

CHARACTERIZATION AND IDENTIFICATION OF
HEPATOCYTE STIMULATING FACTOR (HSF)
AS INTERFERON BETA 2 ($\text{IFN}\beta_2$) AND ITS ROLE
IN THE INITIATION OF THE
ACUTE PHASE RESPONSE OF LIVER

by

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of Liver

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ABSTRACT

The acute phase response in mammals to tissue injury or infection is characterized by a number of systemic effects including fever, neutrophilia and increases in serum levels of liver-derived (synthesized by hepatocytes) acute phase proteins. Soluble mediators or cytokines released by cells of the monocyte/macrophage lineage have been implicated in the initiation of the increased synthesis of protein by hepatocytes and include Hepatocyte Stimulating Factor (HSF) and Interleukin-1 (IL-1). The nature of HSF and IL-1 and their activities in inducing acute phase protein synthesis in vitro by primary cultures of rat hepatocytes and by human Hep-G2 cells was examined. Human peripheral blood monocyte (PBM) -derived HSF showed different characteristics than IL-1 upon separation by chromatography and gel electrophoresis. HSF strongly stimulated some acute phase proteins (rat α_2 -macroglobulin and α_1 -cysteine protease inhibitor, human fibrinogen, and α_1 -antichymotrypsin) whereas IL-1 strongly stimulated human α_1 -acid glycoprotein.

Human PBM derived HSF showed biochemical similarities to another cytokine, Interferon β_2 (IFN β_2), that had previously been cloned from fibroblasts and from T-

lymphocytes. HSF and IFN β_2 showed immunological similarities on the basis of antibody binding and activity inhibition assays. Cloned IFN β_2 from T-cells showed potent inducing activity of acute phase protein synthesis and stimulated maximally the same proteins that did HSF. Both human fibroblast cultures and PBM₁ cultures secreted HSF activity and possessed mRNA species that hybridized to a cDNA probe for IFN β_2 . These data suggest that human PBM derived HSF and human IFN β_2 are identical and that fibroblasts and T-lymphocytes as well as monocytes are capable of releasing Hepatocyte Stimulating Factor/Interferon β_2 .

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List of Abbreviations

a.a.	amino acids
ACTH	adrenocorticotropin
amo	alveolar macrophage
APP	acute phase proteins
α_1 -ACH	alpha-1-antichymotrypsin
α_1 -AGP	alpha-1-acid glycoprotein
α_1 -AT	alpha-1-antitrypsin
α_1 -CPI	alpha-1-cysteine protease inhibitor
α_2 -macro	alpha-2-macroglobulin
anti-crude	antibody against crude PBM supernatant
anti-IL-1	antibody against human Interleukin-1
anti-IFN β_1	antibody against Interferon Beta-1
anti-IFN β_1 & β_2	antibody against Interferon Beta (1 and 2)
anti-TNF	antibody against Tumour Necrosis Factor
ADP	adenosine diphosphate
BCG	Bacille Calmette-Guérin
BSF-2	B-cell stimulatory factor two
C3	the third component of complement
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
Ci	Curies
cm	centimetres

CON A	concanavalin A
Co.V	coefficient of variation
CRP	C-reactive protein
DEX	dexamethasone
DNA, cDNA	Deoxyribonucleic acid, complementary DNA
DTT	dithiothreitol
EDTA	ethylene-diaminetetra-acetic acid
ELIZA	enzyme linked immuno-adsorbent assay
FCS	fetal calf serum
Fib	fibroblast
fig.	figure
g	force of gravity
Hepes	4-[2-hydroxy-ethyl]-1-piperazin- ethanesulphonic acid
HGF	hybridoma/plasmacytoma growth factor
HP	haptoglobin
HPLC	high performance liquid chromatography
HSF	hepatocyte stimulating factor
HTLV	human T-cell leukemia virus
IFN α , β , γ	Interferon alpha, beta, gamma
IL-1	Interleukin-1
i.m.	intramuscular
i.p.	intraperitoneal
IVT	<u>in vitro</u> translation
kb	kilobases
kd	kilodaltons

L	litres
LAF	lymphocyte activating factor
LEM	leukocyte endogenous mediator
LP	leukocytic pyrogen
LPS	lipopolysaccharide
LT	Lymphotoxin
M	molar
ME	mercaptoethanol
MEM	minimal essential medium
mg	milligrams
μ Ci	microcuries
μ g	micrograms
μ l	microlitres
min	minutes
ml	millilitres
mM	millimolar
mm	millimetres
MMTV	mouse mammary tumour virus
MOPS	morpholinopropanesulfonic acid
NK	natural killer
NRS	normal rabbit serum
PBM	peripheral blood monocyte
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PHA	phytohaemagglutinin
pI	isoelectric point

RER	rough endoplasmic reticulum
RIPA	radio immunoprecipitation assay buffer
RNA, mRNA	ribonucleic acid, messenger RNA
RR	rabbit reticulocyte
SAA	serum amyloid A protein
SAP	serum amyloid P protein
S.C.	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
SSC	sodium chloride, sodium citrate solution
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)-aminomethane
VSV	Vesicular Stomatitis Virus
WG	wheat germ

INTRODUCTION

Acute inflammation is a complex biological phenomenon of which a thorough understanding has not yet been attained. Since inflammation is apparent in most if not all diseases of humans, it is of much clinical interest. Acute inflammation is a general term that includes an injurious agent or stimulus; the damage it invokes on tissue; and the acute host reaction to this process, termed the acute phase response. This involves local and systemic effects and as part of the systemic response, there are well-documented increases in concentrations of a series of liver-derived serum proteins or "acute phase proteins". The intricacies of control of this response as well as the purpose and involvement of acute phase proteins in disease, are two areas of increasing interest to basic researchers and clinicians.

The Acute Phase Response

Upon tissue injury, the success or failure of an organism to survive will depend on the extent of cell damage as well as the biological response to that damage in facilitating wound healing, repair and prevention of further damage. The acute phase response is non-specific and occurs relatively rapidly after the injurious stimulus. It is complex, involving cells and mediators or hormones that

interact to produce a number of physiological effects. In addition there are interactions between serum protease systems (such as complement, kinin-forming and coagulation cascades) which are initiated during this response (Sundsmo & Fair, 1983). Many aspects of the acute phase response are regarded as beneficial, such as; facilitating removal of the noxious agent; stoppage of bleeding; demarcation, removal and absorption of dead tissue and waste products; inactivation of excessive digestive proteinase action at the site of inflammation, thereby enhancing repair of affected tissue (Koj, 1985b). Stimuli of the acute phase response include physical or chemical trauma, infectious diseases involving pathogenic bacteria or viruses, and immune-mediated mechanisms such as hypersensitivity. The quality of the acute phase response to various stimuli is consistent although generally the magnitude of the response depends on the extent of tissue destruction (Koj, 1985b; Kusher, 1982). Chronologically, a local acute phase response occurs within hours of tissue damage and is followed by systemic manifestations which are most evident 1 to 2 days later (Ryan & Majno, 1977). The acute phase response usually subsides over a short period, however, if the injurious agent is not removed, aspects of the acute phase response may persist much longer.

The overall physiologic importance of the acute phase response is not clear. However, it is reasonable to

postulate that it is advantageous for host survival since it is conserved through many species such as all mammals yet examined, chickens (Amrani et al., 1986), and at least 1 species of fish, the plaice (White and Fletcher, 1982).

The Local Response

The events in the immediate area of a wound or infection result in the classical signs and symptoms of inflammation such as redness, swelling, heat, and pain. These may be attributable to changes in blood vessel permeability and to changes in blood vessel calibre and flow including transient vasoconstriction followed by vasodilation. These effects result in extravasion of fluid and red blood cells. Upon transection of blood vessels, the coagulation pathway is initiated by exposure of basement membrane components involving aggregation of platelets, platelet plug formation and fibrin deposition. Also involved is the margination and infiltration of blood neutrophils (predominantly early, within 1-3 hours) and monocytes or macrophages (the predominant cell infiltrate at 24-48 hours) (Ryan and Majno, 1977). The normal course of events thereafter involves degradation and removal of dead tissue by phagocytic cells and growth of fibroblasts and other cells in the reconstruction of tissue with concomitant decreases in inflammatory cell infiltrate and vessel

permeability.

The Systemic Response

The systemic acute phase response to tissue damage or infection involves a variety of organ systems. Fever is a symptom used often as an indication of infection and there are apparently few situations where the febrile response is not present (Dinarello, 1984). Temperature set points controlled by the hypothalamus are influenced by blood born and possibly neural messages that arise due to inflammation. Also well-established is the increased numbers of neutrophils in blood in response to infectious diseases or tissue damage such as in surgery.

The systemic response is also characterized by increases in serum concentrations of a number of proteins. These increases (as well as fever and neutrophilia) have become a hallmark of systemic inflammation. In humans, one of these proteins, C-reactive protein (CRP), was first recognized by its ability to precipitate C-polysaccharides derived from Pneumococcus. The descriptions of Abernethy and Avery (1941) were among the first documentation of changes in serum proteins. They observed increased C-reactive protein in sera of patients with febrile infectious diseases. Darcy (1960) later reported similar observations in rat serum, showing increased specific plasma protein

concentrations upon injury and inflammation in the animal. Further studies in the 1960s and 1970s have identified numerous plasma proteins in various species that change in inflammatory states. These are now generally referred to as acute phase proteins (Koj, 1974; Koj and Gordon, 1985).

Over the same time period, it has become apparent that other changes in metabolism occur during the acute phase response that include: aminoacidemia, proteinuria, net nitrogen catabolism, gluconeogenesis, hypoferrremia, hypozincemia, hypercupremia, increases in glucocorticoids, and altered activity of various liver enzymes (Kushner, 1982; Dinarello, 1984; Koj and Gordon, 1985; Baynes, 1986). The liver also shows ultrastructural changes consistent with increased synthesis, transport, glycosylation and secretion of protein. These include increase in weight, expansion of smooth endoplasmic reticulum, proliferation of golgi complex and increased synthesis of tubulin, cytoplasmic actin and RNA, particularly ribosomal RNA (Kushner, 1982; Jamieson et al, 1986).

Whereas the local response acts at the site of tissue damage, and with a shorter initiation, systemic responses are more prolonged and widely distributed. For example, in a rat model of lung pleurisy, thromboxane and prostacyclin mediators in pleural exudate are evident earlier (approximately 2 hours) and are more transient than changes in acute phase protein concentrations (1-2 days) (Tissot et

al., 1984). The systemic nature of the fever or liver responses reflect the centralization of "effector" organs and tissues. This may facilitate healing and preparation for further damage or infection at other sites as well as the initial one.

Hypothesis

The mechanisms involved in the control of the acute phase response of the liver have not been fully elucidated. The problems of particular interest in the set of experiments completed here involve the initiation of the liver acute phase protein synthesis. There are many different functions served by these acute phase proteins whose synthesis appears to be stimulated by products of cells of the monocyte-macrophage lineage. Historically, Interleukin-1 (IL-1), a polypeptide released by activated monocytes and a number of other cells, has been thought by many to be the molecule responsible for initiating this hepatocyte response. In more recent years, it has become evident that other factors, possibly distinct from IL-1 including a factor described as Hepatocyte Stimulating Factor (HSF), can stimulate in vitro synthesis of acute phase proteins by hepatocytes. Neither the role that these molecules play in specifically inducing the synthesis of acute phase proteins nor the relationship between them has been precisely defined. In order to characterize HSF, its

relationship to IL-1 and to examine its action on hepatocytes in vitro the following hypothesis was postulated:

A) HSF derived from human peripheral blood monocytes is a polypeptide cytokine that is distinct from IL-1 and other cytokines.

B) This HSF is the molecule which is primarily responsible for induction of acute phase protein synthesis by hepatocytes.

To examine the hypothesis, in vitro hepatocyte cultures were used as target cells for in vitro generated cytokines from human peripheral blood monocytes. Production, characterization and purification of human HSF to homogeneity was attempted in the hope of obtaining a partial amino acid sequence which would then be used to clone the HSF gene. However, this approach subsequently became unnecessary as other approaches including comparison of separation characteristics, binding and inhibition by specific antibodies, and analysis of activity on different hepatocyte acute phase proteins proved fruitful in identifying HSF and its extent of action on the hepatocyte acute phase response.

REVIEW OF LITERATURE

The Acute Phase Response of The Liver

The Acute Phase Proteins

Protein production is perhaps the most studied of the liver metabolic alterations observed in the acute phase response. Liver output of several proteins during the acute phase response represents a major change in gene regulation and protein synthesis by hepatocytes. These serum proteins have been grouped together and termed acute phase reactants or more commonly, acute phase proteins (APP) (Kushner, 1982; Koj, 1974; Koj, 1985). Table 1 presents APP that occur in various species and their relative changes during the acute phase response. C-reactive protein (CRP) and serum amyloid A (SAA) are two APP that increase markedly (100 to 1000 fold) in serum concentration upon inflammation in man. α_2 -macroglobulin (α_2 -macro) in rats and SAA in mice also undergo substantial increases upon inflammation. Other acute phase proteins increase less markedly (2 to 10 fold) in serum such as, in rats, haptoglobin (HP), hemopexin, fibrinogen and α_1 -acid glycoprotein (α_1 -AGP) also referred to as orosomucoid. Another serum protein in rats, α_1 -cysteine protease inhibitor (α_1 -CPI) also previously called α_1 -acute phase globulin or Major Acute Phase protein, displays 8-10 fold increases. In man, 2-10 fold increases in α_1 -AGP, fibrinogen, HP, α_1 -antichymotrypsin (α_1 -ACH) and α_1 -

antitrypsin (α_1 -AT)[also been termed α_1 -protease inhibitor] have been observed. Mice show considerable increases in serum amyloid P component (SAP). Ceruloplasmin and the third component of complement (C3) have shown 1 to 2 fold increases in serum concentrations in man and mice. Plasma concentrations of protein will depend upon rate of synthesis and secretion, distribution in the vascular and extravascular space, rate of catabolism and rate of utilization. Furthermore, the kinetics of protein levels may depend on the particular type of injury or disease, or even on previous inflammatory state (Macintyre, 1982). Thus the acute phase proteins represent a heterogeneous group with variable kinetics and changes in magnitude.

Other liver derived proteins show a fall in serum concentrations such as albumin and transferrin in most species and α_2 -HS glycoprotein in humans (Koj, 1985b; Lebreton et al., 1979). As suggested by Lebreton and co-workers (Lebreton et al., 1979) these are now generally referred to as negative acute phase proteins.

TABLE 1: Examples of Acute Phase Proteins

100 to 1000 fold increases

	Species	Reference
C-Reactive Protein (CRP)	man, rabbit	Kushner <u>et al.</u> , 1981
Serum Amyloid A (SAA)	man, mouse	McAdam <u>et al.</u> , 1978 Sipe <u>et al.</u> , 1982
α_2 -macroglobulin (α_2 -macro)	rat	Van Gool <u>et al.</u> , 1974, 1984

2 to 10 fold increases

α_1 -cysteine protease inhibitor (α_1 -CPI) or Major Acute Phase protein or α_1 -acute phase globulin	rat	Schreiber <u>et al.</u> , 1982 Schreiber <u>et al.</u> , 1986
α_1 -acid glycoprotein (α_1 -AGP) or orosomucoid	man, rat	Aronson <u>et al.</u> , 1972 Jamieson <u>et al.</u> , 1983
fibrinogen	rat, mouse, rabbit, man	Koj, 1985 Fuller <u>et al.</u> , 1982
haptoglobin (HP)	man, rat	Aronson <u>et al.</u> , 1972
hemopexin	rat, mouse	Baumann <u>et al.</u> , 1987c
α_1 -antichymotrypsin (α_1 -ACH)	man	Aronson <u>et al.</u> , 1972
α_1 -antitrypsin (α_1 -AT) or α_1 -protease inhibitor	man	Aronson <u>et al.</u> , 1972
Serum amyloid P (SAP)	mouse	Le <u>et al.</u> , 1982 Mortensen <u>et al.</u> , 1983

1 to 2 fold increases

ceruloplasmin	man, rabbit, rat	Koj, 1974
C3	man, mouse	Pepys <u>et al.</u> , 1983

decrease

albumin	man, rabbit, rat,	Billingham <u>et al.</u> , 1976
transferrin	rat, man	Koj, 1974; 1984

Functions of Acute Phase Proteins

Many disease states in humans have been documented as being associated with increased acute phase protein concentrations in serum. As already mentioned, typical post-operative sera show elevated concentrations of CRP, SAA, α_1 -AT and α_1 -AGP (Aronson et al., 1972; Fleck & Meyers, 1982; Crockson et al., 1966). Increases in APP are also seen in clinically evident bacterial infections (Mackie et al., 1979) as well as various cancers depending on the various stages and extent of metastases (Cooper & Stone, 1979). Some increases could be due to superimposed bacterial infections (Kushner, 1987). Animals undergoing surgery (Van Gool et al., 1984), experimental bacterial infections (Baltz et al., 1982) or parasite infections (Gauldie et al., 1985; Stadnyk et al., 1987) all show increases in serum APP. Although the presence of APP is evident, the functions these molecules serve in vivo are not all easily apparent, or in some cases not known at all (for example, SAA).

The acute phase proteins have been shown to possess a variety of activities. These include some actions that appear to aid the host response to tissue damage such as inhibition of proteinases, modulation of the blood clotting and fibrinolysis pathways, binding and removing foreign particles or toxic substances and modulation of immune responses.

Tissue destruction and cellular death leads to the release of neutral proteinases (such as collagenase, elastase, cathepsin G) from phagocytic cells which digest and remove necrotic tissue. These may also activate zymogens involved in the clotting, complement or kinin-forming cascades. The concept that the actions of the proteases are held in check from causing untoward damage by serum protease inhibitors, most of which are acute phase proteins, helps explain the pathogenesis of some diseases. The major serum inhibitors in humans are α_1 -antitrypsin (α_1 -AT or α_1 -protease inhibitor) which constitutes 70% of plasma antitrypsin activity, α_1 -antichymotrypsin (α_1 -ACH) and α_2 -macroglobulin. In vitro, α_2 -macroglobulin (725,000 daltons) has the broadest activity inhibiting all serine proteases and some cysteine and metallothioneine proteases. α_1 -AT, a 54,000 dalton molecule, has a more limited specificity to leukocyte elastase. α_1 -Antichymotrypsin (α_1 -ACH) with a molecular weight of 68,000, has specificity toward cathepsin G and chymotrypsin (Travis et al., 1978; Koj, 1985). α_1 -Macroglobulin is large in size and therefore shows less penetration into tissue fluids (from blood) than the smaller α_1 -AT or α_1 -ACH molecules. In rats, α_2 -macroglobulin and α_1 -cysteine protease inhibitor (α_1 -CPI) are both strong acute phase proteins. The serum usually contains excess inhibitory capacity but flooding the system such as in acute pancreatitis (characterized by the release

of pancreatic enzymes into the bloodstream and peritoneal cavity) with excess proteases has been postulated as a cause of the observed systemic effects (Goodman et al., 1986). Deficiency in α_1 -AT is associated with an imbalance of protease-antiprotease interaction in lung tissue. In individuals with hereditary α_1 -AT deficiency, there is a high incidence of early onset emphysema (Sharp, 1971; Carrell et al., 1982; Courtney et al., 1984). Shapira et al. (1977) has shown an altered interaction between trypsin and α_2 -macroglobulin purified from 3 patients with cystic fibrosis compared to α_2 -macro purified from normals. This alteration of α_2 -macroglobulin function may be involved in some manifestations of the disease. These are several examples suggesting the importance of the protease-inhibitors in maintaining a homeostatic balance with proteases.

Some acute phase proteins play roles in blood clotting and fibrinolysis. Cleavage of fibrinogen to fibrin is a central element in formation of a stable platelet plug upon transection of blood vessels. The consolidated plug becomes mostly fibrin, and later, fibrinolytic enzymes digest and remove the plug. Thus the increase in fibrinogen output by liver during the acute phase response would enable greater clotting potential in response to tissue injury. α_1 -AGP has been found to have inhibitory activity in vitro of ADP-induced platelet aggregation (Snyder & Coodley, 1976)

at concentrations (2 mg/ml) that may be reached during the acute phase response (Costello et al., 1979). On the other hand, Anderson et al. (1980) showed an antiheparin effect of α_1 -AGP in vitro. Thus the net effects on the clotting cascade by α_1 -AGP in vitro are difficult to assess. Antiplasmin or α_2 -plasmin inhibitor, a positive acute phase protein in humans (Koj et al., 1985) appears to play a role in vivo in fibrinolysis. Antiplasmin deficiency is associated with severe bleeding disorders (Aoki et al., 1979) that are well managed clinically with replacement therapy.

C-reactive protein (CRP) was named for its ability to precipitate C-polysaccharide derived from the cell wall of Pneumococcus. Its action in binding to bacteria and bacterial products facilitates their removal by the reticuloendothelial system. Kindmark (1972) has shown that CRP binds specifically to several pathogenic strains of bacteria and stimulates the phagocytosis of these (such as Diplococcus pneumoniae, Staphylococcus aureus, Klebsiella aerogenes) by peripheral blood leukocytes in vitro (Kindmark, 1971). Yother et al. (1982) have shown that human CRP could protect mice in vivo (80% survival versus 30% control survival) from mortality due to Streptococcus pneumoniae infection. Thus CRP appears to play a vital role in host resistance to some natural bacterial infections.

α_1 -Acid glycoprotein in addition to affecting

platelet function, may also have other activities against pathogens. Friedman (1983) found that α_1 -AGP, in concentrations similar to those found in inflammation, caused an 80% inhibition of in vitro multiplication of Plasmodia folciparum (malaria). α_1 -AGP apparently acted by blocking parasite-red blood cell interaction in vitro but had no effect on maturation of the parasite. α_1 -Antitrypsin or α_2 -macroglobulin did not show this activity. α_1 -AGP, a highly glycosylated APP, may modulate infection by various agents that recognize sialic acid containing cell surface components such as influenza virus, measles virus or herpes-virus due to inhibition of binding to cells. This remains to be examined experimentally.

Other APP have been shown to possess anti-inflammatory action. α_2 -macroglobulin inhibited carrageenin induced rat paw oedema (Van Gool et al., 1974) and has also been shown to inhibit polymorphonuclear leukocyte chemotaxis (Van Gool, et al., 1982). Ceruloplasmin has been shown to inhibit superoxide anion dependant enzymatic reactions (Goldstein et al., 1982). These APP and others may modulate the acute phase response at the local level with respect to permeability changes, free radical activity, and recruitment of neutrophils.

Acute phase proteins have also exhibited immuno-modulating effects. In addition to inhibiting proteolytic enzymes such as elastase, trypsin and some cathepsins, α_1 -

antitrypsin inhibited human mononuclear cell proliferative responses to lectins such as phytohaemagglutinin and concanavalin A (Briet et al., 1983). α_1 -AT also inhibited plaque-forming cell responses in vitro and in vivo in spleens of mice (Arora et al., 1978). Furthermore Hudig et al. (1981) saw decreases in in vitro human natural killer cell activity due to α_1 -AT or α_1 -antichymotrypsin. In addition, Gravagra et al. (1982) showed similar results of α_1 -ACH effect on human natural killer cell and antibody dependant cellular cytotoxicity assays, as did Adés et al. (1982) with α_2 -macroglobulin and α_1 -AT. Murine SAA was found to inhibit plaque forming cell responses in mice (Bensón & Aldo-Benson, 1979) and Kinsella & Fritzler (1980) found that CRP, while capable of activating the complement pathway, also inhibited proliferation of human T cells and production of lymphokines.

Thus, the acute phase proteins represent a variety of molecules with a broad spectrum of activities. Acute phase proteins inhibit protease activity, inhibit free radical action, modulate clot formation and fibrinolysis, bind and facilitate removal of foreign particles, and appear to modulate antibody and cell mediated immune responses. These effects would lead to decreased inflammation at the local level and thus dampen destructive action and facilitate wound healing.

Models for the Acute Phase Response

Various systems have been employed in characterizing the acute phase response. Analysis in clinical disease or trauma due to surgery or burns has been useful. Experimental models for in vivo analysis involve injection of sterile chemical irritants, injection of endotoxins as well as inoculation with live pathogenic organisms. Turpentine injection (s.c.) (Kushner and Feldman, 1978; Jamieson et al., 1983; Schreiber et al., 1982), celite injection (Glibetic & Baumann, 1986), carrageenin injection (Van Gool et al., 1974) and casein injection in mice (McAdam & Sipe, 1976), calcium phosphate induced pleurisy in rats (Tissot et al., 1984), and etiocholanolone injection in humans (McAdam et al., 1978) are some examples of irritant induced models. The quality or quantity of the acute phase response is not necessarily equal among these. Thus, detailed comparisons of data between models and even between laboratories is often difficult. Other models utilize injection of certain amounts of endotoxin (Sipe et al., 1982) of which there is still variation due to the mode of preparation. Furthermore, variation in sensitivity to endotoxin is evident between strains and between species (for example, C3H/HeJ mice are lipopolysaccharide (LPS) non-responders in comparison to other strains, and rats are

relatively endotoxin-tolerant). In still other studies, infection of mice and rats with parasites (Lamontagne et al., 1985a; Lamontagne et al., 1985b; Gauldie et al., 1985a) and inoculation of mice with various bacterial strains (Balt et al., 1982b) have been used successfully in examining the acute phase response. The problems involved in variability and the obvious complexity in in vivo models has led to the development of in vitro assays for the acute phase response.

In vitro hepatocyte culture systems have been developed which have allowed further investigation of the liver's role in the acute phase response. Hepatocytes have been isolated and cultured from mouse liver (Baumann et al., 1983; Ramadori et al., 1985), rat liver (Rupp and Fuller, 1979; Koj et al., 1984; Koj et al., 1985; Bauer et al., 1984) and rabbit liver (Kushner et al., 1980). Rat liver slices have also been used to study liver response in vitro (Jamieson et al., 1983; Woloski et al., 1983). Although primary human liver cells are not readily available, human hepatoma cell lines have been characterized and used in the analysis of liver responses. Examples include the Hep-G2 cell line (Baumann et al., 1984 and 1987) and the Hep 3B2 cell line (Darlington et al., 1986). Rat hepatoma cell lines such as HTC and H-35 have also been used (Baumann et al., 1983; Vannice et al., 1984; Baumann et al., 1987).

Hepatocyte Synthesis of Acute Phase Proteins

A number of approaches have led to the conclusion that the changes in serum APP are driven by hepatocyte synthesis. The experiments of Gordon and Koj (1968), John and Miller (1969) and Hurlimann et al. (1966), were early demonstrations that the liver both in vivo and in vitro, was the source of various APP. Immunoprecipitation of de novo radiolabelled protein in serum and in rabbit liver culture supernatants, as well as evidence of production by perfused liver in vitro, and later, evidence of labelled amino acid uptake and incorporation into protein by liver, showed the important role of hepatocytes in this respect (Hurlimann et al., 1966; Gordon and Koj, 1968; John and Miller, 1969; Wannamacher et al., 1975). Although there is evidence of extra-hepatic synthesis α_1 -AT (Lamontagne et al., 1985b) by alveolar macrophages and monocytes, and α_1 -ACH by alveolar macrophages (Burnett et al., 1984), the contribution of such extra-hepatic synthesis to serum changes are probably minimal and overshadowed by the liver response. However, synthesis by the alveolar macrophage may result in local effects on surrounding cells or fluid containing mediators and enzymes (Lamontagne et al., 1985b).

Immunofluorescence and immunochemical techniques have confirmed hepatocyte synthesis of APP. Benson and Kleiner (1980) have shown a localization of SAA immuno-staining (in liver taken from casein inflamed mice) in periportal regions

at 8 hours but spreading over the entire liver lobule by 24 hours. Treatment with casein and cholchicine (inhibitor of secretion) resulted in higher liver staining for SAA and lower serum levels suggesting that the SAA synthesized by the liver contributes to serum changes. Gauldie et al. (1981) demonstrated α_1 -AT immunochemically in mouse liver. Kushner and Feldmann (1978) showed that the only source of liver CRP in rabbits undergoing turpentine inflammation was the hepatocyte rather than other structural and endothelial cells of liver. Ultrastructure studies demonstrated localization to rough and smooth endoplasmic reticulum and Golgi apparatus as expected for a secreted protein. Courtoy et al. (1981) showed that serial histological sections of turpentine inflamed rat liver stained for fibrinogen, α_1 -AGP, α_2 -macro and haptoglobin. They found that all hepatocytes examined secreted all four APP suggesting that individual hepatocytes are not specialized in the synthesis of a single plasma APP. Similar to others, a periportal distribution early after inflammatory stimulus, and more general distribution at 24 hours was observed. The total liver output of APP may therefore depend on the extent of individual hepatocyte activation and gene regulation, as well as recruitment of cells synthesizing these proteins.

Studies on mRNA levels in hepatocytes during inflammation confirm these results. Investigations in rats by Ricca et al. (1981) have demonstrated marginal increases

in α_1 -AGP mRNA at 4 hours but up to 90-fold increases at 36 hours after turpentine. Baumann et al. (1983(a)) found 14 fold increases in α_1 -AGP mRNA in rat liver 48 hours after turpentine injection. Schreiber et al. (1986) have examined mRNA increases over time for a number of APP in rats including α_1 -AGP, α_2 -macro, fibrinogen and α_1 -CPI. Ramadori et al. (1985) have found increases in mouse liver mRNA for SAA and SAP during inflammation. Northemann et al. (1983) measured in vitro translatable mRNA (in rabbit reticulocytes) from inflamed rat liver. Immunoprecipitation of specific proteins from the translated material showed markedly more α_1 -AGP, α_2 -macro, less of an increase in α_1 -AT and a drop in albumin. Furthermore, Stadnyk, Baumann and Gauldie (unpublished observations) have shown increases in rat liver RNA for several APP during infection with Nippostrongylus brasiliensis. Taken together, these studies demonstrate the production of APP is predominantly by hepatocytes and leads to the serum changes. The increases in synthesis are associated with alterations of mRNA content in hepatocytes. The increases may be due to increased transcription or increased stability of mRNA.

Initiation of The Acute Phase Response

Considering the different modes of communication possible within an organism, initiation of APP induction could result from direct stimulation of liver by inflammatory agents, or by host-mediated signals. These signals could directly or indirectly originate from the inflamed tissue and travel via electrical conduction (nerves) or as soluble mediators through the blood stream. Two lines of evidence suggest that host-derived humoral products initiate the acute phase response. One is that passive transfer of sterile plasma or plasma proteins (from affected animals) can stimulate aspects of the acute phase response. For example, Homburger (1945) found that pus obtained from turpentine-induced sterile abscesses of dogs could increase plasma fibrinogen levels of normal dogs upon i.m. injection. The authors found that the responsible agent was heat labile and acid precipitable suggesting it was proteinaceous in character. The other line of evidence is supported by experiments showing that in vitro generated products of host cells, upon transfer to a recipient, are capable of inducing components of the acute phase response. More recently, the identities of some of these soluble mediators have become evident and include (addressed in more detail below) Interleukin-1 (IL-1), hepatocyte stimulating

factor (HSF), tumour necrosis factor (TNF) and glucocorticoids. These hormones or cytokines, as suggested by Koj (1974), are thought to be released at the site of or due to events of local inflammation, travel through blood or lymph, and act on hepatocytes directly through putative receptors, or act on other systems which in turn affect the acute phase response.

Cytokines and The Acute Phase Response

The early work of Homburger (1945) did not receive much attention. Later, Bennet and Beeson (1953) found that extracts and supernatant fluids from exudate cells of sterile peritonitis could induce fever in normal rabbits. They introduced the term Leukocytic Pyrogen (LP) to describe the product of leukocytes responsible for this activity. Over the next 25 years, rabbit LP was studied in many laboratories but it wasn't until Kampschmidt and colleagues found an effect of LP on serum concentrations of iron and zinc, that LP was thought of as more than a pyrogen. It must be considered that most of these studies were done with crude preparations which could contain other factors. Kampschmidt and co-workers found that crude LP induced decreases in serum Fe and Zn (Kampschmidt et al., 1970) and later in rats showed increases in acute phase globulins and increased neutrophilia (Eddington et al., 1971). Looking

further, injection of both crude LP and E. coli derived endotoxin in rats gave Kampschmidt et al. (1974) evidence of increased fibrinogen and haptoglobin in serum and furthermore increased fibrinogen synthesis in rat liver slices in vitro. Similar findings by Wannemacher et al. (1975) prompted the term leukocyte endogenous mediator (LEM) to indicate the variety of activities present in leukocyte supernatant preparations.

Merriman et al. (1977) published work showing co-purification and similar characteristics (molecular size, isoelectric point, kinetics of induction) of LP and LEM. Similar results of LP/LEM induced stimulation of CRP, fibrinogen and haptoglobin were observed in rabbit serum (Bornstein, 1982). Hanson et al. (1980) have shown that neutrophils were not a strong source of LP/LEM and that macrophages were responsible for production of LP/LEM in leukocyte cultures. It soon became evident that lymphocyte activating factor (LAF), first described by Igal Gery and coworkers (Gery et al., 1972a, 1972b) as a macrophage derived product that potentiated mitogen stimulation T lymphocytes, also showed similar characteristics to LP/LEM. The term Interleukin-1 (IL-1) was ascribed to the molecule(s) that showed LP/LEM/LAF activities (Aarden et al., 1979). Thus, the molecule IL-1, a 15-20 kilodalton molecule (LEM, LP, LAF) appears to play a role in the induction of the acute phase response of the liver.

Another activity first described by Ritchie and Fuller (1981) and by then by Baumann et al. (1983) and Koj et al. (1984) was termed hepatocyte stimulating factor (HSF) on the basis of its ability to stimulate hepatocyte protein synthesis in vitro. Therefore, there are at least 2 cytokines, HSF and IL-1 that have been implicated in the initiation of the liver APP response. Both HSF and IL-1 are released by activated monocyte/macrophages. These cells apparently serve as a primary source of APP inducing cytokines.

Interleukin-1 and The Acute Phase Response

Purification of IL-1 to homogeneity has revealed molecular weights of between 13-23 kilodaltons and both acidic (pI 5) and neutral (pI 7) isoelectric points (Dinarello, 1984). Molecular cloning studies have confirmed the existence of 2 species of IL-1, one of isoelectric point pI=5, the other of pI=7, from human peripheral blood monocytes (PBM). Auron et al. (1984) published the cDNA sequence of the pI=7 IL-1 encoding a 31 kilodalton (kd) precursor protein. Lomedico et al. (1985) simultaneously characterized a cDNA sequence from the mouse macrophage cell line P388-D1 that encoded a 31 kd precursor of a 17 kd pI=5 extracellular protein. Further studies by March et al. (1986), Gubler et al. (1986) and Wingfield et al. (1986)

identified both human pI=5 and pI=7 cDNA sequences. These are now termed IL-1 α and IL-1 β respectively and show 45% homology at the nucleic acid level. The corresponding mouse IL-1 β (pI=7) has recently been cloned by Gray et al. (1986).

Activities now ascribed to IL-1, in addition to LAF, EP or LEM activities, are numerous. These have been addressed in detail in a number of recent reviews (Dinarello, 1984b; Oppenheim et al., 1986; Le and Vilcek, 1987). Among many interesting activities and actions with possible clinical implications are effects on proliferation of endothelial cells, synovial cells, inhibition or stimulation of fibroblasts and activation of osteoclasts and cartilage resorption. IL-1 has effects on many cells of the immune system in vitro including NK cells, T cells, B cells and neutrophils. Furthermore, many different cell types show ability to generate IL-1 including monocytes/macrophages, endothelial cells, astrocytes, epithelial cells, B lymphoblasts, keratinocytes and a number of continuous cell lines. The wide range of activities of IL-1 may reflect possession of similar receptors for IL-1 on different cell types. Human IL-1 α and β apparently bind to the same receptor on human VDS-O cells (Matsushima et al., 1986a) and mouse EL-4 cells (Kilian et al., 1986) which supports this idea. The presence of IL-1 receptors correlates with responsiveness to IL-1 (Le and Vilcek, 1987).

As already mentioned, most early studies with LP/LEM/IL-1 utilized crude or only partially purified cell supernatants. These showed in vivo activity but investigators could not rule out the role of contaminating protein nor an indirect effect on liver (for example, induction of a second hepatocyte specific messenger). Furthermore, contamination of injected material with bacterial endotoxin cannot be easily controlled. Only more recently has IL-1 been rendered relatively pure. Upon injection into mice purified IL-1 causes SAA serum increases (Sztein et al., 1981; Sipe et al., 1982). These effects complemented earlier studies (Sipe et al., 1979) in which endotoxin injection caused SAA increases in a LPS-sensitive mouse strain (C3H/HeN) but not a non-responsive strain (C3H/HeJ). Transfer of plasma proteins of stimulated mice to non-responder mice caused an SAA response. This suggested a soluble mediator (then termed SAA-inducer) was responsible which subsequently was found to be closely related to IL-1 (Sztein et al., 1981). Later experiments by Matsushima et al. (1985), and Wood et al. (1985) confirmed that highly purified IL-1 pI=5 and pI=7 forms could induce SAA responses in both strains of mice. Recent experiments with recombinant human IL-1 have confirmed many bioactivities ascribed to natural IL-1 such as fever induction, Interleukin-2 production, LAF activity and fibroblast prostaglandin E₂ production (Dinarello et al.,

1986). Recombinant murine IL-1 injection in vivo resulted in serum SAA response as well as increased SAA mRNA (Ramadori et al., 1985).

Assay of IL-1 on isolated hepatocyte in vitro cultures displays a limited capacity by the cytokine to stimulate APP production when compared to crude PBM supernatant. Purified human IL-1 induced rat α_1 -AGP (Gauldie et al., 1985) but not a α_2 -macroglobulin or α_1 -CPI (which are typical APP of rats in vivo). In contrast, Bauer et al. (1985) suggested stimulation rat α_2 -macroglobulin by murine recombinant IL-1. Ramadori et al. (1985) showed a simultaneous increase in SAA mRNA and decrease in albumin mRNA in mouse hepatocyte cultures. Gauldie et al. (1987) have recently confirmed using recombinant material that human IL-1 will stimulate α_1 -AGP but has little effect on α_2 -macroglobulin or α_1 -CPI in rat hepatocyte cultures.

In the human hepatoma cell line Hep-3B2, Darlington et al. (1986) found stimulation of C3 by purified human IL-1 and recombinant murine IL-1 α but no stimulation of fibrinogen or CRP (even at high doses) whereas human PBM conditioned medium showed potent induction of CRP. Perlmutter et al. (1986a) found stimulation of C3 and factor B mRNA in human Hep-G2 cells and Hep-3B cells by recombinant IL-1. Thus IL-1 alone may not be able to elicit the full spectrum of APP response by hepatocytes in vitro. Other factors appear to be necessary. If the in vitro hepatocyte

assays reflect in vivo hepatocyte responses accurately, IL-1 may act directly to induce SAA in mice and α_1 -AGP in rats but probably acts indirectly or in combination with other components for expression of other acute phase proteins in vivo.

Hepatocyte Stimulating Factor and The Acute Phase Response

Although Kampschmidt et al. (1974) described one activity of LEM as stimulating fibrinogen in rat liver slices, it was Fuller's group who coined the name hepatocyte stimulating factor (HSF) to describe a protein from leukocytes that stimulated in vitro rat hepatocyte synthesis of fibrinogen (Ritchie and Fuller, 1981; Fuller and Ritchie, 1982, Ritchie and Fuller, 1983). They used ELISA analysis of fibrinogen in fetal and adult hepatocyte supernatants to study crude leukocyte LP/LEM. In examining further, Fuller and Ritchie (1982) found that monocytes were the source of HSF and that HSF eluted at 30 kilodaltons (kd) on gel chromatography (Ritchie and Fuller, 1983). This was a preliminary indication that HSF was a separate entity from IL-1/LEM/LAF which eluted primarily in the 15-20 kd range. Furthermore, they tested purified IL-1 pI=7 and found no stimulation of fibrinogen synthesis.

Elsewhere, Koj, Gauldie and collaborators developed a similar in vitro rat hepatocyte assay of stimulation of a

more complete set of proteins including α_2 -macroglobulin, α_1 -macroglobulin, fibrinogen, α_1 -AGP, α_1 -CPI, albumin and transferrin (Koj et al, 1984; Koj et al, 1985a). Analysis of the molecular size of PBM derived HSF activity confirmed studies by Fuller in that a PBM cytokine of approximately 30 kd and pI=5-5.2 stimulated fibrinogen maximally and in addition, stimulated α_1 -CPI and α_2 -macroglobulin, and inhibited albumin and transferrin synthesis. Similar results for α_2 -macroglobulin were seen by Bauer et al. (1984).

In the purification of HSF, the specific bioactivity of IL-1, did not follow that of HSF (Koj et al., 1984; Woloski and Fuller, 1985) and further, the mouse macrophage cell line P388-D1 secreted HSF and IL-1 differentially under various stimulation regimes. In a rat model of macrophage activation by in vivo infection with Nippostrongylus brasiliensis, Gauldie et al. (1985a) and Lamontagne et al. (1985a) showed differential release of IL-1 and HSF by macrophages at separate days after infection. In a model of bleomycin-induced rat lung fibrosis, Jordana et al. (1987) found that alveolar macrophages, retrieved at various times after bleomycin installation, secreted LAF activity predominantly early (6-8 hours) but released HSF in greater amounts at later times (maximum 28 days). Although these data suggest that HSF is unique from IL-1/LEM, the results were not definitive. For example, the relationship of HSF

to the precursor IL-1 α (precursor 31 kd pI=5) or TNF (precursor 25 kd, 17 kd pI=5.3 mature protein) was not clear. Various groups are involved in purification of HSF but to date, homogeneous preparations of HSF from human PBM had not been available.

Hepatocyte stimulating factors have been detected from sources other than human PBM including mouse peritoneal exudate macrophages (Baumann et al., 1983; Baumann et al., 1984), rat alveolar macrophages (Richards et al., 1985; Lamontagne et al., 1985a) and cell lines such as P388-D1 (a mouse histiocytic leukemia line) and U937 (a human monocytic leukemia line) (Woloski and Fuller, 1985). Isolated Kupffer cells from rat liver have also yielded factors stimulating synthesis of fibrinogen (Ritchie and Fuller, 1983) and α_2 -macroglobulin (Bauer et al., 1984) of rat hepatocytes. Baumann et al. (1984) have shown HSF activity from human epidermal cells. The relationship of each of these molecules to PBM derived HSF is not yet evident.

The human squamous carcinoma cell line COLO-16 releases a number of HSFs which have been studied in detail by Baumann and colleagues (Baumann et al. 1983; 1984; 1986a). Three forms of COLO-16 HSF (designated HSF-I, II and III) were characterized and showed little LAF activity. The activities were distinct in molecular size (Mr of 17, 30 and 50 Kd). The relationship between these COLO-16 HSFs,

mouse peritoneal macrophage HSF (Mr of 50 Kd) and the HSF from human PBM is not known. For the purposes of this composition, the source of HSF is henceforth included as a prefix and HSF will be defined as cytokines that induce the synthesis of a spectrum of APP that is characteristic of the acute phase response.

> Since highly purified HSF is not available in large amounts, studies of in vivo activity of this cytokine have not been published. Assuming that PBM HSF is unique, a large part of the activity seen in crude or partially purified LEM/IL-1 preps may have been due to HSF. Another possibility is that HSF production by host cells (such as macrophages or Kupffer cells) is stimulated by injection of IL-1 in vivo.

Purified preparations of human PBM HSF have shown inducing activity of sialyltransferase enzymes in rat liver (Woloski et al., 1986). Interestingly PBM HSF has also been shown to stimulate in vitro production of adrenocorticotrophic hormone (ACTH) from the mouse pituitary cell line AT t-20 (Woloski et al., 1985 (b)) whereas cloned murine IL-1 was not as effective. In light of the characteristic increase in serum cortisol during inflammation in rats (Woloski et al., 1983; Van Gool et al., 1984) and humans (Fleck and Myers, 1985), HSF and possibly IL-1 may affect pituitary ACTH production in vivo. Certainly, the identification and characterization of the various HSFs described would aid the

study of the acute phase protein response of liver and glucocorticoid increases during the acute phase response.

Glucocorticoids and The Acute Phase Response

Heim and Ellenson (1965) found that adrenalectomy strongly inhibited the serum rise of α_2 -macroglobulin in rats injected with endotoxin, and that corticosterone injection could reconstitute the response. Furthermore, there is a rise in corticosteroid hormones during the acute phase response (evident 3 to 8 hours after stimulus) in rats (Van Gool et al., 1984) and humans undergoing surgery (Fleck & Meyers, 1982). This suggested a role for glucocorticoids in the liver APP response. Szafarczyk et al. (1974) showed that serum haptoglobin increases in turpentine inflamed rats depended on the time (in circadian rhythm) of injection, and suggested that the highest haptoglobin response was present when the adrenal was maximally reactive. Thompson et al. (1976) showed that adrenalectomized and hypophysectomized rats did not respond to crude LEM with increases in serum α_2 -macroglobulin, although SHAM operated animals did. A supplement of 5 mg/day of cortisol restored the response. In vitro perfused liver showed increases in RNA content only to combinations of crude LEM and cortisol. In another in vivo study, Van Gool et al. (1984) found that orally administered corticosterone, cortisol and dexamethasone (the

most potent), stimulated α_2 -macroglobulin to serum levels seen in surgical trauma. Interestingly, s.c. administration of adrenalin also caused a similar response. Combination of catecholamine with corticosterone resulted in very high levels of α_2 -macroglobulin. Although conclusions regarding direct action of either of these hormones could not be drawn from these experiments, Koj et al. (1984) and Bauer et al. (1986) have shown that adrenalin had no effect on rat hepatocytes in vitro. This suggests that the effects of adrenalin s.c. seen by Van Gool et al. (1984) involved other factors acting on liver.

Baumann et al. (1983a) have examined the influence of dexamethasone on α_1 -AGP levels in rats in vivo and found expression of α_1 -AGP mRNA in liver of turpentine inflamed rats was augmented by dexamethasone (DEX) treatment. Other factors were clearly involved since α_1 -AGP mRNA was evident even in adrenalectomized inflamed rats. In contrast, α_2 -macroglobulin was dependant on the presence of both glucocorticoids (either in normal rats or adrenalectomy and DEX treatment) and the inflammatory stimulus. On the other hand, Moshage et al. (1985) saw no changes in albumin mRNA in vivo or in vitro due to DEX.

The effect of glucocorticoids in vitro on hepatocyte APP production has been examined mostly in rats. Gross et al. (1984) have shown that DEX at concentrations of 10^{-8} to 10^{-7} M resulted in stimulation of α_2 -macroglobulin in rat

hepatocyte cultures. α_1 -AGP production was seen in the absence of DEX and along with α_1 -AT, was stimulated marginally by DEX (10^{-7} M). Albumin production was unchanged. Hoffman et al. (1986) found enhancement of α_1 -AT but not α_2 -macroglobulin by macrophage conditioned supernatants in DEX-free media. Koj et al. found that DEX at $1 \mu\text{M}$ was necessary for maximum α_2 -macro synthesis by stimulated rat hepatocytes in vitro. Along with the data of Baumann et al. (1983a), it thus appears that glucocorticoid is necessary for expression of α_2 -macroglobulin and is stimulatory but not necessary for other APP in rats (α_1 -AGP, α_1 -AT). In contrast, mouse hepatocyte cultures were not affected by DEX in production of α_1 -AGP, β -haptoglobin, SAA or hemopexin (Baumann et al., 1983b). Thus, species differences and differences between some APP are evident in glucocorticoid mediated regulation of protein production. Furthermore, the in vivo significance of DEX must be considered since the predominant natural molecule in rats is corticosterone, not DEX.

Some investigators have examined the effect of other hormones on liver protein synthesis. Neither glucagon ($0.1 \mu\text{M}$) nor adrenalin ($10 \mu\text{M}$) resulted in altered protein synthesis of α_2 -macroglobulin, α_1 -AGP or fibrinogen by rat hepatocytes in vitro (Koj et al., 1984; Bauer et al., 1986). Specific effects of insulin on acute phase protein induction have not been reported, and adrenalin and glucagon appear

not to have direct effects in altering hepatocyte acute phase protein synthesis.

Tumour Necrosis Factor and The Acute Phase Response

Tumour Necrosis Factor (TNF) was first described by Carswell et al. (1975) as a serum component of BCG-primed, LPS stimulated mice that produced haemorrhagic necrosis of LPS-sensitive mouse sarcomas. In addition, this serum was cytotoxic for mouse L cells (transformed fibroblasts). These have since become standard assays of TNF activity (Old, 1985). The characterization and purification of this monocyte-derived factor resulted ultimately in molecular cloning of TNF by various groups (Aggarwal et al., 1984; Gray et al., 1984; Wang et al., 1985). Human TNF is a 17 kd pI=5.3 ~~p~~rotein derived from a 25 kd precursor. TNF shows functional and structural similarity to lymphotoxin (LT) (Pennica et al., 1984). LT (produced by activated lymphocytes) is thought to play a role in lymphocyte mediated killing. Cloned LT is cytotoxic for L cells and causes haemorrhagic necrosis in TNF assays. TNF α and TNF β designations have been given to the monocyte-derived TNF and lymphocyte-derived LT respectively.

Elsewhere, Beutler and colleagues (Beutler et al., 1985a) published work showing very high homology of murine cachectin to human TNF. Cachectin was characterized as a

macrophage-derived product, responsible for wasting and high lipidemia in LPS-injected mice. It also inhibited lipoprotein lipase activity of 'the fat cell line 3T3-L1 in vitro. Purification and cloning of cachectin has confirmed the identity of human cachectin and human TNF α (Beutler et al., 1985(b); Beutler et al., 1986).

In a recent review, Le and Vilcek (1987) point out the extensive similarities of activities between TNF α and IL-1 with regard to cachectin activity, T cell activation, pyrogenic action, osteoclast activation, cartilage resorption, cytotoxicity for tumour cells and effects on endothelial cells. The receptors for TNF and IL-1 appear to be different since TNF does not compete with binding of labelled IL-1 to IL-1 receptors (Matsushima et al., 1986) and IL-1 does not inhibit TNF binding to 3T3-L1 cells (Beutler et al., 1985(b); Kull et al., 1985). The sharing of so many activities is surprising since different receptors are utilized by TNF and IL-1. TNF and IL-1 may utilize similar intracellular second messenger systems which may explain this. It is also possible that TNF and IL-1 induce expression of each other in the target cells.

One might predict then that the effect of TNF on hepatocyte acute phase protein expression is similar to IL-1. However, little information regarding TNF and APP induction is yet available. Perlmutter et al. (1986a) found that recombinant human TNF α and IL-1 β could stimulate

immunoprecipitable Factor B, C3, ACH and (marginally) AGP in human Hep-G2 and Hep-3B cell lines although the increases were not impressive (1.5-2 fold) in that assay system. TNF and IL-1 could also inhibit albumin and transferrin production 2 to 3 fold, but did not affect α_1 -AT, C2 or C4 in Hep-G2 cells. Neither TNF nor IL-1 gene expression not detectable in Hep-G2 cells (Perlmutter et al., 1986a) and thus the affect of each cytokine appeared to be independent of the expression of IL-1 or TNF in the target cells. Darlington et al. (1986) showed recombinant TNF and IL-1 stimulated release of C3 (10 fold) in the Hep-3B2 cell line but unlike IL-1, TNF did not inhibit fibrinogen or albumin synthesis. Crude PBM supernatant was able to stimulate CRP synthesis (albeit very minor) in this cell line where as neither TNF or IL-1 could enhance CRP. This suggested that some other factor (not IL-1) in PBM supernatant (which contains IL-1, TNF and HSF and possibly others) was responsible for CRP induction. Furthermore, in experiments on primary rat hepatocytes, Koj et al. (1987) have recently shown that over a wide range of concentrations, human recombinant IL-1 α , IL-1 β and TNF α induced only slight increases in α_1 -AGP and decreases in albumin whereas crude human PBM supernatant caused much greater increases in fibrinogen and α_1 -CPI. Thus, although TNF and IL-1 stimulate some APP, there appears to be limited capacity of either of these cytokines to stimulate a wide spectrum of APP.

Regulation of Acute Phase Protein Gene Expression

Secretion of APP by hepatocytes occurs via transcription of RNA from DNA, processing of RNA to mature mRNA in the nucleus, mRNA transport to the cytoplasm, translation of protein from the mRNA by ribosomes, modification and glycosylation and finally secretion to the extracellular space. Regulation of gene expression could occur throughout any of these processes. The liver represents a readily obtainable organ that is predominantly composed of large numbers of its parenchymal cell, the hepatocyte. Accordingly, rat, mouse and rabbit livers have been used in various models of regulation of gene expression. Of investigators now studying this in detail at the molecular level, relatively few have examined the transcriptional/translational control of acute phase proteins by separate cytokines. This is due in part to the lack of purified materials.

Since glucocorticoids are available in high purity, and since they have effects on many tissues including liver, the modulation of liver gene expression by glucocorticoids has provided models for the molecular control of transcription. Glucocorticoids in general, interact with specific receptors and form complexes within the cytoplasm. These complexes move to the nucleus where they can

selectively alter transcription of specific genes (Yamamoto and Alberts, 1976).

Baumann et al. (1983a) have compared DEX-stimulated α_1 -AGP mRNA levels to mouse mammary tumour virus (MMTV) mRNA levels (with known glucocorticoid dependence) in a rat hepatoma HTC cell line. They found comparable increases in each mRNA and that the increase was inhibited by actinomycin D (inhibitor of transcription). Interestingly, Baumann and colleagues also found that puromycin inhibited mRNA accumulation of α_1 -AGP but not MMTV. Thus protein synthesis was required for the glucocorticoid mediated elevation of α_1 -AGP mRNA. Reinke and Feigelson (1984) showed similar effects. Upon cloning and transfecting the rat α_1 -AGP gene into mouse L cells, they found α_1 -AGP mRNA levels responsive to DEX and induction was inhibited by cycloheximide. These data suggested that protein expression was required for increases in transcription rate or increases α_1 -AGP mRNA stability. Kulkarni et al. (1985) showed evidence that DEX-treated rats and turpentine inflamed rats exhibited similar increased rates of α_1 -AGP mRNA transcription as measured by nuclear run-off assays. This suggested the effect of DEX may have been due solely to increases in transcriptional rates. Vannice et al. (1984) on the other hand, showed that in rat HTC cells in vitro, little difference existed in nuclear run-off experiments on DEX-treated and untreated cells but much lower nuclear and

cytoplasmic α_1 -AGP mRNA was evident in the untreated HTC cells. These authors concluded that DEX affected the nuclear stability of the α_1 -AGP mRNA transcript. Baumann & Maquat (1986b) have studied regions of the rat α_1 -AGP gene and found that regions between 120 and 42 base pairs upstream from the transcriptional start site were important for responsiveness to dexamethasone suggesting that glucocorticoids can increase α_1 -AGP gene expression at the transcriptional level.

Glucocorticoids may also play a role in post-transcriptional regulation of rat α_2 -globulin. Fulton et al. (1985) showed that upon dividing hepatocyte cellular RNA into nuclear, polysomal and cytosolic fractions, that adrenalectomized rats yielded high nuclear and low polysomal fraction content of α_2 -globulin mRNA (opposite to normal rats). Upon injection of DEX, within 2 hours a redistribution into predominantly the polysomal fraction was seen, suggesting that glucocorticoids were involved in regulating transport of RNA from nucleus to cytoplasm.

Various investigators have examined transcription parameters in vivo in inflamed animals (which of course may involve the net result of numerous cytokines and hormones). Ricca et al. (1981) showed a 90 fold increase in α_1 -AGP mRNA and Princen et al. (1981) found marked increases in fibrinogen mRNA as well as decreases in albumin mRNA in turpentine inflamed rats. Cole et al. (1985) showed that

rat α_1 -CPI mRNA levels paralleled changes in amount of α_1 -CPI in serum in inflamed rats. They suggested that the efficiency of translation was not affected but that transcription or mRNA stability was altered. Birch and Schreiber. (1986) have further measured transcription in nuclear run-off assays of inflamed rat hepatocyte nuclei. Changes in transcription matched well with mRNA levels found previously (Schreiber et al., 1986) for albumin α_1 -CPI, fibrinogen and transferrin. α_2 -macroglobulin on the other hand showed only 4 fold transcriptional increase but a 100-1000 fold increase in mRNA. This had been observed previously by Northemann et al. (1985), and suggested that post-transcriptional events were involved. α_1 -AGP transcription was not increased (Birch and Schreiber, 1986) which was in direct contrast to results of Kulkarni et al. (1985). This discrepancy was not addressed although there were differences in animal strains and dose of turpentine used. In other species, murine SAA mRNA has been found by Lowell et al. (1986) to be controlled transcriptionally and post-transcriptionally in LPS induced inflammation. SAA mRNA transcripts increased 2000 fold and in vitro nuclear run-off assays revealed approximately 300 fold increase in transcription. For SAA the authors suggested that rate of transcription as well as possibly mRNA stability was increased however low sensitivity of in vitro transcription assays could mask the real contribution of transcription.

Collectively, these data summarized, point to increased transcriptional rate of some acute phase proteins during the inflammatory response, and to increased mRNA stability or translation efficiency for other APP such as α_2 -macroglobulin. Furthermore, glucocorticoids have a stimulatory effect on α_1 -AGP (rat) mRNA levels and are necessary for rat α_2 -macroglobulin expression. Increases in mRNA stability may act in addition with increased transcription of particular APP-genes to further increase mRNA accumulation.

Perlmutter et al. (1986(b)) have examined the effect of recombinant IL-1 on expression of Factor B and C2 genes transfected into mouse L cells. IL-1 stimulated Factor B but not C2 even though the genes were closely linked. This is consistent with IL-1 stimulation of Factor B but not C2 or C4 in Hep-G2 cells and points to the probability of specific DNA regions necessary for response to IL-1. Transfection of an α_1 -AGP gene into mouse L cells has revealed that a 5 prime flanking region is important for responsiveness to glucocorticoids (Reinke and Fiegelson, 1984) suggesting the presence of specific DNA regions that are responsive to glucocorticoids in the α_1 -AGP gene.

The events that follow ligand-receptor interaction often include second messenger molecules that relay activating or deactivating signals within the cell. Included in various receptor-linked interactions are

modulation of cyclic AMP or cGMP; activation of protein kinase C and mobilization of intracellular Ca^{++} ions through phosphatidyl inositol metabolism. The liver in vitro provides a good system for examining these receptor-linked intracytoplasmic processes. Some investigators have examined intracellular events associated with various agents' actions on hepatocytes. However, the intracellular events involved in altered output of acute phase proteins has not been examined in detail. In one study, Wannemacher et al. (1975) found no evidence of altered cAMP levels in rat livers treated with crude or partially purified LEM (containing IL-1 and HSF). This suggested that APP induction by either of these molecules (IL-1, HSF) was not mediated by cAMP. With increased availability of purified and recombinant cytokines, the investigation of receptors, receptor-ligand interactions and the second messenger events that are involved in gene regulation should be more easily examined.

Purpose of this Study

The mechanisms involved in the control of the acute phase response of the liver are not fully defined however a number of cytokines and hormones appear to be involved in the stimulation of hepatocyte acute phase protein synthesis. The purpose of this study was to determine the molecular nature of the protein termed HSF derived from human peripheral blood monocytes and to determine the role of HSF

in stimulating the synthesis of acute phase proteins by hepatocyte cultures in vitro. The determination of such properties of HSF will aid the understanding of the interactions involved in cytokine control of the systemic response of acute ~~in~~ inflammation. Future studies of HSF activity in vivo may allow manipulation of models of acute inflammation that could serve as a basis for establishing clinical therapy.

MATERIALS AND METHODS

Analysis of sample protein content

To determine the amounts of albumin, α 2-macroglobulin, α 1-CPI and α 1-AGP in rat hepatocyte culture supernatants, or rat serum samples, rocket electrophoresis was performed as originally described by Weeke et al. (1973) with minor modifications. One gram of agarose (Seakem, Mandel Sci. Co., Rockwell, Ont.) was dissolved by boiling in 100 ml barbitol buffer [1.3% Na barbital (w/v), 0.2% barbitol (w/v), 5.6% glycine (w/v), 4.5% tris (w/v)]. After cooling to 65° C, monospecific antibody was added and 12 ml was poured onto 2" x 3" glass plates and allowed to harden. Wells were punched out, the plugs removed and 7-10 μ l of sample was added to each well. The plates were then placed in electrophoresis chambers with 1% agarose wicks overlapping the top and bottom of each plate and the reservoir (barbitol buffer) buffer. The gels were electrophoresed at 50 milliamps at 4 C for 8 to 12 hours. The gels were then soaked in saline for at least 2 hours, pressed onto cut sheets of gel-bond (FMC Bioproducts, Rockland, Maine, USA), dried, and then stained with Coomassie blue R250 (2% w/v in 40% ethanol (v/v), 10% acetic acid (v/v)) and destained. The peak heights (measured from the wells) reflect various amounts of protein in the sample. By running standard samples of known concentrations, the

concentration in unknown samples could be extrapolated from a standard curve. Hepatocyte culture protein output was expressed as ug per 10^6 cells per 24 hrs. For serum samples, concentrations (mg/ml) were calculated. Analysis of individual proteins was accomplished through the incorporation (into the 65° gel mix) of monospecific rabbit antisera to rat albumin (150 μ l), α 1-AGP (250 μ l), α 2-macroglobulin (80 μ l) and α 1-CPI (40 μ l) that had been prepared by established procedures (Koj et al., 1984).

Generation of Cytokines

Peripheral Blood Monocyte supernatant

Crude supernatants from human peripheral blood monocytes (PBM) were obtained in the following manner. Fifteen ml aliquots of heparinized human blood (Canadian Red Cross, Hamilton, Ont.) were diluted 1:2 in phosphate buffered saline (PBS) (0.3 M sodium phosphate, 0.85% w/v NaCl, pH 7.2). Twenty-two ml of 50% Hypaque solution was added to 80 ml of 7.55% w/v (in H₂O) Ficoll (Pharmacia, Upsala, Sweden) and then autoclaved. Ten ml of the ficoll-hypaque solution (buoyant density 1.075 grams/ml) was layered underneath the PBS-blood mixture in 50 ml sterile polypropylene conical tubes (Corning Glassworks, Corning, NY, USA) before centrifugation at 500 x g (at room temperature) for 20 minutes. The mononuclear cell layer (at the interface) was removed, collected and resuspended in,

PBS. The cells were centrifuged, washed 3 times in PBS and resuspended in RPMI medium (Central Resource Centre, McMaster University) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 ~~ug~~/ml streptomycin, 0.09 mM Na~~2~~ bicarbonate and 5% FCS v/v (Gibco, Grand Island, NY, USA) before counting in a hemocytometer. The cells were adjusted to 10×10^6 cells/ml and plated (10 ml/dish) into 100 x 15 mm petri-dishes (Corning). After incubation at 37°C, 6% CO₂, 100% relative humidity atmosphere for 2 hours, nonadherent cells were washed off thoroughly with warm (37°C) PBS leaving approximately 10×10^6 adherent cells/plate. Fresh complete RPMI with 10 ~~ug~~/ml Lippopolysaccharide (LPS) (TCA extracted, Sigma, St. Louis, MS, USA) was added before incubation for 18-20 hours at 37°C, 6% CO₂. The supernatants were then decanted, pushed through a 0.22 μ m filter (Millipore, Bedford, MA, USA) and dialyzed (using Spectopore tubing, molecular weight cut-off 6 to 8,000, A. Thomas Co., Philadelphia, PA, USA) for 48 hours against 2 changes of PBS. The PBM supernatants were then refiltered and frozen (-20°C) for future analysis. Some samples were concentrated using aquacide (Calbiochem, Behring Diagnostics, La Jolla, CA, USA) absorption of fluid through dialysis tubing and then redialysis. Other samples were dialyzed against 0.1 M ammonium acetate, frozen, lyophilized and reconstituted to 8-10 times the original concentration in PBS. Samples were titrated in the HSF

assay.

Fibroblast Supernatant

Primary fibroblast cell lines established from human lung explants of normal and fibrotic lung patients, have been grown and characterized in the laboratory of J. Gauldie (McMaster University, Hamilton, Ont.). Cytokines from normal and fibrotic cell lines were obtained from a 24 hour culture of fibroblasts. Fibroblasts were grown to confluency in 150 cm² flasks (Corning) in minimal essential medium (MEM) (Central Resource Centre, McMaster University) supplemented with 10% FCS and then lifted off the plate by trypsinization (0.05% w/v trypsin, 0.02% w/v EDTA, Gibco, Grand Island, NY, USA). The lifted cells were centrifuged, washed and resuspended in complete MEM, counted and seeded into 35 x 10 mm Corning petridishes at 250,000 cells/ml/dish. The cultures were incubated for 24 hours (37°C, 6% CO₂) to allow adherence after which they were washed thoroughly with PBS and replenished with complete MEM medium. The cells were incubated a further 24 hours and the crude fibroblast (FIB) supernatants were harvested and prepared as were PBM supernatants.

Rat Alveolar Macrophage Supernatant

Alveolar macrophages were obtained by broncho-alveolar lavage of Sprague-Dawley rats essentially as described by Brain and Frank (1968). Rats were given a lethal dose ($200 \mu\text{l}/100$ gram body weight) of Somnitol (MCT Pharmaceuticals, Mississauga, Ont.). The abdominal cavity was exposed and the aorta severed to exsanguinate the animal after which the diaphragm was punctured to collapse the lungs. The trachea was exposed and cannulated with tubing from a 21 gauge butterfly and secured with surgical thread. Ten volumes (5 ml) of warm (37°C) sterile PBS were carefully infused and withdrawn in sequence and collected in 50 ml tubes (Corning) on ice. The cells were centrifuged at $200 \times g$, 10 min at 4°C and washed twice before counting. The rat cells were then resuspended at 2×10^6 cells/ml in complete RPMI medium and plated (10 ml/dish) into 100 x 15 mm petridishes (Corning) and incubated at 37°C , 6% CO_2 for 2 hours. Nonadherent cells were washed off and fresh complete RPMI containing $10 \mu\text{g}/\text{ml}$ LPS was added. After a 24 hour further incubation, the supernatants were harvested and prepared as described for PBM supernatants.

Time Course of Release

Preparation of cells was as described above, and equal volumes of medium (complete RPMI with $10 \mu\text{g}/\text{ml}$ LPS for

PBM, complete MEM for FIB) were taken off and added again at 1 hour intervals. The supernatants were kept separate and analyzed for activity in the HSF or LAF assays.

Radiolabel of de novo protein synthesis

For production of de novo synthesized protein, PBM cultures were pulsed with ^{35}S -methionine in methionine-free medium and chased with complete media containing at least 1000 fold concentration of unlabelled methionine. Adherent PBM cultures were washed thoroughly with warm PBS, then replenished with medium 199 without methionine (Gibco), and supplemented with 150 $\mu\text{Ci}/\text{ml}$ of L- ^{35}S -methionine (1000-1200 Ci/mM, New England Nuclear, Boston, MA, USA) and LPS (10 $\mu\text{g}/\text{ml}$). After 4-6 hours (37°C , 6% CO_2) the 199 medium was replaced with complete medium. After a further 20 hours, (37°C , 6% CO_2) the supernatants were filtered, dialyzed, lyophilized and resuspended to 8 times concentration in PBS, aliquoted and stored at -70°C .

Hepatocyte Stimulating Factor (HSF) assay

Rat Hepatocyte Isolation

HSF activity was assayed by analysis of acute phase protein production in in vitro primary rat hepatocyte cultures according to previously established techniques (Koj et al., 1984; Koj et al., 1985). Isolation of hepatocytes was performed as described previously (Sweeney et al.,

1978) with minor modifications. Sprague-Dawley rats (150-200 grams from Charles River, Montreal, Que.) were anaesthetized with an i.p. injection of Somnitol at 150 μ l/100 gram body weight diluted in 1 ml PBS) and then 1 ml heparin (Hepalean 2500 units/ml, Organon, Toronto, Ont.) was injected i.p.. The abdomen was swabbed with alcohol, opened and the portal vein was exposed. Surgical thread was used to secure a cannula so that the tip was just barely protruding into the bifurcation to the liver lobes. The liver was perfused with 37° aerated Hank's BSS (Calcium and Magnesium free, Gibco, 0.46% w/v 4-(2-hydroxyethyl)-1-piperazin-athansulfonsaure [Hepes] buffered at pH 7.2, with 25 units/ml heparin) at a flow rate of 30-35 ml/min using a Fischer Scientific peristaltic pump. The aorta was quickly severed below the kidneys and pinched off several times to allow the lobes to fill with perfusate. The liver was then carefully cut away from surrounding tissue and at 10 min from start time, the perfusate was switched to aerated Hank's BSS containing 67 mg/100 ml CaCl_2 , 100 mg/100 ml bovine serum albumin, and 70 mg/100 ml collagenase (Sigma Chem. Co., St. Louis, MS, USA). Care was taken to use collagenase batches with at least 300 units/mg of collagenase, 100-200 units/mg of caseinase, and less than 0.03 units/mg of tryptic activity. The liver was transferred to the collagenase bath and digested for 10 minutes. The liver was removed and teased apart in a petri

dish on ice into William's E medium supplemented with 1 μ M dexamethasone (Hexadrol, Organon, Toronto, Ont.), 1 μ M insulin (Sigma), 100 units/ml penicillin and 100 μ g/ml streptomycin (Gibco), 50 μ g/ml gentamycin (Schering, Pointe Claire, Que.) 0.09 mM bicarbonate buffer, and 2 mM L-glutamine. The cells were filtered through a tissue screen into 50 ml conical tubes and allowed to settle for 15 min at unit gravity on ice. The supernatant was decanted and the cells washed twice in the complete William's E medium. Hepatocytes were then counted and resuspended in complete William's E to 5×10^5 cells/ml and plated at 2×10^5 cells/well in Nunclon T-24 well plates (NUNC, Intermed, Denmark) that had been precoated with a 1:2 dilution (in H_2O) of rat-tail collagen (Vitrogen 100, Collagen Corp., Palo Alto, CA, USA) and dried. The cells were incubated for 2 hrs. at $37^\circ C$, 6% CO_2 and nonadherent cells were washed off and 200 μ l fresh complete William's E was added.

HSF Assay (rat hepatocytes)

50 μ l of test samples were added to separate wells of the T-24 plates and the cultures (250 μ l total volume) were incubated for 72 hours, $37^\circ C$, 6% CO_2 with daily replacements of medium and sample. Supernatants of 24 to 72 hours incubation were saved and kept at $4^\circ C$ or $-20^\circ C$ for future analysis of acute phase protein content by rocket electrophoresis.

HSF Assay (human Hep-G2 cells)

The experiments involving Hep-G2 cell culture were done in collaboration with Dr. Heinz Baumann at Roswell Park Memorial Institute, Buffalo, New York. Hep-G2 cells were obtained from Dr. B. Knowles, Wistar Institute, and cultured in MEM containing 10 % FCS v/v in 150 cm² flasks (Corning). For HSF assay, the cells were trypsinized, lifted and plated onto T-24 Nunclon plates (NUNC). When the cells reached 80 to 100% confluency, 500 μ l of MEM and test sample was added per well and the plates cultured for 18 hours (37°C, 6% CO₂). The medium was then replaced by 400 μ l of fresh test medium and allowed another 24 hours incubation. Between 5 to 50 μ l of Hep-G2 supernatant was applied to rocket electrophoresis analysis using monospecific rabbit antibodies against human fibrinogen, α_1 -AGP, haptoglobin, complement C3, α_1 -antitrypsin (Accurate Chemical, Westbury, New York) and goat anti-human α_1 -antichymotrypsin (prepared in Dr. Baumann's laboratory). The results were expressed as μ g protein secreted per 24 hours per 10⁶ cells.

HSF Assay (rat H35 cells)

These experiments were also done in collaboration with Dr. Heinz Baumann. Reuben H-35 cells were cloned and 50 sublines were screened for response to HSF and one clone was selected that responded with synthesis of several plasma proteins. H-35 cells were grown to 80-90% confluency in 6

well culture plates (10 cm²/well). The wells were washed with serum free DMEM (Gibco) and 1 ml of test material in DMEM was added and the cells were cultured for 48 hours at 37°C after which time the medium was removed, dialyzed for 6 hours against H₂O and lyophilized. The material was then resuspended in 50 μ l buffer for immunoelectrophoresis.

Assay of Interleukin-1 Activity, (Lymphocyte Activating Factor, LAF)

LAF activity was assayed in a standard phytohaemagglutinin (PHA) co-stimulation of proliferation assay on mouse thymocytes as described by Simon and Willoughby (1981). This test has been well documented as a measure of IL-1 activity on T cells (Dinarello, 1984; Oppenheim et al., 1986). Five to ten thymus glands were isolated from 6 to 8 week old C3H/HeJ mice (Jackson, Bar Harbour, Michigan, USA) and pushed through a tissue screen to make a single cell suspension in F-15 MEM (Central Resouce Centre, McMaster University) containing 5% FCS v/v, 50 μ M 2-mercaptoethanol, 1 mM Na pyruvate, 0.09 mM Na bicarbonate, 10 mM Hepes, 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin. The cells were then centrifuged at 200 x g for 10 minutes and washed again in complete F-15 MEM. Upon resuspension, the thymocytes were adjusted to 1.5×10^7 cells/ml, and PHA (Difco, Detroit, MI, USA) was added to 1 μ g/ml and 100 μ l of the cell

suspension then aliquoted to each well of 96 well Falcon microtitre plates (Becton-Dickinson & Co., Oxnard, CA, USA). The cultures were incubated at 37°C 6% CO₂ for 30 min before 100 μ l of test sample was added, and then incubated for a further 48 hours. Each well was then pulsed with 50 μ l of complete medium containing 10 μ Ci/ml of [methyl-H³]-thymidine (30-40 Ci/mM, New England Nuclear). After another 18-24 hours, a minimash harvester was used to transfer contents of individual wells to filter paper. The paper was then dried and individual spots punched out and counted in 5 ml of scintillation fluid (19.6 grams 2,5-diphenyloxazole and 0.4 grams 1,4-di-2-(5-phenyl-oxazolyl)-benzene in 4 L toluene) using a Beckman liquid scintillation counter. Positive control dilutions and negative control samples (PBS only) were included in each assay. The amount of radioactivity counted reflected relative amounts of thymidine incorporation into DNA and thus was a measure of proliferation. LAF units were calculated from the volume of cytokine preparation that gave 1/2 of maximum response.

Purification of HSF

High Performance Liquid Chromatography (HPLC)

Crude preparations of cytokines (PBM and FIB supernatants) were separated on the basis of size using Gilson HPLC equipment and Altex TSK-2000 sw or TSK-3000 sw molecular sieve columns (Beckman Toronto, Ont.). 500 μ l of

cytokine preparations were loaded and chromatographed using Tris buffered saline (.01 M Tris pH 7, 0.15 M NaCl) at a flow rate of 1 ml/min. 500 ~~μ~~l fractions were collected, filtered and frozen (-20°C) for future analysis in HSF or LAF assays. Proteins of known molecular weight (BSA, ovalbumin, chymotrypsinogen, cytochrome C) were run and peak elution times were used to calibrate the molecular sieve columns.

Isoelectric Chromatofocussing

Crude PBM supernatants were chromatographed using Polybuffer exchange resin purchased from Pharmacia (Uppsala, Sweden). A 100 ml bed volume of resin was packed in a 2.5 cm diameter glass column and equilibrated with 10 bed volumes of 0.025 imidazole-HCl pH 7.4. The sample (5 ml PBM supernatant concentrated 8x) containing 1 gram of protein was applied to the top of the bed and allowed to penetrate. Polybuffer 74-HCl (pH 4) at a 1:8 dilution was used to elute the proteins from the gel. Five ml fractions were collected at a flow rate of 25 ml per hour. The pH of each fraction was measured using a pH meter, pushed through a 0.22 ~~μ~~m filter (Millipore) and dialysed using Spectropore dialysis tubing against PBS. Each fraction was then refiltered and frozen (-20°C) or tested immediately in the HSF and LAF assays.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of cytokines on the basis of migration through polyacrylamide containing sodium-dodecyl sulphate (SDS) (Laemmli, 1970) was performed in the following manner. A discontinuous acrylamide/bis-acrylamide (ratio 29:1 w/w) gel (lower gel 12% acrylamide, 4.5% w/v Tris pH 8.8, 0.01% SDS; upper gel 4% acrylamide, 1.5% Tris pH 6.5, 0.01% SDS) was poured, allowed to polymerize and mounted in a Biorad Protein II electrophoresis chamber (Biorad Laboratories, Richmond, CA, USA). 100 μ l of sample cytokine, diluted 2:1 in running buffer (1% v/v 2ME, 2.3% w/v SDS, 0.001% bromophenol blue and 0.062 M Tris pH 6.8) was loaded in each well and the proteins were electrophoresed at 50 volts and constant amperage overnight using glycine-tris reservoir buffer (1.48% w/v glycine, 0.3% w/v tris and 0.1% SDS). Molecular weight markers (Biorad) were run in adjacent wells. For autoradiograph analysis, gels were fixed (40% methanol, 10% acetic acid in H₂O) for 1 hour while shaking and then dried onto Whatman filter paper using a Biorad gel dryer. Kodak XRP-1 or XAR-5 film was then placed next to the dried gel and exposed at -70°C. The film was developed using a Kodak automatic developing machine. For elution of bioactivity, freshly run gels were equilibrated for 30 minutes in 0.01 M Tris buffer (pH 7) and then sliced (5mm) horizontally. The slices were placed in Eppendorf

tubes with 500 μ l of PBS and 10% FCS v/v, and shaken overnight at 4°C. The fluid was then removed, filtered, dialyzed and frozen at -20°C or assayed immediately in HSF or LAF assays.

Treatment with Temperature, Proteases and Reducing Agents

Crude cytokine preparations (PBM supernatant) were treated under various conditions and assayed for HSF activity. Separate aliquots were incubated at 4°C, 23°C, 37°C, 56°C and 80°C for 1 hour before assay to examine temperature lability. Equal volumes of PBM were also treated with various amounts (0, 0.1, 0.5 mg/ml) of trypsin (Gibco), pronase E (Merck, Darnstadt, Germany) or protease (type 5, Sigma) and incubated at 37°C for 2 hours. The reactions were inactivated by boiling at 100°C for 1 min and then assayed. Various reducing agents were incubated with crude PBM (dithiothreitol 10 μ g/ml, 2-mercaptoethanol [2-ME] 1% v/v, 1% w/v SDS) for 2 hr and the mixtures were dialyzed and filtered before assay. Tris (pH 7) and Potassium acetate (pH 4.5) were added in final concentrations of 0.01 M and 0.1 M to separate aliquots of a PBM preparation for 2 hours before HSF assay.

Inhibition of HSF Bioactivity by Antibody

Cytokines and various antisera or antibody preparations were preincubated for 2 hours before assaying HSF or LAF activity. PBM supernatants were mixed with PBS and antibodies to give a final dilution of 1/20 PBM (a suboptimal amount of stimulation in the HSF assay). For inhibition of fibroblast cytokines, a 1/5 final dilution of FIB supernatant was used. Various amounts of antibody were used and results show data with antibody in excess. The samples were incubated 2 hours at 37°C before assay and kept at 4°C between additions of cytokine in HSF assay or at -20°C for future analysis (-20°C storage did not affect the results of antibody inhibitions). A decrease in the rat hepatocyte synthesis of APP or Hep-G2 synthesis of APP was accredited to the specific binding of antibody to APP-inducing cytokines.

The antibody preparations used were:

- 1) sheep polyclonal anti-human IFN β (NIH reference # G-028-501-568) (partially purified antibody and has specificity for both IFN β_2 and IFN β_1) DESIGNATED sheep anti-IFN β_2 & β_1 .
- 2) sheep polyclonal control antibody (NIH reference # G-029-501-568) (partially purified antibody) DESIGNATED Control Ab.
- 3) rabbit polyclonal anti-human IL-1 was obtained from Dr. R. Newton (Dupont Chemicals) (IgG fraction) DESIGNATED anti-crude supernatant.
- 4) rabbit polyclonal anti-human recombinant IL-1 β was a gift of Dr. K. Matsushima, Frederick Cancer research Facility (serum). DESIGNATED Anti-IL-1 β .

- 5) rabbit polyclonal anti-human recombinant IL-1 α was a gift of Dr. K. Matsushima (serum). DESIGNATED anti-IL-1 α .
- 6) rabbit polyclonal anti-human recombinant TNF was a gift from Dr. M. Palladino, Genentech (serum). DESIGNATED anti-TNF.
- 7) rabbit polyclonal anti-human purified IFN γ was a gift of Dr. Chadha (Roswell Park Memorial Institute) (serum).
- 8) bovine polyclonal anti-human IFN γ was a gift from Dr. J. Vilcek (Rockefeller University) (serum). DESIGNATED bovine anti-IFN γ _{2&8}₁.
- 9) normal rabbit serum (NRS)
- 10) fetal calf serum (FCS)

Immunoprecipitations of Radiolabelled Protein

Immunoprecipitations were performed according to the method of Harnish et al. (1981). 100 μ l of L-[³⁵S]-methionine labelled and 8x concentrated PBM supernatants (containing 0.5 to 1 x 10⁶ cpm) were added to 900 μ l of RIPA buffer (0.1% w/v SDS, 1% w/v deoxycholate, 1% v/v Triton X-100, 100mM NaCl, and 10 mM Tris pH 7.5) along with 1 to 20 μ l of antibody preparations in 1.5 ml sterile Eppendorf tubes. Protein A-sepharose beads (CL-4B, Pharmacia) equilibrated in RIPA buffer and adjusted to 1:1 v/v in RIPA buffer, were then added (200 μ l/tube) and the tubes were then rotated gently in a rotorack for 3 hours at 4°C. The beads were then pelleted (2 minutes at 14,000 x g), the supernatant separated and the beads resuspended in 1 ml of RIPA buffer. The beads were pelleted and washed in this manner four more times. 100 μ l of sample buffer (for SDS-

PAGE) was added to the pellet of the last wash, and the mixtures boiled for 10 minutes. The beads were pelleted once more and the supernatants taken for loading onto SDS-PAGE. The gels were electrophoresed, fixed, dried and exposed to x-ray film as described under SDS-PAGE.

Extraction of Polyadenylated RNA

Extraction of total RNA was done according to the CsCl cushion procedure of Chirgwin *et al.* (1979) with minor modifications. PBM cultures were stimulated with 10 μ g/ml LPS for 4 hours at which time the monolayers were washed extensively with warm (37°C) PBS. After adjusting the pH of guanidinium solution to 7 (50% w/v guanidinium isothiocyanate, 0.5% w/v lauryl sarcosine, 0.3% antifoam reagent [Sigma], 0.7% v/v 2-ME, 0.03 M Na citrate pH 7) two ml were applied to each 100 x 15 mm plate and mixed vigorously with a pipette to dislodge cell components adhered to the plastic. Solution from 30 to 40 plates was pooled and stored at 4°C. Six ml of this mixture was then layered over 4 ml of 5.7 M CsCl (in 25 mM Na acetate pH 5) in each of several 12 ml ultracentrifuge tubes. The tubes were then centrifuged at 27,000 rpm for 16 hours in a Beckman ultracentrifuge at 18°C. The upper layer (containing protein) and middle layer (containing DNA) were carefully taken off and discarded. The tubes were inverted, allowed to drain and the pellet (often not visible)

resuspended in 0.1% SDS. Pellets from up to six tubes were pooled and the RNA was precipitated using 66% ethanol. To the RNA, 2 M potassium acetate pH 5.5, 5 M NaCl and 100% cold absolute ethanol were added to final concentrations of 0.3 M KAc, 0.1 M NaCl and 66% ethanol. The solution was distributed into Eppendorf tubes, and precipitated at -20°C for 2 hours or -20°C overnight. The tubes were spun at $14,000 \times g$, 30 minutes at 4°C and the supernatants were decanted and the pellet dried in a speed-vac rotating vacuum drier. The RNA pellets were then resuspended in 50 μl of sterile dH_2O , pooled, and reprecipitated. After the second precipitation and centrifugation, the RNA pellet was resuspended in 100 μl of dH_2O and an aliquot was taken for optical density readings. Optical density at 260 nm was used to estimate the concentration of RNA.

Selection for messenger RNA (mRNA) was obtained through a modified technique of oligo-dT cellulose chromatography (Harnish, personal communication). Oligo-dT-cellulose beads (Pharmacia) were equilibrated with 4 washes of binding buffer (0.5 M LiCl, 0.1% w/v SDS, 1 mM EDTA, 0.05 M Tris pH 7.5) in Eppendorf tubes. RNA was added, mixed gently and incubated at 23°C for 15 minutes. The beads were then pelleted (30 seconds, $14,000 \times g$), the supernatant decanted and 1 ml of buffer added. The tube was mixed gently and repelleted. This procedure was repeated until the O.D. 260 of the wash was 0.000. To the pellet, 1 ml of

low salt elution buffer (0.1% w/v SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.05 M Tris pH 7.5) was added and the suspension incubated at room temp for 5 min. The tube was respun and washed with 5 more 1 ml amounts of elution buffer. O.D.₂₆₀ was used to follow the RNA concentrations in the washes. To the washed pellet, 1 ml of dH₂O 65°C was added and the procedure repeated at least thrice more with 65°C H₂O. The washes with the highest O.D.₂₆₀ from the elution buffer wash were pooled as were the washes from the H₂O wash. The RNA in these fractions were ethanol precipitated as before.

In Vitro Translation (IVT)

IVT was carried out with wheatgerm lysate and rabbit reticulocyte lysate IVT kits (Bethesda Research Laboratory, Bethesda, Maryland, USA) using the included recommended protocol. Ten μ l of rabbit reticulocyte lysate (RR) was mixed with; (for RR), 3 μ l of 10x reaction mixture containing 250 mM Hepes pH 7.2, 400 mM KCl, 100 mM creatine phosphate, 500 μ M amino acids (except methionine); 1.3 μ l of K acetate 2 M, pH 7.2; 2 μ l of Mg acetate 20 mM pH 7.2, 10 μ Ci of L-[³⁵S]-methionine (1000-1200 Ci/mM, New England Nuclear), 15 μ l poly A selected RNA in H₂O; and H₂O to a final reaction volume of 30 μ l. For WG, 10 μ l of WG extract was mixed with; 3 μ l reaction mixture (10x) containing 200 mM Hepes pH 7.5, 300 mM K acetate, 1 mM Mg acetate, 12 mM

ATP, 1 mM GTP, 55 mM creatine phosphate, 2 mg/ml creatine kinase, 800 μ M spermidine phosphate, and 500 μ M of amino acids except methionine; 2 μ l of K acetate 500 μ M pH 7.5; 0.9 μ l Mg Acetate 20 mM pH 7.5; 10 μ Ci of 35 S-methionine (New England Nuclear); RNA (poly-A selected) in H₂O; and H₂O to a final reaction volume of 30 μ l in eppendorf tubes.

The tubes were mixed gently and incubated at 25°C for WG, 37°C for RR. The reactions were stopped (ice bath) and samples were frozen (-70°C) for future HSF assay or run on SDS-PAGE.

Northern Blot Analysis

Northern analysis of RNA was done according to the protocol of Maniatis *et al.* (1982). Agarose (1% w/v) was dissolved in H₂O, cooled to 65°C and 5x gel buffer (0.2 M MOPS pH 7, 50mM Na acetate, 5mM EDTA pH 8) and formaldehyde was added to give final concentrations of 1x gel buffer and 2.2 M formaldehyde. The formaldehyde gel was poured into sub-gel apparatus and allowed to harden in a fume hood. RNA samples (20 μ g/10 μ l) were mixed with 4 μ l 5x gel buffer, 7 μ l formaldehyde, 20 μ l formamide and incubated at 55°C for 15 min before loading. The gel was electrophoresed overnight at 25 volts using 1x gel buffer. The gel was then soaked in several changes of H₂O, then placed in 50mM NaOH and 10 mM NaCl for 45 min. The gel was neutralized with 0.1 M Tris pH 7.5 for 45 min and was then soaked for 1 hour in 20x [sodium

chloride, sodium citrate] SSC (175.3 grams NaCl, 88.2 grams Na citrate in 1 L H_2O and adjusted to pH 7). The gel was placed on a stack of Whatman filter papers, and a nitrocellulose (Biorad) filter (wetted in 2x SSC) placed over top. Several thicknesses of Whatman paper and then paper towel was placed over top. The stack was placed in a dish containing 10x SSC (used as a reservoir) and transfer was carried on for 4 to 6 hours. The filter was then removed, washed in 3x SSC, air dried, baked for 2 hours at 80°C and stored for future hybridization. The blot was then prehybridized overnight (42°C) in Omniblot apparatus envelopes (American Bionetics, Emeryville, CA, USA) containing 40% formamide (deionized), 2 x SSC, 100 μ g/ml single stranded DNA, 0.1 mM Na pyrophosphate, 10 mM TRIS pH 7.5, 0.1% SDS, 1 mM EDTA and 5x Denhardt's solution. Denhardt's solution was stored at -20° as a 100x stock containing 2 grams Ficoll, 2 grams polyvinylpyrrolidone and 2 grams of BSA per 100 ml H_2O . After 18-20 hours, P^{32} labelled plasmid probe was added (see below) and the blots incubated for a further 18-20 hours (42°C). The blots were then washed (2x SSC, 0.1% SDS at 45°C) in the Omniblot apparatus (500-1000 ml per filter) and then removed, blotted on Whatman, wrapped in cellophane and exposed to XAR-5 film overnight.

The plasmid probe was provided by Dr. P. Seghal (Rockefeller University, NY, USA) and contained most of the

coding sequence (minus codons encoding 30 amino acids at 5' end) of IFN β 2 cDNA, inserted into a Pst 1 site of a PUC-9 plasmid (May et al., 1986; Seghal et al., 1987). The plasmid was grown in large scale in Dr. D. Harnish's laboratory (McMaster University, Hamilton, Ont.). For probing the northern blots, the plasmid was labelled by a modified primer-extension technique using an OLB reagent kit purchased from Pharmacia. 50 nanograms of plasmid DNA in 32 μ l H₂O was boiled for 10 minutes. The tubes were moved to 37°C and very quickly and 10 μ l of reagent mix (containing hexadeoxyribonucleotides, Mg, Tris, 2-ME, dATP, dTTP, dGTP, Hepes) was added followed by 2 μ l of 3 mg/ml nuclease free BSA, 5 μ l containing 50 Ci of [α -³²P]-dCTP (3000 Ci/mmol, New England Nuclear), and 2 μ l of 1 unit/ μ l Klenow fragment. The reaction was left at room temperature for 3-4 hours or overnight. When finished, percent incorporation of label into TCA precipitable material was checked (generally between 60-90%), then 1 ml of prehybridization buffer was added, the tubes boiled for 5 minutes and then added to the prehybridized filters.

RESULTS

Rocket Analysis of Acute Phase Proteins

Major acute phase proteins in the rat in response to irritant-induced inflammation include α_1 -CPI, α_1 -AGP, and α_2 -macroglobulin, fibrinogen, haptoglobin, hemopexin and C3 and negative acute phase proteins include albumin and transferrin (Koj, 1985). This is well-documented in turpentine-induced inflammation, however the physiological significance of this stimulus is not clear. A more natural stimulus (trauma) was used to examine the response in rats and compared to turpentine-induced levels. Major changes were evident in concentrations of α_2 -macroglobulin, α_1 -AGP, and α_1 -CPI using rocket electrophoresis. Fig.1 represents rat serum sample levels of α_1 -AGP at day 0 to 10 after surgical trauma (1-2 cm incision). By preparing standard curves with known concentrations of purified antigen, estimations of concentration were extrapolated (data not shown). Fig. 2 presents data for α_1 -AGP, α_1 -CPI and α_2 -macroglobulin. Rat serum sample albumin levels did not change outside the normal value SD (data not shown). Marked changes are evident for all three proteins, α_2 -macro increasing 4-fold by day 3, α_1 -CPI increasing 3-fold and α_1 -AGP increasing 4 fold, both by day 2. These increases were not seen in control (tail bleed only, data not shown) animals and furthermore, were comparable to those observed

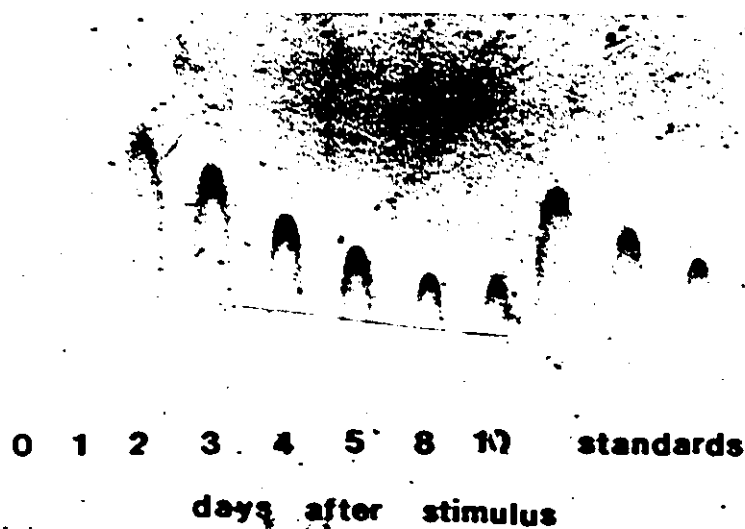


Figure 1: Rocket electrophoresis of rat serum samples taken at various times after surgical trauma. Serum samples were obtained by tail bleeding at 0 to 10 days after trauma and electrophoresed into a gel containing rabbit anti- α_1 -AGP. Standard dilutions of known α_1 -AGP (purified) amounts appear in the last 4 wells.

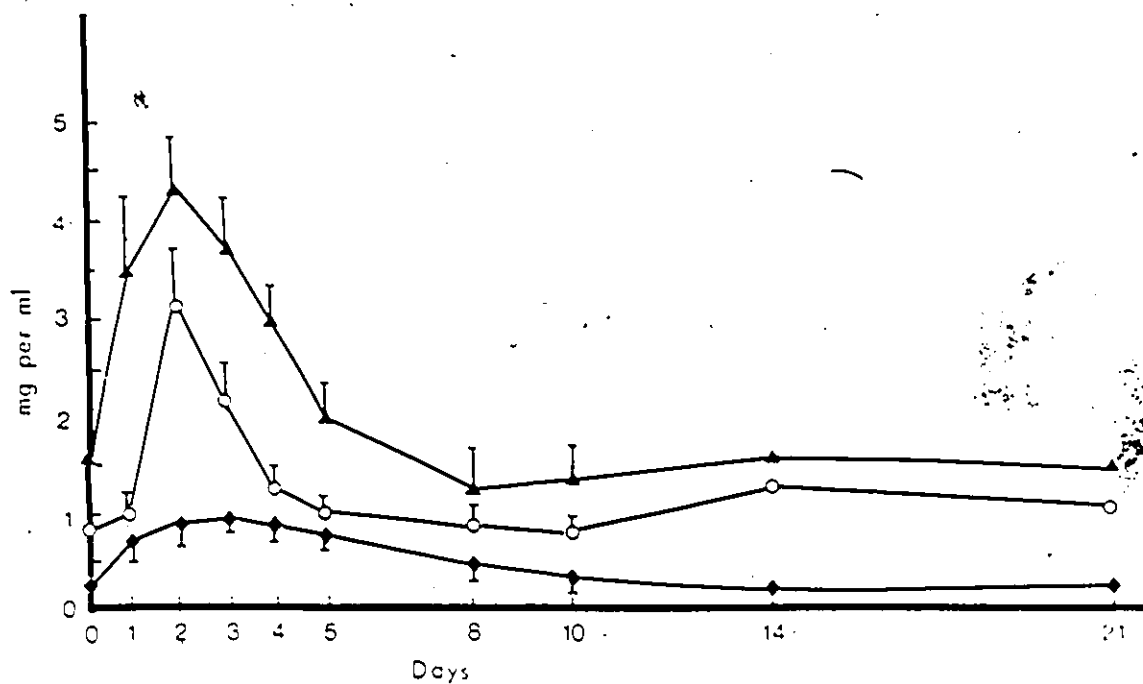


Figure 2: Levels of acute phase proteins in rat serum after surgical trauma. Rocket electrophoresis was used to quantitate serum levels of α_1 -CPI (\blacktriangle), α_1 -AGP (\circ) and α_2 -macroglobulin (\blacklozenge) at days 0 to 21. Error bars represent the standard deviation from the mean values of 5 rats treated similarly.

in turpentine induced inflammation. 100 ~~μ~~l turpentine per 100 gram of rat, injected s.c., produced increases of .2 to 3 mg/ml for α_1 -AGP and from <1 mg/ml to 2 mg/ml for α_2 -macroglobulin and from 1.2 to 10 mg/ml for α_1 -CPI at 48 hours after injection. The turpentine induced concentrations were comparable with those described by Schreiber et al. (1986). The differences in magnitude between turpentine and surgical trauma may be due to the extent of tissue damage or cell activation and thus the release of factors that increase hepatocyte APP in each case. Van Gool et al. (1984) showed α_2 -macroglobulin changes from .05 to 2.8 mg/ml in rats due to in vitro response to cytokines. However, conclusions regarding all acute phase proteins should be guarded against unless all are measured.

The In Vitro HSF Assays

Rat hepatocytes upon culturing in the conditions described in methods, adhere to collagen and survive for periods of up to 5 days. The cells form a monolayer and viability remains high (>80%) for 3-4 days after which viability declines (as assessed by trypan blue exclusion). In the presence of William's E medium supplemented with dexamethasone and insulin, rat hepatocytes were able to

adhere to collagen and secrete APP more efficiently (Laishes & Williams, 1976; Rupp and Fuller, 1979; Koj et al., 1984; Gross et al., 1984; Fuller & Ritchie, 1982) than without. Both hormones were included in the complete Williams' E medium.

As suggested by Koj (1974), and as demonstrated by various investigators, cells of the monocyte/macrophage lineage release molecules or cytokines that affect hepatocyte APP synthesis in vitro (Fuller & Ritchie, 1982; Koj et al., 1984) and can elicit increases in serum concentrations of APP in vivo (Kushner, 1982; Bornstein, 1982; Sztein et al., 1981). Accordingly, human monocytes were used as a source of cytokines and were added to the in vitro assays.

Fig. 3(a) displays rocket electrophoretic analysis of hepatocyte supernatants. Upon adding increasing amounts of PBM cytokine (1.6, 3.1, 6.3, 12.5, 25 and 50 μ l) to rat hepatocytes, output of α_2 -macroglobulin is progressively greater and albumin progressively decreased.

To estimate statistical significance of measurements in the rat hepatocyte rocket analysis, peak heights from separate assays were gathered and analyzed for variation. In table 2, standard deviation (SD), and coefficient of variation (calculated as a percent [$SD \text{ mean} \times 100$]) are presented for α_2 -macroglobulin, α_1 -CPI, α_1 -AGP and albumin levels in both stimulated and unstimulated rat hepatocytes.

The coefficients of variation (Co.V) were all under 10% for analysis of output by different culture wells on different electrophoresis plates under either stimulatory or non stimulatory conditions. Therefore, the differences in APP output of α_2 -macroglobulin (>+300% change and albumin reduced by 50%) by stimulated hepatocytes is highly significant. The change in α_1 -CPI output (+30%) is significant whereas the α_1 -AGP difference is questionable. In the purification and characterization of HSF (fig. 7 to 10) peaks of activity clearly showed +100% change or greater for α_2 -macroglobulin and α_1 -CPI.

The use of DEX in the culture medium (10^{-6} M) may account for the lack of enhanced response by α_1 -AGP in vitro as opposed to the 3 fold stimulation seen in vivo. Baumann et al. (1983) and Gross et al. (1984) have shown stimulation of α_1 -AGP by glucocorticoid in culture and in vivo. Thus, in this culture system, stimulation of α_1 -AGP may have already been close to maximum and therefore the presence of stimulating factors were not evident. However, DEX was required for expression of α_2 -macroglobulin and to maintain culture viability.

Table 3(a) shows the output of rat hepatocyte and Hep-G2 cells calculated on the basis of μg secreted per 10^6 cells over a 24 hour period. Row 1 shows that albumin output under control conditions (addition of PBS) was

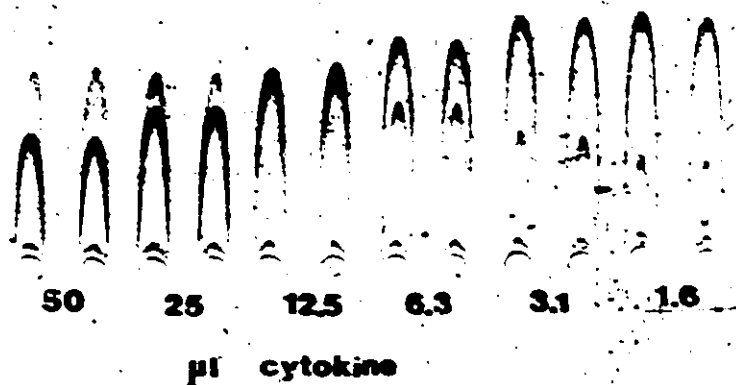


Figure 3: Rocket Electrophoresis of Rat Hepatocyte Culture Supernatants. Various amounts of PBM supernatant (cytokine) were added in duplicate to the in vitro HSF assay (total volume 250 μ l). Hepatocyte supernatants were assayed for albumin (dark precipitin lines) and for α_2 -macroglobulin (light precipitin line) content simultaneously.

Table 2: Acute Phase Proteins in Stimulated and Unstimulated Rat Hepatocyte Culture Supernatants

2(A)		<u>Unstimulated</u>		
APP	x	n	SD	Co.V
	(mm)		(mm)	(%)
α_2 -macro	8.1	8	0.6	7.8
α_1 -CPI	10.1	8	0.6	6.3
albumin	29.5	8	1.7	9.8
α_1 -AGP	15.8	8	0.5	2.9

Rocket peak heights for α_2 -macroglobulin, α_1 -CPI, albumin and α_1 -AGP were measured in 8 stimulated (A) and 8 unstimulated (B) culture wells. The mean (x), standard deviation (SD) and coefficient of variation (Co.V) were calculated. Co.V was expressed as a percent.

2(B)		<u>Stimulated</u>		
APP	x	n	SD	Co.V
	(mm)		(mm)	(%)
α_2 -macro	27.3	8	1.4	5.5
α_1 -CPI	13.5	8	0.5	3.9
albumin	16.1	8	1.0	6.2
α_1 -AGP	17.3	8	0.9	5.5

normally high ($40 \mu\text{g}/10^6 \times 24 \text{ hr}$) whereas α_2 -macro was normally low ($7 \mu\text{g}/10^6 \times 24 \text{ hr}$). α_1 -CPI and α_1 -AGP levels are 25 and $12 \mu\text{g}/10^6 \times 24 \text{ hr}$ respectively. Upon addition of PBM supernatant, α_2 -macroglobulin output increased to 50 (+700%), albumin dropped to 13 (-65%), α_1 -CPI increased to 30 (+20%) and α_1 -AGP increased minimally to 14 (+15%).

Hep-G2 cells (table 3(b)) under control conditions secreted much less APP than did the rat hepatocyte cultures ($0.1 \mu\text{g}/10^6 \times 24 \text{ hr}$ fibrinogen, 1.3 antichymotrypsin, 0.4 haptoglobin and $0.3 \alpha_1$ -AGP). Upon stimulation with PBM supernatant, fibrinogen output increased approx. +400%, antichymotrypsin approximately +800%, haptoglobin approximately +500% and α_1 -AGP approximately +1800%. Upon assay of LAF activity, strong LAF was found in PBM supernatants as expected since PBM are an excellent source of IL-1 (Oppenheim *et al.*, 1986). Thus PBM supernatant contained strong HSF activity and LAF activity as measured by these in vitro assays.

In the rat hepatocyte system, α_2 -macroglobulin was picked to follow the activity of HSF because of its marked sensitivity. Koj *et al.* (1984) and Koj *et al.* (1985) showed that α_2 -macroglobulin α_1 -CPI and fibrinogen were stimulated by cytokines of similar molecular size and that this was consistent with results presented by Fuller & Ritchie (1982) and Ritchie & Fuller (1983). Thus, α_2 -macroglobulin is a reasonable marker with which to follow HSF. HSF activity,

Table 3: Stimulating Activities in PBM and Fibroblast Supernatants

3(A)	APP output by Rat Hepatocytes ($\mu\text{g}/10^6$ 24 hr)				LAF (units/ml)
	α_2 -macro	α_1 -CPI	alb	α_1 -AGP	
control PBS	7	25	40	12	<10
PBM sup.	50	30	13	14	250

PBS and PBM supernatants (sup.), diluted 1:20 in PBS were used to stimulate rat hepatocyte cultures. Production of APP by hepatocytes were expressed as μg per 10^6 cells in 24 hr. LAF content was measured in the thymocyte proliferation assay and expressed as units/ml.

3(B)	APP output by Hep-G2 ($\mu\text{g}/10^6$ 24 hr)			
	Fib	ACH	HP	AGP
control PBS	0.1	1.3	0.4	0.3
PBM sup.	0.4	11.6	2.2	5.8

PBS and PBM sup. were used to stimulate Hep-G2 cells. Production of APP was expressed as $\mu\text{g}/10^6 \times 24$ hr.

for the purpose of this work, was defined using α_2 -macroglobulin. Units/ml were calculated as the reciprocal of the dilution needed to induce 1/2 maximum output of α_2 -macroglobulin by rat hepatocytes. Maximum response was that induced by excess PBM supernatant. Figure 4 shows a curve generated by addition of different dilutions of PBM supernatant. Two different preparations contained 305 units/ml (a) and 62 units/ml (b) of HSF. In general, PBM supernatant preparations ranged between 250 and 500 HSF units/ml. Analysis of 1 preparation on 3 different hepatocyte isolations showed an average of 62 HSF units/ml and standard deviation of 3. Thus the calculation of units gave relatively consistent values. For samples that were not able to stimulate α_2 -macroglobulin to 1/2 maximum even at addition of 1/1 dilution, the HSF units are marked as <5 and represent low if any HSF activity.

General Characteristics of HSF

In undertaking characterization of the HSF molecule(s) from PBM, sensitivity to temperature, reducing agents and proteases was examined. Table 4 shows that HSF activity was not affected by 1 hr incubations at 4°, 23°, 37°, 56°C but was destroyed at 80°C (<10% recovery). As a general observation, HSF activity was stable at 4°C for months if stored as a crude supernatant. Treatment of crude

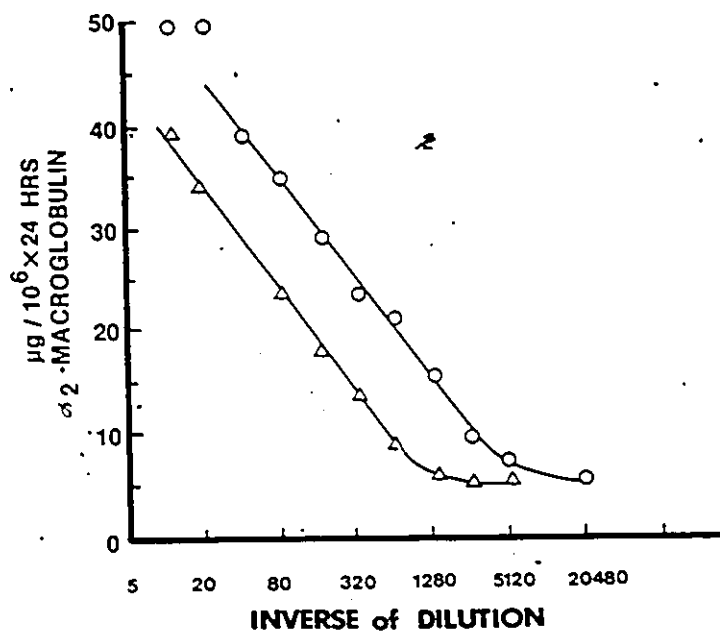


Figure 4: α_2 -macroglobulin Induction and PBM Concentration. The reciprocal of the dilution of PBM added to the HSF assay was plotted against the α_2 -macroglobulin output by rat hepatocytes. Two different preparations of PBM contained (a) 305 HSF units/ml and (b) 63 HSF units/ml. Maximum response to PBM cytokines was 50 $\mu\text{g}/10^6$ cells x 24 hours.

supernatant with 10 mM DTT or 2-ME did not markedly alter HSF activity (98% and 81% recovery of control activity). This suggests that HSF did not have disulphide bonds that were important for the integrity of its bioactivity. Preincubation with 1% SDS and then dialysis inhibited bioactivity. The reason for this could have been interference in hepatocyte synthesis upon incomplete dialysis of the PBM supernatant. The activity appears to be destroyed by high concentration of Tris (0.1 M, pH 7.0) but not high concentration of sodium acetate (0.1 M, pH 5.5) or lower concentrations (0.01 M) of Tris (table 4).

HSF activity in crude PBM supernatant proved to be sensitive to incubations with 0.1 mg/ml of trypsin, pronase and protease (all resulted in <20% recovery of control activity). The lower control HSF value (25 units/ml) may have been due to inactivation of some HSF by the boiling procedure. However, clearly the addition of enzymes inhibited the remaining activity. HSF activity was not lost upon extensive dialysis using Spectropore tubing with a molecular weight exclusion of 6-8,000 daltons. These features are consistent with the high molecular weight (>8 kd) proteinaceous character that has been assigned to HSF (Fuller & Ritchie, 1982; Koj, et al., 1984; 1985).

Table 4: Effect of Various Treatments on Crude PBM HSF Activity

	Treatment	HSF units	% Recovery (of control)
temperature (1hr)	4°C	68	100
	23°C	63	93
	37°C	58	86
	56°C	64	95
	80°C	<5	<10
chemical agent	none	80	100
	10mM DTT	78	98
	10mM 2-ME	65	81
	0.1 M Tris (pH 7)	<5	<10
	0.01 M Tris (pH 7)	75	94
	0.1M Na Ac (pH 4.5)	75	78
	1% SDS	<5	<10
protease	none	20	100
	0.1mg/ml trypsin	<5	<20
	0.1mg/ml pronase	<5	<20
	0.1mg/ml protease	<5	<20

PBM supernatant preparations were incubated under the various conditions and assayed for HSF activity using rat hepatocytes. The recovery was expressed as a percent of the HSF units in untreated control preparations.

Various cell preparations were tested for HSF activity in order to determine the best source for purification on a large scale. Table 5 shows that 24 hour conditioned medium of alveolar macrophages from mice, rats, rabbits and humans all contained HSF upon stimulation with 10 μ g/ml LPS. Human peripheral blood mononuclear adherent cells as well as human alveolar macrophages released the most potent activity on a per cell basis. Two cell lines, known to produce copious amounts of IL-1, the THP-1 cell line (Matsushima et al., 1986) and SK-hep 1 cell line (Doyle et al., 1986), produced minimal, if any, HSF activity. The HL-60 promyelocytic cell line, if stimulated with TPA and/or LPS produced little HSF activity. These results demonstrated that HSF was released by many cells of the monocyte-macrophage lineage. Others have seen release of HSF by PBM (Ritchie & Fuller, 1983; Koj et al., 1984; rat kupffer cells (Bauer et al., 1984; Sanders and Fuller, 1983) and rodent peritoneal exudate cells (Baumann et al., 1983; Koj et al., 1987). The results also show that rat hepatocytes respond to HSF molecules from multiple species. This suggests structural homology between HSFs from mice, rats, rabbits and humans, in that a putative receptor(s) on rat hepatocytes recognize the various factors.

Table 5: Sources of Cytokines with HSF Activity

	HSF Activity (units/ml)
mouse alveolar macrophage	38
rat alveolar macrophage	33
rat alveolar macrophage in the presence of DEX (10^{-8} M)	<5
rabbit alveolar macrophage	18
human alveolar macrophage	223
human peripheral blood monocyte	256
HL-60 cell line	<5
THP-1 cell line	<5
Sk-hep 1 cell line	9

LPS-stimulated (10^{-9} g/ml) 24 hour supernatants from several cell types were harvested, dialyzed, filtered and assayed for induction of α_2 -macroglobulin in rat hepatocytes. The cells were incubated at a concentration of 1×10^6 /ml. Dexamethasone (DEX) was added at 10^{-6} M to 10^{-8} M to rat alveolar cell cultures (only 10^{-8} M data is shown).

Table 5 also displays the effect of DEX on rat alveolar macrophage secretion of HSF. Concentrations of 10^{-6} to 10^{-8} M resulted in potent inhibition of HSF release (<5 vs 32 units/ml without DEX). Woloski and Fuller (1985) have shown a similar effect on HSF release by human PBM.

Cell lines such as THP-1 and SK-HEP 1 released large amounts of IL-1 (Matsushima et al., 1986; Kovacs et al., 1986) but in the experiments described here produced little HSF, suggesting that HSF and IL-1 are released differentially, at least as detected in the present assays. Since PBM supernatants contained potent activity and human blood was available in quantity from the Canadian Red Cross (Hamilton, Ontario), PBM supernatants were selected as the most practical source of HSF.

To examine the time course of HSF release from PBM, supernatants were taken and replaced on a per hour basis and the results depicted in Fig. 5. After LPS stimulation, amount of HSF released per. hour increased to a maximum between 3 and 4 hours, and then declined but not to background levels at 5 to 6 hours. The total HSF units of equal volumes of sample were calculated as a percent of the total HSF release over 24 hours in a separate but parallel plate. These percentages appear just below the bars and show that between 2 and 3 and between 3 and 4 hours, that 19 and 20% of the total activity was released. Over 5 hours of stimulation, over 60% of the 24 hour activity was

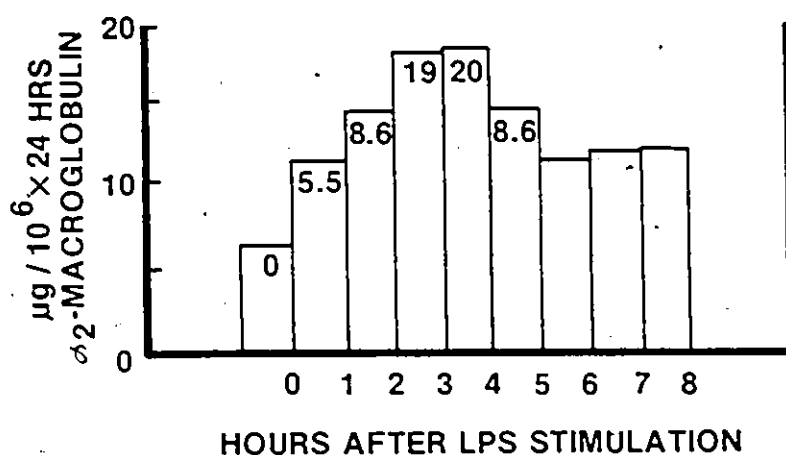


Figure 5: Rate of HSF Production by PBM after Stimulation with LPS. Adherent PBM were stimulated with LPS and the medium replaced on an hourly basis. Each supernatant was filtered dialyzed and assayed for α_2 -macroglobulin induction in rat hepatocytes. The percentage of each sample of total HSF activity released over 24 hours was calculated and is shown underneath the bars.

released. Peak LAF release per hour correlated exactly to this time frame (data not shown). Thus, LPS stimulates HSF in similar fashion to IL-1 in this respect.

Having identified a reasonable source of HSF and some of its properties, the purification of HSF was then begun -- using various techniques.

Analysis of Chromatographic Fractionation of Cytokines

Analysis of HPLC fractionated PBM supernatant allowed an estimation of the molecular size of the major HSF species in PBM. Fig. 6 shows analysis of hepatocyte supernatants upon addition of samples from an HPLC separation. α_2 -macroglobulin production was maximum in response to fraction 14 in this case. Thus a stimulated hepatocyte under these conditions showed marked changes in protein output that was clearly detectable. Figure 7 shows that the major peaks of α_2 -macroglobulin ($17 \mu\text{g}/10^6$ cells x 24 hr versus background $2 \mu\text{g}/10^6$ x 24 hr) and α_1 -CPI ($28 \mu\text{g}/10^6$ x 24 hr versus $5 \mu\text{g}/10^6$ x 24 hr) stimulation co-migrated in fraction 22 which corresponded approximately to the 31 kd peak of molecular weight marker carbonic anhydrase. This agrees with data published previously by Koj et al. (1984) and Ritchie & Fuller (1983) showed HSF elution at 30 kd. α_1 -AGP on the other hand showed high background ($15 \mu\text{g}/10^6$ x 24 hr) and peaked at fraction 26 corresponding to molecular size between 13 and 23 kd molecular weight marker peaks. The significance of this peak is not as pronounced as the 30 kd

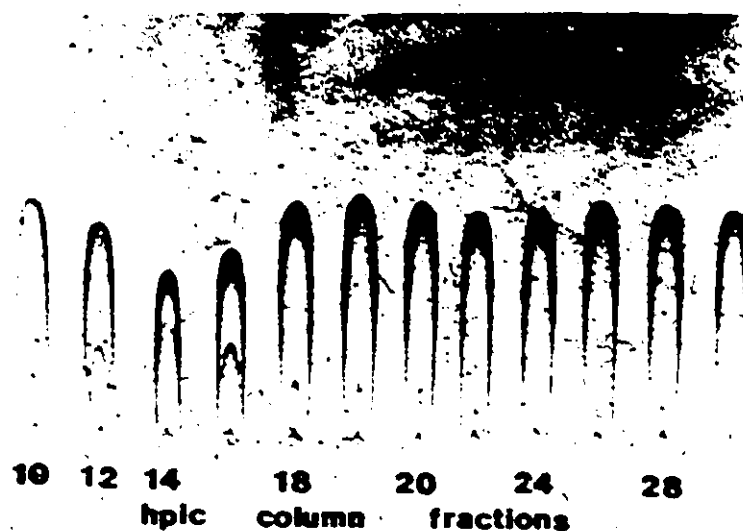


Figure 6: Rocket Electrophoresis of Hepatocyte Supernatants Upon Stimulation by HPLC Fractions. PBM supernatant was fractionated using the TSK-3000 column and added to the HSF assay. Hepatocyte supernatants were analyzed for output of albumin (dark precipitin lines) and α_2 -macroglobulin (light precipitin lines).

α_2 -macroglobulin and α_1 -CPI inducing peak. Assay of LAF activity in the fractions showed maximal activity (white bars) in fraction 25, corresponding to (approximately) the α_1 -AGP stimulating peak. Albumin production was maximally inhibited due to fraction 22 (corresponding to 30 kd) but inhibition was spread over fractions 18 to 28. Assay of fractions of unconditioned RPMI medium showed no change in any APP. These data suggest that multiple APP-stimulating activities are present in PBM supernatant and differ in their migration upon HPLC and that different molecular entities control α_2 -macroglobulin and α_1 -CPI induction than α_1 -AGP induction and LAF.

Furthermore, the output of α_1 -CPI in response to fractions 21 to 23 (30 kd in size) was greater than the output due to crude PBM supernatant alone. In this HSF assay crude PBM gave maximal $20 \mu\text{g}/10^6 \times 24 \text{ hr}$ whereas fraction 22 resulted in $30 \mu\text{g}/10^6 \times 24 \text{ hr}$. This suggested that there were inhibitors of maximal α_1 -CPI stimulation in crude supernatant, that were separated away upon HPLC from HSF.

Recent publications have shown that although the majority of extracellular LAF activity is 17 kd in size, intracellular LAF activity eluted at higher molecular weights (23-25 kd, 45 kd) as well as 17 kd (Lepe-Zuniga et al, 1985; Matsushima et al., 1986c). To examine intracellular HSF, PBM cell lysates were separated by HPLC. Intracellular HSF eluted at 30 kd whereas LAF activity peaked at 13-20 kd,

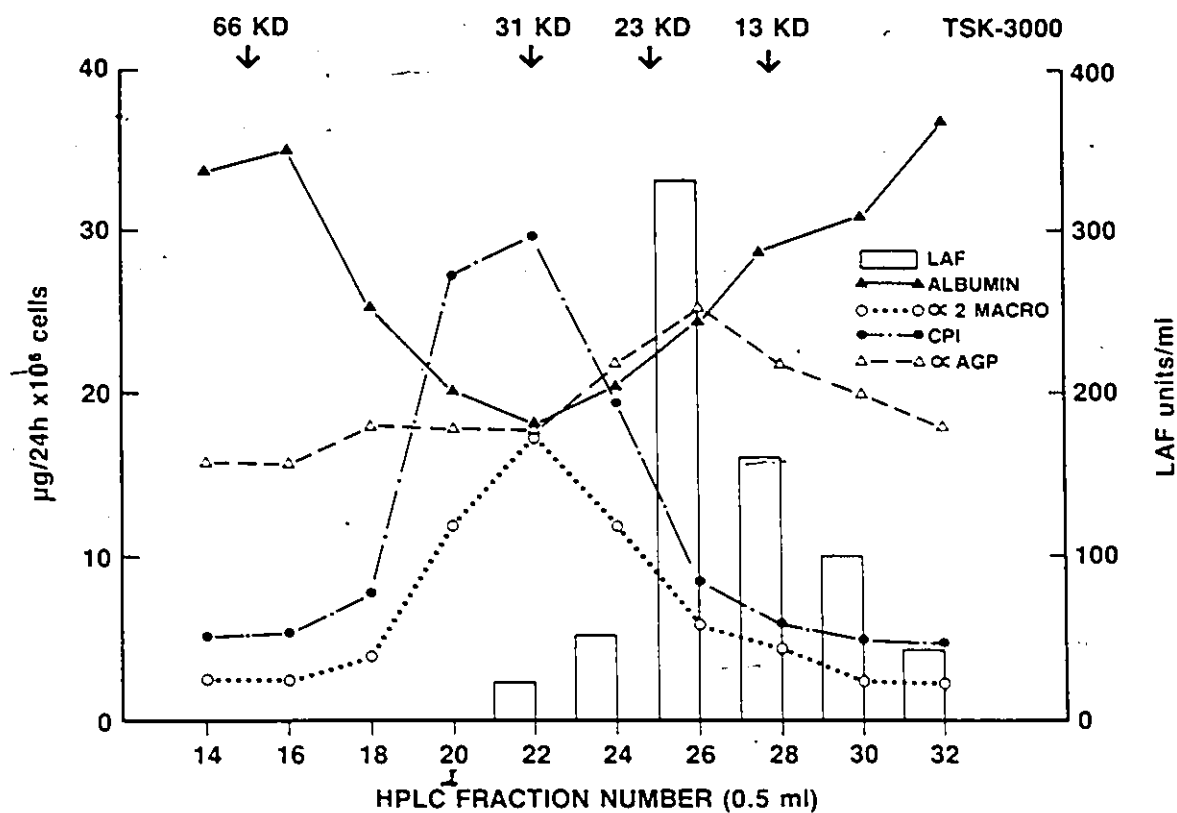


Figure 7: Fractionation of PBM Supernatant by molecular Sieve HPLC. Fractions from HPLC using the TSK-3000 column were assayed for induction of rat α_2 -macroglobulin (\bigcirc), α_1 -CPI (\bullet), α_1 -AGP (\triangle) and albumin (\blacktriangle) in the HSF assay. Fractions were also assayed in the thymocyte proliferation assay and LAF units were calculated (white bars).

25-30 kd and 40-50 kd. This suggested that LAF displayed multiple molecular forms within the cell but that HSF did not.

To examine the molecular size of PBM cytokines active on hepatoma cells, HPLC fractions were added to Hep-G2 cell cultures as well as rat H-35 cells. Fig. 8 depicts the production of APP by Hep-G2 and clearly shows that fractions corresponding to approximately 30 kd were maximally active in stimulating fibrinogen, antichymotrypsin and haptoglobin. α_1 -AGP on the other hand was maximally stimulated by fractions corresponding to lower molecular size.

Fig. 9 shows that α_1 -CPI and α_2 -macroglobulin were stimulated in H-35 cells by the same fractions as were α_1 -CPI and α_2 -macro in rat hepatocytes, and α_1 -antichymotrypsin in Hep-G2 cells. Interestingly, H-35 cells were stimulated for AGP and C3 production by fractions with molecular size in between HSF and IL-1. Thus there could have been an additional factor in PBM responsible for this peak, or a combination of multiple factors is responsible.

These results confirm and extend the findings of others (Fuller & Ritchie, 1982; Koj et al., 1984; Woloski & Fuller, 1985) that HSF migrates differently than IL-1 on HPLC separation. They also suggest that human PBM HSF stimulates certain APP maximally in rat hepatocytes, H-35 rat hepatoma cell line, and in human Hep-G2 cells.

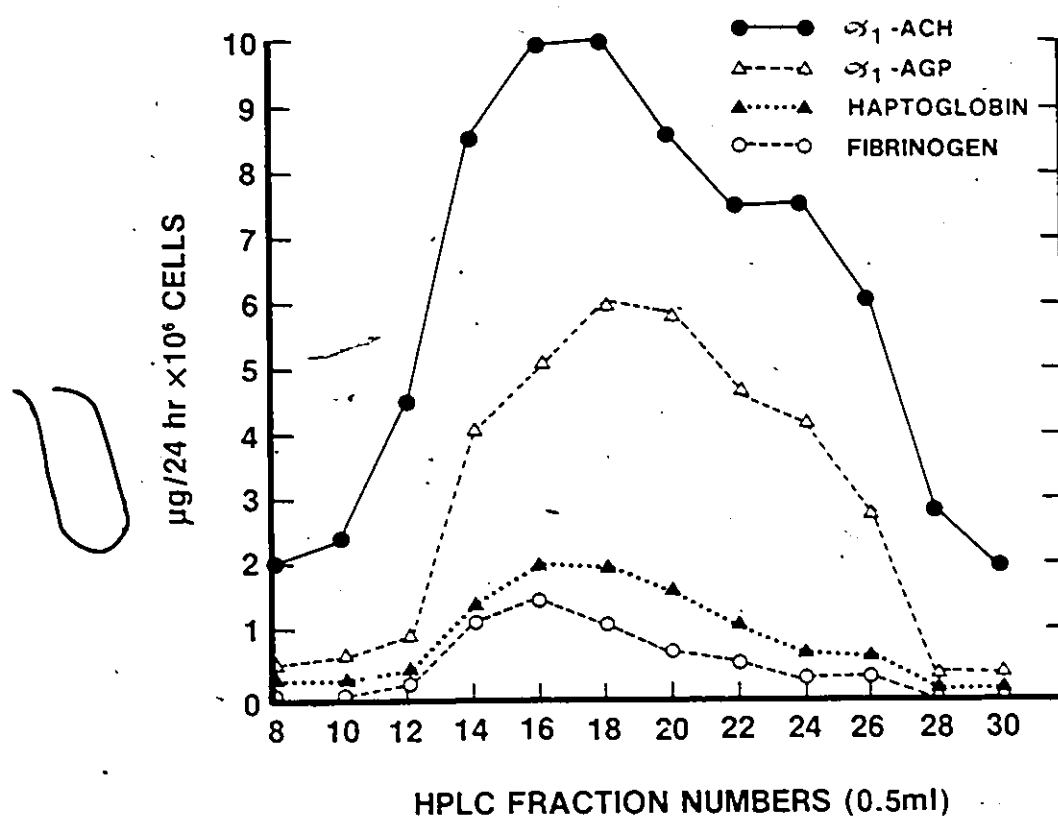


Figure 8: Activity of Fractionated PBM Supernatant on Hep-G2 cells. Fractions from HPLC using the TSK-2000 column were assayed for induction of fibrinogen (○), antichymotrypsin (●), α_1 -AGP (▲) and haptoglobin (△) in Hep-G2 cell culture.

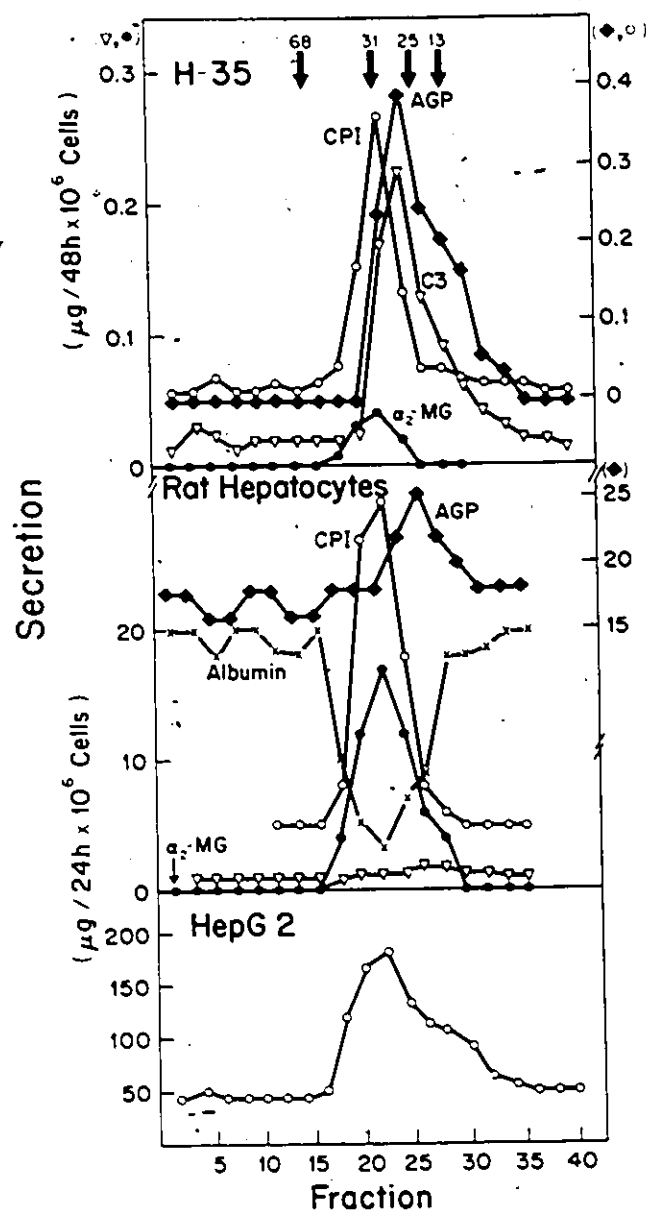


Figure 9: Activity of Fractionated PBM Supernatant on Rat Hepatocytes, H-35 cells and Hep-G2 cells. The same fractions from TSK-3000 HPLC were used to stimulate the three hepatocyte cell types. Molecular weight marker peaks (68, 31, 25 and 13 kD) are shown as arrows on top.

Furthermore , intracellular HSF appears similar in size to extracellular HSF on the basis of its elution from molecular seive HPLC.

Isoelectric point of HSF

The isoelectric point of PBM HSF was estimated by analysis of fractionated PBM on a chromatofocussing column. Fig. 10 shows that peak α_2 -macro and α_1 -CPI stimulation was elicited by molecules eluted at pH 4.8 to 5.2 which is consistent with the findings of Koj et al. (1984) and Fuller & Ritchie (1982). Maximum LAF activity (white bars) was evident in fractions at pH=6.8 to pH=7. This corresponds to the isoelectric point of human IL-1 β (Auron et al., 1984). Although both IL-1 α (pI=5) and IL-1 β are released by PBM, IL-1 β is responsible for 80 to 90% of the LAF- activity in PBM (Oppenheim et al., 1986). Thus the data is consistent with the literature and shows HSF with a pI of 5 to 5.1. α_1 -AGP production by these rat hepatocytes was very high in all samples and no peak of α_1 -AGP was evident at pI=6.8 to 7 as one might expect if indeed IL-1 β stimulated AGP. However, the α_1 -AGP response may have been already maximally stimulated (as might have been the case in molecular sieve HPLC) and therefore further stimulation was not evident.

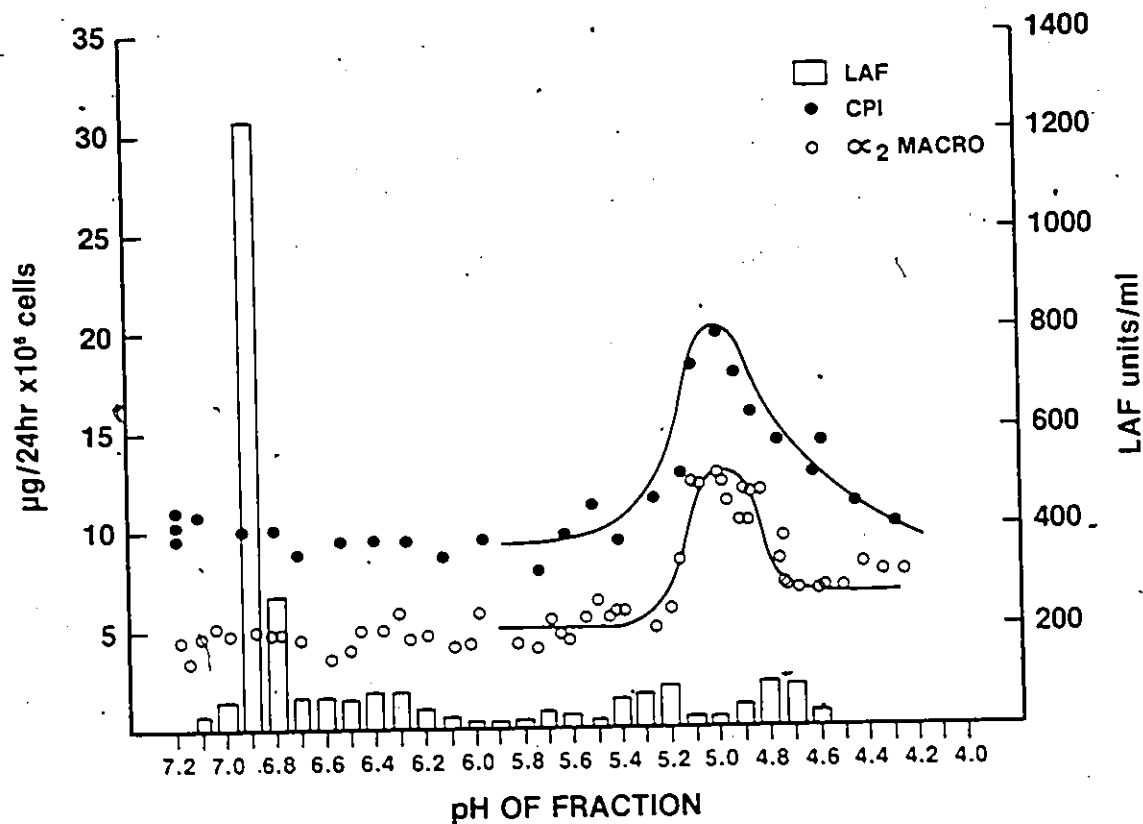


Figure 10: Chromatofocussing of HSF. Fractions from the chromatofocussing column were filtered and the pH measured. Fractions were then dialyzed against PBS (pH 7.2) for two days before assay for induction of α_2 -macroglobulin (○) and α_1 -CPI (●) in the HSF assay. The original pH of the fractions is marked on the x-axis. LAF content of fractions was also measured (white bars).

Although various purification regimes were attempted (which included HPLC separations on anion exchange, hydroxyapatite and molecular sieve columns) and allowed increases in specific activity, the percent yield of HSF activity from crude supernatant was very low (<1%) and impractical for large scale purification. This might have been due to increases in HSF lability once separated from other proteins. Another possibility is that proteases co-eluted with HSF. Cofactors necessary for maximum HSF activity (as detected by in vitro culture) may be separated out. Since the yield of protein was so low (undetectable by total protein assays), analysis of protein homogeneity by standard approaches (such as single band staining in SDS-PAGE) was not successful even with silver staining. A more sensitive method of detection was needed, therefore radiolabelled de novo synthesized PBM cytokines were analyzed.

Analysis by Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Analysis of ³⁵S-methionine radiolabelled protein allowed for more sensitive detection of de novo synthesized proteins. There were numerous PBM products detected in PBM supernatant that were increased upon stimulation by LPS (see Fig. 12 on left). At this level of analysis, multiple bands

were present in the lower molecular weight range between 10 and 60 kd.

To test which band(s) may correspond to HSF, the labelled crude supernatant was separated by HPLC, each fraction lyophilized, tested for HSF and run on SDS-PAGE. Fig. 11 shows that fractions with the maximum α_2 -macroglobulin stimulating activity, contained many labelled proteins. They also contained strong signals in the range between 23 and 30 kd which were not apparent in other fractions. This suggested indirectly that HSF migrated between 23 and 30 kd on SDS-PAGE.

To confirm this, labelled PBM supernatant was electrophoresed in a 12% gel (SDS-PAGE) and HSF activity was assayed in each of several slices cut out of the gel. Fig. 12 shows that maximum HSF eluted from gel slices corresponding to between 25 and 29 kd. α_1 -CPI stimulation corresponded exactly with the α_2 -macro stimulation (data not shown). LAF activity was eluted in two peaks, one at approximately 23 kd, the other larger peak at approximately 17 kd. The migration of LAF is in agreement with the molecular size of IL-1 α and IL-1 β (mature 17 kd, intermediate species 23-24 kd and precursor 31 kd, Auron et al., 1984; Oppenheim et al., 1986). The migration of HSF (maximum between 25 and 27 kd) was consistent with the interpretation of Fig. 11. The reason for the discrepancy between migration on HPLC verses SDS-PAGE is not clear.

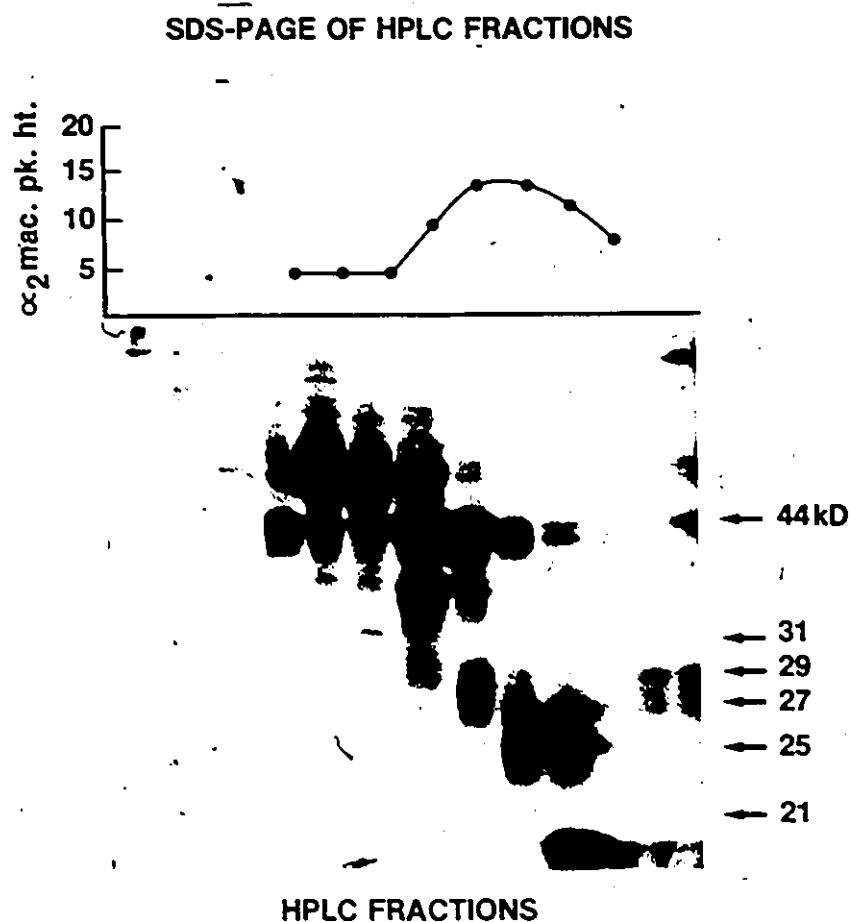


Figure 11: SDS-PAGE of HPLC Fractions. ^{35}S -radiolabelled PBM proteins were fractionated using molecular sieve HPLC (TSK-3000 column) and even fractions were assayed for induction of α_2 -macroglobulin in the HSF assay. Odd fractions were frozen, lyophilized, resuspended in sample buffer and loaded onto a 12% polyacrylamide gel. Molecular weight marker migrations are shown by arrows.

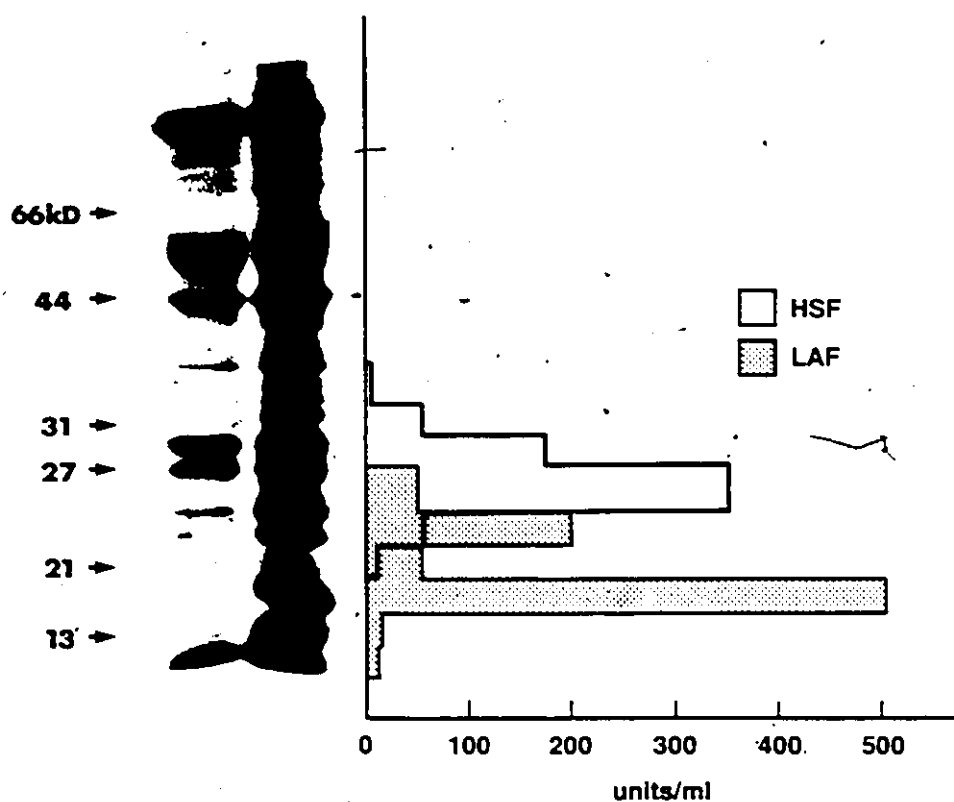


Figure 12: Elution of HSF and LAF from SDS-PAGE Gel Slices. PBM supernatant was loaded in lanes adjacent to S^{35} -radiolabelled PBM (-, no LPS, +, LPS simulated culture) and electrophoresed into a 12% acrylamide gel. Horizontal slices (5 mm thickness) were then assayed for HSF (open box) and LAF (stippled box) content.

Inhibition of HSF by Antibody

Although HSF appeared unique from other known cytokines released from human monocytes such as TNF and IL-1, it became apparent that the characteristics of HSF (30 kd on HPLC, pI=5, 25-29 kd on SDS-PAGE, monocyte-derived) were very similar to those of hybridoma growth factor (HGF) in these properties. (P. Landsorp, personal communication). HGF has shown identity to the "26 kd protein" (Van Damme et al. 1987) which in turn has a cDNA sequence identical to B cell stimulating factor 2 (BSF-2) (Hirano et al., 1986) and interferon β -2 (IFN β_2) (Zilberstein et al., 1986) as described in more detail in Appendix 1. Interferon β_2 had originally been characterized as a product of the fibroblast cell line FS-4. The possibility that HSF shared identity with IFN β_2 was therefore tested. This was accomplished using antiserum to IFN β and by the assay of activity in recombinant material.

A sheep antibody to IFN β (anti-IFN β_2 & β_1) that recognized both IFN β_1 and IFN β_2 and neutralized HGF activity at low concentrations of antibody (P. Landsorp, personal communication) was incubated with crude PBM supernatant and the mixture assayed for HSF on rat hepatocyte and Hep-G2 cell cultures. In addition, a number of antibody preparations to other cytokines were included. These were: rabbit anti-recombinant IL-1 α and rabbit anti-recombinant IL-1 β , rabbit anti-recombinant TNF, rabbit anti-purified

IFN β_1 and rabbit anti- crude PBM supernatant. Fig. 13 shows the actual rocket analysis of a typical experiment and tables 6 and 7 show calculations of APP output.

PBM (at a dilution of 1/20 added to hepatocytes) in lane 1 (fig. 13) produces markedly more of each of the APP than PBS (negative control in lane 7). Preincubation with anti TNF (lane 2) or anti IFN β_1 (lane 3) did not affect the PBM stimulation of any of the APP shown. Anti IL-1 α and anti IL-1 β preincubation (lane 4) had inhibitory activity of α_1 -AGP and haptoglobin induction in Hep-G2 cells (but not complete inhibition) and produced no modulation of α_2 -macroglobulin in rat hepatocytes. Interestingly, preincubation with anti-IL-1 α and anti-IL-1 β (lane 4) produced an enhancement of fibrinogen induction in Hep-G2 cells and enhancement of α_1 -CPI induction in rat hepatocytes. Preincubation with anti-IFN β_2 & β_1 (with both IFN β_1 and IFN β_2 specificity (lane 5) inhibited α_1 -AGP induction partially, haptoglobin partially, but inhibited fibrinogen induction completely in Hep-G2 cells. In rat hepatocytes, anti-IFN β_1 & β_2 inhibited the α_2 -macroglobulin and α_1 -CPI induction to nearly background levels (lane 5) and inhibited the albumin down regulation. Combination of anti IFN β_1 & β_2 with anti-IL-1 α and anti-IL-1 β (lane 6) resulted in complete inhibition of α_1 -AGP, fibrinogen and haptoglobin induction in Hep-G2 cells. In rat hepatocytes, the combination inhibited α_2 -macroglobulin and α_1 -CPI

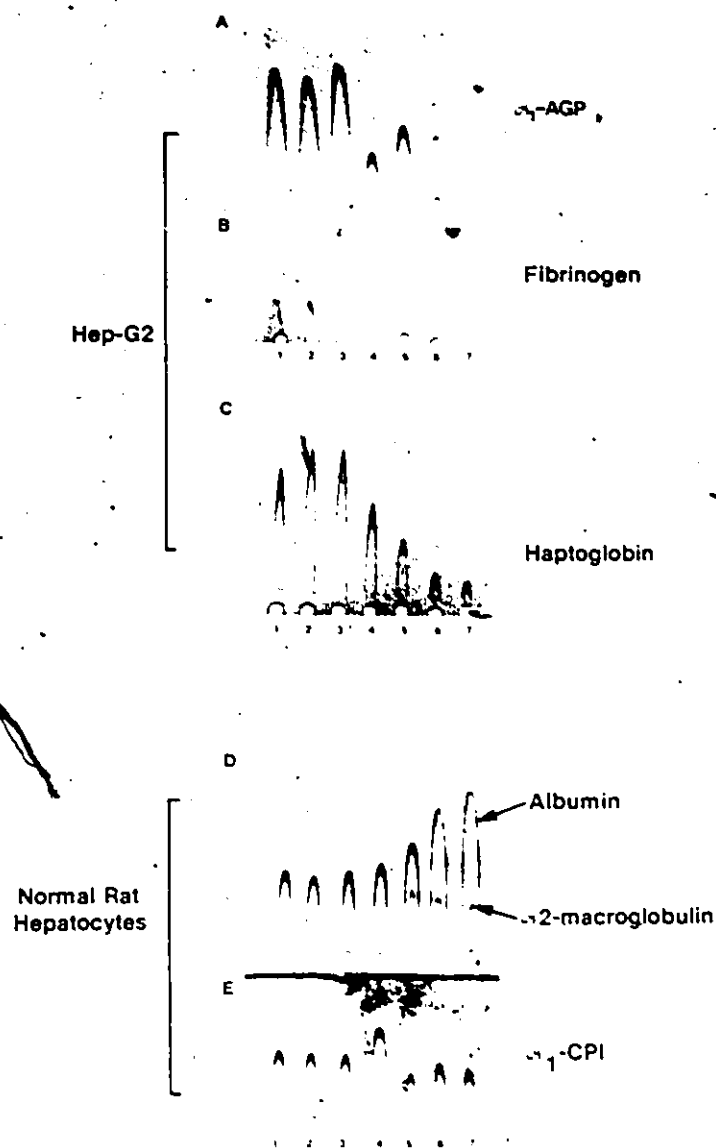


Figure 13: Inhibition of APP Induction by Antibody. PBM supernatant was preincubated with PBS (lane 1), anti-TNF (lane 2), anti-IFN β_1 (lane 3), anti-IL-1 α and anti IL-1 β (lane 4), anti-IFN β_2 & β_1 (lane 5), anti-IFN β_2 & β_1 and anti-IL-1 α (lane 6). Lane 7 represents background APP output (no stimulation). Rockets were run to analyze the APP as marked on Hep-G2 (A,B,C) and rat hepatocytes (D,E).

induction, and albumin down regulation to background levels.

Table 6 presents the results of a similar experiment in terms of absolute amounts of APP produced by rat hepatocytes. Consistent with the results of fig. 13, α_2 -macroglobulin induction by PBM supernatant was inhibited by anti IFN β_1 & β_2 antiserum ($12 \mu\text{g}/10^6 \times 24 \text{ hr}$) as well as anticrude antibody (recognizes many species in PBM supernatant), but not by FCS, anti-TNF, or anti-IFN β_1 ($50 \mu\text{g}/10^6 \times 24 \text{ hr}$). α_1 -CPI induction ($32 \mu\text{g}/10^6 \times 24 \text{ hr}$) was inhibited only by anti-IFN β_1 & β_2 ($22 \mu\text{g}/10^6 \times 24$) and by anticrude ($25 \mu\text{g}/10^6 \times 24$). Anti-IL-1 α and anti-IL-1 β resulted in enhanced ($39 \mu\text{g}/10^6 \times 24 \text{ hr}$) α_1 -CPI induction. Albumin down regulation ($15 \mu\text{g}/10^6 \times 24 \text{ hr}$ in response to PBM) was inhibited partially by anti-IL-1 ($21 \mu\text{g}/10^6 \times 24 \text{ hr}$), partially by anti IFN β_2 & β_1 ($28 \mu\text{g}/10^6 \times 24 \text{ hr}$) and almost completely by the combination of both ($33 \mu\text{g}/10^6 \times 24 \text{ hr}$). The LAF content of the preparations (PBM 1/20 contained 250 units/ml) was inhibited by preincubation with anti IL-1 α and β and anticrude ($<10 \text{ units/ml}$) but not by anti IFN β_2 & β_1 . These results show that anti IFN β_2 & β_1 antiserum inhibited the HSF induction of APP in rat hepatocytes but not the LAF activity, in human PBM supernatant.

Table 7 shows results from the experiment shown in Fig. 13 and is consistent with the above interpretation. Fibrinogen output in response to PBM 1/20 ($0.6 \mu\text{g}/10^6 \times 24$

Table 6: Antibody Inhibition of PBM cytokines (1:20)
Assayed on Rat Hepatocytes

Test Ab	APP output ($\mu\text{g}/10^6$ x 24 hr)			LAF Content
	α_2 -macro	α_1 -CPI	alb	
FCS	52	32	15	250
anti TNF	50	32	15	
anti IL-1	50	39	21	<10
anti IFN β_1	48	32	15	
anti IFN β_2 & β_1	12	22	28	250
anti IFN β_2 & anti IL-1	10	25	33	
anticrude	12	25	35	<10
negative control	7	24	38	<10

Table 7: Antibody Inhibition of PBM cytokines (1:20)
Assayed on Hep-G2 Cells.

Test Ab	APP output ($\mu\text{g}/10^6 \times 24 \text{ hr}$)			
	fibrinogen	α_1 -ACH	HP	α_1 -AGP
FCS	0.6	10.4	2.1	6.0
anti TNF	0.6	9.0	1.8	5.5
anti IL-1	1.4	11.8	1.5	1.4
anti IFN β_1	0.6	10.0	1.4	6.0
anti IFN β_2 & β_1	0.1	5.0	0.8	2.6
anti IFN β_2 & anti IL-1	0.1	4.2	0.4	0.4
negative control	0.1	2.0	0.3	0.3

hr) is inhibited by anti-IFN $\beta_2\&\beta_1$, and by the combination of anti-IFN $\beta_2\&\beta_1$ and anti-IL-1 ($0.1\ \mu\text{g}/10^6 \times 24\text{hr}$). Enhancement of fibrinogen induction (to $1.4\ \mu\text{g}/10^6 \times 24\text{ hr}$) was evident with anti IL-1 preincubation. Haptoglobin and ACH induction were affected markedly by anti-IFN $\beta_2\&\beta_1$ ($10.4\ \mu\text{g}/10^6 \times 24\text{ hr}$ down to 5 for ACH; $2.1\ \mu\text{g}/10^6 \times 24\text{ hr}$ down to .8 for haptoglobin). On the other hand, α_1 -AGP induction ($6\ \mu\text{g}/10^6 \times 24\text{ hr}$) was inhibited partially by either anti-IL-1 ($1.4\ \mu\text{g}/10^6 \times 24\text{ hr}$) or anti-IFN $\beta_2\&\beta_1$ ($0.6\ \mu\text{g}/10^6 \times 24\text{ hr}$) and inhibited completely by the combination ($0.4\ \mu\text{g}/10^6 \times 24\text{ hr}$). Thus, in Hep-G2 cells, anti IFN $\beta_2\&\beta_1$ could inhibit human fibrinogen, α_1 -ACH and HP induction activity and partially α_1 -AGP induction activity in PBM supernatant. Anti-IL-1 inhibited α_1 -AGP induction, enhanced fibrinogen induction, but did not greatly affect ACH or HP inducing activity of PBM cytokines.

Partially purified PBM-HSF preparations (fractions corresponding to 30 kd or pI 5) were tested for inhibition by the antibodies. Both preparations (30 kd, pI 5) contained HSF activity that was inhibited by anti-IFN β and by anti-crude supernatant.

Immunoprecipitation of Radiolabelled Cytokines

Since these results showed that anti-IFN β and anti-IL-1 were neutralizing APP inducing activity, an attempt was made to visualize the size of molecules these antibodies

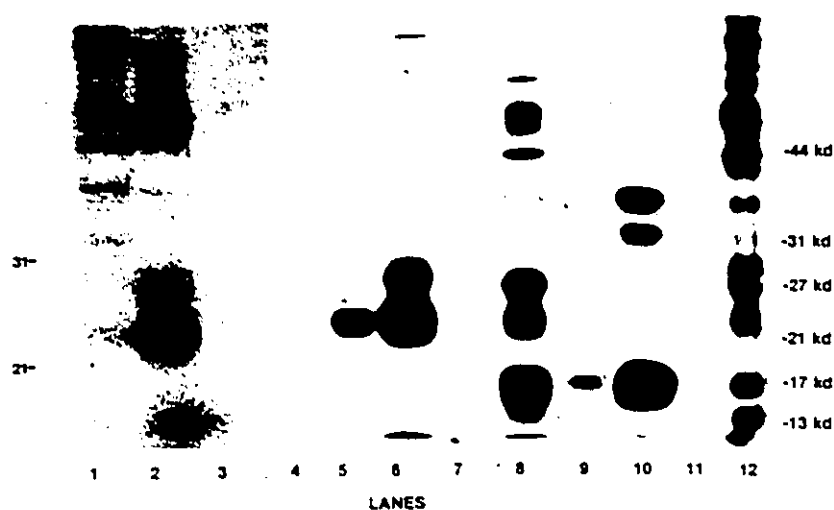


Figure 14: Immunoprecipitation of Radiolabelled Cytokines. S^{35} -radiolabelled PBM cell products were immunoprecipitated with rabbit anti-IFN β_1 (lane 1) bovine anti IFN β_2 & β_1 (lane 2); monoclonal anti-IFN β_1 (lane 3); sheep control antibody (lane 4); sheep anti-IFN β_2 & β_1 (lane 5 & lane 6); normal rabbit serum (lane 7); rabbit anti-crude supernatant (lane 8); rabbit anti-IL-1 α (lane 9) rabbit anti-IL-1 β (lane 10); rabbit anti-TNF (lane 11); (lane 12 contained total PBM products. Samples were run on 12% polyacrylamide SDS-PAGE, dried and autoradiographed.

bound. Fig. 14 shows an autoradiograph of SDS-PAGE analysis of immunoprecipitated, radiolabelled PBM proteins. Control antibody (lane 4) or normal rabbit serum (lane 7) bound very minor bands at high molecular weight but sheep anti- $\text{IFN}\beta_2\&\beta_1$ (lanes 5,6) bound 2 major bands in the 23 to 29 kd range. Rabbit anti-IL-1 α precipitated a band at 17 kd (lane 9). Anti IL-1 β precipitated a very strong signal at 17 kd (lane 10) which was consistent with a greater amount of IL-1 β secreted by monocytes than IL-1 α (Oppenheim et al, 1986) assuming that both products were labelled equivalently and both antibodies bound protein A-sepharose equivalently. Anti IL-1 β also precipitated 31 kd and 37 kd species from PBM (lane 10). Anti TNF antibody precipitated only a very minor species at 17 kd, the molecular size of TNF (lane 11). Anticrude antibody (lane 8), which inhibits both HSF and IL-1 bioactivity in PBM supernatant, precipitated numerous species including 23 to 29 kd bands as well as a 17 kd band. Anti $\text{IFN}\beta_1$ did not inhibit HSF bioactivity and did not show much binding to 23 to 29 kd proteins (lane 1). Bovine anti- $\text{IFN}\beta_2\&\beta_1$ serum showed precipitation of 23 to 29 kd bands as well as higher molecular size species. These results, taken together, show that antibodies that inhibit HSF (anti- $\text{IFN}\beta_2\&\beta_1$, anti-crude) precipitate 23-29 kd proteins, antibodies that inhibit LAF (anti. IL-1, anticrude) precipitate 17 kd proteins, and antibodies to $\text{IFN}\beta_1$, TNF and normal rabbit serum do not

precipitate bands in the 23 to 29 kd range. This suggests (and is consistent with the results of fig. 12) that the molecules responsible for HSF activity migrate in this region and that at least 2 species (approximately 23 and 27 kd) were potential candidates for HSF. This interpretation assumes that HSF is sufficiently labelled with ^{35}S -methionine and that few (if any) contaminating proteins migrate similarly.

To further test the specificity of binding, purified recombinant BSF-2 ($\text{INF}\beta_2$) derived from E. coli was used to compete with labelled PBM proteins in the immunoprecipitation protocol. The recombinant $\text{INF}\beta_2$ (BSF-2) should only bind to specific antigenic binding sites for BSF-2 and thus should compete specifically. Fig. 15 shows that rBSF-2 addition (lane 3) resulted in decrease in binding of both 24 and 27 kd PBM labelled species but that PBS (lane 2) or FCS (lane 4) did not. These results show that recombinant $\text{INF}\beta_2$ (BSF-2) competes with at least 2 species of PBM proteins for binding by anti- $\text{INF}\beta_2\&\beta_1$ antibody. This suggested that the 24 and 27 kd species from human PBM exhibited at least partial identity with human recombinant BSF-2/ $\text{INF}\beta_2$ and supported the suggestion of molecular identity between HSF and $\text{INF}\beta_2$. To further test this hypothesis, recombinant BSF-2 ($\text{INF}\beta_2$) was used in the in vitro hepatocyte assays and examined for APP inducing activity.

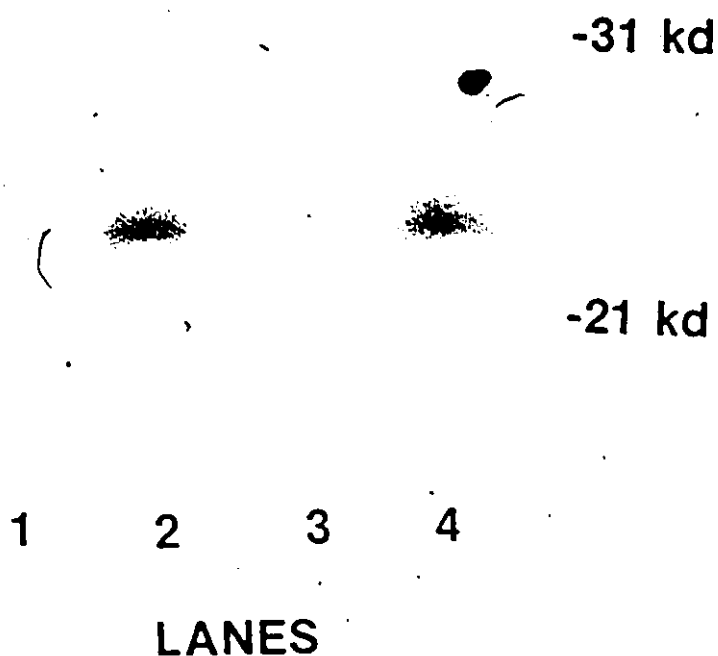


Figure 15: Competition of Binding of Radiolabelled Cytokines by BSF-2 (IFN β_2). Sheep anti-IFN β_2 & β_1 was preincubated with PBS (lane 2); BSF-2 (IFN- β_2) (lane 3) or FCS (lane 4) and then used to immunoprecipitate PBM S³⁵-radiolabelled protein. Lane 1 precipitation contained no antibody. Samples were run on 12% polyacrylamide SDS-PAGE, dried and autoradiographed.

Assay of HSF Activity of Recombinant IFN β_2 /BSF-2

The activity of various dilutions of rBSF-2 is shown in fig. 16 on rat hepatocytes and fig. 17 on Hep-G2 cells. BSF-2 at dilutions which corresponded to BSF-2 units/ml of 160 to 0.4 (assayed by Hirano et al., 1986), resulted in potent α_2 -macroglobulin and α_1 -CPI induction as well as albumin down regulation in rat hepatocytes (fig. 16). Activity was evident at 40 BSF-2 units/ml. In human Hep-G2 cells (fig. 17), BSF-2 units/ml of 1000 down to 16 showed potent induction of haptoglobin, fibrinogen, ACH and α_1 -AT. There was less marked induction of α_1 -AGP and C3 (marked increase only at 1000 units/ml) that was not as high as that induced by conditioned medium from PBM. Thus BSF-2 had potent activity in stimulating rat α_2 -macroglobulin, α_1 -CPI (rat hepatocytes), human haptoglobin, fibrinogen, ACH, α_1 -AT (Hep-G2) but much less activity inducing α_1 -AGP and C3. These results showed that BSF-2 influenced APP in a similar pattern to HSF. Table 8 displays APP output in response to recombinant IL-1 β , recombinant BSF-2/IFN β_2 and partially-purified HSF. Recombinant IL-1 β (250 units/ml) showed strong LAF activity, but was unable to stimulate α_2 -macroglobulin or α_1 -CPI in rat hepatocytes or fibrinogen in Hep-G2 cells. Albumin was reduced somewhat (from 40

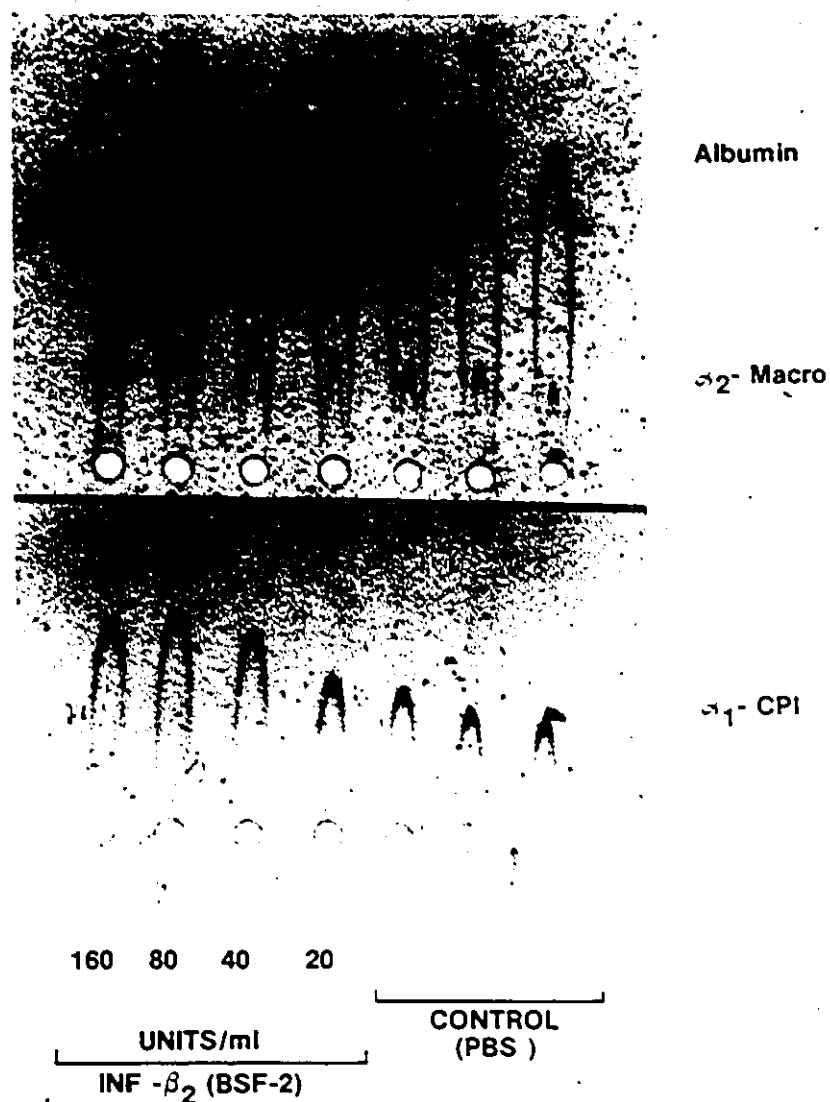


Figure 16: Rocket Analysis of BSF-2/IFN β_2 Effect on Rat Hepatocytes. Various concentrations of purified recombinant BSF-2 were added to rat hepatocyte cultures (A) α_2 -macroglobulin (light precipitin line) and albumin (dark precipitin line) as well as α_1 -CPI (B) were analyzed by rocket electrophoresis. Hepatocytes were stimulated with 160 units/ml (BSF-2 activity), well 1; 80 units/ml, well 2; 40 and 4 units/ml, wells 3 and 4; .4 units/ml, well 5; wells 6 and 7 contain control (non-stimulated) supernatant.

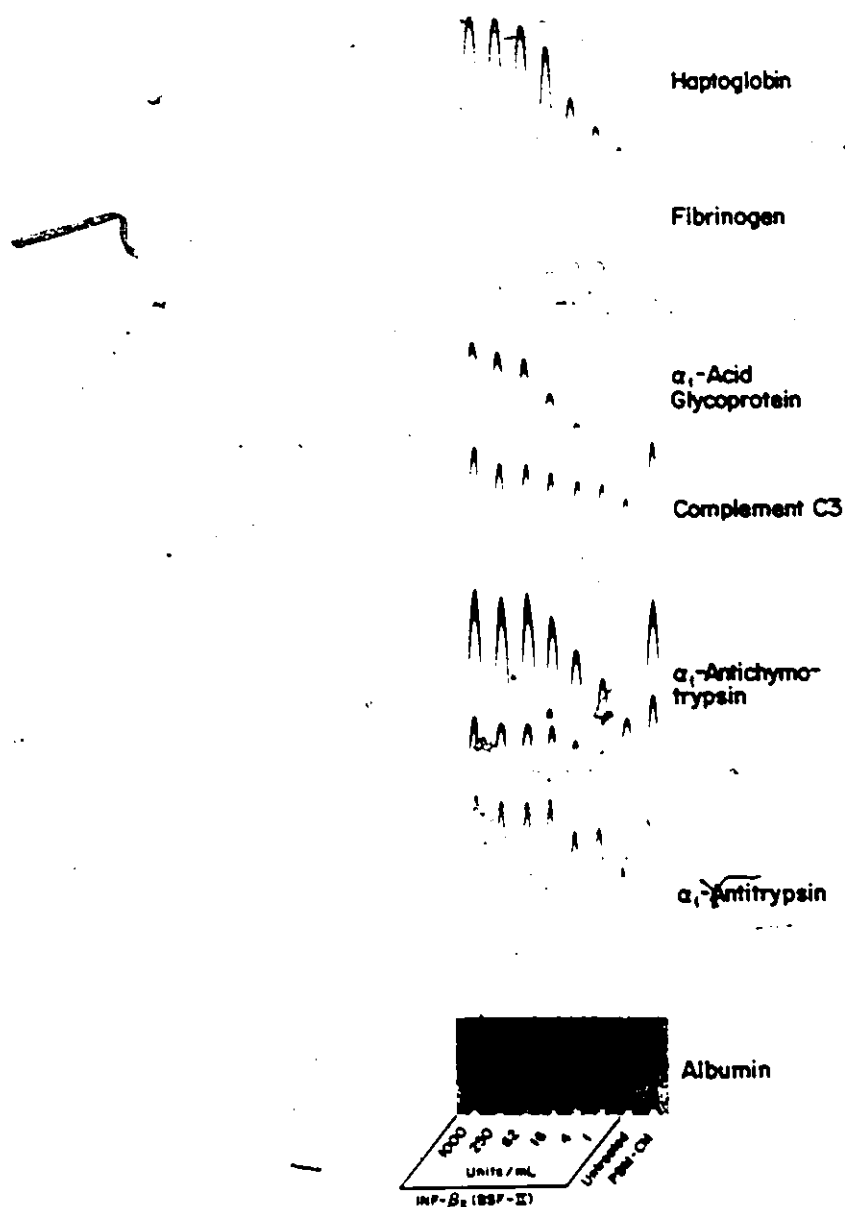


Figure 17: Rocket Analysis of BSF¹2/IFN β_2 Effect on Hep-G2 Cells. Purified recombinant BSF-2 was added to Hep-G2 cells at 1000 units/ml (BSF-2 activity), well 1; 250 units/ml, well 2; 62 units/ml, well 3; 16 units/ml, well 4; 4 units/ml, well 5; 1 unit/ml, well 6; well 7 contained control (unstimulated) supernatant and well 8 contained PBM conditioned medium stimulated supernatant.

Table 8: Activity of Recombinant BSF-2/IFN β_2 and Other Preparations on APP Output

8(a)	Rat hepatocytes (μ g/ 10^6 x 24 hr)			LAF activity (units/ml)
	α_2 -macro	α_1 -CPI	albumin	
HSF (partially purified)	45	35	14	40
r IL-1 β	8	23	33	250
r IFN β_2 /BSF	46	38	14	<10
r IFN β_2 /BSF + anti-IFN β_2	20	28	35	<10
anti-IFN β_2 alone	10	26	40	<10
fibroblast supernatant	42	33	29	<10
control (PBS)	8	25	42	<10
8(b)	Human Hep-G2 (μ g/ 10^6 x 24 hr)			
	fibrin -ogen	ACH	HP	AGP
HSF (partially purified)	1.0	8.0	1.2	1.1
r IL-1 β	<.01	3.1	1.2	1.8
r IFN β_2 /BSF	1.1	10.9	2.2	2.5
r IFN β_2 /BSF + anti-IFN β_2	0.3	3.2	0.3	0.5
anti-IFN β_2 alone	0.2	1.7	0.3	0.2
fibroblast supernatant	1.4	8.0	1.5	0.9

to 33 $\mu\text{g}/10^6 \times 24 \text{ hr}$). On the other hand, recombinant BSF-2/ $\text{IFN}\beta_2$ and HSF (with much lower LAF, <10 and 40 respectively) clearly showed different inducing activities than IL-1 but very similar to each other. α_2 -macroglobulin (46 and 45), α_1 -CPI (38 and 35), and albumin (14 and 14) were stimulated to very similar outputs in rat hepatocytes as were fibrinogen (1.1 and 1.0) and ACH' (10.9 and 8.0) in Hep-G2 cells. Haptoglobin and α_1 -AGP were stimulated by HSF, rIL-1 β and rBSF-2/ $\text{IFN}\beta_2$ in Hep-G2 cells. Antibody to $\text{IFN}\beta_2\&\beta_1$ effectively inhibited the recombinant BSF-2/ $\text{IFN}\beta_2$ activity (row 4) on all APP examined. These results further support the interpretation of identity between HSF and $\text{IFN}\beta_2$ /BSF-2.

Release of HSF by Fibroblasts

Since the results of the antibody inhibition studies, immunoprecipitations and assay of recombinant $\text{IFN}\beta_2$ all suggested identity between HSF and $\text{IFN}\beta_2$, and since fibroblasts are known as a source of $\text{IFN}\beta_2$ (Wiesenbach et al., 1980; Sehgal & Sagar, 1980; Zilberstein et al., 1986; Sehgal et al., 1987), fibroblasts were tested for the release of HSF activity. Table 8(a) shows that crude FIB supernatant induces α_2 -macroglobulin and α_1 -CPI production by rat hepatocytes, and also induces fibrinogen, α_1 -ACH and

Table 9: Comparison of Monocyte and Fibroblast Cytokine Activities

Activity	Monocyte	Fibroblast
rat α_2 -macro induction	250 units/ml	60 units/ml
rat α_1 -CPI induction	++	+++
rat albumin inhibition	++++	++
Hep-G2 fibrinogen induction	+	+++
Hep-G2 α_1 -ACH induction	++++	++++
Hep-G2 HP induction	++++	++
Hep-G2 α_1 -AGP induction	++++	+
LAF	2000 units/ml	<10 units/ml
α_2 -macro induction inhibited by anti-IFN β_2 & β_1	+++	+++
HPLC elution	30 kd	27-30 kd

PBM supernatants and fibroblasts supernatants were compared quantitatively (units/ml) and semi-quantitatively (+, weak; ++, moderate; +++, strong; +++, very strong) in various activities including induction of acute phase protein synthesis by rat hepatocytes and Hep-G2 cells, elution from HPLC, LAF, and modulation by antibody.

haptoglobin synthesis by Hep-G2 cells (table 8(b)). This is a pattern of APP induction consistent with partially purified HSF and recombinant IFN β_2 /BSF-2 activities. FIB supernatant did not contain LAF (<10 units/ml) and furthermore did not induce α_1 -AGP strongly. Fibroblast cultures released HSF constitutively (no LPS stimulation) with 10% FCS present in the culture medium. Table 9 summarizes the characteristics of FIB and PBM cytokine properties. FIB HSF activity (stimulating α_2 -macroglobulin) was inhibited by anti IFN β_2 & β_1 antibody, migrated in the 27 to 30 kd range upon HPLC. Crude fibroblast supernatant stimulated a spectrum of APP in a similar fashion to partially-purified HSF and recombinant IFN β_2 . FIB supernatant had intermediate HP inducing and albumin down regulating activities. FIB supernatant contained no detectable LAF activity, and on a per cell basis, fibroblasts released approximately equivalent amounts of HSF units as PBM.

In Vitro Translation of RNA

Preparations of RNA from PBM and other cell sources of HSF should contain mRNA that codes for HSF molecules. Extractions of RNA from 7×10^8 human PBM and from 6×10^8 adherent alveolar rat macrophages (amo), yielded 550 μ g and 800 μ g of total RNA respectively. Selection for poly A⁺ RNA

resulted in 18 μ g from PBM and 23 μ g from rat alveolar macrophages. These low yields ($30 \mu\text{g}/10^9$ cells) are consistent with previous work (Auron et al., 1984). The poly-A⁺ mRNA was tested in in vitro translation (IVT) systems for the presence of intact mRNA and the presence of mRNA coding for HSF bioactivity. Fig. 18 shows autoradiographs of SDS-PAGE analysis of in vitro translated products. Fig. 18 (a) displays polypeptides translated by the rat alveolar macrophage mRNA (labelled amo) in wheat germ (WG) IVT that are absent in control IVT (no RNA added control, labelled c) and globin RNA directed IVT (labelled globin). Globin RNA directed synthesis of a major band corresponding to the molecular size of rabbit globin (13 kd). Fig. 18(b) shows that PBM mRNA directed synthesis of many species by rabbit reticulocyte lysate IVT (RR) not present in control (no RNA, labelled c) or globin RNA IVT. The bands appear to be relatively sharp suggesting the mRNA is biologically active.

The IVT products were assayed on rat hepatocytes for HSF activity. Fig. 19 shows that PBM IVT products contained marginal but evident α_2 -macroglobulin inducing activity, (PBM), and rat alveolar macrophage (amo) mRNA IVT products showed a greater more definite α_2 -macroglobulin induction. Control IVT (con) and globin IVT products (globin) did not stimulate α_2 -macroglobulin above the background hepatocyte culture output. Thus the HSF activity appeared to be

specifically translated from PBM mRNA and amo mRNA.

Northern Analysis of PBM and FIB RNA

Since the experiments had shown strong evidence of identity between HSF and $\text{IFN}\beta_2$, to further test the existence of $\text{IFN}\beta_2$ production by PBM, the PBM mRNA was tested for presence of RNA that hybridized to a probe for $\text{IFN}\beta_2$. A plasmid probe, provided by Dr. P. Sehgal (Rockefeller University) that contained approximately 3/4 of a full length $\text{IFN}\beta_2$ cDNA (May et al., 1986; Sehgal et al., 1987) was used in Northern blot analysis. In fig. 20, three different preparations of PBM RNA (lanes 1,2,3) showed a strong signal corresponding to approximately 1.3 kilobase (kb) in size. One of the fibroblast RNA preparations provided by Dr. G. McSharry (McMaster University, Hamilton, Ont.) from human lung fibroblasts displayed a strong signal in the 1.3 kb size range and another two preparations showed a weak but evident signal. The bands were not sharp suggesting that the RNA was partially degraded since the size marker bands were discrete. These results confirm the presence of mRNA species in both PBM and fibroblasts that hybridize to this $\text{IFN}\beta_2$ cDNA probe. This suggests that $\text{IFN}\beta_2$ mRNA is present in PBM as well as in FIB.

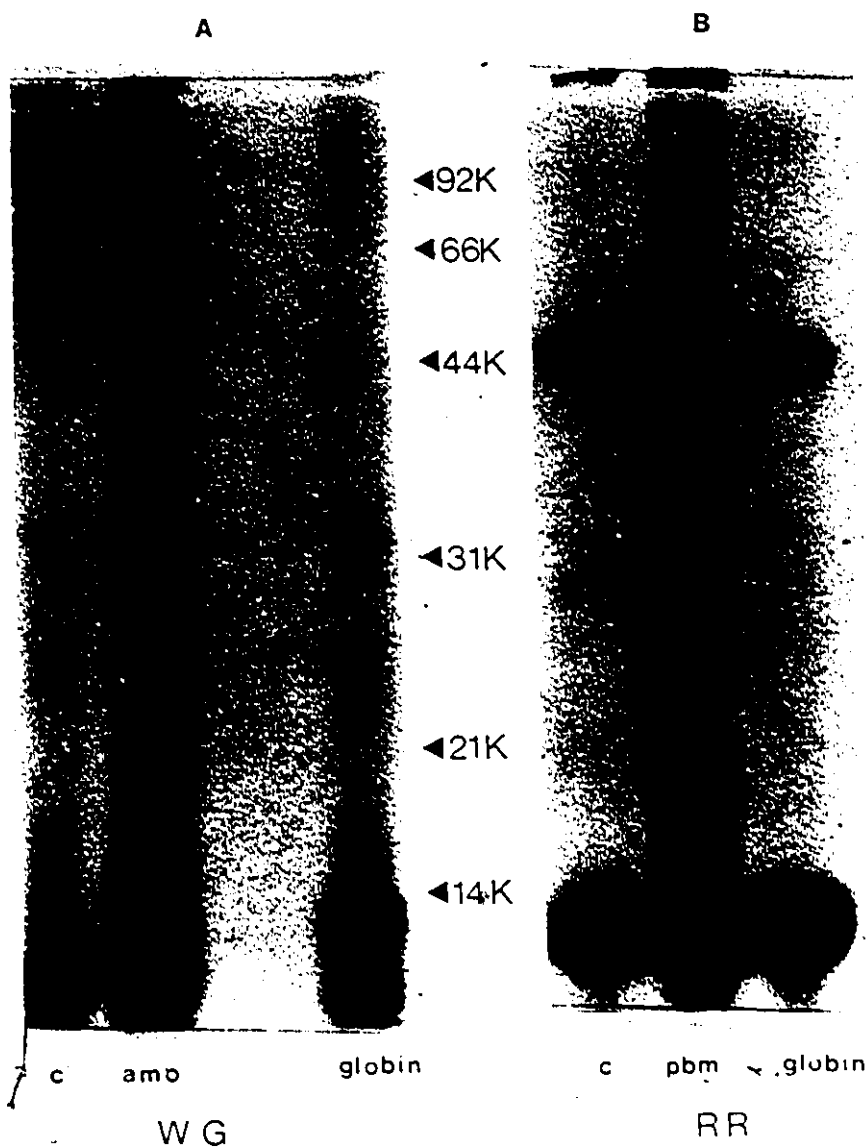


Figure 18: SDS-PAGE of In Vitro Translated Products. Preparations of mRNA from rat alveolar macrophages (amo), human PBM (pbm) and rabbit globin were used to program in vitro translation in (a) wheat germ lysate (WG) or, (b) rabbit reticulocyte lysate (RR). Migration of molecular weight markers (shown by arrows) were run alongside the samples in a 12% polyacrylamide gel which was dried, fixed and exposed to x-ray film.

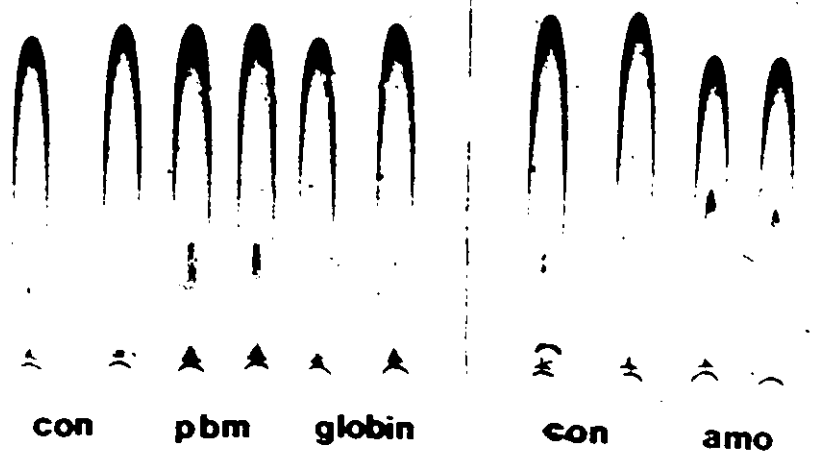


Figure 19: HSF Assay of In Vitro Translated Products. In vitro translated products were used to stimulate rat hepatocytes in duplicate in the HSF assay. α_2 -macroglobulin (light precipitin lines) and albumin (dark precipitin lines) were analyzed in response to translations programmed by human PBM mRNA (pbm), rat alveolar macrophage mRNA (amo), rabbit globin mRNA (globin) and no mRNA (con).

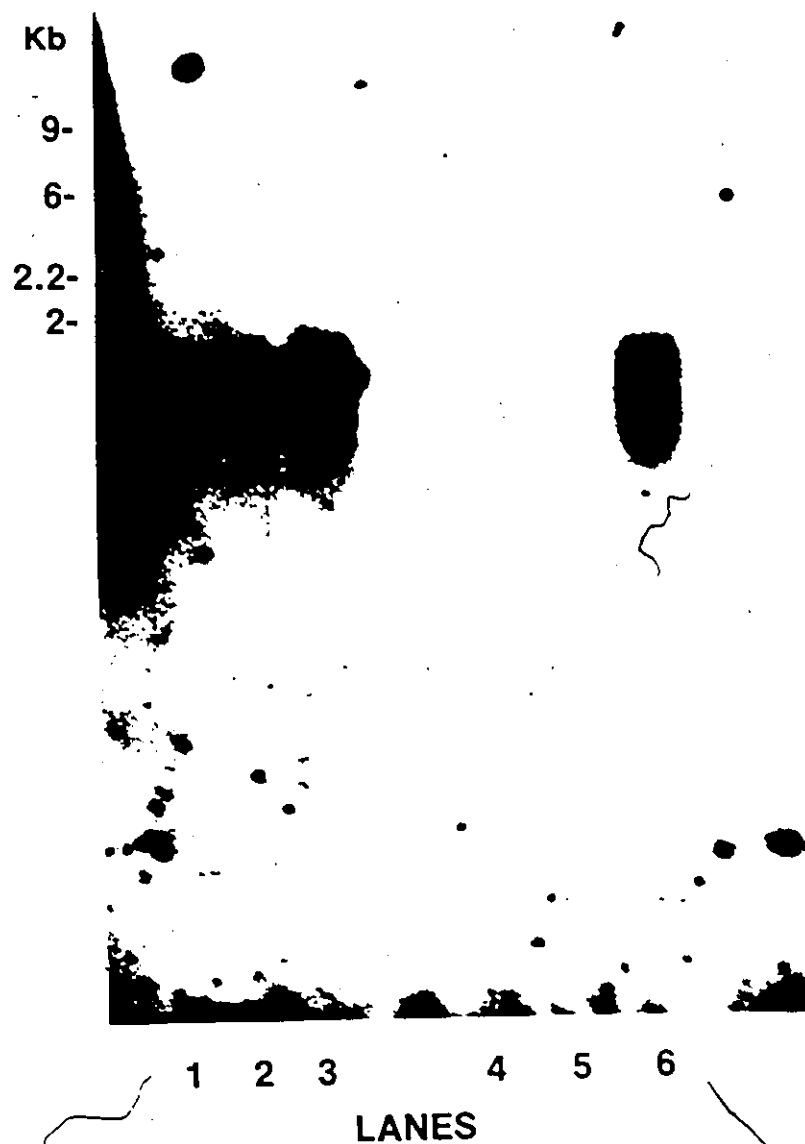


Figure 20: Northern Blot of PBM and FIB RNA. Three preparations of PBM mRNA (lanes 1, 2 and 3) and 3 fibroblast mRNA preparations (lanes 4, 5 and 6) were probed with the IFN β_2 cDNA-containing plasmid. Migration of size markers are shown on left.

DISCUSSION

The acute phase response encompasses a number of physiologic events that occur in response to injury, infection and tissue damage. As part of the acute phase response there are increases in hepatocyte synthesis and output by the liver of several proteins termed acute phase proteins (APP). Some acute phase proteins appear to function in modulating inflammation. The mechanisms involved in the control of this liver response have not been fully elucidated. However, it does appear that soluble mediators or cytokines such as Hepatocyte Stimulating Factor and Interleukin-1 (released at the site of inflammation by cells of the monocyte/macrophage lineage) have a role in initiating the acute phase response. The analysis of the liver response in vitro has shed light on the nature of the cytokine-induced initiation of liver acute phase protein synthesis.

Evidence is provided showing that the previously cloned molecule human Interferon β_2 (IFN β_2) shows functional and immunologic identity with human peripheral blood monocyte derived Hepatocyte Stimulating Factor (PBM HSF) and is likely the molecule responsible for the major induction of the acute response by the liver. This represents a significant step in the analysis of the acute phase response. The usefulness of in vitro HSF assays are

highlighted in determining the roles of HSF/IFN β_2 and Interleukin-1 (IL-1) in stimulating acute phase protein synthesis by rat hepatocytes and human Hep-G2 cells. The main findings presented include:

- 1) PBM HSF elutes at 30 kd and at pI=5 upon chromatographic separation
- 2) PBM HSF migrates at between 23 and 29 kd in SDS-PAGE
- 3) PBM HSF bioactivity is inhibited by antisera to IFN β_2 & β_1 but not by antisera to IL-I, TNF or IFN β_1
- 4) recombinant purified IFN β_2 /BSF-2 has potent APP inducing activity of identical nature to HSF and this APP synthesis stimulating activity is inhibited by anti-IFN β_2 & β_1
- 5) anti-IFN β_2 & β_1 specifically immunoprecipitates 24 and 27 kd proteins from PBM supernatants and this precipitation is inhibited by recombinant IFN β_2 /BSF-2
- 6) partially purified HSF, IFN β_2 /BSF-2 and fibroblast supernatant stimulate rat α_2 -macroglobulin and α_1 -CPI, and human fibrinogen and α_1 -ACH maximally whereas recombinant purified IL-I stimulates human α_1 -AGP maximally
- 7) IL-I appears to inhibit rat α_1 -CPI and human fibrinogen induction by HSF
- 8) PBM supernatant contains HSF/IFN β_2 /BSF-2 and IL-I

whereas FIB supernatant contains only HSF/IFN β_2 /BSF-2 with regards to APP-inducing cytokines

- 9) both fibroblast and PBM mRNA contain sequences that hybridize to IFN β_2 specific cDNA sequences.

The Hepatocyte Stimulating Factor Assays

The value of analysis of both rat hepatocyte and human Hep-G2 cultures was evident in these experiments. The rat hepatocyte output of acute phase proteins in response to cytokines correlated with APP changes seen in vivo with respect to increases in α_2 -macroglobulin and α_1 -CPI in rat serum. The in vitro results were based on relative differences within an HSF assay, which were clearly evident. α_2 -macroglobulin showed 700% increases upon stimulation by crude PBM supernatant (table 3a) or fractionated cytokines (fig.7). α_1 -CPI increased 20% (table 3a) in response to crude supernatant and 50% in response to separated PBM cytokines. However, only slight increases in rat α_1 -AGP synthesis by rat hepatocyte cultures were observed. This may have been due to an elevated basal level of α_1 -AGP production caused by the hepatocyte isolation procedure or by the incorporation of DEX in culture medium. Others have shown that DEX increased α_1 -AGP expression by rat hepatocytes in vitro and in vivo (Baumann et al., 1983a; Baumann et al., 1984; Gross et al., 1984). DEX has also been

shown to be necessary for α_2 -macroglobulin expression in rat hepatocytes (Koj et al., 1984; Baumann et al., 1984; Bauer et al., 1987). Since others have shown increases in mRNA of APP in response to cytokines (Ramadori et al., 1985; Baumann et al., 1983a, 1984), the increased APP in media was assumed to be due to changes in transcription and synthesis, not simply secretion of presynthesized protein. Baumann et al. (1984) have examined the stimulation of APP by the use of radio-incorporation studies to detect de novo synthesis.

The Hep-G2 cultures proved appropriate for examining human fibrinogen, α_1 -ACH, α_1 -AGP, haptoglobin and α_1 -AT expression. The amount of secreted protein was much lower than primary rat hepatocyte cultures (table 3b). This may have reflected differences due to species (human and rat) or more likely, a limitation of the use of hepatoma cells in general since rat hepatoma cells acted similarly. Figure 9 shows maximum output of less than $0.4 \mu\text{g}/10^6$ cells x 48 hr for rat α_1 -AGP, α_1 -CPI and α_2 -macro in H-35 cells compared to stimulated rat primary culture hepatocyte output of 30 and $50 \mu\text{g}/10^6$ cells x 24 hr for α_1 -CPI and α_2 -macro. Hep-G2 cells do not secrete detectable CRP or SAA, two major APP in man. This may be due to abnormal gene regulation in hepatomas or that human hepatocytes in vivo are exposed to other factors necessary for production of these two proteins. However, Goldman and Liu (1987) showed CRP increases in response to PBM supernatant in PLC/PRF/5 human

hepatoma cells. Darlington et al. (1986) showed low but detectable levels of CRP in Hep-3B2 cells stimulated with PBM cytokines. Thus Hep-G2 cells are useful for examining some but not all APP in humans.

The Nature of Hepatocyte Stimulating Factor

Crude PBM conditioned media produced potent APP inducing activity (α_2 -macroglobulin, α_1 -CPI, table 3a) in rat hepatocytes and Hep-G2 cells (fibrinogen, α_1 -AGP, α_1 -ACH, haptoglobin, table 3b) that was consistent with other studies (Fuller & Ritchie, 1982; Ritchie & Fuller, 1983; Baumann et al., 1983a; 1984; Darlington et al., 1986; Bauer et al., 1984). This stimulation of α_2 -macroglobulin secretion was used as a marker of HSF activity since it was apparently enhanced only by the 30 kd cytokine from PBM (see fig.7) as seen previously (Koj et al., 1984; Koj et al., 1985). Although α_1 -CPI was stimulated by 30 kd cytokines (50% increase), inhibitors in crude PBM were evident since separation revealed greater α_1 -CPI induction than unfractionated PBM supernatant.

HSF activity in crude supernatant was stable at 55°C, labile at 80°C, destroyed by protease incubation but not appreciably affected by reducing agents (table 4). Ritchie and Fuller (1982) also found that HSF activity was stable at 56° and was not affected by the reducing agent dithiothreitol. Purified BSF-2 (IFN β_2) activity was eluted

from SDS-PAGE under reducing as well as non-reducing conditions (Hirano et al., 1985) which is consistent with the properties of HSF shown here.

Maximum PBM HSF activity was released at 3-4 hours after in vitro LPS stimulation (fig.5). This suggested that mRNA levels for HSF were maximal around this time and thus RNA was harvested at approximately 3 hours. Other studies examining the time course of release of HSF (IFN β_2 /HGF) from monocytes are not yet apparent. In examining the release of HSF, various cells of the monocyte/macrophage lineage were found to produce HSF (table 5) whereas cell lines such as THP-1 and SK-hep1 produced little HSF. Others have shown that these cells secrete copious amounts of IL-1 (Oppenheim et al., 1986; Doyle et al., 1986) suggesting differential release of HSF and IL-1. Similar observations of differential release of HSF and IL-1 from mouse P388-D1 cells (Woloski & Fuller, 1985), as well as alveolar macrophages from bleomycin-treated rats (Jordana et al., 1987) support the concept of a distinct nature of HSF from IL-1.

PBM derived HSF was maximally detectable in fractions corresponding to 30 kd upon HPLC separation (fig. 7) and at pI=5 upon chromatofocussing (fig. 10). This is consistent with data of others (Koj et al., 1984; Ritchie & Fuller, 1983) and shows difference of HSF peaks from the predominant form of LAF in human PBM supernatants (which is IL-1 β , 17-23

kd pI=7) using these techniques. This is also consistent with 30 kd, pI=5 characteristics of $\text{IFN}\beta_2$ as shown by Aarden et al (1985) for HGF activity from monocytes, and by Hirano et al. (1985) for BSF-2 activity from T-cells. HSF was found to exist intracellularly (from cell lysates) as a 30 kd cytokine. IL-1 existed in multiple forms in cell lysates including sizes of 13-20 kd and 40-45 kd which was consistent with previous work by others (Lepe-Zuniga et al., 1985; Matsushima et al., 1986c) and further suggested that HSF was separate from IL-1. However, there was considerable cross-contamination of LAF (IL-I) in HSF fractions (fig. 7) and various other de novo synthesized PBM products (fig. 11). The possibility that HSF was a high molecular weight form of IL-I or a dimer or a precursor for IL-I or $\text{TNF}\alpha$ could not be definitively ruled out solely on the basis of this data.

Purification of HSF was hampered by the very low recovery of activity after chromatographic separation. Although the activity was relatively stable as a crude supernatant, HSF may have been labile in the absence of other proteins. Due to the need of very large volumes of starting material (human blood), purification of human PBM HSF on a large scale was impractical.

With the recognition of similar characteristics between HSF and the monocyte-derived hybridoma growth factor (HGF) which has been shown to be $\text{Interferon}\beta_2$, a series of

studies were undertaken to examine the relationship between HSF and IFN β_2 .

The -Relationship of Hepatocyte Stimulating Factor to Interferon β_2

The inhibition of HSF bioactivity by two different sources of antisera to IFN β_2 & β_1 but not to IFN β_1 , TNF or IL-I (fig. 13 and tables 6, 7) suggests that HSF and IFN β_2 share immunologic determinants. Anti IFN β_2 & β_1 inhibited α_2 -macroglobulin, α_1 -CPI induction and albumin inhibition in rat hepatocytes as well as human fibrinogen, α_1 -ACH, haptoglobin induction in Hep-G2 in a potent manner. The anti-IFN β_2 & β_1 preparations were not monospecific since they precipitated more than one species of protein from PBM (fig. 14) and are known to recognize both IFN β_2 and IFN β_1 (Seghal et al., 1987). However, since both IFN β_2 & β_1 antisera (from sheep and cow), but not a specific anti-IFN β_1 , bound to species in the 23 to 29 kd range (fig. 14) and HSF bioactivity could be eluted from gel slices in this region, the 24 and 27 kd bands were possible candidates for the HSF molecule(s). The 24 and 27 kd bands were similar in size to the 22 and 26 kd proteins seen by Content et al. (1982) and Haegmann et al. (1986) in their analysis of the 26 kd protein secreted from fibroblasts. Recently it has been recognized that the cDNA sequence of the 26 kd protein, BSF-2 and IFN β_2 were identical (Billiau, 1986;

Billiau, 1987; Seghal et al., 1987) (see appendix 1).

To further test the identity of $\text{IFN}\beta_2$ and HSF, purified recombinant $\text{IFN}\beta_2$ was employed. BSF-2 (or $\text{IFN}\beta_2$) was kindly supplied by Dr. Kishimoto (Osaka University, Japan) and tested in the HSF assays. BSF-2/ $\text{IFN}\beta_2$ was potently active on rat hepatocytes and Hep-G2 (fig. 16, 17 and table 8a and 8b) at 40 to 160 units/ml of BSF-2 activity and stimulated similar APP production as did PBM derived HSF. The anti $\text{IFN}\beta_2\&\beta_1$ antiserum totally inhibited the APP induction by BSF-2/ $\text{IFN}\beta_2$ (table 8). Furthermore, BSF-2/ $\text{IFN}\beta_2$ was found to specifically compete with both the 24 and 27 kd PBM derived proteins for binding with the antibody (fig. 15). Assuming that purified recombinant BSF-2/ $\text{IFN}\beta_2$ (expressed in E. Coli) is free of human PBM products, this indicates at least partial immunologic identity between $\text{IFN}\beta_2$ (BSF-2) and HSF. These data suggest that PBM supernatant contains two species with at least partial antigenic identity to $\text{IFN}\beta_2$ and that both may be biologically active in HSF assays since maximum activity eluted from gel slices corresponded to molecular weight between these two bands and was spread over the 23 to 29 kd range. These two species may be equivalent to the 22 and 26 kd protein species from monocytes as studied by Content et al. (1982) and Haegman et al. (1986), and thus may represent different degrees of glycosylation - or degrees of peptide cleavage of the same gene product ($\text{IFN}\beta_2$). Glycosylation

may not be essential for bioactivity since the E. coli derived recombinant IFN β_2 /BSF-2 was very active.

These various data combined, strongly suggest that PBM HSF and IFN β_2 /BSF-2 are identical. Confirmation of this identity could occur through independent purification and/or cloning of HSF and comparison of the amino acid sequences or nucleic acid sequences. IFN β_2 cDNA has been cloned and sequenced by several groups (Appendix 1) and expressed in E. coli as well as chinese hamster ovary cells. Molecular characteristics of the cloned product revealed features consistent with properties of HSF and the 26 kd protein. IFN β_2 consists of a 212 AA precursor with a typical hydrophobic signal sequence that is cleaved to 184 AA with 2 potential glycosylation sites. This predicts species of molecular weights ranging from 19 kd to 23-26 kd depending on the degree of glycosylation. IFN β_2 from T cells appears to be an unglycosylated 19 kd form (Hirano et al., 1985) but is apparently higher in molecular weight when derived from monocytes and fibroblasts (Haegmann et al., 1986; Zilberstein et al., 1986). The cDNA sequence shows some homology to IFN β_1 at the carboxy-terminal (Hirano et al., 1986) which may explain the cross-reaction of anti-IFN β antibodies to both β_1 and β_2 species. It is possible that the limited homology of the amino-terminal sequences to G-CSF means that the growth stimulatory effects for B-cells may reside at the amino-terminal portion of the protein.

Further studies are necessary to determine the particular portion of the molecule that is important for HSF activity but it appears that the site that confers interferon activity may not be involved since antibody that inhibited IFN did not inhibit HSF.

Since the data shows that $\text{IFN}\beta_2$ /BSF-2 has HSF activity, then HSF should have B-cell stimulating activities. This would further support the identity of HSF and $\text{IFN}\beta_2$. Although these experiments are still to be completed, Rossen et al. (1985) have previously shown that human monocyte derived products that eluted at 30 kd upon HPLC were capable of stimulating immunoglobulin synthesis by B-cell lines. This activity was similar to that described for BSF-2/ $\text{IFN}\beta_2$ by Hirano et al. (1985).

Effect of Hepatocyte Stimulating Factor/Interferon β_2 and Interleukin-1 on Hepatocytes

Various data presented here show that HSF/ $\text{IFN}\beta_2$ maximally stimulates a spectrum of APP distinct from IL-1. Table 8 shows that partially-purified HSF from PBM, and purified recombinant $\text{IFN}\beta_2$, strongly stimulate α_2 -macroglobulin and α_1 -CPI in rat hepatocytes as well as human fibrinogen, α_1 -ACH and (moderately) HP in Hep-G2 cells. IL-I (recombinant) stimulates human α_1 -AGP and C3 predominantly (Hep-G2 cells) but not the others. Figure 17 shows that although HSF/ $\text{IFN}\beta_2$ does stimulate α_1 -AGP and C3,

it does so at much higher concentrations than needed for α_1 -ACH and fibrinogen. This differential spectrum of APP induction by IL-I and HSF/IFN β suggest that each acts in separate pathways to alter gene expression in hepatocytes. IL-I may act on receptors on hepatocytes similar to those described on other cells (Matsushima et al., 1986a) whereas HSF/IFN β_2 may act on distinct hepatocyte receptors. There is evidence that IFN β_2 acts at a similar receptor as IFN β_1 to enhance antiviral activity (Le & Vilcek, 1987) but the lack of IFN β_1 action on hepatocytes (Bauer et al., 1985) and the lack of HGF activity of purified IFN β_1 (Van Damme et al., 1987a) suggest that use of a common receptor does not occur in APP induction. The ability of HSF to cross species barriers (table 5) suggests that the putative hepatocyte receptor for HSF/IFN β_2 was able to recognize homologous structures of the molecule derived from different species. HGF activity of IFN β_2 was also able to cross species barriers (Van Damme et al., 1987b). The events that take place after the putative receptor-ligand interaction are still to be elucidated.

IL-I appeared to have an inhibitory effect on human fibrinogen induction in Hep-G2 and rat α_1 -CPI induction in rat hepatocytes. α_1 -CPI output was enhanced upon separation of HSF by HPLC from other molecules in crude PBM supernatant. Furthermore, antisera to IL-I allowed increased output of rat α_1 -CPI and human fibrinogen (fig.

13) in response to crude PBM supernatant. Finally, assay of recombinant IL-1 β on Hep-G2 cells resulted in a marked decrease in fibrinogen output compared to control (table 8). Koj et al. (1987) have recently reported a similar phenomenon of recombinant IL-1 β inhibition of rat α_1 -CPI output in response to crude cytokines. Other authors have shown that recombinant IL-1 α and IL-1 β did not induce rat α_2 -macroglobulin, haptoglobin or α_1 -AT APP production by normal rat hepatocytes in vitro (Koj et al., 1987). Human fibrinogen and CRP production by human hepatoma cells was not stimulated by recombinant IL-1 (Darlington et al., 1986; Goldman & Liu, 1987) however crude PBM supernatant showed strong induction of these APP. These results taken together, suggest that IL-1 itself does not induce particular acute phase proteins but that another component in PBM supernatant is responsible for a major portion of activity.

The presence of HSF/IFN β_2 in PBM supernatant probably represents the 30 kD CRP-inducing activity of PBM cytokines seen by Goldman & Liu (1987) as well as a 30 kD factor that stimulated B cells as seen by Rossen et al. (1985) and possibly numerous other earlier studies using crude PBM or leukocyte supernatants. The combination of anti IFN β_2 & β_1 with anti IL-1 removed essentially all APP inducing activity found in PBM supernatants as assayed on Hep-G2 and rat hepatocytes (fig. 13, tables 6 and 7) suggesting that HSF/IFN β_2 and IL-1 are the two cytokines responsible for (PBM

supernatant hepatocyte-specific activity in vitro.

If the modulation by IL-I of Hep-G2 APP output reflects in vivo activity, it is interesting that IL-I acts on liver cells to increase α_1 -AGP (inhibition of platelet aggregation, Snyder & Coodley, 1976) and decrease fibrinogen both of which effects could inhibit platelet plug formation. HSF/IFN β_2 might therefore have opposite effects. One might predict then that other APP involved in fibrinolysis such as human α_2 -antiplasmin (a positive APP) would be inhibited by IL-I in vitro and enhanced by HSF/IFN β_2 . However, these isolated effects of IL-I may not be important in vivo since both human fibrinogen and rat α_1 -CPI are positive APP during inflammatory stimuli. If the IL-I response by isolated hepatocytes and hepatomas are not due to phenotypic changes during isolation procedures or culture conditions, one could speculate that the in vivo serum response reflects a dominance of HSF/IFN β_2 over IL-I mediated effects in vivo on liver APP induction. This could be due to concentration differences during inflammation and/or differences in receptor affinities that bind these molecules.

Both IL-I and HSF/IFN β_2 caused a decrease in albumin synthesis by hepatocytes in culture (fig. 7, tables 3 and 8) and this is consistent with decreased serum albumin concentrations seen in vivo in inflammation (Jamieson et al, 1983; Schreiber et al., 1982) as well as decreased albumin mRNA expression in vivo and in vitro (Baumann et

al., 1983a; Ramadori et al. 1985; Birch and Schreiber, 1986). Whether this is due to specific modulation of albumin gene transcription or to nonspecific effects of a switch to APP production is not clear. IL-I and HSF may act by similar mechanisms on modulation of albumin but if so probably activate a particular pathway through different receptor-ligand interactions.

The various modifications of APP expression suggest that multiple systems of gene regulation are present in hepatocytes. Rat α_1 -CPI was increased by HSF but decreased by IL-I, α_2 -macroglobulin was increased by HSF and glucocorticoid presence was necessary, human α_1 -AGP was increased by IL-I and DEX, and albumin was inhibited by both IL-I and HSF. These various responses may have been rendered specific by particular responsive regions in the DNA. Factor B mRNA transcriptional response to IL-I appeared to depend on 5' flanking regions of the Factor B gene (Perlmutter et al., 1986b). α_1 -AGP gene transcription appeared to utilize 5' flanking regions in response to glucocorticoid (Baumann & Maquat, 1986b). The requirement of α_2 -macroglobulin expression for DEX may reflect such glucocorticoid response elements that act in a permissive fashion. With the increased availability of purified cytokines and the identification of HSF as IFN- γ , a more detailed analysis of gene regulation in hepatocyte APP synthesis will be more readily accomplished.

Control of the Acute Phase Response of Liver

The initiation of the acute phase response was thought to be controlled primarily by cytokines released at the site of inflammation by cells of the monocyte/macrophage lineage. Both HSF and IL-1 are produced by these cells. However, since other cells release $\text{IFN}\beta_2$ in culture such as fibroblasts, supernatants from these cells were tested for HSF and were found to contain potent activity in stimulating APP synthesis.

Fibroblast supernatant produced a similar spectrum of maximum APP induction to $\text{IFN}\beta_2$ (table 8) and this was strongly inhibited by anti- $\text{IFN}\beta_2/\beta_1$ antibody, but not anti-IL- α & β . FIB supernatant did not contain appreciable LAF activity and accordingly did not affect α_1 -AGP output by Hep-G2 and had potent fibrinogen stimulating action. Thus the fibroblast appeared to release significant levels of the APP-inducing cytokine, HSF/ $\text{IFN}\beta_2$. Since fibroblasts release $\text{IFN}\beta_2$ /HSF in response to serum factors, PDGF, cytokines and other factors (Sehgal et al., 1987; Kohase et al., 1986; Van Damme et al., 1987a), the fibroblast itself may release sufficient $\text{IFN}\beta_2$ /HSF upon tissue damage, to induce APP synthesis. This may mean that any tissue containing $\text{IFN}\beta_2$ secreting fibroblasts, is capable of eliciting the liver acute phase response. Thus the generally accepted concept that cells of the monocyte/macrophage are primarily

responsible for the biological events that take place during the acute phase response is probably an oversimplification. With the added information of HSF/IFN β_2 release by fibroblasts it may be that these cells play an important role in APP induction due to tissue damage in vivo.

IFN β_2 appears to be unique among IFNs in that it is inducible not only by classical IFN inducers (double stranded RNA and cycloheximide, virus infection), but also other cytokines. Both TNF and IL-I stimulate IFN β_2 mRNA expression (Kohase et al., 1986; May et al., 1986; Kohase et al., 1987; Van Damme et al., 1987a). This enhancement of fibroblast IFN β_2 may represent an additional amplification mechanism of IFN β_2 release at sites of inflammation. Alternatively, or possibly in addition, IL-I and TNF may activate Kupffer cell secretion of HSF/IFN β_2 . This cell, known to produce an HSF activity (Bauer et al., 1984; Sanders & Fuller, 1983) that is likely to be IFN β_2 (Gauldie et al., unpublished) is ideally situated for interaction with hepatocytes.

In addition to fibroblasts and monocytes, activated T-cells (HTLV-I infected, or PHA, CON A treated) release CRP-inducing activity (Goldman & Liu, 1987) as well as IFN β_2 /BSF-2 (Hirano et al., 1985 Hirano et al., 1986). One might predict that immune responses involving activation of T cells have the potential to induce the acute phase response of liver. Virus infection involving fibroblasts

and/or T cells could also induce this response by liver. Unfortunately, there is little if any available literature at present on virus infections and APP serum levels to support or refute this suggestion.

The interpretations presented here regarding PBM HSF/IFN β_2 and IL-I regulation of APP in vitro does not preclude other cytokines or mechanisms that also may influence hepatocytes in vitro or in vivo. Indeed, other molecules such as COLO-HSF I and II (Baumann et al., 1984; Baumann et al., 1986) may be separate entities from PBM HSF/IFN β_2 but fully capable of inducing APP synthesis. Van Gool et al. (1984) showed that adrenalin implants elicited strong α_2 -macroglobulin responses in rats, however, Koj et al. (1984) and Bauer et al. (1987) found no stimulation by adrenalin of α_2 -macroglobulin synthesis by rat hepatocytes in vitro. This suggests that adrenalin acts through an indirect mechanism possibly through induction of HSF/IFN β_2 secretion by other cells. In this respect it would be interesting to examine HSF release by Kupffer cells monocytes and T-cells in response to adrenalin.

Assuming identity between PBM HSF and IFN β_2 , HSF's effect on pituitary cells of increased ACTH production (Woloski & Fuller, 1985) should be mimicked by purified or cloned IFN β_2 . The theoretical result of this interaction in vivo would be increased adrenal gland production of glucocorticoids which can stimulate some APP [for example

rat α_1 -AGP in vivo (Baumann et al., 1984), rat α_1 -AGP and α_1 -AT in vitro (Gross et al., 1984)] and is necessary for expression of rat α_2 -macroglobulin. Endogenous glucocorticoids may possibly also serve in an anti-inflammatory fashion similar to pharmacologically administered steroids. However the increase during acute inflammation is transient and relatively low compared to levels obtained in therapy.

Although the mechanisms involved in the initiation of the liver response are becoming apparent, the nature of the processes responsible for the decline of liver APP synthesis are not known. The increased glucocorticoid blood levels may provide a feedback inhibition to halt cytokine synthesis. Table 5 shows that rat alveolar macrophages were inhibited by the glucocorticoid analogue DEX. DEX has been shown to inhibit IL-1 secretion (Dinarello, 1984) and HSF secretion (Woloski & Fuller, 1985) by human PBM. Staruch & Wood (1985) have found that DEX treatment of mice inhibited the appearance of IL-1 in serum in vivo. Thus a decline in available HSF and IL-1 may result in decreased liver stimulation.

Alternatively, the shutdown of APP production and return to normal metabolism by the liver may be due to other unidentified inhibitors, or simply due to the lack of HSF/IFN γ release upon healing of damaged tissue, or possibly even down regulation of hepatocyte receptors for

cytokines that stimulate APP synthesis.

The evidence provided here, including analysis of biochemical properties, analysis of immunologic identity and analysis of functional activity in induction of acute phase protein synthesis, led to the interpretation that human PBM derived Hepatocyte Stimulating Factor is most likely identical to human Interferon β_2 . This confirms proposals of others that HSF is distinct from cytokines such as Interleukin-1 and Tumour Necrosis Factor which themselves have acute phase protein synthesis inducing activity, albeit apparently less than HSF. Since other cell types, in addition to monocytes, release IFN β_2 , it appears that many tissues have the potential to initiate this response. In vitro, HSF/IFN β_2 appears to be responsible for a majority of activity on hepatocytes in PBM supernatant. Thus, in vivo, and in concert with IL-1, HSF may account for many aspects of the systemic acute phase response including fever, neutrophilia and increases in acute phase proteins and glucocorticoids. The role these cytokines and the liver response play in inflammatory diseases is not yet evident. It may be desirable to examine animal models of inflammation with the use of these cytokines in order to establish the potential of manipulation of the liver response.

CONCLUSIONS

The response of mammals to tissue injury include a number of systemic effects including increased liver synthesis of acute phase proteins. The initiation of the acute phase response of the liver involves soluble cytokines such as Hepatocyte Stimulating Factor (HSF) and Interleukin-1 (IL-1). These cytokines stimulate the in vitro synthesis by hepatocytes of acute phase proteins. The experiments outlined here provide evidence that HSF derived from human peripheral blood monocytes (PBM) is distinct from Interleukin-1. PBM HSF however is not unique, but rather the evidence strongly suggests that PBM HSF is identical to Interferon β_2 (IFN β_2) of fibroblasts which has also been shown by others to be identical to B-cell Stimulatory Factor-2 (BSF-2) of T-lymphocytes, the 26kd protein of fibroblasts and Hybridoma Growth Factor (HGF) of monocytes.

HSF/IFN β_2 strongly stimulates in vitro synthesis of α_2 -macroglobulin and α_1 -CPI by primary rat hepatocyte cultures as well as fibrinogen, α_1 -ACH, α_1 -AT and haptoglobin by human Hep-G2 cells. IL-1 on the other hand does not induce these proteins strongly but does enhance human α_1 -AGP in Hep-G2 and furthermore inhibits rat α_1 -CPI (rat hepatocytes) and human fibrinogen induction (Hep-G2) in vitro. Thus, HSF/IFN β_2 is the major molecular species

responsible for acute phase protein induction in PBM supernatants as analyzed in vitro.

Human fibroblast cultures also release HSF/IFN β_2 with potent acute phase protein -inducing activity but do not secrete detectable Interleukin-1 levels. These interpretations suggest that HSF/IFN β_2 may be a major inducer of hepatocyte acute phase protein synthesis in vivo and that fibroblasts and monocytes are capable of eliciting the acute phase response of the liver.

REFERENCE MATERIAL

Appendix 1

Interferon β_2 (IFN β_2)

Interferons are a heterogeneous group of proteins that are capable of rendering cells resistant to virus infection. Interferons are classified into three types; interferon - alpha (IFN α), IFN-beta (IFN β) and IFN-gamma (IFN γ) on the basis of their primary sources (leukocyte, fibroblast and lymphocyte respectively, Toy, 1983). IFN α and IFN β are related to each other but have no apparent structural homology to IFN γ . Nevertheless, all IFNs share biological activities.

Although many genes exist for IFN α (all with strong homology) (Goeddel et al., 1981), prior to 1979-1980, IFN β was apparently encoded by only 1 gene. However, in 1980, Weissenbach et al. (1980) recognized 2 species of mRNA (.9 kilobase and 1.3 kb) from double-stranded RNA stimulated human foreskin fibroblast cells FS-4, that were capable of displaying IFN activity once injected into frog (Xenopus Laevis) oocytes. These results were confirmed by Sehgal and Sagar (1980). Both products were inhibited by anti-IFN β antisera. Furthermore, the .9 kb mRNA hybridized to the IFN β cDNA of Taniguchi (Taniguchi et al., 1980) but the 1.3

kb mRNA hybridized to the cDNA produced by Revel and colleagues (Weissenbach et al., 1980). Thus the .9 kb mRNA was designated IFN β_1 , and the 1.3 kb mRNA designated IFN β_2 .

In 1982, Content et al. (1982) published work showing that 22 kd and 27 kd proteins were secreted by cycloheximide and actinomycin D induced fibroblasts that were precipitable with anti IFN β antiserum. They also found that mRNA from these cells would translate (frog oocytes) a 26 kd protein similar to the 22 and 27 kd products. However, in contrast to Weissenbach et al. (1980), they could not find antiviral activity nor ppp(A2' p5')n A synthetase-inducing activity in, either in vitro translated 26 kd or the natural 22 and 27 kd products. The reason for this discrepancy is not clear. However in retrospect, Billiau (1987) and Revel & Zilberstein (1986) suggest that comparison of specific IFN activity shows IFN β_2 with 50 to 100 times less than that of IFN β_1 (as assayed against VSV). At the time however, Content et al. named their factor the 26 kd protein on the basis of the lack of IFN activity.

More recently, the cDNA sequences of IFN β_2 was published by Zilberstein et al. (1986) and codes for a 212 amino acid protein (23.7 kd) with an amino-terminal hydrophobic region that probably serves as a signal peptide, resulting in a mature extracellular protein of approximately 180 amino acids. The cDNA clones were expressed in plasmids with the SV-40 early gene promoter and yielded biological

IFN activity upon transfection into Chinese Hamster Ovary cells. The protein's activity was neutralized by anti-IFN β antiserum but not anti-IFN α or anti-IFN γ . Haegman et al. (1986) published the nucleic acid sequence of the 26 kd protein and found it to be identical to the IFN β_2 cDNA (and did not assay IFN in this particular paper).

Also at about the same time, Hirano, Kishimoto and colleagues published a cDNA sequence for a B cell stimulating factor (BSF-2) from activated human T cells (Hirano et al., 1985; 1986). This factor (from TCL-Na 1 cells) stimulates immunoglobulin secretion in Epstein-Barr virus transformed B cell lines as well as staph A activated human B cells. Hirano et al. (1986) showed that the cDNA sequence coded for a mature protein (signal cleaved) with identical amino acid sequence as that derived from amino-terminal amino acid sequence of purified BSF-2. From this T cell line, the cDNA predicted 19 and 22 kd (mature and precursor) proteins. The cDNA sequence was noted to be identical to the 26 kd protein and IFN β_2 sequences (Billiau, 1986; Sehgal et al., 1987).

Another factor, hybridoma/plasmacytoma growth factor (HGF) is secreted by CON A-stimulated Peripheral Blood Monocytes (Aarden et al., 1985) and by fibroblasts induced by similar methods for the production of the 26 kd protein (Van Damme et al., 1987(a)). HGF stimulates growth of rat-mouse or mouse-mouse hybridomas and mouse plasmacytoma cell

lines. Van Damme et al. (1987(b)) showed that 26 kd protein cDNA could be used to enrich for HGF mRNA by hybridization and furthermore showed amino-terminal sequence (12 amino acids) of purified HGF was identical to the 26 kd sequence. As concluded by others, the results of these independent works show identity of nucleic acid sequences for $\text{IFN}\beta_2$, 26 kd protein, BSF-2 and most likely HGF and therefore all activities are due to the same molecule (Billiau, 1987; Paupart et al., 1987; Sehgal et al., 1987). This unfolding of the $\text{IFN}\beta_2$ story points to the usefulness of cloning and sequencing technology in the study of cytokines. That molecules characterized by different activities have eventually been found identical is not unprecedented (for example, IL-1 = LAF, LEM, LP, 22kd IFN inducing protein, catabolin).

Genetic analysis has revealed that the $\text{IFN}\beta_2$ is located on chromosome 7 (Sehgal et al., 1986). Sequence analysis shows a 212 amino acid (a.a.) precursor and 184 a.a. mature protein with 2 potential glycosylation sites. There is some homology at the amino-terminal with G-CSF, and a 35 amino acid stretch in the carboxy-terminal with $\text{IFN}\beta_1$ and $\text{IFN}\alpha$ (Hirano et al., 1986; Sehgal et al., 1987). The cross reactivity of neutralizing antibodies between $\text{IFN}\beta_1$ and $\text{IFN}\beta_2$ may be due to structural and antigenic similarity within this particular region of the protein.

Interferon β_2 can be induced from various cell

sources and under various stimulatory regimes. It appears to be unique among interferons in that it is induced by other cytokines. A list is compiled in table 10.

In addition to these human cell derived IFN β_2 , Van Snick et al. (1987), and Nordan and Potter (1986) have characterized HGFs from murine cells. A product from the mouse P388-D1 cell line (25kd, pI=6 to 6.5) stimulated the proliferation of various plasmacytoma cell lines (Nordan and Potter, 1986). Van Snick et al. purified a factor from mouse T cells they termed Interleukin-HP1 (22 to 27 kd, pI=6 to 7) that had potent HGF activity. Amino-terminal sequencing did not reveal homogeneity to other cytokines nor to the human 26 kd protein/HGF. Complete sequencing is needed to determine if this is a mouse homologue of human IFN β_2 /HGF/BSF-2.

Table 10: Cell sources and stimulatory agents for IFN β_2

Cell Source	Stimulatory Regime	Reference
Fibroblasts	constitutive release	Kohase <u>et al.</u> , 1986 Sehgal <u>et al.</u> , 1987
	double-stranded RNA	Weisenbach <u>et al.</u> , 1980
	and	Sehgal <u>et al.</u> , 1980
	cycloheximide	Content <u>et al.</u> , 1982
	PDGF	Sehgal <u>et al.</u> , 1987
	FCS	Kohase <u>et al.</u> , 1986
	IFN β_1	Kohase <u>et al.</u> , 1986
	TNF	Zilberstein <u>et al.</u> , 1986
	IL-1 & cycloheximide	Kohase <u>et al.</u> , 1986 Van Damme <u>et al.</u> , 1987 Zilberstein <u>et al.</u> , 1986
	LPS	Sehgal (submitted)
Lymphocytes	PHA, CON A, TPA HTLV-1 infection	Hirano <u>et al.</u> , 1985 Hirano <u>et al.</u> , 1985
Monocytes	CON A, LPS	Aarden <u>et al.</u> , 1985

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