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**Brain Cytokine Component Regulation during Interferon Administration and
Pathophysiological Conditions**

**A thesis submitted to the Department of Psychology and Faculty of Graduate Studies
and Research of Carleton University in partial fulfillment for the degree of**

**Doctor of Philosophy in Psychology
Specialization in Neuroscience**

© Nicolas P. Turrin

Department of Psychology

Carleton University (2002)

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Acceptance of the thesis:

"Brain Cytokine Component Regulation During Interferon Administration and
Pathophysiological Conditions"

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in Partial fulfillment of the requirements for
the degree of Doctor of Philosophy

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ABSTRACT

Immune activation involves the synthesis of cytokines in the periphery as well as in the brain. Cytokines are soluble mediators first discovered to play an immunoregulatory role. Their presence in nervous tissue lead to research showing their involvement in neural processes. Studies suggest that cytokines play a role in neuroinflammation, neurodegeneration, neurophysiology and the neurological manifestations associated with immunotherapy and disease. Cytokines act directly on nerve cells or in cascades that stimulate or inhibit the synthesis of other cytokines as well as their own. Since their actions are crucial to immunotherapy and disease progression, it becomes imperative to understand the brain cytokine profile associated with such states. Since most cytokines do not act alone, deciphering cytokine-cytokine, cytokine-neuropeptide and cytokine-neurotransmitter interactions could clarify their individual role in neurological processes. A better understanding of these interactions could lead to strategies of intervention to alleviate the detrimental neurological manifestations (such as anorexia) associated with chronic cytokine activation. The studies of this thesis addressed some of these issues. The cytokines, neuropeptide, and associated components mRNA profiles in the brain of rats during a model of interferon-alpha immunotherapy, and during models of disease (endotoxin administration and tumor progression) were assessed. When relevant, the cytokine profiles in the blood and peripheral organs were characterized to determine mechanisms of periphery-to-brain, and *vice versa*, cytokine activation. It was observed that pro-inflammatory cytokines are upregulated in the brain in response to interferon- α treatment, endotoxin as well as in

tumor-bearing rats. The anti-inflammatory cytokines were up-regulated in most brain regions following IFN α treatment. However, following LPS treatment and in the tumor-bearing rats, these inhibitory cytokines showed little change in mRNA level. Furthermore, an intervention that used NPY, a potent orexigenic peptide, to counteract the interferon-alpha-associated anorexia was studied. NPY was able to counteract the anorexigenic effects of IFN α . Since the anorexia reappeared following the effects of NPY, it is possible that NPY is able to act on the anorexigenic effects of IFN α without affecting its immune properties.

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PUBLISHED PAPERS

Study 2 and 3 of this thesis are primarily based on the following papers, which will be referred in the text by their roman numerals.

- I. Turrin NP, Flynn MC, Plata-Salamán CR. Neuropeptide Y counteracts interferon-alpha-induced anorexia. *Neuroimmunomodulation*, 6:361-366; 1999.
- II. Turrin NP, Gayle D, Ilyin SE, Flynn MC, Langhans W, Schwartz GJ, Plata-Salamán CR. Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal administration of bacterial lipopolysaccharide. *Brain Research Bulletin*, 54:443-453; 2001.

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ABBREVIATIONS

AcP	Accessory protein
ACTH	Adrenocorticotrophic hormone
AP	Activating protein
APR	Acute phase response
BBB	Blood-brain barrier
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRH	Corticotropin releasing hormone
CVO	Circumventricular organ
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HPA	Hypothalamus-pituitary-adrenal
ICAM	Intercellular adhesion molecule
ICV	Intracerebroventricular
IFN	Interferon
IgM	Immunoglobulin M
IL	Interleukin
IL-1R	IL-1 receptor

IL-1Ra	IL-1 receptor antagonist
IP	Intraperitoneal
JAK	Janus kinase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MCA	Methylcholanthrene
MDP	Muramyl dipeptide
NF- κ B	Nuclear factor- κ B
NIK	NF- κ B-inducing kinase
NPY	Neuropeptide Y
OB-R	Leptin receptor
POMC	Pro-opiomelanocortin
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TNF	Tumor necrosis factor

VAF

Virus antibody free

VCAM

Vascular cell adhesion molecule

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GLOSSARY

Activating protein 1: Transcriptional regulatory factor involved in the expression of several pro-inflammatory cytokines.

Acute phase proteins: Serum proteins that increase during acute inflammation.

Acute phase response: A nonspecific response by an individual stimulated by IL-1 β , IL-6, IFN and TNF- α . C-reactive protein may show a striking rise within a few hours. Infection, inflammation, tissue injury, and often neoplasm may be associated with APR. The liver produces acute-phase proteins at an accelerated rate, and the endocrine system is affected with elevated gluconeogenesis and impaired thyroid function.

Adrenocorticotrophic hormone: Polypeptide hormone secreted by the anterior pituitary; stimulates cells of the adrenal cortex to secrete cortisol.

Alpha 1 (α 1)-a glycoprotein: Plasma protein whose elevated levels are associated with inflammation, pregnancy and disease.

Alpha 1 (α 1)-antitrypsin: Protease inhibitor present in the serum that is produced by the liver, especially during the acute phase response.

Alpha 1 and 2 (α 1, 2) macroglobulins: Large plasma anti-proteases with very broad spectrum of inhibitory activity against all classes of proteases.

Antigen: Any foreign material that is specifically bound by a specific antibody or specific lymphocytes.

Arginine vasopressin: A peptide hormone released from the posterior pituitary lobe but synthesized in the hypothalamus. Has anti-diuretic properties and can act on the HPA axis.

Autocrine: The action of a chemical on the same cell that synthesized it.

Beta-amyloid: Principle component of amyloid fibrils deposited during Alzheimer's disease.

Cachexia: A wasting away syndrome (progressive loss of body weight accompanied by weakness) that characterizes the clinical course of many cancers. The cytokine, TNF- α is thought to be an important mediator of this syndrome.

Chemokine: Member of the group of low molecular weight (6-14 kDa) cytokines describes for their chemoattractant properties.

Ciliary neurotrophic factor: A hormone related to the IL-6 family. It has several functions in the nervous system, which are similar to those of leukemia inhibitory factor (see below).

Complement: Regulatory proteins and receptors found on blood and tissue cells. The complement system is activated by the reaction between antigen and antibody. It consists of a series of serum enzymes, the end result of which is lysis of the target or enhanced phagocytosis by phagocytic cells. Complement proteins are significant, nonspecific mediators of humoral immunity.

Corticotropin releasing hormone: Hypothalamic hormone that stimulates ACTH secretion by the anterior pituitary.

Cytokines: Immune system proteins that are biological response modifiers. They coordinate antibody and T cell immune system interactions and amplify immune reactivity. Cytokines include monokines (e.g., IL-1, TNF- α) synthesized by macrophages, and lymphokines (e.g., interleukins, gamma interferon, lymphotoxin) produced by activated T lymphocytes and natural killer cells.

Endotoxin: A Gram-negative bacterial cell wall lipopolysaccharide that is heat stable and causes neutrophils to release pyrogens. It may produce septic shock and causes blood coagulation, stimulates cytokine and complement cascades and can lethal consequences.

Glucocorticoid: One of a group of hormones (e.g., cortisol) of the adrenal cortex that are important in protein and carbohydrate metabolism, secreted especially in times of stress.

Gp 130: A 130 kDa membrane-bound glycoprotein part of the function complex with IL-6-family receptors and, upon binding of the receptor's specific ligand, signals that this binding has occurred.

Haptoglobin: Diverse group of acute phase globular proteins that function as antioxidants and are named for their ability to combine with hemoglobin in the blood.

Immunoglobulin M: Antibody composed of five identical subunits predominantly found during the early phase of an immune response. It is also found on the surface of naïve B cells.

Intercellular adhesion molecule-1: Molecule found on the surface of endothelial cells that regulate cell adhesion and trafficking.

Interferon alpha: Cytokine produced by monocytes, macrophages, lymphoblastoid and mesenchymal cells. It is recognized for its antiviral properties and its ability to inhibit the proliferation of B cells, monocytes, fibroblasts, and transformed cells.

Interferon gamma: Synthesized by activated T-helper cells. Activates killer T cells, NK cells and macrophages.

Interleukins: A group of cytokines synthesized by lymphocytes, monocytes and other cells, which promote growth of T and B cells and other hematopoietic stem cells, and have a variety of other biological functions.

Interleukin-1: Produced by monocytes and macrophages; it stimulates acute phase protein synthesis and promotes T-cell proliferation; it is also an endogenous fever-producing substance. IL-1 is comprised of two principal polypeptides: IL-1 α and IL-1 β .

IL-1 receptor antagonist: Molecule that binds to the IL-1 type I receptor and blocks the binding and biological activity of IL-1.

IL-2: Secreted by T-helper cells. IL-2 has an autocrine effect, acting on the CD4 cells that produce it, but it mainly affects lymphocytes. IL-2 (formerly known as T-cell growth factor) is a factor required for the growth of some T cells, B cells, and promotes synthesis of antibody by B cells. IL-2 also promotes growth of NK cells, and potentiates their cytolytic action.

IL-4: (formerly known as B-cell growth factor) An anti-inflammatory cytokine that suppresses the production of IL-1 and TNF- α , and chemokines. Produced exclusively by T cells.

IL-6: (formerly known as B cell differentiation factor): produced by vascular endothelial cells, phagocytes, fibroblasts, activated T lymphocytes, and neoplasms. IL-6 is secreted in response to IL-1 or TNF. It mainly acts on B cells and hepatocytes. Its main function is to cause B lymphocytes to differentiate into cells that synthesize antibodies. IL-6 also induces hepatocytes to form acute-phase proteins including fibrinogen.

IL-8: Chemokine, produced by different cells, that is chemoattractive for and activates neutrophils. Its production is increased by IL-1 and TNF- α .

IL-10: Anti-inflammatory cytokine that down-regulates the synthesis of IL-1, IFN γ , IL-2, TNF- α , and other pro-inflammatory cytokines and up-regulates anti-inflammatory factors such as the production of IL-1Ra and the shedding of TNF receptors. Mostly produced by Th2 cells, monocytes, and epithelial cells.

IL-13: Anti-inflammatory cytokine, closely related to IL-4, that supports B-cell growth and development and down-regulates IL-1, TNF- α , and chemokine production.

Intracrine: The ability of a chemical to act within the cell that produces it.

Janus kinase: Phosphorylating enzyme involved in gp130-dependent signal transduction by IL-6 family cytokines.

Leptin: Cytokine produced by the obese (*ob*) gene. Leptin is produced by fat cells and regulates food intake through signals to the hypothalamus. It may also act to regulate energy metabolism and reproductive function.

Leukemia inhibitory factor: A 20 kDa lymphoid factor related to IL-6 in terms of molecular structure. It is involved in development and maintenance of stem cells and bone remodeling through the induction of mitogenesis of numerous cells demonstrated in cell culture.

Leukocyte: White blood cell. The principle types include the granulocytes (neutrophils, eosinophils and basophils), lymphocytes (T and B cells) and monocytes (macrophages).

Lymphocytes: Small cell with virtually no cytoplasm, and bears antigen-specific receptors; found in blood, in all tissue, and in lymphoid organs, such as lymph nodes, spleen, Peyer's patches. Lymphocytes are divided into 2 principal groups: T and B lymphocytes. In addition, NK cells, which are large granular lymphocytes, comprise a small percentage of the lymphocyte population. Lymphocytes are distinguished by the expression of distinctive surface molecules that have precise roles in immune reaction.

Mitogen: A substance often derived from plants, which causes DNA synthesis and induces blast transformation and division by mitosis. Lectins, representing plant-derived mitogens, are widely used in clinical and experimental immunology to evaluate T and B lymphocyte function in vitro. Phytohemagglutinin and concanavalin A are principally T cell mitogens. Staphylococcal protein A is the mitogen used to induce B lymphocyte transformation. Pokeweed mitogen is a B and T cell mitogen.

Mitogen activated protein kinases: Group of protein kinases that act in sequence during intracellular signaling by numerous families of receptors. Ligand binding and G-protein activation leads to activation of MAPK3, which phosphorylates MAPK2 (also known as MEK), which in turn phosphorylates MAPK.

Natural killer cells: Cells that attack and destroy tumor cells and certain virus-infected cells. NK cells induce lysis through the action of antibody. Immunologic memory is not involved, in that previous contact with the antigen is not necessary.

Nuclear factor-Kappa B: A ubiquitous transcriptional factor activated by phosphorylation of its regulatory factor, I κ B. When activated, NF- κ B enters the nucleus and binds to transcription elements on the cell's DNA. Genes activated by NF- κ B are involved in immune or inflammatory responses and cell growth control.

NF- κ B-inducing kinase: An enzyme that activates NF- κ B as part of the signal transduction process.

Paracrine: Local effects of a chemical acting on cells in its immediate vicinity.

Plasma: Transparent yellow fluid that constitutes 50-55% of blood volume. It is 92% fluid, 7% protein, and 1% inorganic salts, sugars, lipids, hormones and gases.

Pleiotropy: The tendency of a cytokine to have multiple target cells and multiple actions.

Prostaglandin: Biologically active lipids, with hormonal actions, derived from arachidonic acid by actions of the enzyme, cyclooxygenase. Found in virtually all mammalian tissues, prostaglandins have numerous actions including decreasing blood pressure, stimulation muscle contraction, regulating inflammation and blood clotting and modulating immune responses.

Pyrogen: A substance that induces fever. It may be endogenously produced, such as by IL-1 or may result from exogenous infection (e.g. bacterial or viral insults).

Sensitization: A process whereby an animal is exposed to some challenge and then reexposed to a milder form of the insult after some time delay. A sensitization is manifested if the biological or behavioral response to the reexposure stimulus is significantly greater than that which would be observed after a single exposure to the particular challenge. Thus, the underlying biological system(s) can be said to be primed by the initial stimulus exposure and such effects often increase with the passage of time following the treatment. In immunological terms, sensitization refers to a situation in which exposure to an antigen primed the immune system such that a second exposure to the same antigen results in a greater response (e.g. elevated antibody levels).

Serum: Plasma from which fibrinogen and clotting factors have been removed.

Signal transduction and activators of transcription: Latent transcription factors that are activated following stimulation of gp 130 by IL-6 superfamily cytokines.

Synergism: Situation in which the combined effects of two or more substances result in an effect greater than the additive effects of the individual actions of the substances. For

instance, with respect to plasma corticosterone release, IL-1 + TNF- α results in a much greater rise (synergistic) of corticosterone than if the cytokines were administered individually and their effects summed (e.g. IL-1 alone = 10 units, TNF- α alone = 5 units, IL-1 + TNF = 15 units if they act in an additive fashion, but if IL-1 + TNF- α = 30 units then they may be said to act in a synergistic fashion). Cytokines typically have numerous synergistic effects.

Thromboxane: Arachidonic acid metabolites produced by the action of thromboxane synthetase on prostaglandin cyclic endoperoxides. It is a potent inducer of platelet aggregation and release.

Transforming growth factor-beta: A cytokine which induces the proliferation of many cell types including epidermal and epithelial cells. It also facilitates the growth of selected transformed cells.

Tumor Necrosis Factor: TNF- α is a monokine produced by macrophages stimulated with bacterial endotoxin; it participates in inflammation, wound healing and tissue remodeling. The cytokine also has potent anti-tumor properties and is associated with cachexia. More recently, this cytokine has been shown to be active in the central nervous system in terms of neurochemical and neuro-hormonal actions.

Vascular cell adhesion molecule: Molecule on the surface of endothelial cells that regulate cell adhesion and trafficking.

GENERAL INTRODUCTION

During the late sixties and early seventies, growing evidence supporting the influence of the central nervous system (CNS) on immune functioning began to emerge. This evidence came from discoveries suggesting a role for the brain in the regulation of viral, bacterial as well as oncological ailments. A decade later, this trend led to breakthrough research suggesting that bi-directional communication occurred between these systems so that the immune factors also influenced behavior and central neuronal processes [see Ader et al., Anisman et al., 1996, Haas and Schauenstein, 1997, Plata-Salamán, 1991, for review].

Immune tissue, such as lymph nodes, receives innervation from the CNS [Ader et al., 1991]. Conversely, the brain houses microglial cells, which behave like immune cells and phagocytes [Zielasek and Hartung, 1996]. In addition to these neural and cellular mechanisms of communication between the brain and the immune system, humoral mediators also exist to permit interactions between them. These factors include hormones (especially those of the hypothalamic-pituitary-adrenal (HPA) axis), neuropeptides and, importantly, cytokines [see Bellinger et al., 1990, Plata-Salamán, 1991, Turnbull and Rivier, 1999, for review].

Cytokines and their receptors in the brain

Cytokines are generally glycoproteins that act as soluble cell-to-cell signaling molecules. Earlier on, they were thought to be secreted by, and to act solely on, immune cells such as macrophages and lymphocytes. It has now been established that most cells of

the body express cytokines and/or their receptors, including the cells of the nervous system. In the brain, cytokine expression is apparent in neurons, glial cells, such as microglia and astrocytes, as well as endothelial cells of the cerebrovasculature and brain macrophages [Bartfai and Schultzberg, 1993, Haas and Schauenstein, 1997]. Cytokine receptors are ubiquitously present on most cells of the CNS; thus, cytokines can act directly on neurons to alter their activity.

Pro-inflammatory and anti-inflammatory cytokines

The neuroinflammatory response that results from both central and peripheral infections represents a typical model of the action of cytokines in the CNS. Cytokines are classified as being either pro-inflammatory (stimulatory) or anti-inflammatory (inhibitory). However, the specific role of individual cytokines during inflammation (or injury) can shift from beneficial to deleterious depending on the nature of the pathological state in which a cytokine is involved and its target cell population [Akaneya et al., 1995, Strijbos and Rothwell, 1995]. This shift can be observed for various interleukins (ILs) [Papanicolaou et al., 1996], interferons (IFNs) [Aman et al., 1993, Friedman et al., 1996], tumor necrosis factor-alpha (TNF- α) [Souba, 1994, Yamaguchi et al., 1998] and transforming growth factor-beta 1 (TGF- β 1) [McGee et al., 1995, Möller et al., 1994].

Pro-inflammatory cytokines, which include IL-1, IL-6, IL-8, TNF- α , IFN α and IFN γ , can stimulate the occurrence of neurological symptoms (e.g., anorexia) and physiological signs (e.g., fever) observed during infection [Souba, 1994, Janský et al., 1995,

Plata-Salamán, 1996a]. These cytokines are also referred to as being stimulatory, since they activate the immune and inflammatory responses, the synthesis of acute phase proteins, the neuroendocrine system (e.g., HPA axis) and other regulatory processes [Buckingham et al., 1996, Cannon, 1998, Kaibara et al., 1998, Plata-Salamán, 1999, Sternberg, 1997, Turnbull and Rivier, 1999]. Immunologically, the term pro-inflammatory is broader relative to the designation Th1 cytokines (e.g., IFN γ , IL-2) [Sternberg, 1997, Woerly et al., 1999], which refers to cytokines released by the Th1 T-helper lymphocytes to activate cellular immunity and induce an inflammatory response.

The cytokines IL-4, IL-10, TGF- β 1 and the IL-1 receptor antagonist (IL-1Ra) are designated anti-inflammatory since they can ameliorate or block the neurological manifestations induced by inflammation. They are also known as inhibitory cytokines since they can inhibit or block the induction and/or action of pro-inflammatory cytokines [Ilyin and Plata-Salamán, 1997, Plata-Salamán and French-Mullen, 1992, Plata-Salamán et al., 1998b, Swiegel et al., 1997, Tadmori et al., 1994]. The term anti-inflammatory is broader than that of Th2 cytokines (e.g., IL-4, IL-10, IL-13) [Sternberg, 1997, Woerly et al., 1999], which typically refers to cytokines released by Th2 T-helper lymphocytes, and is primarily involved in humoral (antibody-related) responses and suppression of inflammation.

The administration of exogenous pro-inflammatory cytokines into the CNS can mimic inflammatory and immunological processes (including the generation of neurological and neuropsychiatric manifestations) observed during neuropathophysiological conditions.

Moreover, subsequent or prior administration of their anti-inflammatory counterparts can block or reverse the action of pro-inflammatory cytokines [Krueger et al., 1995, Liew, 1995, Plata-Salamán and French-Mullen, 1992, 1994, Swiergiel et al., 1997]. In general, administration of anti-inflammatory cytokines by themselves does not induce the reverse effect of pro-inflammatory cytokines; inhibitory cytokines ameliorate or block the neurological manifestations of the pro-inflammatory cytokines, without having significant activity of their own.

Cytokine action and signaling

Cytokines exert their effects on their targets using intracrine (within the cell they are synthesized), autocrine (on the receptors of the same cell from which they are released), paracrine (on nearby cells) or endocrine (on distal organs by travelling through the blood stream) pathways. Cytokines exhibit pleiotropy and redundancy: a cytokine can have multiple functions and these functions can be mimicked (in part) by other cytokines.

Cytokines can act in *sequence*, where a cytokine triggers the synthesis or release of one or several other cytokines, which can regulate the synthesis and release of the triggering cytokine as well as other upstream and downstream cytokines [Plata-Salamán, 1999]. They can also act in concert and in *parallel*, where regulatory patterns will depend on the cooperative or antagonistic effects of multiple cytokines in response to a neurophysiological or neuroimmunological process [Ilyin et al., 1997, Plata-Salamán and Ilyin, 1997, Sternberg, 1997]. Moreover, the timing, location, and cellular cast [neurons, glia, immune cells,

endothelial and other cells (e.g., mast cells) of the cerebrovasculature, and foreign cells or particles (e.g., bacteria, virus, and parasites)] involved in cytokine production and action in the brain are all of importance [Elias and Zitnik, 1992, Henderson et al., Ilyin and Plata-Salamán, 1996, Plata-Salamán, 1991].

Cytokine interactions and cytokine balance

Each cytokine plays a distinctive role in CNS processes, but a similar profile of interaction occurs among cytokines. For instance, IL-1 and TNF- α exhibit synergistic interactions, resulting in an effect that is significantly greater than the sum of the individual cytokines' effects, in neural and non-neural models [Bluthé et al., 1994, Chao et al., 1995, Elias et al., 1994, Gayle et al., 1998b, Jeohn et al., 1998, Plata-Salamán, 1998c, Rockett et al., 1994, Sonti et al., 1996b]. IL-1 and IL-1Ra, on the other hand, exhibit antagonism in every model that has been tested [Dinarello, 1997, 1998, Krueger et al., 1995, Plata-Salamán and French-Mullen, 1992, 1994].

The role of other neuroactive endogenous substances such as neurotransmitters, (neuro)peptides and hormones in cytokine signaling should not be overlooked and, in certain cases, this modulation is critical [Sonti et al., 1996a, Plata-Salamán, 1997, Zumwalt et al., 1999]. For instance, in response to the activation of the HPA axis by pro-inflammatory cytokines [Buckingham et al., 1996, Turnbull and Rivier, 1999], central and peripheral signaling mechanisms are activated that, in turn, regulate cytokine production and action through negative regulatory feedback mediated by glucocorticoids [Turnbull and Rivier,

1999]. Emphasis on a holistic approach to study the regulation of central signaling in response to brain chemical interactions is therefore warranted. The initial model proposed included reciprocal neurotransmitter ↔ peptide / neuropeptide ↔ cytokine interactions [Plata-Salamán, 1997].

The interactions between pro- and anti-inflammatory cytokines play a crucial role in the expression of neurological manifestations associated with disease. For example, data obtained in inflammatory models show that anorexia correlates well with a balance toward pro-inflammatory cytokines. That is, challenges that induce both pro-inflammatory and anti-inflammatory cytokines in the brain such as bacterial products from Gram negative bacteria (lipopolysaccharide; LPS) or from Gram positive bacteria (muramyl dipeptide; MDP), show a clear relationship between anorexia and the magnitude of pro-inflammatory over anti-inflammatory cytokine induction [Gayle et al., 1998a Ilyin et al., 1998a, Plata-Salamán et al., 1998b]. During the central infusion of LPS or MDP, both pro- and anti-inflammatory cytokines are up-regulated in brain regions [Gayle et al., 1998a, Plata-Salamán et al., 1998b]. However, during LPS administration, which causes significantly greater anorexia than MDP, the up-regulation of pro-inflammatory cytokines is significantly more prominent relative to that induced by MDP [Gayle et al. 1998a, Plata-Salamán et al., 1998b]. In contrast, MDP induces a greater up-regulation of anti-inflammatory cytokines and significantly less anorexia relative to LPS [Gayle et al., 1998a Plata-Salamán et al., 1998b]. This suggests that, during neuroinflammation caused by central bacterial infections, it is not just the cytokine levels *per se*, but also the ratio between pro- and anti-inflammatory cytokines that could determine the

magnitude of neurological manifestations and the underlying neuropathophysiological process [Plata-Salamán, 1999]. The interaction between pro- and anti-inflammatory cytokines is essential to maintain an optimal inflammatory response; since various pro-inflammatory cytokines, such as IL-1, only need to occupy a minimal number of receptors to induce full biological activity, and greater amounts of the inhibitory factors (in this case IL-1Ra) are needed in order to modulate their activity.

Convergence in signal transduction pathways

Many cytokines exert actions similar to those of other cytokines since they involve pathways that converge onto common downstream signaling components. For example, the homologous signaling components that exist between the leptin receptor (OB-R) and the IL-6 subfamily gp 130 and Janus kinase (JAK)/ signal transducers and activators of transcription (STATs). Common downstream signals between cytokines are critical, especially when they share modulation of the same promoting elements [Abraham, 1996, Haque and Williams, 1998, Meager, 1998, Wajant et al., 2001].

As mentioned earlier, IL-1 and TNF- α interact synergistically in a variety of models. The synergism between IL-1 and TNF- α involves convergence of their signal transduction pathways. TNF- α gene transcription is regulated by multiple transcription factors including activating protein-1 (AP-1) [Newell et al., 1994] and nuclear factor- κ B (NF- κ B) [Barnes, 1997], while IL-1 activates NF- κ B [Wajant et al., 2001]. In addition, both transcription

factors are activated by mitogen-activated protein (MAP) kinase cascades and are predominant mediators of IL-1 and TNF- α action [Eder, 1997].

IL-1 and TNF- α act via distinct and specific signaling receptors. Thus, any post-receptor convergence has the potential to amplify downstream processes. Studies that support this claim report that both IL-1 and TNF- α signaling systems activate NF- κ B-inducing kinase (NIK), a MAP3K related kinase [Eder, 1997, Malinin et al., 1997]. Additionally, IL-1 and TNF- α synergistically activate MAPK. Further, NF- κ B is activated by IL-6 [Blackwell and Christian, 1997], and a synergistic response between IL-1, TNF- α and IL-6 may be due to the convergence of signal transduction mechanisms involving JAK/STAT (IL-6) and MAPK/NF- κ B (IL-1, TNF- α) pathways.

Consequences of cytokine action in the CNS

Pathophysiological consequences

Following an immune challenge, such as bacterial infections, trauma and sepsis, pro-inflammatory cytokines (IL-1 β , TNF- α , and IFNs, for example) are up-regulated in peripheral organs, the blood and the brain. Consequently, physiological and behavioral signs, known as sickness behavior, appear and are closely related to the extent of cytokine activation [Plata-Salamán et al., 1998b, Turrin and Plata-Salamán, 2000]. Fever [Kluger et al., 1998], anorexia [Plata-Salamán, 1998c], depressed locomotor activity (including exploration) [Cirulli et al., 1998], sleep disturbances [Krueger et al., 1995], and anhedonia [Borowski et al.,

1998] are examples of symptoms that characterize sickness behavior. Disturbances in the HPA axis response, which modulates pro-inflammatory cytokine activation, are also reported in models of cytokine activation [Papanicolaou et al., 1996, Reul et al., 1998, Turnbull and Rivier, 1999].

Psychological/psychiatric consequences

Elevated pro-inflammatory levels, especially during immunotherapy, have been associated with mood disturbance [Hurlock, 2001], impaired learning [Jankowsky et al., 2000], as well as deficits in other cognitive tasks [Lerner et al., 1999]. The involvement of cytokines in psychiatric disorders, such as schizophrenia and major depression, is also reported [Hurlock, 2001, Kronfol and Remick, 2000]. Increased suicidal idealization and attempts is also a risk factor during sustained IFN therapy [Vial et al., 2000].

Neurotoxic, neurodegenerative and neurological consequences

As an immune response develops, the levels of pro-inflammatory cytokines rise in the CNS. This can trigger the positive feedback loop of an individual cytokine, which in turn can sustain or further elevate its level or that of other pro-inflammatory cytokines in the brain [Ilyin et al., 1996, Plata-Salamán and Ilyin, 1997]. The continuous elevation in brain cytokine levels can lead to deleterious neurological consequences and ultimately cause neuronal damage. These effects are believed to occur through overstimulated NMDA receptor activity [Chao et al., 1995], reactive oxygen and nitrogen species [Hu et al., 1998,

Liew, 1995, Rossi and Bianchini, 1996], as well as an increase in the neuron's metabolism [Hu et al., 1997, Jeohn et al., 1998].

Cytokines are involved in neurodegenerative disorders, such as Alzheimer's disease [Mrak et al., 1995, Sternberg, 1997, Szelényi, 2001]. Their interaction with beta-amyloid protein could be a cause for the neuronal cell death seen in this condition [Bales et al., 1998, Forloni et al., 1997, Hu et al., 1998, Rossi and Bianchini, 1996]. Cytokines are also associated with seizures, although there is still some debate on whether they play a neuroprotective or neurodegenerative role during and after seizure episodes [Jankowski et al., 2000, Plata-Salamán et al., 2000].

Potential role of cytokines in homeostatic functions

There is some evidence which suggests that some cytokines could be involved in basic homeostatic functions such as sleep [Krueger et al., 1995, 1998], feeding [Plata-Salamán, 1998a,b, 2000b] thermoregulation [Klueger et al., 1998, Leon et al., 1998], ovulation [Cannon and Dinarello, 1985] and exercise [Pederson et al., 1998]. Based on neuropathophysiological studies, a possible role for TNF- α [Fiore et al., 1996], IL-1 β [Gibertini et al., 1995] and IFN α [Licinio et al., 1998] in cognition has also been suggested.

Leptin is considered a pro-inflammatory cytokine since its expression and release are regulated by inflammation [Berkowitz et al., 1998, Moshlyedi et al., 1998] and it can regulate the expression of other cytokines [Loffreda et al., 1998] and action of monocytes [Santos-

Alvarez et al., 1999]. In adipose tissue, leptin and TNF- α interact and are believed to be involved in insulin resistance and obesity [Mantzoros et al., 1997, Sinha and Caro, 1998]. Leptin is also believed to be a factor reflecting immune and reproductive status [Sinha and Caro, 1998].

It is important to consider that the basal levels of cytokines in the CNS are exceedingly low, and many are undetectable. Typically, their actions become evident following immune or pathophysiological challenges, although in most instances their function remains to be elucidated.

Objectives of the current thesis

The mechanisms underlying the action of cytokines in the brain during health and disease are still unclear. Due to the redundancy of their actions, the specific role of each cytokine is sometimes hard to decipher. For example, the absence of a cytokine in response to challenge may be overshadowed through compensation by other cytokines with similar actions. Furthermore, since most cytokines have multiple cellular targets and receptors, their presence in a particular region of the CNS does not assure a specific action that might be evident in some other region. Lastly, most cytokines trigger secondary cytokine cascades ultimately leading to effects in which their contribution is hard to dissociate from that of other cytokines. The studies included in this report addressed some of these concerns and had the following objectives:

1. *To determine the cytokines and associated component profiles in specific brain regions during immunotherapy and disease*

In an attempt to characterize the role that cytokines play in the brain during immune activation, the studies investigated several rat models of central cytokine action, during immunotherapy and disease. More specifically, they examined the expression of pro- and anti-inflammatory cytokines and associated components in specific brain regions following a

stimulatory cytokine (IFN α), or two different immune challenges, one acute (the bacterial endotoxin LPS) and one chronic (a tumor).

2. To characterize the contribution of cytokine-cytokine and cytokine-neuropeptide interactions to neurological manifestations associated with immunotherapy and disease

In order to gain a better understanding of the role played by cytokines in the brain and the implication of their neurological consequences, several studies assessed the concurrent changes of cytokine, neuroendocrine and behavioral factors. This included the analysis of multiple cytokines and their signaling components simultaneously in different brain regions (as well as in the blood and peripheral organs, when necessary). Due to the importance of cytokine-(neuro)peptide interactions in both normal and pathological brain activities, the expression of selected neuropeptides and signal-transduction proteins was studied as well. Furthermore, as it is crucial to examine both the molecular and behavioral consequences of cytokines' actions in brain concurrently, these studies included a behavioral marker of pro-inflammatory cytokine activity, namely that of anorexia. By assessing the simultaneous expression of these components, it was possible to infer potential synthesis within the brain or organ, and cytokine-cytokine and cytokine-neuropeptide interactions that could play an important role in the models studied.

3. *To counteract IFN α -induced anorexia using Neuropeptide Y administration*

By understanding the role that cytokines play in the brain in these models, possible interventions can be developed in order to ablate detrimental neurological and behavioral consequences associated with the central action of cytokines. It will be recalled that NPY may interact with cytokine activity, and hence one of the following studies addressed the effect of NPY intervention in the treatment of interferon-induced anorexia.

4. *To clarify the possible underlying mechanisms of communication between the brain and the periphery during disease*

As peripheral factors may influence central cytokine activity, and vice versa, several experiments assessed the expression of cytokines in both the brain and periphery during immune challenges. These challenges are thought to model disease, and their use may be effective in elucidating the mechanisms involved in immune-to-brain signaling and vice-versa. As immune-to-brain communication may be crucial in the normal progression of disease, such experiments may be fundamental in developing a more holistic perspective of a systemic brain-immune response to disease.

PART I. INTERFERON ALPHA STUDIES

Introduction

IFN α is a cytokine used for the effective treatment of malignancies [Dorr, 1993, Gutterman, 1994, Tsavaris et al., 1996], chronic infectious diseases such as hepatitis B [Gutterman, 1994, Haria and Benfield, 1995] and C [Carithers and Emerson, 1997, Dusheiko, Quin, 1997] and subacute sclerosing panencephalitis [Cianchetti et al., 1998, Miyazaki et al., 1991, Tomoda et al., 1997] due to its antiviral, antimicrobial and antiproliferative properties [Dorr, 1993, Tanneberger and Hrelia, 1996, Viscomi et al., 1995]. Along with its immunomodulatory properties, IFN α plays a neuromodulatory role reflected in the neurological manifestations associated with its use in immunotherapy. These manifestations can be: i) neuropathological, such as neuroendocrine dysfunctions and seizures [Dafny, 1998, Jankowsky and Patterson, 2001, Lerner et al., 1999, Vial et al., 2000]; ii) neuropsychiatric, and iii) behavioral, including depression, cognitive deficits and insomnia [Dafny, 1998, Lerner et al., 1999, Vial et al., 2000] as well as anorexia and sickness-like symptoms [Dafny, 1998, Lerner et al., 1999, Vial et al., 2000]. During immunotherapy, IFN α is infused repeatedly intraventricularly or intrathecally several times a week [Cianchetti et al., 1998, Miyazaki et al., 1991, Tomoda et al., 1997]. The IFN α -induced neurological manifestations can be exacerbated over time and synergize with the manifestations associated with the cancer or chronic disease. For example, mood disorders, such as depressive symptoms that accompany cancer, are worsened by similar symptoms induced by IFN α [Capuron et al., 2001, Meyers and Valentine, 1995]. Indeed, these side effects of the

immunotherapy frequently become a treatment-limiting factor and cause for attrition [Lerner et al., 1999, Tomoda et al., 1997, Vial et al., 2000].

Study 1: Cytokines in the brain following IFN α treatment

It has been established that IFN α can act directly on various brain regions, such as the hippocampus and hypothalamus [Plata-Salamán, 1991, Saphier et al., 1994, Reyes-Vásquez et al., 1997, Vial et al., 2000]. However, it also acts indirectly through secondary cytokine pathways, triggering the synthesis and release of pro-inflammatory cytokines such as TNF- α and IL-1 β [Lerner et al., 1999, Vial et al., 2000]. These two cytokines can act directly on cells of the CNS to modulate neurological manifestations such as anorexia, sleep disorders, neurodegeneration and neuroendocrine dysfunctions associated with chronic diseases, infections and cancer [Plata-Salamán, 1996a, Plata-Salamán et al., 1998a, Turrin and Plata-Salamán, 2000]. IL-1 β and TNF- α are known to stimulate each other's synthesis and to act synergistically in the CNS to exert their actions [Turrin and Plata-Salamán, 2000]. Consequently, it is imperative to understand the molecular mechanisms involved in IFN α -induced neurological manifestations and the role played by secondary cytokine pathways in the expression of these manifestations.

In the first of two studies, the cytokine mRNA profiles (as index of local cytokine production within specific brain regions) in response to the intracerebroventricular (ICV) administration of IFN α are investigated. This included the analysis of two pro-inflammatory cytokines (IL-1 β and TNF- α), and two anti-inflammatory or inhibitory

cytokines (IL-1Ra and TGF- β 1). In addition, we assessed several IL-1 β system-associated components [IL-1 receptor type I or signaling receptor, and IL-1 receptor accessory protein (IL-1R AcP) I and II, which increase the binding affinity of IL-1 β for IL-1RI when the two proteins are co-expressed [Greenfeder et al., 1995] and correlate with IL-1 responsiveness [Wesche et al., 1996]]. Although it has already been established that IFN α is able to induce IL-1 β and TNF- α [Taylor and Grossberg, 1998, Tilg and Peschel, 1996], this experiment represented the first attempt to measure concurrently these two pro-inflammatory cytokines together with anti-inflammatory cytokines and some signaling components for IL-1 β in a central model of IFN α immunotherapy.

Neuropeptides and neuropeptide-cytokine interactions are also critically involved in neuro-immune regulatory network modulation. IFN α is known to alter opioid signaling in the brain by directly binding to opioid receptors [Dafny, 1998, Lerner et al., 1999, Vial et al., 2000]. Additionally, IFN α has been shown to cause seizures, which might involve different substrate that use the IL-6 subfamily receptor members and gp 130 as signal transducers [Jankowsky and Patterson, 2001] that are also able to induce anorexia when activated by the proper ligands [Plata-Salamán, 1996b]. In this study, several neuroimmunomodulatory components were examined, including i) gp 130, ii) NPY, iii) pro-opiomelanocortin (POMC), a precursor for various feeding-enhancing opioid peptides that are thought to modulate cytokine actions [Plata-Salamán et al., 1998b], and iv) preprodynorphin, the precursor for dynorphin, an orexigenic opioid also involved in cytokine activity [Kong et al.,

1997]. Examining these components concomitantly with the cytokines can provide information on potential neurochemical interactions and elucidate possible neurological mechanisms involved in IFN α -associated neurological manifestations.

Study 2: NPY intervention to counteract IFN α -induced anorexia

Interferon- α immunotherapy for various cancers and viral diseases is associated with significant anorexia [Borden and Parkinson, 1998 Gottrand et al., 1996], an effect also evident in experimental models using healthy animals [Plata-Salamán, 1992, Segall and Crnic, 1990]. As mentioned earlier, IFN α acts directly in the CNS to induce anorexia. This action involves the hypothalamus, which is of course known to be an important feeding-regulatory brain site.

It is now well established that IFN α -induced adverse neurological effects can become a limiting factor during immunotherapy [Meyers et al., 1991]. In fact, IFN α -induced anorexia during immunotherapy can synergize with the anorexia associated with chronic diseases such as cancer, hepatitis and HIV. Hence, it would be important to counteract IFN α -induced anorexia in order to improve the immunotherapeutic protocols and quality of life of patients, and to reduce the rate of attrition from IFN α immunotherapy.

NPY is a 36 amino acid peptide found in the hypothalamus, and acts directly in this brain site to induce a robust, short term increase in feeding [Kalra, 1997, Stanley and Thomas, 1993]. Previous studies showed that NPY blocks and reverses the suppression of

food intake induced by IL-1 β , a potent anorexigenic cytokine [Sonti et al., 1996a]. These data suggest that NPY could modulate the anorexia induced by other cytokines, an effect that could have important implications for IFN α immunotherapy-associated anorexia.

In the second study, the effectiveness of NPY to counteract the anorexia induced by an immunotherapeutically relevant dose of IFN α was studied. NPY was administered on different schedules: concomitantly with IFN α , and either three or ten hours following IFN α treatment. The short- and long-term feeding responses of rats maintained *ad libitum* were investigated following the treatments to assess the effectiveness of NPY to block the IFN α -induced anorexia.

STUDY 1: Cytokines in the brain following IFN α treatment

Materials and Methods

Subjects and maintenance

Male Wistar (virus antibody free (VAF)) rats (from Charles River Laboratories, PA), weighing between 250 and 275 g at the beginning of the experiments, were used. Rats were randomly assigned to groups and placed in individual cages. They were maintained *ad libitum* on powdered rat food (Labdiet, PMI feeds, Inc., St. Louis, MO) and tap water. Room temperature was maintained at 21 ± 2 °C, and artificial illumination was from 0600 to 1800 h (12:12 h light : dark cycle). All animals were handled daily. After 10-14 days of adaptation to their home cage, brain cannulas were implanted.

Implantation of brain cannulas

Under intraperitoneal (IP) ketamine (100 mg/kg) plus xylazine (5 mg/kg) anesthesia, a 23-gauge stainless steel guide cannula (18 mm long, 0.64 mm o.d., 0.39 mm i.d.) was implanted into the third cerebral ventricle at stereotaxic coordinates: 2.1 mm posterior and 0.0 mm lateral with respect to bregma, and 7.5-8.0 mm ventral from the brain surface (Paxinos and Watson, 1986). The location of the cannula tip in the third ventricle was verified by the free outflow of cerebrospinal fluid through the guide cannula. A sterile 29-gauge stainless steel obturator was used to ensure that the cannula remained patent.

Intracerebroventricular microinfusion

Microinfusions were made into the third cerebral ventricle because of the importance of the hypothalamic regions in the regulation of feeding and in the action of IFN α . Test solutions were administered following a recovery period of at least fourteen days after surgery. ICV microinfusions (10 μ l/rat, into unrestrained, undisturbed animals) were at the rate of 1 μ l per 60 sec by using an infusion pump (Harvard Apparatus, South Natick, MA). The timing selected for the infusion (0800 h) was based on previous studies on the behavioral profile exhibited by rats receiving IFN α (i.e., anorexia) [Jang et al., 1992, Koren et al., 1993, Koyanagi et al., 1997, Segall and Crnic, 1990].

Randomly assigned groups received 1350 IU of either active, or heat-inactivated recombinant rat IFN α (Biosource International, Camarillo, CA), dissolved in 10 μ l of sterile physiological saline (0.15 M NaCl) containing 2.0 μ g/10 μ l albumin--the normal concentration of albumin in the cerebrospinal fluid [Burtis and Ashwood, 1994]. Ultra-free fatty acid bovine serum albumin was added because of its properties as a stabilizing agent and carrier protein for IFN α [Plata-Salamán, 1992]. The same IFN α lot and stock solutions were used for all experiments. Heat treatment (160°C for 6 h) was followed by heat drying and reconstitution of the volume with sterile water for injection, which was filtered through a 0.1 μ m (Millipore) syringe filter.

The dose of IFN α used in these experiments was based on an immunotherapeutic dose administered ICV or intrathecally for the treatment of subacute sclerosing panencephalitis [Cianchetti et al., 1998, Miyazaki et al., 1991, Tomoda et al., 1997]. A dose of one million IU of IFN α is within the immunotherapeutic dosage range in humans. This dose was recalculated for the rat considering brain weight and amount of cerebrospinal fluid, that is, an estimated total of 1640 g in human (1500 g for brain and 140 ml for cerebrospinal fluid) [Carpenter, 1991], and 2.22 g in rat (1.92 g for brain and 0.3 ml for cerebrospinal fluid) [McNab and Eisenberg, 1989, Plata-Salamán, 1992] yielding a difference in proportion of 738.7 times. Therefore, a proportional dose is one million IU IFN α /738.7 = 1354 IU IFN α . Although this is estimation, it provides a justification for the dose of 1350 IU of IFN α used in this study.

Processing of animals and dissection of brain regions

Following ICV infusion, rats from both groups were sacrificed at 2000 (12 hr post-infusion) or at 0800 (24 hr post-infusion) and their brains were quickly removed. Each brain was immediately placed in oxygenated phosphate-buffered saline solution at 2-4°C. Brains were rinsed several times and selected regions (cerebellum, parieto-frontal cortex, hippocampus, and hypothalamus) were dissected. Each brain region sample was immediately homogenized in guanidine thiocyanate/phenol solution (Tri Reagent, Molecular Research Center, Inc., Cincinnati, OH) using a microtissue grinder, then frozen at -85°C.

The brain samples were number coded for further processing and analyses. The complete brain dissection, homogenization and storage took less than 6 minutes.

RNA isolation and analysis of IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, NPY, dynorphin, POMC, gp 130 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs

Total cell RNA was extracted from the Tri Reagent homogenate as outlined in the manufacturer's protocol. RNA concentration was determined by spectrophotometry at an absorbance of 260 nm. RNA integrity was assessed through agarose gel electrophoresis and ethidium bromide staining. The levels of rat GAPDH mRNAs were RNase protection assayed for consistency and to confirm that equal amounts of total cell RNA were used for each assay.

Probe synthesis was conducted with specific rat cDNAs in 1 mM each of CTP, ATP, and UTP; and 9.38 μ M of [32 P]GTP (800 Ci/mmol) and 25 μ M of unlabeled GTP. [32 P]GTP not incorporated into the probe was removed by two ethanol precipitations in the presence of 2.5 M ammonium acetate. RNase protection assays were used to detect the IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, dynorphin, gp 130, POMC, NPY and GAPDH mRNAs.

Hybridization reactions containing 12.0 μ g of total cell RNA and 2.5×10^4 cpm of each IL-1 β , IL-1RI, IL-1R AcP, IL-1Ra, TGF- β 1, dynorphin, gp 130, POMC, and NPY and 1.5×10^4 cpm of TNF- α antisense probes, 3.0 μ g of total cell RNA and 2.0×10^5 cpm of

GAPDH antisense probe in 30 μ l of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4)) were heated to 85°C for 5 min and then incubated at 48°C for 12 to 18 h. After hybridization, 280 μ l of RNase digestion buffer (50 mM sodium acetate (pH 4.5), 2 mM EDTA) was added with 30 U of T1 RNase (Sigma, St. Louis, MO) for all assays followed by incubation at 30°C for 60 min. RNA was precipitated with 70 μ g of yeast transfer RNA by the addition of Tri reagent in ethanol. RNA was dissolved in loading buffer (80% formamide, 2 mM EDTA (pH 7.4) containing 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 85°C for 5 min, and resolved on 5% acrylamide/8 M urea gels using TBE-buffer (89 mM Tris pH 8.0, 89 mM boric acid and 2.7 mM EDTA). Gels were autoradiographed and results were quantified with an image analyzer (Image Quant, Molecular Dynamics, Sunnyvale, CA). Densitometric values for each mRNA analyzed were converted to percentage values of the total values for a particular mRNA.

Riboprobe templates

Rat IL-1 β (rIL-1 β) expression plasmid containing the entire mature peptide coding sequence of rat IL-1 β , with one extra Met codon at the 5' end cloned into plasmid pET-21d (Novagen, Madison, WI) between EcoRI and NcoI sites was used. Plasmid rat riboprobe IL-1 β (rRIL-1 β) was generated by cloning IL-1 β containing XbaI-EcoRI fragment of the rat IL-1 β expression plasmid into pGEM-2 between XbaI and EcoRI sites. Plasmid rRIL-1 β was linearized with HindIII, and transcribed with SP6 RNA polymerase to generate an

approximately 570 nucleotides-long antisense probe, which protects 492 nucleotides in the rat IL-1 β mRNA.

Plasmid rat IL-1RI 3' clone containing a 3' fragment (nucleotides 473-1826 of accession # M95578) of the rat IL-1RI cDNA cloned into the SmaI site of pBluescript SK (Stratagene) was used. Linearization of this plasmid with HindIII and transcription with T3 RNA polymerase produced an approximately 520 nucleotides-long antisense probe which protects 436 nucleotides in the rat IL-1RI mRNA.

Rat IL-1R AcP cDNA (nucleotides 874-1212 of accession # U48592) cloned into EcoRV site of pBluescript SK was used. Linearization of this plasmid by HindIII and transcription by T3 RNA polymerase resulted in an approximately 420 nucleotides-long antisense probe which protects 339 nucleotides in the membrane bound form of rat IL-1R AcP; additionally, this probe protects a shorter fragment corresponding to the soluble form of IL-1R AcP [Greenfeder et al., 1995].

Plasmid pBS-RA containing a fragment (nucleotides 3-451 of accession # M63101) of the rat IL-1Ra cDNA cloned into the SmaI site of pBluescript SK was linearized with XmnI, and transcribed with T3 RNA polymerase to produce an approximately 250 nucleotides-long antisense probe which protects 207 nucleotides in the rat IL-1Ra mRNA.

Plasmid prTNFtrans (T7) containing the peptide coding portion of rat TNF- α cDNA (nucleotides 1-708 of accession # S40199) was used. Linearization of this plasmid by EcoRI

and transcription with SP6 RNA polymerase produced an approximately 790 nucleotides-long antisense probe which protects 708 nucleotides in the rat TNF- α mRNA.

Rat TGF- β 1 cDNA (accession # X52498) cloned into pBluescript II KS vector (Stratagene Cloning Systems) was used. Linearization of this plasmid by BamHI and transcription with T3 RNA polymerase produced an approximately 320 nucleotides-long antisense probe, which protects 244 nucleotides in the rat TGF- β 1 mRNA.

Rat gp 130 cDNA (accession # M92340) cloned into EcoRI site of pBS vector was used. This cDNA was linearized with BsaI and transcribed with T3 RNA polymerase to produce an approximately 590 nucleotides-long antisense probe that protects 530 nucleotides in the rat gp 130 mRNA.

Plasmid pBLNPY-1 containing a 511 bp insert (accession # M20373) comprising most of the cDNA of rat prepro-neuropeptide Y ligated into the EcoRI site of vector Bluescribe M13(-) was used. Linearization of the plasmid with Bbs I and transcription with T3 RNA polymerase produced an approximately 242 nucleotides-long antisense probe that protects 180 nucleotides in the rat NPY mRNA.

Rat POMC cDNA cloned between BamHI and EcoRI sites of pBluescript KS(+) vector was used. Plasmid was linearized with XmnI and T3 RNA polymerase transcription resulted in an approximately 320 nucleotides-long antisense probe that protects 264 nucleotides in the rat POMC mRNA (predominant form).

Rat antisense template pTRI-GAPDH (Ambion; which contains a 316 bp fragment of the rat glyceraldehyde 3-phosphate dehydrogenase gene) was used to generate the GAPDH antisense probe.

Preparation of rat preprodynorphin antisense template

Primers 5'-AGAAGCCTGCCAGCGACAAAG-3' (corresponding to nucleotides 717-737 of rat dynorphin gene exon 1; accession # M32781) and 5'-CAGAGATGCAATT-AACCCTCACTAAAGGGAGAGTCCTCGTCCCCAGTCATCTC-3' (corresponding to nucleotides 453-473 of rat dynorphin gene exon 4; accession #M32784 and including an additional sequence comprising T3 RNA polymerase promoter) were designed to amplify an approximately 640 bp fragment of the rat preprodynorphin cDNA. Reverse transcription was performed with 1 µg of total rat brain RNA and random hexamers in a final volume of 20 µl using RNA PCR Core Kit according to the manufacturer's protocol (Perkin Elmer, Foster City, CA). Amplification was performed by using Taq2000 DNA Polymerase according to the manufacturer's protocol in a final volume of 100 µl; this was subjected to 35 cycles of denaturation at 95°C for 60s, annealing at 60°C for 70s and extension at 72°C for 90s. The PCR product was purified by the Wizard PCR Preps DNA Purification System (Promega, Madison, WI) and used directly for rat preprodynorphin antisense probe synthesis. The identity of the PCR product was confirmed by sequencing using the Sanger method with T3 RNA polymerase promoter specific primers.

Data analyses

Results are expressed as means $\pm$ SE. Data were analyzed by Analyses of variance (ANOVA) followed by Bonferonni corrected t tests for significant main effects or simple effects of significant interactions. Differences were considered significant for $p \leq 0.05$.

Results

RNase Protection Assay

The same animals were used to analyze all components studied at each time point: IL-1 β , IL-1RI, IL-1R AcP I and II, IL-1Ra, TNF- α , TGF- β 1, dynorphin, gp 130, POMC and NPY mRNA levels. Sample gels of the RNase protection assay for TNF- α , IL-1 β , IL-1RI, AcP I and II, TGF- β 1 and IL-1Ra (Figure 1A) and for dynorphin, gp 130, POMC and NPY (Figure 1B) are given. As shown, samples of both the vehicle (heat-treated IFN α)- and IFN α -treated animals from different brain regions at the 2000 h (night-time) and 0800 h (day-time) time points were analyzed concomitantly. Consistency was verified by analyzing all samples individually, from which all means $\pm$ SE were generated. Each individual sample was obtained from a different rat, and all rats provided all four brain regions; the same samples were analyzed with all antisense probes as described in the Materials and Methods. GAPDH mRNA was relatively constant between treatments within a brain region suggesting consistency of processing and that equal amounts of total RNA were used. In addition, to verify consistency of the assay, serial dilutions of a test RNA sample were also analyzed (not shown). The specificity of the probes used has been demonstrated previously by various

procedures including control experiments on hybridization specificity in which no signal corresponding to IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, dynorphin, gp130, POMC or NYP was detected [Gayle et al., 1998a, 1999, Ilyin and Plata-Salamán, 1996, Ilyin et al., 1996, Plata-Salamán and Ilyin, 1997, Plata-Salamán et al., 1998b, 1999].

IL-1 β mRNA levels

Analyses of variance indicated a main effect for interferon in the cerebellum, cortex, hippocampus and hypothalamus ($F(1, 28) = 13.698, 9.441, 4.188, 27.636$, respectively). Pair-wise comparisons indicated that IFN α significantly increased IL-1 β mRNA in the cerebellum ($p < 0.003$), parieto-frontal cortex ($p < 0.002$) and hypothalamus ($p < 0.05$) of rats 12 hours post-infusion compared to their heat-treated controls (Figure 2A). The most significant induction after 12 hours was seen in the cerebellum, with an approximately five-fold increase in IL-1 β mRNA levels (12.9 arbitrary units for the IFN α -infused group versus 2.4 units for the control group). Twenty-four hours post-infusion, IL-1 β mRNA levels were elevated in the IFN α infused group within the hippocampus and hypothalamus relative to the heat-treated group (Figure 2B). At this time point, the most notable upregulation for IL-1 β was seen in the hypothalamus, with an over six-fold stronger signal in IFN α infused animals relative to controls (27.8 versus 4.3 arbitrary units, respectively). IL-1 β levels remained unchanged over time in most regions, whereas in the cortex the signal declined somewhat over the 24 hr period ($F(1, 28) = 4.492, p < 0.05$).

TNF- α mRNA levels

Intracerebroventricular IFN α infusions induced TNF- α mRNA increases relative to heat-treated controls in all brain four regions 12 hours post-infusion, but this effect was far less pronounced at 24 hr. As a result, significant interactions were evident between the Cytokine treatment and Time for TNF- α within the cerebellum ($F(1, 28) = 5.85$, $p < 0.05$), parieto-frontal cortex ($F(1, 28) = 11.057$, $p < 0.005$), hippocampus ($F(1, 28) = 7.138$, $p < 0.02$), and in the hypothalamus this effect approached statistical significance ($F(1, 28) = 3.91$, $p < 0.058$) (Figure 2C). Bonferonni corrected t tests confirmed that IFN α markedly increased the signal at 12 hr in each of the brain regions examined. The strongest induction was in the hippocampus, with a four-fold increase compared to the control group (14.6 versus 3.4, respectively). At 24 hours following the infusion of IFN α , all brain regions showed TNF- α mRNA levels that were not significantly different from controls (Figure 2D).

TGF- β 1 mRNA levels

A main effect of the cytokine treatment on TGF- β 1 mRNA levels was apparent in the four brain region studied ($F(1, 28) = 14.942$ (cerebellum), 27.956 (cortex), 9.965 (hippocampus), 29.159 (hypothalamus). The TGF- β 1 mRNA levels were up-regulated in the cerebellum ($p < 0.05$), parieto-frontal cortex ($p < 0.001$), and hypothalamus ($p < 0.05$) of IFN α infused rats relative to their heat-treated controls 12 hours following the infusion

(Figure 3A). These effects were evident both 12 and 24 hr following IFN α treatment. Within the hippocampus there was a significant increase of TGF- β 1 in cytokine treated animals ($p < 0.01$) and TGF- β 1 varied over Time ($p < 0.05$). Although the interaction was not significant, as seen in Figure 3B, and confirmed by the post hoc tests, the increase of TGF- β 1 was marginal at 12 hr, but more pronounced at the 24 hr time point.

IL-1Ra mRNA levels

A main effect of the cytokine treatment on IL-1Ra mRNA levels was apparent in the four brain region studied ($F(1, 28) = 15.919$ (cerebellum), 8.277 (cortex), 11.67 (hippocampus), 21.947 (hypothalamus). In the night-time, IFN α -infused animals showed greater levels of IL-1Ra mRNA than their heat-treated controls in the cerebellum ($p < 0.05$), parieto-frontal cortex ($p < 0.01$), and hypothalamus ($p < 0.002$) (Figure 3C). All regions showed an approximately 2- or 3-fold increase in signal. In each of the brain regions, except the cortex, the effects were still apparent 24 hours post-infusion (Figure 3D).

IL-1R type I, AcP I and II mRNA levels

In response to intracerebroventricular IFN α , IL-1RI and AcP I mRNAs were only up-regulated in the fronto-parietal cortex ($p < 0.05$, for both) compared to controls at the 12 hr time point and in the hypothalamus ($p < 0.05$, for both) at the 24 hr time point. IL-1R

Figure 1. Sample gels of mRNA from male Wistar rats treated with the intracerebroventricular administration of vehicle (heat-treated IFN α [minus signs]) or active IFN α [1350 UI, plus signs]. Brain tissue samples were collected 12 h or 24 h [symbols in parentheses] after infusions. Figure shows that samples from each treatment and brain region were processed using all antisense probes concomitantly, as described in Materials and Methods. **A.** Tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-1 receptor type I (IL-1RI), IL-1R accessory protein type I (AcP (I)) and type II (AcP (II)), IL-1 receptor antagonist (IL-1Ra), transforming growth factor- β 1 (TGF- β 1) and GAPDH mRNA levels in the cerebellum (CLL), parieto-frontal cortex (CTX), hippocampus (HIP) and hypothalamus (HYP). **B.** Dynorphin, gp 130, pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and GAPDH levels in the hypothalamus.

Figure 2. IL-1 β mRNA levels 12 h (A) and 24 h (B) and TNF- α mRNA levels 12 h (C) and 24 h (D) following the intracerebroventricular infusion of heat-inactivated (open bars) or active IFN α , 1350 UI (closed bars). Values (means $\pm$ SE; $n=8$ for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus. *: simple main effect of treatment ($p < 0.05$); †: significant interaction ($p < 0.05$); *: simple main effect of time ($p < 0.05$).

Figure 3. TGF- β 1 mRNA levels 12 h (A) and 24 h (B) and IL-1Ra mRNA levels 12 h (C) and 24 h (D) following the intracerebroventricular infusion of heat-inactivated (open bars) or active IFN α , 1350 UI (closed bars). Values (means $\pm$ SE; $n=8$ for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus. *: simple main effect of treatment ($p < 0.05$); *: simple main effect of time ($p < 0.05$).

AcP II was induced in the hypothalamus ($F(1, 28) = 26.610, p < 0.001$) and the expression of IL-1R AcP II was lower 24 hr after infusion than at 12 hr ($F(1, 28) = 5.757, p < 0.005$).

Dynorphin, gp 130, POMC and NPY mRNA levels

Following IFN α infusions, there were no significant changes in mRNA levels of dynorphin, gp 130, POMC or NPY in any of the brain regions examined.

Discussion

Following ICV infusion of IFN α at an immunotherapeutic dose, the mRNAs of pro-inflammatory cytokines, IL-1 β and TNF- α , were up-regulated in multiple brain regions both 12 and 24 hours following infusion. The two cytokines showed a different temporal pattern of expression: relative to controls, IFN α treated animals showed elevated TNF- α mRNA levels in all brain regions examined. The TNF- α then returned to baseline levels at 24 hours post-infusion. In contrast, the IL-1 β mRNA profile remained up-regulated between the 12 and 24 hour measurements. The differential mRNA expression between TNF- α and IL-1 β follows the expected cascade of pro-inflammatory cytokine activation, with TNF- α being consistently expressed earlier than IL-1 β following most immune challenges [Abbas et al., 1991]. These profiles support the ability of centrally administered IFN α to regulate the expression of cytokines within specific regions in the brain in a similar manner to that of other cytokines or immunoactive substances that yield similar neurological

and behavioral manifestations [Plata-Salamán, 1996a, Plata-Salamán et al., 1998a, Turrin and Plata-Salamán, 2000].

The mRNAs of anti-inflammatory cytokines, TGF- β 1 and IL-1Ra, like that of the pro-inflammatory cytokines, were elevated in the brain at both time points after IFN α administration. This could reflect an effort by the brain to modulate the elevated pro-inflammatory cytokine levels (for both IL-1 β and TNF- α). Additionally, the IL-1Ra mRNA induction profile in the four brain regions was very similar to that of IL-1 β , supporting the existence of a negative feedback loop through which IL-1Ra would modulate the actions of IL-1 β within the brain during IFN α immunotherapy. The balance between the pro- and anti-inflammatory cytokines has been suggested to play an important role in the pathophysiological and neurological manifestations associated with disease and immunotherapy [Plata-Salamán et al., 1998b, Turrin and Plata-Salamán, 2000].

The mRNAs of the IL-1 signaling components (IL-1RI, AcP I and II) remained relatively stable in most brain regions following IFN α infusion. These components were found to be up-regulated in other models of brain cytokine activation; however, these involved either chronic preparations (such as a tumor model [Plata-Salamán et al., 1998a]), or acute application of endotoxins that caused the massive upregulation of IL-1 β in the brain and peripheral organs [Plata-Salamán et al., 1998b]. Therefore, we suggest that the regulation of these components occurs only following chronic or drastic immune challenges that lead to major dysregulation of the immune response of the brain.

Interestingly, no changes were detected in dynorphin, gp 130, POMC or NPY levels in response to ICV IFN α . Although these neuropeptides are involved in the systems affected by IFN α , the inability of this cytokine to regulate their expression could indicate its specificity of action through the pro-inflammatory cytokine pathways. Having distinct regulatory pathways between the cytokine and these neuropeptides could offer the possibility of regulating some of the neurological consequences (e.g., anorexia, mood disturbances) of IFN α administration. This possibility will be explored in Study 2.

As indicated earlier, IFN α immunotherapy is associated with neurological manifestations that include anorexia, mood disorders, cognitive deficits as well as neurological disorders such as seizures [Jankowsky and Patterson, 2001, Lerner et al., 1999, Vial et al., 2000]. There has been evidence that the action of IL-1 β and TNF- α in the brain could be related to the induction of these manifestations. Indeed, the finding that ICV infusion of IFN α led to the induction of IL-1 β and TNF- α mRNA in brain, is consistent with this supposition. Given that IFN α may also directly modulate the activity of glucose-sensitive neurons within the hypothalamus, the neurological manifestations associated with central administration of this cytokine could reflect a combination of the direct action of IFN α on neural cells and the indirect action of others cytokines downstream from it.

Implications

Clearly, the present findings provide evidence that IFN α may influence both IL-1 β and TNF- α within the brain. Further research is needed to clarify the interaction of IFN α with other cytokine and neuropeptide systems in order to elucidate other possible modes of action of this cytokine. Understanding the underlying mechanisms involved in IFN α immunotherapy-induced neurological manifestations, such as anorexia, could lead to interventions that could ease the side effects of immunotherapy and ameliorate the patient's quality of life and therapy outcome. The detrimental neurological manifestations associated with IFN α immunotherapy can exacerbate the symptoms of the pathological or oncological ailment it treats. For example, cancer-induced anorexia can be worsened by immunotherapy, which could lead to cachexia [Lerner et al., 1999, Meyers et al., 1991, Vial et al., 2000]. IFN α did not influence NPY expression in the brain, suggesting that this peptide was likely independent of the actions of the cytokine. Yet, given that NPY is an orexigenic peptide, it may prove useful in countering some of the side effects associated with IFN α . In the next study, NPY was administered centrally in conjunction with IFN α to attempt to abate the IFN α -induced anorexia.

STUDY 2: NPY intervention to counteract IFN α -induced anorexia

Materials and Methods

Subjects and maintenance

Male Wistar (VAF) rats (from Charles River Laboratories, PA), weighing between 250 and 275 g at the beginning of the experiments, were used. Rats randomly assigned to groups, were individually caged, and maintained *ad libitum* on powdered rat food (Labdiet, PMI feeds, Inc., St. Louis, MO) and tap water. Room temperature was maintained at $21 \pm 2^{\circ}\text{C}$, and artificial illumination was from 0600 to 1800 h (12:12 h light:dark cycle). All animals were handled daily. After several days of adaptation to their home cage, brain cannulas were implanted.

Food intake

Food intake was measured to within 0.1 g. Before and after the brain cannula implantation, the rats were fed *ad libitum* daily, except between 1630 and 1800 h. At this time food was removed to be weighed and replaced, rats handled, and body weights determined. Pre-measured food and water were presented to the animals at 1800 h. Food and water intakes were measured at 2000 h (2-h nighttime consumption), at 0600 h (total nighttime consumption from 1800 to 0600 h), and at 1630 h (total daily consumption from 1800 h to 1630 h). Furthermore, when the rats received a microinfusion of NPY at 0800 or at 1100 h,

additional measurements were done at 0900 and 1000 h, or 1200 and 1300 h, respectively (1-h and 2-h daytime measurements).

Implantation of brain cannulas

Under IP ketamine (100 mg/kg) plus xylazine (5 mg/kg) anesthesia, a 23-gauge stainless steel guide cannula (18 mm long, 0.64 mm o.d., 0.39 mm i.d.) was implanted into the third cerebral ventricle at stereotaxic coordinates: 2.1 mm posterior and 0.0 mm lateral with respect to bregma, and 7.5-8.0 mm ventral from the brain surface (Paxinos and Watson, 1986). The location of the cannula tip in the third ventricle was verified by the free outflow of cerebrospinal fluid through the guide cannula. A sterile 29-gauge stainless steel obturator was used to ensure that the cannula remained patent.

Intracerebroventricular microinfusion

Microinfusions were made into the third cerebral ventricle because of the importance of the hypothalamic regions in the regulation of feeding and in the action of both IFN α and NPY. Test solutions were administered following a recovery period of at least fourteen days after surgery. ICV microinfusions (10 μ l/rat, into unrestrained, undisturbed animals) were delivered through using an infusion pump (Harvard Apparatus, South Natick, MA) at the rate of 1 μ l per 60 sec. The schedules for the infusions are shown in Figures 4 through 7. The timing selected for the infusions was based on previous studies on the behavioral profile exhibited by rats receiving IFN α (i.e., anorexia) [Jang et al., 1992, Koren et al., 1993,

Koyanagi et al., 1997, Segall and Crnic, 1990] or NPY (i.e., increased food intake) [Stanley and Thomas, 1993].

Randomly assigned groups received 1350 IU of either active, or heat-inactivated recombinant rat IFN α (Biosource International, Camarillo, CA), dissolved in 10 μ l of sterile physiological saline (0.15 M NaCl) containing 2.0 μ g/10 μ l albumin--the normal concentration of albumin in the cerebrospinal fluid [Burtis and Ashwood, 1994]. Ultra-free fatty acid bovine serum albumin was added because of its properties as a stabilizing agent and carrier protein for IFN α [Plata-Salamán, 1992]. Other groups received either vehicle (sterile physiological saline solution, 10 μ l), or 5.0 μ g of NPY (Research Biochemicals International, Natick, MA) in 10 μ l vehicle. For the concomitant IFN α plus NPY administration, both compounds were dissolved in 10 μ l of physiological saline plus albumin, as described earlier. The same IFN α and NPY lot and stock solutions were used for all experiments. Heat treatment (160°C for 6 h) was followed by heat drying and reconstitution of the volume with sterile water for injection/albumin solution which was filtered through a 0.1 μ m (Millipore) syringe filter.

The dose of IFN α used in these experiments was based on an immunotherapeutic dose administered ICV or intrathecally for the treatment of subacute sclerosing panencephalitis [Cianchetti et al., 1998, Miyazaki et al., 1991, Tomoda et al., 1997]. A dose of one million IU of IFN α is within the immunotherapeutic dosage range in humans. This

dose was recalculated for the rat considering brain weight and amount of cerebrospinal fluid, that is, an estimated total of 1640 g in human (1500 g for brain and 140 ml for cerebrospinal fluid) [Carpenter, 1991], and 2.22 g in rat (1.92 g for brain and 0.3 ml for cerebrospinal fluid) [McNab and Eisenberg, 1989, Plata-Salamán, 1992] yielding a difference in proportion of 738.7 times. Therefore, a proportional dose will be one million IU IFN α /738.7 = 1354 IU IFN α . Although this is estimation, it provides a justification for the dose of 1350 IU of IFN α used in this study. The dose of NPY was selected based on previous studies that established its robust feeding effect in rats [Kalpa, 1997, Sonti et al., 1996a, Stanley and Thomas, 1993], and its antagonist effect on IL-1 β -induced anorexia [Sonti et al., 1996a].

Data analyses

Results are expressed as means $\pm$ SE. Data were analyzed by mixed measures ANOVA followed by paired and two-sample t tests, or Mann-Whitney test when data did not pass normality (Kolmogorov-Smirnov) and equal variance (Levene Median) tests. Power of the performed test was > 0.8 to detect significant effects at $p < 0.05$.

Results

Effect of IFN α on food intake

In a preliminary experiment, the effectiveness of IFN α to induce anorexia when administered during the day (0800 h, $n = 7$) or just before the evening (1730 h, $n = 10$) was determined. This experiment showed that IFN α was significantly more potent in provoking nighttime anorexia (active period in the rat), when the cytokine was administered at 0800 h. Accordingly, this time of administration was used in subsequent studies.

Figure 4 shows the effect of the acute ICV microinfusion of IFN α on food intake. There was a significant interaction between cytokine treatment and time ($F(1, 16) = 25.911$, $p < 0.001$) consequently, two-way comparisons were done. In response to IFN α administration, both the 2-h and 12-h nighttime food intake were significantly decreased (50% and 37%, respectively) relative to the baseline (the day before infusion or pre-infusion day) ($n = 17$, paired t test, $p < 0.001$ for 2-h and 12-h test periods). The decrease in food intake during these two sampling periods indicates that IFN α induced significant anorexia during the 10 to 22 h period following the ICV microinfusion. Heat-inactivated IFN α , in contrast, had no effect (data not shown).

Effect of NPY administered before nighttime on IFN α -induced anorexia

Figure 5 shows the effect of the ICV microinfusion of heat-inactivated or active IFN α , followed by the ICV administration of NPY. Mixed design ANOVA yielded a significant interaction between cytokine treatment and time ($F(1, 15) = 9.953$, $p < 0.01$). Paired t test revealed that both NPY-treated groups increased their food intake significantly during the 2-h nighttime period relative to the preinfusion day ($p < 0.001$ for heat-inactivated, and $p < 0.005$ for active IFN α -treated group). However, there was no significant difference in 2-h food intake between the heat-inactivated and active IFN α -treated groups ($p = 0.4$). Evidently, NPY was equally effective in inducing the short-term feeding response in both conditions, indicating that this peptide counteracted the anorectic effect of IFN α .

During the 12-h nighttime period, food intake in the heat-inactivated IFN α -treated group did not change from the preinfusion day. By contrast, the group that received active IFN α showed a significant decrease in food intake ($p < 0.001$) (Fig. 5). Furthermore, this decrease was also significantly different from heat-inactivated IFN α (fig. 5, $p < 0.005$). Thus, it seems that IFN α -induced anorexia reappeared following the short-term actions of NPY. This suggests that NPY was able to counteract the IFN α -induced anorexia, but only during the period of NPY activity, after which IFN α -associated anorexia reappeared.

Effect of NPY administered during the daytime on IFN α -induced anorexia

Figure 6 shows the effect of the ICV microinfusion of heat-inactivated or active IFN α at 0800, followed by a second microinfusion of NPY or vehicle at 1100. Food intake was measured at 1 h and 2 h thereafter. NPY significantly increased feeding in both the heat-inactivated and active IFN α conditions. There were no significant differences in the 1-h or 2-h food intake between the NPY-treated groups that received either heat-inactivated IFN α or active IFN α . Thus, it appears that during the day NPY stimulated food intake following an ICV microinfusion of IFN α . Consistent with the well known short-term feeding increase elicited by NPY [Forloni et al., 1997, Friedman et al., 1996, Gayle et al., 1998a], Figure 6 shows that almost all food intake occurred during the first hour following NPY administration.

Effect of the concomitant administration of NPY and IFN α

For this experiment, IFN α was administered concomitantly with NPY (Fig. 7). Again, NPY significantly increased food intake relative to vehicle ($F(1, 6) = 63.985$, $p < 0.001$), irrespective of the IFN α condition, and no changes in food intake occurred between the 1 and 2h measurements. Thus, NPY effectively induces a robust feeding response even when both compounds are administered concomitantly.

Figure 4. Effect of ICV microinfusion of active IFN α (1350 UI / rat) on nighttime 2 h (12 h post-infusion) and 12 h (22 h post-infusion) food intakes. The schedule is shown on top. Bars represent mean $\pm$ SE (n =17), g eaten on the pre-infusion and infusion days. *, p < 0.001, between pre-infusion and infusion days.

Figure 5. Effect of the ICV microinfusion of NPY (5 μ g/rat) in animals that received heat-inactivated or active IFN α (1350 UI/rat). Nighttime 2h and 12 h food intakes were measured. The schedule is shown on top. Bars represent mean $\pm$ SE differences in g eaten between the pre-infusion and infusion days. The number of rats is shown above/below bars. *: $p < 0.005$, between pre-infusion and infusion days, and between heat-inactivated and active IFN α . At the 2 h measurement, both groups exhibited a significant increase in food intake compared to the preinfusion day (see text).

Figure 6. Effect of the ICV microinfusion of NPY (5 μ g/rat) three hours after administration of heat-inactivated or active IFN α (1350 UI/rat). 1 h and 2 h food intakes were measured. The schedule is shown on top. Bars represent mean $\pm$ SE differences in g eaten. The number of rats is shown above bars. *: $p < 0.005$, between NPY and saline.

Figure 7. Effect of the concomitant ICV infusion of IFN α (1350 UI/rat) and NPY (5 μ g/rat) on 1 h and 2 h food intakes. The schedule is shown on top. Bars represent mean $\pm$ SE differences in g eaten. The number of rats is shown above bars. *: $p < 0.001$, between IFN α + saline and IFN α + NPY.

Discussion

Interferon- α induced significant anorexia when administered centrally into the third cerebral ventricle of healthy rats maintained *ad libitum*. NPY counteracted the IFN α -induced anorexia when administered three or ten hours following IFN α , or when it was administered concomitantly with IFN α . Interestingly, IFN α -induced anorexia reappeared following the short-term NPY action. These data suggest that IFN α was still active throughout the NPY-induced feeding enhancement. In the first study of this thesis, no changes of NPY mRNA were found in the brain following the ICV administration of IFN α , which could further suggest that NPY and IFN α act independently on the hypothalamus to modify ingestive behavior. This is important for the immunomodulation produced by IFN α immunotherapy.

Interferon- α stimulates the production of IL-1, TNF- α and IL-6 [Taylor and Grossberg, 1998, Tilg and Peschel, 1996], which are cytokines with anorexigenic activity [Plata-Salamán, 1998a]. This raises the possibility that proinflammatory cytokine production can participate in IFN α -induced anorexia. IFN α , as IL-1 and TNF- α , has the ability to modulate glucose-sensitive neurons in the hypothalamus [Kuriyama et al., 1990, Plata-Salamán, 1991, Reyes-Vásquez et al., 1997], which are proposed to play an important role in the control of feeding [Plata-Salamán, 1996a]. This IFN α -associated neuronal action is direct and specific [Kuriyama et al., 1990, Plata-Salamán, 1991, Reyes-Vásquez et al., 1997],

and hence the anorexia may likewise involve via direct modulation of neuronal activity, although a role for indirect mechanisms should not be dismissed.

Neuropeptide Y blocked and reversed IL-1 β -induced anorexia in the normal Wistar rat [Sonti et al., 1996a]. Since IFN α induces the production of IL-1 β , it is possible that both IFN α and NPY might regulate feeding through IL-1-associated mechanisms. Moreover, the hypothalamus may represent a neuroanatomical substrate for the NPY-induced inhibition of IFN α -associated anorexia, since this site includes both, NPY-orexigenic [Kalpa, 1997, Stanley and Thomas, 1993] and IFN- α -anorexigenic [Plata-Salamán, 1991, Reyes-Vásquez et al., 1997, Segall and Crnic, 1990] mechanisms.

It is clear that IFN α was more effective in provoking anorexia when administered at the beginning of the day. This is consistent with previous chronobiological studies on IFN- α in rodents in which the strongest fever response in normal mice was evident when administered during the daytime [Koyanagi and al., 1997]. Administration of IFN α at 4-h after lights onset also produced maximum anti-tumor activity in a rodent model of cancer [Koren et al., 1993]. Together with the present results, these data indicate that biological activity and toxicity of IFN α is significantly influenced by the time of administration within the circadian cycle in both normal and disease animal models. Indeed, these findings are consistent with human studies that have reported the importance of chronotherapeutic

schedules for IFN α [Deprés-Brummer et al., 1991] and may have important implications for immunotherapeutic treatments.

As previously reported [Segall and Crnic, 1990], ICV administration of IFN α induced a long-lasting anorectic effect. The NPY-induced short-term feeding increase is also consistent with previous findings [Kalpa, 1997, Sonti et al., 1996a, Stanley and Thomas, 1993]. This short-term NPY effect could also have implications for IFN α immunotherapy. For example, in conditions where IFN α is administered ICV or intrathecally, the NPY short-term effect could be timed to coordinate with the eating schedule of a patient receiving IFN α therapy. This will maximize the feeding induction properties of NPY, while not influencing hunger at times where it would be inappropriate (during sleeping periods, for example). On the other hand, strategies to lengthen an inhibition of the IFN α -induced anorexia by NPY could involve sustained NPY administration, since no desensitization to NPY occurs during recurrent perfusion [Paez and Myers, 1991]. Furthermore, Y receptor agonists with a more sustained effect relative to NPY could also be studied in preclinical and clinical models. These possibilities should be considered as prospective approaches for IFN α -induced anorexia, in particular in conditions where the immunotherapeutic protocol requires ICV or intrathecal administration.

PART II: DISEASE MODELS OF CYTOKINE ACTION

Introduction

Models of peripheral immune challenge or inflammation have shown cytokine induction within the brain [Eriksson et al., 1999, Gabellec et al., 1995, Konsman et al., 1999, Luheshi et al., 1997, Quan et al., 1998, van Dam et al., 1998, Wong et al., 1997]. The exact mechanisms by which brain cytokines are regulated during peripheral infections are still under debate. Several possible models are available for central cytokine regulation, including: i) intermediate soluble factors (e.g., prostaglandins or cytokines themselves) produced and released within the CNS by microglia and/or endothelial cells of the cerebrovasculature or from circumventricular organs (CVOs) [Day and Akil, 1996, Herkenham et al., 1998, Konsman et al., 1999, Maness et al., 1998]; ii) transport of cytokines across the blood-brain barrier (BBB) and/or CVOs, or direct activation of CVO neurons [Banks et al., 1995, Maness et al., 1998]; and iii) neural afferent signals inducing CNS cytokine production [Goehler et al., 1999, Maier and Watkins, 1998, Maier et al., 1998]. While there has been support for the view that activation of vagal afferents plays a critical role in subserving some central cytokine effects [Goehler et al., 1999, Maier et al., 1998], this model has not been supported by experiments using selective subdiaphragmatic deafferentation or celiac superior mesenteric ganglionectomy [Porter et al., 1998b, Schwartz et al., 1997]. In light of the latter findings, it needs to be considered that that humoral mediation may play a critical role in subserving the cytokine actions.

The humoral mediators present during a peripheral immune challenge can involve endogenous soluble factors secreted by the immune cells of the host, in addition to exogenous factors that make up bacterial cell walls (e.g., LPS) and factors released by tumor cells. The relative contribution of host versus non-host factors during an immune response to the activation of brain cytokines remains to be established. Likewise, the brain cytokine profile associated with most peripheral immune challenges has not been characterized. Consequently, it is important to elucidate possible similarities and differences between the brain cytokine profile elicited by various peripheral challenges in order to understand the role of the individual peripheral and central mediators of the immune response.

The second part of this thesis investigated the cytokine mRNA profile associated with two models of disease: an acute bacterial endotoxin (LPS) IP injection and the chronic progression of a tumor in anorectic rats. By examining these two distinct models, the role of individual cytokines and their components during acute and chronic disease may be clarified. Furthermore, the study of possible interactions between cytokines, their components, and neuropeptides could elucidate distinct brain activation profiles for acute and chronic immune challenges. Differential profiles could explain the behavioral and neurological manifestations that are distinctively associated with bacterial infections and cancer.

Study 3: Cytokine mRNA profiles following LPS administration

Gram-negative bacterial infections are associated with immunological and neurological manifestations including anorexia, fever, sleep disturbances, learning disabilities,

neurological dysfunction and injury. Bacterial endotoxin (or LPS) is responsible for multiple pathophysiological changes associated with Gram-negative infections [Holst et al., 1996]. LPS acts through pro-inflammatory cytokines including IL-1 β and TNF- α [Dinarello, 1999, Hernandez and Hidalgo, 1998, Langhans, 1996, Plata-Salamán, 1991]. Moreover, the exogenous administration of a competitive endogenous inhibitor, IL-1Ra, blocks or ameliorates the CNS effects induced by (central) LPS and IL-1 β [e.g. Cartmell et al., 1999, del Rey et al., 1998, Habu et al., 1998, Plata-Salamán, 1998c].

Brain administration of LPS induces IL-1 β and other cytokines in specific brain regions [De Simoni et al., 1995, Gayle et al., 1998a, 1999, Plata-Salamán et al., 1998b]. Peripheral injections of LPS are also able to induce central cytokine production (and central cytokines mediate the CNS effects induced by peripheral LPS) [Cartmell et al., 1999, Jacobs et al., 1997, Kakizaki et al., 1999, Pitossi et al., 1997, Satta et al., 1998], and modulation of their components (ligands, receptors, signal transducing proteins, inhibitory mechanisms) [Gabellec et al., 1996a,b, van Dam et al., 1998]. It should be noted, however, that several of these studies used extremely high doses of LPS (in some cases mg/kg) [e.g., Gabellec et al., 1996a, Kakizaki et al., 1999, Kozak et al., 1995]. Moreover, these studies did not simultaneously characterize multiple cytokine systems and their components within specific brain regions in the same animal. The characterization of brain cytokine component profiles following systemic LPS administration is pivotal to understand peripheral-to-central immune signaling as well as the neurological and neuropsychiatric manifestations associated with

bacterial product and peripheral cytokine challenges [Langhans, 1996, Mullington et al., 2000, Pernerstorfer et al., 1999, Plata-Salamán 1998c].

In the present study, the cytokine mRNA profiles (as evidence for local cytokine production within the brain) in response to the intraperitoneal administration of LPS were investigated in several brain regions (cerebral cortex, cerebellum, hippocampus and hypothalamus) and peripheral organs (liver, spleen and adipose tissue) in response to IP administration of LPS. The cytokines and modulators included two pro-inflammatory cytokines (IL-1 β and TNF- α), two anti-inflammatory or inhibitory cytokines (IL-1Ra and TGF- β 1), IL-1 β system-associated components [IL-1 receptor type I or signaling receptor, and IL-1R AcP I and II—which increase the binding affinity of IL-1 β for IL-1RI when the two proteins are co-expressed [Greenfeder et al., 1995]; IL-1R AcP expression also correlates with IL-1 responsiveness [Wesche et al, 1996]], gp 130 (a common signal transducer among receptors for members of the IL-6 receptor subfamily [Plata-Salamán, 1996b]), and OB-R (which shows homology with gp 130 and responds to leptin, an adiposity cytokine signal that inhibits feeding and that can be modulated by cytokines [Grunfeld et al., 1996]). The simultaneous analysis of IL-1 β system components, TNF- α , TGF- β 1, and gp 130 mRNAs can provide information on multiple cytokine components and on potential cytokine-cytokine interactions. The cytokine-associated mRNAs were examined in the same brain region and peripheral organ samples.

Cytokine-neuropeptide and cytokine-endogenous opioid interactions may play a role in cytokine-associated CNS activities [Kong et al., 1997, Plata-Salamán, 1997, Sonti et al., 1996a]. Thus, mRNA levels for NPY and POMC, an opioid peptide precursor, in response to IP LPS were also studied. Since LPS induces anorexia, analysis of POMC gene expression also could provide insights into potential changes of melanocortin-related mechanisms.

Study 4: Cytokine action in the brain of tumor-bearing rats

The progression of cancer involves a complex, multifaceted mechanism of chemical, cellular and systemic events. Tumor development can also be accompanied by neurological manifestations that include depression, cognitive deficits, anxiety and anorexia. The synthesis of cytokines within the brain in response to intracranial or peripheral tumor progression is implicated in cancer induced behavioral manifestations [Noguchi et al., 1996, Plata-Salamán, 1996a]. Cytokines such as IL-1 β and TNF- α were previously shown to be critically involved in these tumor-induced immune and behavioral changes [Gelin et al., 1991, Murray et al., 1997, Plata-Salamán et al., 1998a, Strassmann et al., 1993]. Studies have reported that administration of an IL-1 antagonist alleviates anorexia in animal cancer models and the administration of appropriate cytokines mimics neurological manifestations observed in cancer patients [Strassmann et al., 1993]. Together, these findings support the involvement of the IL-1 β system in tumor-related pathology.

Cytokines have a very brief half-life and thus cannot solely act in an endocrine fashion; instead, they rely on paracrine, autocrine, and intracrine pathways, which cannot be detected in circulation. Most of the cytokines act predominantly through paracrine mechanisms, which would suggest their involvement in cancer-related clinical manifestations to be mostly due to their local synthesis within the organ, such as the brain.

In a previous study [Plata-Salamán et al., 1998a], a radical new model of neuropsychiatric manifestations associated with cancer such as anorexia was proposed; prostate adenocarcinoma tumor cell-bearing rats exhibiting anorexia were shown to express IL-1 β , its receptor and receptor antagonist mRNAs in the brain as well as in peripheral organs. These findings raised the possibility that the IL-1 β system upregulation in the brain could be relevant to the tumor-induced anorexia manifested by the rats. Furthermore, the *in vivo* characterization of cytokine components in specific brain regions stressed the importance of using such a model to understand the underlying molecular mechanisms involved in cancer-associated neurological manifestations.

Because of the importance and implications of this model, the concept needed to be tested on yet a different cancer model. In the fourth study, rats inoculated with methylcholanthrene (MCA)-induced sarcoma cells were used to assess behavioral and molecular changes accompanying tumor development. These rats were continuously monitored for the initiation and progression of anorexia. After establishment of early anorexia, the control and tumor-bearing rats were sacrificed, and their brains and peripheral

organs (as well the tumor, where applicable) were dissected. The production of the anorexigenic cytokines IL-1 β , TNF- α and IFN γ (measured for the first time in such a model) was assessed. Local synthesis of IL-1 β in the brain and modulation of specific components of IL-1 β system, IL-1Ra and IL-1R AcP II, in the liver of tumor bearing rats were also investigated. To understand fine regulation of IL-1 β centrally, local mRNA levels of IL-1RI, IL-1R AcPs and IL-1Ra, factors previously shown to be important for the modulation of IL-1 β bioactivity [Dinarello, 1996, Plata-Salamán, 1994, Wesche et al., 1996], were monitored within the brain. TGF- β 1, which is proposed to stimulate tumor growth [Ilyin et al., 1998b] and inhibit IL-1 β and TNF- α activity [Plata-Salamán and Ilyin, 1997], was also examined. The simultaneous analysis of these cytokines and their components provided an opportunity to study possible interactions and feedback systems involved in tumor progression as well as their behavioral and neurological consequences.

Evidence shows that neuropeptides and neuropeptide-cytokine interactions are also critically involved in neuro-immune regulatory network modulation. For example, NPY can block and even reverse anorexia induced by IL-1 β treatment [Sonti et al., 1996a]. In this study, several components important in feeding regulation were examined: i) gp 130, a transducer among receptors for IL-6 subfamily receptor members shown to induce anorexia when activated by the appropriate ligands [Plata-Salamán, 1996b]; ii) NPY, a feeding enhancing peptide proposed to participate in the maintenance of normal feeding [Sonti et al., 1996a]; iii) POMC, an opioid peptide precursor for various feeding-enhancing opioid

peptides that are thought to modulate cytokine activity [Plata-Salamán et al., 1998b]; and iv) preprodynorphin, the precursor for dynorphin, an orexigenic opioid also involved in cytokine activity [Kong et al., 1997]. Examining these components concomitantly with the cytokines can provide information on potential chemical interactions and may elucidate possible neurological mechanisms involved in the tumor-associated anorexia observed in animal and human models of oncology.

STUDY 3: Cytokine mRNA profiles following LPS administration

Materials and Methods

Subjects and maintenance

Male Wistar (VAF) rats weighing between 250 and 275 g at the beginning of the experiments were used. Rats were randomly assigned to groups, housed individually, and maintained ad libitum on powdered rat food (Labdiet, PMI Feeds, Inc., St. Louis, MO) and tap water. Lights were on from 0600 to 1800 h and room temperature was kept at $23 \pm 1^{\circ}\text{C}$. All rats were handled daily.

Bacterial lipopolysaccharide administration

The rats were injected IP using either vehicle (sterile physiological saline) or 100 $\mu\text{g}/\text{kg}$ of bacterial lipopolysaccharide (*Escherichia coli* serotype O111:B4; Sigma, St. Louis, MO; Lot 78H4086) in sterile physiological saline. The same lot and stock solutions were used for all experiments. The concentration of LPS selected was based on previous studies that used acute IP administration [Langhans et al., 1991, 1993, Porter et al., 1998a]. The time selected for tissue sampling (5-h after the acute IP administration) was based on previous studies concerning the cytokine and behavioral profiles exhibited by rats receiving LPS [Langhans et al., 1991, 1993, Plata-Salamán et al., 1998b].

Dissection of brain regions and peripheral organs

Five hours following the IP injection, rats were sacrificed and their brains were quickly removed (< 30 s from the time of decapitation). Trunk blood was also taken from the animals. Each brain was immediately placed in oxygenated phosphate-buffered saline solution at 2-4°C. Brains were rinsed several times and selected regions (cerebellum, parieto-frontal cortex, hippocampus, and hypothalamus) were dissected. Liver, spleen and epididymal adipose tissue samples were also taken immediately from the rats. Each brain or peripheral sample was immediately homogenized in guanidine thiocyanate / phenol solution (Tri Reagent, Molecular Research Center, Inc., Cincinnati, OH) using a microtissue grinder, and frozen at -85°C. The samples were number coded for further processing and analyses. The complete brain and organ dissection, homogenization and storage took less than 6 minutes.

RNA isolation and RNase protection assay

Total cell RNA was extracted from the Tri Reagent homogenate as outlined in the manufacturer's protocol. RNA concentration was determined by spectrophotometry at an absorbance of 260 nm. The RNA integrity was assessed using agarose gel electrophoresis and ethidium bromide staining. The level of rat GAPDH mRNA and β -actin mRNA were RNase protection assayed to confirm consistency and that an equal amount of total cell RNA was used for each assay.

Probe synthesis was conducted with specific rat cDNAs in 1 mM each of CTP, ATP, and UTP; and 9.38 μM of [^{32}P] GTP (800 Ci/mmol) and 25 μM of unlabeled GTP. [^{32}P]GTP not incorporated into the probe was removed by two ethanol precipitations in the presence of 2.5 M ammonium acetate. RNase protection assays were used to detect the IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, OB-R, POMC, NPY, GAPDH and β -actin mRNAs.

Hybridization reactions containing 10.0 μg of total cell RNA and 2.5×10^4 cpm each of IL-1 β , IL-1RI, IL-1R AcP, IL-1Ra, and TGF- β 1 antisense probes, and 1.5×10^4 cpm of TNF- α antisense probe; 10.0 μg of total cell RNA and 2.5×10^4 cpm each of gp 130, OB-R, POMC, and NPY antisense probes, or 3.0 μg of total cell RNA and 2.0×10^5 cpm each of GAPDH and β -actin antisense probes in 30 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4)) were heated to 85 $^{\circ}\text{C}$ for 5 min and then incubated at 48 $^{\circ}\text{C}$ for 12 to 18 h. After hybridization, 280 μl of RNase digestion buffer (50 mM sodium acetate (pH 4.5), 2 mM EDTA) was added with 30 U of T1 RNase (Sigma) for all assays followed by incubation at 30 $^{\circ}\text{C}$ for 60 min. RNA was precipitated with 70 μg of yeast transfer RNA by the addition of Tri reagent in ethanol. RNA was dissolved in loading buffer [80% formamide, 2 mM EDTA (pH 7.4) containing 0.05% bromophenol blue and 0.05% xylene cyanol], denatured at 85 $^{\circ}\text{C}$ for 5 min, and resolved on 5% acrylamide / 8 M urea gels using TBE-buffer (89 mM Tris pH 8.0, 89 mM boric acid and 2.7 mM EDTA). Gels were autoradiographed and results were quantified with an image analyzer (Image

Quant, Molecular Dynamics, Sunnyvale, CA). Densitometric values for each mRNA analyzed were converted to percentage values of the total values for a particular mRNA.

In control experiments on hybridization specificity, 10.0 μ g of yeast transfer RNA were hybridized and processed as described above. No signal corresponding to IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, OB-R, POMC, or NPY was detected.

Riboprobe templates

Rat IL-1 β expression plasmid containing the entire mature peptide coding sequence of rat IL-1 β , with one extra Met codon at the 5' end cloned into plasmid pET-21d (Novagen, Madison, WI) between EcoRI and NcoI sites was used. Plasmid rat riboprobe IL-1 β (rRIL-1 β) was generated by cloning IL-1 β containing XbaI-EcoRI fragment of the rat IL-1 β expression plasmid into pGEM-2 between XbaI and EcoRI sites. Plasmid rRIL-1 β was linearized with HindIII, and transcribed with SP6 RNA polymerase to generate an approximately 570 nucleotides-long antisense probe that protects 492 nucleotides in the rat IL-1 β mRNA.

Plasmid rat IL-1RI 3' clone containing a 3' fragment (nucleotides 473-1826 of accession # M95578) of the rat IL-1RI cDNA cloned into the SmaI site of pBluescript SK (Stratagene) was used. Linearization of this plasmid with HindIII and transcription with T3 RNA polymerase produced an approximately 520 nucleotides-long antisense probe that protects 436 nucleotides in the rat IL-1RI mRNA.

Rat IL-1R AcP cDNA (nucleotides 874-1212 of accession # U48592) cloned into EcoRV site of pBluescript SK was used. Linearization of this plasmid by HindIII and transcription by T3 RNA polymerase resulted in an approximately 420 nucleotides-long antisense probe that protects 339 nucleotides in the membrane bound form of rat IL-1R AcP; additionally, this probe protects a shorter fragment corresponding to the soluble form of IL-1R AcP [Greenfeder et al., 1995].

Plasmid pBS-RA containing a fragment (nucleotides 3-451 of accession # M63101) of the rat IL-1Ra cDNA cloned into the SmaI site of pBluescript SK was linearized with XmnI, and transcribed with T3 RNA polymerase to produce an approximately 250 nucleotide-long antisense probe that protects 207 nucleotides in the rat IL-1Ra mRNA.

Plasmid prTNFtrans(T7) containing the peptide coding portion of rat TNF- α cDNA (nucleotides 1-708 of accession # S40199) was used. Linearization of this plasmid by EcoRI and transcription with SP6 RNA polymerase produced an approximately 790 nucleotides-long antisense probe which protects 708 nucleotides in the rat TNF- α mRNA.

Rat TGF- β 1 cDNA (accession # X52498) cloned into pBluescript II KS vector (Stratagene) was used. Linearization of this plasmid by BamHI and transcription with T3 RNA polymerase produced an approximately 320 nucleotides-long antisense probe that protects 244 nucleotides in the rat TGF- β 1 mRNA.

Rat gp 130 cDNA (accession # M92340) cloned into EcoRI site of pBS vector was used. This cDNA was linearized with BsaI and transcribed with T3 RNA polymerase to produce an approximately 590 nucleotides-long antisense probe that protects 530 nucleotides in the rat gp 130 mRNA.

Leptin receptor riboprobe template was generated by cloning a HincII-BamHI fragment of rat leptin receptor (Ob-Rb isoform) cDNA into respective sites of pGEM-2. The leptin receptor riboprobe was linearized using HindIII and SP6 transcription resulted in an approximately 450 nucleotides-long antisense probe that protects 415 nucleotides (corresponding to nucleotides 686-1101 of accession # U52966) in the rat OB-R mRNA.

Rat POMC cDNA cloned between BamHI and EcoRI sites of pBluescript KS(+) vector was used. Plasmid was linearized with XmnI and T3 RNA polymerase transcription resulted in an approximately 320 nucleotides-long antisense probe that protects 264 nucleotides in the rat POMC mRNA (predominant form).

Plasmid pBLNPY-1 containing a 511 bp insert (accession # M20373) comprising most of the cDNA of rat prepro-neuropeptide Y ligated into the EcoRI site of vector Bluescribe M13(-) was used. Linearization of the plasmid with BbsI and transcription with T3 RNA polymerase produced an approximately 242 nucleotides-long antisense probe that protects 180 nucleotides in the rat NPY mRNA.

Rat antisense template pTRI-GAPDH (Ambion; which contains a 316 bp fragment of the rat glyceraldehyde 3-phosphate dehydrogenase gene) was used to generate the

GAPDH antisense probe. Antisense template pTRI- β -actin-125-Rat (Ambion; which contains a 126 bp cDNA fragment of the rat β -actin gene) was used to generate the β -actin antisense probe.

Rat TNF- α ELISA

The blood samples were analyzed with a rat ultra-sensitive TNF- α ELISA according to the manufacturer's protocol (Cytoscreen™ Immunoassay kit, BioSource International, Inc., Camarillo, CA). The sensitivity of the assay is < 0.7 pg / ml (range = 2.3-150 pg / ml), and there is no cross-reactivity with other cytokines.

Data analyses

Results are expressed as means $\pm$ SE unless otherwise stated. Data were analyzed ANOVA followed by Bonferroni corrected t tests to control for family-wise error. When data did not pass the normality (Kolmogorov-Smirnov) and equal variance (Levene Median) tests, Kruskal-Wallis ANOVA on ranks was applied (followed by post hoc tests).

Results

RNase Protection Assay

The same animals were used to analyze all components studied: IL-1 β , IL-1RI, IL-1R AcP I and II, IL-1Ra, TNF- α , TGF- β 1, gp 130, OB-R, POMC, and NPY mRNA levels. A sample gel of the RNase protection assays is shown in Figure 8. As shown, samples from

both the vehicle (controls)- and LPS-treated animals from different brain regions (Figure 8A) and peripheral organs (Figure 8B) were analyzed concomitantly. Consistency was verified by analyzing all samples individually, from which all means $\pm$ SE were generated. Each individual sample was obtained from a different rat, and all rats provided all four brain and three peripheral organ samples; the same samples were analyzed with all antisense probes as described in the Materials and Methods. GAPDH and β -actin mRNAs were relatively constant between treatments within a brain region, suggesting consistency of processing and that equal amounts of total RNA were used. In addition, to verify consistency of the assay, serial dilutions of a test RNA sample were also analyzed (not shown). The specificity of the probes used has been demonstrated previously by various procedures, including control experiments on hybridization specificity in which no signal corresponding to IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, OB-R, POMC, or NPY was detected [Gayle et al., 1998a, 1999, Ilyin and Plata-Salamán, 1996, Ilyin et al., 1996, Plata-Salamán and Ilyin, 1997, Plata-Salamán et al., 1998a,b, 1999].

IL-1 β mRNA levels

IP administered LPS significantly induced the expression of IL-1 β in cerebellum ($F(1, 14) = 22.528$, $p < 0.001$), parieto-frontal cortex ($F(1, 14) = 48.003$, $p < 0.001$) and hippocampus ($F(1, 14) = 13.224$, $p = 0.003$) (Figure 9). The same trend was observed in the hypothalamus. The cerebellum and parieto-frontal cortex showed an induction of similar

magnitude (i.e., the difference between control and LPS-treated animals), 14.7 and 16.0 arbitrary units, respectively.

IP LPS significantly induced IL-1 β mRNA expression in the peripheral samples compared to vehicle treatment: liver ($F(1, 14) = 846.967$, $p < 0.001$), spleen ($F(1, 14) = 11.294$, $p < 0.001$), and adipose tissue ($F(1,14) = 11.294$, $p < 0.001$) (Figure 9). The magnitude of induction was greatest in the spleen (41.0 arbitrary units difference between control and LPS treatments) > liver (34.2) > adipose tissue (9.5).

IL-1Ra mRNA levels

There was no significant induction of IL-1Ra mRNA in the cerebellum, hippocampus, or hypothalamus in response to IP LPS (Figure 10). Only the parieto-frontal cortex showed a significant LPS-stimulated up-regulation of IL-1Ra mRNA levels ($F(1, 14) = 8.56$, $p < 0.01$).

IP LPS also induced a robust up-regulation of IL-1Ra mRNA in the liver (magnitude of induction relative to controls = 38.6 arbitrary units, ($F(1, 14) = 1058$, $p < 0.001$) and spleen (25.7 ($F(1, 14) = 364.872$, $p < 0.001$), and a milder, but significant increase of 5.7 arbitrary units over control in the adipose tissue ($F(1, 14) = 24.649$, $p < 0.001$).

IL-1RI mRNA levels

Intraperitoneal LPS significantly induced cerebellar ($F(1, 14) = 15.947, p < 0.001$), cortical ($F(1, 14) = 23.2, p < 0.001$) and hippocampal ($F(1, 14) = 26.219, p < 0.001$), but not hypothalamic, IL-1RI mRNA expression (Figure 11). The cerebellum and parieto-frontal cortex exhibited the highest magnitudes of induction.

Analysis of peripheral expression also revealed a significant LPS-induced up-regulation of IL-1RI mRNA in liver ($F(1, 14) = 140.003, p < 0.001$), spleen ($F(1, 14) = 218.606, p < 0.001$) and adipose tissue ($F(1, 14) = 23.015, p < 0.001$). The liver showed the highest magnitude of induction.

IL-1R AcP I and II mRNA levels

Intraperitoneal LPS significantly induced brain IL-1R AcP I mRNA expression (Figure 12) in the cerebellum ($F(1, 14) = 5.294, p < 0.04$) and parieto-frontal cortex ($F(1, 14) = 5.491, p < 0.04$); no changes, in contrast, were observed in the hippocampus or hypothalamus. All three peripheral tissues showed significant LPS-induced up-regulation of IL-1R AcP I mRNA ($F(1, 14) = 251.544$, and $97.689, p < 0.001$, liver and spleen, respectively; ($F(1, 14) = 14.615, p = 0.002$, adipose tissue). The liver and spleen showed similarly high magnitudes of induction while the adipose tissue exhibited a lower, but still robust increase.

Figure 13 shows a similar trend for the IL-1R AcP II mRNA profile. IP LPS increased IL-1R AcP II mRNA levels in cerebellum ($F(1, 14) = 7.079$, $p < 0.02$), parieto-frontal cortex ($F(1, 14) = 33,939$, $p < 0.001$), liver ($F(1, 14) = 23.001$, $p < 0.001$), spleen ($F(1, 14) = 103.25$, $p < 0.001$), and adipose tissue ($F(1, 14) = 7.762$, $p < 0.02$). The spleen exhibited the highest magnitude of induction relative to the vehicle-treated group.

TNF- α mRNA levels

There was an LPS-stimulated increase of TNF- α mRNA expression (Figure 14) in the parieto-frontal cortex ($F(1, 14) = 17.313$, $p < 0.001$) and hippocampus ($F(1, 14) = 29.813$, $p < 0.001$), but not in the cerebellum or hypothalamus. LPS treatment also significantly induced TNF- α mRNA expression in the liver ($F(1, 14) = 18.262$, $p < 0.001$) and spleen ($F(1, 14) = 10.599$, $p < 0.001$). Interestingly, the magnitude of induction in the spleen (52.8 arbitrary units over control levels) was much greater than that exhibited in the liver (12.0 arbitrary units).

TGF- β 1 mRNA levels

Analysis of TGF- β 1 mRNA levels showed a significant LPS-induced expression in the hippocampal ($F(1, 14) = 5.615$, $p < 0.04$) and liver ($F(1, 14) = 73.521$, $p < 0.001$) samples (Figure 15). No significant changes were observed in the other samples examined.

Gp 130, NPY, OB-R, and POMC mRNA levels

In contrast to the cytokine mRNA changes, there was no significant LPS-induced modulation of hypothalamic mRNA levels of gp 130 (23.8 ± 3.9 versus 26.2 ± 3.4 (mean $\pm$ SD) arbitrary units for vehicle and LPS, respectively, $n = 8$ each), NPY (24.9 ± 1.2 versus 25.1 ± 3.2 arbitrary units for vehicle and LPS, respectively, $n = 8$ each), OB-R (24.1 ± 4.4 versus 25.9 ± 7.0 arbitrary units for vehicle and LPS, respectively, $n = 8$ each), or POMC (24.0 ± 2.5 versus 26.0 ± 3.2 arbitrary units for vehicle and LPS, respectively, $n = 8$ each).

TNF- α protein levels

This study also determined TNF- α protein levels in the serum of vehicle and LPS-treated rats. Animals injected IP with LPS showed high levels of TNF- α protein (mean = 56 pg/ml) relative to control animals (mean = 3 pg/ml) at 5 h post-LPS administration, that is, at the point of analysis of all brain and peripheral samples.

Figure 8. Sample gels of samples from male Wistar rats treated with acute IP administration of vehicle (physiological saline; control) or bacterial lipopolysaccharide (LPS 100 µg/kg). Brain and peripheral tissue samples were collected 5 h after vehicle or LPS administration. Figure shows that samples from each treatment and brain region or organ were processed simultaneously and analyzed with all antisense probes as described in Materials and Methods. **A.** Tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-1 receptor type I (IL-1RI), IL-1R accessory protein type I (AcPI) and type II (AcPII) and transforming growth factor- β 1 (TGF- β 1) mRNA levels in the cerebellum (lane 1), parieto-frontal cortex (lane 2), hippocampus (lane 3), and hypothalamus (lane 4). **B.** Same as **A.**, for liver, spleen, and adipose tissue.

Figure 9. IL-1 β mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 μ g/kg (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: p < 0.001; ‡: p < 0.01 from vehicle.

Figure 10. IL-1Ra mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 μ g/kg (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: p < 0.001; ‡: p < 0.05 from vehicle.

Figure 11. IL-1RI mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 µg/kg (closed bars). Values (mean ± SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: $p < 0.001$; ‡: $p < 0.01$ from vehicle.

Figure 12. IL-1R AcP I mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 µg/kg (closed bars). Values (mean ± SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: $p < 0.001$; ‡: $p < 0.01$; †: $p < 0.05$ from vehicle.

Figure 13. IL-1R AcP II mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 μ g/kg (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: $p < 0.001$; †: $p < 0.05$ from vehicle.

Figure 14. TNF- α mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 μ g/kg (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: $p < 0.001$ from vehicle.

Figure 15. TGF- β 1 mRNA levels in response to the IP administration of vehicle (controls, open bars) or LPS, 100 μ g/kg (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; CTX, parieto-frontal cortex; HIP, hippocampus; HYP, hypothalamus; LIV, liver; SPL, spleen; ADP, adipose tissue. *: $p < 0.001$; †: $p < 0.05$ from vehicle.

Discussion

When assessed 5 hours after the IP administration of LPS pro-inflammatory cytokine (IL-1 β and TNF- α) mRNAs were up-regulated in the cerebral cortex, hippocampus and cerebellum, as well as in peripheral organs. LPS-treated animals also exhibited high levels of circulating TNF- α protein compared to vehicle-treated controls. Other components of the IL-1 β system (IL-1RI, IL-1 AcP I and II) were also up-regulated in brain regions and periphery. The results exemplify the ability of peripheral immune signals to communicate with the CNS and activate a cytokine response within specific brain regions. The data also show that neither gp 130, OB-R, NPY, nor POMC mRNA was modulated during the testing conditions. This suggests that although this model is associated with multiple transcriptional changes within brain region, they were specific to cytokine components.

Anti-inflammatory cytokine (IL-1Ra and TGF- β 1) mRNAs were also up-regulated within the brain, but only in the cerebral cortex for IL-1Ra and hippocampus for TGF- β 1, while both were up-regulated in the liver; and IL-1Ra was up-regulated in the spleen and adipose tissue. IL-1Ra and TGF- β 1 mRNA up-regulation observed in brain regions, however, was mild in comparison to the robust pro-inflammatory cytokine mRNA response. Based on previous reports (Plata-Salamán et al., 1998b), the lack or a mild response of IL-1Ra and TGF- β 1 modulation could be associated with an imbalance towards pro-inflammatory cytokines. The net outcome of this imbalance could participate in the

magnitude of the CNS (neurological and neuropsychiatric) manifestations; that is, an inadequate up-regulation of anti-inflammatory components will preclude compensation through negative feedback mechanisms. This is also consistent with previous studies that focused on the relationship between pro-inflammatory to anti-inflammatory cytokine balance in the brain and neurological manifestations [Plata-Salamán and Ilyin, 1997, Plata-Salamán et al., 1998b, Turrin and Plata-Salamán, 2000]. Such imbalance could be indicative of a breakdown of the adaptive immune response. If the anti-inflammatory response is inadequate to subdue the up-regulation of pro-inflammatory cytokines, a chronic inflammatory response could ensue, which could lead to inflammatory disorders and/or autoimmune complications.

Previous models that used centrally administered LPS (into the third cerebral ventricle, 500 ng LPS/rat) [Gayle et al., 1998a, 1999, Plata-Salamán et al., 1998b] show a similar profile of pro-inflammatory cytokine component mRNA expression in specific brain regions. The profile in the present investigation was different, however, in relation to the magnitude of cytokine mRNA component up-regulation as well as brain regional over-expression. In particular, in these earlier studies, LPS administered centrally induced a large up-regulation of cytokine mRNAs in all brain regions examined (e.g., cerebellum, hippocampus, hypothalamus) [Gayle et al., 1998a, Plata-Salamán, 1998b]. Moreover, the central administration of LPS also induced up-regulation of IL-1Ra mRNA [Gayle et al., 1998a, Plata-Salamán, 1998b]; however, the ratio IL-1 β to IL-1Ra was significantly larger [Gayle et al., 1998a, Plata-Salamán, 1998b] and the net pro-inflammatory cytokine profile

correlated with the magnitude of anorexia. The larger over-expression of brain cytokine components in these studies likely stems from the fact that LPS was administered centrally, rather than through the IP route. In effect, while it is often thought that the actions of LPS stem from the peripheral activation of cytokines, it seems that LPS may have direct actions in brain, possibly through the promotion of central cytokine synthesis. In effect, regardless of whether LPS is applied directly to the brain (central administration) or requires cascade of events (e.g., following peripheral administration), including activation of humoral and / or neural signals, the overall net result is the same, namely, pro-inflammatory cytokine induction within the brain. The similarities in brain cytokine component mRNA over-expression elicited by the two routes of administration may be related to the similar neurological manifestations that occur following these treatments.

The pathway for peripheral LPS-induced modulation of brain cytokine components is still unresolved. The data showing increased TNF- α protein levels in the circulation of LPS-treated rats exhibiting up-regulation of pro-inflammatory cytokine components in the periphery and brain, suggests the involvement, at least to some extent, of circulating cytokines as mediators in the present model. In fact, circulating TNF- α has been proposed to play an important role in triggering adrenocorticotrophic hormone (ACTH) secretion in response to the IP administration of LPS [Kakizaki et al., 1997]. Of course, as other cytokines are also activated in response to LPS, these in conjunction with TNF- α may play a fundamental role in the elicitation of the neuroendocrine and central cytokine variations. This, of course, does not belie the possibility that local production of cytokine components

in the brain could be involved in the induction and/or maintenance of neurological manifestations observed during peripheral LPS administration.

Implications

The ability of peripherally administered bacterial LPS to produce an up-regulation of brain IL-1 β system components, as well as TNF- α , and to a lesser extent TGF- β 1 mRNAs, suggests the presence of mechanisms in the periphery that are able to signal the brain and modify the cytokine profile within specific CNS regions. Whether the nature of these mechanisms is humoral or neural is still to be determined; however, the concomitant up-regulation of pro-inflammatory cytokine component mRNAs (indicators of local transcriptional modulation) in peripheral organs and discrete brain regions associated with increased circulating TNF- α protein levels supports a humoral role in the present model. TNF- α has been shown to induce its own production and that of IL-1 β from various immune and brain cell types [Dinarello, 1996, Plata-Salamán, 1991], and therefore, TNF- α could be a possible soluble factor involved in the induction of cytokines. The same applies to IL-1 β , the predominant soluble form of IL-1 [Dinarello, 1996]. The brain cytokine profile activated by peripheral LPS injections could have implications in the neurology associated with this model of peripheral challenge. That is, the peripheral administration of bacterial LPS to human healthy volunteers results in a dose-dependent induction of neurological manifestations [Mullington et al., 2000, Pernerstorfer et al., 1999]. The possible relationship of brain regional cytokine component up-regulation and neurobehavioral

consequences in response to LPS administration has been discussed [Gayle et al., 1999, Plata-Salamán et al., 1998b]. Overall, cytokine-cytokine interactions with positive ($\text{IL-1}\beta \leftrightarrow \text{TNF-}\alpha$) [Sonti et al., 1996b] and negative ($\text{IL-1Ra} \rightarrow \text{IL-1}\beta$; $\text{TGF-}\beta \rightarrow \text{IL-1}\beta / \text{TNF-}\alpha$) [Cartmell et al., 1999, Dinarello, 1996, Plata-Salamán, 1998c] feedback, or a balance between pro-inflammatory and inhibitory cytokines, may be pivotal for the modulation of the cascade of events associated with the neurological manifestations stemming from peripherally or centrally administered LPS. A balance towards a pro-inflammatory cytokine profile could be reflected in the magnitude of neurological manifestations and complications [Plata-Salamán et al., 1998b, Turrin and Plata-Salamán, 2000]. The findings in the present *in vivo* model are consistent with this proposal.

A peripheral model of disease involving rats bearing prostate adenocarcinoma tumor cells also showed activation of cytokine components in specific brain regions and this activation was associated with the development of early cancer anorexia [Plata-Salamán et al., 1998a]. In a like fashion, there could be a relationship with the known clinical manifestations that occur during Gram-negative infections or during LPS challenge [Langhans, 1996, Mullington et al., 2000, Pernerstorfer et al., 1999]. The similarities between two distinct pathophysiological models, oncological and LPS challenge, suggest that although they may act differently, both are able to trigger brain production of pro-inflammatory cytokine components that could act as mediators of neurological and neuropsychiatric manifestations. The results from an acute inflammatory (LPS challenge) and chronic (oncological) model add validity to the importance of brain cytokine production

in response to pathophysiological processes. Hence, the last study of this thesis examined another chronic model using tumor-bearing rats to validate this proposal.

STUDY 4: Cytokine Regulation and Action in the Brain of Tumor-Bearing Rats

Materials and Methods

Subjects and maintenance

Sixteen male Fischer 344 rats (Charles River, Inc., Wilmington, MA, USA) with an initial body weight of 260-300 g were housed individually in holding cages for 7-10 d to acclimatize them to the study surroundings. The environmental conditions were kept constant: 12-h (0600-1800 h, 1800-0600) light/dark cycles; room temperature $26 \pm 1^{\circ}\text{C}$; and 45% humidity. Rats had free access to water and fresh coarsely ground rat chow (Diet #5008, Ralston Purina Co., St. Louis, MO, USA), that yielded 3.5 kcal/g. Food intake was gravimetrically measured between 0800 and 0900 h each day, and a fresh supply of water and rat chow provided from the same initial stock.

Tumor model

After the 10-day acclimation periods, rats were randomly assigned to either a non-tumor-bearing control group or a tumor-bearing experimental group. Controls received a subcutaneous inoculation of 1.0 ml of 0.9 N sterile saline into the right flank. The tumor-bearing rats were given a 1.0 ml inoculum containing 10^6 MCA-induced sarcoma cells from a MCA tumor freshly excised from a donor. The MCA tumor cells were prepared according to the method of Madden and Burke [1961]. This tumor characteristically grows locally within 10-12 days [Meguid et al., 1999]. After inoculation, the rats were placed in individual

cages and given free access to food and water. Food intake was measured daily as mentioned earlier.

Processing of animals and dissection of brain regions

When the tumor bearing rats showed a significant decrease of food intake (approximately 50% less) compared to their control counterparts, both groups were sacrificed and quickly frozen at -85°C until processing for mRNA analyses.

The brains, liver and tumor samples were removed from the frozen animals. The brains were rinsed several times and selected regions (cerebellum, hippocampus, and hypothalamus) were dissected. Each sample was immediately homogenized in guanidine thiocyanate/phenol solution (Tri Reagent, Molecular Research Center, Inc., Cincinnati, OH) using a microtissue grinder, and then frozen at -85°C. The samples were number coded for further processing and analyses. The complete brain and liver dissection, homogenization and storage took less than 6 minutes.

RNA isolation and analysis of IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, dynorphin, NPY, POMC, actin and cyclophilin mRNAs

Total cell RNA was extracted from the Tri Reagent homogenate as outlined in the manufacturer's protocol. RNA concentration was determined by spectrophotometry at an absorbance of 260 nm. RNA integrity was assessed through agarose gel electrophoresis and ethidium bromide staining. The levels of rat actin and cyclophilin mRNAs were RNase

protection assayed to confirm consistency and that equal amounts of total cell RNA were used for each assay.

Probe synthesis was conducted with specific rat cDNAs in 1 mM each of CTP, ATP, and UTP; and 9.38 μ M of [32 P]GTP (800 Ci/mmol) and 25 μ M of unlabeled GTP. [32 P]GTP not incorporated into the probe was removed by two ethanol precipitations in the presence of 2.5 M ammonium acetate. RNase protection assays were used to detect the IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, dynorphin, NPY, POMC and cyclophilin mRNAs.

Hybridization reactions containing 12.0 μ g of total cell RNA and 2.5×10^4 cpm of each IL-1 β , IL-1RI, IL-1R AcP, IL-1Ra, TGF- β 1, gp 130, dynorphin, NPY and POMC and 1.5×10^4 cpm of TNF- α antisense probes; 5.0 μ g of total cell RNA and 3.0 μ g of total cell RNA and 2.0×10^5 cpm of each actin and cyclophilin antisense probe in 30 μ l of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4)) were heated to 85°C for 5 min and then incubated at 48°C for 12 to 18 h. After hybridization, 280 μ l of RNase digestion buffer (50 mM sodium acetate (pH 4.5), 2 mM EDTA) was added with 30 U of T1 RNase (Sigma, St. Louis, MO) for all assays followed by incubation at 30°C for 60 min. RNA was precipitated with 70 μ g of yeast transfer RNA by the addition of Tri reagent in ethanol. RNA was dissolved in loading buffer (80% formamide, 2 mM EDTA (pH 7.4) containing 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 85°C for 5 min, and resolved on 5% acrylamide/8 M urea gels using TBE-buffer (89 mM Tris pH

8.0, 89 mM boric acid and 2.7 mM EDTA). Gels were autoradiographed and results were quantified with an image analyzer (Image Quant, Molecular Dynamics, Sunnyvale, CA). Densitometric values for each mRNA analyzed were converted to percentage values of the total values for a particular mRNA.

In control experiments on hybridization specificity, 12.0 μ g of yeast transfer RNA was hybridized and processed as described above. No signal corresponding to IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, dynorphin, NPY, POMC was detected.

Riboprobe templates

Rat IL-1 β (rIL-1 β) expression plasmid containing the entire mature peptide coding sequence of rat IL-1 β , with one extra Met codon at the 5' end cloned into plasmid pET-21d (Novagen, Madison, WI) between EcoRI and NcoI sites was used. Plasmid rat riboprobe IL-1 β (rRIL-1 β) was generated by cloning IL-1 β containing XbaI-EcoRI fragment of the rat IL-1 β expression plasmid into pGEM-2 between XbaI and EcoRI sites. Plasmid rRIL-1 β was linearized with HindIII, and transcribed with SP6 RNA polymerase to generate an approximately 570 nucleotides-long antisense probe, which protects 492 nucleotides in the rat IL-1 β mRNA.

Plasmid rat IL-1RI 3' clone containing a 3' fragment (nucleotides 473-1826 of accession # M95578) of the rat IL-1RI cDNA cloned into the SmaI site of pBluescript SK (Stratagene) was used. Linearization of this plasmid with HindIII and transcription with T3

RNA polymerase produced an approximately 520 nucleotides-long antisense probe which protects 436 nucleotides in the rat IL-1RI mRNA.

Rat IL-1R AcP cDNA (nucleotides 874-1212 of accession # U48592) cloned into EcoRV site of pBluescript SK was used. Linearization of this plasmid by HindIII and transcription by T3 RNA polymerase resulted in an approximately 420 nucleotides-long antisense probe which protects 339 nucleotides in the membrane bound form of rat IL-1R AcP; additionally, this probe protects a shorter fragment corresponding to the soluble form of IL-1R AcP [Greenfeder et al., 1995].

Plasmid pBS-RA containing a fragment (nucleotides 3-451 of accession # M63101) of the rat IL-1Ra cDNA cloned into the SmaI site of pBluescript SK was linearized with XmnI, and transcribed with T3 RNA polymerase to produce an approximately 250 nucleotides-long antisense probe which protects 207 nucleotides in the rat IL-1Ra mRNA.

Plasmid prTNFtrans (T7) containing the peptide coding portion of rat TNF- α cDNA (nucleotides 1-708 of accession # S40199) was used. Linearization of this plasmid by EcoRI and transcription with SP6 RNA polymerase produced an approximately 790 nucleotides-long antisense probe which protects 708 nucleotides in the rat TNF- α mRNA.

Rat TGF- β 1 cDNA (accession # X52498) cloned into pBluescript II KS vector (Stratagene Cloning Systems) was used. Linearization of this plasmid by BamHI and

transcription with T3 RNA polymerase produced an approximately 320 nucleotides-long antisense probe, which protects 244 nucleotides in the rat TGF- β 1 mRNA.

Rat gp 130 cDNA (accession # M92340) cloned into EcoRI site of pBS vector was used. This cDNA was linearized with BsaI and transcribed with T3 RNA polymerase to produce an approximately 590 nucleotides-long antisense probe that protects 530 nucleotides in the rat gp 130 mRNA.

Plasmid pBLNPY-1 containing a 511 bp insert (accession # M20373) comprising most of the cDNA of rat prepro-neuropeptide Y ligated into the EcoRI site of vector Bluescribe M13(-) was used. Linearization of the plasmid with Bbs I and transcription with T3 RNA polymerase produced an approximately 242 nucleotides-long antisense probe that protects 180 nucleotides in the rat NPY mRNA.

Rat POMC cDNA cloned between BamHI and EcoRI sites of pBluescript KS(+) vector was used. Plasmid was linearized with XmnI and T3 RNA polymerase transcription resulted in an approximately 320 nucleotides-long antisense probe that protects 264 nucleotides in the rat POMC mRNA (predominant form).

Rat antisense template pTRI- β -actin-125-Rat (Ambion; which contains a 126 bp cDNA fragment of rat β -actin gene) was used to generate the β -actin antisense probe. Rat antisense template pTRI-Cyclophilin (Ambion; which contains a 103 bp cDNA fragment of the rat cyclophilin gene) was used to generate the cyclophilin antisense probe.

Preparation of rat preprodynorphin antisense template

Primers 5'-AGAAGCCTGCCAGCGACAAAG-3' (corresponding to nucleotides 717-737 of rat dynorphin gene exon 1; accession # M32781) and 5'CAGAGATGCAATTA-ACCCTCACTAAAGGGAGAGTCCTCGTCCCCAGTCATCTC-3' (corresponding to nucleotides 453-473 of rat dynorphin gene exon 4; accession #M32784 and including an additional sequence comprising T3 RNA polymerase promoter) were designed to amplify an approximately 640 bp fragment of the rat preprodynorphin cDNA. Reverse transcription was performed with 1 µg of total rat brain RNA and random hexamers in a final volume of 20 µl using RNA PCR Core Kit according to the manufacturer's protocol (Perkin Elmer, Foster City, CA). Amplification was performed by using Taq2000 DNA Polymerase according to the manufacturer's protocol in a final volume of 100 µl; this was subjected to 35 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 70 s and extension at 72°C for 90 s. The PCR product was purified by the Wizard PCR Preps DNA Purification System (Promega, Madison, WI) and used directly for rat preprodynorphin antisense probe synthesis. The identity of the PCR product was confirmed by sequencing using the Sanger method with T3 RNA polymerase promoter specific primers.

Analysis of IFN γ mRNA expression in hypothalamus and tumor by RT PCR

Primers 5'-ATGAGTGCTACACGCCGCTCTTGG-3' (corresponding to nucleotides 1-25 of rat interferon gamma gene exon 1) and 5'-GAGTTCATTGACAGCTT-TGTGGTGG-3' (corresponding to nucleotides 18-42 of rat interferon gamma gene exon 4)

were designed to amplify 405 bp fragment of the rat interferon gamma cDNA. Reverse transcription was performed with 1 µg of total rat RNA from hypothalamus or tumor and random hexamers in a final volume of 20 µl using RNA PCR Core Kit according to the manufacturer's protocol (Perkin Elmer, Foster City, CA). Amplification was performed by using AmpliTaq DNA Polymerase according to the manufacturer's protocol in a final volume of 100 µl; this was subjected to 35 cycles of denaturation at 95°C for 60 s and annealing-extension at 60°C for 90 s. The PCR product was purified by the Wizard PCR Preps DNA Purification System (Promega, Madison, WI) and ligated into SrfI site of pCR-Script SK(+) vector according to the manufacturer's protocol (pCR-Script Amp SK(+) Cloning Kit, Stratagene Cloning Systems, La Jolla, CA). Following transformation of *E. coli* Epicurian Coli XL1-Blue MRF' Kan supercompetent cells (Stratagene), separate colonies were selected, grown overnight, and plasmid DNA was purified by the Wizard Plus Minipreps DNA Purification System (Promega). The identity of the insert was confirmed by sequencing the plasmid DNA from individual colonies using the Sanger method with T3 vector specific primers.

Data analyses

Results are expressed as means $\pm$ SE. Food consumption was analyzed by mixed measures ANOVA followed by Newman-Keuls multiple comparisons of significant main effects or simple effects of significant interactions. The mRNA data comparing tumor and nontumor bearing rats were analyzed by t tests ($\alpha = 0.05$). Kruskal-Wallis ANOVA on ranks

was applied (followed by post hoc tests) when the data did not pass the normality (Kolmogorov-Smirnov) and equal variance (Levene Median) tests.

Results

Food Intake

Food intake, over days, as a function of the tumor condition is presented in Figure 16. Mixed measures ANOVA revealed a significant interaction between tumor condition and time ($F(10, 140) = 11.090, p < 0.0001$). As shown, food intake for the tumor-bearing rats was significantly lower than among control animals ($p < 0.05$) on the seventh day following the inoculation with tumor cells. After sustaining this anorexia to day 11 post-inoculation, where food intake was reduced to about 50% of that of control animals, rats were sacrificed for molecular analysis.

RNase-protection assay of IL-1 β , IL-1RI, IL-1R AcPs, IL-1Ra, TNF- α , TGF- β 1, gp 130, NPY, dynorphin and POMC mRNAs.

Analyses were performed for mRNA variations within the IL-1 β system, TNF- α , TGF- β 1, gp 130, NPY, POMC and dynorphin in selected brain regions, liver and tumor. Fig. 17A presents an example of the RNase protection assays used in brain samples. As shown, different brain region samples from tumor bearing and control animals were analyzed concomitantly. Fig. 17B shows levels of IL-1R AcP II and IL-1Ra mRNA in the liver from tumor bearing and control animals. Consistency was verified by analyzing all

samples individually, from which all means $\pm$ SE were generated. It should also be noted that each individual sample was obtained from a different rat, that the same rat provided all three brain regions, and that the same samples were analyzed with all antisense probes as described in Methods section. The levels of rat actin and cyclophilin mRNA were relatively constant between treatments within a brain region, suggesting consistency of processing and that equal amounts of total RNA were used. Moreover, the specificity of the probes used has been demonstrated previously [Gayle et al., 1998a, 1999, Ilyin and Plata-Salamán, 1996, Ilyin et al., 1996, Plata-Salamán and Ilyin, 1997, Plata-Salamán et al., 1998a,b, 1999].

Interleukin-1 β and tumor necrosis factor- α mRNAs in brain regions and tumor in response to cancer progression

The profile of IL-1 β mRNA in brain regions obtained from tumor bearing and control animals is summarized in Fig 18. The differences in the levels of IL-1 β mRNA were significant in the cerebellum ($p=0.001$), hippocampus ($p=0.002$), and hypothalamus ($p=0.001$); these brain regions showed a significant IL-1 β mRNA induction in the tumor bearing group relative to the control. The cerebellum from tumor bearing rats exhibited the highest levels of IL-1 β mRNA. TNF- α mRNA expression in the brain was not detectable, however the tumor itself exhibited high levels of both IL-1 β and TNF- α mRNAs (data not shown).

Transforming growth factor- β 1 mRNA in brain regions in response to treatments

It will be recalled that in several models of inflammation, TGF- β 1 is upregulated in response to elevated proinflammatory cytokines such as IL-1 β and TNF- α . Commensurate with the absence of any effects on TNF- α , there was no significant change in the level of TGF- β 1 in any of the brain regions examined (see Figure 19).

Interferon-gamma mRNA in the hypothalamus and tumor in response to cancer development

In order to measure IFN γ mRNA expression, RT PCR rather than RPA was employed. As seen in Figure 25, IFN γ mRNA expression was detected in the tumor, but not in the hypothalamus of either the tumor bearing or in control rats.

Interleukin-1 receptor type I mRNA in brain regions in response to treatments

As shown in Fig. 20, the tumor bearing rats exhibited significantly increased IL-1RI mRNA levels relative to the control group in the cerebellum ($p=0.008$), hippocampus ($p=0.001$) and hypothalamus ($p=0.006$). There were small differences of IL-1RI mRNA expression among the different brain regions in the control group, but none were significant.

Interleukin-1 receptor accessory proteins mRNA in brain regions and liver in response to treatments

Levels of IL-1R AcP I (membrane bound) mRNA and interleukin-1 receptor AcP II (soluble form) were not significantly changed in the cerebellum, hippocampus and hypothalamus (see Figs. 21 and 22). In contrast, the level of IL-1R AcP II mRNA was

significantly upregulated in the liver of tumor bearing rats compare to controls ($p=0.001$) (see Fig. 23).

Interleukin-1 receptor antagonist mRNA in brain regions and Liver in response to treatments

Despite IL-1 mRNA levels being consistently elevated, IL-1Ra mRNA was not detectable in brain regions from either the tumor bearing or control animals. Significantly increased levels of IL-1Ra mRNA were, however, detected in the liver of tumor bearing animals ($p=0.001$) (see Fig.23).

Glycoprotein 130, neuropeptide Y, dynorphin and pro-opiomelanocortin mRNAs in hypothalamus in response to tumor development

Several of the hormones thought to be involved in signal transduction or in subserving feeding effects were analyzed together with the cytokine mRNAs. As depicted in Figure 24, there were no significant differences between control and tumor bearing rats in the levels of gp 130, Dynorphin, NPY and POMC mRNA within the hypothalamus.

Figure 16. Total daily food intake in male Fischer 344 rats bearing MCA-induced sarcoma tumor cells and controls. Presurgery food intake (PreS) shown is the average food eating for the 3 days preceding tumor cell inoculation. Rats were sacrificed on the last day, and these rats were used for molecular analysis. Values are mean $\pm$ SE; $n=8$. Asterisk with arrows indicate range of days where tumor-bearing animals ate significantly less than the controls ($p < 0.05$).

Figure 17. Sample gels of samples taken from male Fischer 344 rats inoculated with MCA-induced sarcoma cells [plus signs in **A.**, lanes 1-3 in **B.**] or controls [minus signs in **A.**, lanes 4-6 in **B.**]. Brain and liver samples were collected 11 days after inoculations. Figure shows that samples from each treatment and brain region or organ were processed simultaneously and the same samples were analyzed with all the antisense probes as described in Materials and Methods. **A.** Interleukin-1 β (IL-1 β), IL-1 receptor type I (IL-1RI), IL-1R accessory protein type I (AcPI) and type II (AcPII), transforming growth factor- β 1 (TGF- β 1), and cyclophilin mRNA levels in the cerebellum (CLL), hippocampus (HIP) and hypothalamus (HYP). **B.** AcP II, IL-1 receptor antagonist (IL-1Ra), and actin mRNA levels in the liver.

Figure 18. IL-1 β mRNA levels in control (open bars) or tumor bearing rats (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; HIP, hippocampus; HYP, hypothalamus. *: $p < 0.002$ from controls.

Figure 19. TGF- β 1 mRNA levels in control (open bars) or tumor bearing rats (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; HIP, hippocampus; HYP, hypothalamus.

Figure 20. IL-1RI mRNA levels in control (open bars) or tumor bearing rats (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; HIP, hippocampus; HYP, hypothalamus. *: $p < 0.008$ from controls.

Figure 21. IL-1R AcP I mRNA levels in control (open bars) or tumor bearing rats (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; HIP, hippocampus; HYP, hypothalamus.

Figure 22. IL-1R AcP II mRNA levels in control (open bars) or tumor bearing rats (closed bars). Values (mean $\pm$ SE; $n=8$ for each group) were standardized to arbitrary units. Abbreviations: CLL, cerebellum; HIP, hippocampus; HYP, hypothalamus.

Figure 23. IL-1RI, IL-1R AcP II and IL-1Ra mRNA levels in the liver of control (open bars) and tumor bearing rats (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units. *: $p < 0.001$ from controls.

Figure 24. Dynorphin (DYN), Gp 130, POMC and NPY mRNA levels in the hypothalamus of control (open bars) and tumor bearing rats (closed bars). Values (mean $\pm$ SE; n=8 for each group) were standardized to arbitrary units.

Figure 25. Sample gel of RT-PCR product showing IFN- γ mRNA expression in the hypothalamus of control (lane 1) and tumor bearing rats (lane 2), as well as in the tumor tissue (lane 3). IFN- γ was only detected in the tumor tissue

Discussion

In this rat model of MCA-induced sarcomas, tumor development caused anorexia, coupled with increased levels of IL-1Ra and IL-1R AcP II in the liver of tumor bearing rats. The tumor itself exhibited high levels of TNF- α and IL-1 β mRNA and detectable levels of IFN γ mRNA. The localization of the IFN γ expression within the tumor tissue could be due to the expression of the cytokine by immune cells (NK cells and cytotoxic T-cells, for example) that migrated to the tumor area as well as by the sarcoma cells themselves. In addition, in tumor bearing rats, significantly increased levels of IL-1 β and IL-1RI mRNA were detected in the cerebellum, hippocampus and hypothalamus, indicating that the effects of the tumor, whether direct or indirect, were not restricted to peripheral tissues. However, unlike these cytokines, IL-1Ra and IFN γ mRNAs were not detected in the brain, nor was there any variation in the levels of IL-1R AcPs, TGF- β 1, gp 130, dynorphin, NPY, or POMC in any of the brain region examined.

The up-regulation of IL-1 β mRNA in the brain suggests the involvement of IL-1 β in the early anorexia exhibited by the sarcoma tumor cell bearing rats. Previous work has shown that IL-1 β can participate in the induction and progression of disease (including cancer) induced cachexia [Gelin et al., 1991, Plata-Salamán, 1996a, Strassmann et al., 1993]. This investigation supported an earlier study that reported upregulation of IL-1 β mRNA in peripheral organs and discrete brain regions of rats responding to a peripheral tumor [Plata-Salamán et al., 1998a]. The findings suggested that cytokines play an important role in tumor

biology. Cytokines produced by the tumors and associated immune cells may act as growth factors involved in the progression of the tumor. In contrast, cytokines released by the tumor tissue may induce local production of cytokines and/or cytokine inhibitory factors in other distal organs. Indeed, in this study, the liver of tumor bearing animals showed high levels of IL-1Ra, a cytokine with anti-inflammatory properties that can block the biological activity of IL-1 β by competitive inhibition [Ilyin et al., 1996, Plata-Salamán and Ilyin, 1997, Plata-Salamán et al., 1998b], and IL-1R AcP II, a soluble molecule with potential inhibitory activity on the IL-1 system.

Upregulation of IL-1 β and its signaling receptor IL-1RI in the cerebellum, hippocampus and hypothalamus of tumor bearing rats indicates local production of IL-1 β in the brain and the possible existence of paracrine interactions between these brain regions. TNF- α and IL-1 β have the ability to induce their own expression and these factors have synergistic activity [Turrin and Plata-Salamán, 2000]. That is, IL-1 β can induce synthesis and secretion of TNF- α and *vice versa*. However, in this study, no detectable up-regulation of TNF- α in the brain was observed. Consequently, the data suggest that the mechanism associated with the tumor progression in the periphery induced a differential modulation of cytokine immunoregulatory network in brain. The results support critical involvement of cytokines in tumor-host interactions. Previous experiments have shown that IL-1 β and its receptor are expressed by several types of peripheral [Carson et al., 1998, Ozaki et al., 2001, Plata-Salamán et al., 1998a] and central tumors [Ilyin et al., 1998b, 1999, Negri et al., 2001].

Chronic ICV administration of IL-1 β , at doses that yield estimated pathophysiological concentrations, induced anorexia and resulted in significant body weight loss [Plata-Salamán et al., 1996]. Additionally, the application of a specific IL-1 β antagonist blocked or alleviated neurological manifestations in animal tumor bearing models [Laviano et al., 2000]. While these findings may reflect the actions of cytokines on biological processes governing feeding or satiety, other possibilities cannot be dismissed. While the local expression of IL-1 β in hypothalamus may be directly related to the observed anorexia, it may also be secondary to the stress associated with the anorexia. To be sure, psychogenic and neurogenic stressors have reliably been shown to alter central cytokine expression. IL-1 β mRNA has been reported to be upregulated in the brain following inescapable shock in adrenalectomized rats [Nguyen et al., 1998], while amygdala kindling was shown to upregulate IL-1 β , TGF- β 1, and TNF- α levels in the brain of rats [Plata-Salamán et al., 2000]. This raises the possibility of a generalized response to stressors in the brain, although the mechanisms by which the brain responds to stressors of psychogenic and immune nature might differ.

While these data point to the similarity between the effects of stressors and immunogenic stimuli, it seems that stressors and cytokines may activate different regions of the amygdala. Ericsson et al. [1994] reported profound *c-fos* activation of the central amygdala in response to footshock, while systemic IL-1 β provoked the strongest effect within the medial nucleus. Little information exists concerning the role of the medial

amygdala in the response to cytokines, relative to those elicited by stressors; however, it was suggested that the medial nucleus may play a more important role than the central nucleus in the neuroendocrine response to restraint stress [Dayas et al., 1999]. Given that cytokines and stressors may contribute differentially to amygdaloid stimulation, these challenges may affect different phases of anxiety/fear responses.

Although cytokines influence amygdala neuronal activity, there is limited information concerning the anxiogenic effects of cytokine treatments. The i.c.v. administration of IL-1 β or TNF- α elicited anxiogenic-like effects, but this has been evaluated in only a limited number of situations [Connor et al., 1998]. Moreover, it is not clear that the anxiety-like effects associated with IL-1 β treatment stemmed from the central actions elicited by the cytokine, and instead may have been secondary to the malaise engendered by the treatment.

Others studies, including Study 2 within the present thesis, showed that cytokine-neuropeptide interactions are important in cytokine-induced anorexia [Sonti et al., 1996a]. The current study examined components associated with feeding: gp130, dynorphin, NPY and POMC mRNAs. None of these differed significantly in any brain region between control and tumor-bearing rats. Thus, it appears that early anorexia in the MCA-induced sarcomas model is not correlated with changes in hypothalamic mRNA for these hormone and/or signaling molecules. This contrasts with an earlier study that suggested that the concentration of NPY within the hypothalamus was lower in anorectic rats bearing MCA-induced sarcomas [Chance et al., 1994]. Since local NPY mRNA levels were measured in the

present experiment, instead of protein levels, together these two studies suggest that post-transcriptional mechanisms of modulation of the release of NPY could play a role in regulating the concentration of this neuropeptide within the hypothalamus.

Implications

In conjunction with a previous study using a rat peripheral tumor model (prostate adenocarcinoma) [Plata-Salamán et al., 1998a], the present findings suggest that different tumors will exhibit characteristic cytokine and neuropeptide profiles in the brain and in peripheral organs. Other work [Ilyin et al., 1998b] also showed different cytokine expression by the tumor tissue itself. These characteristic *in vivo* profiles could contribute to the elucidation of the specific cytokine mechanisms induced by various types of cancer in different organs, including the brain, and may be effective in defining their associated clinical manifestations. Understanding cytokine and neuropeptide expression in the brain during cancer, particularly if coupled with the array of neurotransmitter changes elicited by cytokines, may provide important information with respect to the neurological manifestations (anorexia, depression, cognitive deficits, etc.) often seen during cancer progression. Of course, the pathways governing cytokine-neurochemical processes are multidirectional, and no doubt complex. Clarification of peripheral-to-brain signaling is needed to comprehend how peripheral tumors interact with the brain to evoke the neurological, autonomic and immune responses associated with cancer pathology.

GENERAL DISCUSSION

Cytokines and their signaling components serve as essential mediators between cells during the immune response, and play a major role in the brain in the generation of disease-associated neurological manifestations. A dysregulation of the balance between pro- and anti-inflammatory cytokines is a cardinal sign of chronic immune activation and can lead to exacerbation of the symptoms involved in the disease process [Gayle et al., 1998a, Plata-Salamán et al., 1998b]. For example, the anorexia and increase in metabolism associated with cancer, if left unchecked, can lead to cachexia. Furthermore, cytokines used immunotherapeutically to treat chronic disease also induce cytokines within the brain and cause neurological manifestations similar to the disease being treated, and may consequently aggravate the outcome of treatment and lead to attrition [Lerner et al., 1999, Tomoda et al., 1997, Vial et al., 2000].

The aims of this thesis were to determine the contribution of various pro- and anti-inflammatory cytokines and neuropeptides to disease, immunotherapy and their associated neurological manifestations. Interactions between cytokines, their components and neuropeptides as well as their periphery-to-brain relationships were also studied. Finally, Neuropeptide Y was used in an attempt to counteract the anorexia associated with interferon- α immunotherapy.

The present thesis examines three models of immune activation in the brain: the acute central infusion of IFN α , the peripheral administration of LPS, and the application of MCA-induced sarcoma cells. These provided common features elicited under challenge

conditions, suggesting ubiquity across the neuroimmune responses to threats, and illustrated several distinct differences between the various treatments. Each of these challenges will first be discussed independently, and then an appraisal will be offered concerning the commonalities between the outcomes of these treatments and their general implications. The cytokine and component mRNA profiles from each of these models have been summarized in Table 1 below.

Area Model	CLL	CTX	HIP	HYP	LIV
IFNα					
TNF- α	↑	↑	↑	↑	NA
IL-1 β	↑	↑	-	↑	NA
IL-1RI	↑	-	-	-	NA
IL-1R Acp I	↑	-	-	-	NA
IL-1R Acp II	-	-	-	↑	NA
IL-1Ra	↑	↑	-	↑	NA
TGF- β 1	↑	↑	-	↑	NA
LPS					
TNF- α	-	↑	↑	-	↑
IL-1 β	↑	↑	↑	-	↑
IL-1RI	↑	↑	↑	-	↑
IL-1R Acp I	↑	↑	-	-	↑
IL-1R Acp II	↑	↑	-	-	↑
IL-1Ra	-	↑	-	-	↑
TGF- β 1	-	-	↑	-	↑
TUMOR					
TNF- α	-	NA	-	-	-
IL-1 β	↑	NA	↑	↑	-
IL-1RI	↑	NA	↑	↑	-
IL-1R Acp I	-	NA	-	-	-
IL-1R Acp II	-	NA	-	-	↑
IL-1Ra	-	NA	-	-	↑
TGF- β 1	-	NA	-	-	-

Table 1: Summary of mRNA profile in the three models studied. Arrow indicates significant upregulation of the transcript in area (CLL: cerebellum, CTX: parieto-frontal cortex, HIP: hippocampus, HYP: hypothalamus, LIV: liver) studied (Night time measurement was used for IFN α model) compared to control. NA : not applicable / measured. - : no change / not detectable.

Interferon alpha studies

Assessment of IFN α is of particular interest not only because the cytokine plays an integral role in the cascade of events associated with immune activation, but also because this cytokine has seen increasing use, alone or in conjunction with other chemotherapeutics [Miyamoto et al., 2000, Verastegui-Aviles et al., 1999], in the treatment of Hepatitis B and C, subacute sclerosing panencephalitis and malignancies (e.g., melanomas, leukemias) [Nishigushi et al., 2001, Pfeffer et al., 1998, Poynard et al., 1999, Tomodo et al., 2001]. Recent findings have also supported its use for the treatment of HIV [Brassard et al., 2002]. Despite the apparent positive outcomes derived from IFN α , this cytokine provoked profound neuropsychiatric and neurocognitive effects, including anxiety, depression, suicidal ideation, manic and psychotic symptoms [Debien et al., 2001, Hurlock, 2001, Menkes and MacDonald, 2000, Meyers et al., 1991, Vial et al., 2000]. Indeed, the symptoms may be sufficiently severe to require discontinuation of therapy [Lerner et al., 1999, Tomoda et al., 1997, Vial et al., 2000]. Surprisingly, despite these psychological effects, very little information has been reported concerning the brain changes associated with this cytokine. Studies have reported that IFN α is able to act in brain by direct modulation of the activity of neurons in the hypothalamus, amygdala, thalamus, hippocampus and somatosensory cortex [Dafny et al., 1996, Mendoza-Fernández et al., 2000, Reyes-Vázquez et al., 1994, 1997, Saphier et al., 1994, Saphier, 1995]. In the hypothalamus, neurons modulated by IFN α include gluco-sensitive neurons [Reyes-Vázquez et al., 1997], as well as CRH-secreting

neurons of the PVN [Saphier, 1995], which could relate to the feeding and neuroendocrine effects associated with the actions of IFN α , respectively. Furthermore, a single central administration of IFN α was able to modulate the brain levels of monoamines, decreasing the expression of serotonin and noradrenalin in the frontal cortex [Kamata et al., 2000]. Peripheral administration of the cytokine is also able to modulate dopaminergic activity in the brain, but repeated administration was needed to engender such an outcome [Shuto et al., 1997]. These effects of interferons on central monoamine function have been proposed to be related to the neurotoxic effects associated with its use in therapy [Kamata et al., 2000, Menkes and MacDonald, 2000, Shuto et al., 1997].

Analysis of the effects of IFN α on cytokine expression in cell culture and in plasma, indicated that the cytokine could up-regulate the activity of pro-inflammatory cytokines (e.g., IL-1 β and TNF- α), anti-inflammatory cytokines (IL-1Ra), as well as their associated components [for review, see Brassard et al., 2002, Taylor and Grossberg, 1998]. However, the effects of interferon on brain cytokine levels have not been reported. The results of the present investigation are the first to show changes in the mRNA levels of cytokines and their components in the brain and periphery. IL-1 β and TNF- α , pro-inflammatory cytokines, were up-regulated in the brain following IFN α treatment. Given the previously demonstrated effects of these cytokines on neuronal functioning, it is possible that IFN α provokes neurological and psychiatric disturbances by influencing the firing rate of neurons in areas of the brain involved in mood, feeding, memory and cognition. In this respect, IFN α may influence monoamine

activity, as well as other neurotransmitter systems, hence influencing mood and motivational processes. Alternatively, it may act indirectly, affecting these systems by stimulating other neurologically active cytokines, such as IL-1 β and TNF- α .

In addition to stimulation proinflammatory cytokine activity, it appeared that the central infusion of IFN α also up-regulated the levels of anti-inflammatory cytokines IL-1Ra and TGF- β 1. This would suggest that in response to IFN α the pro- and anti-inflammatory cytokine balance is maintained within the brain. Other models of central immune challenge studied previously [Gayle et al., Ilyin et al., 1998a, Plata-Salamán et al., 1998b] demonstrated that a relationship exists between the magnitude of the anorexia and the pro- and anti-inflammatory cytokine balance in the brain: the central administration of LPS, which was associated with severe anorexia, showed a clear shift toward the expression of pro-inflammatory cytokines, whereas, MDP, associated with milder anorexia, did not provoke such a shift.

The pleiotropic effects of IFN α on the brain could have implications regarding the management of its neurotoxic consequences. For example, the use of anti-inflammatory drugs to control the pro-inflammatory effects of IFN α on cytokine expression in the brain, could prevent the impact otherwise provoked by IL-1 β and TNF- α activation. However, such treatment would not be successful in attenuating the direct effect of the cytokine on neuronal firing, and could impede its therapeutic effects. Interestingly, the current study showed that IFN α does not appear to modulate dynorphin, gp 130, POMC or NPY levels in the brain. This suggests some degree of specificity in the action of IFN α , hence permitting the

opportunity to regulate some of the consequences of IFN α therapy (e.g., mood disturbances and anorexia) through the independent regulation of these neuropeptide systems. This possibility was explored in Study 2.

The results of the second study showed that NPY was able to counteract the anorexigenic effects of centrally infused IFN α . Since the anorexia reappeared following the initial orexigenic actions of NPY, it seems that the positive attributes of the treatment persisted only so long as the peptide was present and active. This has implications for interferon- α based immunotherapy; in that the short-term feeding effects of NPY could permit a customized treatment to counteract the IFN α -induced anorexia and enable the patient to feel motivated to eat only when it would be appropriate.

Some of the neurotoxic effects related to IFN α occur only after prolonged treatment with the cytokine [Shuto et al., 1997, Vial et al., 2000] and synergize with some of the neurological consequences associated with the disease being treated [Kurzrock, 2001, Valentine and Meyer, 2001]. In order to assess whether and how NPY facilitates IFN α immunotherapy, it would be advantageous to apply repeated IFN α using a viral infection or tumor model (such as the one from Study 4). Such an investigation may be useful in clarifying the interaction between the IFN α treatment and the chronic disease in promoting neurological manifestations and brain cytokine profiles. Furthermore, the ability of repeated NPY to counteract some of the neurological manifestations could be assessed to determine whether any tolerance or sensitization to the neuropeptide develops.

In addition to its orexigenic properties, NPY is known to induce anxiolytic effects [Antonijevic et al, 2000, Ehlers et al., 1997, Kask et al., 2002], possibly through actions on CRH [Britton et al., 2000] Inasmuch as cytokines may induce anxiety, as described earlier, anxiolytic properties of NPY would be of obvious therapeutic value. At this juncture it would seem propitious to assess the anxiolytic effects associated with NPY in conjunction with IFN α treatment, particularly as IFN α acts on CRH neurons [Saphier, 1995]. In effect, it is possible that NPY could have effects in suppressing some of the mood disturbances associated with IFN α through CRH actions. Clearly, if effective, NPY could improve patient's mood, and consequently lead to more positive outcomes from immune therapy.

Disease models of cytokine action

Cytokine mRNA profiles following LPS administration

The administration of endotoxin, such as LPS, is well established as an acute model of inflammation. Furthermore, the peripheral administration of LPS had been shown to modulate cytokines both in the brain and periphery. For instance, Jacobs et al. [1997] reported the upregulation of IL-1 β mRNA in the hypothalamus following LPS administration. Likewise, Kakizaki et al. [1999] detected higher levels of IL-1 β and TNF- α proteins in the plasma and higher levels of TNF- α in the PVN of rats up to 2 and a half hours following both intraperitoneal and intravenous LPS administration. Similar results in TNF- α and IL-1 β expression following LPS were reported in other studies [Pitossi et al., 1997, Satta et al., 1998]. However, none of these simultaneously assayed the contribution of multiple cytokine systems

(pro- and anti-inflammatory) and their associated components in specific brain regions and peripheral organs. Study 3 was conducted in order to understand the contribution of these cytokines and the related components to the peripheral-to-brain immune communication that occurs during a bacterial insult, and to clarify their role in the neurological manifestations associated with this immune challenge.

In Study 3, it was observed that 5 hr after the systemic administration of LPS, IL-1 β and TNF- α mRNA were up-regulated in multiple brain regions and peripheral organs (liver, spleen, adipose tissue). At this time TNF- α protein was also found to be elevated in the sera of LPS-treated rats. In contrast, the anti-inflammatory cytokines, IL-1Ra and TGF- β 1, showed significant up-regulation only in the cerebral cortex and hippocampus, respectively, and in the peripheral organs. As mentioned earlier, a shift of balance toward the pro-inflammatory cytokines could be indicative of more severe neurological consequences due to the lack of modulation by their anti-inflammatory counterparts. Furthermore, the rats injected with LPS showed an up-regulation of some of the IL-1 signaling components studied (IL-1RI, IL-1 AcP I, and II) both in the brain and in the peripheral organs, demonstrating the ability of strong peripheral immune challenges to communicate with the brain and not only be able to regulate cytokine expression, but also that of its signaling molecules. However, as observed in Study 1, none of the mRNA levels from neuropeptide components studied were affected by the endotoxin, further supporting the specificity of the cytokine response.

The model of peripheral LPS challenge yielded a similar cytokine profile to the one observed during central infusion of the bacterial endotoxin, although the induction of the pro-

inflammatory cytokines by the peripheral challenge was milder than that seen in the central model [Gayle et al., 1998a, Plata-Salamán, 1998b]. This suggests that LPS has direct actions within the CNS, without necessitating the recruitment of peripheral immune signals. Therefore, irrespective of whether it results from the direct action (ICV infusion) of LPS or of the cascade of events (neural and/or humoral) that follows its peripheral administration, the net consequence of an LPS challenge leads to pro-inflammatory cytokine induction within the brain, and similar neurological manifestations.

Although, this study determined the cytokine profile following LPS challenge both in the brain and periphery, the pathway by which the immune signal reaches the brain is still unresolved. However, several possibilities can be proposed in this respect. It will be recalled that cytokines are relatively large molecules that do not readily enter the brain. However, they may gain access at restricted sites and thus have direct actions on CNS processes. In particular, IL-1 β [Banks et al., 1989] and TNF- α [Gutierrez et al., 1993] have saturable carrier mediated transport mechanisms, which pump these cytokines into the brain. Further, entry into the brain may occur at circumventricular areas, which lack an efficient BBB [Banks et al., 1989, Quagliarello et al., 1991], ultimately reaching various brain nuclei through a process of volume diffusion [Konsman et al., 2000, Laflamme and Rivest, 1999, Lee et al., 1998]. Interestingly, IL- β and TNF- α themselves may disrupt the BBB [Quagliarello et al., 1991], thereby increasing accessibility of the CNS. Parenthetically, the view was also expressed that cytokines may affect the BBB by the induction of adhesion molecules, such as ICAM-1 and VCAM-1 in the brain endothelium and astrocytes, which may guide

inflammatory leukocytes into the brain parenchyma [Merill and Benveniste, 1996].

In response to systemic insults, cytokines and other immune signals (such as endotoxin) can bind to the endothelium of the brain microvasculature, which will in turn produce intermediate signaling mediators, such as NF- κ B and cyclooxygenase-2 [Laflamme and Rivest, 2001, Rivest et al., 2000]. These mediators may be responsible for the synthesis of cytokines and perpetuation of the immune response within the brain.

In addition to direct actions, cytokines may influence CNS processes indirectly through stimulation of afferent fibers of the vagus nerve [Dantzer et al., 1996, Maier and Watkins, 1998, Watkins et al., 1995]. Receptors for IL-1 are present on the nodose ganglion which sends afferent projections to the brainstem NTS, and following cytokine or endotoxin challenge, *c-fos* expression is elevated in these regions [Ek et al., 1998, Gaykema et al., 1998]. It appears that activation of vagal branches may stimulate the *de novo* synthesis of cytokines within brainstem and hypothalamic nuclei [Dantzer et al., 2001, Gaballec et al., 1995, Hopkins and Rothwell, 1995].

In contrast to IL-1 β and TNF- α , the available data concerning IFN α access to the brain are limited. As discussed earlier, whether the cytokine is applied peripherally and centrally, many of the neurological effects induced are similar, suggesting some common routes of action between systemic and central administration. Owing to its ability to induce an early activation of immune cells [Brassard et al., 2002], IFN α could regulate brain cytokine synthesis indirectly through its effects on macrophages, NK cells and T-cells that subsequently gain access to the CNS. In addition, Study 1 of this thesis demonstrated the ability of IFN α to directly act within

the brain to cause up-regulation of pro-inflammatory cytokine synthesis, suggesting the possibility that it passes into the brain, possibly at areas where the BBB is compromised or absent. Alternatively, as IFN α shares common signal transduction mechanisms (the JAK / STAT signaling molecules, for example) that are used by other cytokines and neuroimmune signals [Brassard et al., 2002, Prejean and Colamonici, 2000], it could bind to the cells of the BBB and microvasculature causing the synthesis of these cytokines and neuroimmune signals within the brain.

Cytokine regulation and action in the brain of tumor-bearing rats

The first two models described, central infusion of IFN α and the systemic injection of LPS, document the consequences of acute immune challenges on cytokine expression in the brain and periphery. However, most pathological threats and immune therapy regimens generate or involve a chronic state of cytokine production, which could be related to ensuing neurological manifestations. For example, the progression of a tumor will be accompanied by sustained, elevated cytokine production (by the host and the tumor) and the increased occurrence of anorexia / cachexia, mood, and cognitive disturbances [Negri et al., 2001, Plata-Salamán, 2000a, Valentine and Meyers, 2001]. These long-term psychological consequences of immune activation are proposed to be associated with a shift in balance between pro- and anti-inflammatory cytokines, favoring the presence of pro-inflammatory signals [Kurzrock, 2001, Plata-Salamán, 2000a]. Study 4 of this thesis sought to measure expression of cytokines and associated products in the brain, tumor and liver of rats bearing MCA-induced sarcoma cells. This study was conducted to further understand the role of pro- and anti-inflammatory

cytokines, and neuropeptides in a chronic model of immune activation and its associated anorexia.

In Study 4, the rats bearing the MCA-induced sarcoma cells developed significant anorexia and showed distinctive cytokine mRNA profile in the brain, liver and tumor tissue itself. In this respect, IL-1 β and its signaling receptor mRNAs were up-regulated in several brain regions of the tumor-bearing rats, a finding consistent with the effects of IL-1 β in several tumor models. Earlier peripheral models using rats bearing prostate adenocarcinoma cells [Plata-Salamán et al., 1998a] or ovarian carcinoma cells [Carson et al., 1998], as well as mice bearing Ehrlich's ascites tumor cells [Ozaki et al., 2001] all showed up-regulation of IL-1 β mRNA within the brain of the animals. Central tumor models involving the infusion of C6 glioma cells [Ilyin et al., 1999] and human carcinoma and glioma cells [Negri et al., 2001] into the CNS of rodents also demonstrated the induction of IL-1 β mRNA, supporting an ubiquitous response of IL-1 β in the brain during tumor progression. The absence of IL-1Ra expression or TGF- β 1 upregulation within the brain suggests a shift toward the pro-inflammatory cytokines within the brain, which as mentioned earlier, could relate to the magnitude of the neurological manifestations associated with this tumor model. Taken together, this brain cytokine mRNA profile supports the ability of the tumor and its associated immune cell mass to modulate central cytokine expression through endocrine and/or neural signaling. Consequently, this neuroimmune activated central state could direct the systemic response to the tumor, although evidence supporting direct implication of the pro-inflammatory cytokine in this regard is still lacking [Cravo et al., 2000].

The tumor tissue exhibited high levels of TNF- α and IL-1 β , and detectable levels of IFN γ , while IL-1Ra and IL-1R AcP II mRNA levels were elevated in the liver of the tumor-bearing rats. This might suggest that immune cells have migrated to the tumor site, causing the increased release of the pro-inflammatory cytokines, and supports the possibility that the tumor also releases cytokines [Csiszar et al., 2001, Erroi et al., 1989]. Interestingly, neither IL-1 β nor TNF- α was detected in the liver of the tumor-bearing rats. The presence of IL-1Ra and IL-1R AcP II mRNA expression within the organ could reflect suppression of the IL-1 β response by an antagonist and and/or by an inhibitory molecule, possibly in an effort by the system to contain the inflammatory response to the site of the tumor, thereby minimizing systemic toxicity. A model of a peripheral tumor in mice showed a similar imbalance [Ozaki et al., 2001], wherein the appearance of pro-inflammatory cytokine mRNA in the liver occurred only 28 days after the implantation of tumor cells. The presence of pro-inflammatory cytokine mRNA at a later time (36 days) is also supported by another peripheral tumor model in the rat [Plata-Salamán et al., 1998a]. Taken together, these studies suggest that there is a drive by the immune system to keep the response to the tumor localized; however, as the tumor and its associated immune mass grows, there may be a gradual systemic perpetuation of the cytokine response through paracrine and endocrine signaling, leading to chronic cytokine activation in the brain and periphery. Such a cytokine activation has been related to the physiological (e.g. cachexia) and psychological (e.g. mood disorders) states associated with tumor progression [Cravo et al., 2000, Kurzrock, Valentine and Meyers, 2001].

The cognitive and motor deficits associated with cancer include decreased performance in verbal and visuo-spatial memory tasks, impaired frontal lobe executive functions, and poor motor coordination [Ciesielski et al., 1994, Cole et al., 2000, Dropcho, 2002, Juntunen et al., 1997, Kingma et al., 2001, Meyers et al., 1995, Stubgen, 1995, Younger, 2000]. These are accompanied by irreversible metabolic changes affecting glucose, lipid and protein synthesis and utilization [Cravo et al., 2000]. It still remains to be clarified whether the upregulation of cytokines within discrete brain regions are responsible for the neurological, physiological and psychological disturbances. The physiological and neuroendocrine role of pro-inflammatory cytokine up-regulation in the hypothalamus has already been discussed. Furthermore, increased levels of IL-1 β observed in the hippocampus may play a role in supporting some of the cognitive deficits observed in cancer patients [Lynch, 2002, Matsumoto et al., 2001], while elevated cerebellar IL-1 β could relate to motor dysfunctions associated with cancer progression [Vernet-der Garabedian et al., 1998]. To be sure, this does not exclude the role of other mechanisms that could contribute in this respect, but suggests that cytokines may either play a role in influencing these systems or may be a marker of abnormal functioning within these distinct regions during tumor progression.

Cytokine signaling associated with diverse challenges

Overall, the present findings indicated that the pro-inflammatory cytokines are upregulated in several of the brain regions studied in response to IFN α treatment, LPS as well as in MCA-induced tumor-bearing rats. Despite the differences with respect to the site of inoculation, the duration of treatment, and the nature of the challenge, the three models of

cytokine activation showed striking similarities in their effects on brain cytokine mRNA profiles. They all displayed upregulation of pro-inflammatory cytokine mRNAs, while showing some conservation of their signaling components and neuropeptides mRNAs. Thus, it is likely that convergence of their neuro-immune signal exists. The cytokine IFN α shares common signaling mechanisms as other pro-inflammatory cytokines [Prejean and Colamonici, 2000] and activates immune cells known to produce or cause the production of these cytokines [Brassard et al., 2002]. The endotoxin, LPS, is thought to bind to the serum LPS binding protein and may communicate with CNS processes via Toll-like receptors associated with CD14, both found on the surface of cells making up the CVOs [Laflamme and Rivest, 2001]. These transduction events would ultimately lead to NF- κ B activation and pro-inflammatory cytokine production in the brain parenchyma [Laflamme and Rivest, 2001]. The MCA-induced sarcoma cells, for their part, could cause the transcription of brain cytokines through the release of cytokines and other neuroendocrine signals, such as prostaglandins, into the bloodstream that would reach the brain through mechanisms described earlier in this discussion. These signals could be released by the tumor cells and/or the immune cells invading the tumor site [Csiszar et al., 2001, Erroi et al., 1989].

The possibility that neural afferents contribute to the periphery-to-brain communication [Dantzer, 2001] in the LPS and tumor models is certainly not dismissed. Furthermore, the recruitment of other peripheral effectors such as acute phase proteins [Johnson et al., 1999], circulating immune cells, and the effect of a systemic immune response on the release of neuroendocrine signals from other organs (e.g., liver, pancreas, spleen) [Cravo

et al., Wright et al., 2000] may also play a neuromodulatory role in brain cytokine functioning. In fact, the contribution of such signals could explain the difference in the magnitude of cytokine mRNA induction seen between the central (IFN α) and the two peripheral (LPS and tumor) models studied in this thesis; the strongest induction of cytokines in the brain was observed in the LPS model, where there was up to a 16-fold induction of the pro-inflammatory cytokines within some brain regions, whereas in the other two models, the induction rarely exceeded a 2-3 fold induction compared to their controls. However, these effects may be dependent on the dose and route of administration of the challenges. The different mRNA profiles observed between the LPS and tumor models also exemplify the nature of the challenge involved. In particular, LPS administration triggers an acute phase response, which is present during the early, non-specific stage of a response to a pathogen and is accompanied by a severe inflammatory that focuses on minimizing the invasion of the pathogen. In contrast, the neuroimmune response to the tumor involves a long term adaptive immune response, comprising the mobilization of specific immune cells to the tumor site and thus, spares the rest of the host from chronic inflammation. This is supported by up-regulation in anti-inflammatory signals and the absence of pro-inflammatory signals observed in the chronic tumor model (Study 4).

Protracted effects of cytokine activation

The present investigation focused not only on the immediate effects observed following a challenge (IFN α and LPS), but also the cytokine variations that occur while the challenge is

ongoing (tumor progression). It is understood, however, that some of the effects provoked by acute or prolonged cytokine activation can engender consequences that span days and even weeks. For example, IL-1 β and TNF- α provoke the sensitization of HPA activity such that later reexposure to the cytokine resulted in augmented neuropeptide and hormonal activity in a time-dependent manner [Hayley et al., 1999, 2001b, Schmidt et al., 1995]. In particular, while the cytokines increased the co-localization of CRH and AVP within the median eminence, this outcome only became apparent 4 days after IL-1 β administration and peaked 1-2 weeks following the treatment [Schmidt et al., 1995]. A second administration of IL-1 β (eleven days after the initial challenge) elevated plasma ACTH and corticosterone levels [Schmidt et al., 1995], suggesting that the cytokine provoked a functionally hyper-responsive HPA axis.

Systemic administration of TNF- α likewise induced a time-dependent sensitization of HPA activity. However, the temporal profile of induction of the hypothalamic peptide immunoreactivity did not parallel the expression of the corticosterone sensitization. Specifically, while median eminence CRH and AVP immunoreactivities were maximally elevated 7-14 days following the initial TNF- α injection, the neuropeptide expression was comparable to baseline by 28 days [Hayley et al., 2001b]. However, reexposure to TNF- α 28 days following initial administration of the cytokine provoked a pronounced sensitization of corticosterone release, an effect that was absent at earlier reexposure intervals (1, 7 and 14 days) [Hayley et al., 1999]. Thus, factors in addition to hypothalamic CRH and AVP may influence the TNF- α -induced hormonal sensitization. For instance, the cytokine may have had direct actions upon the adrenal or pituitary gland, both of which contain high TNF- α

receptor densities [Kobayashi et al., 1997]. Likewise, other peripherally acting inflammatory factors (e.g. histamine) may mediate some of the sensitizing effects of TNF- α . Indeed, it was recently observed that systemic antihistamine treatment (H1 and H2 antagonists) ameliorated both the corticosterone and sickness sensitization effects of TNF- α [Kelly et al., 2001]. As well, in animals treated with TNF- α , later intracerebroventricular reexposure to the cytokine did not sensitize HPA activity, irrespective of whether the initial injection was centrally or peripherally administered, suggesting that peripheral factors and/or targets are involved [Hayley et al., 2002].

It is important to note that mice displaying the TNF- α induced HPA sensitization, like the treated rats in the present investigation, also showed signs of marked illness (e.g. ptosis, piloerection, lethargy, cyanosis of the extremities) [Hayley et al., 2001a]. Although it is tempting to speculate that stress or other factors associated with the illness may be related to the provocation of the corticosterone sensitization, it was observed that the two processes were independent of one another [Hayley et al., 2001a].

As mentioned earlier, the administration of IFN α , both centrally and systemically, elicits several behavioral changes that include lethargy, hypersomnia, mood and cognitive disturbances. Although most of these symptoms abate following the cessation of IFN α , interferon neurotoxicity may be irreversible, especially following chronic, high dose therapy [Greenberg et al., 2000, Seno et al., 1999, Valentine et al., 1998]. Bipolar and manic syndromes may appear with pauses in IFN treatment for melanomas and are thought to occur up to 6 months following the end of treatment [Greenberg et al., 2000]. Furthermore,

Meyers, Scheibel and Forman [1991] reported deficits resembling frontal-subcortical dysfunctions (e.g. rigidity, tremor) and neuropsychological disturbances (e.g. fatigue, depression, anxiety) that were present up to 3 years following the end of the IFN α treatment. Inasmuch as these symptoms were not an immediate effect of the immunotherapy raises the possibility that the development of these syndromes involves processes akin to the sensitization effects described with respect to IL-1 β and TNF- α . At this point, however, data are unavailable as to whether IFN induces a comparable sensitization effect.

It may be significant that IFN expression occurs as an early response to infection and precedes the majority of other inflammatory cytokines [Brassard et al., 2002], raising the possibility that it could develop as a priming stimulus during the initial immune response to a challenge [Biron 1998, von Hoegen, 1995]. In fact, such a priming effect was demonstrated when a low-dose IFN- α treatment preceding viral, endotoxin, or poly(I:C) resulted in increased protection from the challenges [Beilharz et al., 1997, Havell and Spitalny, 1983], indicating that the interferon was able to increase immune responsivity. It remains to be established whether this immune priming also results in enhanced neurological manifestations associated with IFN α , but priming does offer the opportunity for IFN α to act in synergy with other early cytokines, such as IL-2, that influence CNS functioning.

Although the mechanism by which IFN α causes the long-term effects is still not clearly understood, it is possible that IFN α causes neurotoxicity through its effect on the neuroendocrine, neurotransmitter and/or cytokine systems [Valentine et al., 1998]. The

interferon is thought to cause perturbations of the HPA [Saphier et al., 1994] and hypothalamic-pituitary-thyroid axis [Jones et al., 1998] that are similar to those observed in depression [McDaniel and Nemeroff, 1993, Nemeroff et al., 1992]. Furthermore, IFN α can act through the opioid system to cause symptoms such as catatonia and behavioral slowing, which can be reversed through the use of opioid antagonists [Birmanns et al., 1990, Blalock and Smith, 1981]. This suggests that opioid-related IFN α functions could also mediate some long-term effects of the cytokines.

Clearly, the suggestion that IFN induces effects on mood states through a sensitization of neurochemical functioning is highly speculative, and indeed alternative explanations are available. For instance, IFN α may act as a dopamine agonist through activation of opioid mechanisms [Ho et al., 1992]. However, the neurotoxicity associated with the cytokine (e.g. psychomotor slowing, cognitive disturbances) is more consistent with dopamine antagonism. Nevertheless, long-term activation of the dopaminergic system through chronic treatment using IFN α could result in dopamine depletion, receptor downregulation and subsensitivity, leading to effects similar to those elicited by a dopamine antagonist. Furthermore, patients treated with IFN α also exhibit alterations in peripheral adrenergic receptors and serum tryptophan levels, leading to speculations that IFN α induced serotonin depletion and noradrenergic dysfunction could be associated with the depressive and cognitive symptoms evident with long-term cytokine treatment [Brown et al., 1991, Vergassola et al., 1990].

The indirect effects of IL-1 β , IL-2 and TNF- α that can be induced by IFN α could

also lead to the neuropsychiatric complications observed in long-term treatment involving the cytokine. As will be described shortly, multiple sources of evidence have, indeed, supported the view that cytokines may play a provocative role in the evolution of depressive illness. Certainly, no single line of research has provided conclusive evidence for such a link, but together this body of work provides a *prima facie* case implicating immune activation in the provocation of depression. Major depression in humans, particularly in patients presenting with severe melancholic illness, has been associated with variations of immune functioning, including a reduction of mitogen-stimulated lymphocyte proliferation and reduced NK cell activity [Herbert and Cohen, 1993, Irwin, 1999, Maes 1995, 1999]. Contrary to the position that depression was associated with the suppression of nonspecific immunity, the view was advanced that affective disturbances may be secondary to activation of the inflammatory immune response [Licinio and Wong, 1999, Maes, 1995, 1999]. In this respect, depressed patients were found to present with signs of immune activation, reminiscent of an acute phase response, including increased plasma concentrations of complement proteins, C3 and C4, IgM, and positive acute phase proteins, haptoglobin, α 1-antitrypsin, α 1 and α 2 macroglobulin, whereas negative acute phase proteins were reduced [Maes, 1999, Sluzewska, 1999]. Further, major depressive illness was accompanied by elevated activated T cells ($CD25^+$ and $HLA-DR^+$), secretion of neopterin, prostaglandin E2 and thromboxane [Maes, 1995, 1999].

Commensurate with the view that cytokines are fundamental in depression, it was reported that severe affective illness was accompanied by elevated circulating cytokines or

their soluble receptors, including IL-2, soluble IL-2 receptors (sIL-2R), IL-1 β , IL-1 receptor antagonist IL-1Ra, IL-6, soluble IL-6 receptor sIL-6R, and IFN γ [Berk et al, 1997, Frommberger et al., 1997, Maes, 1995, 1999, Mullar and Ackenheil, 1998, Nassberger and Traskman-Bendz, 1993, Sluzewska et al., 1995, Smith, 1991] as well as increased production of IL-1 β , IL-6 and TNF- α in response to mitogen challenge, [Anisman et al., 1999a,b, Maes, 1995, 1999]. Interestingly, with alleviation of depression in response to antidepressant treatment, normalization was evident with respect to levels of IL-1 β , IL-6 and α 1-acid glycoprotein [Frommberger et al., 1997, Sluzewska et al., 1995], whereas no such changes were apparent concerning the upregulated production of sIL-2R, IL-6 and sIL-6R in major depression [Maes, 1999]. Likewise, antidepressant treatment did not diminish the elevated serum levels of the IL-6, and that of anti-inflammatory cytokines, IL-10 and IL-1Ra [Kubera et al., 2000]. In effect, these cytokines may potentially act simply as trait markers of the illness, [Anisman et al., 1999b, Maes 1999], although it ought to be considered that sustained treatment may be necessary to achieve normalization of cytokine functioning.

The aforementioned studies, in the main, are correlational, and thus it cannot be determined whether the cytokine elevations are secondary to the illness (i.e., being directly or indirectly brought on by the depression), or contribute to the provocation of the disorder. Yet, it has been reported that in humans undergoing immunotherapy high doses of IL-2, IFN α and TNF- α induce neuropsychiatric symptoms, including depression. As such, these data imply that the affective changes were related to the cytokine treatment [Caraceni et al., 1992, Denicoff et al., 1987, Maes et al., 2001, Meyers and Valentine, 1995]. Importantly, it

was reported [Musselman et al., 2001] that depressive symptoms provoked by IFN α were attenuated by treatment with the selective serotonin reuptake inhibitor, paroxetine. Together, these findings provide good reason to suspect an etiological role for cytokines in depressive illness. Yet, it is important to consider that the populations being assessed in the latter studies were undergoing considerable strain (e.g., the distress regarding their illness), and hence depression may reflect the interactive effects of the distress and the cytokine treatments.

Implications

It seems probable that understanding the brain cytokine profile associated with immunotherapy and disease may be useful in clarifying the role that individual cytokines play in the pathophysiological symptoms and neurological manifestations associated with such states of cytokine activation. By including a concurrent analysis of multiple cytokines, neuropeptides, and other related molecules, these studies showed the existence of possible interactions between these various components. For example, during ICV IFN α administration, the temporal relationship between IL-1 β and TNF- α was supported by the later up-regulation of IL-1 β compared to that of TNF- α . Additionally, this multi-component analysis suggests the presence of modulatory mechanisms that could exert negative feedback on the pro-inflammatory cytokine system. Three of the studies showed the up-regulation of inhibitory cytokines, IL-1Ra and TGF- β 1, concomitant with that of the pro-inflammatory cytokines IL-1 β and TNF- α . This indicates that there are

immunoregulatory systems in place within the brain which may serve to moderate the overstimulation caused by the neuroinflammatory response. However, as mentioned earlier, it also appears likely that an imbalance between pro- and anti-inflammatory cytokines usually exists during pathology. Therefore, understanding the dynamics between these opposing systems could suggest possible pharmaceutical strategies to be used in countering this imbalance.

Although these studies, like others, may be useful in disentangling the role that each cytokine plays in the nervous system, their precise involvement during neurological/neuropathological processes is, of course, highly complex. For example, studies using cytokine knockout models have led to confounding results, possibly owing to the redundancy encountered among the function of cytokines. Furthermore, the relationship between the expression of cytokines by a cell, their release, and binding to the same cell or other cells is not fully understood. Since cytokines undergo some post-translational modifications and have short-half lives, their expression does not necessarily mean that they will act outside of the cell in which they were induced. Conversely, since they can trigger secondary cytokine pathways, their absence from a specific area does not indicate that they have had no effect at that site or that they did not have an earlier influence on the cytokine profile within that area. Therefore, it is necessary to focus on using time series analyses to understand the sequence of the cytokine cascades within different areas and regions of the brain. Finally, studying the secondary messenger systems (e.g., NF- κ B, MAPKs) and receptor dynamics (regional expression and down-regulation) involved in the regulation of

cytokine expression and release could also lead to a better understanding of the brain cell cytokine behavior.

The goal of dissociating the underlying molecular mechanisms of cytokines in the brain may contribute to the understanding of how they relate to neurology and behavior. Cytokine imbalance is thought to be involved in the dysregulation of feeding, drinking, thermoregulation, sleep, mood and cognition. Identifying the correspondence between the cytokine actions and the specific behavioral/cognitive disturbances may be fundamental in identifying targets for interventions to treat disease, and may prove useful in facilitating and optimizing treatment efficacy by ameliorating the adverse side effects of the treatments. Clearly, further research on the role of cytokines in the CNS should, among other things, emphasize integrative strategies including molecular, cellular, neural network, behavioral as well as neurological/neuropsychiatric aspects of cytokine action in the brain.

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