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Interleukin 4 inhibits the production of some acute-phase proteins by human hepatocytes in primary culture

Pascal Loyer[a,*], Gennady Ilyin[a], Ziad Abdel Razzak[a], Jacques Banchereau[b], Jean-François Dezier[a], Jean-Pierre Campion[a], Christiane Guguen-Guillouzo[a], André Guillouzo[a]

[a]*Institut National de la Santé et de la Recherche Médicale; Unité de Recherches Hépatologiques U-49, Hôpital Pontchaillou, 35033 Rennes Cedex, France*

[b]*Schering-Plough, Laboratoire de Recherches Immunologiques, 27 Chemin des Peupliers, 69571 Dardilly Cedex, France*

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Interleukin 4 (IL4) has been shown to exhibit anti-inflammatory effects by inhibiting the secretion by monocytes of proinflammatory cytokines such as interleukin 1 (IL1), interleukin 6 (IL6), and tumor necrosis factor (TNF) and by inducing the secretion of the IL1 receptor antagonist. We investigated the role of this cytokine on the production of acute-phase proteins in primary human hepatocyte cultures. Cells were exposed to either IL4 and/or IL6, the most potent mediator of hepatic acute phase proteins. IL4 led to decreased production of haptoglobin, C-reactive protein and albumin while α1-antitrypsin and fibrinogen remained unaffected. These inhibitory effects of IL4 were also observed at the mRNA level. In addition, IL4 inhibited the IL6-induced production of haptoglobin although it had no effect on the induced C-reactive protein and fibrinogen. Our results demonstrate that IL4 can affect the production of a subset of acute-phase proteins by human hepatocytes and can antagonize some of the effects of IL6. These observations reinforce the notion that IL4 can be considered as an anti-inflammatory cytokine.

Interleukin 4; Interleukin 6; Acute phase protein; Human hepatocytes; Primary culture

1. INTRODUCTION

The acute-phase response is characterized by a set of hepatic disturbances which include marked variations in the concentration of some plasma proteins [1–4], impairment of drug metabolism [5,6] and alterations of lipid metabolism [7].

Acute-phase protein patterns vary from one species to another. Thus, C-reactive protein (CRP) and serum amyloid A show the highest increase in man [8] whereas in the rat, α_2-macroglobulin and α_1-acid glycoprotein exhibit the most substantial increases. In many species, plasma concentrations of fibrinogen, α_1-antitrypsin, haptoglobin and α_1-anti-chymotrypsin also increase and, simultaneously, albumin and transferrin plasma levels decrease [1,9–13].

The acute-phase response is mediated by cytokines [14], six of which have been reported to modify production of plasma proteins by the hepatocyte. The most potent mediators are IL6 [8–10,13,15], interleukin-1 (IL1) [13] and tumor necrosis factor α (TNFα) [13]. IL6 induces the broadest range of proteins [15]. By contrast, interferon γ (IFNγ) down-regulates only a few proteins [1]. Two additional cytokines, i.e. leukemia inhibitory factor (LIF) [16] and transforming growth factor β (TGFβ) are now recognized to be capable of modulating production of acute-phase proteins [17–20]. However, it has now become apparent that changes in many hepatic plasma proteins during the acute-phase response are not mediated by a single cytokine but rather by a combination of cytokines [19].

Interleukin 4 (IL4) produced by T lymphocytes [21] and mast cells [22], displays a wide range of biological effects [23,24] as suggested by the expression of its specific receptor in nearly all the cell types [25,26]. Up to now, the effects of IL4 on the production of acute-phase proteins by hepatocytes have not been studied. The only effect reported on hepatic cells, is the inhibition of the lipogenesis stimulation by IL1, IL6 and TNFα in mouse hepatocytes [27]. These effects as well as the inhibition of IL1, TNFα, prostaglandin E2 [28,29] and stimulation of IL1 receptor antagonist (IL1ra) production by IL4 in human monocytes [30], suggest that IL4 could act as an anti-inflammatory mediator. This led us to investigate whether other liver functions, including production of plasma proteins that are increased during acute-phase response, could be modulated by IL4. In the present report, we show that IL4 can affect the production of various plasma proteins in human hepatocytes and negatively regulate some of the effects induced by IL6.

2. MATERIALS AND METHODS

2.1. *Materials*

Cell culture reagents were purchased from Gibco (Cergy-Pontoise, France). Recombinant human IL4 (specific activity: 10^7 U/ml) was

*Corresponding author. Fax: (33) (99) 54 01 37.

provided by Schering-Plough Research Institute (Kenilworth, NJ). Purified recombinant human IL6 used was provided by Genzyme (Cambridge, UK) and used at 50 U/ml. Semi-purified IL6 obtained from transfected COS cells, was also used in some experiments. When tested in parallel, both purified- and semi-purified IL6 batches gave similar quantitative results (not shown).

Antisera from rabbit anti-CRP, anti-haptoglobin, anti-α_1-antitrypsin and anti-fibrinogen were from Hoechst-Behring (France), goat anti-CRP and anti-haptoglobin from Calbiochem and rabbit anti-albumin from Cappel (USA).

The human albumin and β-fibrinogen cDNAs were gifts from A. Dugaiczyk and G. Marguerie, respectively. cDNA probes for haptoglobin, CRP and α_1-antitrypsin were purchased from the American Type Culture Collection (ATCC, Rockville, USA). 5'-[α-^{32}P]triphosphate deoxycytidine (3000 Ci/mmol) was from Amersham (Les Ulis, France).

2.2. *Cell cultures*

Human hepatic tissue biopsies were obtained from seven donors (HLI-7) in agreement with French legislation. The group of donors was composed of three females (HL1 = 53, HL3 = 41 and HL5 = 73 years old) and four males (HL2 = 46, HL4 = 64, HL6 = 52 and HL7 = 50 years old). Hepatocytes were isolated by a two-step collagenase perfusion procedure as previously described [32]. The average cell viability was 81 ± 6%. Hepatocytes were seeded at 1.5×10^5 cells/well in 24 multi-well plates for protein concentration determination, or at 10^7 cells/75 cm^2 flask for total RNA preparation, in a mixture of 75% minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum and, per ml: 100 IU penicillin, 100 μg streptomycin sulphate, 1 mg bovine serum albumin and 5 μg bovine insulin. After cell attachment (e.g. around 10 h later), the medium was renewed with the same medium deprived of fetal calf serum and supplemented with 7×10^{-5} M hydrocortisone hemisuccinate. At this time, the cytokines were added and it was changed every day thereafter. Conditioned media were collected and frozen prior to determination of plasma protein concentrations.

2.3. *ELISA assays*

Concentration of CRP and haptoglobin in culture medium was determined by a sandwich enzyme immunoassay (ELISA). 96-well microtiter plates were coated with goat anti-CRP or anti-haptoglobin antibodies diluted to 1/2500 and 1/2000, respectively, in 0.1 M carbonate buffer, pH 9.6, for 18 h at 4°C. Wells were then washed two times with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20. Blocking was performed for 60 min at room temperature with PBS containing 3% (w/v) bovine serum albumin. After washes, standards or samples were added to the wells, followed by an 18 h incubation at 4°C. After three PBS-Tween washes, rabbit antibodies against CRP (1/750 dilution in PBS-Tween) or against haptoglobin (1/1000 dilution) were added and incubated for 120 min at 37°C. After three washes, the wells were incubated with peroxidase-conjugated anti-rabbit IgG (Sigma, 1/25000 dilution in PBS-Tween) for 60 min at 37°C. Plates were washed three times with PBS-Tween and 100 μl of the peroxidase substrate solution (citrate-phosphate buffer, pH 5.0, containing 0.4 mg of *O*-phenylendiamine/ml and 0.01% H_2O_2, v/v) were added to each well. Color development was stopped by the addition of 50 μl of 2.5 M sulfuric acid and plates were read at 492 nm using a microplate photometer (Titertek Multiscan MCC/340, Labsystems, Finland). Standards for the determination of CRP and haptoglobin levels, N/T CRP control serum and N/T protein standard serum were from Behring.

2.4. *Immunonephelometry*

Albumin, α_1-antitrypsin and fibrinogen production was quantified in the culture medium by laser immunonephelometry [33]. The validity of this method was confirmed by the linearity of the reference curve obtained by addition to the medium of increasing amounts of standards. Results were expressed as ng/ml/24 h.

2.5. *Statistical analysis*

Values (as percent of variation with the corresponding control value), were analysed by Student's *t*-test, and the criterion for significance of the differences between IL4- or IL6-treated cultures versus controls was $P < 0.05$.

2.6. *RNA analysis*

Total RNAs were extracted by the guanidium thiocyanate procedure [34] and quantified. For each sample, 2 μg of total RNA were subjected to electrophoresis and stained with ethidium bromide to check quality and quantification of the RNAs. For dot blot hybridization, aliquots of 10 and 5 μg RNA were spotted onto nylon N$^+$ membrane (Amersham) that had been pretreated with 20 × standard saline citrate. For Northern blot analysis, 20 μg of total RNA were separated by electrophoresis in 1.5% agarose gels, in 10 mM phosphate buffer, pH 7.4 containing 1.1 M formaldehyde and then transferred onto nylon N$^+$ membranes. RNA amounts in each lane and transfer efficiency were verified by staining the gel with ethidium bromide. The Northern and dot blots were prehybridized according to Andrews et al. [35]. Hybridizations with 20 to 25×10^6 cpm/membrane of [α-^{32}P]dCTP nick-translated DNA probes were carried out for 18 h. Post-hybridization washes were performed at 65°C in 1 × standard saline citrate, 0.1% SDS. Filters were autoradiographied using hyperfilms MP (Amersham, UK) with DuPont lightning Plus intensifying screens at −80°C.

3. RESULTS

The effect of IL4 on haptoglobin production was investigated in human hepatocyte primary cultures incubated with various concentrations of the cytokine ranging from 1 to 150 U/ml for 4 days (Fig. 1). Hepatocyte cultures were also exposed to IL6 (50 U/ml) which stimulatory effect on the production of acute phase protein is well known. The medium was totally renewed every day and the levels of proteins were measured in the culture supernatants harvested daily. After three

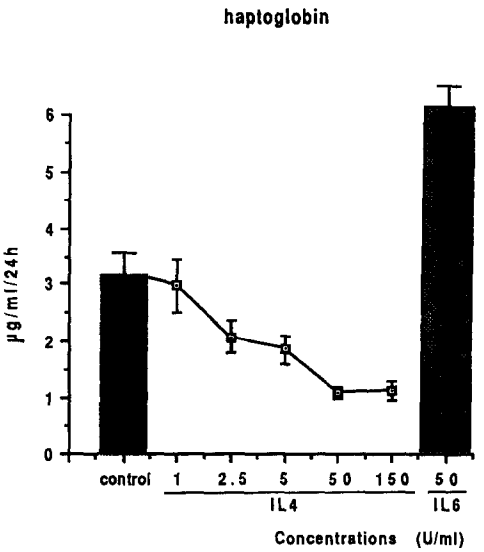

Fig. 1. Dose–response of haptoglobin production to IL4 exposure. IL4 was added daily during 3 days, at 1, 2.5, 5, 50 and 150 U/ml to determine dose–response effects on haptoglobin level at day 3. IL6 was added daily at 50 U/ml. The values are the mean ± S.D. (bars) of four dishes, and are expressed as μg/ml/24 h.

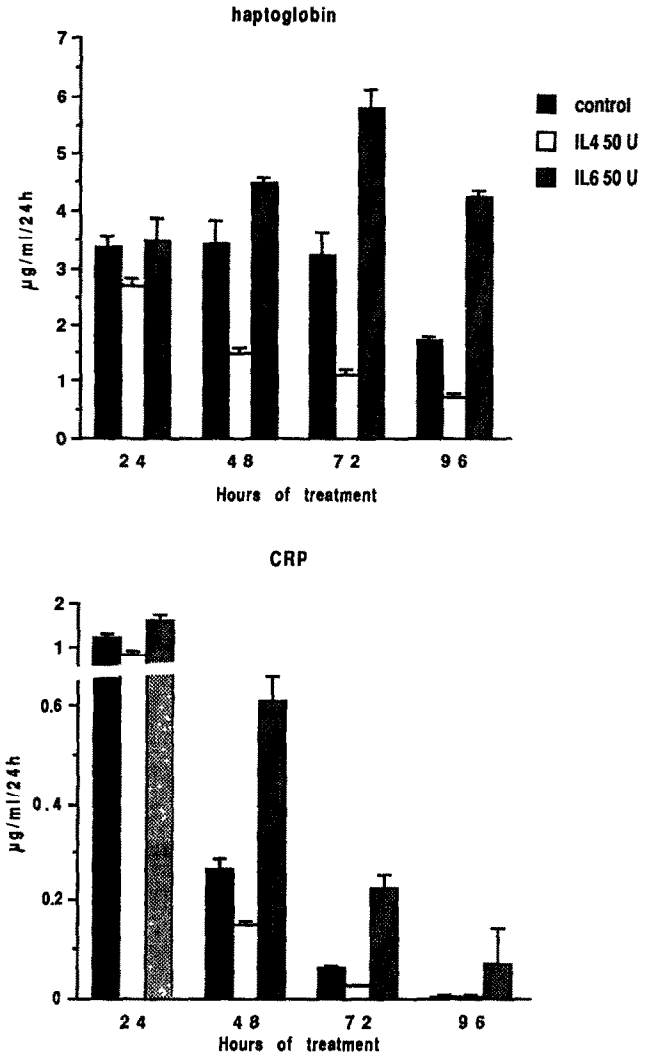

Fig. 2. Time-course analysis of the effects of IL4 and IL6 on haptoglobin and C-reactive protein production. Hepatocytes were treated 10 h after plating with IL4 (50 U/ml) or IL6 (50 U/ml) and daily thereafter during 4 days. The 24 h-conditioned media from control, IL4- and IL6-cultures were collected during the 4 days and used for determination of haptoglobin and C-reactive protein production rates.

days of treatment, haptoglobin secretion was decreased by IL4 in a dose-dependent manner; in contrast, it was increased by IL6. IL4 had a maximal inhibitory effect (60%; mean of 2 experiments) on haptoglobin secretion when used at a concentration of 50 U/ml and above. No significant difference was observed with a higher concentration (i.e. 150 U/ml). Consequently, IL4 was used at 50 U/ml for further experiments.

The kinetics of the modulatory effects of IL4 (50 U/ml) and IL6 on haptoglobin and CRP production were then studied during a 4-day culture (Fig. 2). IL6 did not modify haptoglobin production during the first 24 h, and only slightly increased it after 48 h. The secretion of haptoglobin was markedly augmented (2 to 3-fold) by IL6 after 72 and 96 h. IL6 also increased CRP production, whatever the culture time. However, IL6 did not prevent the decrease at 48, 72 and 96 h (< 0.1 µg/ml/24 h). Haptoglobin and CRP production were both slightly down-regulated by IL4 during the first day. IL4 had a strong inhibiting effect on the production of these two proteins after 72 h. The decreased production could still be observed at day 4 with haptoglobin but not with CRP which secretion was virtually abolished at that time.

Hepatocytes from seven different donors were incubated with IL4 and IL6 for 3 days, and the secretion of albumin, fibrinogen and α_1-antitrypsin was compared with that of haptoglobin and CRP. The effects measured at day 3 are presented in Table I. IL6, used as a reference mediator, induced a typical response characterized by an increased level of haptoglobin, fibrinogen, CRP and α_1-antitrypsin, and a depressed production of albumin. The strongest effect was observed for fibrinogen, the concentration of which reached 5-fold the control value after a 3-day exposure. A 30% decreased secretion of albumin was found after 72 h. IL4 did not alter production of fibrinogen and α_1-antitrypsin, whereas it depressed haptoglobin and CRP. Surprisingly, IL4 decreased the production of albumin as much as did IL6.

The effects of IL6 and IL4 on the expression of haptoglobin, albumin, β-fibrinogen and α_1-antitrypsin were also analysed at mRNA levels by dot blotting using specific cDNA probes (Fig. 3A). Total mRNA was extracted from hepatocytes cultured for 4 days with IL6 or IL4 and probed successively with the different specific cDNAs. In IL6-treated cells, mRNA levels of β-fibrinogen, α_1-antitrypsin and haptoglobin were increased by 9-, 4- and 5-fold, respectively, while the albumin mRNA level dropped to 10% of the control value. Albumin and haptoglobin mRNA levels were decreased, respectively 2- and 3-fold by IL4 treatment (50 U/ml), whereas β-fibrinogen and α_1-antitrypsin mRNA levels were unaffected.

The decrease of CRP protein observed from 48 h of culture, was also found at the mRNA level but after three or four days CRP mRNAs were only detectable as very faint bands which did not permit accurate comparisons (not shown). Thus, the effects of IL6 and IL4 on CRP transcripts were evaluated by Northern-blot analysis after a 24 h treatment (Fig. 3B). A marked increase of CRP mRNA was observed in 24 h control cultured cells compared to their freshly isolated counterparts. This increase was transient and CRP mRNA became undetectable in 72 and 96 h control cultures (not shown). After 24 h of treatment, the levels of CRP mRNA were increased by IL6 and decreased by IL4. Thereafter, the membrane was hybridized with haptoglobin cDNA probe and variations of haptoglobin mRNA levels were compared to those observed for CRP. No early induction of haptoglobin mRNA was observed when isolated hepatocytes were placed in culture. A 24 h exposure to IL4 considerably reduced hap-

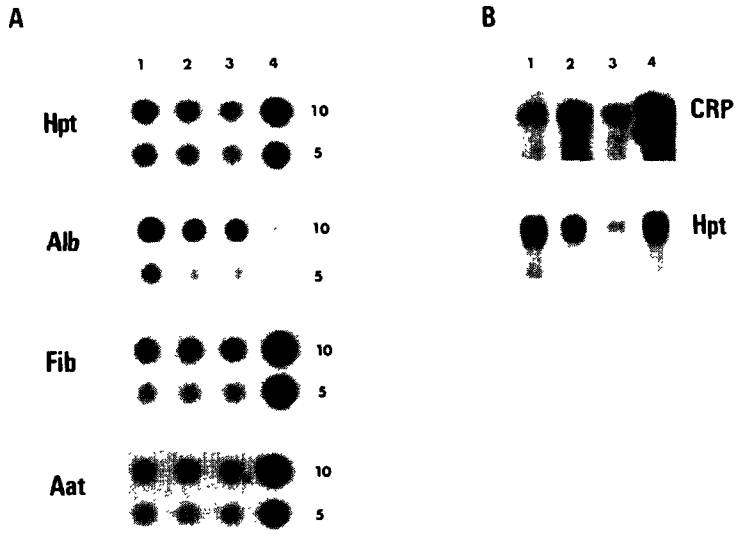

Fig. 3. Effects of IL4 and IL6 on levels of haptoglobin, albumin, β-fibrinogen, α_1-antitrypsin and C-reactive protein mRNAs. (A) After 96 h of culture, total RNA was isolated in control (lane 1), IL4 (5 and 50 U/ml, lanes 2 and 3), and IL6 (recombinant semi-purified cytokine, lane 4)-treated cultures and subjected to dot blot analysis (10 and 5 μg) with haptoglobin (Hpt), albumin (Alb), fibrinogen (Fib), and α_1-antitrypsin (Aat) specific cDNA probes. (B) Northern blot analysis of CRP and haptoglobin (Hpt) mRNA levels in freshly isolated hepatocytes (lane 1), and in 24 h control cultures (lane 2), IL4 (50 U/ml, lane 3) and IL6 (50 U/ml, lane 4)-treated cultures.

toglobin mRNA levels. In contrast, incubation with IL6 increased them.

In order to better define the potential role of IL4 during the acute phase response, the effects of IL4 on IL6-stimulated hepatocytes were investigated in two experiments. Hepatocyte cultures were exposed to a single dose of IL6 (50 U/ml) or IL4 (50 U/ml) or simultaneously treated with both IL6 and IL4 (50 U/ml each) and the levels of CRP, fibrinogen and haptoglobin were measured after 2 and 3 days of exposure (Fig. 4). As observed in Fig. 2, treatment by IL4 decreased haptoglobin while IL6 stimulation triggered overexpression of this protein. Simultaneous treatment with IL6 and IL4 resulted in a complete inhibition of haptoglobin induction by IL6 ($P < 0.05$). In contrast, IL4 did not affect stimulation of CRP and fibrinogen production by IL6 (not shown).

4. DISCUSSION

In the present study, using primary cultures of normal human hepatocytes, we confirmed that IL6 acts as

Table I

Haptoglobin, albumin, C-reactive protein, fibrinogen and α_1-antitrypsin concentrations in human hepatocyte cultures treated with IL4 or IL6

	IL4					IL6				
	Mean % vs. control	S.D. (%)	range (%)	*n*	*P*	Mean % vs. control	S.D. (%)	Range (%)	*n*	*P*
Haptoglobin	45	16	25–69	26* 7‡	0.0013	185	43	150–287	26* 7‡	0.0001
Albumin	71	3.1	67–74	20* 5‡	0.001	69.7	5	63–75	20* 5‡	0.0001
C-reactive protein	54	20	42–78	13* 3‡	0.001	275	50	220–400	9* 3‡	0.0001
Fibrinogen	120	43	80–150	12* 3‡	0.055	506	260	220–725	12* 3‡	0.005
α_1-Antitrypsin	104	9	90–113	12* 3‡	0.33	263	37	244–470	8* 3‡	0.0001

Results are the average of tetraplicate ($n = 4$) cultures of independent experiments and expressed as percent of variation (mean ± S.D.) from the corresponding control value average (100%). IL4 (50 U/ml) and IL6 (50 U/ml) or semi-purified) were used and plasma protein levels were determined at day 3. The values which composed these different averages were analysed by the Student's *t*-test, and the criterion for significance of the differences between control and treated cultures was $P < 0.05$; *n*: *number of independent protein concentration determinations, ‡number of independent cultures; Range: minimal and maximal percent of variation vs. control (100%).

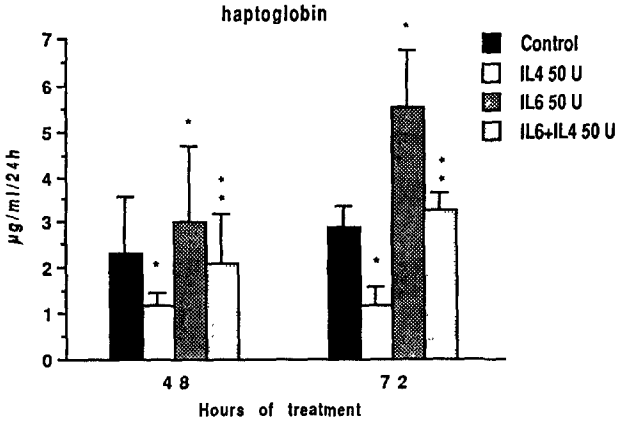

Fig. 4. IL4 effect on haptoglobin stimulation induced by IL6. Haptoglobin concentrations were determined in control, IL4 (50 U/ml), IL6 (semi-purified) and IL6 + IL4-treated (50 U/ml) cultures. Values are expressed as µg/ml/24 h, are the mean ± S.D. (bars) of two independent experiments in tetraplicate and were analysed by Student's *t*-test. $*P < 0.05$ IL4 or IL6 vs. control; $**P < 0.05$ IL6 + IL4 vs. IL6.

a very potent mediator of the hepatic acute phase response. The stimulation of fibrinogen, CRP, haptoglobin and α_1-antitrypsin by IL6 found in human hepatocytes demonstrated that these cells retained in vitro the capacity to respond to inflammatory mediators. More importantly, we demonstrated that IL4 can also affect the production by hepatocytes of some acute-phase proteins. IL4 inhibited the production of three of the five acute-phase proteins studied, i.e. haptoglobin, CRP and the negative acute-phase protein albumin. These effects were shown both at mRNA and protein levels.

The selectivity of IL4 effect on the production of only some proteins as well as the absence of general alteration of the total protein content and cell morphology, allow us to eliminate the possibility of a toxic effect of IL4 on hepatocytes. In addition, in a parallel study, we have recently shown that IL4 is also able to markedly affect cytochrome P450 expression in cultured human hepatocytes [31]. To our knowledge, this is the first evidence that IL4 can affect hepatic functions in humans.

Our findings also show that CRP, the most induced acute phase protein in man, was strongly enhanced following hepatocyte seeding while fibrinogen, haptoglobin and α_1-antitrypsin were not altered. The transient increase in CRP, also reported by Moshage et al. [12], appears to be related to the dissociation process and the adaptation of human hepatocytes to culture.

Potential anti-inflammatory effects of IL4 have been shown previously. Indeed, IL4 has been shown to inhibit production of IL1, TNFα and prostaglandin E2 [28,29] and to induce IL1 receptor antagonist secretion [30] by human monocytes. Grunfeld et al. [27] have confirmed that IL4 acts as anti-inflammatory mediator since it was able to inhibit stimulation by TNFα, IL1 and IL6, of hepatic lipogenesis in mouse in vivo. However, they did not observe any effect of IL4 by itself on lipogenesis. Our data show that IL4 can decrease haptoglobin, CRP and albumin production regardless of stimulation by recombinant IL6, and can modulate IL6-mediated induction of haptoglobin. Moreover, we have not shown by ELISA, the presence of endogenous IL6 (not shown), strongly suggesting that the effects of IL4 on haptoglobin, CRP and albumin are direct and not due to modulation of an endogenous IL6-response.

The fact that IL4 is able to act on negative (albumin) as well as on positive (CRP, haptoglobin) acute-phase proteins, and to antagonize some effects of IL6, leads us to consider IL4 as a modulator of hepatic plasma protein production and support the idea of its anti-inflammatory function. The data collected on the IL4 function raise the possibility that this cytokine (1) could be produced by liver-associated lymphocytes including large granular (i.e. Pit cells) and agranular lymphocytes [37,38], since these cells display natural killer and lymphokine-activated killer activities [38]; (2) could act on other hepatic functions (including plasma proteins and cytochrome P450 enzymes) and may antagonize and/or cooperate with other cytokines; (3) could be involved in the development of some liver diseases. Indeed, Schlaak et al. [39] have recently reported elevated IL4 production by liver infiltrating T cells in autoimmune hepatitis. Thus, it would be extremely important to determine whether lymphocytes located in normal and pathologic livers, produce IL4 and what is the incidence of this production on hepatic functions.

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