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We have read this thesis
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REGULATION OF INTERLEUKIN 1 PRODUCTION BY α + β INTERFERONS: EVIDENCE FOR BOTH DIRECT AND INDIRECT ENHANCEMENT

A Thesis

Presented for the

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Degree

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Robert Victor Candler

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DEDICATION

This thesis is lovingly dedicated to my parents, Bobby and Zuann Candler, who gave me the encouragement and inspiration to work toward my own goals.

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ABSTRACT

Pretreatment of murine macrophages with interferon (IFN) influences a variety of their responses to other mediators. For example, bone marrow derived macrophage progenitors pretreated with IFN $\alpha + \beta$ display augmented proliferation upon subsequent exposure to specific macrophage-type colony-stimulating factor (M-CSF). Similar pretreatment of macrophage-enriched cultures from proteose-peptone elicited peritoneal exudate cells results in enhanced production of the immunoregulatory polypeptide interleukin 1 (IL-1) in response to lipopolysaccharide (LPS). Therefore, the present investigation was performed to determine if exposure of mature murine exudate macrophages and macrophage cell line cultures would, in addition, augment M-CSF stimulated IL-1 production. The results indicate that preincubation with IFN $\alpha + \beta$ enhances IL-1 production in response to M-CSF as well as to LPS. This pretreatment regimen elicited enhanced macrophage responsiveness to sub-optimal doses of CSF as well as LPS.

LPS hyporesponsive cells from C3H/HeJ mice or CSF hyporesponsive $P388D_1$ monocytic tumor cells produced an apparently synergistic level of IL-1 in response to the two stimuli combined. Prior exposure to IFN $\alpha + \beta$ enhanced this apparently synergistic response.

An unexpected finding in this investigation was that IFN $\alpha + \beta$ pretreatment dosages exceeding 1000 U/ml directly stimulated IL-1 production by exudate macrophages. Direct and augmented stimulation of IL-1 production by IFN $\alpha + \beta$ could be neutralized by addition of highly specific anti IFN $\alpha + \beta$ antiserum or by heat treating the preparation at 56°C for 45 minutes.

There is a correlation between the local presence of IFN and both inflammation and fever. This observation is discussed in regard to the IFN enhanced production of IL-1.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	19
	Mice	19
	Reagents	19
	Macrophage Cultures	21
	Interleukin 1 (IL-1) Induction	22
	Interferon (IFN) Pretreatment	22
	IFN Inactivation/Neutralization	23
	Interleukin 1 (IL-1) Assay	23
	Calculations	24
III.	RESULTS	25
	CSF Induction of IL-1: Effect of Anti-IFN $\alpha + \beta$ and Indomethacin	25
	LPS Induction of IL-1: Effect of IFN $\alpha + \beta$ Pretreatment	27
	IFN $\alpha + \beta$ Enhancement of CSF-induced IL-1 Production	30
	IFN $\alpha + \beta$ Enhancement of IL-1 Production Stimulated by LPS and CSF	30
	IFN $\alpha + \beta$ Stimulation of IL-1 Production	35
IV.	DISCUSSION	43
LIST OF REFERENCES		50
VITA		64

LIST OF TABLES

TABLE PAGE

1. Influences of anti-IFN α + β antiserum and indomethacin on CSF-induced IL-1 production 26
2. IFN enhancement of IL-1 production in response to LPS . . . 28
3. Effect of IFN pretreatment on LPS dose response for IL-1 production. 29
4. IL-1 production by CSF-treated peritoneal macrophages following 18 hour preincubation with IFN α + β. 31
5. Effect of IFN pretreatment on IL-1 production by C3H/HeJ macrophages 33
6. Effect of IFN pretreatment on IL-1 production by $P388D_1$ cells exposed to LPS and/or CSF. 34
7. IL-1 production by peritoneal macrophages stimulated with LPS and CSF 36
8. IL-1 production in response to direct IFN stimulation of $P388D_1$ cells 39
9. IL-1 production by C3H/HeJ macrophages in response to direct IFN stimulation 41
10. A comparison of stimulation and neutralization of production with two preparations of IFN α + β. 42

CHAPTER I

INTRODUCTION

Mononuclear phagocytes (macrophages and monocytes) are multifunctional cells capable of influencing a variety of normal host functions. These regulatory activities are, however, not predetermined but, rather, are influenced by environmental factors including molecular signals produced by other cell types. As an example, interferons influence a wide variety of mononuclear phagocyte functions. Recently, Moore et al. (79, 83) reported that certain classes of interferons enhanced the response of mononuclear phagocyte precursors to the proliferative effects of their specific growth factor. This growth factor, macrophage-type colony stimulating factor (M-CSF), is known also to be a regulator of certain non-proliferative functions of more mature mononuclear phagocytes. Therefore, it is possible that interferons may similarly influence the responsiveness of mature mononuclear phagocytes to the regulatory influences of this dual functioning growth factor. Research to be reported in this thesis deals with this potentially important aspect regarding regulation of the mononuclear phagocyte system.

Macrophages are a diversified group of monocytic leukocytes which affect many important biological functions. Because of their ubiquity, phagocytic and detoxifying abilities, they constitute the primary host surveillance system (138). Macrophages act to protect the host from harmful infections before the establishment of a specific immune response (138). As phagocytic cells, macrophages can ingest foreign

particles in both a non-specific and specific manner via surface Fc and/or C3b receptors (118).

Monocytes arise from precursors in the bone marrow, circulate in the peripheral blood, randomly immigrate into various tissues and differentiate into macrophages (119). Movement of monocytes into the tissues is accompanied by a series of maturational events which lead to structural and functional alterations resulting in differentiation into macrophages. Examples of tissue macrophages include histiocytes of connective tissue and skin, liver Kupffer cells, alveolar macrophages, and macrophages of lymph nodes, spleen, and serous cavities (pleural and peritoneal macrophages). Related cell types can be found as bone osteoclasts, and in nerve tissue as the microglial cells (118). Other "macrophage-like" cells exist, such as Langerhans cells, that have a potential role in immunity (118).

Monocyte immigration into tissues can result also in response to chemotactic stimuli generated locally during an inflammatory response (138). Associated with inflammatory and immunological responses is a typical activation phenomenon for both newly immigrating monocytes and resident macrophages. Initially, Mackaness used the term activation to describe increased bactericidal activity of macrophages in animals infected with facultative intracellular bacterial pathogens (67, 68, 69). Macrophages can be activated by antigen or mitogen stimulated lymphocytes through the action of soluble lymphocyte secretions called lymphokines (97). There is also evidence demonstrating that macrophages may be activated through lymphokine independent mechanisms. Lipopolysaccharide (LPS) and muramyl dipeptide (MDP), both bacterial

products, have the ability to directly activate macrophages (100). Activation can also take place with a short incubation with proteolytic enzymes such as trypsin, chymotrypsin, pronase and papain (48). This suggests that macrophages may be activated by proteases from neutrophils or in serum.

A compilation of characteristics of activated macrophages was made by Mackaness in 1970 (cited in 25). These included enhanced spreading on glass and phagocytic activity, increased content of acid hydrolases, increased respiratory and mitotic rate along with increased digestive capacity. Activated macrophages had increased size and a more complex morphology. Biochemically, many changes were noted in activated macrophages. In addition to the aforementioned hydrolase enhancement, levels of collagenase, elastase and plasminogen activator were also increased (55, 133, 134, 145, 146). A decrease in the activity of membrane associated ecto-5'-nucleotidase, which releases adenosine has also been reported (54).

There is a burst of oxidative metabolism associated with phagocytosis by both neutrophils and macrophages (49), and activated macrophages display an augmented response over that of resting cells (49). Specific metabolic events in this process have been defined through studies of phagocytosis with neutrophils (49). Initial oxygen consumption is directed by an oxidase which converts the molecule to the superoxide anion form, which then combines with two protons to form hydrogen peroxide and oxygen. The potent oxidant, hydroxyl radical, is formed through the interaction of superoxide anion and H_2O_2 in the presence of metal ions (Haber-Weiss reaction) (49). Glucose oxidation

takes place through the hexose monophosphate (HMP) shunt stimulated through the production of $NADP^+$ formed during phagocytosis (49). Several of these findings have been repeated in direct studies with macrophages (49). There is a correlation between these processes and enhanced antimicrobial activity. For example, several investigators have demonstrated that the ability to kill Toxoplasma gondii varied directly with the macrophages' ability to generate oxygen metabolites (87, 148). Macrophage killing of tumor cells can also be associated with increases in oxygen metabolite levels. Nathan et al. demonstrated that only hydrogen peroxide releasing, stimulated macrophages could lyse certain tumor cell lines (88).

Macrophages have in addition attained recognition as important secretory cells. As mentioned before, they can secrete a series of proteolytic enzymes, including plasminogen activator (55, 133, 134, 145, 146). Immune regulatory molecules are also secreted by macrophages. Macrophage-produced prostaglandins appear to modulate macrophage mediated immune suppressive mechanisms (31). Colony Stimulating Factor (CSF) is another product which serves as a myelopoietic growth factor among other functions (95). Macrophages additionally are a source of interferons which suppress viral replication (71, 92, 93) and modulate cell growth and differentiation within the immune system (118). Interleukin-1 (IL-1) is another multi-functional macrophage mediator, initially found to be essential for T-lymphocyte activation (33). Interestingly, macrophages can secrete seemingly antagonistic molecules. For example, plasminogen

activator induces fibrinolysis, (133) while other factors are produced which induce the clotting cascade (104).

As mentioned before, macrophages have been found to be both enhancing and suppressive in immune regulation. This duality of function is presumably because of the heterogeneity of macrophage populations. Indeed, several investigators found that within a given population of peritoneal macrophages there was a variation in the ability to ingest and kill bacteria (45, 67). A partial explanation for this functional heterogeneity is that the mode of activation may affect function. For example, thioglycollate-elicited macrophages display several enhanced or newly expressed functions when compared to simple resident cells (97). Mackaness demonstrated that exudate macrophages obtained following intraperitoneal injection of the Calmette-Guerin bacillus (BCG) are in a state of "enhanced" activation in their ability to kill bacteria, parasites and tumor cells (70). Similarly, only lymphokine activated macrophages displayed both enhanced killing properties and secretion of plasminogen activator (96).

Whether macrophages arise from several branched yet related precursors or from a single precursor in a linear fashion is still unknown. In both models, the environment influences the stability of a particular phenotype. Not only homeostasis of macrophages, but the expression of the antagonistic functions require a complex system of regulatory mediators (118). These mediators can be categorized as activation, differentiation and mitogenic signals (reviewed in 118). Activation signals are relatively short lived with induction of

function possible without cell cycling or differentiation. Differentiation signals generally require mitotic cycles to rearrange the genetic program. Mitogenic signals are required for clonal expansion of subpopulations, phenotypes or progenitors. This usually involves immature macrophages since, as a generality, proliferative capacity decreases with increasing cellular differentiation (117). Leukocyte hormones consisting of lymphokines, monokines and cytokines, depending on function, can be placed into one of these categories. Successful control of the mononuclear phagocyte system to control both infection and neoplasia, therefore, may depend upon complex activation or suppression of certain subpopulations of cells.

What are these mediators and how do they work? Macrophage inhibitory factor (MIF) is such a mediator. This factor has been obtained by treating lymphocytes with antigen, removing the supernatant media and performing molecular exclusion chromatography (105, 106). Macrophages incubated with MIF exhibited an enhanced adherence to culture dishes with increased membrane ruffling, motility and spreading (89). Conflicting data pertaining to phagocytic rate were seen in several studies (89, 91, 107). However, MIF treatment of macrophage cultures did result in increased HMP shunt activity (89) and enhanced bacteriostasis (30). Piessens et al. (102) also demonstrated that macrophage activation by lymphokine preparations enhanced their tumoricidal activity against syngeneic tumor cells. This presumably takes place through the release of the cytotoxic molecules including enzymes (26) and tumor necrosis factor (TNF) (cited in 80).

Macrophage activating factor (MAF) is a lymphokine which enhances cell adherence and glucose oxidation (20). It is unclear at this time whether MIF and MAF are the same molecule. Indeed, both mediators have molecular weights between 65,000 and 25,000 (18). Other methods of separation have failed to distinguish the two molecules (17). It remains to be seen if these findings represent two activities on separate molecules or a single molecular species.

Colony stimulating factors have been suggested as possible macrophage activators. Colony stimulating factors are myeloproliferative agents which stimulate the clonal proliferation and differentiation of committed granulocyte/macrophage progenitors in vitro (103). They are found in serum, urine and nearly all tissues (95). Specifically, T-lymphocytes (101), macrophages (95), endothelial cells (95) and fibroblasts (121) produce CSF.

There are four different classes of CSF which have been purified to at least near homogeneity (95). Most, if not all, can stimulate granulocyte and/or macrophage colony formation in semi-solid cultures of murine bone marrow (95). They are stable glycoproteins active at low concentrations of 10^{-11} to 10^{-13} M (95). Macrophage-type CSF (M-CSF or CSF-1) is a 70,000 molecular weight glycoprotein consisting of two disulfide bonded subunits (122). This CSF is found in normal murine tissues and can be purified from L-cell fibroblast conditioned media (122). This mediator specifically stimulates macrophage precursors to proliferate (13). Granulocyte-macrophage CSF (GM-CSF or CSF-2) has been purified from lung-conditioned medium of LPS-injected mice (11) and is a 23,000 MW glycoprotein with no

disulfide-bonded subunits (95). This mediator stimulates colony formation from both granulocyte and macrophage precursors in addition to bi-potential granulocyte/macrophage precursors (95). Granulocyte CSF (G-CSF or CSF-3) has also been purified from lung-conditioned medium of LPS-injected mice (94) and is a 25,000 MW glycoprotein monomer with internal disulfide bonds (94). Granulocyte CSF primarily stimulates subsets of granulocyte colony-forming cells (95). Finally, multi-CSF has been purified from medium conditioned by WEHI-3B cells. It has a molecular weight in the 23,000 to 28,000 range (95). It stimulates a variety of colony forming cells and, thus, has been given several additional names, including burst promoting activity (BPA) (95), interleukin 3 (IL-3) (42), P-cell stimulating factor (PSF) (19), hematopoietic cell growth factor (HCGF) (3), mast cell growth factor (MCGF) (152) and erythroid, megakaryocyte and eosinophil colony stimulating factors (E-CSF, MEG-CSF, EO-CSF) (12). All of these different CSF are required continuously for each cell division. In the absence of CSF, cells die at a rate of 5 to 10% per hour (76). The signal for CSF production appears to be infection or other membrane perturbation. Indeed, producing cells respond to LPS (75, 117), retrovirus infection (57) and treatment of the tumor promoter tetradecanoyl phorbol acetate (TPA) (95) in addition to specific antigen (101).

By definition, CSF were originally found to exert their effect on immature myelopoietic progenitor cells. However, while the peripheral sources are well documented, their role <u>in vivo</u> is unclear. Injection of purified CSF preparations into animals neither enhances non-specific

resistance nor myelopoiesis (78). Perhaps circulating CSF may be unimportant in myelopoiesis in light of evidence that CSF action may be localized, short-range communications possibly involving cell-to-cell contact (22).

Rather than acting as hematopoietic agents, perhaps peripheral CSF activities are exerted upon mature cells. There is evidence that M-CSF will stimulate less mature macrophages to proliferate (121); however, M-CSF also stimulates macrophage RNA and protein synthesis (95), and production and release of interleukin 1 (IL 1) (82), E-prostaglandins (60) and plasminogen activator (37, 63). M-CSF has been reported to enhance macrophage killing of tumor cells (149); however, this observation remains controversial (80). GM-CSF possesses some of these properties as well as the ability to stimulate macrophages to kill intracellular Leishmania tropica (38).

CSF appears to increase macrophage sensitivity to activation with other mediators. Macrophages produce high levels of interferon in response to challenge with virus when incubated with M-CSF (61). Similarly treated macrophages readily produce tumor necrosis factor when stimulated with LPS (144). Moore et al. (80) demonstrated that M-CSF conditioned macrophages required 10-fold fewer cells challenged with 1000 times less LPS to produce interferon levels comparable to freshly obtained cells. CSF may, therefore, cause indirect macrophage activation through the release of interferon. There is speculation that CSF-induced production of interferon could be activating other macrophages to produce elevated levels of interferon (36, 113). Moore et al. (80) also supported this idea in their investigation. For

example, addition of anti-interferon to CSF stimulated cultures abrogated the enhanced production of interferon in response to polyinosinic polycytidylic acid (80).

Interferon is a mediator that is produced by macrophages (71), as well as a variety of other cell types, that also has the ability to influence macrophage functions. Interferon was discovered by Isaacs and Lindenman in 1957 (44) as an agent that inhibits viral replication. Since then, interferon has been found to possess many activities related to immunoregulation, hormonal and tumor control (46). Human and murine interferons can be classified into three groups. They are leukocyte (IFNα), fibroblast (IFNβ) and immune (IFNγ) interferons. IFNα and IFNβ can be induced in their respective cell types by treatment with viruses or polyribonucleotides (28, 43). IFNγ is induced in T-lymphocytes by T-cell mitogens or specific antigens (147, 170, 109, 125).

Interferon is produced by virus infected cells during the early stages of infection (123). It is secreted by the cell and interacts with specific receptors on the surface of other cells (46). The evidence with regard to anti-viral action suggests that it blocks both transcription of viral RNA and translation of viral proteins (46).

Recent studies have demonstrated interferon's role in modulation of the immune response. Very small amounts (20-60 units/ml) of interferon will suppress the <u>in vitro</u> plaque-forming cell response (a measurement of antibody production) by 90% or more (48). Interferon can cooperate with interleukin-2 (IL-2) to enhance the killing of target cells by cytotoxic T-cells (47). Natural killer (NK) cells can

be stimulated for enhanced killing of tumor cells through treatment with interferon (48). It is presumed that therapeutic administration of interferon as a treatment for human cancers modulates the immune system to combat tumors.

Interferons' role as a macrophage activator/differentiator is important in regard to the present investigation. Treatment of macrophages with interferon has a variety of effects depending upon conditions. In vitro addition of IFNβ to murine macrophages resulted in increased spreading and vacuolization (113). Similar changes were observed in human macrophages treated with IFNα and IFNβ (138). Preparations of interferon of variable purity induced in vitro macrophage tumoricidal and tumoristatic activities (138). Similar in vivo observations have been reported (138). Their finding suggests the possibility of another mechanism by which cancer patients derive benefit from therapeutic administration of interferon. Indeed, monocytes taken from greater than 80% of patients receiving recombinant IFNα treatment demonstrated a significant increase in tumoristatic activity in vitro in comparison to peripheral blood monocytes from normal volunteers (cited in 138).

There is also evidence that IFNγ and MAF are identical molecules. Roberts and Vasil (cited in 138) found that antiviral and macrophage activating abilities co-eluted in an intense purification regimen including ammonium sulfate precipitation and several sequential chromatographic steps. They, along with others, found that both activities could be deactivated upon pH 2 treatment or heating at 56°C (138). In addition, MAF activity is separable into two peaks of 45,000

and 23,000 MW. Interferon lies within the higher molecular weight peak (138). Conversely, others (9, 10, 127) have found no correlation between the two activities in supernatant media from splenic or peripheral blood leucocyte cultures. Similarly, although IFNβ has been found to have macrophage activating ability, the pattern is markedly different from that of MAF. For example, MAF preparations require a second signal, such as trace quantities of LPS, to induce cytolytic macrophages while IFNβ requires no such signal (128).

Interferon also induces changes in surface antigen expression which can alter macrophage function. Vogel et al. demonstrated that purified IFN β, IFNγ (140) and IFNα (136) significantly enhanced Fc mediated phagocytosis. This enhancement increased the macrophages' ability to phagocytose opsonized sheep erythrocytes. Additionally, Vogel et al. (137) demonstrated that the number of Fc receptors per cell was increased approximately four-fold in interferon treated macrophage cultures. Another example of interferon modulation of the cell surface is expression of Mac-1 antigen. Mac-1 antigen on the surface of macrophages is representative of less differentiated cells (40). Treatment of macrophages with IFN led to a dose-dependent reduction of expression of Mac-1 (140). Interferonγ treatment can in addition enhance the expression of Ia antigen (124). Ia expression on macrophages is a requirement for successful antigen presentation to T-lymphocytes and also a marker of differentiation (4, 5). Human cloned IFN was found to induce DR antigen (human analog to mouse Ia) on human monocytes and a monocytic cell line. In summary, interferon modulates the expression of surface markers associated with early

stages of macrophage differentiation (e.g. FcR, Mac-1), while markers associated with an activated state (e.g. C3b, Ia) may require other factors (or in addition to interferon) for their expression (138).

There have been studies using interleukin-1 (IL-1) secretion as an indicator of macrophage activation. This immunoregulatory molecule is produced by macrophages and macrophage-like cells including alveolar, liver (Kupffer cell), bone marrow, glial, splenic, peritoneal, renal, placental and corneal sources (23). Recently, other cell types including keratinocytes (65), peridontal epithelial cells (15) and human large granular lymphocytes (112) have been found to secrete either IL-1 or molecules biochemically and functionally similar to IL-1. IL-1 is a globular protein of molecular weight 12,000 to 16,000 and is active at low concentrations (less than 10^{-10}) (98).

Resting monocytes produce only small, constitutive amounts of IL-1 (98), while a variety of natural and synthetic compounds can stimulate production. For example, lymphocytes treated with mitogens or antigen can stimulate macrophages through either a cell-to-cell contact mechanism or through the release of lymphokines (98). Agents, such as LPS, mycobacterial peptidoglycolipids and antigen-antibody complexes can directly stimulate macrophages to produce IL-1 (98). As previously mentioned, CSF can also augment IL-1 production (82). Phorbol myristic acetate (PMA), pyran co-polymer, latex particles and other synthetic products are also IL-1 inducers (132). The mechanism of stimulation is unclear except that most of the molecules mentioned as inducers perturb macrophage membranes or cause an elevation of intracellular cGMP levels (132).

Interleukin 1 was originally described as an activity directly mitogenic for murine thymocytes, but not for immunocompetent peripheral lymphocytes (32). It also augments, in a synergistic fashion, the proliferative effects of mitogens including Concanavalin A and phytohemagglutinin (Con A and PHA) for murine thymocytes and splenic T-lymphocytes (32). These stimulatory activities take place through the indirect release of the lymphokine, IL-2. According to Stadler et al., IL-1 along with antigenic or polyclonal stimulants will shift a specific sub-population of helper T-lymphocytes from the resting G_0 phase into G_1 resulting in IL-2 production (120). IL-2 then stimulates other activated lymphocytes to proliferate. In summary, IL-1 serves as the response signal while IL-2 is the growth factor.

Much interest has been generated over the existence of identical or similar molecules which have other properties in addition to being stimulatory to thymocytes. IL-1 is closely related to or identical to leukocytic endogenous mediator (LEM). Initially, LEM was thought to be strictly a neutrophil product, but more recent investigations revealed that production could be due to contaminating macrophages (24, 38). LEM elicits fever in experimental animals and is inseparable from the classic fever-inducing molecule known as endogenous pyrogen (EP) (73). Rabbit LEM/EP injected into rats, rabbits and mice significantly decreases plasma iron and zinc levels while increasing the level of copper (50). Also, LEM/EP induces neutrophilia by modifying the rate at which neutrophils appear in the peripheral circulation (52). Multiple injections increase the levels of immature forms of neutrophils (51). There has been speculation that LEM could be a

peripheral signal of granulopoiesis by stimulating endogenous production of CSF in the bone marrow (52).

Similarities first seen between LEM and IL-1 were biochemical. Indeed, the molecular weights and isoelectric points are very similar between the two molecules (50). Also, susceptibility of the activities' inactivation were similar (50). Later, data were presented demonstrating that the activities were produced simultaneously (23). LEM/EP was found to have lymphocyte activating ability in enhancing the thymocyte response to low concentrations of PHA (108). Murphy et al. demonstrated that not only are the activities inseparable chromatographically, but antiserum prepared against LEM/EP inhibited thymocyte proliferation augmented by IL-1 (86).

Still other IL-1-like molecules have been discovered. Epidermal keratinocytes produce a factor (Epidermal T-cell Activating Factor or ETAF) which is analogous to IL-1 (65, 66, 111). In addition to classic thymocyte stimulating activity, ETAF is chemotactic for neutrophils and monocytes (64, 111) and is directly mitogenic for fibroblasts (15). Krane et al. (58) have reported a macrophage elicited factor which induces prostaglandin synthesis in cultured synovial cells. This factor, called mononuclear cell factor (MCF), co-purifies with IL-1.

IL-1 has an apparent role in the modulation of acute-phase proteins such as fibrinogen, haptoglobin, C-reactive protein and serum amyloid A. These proteins can be detected at elevated levels days after injection of microbial products or inflammatory agents (23). There is evidence that these molecules may have immunopathological as well as immunoregulatory properties (6). Macrophages produce an

inducer of serum amyloid A synthesis which is identical to IL-1 (126). IL-1 itself has been found to increase levels of serum fibrinogen and haptoglobin 5 hours after injection (74). Another process observed in conjunction with fever and infection is protein degradation in skeletal muscles (34). IL-1 enhances this degradation presumably through the endogenous production of prostaglandin E_2 (PGE_2) (34). IL-1 has additional immunoregulatory properties. It apparently acts as a B-lymphocyte activator essential for antibody production (150, 151), and can synergize with IL-2 and interferon to stimulate natural killer cell killing of tumor cells (21).

Are these activities associated with one or a family of closely related molecules? Though the activities are very similar biochemically and functionally, no amino acid sequence comparison has been reported to provide definitive evidence. Perhaps IL-1 may be IFN-like in that it could be under multiple gene control. IL-1 could be a family of closely related molecules with homologous gene sequences (23).

The present investigation involves the establishment of a relationship between IFN/CSF activation and IL-1 production. Immature macrophages are responsive to IFN/CSF activation. Indeed, Moore et al. demonstrated that highly purified IFN α + β pretreatment of bone marrow, enriched for committed precursors, enhanced the proliferative response to sub-optimal doses of CSF (79, 83). This pretreatment effect contrasts with previous investigations demonstrating the anti-proliferative effect observed by incubating marrow cultures in the presence of interferon (41, 56).

Several studies involving the C3H/HeJ mouse are directly pertinent to this investigation. This mouse strain is markedly hyporesponsive to LPS due to a mutation in a single gene on the fourth chromosome (143). Macrophages from these mice fail to produce IL-1 or PGE_2 in response to concentrations of LPS which elicit enhanced levels of the mediators from cells of normal mice (139). Macrophages of these abnormal mice are additionally hyporesponsive to the general activating effects of LPS including enhanced tumoricidal activity (16) and release of oxygen metabolites (100). Vogel and Rosenstreich (139) used these hyporesponsive mice to demonstrate that IFN has a role in macrophage differentiation/ activation. Preincubation of hyporesponsive macrophages with IFN containing preparations resulted in enhanced levels of IL-1 in response to LPS (139). In a similar study, Arenzana-Seisdedos and Virelizier (2) demonstrated that pre-incubation of normal human macrophages with IFNα or IFNβ made them more responsive to LPS, especially at doses (0.02 μg/ml) which normally do not induce an IL-1 response.

Vogel and coworkers demonstrated that IFN treated C3H/HeJ macrophages could be induced to phagocytose IgG coated sheep erythrocytes via enhanced expression of Fc receptors (136, 137, 138). Additionally, Vogel (135) surmised that endogenous production of IFN plays a role in autodifferentiation in the normal mouse. Treatment of normal murine macrophage cultures with anti-IFN $\alpha + \beta$ reduced their ability to phagocytose via Fc. Also, co-culture of hyporesponsive macrophages with normal macrophages or transfer of normal macrophage conditioned media to hyporesponsive macrophages rendered them able to

phagocytose via Fc (135). Co-stimulation of C3H/HeJ macrophages with CSF and LPS resulted in levels of IL-1 production in excess of the two individual stimulatory abilities (84). Again, CSF induced interferon may be inducing macrophages to become more responsive to LPS stimulation.

Research to be reported in this thesis was initiated to determine if IFN α + β pretreatment of murine macrophages influences their capacity to produce IL 1 upon subsequent M-CSF stimulation as has been reported for LPS stimulation (2, 139). In brief, data to be presented in this thesis demonstrate that murine macrophages can be primed for enhanced LPS and CSF elicited IL-1 production with IFN α + β preincubation. Such pretreatment makes macrophages more responsive to lower doses of the stimulants. Another interesting observation to be reported is that high doses of interferon alone can stimulate production of IL-1.

CHAPTER II

MATERIALS AND METHODS

Mice

Five to seven week old female mice were used in all experiments. C3H/AnF mice were purchased from Cumberland View Farms, Clinton, TN. C3H/HeJ mice were obtained from the breeding facilities at The University of Tennessee Memorial Research Center and Hospital, Center for the Health Sciences, Knoxville, TN. Prior to use the animals were maintained at 5 to 10 per cage in the animal facilities of Walters Life Sciences Building. Animals were provided with standard laboratory food and acidified water ad libitum.

Reagents

CSF. Highly purified M-CSF was prepared in this laboratory by D. G. Deana and F. J. Pitruzzello by the method of Waheed and Shadduck (141). Briefly, 10 L of serum-free medium conditioned by L929 cells were concentrated on a Millipore Pellicon Cassette System (Millipore, Bedford, MA) to approximately 100 ml. A sequential 40% and 50% ethanol precipitation was performed at 5°C. After the respective precipitates had been removed by centrifugation, the supernatant fluid was sequentially purified using DEAE-Sephacel (Pharmacia, Uppsala, Sweden), Con A Sepharose (Sigma) and Sephacryl S-200 (Pharmacia) columns. Fractions in the 100,000 to 150,000 molecular weight range from the final S-200 column were pooled, concentrated and assayed for myelopoietic activity using a soft-agar assay (85). This CSF preparation had a specific activity of 5.0×10^7 units/mg of protein

(one unit is that amount required to stimulate proliferation of one colony) and contained <100 pg of LPS/50,000 U as measured by enhancement of superoxide production by macrophages stimulated with phorbol myristic acetate (100).

LPS. Lipopolysaccharide (LPS) was extracted from Escherichia coli K235 by the method of McIntire et al. (73). The preparation was free of protein and was available as a standard laboratory preparation.

Interferon. Highly purified murine IFN α + β was obtained from Enzo Biochem (New York, NY). This IFN was prepared in serum-free Joklik modified Eagles Minimal Essential Medium by Newcastle Disease Virus activation of C243-3 mouse fibroblasts according to the procedure of Tovey et al. (129). It was further purified by affinity chromatography according to a modified procedure of Aguet and Blanchard (1). This material exhibited three bands in SDS-polyacrylamide gels, correlating to molecular weights, 30,000, 24,000 IFNβ and 19,000 IFNα. The preparation had a specific activity of 5.0×10^8 U/mg of protein.

IFN α + β was also obtained from Lee BioMolecular (San Diego, CA). The IFNα had an apparent molecular weight of 28,000 and the IFNβ demonstrated a 36,000 molecular weight. Total IFN content in this preparation was composed of approximately 10% IFNα and 90% IFNβ. This preparation had a specific activity of 9.8×10^6 U/mg of protein.

Anti-IFN. Anti-IFN α + β antiserum was obtained also from Enzo Biochem. Specifically, anti-mouse IFN α + β was prepared by repeated

immunization of rabbits with Ehrlich Ascites tumor cell IFN having a specific activity of 5 x 10^7 U/mg of protein. The antiserum was heat inactivated at 56°C for thirty minutes. At a dilution of 1:1000, this antiserum neutralized at least 300 U/ml of IFN α + β (78).

Indomethacin. Indomethacin (Sigma, St. Louis, MO) was prepared by initially dissolving approximately 10 mg in a 0.1 M sodium carbonate solution. Medium was added immediately to make the concentration of indomethacin 1.0 mg/ml. The solution was serially diluted to make a stock solution of 1.0 μg/ml (0.3 μM) and sterilized through a 0.2 micron filter apparatus (Gelman, Ann Arbor, MI).

Macrophage Cultures

Peritoneal exudate cells were elicited by first injecting each mouse with 2 ml sterile 10% proteose-peptone broth (Difco Laboratories, Detroit, MI) by the intraperitoneal route. After 4 days, the peritoneal cavities were lavaged with 5 ml of Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution, (HBSS, GIBCO, Grand Island, NY). The peritoneal lavage fluids were pooled, and the exudate cells pelleted by centrifugation (250 x g relative centrifugal force) (International Equipment Company, Needham Heights, MA). All cells washed by centrifugation were done in this manner. Cells were washed twice and resuspended at the desired viable cell concentrations (1.0 - 1.5 x 10^6 cells/ml) in RPMI medium 1640 (GIBCO) supplemented with 10% lipopolysaccharide-free (<1.0 ng/ml) fetal bovine serum (FBS, Hy-Clone,

Hy-Clone, Sterile Systems Inc., Logan, UT), sodium bicarbonate (5.6 mM), antibiotics (Penicillin, 100 U/ml, Streptomycin 100 μg/ml, GIBCO) and L-glutamine (200 mM, GIBCO). The cells were allowed to adhere for 2 hr at 37°C in a volume of 1 ml in 24-well plastic plates (GIBCO). Non-adherent cells were removed by vigorous washing with HBSS and fresh medium containing desired additions was added to a total volume of 0.5 ml. Cells of the $P388D_1$ murine macrophage cell line (77) were also used. These cells were grown at 37°C in the same medium and conditions described above and passaged by 1:5 split at 5 day intervals.

Interleukin-1 (IL-1) Induction

Cultures to be stimulated for IL-1 production were washed with fresh medium and were adjusted to 0.5 ml total volume with the following additions made in complete medium: stimulants (CSF and/or LPS), anti IFN α + β (1:1000 dilution) and indomethacin (0.3 μM). Control cultures were those incubated without the addition of IL-1 stimulants. After these additions, the cultures were allowed to incubate in a 7% CO_2 atmosphere at 37°C for 48 hr at which time the supernatant fluids were harvested and stored at -80°C until the activities were assayed.

Interferon (IFN) Pretreatment

Highly purified murine α + β interferons (IFN α + β) were added at desired concentrations to macrophage cultures after the adherence period. The preincubation was performed in a 7% CO_2 atmosphere at 37°C for 17 to 24 hrs. The cells were then washed with fresh medium.

Controls were cell cultures, with no IFN addition, allowed to incubate the same period of time.

IFN Inactivation/Neutralization

Heat inactivation was performed with the stock solution of IFN (2.5×10^4 U/ml). The stock solution was held in a hot water bath for 45-60 minutes at 56°C. In neutralization experiments, anti-IFN at a final dilution of 1:1000 or 1:250 was added during the preincubation period. Control cultures were treated with heated complete RPMI 1640 medium. Additional controls were treated with the anti-IFN antiserum. All supernatant fluids were dialyzed overnight against fresh medium with 8,000 M.W. exclusion membrane tubing (Spectrum, Los Angeles, CA).

Interleukin 1 (IL-1) Assay

Stimulated macrophage supernatant fluids were assayed for IL-1 activity as described by Oppenheim et al. (99). IL-1 activity was determined as enhanced stimulation of mitogenesis by C3H/HeJ thymocytes exposed to a submitogenic dose of phytohemagglutinin (PHA, GIBCO). Five-fold serial dilutions of the supernatant fluids in RPMI medium 1640 (GIBCO) supplemented with 5% processed low mitogenic serum (AMF Biological and Diagnostic, Seguin, TX), sodium bicarbonate (5.6 mM), antibiotics (Penicillin 100 U/ml, Streptomycin 100 µg/ml, Gibco), L-glutamine (200 mM, GIBCO) and 2-mercaptoethanol (2×10^{-5} M) were added to individual wells of 96 well plates (Corning, Corning, NY) in a volume of 100 µl/well. Cells were obtained by thymectomizing mice and pressing the thymuses through sterile 80-mesh screens into assay media. The resulting single cell suspension was pelleted by centrifugation and

of Tris-NH_4Cl (0.17 M Tris buffer, Sigma, containing 0.16 M NH_4Cl, pH 7.2) and incubated 2.5 minutes to lyse red blood cells (62). Fresh media were then added to make the total volume 10 ml. The cells were washed by centrifugation and resuspension three times to remove residual Tris-NH_4Cl. Finally, the cells were resuspended at 1.5 x 10^7 cells/ml. 100 µl of cells were added to supernatant fluids dilutions and 25 µl of PHA (1/5 dilution of stock, GIBCO) were also added. The cultures were incubated at 37°C in 7% CO_2 for 72 hrs. One µCi of tritiated thymidine (^3H-TdR, ICN Chemical and Radioisotope, Irvine, CA) was added to each well during the last 4 hr of culture. Cells were harvested onto glass fiber filters (Flow Laboratories, McLean, VA) with a Skatron harvester (Skatron division of Flow Laboratories). Incorporation of radiolabeled thymidine was measured by liquid scintillation spectrometry using a Beckman LS7000 spectrometer (Beckman, Fullerton, CA).

Calculations

Experiments were performed in triplicate. Presented data represent the average of each experimental group. Standard error of the mean was determined by the formula:

$$\text{S.E.M.} = \frac{\text{Standard Deviation}}{\sqrt{N}}$$

Significance was determined by using Student's t-test (29).

CHAPTER III

RESULTS

CSF Induction of IL-1: Effect of Anti-IFN α + β and Indomethacin

Initial experiments were performed to determine optimal conditions for CSF-induced IL-1 production and detection. Interferons and PGE, both anti-proliferative agents (56, 59, 60), are produced by stimulated macrophages (60, 71, 92, 93). Therefore, anti-IFN and indomethacin [an inhibitor of PGE synthesis (59)] were tested for augmentation of IL-1 detection. The final concentrations of these reagents, anti-IFN α + β at 1:1000 and indomethacin at 0.3 μM, had been found by Moore et al. (81) to be sufficient to block the activity and production of these two agents. As in previous investigations, CSF at a concentration of 5000 U/ml (82) stimulated production of IL-1 (Table 1). However, addition of the two reagents to CSF-treated cultures resulted in an approximately three-fold enhancement of IL-1 activity over that stimulated by CSF alone ($p \leq 0.05$). The effects of the combined anti-IFN α + β and indomethacin treatment without CSF stimulation were variable (Exps. 1 and 2, Table 1). In the first experiment, the two reagents together resulted in IL-1 activity similar to that of CSF alone. However, in the second experiment, a different pattern was detected. There was a modest increase in IL-1 production in response to CSF alone, and, in addition, no increase with indomethacin, anti-IFN α + β or the combination of the two without CSF. Based on these findings, both indomethacin and anti-IFN α + β were added to macrophage

Table 1. Influences of anti-IFN α + β antiserum and indomethacin on CSF-induced IL-1 production

Treatment	IL-1 activity (cpm ± sem) Exp. 1	Exp. 2
0	286 ± 109	2439 ± 356
indomethacin	245 ± 159	1386 ± 288
anti-IFN α + β	396 ± 467	554 ± 222
indomethacin + anti-IFN α + β	1032 ± 85	2074 ± 382
5000 U CSF/ml	1095 ± 351[a]	3157 ± 977[N.S.]
5000 U CSF/ml + indomethacin	771 ± 344	2324 ± 782
5000 U CSF/ml + anti-IFN α + β	841 ± 61	2413 ± 1943
5000 U CSF/ml + indomethacin and anti-IFN α + β	2782 ± 370[b]	9590 ± 466[b]

Macrophage-enriched adherent cells from 1.5 x 10^6 proteose-peptone elicited C3H/AnF peritoneal exudate cells were incubated with the indicated materials for 48 hr. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN α + β antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for their respective PHA controls (Exp. 1 - 1088 ± 58, Exp. 2 - 2011 ± 152).

[a]Values represent significant enhancement at $p \leq 0.05$ over controls.

[b]Values are significantly higher than all values at $p \leq 0.05$.

N.S. = No significance.

cultures during the IL-1 production phase of all further experiments to be reported.

LPS Induction of IL-1: Effect of IFN $\alpha + \beta$ Pretreatment

Arezana-Seisdedos and Virelizier (2) and Vogel and Rosenstreich (139) have reported that preincubation of human and murine mononuclear phagocytes, respectively, with IFN enhances the subsequent production of IL-1 by these cells upon stimulation with LPS. A similar experiment performed in this investigation yielded an equivalent response (Table 2). Interleukin-1 production in response to LPS was significantly ($p \leq 0.05$) enhanced at all IFN preincubation doses, but was maximal in the dose range between 312 and 625 U/ml IFN $\alpha + \beta$. An additional observation, to be considered in depth later, was that overnight incubation (approximately 17 hours) with increasing doses of IFN resulted in an increase in supernatant IL-1 from cells receiving no further stimulation (Table 2).

Because the limited range of IFN concentrations between 312 and 625 U/ml resulted in the greatest enhancement of LPS responsiveness, experiments were performed to ascertain if IFN pretreatment could enhance IL-1 production in response to a range of LPS concentrations. Pretreatment with 325 U/ml IFN $\alpha + \beta$ slightly enhanced the production of IL-1 in response to the highest dose of LPS (1.0 μg/ml) (Table 3). More interesting, however, was the finding that the same pretreatment regimen could make macrophages responsive to lower concentrations of LPS (Table 3). In fact, as the concentration of LPS decreased ($p \leq 0.05$), the difference between IFN-treated and non-treated cells became more apparent. For example, at the lowest LPS dose tested (0.001

Table 2. IFN enhancement of IL-1 production in response to LPS

IFN α + β U/ml Pretreatment	IL-1 activity (cpm ± sem) stimulus None	stimulus LPS	cpm LPS-cpm control
0	471 ± 55	1,434 ± 89[b]	983
156	1,370 ± 50[a]	4,384 ± 368[b]	3,014
312	1,662 ± 102[a]	6,423 ± 317[b]	4,761
625	1,686 ± 150[a]	6,694 ± 888[b]	5,008
1250	2,206 ± 128[a]	5,774 ± 292[b]	3,568
2500	2,854 ± 105[a]	6,006 ± 423[b]	3,152

Macrophage-enriched adherent cells from 1.5 x 10^6 proteose-peptone elicited C3H/AnF peritoneal exudate cells were incubated with various doses of IFN α + β for 18 hours. After removal of IFN containing medium and washing with fresh medium, cells were incubated with LPS (1.0 μg/ml) for 48 additional hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN α + β antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for the PHA control (452 ± 29).

[a]Values represent significant enhancement at $p \leq 0.05$ over control.

[b]Values represent significant enhancement at $p \leq 0.05$ over corresponding pretreated controls.

Table 3. Effect of IFN pretreatment on LPS dose response for IL-1 production

Pretreatment/Stimulus	IL-1 activity (cpm ± sem)	
0/0	2,578 ± 534	
IFN/0	2,940 ± 1,309	N.S.
0/0.001 μg	2,723 ± 383	
IFN/0.001 μg	6,144 ± 452	$p \leq 0.05$
0/0.01 μg	3,532 ± 396	
IFN/0.01 μg	9,352 ± 401	$p \leq 0.05$
0/0.1 μg	5,845 ± 1,877	
IFN/0.1 μg	11,950 ± 932	$p \leq 0.05$
0/1.0 μg	16,873 ± 2,109	
IFN/1.0 μg	19,687 ± 3,488	N.S.

Macrophage-enriched adherent cells from 1.5×10^6 proteose-peptone elicited C3H/AnF peritoneal exudate cells were incubated with 325 U/ml IFN $\alpha + \beta$ as described in Table 2. IFN free cells were incubated with various doses of LPS for 48 additional hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN $\alpha + \beta$ antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for the PHA control (3,392 ± 323).

N.S. = No significance.

μg/ml), macrophages which received no IFN pretreatment gave a negligible response to LPS while cells which were pretreated with 325 U/ml of IFN gave a significant ($p \leq 0.05$) response (Table 3).

IFN α + β Enhancement of CSF-induced IL-1 Production

The preceding experiments confirmed prior reports that IFN pretreatment can enhance LPS-induced IL-1 production. Further observations on the direct stimulation of IL-1 production by exposure to high dosages of IFN α + β will be reported in a following section. Herein, the effect of IFN α + β pretreatment on CSF-induced IL-1 production is reported. The same pretreatment regimen (325 U/ml IFN α + β overnight) caused an enhanced ($p \leq 0.05$) production of IL-1 in response to the highest dose of CSF (5,000 U/ml) (Table 4). Also, IFN α + β pretreatment dramatically ($p \leq 0.05$) increased IL-1 production in response to 2,500 U/ml CSF (Table 4). Though the IFN/CSF enhancement did not cover the range of concentrations that LPS displays, the IFN α + β pretreatment did enhance macrophages responsiveness to doses of CSF which normally do not elicit IL-1 (78).

IFN α + β Enhancement of IL-1 Production Stimulated by LPS and CSF

Moore et al. (84) have reported that treatment of LPS-hyporesponsive macrophages from C3H/HeJ mice with a combination of LPS and M-CSF stimulates apparently synergistic production of IL-1. The preceding experiments had demonstrated that pretreatment of exudate macrophages with IFN α + β can enhance IL-1 production stimulated by either LPS or CSF. Therefore, the following experiments were performed to determine if IFN α + β pretreatment influences IL-1 production in

Table 4. IL-1 production by CSF-treated peritoneal macrophages following 18 hour preincubation with IFN $\alpha + \beta$

Pretreatment/Stimulus	IL-1 activity (cpm $\pm$ sem)	
0/0	2,578 $\pm$ 534	N.S.
IFN/0	2,940 $\pm$ 1,309	
0/625 U CSF	1,317 $\pm$ 580	N.S.
IFN/625 U CSF	2,213 $\pm$ 119	
0/1,250 U CSF	2,700 $\pm$ 497	$p \leq 0.05$
IFN/1,250 U CSF	5,385 $\pm$ 157	
0/2,500 U CSF	2,192 $\pm$ 153	$p \leq 0.05$
IFN/2,500 U CSF	4,798 $\pm$ 92	
0/5,000 U CSF	5,788 $\pm$ 255	$p \leq 0.05$
IFN/5,000 U CSF	8,177 $\pm$ 145	

Macrophage-enriched adherent cells from 1.5×10^6 proteose-peptone elicited C3H/AnF peritoneal exudate cells were incubated with 325 U/ml IFN $\alpha + \beta$ as described in Table 2. IFN-free cells were incubated with various doses of LPS for 48 additional hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN $\alpha + \beta$ antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means $\pm$ standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for the PHA control (3,392 $\pm$ 323).

N.S. = No significance.

response to combined stimulation with LPS and CSF. Two different responding cell types were used. These included CSF-responsive but LPS hyporesponsive C3H/HeJ macrophages and CSF-hyporesponsive but LPS responsive cells of the murine $P388D_1$ cell line.

The C3H/HeJ macrophages gave a moderate response to CSF and a slight additional enhancement with IFN $\alpha + \beta$ pretreatment (Table 5). Also, IFN $\alpha + \beta$ pretreated cells produced four-fold as much IL-1 as the non-pretreated cells (Table 5). When the macrophages were co-stimulated with CSF and LPS there was an apparent synergistic response. Indeed, this treatment resulted in IL-1 production levels in excess of ten times the control value (Table 6). Pretreatment with IFN $\alpha + \beta$ resulted in further activation. Pretreatment of macrophages with 500 U/ml IFN $\alpha + \beta$ resulted in a 16-fold increase of IL-1 in comparison to cells which had been identically pretreated but received no further stimulation (Table 5).

Similar treatment of $P388D_1$ cells led to several interesting findings. These cells were normally responsive to LPS, but non-responsive to CSF stimulation (Table 6). Also, these CSF-hyporesponsive cells displayed the apparent synergism upon CSF/LPS co-stimulation (Table 6). Pretreatment with 500 U/ml IFN $\alpha + \beta$ resulted in a four-fold enhancement of the LPS response and a slight enhancement of the CSF response (Table 6). There was also a mild elevation ($p \leq 0.05$) of IL-1 production in response to CSF/LPS co-stimulation over non-pretreated cells (Table 6) as seen with C3H/HeJ macrophages. The IFN $\alpha + \beta$ enhanced responses to LPS, CSF and combined CSF/LPS stimulation were neutralized by addition of anti-IFN $\alpha + \beta$

Table 5. Effect of IFN pretreatment on IL-1 production by C3H/HeJ macrophages

Pretreatment/Stimulus	IL-1 activity (cpm ± sem)	
0/0	383 ± 46	N.S.
IFN/0	582 ± 104	
0/CSF	1,086 ± 67	N.S.
IFN/CSF	1,571 ± 324	
0/LPS	413 ± 184	$p \leq 0.05$
IFN/LPS	1,653 ± 73	
0/CSF + LPS	3,813 ± 713[a]	
IFN/CSF + LPS	9,225 ± 1,418[a]	

Macrophage-enriched adherent cells from 1.5×10^6 proteose-peptone elicited C3H/HeJ peritoneal exudate cells were incubated with 500 U/ml IFN $\alpha + \beta$ as described in Table 2. IFN free cells were incubated with LPS (1.0 μg/ml) and/or CSF (5000 U/ml) for 48 additional hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN $\alpha + \beta$ antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for the PHA control (1,384 ± 97).

[a]Displays synergistic response over the additive effects of the individual stimulants.

N.S. = No significance.

Table 6. Effect of IFN pretreatment on IL-1 production by $P388D_1$ cells exposed to LPS and/or CSF

Pretreatment	IL-1 activity (cpm ± sem) Stimulant			
	0	LPS	CSF	LPS + CSF
0	3,167 ± 245	10,779 ± 2,760[a]	3,193 ± 273[N.S.]	32,172 ± 689[a,b]
500 U IFN/ml	3,317 ± 586	42,446 ± 7,167[a]	6,563 ± 452[a]	60,869 ± 2,276
1:1000 anti-IFN	3,484 ± 218	12,123 ± 1,043	3,547 ± 601	31,944 ± 4,482
500 U IFN/ml + 1:1000 anti-IFN	3,374 ± 228	11,384 ± 1,134	3,148 ± 638	24,918 ± 530

1.5×10^6 cells were incubated with the indicated dose of IFN α + β as described in Table 2. Neutralization took place with addition of anti-IFN α + β antiserum at a dilution of 1:1000 during this period. IFN-free cells were incubated with LPS (1.0 μg/ml) and/or CSF (5000 U/ml) for 48 additional hours. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:25. The PHA background is 393 ± 28.

[a]Values represent significant enhancement at $p \leq 0.05$ over controls.

[b]Displays a synergistic response over the additive effects of the individual stimulants.

N.S. = No significance.

during the pretreatment period (Table 6). This specific neutralization demonstrated that the additional activation was IFN α + β-induced and not due to contaminants such as LPS.

An interesting point can be emphasized. C3H/HeJ macrophages normally respond to CSF but not LPS, whereas $P388D_1$ cells respond to LPS but not CSF. In both systems, the combination resulted in an apparent synergism. What would be the result if the cells used in the experiments were normally responsive to both stimulants such as C3H/AnF macrophages? Results given in Table 7 indicate that there is no synergistic response with cells from these mice. Response to the combined stimuli was additive over a wide range of LPS dosages.

IFN α + β Stimulation of IL-1 Production

Results presented earlier in Table 2 indicated the possibility that IFN α + β may directly stimulate production of IL-1. Subsequent experiments were performed to determine if IFN α + β can directly stimulate IL-1 production. A variety of responder cells, including peritoneal exudate macrophages from C3H/AnF and C3H/HeJ mice, as well as $P388D_1$ cells were used.

While lower concentrations (312 to 625 U/ml) of IFN α + β induced macrophages to produce elevated levels of IL-1 in response to LPS and CSF, higher doses directly stimulated its production. With C3H/AnF macrophages, IL-1 levels began to rise significantly ($p \leq 0.05$) at concentrations of IFN α + β greater than 625 U/ml (Figure 1). An interferon dose response with $P388D_1$ cells indicated that these cells would not respond to direct interferon stimulation (Table 8). This

Table 7. IL-1 production by peritoneal macrophages stimulated with LPS and CSF

µg LPS/ml	CSF	IL-1 activity cpm ± sem)	
-	-	2,585 ± 326	$p \leq 0.05$
-	5,000	5,749 ± 462	
10	0	5,123 ± 134	N.S.
10	5,000	6,731 ± 782	
1.0	0	6,622 ± 385	N.S.
1.0	5,000	7,649 ± 882	
0.1	0	8,728 ± 694	N.S.
0.1	5,000	6,065 ± 926	
0.01	0	4,528 ± 1,043	N.S.
0.01	5,000	7,183 ± 566	

Macrophage-enriched adherent cells from 1.5×10^6 proteose-peptone elicited C3H/AnF peritoneal exudate cells were incubated with the indicated doses of LPS and/or CSF for 48 hours. Indomethacin was added at a final concentration of 0.3 µM and anti-IFN α + β antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for the PHA control (703 ± 34).

N.S. = No significance.

Figure 1. Direct IFN stimulation of IL-1 production.

Macrophage-enriched adherent cells from 1.5 x 10^6 proteose peptone elicited C3H/AnF peritoneal exudate cells were incubated with indicated doses of IFN α + β for 18 hours followed by washing with fresh medium. IFN-free cells were incubated with medium for an additional 48 hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN (α + β) antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. ●--●--●-- Exp. 1. and --▲-- Exp. 2. Incorporation values are corrected for their respective PHA controls (Exp. 1 3392 ± 323, Exp. 2 459 ± 29).

*Values represent significant enhancement at $p \leq 0.05$ over controls.

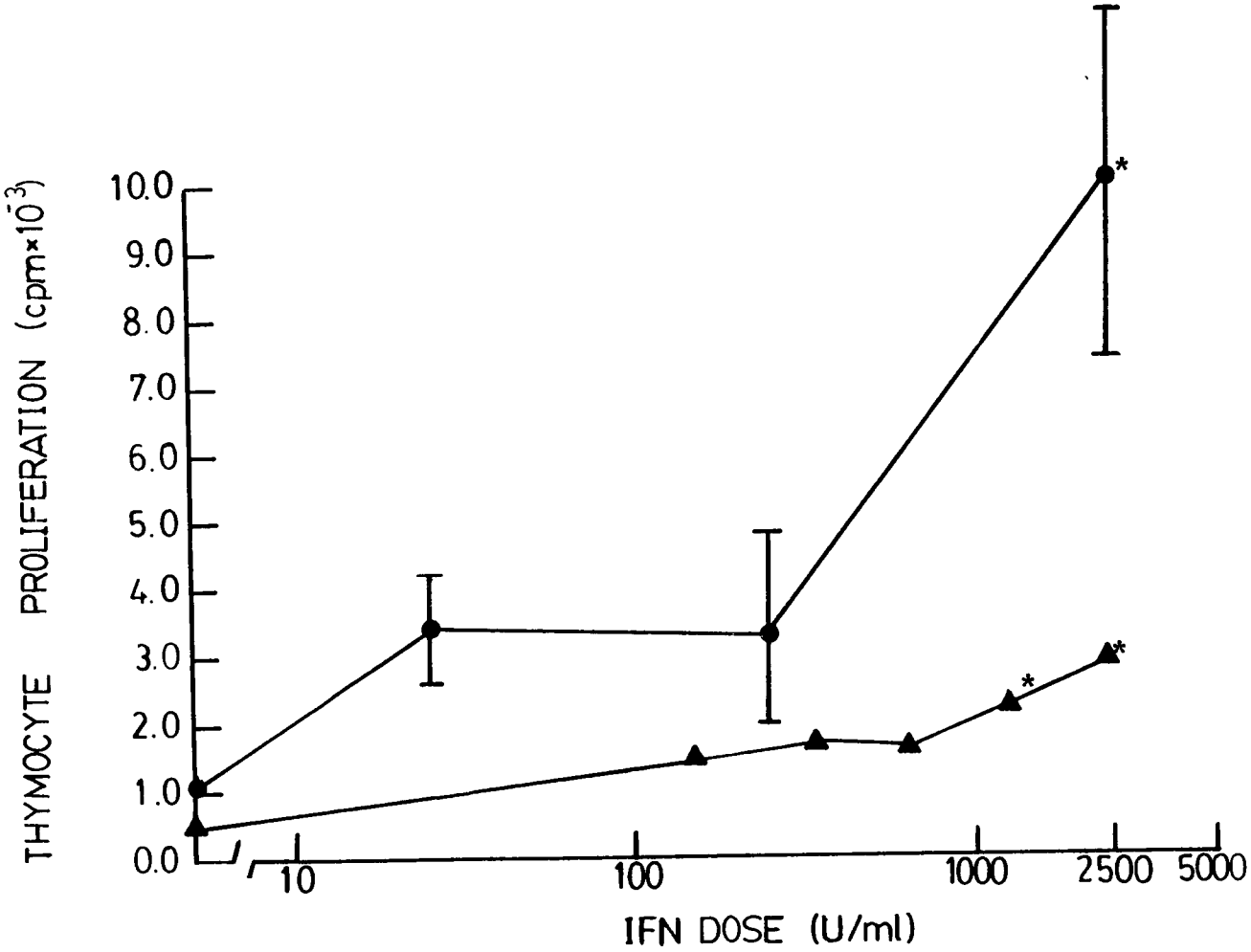

THYMOCYTE PROLIFERATION (cpm×10⁻³)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
10
100
1000
2500
5000
IFN DOSE (U/ml)
*
*
*

Fig. 1.

Table 8. IL-1 production in response to direct IFN stimulation of $P388D_1$ cells

IFN Dose (U/ml)	IL-1 activity (cpm ± sem)
0	2,477 ± 258
10	2,501 ± 119
100	2,403 ± 427
1,000	2,007 ± 130
2,500	3,221 ± 304
5,000	2,423 ± 79

1.5×10^6 cells were incubated with various doses of IFN $\alpha + \beta$ as described in Table 2. IFN-free cells were incubated with medium for additional 48 hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN $\alpha + \beta$ antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^3H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. PHA background is 559 ± 44.

finding provided additional evidence that the observed stimulation is due to IFN $\alpha + \beta$ exclusively, since this cell line is responsive to LPS and should produce IL-1 if there is contamination with this molecule. C3H/HeJ macrophages responded to the IFN $\alpha + \beta$ treatment with maximum IL-1 production at 2,500 U/ml concentration (Table 9). Heating the IFN $\alpha + \beta$ preparation at 56°C for 45 minutes abrogated its ability to stimulate production of IL-1 (Table 9). Although heat treatment of medium alone resulted in higher IL-1 levels, the characteristic IFN $\alpha + \beta$ dose response was not observed. Perhaps the IL-1 production enhancement was due to creation of heat aggregated protein during the heat treatment. Heat treatment of IFN $\alpha + \beta$ served as an additional control in that IFN $\alpha + \beta$ is sensitive to heat, while LPS is not.

An IFN $\alpha + \beta$ preparation purchased from Lee Biomolecular Inc. also directly stimulated production of IL-1 (Table 10). An anti-IFN $\alpha + \beta$ preparation produced against the Enzo IFN $\alpha + \beta$ not only abrogated the IL-1 response to Enzo IFN $\alpha + \beta$, but also neutralized the Lee product (Table 10). This comparison further demonstrates that IFN $\alpha + \beta$ is indeed the activator since both preparations stimulated the response and specific antiserum to one product neutralized the other.

Table 9. IL-1 production by C3H/HeJ macrophages in response to direct IFN stimulation

IFN Dose (U/ml)	IL-1 activity (cpm ± sem)	
	Normal IFN	Heat-inactivated IFN
0	551 ± 303	1,834 ± 140
10	702 ± 36	1,973 ± 229
100	753 ± 137 N.S.	1,993 ± 143
1,000	1,049 ± 83[a]	1,110 ± 348
2,500	2,343 ± 633[a]	1,088 ± 4
5,000	1,293 ± 367 N.S.	1,478 ± 364

Macrophage-enriched adherent cells from 1.5 x 10^6 proteose-peptone-elicited C3H/HeJ peritoneal exudate cells were incubated with indicated doses of IFN α + β or IFN α + β heat-inactivated at 56°C for 45 minutes as described in Table 2. IFN-free cells were incubated with medium for an additional 48 hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN α + β antiserum was added at a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernatant fluid dilutions of 1:5. Incorporation values are corrected for PHA control (1,256 ± 162).

[a]Values represent significant enhancement at $p \leq 0.05$ over controls.

N.S. = No significance.

Table 10. A comparison of stimulation and neutralization of production with two preparations of IFN α + β

Treatment	IL-1 activity (cpm ± sem) Preparation A. Enzo	B. Lee
0	2,407 ± 5	793 ± 268
anti-IFN (1/250 dilution)	2,171 ± 301	388 ± 127
625 U/ml IFN	5,109 ± 80	1,575 ± 217
625 U/ml IFN + anti-IFN (1/250 dilution)	2,172 ± 320 $p \leq 0.05$	0 $p \leq 0.05$
1250 U/ml IFN	4,928 ± 272	2,136 ± 109
1250 U/ml IFN + anti-IFN (1/250 dilution)	2,495 ± 992 $p \leq 0.05$	103 ± 211 $p \leq 0.05$

Macrophage enriched adherent cells from 1.5 x 10^6 proteose-peptone elicited C3H/AnF peritoneal exudate cells were incubated with the indicated additions for 18 hours after which the cells were washed with fresh medium. IFN free cells were incubated with medium for an additional 48 hours. Indomethacin was added at a final concentration of 0.3 μM and anti-IFN (α + β) antiserum a + a final dilution of 1:1000. Values given are thymocyte incorporation of ^{3}H-thymidine and represent the means ± standard error of triplicate determinations at supernate dilutions of 1:5. Incorporation values are corrected for their respective PHA controls (Enzo - 448 ± 100, Lee - 1,875 ± 97).

CHAPTER IV

DISCUSSION

Incorporation of indomethacin and anti-IFN $\alpha + \beta$ in the medium used during the stimulatory phase of IL-1 production, significantly ($p \leq 0.05$) enhanced detectable supernatant IL-1 activities. Vogel (135) has determined that indomethacin-augmented IL-1 activity results from inhibition of synthesis of antiproliferative PGE rather than by enhancement of IL-1 production. Similarly, Moore (78) has determined that anti-IFN exerts its augmentation by neutralization of IFN rather than directly effecting IL-1 production. Used in combination in this investigation, these two reagents significantly augmented the detection of supernatant IL-1 activity permitting direct supernatant assay avoiding the necessity of prior sample dialysis.

This investigation was performed to determine if IFN $\alpha + \beta$ influences the responsiveness of mononuclear phagocytes to macrophage-specific M-CSF. The results clearly indicate that prior exposure of exudate macrophages to IFN $\alpha + \beta$ enhances IL-1 production upon subsequent stimulation with the growth factor. This effect was remarkably similar to IFN-augmented proliferation of committed macrophage precursors to CSF previously reported by Moore and coworkers (79, 83). Thus, IFN $\alpha + \beta$ apparently influences the responsiveness of both immature and mature cells of this lineage to the mononuclear phagocyte-specific growth/stimulatory factor.

Additional findings reported herein confirm previous observations (2, 139) that IFN α + β can augment IL-1 production in response to LPS. This enhancement, as additionally noted for CSF, was most dramatic at suboptimal stimulatory doses of LPS.

The mechanism(s) by which IFN α + β enhances CSF and LPS responsiveness remains unknown. Two distinct possibilities involve expression of specific receptors. Perhaps short exposure of macrophages to certain concentrations of IFN enhances the expression of CSF and LPS receptors. When considering evidence presented by others, this idea seems feasible. IFN appears to be instrumental in the normal regulation of surface marker expression. Vogel et al. (137) demonstrated that IFN treatment of macrophages results in a significant increase in expression of Fc receptors as well as a functional increase in Fc mediated phagocytosis (136, 137, 140). Interferons also influence expression of macrophage surface Mac-1 and Ia antigens. There is a decrease in Mac-1 expression (140) and an increase in Ia expression (124). The significance of this finding is that interferon influences differentiation as well as functional surface associated molecule expression. Mac-1 antigen expression is apparently limited to less differentiated mononuclear phagocytes (40) while Ia antigen expression is linked to a functionally differentiated state (4). Additional experiments by Vogel (135) has indicated that endogenous production of IFN by ostensibly normal murine macrophages is at least partly responsible for positive regulation of Fc receptor expression. Conversely, macrophages of the genetically defective C3H/HeJ strain expressed a lower number of Fc receptors due to a putative defect in

production of endogenous interferon (135). A second possible explanation for interferon enhancement of CSF and LPS responses may be that such treatment simply prevents loss of responsiveness or receptors upon in vitro culture.

The apparently synergistic production of IL-1 observed with LPS-hyporesponsive C3H/HeJ macrophages following stimulation with both LPS and CSF raises speculation as to the normal process of IL-1 stimulation. Why did these cells respond to co-stimulation with CSF and LPS by producing IL-1 levels in excess of their additive effects while normal macrophages (C3H/AnF) did not? C3H/HeJ macrophages normally can not be activated by LPS (139), but, perhaps, incubation with this molecule could alter critical surface characteristics, possibly membrane fluidity, resulting in enhanced cellular responsiveness to other mediators such as CSF. Another explanation may be that production of IL-1 involves the prior release of an intermediate product. To illustrate this, CSF induces macrophages to produce interferon (80) which may act endogenously to activate the LPS hyporesponsive macrophages. Thus, through endogenous mediation these cells may produce enhanced levels of IL-1 in response to CSF and LPS. This mechanism, however, cannot explain the presented data since anti-IFN was included in the culture medium during stimulation. C3H/AnF cells may not respond to co-stimulation because they normally can be activated with either one mediator or the other. Endogenous CSF may also serve as an intermediate in the normal response to LPS. Normal macrophages rapidly produce CSF in response to LPS (95). This CSF could function endogenously to stimulate production of IL-1. If

this is indeed true, additional CSF should not further stimulate these cells since endogenous CSF has fully activated them.

The concept of CSF acting as an endogenous mediator of LPS responsiveness has been suggested previously by Kurland et al. (60), and, as such, is not an unsupported hypothesis. Colony-stimulating factors are known stimulants of macrophage PGE synthesis (60). Kurland et al. with supporting data, suggested that at least part of the total PGE produced by LPS-stimulated macrophages resulted from secondary endogenous stimulation of the culture by CSF produced upon LPS stimulation. While the basis(es) for IFN $\alpha + \beta$ enhancement remains undetermined, detection of a similar augmentation with the $P388D_1$ cell line provides an excellent tool for future investigation. With this cell line and with macrophage-enriched exudate populations, questions such as those outlined regarding the influence of IFN $\alpha + \beta$ on CSF receptors become feasible to investigate. In the precursor response system of Moore and Rouse (83) where the frequency of responsive cells is $\leq$1 cell in 100,000, analysis of these questions was impractical.

Another major finding in this investigation is that IFN $\alpha + \beta$ directly stimulated production of IL-1 at high doses ($\geq$1000 U/ml). This confirms recent investigations by Boraschi and co-workers except that their experiments involved direct stimulation with recombinant IFNα and IFNγ (8). A noteworthy observation in the present investigation was that the stimulation took place in two phases. At the lower range of doses, interferon gradually increased the production of IL-1, but at approximately 1000 U/ml of IFN, production increased

sharply. This might indicate the endogenous production of another intermediate which could additionally stimulate IL-1 production. However, it may also be directly related to toxicity. Doses of IFN α + β in excess of 1000 U/ml were noted to be generally toxic for macrophages and have been reported to disrupt cytoskeletal structures (130). These observations pose the interesting question of whether the direct stimulation for IL-1 represents newly stimulated production and release of IL-1 or simply release of preformed mediator (32). Macrophage-disruptive agents such as silica (32) act to enhance release of IL-1, and it is distinctly possible that excessive levels of IFN α + β may act similarly.

A logical extension of these findings is their potential relationship to viral infections. IFN is one of the earliest appearing mediators in response to viral infections (46). Perhaps this release has analogous activating abilities as endotoxin in bacterial infections. Through this rapid production of interferon and subsequent enhancement of IL-1 production, the host may mount accelerated cellular and humoral immune responses. IL-1 is an essential modulator of both T and B lymphocyte proliferation (32, 150, 151). This mechanism for IL-1 enhancement may also influence the febrile response associated with acute viral infections. As mentioned previously, IL-1 is believed to be identical to the fever-inducing molecule, EP (73), and the ubiquitous presence of LPS and frequent presence of CSF in tissues and tissue fluids (95) provides the additional possibilities of indirect as well as direct induction of IL-1 during viral infection.

Interleukin-1 elicited either directly or indirectly by IFN may play a role in local inflammatory processes. Indeed, Isaacs et al. (116) found IFN in every sample of nasal secretion from patients with rhinovirus infection. Also, administration of purified IFNγ to nasal mucosa of health volunteers resulted in a significant increase of nasal secretions (cited in 116). Observations of skin reactions in response to interferon are well documented (7, 114, 115, 116). Immediate flare reactions in response to injections of crude IFNα and IFNβ in humans gradually faded within an hour but were replaced with a dense erythema which peaked at 4 to 8 hours and persisted up to 48 hours or longer (115). Skin biopsies demonstrated immediate polymorphonuclear leucocyte infiltration, but by 4 hours, the infiltrate was primarily composed of mononuclear cells (116). Further studies with more highly purified IFN have indicated that the inflammatory response was due to the IFN itself and not to the presence of contaminants (114, 115). Since the skin contains not only macrophages but also cells with certain macrophage-like properties (Langerhans cells and keratinocytes), an attractive supposition is that interferon directly stimulates the localized production of IL-1 by these cells and that this IL-1, as a chemoattractant (64, 111), may influence the observed skin inflammation. Furthermore, intramuscular injections of purified IFN elicit a rise in the circulating polymorphonuclear cell count reported to be maximal at 8 hours (116). Again, release of IFN-induced IL-1, a neutrophil releasing agent (51), may be the cause of this neutrophilia.

Finally, many investigators have observed mild to severe fever reactions in patients who have received interferon treatment (7). The febrile response was seen with use of either IFNα and IFNβ of various purities (7). Both highly purified recombinant human IFNα and human IFNα produced by lymphoblastoid cells were found to be pyrogenic (7). These observations along with the association of IFN and fever in viral infections are highly suggestive that interferon inducible IL-1 may be responsible for these pathological, but potentially beneficial, effects of IFN.

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LIST OF REFERENCES

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VITA

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