α, or anti-IL-6 antibodies on CM stimulation

To determine the relative contributions of IL-6, IL-1β, and TNF-α to the stimulation of CRP, SAA, and fibrinogen release elicited by a cytokine-rich conditioned medium, hepatocytes and PLC/PRF/5 cells were stimulated by CM in presence or absence of IL-1Ra, anti-TNF-α, and anti-IL-6 antibodies. The CM, whose concentration in TNF-α, IL-1β and IL-6 were 3·2 ng/ml, 11 ng/ml, and 175 ng/ml, respectively, was used in a concentration of 1:20. Tables 1 and 2 show the results of APP measured in the culture supernatants of hepatocytes and PLC/PRF/5. The production of CRP was completely blocked by adding anti-IL-6 antibodies to CM-stimulated hepatocytes. On the other hand, IL-1Ra and anti-TNF-α antibodies, at concentrations which completely suppressed the effect of 1 ng/ml IL-1β and 0·5 ng/ml TNF-α, respectively, on the production of IL-6 by human skin fibroblasts (Fig. 5), were totally ineffective to reduce the CM-induced level of CRP. In fact, IL-1Ra had a tendency to increase the stimulatory effect of CM. In contrast, the production of CRP by PLC/PRF/5 cells was significantly lowered by IL-1Ra as well as by anti-IL-6 antibodies, and completely blocked when both IL-1Ra and anti-rhIL-6 antibodies were added. Similar results were observed for SAA with primary hepatocytes. The production of fibrinogen was completely blocked by anti-IL-6 antibodies in both normal hepatocytes and hepatoma cells, and the addition of IL-1Ra to CM to both culture types significantly increased the fibrinogen concentrations. Anti-TNF-α antibodies had no significant effect on the fibrinogen levels produced by the two types of cells.

Immunoprecipitation of ^{35}S-methionine-labelled CRP

To substantiate further the results obtained by ELISA and determine the effect of IL-6 and IL-1β on *de novo* synthesis of CRP in primary human hepatocytes, we performed immunoprecipitations of supernatants from ^{35}S-methionine-labelled cells. The results show that *de novo* synthesis of CRP was

Table 2. Levels of C-reactive protein (CRP) and fibrinogen in the culture supernatants of conditioned medium (CM)-stimulated PLC/PRF/5 cells with or without addition of IL-1Ra, anti-TNF-α or anti-rhIL-6 antibodies

Anti-rhIL-6 (μg/ml)	0	0	0	0	0	1	5	5
IL-1Ra (ng/ml)	0	0	20	100	0	0	0	100
Anti-rhTNF-α (μg/ml)	0	0	0	0	1	0	0	0
CM (1:20)	0	+	+	+	+	+	+	+
CRP (ng/ml)	0·44 ± 0·17	9·98 ± 0·74	6·31 ± 2·38	4·79 ± 1·13	12·33 ± 0·39	4·02 ± 0·93	1·56 ± 0·25	0·77 ± 0·14
Fibrinogen (μg/ml)	2.5 ± 0·14	7·3 ± 0·84	8·7 ± 0·21	10·0 ± 0·62	6·6 ± 0·08	3·4 ± 0·28	2·3 ± 0·73	2·8 ± 0·45

Values represent mean ± s.d. of two stimulations analysed in duplicate by specific immunoassays.

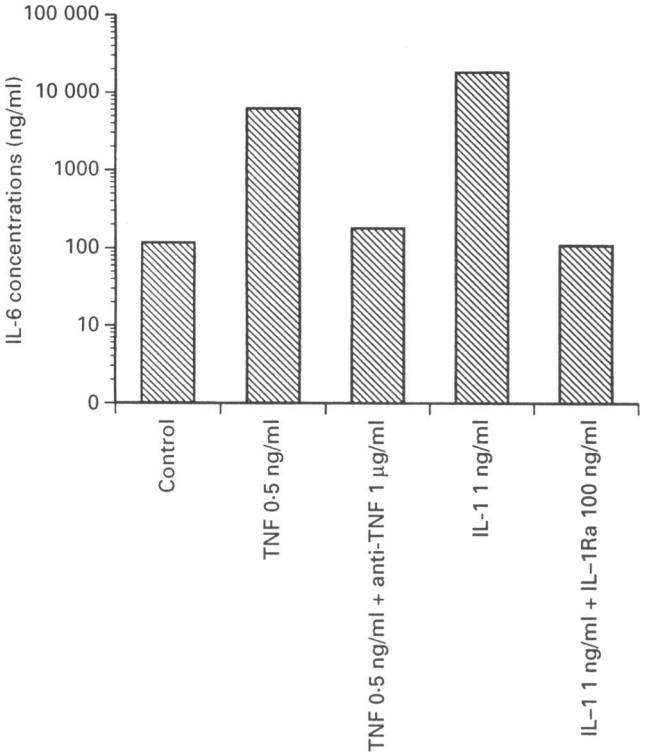

Fig. 5. Effect of IL-1Ra and anti-tumour necrosis factor-alpha (TNF-α) antibodies on IL-6 production by skin fibroblasts. Skin fibroblasts were incubated with IL-1β ± IL-1Ra or with TNF-α ± anti-TNF-α antibodies. After 24 h culture, the supernatants were collected and tested for their content in IL-6 by the B9 cell proliferation assay. Values represent the mean of IL-6 levels assessed in triplicate.

induced by 1 ng/ml IL-6 or CM. The addition of anti-IL-6 antibodies completely blocked the synthesis of CRP. By contrast, the production of CRP was not inhibited, but rather slightly increased, by the addition of 100 ng/ml IL-1Ra (Fig. 6).

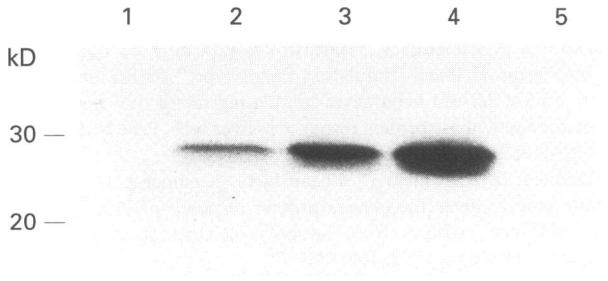

Fig. 6. Determination of C-reactive protein (CRP) synthesis by immunoprecipitation. Human primary hepatocyte cultures unstimulated (lane 1); stimulated with 1 ng/ml IL-6 (lane 2), or with supernatant of activated monocytes at dilution 1 : 20 (lane 3), or with supernatant of activated monocytes at dilution 1 : 20 + 100 ng/ml IL-1Ra (lane 4), or with supernatant of activated monocytes at dilution 1 : 20 + 1 μg/ml anti-IL-6 antibodies (lane 5) were cultured for 16 h. Cells were then labelled with ^{35}S-methionine in methionine-free medium for 8 h and the supernatants collected for immunoprecipitation.

DISCUSSION

Our results show that neither IL-1Ra nor anti-TNF-α antibodies had inhibitory effect on the production of SAA or CRP by CM-stimulated normal human hepatocytes. These data contrast with previous studies on hepatoma cells. The production of SAA by HuH-7 cells, a human tumoral cell line, could be induced by IL-1 alone and reversed by IL-1Ra [20]. In other cell lines, such as Hep 3B or Hep G2, numerous reports have shown that the production of SAA or CRP can not be induced by IL-6 alone, but requires a combination of IL-6 and IL-1 [10,11]. In Hep 3B, IL-1Ra has been reported to block CM-induced SAA and CRP synthesis completely [13,14]. In primary hepatocytes, however, the present results as well as previous reports show that IL-6 by itself is competent for maximal stimulation. These data emphasize the fact that some class 1 APP, such as SAA and CRP, are strikingly differently regulated in normal hepatocytes and in a hepatoma cell line. Furthermore, our results show also that the amounts of CRP and SAA produced by hepatoma cells were clearly lower than those obtained with primary cells. The regulation of fibrinogen, a typical class 2 APP, was comparable in the two types of cells. In our own studies with the Hep G2 tumoral cell line, we were unable to induce the production of CRP even with combinations of IL-6 and IL-1β (data not shown), which indicates a variability in the production of CRP by the different cell lines. Taken together, these results suggest that, compared with human hepatocytes in primary culture, tumoral hepatoma cells lose part of their responsiveness to IL-6. The fact that distinct hepatoma cell lines react differently to IL-6 implies that IL-6 responsiveness might vary with the differentiation state of tumoral cells. Studies in progress indicate that a deficiency in the 80-kD soluble IL-6 receptor might account, at least partly, for the lack of the responsiveness of the different hepatoma cells to IL-6. In any event, these findings emphasize the fact that results obtained in hepatoma cells should be interpreted with caution. One should also be aware that the production of APP by primary hepatocytes could be influenced by culture conditions and the trauma of initial culture adaptation, and that those cells might therefore not constitute a perfect model of the *in vivo* situation. However, as shown in our results, the production of APP, including CRP and SAA, is easily inducible even with low concentrations of IL-6 in primary cells, in contrast to hepatoma cells, and these cells certainly represent the best model available for the study of APP regulation. Cultures of primary hepatocytes are contaminated by non-parenchymal cells, which could be the source of IL-6 and of other biologically active cytokines and/or cytokine inhibitors. However, the percentage of such cells was low in our cultures. In addition, the absence of detectable levels of IL-6 in the supernatants of primary hepatocytes incubated with IL-1β or TNF-α (data not shown), indicates that the biological interference of contaminating cells was rather weak, if existent, in our study.

Our findings in human normal hepatocytes show that the usual definition of class 1 and class 2 APP may not be entirely satisfactory. As the production of both class 1 and class 2 APP is fully inducible by IL-6 in primary hepatocytes, it would appear that the two classes of APP would best be distinguished by the absence of significant inhibitory effect of IL-1β on the synthesis of class 1 APP and effective IL-1β inhibition of the production of class 2 APP. Further studies including other APP

should, however, be performed in order to support this suggestion.

A correlation between serum CRP and IL-6 levels has already been reported in some inflammatory conditions [21,22]. However, in systemic lupus erythematosus (SLE), a highly inflammatory rheumatic disease, we found that serum CRP concentrations—which are well known to remain generally low—did not correlate with IL-6, whose serum concentrations appeared high [18]. The causes of this altered APP response to IL-6 was tentatively attributed to the presence of elevated circulating serum levels of cytokine antagonists found in SLE and polymyositis-dermatomyositis [16,17]. This role should not be excluded, despite the fact that our results indicate that neither IL-1β nor TNF-α have any detectable role on the regulation of SAA or CRP synthesis by human hepatocytes *in vitro*. The production of IL-6 by fibroblasts, macrophages, and endothelial cells present in the human liver can take place under the stimulation of circulating IL-1β or TNF-α, and this paracrine stimulation of APP synthesis might therefore be down-regulated by IL-1Ra or TNF-sR. Other cytokines, such as IL-11, LIF, and Oncostatin M, which have been shown to induce the synthesis of some APP in hepatoma cell lines, may also contribute to the variation of APP responses in different rheumatic diseases. However, the serum levels of these cytokines in patients with rheumatic inflammatory diseases and their effects in primary hepatocytes are unknown so far.

We found that IL-1Ra enhances the levels of fibrinogen in the supernatants of normal hepatocytes stimulated by CM. This finding is consistent with previous reports on hepatoma cell line cultures [14,20] and the inhibitory effect of IL-1β on fibrinogen synthesis [14,15]. Fibrinogen is a high molecular plasma protein, which influences the sedimentation of erythrocytes. The fact that fibrinogen is regulated differently from SAA or CRP may provide an explanation for the dissociation between serum CRP levels and erythrocyte sedimentation rate in SLE [23].

In accordance with a previous study [24], we observed that TNF-α slightly decreases the production of SAA and CRP by hepatocytes stimulated by both IL-6 and IL-1β. This effect was, however, not significant even when high concentrations of TNF-α (100 ng/ml) were added (data not shown). In addition, our results show that the production of CRP by normal hepatocytes was increased by adding IL-1Ra on cells incubated with CM, suggesting a possible inhibitory effect of IL-1 on CRP synthesis. This finding is consistent with the mild, although not significant, inhibitory effect of IL-1, when added to IL-6. This finding has not been reported by other investigators, and might be related to the doses of IL-1 or the conditions of cell isolation and culture [15,25]. This effect of IL-IRa on CRP production was more pronounced when determined by immunoprecipitation than by ELISA. The difference between these techniques might be related to the semiquantitative nature of immunoprecipitations and to the kinetics of CRP production that was differently analysed by the two techniques.

In conclusion, our results show that, in the presence of IL-6, IL-1β and TNF-*a* exert no significant effect on the synthesis of SAA and CRP by normal hepatocytes, and that APP are differently regulated in tumour or non-tumour cells. These findings suggest that the usual definition of class 1 and class 2 APP based on their regulation in hepatoma cells may require re-evaluation in the light of the effects of cytokines observed in primary cultures of human hepatocytes.

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REFERENCES

1 Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute-phase response. Biochem J 1990; **265**:621–36.
2 Baumann H, Schendel P. Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. J Biol Chem 1991; **30**:20424–7.
3 Baumann H, Wong GG. Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. J Immunol 1989; **143**:1163–7.
4 Richards CD, Brown TJ, Shoyab M, Baumann H, Gauldie J. Recombinant oncostatin M stimulates the production of actute phase proteins in Hep G2 cells and rat primary hepatocytes *in vitro*. J Immunol 1992; **148**:1731–6.
5 Schooltink H, Stoyan T, Roeb E, Heinrich PC, Rose-John S. Ciliary neurotrophic factor induces acute-phase protein expression in hepatocytes. FEBS Lett 1992; **314**:280–4.
6 Taylor AW, Ku N-O, Mortensen RF. Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-β. J Immunol 1990; **145**:2507–13.
7 Magielska-Zero D, Bereta J, Czuba-Pelech B, Pajdak W, Gauldie J, Koj A. Inhibitory effect of human recombinant interferon gamma on synthesis of acute phase proteins in human hepatoma Hep G2 cells stimulated by leukocyte cytokines, TNFα and IFNβ2/BSF-2/IL-6. Biochem Int 1988; **17**:17–23.
8 Baumann H, Gauldie J. Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. Mol Biol Med 1990; **7**:147–60.
9 Baumann H, Prowse KR, Marinkovic S, Won K-A, Jahreis GP. Stimulation of hepatic acute phase response by cytokines and glucocorticoids. Ann NY Acad Sci 1989; **557**:280–95.
10 Ganapathi MK, Rzewnicki D, Samols D, Jiang S-L, Kushner I. Effect of combinations of cytokines and hormones on synthesis of serum. A amyloid and C-reactive protein in Hep 3B cells. J Immunol 1991; **147**:1261–5.
11 Smith JW, McDonald TL. Production of serum amyloid A and C-reactive protein by HepG2 cells stimulated with combinations of cytokines or monocyte conditioned media: the effects of prednisolone. Clin Exp Immunol 1992; **90**:293–9.
12 Gauldie J, Richards C, Harnish D, Lansdorf P, Baumann H. Interferon β2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci USA 1987; **84**:7251–5.
13 Damtew B, Rzewnicki D, Lozanski G, Kushner I. IL-1 receptor antagonist affects the plasma protein response of Hep B3 cells to conditioned medium from lipopolysaccharide-stimulated monocytes. J Immunol 1993; **150**:4001–7.
14 Rokita H, Loose LD, Bartle LM,Sipe JD. Synergism of interleukin 1 and interleukin 6 induces serum amyloid A production while depressing fibrinogen: a quantitative analysis. J Rheumatol 1994; **21**:400–5.
15 Castell JV, Gomez-Lechon MJ, David M *et al.* Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. FEBS Lett 1989; **242**:237–9.
16 Gabay C, Gay-Crosier F, Roux-Lombard P *et al.* Elevated serum levels of interleukin-1 receptor antagonist in polymyositis-dermatomyositis: a biological marker of disease activity with a possible role in the lack of acute-phase protein response. Arthritis Rheum 1994; **37**:1744–51.

17 Guerne P-A, Cekir N, Moral F *et al.* Levels of TNF soluble receptors are markedly elevated in systemic lupus erythematosus in contrast to rheumatoid arthritis. Highly significant correlation with disease activity. Arthritis Rheum 1993; **36**:(Suppl.):S147 (Abstr.).

18 Gabay C, Roux-Lombard P, De Moerloose Ph, Dayer J-M, Vischer T, Guerne P-A. Absence of correlation between interleukin-6 and C-reactive protein blood levels in systemic lupus erythematosus compared with rheumatoid arthritis. J Rheumatol 1993; **20**:815–21.

19 Highton J, Hessian P. A solid-phase enzyme immunoassay for C-reactive protein: clinical value and effect of rheumatoid factor. J Immunol Methods 1984; **68**:185–92.

20 Bevan S, Raynes JG. IL-1 receptor antagonist regulation of acute phase protein synthesis in human hepatoma cells. J Immunol 1991; **147**:2574–8.

21 Houssiau FA, Devogelaer J-P, Van Damme J, Nagant de Deux-chaisnes C, Van Snick J. Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritis. Arthritis Rheum 1988; **31**:784–8.

22 Metsärinne KP, Nordström DCE, Kontinnen YT, Teppo A-M, Fyhrquist FY. Plasma interleukin-6 and renin substrate in reactive arthritis, rheumatoid arthritis, and systemic lupus erythematosus. Rheumatol Int 1992; **12**:93–96.

23 Pepys MB, Lanham JG, De Beer FC. C-reactive protein in SLE. Clin Rheum Dis 1982; **8**:91–101.

24 Yap SH, Moshage HJ, Hazenberg BPC *et al.* Tumor necrosis factor (TNF) inhibits interleukin (IL-1 and/or IL-6) stimulated synthesis of C-reactive protein (CRP) and serum amyloid A (SAA) in primary cultures of human hepatocytes. Biochemica et Biophysica Acta 1991; **1091**:405–8.

25 Moshage HJ, Roelofs HMJ, Van Pelt JF *et al.* The effect of interleukin-1, interleukin-6 and its relationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. Biochem Biophys Res Commun 1988; **155**:112–7.