Heterogeneous modulation of acute-phase-reactant mRNA levels by interleukin-1 β and interleukin-6 in the human hepatoma cell line PLC/PRF/5

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The acute-phase response to tissue injury and inflammation is accompanied by a dramatic increase in the hepatic synthesis of plasma proteins known as acute-phase reactants (APRs). This response is mediated by cytokines produced in part by activated macrophages at the site of inflammation; glucocorticoids have also been implicated as playing a regulatory role. The effects of the cytokines interleukin (IL)-1 β and -6, alone or in combination, and in the absence or presence of the synthetic glucocorticoid dexamethasone, on the levels of APR mRNAs in the human hepatoma cell line PLC/PRF/5 were analysed. The accumulation of APR mRNAs [the complement components C3, factor B and C1 inhibitor; the major APRs C-reactive protein (CRP) and serum amyloid A protein and the CRP analogue serum amyloid P protein] was determined in dose-response and time-course studies. The APRs differed from each other in their responses to IL-1 β alone, IL-6 alone, and IL-1 β plus IL-6. Dexamethasone enhanced the cytokine-driven induction of a subset of APR mRNAs. These studies detail the heterogeneity of the 'in vitro' acute-phase response to defined mediators.

INTRODUCTION

The acute-phase response is the systemic reaction to tissue damage and inflammation caused by trauma, infections and other acute insults. Among the physiological changes that occur is an increase in the hepatic synthesis of a number of plasma proteins known as acute-phase reactants (APRs) [1]. The increase in plasma concentration of particular APRs ranges from 50% to 1000-fold. In man, the most dramatic increases are seen for the APRs serum amyloid A (SAA) and C-reactive protein (CRP). APRs that increase in concentration to a lesser extent are the complement components C3 and factor B (FB), proteinase inhibitors such as cysteine proteinase inhibitor, α_1 -proteinase inhibitor, C1 inhibitor (C1-inh), α_1 -antitrypsin (α_1 -AT) and α_1 -antichymotrypsin (ACH); and α_2 -macroglobulin, α_1 -macroglobulin, haptoglobin (HP), haemopexin, fibrinogen, caeruloplasmin and α_2 -acid glycoprotein (AGP) (for a review, see [2]). Albumin and transferrin decrease in plasma concentration and are termed 'negative' APRs.

Induction of the acute-phase response *in vivo* is mediated in part by cytokines produced by activated macrophages at the site of inflammation [1,3]. Induction of APRs *in vitro* is readily achieved in rat, mouse or human hepatoma cells or primary hepatocytes by incubation with medium from lipopolysaccharide-stimulated peripheral-blood monocytes (MoCM). Among the regulatory cytokines that are present in MoCM are interleukin-1 (IL-1 α and - β), interleukin-6 (IL-6), cachectin/tumour-necrosis factor, interferon- γ [4,5] and transforming growth factor β [6]. Treatment of cultured human hepatoma cells with either recombinant IL-1 β or recombinant IL-6 induces the synthesis of several APRs, including C3, caeruloplasmin, α_1 -AT, ACH, HP and AGP [7–12]; either IL-1 β or IL-6 will down-regulate the mRNA and protein synthesis of the negative acute-

phase proteins albumin and transferrin [7,8,10,12,13]. However, other APRs respond differently to IL-1 β and IL-6. For example, IL-6 induces the synthesis of fibrinogen, CRP, and SAA [9,13–15], whereas IL-1 β inhibits fibrinogen synthesis [8,9,11] and has been reported not to modulate CRP and SAA synthesis [7,8,16]. In many cases the individual effect of these cytokines on particular APRs *in vitro* does not account for the magnitude of induction observed during the acute-phase response *in vivo*. Therefore treatment of hepatoma cells with combinations of cytokines may elucidate the co-operative interactions of these cytokines and provide a model system that more accurately reflects the acute-phase response *in vivo*. In the present paper we describe the 'in vitro' response of the human hepatoma cell line PLC/PRF/5 to IL-1 β and IL-6, alone and in combination, and in the presence or absence of dexamethasone.

MATERIALS AND METHODS

Cell culture

Human PLC/PRF/5 hepatoma cells were a gift from Dr. Richard A. Mortensen (Department of Microbiology, Ohio State University, Columbus, OH, U.S.A.). Cultures were maintained in an air/CO $_2$ (19:1) atmosphere at 37 °C in Eagle's Minimal Essential Medium (EMEM; MA Bioproducts, Walkersville, MD, U.S.A.) with 10% (v/v) bovine calf serum (Hyclone, Logan, UT, U.S.A.) supplemented with 0.01 mM-sodium pyruvate 0.01 M-Hepes, gentamicin (50 μ g/ml; MA Bioproducts), and 0.01 mM non-essential amino acids (Sigma, St. Louis, MO, U.S.A.).

Cytokine treatment

Recombinant human IL-1 β [17] was the gift of Dr. D. E. Tracey (Upjohn Co., Kalamazoo, MI, U.S.A.).

Abbreviations used: APR, acute-phase reactant; C1-inh, C1 inhibitor; CRP, C-reactive protein; SAA, serum amyloid A protein; SAP, serum amyloid P protein; FB, factor B; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; MoCM, monocyte conditioned medium; α_1 -AT, α_1 -antitrypsin; FB, factor B; ACH, α_1 -antichymotrypsin; HP, haptoglobin; AGP, α_2 -acid glycoprotein; EMEM, Eagle's Minimal Essential Medium; 1 $\times$ SSC, 0.15 M-NaCl/0.015 M-sodium citrate; LAF, lymphocyte-activating factor.

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Table 1. Oligonucleotides used in Northern-Blot analysis

Serum protein	Region*	Reference
C1-inh	372–381	[19]
C3	251–260	[20]
FB	241–250	[21]
SAA	16–25	[22]

* Region of mRNA to which oligonucleotide is complementary-numbered according to reference listed.

Recombinant human IL-6 [18] was the gift of Dr. G. Wong (Genetics Institute, Cambridge, MA, U.S.A.). Cells were plated on to six-well tissue-culture plates (Falcon, Lincoln Park, NJ, U.S.A.) and grown to confluence. IL-1 β (1×10^7 LAF units/mg) and IL-6 (1×10^6 T1165 units/mg) were diluted in culture medium to appropriate concentrations and added to the cells, alone or in combination, at 1.0 ml/well. Dexamethasone (Sigma) was added to a final concentration of 10^{-8} M where appropriate. Plates were incubated at 37 °C and the cells were harvested at specific intervals and quick-frozen in liquid N₂ before RNA preparation.

DNA probes

Oligonucleotide probes were made using an Applied Biosystems 380B DNA synthesizer (Foster City, CA, U.S.A.), were 30 b in length, and contained 50% G+C (Table 1). Oligonucleotides were end-labelled with [γ -³²P]ATP (New England Nuclear; 6000 Ci/mmol) to a specific radioactivity of $(1-5.8) \times 10^8$ d.p.m./ μ g.

cDNA probes for CRP [23] and SAP [24] were radiolabelled with [α -³²P]dCTP (New England Nuclear; 6000 Ci/mmol) to specific radioactivities of $(1.1-3) \times 10^9$ d.p.m./ μ g by using an oligolabelling kit (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.).

Labelled probes were separated from unincorporated dNTPs using prepacked Sephadex G-50 nick columns (Pharmacia Fine Chemicals).

RNA extractions and Northern-blot analysis

RNA was extracted by the LiCl/urea method [25]. For Northern-blot analysis, 10 μ g of each sample was electrophoresed on a denaturing 1%-agarose/formaldehyde gel and transferred to nitrocellulose acetate membranes (Schleicher and Schuell, Keene, NH, U.S.A.). The membranes were baked at 80 °C for 2 h and prehybridized for 3 h in $3 \times$ SSC/ $3 \times$ Denhardt's/tRNA (100 μ g/ml; Sigma)/0.1 mM-EDTA (pH 7.5)/0.2% SDS/45% deionized formamide/0.05% sodium pyrophosphate. Radiolabelled probes were added to prehybridized membranes and hybridized at 30 °C (oligonucleotide probes) or 50 °C (cDNA probes) for 24 h. Membranes probed with oligonucleotides were washed for 20 min at 55 °C in $6 \times$ SSC/0.1% SDS/0.05% sodium pyrophosphate. Membranes probed with cDNAs were washed for 30 min at room temperature in $2 \times$ SSC/0.1% SDS and 60 min at 55 °C with $0.2 \times$ SSC/0.1% SDS. The membranes were exposed to X-ray film (Kodak, Rochester, NY, U.S.A.) at -70 °C in the presence of intensifying screens.

Gel scans

The intensities of 18 S RNA bands on photographs of ethidium bromide-stained formaldehyde gels and of mRNA bands on autoradiographs were measured by using an LKB Ultrascan XL

laser densitometer. The densitometry measurements of 18 S bands were used to correct for minor loading variations and the measurements of autoradiograph band intensity were adjusted appropriately before plotting data.

RESULTS AND DISCUSSION

Confluent PLC/PRF/5 cells were treated with various concentrations of IL-1 β and IL-6, alone or in combination. Experiments were performed in the presence or absence of 10^{-8} M-dexamethasone, a concentration at which dexamethasone-dependent responses were found in preliminary experiments to be maximal (results not shown) and which approximates to the levels of glucocorticoids present during the acute-phase response *in vivo* [26]. The cells were treated with cytokines and/or dexamethasone for 20 h at 37 °C, and total RNA was harvested for Northern-blot analysis. Fig. 1 shows the heterogeneity of the response to IL-1 β , IL-6 and dexamethasone with respect to the accumulation of mRNA for C3, FB, C1-inh, the major APRs SAA and CRP, and the CRP analogue SAP.

C3

C3 mRNA levels increased severalfold in response to either IL-1 β alone (Fig. 1, lanes G-I, and Fig. 2a; a maximum 4.5-fold increase with 10 ng of IL-1 β /ml) or IL-6 alone (Fig. 1, lanes N-P; a maximum 5.4-fold increase with 100 ng of IL-6/ml). High concentrations of IL-1 β appeared to be less effective in mediating the increase. The response to low concentrations of IL-6 was enhanced by dexamethasone (Fig. 1, lanes K-M; 3-fold for 10 ng of IL-6/ml). However, the highest concentration of IL-6 (Fig. 1, lane P) gave rise to substantially higher levels of C3 mRNA in the absence of dexamethasone. The addition of 10 ng of IL-1 β /ml and 10 ng of IL-6/ml in combination had essentially an additive effect, increasing C3 mRNA levels to 7-fold over background levels (Fig. 1, lane J); this increase was slightly enhanced by the addition of dexamethasone (Fig. 1, lane E), possibly reflecting the capacity of dexamethasone to enhance the effect of IL-6 at the concentration used. Induction of C3 protein in PLC/PRF/5 cells by treatment with 100 ng of IL-1 β /ml alone or 100 ng of IL-6/ml alone has previously been reported by Ramadori *et al.* [10]; the C3 levels were increased in that study 236 and 243% over control levels respectively. A 2-fold induction of C3 protein synthesis in response to MoCM in HepG2 cells has been reported by Baumann *et al.* [11], whereas a 20-fold increase in C3 protein in Hep3B2 cells in response to MoCM has been reported by Darlington *et al.* [8].

The time course of induction of C3 mRNA levels in response to 5 ng of IL-1 β /ml and 50 ng of IL-6/ml, singly and in combination, and in the presence of 10^{-8} M-dexamethasone, is shown in Fig. 3. C3 mRNA levels decreased immediately after the addition of minimal essential medium containing dexamethasone only (i.e. in the absence of cytokine), but returned to background levels by 36 h. This may reflect a down-regulation of C3 by dexamethasone in the absence of inflammatory cytokines. The role of glucocorticoids as anti-inflammatory agents may therefore involve direct action on hepatocytes in addition to the established inhibition of cytokine production by monocytes/macrophages [27]. C3 mRNA levels also decrease immediately after the addition of 5 ng of IL-1 β /ml, suggesting that the down-regulatory effect of dexamethasone is dominant in the early stages of IL-1 β -driven induction. C3 mRNA levels began to increase by 8 h and peaked at 24 h; elevated C3 mRNA levels were maintained at least until 48 h. No initial down-regulation by dexamethasone was observed in the IL-6-treated cells; C3

Fig. 1. Northern blots showing C3, FB, C1-inh, SAA, CRP and SAP mRNA levels after treatment of PLC/PRF/5 cells with defined concentrations of IL-1β and IL-6, alone or in combination, and in the presence or absence of 10⁻⁸ M dexamethasone ('Dex')

Note that lane B is absent in the C1-inh blot.

mRNA levels peaked at 36 h in response to treatment with IL-6 and levels remained elevated at 48 h. The addition of IL-1β and IL-6 in combination resulted in an increase in C3 mRNA levels of a magnitude greater than that observed with either cytokine alone, that began at 8 h, peaked between 12 and 36 h, and was maintained at least to 48 h (Fig. 3).

Factor B

Factor B mRNA was not detected in untreated cells and was not measurably induced by the addition of IL-1β alone or IL-6 alone, either in the presence or absence of dexamethasone (Fig. 1). However, IL-β and IL-6 in combination, in the presence or absence of dexamethasone, resulted in significantly elevated FB mRNA levels (Fig. 1, lanes E and J). In the time-course experiment (Fig. 4), FB mRNA levels did not respond to IL-1β alone; incubation with IL-6 alone gave barely detectable FB mRNA levels at 36 h. However, IL-1β and IL-6 in combination caused a significant increase in FB mRNA levels that was first apparent at 8 h and which peaked at 36 h; at 36 h, FB mRNA levels were increased 2.5-fold over those observed at 8 h.

C1-inh

C1-inh mRNA levels increased 2-fold after treatment with IL-6 alone (Fig. 1, lanes N–P, and Fig. 2b). Dexamethasone enhanced this response (Fig. 1, lanes K–M). Treatment with IL-1β alone in the presence or absence of dexamethasone mediated a slight reduction in C1-inh mRNA relative to endogenous levels, suggesting that IL-1β may down regulate C1-inh mRNA accumulation (Fig. 1, lanes C–D and G–I). This is supported by the observation that IL-1β completely abolished the IL-6-driven induction of C1-inh mRNA when cells were treated with both

cytokines (Fig. 1, lanes E and J). Therefore IL-1β can suppress one of the regulatory functions of IL-6 *in vitro*.

SAA

Endogenous SAA mRNA was not detected in our studies. SAA mRNA was observed after treatment with IL-1β alone (Fig. 1, lanes G–I and Fig. 2c); however, treatment with dexamethasone in addition to IL-1β (Fig. 1, lanes B–D) resulted in SAA mRNA levels 10-fold higher than those induced by IL-1β in the absence of dexamethasone. These findings contrast with those of Ganapathi *et al.* [7], who did not find increased SAA mRNA levels in response to IL-1β in Hep3B cells.

IL-6-induced SAA mRNA to barely detectable levels in our studies; Castell *et al.* [14], however, have shown an increase in SAA mRNA levels in human primary hepatocytes in response to IL-6, which could be enhanced by dexamethasone. In the absence of dexamethasone, IL-1β and IL-6 in combination caused an increase in SAA mRNA levels 5.8-fold over levels induced by IL-1β alone (Fig. 1, lane J). Dexamethasone enhanced this effect a further 7-fold (Fig. 1, lane E). Again, these findings contrast with those of Ganapathi *et al.* [13], who did not find an IL-1β/IL-6 co-operative induction of SAA mRNA in the NPLC/PRF/5 cell line, which is derived from the passage of PLC/PRF/5 cells through nude mice; they did, however, describe a synergistic IL-1β/IL-6-driven induction of SAA mRNA in Hep3B cells.

In the time-course experiment (Fig. 5), SAA mRNA was absent, or below detectable levels, in the control cells incubated with 10⁻⁸ M-dexamethasone alone. Therefore dexamethasone in the absence of cytokines does not itself induce SAA mRNA accumulation. In response to IL-1β, SAA mRNA levels increased slightly by 12 h, reached a peak between 24 and 36 h, and

Fig. 3. Northern blot of C3 mRNA accumulation in PLC/PRF/5 cells over 48 h after various treatments

Control, 10^{-8} M-dexamethasone; IL-1β, 5 ng of IL-1β/ml + 10^{-8} M-dexamethasone; IL-6, 50 ng of IL-6/ml + 10^{-8} M-dexamethasone; and IL-1β+IL-6, 5 ng of IL-1β/ml + 50 ng of IL-6/ml + 10^{-8} M-dexamethasone.

Fig. 4. Northern blot of FB mRNA accumulation in PLC/PRF/5 cells over 48 h after various treatments

Control, 10^{-8} M-dexamethasone; IL-1β, 5 ng of IL-1β/ml + 10^{-8} M-dexamethasone; IL-6, 50 ng of IL-6/ml + 10^{-8} M-dexamethasone; and IL-1β+IL-6, 5 ng of IL-1β/ml + 50 ng of IL-6/ml + 10^{-8} M-dexamethasone.

remained elevated at 48 h. IL-6 caused a marginal induction of SAA mRNA to barely detectable levels between 8 and 36 h. IL-1β and IL-6 in combination had a dramatic effect: SAA mRNA levels were elevated by 4 h and increased to a peak concentration at 24 h that was 39-fold greater than that observed at 4 h. High levels of SAA mRNA were maintained at the 48 h time point (Fig. 5).

CRP

CRP mRNA was not detected in untreated cells, but became detectable after treatment with IL-6 (Fig. 1, lanes N–P, and Fig. 2d); this effect was enhanced by the presence of dexamethasone (Fig. 1, lanes K–M). IL-1β did not induce CRP mRNA when added alone, either in the presence or in the absence of dexamethasone. However, IL-1β had a dramatic enhancing effect on the IL-6-induced response in the presence or absence of

Fig. 2. Effect of various concentrations and combinations of IL-1β, IL-6 and dexamethasone on levels of (a) C3, (b) C1-inh, (c) SAA, (d) CRP and (e) SAP mRNA

Levels of mRNA are plotted as a proportion of those seen in untreated controls and were calculated by scanning densitometry of the data depicted in Fig. 1 as outlined in the Materials and methods section. ●, IL-1β + 10^{-8} M-dexamethasone; ○, IL-1β alone; ■, IL-6 + 10^{-8} M-dexamethasone; □, IL-6 alone. Solid bar, IL-1β (10 ng/ml) + IL-6 (10 ng/ml) + 10^{-8} M-dexamethasone; clear bar, IL-1β (10 ng/ml) + IL-6 (10 ng/ml) without dexamethasone.

Fig. 5. Northern blot of SAA mRNA accumulation in PLC/PRF/5 cells over 48 h after various treatments

Control, 10^{-8} M-dexamethasone; IL-1 β , 5 ng of IL-1 β /ml + 10^{-8} M-dexamethasone; IL-6, 50 ng of IL-6/ml + 10^{-8} M-dexamethasone; and IL-1 β +IL-6, 5 ng of IL-1 β /ml + 50 ng of IL-6/ml + 10^{-8} M-dexamethasone.

Fig. 6. Northern blot of CRP mRNA accumulation in PLC/PRF/5 cells over 48 h after various treatments

Control, 10^{-8} M-dexamethasone; IL-1 β , 5 ng of IL-1 β /ml + 10^{-8} M-dexamethasone; IL-6, 50 ng of IL-6/ml + 10^{-8} M-dexamethasone; and IL-1 β +IL-6, 5 ng of IL-1 β /ml + 50 ng of IL-6/ml + 10^{-8} M-dexamethasone.

dexamethasone, increasing CRP mRNA levels 3.2-fold and 2-fold respectively over levels induced by treatment with IL-6 (10 ng/ml) alone (Fig. 1, lanes E and J). These results are in agreement with those of others who have found that CRP is not induced by IL-1 β in PLC/PRF/5 cells [16] or Hep3B cells [7,16]. Induction of CRP mRNA levels in human hepatoma cells by IL-6 has been reported by Ganapathi *et al.* [13], who also observed that the addition of IL-1 β together with IL-6 did not increase the magnitude of IL-6-driven CRP mRNA induction in NPLC/PRF/5 cells; however, they did find enhanced induction of CRP mRNA after IL-1 β /IL-6 co-treatment of Hep3B cells [13].

The time-course experiment confirmed the absence of CRP mRNA induction by IL-1 β (Fig. 6). CRP mRNA was, however, induced by IL-6; levels peaked at 24 h and had returned to background levels by 48 h. Treatment with IL-1 β and IL-6 in combination, in the presence of dexamethasone, showed a dramatic increase in CRP mRNA levels which were detectable by 8 h, reached a peak at 24 h to levels that were 18.4-fold higher than those observed at 8 h, and returned almost to background levels by 48 h. The contrast between our results and those of others with regard to CRP mRNA induction further demonstrates the variability between different human hepatoma cell lines and between sub-clones of the same cell line.

SAP

Although SAP is not considered a classical APR in humans, it

is in the mouse, as demonstrated both *in vivo* [28,29] and *in vitro* [30]. SAP resembles CRP in gross structure, amino acid sequence and gene organization. Both are members of the pentraxin family of proteins and are probably derived from a common ancestral gene [31]. The genes encoding SAP and CRP have remained in close linkage in human and mouse [32]. Consequently, their differential expression in these species during the acute-phase response has been a paradox. Therefore an analysis of SAP mRNA modulation was included in this study.

SAP mRNA levels increased 1.9-fold in response to IL-6 (100 ng/ml) in the absence of dexamethasone (Fig. 1, lane P, and Fig. 2e). A comparable level of induction was observed with only 10 ng of IL-6/ml when dexamethasone was present (Fig. 1, lane K). In contrast, treatment with IL-1 β led to dramatically decreased levels of SAP mRNA. For example, treatment with 50 ng of IL-1 β /ml alone in the absence of dexamethasone resulted in SAP mRNA levels that were one-tenth those of background (Fig. 1, lane H). The IL-1 β -mediated reduction of SAP mRNA levels was diminished to only one-third the background level in the presence of dexamethasone (Fig. 1, lane C). These effects do not appear to be concentration-dependent with regard to the three concentrations of IL-1 β used in the study. As in the case of C1-inh mRNA, IL-1 β inhibited the IL-6-driven induction of elevated SAP mRNA levels (Fig. 1, lanes E and J). Indeed, the down-regulatory effect of IL-1 β on SAP mRNA appears to supersede the up-regulatory effect of IL-6. IL-1 β has previously been shown to inhibit the response of rat α_2 -macroglobulin, fibrinogen and cysteine proteinase inhibitor to IL-6 [33]. The mechanism(s) by which IL-1 β down-regulates the expression of the SAP gene and modulates its response to IL-6 is not known and requires further analysis. Although plasma SAP levels have not been reported to be elevated during the acute-phase response in humans, particular combinations and/or levels of cytokines, glucocorticoids and other factors may result in induction of SAP under certain circumstances. The control of SAP mRNA and protein synthesis will require further studies, *in vitro* and *in vivo*, to define the true behaviour of this pentraxin during the acute-phase response.

Concluding remarks

This study characterizes the heterogeneous responses of a number of APRs after treatment of the human hepatoma cell line PLC/PRF/5 with the inflammatory mediators IL-1 β , IL-6 and the synthetic glucocorticoid dexamethasone, alone and in combination. Some APRs (C3) are up-regulated by either cytokine, whereas others (SAA, CRP) are up-regulated by one, but not the other. In some cases (SAP, C1-inh) IL-1 β down-regulates APR mRNA accumulation and suppresses the IL-6-driven APR mRNA induction. In other cases (C3, FB, SAA, CRP) the two cytokines have an additive or synergistic effect when added in combination. Dexamethasone modulated the IL-1 β and IL-6-driven response of a subset of the APRs in these studies.

We have therefore established that different cytokines or combinations of cytokines and glucocorticoids affect APRs to different extents and in different ways. APR gene regulation is clearly highly complex. A number of intracellular signal-transduction pathways, for example activation of protein kinase C [34] and stimulation of diacylglycerol [35], are likely to be involved, and the identification of *cis*-acting genetic elements and isolation of specific DNA-binding proteins will probably be required to permit an understanding of the precise mechanisms underlying the heterogeneous nature of APR gene expression. Such studies, using PLC/PRF/5 cells as an '*in vitro*' model system, should form the basis for future analyses of the complex physiological parameters that control the acute-phase response *in vivo*.

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