IN VIVO AND *IN VITRO* REGULATION OF THE IL-6 SUBFAMILY OF CYTOKINE RECEPTORS IN THE LIVER

by

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RECEPTORS OF THE IL-6 CYTOKINE FAMILY

ABSTRACT

Interleukin 6 (IL-6) is a pleotropic cytokine that has many important physiological roles during inflammation. One function is the regulation of the acute phase response of the liver. In order to understand the role of IL-6 as an inflammatory mediator in the acute phase response, it was necessary to study the effect that this molecule had on the regulation of its own receptors, IL-6 receptor (IL-6R, gp80 or α-receptor), and signal transducing protein, gp130, both *in vivo* and *in vitro.*

To investigate the role that IL-6 and corticosterone play in the acute phase response and receptor regulation, we examined serum IL-6 levels, serum corticosterone levels, acute phase protein levels, and the expression of hepatic IL-6R (gp80) and gp130 mRNA levels, in three different models of acute inflammation. Rats were treated with either Freund's complete adjuvant (FA) via intraperitoneal injection, LPS via intravenous injection, or turpentine via subcutaneous injection. All three models showed increased levels of serum IL-6 activity with LPS-treated rats inducing the quickest and greatest response (>100 ng/ml within 3h). Serum corticosterone levels increased by 3h after all treatments, and serum levels of acute phase proteins were detected within 12-24h. The expression of the IL-6R (gp80) mRNA increased as early as 3h after treatment and mRNA levels began to decline by 6-12 h. The gp130 mRNA levels increased 2-3 fold within 24h, and the time of maximum increase differed depending on the treatment that the rat received.

Another cytokine, Leukemia Inhibitory Factor (LIF), and IL-6 share many functions *in vitro,*

and this redundancy is thought to occur as a result of their two alpha receptors, LIF-R and IL-6R, respectively, interacting with the same signal transducing molecule, gp130. We examined the increase of LIF-R mRNA levels in the three models of acute inflammation, to determine the role of LIF during the acute phase response in comparison to IL-6. We found a maximum 2-3 fold increase in mRNA levels in comparison to controls. Maximum LIF-R mRNA levels varied depending on the type of treatment the rats received.

Overall analysis of all mRNA levels studied, showed that maximum IL-6R (gp80), gp130, LIF-R and Cysteine Proteinase Inhibitor (CPI-an acute phase protein whose expression is regulated by IL-6) mRNA levels peaked at different times depending on the type of acute inflammation induced. Although IL-6R mRNA levels reached maximum levels quickly (3-6h) in all three models of acute inflammation, the maximum induction of gp130 and LIF-R differed depending on the type of treatment the rats received. In all three acute inflammatory models, IL-6R and LIF-R mRNA levels did not peak at the same time for any of the treatments given. This suggests that although IL-6 and LIF may have similar functions *in vitro*, their role *in vivo* in inflammation is unique. Staggering of maximum LIF-R and IL-6R expression in the liver may ensure that cells are receptive to continual messages to make acute phase proteins.

To further investigate the individual effects of raised corticosterone and IL-6 on the expression of these receptors, rats were injected with either dexamethasone (DEX) or purified recombinant IL-6 (rIL-6) via intraperitoneal injection. Rats injected with rIL-6 showed a dramatic increase in both IL-6R (gp80) and gp130 mRNA levels, as early as 1h after treatment. Dexamethasone had a significant, but less dramatic effect, on IL-6R (gp80) mRNA levels and no effect on gp130 message. An acute phase protein response was only seen when rats were injected

with rIL-6 and not DEX. Neither rIL-6 or DEX treated rats had any effect on LIF-R mRNA levels.

To continue these studies, we investigated the long term *in vivo* effects of prolonged exposure to purified rIL-6 on the expression of these hepatic cytokine receptors. Repeated injections of rIL-6 did not cause decreased mRNA levels, instead increased IL-6R (gp80), gp130, and CPI mRNA levels (2-3 fold) were seen. Serum CPI protein levels increased gradually over a nine day period from 1.4 mg/ml to 5.8 mg/ml. LIF-R mRNA levels remained unaffected by the repeated injections of rIL-6.

The steady-state mRNA levels of the interleukin-6 receptor (IL-6R, gp80) and its signal transducing molecule, gp130, were examined in the rat hepatoma cell line, H-35, stimulated by cytokines IL-6, IL-1, Oncostatin M, and/or DEX. In contrast to our *in vivo* findings, *in vitro* DEX seemed to be the major stimulator of IL-6R mRNA expression, whereas IL-6 seemed to have little effect on the expression of its own receptor mRNA levels. However, the presence of other cytokines also influenced the corticosteroid (dexamethasone-DEX) mediated stimulation of IL-6R expression. Oncostatin M, a third cytokine related to IL-6, stimulated IL-6R mRNA levels, and this stimulation was additive with the DEX-mediated stimulation of IL-6R mRNA levels. In contrast, Interleukin 1 (IL-1) inhibited the DEX-mediated stimulation of IL-6R mRNA. At the same time, IL-1 also stimulated the presence of a second smaller mRNA transcript. This mRNA species contained the extracellular domain but lacked both the transmembrane and cytoplasmic domains of the IL-6R, suggesting alternate splicing, possibly coding for a soluble form of gp80.

The expression of the gp130 molecule was not regulated to any major extent *in vitro*, and cytokines IL-6, Oncostatin M, and IL-1 all stimulated the expression of the signal transducing molecule, gp130, approximately 2 fold.

Cysteine Proteinase Inhibitor mRNA and protein levels were elevated by combinations of

cytokines: IL-6+DEX, IL-6+IL-1+DEX, OSM, OSM+DEX. However, IL-1 again seemed to inhibit the IL-6+DEX mediated stimulation of CPI mRNA, possibly through inhibition of IL-6R expression and induction of a possible soluble form of the receptor.

This study shows that the combination of cytokines and hormones that interact with the hepatocyte and in turn, regulate the expression of these different receptors is very complex. *In vitro*, we demonstrated that both IL-1 and Oncostatin M are involved in the regulation of the IL-6 receptor complex, and that different combinations of cytokines can affect the complexity and magnitude of the hepatic acute phase response. *In vivo*, we demonstrated that depending on the type of acute inflammation induced, that a variety of combinations of cytokines and hormones are released, and these in turn regulate the diverse receptors on the hepatocyte, resulting in different acute phase response kinetics. Therefore, even though all of these receptors belong to a family and have similar functions, the regulation of these receptors is unique and may lead to altered cell responses in the different models of inflammation.

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"These things are sent to try us,
forge our character,
and make us strong."

This is one verse that I will always remember, and I give my deepest thanks to my supervisor, Dr. Jack Gauldie, for his unending support, understanding, and sense of humour. He has been a true mentor, and has made scientific research an exciting and rewarding experience. I greatly appreciate the support and advice of my committee members, Dr. Del Harnish and Dr. Jean Marshall. I would also like to thank other members in our Lab, including Dr. Carl Richards, Dr. Manel Jordana, Donna Green, Liz Scheid, and Laurie Nelson for their support and advice. A special thanks to Jerry Schulman, Jane-Ann Schroeder, and Duncan Chong for their technical help, advice, and friendship.

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PREFACE

This dissertation consists of seven chapters. Chapter one, the Introduction, summarizes background and current literary research that relates to the field under investigation in this thesis. Chapters two to five consist of three published scientific papers and one manuscript. Each of these chapters investigates different aspects of cytokine receptor regulation on the hepatocyte. Finally, chapter six, summarizes the findings in this thesis, highlighting possible implications deduced from the results obtained. Additional information on the IL-6 receptor (gp80) is presented in Appendix A. Appendix B contains a paper that emerged as a result of a collaboration with another group. The author of this thesis did many of the experiments in this paper. The references cited in both the Introduction and the Summary are listed in the reference section at the end of the thesis.

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LIST OF ABBREVIATIONS

AGP	α_1-acid glycoprotien
APP	acute phase response
APRF	acute phase response factor
cDNA	complementary deoxyribonucleic acid
CPI	cysteine proteinase inhibitor
CNTF	ciliary neurotropic factor
CytRE	cytokine response element
DEX	dexamethasone
FA	freund's complete adjuvant
FCS	fetal calf serum
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	hour
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin-1
IL-4	interleukin-4
IL-6	interleukin-6
IL-6R	interleukin-6 receptor (gp80)
IL-6REBP protein	interleukin-6 receptor response element binding
IL-7	interleukin-7

IL-11	interleukin-11
i.p.	intraperitoneal
i.v.	intravenous
JAK	Janus family of tyrosine kinases
kb	kilobase
kDa	kilodalton
LIF	leukaemia inhibitory factor
LIF-R	leukaemia inhibitory factor receptor
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
ml	milliliter
NaOH	sodium hydroxide
NF-IL6	nuclear factor - interleukin-6
ng	nanogram
OSM	oncostatin M
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
rIL-6	recombinant interleukin-6
RNA	ribonucleic acid
s.c.	subcutaneous
TNF	tumor necrosis factor
µg	microgram

Chapter one

INTRODUCTION

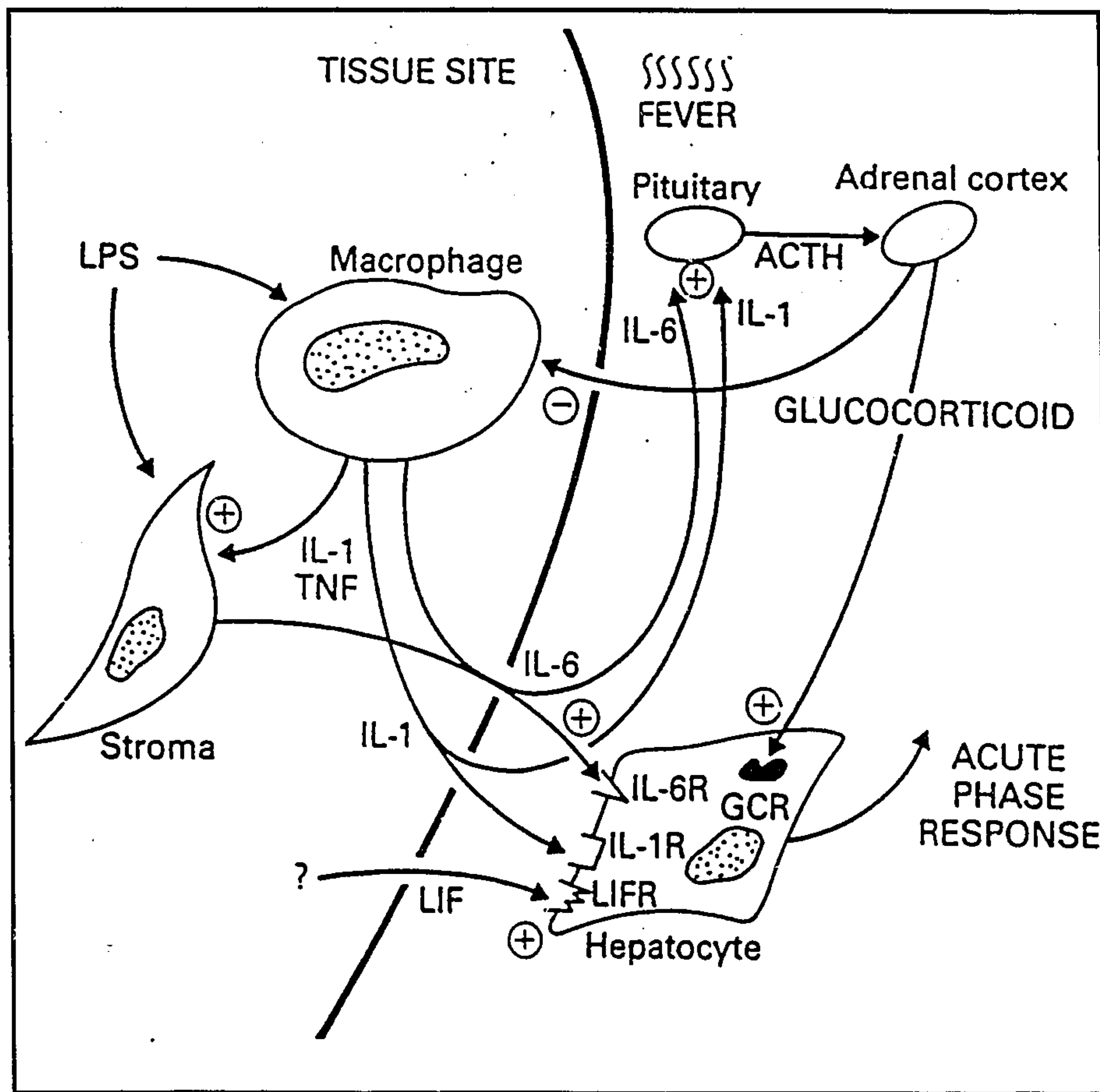
1. INFLAMMATION AND THE ACUTE PHASE PROTEIN RESPONSE

1.1 Inflammation

Inflammation is the physiological and homeostatic response of an organism to tissue injury, trauma, or infection. At the site of injury or infection, inflammatory cells such as monocyte/macrophages are stimulated to release many hormone-like soluble mediators, known as cytokines. These cytokines act both locally and systemically to initiate a complex series of reactions that regulate the inflammatory response, resulting in tissue repair, cessation of infection, and the return of the body to normal function.

The cytokines act in sequence and in cascade. Locally they can induce changes such as edema, heat, and pain, and systemically have broad activities. One of the major target organs for the systemic cytokines such as Interleukin 1, Tumor Necrosis Factor, or Interleukin 6, is the liver (Scheme 1). The hepatocyte expresses specific high affinity receptors for many of these factors. This cell is the primary source for a series of plasma proteins which are present in increased amounts during inflammation. The hepatocyte is stimulated by these cytokines to synthesize these acute phase proteins (APP). Many functions of the acute phase proteins involve control of tissue damage, removal of infectious agents and initiation of tissue repair (for reviews see Koj *et al.*, 1993; Baumann & Gauldie, 1994*a;* Richards & Gauldie, 1994).

To understand the role of the liver during inflammation, it is necessary to study the interactions that liver cells or hepatocytes have with their environment. Many cytokine receptors expressed on the surface of the hepatocyte are regulated by various factors in circulation, to either increase or decrease receptor cell surface expression. We set out to study the regulation of some of



Scheme 1: Cytokine interaction with the hepatocyte and control of the acute phase response. At site of tissue injury or infection, activated macrophages release cytokines such as interleukin 1, or interleukin 6 which act both locally and systemically. One of the major target organs systemically is the liver. This stimulates the liver to synthesize a series of protein called acute phase proteins. These proteins then act at the tissue site to control tissue damage, aid in removal of infectious agents, and initate tissue repair. (This figure was copied with permission from Richards & Gauldie 1994)

some of these receptors on the hepatocyte, how this regulation may result from interaction between the cytokines, and how this regulation affects the outcome of the acute phase protein response.

1.2 Acute Phase Proteins

Several cytokines released during inflammation stimulate the liver to synthesize acute phase proteins (APP). It has been shown *in vitro* that interleukin-6 (IL-6) and interleukin-1 (IL-1) in the presence of corticosteroid can account for the full acute phase response in the liver (for reviews see Baumann & Gauldie, 1994; Gauldie, 1991; Fey & Gauldie, 1990). There are many other cytokines that stimulate the same APPs as IL-6. These IL-6-like cytokines include interleukin-11 (IL-11), leukaemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotropic factor (CNTF).

In general, there are two groups of cytokines that stimulate two types of APPs in the liver (See Table 1). Type I APPs are stimulated by IL-1 and TNF and their stimulation can be synergistically enhanced by IL-6 or IL-6-like cytokines. Type I APPs include α_1-acid glycoprotein, serum amyloid A, C-reactive protein, and complement component C_3 (for reviews see Baumann & Gauldie, 1994*a;* Richards & Gauldie, 1994).

Type II APPs are stimulated by IL-6 or IL-6-like cytokines and IL-1-like cytokines usually do not effect these APPs. However, in some cases, IL-1 may have an inhibitory effect on Type II APPs. Some examples include fibrinogen, thiostatin (rat), and α_2-macroglobulin (rat) (Richards *et al.*, 1991; Baumann & Gauldie, 1990*b*).

Other hormones, growth factors, and cytokines, seem to influence the IL-1/IL-6 regulation of APPs (Baumann & Gauldie, 1994). Generally, the presence of glucocorticoids enhances the expression of Type I and Type II APPs (Gauldie, 1991; Fey & Gauldie 1990). Glucocorticoids can

TABLE I

TYPE 1 APPs (TNF/IL-1)	TYPE II APPs (IL-6/IL-11/OSM/LIF/CNTF)
C-reactive protein	Fibrinogen
Serum amyloid A	α_2 Macroglobulin (rat)
α_1 Acid glycoprotein	Thiostatin (rat)
Complement C3	α_1 Antitrypsin
Haptoglobin (rat)	α_1 Antichymotrypsin
Hemopexin (rat)	Haptoglobin (human)
	Hemopexin (human)
	Ceruloplasmin
	α_1 Proteinase inhibitor

TABLE I: Acute phase proteins (APP) Type I and Type II. Acute phase proteins can be divided into two groups dependant on which cytokines they are stimulated by. Type I APPs' are stimulated by IL-1 and TNF, and IL-6-like cytokines can synergistically enhance their synthesis. Type II APPs' are stimulated by IL-6-like cytokines (IL-6, IL-11, OSM, LIF, CNTF) and IL-1 may have an inhibitory effect on some of the type II APPs. There are species differences between type I and type II APPs. (Modified from Richards et al., 1991)

stimulate some APPs directly such as α_1-acid glycoprotein, but this stimulation is small compared with the above cytokines (Baumann *et al.*, 1983). Other cytokines such as TGFβ, (which does not regulate APPs alone) enhance IL-6 stimulation of thiostatin and hemopexin, and attenuate the effects of other cytokine stimulation of APPs (Campos *et al.*, 1993). In contrast, hormones such as insulin are nonspecific inhibitors of both cytokine and glucocorticoid stimulation of APPs in rodents (Campos *et al.*, 1992).

Studies done on IL-6-deficient mice have revealed that IL-6 plays a major role in acute phase protein production (Kopf *et al.*, 1994; Fattori *et al.*, 1994). When these mice are injected with turpentine, their acute phase protein response is severely compromised. Mice injected with LPS are moderately affected. All APP levels can return to normal after the injection of rIL-6 (Kopf *et al.*, 1994). Therefore, though many other cytokines are stimulated during inflammation to produce APPs, only in the presence of IL-6 is a full acute phase protein response launched. To understand the role of IL-6 as an inflammatory mediator in the acute phase response, it was necessary to study the effect that this molecule could have on the regulation of its own receptor complex (gp80 and gp130), both *in vivo* and *in vitro*.

2. RECEPTORS AND MODULATION

2.1 The Class I Family of Cytokine Receptors

The IL-6-like cytokines, including IL-11, LIF, OSM, and CNTF have many functions in common (See Table 2). Some examples include neuronal differentiation and survival, and M1 leukaemic differentiation (for reviews see Hirano *et al.*, 1994; Kishimoto *et al.*, 1992; Miyajima *et*

TABLE 2

	IL-6	LIF	0SM	IL-11	CNTF
ACUTE PHASE PROTEIN INDUCTION	X	X	X	X	X
NEURONAL DIFFERENTIATION	X	X	X	X	X
M1 MYELOID CELL DIFFERENTIATION	X	X	X	X	X
MEGAKARYO-CYTE DIFFERENTIATION	X	X	ND	X	ND
HEMATOPOIETIC STEM CELL GROWTH	X	X	ND	X	ND
BONE REMODELLING	X	X	ND	X	ND
B CELL DIFFERENTIATION INTO PLASMA CELLS	X	O	ND	X	ND

TABLE 2: Overlapping biological activities of IL-6-like cytokines. These cytokines all use gp130 as a signal transducing molecule in their receptor complexes. The common signaling pathways initiated by this gp130 molecule may explain the overlapping functions found among these cytokines. x = response; o = no response; ND = not detemined. (Modified from Hirano et al., 1994; Kishimoto et al., 1992; Miyajima et al., 1992)

al., 1992). It has been proposed that the use of a common signal transducing molecule, gp130, is the reason for the similar functions seen among these cytokines (for reviews see Hirano *et al.*, 1994; Sato *et al.*, 1994; Taga & Kishimoto, 1992; Miyajima *et al.*, 1992).

The main focus of this study is the regulation of the IL-6 receptor complex, which includes an alpha component or gp80 molecule (IL-6R), and the gp130 molecule (See Figure 1). We also investigated the regulation of the LIF receptor complex, which includes the LIF binding protein (LIF-R) and the gp130 molecule. We examined how these receptors were regulated during acute inflammation *in vivo*. We also investigated how individual cytokines regulate the IL-6 receptor complex *in vitro*.

The IL-6R, LIF-R, and signal transducer gp130 have been cloned and characterized (Yamasaki *et al.*, 1988; Hibi *et al.*, 1990; Gearing *et al.*, 1991) (See Figure 1). These molecules belong to a larger family of class I cytokine receptors or the haematopoietic cytokine receptor family (for reviews see Kishimoto *et al.*, 1992; Taga & Kishimoto, 1992; Miyajima *et al.*, 1992). This family contains an extracellular homologous region of 200 amino acids that form two barrel-like structures consisting of 7 anti-parallel β-strands (similar to the fibronectin type III domain). Also, the amino terminal domains contain 4 conserved cysteine residues, and the carboxy terminal domain contains a conserved WSXWS (Trp-Ser-X-Trp-Ser) motif (Bazan 1990). It is the hinge region between the two barrels and WSXWS motif that seem to be involved in ligand binding (Yawata *et al.*, 1993).

The family of class 1 cytokine receptors is subdivided into subfamilies based on the use of a common β-subunit (Sato *et al.*, 1994). IL-6R and LIF-R belong to the IL-6 subfamily and they share the common β-subunit, gp130 (See Figure 2, pg.11) Other cytokines involved in this family include IL-11, OSM and CNTF. These cytokines bind their own receptors (IL-6R/IL-11R) (Taga *et al.*,

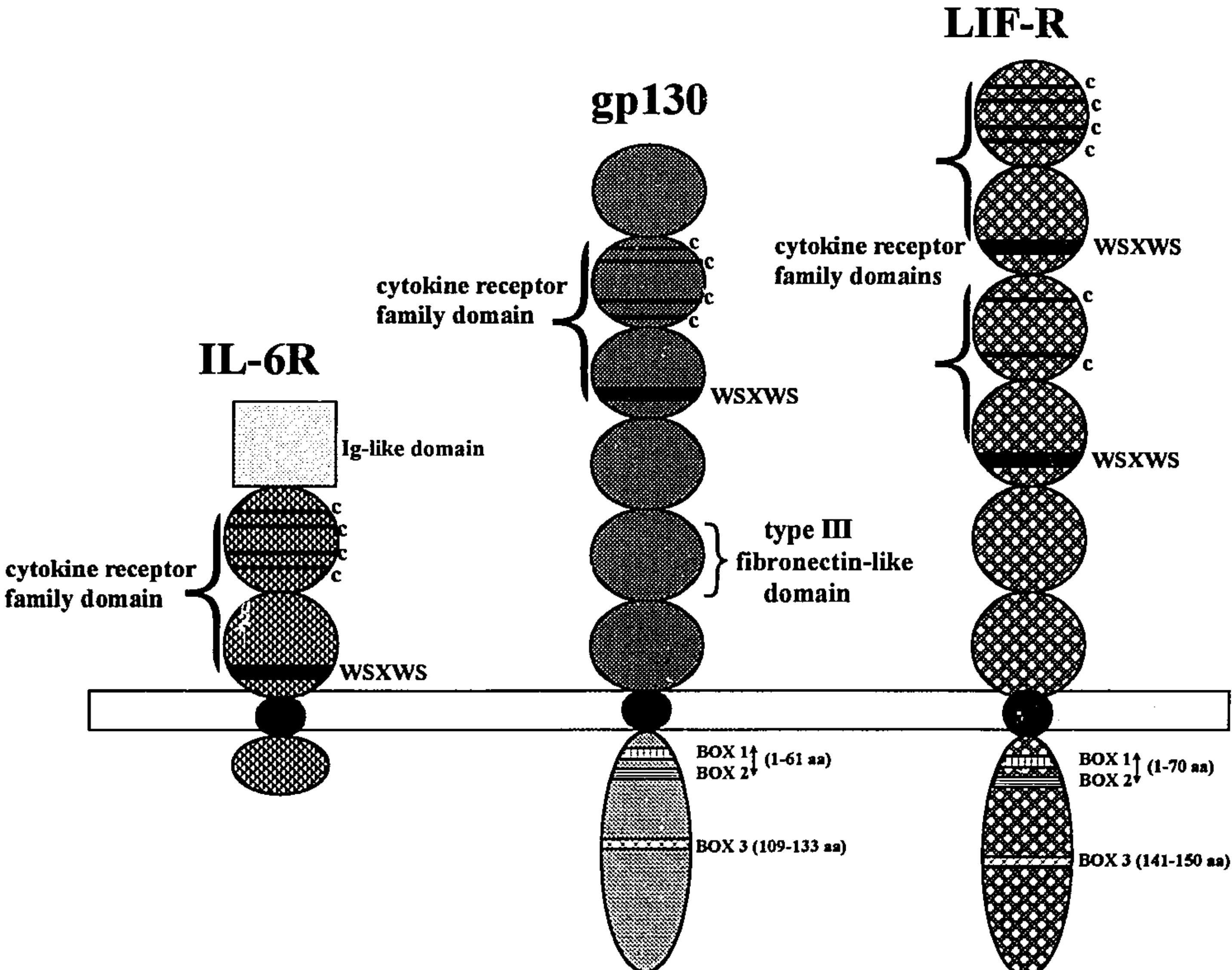


FIGURE 1: Schematic representation of the IL-6R, gp130, and LIF-R . These receptors belong to the Cytokine Receptor Family and all share the common motifs of conserved cysteine residues and the WSXWS sequence. These molecules are largely made up of repeating type III fibronectin-like domains. The gp130 and LIF-R are very similar in both their extracellular and cytoplamic domains. Both contain similar conserved areas in their cytoplasmic domains (Box 1- Box 3) required for signal transduction (Modified from Kishimoto et al., 1995).

1989; Hilton *et al.*, 1994; Yin *et al.*, 1993) or a shared receptor (OSM/LIF/CNTF receptor) (Gearing *et al.*, 1992*b*; Liu *et al.*, 1992; Ip *et al.*, 1992; Stahl *et al.*, 1993) with low affinity, and these complexes are then converted to high-affinity bindings sites by association with the β-subunit, gp130 (Gearing *et al.*, 1992*b*; Gearing *et al.*, 1991; Hibi *et al.*, 1990; Taga *et al.*, 1989). The IL-6/IL-6R complex induces disulphide-linked homodimerization of gp130 (Murakami *et al.*, 1993) and LIF/CNTF receptor complexes induce heterodimerization of gp130 (Davis *et al.*, 1993*b*). These steps are essential for activation of associated tyrosine kinases and signal transduction (Sato *et al.*, 1994).

2.2 Signal Transduction Pathways

The signal transduction pathways stimulated by the IL-6 cytokine subfamily, and ultimately the APP genes, are currently under investigation (for reviews see Baumann & Gauldie, 1994; Kishimoto *et al.*, 1994; Hirano *et al.*, 1994). The homo- and heterodimerization of the cytokine receptor complexes induce rapid tyrosine phosphorylation of the gp130 molecule and other cellular proteins (Davis *et al.*, 1993*b*; Murakami *et al.*, 1993; Stahl *et al.*, 1994; Matsuda *et al.*, 1994*b*; Feldman *et al.*, 1994; Yin *et al.*, 1994*a*). One signaling pathway may involve the Janus kinase family of protein tyrosine kinases (JAK-TYK) (for reviews see Ihle *et al.*, 1994; Darnell *et al.*, 1994). Cytokines IL-6, IL-11, LIF, and CNTF were shown to activate and tyrosine phosphorylate JAK2 (Narazaki *et al.*, 1994; Yin *et al.*, 1994*b*; Stahl *et al.*, 1994; Berger *et al.*, 1994). Recent studies have also shown that JAK1, JAK2 and TYK2 are physically associated with gp130 and LIF-R in the absence of ligand. Then, after ligand binding, these tyrosine kinases are activated (Lütticken *et al.*, 1994; Stahl *et al.*, 1994; Yin *et al.*, 1994*b*).

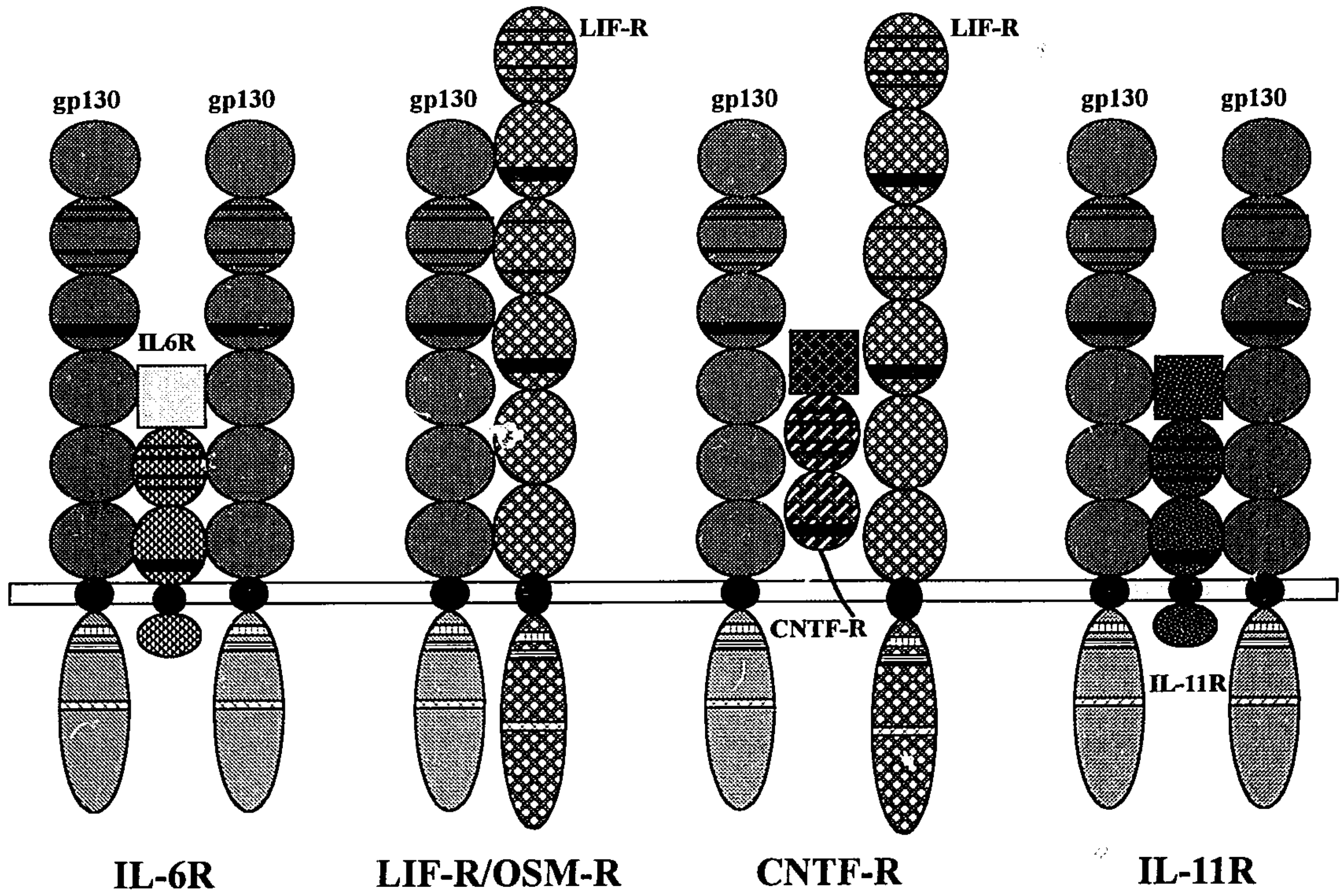


FIGURE 2: THE IL-6R SUBFAMILY OF THE CLASS I OF CYTOKINE RECEPTORS. The common beta subunit in this family is the gp130 molecule. All receptors contain the common motifs among class I cytokine receptors, the conserved cysteine residues and the WSXWS sequence motif shown as thin and thick bars, respectively. Shaded circles represent the folding structure similar to firbronectin type III domains, squares represent Ig-like domains. (Modified from Kishimoto et al., 1995).

Although these cytokines can stimulate all three members of the JAK-TYK family, the patterns of tyrosine phosphorylation they induce are distinct and different depending on the cell lines involved (Stahl *et al.*, 1994; Berger *et al.*, 1994). For example, stimulation of SK-MES cells with IL-6 or OSM results in phosphorylation of JAK2, whereas stimulation of U266 cells with IL-6 results in JAK1 and TYK2 phosphorylation (Stahl *et al.*, 1994).

Baumann *et al.*, 1994*b/c*, have provided evidence through chimeric studies done with gp130 and LIF-R that at least two distinct pathways exist that regulate the APPs. One pathway targets the cytokine response element (CytRE of Type I RE) on the rat AGP gene, and the other pathway targets the IL-6 response element (IL-6RE or Type II RE).

The Type I response element (T(T/G)NNGNAA(T/G)) is the binding site for the nuclear factor NF-IL6 (for reviews see Chen-Kiang *et al.*, 1993; Akira *et al.*, 1992). NF-IL6 regulates APPs of both classes and is activated by a ras-dependant MAP kinases (Nakajima *et al.*, 1993). NF-IL6 and IL-6REBP (binds Type II RE) are members of the C/EBP family of leucine zipper proteins (Poli *et al.*, 1990; Akira *et al.*, 1990).

The Type II response element was first discovered through mutagenesis studies done on the α_2-macroglobulin gene (Hattori *et al.*, 1990; Ito *et al.*, 1989). This hexanucleotide motif (CTGGGA) found mainly on Type II acute phase proteins binds two different nuclear factors called IL-6 response element binding protein (IL-6REBP) and acute phase response factor (APRF) (Wegenka *et al.*, 1993; Hocke *et al.*, 1992). Recent studies have shown that IL-6-like cytokines induce the phosphorylation and DNA binding of APRF (Akira *et al.* 1994; Lütticken *et al.*, 1994; Wegenka *et al.*, 1993). APRF belongs to the family of STAT related proteins (STAT3) (Wegenka *et al.* 1994; Akira *et al.*, 1994), and this factor associates with gp130 and JAK1 (Lütticken *et al.*, 1994). Stahl *et al.*, 1995, have

recently demonstrated through chimeric studies that APRF/STAT3 binds certain tyrosine motifs found on the cytoplamic domains of gp130 and LIF-R. Dimerization of the receptor molecules allows for phosphorylation and activation of the associated Jak kinases, which in turn phosphorylate tyrosine residues on the cytoplasmic domains of gp130 and LIF-R. These phosphorylated tyrosines act as docking sites for SH_2 domains found on APRF/STAT3. This allows the associated Jak kinase to phosphorylate and activate APRF/STAT3. This molecule then dimerizes and translocates to the nucleus where it binds to the appropriate DNA sequence (Stahl *et al.*, 1995; Lütticken *et al.*, 1994; Akira *et al.*, 1994)

3. THE IL-6 RECEPTOR (gp80, α-subunit)

3.1 Cloning and Characterization

The human and rat alpha component of the IL-6 receptor complex (IL-6R) has been cloned and characterized (Figure 3) (Yamasaki *et al.*, 1988; Baumann *et al.*, 1990*a*). The human IL-6R was originally cloned from the human NK-cell line YT. The protein contains 468 amino acids, including a 19 amino acid signal peptide, a 28 amino acid transmembrane domain, and a short cytoplasmic domain of 82 amino acids, that

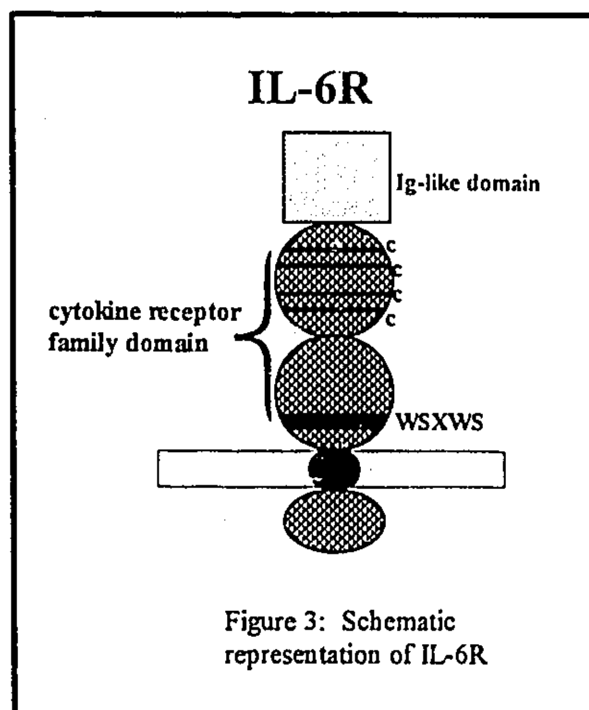


Figure 3: Schematic representation of IL-6R

lacks any tyrosine kinase domains (Yamasaki *et al.*, 1988).

The rat IL-6R was cloned from an inflammed rat liver library. Two major clones were pulled from this library, both of which differed only in their long 3' UTR. There was 71% nucleotide sequence homology with human IL-6R in the coding region and 36% homology in the non-coding region (Baumann *et al.*, 1990*a*).

IL-6R belongs to the Class I cytokine receptor family, (for reviews see Sato *et al.*, 1994; Taga & Kishimoto, 1992; Miyajima *et al.*, 1992) and the IL-6 subfamily, where gp130 is the common β subunit (Hirano *et al.*, 1994). The extracellular domain is comprised of two fibronectin type III modules (See Figure 3). These two fibronectin type III modules form two barrel-like structures with a hinge region between them (Bazan, 1990). It is this hinge region that seems to have amino acids critical for IL-6 binding. Site-directed mutagenesis studies done on the hinge region and WSXWS motif either reduce considerably or abolish the binding capacity of this receptor. These same studies have also shown that the proximal half of the second barrel seems to be involved in gp130 binding. IL-6R is also composed of an Ig-like domain which does not seem to be involved in IL-6 or gp130 binding (Yawata *et al.*, 1993).

3.2 IL-6R mRNA Regulation *In vitro* and *In vivo*

Not all cells respond to IL-6 stimulation and studies have shown that the IL-6R is only expressed on certain cells. IL-6R can be found on hepatocytes, astrocytes, endothelial cells, monocytes, glial cells, and neuronal cells (Sonne *et al.*, 1990; Peterson *et al.*, 1990; Schöbitz *et al.*, 1992). IL-6R is also expressed on resting T cells and activated B cells (Taga *et al.*, 1987).

Many groups have looked at the regulation of the IL-6R in liver cells. Dexamethasone

(corticosteroid analogue) (Dex) seems to be the major regulator of IL-6R mRNA levels *in vitro* in both human and rodent systems (Nesbitt & Fuller, 1992; Rose-John *et al.*, 1990; Wang *et al.*, 1992). These results were also confirmed by our group (Geisterfer *et al.*, 1995*b*) in rat H-35 hepatoma cells. However, recent studies have also shown that many cytokines have an effect on the Dex-mediated stimulation of IL-6R mRNA, with some cytokines enhancing IL-6R mRNA levels, while others inhibit them. Studies done by Rose-John *et al.*, 1990, have shown that IL-6 has no effect on IL-6R mRNA levels in human HEPG2 cells, while Nesbitt and Fuller, 1992, found that IL-6 inhibited the Dex-mediated stimulation of IL-6R mRNA in rat primary hepatocytes. Recently, other groups have shown that TGFß (Campos *et al.*, 1993) and TNFα (Schooltink *et al.*, 1991) enhance the induction of IL-6R mRNA by Dex. Our group looked at the effects of the cytokines IL-1 and OSM on the levels of IL-6R and gp130 expression (Geisterfer *et al.*, 1995*b*).

In vivo, as expected, IL-6R mRNA levels are increased by different inflammatory substances including LPS, Turpentine, and FA (Geisterfer *et al.*, 1993; Baumann *et al.*, 1990*a*; Nesbitt & Fuller, 1992) Although the time of maximum mRNA induction varies somewhat, the *in vivo* regulation studies done by the different groups are quite consistent.

Although *in vitro*, Bauer *et al.*, 1989, have shown that IL-6 can up-regulate IL-6R in human fetal hepatocytes, in general other groups have shown that IL-6 alone has no effect on liver cells in both human and rodent systems (Nesbitt & Fuller, 1992; Rose-John *et al.*, 1990; Wang *et al.*, 1992). *In vivo,* however, IL-6R mRNA levels increase dramatically in rodent livers as little as one hour after rIL-6 injection (Geisterfer *et al.*, 1993; Saito *et al.*, 1992). In comparison, Dex induced a much milder and slower rise in IL-6R mRNA levels *in vivo* (Geisterfer *et al.*, 1993).

One explanation for the different results seen between *in vivo* and *in vitro* systems is that *in*

vivo other factors may be present that respond to rIL-6 to enhance IL-6R mRNA levels that are not present *in vitro*. Another explanation could be that cells *in vitro* behave differently than cells *in vivo*.

3.3 The Soluble Form of the IL-6R

Although IL-6R is considered to be a membrane receptor, a soluble form of the receptor has been found in many body fluids. It has been shown that soluble IL-6R receptor exists in normal human serum at ~75 ng/ml (Montero-Junian *et al.*, 1994; Frieling *et al.*, 1994) and in normal urine at ~1-3 ng/ml (Novick *et al.*, 1989: Frieling *et al.*, 1994). Also, many diseased states have shown abnormal levels of the soluble receptor in body fluids. For example, patients with HIV and multiple myeloma have significantly higher levels of soluble IL-6R levels compared to controls (Honda *et al.*, 1992; Gaillard *et al.*, 1993). In contrast, patients with systemic juvenile rheumatoid arthritis have significantly lower soluble IL-6R levels (De Benedetti *et al.*, 1994).

The role of the soluble IL-6R in disease and inflammation is unknown, however, it has also been shown that a genetically engineered soluble IL-6R lacking transmembrane and cytoplasmic domains has agonist activity and binds gp130 in the presence of IL-6 to produce a signal within the cell (Taga *et al.*, 1989; Hibi *et al.*, 1990; Mackiewicz *et al.*, 1992*a/b*; Diamant *et al.*, 1994). This is in contrast to the soluble forms of other cytokine receptors such as soluble TNFα receptor (Howard *et al.*, 1993), soluble IL-4 receptor (Mosley *et al.*, 1989; Sato *et al.*, 1993), and soluble IFNγ receptor (Ozmen *et al.*, 1993) where the role of the receptor is antagonistic.

There have been conflicting results in the literature as to the source of this soluble receptor. Using RT-PCR, different groups (Lust *et al.*, 1992; Horiuchi *et al.*, 1994) have found message that encoded for the soluble IL-6R in various cell lines. The soluble receptor mRNA lacked the

transmembrane domain of the coding region. Further studies showed similar results with other human cell lines. Horiuchi *et al.*, 1994, precipitated a 45 kDa protein from culture supernatants of MT-2, MT-4, and U937 cell lines using three specific anti-IL-6R antibodies. Our group has also shown through Northern blot analysis that IL-1 stimulated H-35 cells induced an mRNA species that may encode for an alternately spliced mRNA encoding the soluble IL-6R (Geisterfer *et al.*, 1995*b*). Other cytokine receptors, such as IL-4 and IL-7 receptor, have soluble forms generated through alternate splicing (Goodwin *et al.* 1990; Mosley *et al.*, 1989).

In contrast to these results, Müllberg *et al.* (1992, 1993*a/b*, 1994) using a IL-6R transiently expressed in COS-7 and MDCK cells, have shown that a soluble form of IL-6R can be derived by proteolytic cleavage of the intact membrane bound IL-6R, and that this cleavage is enhanced by phorbol-12-myristate-13-acetate (PMA), a protein kinase C activator. They also demonstrated that the generation of the soluble IL-6R was not dependant on protein *de novo* synthesis. Recently however, Körholz *et al.*, 1994, demonstrated that although PMA significantly enhanced soluble IL-6R release from human B-cell line SKW6.4, the release was dependant on *de novo* synthesis. Using RT-PCR, they showed an increased expression of mRNA encoding the soluble form of the IL-6R. The reason for the differences seen between the above two sets of results could be due to the fact that Müllberg *et al.* used an artificially induced system for the expression of the IL-6R, and therefore, these cells responded differently to PMA in comparison to the B cells which naturally express IL-6R.

4. THE SIGNAL TRANSDUCING RECEPTOR, gp130.

4.1 Cloning and Characterization

The human, murine, and rat gp130 molecules have been cloned and characterized (Hibi *et al.*, 1990; Wang *et al.*, 1992; Saito *et al.*, 1992). The human gp130 contains extracellular region of 597 amino acids, including a 22 amino acid signal sequence, a transmembrane region of 22 amino acids, and a cytoplasmic region of 277 amino acids (Hibi *et al.*, 1990). Rodent gp130 cDNAs have shown that this molecule is highly conserved between species. The human, rat and murine gp130 share 76%-78% homology at the amino acid level. A higher degree of homology exists between the cytoplasmic domains (~85%) than the extracellular domains (~75%) (Wang *et al.*, 1992; Saito *et al.*, 1992).

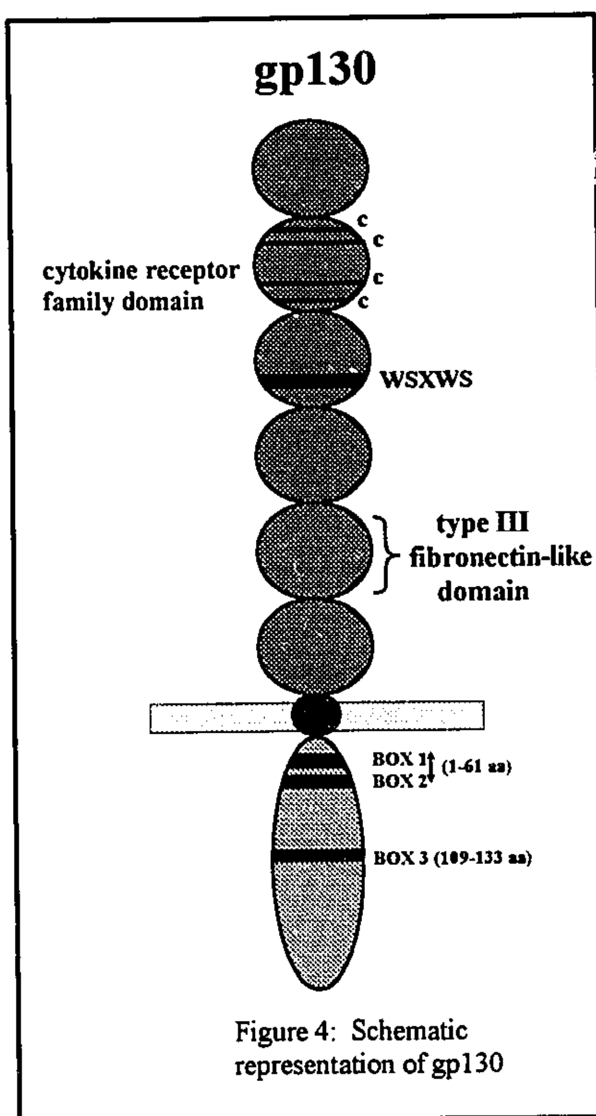


Figure 4: Schematic representation of gp130

This molecule belongs to the Class I cytokine receptor family and gp130 molecule is the signal transducing molecule for the IL-6 subfamily of cytokine receptors (See Figure 1 & 4)(for reviews see Taga & Kishimoto, 1992; Miyajima *et al.*, 1992; Hirano *et al.*, 1994). The extracellular region forms six units of fibronectin

type III modules. It also contains elements particular to the cytokine receptor family, the four conserved cysteine residues and the WSXWS motif. It is the extracellular domain of gp130 that is involved in the high affinity IL-6R sites (Taga *et al.*, 1987). Binding of IL-6 to IL-6R induces homodimerization of gp130 and binding of LIF and CNTF/CNTF-R to LIF-R induces heterodimerization of gp130 (Murakami *et al.*, 1993; Davis *et al.*, 1993*b*) (See Figure 2). This step is crucial for signal transduction because tyrosine kinase activity is associated with dimerized gp130, not monomeric gp130.

4.2 Signal Transduction of gp130

Chimeric and mutagenesis studies have shown that the cytoplasmic domain of gp130 contains different areas required for signal transduction (Murakami *et al.*, 1991; Baumann *et al.*, 1994*b/c*; Stahl *et al.*, 1995) (See Figure 1 & 4). In the cytoplasmic region, a 61 amino acid region proximal to the cell membrane, is sufficient for mediating a growth signal in transfected BF303 cells. This stretch of amino acids contains two short segments (designated Box 1 and Box 2) that are highly conserved among members of the cytokine receptor family (Murakami *et al.*, 1991). Baumann *et al.*, 1994*b*, demonstrated that an additional cytoplasmic sequence (designated Box 3) was also required for the full activation of acute phase genes. The Box 1 and Box 2 motifs may activate signaling pathways requiring the protein tyrosine kinase JAK1, JAK2, and TYK2 (Murakami *et al.*, 1991), whereas the Box 3 motif is required for the signaling pathway that involves the IL-6RE consensus sequence (Baumann *et al.*, 1994*b*).

Stahl *et al.*, 1995, have recently demonstrated that receptor dimerization causes phosphorylation of five tyrosine residues on the cytoplasmic domain of gp130. It is likely that the

Janus family of tyrosine kinases are the ones that phosphorylate these tyrosine motifs, and as a result of this phosphorylation, these residues become docking sites for downstream signal transducing molecules. This group demonstated that two of the phosphorylated tryosine motifs were specific for STAT3 and protein tyrosine phosphatase (PTP1D). These molecules bound these docking sites via their SH_2 domains, and as a result were activated by phosphorylation via the associated tyrosine kinases.

One group has also demonstrated through mutagenesis studies, that a 10 amino acid sequence, containing a di-leucine motif, may be required for efficient internalization of IL-6R and IL-6. It has been suggested that this di-leucine motif directs the receptor internalization (Dittrich *et al.*, 1994).

4.3 gp130 mRNA regulation

Northern blot analysis has shown that gp130 mRNA is ubiquitously expressed (Hibi *et al.*, 1990; Wang *et al.*, 1992; Saito *et al.*, 1992). Analysis of murine and rat liver mRNA have revealed the existence of two mRNA species at ~7.5kb and ~9kb. Since only one message exists for the human gp130 mRNA, it has been suggested that the difference between the human and rodent mRNAs may be a species-specific phenomenon (Hibi *et al.*, 1990). The two mRNA messages found in rodents most likely represent differential splicing or polyadenylation of the two transcripts (Wang *et al.*, 1992; Saito *et al.*, 1992).

Both *in vitro* and *in vivo* studies done on the gp130 signal transducing molecule have shown that mRNA levels are not as highly regulated as the IL-6R mRNA levels (Wang *et al.*, 1992; Saito *et al.*, 1992; Schooltink *et al.*, 1992). Schooltink *et al.*, 1992, have shown that in human HEPG2 cells, gp130 is stimulated by IL-6, but not by Dex. However, a combination of IL-6+Dex stimulates

the expression of gp130 to the greatest degree. In contrast, rat primary hepatocytes gp130 levels remained fairly constant when cells were exposed to the same conditions (Nesbitt & Fuller, 1992).

In vivo, IL-6 seems to be a major regulator of gp130. Saito et. al., 1992, have shown that at 1h the gp130 mRNA expression increased approx. 2 fold after injection of hIL-6 into mice, and our group showed similar results in rats (Geisterfer *et al.*, 1995*a*).

4.4 The Soluble Form of gp130

A soluble form of the gp130 molecule also exists (Matsuda *et al.*, 1994*a*; Narazaki *et al.*, 1993,). Matsuda *et al.*, 1994*a*, have shown that soluble gp130 is present in ascitic fluids of tumour bearing mice. Recently, Narazaki *et al.*, 1993, found two soluble forms of gp130 (90 & 110 kd) in human serum. Whether these soluble molecules are produced by proteolytic shedding or from alternately spliced mRNA encoding a soluble form of the gp130 molecule or both, still needs to be determined. It has been shown that anti-gp130 monoclonal antibodies and soluble forms of gp130 block the biological activities of IL-6 and IL-6-like cytokines (Taga *et al.*, 1992*b*; Narazaki *et al.*, 1993). Therefore, unlike the alpha component of the IL-6 receptor complex, the soluble form of gp130 acts as an antagonist.

5. THE LIF RECEPTOR

5.1 Cloning and Characterization

The human and murine leukaemia inhibitory factor receptor (LIF-R) have been cloned and characterized. The human LIF-R protein (1097 amino acids) contains a 44 amino acid signal sequence, a 789 amino acid extracellular domain, a 26 amino acid transmembrane domain, and a 338 amino acid cytoplasmic domain. Murine LIF-R shares 76% amino acid homology with the human LIF-R. However, the murine LIF-R cDNA contains a stop codon between the 4th and 5ft type III fibronectin-like module, and it is predicted that this cDNA encodes for the soluble LIF-R (Gearing *et al.*, 1991).

LIF-R also belongs to the cytokine receptor family (See Figure 1 & 5). The extracellular domain has six units of the type III fibronectin like modules. This extracellular domain also contains two conserved WSXWS motifs and two domains with conserved cysteine residues (Gearing *et al.*, 1991).

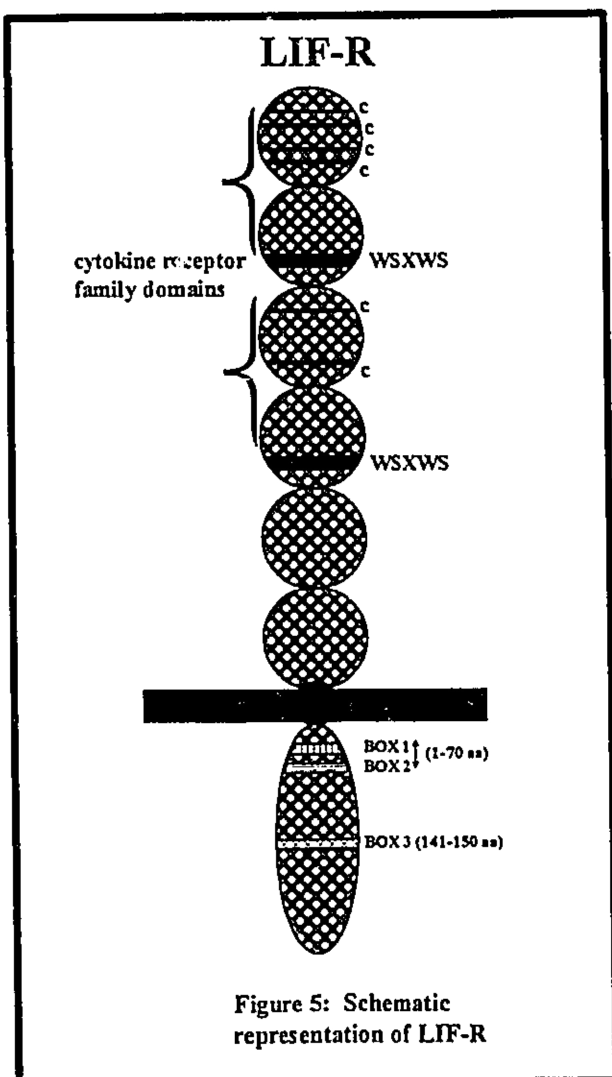


Figure 5: Schematic representation of LIF-R

5.2 Ligand Binding and Signal Transduction

LIF only binds LIF-R with low affinity, whereas co-expression of both LIF-R and gp130 results in both low and high affinity binding sites (Gearing *et al.*, 1992*b*). Interestingly, like IL-6, human LIF can bind both the human and murine LIF-R, but murine LIF cannot bind the human LIF-R. Using chimeric forms of both human and murine LIF-R, revealed that binding of murine LIF to murine LIF-R involved a different site than binding of human LIF to human LIF-R. However, binding of human LIF to murine LIF-R involved both sites (Layton *et al.*, 1994).

The LIF-R/gp130 complex is activated by other cytokines such as OSM and CNTF. OSM can bind the same receptor complex as LIF with high affinity and competes with LIF for the same binding sites (Gearing *et al.*, 1992*b*). CNTF, like IL-6, binds to its alpha subunit first (CNTF-R) and this complex then binds LIF-R and gp130 (Baumann *et al.*, 1993; Davis *et al.*, 1993*a*; Stahl *et al.*, 1993) (See Figure 2). Stimulation of cells with LIF or CNTF give rise to identical sets of tyrosine-phosphorylated proteins (Stahl *et al.*, 1993). However, when LIF stimulated cells are compared to OSM stimulated cells, different patterns of tyrosine phosphorylated proteins are seen. This observation suggests that another receptor may bind OSM that does not bind to LIF (Thoma *et al.*, 1994).

LIF binding induces heterodimerization between LIF-R and gp130, and this in turn activates a variety of intracellular signaling molecules, some of which have already been discussed (Stahl *et al.*, 1994; Davis *et al.*, 1993*b*). Both the transmembrane and cytoplasmic domains of LIF-R are closely related to the gp130 molecule (Gearing *et al.*, 1991; Baumann *et al.*, 1994*b*). Due to the homology seen in the cytoplasmic domains of these receptors, it has been suggested that they may initiate the same signal transducing pathways (Baumann *et al.*, 1994*b/c*).

Baumann *et al.* (1994*b/c*) have shown that LIF-R has similar Box 1, Box 2, and Box 3 motifs in its cytoplasmic domain as the gp130 molecule, and that these motifs are necessary for signaling. The first two sequence motifs are located within the first 70 amino acids of the cytoplasmic domain and the third critical motif (Box 3) is located between residues 141 and 150.

Stahl *et al.* (1994/1995) have shown that the JAK family of kinases are associated with LIF-R and that dimerization results in the phosphorylation of certain tyrosine motifs located in the cytoplasmic domain of the LIF-R. One particular tyrosine motif (Y_3) is critical for the phosphorylation and activation of a downstream signal transducing molecule STAT3. The phosphorylated tyrosine acts as a docking site for STAT3 so that it can be activated by the associated tyrosine kinases.

5.3 The Soluble LIF Receptor

Soluble forms of LIF-R have been found in normal mouse serum at ~1 ug/ml. Isolation of this soluble LIF-R from mouse serum yields a 90 kDa protein. However, expression of the murine LIF-R cDNA (isolated from mouse liver library) in COS-7 cells, yields both a membrane bound and soluble 130 kDa protein (Gearing *et al.*, 1991). This suggests that either additional mRNA processing must exist in cells before the message is translated into protein, or that the origin of the 90 kDa protein may be different from the 130 kDa protein (i.e. proteolytic cleavage). Although soluble LIF receptors have been found in serum, unlike IL-6R and CNTF-R, soluble LIF-R is an antagonist and blocks biological activity (Layton *et al.*, 1992; Gearing *et al.*, 1991). Mouse serum contains enough soluble LIF-R to inhibit biological activity of LIF up to 1 ng/ml (Layton *et al.*, 1992).

6. RESEARCH OBJECTIVES

IL-6 is an important mediator in inflammation and the acute phase response. In order to understand the role that IL-6 plays in the regulation of the acute phase protein synthesis by the liver, it was necessary to study the interaction that this cytokine has with liver cells or hepatocytes through high affinity specific receptors expressed on their cell surface. These cell surface receptors are regulated by many factors in their environment, to either enhance or inhibit a response, and we set out to study the regulation of some of these receptors (IL-6R, gp130, and LIF-R) on the hepatocyte *in vivo*. Such regulation would, in turn, modify the hepatic acute phase protein response and affect the site of inflammation.

We began this study by investigation of the regulation of the expression of the family of haematopoietic cytokine receptors, IL-6 Receptor (gp80), the IL-6 signal transducing receptor, gp130, and the LIF Receptor during inflammation, and detemination as to whether these receptors are regulated in a unique fashion depending on the type of inflammation induced - acute systemic caused by LPS injection; acute local caused by subcutaneous injection of turpentine; or chronic caused by intraperitoneal injection of Freunds Complete Adjuvant.

Having seen a relationship *in vivo* between circulating cytokine levels and IL-6R (gp80) expression in the liver, we then determined whether administration of IL-6 or corticosteroid alone

would affect the expression of the IL-6 Receptor.

As data generated *in vivo* differed from earlier published results with human liver cells, we pursued *in vitro* rat hepatoma cell studies to explore how this cytokine family (IL-6, LIF, and OSM) would affect IL-6 Receptor expression in a representative cell system *in vitro*. In addition, as these cytokines share interactions and signal transduction through a common receptor component, gp130, we examined whether "cross-talk" existed within this haematopoietic cytokine/receptor family. Could on cytokine (IL-6) regulate the expression of the receptor for another (LIF-R)?

Chapter two

REGULATION OF IL-6 AND THE HEPATIC IL-6 RECEPTOR IN ACUTE INFLAMMATION IN VIVO

The following paper, that was written by the author of this thesis, was published in Cytokine, 5:1-7, 1993. It deals with the regulation of the IL-6, corticosteroid, acute phase protein production, and hepatic IL-6R (alpha component, gp80) mRNA levels, during three different models of acute inflammation *in vivo*. It also deals with the regulation of the hepatic IL-6R mRNA levels *in vivo* when rats are exposed to rIL-6 or corticosteroid injections.

The research for this study was performed by the author of the thesis with help from laboratory technicians for cytokine activity assays, and determination of acute phase protein production. The supervision from Dr. Gauidie, advise from Dr. Richards, and information and technical help from Drs. Baumann, Fey and Gywnne resulted in the multiple authorship in this paper.

REGULATION OF IL-6 AND THE HEPATIC IL-6 RECEPTOR IN ACUTE INFLAMMATION IN VIVO

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The serum levels of interleukin 6 (IL-6) and the expression (mRNA) of the 80 kDa IL-6 receptor (IL-6R) were examined in three different models of acute inflammation. Rats were treated with either Freund's complete adjuvant (FA) via intraperitoneal injection, LPS via intravenous injection, or turpentine via subcutaneous injection. Using bio- and specific immunoassays, rat serum levels of IL-6, corticosterone, and acute phase proteins were quantified. LPS treatment induced the quickest and greatest serum IL-6 response (>100 ng/ml within 3 h). In comparison, sera from turpentine and FA-treated rats contained much lower levels of IL-6 activity (<10 ng/ml). Serum corticosterone levels increased by 3 h after injection in all three models, and equivalent raised serum levels of acute phase proteins were detected within 12–24 h. The expression of IL-6 receptor mRNA in hepatocytes increased markedly as early as 3 h after treatment and message levels began to decline by 6–12 h in all three models.

To analyze the individual effects of raised corticosterone and IL-6 on the expression of hepatic IL-6R mRNA, rats were injected with either dexamethasone (Dex) or purified recombinant rat IL-6 (rIL-6) via intraperitoneal injection. Rats injected with rIL-6 showed highly induced IL-6R mRNA levels as early as 1 h after injection, and Dex-injected rats showed a significant but less dramatic rise in IL-6R message levels. Dex- or rIL-6-injected rats demonstrated a distinct profile of acute phase protein response different from that seen in the three experimental models. Regulation of IL-6R gene expression in the liver in vivo depends on a complex interaction between the hepatocyte and a combination of cytokines and other hormones.

Interleukin 6 (IL-6) is a multifunctional cytokine involved in the immune response, the acute-phase reaction in inflammation and growth and differentiation. This cytokine is produced by a variety of cell types after appropriate stimulation, such as occurs with infection or trauma. Monocytes, macrophages, endothelial cells, and fibroblasts are probably the major sources of IL-6 during systemic inflammation. The biosynthesis of this important mediator of inflammation and its pleiotropic functions in the host defense against tissue injury and injection have been extensively reviewed (for recent reviews see refs 1–8). Although IL-6 acts on many different cell types in vitro, the liver seems to be one of the major target organs for IL-6 in vivo[9,10] and IL-6 induces acute phase protein synthesis in liver during the acute inflammatory response to tissue injury and infection.[11–15]

In order to understand the role of IL-6 as an inflammatory mediator in the acute phase response, it is necessary to study the interaction of this molecule with its receptor(s) on target cells. IL-6 confers its signal to target cells by binding to a specific IL-6 receptor on the cell surface which consists of two chains, a ligand binding chain of *c*. 80 kDa (IL-6R)[16] and a 130 kDa non-ligand binding glycoprotein essential for signal transduction (gp130).[17] The gp130 glycoprotein apparently associates with the IL-6R ligand binding chain only after binding IL-6 resulting in the

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formation of high-affinity IL-6 binding sites. Both the ligand binding chain IL-6R[16,18] and the gp130 glycoprotein have been cloned and characterized.[19]

The studies reported here examine the effect of experimental models of acute inflammation in the rat, and the administration of rIL-6 and/or corticosteroid (dexamethasone [Dex]) on the regulation of IL-6 in serum, and the expression of the 80 kDa IL-6R mRNA in hepatocytes. Different in vivo models were investigated: rats were treated with Freund's complete adjuvant (FA) via intraperitoneal (i.p.) injection, LPS via intravenous (i.v.) injection, turpentine via subcutaneous (s.c.) injection, rIL-6 via i.p injection and the corticosteroid analogue dexamethasone via i.p. injection. We examined the change in serum IL-6 levels, corticosterone levels, and acute phase protein levels, as well as hepatic IL-6R mRNA levels over a 24 h time period. Results indicate that both the production of IL-6 and the regulation of expression of hepatic IL-6R mRNA occur rapidly during acute inflammation and that both IL-6 and corticosteroid are involved in the regulation of hepatic IL-6R message levels.

RESULTS

FA-, LPS- and Turpentine-Injected Rat Models

Serum IL-6 Activity

Serum IL-6 activity levels peaked at different times in the different models (Fig. 1A). LPS-treated rats had peak serum levels of IL-6 activity by 3 h (*c.* 120 ng/ml), whereas peak serum levels of IL-6 activity for turpentine and FA-treated rats occurred by 12 h. LPS-treated rats showed 16-fold greater amounts of IL-6 activity in comparison to turpentine-treated rats (*c.* 7.5 ng/ml), and 170 times the amount of IL-6 activity in comparison to FA-treated rats (*c.* 0.74 ng/ml).

Serum Corticosterone Levels

In all three models of acute inflammation the corticosterone levels were increased 2–3-fold by 3 h in comparison to normal (Fig. 1B). These levels remained raised at 18 h in LPS- and turpentine-treated

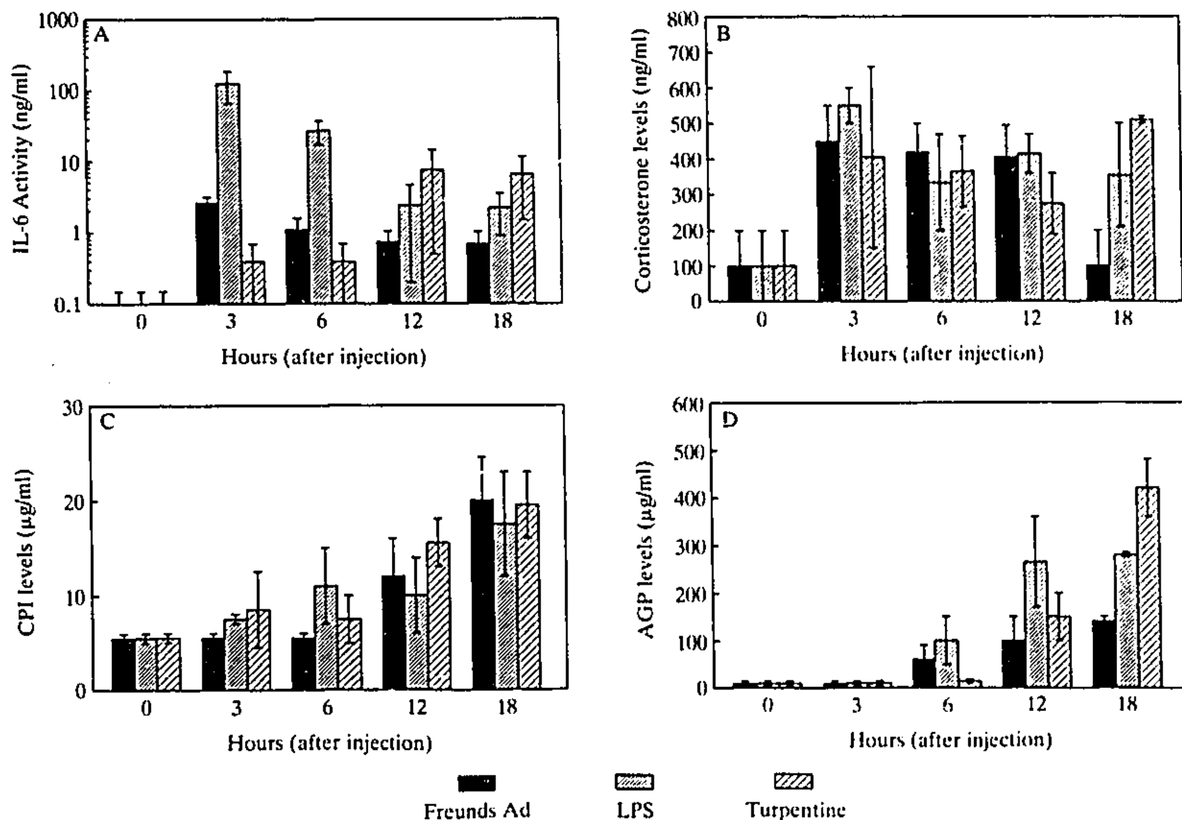

Figure 1. Serum levels of (A) IL-6, (B) corticosterone, (C) CPI and (D) AGP, of FA-, LPS- and turpentine-treated rats.

Samples for each time point represent the average amount (with range) calculated from two rats injected with the eliciting agent.

rats, but declined to control levels in FA-treated animals at this time point.

Acute Phase Protein Response

Serum levels of cysteine proteinase inhibitor (CPI) (Fig. 1C) and α1-acid glycoprotein (AGP) (Fig. 1D) gradually increased in all three experimental models, with highest protein levels measured at 18 h. The levels of CPI reached at 18 h (17.5–20.0 μg/ml) were approximately equivalent in all three models, whereas maximum AGP levels varied between the three models (140–420 μg/ml), possibly due to the fact that CPI and AGP respond differently to combinations of cytokines[13–15] and that FA, LPS and turpentine, may elicit distinct combinations of cytokines that act upon the liver.

CPI mRNA in Liver

Northern Blot analysis of hepatic poly(A)+ demonstrated the presence of one predominant message for CPI at 1.5 kb. All three models of acute inflammation demonstrated increased levels of CPI message over 18 h (Fig. 2A). CPI mRNA was increased 17.7 fold in FA-treated rats, 11.2 fold in LPS-treated rats, and 5.2 fold in turpentine-treated rats at 18 h. There was some temporal differences in CPI mRNA expression in the liver. In LPS-treated rats, CPI mRNA expression peaked at 6 h, whereas in both FA- and turpentine-treated rats mRNA expression peaked later at 18 h. Although mRNA levels differed considerably, serum CPI protein levels were similar in all three models of inflammation.

IL-6R mRNA in Liver

Northern Blot analysis of 30 μg poly(A)+ demonstrated the presence of one predominant message at 5.1 kb and IL-6R mRNA levels increased rapidly in all three animal models (Fig. 2B). The IL-6R mRNA levels increased 5.9-fold and 6.4-fold at 3 h and 6 h respectively, after injection of FA. In LPS-treated rats, IL-6R mRNA levels increased 8.4 and 8.6 fold at 3 h and 6 h. Turpentine-treated rats showed only a modest 2.8-fold increase. In all three models, the raised receptor mRNA levels begin to decline at 6–12 h. Although the peak levels of IL-6R mRNA differed between the three models, the overall pattern of regulation was the same.

Dex and rIL-6-Injected Rats

Serum IL-6 Activity and Corticosterone Levels

Rats injected with rIL-6 protein (50 μg) showed IL-6 serum activity levels of 0.16 ng/ml at 1 h and 0.12 ng/ml at 4 h after injection. Serum IL-6 levels returned to normal by 12 h after injection (Fig. 3A).

Rats injected with Dex showed no increase in serum IL-6 levels at any of the time points, but showed a dramatic drop in endogenous corticosterone levels that started to recover 12 h after Dex injection (data not shown).

Acute Phase Protein Response

Rats injected with Dex or rIL-6 demonstrated a different acute protein phase response in comparison with FA-, LPS- and turpentine-treated rats.

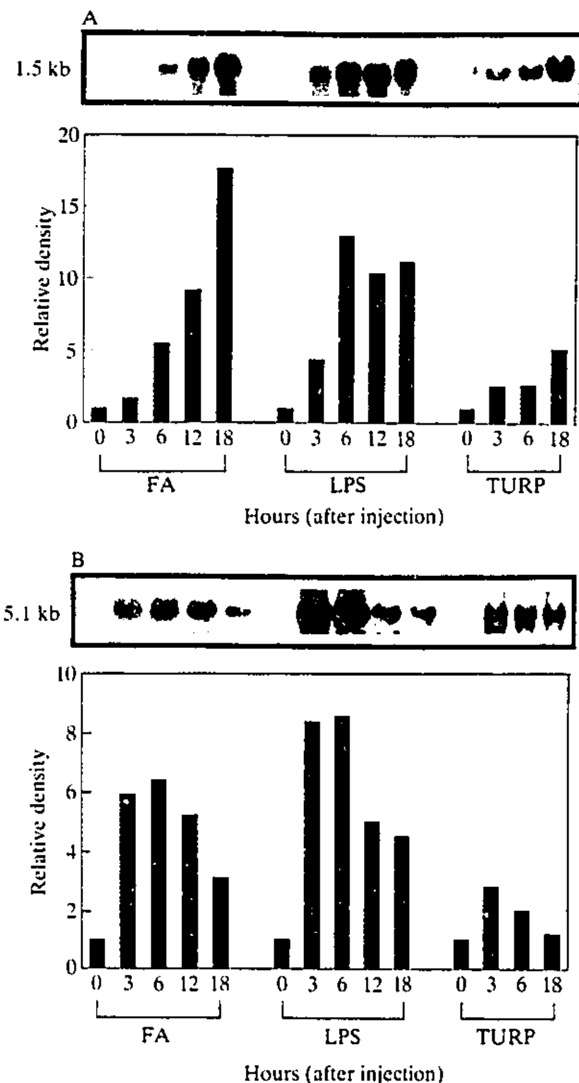

Figure 2. Northern Blot and Densitometer scans of hepatic mRNA are shown for (A) CPI and (B) IL-6R of FA-, LPS- and turpentine-treated rats.

Each lane represents 30 μg of Poly (A)+. Standardization was done by reprobing the blots with GAPDH and IL-6R mRNA densitometer scans were normalized to GAPDH mRNA densitometer scans.

CPI protein did not increase in Dex-injected rats, whereas rIL-6-injected rats demonstrated peak CPI serum levels at 12 h (c. 20 μg/ml) comparable to CPI protein levels from FA-, LPS- and turpentine-treated rats (Fig. 3B). Both Dex- and rIL-6-injected rats recorded increased protein levels of AGP but Dex-treated rats demonstrated a much better AGP response in comparison to rIL-6-injected rats, where a significant increase in AGP protein concentration was seen only at 12 h (Fig. 3C). However, protein levels were much lower than the AGP protein levels from FA-, LPS- and turpentine-treated rats.

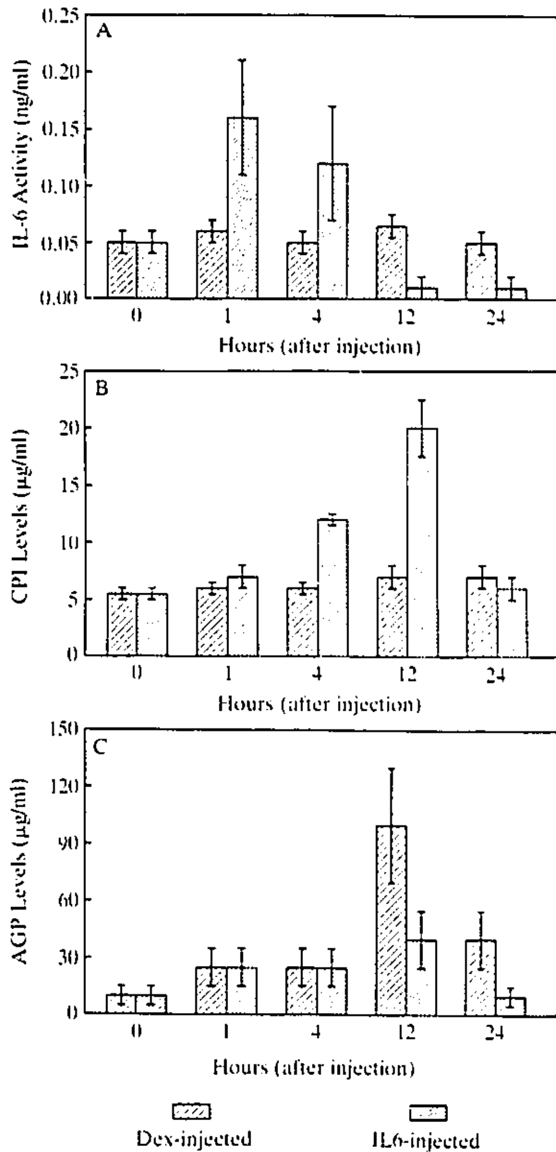

Figure 3. Serum levels of (A) IL-6, (B) CPI and (C) AGP of Dex-, and rIL-6-injected rats.

Samples for each time point represent the average amount (with range) calculated from two rats injected with the eliciting agent.

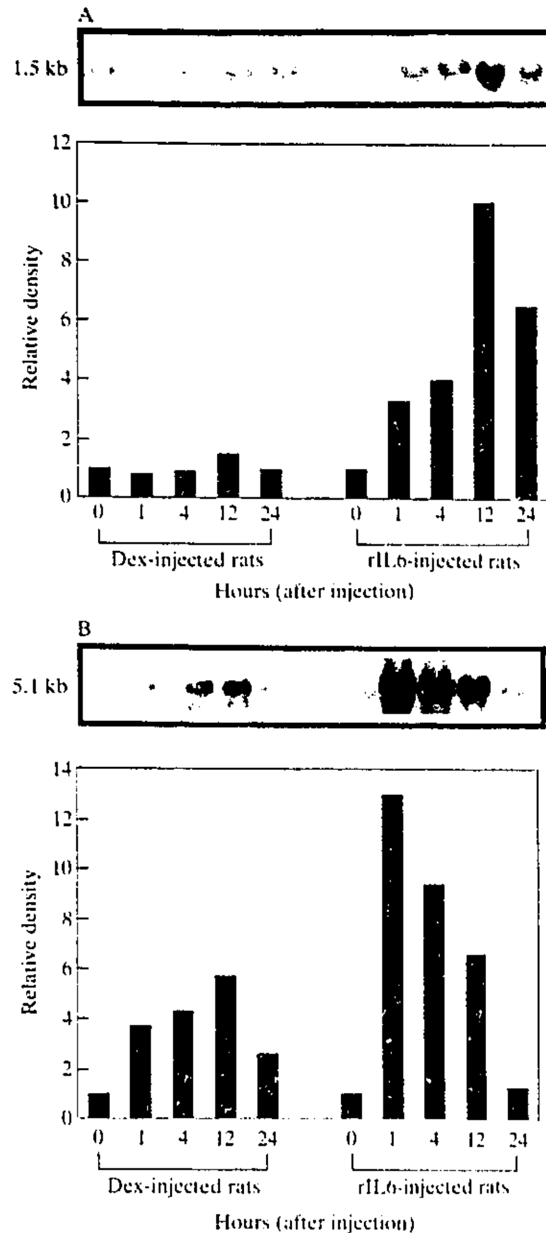

Figure 4. Northern Blot and Densitometer scans of hepatic mRNA are shown for (A) CPI and (B) IL-6R of Dex- and rIL-6-injected rats.

Each lane represents 30 μg of Poly (A)+. Standardization was done by reprobing the blots with GAPDH and IL-6R mRNA densitometer scans were normalized to GAPDH mRNA densitometer scans.

CPI mRNA in Liver

The expression of hepatic CPI mRNA remained the same for all time points after Dex injection consistent with serum CPI protein levels (Fig. 4A). However, rIL-6-injected rats demonstrated a 5.6-fold increase in mRNA concentration in comparison with normal at 12 h correlating with changed plasma levels.

IL-6R mRNA in Liver

The expression of hepatic IL-6R mRNA levels increased to a maximum of 5.7-fold by 12 h after Dex injection (Fig. 4B). Rats injected with rIL-6 protein showed a much earlier and greater increase. One hour after injection of rIL-6, IL-6R mRNA levels increased 13-fold in comparison with normal liver tissue mRNA. Message levels then decreased to normal by 24 h.

DISCUSSION

We have examined three experimental models of acute inflammation in the rat. Each of these stimuli induced the synthesis and release of acute phase proteins from the liver within 12–24 h, and increased the expression of hepatic IL-6R mRNA.

The serum cytokine (IL-6) levels of the three models differed in intensity and kinetics of change, with LPS inducing the quickest and greatest serum IL-6 response. Although large differences in serum IL-6 activity were seen, the overall acute phase protein response was similar between the FA-, LPS- and turpentine-treated rats, indicating that saturating levels of IL-6 protein for hepatic responses had been produced in all three models. In vitro, maximum responses to IL-6 are seen at 10 ng/ml.[6,8]

Expression of hepatic IL-6R message also increased very rapidly after injection of the eliciting agents. Peak hepatic IL-6R mRNA induction was seen 3–6 h after injection in FA-, LPS- and turpentine-treated rats and message levels begin to decline by 12 h. These results are similar to message levels found by Baumann et al.[18] in FA-treated rats, and Nesbitt & Fuller[20] in turpentine-treated rats. Although the increased levels of IL-6R mRNA expression differed between the three models, the overall pattern of regulation was similar.

In this paper, we investigated whether individually, rIL-6 or the corticosteroid analogue dexamethasone could increase IL-6R message levels in vivo. Rats injected with rIL-6 showed highly induced hepatic IL-6R message levels as early as 1 h after injection. In comparison, rats injected with Dex showed a significant although slower and less dramatic rise in IL-6R message levels.

Although IL-6R message levels were induced, the acute phase protein response varied between rIL-6- and Dex-injected rats. Rats injected with rIL-6 demonstrated a strong CPI response similar to levels seen in vivo in an acute inflammatory response, but AGP levels were low, whereas Dex-injected rats demonstrated a moderate AGP response but no CPI response. CPI and AGP respond differently to combinations of cytokines. CPI responds to IL-6 alone, whereas AGP is induced synergistically by IL-1 and IL-6 in the presence of corticosteroid,[13–15] and may also be induced by glucocorticoids alone as reported previously.[21–23] The fact that IL-6R mRNA was so highly and quickly induced in rIL-6-injected rats suggests that IL-6 itself may directly increase the expression of hepatic IL-6R mRNA. This is consistent with changes seen in vitro with HEPG2 cells in our hands. We found that IL-6 in the presence of Dex (10^{-6} M) increased IL-6R message significantly (3–4-fold) whereas Dex alone only increased IL-6R message a small amount (1-fold) in these cells (data not shown). Rose-John et al.[24] also found that treatment of HEPG2 cells with Dex leads to a time and dose-dependent increase of IL-6R message. Nesbitt & Fuller[20] found that treatment of rat primary hepatocytes cultures with Dex increased receptor mRNA levels 2.7-fold above normal. In contrast to our in vivo results, these authors reported that IL-6R message levels did not increase when primary hepatocytes were treated with rIL-6 and/or rIL-1 in vitro. They also demonstrated that when these same cytokines were added to primary rat hepatocyte cultures, they diminished the inductive effect that Dex has on the receptor mRNA message and protein levels. These in vitro data were supported by ligand binding studies. However, in addition to in vivo and in vitro differences, there may be considerable differences in experimental conditions such as time of examination and/or amounts of ligand, that may account for these apparent discrepancies. Moreover, Bauer et al.[25] reported that in primary cultures of fetal human hepatocytes, IL-6 increased expression of IL-6R mRNA directly. Therefore, there is both in vivo and in vitro evidence that both rIL-6 and Dex may directly regulate IL-6R mRNA.

We have previously shown that IL-6 acts as an exocrine or paracrine hormone with respect to hepatic response in inflammation [refs 26, 34]. The response of the hepatocyte in enhanced expression of IL-6R mRNA is rapid and of considerably greater magnitude than seen in vitro. The contribution of matrix to presentation of cytokines as noted by Nathan & Sporn[27] may play a significant role in response of cells in vivo. In addition, there may be other cytokines induced during inflammation that can impact on hepatic function.[13] While the data show only enhanced mRNA for IL-6R and need to be confirmed by suitable ligand binding studies, they imply that a system involved in a crucial part of the defense mechanism in inflammation, the hepatic acute phase response, not only responds to the circulating mediator, but in the short term also enhances the sensitivity of the system for the ligand by increasing the expression of the ligand receptor.

In conclusion, all three models of acute inflam-

mation rapidly increase the expression of IL-6R mRNA, and rIL-6 and Dex may play a role in this regulation. Although rIL-6 and Dex may directly effect IL-6R message levels, an increase of IL-6R message alone does not induce a full acute phase response in the liver, and regulation of the acute phase response in the liver in vivo during inflammation must depend on a complex interaction between the hepatocyte and a combination of cytokines and other hormones.

MATERIALS AND METHODS

Animals and Materials

Male Sprague-Dawley rats (*c.* 200 gm) were treated with Freund's complete adjuvant (Gibco Labs) (500 µl i.p. injection), LPS (Sigma) (200 µg i.v. injection), turpentine (200 µl s.c. injection), rIL-6 protein (*E. coli*, purified IL-6 from Allelix Biopharmaceuticals, Inc.) (50 µg i.p. injection), and dexamethasone (5 µg i.p. injection). The levels of stimuli were chosen to elicit a strong acute phase response. Rats were sacrificed at various time points up to 24 h and both serum and liver tissue samples taken. Serum cytokine and protein levels are the average of data from two rats injected for each time point and mRNA was extracted from pooled liver tissue samples from two rats.

The IL-6R probe, 1387 bp, covering the entire open reading frame of the receptor, was prepared by PCR from rat fibroblast poly(A)+ RNA and has a coding sequence with >99% homology to the rat IL-6R sequence derived from rat liver reported earlier by Baumann et al.[18]

Serum IL-6 Assay

Serum IL-6 activities were assayed by measuring the IL-6-dependent proliferation of the murine plasmacytoma cell line, B9, as described by Aarden et al.[28,29] with minor modifications as described by Mosmann.[30] Rat serum samples were not heat inactivated, as rodent IL-6 is susceptible to heat denaturation unlike the human cytokine. No cytotoxicity was noted with any rat serum samples. Specificity was confirmed by inhibition with rabbit antiserum raised against purified rat rIL-6. This antiserum inhibited recombinant and natural rat and mouse IL-6 in both the B9 and rat hepatocyte assay.[31]

Corticosterone Serum Levels

Serum corticosterone levels were determined by an RIA kit (ICN Biomedicals, Inc.). The assay had a sensitivity of 20 ng/ml.

Acute Phase Protein Serum Levels

The amounts of acute phase proteins secreted into the blood were determined by rocket immunoelectrophoresis using mono-specific antisera for rat acute phase proteins, cysteine proteinase inhibitor (CPI), and α1-acid glycoprotein (AGP) as previously described.[31]

mRNA Preparation and Northern Blot Analysis

RNA was extracted from liver tissue and prepared according to the method of Chomczynski & Sacchi.[32] Poly(A)+ RNA was prepared by oligo d(T) sepharose chromatography. We compared the level of IL-6R expression in total liver and isolated hepatocytes. There was no difference between the preparations and as such, total liver mRNA was used for examination of changes in inflammation. For Northern blot analysis, due to the low level of expression of mRNA for IL-6R in hepatocytes or total liver, 30 µg of poly(A)+ RNA was electrophoresed on 1% agarose-formaldehyde gels, denatured in 50 mM NaOH and transferred to ICN biotrans nylon membrane in 10X SSC. Standardization was done by reprobing the blots with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[33] Hybridizations were performed for 24 h with ^{32}P-labelled specific cDNAs using 1×10^6 cpm of labelled probe per ml hybridization buffer. Filters were exposed to Kodak XAR5 film and −70°C. mRNA levels were measured by quantitative scanning densitometry of autoradiographs using a Haefer Scientific Instrument densitometer and were normalized to constant amounts of GAPDH mRNA. All changes in mRNA intensity on the blots were compared to normal mRNA. Densitometer scans shown in this paper are only representative of the different mRNA concentrations on the Northern blots. Owing to the limitation of this method, and to the variability between different animals, these concentrations should only be considered approximate changes.

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Chapter three

REGULATION OF SIGNAL TRANSDUCER, gp130, AND THE LIF RECEPTOR IN ACUTE INFLAMMATION IN VIVO

The following manuscript, written by the author of this thesis, is *in press* in Cytokine. It continues the study from the first paper, and deals with the mRNA regulation of the IL-6 signal transducing molecule, gp130, and LIF receptor mRNA during three different models of acute inflammation *in vivo*. This manuscript also combines the data from the first paper and this paper to look at overall trends in receptor regulation.

The research for this study was performed by the author of the thesis, and the supervision from Dr. Gauldie resulted in the dual authorship in this paper.

REGULATION OF SIGNAL TRANSDUCER, gp130, AND THE LIF RECEPTOR IN ACUTE INFLAMMATION IN VIVO

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ABSTRACT

Recently, we examined the expression of the IL-6 receptor (gp80) in three different models of acute inflammation [Geisterfer et al, 1993, Cytokine 5:1]. To continue these studies, we examined the mRNA expression of signal transducing molecule, gp130, and the LIF-R as associated members of a receptor family. Rats were treated with either Freund's complete adjuvant (FA) via intraperitoneal injection, LPS via intravenous injection, or turpentine via subcutaneous injection. The levels of gp130 and LIF-R mRNA expression had a maximum 2-3 fold increase over a 24h period. However, the time of the maximum increase differed depending on the treatment the rats received. FA treated rats had a maximum induction of gp130 mRNA levels of 2.2 fold (for the 7.5 kb transcript) and 1.3 fold (for the 9.0 kb transcript) at 12h. LPS treated rats had a maximum increase at 3h where message levels increased 2.5 fold (7.5 kb) and 1.2 fold (9.0 kb). Turpentine-injected rats showed little difference in gp130 mRNA levels at any time after injection compared to controls. Maximum LIF-R mRNA levels also differed depending on the type of treatment the rats received. FA-injected rats showed a 2.1 fold mRNA increase at 3h, whereas LPS treated rats show a maximum 2.4 fold increase at 18h. Turpentine-injected rats showed little increase in mRNA levels compared to controls.

Injection of purified recombinant rat IL-6 (rIL-6) had little effect on LIF-R mRNA levels, but had a dramatic inducing effect on gp130 mRNA levels. Rats were also injected (i.p.) with Dexamethasone (Dex) and this had no effect on either gp130 or LIF-R mRNA level expression.

These *in vivo* results indicate that IL-6 has a major role in the regulation of its own receptors, IL-6R (gp80) and gp130, and the onset of acute phase response. We found that the

maximum IL-6R (gp80), gp130, and LIF-R mRNA levels peaked at different times depending on the type of acute inflammation induced in the rat. It seems that various of combinations of cytokines and hormones are released depending on the type of acute inflammation, and these in turn regulate the expression of diverse receptors on the hepatocyte, resulting in different acute phase kinetics in the various models of inflammation.

INTRODUCTION

IL-6 and LIF are pleotropic cytokines that share many overlapping functions (1). Some examples are bone remodelling, neuronal differentiation and the stimulation of the acute phase response in the liver during inflammation. Both stimulate type 2 acute phase proteins and enhance the stimulation of type 1 acute phase proteins by IL-1 and TNF (2). IL-6 and LIF bind their own receptors IL-6R (gp80) and LIF-R with low affinity, but both cytokines share the same signal transducing molecule, gp130, and this may be the reason they share many similar functions (for reviews 3-5). The low affinity receptor/ligand complex then associates with gp130 to form high affinity binding sites (6-9). The signal transducer molecule, gp130, is also the signal transducing component for other cytokines such as Oncostatin-M (OSM), Interleukin 11 (IL-11) and Ciliary Neurotrophic Factor (CNTF) (10-14). OSM binds the gp130 molecule with low affinity and co-expression of gp130 and LIF-R result in intermediate affinity OSM binding sites (10). Whether there is a primary OSM receptor is still unknown but its presence is suspected.

The IL-6R, LIF-R, and signal transducer gp130 have been cloned and characterized (7,8,15-18), and all belong to the haematopoietic cytokine receptor family (19).

LIF-R is a 190 kDa molecule with both transmembrane and cytoplasmic domains closely related to the gp130 molecule (8). An alternative explanation for the similar functions caused by IL-6 and LIF is that both LIF-R and gp130 initiate a common signal transduction pathway (8). However LIF only binds LIF-R with low affinity, whereas co-expression of both LIF-R and gp130 results in both low and high affinity binding sites (7).

Although IL-6R and LIF-R may use the same signal transducing receptor molecule, the signal that they send into the cells may be different. For example, IL-6 mediated stimulation of thiostatin gene expression in H-35 cells is enhanced by Dex, whereas LIF mediated stimulation of thiostatin gene expression is inhibited by Dex (2).

We have previously examined the regulation of gp80 by IL-6 *in vivo* in experimental inflammation (20) and noted differences from *in vitro* regulation. Here we expand those studies to include examination of gp130 and LIF-R regulation *in vivo* to determine whether the receptor family is regulated in a coordinated manner.

MATERIAL AND METHODS

Animals and Materials

Male Sprague-Dawley rats (c. 200 gm) were treated with three different inflammatory stimuli as previously described (20). Rats were injected with Freund's complete adjuvant (Gibco Labs) (500 μl i.p. injection), LPS (Sigma) (200 μg i.v. injection), and turpentine (200 μl s.c. injection), and dexamethasone (μg i.p. injection). In addition rats were treated with either rIL-6 (Allelix Biopharmaceuticals, Inc.)(50 μg i.p. injection) or dexamethasone (5 μg i.p. injection).

Rats were sacrificed at various time points up to 24h after treatment, and liver tissues were taken for RNA isolation.

Human gp130 (cytoGP130/bluescript) and murine leukaemia inhibitory factor receptor (LIF-R) (pmLIFR21ΔBglII-BamHI) cDNA were supplied by Immunex.

mRNA Preparation and Northern Blot Analysis

RNA was extracted from liver tissue as described by Chomczynski & Sacchi (21). Poly(A)$^+$ RNA was prepared using oligo d(T) sepharose chromatography. For Northern blot analysis, 30 μg of Poly(A)$^+$ RNA was electrophoresed on 1% agarose-formaldehyde gels, transferred, hybridized, and probed as described (20). However, due to the species differences of human gp130 and murine LIF-R probes on rat mRNA, the washing conditions were as follows: 1) when blots were probed with murine LIF-R cDNA blots were washed with 1xSSC, 1%SDS for 10' at R/T, then 3 x 20' in 0.1xSSC, 1%SDS at 55°C. 2) when blots were probed with human gp130 cDNA blots were washed once with 1xSSC, 1%SDS for 10' R/T, once in .5xSSC, 1%SDS for 20' at 50°C, twice in .25XSSC, 1%SDS for 20' at 50°C. Standardization was done by reprobing the blots with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Filters were exposed to Kodak XAR5 film at -70°C. Autoradiographs were scanned by quantitative densitometry (Haefer Scientific Instruments), and mRNA was normalized to constant amounts of GAPDH. Densitometer scans shown in this paper are only representative of different mRNA concentrations and should only be considered approximate changes due to the limitation of this method.

RESULTS

FA-, LPS-, and Turpentine-injected Rat Models

gp130 mRNA in Liver

Northern Blot analysis of 30 μg poly(A)$^+$ demonstrated the presence of two predominant message transcripts at 7.5 kb and 9.0 kb. Wang et al. (17) also found the presence of two predominant messages in rat liver cells. Unstimulated cells generally showed slightly higher levels of 7.5 kb message than 9.0 kb message.

All three models of acute inflammation demonstrated increased levels of gp130 over 18h (Fig. 1), but the maximum increase in RNA levels varied according to the different treatment administered. In FA-injected rats, maximum induction of gp130 was 2.2 fold (7.5 kb) and 1.3 fold (9.0 kb) at 12h. LPS treated rats had a maximum induction at 3h where the 7.5 kb message levels increased 2.5 fold and the 9.0 kb message levels increased 1.2 fold. The maximum increase for turpentine-injected rats was at 18h and this increase was slight in comparison with the other models used. LPS treated rats had normal gp130 mRNA levels by 18h and FA treated rat mRNA levels were returning to normal by 18h, whereas turpentine treated rat mRNA levels were only beginning to increase.

LIF-R mRNA in Liver

Northern Blot analysis of 30 μg poly(A)$^+$ demonstrated the presence of one predominant message for LIF-R at approx. 10 kb (Fig. 2). FA-injected rats showed a 2.1 fold maximum increase at 3h. LPS-injected rats mRNA levels gradually increased 2.4 fold over 18h, whereas

turpentine-injected rats had only a slight increase in mRNA levels. The maximum increase of the LIF-R mRNA varied with the type of acute inflammation induced.

Some of the blots that were probed for LIF-R showed a faint lower band at approx. 3 kb. This could represent a truncated form of mRNA coding for the soluble LIF receptor. A truncated form of the LIF-R lacking the transmembrane domain was cloned from a mouse library (8). Alternate mRNA species have also been found for the human LIF-R (6 kb, 4.5 kb, and a minor 5 kb species). These may represent membrane bound and soluble forms of the LIF-R (8). However in the majority of rat samples, such a band was barely detectable and could not be properly analyzed.

Dex and rIL-6-Injected Rats

gp130 mRNA in Liver

The expression of hepatic gp130 remained the same for all the time points after Dex injection. Levels of gp130 mRNA were barely detectable even though 30 μg of poly(A)$^+$ were on the blot and blots were exposed at -70°C for over a month.

The expression of hepatic gp130 mRNA showed a maximum 2.5 fold (7.5 kb) and 1.5 fold (9.0 kb) 4h after IL-6 injection (Fig. 3). Subsequently mRNA levels decreased and were normal by 24h.

LIF-R mRNA in Liver

The hepatic mRNA for LIF-R showed no detectable increase in control or Dex-injected rats for any of the time points.

There was a small increase in mRNA levels in LIF-R 24h after rIL-6 injection, however, this increase in mRNA was slight, and could still be considered within normal range.

Comparison of IL-6R, LIF-R, gp130, and CPI mRNA levels

Kinetics of mRNA changes in the three acute inflammatory models and IL-6-injected rats, both from this paper and previous papers (20) are summarized in Fig. 4(A-D). Maximum mRNA levels for the cytokine receptors peaked at different time points depending on the type of acute inflammation present. Although IL-6R mRNA levels reached maximum levels quickly (3-6h) in all three models of acute inflammation, the maximum induction of gp130 and LIF-R differed depending on the type of treatment the rats received. For example, in FA stimulated rats, the gp130 mRNA levels peaked later at 12h with a 2.2 fold induction, whereas the LIF-R mRNA levels peaked at 3h with a 2.1 fold induction. In comparison, peak levels were reversed in LPS stimulated rats where gp130 mRNA levels peaked 2.1 fold at 3h, whereas LIF-R mRNA levels peaked later (2.4 fold) at 18h. In the Turp-injected rats, there was only a slight increase in both the gp130 and LIF-R mRNA levels at 18h.

A cytokine alone, such as IL-6, injected into rats gives only a partial profile of what happens during inflammation. IL-6R had a maximum 13 fold induction at 1h, but gp130 mRNA levels only increased 2-3 fold by 4h. LIF-R mRNA levels remained unaffected.

DISCUSSION

These data imply that although LIF and IL-6 have similar functions, and regulate the same set of acute phase proteins, their receptors are regulated differently depending on the type

of inflammation induced. These cytokines may be designed to function in tandem depending on the acute inflammatory response (1) and extensive *in vivo* studies by our group supports this hypothesis. In all three acute inflammatory models, IL-6R and LIF-R mRNA levels do not peak at the same time for any of the treatments given to the rats. This staggering of maximum LIF-R and IL-6R mRNA levels in the liver may ensure that cells send continual messages to make acute phase proteins.

Certain cytokines may have a more prominent role in some types of inflammation. For example, in IL-6-deficient mice the acute phase response is severely compromised in turpentine-injected mice, whereas in LPS-injected mice, the acute phase response is only moderately affected. All responses return to normal in these mice if they are injected with IL-6 (22). These data imply that the turpentine model is solely dependant on IL-6 while LPS involves several cytokines capable of triggering gp130.

In our studies, turpentine-injected rats showed an increase in IL-6R and gp130 mRNA levels, but showed a negligible increase in LIF-R mRNA levels. In contrast, LPS-injected rats showed a significant increase in the level of expression of all three receptors, indicating once more that many cytokines are involved in this endotoxin response. Since IL-6 and LIF stimulate the same set of acute phase proteins, LIF and other cytokines such as OSM and IL-11 may play important roles in LPS-injected rats.

Overall the amount of LIF mRNA present in normal rat hepatocytes seems to be much lower than gp130 and IL-6R. LIF-R mRNA levels are undetectable in normal rat hepatocytes and barely detectable in acutely inflamed liver cells. Distribution studies by Hilton et. al. (23), showed that on the majority of cell types, the number of LIF receptors is low (approx. 150-400

per cell, K_d = 10-200pm). In contrast, fetal and adult murine hepatocytes expressed approximately 4000 receptors/cell (K_d = 100pm). However it is not known whether rat hepatocytes express high numbers of LIF-R, and message levels would indicate that this is not the case. A similar discrepancy between rat and human gp130 mRNA has also been seen (data not shown).

Regulation of gp130 mRNA levels by IL-6 has been demonstrated both *in vivo* and *in vitro* by other groups (18, 24). Saito et al. (18) found that murine gp130 mRNA levels increase approximately two fold in liver tissue 1h after hIL-6 injection. *In vitro* studies done on human HEPG2 cells found that gp130 mRNA levels were stimulated by IL-6 (24).

This study shows that the combination of cytokines and hormones that interact with the hepatocyte *in vivo* and in turn, regulate the expression of the different receptors, is very complex and differ depending on the type of acute inflammation induced. Each inflammatory response produces a slightly different set of cytokines, and this in effect would regulate the receptors differently. Therefore, even though all of these receptors belong to the same family and have similar functions, the regulation of these receptors is unique and may lead to altered cell responses in the different models of inflammation.

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FIGURE LEGENDS

Figure 1 **Northern blot and Densitometer scans of hepatic gp130 mRNA for FA-, LPS, and turpentine-treated rats.** Each lane represents 30 μg of Poly(A)+. Standardization was done by reprobing the blots with GAPDH, and gp130 mRNA densitometer scans were normalized to GAPDH mRNA densitometer scans.

Figure 2 **Northern blot and Densitometer scans of hepatic gp130 mRNA for rIL-6-injected rats.** Each lane represents 30 μg of Poly(A)+. Standardization was done by reprobing the blots with GAPDH, and gp130 mRNA densitometer scans were normalized to GAPDH mRNA densitometer scans.

Figure 3 **Northern blot and Densitometer scans of hepatic LIF-R mRNA for FA-, LPS-, and turpentine-treated rats.** Each lane represents 30 μg of Poly(A)+. Standardization was done by reprobing the blots with GAPDH, and LIF-R mRNA densitometer scans were normalized to GAPDH mRNA densitometer scans.

Figure 4 **Comparison of IL-6R, LIF-R, gp130, and CPI mRNA levels in A) FA-, B) LPS-, C) turpentine-, and D) rIL-6-injected rats.** Kinetics of mRNA from a previous paper (20) and this paper are summarized. Maximum mRNA levels for the different cytokine receptors and CPI peaked at different time points depending on the treatment.

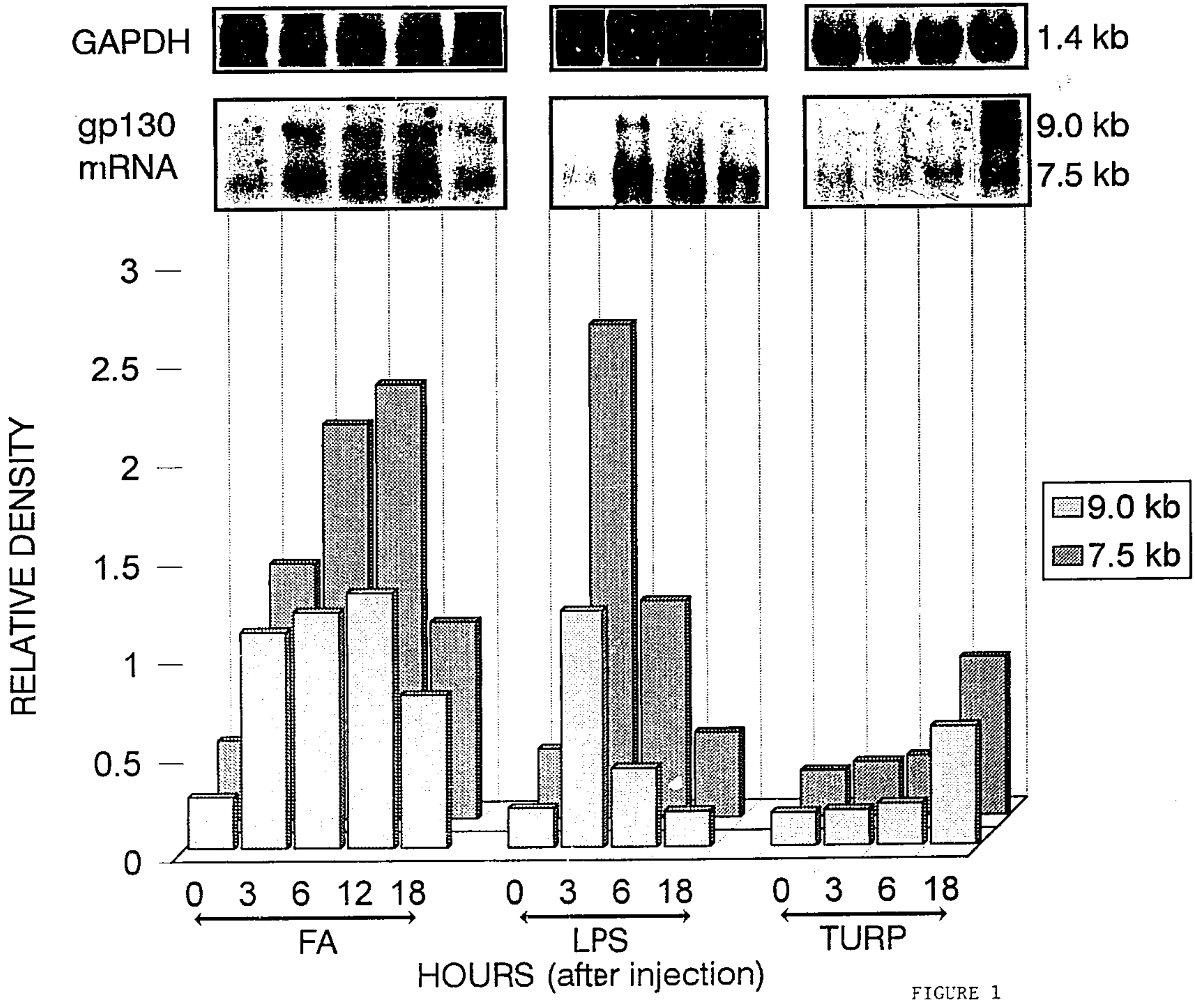

FIGURE 1

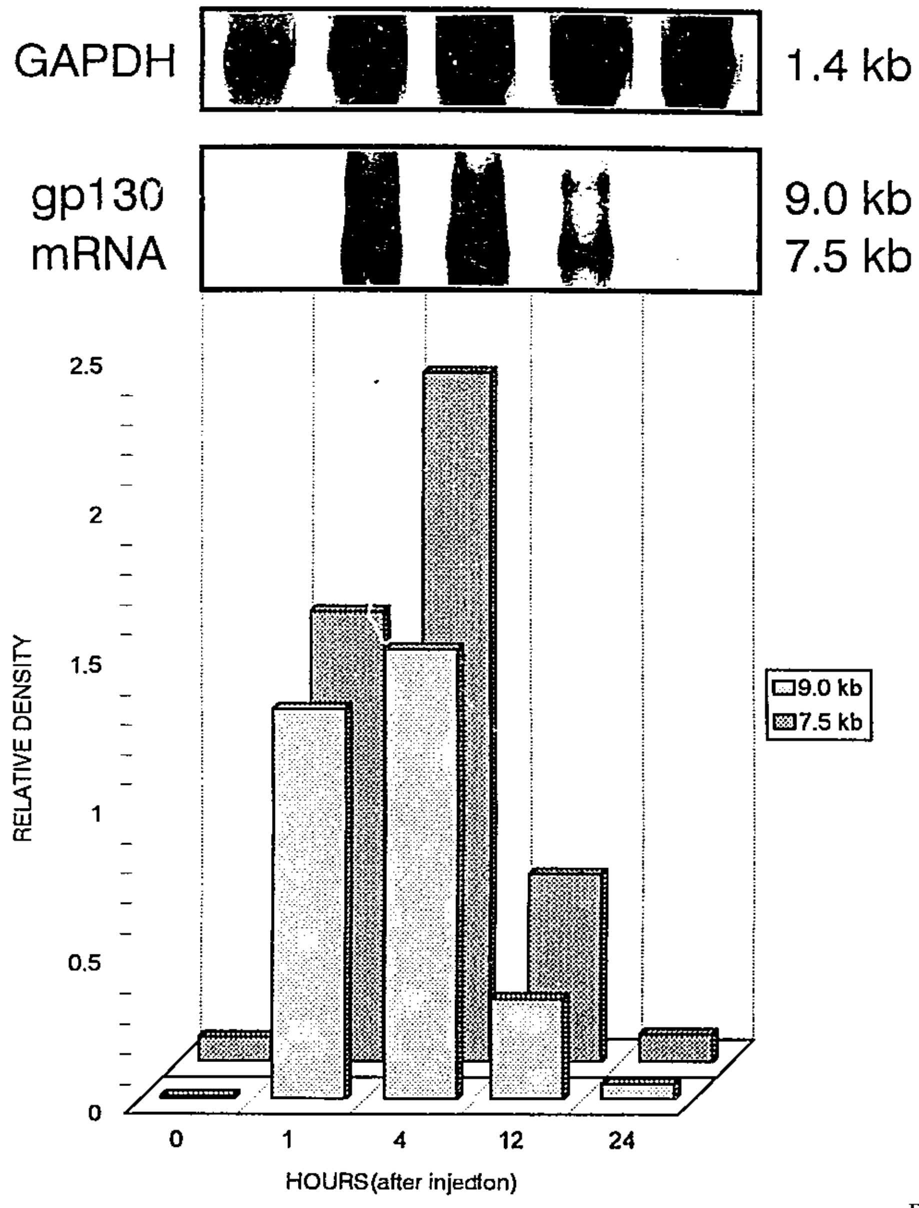

FIGURE 2

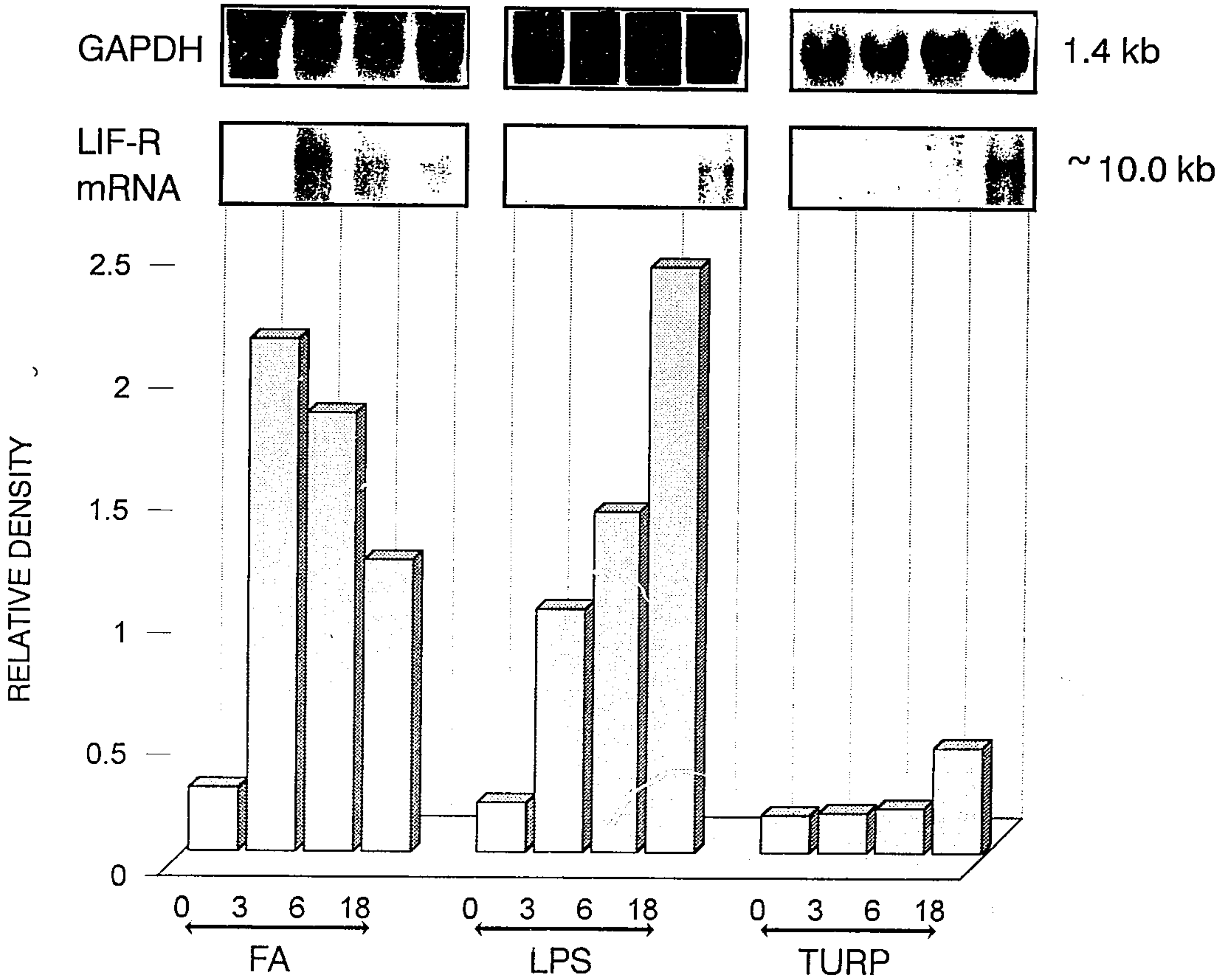

FIGURE 3

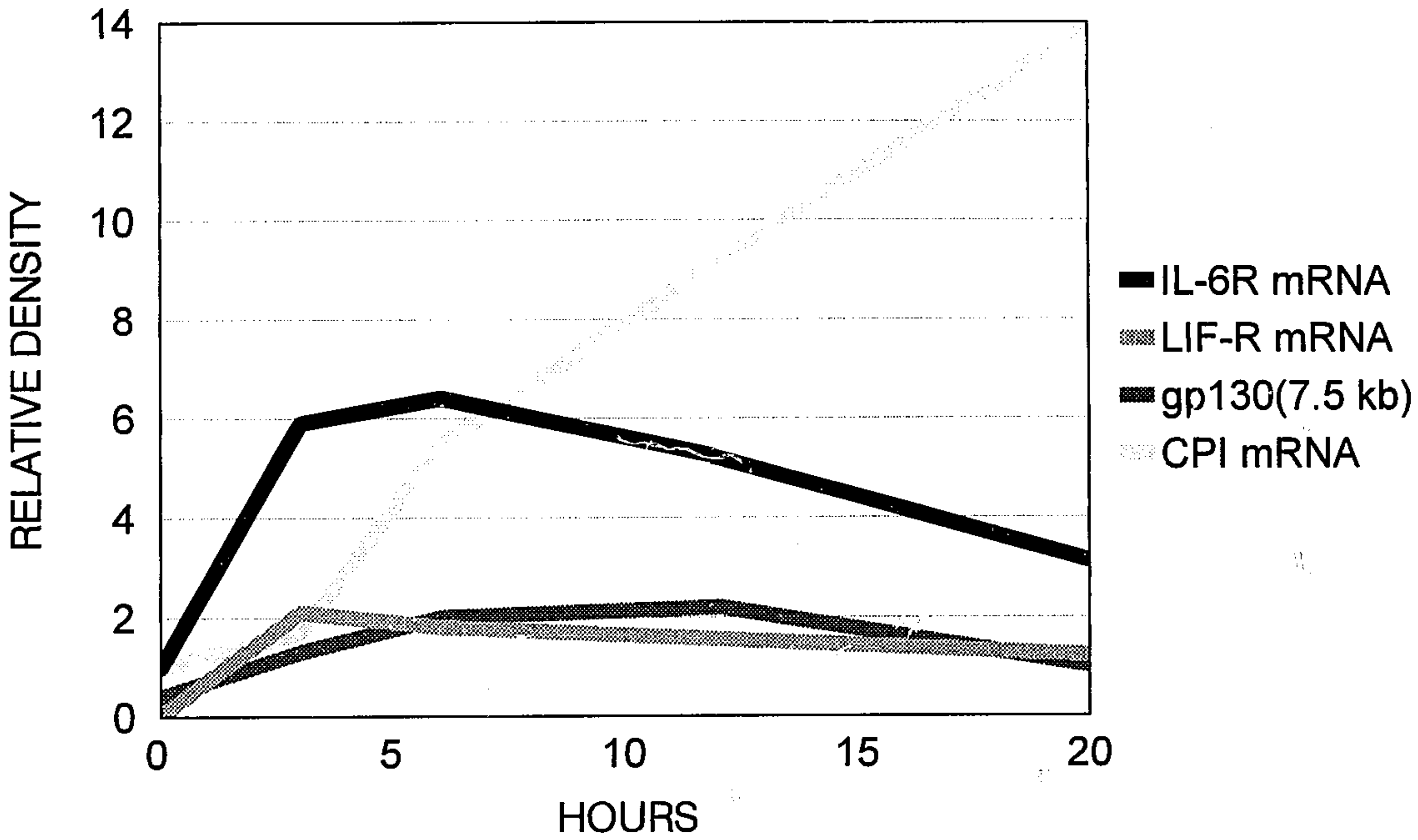

FIGURE 4A

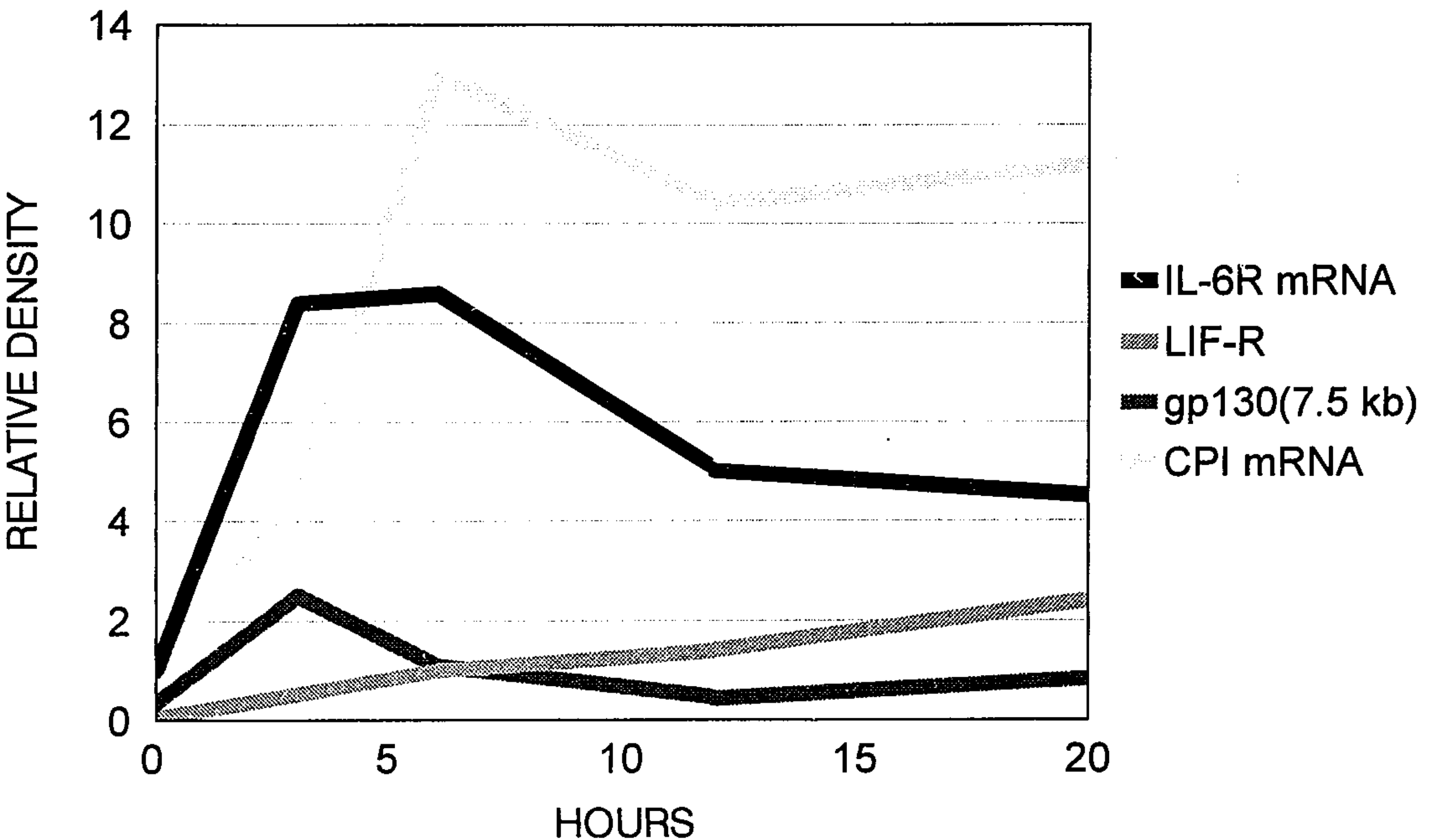

FIGURE 4B

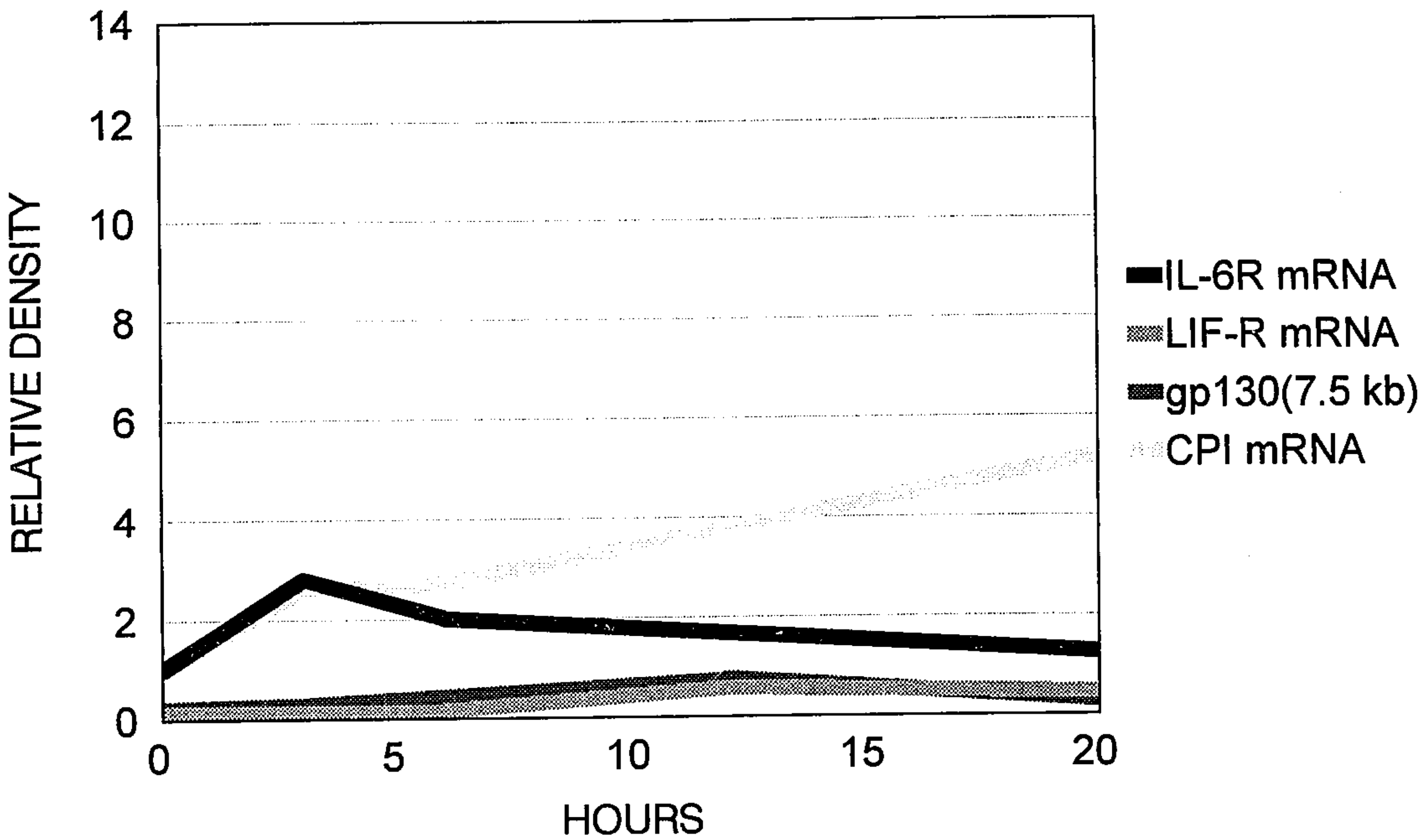

FIGURE 4C

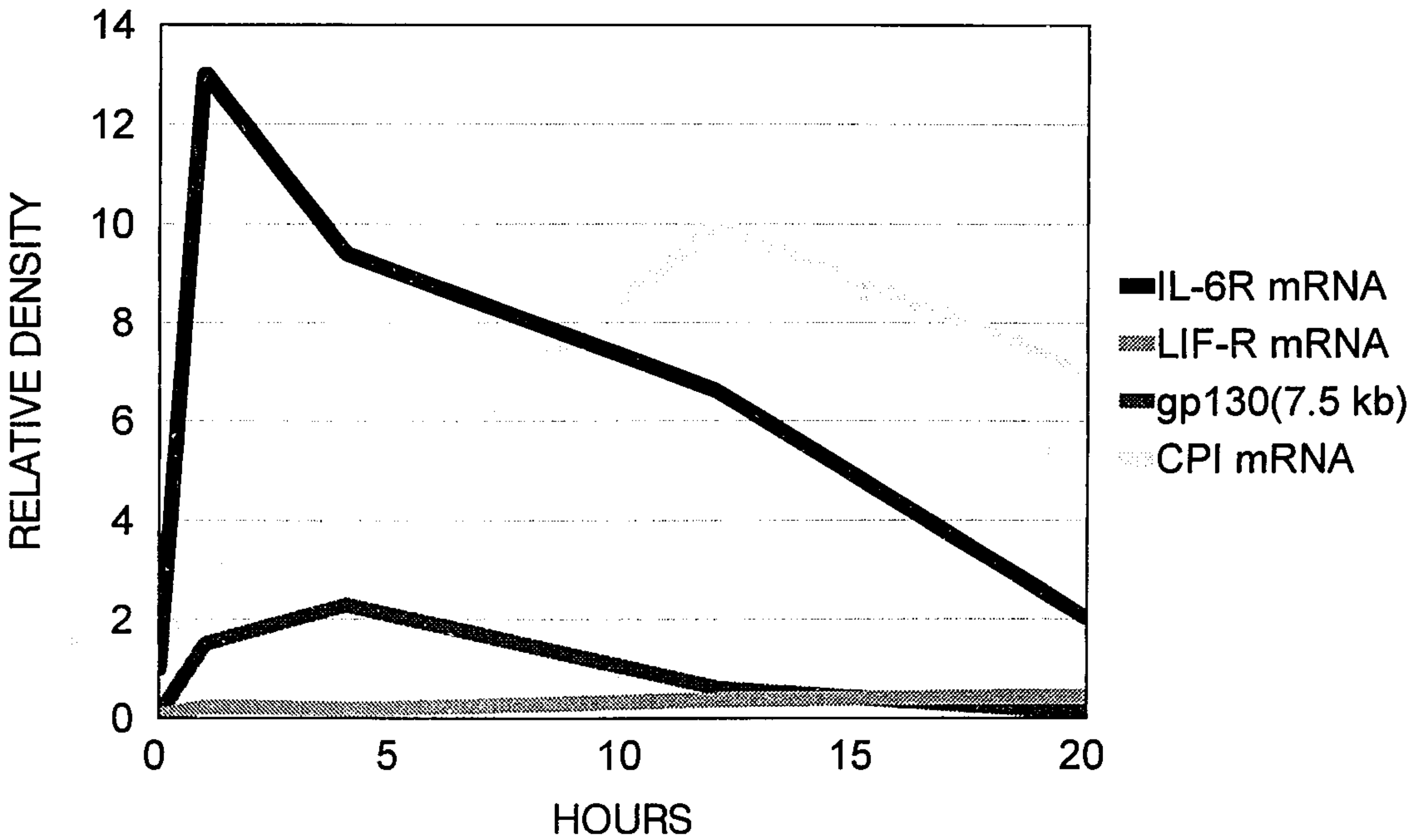

FIGURE 4D

Chapter four

REGULATION OF HEPATIC IL-6R, gp130, LIF-R, AND CPI mRNA LEVELS AFTER MULTIPLE rIL-6 INJECTIONS IN RATS.

The following manuscript was written by the author of this thesis. It continues the study from the first two papers, and deals with the mRNA regulation of the hepatic receptors after multiple rIL-6 injections into rats, to determine if the regulation is the same or different.

The research for this study was performed by the author of the thesis, and the supervision from Dr. Gauldie resulted in the dual authorship in this manuscript.

REGULATION OF HEPATIC IL-6R, gp130, LIF-R, AND CPI mRNA LEVELS AFTER MULTIPLE rIL-6 INJECTIONS IN RATS

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Running Title: IL-6R, LIF-R and gp130 REGULATION

Key Words: IL-6 Receptor/gp130/LIF-R

ABSTRACT

Recently we examined the expression of IL-6R (gp80), gp130, LIF-R, and CPI mRNA in acute inflammation (1,2) *in vivo*. At the same time, we examined serum levels of IL-6, corticosterone, and CPI. To continue these studies, we investigated the long term *in vivo* effects of repeated injections of purified rIL-6 on the expression of these hepatic cytokine receptors. Repeated injections of rIL-6 did not cause receptor down regulation, instead increased IL-6R (gp80), gp130, and CPI mRNA levels (2-3 fold) were seen. Serum CPI protein levels increased gradually over the nine day period from 1.4 mg/ml to 5.8 mg/ml. LIF-R mRNA levels remained unaffected by the repeated injections of rIL-6.

INTRODUCTION

IL-6 is a pleotropic cytokine that has many functions including the regulation of the hepatic acute phase response in inflammation (3). IL-6 stimulates the liver to produce a series of acute phase proteins during inflammation that are important in fighting infection and mediating the homeostatic response of the body. Although many cytokines such as OSM, LIF, and IL-11 mimic IL-6 in stimulating the same set of acute phase proteins (for reviews see 4-6), IL-6 function cannot be totally replaced *in vivo* by these other cytokines. Studies done by Baumann et. al. (7) have shown that in IL-6 knock out mice, turpentine-injected mice had a severly compromised acute phase protein response, and LPS-injected mice had a partially compromised acute phase response. Their acute phase response returned to normal levels after injection of rIL-6.

We have previously studied the effect of rIL-6 on expression of its own receptors, IL-6R and gp130 *in vivo* (1,2). We found that within the first few hours after rIL-6 injection, mRNA levels for

both receptors increased dramatically. We also found that rIL-6 injection had no effect on LIF-R mRNA levels, even though LIF-R and IL-6R use the same signal transducing molecule gp130, and initiate similar signalling pathways (4-6). To continue these studies, we investigated the acute phase response in rats repeatedly injected over several days with rIL-6. We investigated receptor regulation in the hepatocyte to determine if multiple injections caused receptor down regulation, or if the hepatocyte responded to continual stimulation by rIL-6. We also wanted to determine if long term exposure to rIL-6 would initiate any cross-talk with expression of the LIF receptor.

MATERIAL AND METHODS

Animals and Materials

Male Sprague-Dawley rats (c. 200 gm) were treated with purified rIL-6 several times (10 μg s.c. injection each day for 9 days). Blood samples were taken at day 0, 1, 4, 6, and 9 . Rats were sacrificed on the ninth day and both blood and liver samples were taken.

The rat IL-6R cDNA was cloned as described (1). The human gp130 (cytoGP130/bluescript) and murine leukaemia inhibitory factor receptor (LIF-R) (pmLIFR21ΔBglII-BamHI) cDNA were supplied by Immunex.

Acute Phase Protein Serum Levels

The amount of cysteine proteinase inhibitor secreted into the blood was determined by rocket immunoelectrophoresis as previously described (8).

Corticosterone Serum Levels

Serum corticosterone levels were determined as previously described (1).

mRNA Preparation and Northern Blot Analysis

RNA was extracted from liver tissue as described by Chomczynski & Sacchi (9). Poly(A)$^+$ RNA was prepared using oligo d(T) sepharose chromatography. For Northern blot analysis, 30 μg of Poly(A)$^+$ RNA was electrophoresed on 1% agarose-formaldehyde gels, transferred, hybridized, and probed as described (1). However, due to the species differences of the rat IL-6R, human gp130, and murine LIF-R probes, the washing conditions were as follows: 1) when blots were probed with rat IL-6R cDNA blots were washed with 1xSSC, 1%SDS for 10' at R/T, then 3 x 20' in 0.1xSSC, 1%SDS at 65°C. 2) When blots were probed with murine LIF-R cDNA, the blots were washed as in #1 but at 55°C. 3) When blots were probed with human gp130 cDNA blots were washed once with 1xSSC, 1%SDS for 10' R/T, once in .5xSSC, 1%SDS for 20' at 50°C, twice in .25XSSC, 1%SDS for 20' at 50°C. Standardization was done by reprobing the blots with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Filters were exposed to Kodak XAR5 film at -70°C. Autoradiographs were scanned by quantitative densitometry (Haefer Scientific Instruments), and mRNA was normalized to constant amounts of GAPDH. Densitometer scans shown in this manuscript are only representative of different mRNA concentrations and should only be considered approximate changes due to the limitation of this method.

RESULTS

Serum CPI Protein Levels

Rats repeatedly injected with rIL-6 on a daily basis showed a steady increase in serum CPI protein levels over a 9 day period. Normal levels of 1.4 mg/ml CPI increased gradually to 5.8 mg/ml CPI (Figure 1). Control rats injected with PBS had a normal range of CPI throughout the 9 day period.

Serum Corticosterone Levels

Corticosterone levels were measured in control and rIL-6 injected rats (Figure 2). Overall the levels of corticosterone in the control and rIL-6 rats varied greatly over the nine day period. However in general all rats were within normal range (200-500ng/ml) except for the serum taken on the ninth day from rats injected with rIL-6 (675-950ng/ml).

Northern Blot Analysis

1)IL-6R mRNA in Liver

Northern Blot analysis revealed the presence of a predominant message at 5.1 kb. There was an overall 2.6 fold increase in mRNA levels in comparison to controls (Figure 3) over the nine day period.

2) gp130 mRNA in Liver

Northern blot analysis demonstrated the presence of two predominant message at ~7.5 kb and

~9.0 kb. There was approx. a 2 fold increase in mRNA levels and the ratio of the two messages did not change (Figure 3) over the nine days.

3) LIF-R mRNA in Liver

Northern blot analysis revealed the presence of one predominant message at approximately 10 kb. There was no change in message levels over the 9 day period (Figure 3).

4)CPI mRNA in Liver

Northern blot analysis revealed the presence of one predominant message at 1.5 kb. There was a 4 fold increase in mRNA levels over the 9 day period (Figure 3).

DISCUSSION

The increase in hepatic receptor mRNA levels of IL-6R and gp130 observed when rats are injected with rIL-6 over a 9 day period, were similar to results observed when rats were injected only once. There was an approximate 1-3 fold increase over normal mRNA levels of IL-6R, gp130 and CPI in comparison to controls. The mRNA levels were not at a maximum as we had previously seen (1), due to the fact that rats were not sacrificed at times consistent with maximum mRNA levels time.

The serum CPI protein levels gradually increased due to the accumulation of CPI protein over the nine day period. This implied that the receptors on the hepatocyte were not down regulated and hepatocytes were able to respond to the stimulus given repeatedly over the nine day period.

In vitro studies have shown that long term exposure to IL-6 (days) caused down-regulation of receptor function (10). However, there did not seem to be any down-regulation of IL-6R or gp130 mRNA levels leaving hepatic cells unresponsive. Since we did not see down regulation, we can only

speculate that the rIL-6 stimulation over the nine day period was not sufficient to cause receptor down-regulation, or that *in vivo* rIL-6 does not cause receptor down regulation. There was no detectable IL-6 activity levels found in the rat serum after 24h (data not shown), therefore rats probably cleared the IL-6 quickly, and hepatic cells had time to recover, if needed, before the next treatment began.

LIF-R mRNA levels were unaffected. Although LIF-R and IL-6R share the same signal transducing molecule, gp130, and initiate many of the same signalling pathways, these pathways seem to regulate IL-6R mRNA level modulation, whereas LIF-R mRNA level moduation must be influenced by alternate pathways that do not involve IL-6.

Studies on the chronic stimulation with rIL-6 and its effects should be continued in order to determine the long term effects of rIL-6 on its receptors, the hepatocyte, and the acute phase response.

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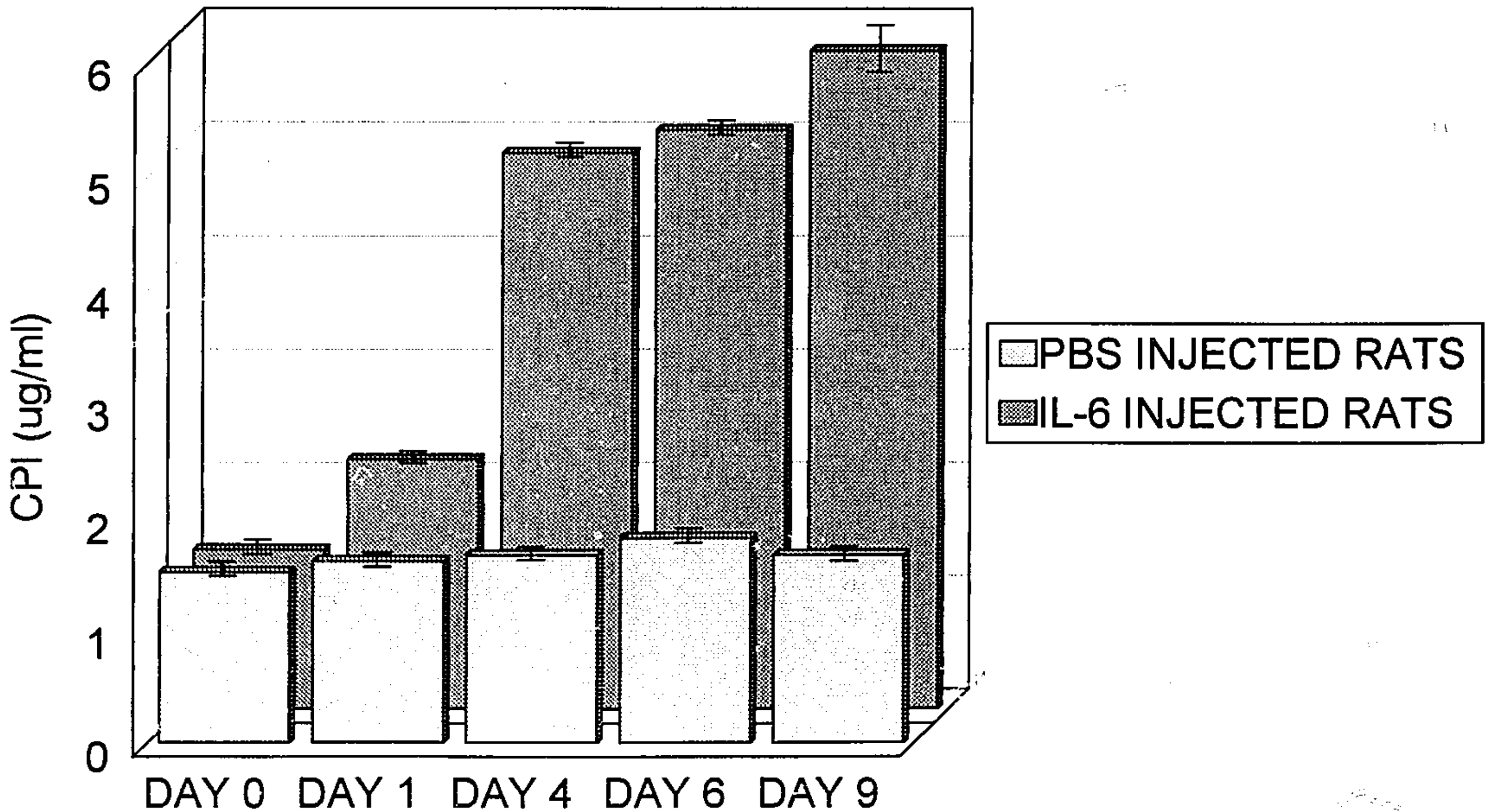

FIGURE 1: Serum levels of CPI protein, Samples for each time point represent the average amount (with range) calculated from two rats injected with rIL-6 or PBS over a 9 day period.

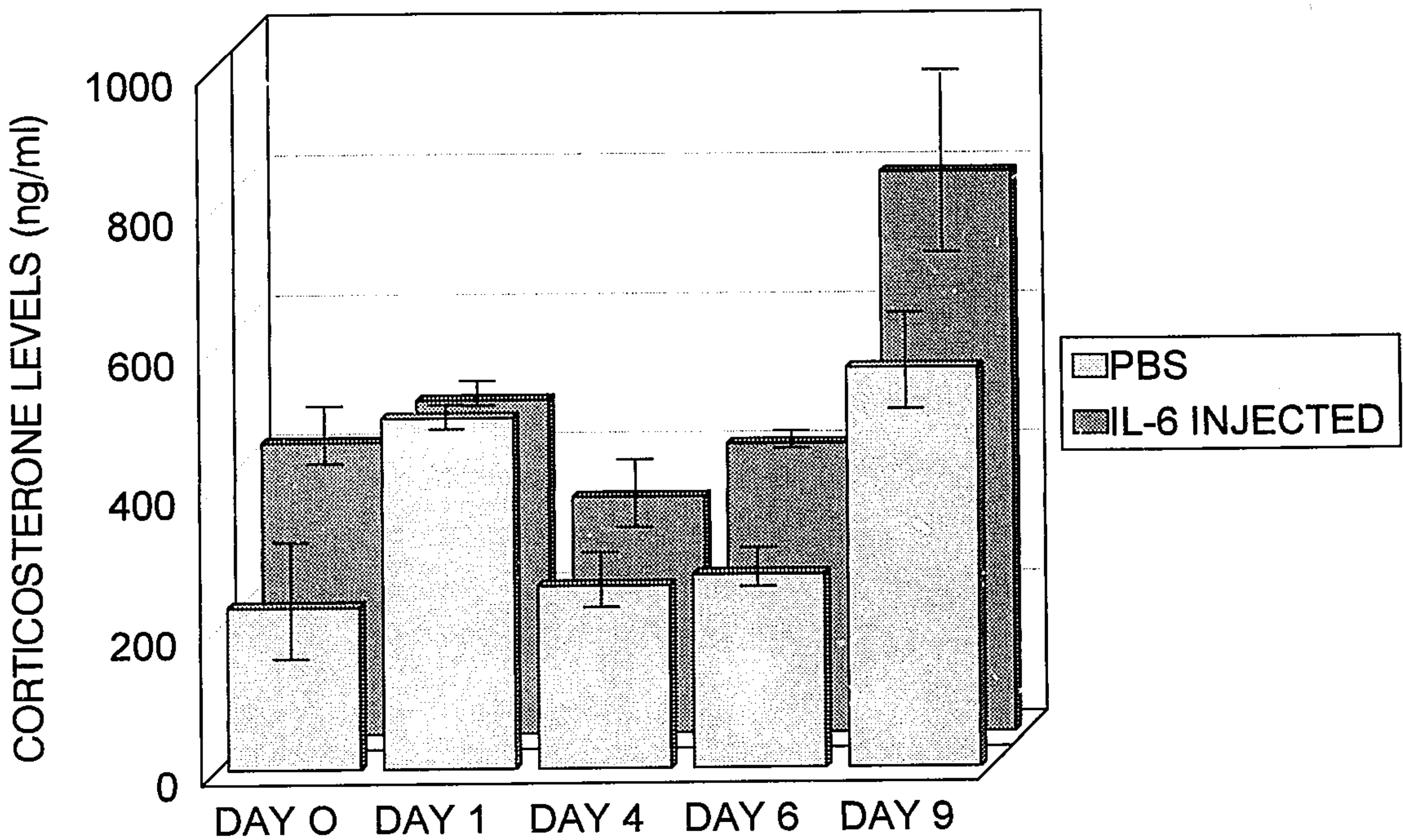

FIGURE 2: Serum levels of corticosterone. Samples for each time point represent the average amount (with range) calculated from two rats injected with rIL-6 or PBS over a nine day period.

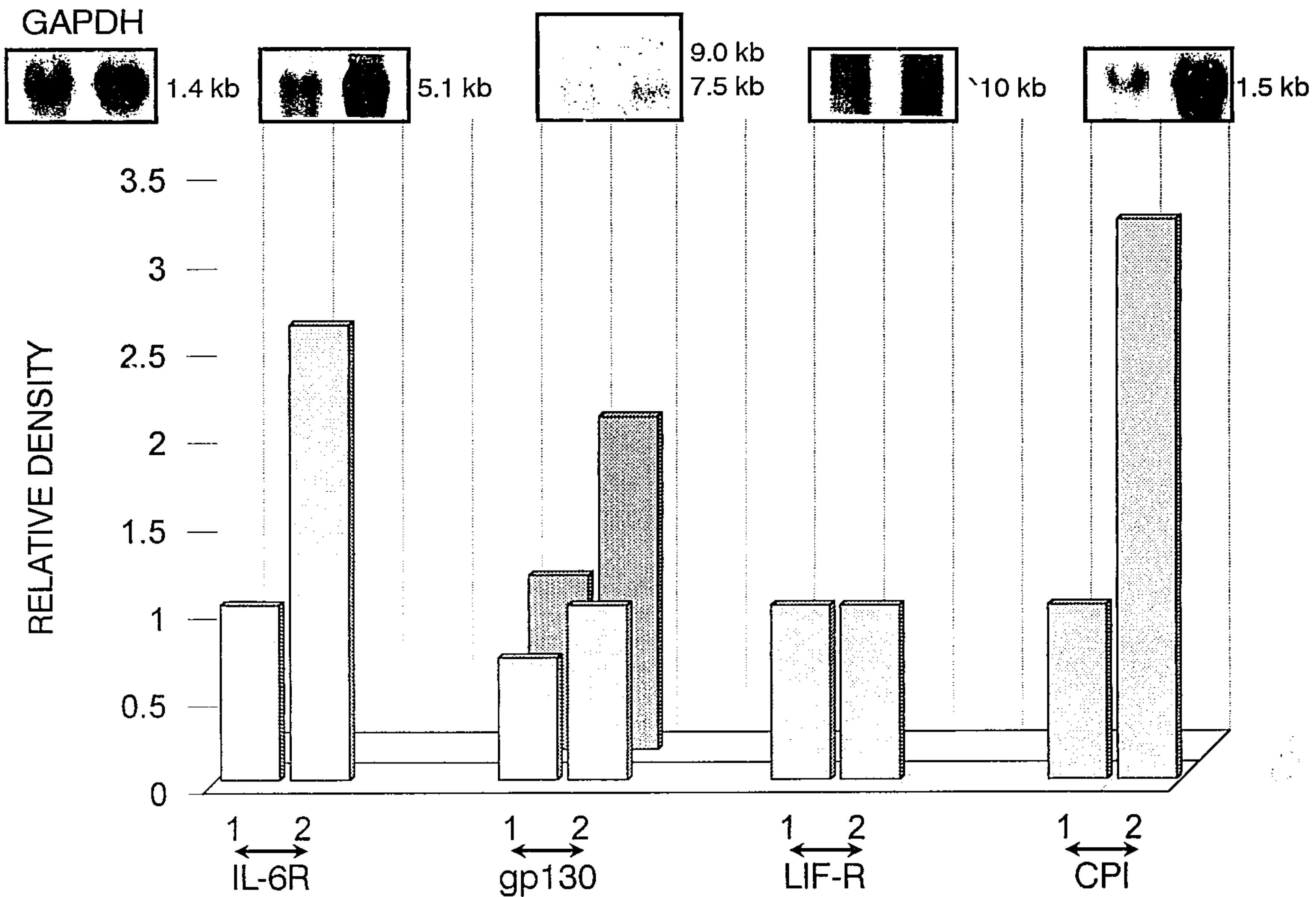

FIGURE 3: Northern Blot and Densitometer scans of hepatic mRNA are shown for A) IL-6R, B) gp130 C) LIF-R and D) CPI. Lane 1 represents control rats injected with PBS and Lane 2 represents rats injected with rIL-6 over a 9 day period. Standardization was done by reprobing the blots with GAPDH and densitometer scans were normalized to GPADH mRNA densitometer scans.

Chapter five

REGULATION OF IL-6R (gp80), gp130, AND CPI mRNA LEVELS IN VITRO IN RAT HEPATOMA CELLS

The following paper entitled, "Cytokines oncostatin M and interleukin 1 regulate the expression of the IL-6 receptor (gp80, gp130)" was written by the author of this thesis, and was published in Cytokine 7:503-509, 1995. It continues the theme of this thesis by looking at the regulation of the IL-6 hepatic receptors *in vitro* and comparing these results with the regulation of the IL-6 hepatic receptors *in vivo.*

The research for this study was performed by the author of the thesis. The supervision from Dr. Gauldie, and the advise of Dr. Richards resulted in the multiple authorship of this paper.

CYTOKINES ONCOSTATIN M AND INTERLEUKIN 1 REGULATE THE EXPRESSION OF THE IL-6 RECEPTOR (gp80, gp130)

M. Geisterfer, C. D. Richards, J. Gauldie

The steady-state mRNA levels of the interleukin 6 receptor (IL-6R, gp80) and its signal transducing molecule, gp130, were examined in the rat hepatoma cell line, H-35, stimulated by cytokines IL-6, IL-1, oncostatin M (OSM) and/or Dexamethasone (Dex). In contrast to our previous findings in vivo [Geisterfer *et al.*, 1993, *Cytokine*, 5:1] in vitro Dex seemed to be the major stimulator of IL-6R mRNA expression, whereas IL-6 seemed to have little effect on the expression of its own receptor mRNA levels. However, the presence of other cytokines influenced the Dex mediated stimulation of IL-6R expression. OSM stimulated IL-6R mRNA levels. At 6 h, cells stimulated with OSM showed a 2.1-fold increase in IL-6R mRNA expression. This stimulation was additive with the Dex-mediated stimulation of IL-6R mRNA levels. In contrast, IL-1 inhibited the Dex-mediated stimulation of IL-6R mRNA. At the same time, IL-1 stimulated the presence of a second smaller mRNA transcript. This mRNA species contained the extracellular domain but lacked both the transmembrane and cytoplasmic domains of the IL-6R, suggesting alternate splicing, possibly coding for a soluble form of gp80. Unlike the gp80 IL-6R molecule, the expression of the gp130 molecule normally expressed as two species of mRNA was not regulated to any major extent in vitro. IL-1 and OSM stimulated both mRNA bands (7.5 and 9.0 kb) approximately 2-fold, whereas IL-6 stimulated mainly the upper 9.0 kb mRNA band. Cysteine proteinase inhibitor (CPI), mRNA and protein levels were elevated by combinations of cytokines; however, IL-1 seemed to inhibit the IL-6 + Dex mediated stimulation of CPI mRNA, possibly through inhibition of IL-6R expression and induction of a soluble form of the receptor. This study showed that both IL-1 and OSM are involved in the regulation of the IL-6 receptor complex, and that different combinations of cytokines can affect the complexity and magnitude of the hepatic acute phase response.

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Several cytokines released during inflammation stimulate the liver to synthesize acute phase proteins (APP). It has been shown in vitro that stimulation by a combination of interleukin 6 (IL-6) and interleukin 1 (IL-1) in the presence of corticosteroid can account for the full acute phase response in the liver. Although IL-6 alone can stimulate expression of all the acute phase proteins, this effect is influenced by the presence of other cytokines such as IL-1. For example, some acute phase proteins such as α1-acid glycoprotein are synergistically stimulated by IL-1 and IL-6, whereas others such as fibrinogen show inhibition of IL-6 stimulation by IL-1.[1–4] How IL-1 mediates these effects is unknown, but one mechanism may be through regulation of the expression of the IL-6 receptor complex.

Other cytokines apparently functionally related to IL-6 such as oncostatin M (OSM), interleukin 11 (IL-11), leukaemia inhibitory factor (LIF) and ciliary neurotropic factor (CNTF) can elicit the same response as IL-6 in liver cells in vitro, and act synergistically with IL-1 on the same set of acute phase proteins.[3–9] These cytokines interact either with their own specific receptor (IL-6, CNTFR)[10] or a shared receptor (OSM/LIF receptor)[12,14] which then combines with gp130 to form in a high affinity complex, and it is this complex that sends a signal into the cell.[10–16] It is also believed that OSM interacts with gp130 directly.[12,13]

We have previously examined the regulation of IL-6R in vivo during inflammation in rat liver.[17] In this current study, we examined the effect of exposure to cytokines on the regulation of IL-6R and gp130 mRNA

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KEY WORDS: gp130/IL-6 receptor

levels in vitro in rat hepatoma cells. This study showed that both OSM, a cytokine sharing the signalling pathway of IL-6, and IL-1 are involved in the regulation of the expression of IL-6 receptor complex molecules.

MATERIALS AND METHODS

Cell lines and materials

Rat H-35 hepatoma (clone T-7-18) (18) cells were cultured in α-MEM, 10% FCS, 1% Pen-Strep. The following cytokines were used at 10 ng/ml:human IL-6 (Immunex, Seattle, WA) and human IL-1α (Immunex, Seattle, WA). Human OSM (Bristol Meyers Squibb, Seattle, WA) was used at 40 ng/ml. The concentration of Dexamethasone (Dex) used in the cell media was 10^{-6} M. Cells were treated with cytokines and/or Dex in α-MEM plus 1% Pen-Strep. All concentrations of cytokines used induced a maximum acute phase response in H-35 cells (based on previous studies).

The rat interleukin 6 receptor (IL-6) cDNA (1330 bp) was prepared by PCR as described.[17] Fragments of IL-6R cDNA were generated to reprobe blots: (1) the extracellular 327 bp BamHI fragment (640–967), and (2) the 317 bp BamHI-EcoRI fragment containing only the transmembrane and cytoplasmic domains of the cDNA (1070–1387) (See Fig. 2A for schematic). A human gp130 cDNA was provided by Immunex Corp. (cytogp130 in Bluescript SK).

Northern blot analysis and mRNA preparation

RNA was extracted from H-35 cells as described by Chomczynski & Sacchi.[19] Poly(A)$^+$ RNA was prepared using oligo d(T) sepharose chromatography. RNA was extracted after 6 h and 24 h of stimulation by the various cytokines and/or Dex. For Northern blot analysis, 5 μg of Poly(A)$^+$ RNA was electrophoresed on 1% agarose-formaldehyde gels, transferred, hybridized, and probed as described.[17] However, due to the species differences of the rat IL-6R and human gp130 probes, washing conditions were as follows: (1) when blots were probed with rat IL-6R cDNA, or fragments thereof, blots were washed with 1 × SSC, 1% SDS for 10' at R/T, then 3 × 20' in 0.1 × SSC, 1% SDS at 65°C. (2) when blots were probed with human gp130 cDNA blots were washed once with 1 × SSC, 1% SDS for 10' at R/T, once in 0.5 × SSC, 1% SDS for 20' at 50°C, twice in 0.25 × SSC, 1% SDS for 20' at 50°C. Standardization was done by reprobing the blots with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rat α-tubulin (Clontech Laboratories, Palo Alto, CA). Filters were exposed to Kodak XAR5 film at −70°C. Autoradiographs were scanned by quantitative densitometry (Hoefer Scientific Instruments, San Francisco, CA), and mRNA was normalized to constant amounts of rat α-tubulin. Densitometer scans shown in this paper are only representative of different mRNA concentrations and should only be considered approximate changes due to the limitations of this method.

Acute phase protein levels

The amount of cysteine proteinase inhibitor (CPI) secreted into the media was determined by rocket immunoelectrophoresis as previously described.[20]

RESULTS

OSM and Dex stimulate IL-6R mRNA levels

Northern blot analysis of 5 μg of poly(A)$^+$ demonstrated the presence of a predominant message at 5.1 kb (Fig. 1). When H-35 cells were stimulated with cytokines IL-1, IL-6 and OSM, only OSM showed a slight 2.1-fold increase in IL-6R mRNA levels at 6 h. This effect seemed to be additive in the presence of Dex. At 6 h and 24 h there was a 5.1- and 7.2-fold increase in message when cells were stimulated in the presence of OSM + Dex (data at 24 h not shown).

Cells treated with Dex (10^{-6}M) showed a 3.3-fold increase at 6 h and a 5.2-fold increase at 24 h (data at 24 h not shown). IL-6R mRNA levels also increased when cells were treated with IL6 + Dex. H-35 cells mRNA levels increased 3.0-fold and 5.6-fold at 6 h and 24 h respectively. There did not seem to be a significant difference between cells treated with Dex in the presence or absence of IL-6.

Effects of IL-1 on IL-6R mRNA levels

Although there was no increase in the 5.1 kb message levels with IL-1 stimulated H-35 cells, a

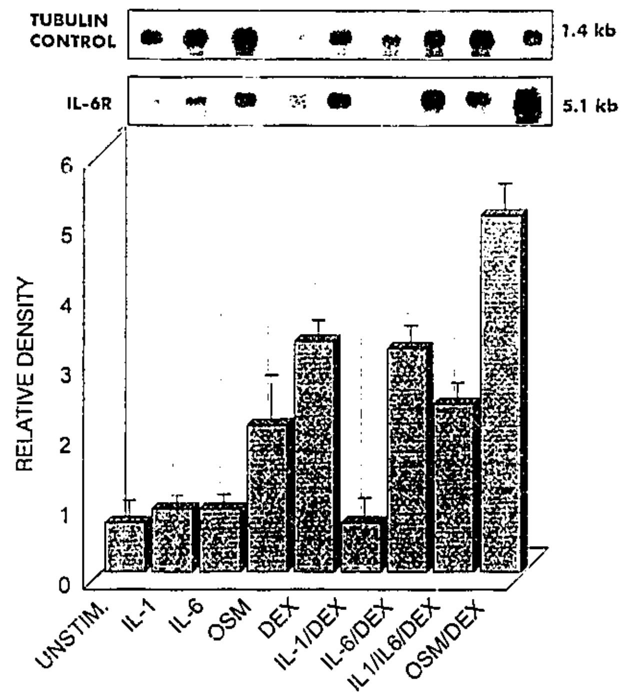

Figure 1. Northern blot and densitometer scan for IL-6R mRNA.

Each lane represents 5 μg of poly(A)$^+$ isolated from H-35 cells stimulated for 6 h under different conditions as shown in the legend and described in Materials and Methods. Standardization involved reprobing the blots for α-tubulin (Northern blot shown above) and IL-6R mRNA densitometer scans were normalized using α-tubulin mRNA as an internal control.

second predominant message appeared approximately 1 kb smaller in size (Fig. 2B). This mRNA message level was approximately 2–3-fold higher than the 5.1 kb IL-6R mRNA message at 24 h. When the same blot was reprobed with different sections of the IL-6R cDNA (Fig. 2A), it was found that this smaller mRNA band did not hybridize with the piece of cDNA that corresponded to the transmembrane and cytoplasmic domains of the IL-6R (Fig. 2B-2). However, the lower band did hybridize to the segment of cDNA corresponding to a portion of the extracellular region of the protein (Fig. 2B-3). This suggests that IL-1 stimulated an alternately spliced form of IL-6R mRNA. This message was present only in cells stimulated with IL-1 alone, whereas cells stimulated with IL-1 + Dex or IL-1 + IL-6 did not show the presence of this second message.

H-35 cells treated with IL-1 + Dex had essentially normal levels of the 5.1 kb IL-6R mRNA (Fig. 1), and no second smaller mRNA transcript was seen. IL-1 inhibited the Dex mediated stimulation of the IL-6R receptor mRNA levels. When H-35 cells were treated with IL-1 + IL-6 + Dex, only a 2.8-and 2.2-fold increase in mRNA levels were seen at 6 h and 24 h. In comparison, cells treated with IL-6 + Dex had a 3.0- and 5.6-fold increase at 6 h and 24 h (data at 24 h not shown). Again IL-1 somehow interfered with the Dex mediated stimulation of IL-6R mRNA. However, the presence of IL-6 counteracted the inhibition of IL-6R mRNA levels caused by IL-1.

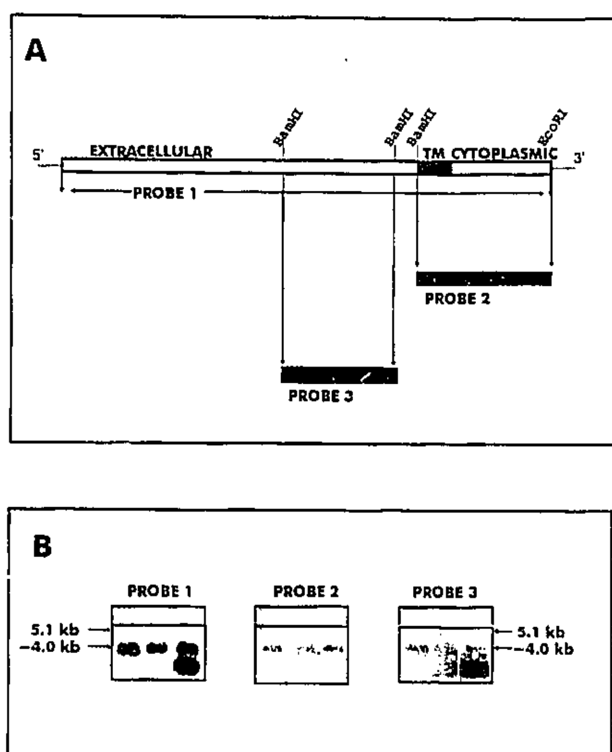

Figure 2.

(A) Schematic diagram of full length IL-6R cDNA and fragments generated to probe northern blots. Probe 1 represents the full length IL-6R cDNA. Probe 2 represents the 317 bp BamHI-EcoRI fragment containing the region of cDNA corresponding to the transmembrane and cytoplasmic domains of the IL-6R. Probe 3 represents the 327 bp BamHI fragment corresponding to a portion of the extracellular domain of the IL-6R. (B) Northern Blot of IL-6R mRNA from H-35 cells stimulated with IL-1α. Lane 1 represents unstimulated H-35 cells. Lanes 2 and 3 represent H-35 cells stimulated with 10 ng/ml of IL-1α for 1 h and 24 h respectively. Probe 1 represents Northern blot probed with the full length IL-6R cDNA. Probe 2 represents the same Northern blot as in A probed with the 317 bp BamHI-EcoRI fragment containing the region of cDNA corresponding to the transmembrane and cytoplasmic domains of the IL-6R as described in part A. Probe 3 represents the same Northern blot as in A probed with the 327 bp BamHI fragment corresponding to a portion of the extracellular domain of the IL-6R as described in part A.

Effect of IL-1 and OSM on gp130 mRNA levels

Northern blot analysis of 5 µg of poly(A)$^+$ demonstrated the presence of two predominant mRNA transcripts at ~7.5 kb and ~9.0 kb for gp130 in unstimulated cells (Fig. 3). Wang *et al.*[21] also found the presence of two prominent messages in rat hepatocytes. Unstimulated cells generally showed slightly higher levels of 7.5 kb message than 9.0 kb message.

When H-35 cells were stimulated with the various cytokines, the overall regulation of the two gp130 mRNA was much less than that seen for the IL-6R mRNA. There was a 2–3-fold increase in gp130 mRNA in comparison to 3–7-fold increase found in the mRNA IL-6R levels.

Northern blot and densitometer scan analysis indicated that the gp130 7.5 kb mRNA band was slightly

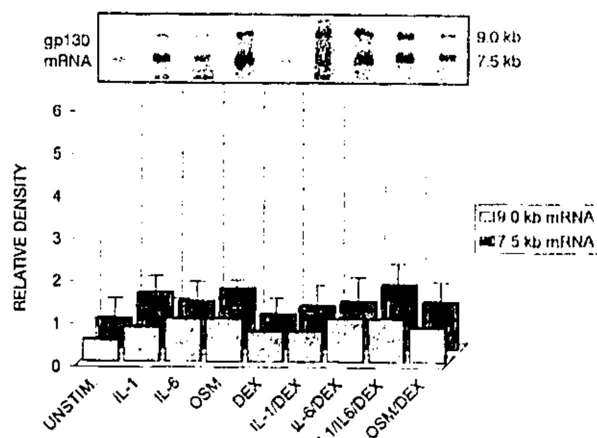

Figure 3. Northern blot and densitometer scans for gp130 mRNA.

The gp130 cDNA hybridized to two predominant messages at 9.0 kb and 7.5 kb. Each lane represents 5 µg of poly(A)$^+$ isolated from H-35 cells stimulated for 6 h under different conditions as shown in the legend and described in Materials and Methods. Standardization as in Fig. 1. This figure has the same scale as the other two Northern blot figures shown to emphasize that the regulation of gp130 mRNA is not as great as IL-6R and CPI mRNA.

more regulated than the 9.0 kb mRNA band. The 9.0 kb mRNA levels were stimulated by IL-1, IL-6 and IL-6 + Dex, whereas the 7.5 kb mRNA was stimulated to some degree by all of the cytokines in the presence or absence of Dex. OSM, in the presence or absence of Dex stimulated the 7.5 kb mRNA message slightly more, whereas IL-6 stimulated mainly the 9.0 kb mRNA. IL-1, however, stimulated both messages in the presence or absence of Dex.

Acute phase protein stimulation—cysteine proteinase inhibitor

The CPI mRNA levels were stimulated in the presence of OSM, OSM + Dex, IL-6 + Dex, and IL-6 + IL-1 + Dex (Fig. 4). The highest levels of CPI mRNA were seen when H-35 cells were stimulated with IL-6 + Dex (3-fold and 7.5-fold increase at 6 h and 24 h). In comparison, when cells were stimulated with IL-1 + IL-6 + Dex, there was only a 1.3- and 1.6-fold increase at 6 h and 24 h (data at 24 h not shown). IL-1 seemed to inhibit the IL-6 + Dex mediated stimulation of CPI also.

OSM is a strong stimulator of CPI synthesis with or without the presence of Dex. When cells stimulated with OSM or OSM + Dex, there was a 5.1-fold and 3.9-fold increase in CPI mRNA levels at 6 h respectively. In addition, at 24 h there was a 3-fold increase in CPI mRNA for OSM stimulation alone and a 4.6-fold increase for OSM + Dex (data not shown).

CPI protein levels were significantly elevated in the media of H-35 cells stimulated with OSM, OSM + Dex, IL-6 + Dex, IL-6 + IL-1 + Dex (data not shown). Cells stimulated by IL-6, IL-1 or Dex alone showed no detectable raised levels of CPI protein.

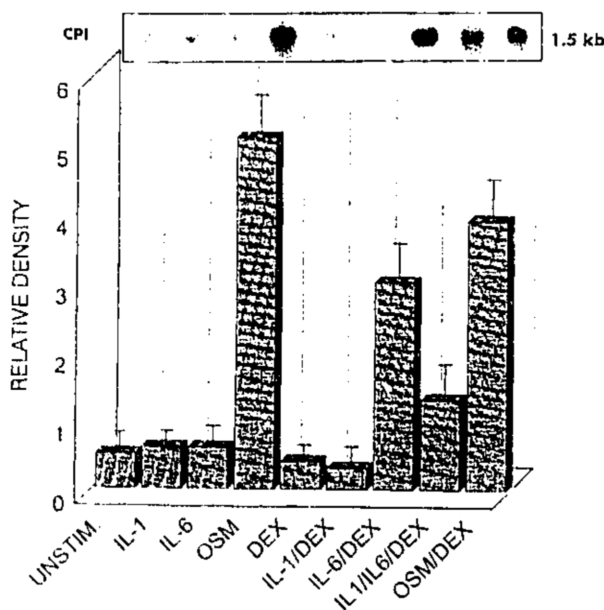

Figure 4. Northern blot and densitometer scan for CPI mRNA.

Each lane represents 5 μg of poly(A)$^+$ isolated from H-35 cells stimulated for 6 h under different conditions as shown in the legend and described in Materials and Methods. Standardization was done by reprobing the blots with α-tubulin (Northern blot shown above) and IL-6R mRNA densitometer scans were normalized using α-tubulin mRNA as an internal control.

DISCUSSION

Several groups have looked at regulation of the IL-6R and its signal transducer gp130 to determine the effect that cytokines and other factors have on the expression of these receptors. Although corticosteroid seems to be the major regulator of IL-6R expression in vitro in both human and rodent systems,[21–24] this effect can be influenced by the presence of other cytokines. Studies done by Nesbitt & Fuller[22] showed that IL-6 inhibited the Dex-mediated stimulation of IL-6R expression in rat primary hepatocytes. Recently, Campos *et al.*[25] showed that other cytokines such as TGF-β stimulate the expression of IL-6R mRNA in H-35 cells. In contrast, in vivo, IL-6 had a profound enhancing effect on the regulation of IL-6R mRNA expression, whereas Dex stimulation of mRNA levels was minor in comparison.[17]

Both in vitro and in vivo studies on the expression of the gp130 signal transducing molecule have shown that steady-state mRNA levels are not as highly regulated as IL-6R gp80 mRNA.[21,22,26,27] Schooltink *et al.*[26] have shown that in human HEPG2 cells, gp130 is stimulated by IL-6 but not by Dex. However, the best stimulation of gp130 mRNA levels was seen with a combination of IL-6 + Dex. In contrast, gp130 mRNA expression remained fairly constant when rat primary hepatocytes were exposed to similar conditions.[22] However, in vivo, IL-6 seems to be a major regulator of gp130 expression. Saito *et al.*[27] showed that at 1 h gp130 mRNA expression increased approximately 2-fold after injection of hIL-6 into mice. The presence of other cytokines, such as OSM and IL-1, also has an effect on mRNA expression levels of the IL-6R receptor complex, as shown by this study. OSM may enhance the IL-6 mediated acute phase response through the stimulation of IL-6R mRNA levels, while in contrast IL-1 may inhibit this effect through the inhibition of the Dex-mediated stimulation of the IL-6R mRNA levels. This influence is not unique to these cytokines alone. For example, H-35 cells treated with TGF-β increased IL-6R mRNA levels 3-fold.[25] Cells treated with TGF-β and Dex showed a maximum 20-fold increase in mRNA levels over control 4 h after treatment. The number of binding sites per cell increased 3-fold by 6 h after treatment.

OSM stimulated IL-6R mRNA levels, and this

effect seemed to be additive with the Dex-mediated stimulation of IL-6R mRNA. Since OSM is believed to be a natural ligand for gp130,[12,13] it may stimulate IL-6R mRNA levels directly through signal transduction pathways different from IL-6, activated by direct binding to gp130. However, OSM also stimulates the same set of acute phase proteins as IL-6; therefore, the additional stimulation of IL-6R mRNA levels may ensure that these particular acute phase proteins are made.

In contrast, IL-1 completely inhibited the Dex-mediated stimulation and partially inhibited the IL-6 + Dex-mediated stimulation of IL-6R mRNA. This inhibition by IL-1 was also shown by Nesbitt & Fuller[22] using rat primary hepatocytes. IL-1 decreased IL-6R mRNA levels ~28% in the presence or absence of Dex. IL-1 also partially inhibits the IL-6 + Dex-mediated stimulation of CPI mRNA. This opposing effect between IL-1 and IL-6 has previously been demonstrated in the expression of several acute phase proteins. We showed that IL-1 inhibited the IL-6-mediated stimulation of fibrinogen (all species), α_2-macroglobulin and cysteine proteinase inhibitor (rat).[1-4] One method by which IL-1 may accomplish this is by decreased regulation of the expression of gp80 IL-6R (as seen in these studies) rendering the cell less responsive to IL-6. This inhibition by IL-1 may shift the hepatic acute phase response so that IL-1-stimulated acute phase proteins are more prominent. However, it should be noted that in the same cells, IL-1 will act synergistically with IL-6 to stimulate certain acute phase proteins, such as α1-acid glycoprotein. The explanation of interfering action of IL-1 on IL-6 function is likely more complex than merely modulating IL-6R expression.

Not only does IL-1 inhibit the Dex-mediated stimulation of the IL-6R mRNA, but it markedly stimulates the appearance of a second smaller mRNA transcript. Preliminary studies have shown that this transcript does not contain the coding region for the transmembrane or cytoplasmic domains of the full length transcript. This transcript could be an alternately spliced mRNA species that presumably may translate into a soluble IL-6R protein. It has been shown that IL-6R soluble receptor exists in normal human serum at ~75 ng/ml[28-30] and in normal urine at ~1 ng/ml.[31] It has also been shown that a genetically engineered soluble IL-6R lacking transmembrane and cytoplasmic domains has agonist activity and binds gp130 in the presence of IL-6 to produce a signal within the cell[10,11,32,33] Whether this agonist activity is retained in vivo is as yet unknown.

Using PCR, Lust *et al.*[34] found message that encoded for both the soluble and membrane bound forms of the human IL-6R in both normal and myeloma cells. The soluble receptor mRNA appeared to lack only the transmembrane domain of the coding region. Also Baumann *et al.*[35] found several unidentified mRNA transcripts of rat IL-6R message through RNase protection assays on primary liver cells and implied that there could possibly be several forms of alternately spliced message for this molecule. In addition, other cytokine receptors such as that for IL-7 and IL-4 have alternately spliced cDNA clones isolated that code for the soluble forms of these receptors.[36,37]

In contrast to these results, other groups, using a transfected engineered truncated cDNA in hepatoma cells, have shown that a soluble form of IL-6R can be derived by proteolytic cleavage of the intact membrane bound IL-6R, and that this cleavage is enhanced by PMA, a protein kinase C activator.[38-41]

Both in vivo and in vitro studies have shown that gp130 mRNA levels are regulated by IL-6[26,27] and Dex.[21] Our studies show that the levels of the two gp130 mRNA are stimulated to the greatest extent by IL-6 + Dex. There is a 2–2.5-fold increase in mRNA levels at 24 h. However, this study also shows that other cytokines such as IL-1 and OSM also regulate the two gp130 molecules. Since gp130 is the signal transducing molecule for many cytokines (IL-6/OSM/LIF/CNTF),[11,15] it may be regulated to some degree by many cytokines and hormones.

The functional role of the two different transcripts for rat gp130 is not yet known; however, in vivo the ratio of the two gp130 messages remained fairly constant,[27] although this does not seem to be the case in vitro. Whereas treatment of H-35 cells with IL-1 and/or Dex stimulated both messages, cytokines such as OSM and IL-6 preferentially stimulate only one of the messages, causing in some cases the ratio of the two messages to change drastically. OSM stimulates mainly the 7.5 kb message, whereas IL-6 stimulates mainly the 9.0 kb message. Since only one message for the gp130 molecule has been reported in human cells,[11] it has been suggested that the two mRNA messages found in rodents most likely represent differential splicing or polyadenylation of the two transcripts.[21,27] Recently, Narazaki *et al.*[42] found the presence of two soluble forms of gp130 (90 & 110 kd) in human serum. These soluble molecules could have been produced by proteolytic shedding or from alternately spliced mRNA message encoding a soluble form of the gp130 molecule or both. However as long as the identity of the two mRNA species remains unclear, we will not know whether this preferential regulation by different cytokines has any effect on a cell.

This study has shown that cytokines such as OSM and IL-1 regulate both the IL-6R gp80 and gp130 molecules, and hence play an important role in the IL-6 mediated stimulation of certain genes in the acute phase response of the liver during inflammation. We have also shown that IL-1 stimulates a second mRNA transcript that could represent an alternately spliced mRNA species translating into the soluble IL-6R. However,

further studies, need to be done to characterize this transcript.

When many cytokines are present as would be the case in vivo, the Dex-mediated stimulation of the IL-6R changes drastically. The presence of many different cytokines could explain why in vivo IL-6 has a profound effect on its own receptor and Dex does not have so great an effect.[17] However, another explanation could be that different mechanisms are at work in vivo than in vitro. Whichever the case, these studies help us define and isolate the complex interactions that occur between the different cytokines and their receptors during the acute phase response.

Acknowledgements

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Chapter six

SUMMARY

SUMMARY

The IL-6 family of cytokines include IL-6, Leukemia Inhibitory Factor (LIF), Oncostatin M, Ciliary Neurotropic Factor (CNTF), IL-11 and the recently described Cardiotrophin. Each of these cytokines result in signal transduction mediated by a common receptor element, gp130. Each cytokine has a specific alpha receptor IL-6R (gp80); LIF-R; OMR etc. Our studies have shown that certain receptors for the IL-6 cytokine family, IL-6R, gp130, and LIF-R, are all regulated *in vivo* during acute inflammation, and that this regulation is complex, depending on the milieu of different cytokines present. We examined the regulation of the cytokine IL-6 during acute inflammation *in vivo* and the role that IL-6 plays in regulating liver function during acute inflammation, and found that all three models of experimental inflammation (acute systemic - LPS; acute local - Turpentine; chronic - FA) induced circulating levels of IL-6 several hours before detectable changes in acute phase proteins (Figure S1A). Although differences in serum IL-6 activity were seen, the acute phase response was similar in all three models of inflammation. This showed that early on saturating levels of IL-6 protein for hepatic responses had been reached in each model.

We also saw a rapid increase in serum corticosterone levels in all three models and this increase had similar kinetics to serum IL-6 activity (Figure S1B). All three models showed early and dramatic IL-6R mRNA level modulation, whereas gp130 and LIF-R mRNA level fluctuations were comparatively small (Figure S1C). This may indicate that IL-6/IL-6R binding is important in generating an early signal for the production of acute phase proteins. Since gp130 is the signal transducing molecule for many receptors including IL-6R (gp80) and LIF-R, we can speculate that the levels of gp130 must remain at constant levels and be ready to interact with any of the α-components once ligand has bound. Therefore, the interaction of the α-components and the

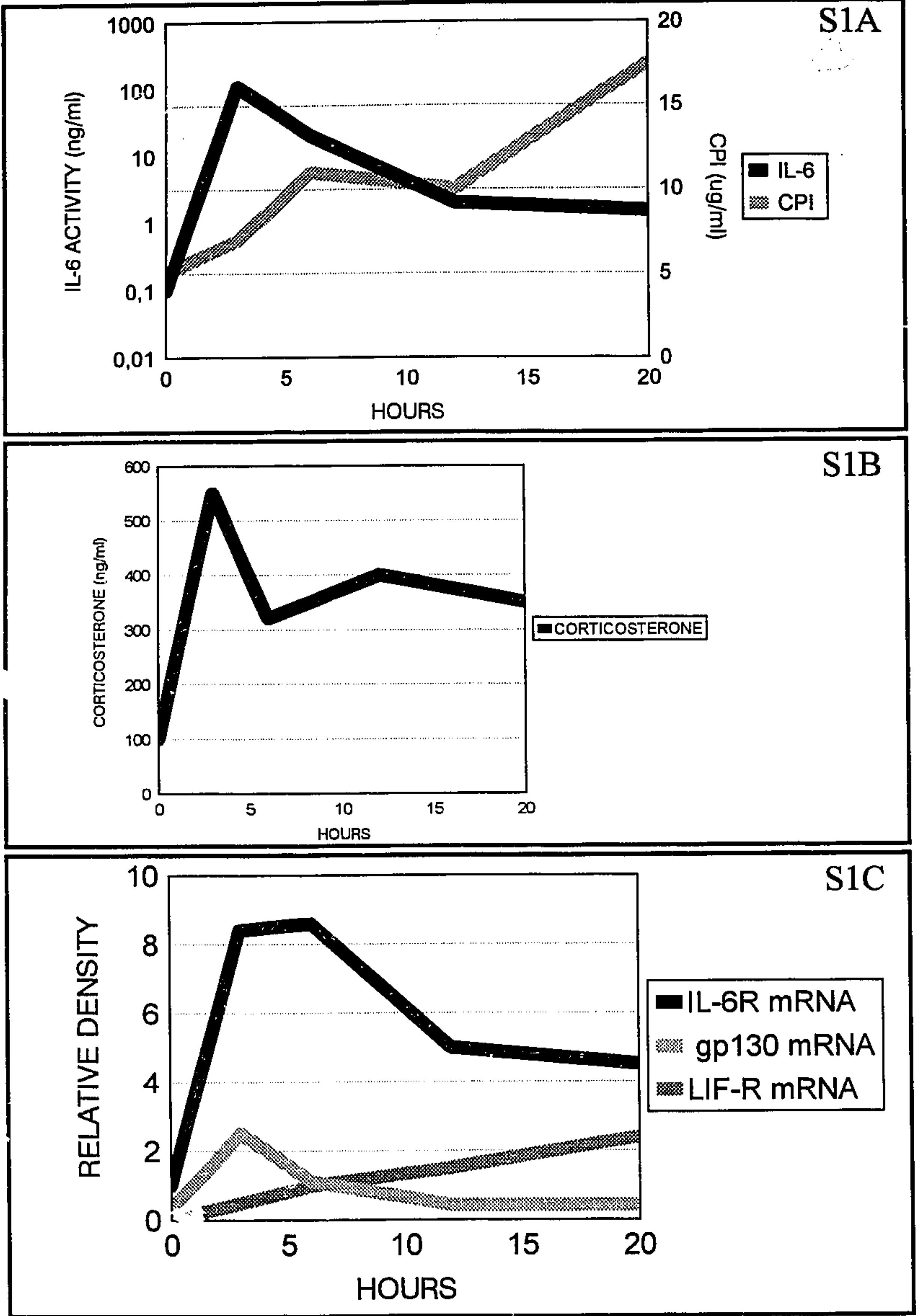


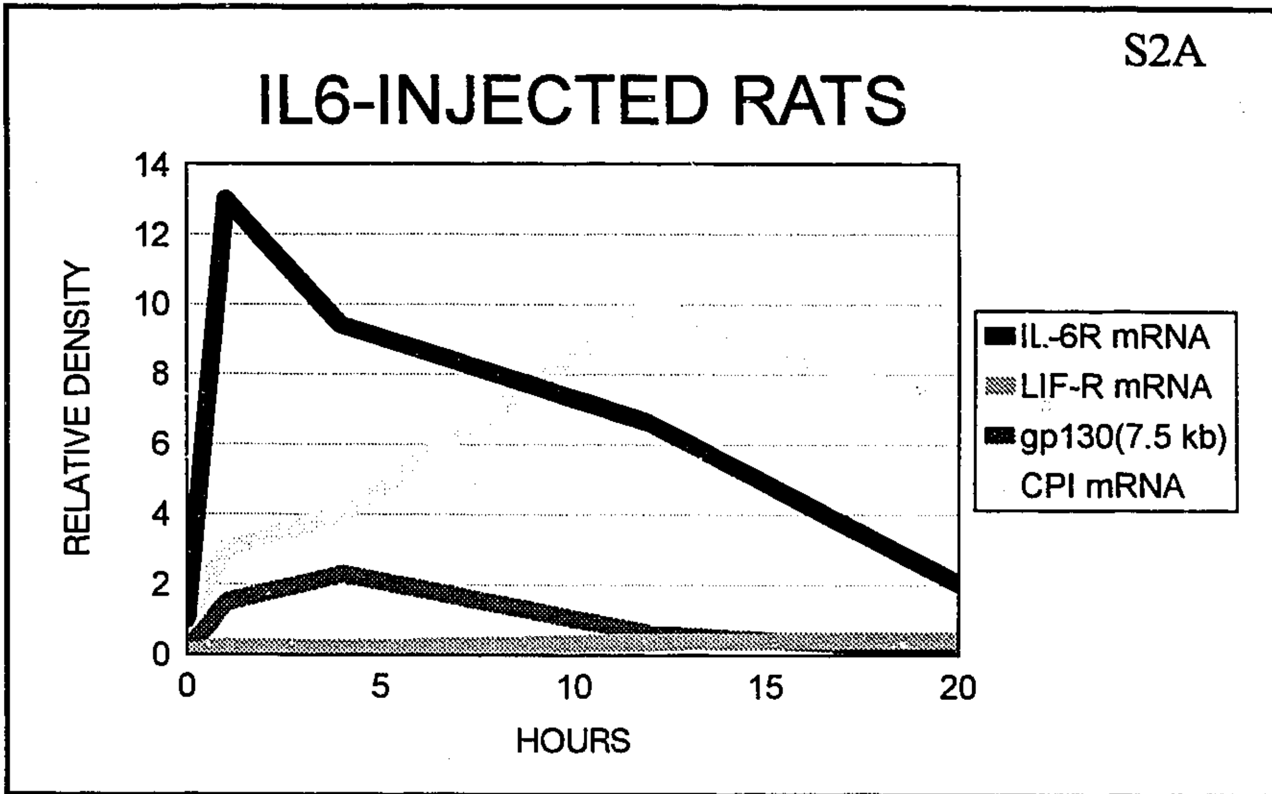
FIGURE S1: Summary of *in vivo* models of acute inflammation. A) Overall serum IL-6 activity and serum cysteine proteinase inhibitor B) Overall serum corticosterone activity. C) Overall liver IL-6R, gp130, and LIF-R mRNA levels. All figures were summarized from the LPS acute systemic inflammatory model.

corresponding ligand are highly regulated and direct the response of the liver *in vivo*.

Other groups such as Kopf et al., 1994, have already verified the importance of IL-6 in the acute phase protein response of the liver. In IL-6-deficient mice, the acute phase response is severely compromised in turpentine-injected mice and moderately affected in LPS-injected mice, and can return to normal only after the injection of rIL-6. Therefore, although many other cytokines are stimulated during inflammation to produce acute phase proteins, only in the presence of IL-6 is a full acute phase response launched.

To continue these studies we examined the effect that recombinant IL-6 or corticosteroid would have on the regulation of these receptors (Figure S2). *In vivo*, our results showed that in addition it its impact on the acute phase protein synthesis, rIL-6 had a rapid and dramatic effect on the mRNA levels of its own receptor, IL-6R (Figure S2A). The mRNA levels of gp130 also increased 1h after injection of rIL-6, but with a much less significant change. Injection of Dexamethasone (DEX) *in vivo* causes a slow but pronounced IL-6R mRNA increase in the liver, but does not affect gp130 or CPI mRNA levels (Figure S2B). Although we found corticosteroid to be a major regulator of IL-6R mRNA levels *in vitro* in rat hepatoma cells (H-35), this was not the case *in vivo*, and IL-6 seems to be the major regulator of IL-6R mRNA expression in the liver *in vivo*.

The changes seen in expression of the receptor *in vivo* are restricted to examination of mRNA and this represents a limitation in the approach. Binding studies for functional assessments are not possible within the organ and protein quantification of receptor presence is not feasible, given the lack of reagents and/or exceptionally low number of receptors known to be expressed on hepatocytes. However, the rapid changes seen in steady state levels of mRNA for the gp80 IL-6R are likely to relate to amounts of receptor protein, and thus to increased presence of the receptor at the hepatocyte



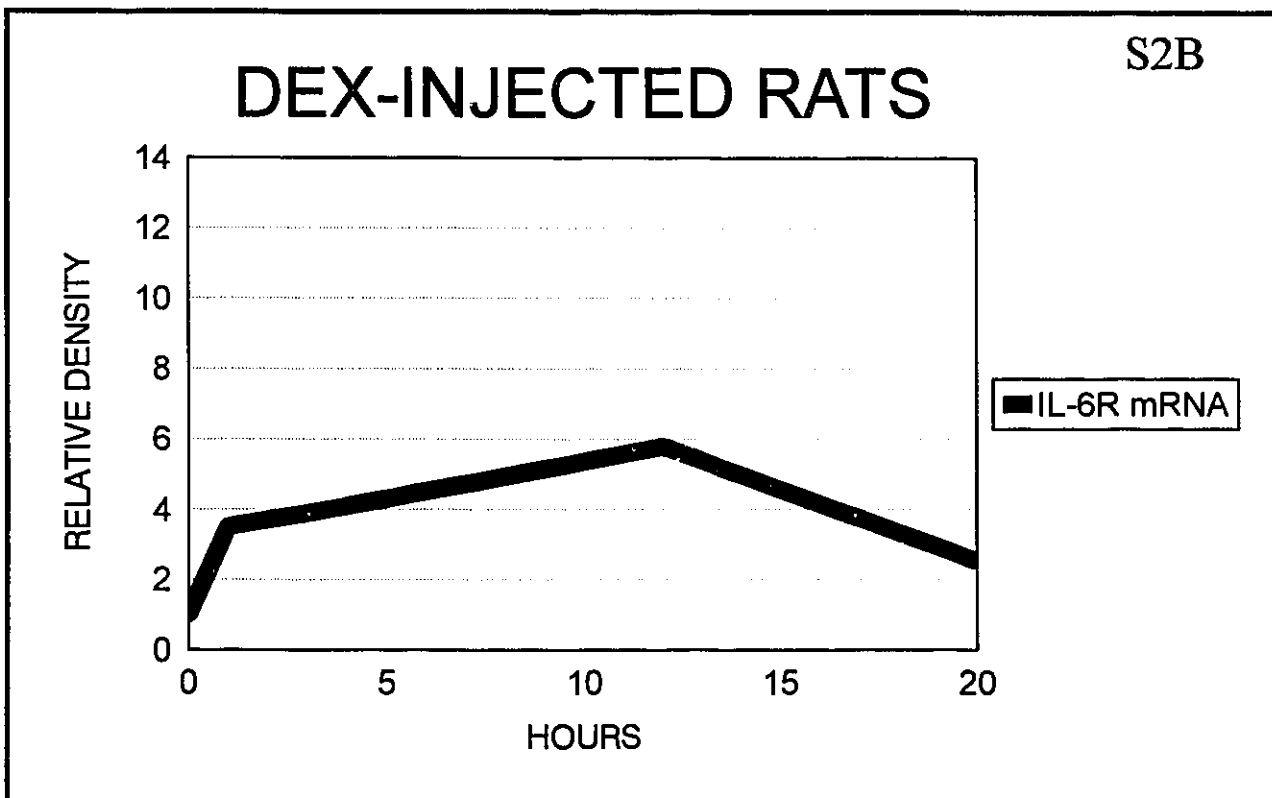


FIGURE S2: Summary of Data obtained from IL-6 and DEX-injected rats. A) IL-6R, LIF-R, gp130, and CPI mRNA levels from IL-6-injected rats. B) IL-6R mRNA levels from DEX-injected rats.

surface. With these reservations in mind, it is still our opinion that IL-6 is the major regulator of the expression of its own receptor and this occurs in a coordinated fashion in the progress of the acute phase response in inflammation

LIF-R expression was not influenced by administration of rIL-6 or DEX, but was regulated slowly *in vivo* during acute inflammation. Because both IL-6R and LIF-R share the same signal transducing molecule, gp130, they share many of the same intracellular pathways. This may account for the functional redundancy seen *in vitro* function between these the two cytokines. Because rIL-6 injected rats show increased mRNA levels in both IL-6R and gp130 (the alpha- and signal transducing receptors), we believed that these same signal pathways would also stimulate LIF-R. However, our studies demonstrate that even when rats are injected with rIL-6 over a period of nine days, LIF-R mRNA levels remain unaffected. Therefore, it must be activation of as yet described alternate pathways that influence LIF-R receptor regulation.

To sort out how different hormones and cytokines influence receptor regulation in the hepatocyte, we continued our studies using the rat hepatoma cell line H-35. To investigate whether "cross-talk" via regulation of their receptors, could happen between several cytokines known to stimulate hepatocyte acute phase protein synthesis, we investigated how Oncostatin M or IL-1 would influence the expression of IL-6R(gp80) and gp130 receptor mRNA levels. We found two things.

First, Oncostatin M can stimulate the expression of IL-6 receptor gp80 and was additive to the increased expression of IL-6R caused by exposure of the cells to corticosteroid. Second, addition of IL-1 to steroid treated cells results in an inhibition of the steroid mediated up regulation of IL-6R. These data clearly suggest "cross-talk" among the liver stimulating cytokines and as many such different cytokines are likely to be present *in vivo*, the overall effect of corticosteroid *in vivo* would

be minor compared to that caused by IL-6.

These studies thus show that although *in vitro*, IL-6 does not appear to effect IL-6R mRNA levels in rat hepatoma cells, *in vivo*, IL-6 has a dramatic influence on the IL-6R mRNA levels. The differences seen between the *in vitro* and *in vivo* results could be explained by the fact that, *in vivo*, other substances may be present that are not present *in* vitro and may change the IL-6R mRNA response. Alternatively, although cell lines like H-35 allow for controlled environments, these cell are transformed lines and can behave differently from primary cells in their natural environment. It is possible for the response of a cell line in tissue culture to be the opposite of a primary cell *in vivo* in its natural environment, and while IL-6 may not directly stimulate H-35 cells to regulate its own receptor IL-6R (gp80) and gp130, *in vivo* primary hepatocytes in their natural environment may be stimulated by IL-6.

In summary, our studies have shown that the regulation of cytokines and their receptors on the hepatocyte during the acute phase response in inflammation is a complex process. The variety of cytokines and hormones released during inflammation depends on the type of acute inflammation and location of acute inflammation induced. These in turn regulate the expression of cytokine receptors on the hepatocyte, resulting in different kinetics of acute phase protein synthesis, allowing for the most sensitive response directed at generating the appropriate conditions for the body to return to homeostatic balance.

APPENDIX A

CLONING OF THE RAT FIBROBLAST IL-6 RECEPTOR (gp80)

The IL-6R(gp80) cDNA was cloned from rat fibroblast poly(A)$^+$ using standard RT-PCR protocols. Due to size restrictions for PCR fragments, the IL-6R cDNA was cloned in two pieces (divided in half at the DraI site), then annealed together (See Figure 1A). The oligos that were used as primers for the RT-PCR reaction contained restriction enzymes sites for convenient subcloning. The 623 bp. (5') PCR fragment and 764 bp. (3') fragment were cloned separately into ptz19R as XbaI-EcoRI fragments (Figure 1B). Several clones containing the two fragments were sequenced and corrected using site directed mutagenesis (standard protocols). A three base pair difference was found in our sequence as compared with the rat liver sequence published by Baumann et al., 1990. We found that these base pair differences were consistant in all the clones sequenced. As a result, we concluded that the rat fibroblast cDNA may be slightly different than the rat liver cDNA or that the published sequence may be incorrect at these sites. However, since the difference between our clone and the published clone was minimal, we decided that this clone was sufficiently close enough in sequence to the published rat liver cDNA sequence to continue with our studies. The corrected fragments were cut out of the vectors with restriction enzymes (5' end with XbaI-DraI and 3' end with DraI-EcoRI) and a 3-way ligation inserted the full length cDNA into the XbaI-EcoRI site of plasmid ptz19R (Figure 1C).

The cDNA and amino acid sequence of our cloned rat fibroblast IL-6R (gp80) is shown in Figure 2. This cDNA does not contain the signal sequence or ATG start codon.

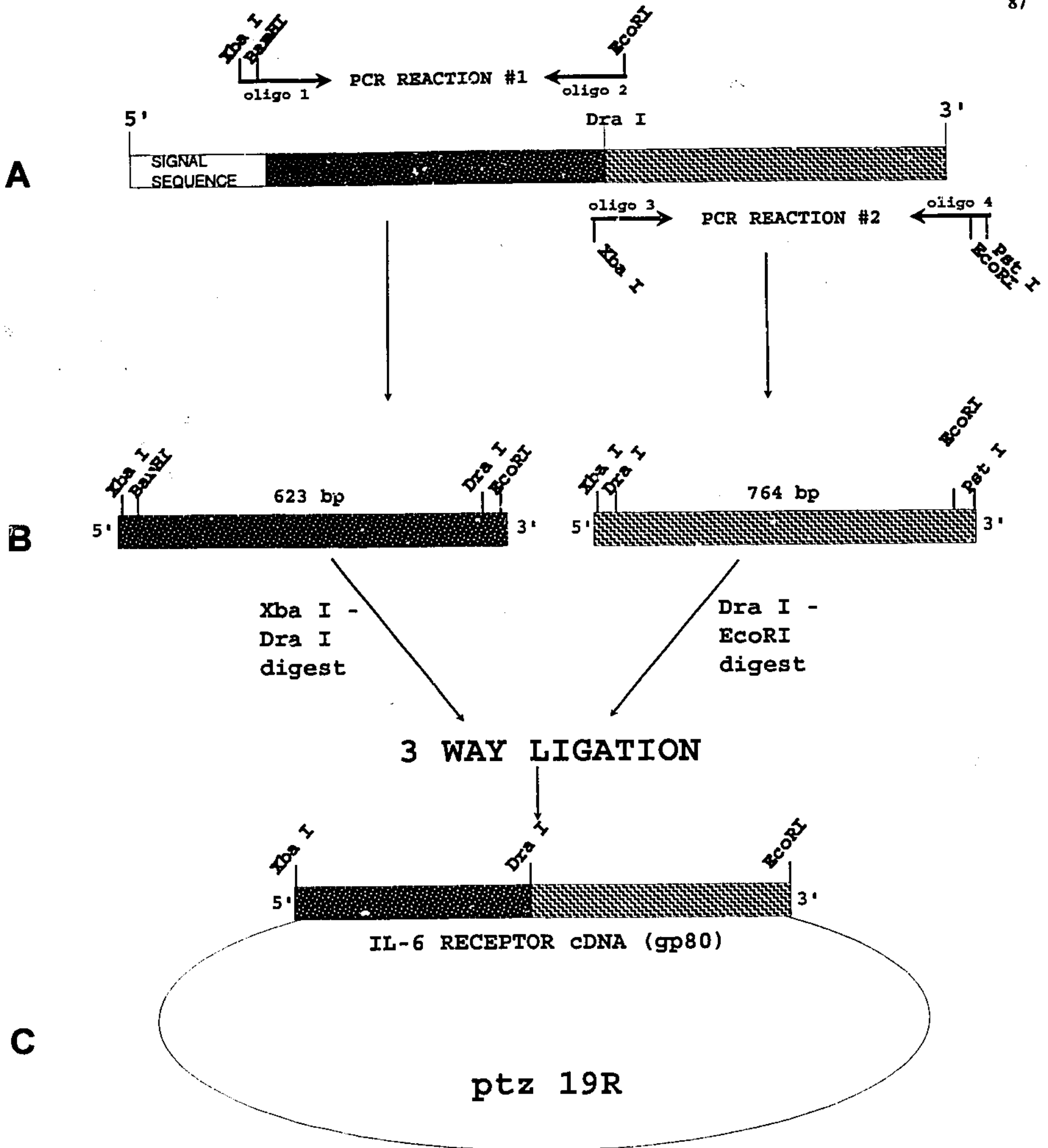


FIGURE 1: SCHEMATIC FOR PCR REACTION AND SUBCLONING OF THE RAT FIBROBLAST IL-6 RECEPTOR (gp80). The cDNA was divided in half at the Dra I site shown in part A, and each half was amplified with oligos containing restriction enzymes (part B). Each half was then cut with restriction enzymes and subcloned into the plasmid ptz19R (part C).

```
   1  ATGCTGGCCGTCGGCTGCACCCTGCTGGTCGCCCTGCTGGCCGCGCCCGCAGTCGCG
   1  M  L  A  V  G  C  T  L  L  V  A  L  L  A  A  P  A  V  A
  58  CTGGTCCTTGGGAGCTGCCGCGCGCTGGAGGTGGCAAATGGTACGGTGACGAGCCTG
  20  L  V  L  G  S  C  R  A  L  E  V  A  N  G  T  V  T  S  L
 115  CCAGGGGCCACTGTTACCCTGATCTGCCCTGGGAAGGAAGCAGCAGGCAATGCTACC
  39  P  G  A  T  V  T  L  I  C  P  G  K  E  A  A  G  N  A  T
 172  ATTCACTGGGTGTACTCAGGCTCACAGAGCAGAGAATGGACTATCACGGGAAACACA
  58  I  H  W  V  Y  S  G  S  Q  S  R  E  W  T  I  T  G  N  T
 229  CTGGTTCTGAGGGCCGTGCAGGTCAATGACACTGGGCACTATTTGTGCTTCTTGGAT
  77  L  V  L  R  A  V  Q  V  N  D  T  G  H  Y  L  C  F  L  D
 286  GATCATCTGGTTGGGACTGTGCCCTTGCTGGTGGATGTTCCCCCAGAGGAGCCCAAG
  96  D  H  L  V  G  T  V  P  L  L  V  D  V  P  P  E  E  P  K
 343  CTCTCCTGCTTCCGGAAGAACCCCCTTGTAAATGCCTTTTGTGAGTGGCATCCAAGC
 115  L  S  C  F  R  K  N  P  L  V  N  A  F  C  E  W  H  P  S
 400  AGCACTCCCTCTCCAACCACGAAGGCTGTGATGTTTGCAAAGAAAATCAACACCACC
 134  S  T  P  S  P  T  T  K  A  V  M  F  A  K  K  I  N  T  T
 457  AATGGGAAGAGTGACTTCCAGGTGCCTTGCCAGTATTCTCAGCAGCTGAAAAGCTTC
 153  N  G  K  S  D  F  Q  V  P  C  Q  Y  S  Q  Q  L  K  S  F
 514  TCCTGCGAGGTGGAGATCCTGGAGGGTGACAAAGTGTACCACATAGTGTCACTGTGC
 172  S  C  E  V  E  I  L  E  G  D  K  V  Y  H  I  V  S  L  C
 571  GTTGCAAACAGTGTTGGAAGCAGGTCCAGCCACAATGTAGTATTTCAGAGTTTAAAA
 191  V  A  N  S  V  G  S  R  S  S  H  N  V  V  F  Q  S  L  K
 628  ATGGTGCAGCCGGATCCACCTGCCAACCTTGTGGTATCAGCCATACCTGGAAGGCCT
 210  M  V  Q  P  D  P  P  A  N  L  V  V  S  A  I  P  G  R  P
 685  CGTTGGCTCAAAGTCAGTTGGCAAGACCCTGAGTCCTGGGACCCAAGTTACTACTTG
 229  R  W  L  K  V  S  W  Q  N  P  E  S  W  N  P  S  Y  Y  L
 742  TTGCAATTCGAGCTTCGATACCGACCTGTATGGTCAAAGACGTTCACGGTGTGGCCG
 228  L  Q  F  E  L  R  Y  R  P  V  W  S  K  T  F  T  G  W  P
 799  CTCCAGGTGGCCCAGCATCAATGTGTCATCCATGATGCCTTGCGAGGAGTAAAGCAT
 267  L  Q  V  A  Q  H  Q  C  V  I  H  D  A  L  R  G  V  K  H
 856  GTGGTGCAGGTCCGAGGGAAGGAGGAGTTTGACATTGGCCAGTGGAGCAAATGGTCC
 286  V  V  Q  V  R  G  K  E  E  F  D  I  G  Q  W  S  K  W  S
 913  CCGGAGGTCACAGGCACTCCTTGGCTAGCAGAGCCCAGGACCACTCCGGCAGGGATC
 305  P  E  V  T  G  T  P  W  L  A  E  P  R  T  T  P  A  G  I
 970  CCGGGGAACCCCACACAGGTCTCTGTTGAAGACTATGACAACCACGAGGATCAGTAC
 324  P  G  N  P  T  Q  V  S  V  E  D  Y  D  N  H  E  D  Q  Y
1027  GGAAGTTCTACAGAAGCAACGAGTGTCCTCGCCCCAGTGCAAGGATCCTCGCCTATA
 343  G  S  S  T  E  A  T  S  V  L  A  P  V  Q  G  S  S  P  I
1084  CCCCTGCCCACATTCCTGGTAGCTGGAGGAAGCCTGGCGTTTGGATTGCTTCTCTGT
 362  P  L  P  T  F  L  V  A  G  G  S  L  A  F  G  L  L  L  C
1141  GTCTTCATCATCTTGAGACTCAAGAAGAAATGGAAGTCACAGGCTGAGAAGGAAAGC
 381  V  F  I  I  L  R  L  K  K  K  W  K  S  Q  A  E  K  E  S
1198  AAGACGACTTCTCCCCCACCGTATCCCTTGGGACCGCTGAAGCCGACCTTCCTCCTG
 400  K  T  T  S  P  P  P  Y  P  L  G  P  L  K  P  T  F  L  L
1255  GTTCCTCTCCTCACCCCATCAGGGTCCCATAACAGCTCTGGGACTGACAACACCGGA
 419  V  P  L  L  T  P  S  G  S  H  N  S  S  G  T  D  N  T  G
1312  AGCCACAGCTGCCTGGGTGTCAGGGACCCACAGTGCCCTAATGACAACAGCAACAGA
 438  S  H  S  C  L  G  V  R  D  P  Q  C  P  N  D  N  S  N  R
1369  GACTACTTATTCCCCAGAT   1388
 457  D  Y  L  F  P  R       462
```

FIGURE 2: Rat Fibroblast amino acid & nucleotide IL-6R sequence. Bold type =signal sequence. Bold underlined =transmembrane domain

APPENDIX B

IL-6 Stimulates Vitronectin Gene Expression In Vivo[1]

Dietmar Seiffert,[2] Margit Geisterfer, Jack Gauldie, Edward Young, and Thomas J. Podor[3,4]

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We tested the hypothesis that vitronectin (Vn) is regulated as an acute phase reactant in response to inflammatory stimuli. In initial experiments, Vn levels were measured during the surgically induced acute phase response in humans. The plasma concentration of Vn increased approximately twofold following elective orthopedic surgery and remained elevated up to 5 days. To examine the mechanism(s) of increased Vn synthesis, hepatic Vn mRNA expression and serum levels were examined in three rat models of acute inflammation: LPS (i.v.), CFA (i.p.), or turpentine (s.c.) injection. The serum concentration of Vn increased approximately twofold 24 h following treatment with turpentine. The expression of Vn mRNA in the liver increased markedly as early as 3 h after treatment in these models and remained elevated up to 18 h. Northern blot analysis of RNA isolated from fractionated liver cells derived from rats treated with LPS indicated that Vn was mainly expressed in hepatocytes, but not in the endothelial or nonparenchymal cell fractions. To analyze the individual effects of raised corticosterone and IL-6 levels on the expression of hepatic Vn mRNA, rats were injected (i.p.) with either dexamethasone or purified recombinant rat IL-6. Vn mRNA expression was elevated within 1 h after IL-6 injection, whereas dexamethasone-injected rats showed unchanged Vn expression. Vn mRNA also was increased in rats chronically injected with IL-6. These results indicate that the Vn gene is up-regulated in acute and chronic inflammation, and this induction is primarily mediated by IL-6. *The Journal of Immunology,* 1995, 155: 3180–3185.

Vitronectin (Vn)[5] is a major adhesive glycoprotein present in plasma and is identical with the S protein of the complement cascade and, thus, inhibits complement-mediated cell lysis (for reviews, see Refs. 1 and 2). It also appears to have a variety of functions in the coagulation and fibrinolytic systems. For example, Vn may regulate the blood coagulation pathway by inhibiting the rapid inactivation of thrombin and factor Xa by antithrombin III in the presence of heparin (3, 4). In addition, Vn binds to and stabilizes type 1 plasminogen activator inhibitor (5–7) and alters its specificity toward thrombin (8, 9).

The liver hepatocyte appears to be the major source of plasma Vn (10). Consistently, plasma levels of Vn are markedly reduced in some patients with disseminated intravascular coagulation, especially in those with liver failure (11), and in patients with degenerative liver disease (12). The regulation of human Vn in other disease states or genetic deficiencies has not been described (13).

We noted that Vn shares functional similarities with APR proteins with respect to lack of significant extrahepatic synthesis and regulatory functions in proteolytic enzyme cascades, including the blood coagulation, fibrinolysis, and complement systems (1, 2). This observation stimulated us to determine plasma/serum Vn levels in patients undergoing hip replacement surgery and in rats undergoing acute inflammatory reactions. Results suggest that rat Vn is regulated as an APR protein. To elucidate the mechanism(s) involved in the up-regulation of serum Vn, we analyzed Vn mRNA levels in livers of rats treated with various acute inflammatory stimuli or purified IL-6 and corticosteroids. The induction of hepatocyte Vn mRNA in rats occurs rapidly during acute inflammation, suggesting that IL-6 may be directly involved in its induction.

Materials and Methods

Patient population and plasma preparation

Blood samples were obtained with informed consent from patients undergoing elective hip replacement surgery as described previously (14).

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[5] Abbreviations used in this paper: Vn, vitronectin; APR, acute phase reactant; CPI, cysteine protease inhibitor.

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Citrated blood was collected preoperatively and every 24 h postoperatively for 5 days. All patients received prophylactic heparin starting 18 h after surgery. Platelet-poor plasma was prepared by centrifugation of blood at 3000 × *g* at 4°C for 15 min. Aliquots were kept at −70°C until assayed.

Analysis of Vn, IL-6, and APR

Human Vn was purified from serum by heparin affinity chromatography (15), and Ab were raised in rabbits using standard procedures (16). The IgG fraction of the resulting antiserum was further purified by Vn affinity chromatography (17). Vn Ag levels in patient plasma were quantified using a sandwich ELISA. Briefly, microtiter wells were coated (5 μg/ml in PBS, 4°C, 16 h) with rabbit anti-human Vn IgG. After washing, wells were blocked with PBS containing 3% BSA and 0.05% Tween-20. Vn-containing samples in PBS with 0.1% albumin and 0.1% Tween-80 were incubated in the wells for 1 h; after washing, bound Ab were detected using biotin-labeled rabbit anti-human Vn, followed by streptavidin-alkaline phosphatase conjugate and the chromogenic substrate p-nitrophenyl phosphate. The change in absorbance of duplicate wells was determined and corrected for the absorbance of wells treated identically except that the incubation step with Vn-containing samples was replaced with assay buffer. A standard curve was constructed using serial dilutions of purified human Vn. Changes in Vn levels in rat serum were determined semiquantitatively by immunoblotting. Pooled serum ($n = 5$) from normal or turpentine-injected rats (24 h after injection) was fractionated by SDS-PAGE (10% acrylamide in the separating gel) under reducing conditions and transferred to nitrocellulose membranes. Vn was detected using rabbit anti-human Vn IgG, followed by biotin-labeled goat anti-rabbit IgG, streptavidin-alkaline phosphatase conjugate, and the chromogenic substrate naphthol phosphate/fast red (Bio-Rad Laboratories, Richmond, CA). The relative intensities of the immunoreactive bands were quantified by laser densitometric scanning. Due to the limitation of this method and the potential variability between different animals, these concentrations should be considered only approximate changes. Quantitation of IL-6 bioactivity was conducted with the B9.9 hybridoma tritiated-thymidine incorporation assay (18). C-Reactive protein was quantified by a nephelometric assay (18). Plasma protein electrophoresis was performed in agarose gels using the Paragon electrophoresis system (Beckman Instruments, Fullerton, CA). The stained albumin and fibrinogen bands were quantified by densitometric scanning.

Rat models of acute inflammation

Male Sprague-Dawley rats (~200 g) were treated with CFA (Life Technologies, Grand Island, NY; 500 μl i.p. injection), LPS (Sigma Chemical Co., St. Louis, MO; 200 μg i.v. injection), turpentine (200 μl s.c. injection), rIL-6 protein (*Escherichia coli*-purified IL-6 from Allelix Biopharmaceuticals, Mississauga, Ont.; 50 μg i.p. injection), and dexamethasone (5 μg i.p. injection). The levels of stimuli were chosen to elicit a strong acute phase response (18, 19). Rats were killed at various time points, and liver tissue and blood were harvested. RNA was extracted from pooled liver tissue samples from two different rats (19).

Liver cell isolation and fractionation: Northern blot analysis

Crude liver cell suspensions from collagenase perfusates of LPS- or saline-treated rats were fractionated for purification of hepatocytes, Kupffer cells, and endothelial cells by metrizamide double layer centrifugation and centrifugal elutriation techniques (18). The purity of the resulting fractions was characterized as outlined previously (18). For Northern blot hybridization, 30 μg of RNA was electrophoresed on 1% agarose-formaldehyde gels, stained with ethidium bromide, denatured in 50 mM NaOH, and transferred to nylon membranes (19). Membrane were hybridized with a random primed ^{32}P-labeled murine Vn cDNA probe (bases 1–650) (10). The membranes were washed in 5% SDS-2X SSC at hybridization temperature (57°C), dried, and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 6 h at −80°C. In some experiments, the membranes were hybridized with a CPI probe (18, 19). All changes in specific RNA intensity on the blots were compared with the ethidium bromide-stained RNA gel using a Pharmacia densitometer (Pharmacia, Piscataway, NJ). Again, due to the limitation of this method and the potential variability between different animals, these concentrations should be considered only approximate changes.

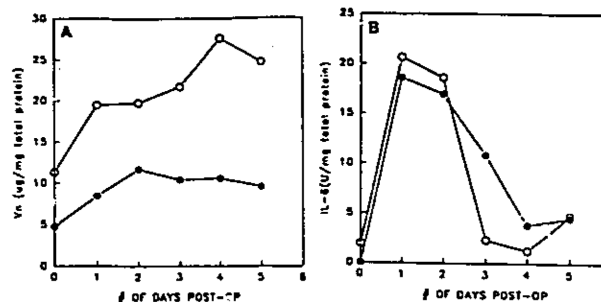

FIGURE 1. Time course of plasma Vn and IL-6 levels in two patients after elective hip surgery. Plasma samples obtained from two representative patients (patient 1, open circles; patient 2, closed circles) at the indicated times after surgery were analyzed for levels of Vn Ag (*A*) and IL-6 (*B*) activities (see *Materials and Methods*). Results are expressed on the basis of total plasma protein concentration to correct for the effects of hemodilution.

Results

Figure 1 illustrates the time course of postoperative changes in Vn and IL-6 for two representative surgical patients. The results have been corrected for the effects of hemodilution. Postoperative plasma levels were elevated to a maximum of 2- to 2.5-fold over preoperative levels within 2 days (Fig. 1*A*). In the total patient group ($n = 11$), Vn Ag levels increased incrementally from days 1 to 5 postoperatively to 131, 172, 170, 183, and 177%, respectively. On the first postoperative day, IL-6 levels were elevated by more than 1000-fold and remained increased for 4 to 5 days (Fig. 1*B*). The postoperative increases in IL-6 were followed by increases in IL-6-responsive APR, such as fibrinogen and C-reactive protein, and decreases in albumin (Table I). These results indicate that the patients underwent a surgically induced acute phase response and suggest that Vn may be regulated as an APR protein.

To characterize the mechanism(s) involved in the regulation of Vn, we employed three experimental models to induce acute inflammation in the rat. In each case, the inflammatory stimuli significantly increased serum IL-6, corticosterone, and CPI levels, indicating that the rats underwent an acute phase response (18, 19). The serum levels of Vn were analyzed by semiquantitative immunoblotting using cross-reacting rabbit anti-human Vn IgG (Fig. 2). The Ab stained a single band of M_r 65,000 in both normal and turpentine-treated rat serum (pool of five different animals; Fig. 2, *inset*, *lane 1*). The migration of purified human Vn is indicated in *lane* 2. Semiquantitative immunoblotting experiments revealed that this band was induced approximately twofold 24 h after turpentine injection compared with that in normal rat serum (Fig. 2).

Table I. *Time course of plasma acute phase reactants in two patients after elective hip replacement surgery*[a]

Plasma Acute Phase Reactant (mg/ml)	No. Days Post-Op 0	1	2	3	4	5
Patient 1						
Albumin	29.8	27.1	20.7	21.4	20.5	20.0
Fibrinogen	3.2	3.8	4.0	5.2	6.7	7.5
C-Reactive Protein	0.30	1.91	2.67	2.26	1.57	0.92
Patient 2						
Albumin	26.8	21.7	18.5	18.5	16.6	17.2
Fibrinogen	5.7	8.6	4.7	4.7	5.2	5.8
C-Reactive Protein	0.30	3.60	2.95	2.44	2.42	2.92

[a] Plasma samples were obtained at various times after surgery and analyzed for the levels of plasma albumin, fibrinogen, and C-reactive protein (see *Materials and Methods*). Patient 1, indicated by open circles in Figure 1; patient 2, indicated by closed circles in Figure 1.

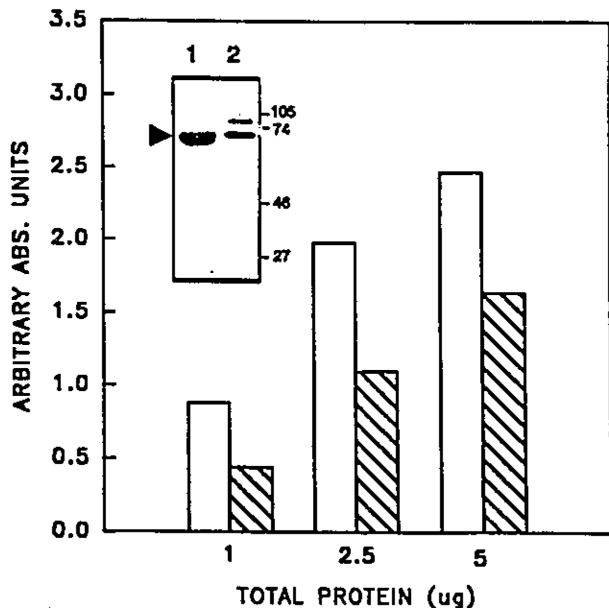

FIGURE 2. Quantification of Vn in rat serum. Pooled rat serum ($n = 5$) from normal (hatched bars) or turpentine-injected (24 h after injection; open bars) rats was fractionated by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and stained with rabbit anti-human Vn antiserum. The relative intensities of the immunoreactive bands at the indicated total protein amounts applied to the gel were quantified by laser densitometric scanning and are expressed as arbitrary units. *Inset,* The polyclonal Ab detected a single band of M_r 65,000 in total rat serum (*lane 1*) by immunoblotting analysis. The migrations of purified human Vn (*lane 2*) and M_r standards are indicated.

The fold increase was similar using the Vn ELISA assay (not shown). Similar results were obtained using serum from rats injected with CFA (not shown). In the case of LPS, serum Vn levels were variable and decreased in some animals due to extravasation of Vn into damaged tissues (T. J. Podor, unpublished observation).

Northern blot analysis of hepatic RNA demonstrated the presence of one predominant message for rat Vn at 1.7 kb

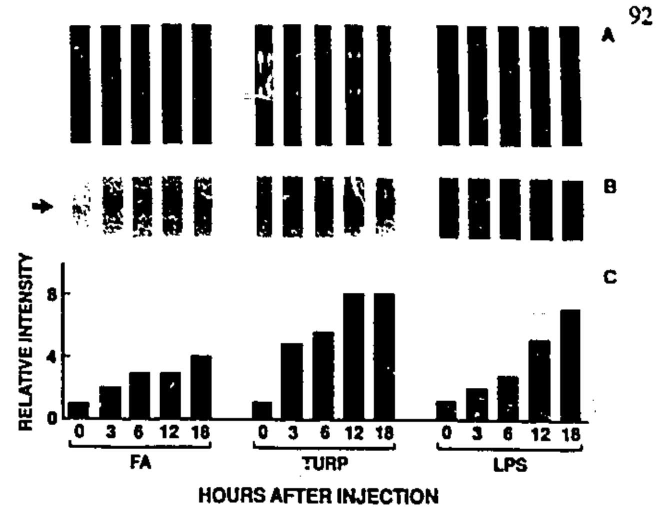

FIGURE 3. Regulation of hepatic Vn mRNA in experimental models of acute inflammation. Hepatic RNA was isolated in duplicate at various time points from rats treated with CFA (FA), turpentine (TP), or LPS. RNA (30 μg/lane) was fractionated by agarose gel electrophoresis, stained with ethidium bromide (*A*), transferred to nylon membranes, and hybridized to a ^{32}P-labeled mouse Vn cDNA probe (*B*). Vn mRNA densitometer scans were normalized to the staining intensity of the RNA gels (*C*). The estimated size of the positive band in B (arrow) relative to the 18S and 28S rRNA is 1.7 kb.

(Fig. 3). In all three models of acute inflammation, Vn mRNA levels were increased two- to fivefold by 3 h compared with normal levels (Fig. 3). These levels remained elevated up to 18 h; the strongest increase in Vn mRNA (approximately eightfold above control levels) was seen in both LPS- and turpentine-treated rats, whereas CFA elicited only a fourfold induction (Fig. 3). The kinetics of induction of Vn and CPI mRNA in turpentine-treated rats were compared (Fig. 4). Both messages were maximally induced at 12 h, whereas a half-maximum increase in Vn mRNA was detectable at 3 h; at this time point, CPI mRNA expression was only 20% of its peak value. Vn mRNA levels remained elevated for up to 18 h, whereas CPI mRNA levels declined (Fig. 4).

Liver cell isolation and fractionation were used to identify the specific Vn-producing cell type under acute inflammatory conditions (Fig. 5). Under control conditions, Vn-expressing cells were highly enriched in the hepatocyte fraction (not shown). This indicates that the hepatocyte is the major Vn-producing cell type in the rat liver consistent with results obtained using murine liver (10). The steady state level of Vn mRNA in the liver from LPS-treated rats was significantly increased, and again, the Vn transcript was only detectable within the detection limit of Northern blot analysis in the hepatocyte fraction (Fig. 5).

To evaluate the roles of IL-6 and corticosteroids in increased hepatic Vn mRNA in acute inflammation, rats were individually injected with either dexamethasone or

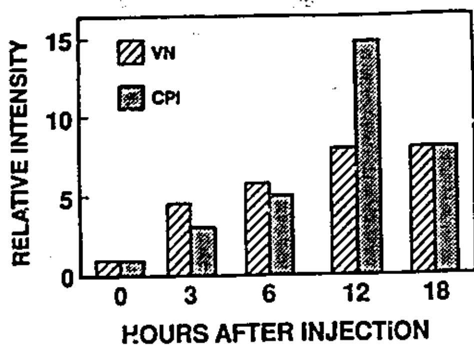

FIGURE 4. Comparison of the kinetics of CPI and Vn mRNA induction during acute inflammation. Densitometer scans of hepatic RNA are shown for CPI and Vn for turpentine-injected rats. mRNA densitometer scans were normalized to the ethidium bromide staining intensity of the RNA gels.

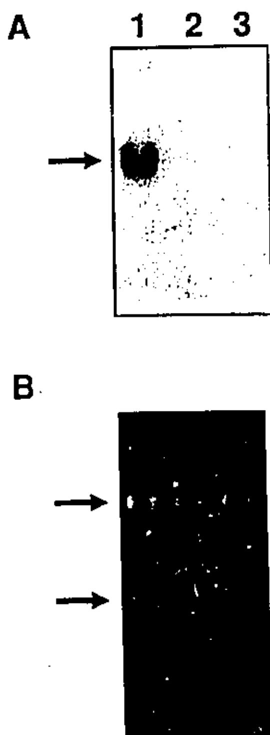

FIGURE 5. Detection of Vn mRNA expression in hepatocyte RNA isolated from LPS-inflamed rats. Total RNA 6 h after injection of LPS was isolated from liver cell fractions enriched for hepatocytes (*lane 1*), Kupffer cells (*lane 2*), or endothelial cells (*lane 3*). RNA (20 μg) was subjected to Northern blotting analysis using a murine Vn cDNA probe (*A*). *B*, Ethidium bromide staining of total RNA before transfer. The arrows denote the relative migration of the Vn transcript (*A*) and the 18S and 28S rRNA (*B*).

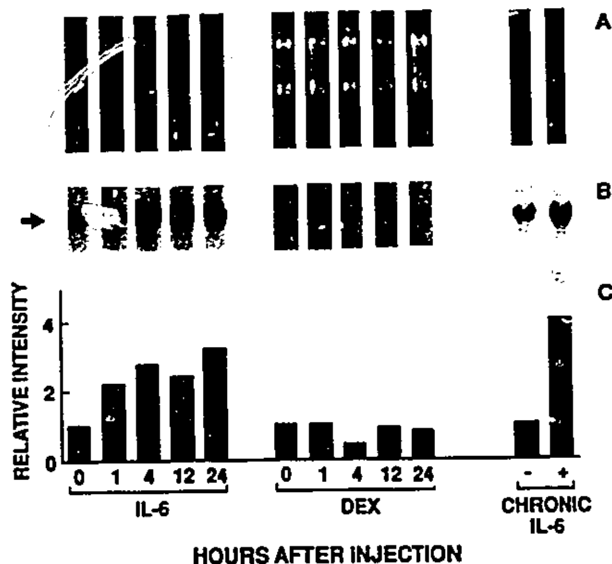

FIGURE 6. Regulation of hepatic Vn mRNA by dexamethasone and IL-6. Rats were injected with a single dose of IL-6 or dexamethasone, and at the indicated time points, hepatic RNA was isolated and analyzed for Vn mRNA as described in Figure 3. Rats also were treated daily for 9 days with IL-6 or saline (control), and the livers were harvested on day 10. *A*, Ethidium bromide-stained gels; *B*, hybridization with Vn cDNA probe; *C*, densitometer scans of Vn mRNA normalized to the staining intensity of the RNA gels. The estimated size of the positive band in *B* (arrow) relative to those of the 18S and 28S rRNA is 1.7 kb.

IL-6. The overall hepatic Vn mRNA levels in rats treated with dexamethasone were unchanged (Fig. 6), whereas the steady state level of hepatic Vn mRNA increased as early as 1 h after the injection of purified IL-6 and remained elevated (approximately threefold increase) up to 24 h (Fig. 6). CPI mRNA levels increased about ninefold at 12 h and started to decline (fourfold above baseline) at 24 h (not shown). When rats were chronically treated with IL-6 for a period of 9 days, hepatic Vn mRNA was significantly higher (average fourfold mean induction; $n = 2$) compared with that in controls injected with saline (Fig. 6).

Discussion

The response of the body to injury or trauma consists of an orderly and orchestrated series of reactions that normally result in the arrest of the process of injury, protection of the rest of the organism against further injury, and the initiation of repair and restorative processes aimed at returning the organism to homeostatic balance (reviewed in Refs. 20–22). As part of the systemic reaction to trauma and inflammation, APR proteins are synthesized in hepatocytes. This synthesis is stimulated at least in part by IL-6 elicited from areas of local inflammation (reviewed in Refs. 20–22). The cytokines IL-1 and TNF-α have a much more limited effect on a small subset of APR proteins (21–23). These observations were recently supported by gene disruption studies of the IL-6 gene. As expected, the inflammatory acute phase response after local sterile tissue

damage (i.e., turpentine injection) or infection with Gram-positive bacteria was severely compromised (24). Surprisingly, the APR was only moderately affected after LPS injection (24), suggesting that LPS, in addition to inducing IL-6, can stimulate cytokines displaying overlapping activities with IL-6.

In the present study, we provide evidence that Vn is regulated as an APR in two different mammalian species. Firstly, the Vn plasma levels increased in patients undergoing elective hip replacement surgery (Fig. 1), an established model for human APR (14). Concomitantly, IL-6 and fibrinogen levels were increased, whereas the plasma levels of albumin were reduced, indicating that the patients indeed underwent a surgically induced acute phase reaction (21). Secondly, the serum level of a single M_r 65,000 protein, cross-reacting with monospecific Ab to human Vn, was up-regulated approximately twofold in acutely inflamed rats (i.e., those treated with turpentine and CFA; Fig. 1). The relative mobility of this positive band is similar to that reported for rat Vn (25), and ligand binding studies indicate that this band also represents the major type 1 plasminogen activator inhibitor binding protein in rat serum (T. J. Podor, unpublished observation). Taken together, these immunologic and functional studies strongly suggest that this protein band is Vn and that Vn levels increase in the blood of acutely inflamed rats.

The molecular mechanism underlying the increased serum Vn level were studied in three rat models of acute inflammation. The steady state level of rat hepatic Vn mRNA was up-regulated in all three models of acute inflammation (Fig. 3). Although some differences in the intensity and kinetics of Vn mRNA induction in the three models were seen, the overall Vn acute phase responses were similar in the CFA-, turpentine-, and LPS-treated rats (Fig. 3). It should be noted that the kinetics of the induction of Vn were similar to those of CPI, a well characterized, relatively late APR protein (Fig. 4). Also, as expected for a typical APR protein, the liver hepatocyte is the major site of biosynthesis (Fig. 5). Taken together, this study identifies Vn as an APR protein in two mammalian species.

To address the role of IL-6 in the up-regulation of Vn, we investigated whether individually, IL-6 or the corticosteroid analog dexamethasone could increase Vn mRNA levels in vivo (Fig. 6). Rats injected with IL-6 showed increased hepatic Vn mRNA levels as early as 1 h after injection (Fig. 6). Hepatic Vn mRNA appears to be unchanged by dexamethasone (Fig. 6), whereas in RNA isolated from the same animal, IL-6 receptor mRNA was induced (19), indicating that the dexamethasone was biologically active. The fact that Vn mRNA was so quickly induced in IL-6-injected rats suggests that IL-6 itself may directly increase the expression of hepatic Vn mRNA. It should be noted, that Vn mRNA levels remained elevated at times when serum IL-6 activity returned to baseline levels. This finding appears to be most striking in the case of the injection of purified IL-6, leading to peak IL-6 levels at 1 h that returned to normal by 12 h (19), whereas Vn mRNA remained elevated up to 24 h (Fig. 6). Moreover, chronic injection of low doses of IL-6 over 9 days maintained the increased steady state expression of Vn mRNA (Fig. 6). A variety of considerations may account for these apparent discrepancies. For example, IL-6-induced transcription of acute phase protein genes is in some cases followed by higher mRNA stability (26). In addition, there may be other cytokines induced during inflammation that impact on hepatic function, or IL-6 may act as an exocrine or paracrine hormone with respect to hepatic responses in inflammation (18, 27). In this respect, it should be noted that leukemia inhibitory factor, oncostatin M, and IL-11 display overlapping activities with IL-6 in vitro (24).

In summary, while the true physiologic role of the acute phase regulation of Vn remains to be elucidated, many of its functions may be directed to control localized extracellular inflammatory reactions and associated tissue damage.

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