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Circulating levels of IL-11 and leukaemia inhibitory factor (LIF) do not significantly participate in the production of acute-phase proteins by the liver

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SUMMARY

To investigate the contribution of IL-11 and LIF to acute-phase protein (APP) production, we first analysed the effects of IL-11 and LIF on production of C-reactive protein (CRP), fibrinogen, and haptoglobin by human primary hepatocytes. We also measured the serum levels of IL-11, LIF, and CRP in serum from patients with inflammatory rheumatic diseases to assess the role of these cytokines in the APP response *in vivo*. We included patients with conditions associated with a high APP response such as rheumatoid arthritis (RA) or spondylarthropathy (SpA), and others usually associated with a weak APP response such as systemic lupus erythematosus (SLE), in order to investigate whether these cytokines could account for the differences in APP responses. Our results showed that IL-11 and LIF induced only minimal stimulation on production of APP by human primary hepatocytes compared with IL-6, known as the major inducer. Serum levels of CRP were elevated in RA and SpA, and significantly higher than in SLE patients. Despite the presence of a high APP response in some of our patients and despite the fact that we used sensitive assays to measure IL-11 and LIF, serum levels of both cytokines were not detected in any of the tested sera. In conclusion, our results show that circulating levels of IL-11 or LIF do not contribute significantly to the production of APP *in vivo*, and that they do not account for the difference in APP response between SLE and other inflammatory rheumatic diseases.

Keywords acute-phase proteins IL-11 leukaemia inhibitory factor IL-6 human primary hepatocytes

INTRODUCTION

The acute-phase proteins (APP) play an important role in body homeostasis during different inflammatory conditions. Numerous cytokines are known to contribute to the induction of their synthesis by the liver. In addition to IL-6, considered to be the major inducer [1,2], IL-1 [3], tumour necrosis factor-alpha (TNF-α) [3], IL-11 [4] and LIF [5] have been shown to stimulate the synthesis of APP. Both LIF and IL-11 share numerous biological effects with IL-6 on different tissues [6–8], including stimulation of APP synthesis by hepatoma cells [4,5]. However, results obtained with tumour cells can be difficult to interpret, since they are not always reproducible with human primary hepatocytes [2,9,10].

Only limited data are available on circulating levels of IL-11 or LIF [11,12] and on their possible role in the APP response *in vivo*, and particularly in patients with rheumatic diseases. In systemic lupus erythematosus (SLE), an autoimmune disease with highly inflammatory flares, the APP response is generally weak [13,14] compared with patients with chronic arthritis such as rheumatoid arthritis (RA) [15] or spondylarthropathies (SpA) [16]. Previous studies have already shown that these rather low APP levels in SLE are explained neither by a relative decrease in IL-6, whose serum levels have been found elevated in SLE [15,17–19], nor by serum levels of other cytokines such as IL-1β and TNF-α [20,21]. This suggests that in SLE either the effects of IL-6 or of other cytokines may be inhibited by some antagonists, or that serum levels of other inducing cytokines such as IL-11 or LIF may be low in comparison with other diseases.

We therefore first examined the role of IL-11 and LIF in the production of APP by human primary hepatocytes, and in parallel also measured circulating levels of these cytokines in serum of patients with various inflammatory diseases in order to determine their contribution to the APP response *in vivo*.

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MATERIALS AND METHODS

Reagents

Recombinant human (rh)IL-6 was a gift from Amgen Corp. (Thousand Oaks, CA). Its activity (7×10^7 U/mg) was determined

using the B9 cell proliferation assay. rhIL-11 was given by the Genetics Institute (Cambridge, MA). Its biological activity ($1{\cdot}5 \times 10^6$ U/mg) was determined using the T10 cell proliferation assay. rhLIF and rhIL-1β were purchased from British Bio-Technology Products (Abingdon, UK). The biological activities of LIF and of IL-1β, as indicated by the supplier, ranged from 3·3 to $6{\cdot}7 \times 10^6$ U/mg and from 1 to $3{\cdot}3 \times 10^8$ U/mg, respectively. All cytokines used in the present study contained <0·2 ng lipopolysaccharide (LPS)/μg protein as determined by the Limulus amoebocyte lysate assay (Chromogenix, Molndal, Sweden). Rabbit anti-human C-reactive protein (CRP) and anti-human fibrinogen antibodies were bought from Dako (Glostrup, Denmark); goat anti-human haptoglobin antibodies from Calbiochem (San Diego, CA); rabbit anti-human haptoglobin antibodies from Hoechst-Behring (Zürich, Switzerland); and peroxidase-conjugated goat anti-rabbit IgG antibodies from Sigma (St Louis, MO).

Human hepatocyte primary cultures

Primary hepatocytes were isolated as described previously [9,10]. Briefly, liver explants were taken from patients undergoing partial hepatectomy. The procedure received the approval of the institutional ethics committee of the Department of Surgery. Written informed consent was obtained from each patient. A wedge of macroscopically normal tissue (15–30 g) was perfused in the operating room, using a technique derived from the one described for rat hepatocytes [22]. The liver biopsy was then incised and liver cells were dissociated in RPMI 1640 supplemented with penicillin, streptomycin, and 10% fetal calf serum (FCS). The cell suspension was centrifuged at 40 ***g*** to separate hepatocytes from other cell types. Viable hepatocytes (75×10^3 cells/0·5 ml per well) were seeded on 48-well culture plates (Corning Glass, Corning, NY). After 48 h of culture the cells were stimulated. Supernatants were collected at 24 h, aliquoted, and kept frozen at −20°C until assessed for APP concentrations. Experiments were performed in duplicate.

ELISA for CRP and fibrinogen

The highly sensitive sandwich ELISA procedures used to measure the concentrations of CRP and fibrinogen in the cell supernatants were previously described [9].

ELISA for haptoglobin

The ELISA procedure used to measure the concentrations of haptoglobin was adapted from a previously described method [23]. Plates were coated with goat anti-haptoglobin antibodies and culture supernatants were added to the wells. The standard curve was prepared with human purified haptoglobin (generously given by Hoechst-Behring). Rabbit anti-haptoglobin antibodies were used as second antibody. Plates were washed and anti-rabbit IgG antibodies were added. The reaction with the substrate OPD (Dako) was stopped with sulphuric acid.

Serum studies

The study included sera from 20 patients with SLE, 20 patients with RA, 18 patients with SpA, and 20 healthy blood donors. All sera were aliquoted and stored at $\leqslant 20$°C until assessed for cytokine and CRP contents. A physical examination was performed on each patient at the time of cytokine determination. All SLE patients met 1982 American College of Rheumatology criteria for the classification of SLE [24]. Disease activity was assessed using the systemic lupus activity measure (SLAM) index, which includes 32 relevant clinical and biological parameters [25]. The results of the SLAM index ranged from 1 to 42 (median 8·5) at the time of examination. All RA patients met 1987 American College of Rheumatology criteria for the classification of RA [26]. Sixteen patients had a history of positive latex tests for IgM rheumatoid factor. All patients had clinical signs of active synovitis. One had clinical signs of cutaneous vasculitis. The 18 patients with SpA met the criteria recently proposed by the European Study Group [27]. All had clinical signs of synovitis. Patients with superimposed infection were excluded from this study.

IL-11 and LIF determination

IL-11 and LIF levels were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN). The sensitivity of these assays was 30 pg/ml for both cytokines.

CRP determination

Serum CRP levels were measured by nephelometry using a Behring nephelometer. The detection limit and normal values of CRP in this assay were 3 mg/*l*.

Statistical analysis

Student's *t*-test was used to compare the effects of the different cytokines on the production of APP by hepatocytes; $P < 0{\cdot}05$ was considered significant. Mann–Whitney test was used to compare the levels of CRP. Bonferroni correction was applied to *P* values obtained by the Mann–Whitney test; corrected *P* values <0·05 were considered significant.

RESULTS

Effects of IL-11 and LIF on the production of CRP by human primary hepatocytes

Effects of IL-11 and LIF were first compared with those of IL-6, the major inducer of APP synthesis by the liver. CRP concentrations were measured by ELISA in supernatants from primary hepatocytes stimulated with IL-6, IL-11, or LIF for 24 h. As shown in Fig. 1a, both IL-11 and LIF had only minimal effects on CRP production in comparison with IL-6. The induction produced by IL-11 or LIF, even at high concentrations (100 ng/ml), was always smaller than the effects of low concentrations of IL-6 (0·1 ng/ml).

Effects of IL-11 and LIF in combination with IL-1

CRP is defined as a class 1 APP based on the activating effect of IL-1 on its synthesis by hepatoma cells [28]. The production of other class 1 APP has also been found to be strongly induced by IL-1 in the presence of IL-6 [29] or IL-11 [4] in tumour hepatocytes. In contrast, it has recently been shown that IL-1β, alone or in the presence of IL-6, does not stimulate the production of certain class 1 APP by human primary hepatocytes [9]. To investigate further the role of IL-1 in the presence of other cytokines, we examined the effect of IL-1β in combination with either IL-11 or LIF on primary hepatocytes. Consistent with the results on IL-6, IL-1β, in the presence of IL-11 or LIF, was devoid of any additional effects on CRP production by these cells (Fig. 2a).

Effect of IL-11 and LIF in combination with IL-6

As the effects of either IL-11 or LIF on CRP production were

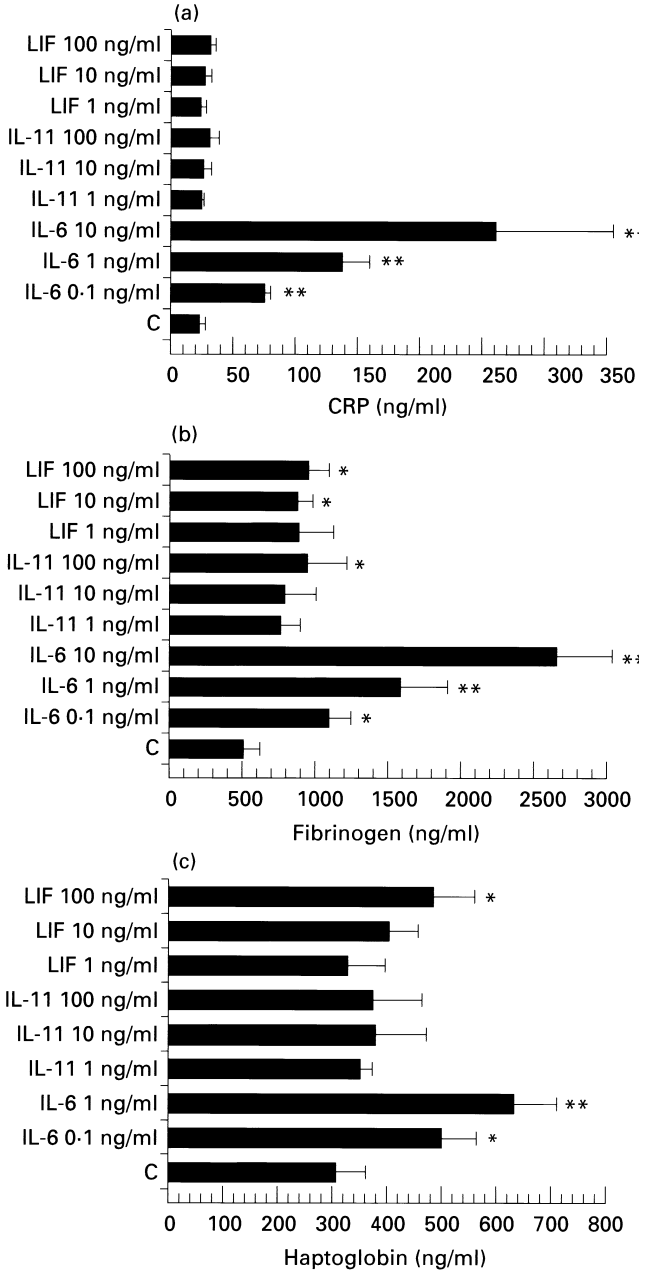

Fig. 1. Human primary hepatocytes were stimulated at the indicated concentrations of IL-6, IL-11, or LIF. After 24 h of stimulation, the cell supernatants were collected and their content in C-reactive protein (CRP), fibrinogen, and haptoglobin tested by specific ELISAs (see Materials and Methods). The values represent the mean ± s.d. of three different experiments. Each experiment was performed and analysed in duplicate. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$ compared with unstimulated cells.

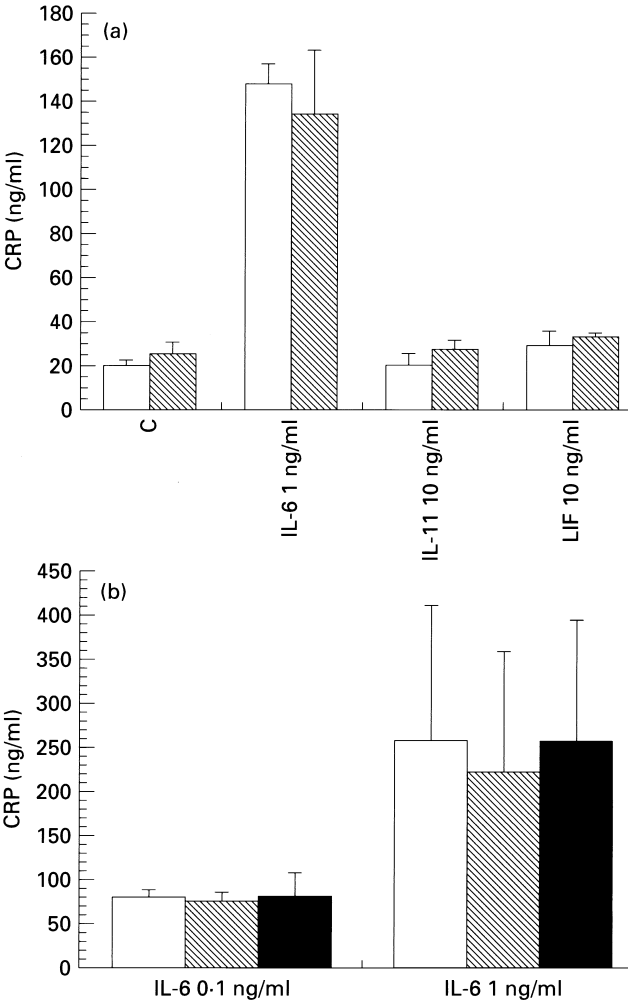

Fig. 2. Human primary hepatocytes were stimulated (a) at the indicated concentrations of IL-6, IL-11, or LIF in the presence (▨) or absence of IL-1β (□); (b) with IL-6 alone (□) and in the presence of IL-11 (▨) or LIF (■). The supernatants were collected and analysed for their content of C-reactive protein (CRP). The values represent the mean ± s.d. of two experiments. Each experiment was performed and analysed in duplicate.

quantitatively weaker than those of IL-6, we attempted to determine whether combinations of IL-11 or LIF with IL-6 may reduce IL-6-induced CRP production by decreasing the ability of IL-6 to interact with their shared gp130 receptor. Alternatively, IL-11 or LIF could play significant roles in enhancing CRP production by synergizing with IL-6. As shown in Fig. 2b, IL-6-induced CRP production was not changed in any way by addition of either IL-11 or LIF, even in excess amounts.

Effects of IL-11 and LIF on the production of fibrinogen and haptoglobin by human primary hepatocytes

Both haptoglobin and fibrinogen are considered to be class 2 APP based on the effects of IL-6-related cytokines and on the absence of stimulation by IL-1 [28]. IL-6, IL-11 and LIF have been shown to stimulate the synthesis of both fibrinogen and haptoglobin by hepatoma cell lines. As shown in Fig. 1b,c, the production of these APP was induced by IL-6, IL-11 or LIF in human primary hepatocytes. However, the quantitative effects of IL-11 or LIF, even in concentrations of 100 ng/ml, never reached those obtained with low concentrations (0·1 ng/ml) of IL-6.

Serum studies

To examine whether circulating levels of IL-11 or LIF participate in the APP response *in vivo*, we measured the concentrations of these cytokines in serum of patients with rheumatic diseases. We included patients with diseases associated with a high APP response (i.e. RA and SpA) and others usually characterized by a weak APP response (i.e. SLE), in order to determine whether

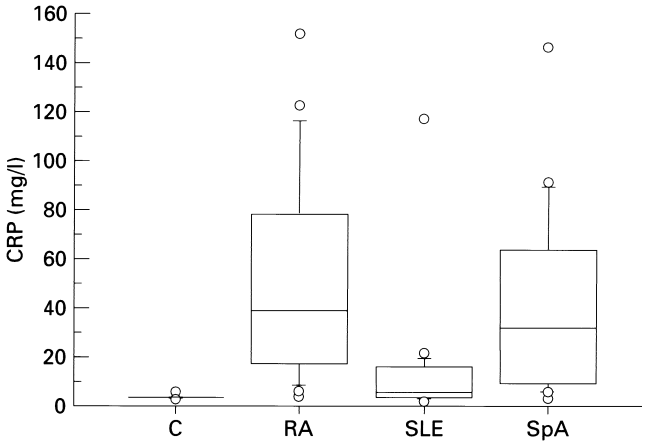

Fig. 3. Levels of C-reactive protein (CRP) in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), spondylarthropathy (SpA), and controls. Concentrations of CRP were determined by nephelometry. The horizontal line within boxes represents the median; boxes show the interquartile range (± 25% of the median), vertical bars show the 95% confidence interval (value above or below these levels were plotted separately).

circulating levels of these cytokines vary between these groups. As shown in Fig. 3, we found that serum levels of CRP were elevated in most RA or SpA patients (median 38·5 mg/*l* and 31·7 mg/*l*, respectively). These values were significantly higher than in controls (median 3 mg/*l*) ($P < 0{\cdot}0001$ for both) and than in SLE patients (median 5·4 mg/*l*) ($P < 0{\cdot}0001$ and $P < 0{\cdot}01$, respectively). Although most patients with RA or SpA had a high APP response and the assays used to measure IL-11 or LIF were very sensitive (detection of cytokine concentrations clearly lower than those added to the cell cultures), both cytokines were not detected in any of the tested serum.

DISCUSSION

This is the first study of the contribution of circulating IL-11 and LIF to the APP response based on both *in vitro* effects on human primary hepatocytes and serum concentrations. Our results clearly show that this contribution is not significant, and that these cytokines do not account for the low CRP levels encountered in SLE in comparison with either RA or SpA patients.

LIF levels have already been studied in various body fluids. Elevated concentrations of LIF were detected in cerebrospinal, peritoneal, and pleural fluids from patients with various infectious diseases [11]. High levels were also found in synovial fluid (SF) from approximately one-quarter of a series of patients with RA and other inflammatory arthritides, but these values did not correlate with clinical or laboratory signs of disease activity [30]. In contrast, serum LIF was detected in only a few patients with septic shock and in none with rheumatic diseases [11]. Accordingly, we could not detect the presence of LIF in any of the sera tested, although most patients had obvious signs of disease activity. Some investigators have found elevated LIF levels in giant cell arteritis, but not in other vasculitis [12]. Interestingly, there were no correlations between LIF and CRP concentrations and, in contrast to IL-6 and CRP, the levels of LIF did not decrease when patients were treated, supporting the hypothesis that although it is present in some inflammatory conditions, LIF does not significantly contribute to the APP response. Similar to IL-6 [31,32] and LIF [33], IL-11 is produced by chondrocytes and synoviocytes and its synthesis is also regulated by IL-1 [8]. The presence of elevated SF or serum IL-11 levels would therefore be expected in patients with inflammatory joint involvement. However, as for LIF, circulating IL-11 levels were not detectable in any of the tested sera.

IL-11 and LIF belong to the same family of cytokines related to IL-6. They exert their biological effects after binding to their receptor at the surface of cells. These receptors are heterodimers consisting of a cytokine-specific α subunit, i.e. gp80 IL-6R for IL-6, and a β subunit, the gp130, common to all these cytokines. This glycoprotein of 130 kD has a long membrane-spanning and intracytoplasmic domain and serves as the signal tranducer [34–37]. The fact that these mediators share a variety of biological effects on different cells is related to their ability to interact with this common receptor subunit. However, quantitative differences in their effects have been demonstrated in several *in vitro* studies [7,34,38]. These variations also affect the expression of APP genes. IL-6 has been found to be a more potent inducer of the expression of different APP genes than either IL-11 or LIF in human hepatoma cells [4,5]. In addition, the effects of these cytokines vary according to the different APP. Production of haptoglobin was only weakly induced by IL-11 compared with LIF [4], whereas synthesis of other APP was equally stimulated by either cytokine. Accordingly, our results on human primary hepatocytes show that the effects of LIF and IL-11 are clearly weaker than those of IL-6, particularly on the production of CRP. In pharmacological doses, however, these cytokines appear to affect the APP response; thus, in cancer patients, treatment with rhIL-11 was associated with a rise in serum CRP levels [39]. However, the doses received by these patients were very high, and indirect effects mediated by rises in levels of other cytokines cannot be excluded. In addition to these quantitative variations, qualitative differences regarding the biological effects of IL-6-related cytokines have been shown on different cells [37,40,41]. Several mechanisms may explain these qualitative or quantitative variations. A complete or relative absence of specific α or β receptors at the surface of cells may decrease cell responsiveness to certain cytokines [34,37]: for instance, the hepatoma cell line Hep3B, which has been shown to be unresponsive to LIF and ciliary neurotrophic factor (CNTF) but responsive to IL-6 or IL-11, does not express LIF-R [42]. Other mechanisms, including activation of different intracellular signals, may also be implicated in the variations of cell responsiveness to the various IL-6-related cytokines.

In conclusion, this study shows that serum levels of IL-11 and LIF are very low and that these cytokines induce only minimal stimulation of APP production by human hepatocytes. Taken together, these results indicate that IL-11 and LIF do not significantly contribute to the APP response, and that the weak APP response in SLE can therefore not be explained by selectively low levels of these cytokines.

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