The Effects of Brain Infusion of Human Immunodeficiency Virus Glycoprotein (gp120) and Peripheral LPS on Splenic Macrophage IL-1β and TNFα and Splenic Sympathetic Nerve Activity

by

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THE EFFECTS OF BRAIN INFUSION OF HUMAN IMMUNODEFICIENCY VIRUS GLYCOPROTEIN (gp120) and PERIPHERAL LPS ON SPLENIC MACROPHAGE IL-1β and TNFα AND SPLENIC SYMPATHETIC NERVE ACTIVITY

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ABSTRACT

It has been shown previously that immune cells produce cytokines such as interleukin-1β (IL-1ß) and tumor necrosis factor α (TNFα) which can signal the central nervous system and result in immune suppression. It has been demonstrated in our lab that an immune challenge, bacterial lipopolysaccharide (LPS) or endotoxin, can activate central autonomic regulatory regions and pathways, and increase sympathetic input to the spleen. In addition, splenic macrophage function has been shown to be regulated by the splenic nerve, and that the sympathetic signal is tonically immunosuppressive and further suppression can be centrally induced. Our previous data indicated that the HIV-1 coat protein, gp120, may disrupt the neural-immune regulatory circuit and our present hypothesis is that gp120 produces autonomic dysregulation which may result in a cytokine environment in the spleen that enhances HIV replication in early infection. gp120, infused into brain ventricles, has been shown to enhance splenic macrophage function, as measured by macrophage cytokine production after LPS stimulation in vitro, rather than suppressing function as do intracerebral ventricular cytokines. However, similar to cytokines or LPS, gp120 does elevate plasma corticosterone. In addition, gp120, coinfused with α-msh, attenuated the increase in splenic NE turnover produced by α-MSH, which is thought to play a role in the sympathetic down regulatory mechanism. Yet, gp120 had no effect on NE turnover when gp120 was infused alone. This data suggested that gp120 may disrupt sympathetic inhibition of splenic macrophage activity. The present study sought to observe in vivo the effect of central gp120 (4μg icv) on LPS ("low dose" 0.1μg iv) stimulated splenic cytokine mRNA production via Northern Blot

analysis of the splenic cytokine mRNAs in rats. Immunocytochemistry was also used to examine TNFα protein in vivo. It was expected that a small peripheral dose of LPS would result in increased cytokine mRNA expression and that central gp120 would significantly enhance this expression. The hypothesis was confirmed as gp120 did significantly increase the levels of both TNFα ($p<.05$) and IL-1β ($p<.025$) mRNAs. This enhancement is consistent with our previous findings that central gp120 significantly increased splenic macrophage production of cytokines after low dose LPS stimulation in vitro. TNFα protein was not significantly elevated but a trend was apparent and the levels were significantly correlated with the mRNA levels ($p<.0001$). As seen previously, plasma corticosterone levels were significantly elevated ($p<.0001$) following central gp120 and low dose LPS relative to vehicle injected controls, but there was no difference in plasma catacholamines, suggesting no change in sympathetic activity. The present study also sought to examine the effect of centrally infused gp120 on the LPS ("high dose" 100μg iv) induced increase in splenic nerve electrical activity in adult male rats. It was predicted that gp120 would attenuate the LPS induced increase in splenic nerve activity. With regard to this latter hypothesis, no inhibition was observed under the conditions of the present experiment. Although gp120 did not attenuate the increase in sympathetic nerve activity induced by intravenous LPS, this does not directly conflict with the ability of gp120 to block the increase in NE turnover produced by central α-MSH injection. Overall, these data provide further evidence that gp120 acts in the brain to alter the splenic cytokine environment by attenuating the suppressive signal provided by the sympathetic nervous system.

CONTENTS

Figures

INTRODUCTION

In the 15 years since the recognition of the bi-directional communication between the brain and the immune system, our attention has been focused on central nervous system (CNS) modulation of splenic immune function by the splenic sympathetic nerve. Sympathetic innervation of lymphatic organs, including the spleen, is well documented (Felten, Felten, Bellinger, Carlson, Ackerman, Madden, Olschowka and Livnat, 1987; Nance and Burns, 1989) and lymphocytes express receptors for the sympathetic transmitter, norepinephrine (NE), and they respond to transmitter binding (Roszman and Carlson, 1991). It has been shown that the brain possesses receptors for cytokines released by activated immune cells, and that cytokines may act as neuromodulators (Farrar, Killian, Ruff, Hill and Pert, 1987). It appears that the CNS, signalled by products of immune cells, provides a restraining influence on the immune response through the hypothalamic-pituitary-adrenal axis (HPA) as well as the sympathetic nervous system (Besedovsky, del Rey, Sorkin, Da Prada and Keller, 1979; Brown, et al.,1991; Madden and Livnat, 1991; Sundar, Cierpial, Kitts, Ritchie and Weiss, 1990). Interleukin-1ß (IL-1β) and tumor necrosis factor α (TNFα), cytokines primarily produced by macrophages, act as neuromodulators and have been shown to have a number of CNS effects (Besedovsky, del Rey, Sorkin and Dinarello, 1986; Breder, Dinarelle & Sapier, 1988; Dinarello, 1988,1991).

It has become apparent that these neural-immune regulatory mechanisms are not readily seen until the immune system has been challenged. Thus, the most constructive approach has been to provide an immune challenge, such as an intravenous low dose of

bacterial or viral antigen, along with the experimental manipulation of the central system. In this manner much has been learned about the CNS modulation splenic immune function during stress. Surgical denervation of the spleen permits one to evaluate the role of the sympathetic nerve in this modulation. In addition, antigenic agents have been administered by other routes in order to elucidate the afferent and efferent components of this regulatory system.

It has long been recognized that many infectious agents have evolved the ability to subvert aspects of the immune system (Cook-Mills, Munchi, Perlman & Chambers, 1992; Marrack & Kappler, 1994). The human immunodeficiency virus (HIV) is one such agent which is well known to utilize immune cells and their products to enhance its own replication (De Simone, Famularo, Cifone & Mitsuya, 1996; Ho, Poverantz and Kaplan, 1987; Milman & D'Souza, 1994; Price, et al., 1988). An autonomic immunomodulating pathway could also be acted upon by naturally occurring infectious agents or their products. HIV and its viral coat proteins gain access to the brain early in infection and it is reasonable to suppose that CNS modulation of immune function would be affected. HIV has been demonstrated to impact on brain function early in the course of the disease, and central effects are often detectable prior to evidence of systemic infection (Diederich, et al., 1988; Navia and Price, 1987). There is no evidence of neuronal HIV infection; however, HIV selectively infects brain macrophages and microglia. Neuronal damage appears to be brought about by macrophage or microglial secretion of cytokines or other toxic agents in response to HIV (Merrill and Chen, 1991) or the toxic effects of viral proteins on microglia, astrocytes or neurons (Magnuson, Knudsen, Geiger, Brownstone

& Nath, 1995). Reports indicate that active infection of a cell is not necessary for cytokine induction. The viral coat protein, glycosylated peptide 120 (gp120), can bind to macrophages and microglia and induce secretion of IL-1β, TNFα and IL-6 (Jordan, Walkins, Kufta and Dubois-Dalcq, 1991; Wahl, et al.,1989).

Work by Sundar and colleagues (1993) confirmed that central gp120 does induce cytokine production in the brain of rats and they observed in vitro a decrease in splenic natural killer (NK) cell activity and T-cell proliferation which they attributed to gp120 initiating a central cytokine induced downregulatory signal. Preliminary evidence in our lab suggests that rather than contributing to the downregulatory signal, gp120 may disrupt the autonomic immunopathway. For example, although gp120 by itself had no effect on NE turnover in the spleen, in studies examining the effect of αMSH and gp120 on splenic NE turnover it was found that gp120 injected centrally attenuated the central α-MSH induced increase in splenic NE turnover. An increase in splenic NE turnover is associated with suppression of splenic macrophage function as measured by splenic cytokine production. More important, central gp120 resulted in increased splenic macrophage production of TNFα, rather than a suppression, an effect similar to that of cutting the splenic sympathetic nerve (Vriend, 1993).

Prior to presentation of the rational and hypotheses of the present study, the following sections will begin with a discussion of the importance of this line of research in Psychoneuroimmunology for the health psychologist. This will be followed by a review of the evidence for a functional sympathetic brain immune pathway and the role of cytokines in the induction of an immunosuppressive signal. In addition, the use of LPS

as a tool to probe the pathway will be examined. The manner in which HIV infection has been shown to affect the autonomic nervous system in patients will be reviewed, as well as, the effects of gp120 on the brain.

Psychoneuroimmunology (PNI)

A sizable body of literature has accumulated linking altered immune function to stress and other psychological experiences (for a review see: Ader, Cohen & Felten, 1991; Glaser & Kiecolt-Glaser, 1994). For instance, increases in Epstein-Barr (EB) virus antibodies have been shown to accompany academic stress in the form of medical school or military academy exams (Kiecolt-Glaser & Glaser, 1991). EB virus is endemic in humans and ordinarily held in check by the immune system. Increases in antibody titers are associated with escape from cellular immunity. However, as perception of stress differs, the effects on immune measures differ as well. EB viral antibody titers and illness were primarily associated with a subject's opinion that he or she was not working up to their own expectations. Further, a stressor viewed as aversive by one subject may be viewed as a challenge to another and the perception may depend on (among other things) previous stress experiences and may be modified with cognitive therapy. Both physical and psychological stressors may be involved and even for mice, the context of a physical stress affects an immune response (Aarstad, Thiele & Seljelid, 1991; Chi, Neumann, Mota-Marquez & Dubbery, 1993; Linthorst, et al., 1997).

Ample evidence exists for conditioned effects on the immune-response of animals and specific mechanisms are being explored. For example, Lysle, Cunnick and Maslonek (1991) reported that β adrenergic receptors are involved in Pavlovian conditioned

immune alterations in immune function as a result of a conditioned aversive stimulus. As well, macrophage derived nitric oxide may be a part of the conditioned mechanism (Cousins-Read, Maslonek, Fecho, Percy & Lysle, 1994). In our lab, a taste-aversion paradigm has been used to condition LPS induced changes in plasma corticosterone, splenic NE levels and IL-2 production (Janz, et al., 1996). The limbic system is the primary mediator of both cognitive appraisal of stress and, with the cortical forebrain, of cognitive and behavioral responses to stress including immune responses (Haas & Schauenstein, 1997). Recent animal studies have demonstrated that the neurochemical changes which occur in the limbic areas during and following restraint stress are independent of HPA activation. Whether or not the HPA is activated appears to depend on the limbic assessment of the experience (Menzaghi, Heinrichs, Pich, Weiss & Koob, 1993). In addition, the limbic system mediates learning associated with an aversive experience and conditioned responses. Functional MRI studies in humans suffering from post-traumatic stress disorder indicate altered limbic system and HPA function (Ingmundson, personal communication, January 11, 1997) and, possibly, conditioned alterations in immune response.

Health psychologists are becoming aware that surgery can be immunosuppressive, depending on the circumstances of the patient and how they view possible outcomes. Recently, it was reported that surgical stress decreased cellular immunity and enhanced humoral immunity and that laproscopic techniques were less immunosuppressive than conventional surgery (Decker, et al., 1996). This may be a result of less physical and perceived trauma.

Preliminary clinical studies in PNI have often resulted in conflicting data. Animal studies have been helpful in identifying which possible dependent measures for human experiments, and have enabled us to gain some understanding of the mechanisms underlying many puzzling clinical phenomena. As well, we have attained some insight into the effects of more chronic social stresses under controlled conditions. For instance, immune suppression has been observed following social defeat and one may hypothesize that inability to adjust to the changing job market may have similar effects clinically. Basic research has also shown that such terms as immune suppression must be strictly defined and that an immune response is a broad term which may include processes which appear counter productive and in conflict. Psychologists need to know where in the response pathways they can intervene and what the biological impact might be in particular situations. What kind of immune challenges are their patients facing and which aspects of immunity would it be useful to enhance or suppress? When is complete relaxation productive and when are more active coping methods useful? For instance, in HIV exposed or high risk populations, enhanced macrophage activity may seem desirable as these cells phagocytize virus. But, once infected, overly stimulated macrophages may produce far more virus than they can eliminate. Acknowledgement of the interaction between the CNS and the immune system is relatively recent. The neuroimmune systems are complex and this complexity is exacerbated by the further imposition of human psychology on CNS function. However, by probing the systems with immune challenging agents such as LPS, as well as various CNS manipulations, we are slowly

gaining some indications of where in the neural-immune pathways we may be able to positively intervene both pharmacologically and psychotherapeutically.

The Sympathetic Brain-Immune Pathway

Cytokine Signalling Pathways to the Brain

Communication between the brain and the immune system is bidirectional. That is, activated immune cells release factors which are capable of signalling the brain (Blalock, 1989; 1994). The brain has been shown to respond to immunization by changes in neuronal firing rates (Besedovsky, Sorkin, Felix and Haas, 1977; Saphier, Abramsky, Mor and Ovadia, 1987). In addition, changes in NE content and turnover were observed in key brain areas such as the hippocampus (Carlson, Felten, Livnat and Felten, 1987) and hypothalamus (Kabiersch, del Rey, Honegger and Besedovsky, 1988), the latter apparently induced by IL-1. In addition, IL-2 and IL-6 have also been found to alter central monoamines (Zalcman, et al., 1994). An immune stimulus, such as an infectious agent or bacterial coat lipopolysaccharide (LPS), results in the release of a cascade of cytokines beginning with TNFα and IL-1. Considerable evidence has accumulated that these cytokines, released by activated macrophages, result in activation of the HPA and an increase in circulating corticosterone followed by immune suppression (Besedovsky et al., 1979, 1986; Besedovsky and del Rey, 1987). Similarly, stress research has shown that endogenously released glucocorticoids are immunosuppressive, consistent with an HPA mediated pathway (Bateman, Singh, Kral and Solomon, 1989).

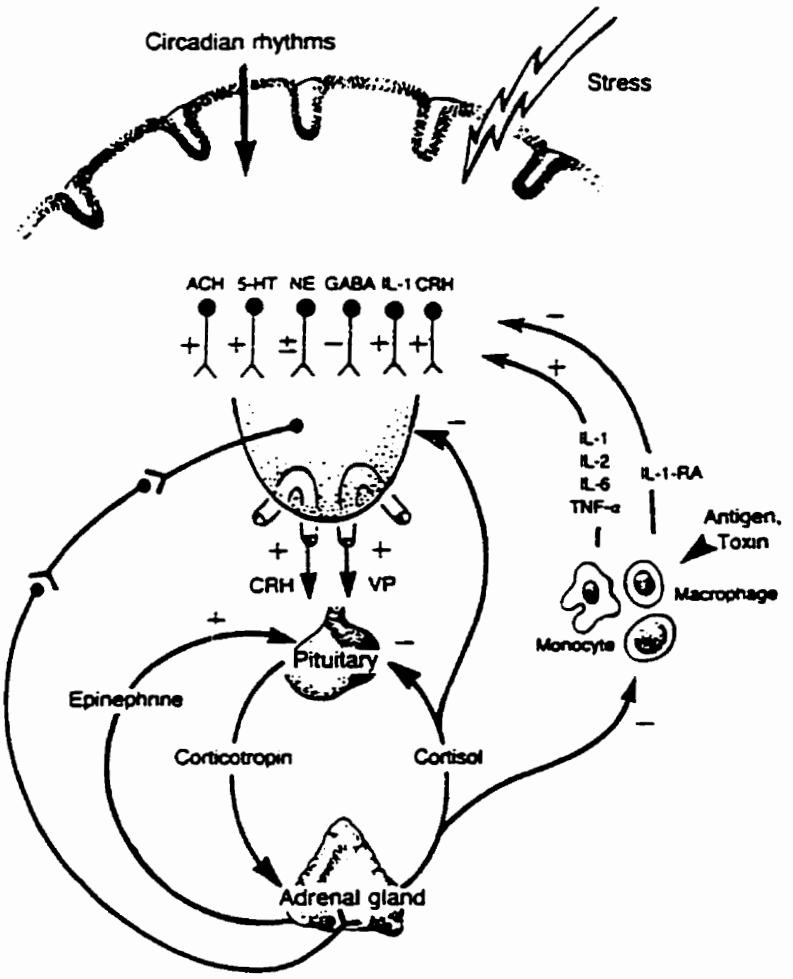

Figure 1. The immunosuppressive HPA pathway (Reichlin, 1993).

IL-1 is the most thoroughly examined of the cytokines induced by infectious agents or LPS and has been shown to produce central effects (Dinarello, 1988; 1996). IL-1 is a 17 kd peptide that is produced by activated immune cells and mediates the acute phase response to infection. This response includes hepatic acute phase protein synthesis, increases in circulating neutrophils and such central effects as fever, increased slow wave and non-REM sleep, and release of neuropeptides such as corticotrophin releasing factor (CRF), ACTH, vasopressin, somatostatin and α-melanocyte stimulating hormone (α-MSH). IL-1 is present in two forms, α and ß, which, though they share little homology,

appear to bind to the same receptor and have similar physiological effects. IL-1α is generally a membrane bound form while IL-1β is freely secreted and appears to act in a paracrine fashion (Dinarello, 1984; 1991).

In the rat, immunoreactive IL-1ß (irIL-1ß) has been found in the hippocampus and the hypothalamus. In the hypothalamus, irIL-1ß was present in the neurons of the paraventricular nucleus with beaded axons running through the lateral hypothalamus toward the medial basal hypothalamus. Positive fibers terminated in the suprachiasmatic nucleus, basal dorsomedial arcuate, internal and external zones of the median eminence and the posterior pituitary. Fibers were also seen in the periventricular nucleus and the parvocellular area of the paraventricular nucleus bordering the third ventricle and also the bed nucleus of the stria terminalis. In addition to the hipppocampus and hypothalamus, irIL-1ß was found in the basal forebrain and the olfactory bulbs (Lechan, et al., 1990). In the human, IL-1 has been found throughout the hypothalamus and the paraventricular nucleus of the thalamus. In addition, cell bodies have been reported near the anteroventral tip of the third ventricle (Breder et al., 1988). Following peripherally injected LPS, CNS levels of IL-1 were reported to be elevated (Wilkinson, Horn, Kasting & Pittman, 1994).

After injection of the cytokine interferonγ (IFNγ) or LPS in the rat brain, IL-1ß mRNA was found at the point of injection and also within perivascular cells in the dorsal recess of the third ventricle ventral to the hippocampus and choroid plexus (Higgins and Olschowka, 1991). These were identified as cells of CNS origin and not infiltrating macrophages. IL-1ß has been shown to bind extensively in the rat brain with a very high

density of receptors in the densely packed neuronal cell layers of the hippocampus and high densities are also present in the cerebral cortex, the pyriform and cingulate cortex, the anterior dorsal thalamus and the ventromedial hypothalamus (Farrar et al., 1987). More recently, IL-1 Receptor 1 mRNA has been found in the anterior olfactory nucleus, medial thalamic nucleus, basolateral amygdaloid nucleus, ventromedial hypothalamic nucleus, arcuate, median eminence, mesencephalic trigeminal nucleus, facial nucleus and the Purkinje cells of the cerebellum (Yabuuchi, Minami, Katsumata & Satoh, 1994). As well, IL-1 R1 mRNA has been reported in hippocampal neurons, the choroid plexus, the endothelium of postcapillary venules and in glial cells surrounding brain arterioles (Wong & Licinio, 1994)

As a lymphokine, the primary effect of IL-1 is the induction of IL-2, a T-cell growth factor which stimulates T-cell proliferation and clonal expansion. IL-1 induces T-cell maturation, enhances B-cell activation and proliferation and enhances natural killer (NK) cell activity. An immune response is initially produced by macrophages, those cells which are first to encounter an immune challenge and are responsible for antigen presentation to T-cells. IL-1 is secreted by peripheral blood monocytes and by lung, peritoneal, splenic, liver, synovial and bone marrow macrophages. Of significance to the present proposal, IL-1 is also expressed in the brain by astrocytes, ameboid microglia and by infiltrating macrophages, and is active in recovery from brain injury (Dinarello, 1984, 1991; Guilian, Baker, Shih and Lachman, 1986). A saturable transport system across the blood brain barrier has been described for circulating IL-1 (Banks, Kastin & Broadwell, 1995). In addition, there are areas where the blood brain barrier is weak or non-existent

and IL-1 may cross and auto-induce further production of itself or an intermediate which may act as a transmitter.

Both stress and infection have been shown to modify IL-1 receptor expression throughout the neuroimmune system. Ether anesthesia induced stress was reported to result in a selective increase in IL-1 receptors in the pituitary and a decrease in receptors for CRF, the major regulator of the stress response. CRF, itself, resulted in a marked increase in IL-1 receptors in the pituitary. Low doses of LPS, although dramatically increasing IL-1 levels, decreased receptors in pituitary and spleen, but not hippocampus. Repeated doses of LPS continued to increase IL-1 further but decreased IL-1 binding in the pituitary, spleen and hippocampus the same as high doses of LPS. Thus, IL-1 is involved in coordinating the brain immune response to both infection and stress (Takao, Hashinmato & De Souza, 1995). As a result of its CNS effects, as well as its presence and binding in the brain, IL-1 may be considered a neurokine as well as a lymphokine and it has been proposed that IL-1 acts as a neuro-immuno-transmitter in the bidirectional communication between the brain and the immune system (Blalock, 1989, 1994).

A similar cytokine neural circuit has been described for TNFα (Breder,Tsujimoto, Terano, Scott & Saper, 1993; Turnbull & Rivier, 1995). TNFα is released prior to IL-1 in the early phase of an immune response by activated macrophages and was initially described as a cytotoxic molecule which in large doses causes hemorrhagic necrosis of tumors in vivo. It has been shown to be identical with cachectin, a protein which causes the metabolic derangement leading to shock and the cachexia or wasting observed during severe disease including AIDS (Ghiara, Boraschi, Nencioni, Ghezzi and Tagliabue, 1987).

In smaller amounts, it has been shown to interact synergistically with IL-1 in response to the same immune stimuli such as LPS. TNFα is also self inductive, and both cytokines are mutually inductive.

Like IL-1, TNFα induces acute phase proteins, stimulates the HPA, acts as an endogenous pyrogen, regulates cell metabolism and acts as a growth factor. In addition, TNFα causes cytotoxic lymphocyte (CTL) differentiation, is a thymocyte comitogen and induces IL-8 during inflammatory responses. The TNFα produced by activated macrophages in turn induces IFNγ production by NK cells. Subsequently, TNFα and IFNγ activate macrophage MHC II expression and tumoricidal activity (Aggarwal & Natarajan, 1996; Bemelmans, van Tits & Buurman, 1996; Bonavida, 1992). The main difference between IL-1 and TNFα is that the latter does not have a direct effect on activation of lymphocytes although it increases expression of HLA and IL-2 receptors on IL-2 dependent lymphocytes. Both cytokines are inhibited by prostaglandins, corticosteroids, IL-4, IL-6 and transforming growth factor ß (TGFß) (Rosenblum and Donato, 1989). Despite overlapping effects with IL-1, a synergistic action with IL-1 indicates that TNFα and IL-1 may have different means of signalling cells. It is assumed that both cytokines are necessary for the full biological effect during an immune response and that the effects observed following exposure to one are attributable to the combined action of both (Neta, Sayers and Oppenheim, 1992). Kluger and colleagues found that subcutaneous injection of turpentine, which results in localized inflammation, produced high fever and reduced locomotor activity in mice (Kluger, Kozak, Leon, Soszynski & Conn, 1995). In contrast, IL-1β knock-out mice show no fever nor a decrease in

locomotor activity. However, the effect of intraperitonial injection of LPS was only partially attenuated in the knock-out mice, suggesting that TNFα and likely IL-6 is also involved in the systemic response to LPS.

Central Circuits

The expression of immediate early response genes such as c-fos has become a powerful tool for structural and functional analysis of the central nervous system (Morgan & Curran, 1989, 1991; Scheng & Greenberg, 1990) and is used extensively for that purpose in our lab. Numerous studies support the observation that c-fos gene expression is induced in neurons which have been strongly stimulated by synaptic input (Quercia & Chang, 1996; Sagar, Price, Kastin & Sharp, 1995; Suzuki, Pilowsby, Minson, Arnolda, Llewellyne-Smith & Chalmers, 1994). For example, c-fos is expressed in dorsal horn neurons of the spinal cord following noxious stimulation of the foot, in the supraoptic nucleus of the hypothalamus following water deprivation and in the hippocampus following seizures (Hunt, Pini & Evans, 1987; Morgan & Curran, 1991). Three hours following central administration of LPS, localized expression of c-fos protein was found in the paraventricular nucleus (PVN) of the hypothalamus (Wan, et al., 1993). In addition, intraperitoneal and intravenous injections of LPS produce a dose and time dependent activation of c-fos protein in the PVN, supraoptic nucleus, and arcuate nucleus as well as the catecholamine cell groups located in the dorsal and ventrolateral regions of the medulla (Wan, et al., 1993; Wan, Wetmore, Sorensen, Greenberg & Nance, 1994). In addition, c-fos staining has been found after larger iv doses of LPS in the nucleus tractus solitarius, parabrachial nucleus, zona incerta and the median nucleus of the

preoptic area, in neurons of the bed nucleus of the stria terminalis, lateral septum, and central and medial nuclei of the amygdala (Sagar et al, 1995). Stress or central injection of CRF induced c-fos in many, but not all, of these same regions. As well, painful stress and immobilization stress were reported to produce similar patterns of c-fos expression in the brain (Imaki, Shibasaki, Hotta & Demura, 1993; Senba, Matsunaga Tohyamy and Noguchi, 1993). In addition, serum corticosterone levels following injections of LPS followed a similar dose-response curve to that found for activation of c-fos protein in the hypothalamus (Wan, et al., 1993).

Consistent with the concept that some of the actions of LPS may be mediated by cytokine release, central and peripheral injections of high doses of IL-1 produce a neuronal pattern of c-fos mRNA and/or protein in the brain comparable to that reported for LPS (Brady, Lynn, Herkenhan & Gottesfeld, 1994; Ericsson, Kovacs & Sawchenko, 1994; Rivest, Torres & Rivier, 1992). Pretreatment with the prostaglandin synthesis inhibitor, indomethacin, blocked the activation of c-fos protein in the brain by both ip and iv injections of LPS (Wan, et al., 1994). Indomethacin and related drugs, inhibit many of the physiological effects of LPS such as fever, corticosterone release, changes in central catecholamine levels and increased splenic nerve activity, as well as most of the physiological effects of IL-1 and TNFα (Endres, et al., 1989; Derijk,Van Kampen, Van Rooijen & Berkenbosh, 1994; Hambra, Muro, Hiraide & Ozawa, 1994; Niijima, Hori, Aou & Oomura, 1991; MacNeil, et al., 1997; Masana, Heyes & Mefford, 1990; Morimoto, Watanabe, Morimoto, Nakamori, Murakimi, 1991; Ovadia, Abramsky &

Weidenfeld, 1989; Rettori, Milenkovic, Beutler, & McCann, 1989; Takahashi, Mishimura, Sakamoto, Ikegaki, Nakanishi & Yoshimura, 1992; Terao,Oikawa & Saito, 1993).

It has been a widely held concept that all immune system related signals reach the CNS directly via the circumventricular organs where the blood brain barrier is weak or, perhaps, by active transport. However, recently it has been demonstrated that the central activating effects of intraperitoneally injected LPS can be mediated by vagal afferents. Subdiaphragmatic vagotomy completely blocked the induction of c-fos protein in the brain following intraperitoneal injections of LPS (Nance, Wan, Wetmore & Greenberg, 1993; Wan, et al., 1994). Subsequently, it has been found that all the behavioral and physiological effects of ip injections of LPS and/or IL-1 examined to date have been shown to be eliminated by subdiaphragmatic vagotomy or selective hepatic deafferentation (Watkins, Goehler, et al., 1994; Watkins, Wiertelak, et al., 1994; Watkins, et al., 1995a, 1995b). Thus, there appear to exist multiple and separate input pathways for cytokine mediated signals to the endocrine and autonomic regulatory regions of the brain.

It has been demonstrated that the NMDA glutamate antagonist, MK801, can block the activation of c-fos protein in the brain by ip and iv injections of LPS, thereby implicating glutamate neural transmission in the central circuits activated by LPS (Wan, et al., 1994). Recently, nitric oxide production has been linked with both glutamate neural transmission and the activation of c-fos protein (Coderre, 1993; Haby, Lisovoski, Aunis & Zwiller, 1994; Herdegen, Rudiger, Mayer, Bravo & Zimmermann, 1994). Preliminary data from our lab indicated that central inhibition of nitric oxide production

with the NOS inhibitor, L-NAME, blocked the central induction of c-fos by iv LPS (Jackson, Janz, Greenberg & Nance, 1995). Consistent with the demonstration that prostaglandin synthesis is a mediator of the effects of LPS on the brain (Wan, et al., 1994), it has been shown that prostaglandin E_2 (PGE_2) activates c-fos protein in the hypothalamus similar to LPS and that this effect can also be blocked by L-NAME. These data suggest that LPS initiates a PGE_2>glutamate>NO>c-fos cascade within the brain. It has been proposed that many of the physiological effects of LPS and IL-1 may be mediated via central CRF neurons and pathways as indicated by the inhibitory effects of CRF antagonists (Bianchi & Panerie, 1995; Irwin, Hauger, Jones, Provencio & Britton, 1990; Irwin, Vale & Rivier, 1990; Saperstein, et al., 1992). In addition, some of the behavioral and physiological effects of LPS have been shown to be unaltered by IL-1 receptor antagonist (IL-1ra) (Kent, Kelley & Dantzer, 1992; Saperas & Tache, 1994). At this time the role of CRF neurons and the possible intermediary action of IL-1 or other cytokines within the suggested LPS->c-fos cascade are unknown. It does appear that oxytocin, arginine vasopressin and α-MSH, neuropeptides often colocalized with CRF, are also involved (MacNeil, et al., 1997; Vriend, et al, 1995). In any case, the PVN, a primary locus of CRF neurons, is intimately associated with pituitary function, autonomic regulatory centers in the medulla, and preganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord, and thus, splenic sympathetic outflow (Ceccateli, Villar, Goldstein & Hockfelt, 1989; Irwin, Vale & Rivier, 1990).

The Splenic Sympathetic Pathway

Conclusive evidence of sympathetic innervation of the lymphoid organs has been demonstrated (Felten et al., 1987; Nance, Hopkins and Bieger, 1987; Vizi, Orso, Osipenko, Hasko & Elenkov, 1995). In rats, it has been shown that the prevertebral celiac-mesenteric ganglia provide a major source of the sympathetic innervation to the spleen (Nance & Burns, 1989). The paravertebral sympathetic chain ganglia provide a second and comparable input to the spleen and by cutting the splenic nerve, it has been established in rats that the splenic nerve constitutes the final efferent pathway. No parasympathetic or afferent input to the spleen in the rat has been observed. Thus neural modulation of the spleen is mediated entirely via the sympathetic nervous system (Nance & Burns, 1989).

Initially, evidence that the sympathetic pathway to the spleen was functionally relevant to the immune system was indirect. Chemical sympathectomy was shown to result in an enhanced immune response in the spleen as measured by an increased number of plaque-forming cells in response to sheep red blood cells (Besedovsky et al., 1979). As well, β-adrenergic antagonists attenuated shock-induced suppression of splenic lymphocyte proliferation following mitogen stimulation, whereas adrenalectomy did not (Cunnick, Lysle, Kucinski & Rabin, 1990). As noted previously, in vitro studies have shown that the mitogenic response of splenic lymphocytes can be inhibited by catecholamine agonists, and immune cells have been shown to express receptors for sympathetic transmitters and to respond to transmitter binding with altered function (Heilig, Irwin, Greqal & Sercarz, 1993; Roszman and Carlson, 1991).

NE has been demonstrated to suppress IL-1 production in splenic macrophages and NE content of the spleen was decreased during immunization at the exponential phase of the immune response (Besedovsky, et al., 1979; Del Rey, Besedovsky, Sorkin, Da Prada & Arrenbrecht, 1981; Green-Johnson, et al.,1996; Koff, Frann, Dunegan & Lachman, 1986; Zalcman, Green-Johnson, Nance, Dyck & Greenberg, 1993). Similar to IL-1, macrophage secretion of TNFα was shown to be influenced by NE and therefore likely modulated by the sympathetic neurvous system (Hu, Goldmuntz & Brosnan, 1991; Sekut, Champion, Page, Menius & Connolly, 1995; Shimizu, Hori & Nakane, 1994; Spengler, Allen, Remick, Strieter & Kunkel, 1990; Spengler, Chensue, Giacherio, Blenk & Kunkel, 1994). Macrophages have been shown to possess adrenergic receptors and selective α- and β-agonists have been observed to potentiate and inhibit macrophage cytokine secretion, respectively, and to modify macrophage cytokine mRNA expression (Abrass, O'Connor, Scarpace & Abrass, 1985; Fukushima, et al., 1993; Hu, et al., 1991; Severn, Rapson, Hunter & Liew, 1992; Spengler, et al., 1990; Spengler, et al., 1994). In addition, macrophages take up extraneuronal NE, may resecrete it in an autocrine or paracrine manner, and are capable of metabolizing NE (Inoue & Creveling, 1993; Spengler, etal., 1994). Splenic NE turnover was increased concurrent with antigen induced increases in sympathetic activity (Fuch, Campbell & Mansen, 1988) and following icv IL-1 injections (Vriend, Zuo, Dyck, Nance & Greenberg, 1993). NE has been shown to change migration of lymphocytes in the spleen and whether it stimulates or inhibites immune activity is a function of cell type, adrenoreceptor subtype and temporal

parameters (Heilig, Irwin, Grewal & Sercarz, 1993; Madden, Felten, Felten, Sundarsson & Livnat, 1989; Madden, et al., 1994; Murray et al., 1993).

As noted previously, studies in Lewis rats demonstrated that although adrenalectomy prevented shock induced suppression of peripheral blood T-cell mitogen response, it had no effect on suppression of the splenic T-cell response. In contrast, β adrenergic blockers attenuated the suppression of splenic T-cell response in a dose dependent manner but had no effect on peripheral blood T-cells (Cunnick et al., 1990). These observations and others suggest that adrenal hormones play a greater role in mediating the suppression of peripheral blood cells and that sympathetic release of NE mediates splenic suppression (Besedovsky and del Rey, 1987; Cunnick et al., 1990; Delru-Perullet, Li, Vitiello & Nevev, 1995).

In a series of studies bearing directly on this proposal, a small dose (5 ng icv) of IL-1β was infused resulting in suppression of the splenic T-cell proliferation response and NK cell activity (Sundar, et al., 1989). In further studies, Sundar and colleagues observed that IL-1β, injected centrally, resulted in immunosuppression within two hours of injection and also decreased IL-2 production by blood and splenic lymphocytes (Sundar et al., 1990). This effect was blocked by central infusion of anti-CRF 30 min prior to IL-1 infusion, and partially blocked by a sympathetic ganglion blocker administered intraperitoneally 60 min prior to IL-1. The authors concluded that two signalling pathways existed, the HPA and the sympathetic nervous system (SNS), both activated by CRF. Subsequently, it was demonstrated in our lab that a central injection of IL-1β increased plasma corticosterone and ACTH and suppressed splenic macrophage

production of IL-1 in response to LPS stimulation in vitro. The suppression of macrophage function was attenuated by prior adrenalectomy (ADX) or splenic nerve cut. In animals that underwent both ADX and nerve cut, macrophage secretion of IL-1 was enhanced (Brown, et al., 1991). Using the same icv IL-1 protocol, NE turnover in the spleen was shown to be significantly increased during that same time frame (Vriend et al., 1993).

In like manner, IFNγ administered centrally has been shown to result in an increase in the electrical activity of the splenic nerve (Katafuchi, Hori & Take, 1991). Peripheral immune stimulation results in activation of the same pathway and there is some specificity in the response. For example, specific doses of intravenous LPS elicited differential responses from splenic and renal nerves (MacNeil, Jansen, Greenberg & Nance, 1995a, 1995b, 1996). The onset of splenic sympathetic nerve activity

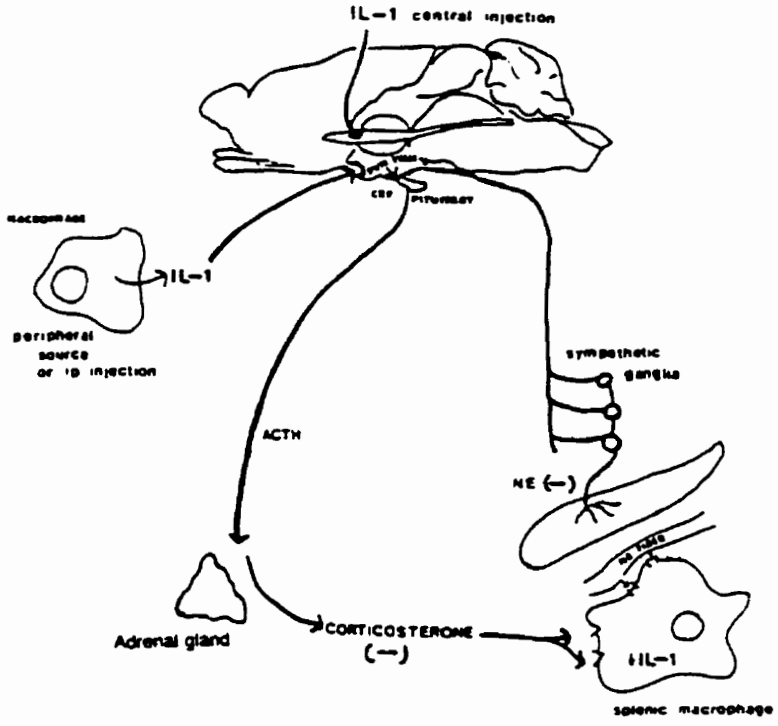

Figure 2. Original model for brain-immune bidirectional pathway.

consistently occurred prior to that observed in the renal nerve. At a 10 μg dose, all splenic nerves tested demonstrated an increase in activity, whereas, the renal nerve response rate was less than 50%. As well, with a higher dose of LPS, splenic nerve activity increased at the time renal nerve activity decreased. Thus, the renal nerve response likely reflected body temperature and blood flow regulation while the response of the splenic nerve likely represented modulation of the immune response to LPS. This result is consistent with other studies utilizing IL-1β intravenously in which injections resulted in a long lasting increase in adrenal and splenic nerve activity, while the renal nerve response was transiently increased and then tonically suppressed (Niijima et al., 1991). It was noted that during the rise in fever, an IL-1 central effect, cutaneous sympathetic nerves are activated while renal and cardiac nerves are inhibited. This pattern reverses during the falling phase of fever. Similarly, NE turnover studies in peripheral organs following intraperitoneal IL-1β demonstrated that NE turnover was selectively increased in the spleen and lung (Akiyoshi, Shimuzu and Saito, 1990). IL-1β injected centrally increased NE turnover in the spleen as previously noted, as well as in the lung, diaphragm and pancreas but not in the heart, liver, kidney or brown adipose tissue (Terao, Okawa and Saito, 1994).

Additional evidence for the functional relevance of the sympathetic splenic nerve was provided by us when we demonstrated that cutting the splenic nerve prevented stress induced inhibition of the plaque forming cell response to sheep red blood cells. As well, splenic nerve section prevented the stress induced inhibition of the splenic T-cell proliferative response to mitogens (Wan, et al., 1993). Figure 4 p.40. Recently, we have

observed a profound drop in splenic NE content during graft versus host reaction (unpublished observation). Graft vs host is known to be mediated by cytokines including IFNγ, IL-1β and TNFα. The depletion of splenic NE after graft inoculation may reflect a robust attempt at suppression of the production of these cytokines via the splenic nerve.

LPS

Commonly known as endotoxin, LPS is a lipopolysacharide molecule which along with other proteins and phospholipids forms the outer coat of gram negative bacteria. The molecule is composed of three parts each with antigenic properties: an hydrophillic core oligosaccharide and an O-specific polysaccharide, and an hydrophobic lipid A portion. The structures of all three portions vary somewhat from one species of bacteria to another. In general , a host animal is exposed to the largest doses of LPS shortly after the peak of infection and very large doses are responsible for septic or endotoxic shock such as hamburger (Salmonella) disease. Shock results from systemic complement activation and a massive over production of cytokines which leads to cardiovascular collapse. Mild infection or smaller doses of LPS efficiently activate the immune system to defend against the infection with activation modified by feedback systems including receptor down regulation, induction of antagonists and sympathetic inhibition. Animals are exposed daily to minute doses of LPS through cuts and scrapes on skin laden with gram negative bacteria and through mucous membranes, such as the gut, as bacteria are ingested and some strains are part of the normal mucosal flora. Similar to gram negative bacteria, gram positive bacteria produce a characteristic toxin, exotoxin, such as

streptococcal exotoxin (or enterotoxin) which is responsible for toxic shock syndrome (Anderson, et al., 1992).

In the circulation, LPS, which is relatively insoluble, binds to LPS binding protein (LBP) which facilitates LPS binding to cellular receptors. Macrophages, T-cells and B-cells have a specific receptor, CD14, for LPS that leads to activation and LPS has long been used experimentally as a B-cell mitogen. In the presence of serum containing soluble CD14, LPS also binds to and activates endothelial cells (Gegner, Ulevitch & Tobias, 1995; Lie, Chen & Morrison, 1990; Viriyakosol & Kirkland, 1995). In particular, LPS is an efficient inducer of macrophage activation and production of IL-1 and TNFα with TNFα being largely responsible for symptoms of endotoxic shock. Most of the circulating LPS-LBP complexes are cleared by the liver slowly over many weeks and excreted in the bile (Freudenberg & Galanos, 1990). Some complexes that have been phagositized by macrophages are carried to the lung and deposited on alveolar surfaces. In addition, a small amount is excreted in the urine .

A range of doses of LPS are administered in the lab to activate the immune system. Very small doses result in limited peripheral macrophage activation and larger doses recruit CNS involvement, as indicated by fever, HPA activation and increased sympathetic outflow (MacNeil, et al, 1995; Meltzer, et al., 1997). In the latter case, large enough amounts of cytokines such as IL-1β and TNFα are produced that the brain is signalled and centrally mediated autonomic downregulatory systems are activated. The neuroimmune system is thought to coordinate a response which eliminates the infectious agent and then terminates before the immune response itself becomes a threat to the

organism (Freudenberg & Galanos, 1990). This threat is substantial as continued production of these cytokines can result in death (Viriyakosol & Kirkland, 1995). Despite the availability of sophisticated critical care facilities in Europe and North America, it is estimated that LPS or gram negative bacteria results in hundreds of thousands of cases of bacteremia each year with up to one quarter of these ending in death (Anderson et al, 1992).

In the brain, LPS has been demonstrated to activate noradrenergic, dopaminergic and adrenergic pathways in the hypothalamus, and dopaminergic and serotonergic systems in the forebrain and limbic areas (Linthorst, Flachskamm, Hosboer & Reul, 1995). As discussed previously, intraperitoneal, intravenous and intracerebral ventricular injections of LPS resulted in characteristic patterns of expression of c-fos protein in areas of the brain which control autonomic and behavioral responses to infection. Systemic LPS was observed to depress cognitive function in that LPS administered to rats during the acquisition phase of an autoshaped task severely disrupted acquisition. When injected after performance was stabilized, LPS had no effect on subsequent performance (Aubert, Vega, Dantzer & Goodall, 1995). As well, LPS was found to induce other sickness behaviors such as decreased exploratory behavior and increased sensitivity to pain and other stimuli. Also, it increased sympathetic activity systemically (Berszi, 1993; Jones, Kovarik & Romano, 1986), but selectively (MacNeil, et al, 1996; Ohashi & Saigusa, 1997), elevated circulating epinephrine, and increased plasma corticosterone (or cortisol in humans). These effects are mediated peripherally by IL-1, TNFα, IL-6, IL-8 and IL-10, as previously discussed. These central effects may be mediated by induction of IL-1,

TNFα and IL-6 in the brain. Following intravenous LPS, IL-1 mRNA has been reported in the hippocampus, the striatum, thalamus and pituitary (Ban, Haour & Lenstra, 1992). TNFα mRNA induction by iv LPS has been observed in the hypothalamus and pituitary, and to a lesser extent, in the hippocampus. Together with IL-6 mRNA, TNFα mRNA has also been found in the pituitary and hypothalamus (Gatti and Bartfi, 1993). Two hours after intravenous LPS, IL-1 immunoreactive staining was dramatically increased in neurons in the region extending from the basal forebrain back to the dorsolateral preoptic area and paraventricular nucleus of the hypothalamus and the mid portion of the amygdala (Rettori, Dees, Hiney, Lyson and McCann, 1994).

Several approaches have been followed to assess the interaction of LPS with the sympathetic nervous system in vivo. One approach is to administer 6-hydroxydopamine (6-OHDA). When given to adult rats, peripheral injections of 6-OHDA produce a peripheral chemical sympathectomy while sparing the central catacholaminergic systems because it does not cross the blood-brain barrier. When LPS was administered after 6-OHDA sympathectomy, the LPS induced suppression of mitogenesis was reversed in the spleen but not in the thymus. In addition, chemical sympathectomy completely inhibited the effects of LPS on ACTH and attenuated the effects on corticosterone (Delrue-Perollet, Li, Vitiello & Neveu, 1995). Although many research groups continue to use 6-OHDA to explore the effects of sympathectomy, it is a very general and nonselective manipulation which knocks out adrenal function, a major modulator of immune response, especially TNFα production after LPS (Meltzer et al. 1997). In addition, it severely compromises regulation of peripheral vascular resistance and, hence, blood pressure and

as a result, may generate uncontrolled central effects. In addition, as previously mentioned, the blood-brain barrier is not complete and areas of the brain critical to immune modulation are located close to these weakened and incomplete portions and may be affected by 6-OHDA.

A more refined approach has been to eliminate specific sympathetic ganglia which innervate organs of interest either by removing the ganglia or disconnecting it from its central input. In this manner, it was found that superior cervical ganglionectomy augmented the hypotension induced by LPS, likely as a result of removing sympathetic input to submandibular glands which secrete factors thought to modulate the immune and vascular responses to LPS. In addition, this ganglionectomy reduced late phase pulmonary inflammation and inhibited LPS induced release of TNFα from alveolar macrophages (Matheson, Davison and Befus, 1994). In our lab, we have concentrated on elucidating the role of the splenic sympathetic nerve in modulating the splenic immune response, either by measuring nerve activity with an electrode or by examining the effect of various central manipulations on splenic cytokines before and after cutting the splenic nerve. The intravenous LPS induced increase in splenic nerve activity and inhibition by indomethacin has been documented electrophysiologically by our group (MacNeil et al, 1995). Splenic nerve cut was first used in our lab to confirm that the nerve contributes, along with the adrenals, to the CNS mediated immunosuppressive signal induced by icv administration of IL-1β (Brown et al, 1991).

Previously we observed that α-MSH given centrally resulted in increased NE turnover in the spleen in a manner similar to centrally injected IL-1. In addition,

following central IL-1, endogenous α-MSH was depleted from the arcuate nucleus of the hypothalamus. In further studies it was determined that central α-MSH also increased splenic nerve activity in a manner similar to high dose LPS iv (Vriend, Janz, Murray, Greenberg & Nance, 1994). Our hypothesis was that endogenous α-MSH was a component of the downregulatory pathway we have been studying. We also observed that gp120 attenuated the increase in splenic NE turnover produced by central injections of α-MSH (Vriend, et al., 1995). Thus, we proposed to test the effect of gp120 on the α-MSH stimulated increase in splenic nerve activity. However, in preliminary studies, we determined that the α-MSH effect was too variable for our purpose. Hence, we returned to using our standard immune and nerve activity stimulus, LPS.

GP 120

HIV

HIV is a viral particle approximately 100 nm in diameter with a lipid envelope composed of viral glycoprotein and cell membrane from the infected cell. It is a retrovirus and is characterized by a dense cylindrical nucleoid containing core proteins, genomic RNA and reverse transcriptase. Its most prominent immunosuppressive effect is the selective depletion of CD4 helper/inducer T-cells. It is the loss of the CD4 cell population in the presence of an opportunistic infection which is diagnosed as acquired immune deficiency syndrome (AIDS).

The infection process begins with receptor mediated endocytosis, viral entry and uncoating. The genomic RNA is transcribed into DNA by reverse transcriptase and the

DNA is integrated into the host genome during cell division by a virally encoded enzyme. At this point replication is restricted until the T-cell is activated by an antigen or allogen and begins to proliferate. T-cell activation results in transcription followed by protein synthesis, post translational processing and glycosylation. Viral proteins and RNA are assembled at the cell surface and viral particles bud off. The T-cell dies in the process (Ho et al., 1987).

The bulk of the viral load is produced by rapid turnover of proliferating CD-4 T-cells (Ho et al, 1995) However, macrophages and other antigen presenting cells, such as dendritic cells and resident tissue macrophages such a brain microglia, serve as long term reservoirs of the virus (Mosier & Sieberg, 1994). These cells continuously assemble virus and store or secrete virus by exocytosis while remaining somewhat resistant to the cytopathic effects of infection for an extended period of time. However, TNFα and IL-1 production is increased and the cells demonstrate an activated phenotype.

In the past, research has approached HIV by attempting to develop a protective vaccine and drugs which disrupt viral replication and protein synthesis. Both approaches have met with difficulty and have prompted increased interest in natural immunity to the disease and more recent studies have focused on two different patient populations. One group is a population which has been exposed to the virus and displays HIV specific cytotoxic T-cells, but has not produced antibody (become seropositive). The other group, referred to as long-term non-progressors, is characterized as being seropositive but remains healthy for an extended period of time with normal CD4 cell counts. The consensus of opinion at this time is that the outcome of exposure to HIV is dependent on

the complex interplay between viral and host factors with host factors being influenced significantly by immune status and/or stress at the time of exposure (Heeney, et al., 1997). These are factors which may specifically affect virus load both before and after seroconversion and virus load is the most critical factor in disease progression (Wei, et al., 1995). Thus, therapeutic efforts should be made as early in the course of infection as possible, preferably before seroconversion and prophylactically in high risk groups when feasible. Otherwise, the rapid turnover and high mutation rate of HIV leads to viral diversity and escape from natural immunity as well as control by therapeutic agents (Ho, et al., 1995).

Brain Involvement

Initially, it was thought that brain involvement occurred as a result of opportunistic infection such as cytomegalovirus (Gray, Gherardi, Scaravilli, 1988). It is now known that meningoencephalitis occurs early in the disease process, often before a patient is aware of other symptoms. The infection is easily overlooked, characterized by headache and upon examination, aseptic meningitis is often diagnosed. This initial brain infection appears to occur in virtually all cases and may be a necessary precursor to the development of AIDS (Diedirich, et al., 1988; Price et al., 1988). As the encephalitis becomes subacute, the patient may present with mild neurological symptoms and no or mild constitutional symptoms. The neurological symptoms may be the primary indication of HIV infection or the only aspect of the disease initially displayed. Thus, the mental health care worker may be the first practitioner the patient seeks (Navia and Price, 1987). Neurological examination may show abnormalities on CAT scan, magnetic resonance

imaging and EEG preceding systemic development of AIDS. These are characterized as slow alpha waves, diffuse theta waves, atrophy and enlarged ventricles and parenchymal lesions or calcification (Perry, 1990).

Neuropsychological testing shows that patients with AIDS often have difficulty with complex sequencing, impaired fine and rapid motor movement and verbal fluency but not vocabulary. There may be diminished performance under time pressure, with problem solving, visual scanning, visual motor integration and with alternation between two or more performance rules or stimulus sets. Depression may coexist with, or result from these signs but does not correlate with impaired performance. This characteristic testing pattern is diagnosed as AIDS dementia complex. Because of the relative sparing of cortical function in the early stages, it has been termed a subcortical dementia and thought to be caused by damage to the hippocampus and basal ganglia. A comprehensive study of HIV positive, but asymptomatic, patients revealed that they consistently scored significantly worse on cognitive tests than a matched group of HIV negative subjects. Complaints about declining cognitive ability were more frequent in the HIV positive patients and correlated with poor test performance (Stern, et al., 1991). Further development of dementia leads to global loss of cognitive ability, mutism, weakness, incontinence and death in patients who have not already succumbed to opportunistic diseases (Price et al., 1988).

Although 60% of all AIDS patients display symptoms of dementia, subacute encephalitis is apparent upon autopsy in virtually all, along with opportunistic infection and lymphoma. Even in young healthy subjects killed by accident or violence and

autopsied, those who were seropositive for HIV also displayed early signs of HIV encephalitis (Johnson, personal communication, March 5, 1997). The usual pathological manifestations at autopsy are gliosis of the cerebral cortex and subcortical nuclei, focal necrosis of white and gray matter, enlargement of oligodendrocyte nuclei, microglial nodules, multinucleated giant cells and demyelination. As noted previously, the nature of memory disturbance indicates that the hippocampus is an early target of HIV damage. The high concentration of hippocampal IL-1 receptors would make this area particularly vulnerable to virus induced cytokine mediated damage. In vitro studies with hippocampal slices indicated that IL-1 inhibited long term potentiation (Katsuki, et al., 1990), and HIV patients display disturbances of theta rhythms, which are recorded from the hippocampus (Perry, 1990).

It has been recognized that autonomic disfunction develops quite early in HIV infection. In studies of HIV positive, but asymptomatic individuals, a variety of abnormalities became apparent relative to seronegative controls. Cold pressor studies indicate that the plasma norepinephrine response is blunted. In HIV positive subjects the NE levels peak earlier than in controls but never reach the level observed in controls (Kumar, Morgan, Syapocznik & Eisdorfer, 1991). Other studies have found autonomic urogenital disfunctions (Welby, Rogerson & Bieching, 1991), othostatic hypotension (Cohen, Miller & Polish, 1991) and abnormal pupil cycle times (MacLean & Dhillon, 1993). Several studies have examined cardiovascular function in AIDs patients and have found decreased stroke amplitudes and lower 24 hour mean systolic and diastolic pressures accompanied by increased heart rate (Ohishi, et al., 1993; Ruttiman, Hilli,

Spinas & Dubach, 1991). Although the severity of dysfunction increases with disease progression, it is apparent prior to the development of AIDs and these autonomic signs do not correlate with other neurologic signs, therapeutic treatments or ongoing opportunistic infections or neoplasms (Aminoff, 1992; Freeman, Roberts, Friedman & Broadbridge, 1990). In addition, AIDS patients were found to exhibit a significant increase in plasma ACTH and cortisol following immunization with tetanus toxoid. However, compared to controls, the response was delayed and lower in magnitude. Adrenal insufficiency was ruled out by the presence of normal or low ACTH levels and the authors concluded that the patients suffered from altered hypothalamic -pituitary function (Catania, et al., 1990). More recently, physicians caring for HIV patients have been advised to monitor for development of ventricular arrhythmias due to autonomic neuropathy and other complications (Newton, 1995; Dalakas & Cupler, 1996; Villa, Foresti & Confalonieri, 1995). Others report that neuropathy of the autonomic system was found in a prospective study of asymptomatic HIV positive patients as well as those with AIDS (Malesse, Ohrmann, Agelink, Brokmeyer & Diener, 1996).

Mechanism of Damage

HIV is now considered a neurotropic slow virus despite the lack of demonstrable infection in neurons. The symptoms of neurological disease may not develop for years but it is known that HIV enters the brain early after virus exposure and viral proteins are found in the brain shortly after viral production begins systemically. Data suggests that the number of activated brain macrophages in the CNS at autopsy is highly correlated with neurological impairment and may be a better indicator of impairment than virus

production per se (Persidsky, et al., 1997). More recent evidence indicates that TNFα levels in the CSF may be an accurate predictor of impairment in patients (Johnson, personal communication, March 5, 1997). Neurons and glia are damaged or functionally altered by cytokines and other toxic products and may suffer 'innocent bystander' destruction. Neurons, though damaged, remain uninfected whereas CD4-bearing microglia and macrophages do (Ho et al., 1987; Merrill and Chen, 1991). A low level of infection and nonproductive infection has been reported for astrocytes in vivo whereas they are readily infected in vitro.

Recently, attention has focused on the highly reactive nitrogen intermediate, nitric oxide (NO) and NMDA receptor-related neurotoxicity as a cause of HIV neuropathology. In our lab we have been examining the role of NO and glutamate as transmitters in the normal neuroimmune response to an immune challenge. These molecules appear to be part of the cytokine signalling molecular cascade in the brain which may also involve PGE_2. However, enhanced production of NO or alteration of astrocyte regulation of glutamate availability as a result of cytokines induced by the presence of HIV is now thought to be a primary cause of neuropathology (Brosnan, et al., 1995; Chao, Hu, Ehrlich & Peterson, 1995; Gendelman, Genis, Jett, Zhai & Nottet, 1994; Mitrovic, et al., 1995; Vitkovic, Chatham, Cunha, 1995). The presence of intact virus in the brain may not be required for damage to occur. There is evidence that the viral proteins accumulate in the brain, bind to specific receptors on macrophages and microglia, and trigger a transmembrane signal. This signal results in cellular disfunction and production of cytokines and other products which are toxic to neurons and astrocytes, as well as

potentiate viral replication in infected cells (Burkinsky, et al., 1995; Merrill and Chen, 1991; Rho, et al., 1995).

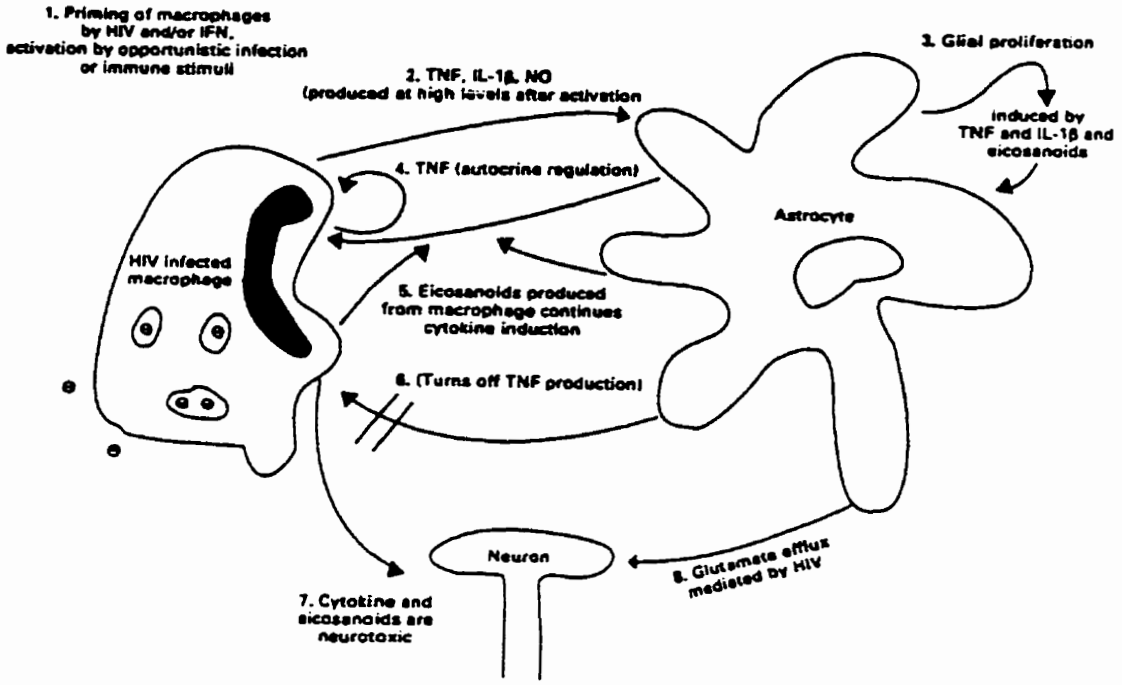

Figure 3. A model system for HIV damage (Gendelman, Genis, Jett, Zhai & Nottet, 1994)

Glycosylated Peptide 120

gp120 is a viral coat protein which is incorporated into the infected cell membrane prior to viral budding. It is the portion of the viral surface which allows HIV to bind to the CD4 antigen on T-cells, macrophages and microglia (Ho et al., 1987 Jordan et al., 1991; Wahl, et al., 1989). Brain derived cell lines, such as glioblastoma and neuroblastoma, have been shown to have specific non-CD4 binding sites for gp120 which

are not blocked by CD4 antibody (Kozlowski, Sandler, Lin & Watson, 1991). A binding site referred to as the V3, located 157 amino acids upstream from the CD4 site on gp120, is thought to be responsible for the neurotropism of the virus and binding of both regions is necessary for efficient viral entry of macrophages and microglia (O'Brian, et al., 1990; Sharpless, et al., 1992). The gp120 epitopes responsible for IL-1β and TNFα induction were found to be in the V3 loop and antibodies to these areas, but not those to CD4, blocked IL-1 and TNFα induction (Merrill, et al., 1992).

Numerous studies indicate that CNS tissues are far more sensitive to the toxic effects of gp120 than immune tissues. In general, sub-picomolar amounts of gp120 result in cytotoxicity in CNS related cells whereas nanomolar levels are required to induce damage in immune tissues. In addition, large amounts of gp120 can be shed by only a few early infected cells (Wahl et al., 1989) and a portion is likely to gain access to the brain. Toxicity to neurons is reported to be specific to particular neuronal populations even after long term exposure (Hill, Mervis, Avidor, Moody & Brenneman, 1993). In transgenic mice, engineered such that gp120 was endogenously expressed by astrocytes, a range of glial and neuronal damage has been observed which was positively correlated with location and intensity of gp120 expression and was consistent with the pathology observed in the brains of HIV patients (Toggas, et al., 1994). Like HIV, the neurotoxicity of gp120 is generally attributed to cytokine induction. Daily doses of 100 ng (icv) of gp120, administered for 7 or 14 consecutive days, resulted in apoptosis in neurons of the neocortex but not the hippocampus. (Bagetta, et al., 1995). This is likely to be a consequence of TNFα induction in the neocortex (Adle-Biasette, et al., 1995;

Benveniste & Benos, 1995; Dubois-Dalq, Altemeyer, Chiron & Wilt, 1995). Recently, chronic central infusion with gp120 was found to increase mRNA for IL-1β, IL-1Ra (IL-1 Receptor antagonist), TNFα and TGFβ$_1$ (Transforming Growth Factorβ$_1$) in rat hypothalamus in vivo. IL-1 RI, IL-1RAcP (Accessory Protein) I and II and TGFα did not change. These data suggest the possibility of a dysregulation of the balance between stimulatory and inhibitory cytokine mechanisms (Ilyin & Plata-Salaman, 1997). In an observation which may be important for autonomic effects, in vitro studies with microglial cells have demonstrated that gp 120 can partially antagonize the β-adrenergic inhibitory modulation of LPS induced production of TNFα (Levi, Patrizio, Bernardo, Petrucci & Agresti, 1993). Further, it was found that gp120 totally prevents β-adrenergic receptor mediated protein phosphorylation in astrocytes in both acute and chronic studies (Bernardo, Patrizio, Levi & Petrucci, 1994). This is indicative of a possible major alteration of astrocyte receptor mediated signal transduction. Other studies using human fetal brain cell aggregates have noted astrocyte dysfunction and death which appeared similar to in vivo alterations observed in experimental hepatic encephalopathy (Pullium, West, Haigwood & Swanson, 1993)

Another form of gp120 related damage appears to be induced by NMDA-receptor mediated neurotoxicity (Dreyer, Kaiser, Offerman and Lipton, 1990; Guilian, Vaca and Noonan, 1990). Recent studies implicate gp120 in NMDA induced toxicity in several ways. In studies utilizing pure cultures of neurons bearing NMDA receptors from rat hippocampus and cortex, results suggested that gp120 was a high affinity ligand for the glycine site on the receptor and a more powerful allosteric modulator of NMDA

receptors than glycine. Thus, in the presence of glutamate, gp120 potentiated the release of norepinephrine evoked by glutamate activation of NMDA receptors (Pittaluga & Raiteri, 1994). In human embryonic prosencephalon and spinal cord cells cultured with attendant astrocytes, microglia and fibroblasts, gp120 markedly increased NMDA induced Ca^{++} responses. In addition, gp120 induced neuronal cell death which was dose dependent and paralleled that induced by adding NMDA but the toxic effects were not additive (Lannuzel, Lledo, Lamghitnia, Vincent & Tardieu, 1995). These results point to a second aspect of NMDA receptor mediated toxicity which gp120 may affect. gp120 has been shown to impair normal astrocyte regulation of glutamate availability and to increase glutamate efflux from astrocytes, providing increased availability of ligand for the NMDA receptor (Benos, Hahn & Ruben, 1994) This activity has been observed in rat and both human adult and embryonic neuronal tissue (Benos, personal communication, June 26, 1995) and may be mediated by macrophage release of arachidonic acid (AA). Arachidonic acid inhibits astrocyte uptake of glutamate and the binding of AA by bovine serum albumin (BSA) blocked the effect of gp120 on glutamate uptake. AA is metabolized to platelet activating factor (PAF), but a PAF inhibitor had no effect on glutamate uptake, suggesting AA itself is the mediator (Dreyer & Lipton, 1995). Prior studies had indicated that PAF may mediate some gp120 induced neuronal toxicity. Following observations that AIDS patients had greatly increased levels of PAF in CSF, it was demonstrated that HIV infected macrophage interactions with astrocytes produced PAF and that treating human fetal cortical cultures and rat retinal ganglion neurons with

PAF resulted in cell death. The neurotoxicity was only partially blocked by using NMDA receptor antagonists (Gelbard, et al., 1994).

At the least, the net result of allosteric potentiation of NMDA receptor evoked intracellular Ca^{--} flow and increased availability of glutamate would be interference with normal NMDA-receptor mediated transmission and brain function. In addition, gp120 could induce glutamate neurotoxicity in situations where ordinarily non-toxic levels of glutamate were present. These data would serve to explain the observation that gp120 exacerbated glutamate induced toxicity resulting from hypoglycemia and brain injury (Barks, Sen, Malink & Silverstein, 1995).

In addition to glutamate mediated changes in calcium ion flow, one study reported Ca^{--} increases in presynaptic terminals in rats that are not blocked by glutamate receptor agonists, removal of glutamate with glutamate dehydrogenase, or by calcium channel blockers (Nath, Padua & Geiger, 1995). This observation could be related to increased K^{+} flow from astrocytes into neuronal extracellular space because gp120 has been shown to activate large-conductance potassium channels in primary cultures of rat astrocytes (Bubien, Beneviste & Benos, 1995).

Another factor which may be involved in cell damage is NO. L-arginine depletion in neuronal cell cultures, as well as addition of nitroarginine, both of which inhibit NO production, prevented gp120 toxicity. Further, antibodies to gp120 have been shown to prevent iNOS (inducible nitric oxide synthase) induction and NO formation. When a free radical trapping reagent was administered it prevented gp120 induced NO formation and brain damage in neonatal rats which had been given chronic subcutaneous injections of

gp120 (Tabatabau, Stewart, Pye, Kotake & Floyd, 1996). As NO is utilized in many brain areas as a neurotransmitter, these results suggest another means by which very small amounts of gp120 could interfere with normal brain function prior to causing outright cytopathology.

At present, we do not know all the steps and pathways which compose the central neuroimmune circuits. We do know that glutamate and NMDA receptor transmission are involved in propagating the signal. It does appear that gp120 in the brain is capable of causing neurotoxicity in cells bearing these receptors which may be part of the signal cascade and, thus, has the potential to disrupt the signal cascade and interfere with the normal CNS modulation of an immune response.

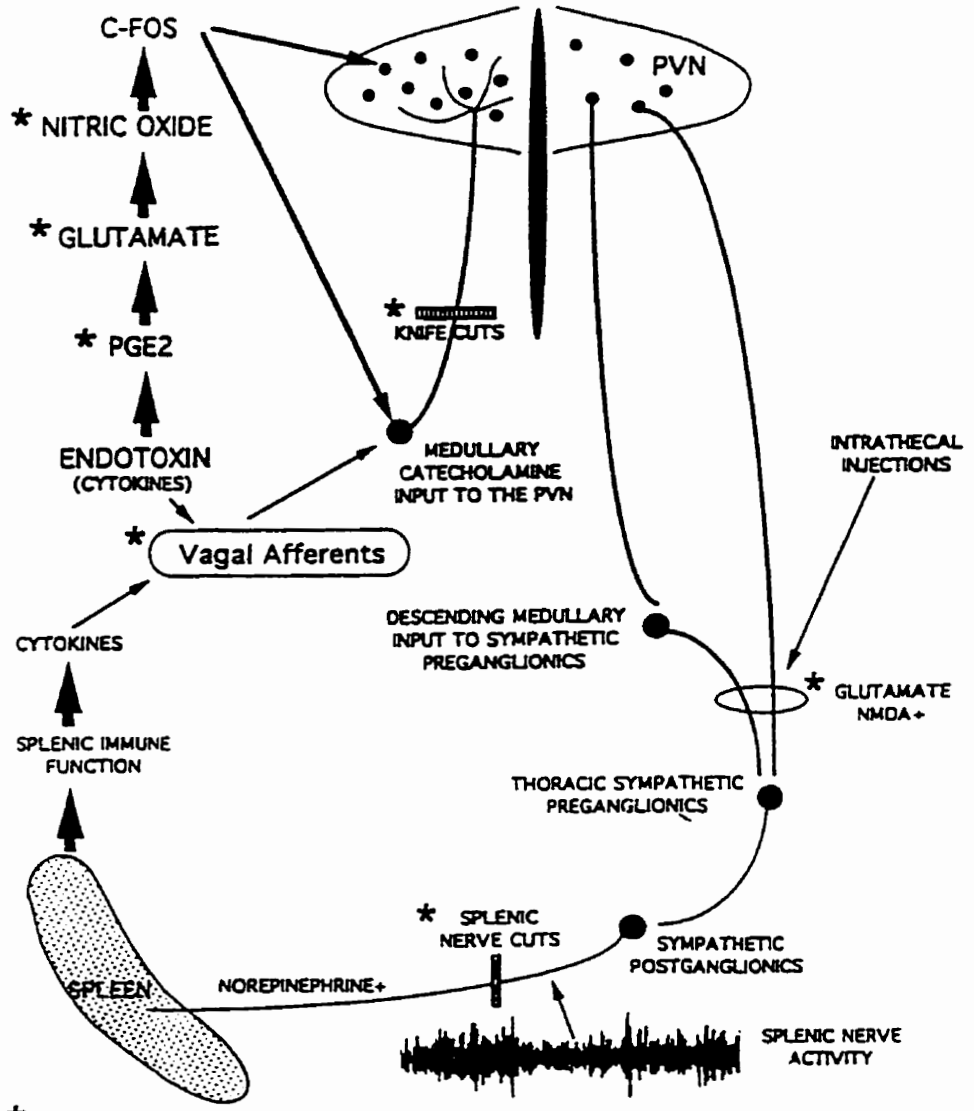

Figure 4. Current model for the brain-immune bidirectional pathway.

The Present Study

Our previous results indicated that gp120 attenuated the α-MSH induced increase in splenic NE turnover and that increased NE turnover is associated with suppression of splenic cytokine production (Brown, et al., 1991; Vriend, 1993). In addition, we observed an increase in the production of splenic macrophage TNFα in vitro following LPS stimulation. This data suggested that despite the likely induction of cytokines in the brain by gp120, the effect of gp120 was not the same as central cytokines. Although, central gp120 did elevate plasma corticosterone similar to IL-1β, possibly via IL-1 induction, it did not suppress splenic cytokine production in the same manner as IL-1β. The present study sought to confirm the impact of gp120 in the brain on the splenic cytokine environment by examining cytokine mRNA and protein in vivo following central gp120, and to further examine the effect of central gp120 on the modulating signal originating in the brain and carried by the splenic sympathetic nerve by means of direct nerve activity recording. The first hypothesis of the present study was that administration of gp120 would potentiate the expression of splenic TNFα mRNA and protein in vivo following immune stimulation by a low dose of LPS (0.1μg iv). The second hypothesis was that gp120 would attenuate the high dose LPS (100μg iv) induced increase in nerve activity the same as it attenuated the α-MSH induced increase in splenic NE turnover.

METHODS

Subjects

The animals used for these experiments were 60 experimentally naive, male Sprague-Dawley rats obtained from Charles River, Quebec, and weighing 300-350 g. The rats were housed individually in polypropylene cages and maintained on a 12:12 lighting regime with food and water ad libitum. Animals were routinely handled so that baseline corticosterone values would remain low during each experiment.

Surgery

Seven days prior to each experiment, animals were anesthetized with nembutal (60 mg/kg ip). Animals were food restricted 12 hours prior to surgery, and immediately prior to anesthesia were injected with 0.02 ml atropine (0.5 mg/ml sc) to reduce respiratory problems during anesthesia. A cannula was stereotaxically implanted in the lateral ventricle of each rat with coordinates 0.8 mm posterior to bregma, 1.3 mm lateral and 4.5 mm deep relative to dura (Paxinos and Watson, 1981). Immediately after surgery the rats were injected im with a broad spectrum antibiotic. Animals were allowed to recover for one week prior to experiments.

Experiment 1: Effects of Central gp 120 and Intravenous LPS on In Vivo Splenic Macrophage Cytokine Production.

Experimental procedure

Groups of animals (n=10 per group) were infused intracerebral ventricularly with 4μg of gp120/20μl vehicle (Intracell), or vehicle over a 10 minute period while

unrestrained. One hour later, animals were injected intravenously with 0.1μg of LPS/0.2ml saline (Sigma) or saline. Two hours after gp120 infusion (one hour post LPS), rats were rapidly sacrificed by decapitation. Plasma was collected and alliquots and stored at -70°C for corticosterone and catacholamine analysis. The spleen was quickly removed, sectioned into several pieces, immediately frozen in liquid nitrogen and stored at -70°C for Northern Blot analysis of TNFα and IL-1β mRNA and immunocytochemistry of TNFα.

Northern Blots

RNA isolation : Spleen tissue (160-200 mg) was homogenized in 2 ml of trizol (50-100 mg/ml). After 5 min at room temperature, 200μl of chloroform per ml of trizol was added. Samples were shaken vigorously and then incubated at room temperature for 3 min. Samples were then split into two eppendorf tubes and spun at 7500g for 15 min in a cold room. The upper phase was removed to another tube and 0.5 ml of isopropanol per ml trizol was added and vortexed. After standing at room temperature for 10 min, the sample was centrifuged at 7500g in a cold room. The supernatant was aspirated and 1 ml of 75% ethanol was added to the pellet and mixed. Samples were then centrifuged at 7500g for 5 min in the cold room. The liquid was aspirated and the pellet air dried for 5-10 min. The pellet was resuspended in 100-200 μl of DEPC-treated water, heated to 70°C, aliquoted and stored at - 70°C.

RNA quantitation: 1μl sample of RNA was placed into 1ml of DEPC-treated water and read in a quartz cuvette at 260/280 nm on a spectrophotometer. Concentration was calculated as the O.D. at 260 nm multiplied by 4×10^{5}.

Blotting: Samples were made up in denaturing solution so that there was 10µg of total RNA in 10µl of denaturing buffer. Samples were heated to 65°C for 10 min, cooled and centrifuged and then loaded on a formaldehyde gel prepared with DEPC-treated water. The gel was run for 3 hours at 50V. After rinsing the gel with DEPC-treated water for 30 min at room temperature, the gel was placed into a tray containing a transfer tray with blotting paper and solution. Then the nylon membrane was applied and two more pieces of blotting paper. The edges were covered with parafilm and the whole covered with a pad of paper towels and weighted glass. The gel was allowed to transfer over night.

Hybridization: The membrane was rinsed in blotting solution, air dried at room temperature and then heated for 2 hours at 80°C. Following a 5 min rinse in blotting solution, the membrane was placed in the prehybridizing solution for 2 hours at 42°C. The solution was drained and Digoxigenin (dig) labelled riboprobe was added at 0.5 to 5µl of probe per 1ml of prehybridization solution and incubated at 60° overnight.

Detection: Blots were rinsed twice in blotting solution with 0.1% SDS at room temperature and twice at 65°C. They were then placed in maleate buffer for 5 min followed by Boehringer Block (BB) for 1 hour. Excess BB was poured off and antibody diluted in BB was then added and the blots were incubated at room temperature for 1 hour. This was followed by rinsing twice in maleate buffer followed by detection buffer. Blots were then placed in a container with anti-dig peroxidase detection solution and incubated for one hour. After removing excess solution from the blots, mRNA was quantitated by densitometry. Relative values were determined by calculating the ratio of

the O.D. units of the mRNA blots to the O.D. units of ethidium bromide staining of rRNA of the ribosomal RNA s28 band. In these studies, the rRNA s28 remains at a constant level and is vualized under uv light to ensure the integrity of the RNA during analysis and as a loading control for the gels. Other methods use various housekeeping genes as a loading control but in experiments resulting in cellular changes similar to phenotype changes as in macrophage activation, levels of these genes may not remain constant. Statistical analysis was to be carried out by ANOVA with a planned comparison of the critical groups. However, two control groups were eliminated as a result of failure to detect mRNA in animals not receiving LPS intravenously, and analysis of the critical groups was carried out by T-test for unpaired groups.

Immunocytochemistry

Portions of frozen spleen were post fixed in PLP (periodate, lysine and 4% paraformaldehyde) for four hours followed by 30% sucrose overnight. Serial 50μm spleen sections were cut on a freezing microtome and sections transferred to 24 well culture plates containing phosphate buffered saline (PBS). Sections were rinsed three times with PBS then placed in primary antibody (Anti-TNFα, 1:5000, Genzyme) in PBS containing 1% Triton X-100 (TTX), 1% normal goat serum and 1% bovine serum albumin (ICC solution). Trays were then securely covered and bagged and placed on rocker tables overnight at room temperature. The following day sections were rinsed in PBS and then placed into a 1:750-1:1000 dilution of AP-conjugated goat anti-rabbit antibody for 2 hours, rinsed and then developed with NBT/BCIP (0.4mM NBT, 0.4mM BCIP and 3 mM levamisole were added to 50nM $MgCl_2$/100mM Tris/100mM NaCl, pH

9.3). After approximately 10 min development, sections were rinsed in PBS, floated onto slides, dried overnight and coverslipped in glycerol gel.

Computerized image analysis was performed with a Nikon microscope, Panasonic Newvicon video camera attached to a Macintosh 7600 PPc computer. Image analysis was accomplished with the public domain software, NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.gov/nih-image/) and a custom written macro (courtesy of Dr. Paul C. Grimm, University of Manitoba). The areas of TNFα staining were segmented and reported as the percentage of positively stained pixels per high power field (% area). A representative area of the center of the tissue section was taken by an operator blind to the identity of the slides. Statistical analysis was to be by ANOVA with a planned comparison of the critical groups. However, those spleens in which TNFα mRNA was not detected were not stained for protein. Therefore, the critical groups were compared by T-test for unpaired groups.

Corticosterone Assay

Vials of plasma and standards (corticosterone diluted to 1 mM in redistilled ETOH and then further diluted with charcoal stripped serum to final concentrations of 0.0, 50, 100, 200, 400, 600, 1000 nmol) were thawed and 10 ul added to 0.5 ml assay buffer (0.82% anhydrous sodium acetate, 0.01% sodium azide, pH 5.2 with 0.01% BSA added). Duplicate 100 ul volumes of unknown and standard dilutions were gently vortexed with 100 ul of antiserum (Sigma; diluted 1:4 with physiological saline containing 0.1% sodium azide then further diluted 1:100 with assay buffer). 100 ul of ^{3}H

corticosterone (New England Nuclear, 250 uCi in 7.5 ml of re-distilled ETOH and further diluted with assay buffer to 25,000 cpm/100 ul) was added to each tube, vortexed and incubated at 4°C for 90 min. After incubation, 1 ml of charcoal/dextran (0.025% dextran T-70 and 0.25% Norit A charcoal in assay buffer) was added to each tube, vortexed and incubated at 4°C for 10 min. Tubes were then centrifuged at 3000 rpm for 15 min at 4°C, and supernatents decanted into vials for scintillation counting on a Beckman ß scintillation counter. Data were expressed as nmol per liter. Statistical analysis was accomplished by T-test.

Catecholamine Analysis

1.0 ml of plasma was taken for alumina extraction using the ESA plasma catecholamine methodology with an internal standard. Samples were separated by HPLC using an ESA model 5700 solvent delivery system with a CSC-S ODS2 5µm column. Analysis and quantitation was accomplished with a Coulochem 5100 A electrochemical detector and Shimadzu CR601 Chromatopac integrator. Data were expressed as ng NE or EP per ml. Statistical analysis was accomplished by T-test.

Experiment 2: Effects of icv gp 120 and iv LPS on Splenic Nerve Activity.

Experimental procedure

Rats were anesthetized with a urethane/chloralose (912 mg and 78mg/kg iv, respectively) and intubated to minimize pulmonary complications. The femoral artery was catheterized to monitor blood pressure and heart rate was monitored via the pulse pressure signal. The femoral vein was also catheterized and a constant saline/anesthetic

supplement (~1ml/hr iv) given to maintain fluid and anesthetic levels throughout the experiment.

Recording of Nerve Activity

A left side laparotomy was used to access the spleen which was then displaced anteriorly to reveal the splenic neurovascular network. A peripheral branch of the splenic nerve was dissected and placed on a bipolar platinum electrode. The preparation was insulated from the surrounding tissue with warm mineral oil. Electrical signals from the nerve were amplified, filtered (30-3000Hz) and displayed on an oscilloscope. A window discriminator was used to select impulses above baseline and to generate a monostable pulse for each spike above the window threshold. The output of the window was quantified by a rate meter and plotted as the total number of spikes/5 seconds. The amplified nerve signal, rate meter output, blood pressure and heart rate were recorded on a strip chart as well as digitized and stored on video tape.

Injections

Following a 30 minute baseline recording, 4 μg of gp 120 (Bartel, Inc.), in 20 μl of vehicle or vehicle alone were infused intracerebral ventricularly (n=10 in each group of animals). Thirty minutes later, 100μg of LPS (Escherichia coli, 055:B5 ; Sigma) dissolved in phosphate buffered saline, pH 7.5, or saline alone was injected via the femoral vein. Splenic nerve activity was then continously recorded for 2-4 hours.

Statistical Analysis

A detectable increase in nerve activity was defined as a sustained increase of greater than 15% above baseline level and expressed as percent increase above baseline.

The time of onset (latency) was taken as the point at which the sustained increase occurs following injection of LPS or saline. One way ANOVAs, LPS or saline versus % increase in nerve activity and LPS or saline versus latency to onset, were to be used to identify significant differences among the groups followed by planned comparisons of the critical groups. However, as no effect of gp 120 on LPS induced splenic nverve activity were observed, the data was not analysed further.

RESULTS

Experiment 1

Northern blot analysis of spleens taken 2 hours post gp120 or saline (one hour post LPS) indicated that mRNA flevels for TNFα and IL-1βin the gp 120 injected animals were significantly elevated compared to saline injected controls. The mRNA levels were calculated as ratios of O.D. units for mRNA / O.D. units of ethydium bromide staining of the rs28 band, and for the critical comparisons were: TNFα mRNA: gp120 = 6.636 ± 1.716 and saline = 2.532 ± 0.525, T= -2.066, $p<0.05$ 1-tail; IL-1β mRNA: gp120 = 16.619 ± 1.298 and saline = 12.670 ± 0.880, T= -2.385, $p< 0.025$ 1-tail. See Figure 5.

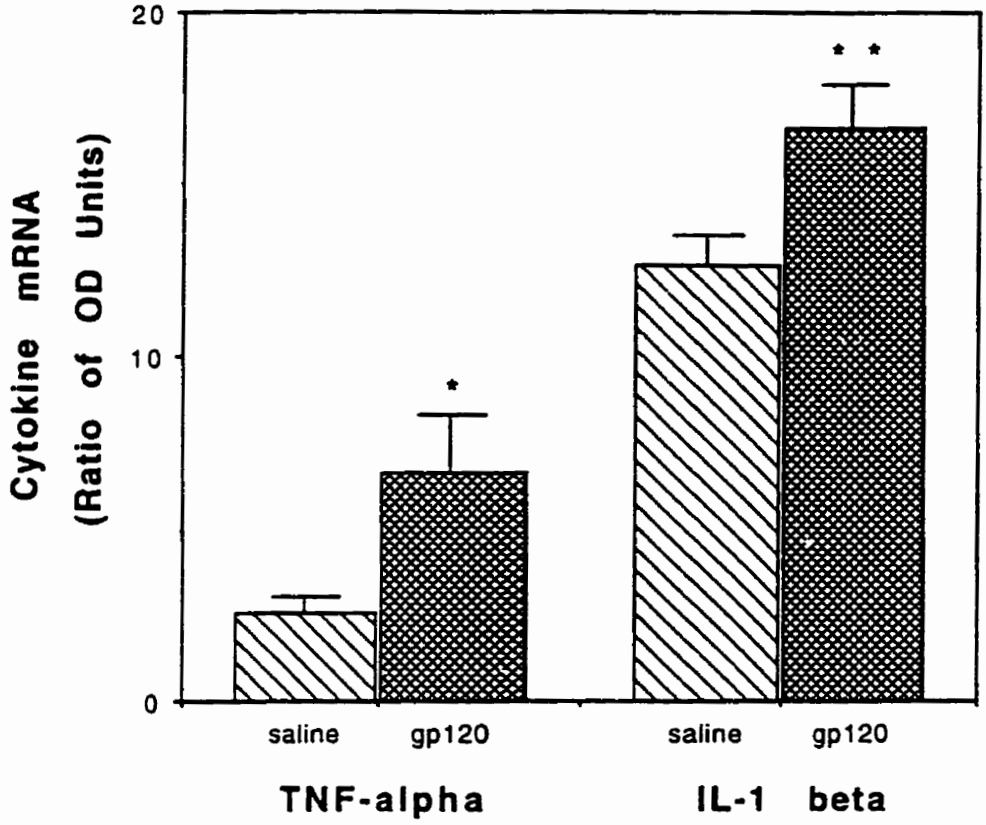

Figure 5. Splenic levels of TNFα and IL-1β mRNA in gp120 treated animals (n=10) relative to saline controls (n=8). All animals received LPS iv. OD units were derived from Northern Blots using dig labelled probes of the mRNA and ethydium bromide staining of the s28 band.. *p<0.05, **p<0.025, 1-tail.

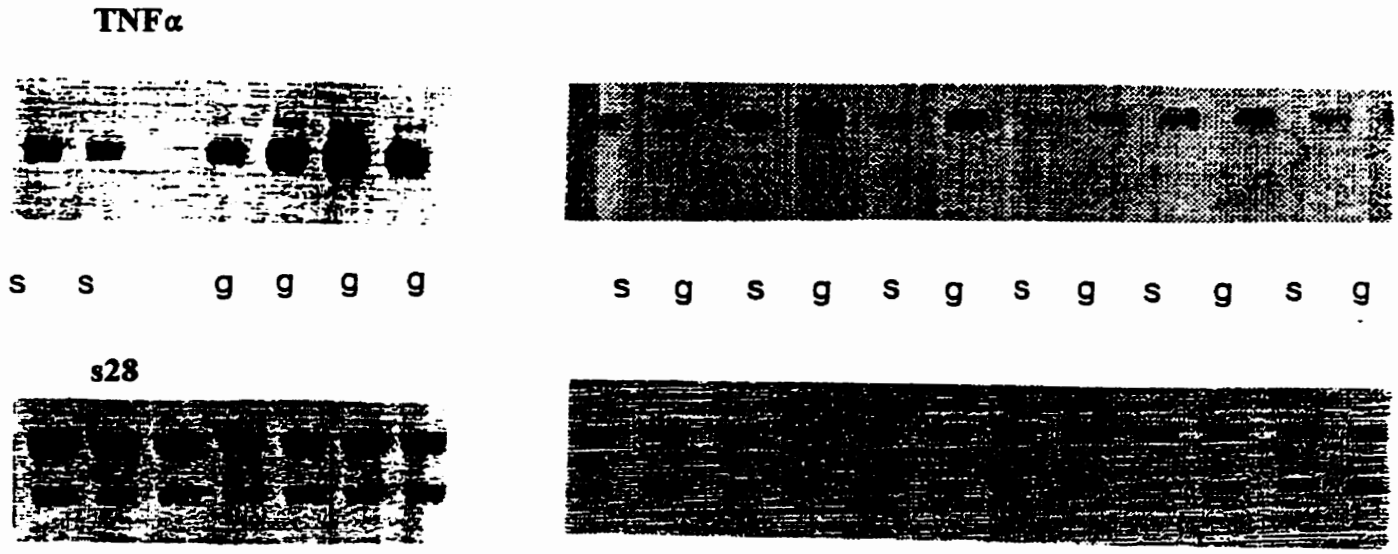

Figure 6. Northern blots of TNFα and IL-1β mRNA (g: gp120 treated, s: saline treated) and ethidium bromide staining of the s28 band loading controls . Data from Exp. 1a and Exp. 1b were collapsed. Note that the animal in lane 3, Exp. 1a did not receive a good LPS iv injection and was excluded from the analysis.

The immunocytochemical staining for TNFα immunoreactive protein in the spleen revealed mean increase in irTNFα, but the effect was not significant. The means for the staining, expressed in % area of staining detected over background, were: gp120 = 0.497 ± 0.280 and saline = 0.193 ± 0.074, T= -0.932, p = 0.3653. However, the TNFα protein values were highly correlated with mRNA levels: adjusted $R^2 = 0.832$, $p < .0001$, n = 18. Since those animals which did not receive intravenous LPS did not have detectable levels of TNFα mRNA, their spleens were not processed for TNFα protein. See Figures 7 and 8.

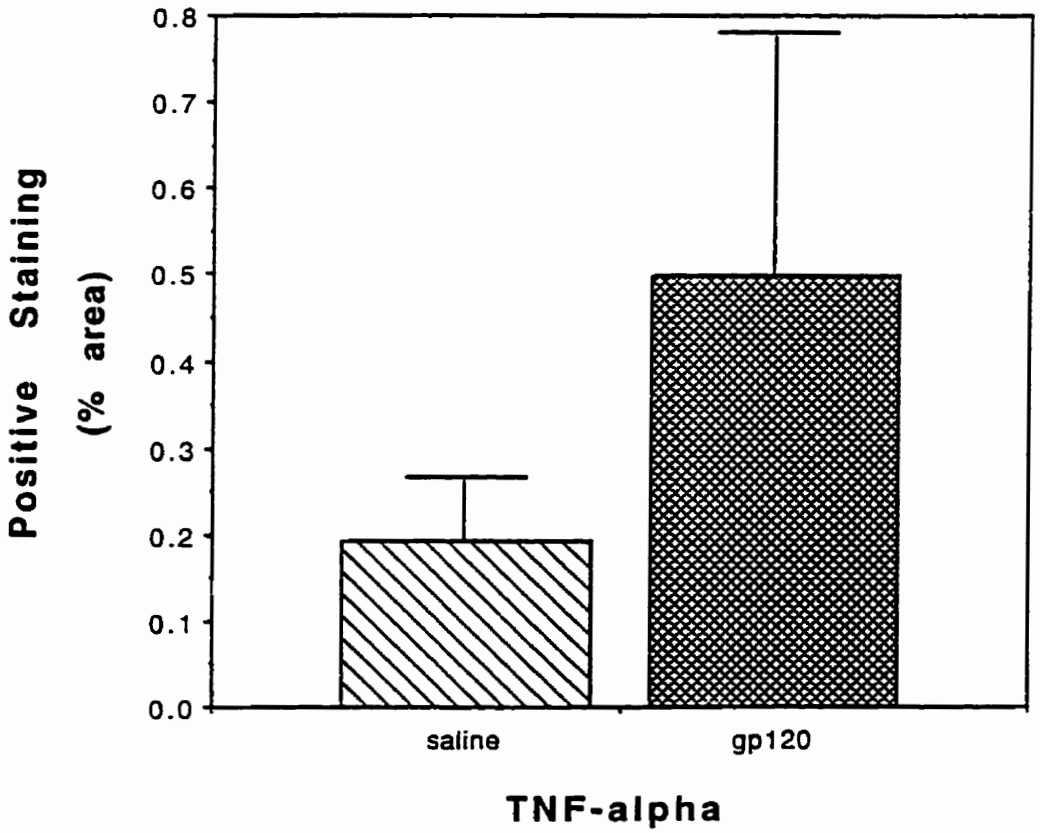

Figure 7. Levels of immunocytochemical staining of immunoreactive TNFα in the spleen, determined by pixel evaluation of tissue sections with NIH Imaging software for gp120 treated animals (n=10), relative to saline controls (n=8). All animals received LPS iv.

Figure 8. Representative photograph of immunocytochemical staining of splene sections for TNF_{α} protein. A. Section from an animal without iv LPS stimulation. B. Section from an animal stimulated with iv LPS.

Plasma corticosterone was significantly elevated in those animals that received intracerebral ventricular gp120 compared to saline controls. The mean values, expressed in mg/dl were: gp120 = 698.50 ± 33.09 and saline = 62.54 ± 53.24, T= -10.146, p <0.0001. In those groups of animals that received low dose intravenous LPS, the corticosterone levels were further elevated. However, the gp120 animals were still significantly elevated compared to saline: gp120 = 953.20 ± 81.50 and saline = 709.20 ± 79.55, T= -2.143, p <0.05, 1-tail. See Figure 9.

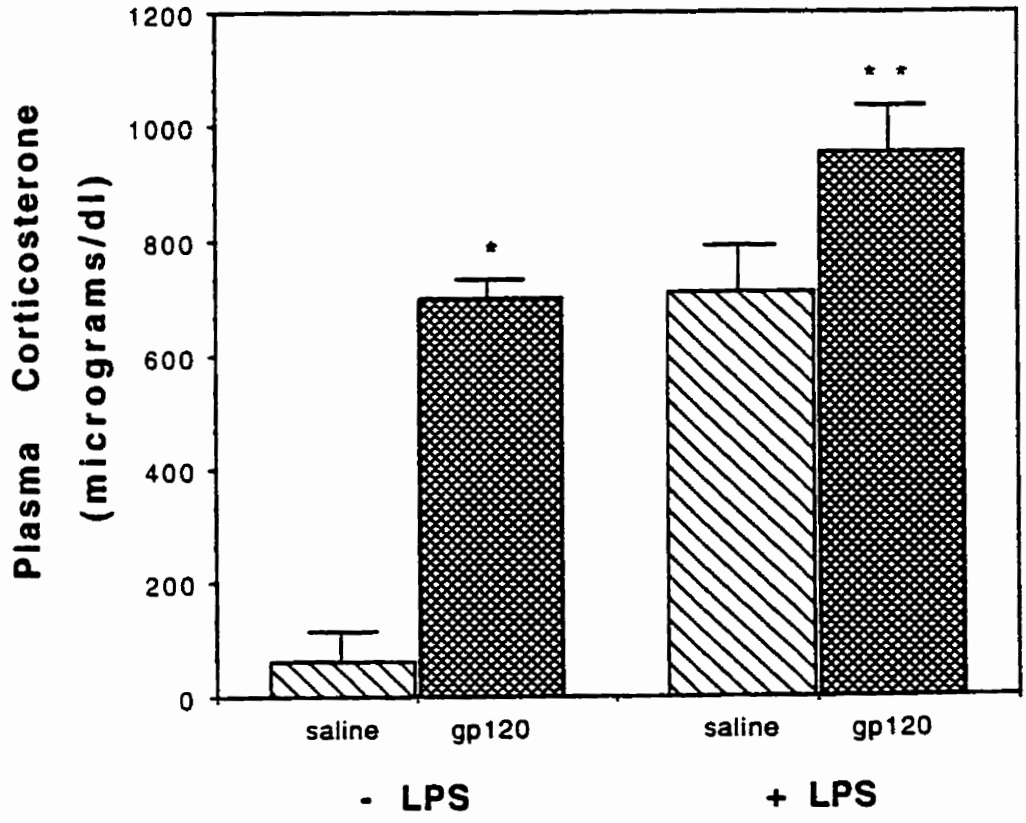

Figure 9. **-LPS**: plasma corticosterone values for gp120 treated animals (n=5) compared to saline controls (n=5) in those animals not receiving LPS iv (*p<0.0001). **+LPS**: plasma corticosterone values for gp120 treated animals (n=5) compared to saline controls (n=5) in those animals receiving LPS iv (**p<0.05, 1-tail)

Plasma catecholamines, norepinephrine and epinephrine, for the two critical groups were the same. The mean values for NE expressed in ng/ml were: gp120 = 9.306 ± 1.433 and saline = 9.643 ±1.936, T= -.140, p = 0.8921. The values for EP were: gp120 = 20.239 ± 2.027 and saline = 19.785 ±1.658, T= -.173, p = 0.8660. These levels of plasma catacholamines reflect no change in overall sympathetic drive or adrenal release of EP despite a significant increase in corticosterone. See Figure 10.

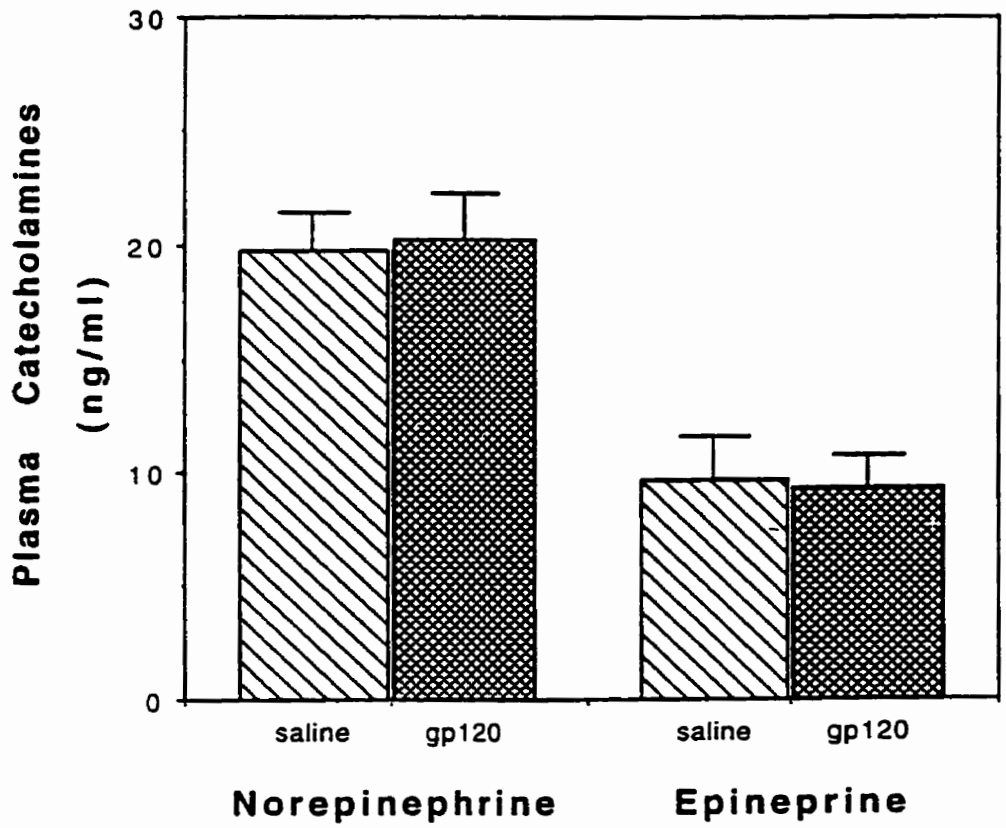

Figure 10. Levels of plasma catecholamines in animals treated with gp120 icv (n=5) compared to saline controls (n=5). All animals received LPS iv.

Experiment 2.

Several groups of animals were used in this experiment which examined the effect of icv gp120 on splenic nerve activity in animals receiving iv LPS 30 min after gp120. Animals which receive 100 μg of LPS experience an increase in splenic nerve activity usually within 10 min of injection. See Figure 10. In this experiment, one group consisted of animals which received gp120 vehicle (PBS, pH7.2) supplied by Intracell. A second group received gp 120. Consistent with our previous data that gp 120 alone had no effect on splenic NE turnover, gp120 alone had no effect on splenic nerve activity. This data is also consistent with the finding in Experiment 1 that there was no increase in overall sympathetic drive as measured by plasma catecholamine levels even with "low dose" intravenous LPS. Contrary to our hypothesis, gp 120 did not attenuate the LPS induced increase in splenic nerve activity. Vehicle alone had no effect on splenic nerve activity and had no effect on the increased activity induced by high dose intravenous LPS. Figure 11 illustrates the effect of LPS on splenic nerve activity. The data were stored for further qualitative analysis at a later date.

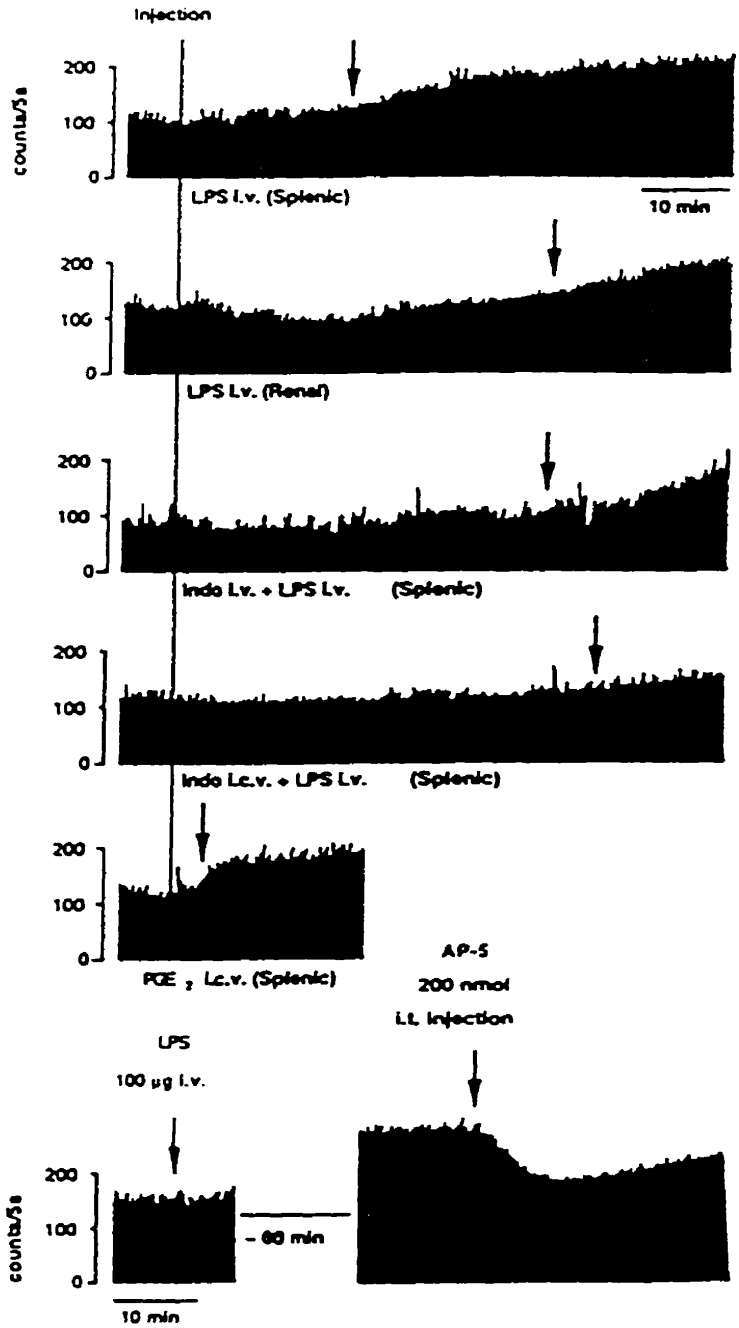

Figure 11. Spenic and renal nerve recordings following 100µg iv LPS or 1.0 µg icv PGE2. Also shown are the effects of iv and icv indomethacin and IT AP-5 in PLS induced increases in activity. We hypothesized that gp 120 would have an effect similar to indomethacin or IT AP-5.

DISCUSSION

The first hypothesis of the present study was that administration of gp120 would potentiate the expression of splenic TNFα mRNA and protein in vivo following immune stimulation by a low dose of LPS (0.1 μg iv). In this study, both TNFα and IL-1β mRNA levels were elevated in the spleen by intravenous "low dose" LPS, and this effect was significantly enhanced in animals pretreated icv with gp120 compared to saline controls. Thus, both increased message and protein in vivo and functional protein secretion in vitro have been observed as a result of acute icv gp120 administration. The second hyposthesis was that gp12 would attenuate the high dose LPS (100μg iv) induced increase in nerve activity as it attenuated the α-MSH induced increase in splenic NE turnover. The data from the present study indicates that under the conditions utilized, centrally administered gp120 does not inhibit the increase in pslenic nerve activity induced by iv LPS In addition, gp120 alone does not have an observable effect on splenic nerve activity.

The most significant finding in the present study is the increased in vivo splenic cytokine mRNA levels produced by central gp 120. Our previous studies on sympathetic regulation of macrophage function evaluated macrophage cytokine production in vitro following in vivo experimental manipulation. In vitro measures utilize the quantitation of cytokines produced and secreted in tissue culture by macrophages taken from the experimental animals and stimulated in culture with a small amount of LPS. The cytokines which are produced and accumulate in the culture media may be considered to be functional proteins, as their quantitation is dependent on the cytokine's effect on a

second set of indicator cells. On the other hand, this method has a major disadvantage in that the cells are removed from their complete natural environment and are lacking normal cell-to-cell interactions. In addition, the process of plating adherent cells in tissue culture has been reported to alter the activation level of the cells (Favre, Bordmann & Rudin, 1977). Therefore, it was important to have confirmation of our previous data by observing an increase in both message and protein in vivo. In the present study, immunocytochemistry (icc) results did not demonstrate a statistically significant difference in TNFα protein between gp120 and saline groups; however, a clear trend toward an increase in TNFα protein was evident in the gp120 group and the icc data correlated highly with the TNFα mRNA data. Similar studies indicated that the time course in mice for TNFα production after LPS is similar to that in rats. However, when animals were pretreated with a β blocker, the time course for protein production shifted. In the control group, TNF was elevated at 45 min post LPS, peaked at 90 min and was still high at 135 min compared to control (Elennkov, et al., 1995). If a similar peak shift occurs in rats, and gp120 is interfering with β adrenergic receptor signalling, the peak levels of TNFα protein may have been missed. In our previous studies, gp120 was administered 2 hours prior to tissue harvest and splenocytes were plated out and then LPS was added to the cultures. Protein accumulation was then determined at one hour and at 18 hours. In the present study, gp120 was administered only one hour prior to LPS and tissues were taken one hour after LPS. One hour post LPS may be too early to see a robust difference between groups. In addition, one would expect to see much less protein in situ as opposed to after accumulation in culture media. In any case, the present

time frame was selected based on mRNA expression which occurs prior to protein sysnthesis.

We do not have data on IL-1β protein in the present study because a suitable rat antibody is not yet available. IL-1β mRNA was significantly elevated; however, both mRNA and protein for the two cytokines are differentially regulated and despite strong signals for IL-1β transcription, little translation may take place (Dinnarello, 1996). TNFα mRNA has been demonstrated to be under a translational blockade in resting cells which is lifted as a result of LPS stimulation or macrophage activation. IFNγ can augment LPS induction, but is not sufficient to induce translation by itself. TNFα is usually transiently increased in the rat by 60 min and enhancement of secretion is thought to be accomplished through increased duration of transcription and an increase in protein translation or stability (Beutler & Krugs, 1995; Burchett, Weaver, Westall, Larsen, Kronheim & Wilson, 1988; Han, Beutler & Huey, 1991). In contrast, IL-1 appears to utilize two separate initiating pathways, with only one being associated with LPS (Fenton, Vermeulen, Clark, Webb & Auron, 1988). The LPS induced pathway is accompanied by production of an inhibitory protein and produces an early but unstable increase in IL-1. The other pathway produces IL-1 more slowly but is not accompanied by an inhibitor and the increase in cytokine is more stable. Little is known about sympathetic modulation of either pathway except that enhanced cAMP levels result in increased IL-1α induced IL-1β mRNA and protein synthesis (Dinarello, 1996). However, the role of IL-1α suggests this activity may be more associated with locally mediated inflammation. In general, it appears that although both TNFα and IL-1β are induced by LPS, TNFα is rapidly secreted during the

initial immune respons and may amplify IL-1β or induce factors necessary for IL-1β gene transcription. Post transcriptional, translational and secretory mechanisms vary greatly between the two cytokines. It cannot be assumed that in our system increased IL-1 mRNA is going to result in increased expression of protein althoug it is likely (Burchet, et al., 1988; Favre, et al., 1997; Turner, Chantry, Buchan, Barrett & Feldman, 1989).

The effect of gp120 on splenic cytokines and NE could be mediated by the splenic sympathetic nerve, and/or by hypothalamic or pituitary factors. It appears very likely that gp120 may affect positively or negatively the release of a pituitary factor or hypothalamic peptide which impacts on NE metabolism in the spleen. For instance, given that splenic macrophages can take up and degrade NE, increased macrophage uptake and degradation of NE in response to a humoral factor could result in a more rapid NE turnover in the spleen. Macrophages have functional receptors for a number of humoral factors and gp120 does engage the HPA. In addition, TNFα but not IL-1β has been shown to reduce Ca^{++} dependent release of NE by noradrenergic terminals. It could be that increased macrophage production of TNFα stimulated by a humoral factor, slows NE outflow and, thus, decreases NE turnover in the spleen (Soliven & Albert, 1992). Alternatively, it may be that gp120 in the brain qualitatively changes the nature of the signal, perhaps changing the nature of sympathetic outflow in the spleen in a manner we were unable to detect. The data from the present study indicates that under the conditions utilized, centrally administered gp120 does not inhibit the increase in splenic nerve activity induced by iv LPS. In addition, gp120 alone does not have an observable effect on splenic nerve activity. The hypothesis that gp120 would attenuate the increase in

splenic nerve activity was based upon the observation that gp120 attenuated the α-MSH induced increase in splenic NE turnover. Although an observable effect would have clearly implicated splenic neuronal activity in the gp120 effect, the lack of an effect does not rule out the role of the splenic nerve. The use of LPS in an intravenous "high dose" to activate the sympathetic signal does not allow a direct comparison with the effects of central α-MSH. One critical future study will involve cutting the splenic nerve. If cutting the nerve prevents the effect of gp 120 on splenic cytokines, then a specific neuronal factor is implicated. We have shown that cutting the splenic nerve enhances splenic macrophage cytokine secretion in vitro but apparently has no effect on in vivo mRNA levels in the spleen (Meltzer, et al. 1997). If cutting the nerve prevents gp120 induced enhancement of cytokine mRNA, the data would suggest that gp120 qualitatively changes the signal carried by the nerve but it would not rule out participation by humoral factors. Splenic NE turnover could be affected via a humoral rather than neural route as discussed previously and macrophage cytokine production could be as well.

Of the many possible humoral factors which may be involved with NE in modulating splenic macrophage activity, changes in plasma glucocorticoids (GC) have been shown to profoundly affect the immune system. ACTH release of corticosterone has long been known to be immunosuppressive and it is well recognized that cytokine induced corticosterone is one arm of the downregulatory CNS-immune mechanism. Specifically, it has been demonstrated that treating animals with dexamethazone will prevent LPS induced increases in serum TNFα and significantly decrease IL-1. The eventual decline in TNFα after LPS stimulation is coincident with the rise in endogenous

corticosterone and the fall in TNFα can be prevented by adrenalectomy or hypophysectomy (Ramachandra, Sehon & Berczi, 1992; Zuckerman, Shellhaas & Bulter, 1989). Similar results were observed when animals were treated with cyanoketone, a GC synthesis inhibitor, or treated with antibodies to ACTH. TNFα levels were restored to normal when stressed animals were treated with RU38486, a GC receptor antagonist (Fratuzzi, Di Santo, Sacco, Benigni & Ghezzi, 1995). In an experiment somewhat similar to the first experiment in the present study, mice were exposed to restraint stress which served to elevate circulating corticosterone levels compared to the control as does gp120. Both groups of mice were then given a peripheral injection of a low dose of LPS which significantly increased cytokine mRNA expression and moderately elevated corticosterone in the unstressed animals. Restraint stress significantly attenuated cytokine mRNA expression in the brain, pituitary and the spleen, with enhanced levels observed in ADX mice (Goujon, et al., 1995). In vitro studies have demonstrated that corticosterone can inhibit cytokine production both at the level of transcription and at the level of post translational processing (Heck, et al., 1994; Lee, et al., 1988; Mukaida, et al., 1994). In contrast, despite significantly increasing plasma corticosterone, central gp120 administration resulted in enhanced cytokine mRNA expression. Clearly, gp120 in the brain has a distinctive effect and does not act like a simple stressor that increases ACTH release, nor does it act by suppressing ACTH.

One possible factor which the above studies have not taken into consideration is the effect of the adrenal steroid, dehydroepiandrosterone (DHEA). DHEA is secreted by mammals in its sulfated prohormone form, often under the same circumstances as

glucocorticoids. It is converted to functional form in various target organs, the most prominant being the lymphoid organs. The spleen and lymph nodes, in particular, contain relatively high and compartmentally specific levels of DHEA sulfatase (Daynes, Dudley & Arania, 1990). Although there is some controversy over the source of DHEA-S in rodents, it is utilized in these immune tissues as well as the brain and gonads (Akwa, Morfin, Robel & Baulieu, 1992; van Weerdon, Bierings, Van Steenbrugge, de Jong & Schroder, 1992).

DHEA has been implicated in a variety of disease processes reflecting its actifity in various systems including immunoincompetence, neoplastic growth, failing neural integrity, hypertension, infertility, aging and eating disorders (Taylor, Scherrer, Weiss & Pitha, 1994; Wright, Porter & Svec, 1990). In aging animals and animals exposed to uv radiation, there is a drop in DHEA and a change in the corticosterone/DHEA ratio as cort levels remain the same or incrase. It is known that high levels of corticosterone decrease IL-2, IL-3 and GM-CSF levels and increase IL-4, IL-5 and IFNγ levels. This shift in cytokine profile is associated with the shift in T-cell population from a TH_1 or cellular mediated immune response to a TH_2 or antibody mediated response and it has been suggested that HIV+ patients have undergone a premature shift in the TH_1 response to the TH_2 response (Mossman & Coffman, 1989). The same pattern is observed in aging animals and may be more related to the change in corticosterone/DHEA ratios than to corticosterone levels alone (Daynes, el al., 1990; Aranio, Woods & Daynes, 1993). In these studies, immunosenescence was shown to be reversed in aged mice receiving DHEA and dependent on the physiologic milieu rather than on the functional integrity of the

immune cells. In patients with AIDS a dramatic decline has been observed in DHEA despite continued high levels of DHEA-S implicating a change in sulphatase levels or function. The decrease correlated significantly with drops in CD-4 cell numbers and was progressive as HIV+ status progressed to full blown AIDS (Wisneiwski, Helton, Morse & Svec, 1993). In addition, HIV+ patients have chronic elevated cort levels with blunted peeks (Villette, Bourin, Doinel, Mansour, Fist, Boudon, Dreauz, Roue, Debord & Levi, 1990). In the present study, circulating levels or corticosterone were highly significantly elevated. Splenic levels of DHEA or DHEA sulfatase were not assessed. However, if DHEA levels were reduced or unchanged as corticosterone increased, the resulting splenic hormone milieu would have enhanced the shift to a TH_2 cytokine profile or enhanced antibody rather than cellular immune response as proposed in AIDS patients and as seen in immunoscenescent animals and animals exposed to uv radiation.

Virtually every pituitary and hypothalamic factor described has been found to have either positive or negative effects on immune responses. The increase or decrease in any one of these factors may account for the effect of gp120 on splenic cytokines. For the thymus, it has been demonstrated that CRH, LHRH, prolactin, growth hormone, somatostatin, TSH, ACTH, FSH, LH and α-MSH all have effects on thymic peptides which are in turn capable of altering T-cell activity and cytokine profiles (Dardinne & Savino, 1994). In the spleen, a likely mediator of the gp120 effect may be met-enkephalin. In mice, ip injection of met-enkephalin suppressed NK cytotoxic activity and the PFC response to SRBC, but enhanced IL-1 secretion, peripheral blood monocyte (PBMC) chemotaxis and macrophage phagocytic function. Moreover, plasma

corticosterone was elevated (Marotti, et al., 1996). One observed effect of gp120 has been reduced pulmonary NK cell activity against metastatic turmor cells, and decreased splenic NK cell activity (Hodgson et al, 1997; Sundar et al, 1993). These observations, as well as our own, would be consistent with a met-enkephalin effect. Macrophages are considered a primary target of opiates, which enhance activity and cytokine secretion. Dynorphin-A, leucine-enkephalin and β-endorphin have also been shown to augment macrophage activity as measured by increased tumoricidal activity, antibody dependent cytotoxicity and superoxide production. It has been suggested that opioid peptides produced in the anterior pituitary during stress may serve to counter some of the immunosuppressive effects of stress on macrophages (Hagi, Uno, Inaba & Muramatsu, 1994).

Endorphins themselves may induce growth hormone and prolactin release which augment immune function and counter some of the suppressive effects of glucocorticoids (Ramachandra et al., 1992). In cellular differentiation, these hormones are thought to serve as immunocompetence factors or necessary first signals which set the stage for response to antigens or adhesion factors. Specifically, growth hormone has been shown to stimulate the production of IL-1 and to augment the production of TNFα in mononuclear phagocytes following LPS exposure. Prolactin treatment also increases phagocytosis by macrophages and superoxide production. Significantly, growth hormone was observed to enhance replication of HIV in acutely infected PBMC in vitro and this enhancement correlated with increased DNA transcription and TNFα secretion (reviewed in Berczi, 1994). In recent in vivo studies of hypothalamic wounding, prolactin was

found to greatly increase TNFα levels at the wound site (De Vito, Stone & Shmgochian, 1995). Endogenous prolactin and prolactin mRNA also increased at the wound site suggesting that prolactin may be involved in cytokine expression in response to injury.

Another possible mediator may be nerve growth factor which is co-secreted with prolactin, secreted during stress and anxiety even prior to HPA axis stimulation and, also, has immunomodulatory activities. It has been proposed as an early immune alert signal, along with prolactin, which serves to prime the immune system for action. However, less is known about the effect of long term secretion on immune response (Missale, et al., 1996). Other possible neuropeptide mediators could be Substance P, vasoactive intestinal peptide (VIP), TSH and somatostatin. For instance, VIP has been observed in macrophages and macrophage receptors for VIP have been detected. In addition, it is thought to enhance cAMP signaling processes which may implicate it in β-adrenergic receptor function (Bellinger, Lorton,Brouxhon, Felten & Felten, 1996). That is, reduced VIP exposure may serve to alleviate β-adrenergic suppression of macrophage cytokine production.

The latter possibility illustrates a point which should be kept in mind when speculating on these mechanisms. The modulating effect of a hormone, transmitter or other factor is likely to vary depending on the presence of other critical molecules. It may serve to augment or attenuate the effect of a second molecule but have no effect on the mechanism in question by itself. In addition, the activational state of immune cells contribute in large measure to the observed effect of the factor under study. As well, the source of the mediator may not be intuitively obvious as immune cells are capable of

either synthesizing many of these factors themselves, or taking up and later secreting factors in a autocrine manner (Fabris, Mocchegiani & Provinciali, 1995 Fabry, Raine & Hart, 1994; Weiss & Littman, 1994). Continuing studies will examine plasma levels of likely humeral factors and utilize specific antagonists or agonists of individual factors and possibly combinations of factors.

In addition to the forgoing considerations, a possibility exists for a direct effect of gp120 on splenic immune cells. It is generally assumed that central injection of a large peptide precludes any systemic exposure because of the blood brain barrier. However, it has recently been demonstrated that although the brain does not have a classical lymphatic drainage system, it is constantly being drained of CSF and a good portion of that CSF makes its way into the cervical lymphatics. Proteins as large as human serum albumin have been injected into the lateral ventricles of rats and tracked to the cervical lymphatics (Cserr & Knopf, 1992). In addition, given that gp120 can antagonize β adrenergic signalling in microglia and astrocytes (Bernardo, et al., 1993; Patrizio, et al., 1993), it is possible that gp120 could have the same effect on macrophages. As discussed previously, ample evidence exists that gp120 induces cytokines directly in these cells. However, within the time frame of the present studies, drainage of gp120 into the lymphatics and a direct effect on splenic macrophages is unlikely. The cervical lymphatic drainage of CSF is slow and the spleen is not directly connected to the lymphatic portal system. However, this possibility may complicate chronic studies if gp120 escapes the lymphoid cells and enters the circulation.

As stated previously, splenic NE turnover and macrophage function could both be affected via a humoral rather than neural route. However, the results may also be attributed to a number of difficulties with the experimental procedures. The time frames of the present studies are the shortest of those used for in vivo studies with gp120. Experiments examining effects on splenic NK cells and mitogen proliferation took tissues at 2 hours post injection (Sundar et al, 1993) but utilized an in vitro low dose of LPS in assays which were carried out over 4 to 18 hours. A recent study examining the retention of lung metastatic tumor cells examined in rats at 6 hours post tumor injection (8 hours post icv gp120) and at 4 weeks (Hodgson, Yermiya & Taylor, 1997). A paradigm which would more closely simulate gp120 exposure in nature would involve chronic administration or daily injections of gp120 for one or two weeks. An osmotic minipump has been used for three day to one week experiments but results have been mixed (Bagetta, el al., 1994; Ilyin & Plata-Salaman, 1997). The size and fragility of the peptide has led most groups to prefer one or two weeks of daily 100 ng icv injections. A dose of 100 ng per day would appear to be too large for our purposes as it results in cortical apoptosis (Bagetta et al, 1996; Bagetta et al., 1995). Our hypothesis predicts the splenic cytokine effect would occur prior to development of overt neuronal cytopathology.

With regard to the lack of an observed effect of gp120 on splenic nerve activity, the nerve activity recording procedure may be problematic. First, it involved extensive acute surgical preparation of the animal prior to the recordings. Anesthesia and surgery are stressful procedures which result in sustained peak levels of plasma corticosterone, as well as, concurrent immunosuppressive effects. Large amounts of corticosterone

activate a negative feedback system in the brain which may blunt the release of CRF. This would alter processing and release of ACTH and related peptides not only in the hypothalamic areas communicating with the median eminence, but also in other areas where these peptides may function as autonomic neurotransmitters. We have tried several approaches to ameliorate this problem, including adrenalectomy (ADX) and various inhibitors. Chronic and acute ADX are problematic because other central processes are activated and blood pressure becomes very difficult to control during the experimental procedure. Likewise, we have not found any inhibitors or receptor blockers which would only dampen the system or provide a physiological state which appears some what normal but non-stressed. Presently, we are preparing to move from an acute surgical preparation to a chronic procedure in which nerve recordings can be collected from conscious undisturbed rats. An additional difficulty presented by the anesthesia has been suggested by other groups using a similar paradigm. It appears that in the anesthetized rat, some hypothalamic-spinal neurons are inactive. This may be a result of inhibitory inputs which are activated by anesthetic drugs and produce neuronal membranes which are in a stable "hard off" condition at the outset of the experiment (Malpas & Coote, 1994). If such a situation occurs in neurons critical to the gp120 effect observed in conscious rats, the mechanism would be hidden.

The chronic preparation would eliminate second problem with the present electrophysiological procedure, that is, limited viability of the nerve. Presently the system demonstrates very well a robust and relatively rapid increase in nerve activity. After baseline nerve activity and blood pressure have been established, the onset of increased

activity can be seen with intravenous LPS within 20 minutes. With central PGE_2 injections, the latency to onset is even shorter. If the effect of interest occurs over time and is expected to be a suppression of activity, it is difficult to distinguish a possible effect from diminished nerve activity as a result of deterioration of the nerve preparation. This makes it relatively easy to accumulate data about onset of increased activity, but more difficult to see a decrease in activity short of total inhibition, such as that provided by indomethacin. Even partial suppression of the initial increase expected after LPS would be difficult to interpret because of the variability in magnitude of sympathetic response to LPS from one animal to the next.

A third difficulty is presented by the nature of the nerve activity analysis. At the time of the present study, we were able to measure gross increases in nerve activity and latency to onset of the increase. In recent studies, we have begun to analyse additional changes in the nature of the signal, such as bursting patterns. The splenic nerve is composed of a bundle of fibers, some of which appear to respond more like the renal nerve and appear to be concerned with vascular control. However, a subpopulation of fibres do not display cardiac related firing patterns and may be more related to the specific immune function of the spleen. We would like to be able to subtract the cardiac related signals from those concerned with immune function, and subsequently look for organ specific changes in the signal carried by the immune related fibers.

The range of response to norepinephrine in the spleen is broader than that suggested by a simple on/off mechanism and supports the need for a more qualitative analysis of the nerve signal. Splenocytes are ordinarily exposed to a wide gradient of NE

concentration. Further, splenic macrophages have been shown to bear α_2 adrenergic receptors which when stimulated by NE result in increased LPS induction of TNFα, as well as TNFα mRNA accumulation (Spengler, Allen, Remick, Strieter & Kunkel, 1990). The biggest increases are seen after the lowest dose of LPS. This observation is consistent with the notion that a higher dose of LPS would result in engagement of CNS mediation, increased sympathetic outflow and, thus, stimulation of the lower affinity β adrenergic receptors which are suppressive. In general, higher levels of NE have been demonstrated to result in overall suppression of macrophage activity as measured by TNFα and IL-1β production and this suppression can be prevented by administration of a β receptor blocker (Elenkov, Hasko, Kovacs & Vizi, 1995; Hasko, Elenkov, Kvetan & Vizi, 1995; Yoshimura, et al., 1997). The use of receptor blockers in these studies has led to some confusion as one must take into consideration receptors located both presynaptically, the neuronal autoreceptors, and postsynaptically on immune cells. α_2 receptors on macrophages and lymphocytes have been characterized for over 10 years and both a high affinity and low affinity form of the receptor has been found on neutrophils (Spengler, et al., 1990). Significantly, α adrenergic stimulation may mediate TNFα processing and secretion (Hu, Goldmuntz & Brosnan, 1991). Administration of an α_2 antagonist inhibited TNFα production and when administered with LPS, it decreased the LPS induced accumulation of TNFα mRNA (Spengler, Shensue, Giacherio, Blenk & Kunkel, 1994). In addition, α_2 receptor agonists were found to augment macrophage tumoricidal and phagocytic activity. As discussed previously, macrophages have been found to take up NE. Stored levels of NE decreased with LPS stimulation in the short

term and it appeared that macrophages may regulate LPS induced TNF production via an autocrine NE mechanism (Spengler et al, 1994). A similar differential catecholamine response has been reported for T-cells (Chambers, Cohen & Perlman, 1993; Sanders, et al., 1997) and B-cells (Carr, Wooly & Blalock, 1992; Li, Kovassi & Revillard, 1990; Sanders & Powel-Oliver, 1992). These effects appear to generally sort out by second messenger systems. β adrenergic receptor stimulation increases cAMP levels whereas α_2 receptor stimulation decreases cAMP. The α_1 receptor, also present on lymphocytes, is associated with the PI system and its function is less clear. It should be kept in mind that within lymphoid compartments, the major ligand available for β adrenergic receptors is NE, the low affinity ligand, and that NE is the high affinity ligand for the α receptors. Circulating EP and NE do not contribute detectable quantities of ligand compared with that provided by the splenic nerve. However, adrenal activation contributes to NE modulation of splenocyte activity by corticosterone regulation of receptor expression. In addition to varying amounts of NE provided by the splenic sympathetic nerve, high intensity stimulation of the nerve results in release of NPY which is co-localized in some synaptic vesicles with NE. NPY is thought to prolong the effects of NE and it is also taken up by macrophages and held for possible release and autocrine regulation (Lundberg, Rudehill & Sollevi, 1986, 1989; Madden, et al., 1994) One would expect, then, a considerable range in qualitative differences in the type of signal carried by the immune related fibers in order to provide the range of transmitter concentrations required for activation of the various lymphocyte receptors for NE as well as NPY. With this in

studies has been digitized and stored pending analysis by more sophisticated techniques as they become available to our lab.

As discussed previously, the gp120 pretreatment time, prior to LPS stimulation of the nerve, may not be appropriate. The central injections of gp120 were administered only 30 minutes prior to the intravenous LPS injections with changes in nerve activity expected within an hour of LPS administration. This time frame is 30 minutes shorter than that used to detect changes in splenic cytokine mRNA and the effect may not be robust enough at 30 minutes to detect electrophysiologically given the procedural problems discussed above. Continuing studies in out lab will proceed to one and two week daily injections prior to nerve recording sessions, as well as the critical nerve cut experiment previously discussed.

The significant increases which we have reported in functional TNFα protein and mRNA levels are novel observations which are contrary to the literature and previous expectations. Prior work in another lab suggested that gp120 would induce cytokines in the brain and trigger the normal down regulation of immune responses which is ordinarily induced by high plasma levels of cytokines during infection. Thus, central gp120 would be projected to suppress splenic macrophage function. However, we have demonstrated that although gp120 elevated plasma corticosterone the same as LPS and cytokines, it enhanced splenic cytokine production. In addition, plasma NE and EP were unaffected by central gp120 in contrast to significant elevations of both NE and EP following high dose intravenous LPS (unpublished observation). Further, central gp120 alone did not increase splenic nerve activity. Collectively, the data from the present study and our

previous studies provide evidence that gp120 in the brain does not activate the normal autonomic downregulatory modulation of splenic immune response and may, instead, disrupt normal CNS-immune modulation.

The disruption of the neuro-immuno-regulatory signal could have grave consequences for the HIV infected individual. The usual response to viral challenge is a predominantly cellular mediated response gradually shifting to an antibody response which provides protection against reinfection. As noted previously, a small population of HIV exposed persons have been identified that appear to have successfully eliminated the virus. They are seronegative, but have HIV specific cytotoxic T-cells. It has been suggested that HIV^{-} patients have undergone a premature shift to antibody production (Mossman & Coffman, 1989; Vyakarnam, Matear, Martin & Wagstaff, 1995) and that this shift is associated with virus load (Ho, et al., 1997). TNFα increases viral replication in infected T-cells and macrophages by activating the transcription factor $NF_{\kappa}\beta$ (Ayehunie, et al., 1993; Poli & Fauci, 1993). IL-1 primes T-cells for clonal expansion and rapid T-cell turnover accounts for much of the virus load. Increased corticosterone results in an upregulation of IL-1 receptors on T-cells and would mitigate against the down regulation of receptors that should result from increased macrophage production of IL-1 (Neta, Sayers & Oppenheim, 1992). In addition, IL-1 induces HIV replication in vitro in a chronically infected promonocyte cell line and enhances TNFα induced replication (Granowitz, Saget, Wang, Dinarello & Skolnik, 1995). The net effect may be a shift away from the clonal expansion of those CD4 T-cells (Th_1) responsible for cellular mediated immunity, including cytotoxic CD8 T-cell clearance of infected cells

and enhanced non-cytotoxic CD8 suppression of HIV replication, toward clonal expansion of those T-cells (Th_2) responsible for initiating antibody production (Barker, Mackewicz & Levy, 1995; Teal & Estes, 1990). In addition, other investigators have shown that gp120 inhibits the function of NK cells, one arm of cellular immunity (Hodgson, et al., 1997; Sundar, et al., 1993). Experience with HIV indicates that antibody production (HIV positive status) inevitably leads to development of AIDS and, as noted previously, HIV positive individuals may have undergone a premature shift to antibody production (Ayehunie, et al., 1993; Mossman & Coffman, 1989; Poli & Fauci, 1993). Taken together, our data suggest that autonomic and/or hypothalamic dysregulation by gp120 may contribute to this process and is yet another means by which HIV may subvert the immune response to enhance its own replication.

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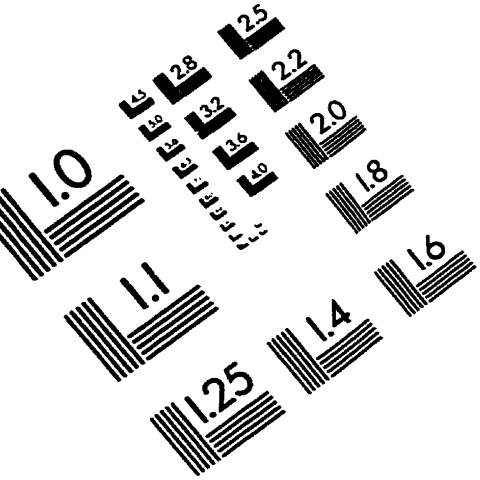

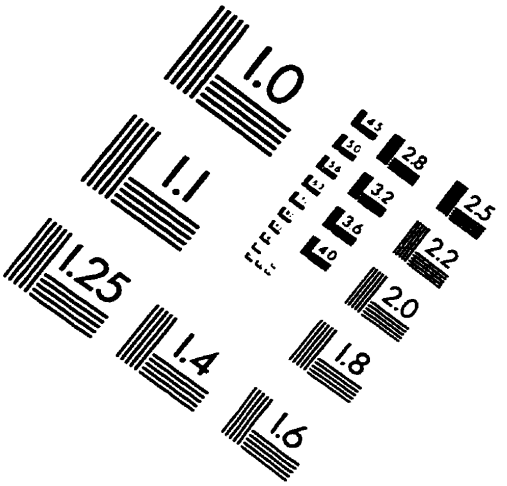

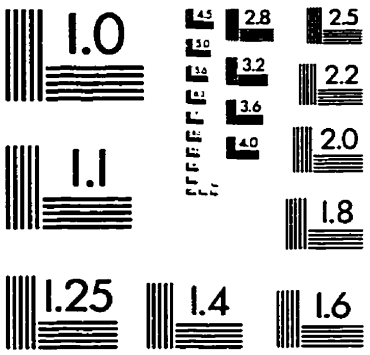

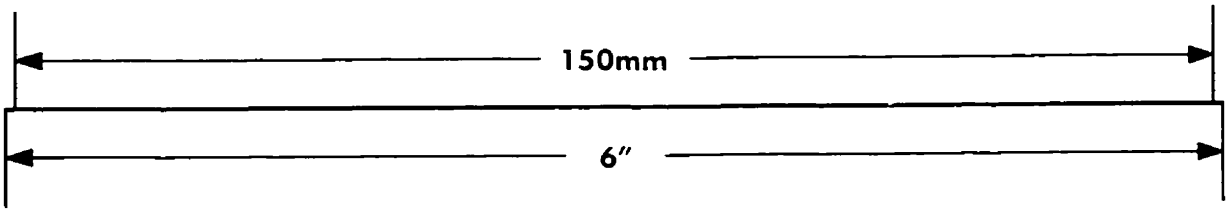

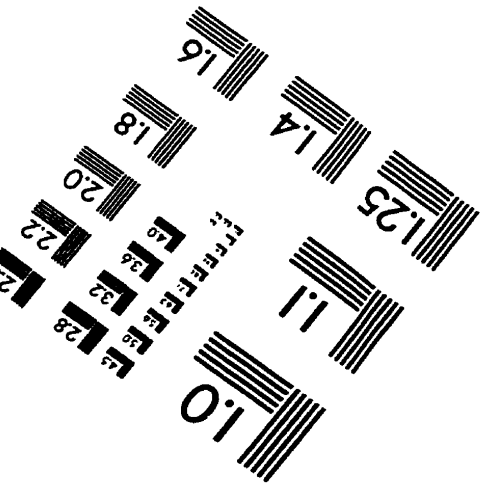

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