Pavlovian Conditioning of LPS Induced Responses: Effects on Corticosterone, Splenic NE, and IL-2 Production

by

Loren J. Janz

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ABSTRACT

Research has shown that cytokines such as interleukin-1, (IL-1) secreted by activated immune cells, can signal the central nervous system to initiate immunosuppression through immunoactive hormones such as corticosterone (Besedovsky, del Rey, Sorkin, & Dinarello, 1986) and sympathetic innervation of lymphoid organs (Brown et al., 1991). Infective agents such as lipopolysaccharide (LPS) can induce immune alteration, IL-1 secretion, and corticosterone production (Berkenbosch, de Rijk, del Rey, & Besedovsky, 1990). Further, both LPS and IL-1 administered intraperitoneally (IP) and intracerebroventricularly (ICV) induce corticosterone release and suppress peripheral interleukin-2 (IL-2) production; effects blocked by sympathectomy or the IL-1 antagonist, α melanocyte stimulating hormone (α MSH; Sundar et al., 1989; Sundar, Cierpial, Kilts, Ritchie, & Weiss, 1990). Based on reports of conditioned IL-1 induced corticosterone production (Dyck et al., 1990) and conditioned LPS induced fever responses (Bull, Brown, King, & Husband, 1991), the present study used a taste aversion paradigm to simultaneously condition LPS-induced alterations in IL-2 production, corticosterone production, and splenic norepinephrine (NE) content in rats.

The design included three groups of animals which received

saccharin and IP LPS in a paired or specifically unpaired manner. In the test the unpaired group (Unpaired) and one of the paired groups were re-exposed (Re-exposed) to the cue and the other not (Not Re-exposed). The remaining groups controlled for the unconditioned effects of LPS (UCS), cues, and fluid deprivation (Negative Control). A robust taste aversion was observed. Moreover, relative to the combined control groups, the Re-exposed group evidenced concurrent suppression of IL-2 production, reduced splenic NE content, and elevated corticosterone production. Due to a small sample size, the various LPS induced responses and conditioning effects did not significantly correlate with each other. Nevertheless, this is the first successful attempt to concurrently condition an immune response along with the hypothesized adrenocortical and sympathetic mediators of this response.

INTRODUCTION

Considerable evidence indicates that the immune system (IS; refer to the Appendix for a complete list of abbreviations used) and the central nervous system (CNS) participate in bi-directional communication (cf. Besedovsky, del Rey, & Sorkin, 1984; Besedovsky et al., 1983; Besedovsky, del Rey, Sorkin, & Dinarello, 1986). Anatomic linkage between these two systems (Felten & Felten, 1991) as well as functional interactions (Ader & Cohen, 1985; Besedovsky, et al., 1983) have been demonstrated.

Anatomic studies have revealed that lymphoid organs, such as bone marrow, lymph nodes, and spleen, are sympathetically innervated (Felten & Felten, 1991). Further anatomical evidence is based on the presence of receptors in the brain for immune derived molecules such as interleukin-1 (IL-1; Farrar, Kilian, Ruff, Hill, & Pert, 1987), sympathetic input to both T-cells and macrophages[1] (Felten, Ackerman, Wiegand, & Felten, 1987), and the presence of catecholamine and

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1 T-cells are comprised of several subpopulations including helper, suppressor, and cytotoxic subsets. They differentiate within the thymus gland and operate against cells bearing intracellular organisms by the release of cytokines such as interleukin-2. They can also activate other cells such as macrophage. Macrophage are derived from bone marrow and are present in many tissues such as the lung, liver, and brain. Macrophage secrete many soluable products such as interferon and interleukin-1 which can enhance or suppress activities of other cells. Macrophage can also kill foreign microbes by phagocytosis and oxygen dependent mechanisms.

neuropeptide receptors on lymphocytes (Ottaway, 1991).

Functional CNS-IS interactions have been demonstrated using a variety of approaches. Lesions in the lateral septal and hippocampal areas of the rat brain have been found to result in altered humoral antibody responses (Nance, Rayson, & Carr, 1987). Other work has evaluated the immunological effects of pharmacologic agents which act primarily on the CNS. For example, Shavit, Terman et al. (1986) reported that a systemic injection of morphine suppressed natural killer cell (NK) activity. Intracerebroventricular (ICV) administration of morphine produced a similar suppression of NK activity which was blocked by pre-treatment with naltrexone, an opioid antagonist. NK activity was unaffected by the systemic administration of N-methylmorphine, an anolog of morphine which is incapable of crossing the blood brain barrier (Shavit, Depaulis et al., 1986). Taken together, both brain lesion and pharmacological studies indicate that CNS perturbations can alter peripheral immunity.

The bi-directional communication hypothesis predicts not only that CNS outputs can alter peripheral immune responses but also that immunoactive agents (e.g., cytokines, bacterial endotoxins) can alter CNS functioning. In this regard, the CNS signaling properties of cytokines such as IL-1 have been intensely studied. Such studies have shown that IL-1 induces a broad range of neurochemical changes

affecting central norepinephrine (NE), dopamine and seretonin activity (Zalcman, et al., 1992). In addition, IL-1 stimulates hypothalamic corticotropin releasing factor (CRF) release (e.g., Sapolsky, Rivier, Yamamoto, Plotsky, & Vale, 1987). Finally, the bacterial endotoxin, lipopolysaccharide (LPS), a potent pathogen and IL-1 stimulatory agent, can induce a series of reactions known as the acute-phase response, as well as two CNS mediated alterations; fever production and slow wave sleep (Krueger, Kubillus, Shoham, & Davenne, 1986).

The effects of aversive stressors on immune function represent another approach to demonstrate functional relationships between the nervous and immune systems. A variety of aversive stressors (e.g., electric shock) have been shown to alter NK cell function (Shavit, 1991) as well as host-resistance to NK sensitive tumors (e.g., Greenberg, Dyck, & Sandler, 1984). Other reports indicate that shock suppresses splenic and peripheral blood lymphocyte responses to a nonspecific T-cell mitogen, an effect which can be blocked by administration of β-adrenergic antagonists (Cunnick, Lysle, Kucinski, & Rabin, 1990). Finally, Wan, Vriend et al. (1993) recently demonstrated that splenic deafferentation was able to block the stress-induced suppression of the plaque-forming cell and T-cell mitogen responses of splenocytes.

Arguably the most dramatic evidence for functional CNS-IS interactions comes from the conditioned alteration of immune

responses. Through associative learning, immunologically neutral environmental cues come to alter peripheral immunity. The most common conditioning procedure uses a conditioned taste aversion paradigm in which a novel tasting solution is paired with an immunopharmacologic stimulus such as cyclophosphamide (CY) or polyinosinic polycytidylic acid (poly I:C). A number of studies indicate that specific parameters of the immune system can be altered by this paradigm (cf. Ader & Cohen, 1991 for a review). Other recent work with autoimmune diseased animals indicates that these animals may be sensitive to immuno-dysregulation and hence learn to adjust their behavior accordingly (cf. Ader & Cohen, 1991; Ader, Grota, Moynihan, & Cohen, 1991).

In the next section, an overview is provided of the literature on conditioned immunomodulation. We will begin with a description of the Pavlovian procedure, summarize early work in the area, and then review the contemporary literature.

CONDITIONED IMMUNOMODULATION

The Pavlovian Procedure

In the Pavlovian paradigm an immunomodulatory agent (unconditioned stimulus; UCS), which unconditionally elicits some immune alteration (unconditioned response; UCR), is paired with an immunologically neutral stimulus (conditioned stimulus; CS) during the training phase. In a subsequent test phase the CS is presented alone, and changes in the immune response produced by cue exposure (conditioned response; CR) in the paired group is assessed relative to controls. The controls typically include groups which are conditioned but not re-exposed to the CS, an unconditioned group which receives either the UCS only, or the CS and UCS in an unpaired manner, as well as positive and negative controls (e.g., Ader & Cohen, 1975; Janz et al., 1991). A positive control group receives the UCS alone in order to evaluate the UCR. A negative control group typically receives a placebo in conjunction with CS exposure in order to establish a baseline measure. While conditioning is most commonly inferred from responses to the CS alone, in some instances a modified UCR to cued versus uncued presentation of the UCS (e.g., conditioned tolerance) has also been presented as evidence of conditioning (Dyck, Greenberg, & Osachuk, 1986; Dyck, Driedger, Nemeth, Osachuk, & Greenberg, 1987).

A brief overview of the conditioned immune alteration literature will now be presented.

Early Conditioning Studies

The earliest attempts to condition immune responses using a Pavlovian paradigm can be found in the Soviet literature in the early 20th century. This literature has been summarized by Ader and Cohen (1985) as well as Spector (1987). These authors describe a series of experiments conducted by Metal'nikov and Chorine, early Russian investigators, who conditioned a number of specific (e.g., antibody levels) as well as non-specific (e.g., inflammatory) responses to CSs such as heat or scratches. For example, a conditioned elevation in peritoneal polymorphonuclear leukocytes was demonstrated by pairing a scratch (CS) with an injection of a foreign protein immunogen (UCS). Following repeated CS-UCS pairings in training, the animals were re-exposed to the CS alone and again the polymorphonuclear leukocyte population in the peritoneal cavity was observed to increase. Later Russian investigators have successfully demonstrated conditioned alteration of immune responses in similar experiments and continue to do so (cf. Ader, 1981; Ader & Cohen, 1985; 1991; Spector, 1987). Although these early studies were lacking in methodological detail and sophistication,

the consistency of the evidence provided a foundation for further investigation. It was left to other investigators to implement the controls necessary to demonstrate conclusively the phenomena of conditioned alteration of immune responses.

Contemporary Studies of Conditioning

Contemporary studies of immunopharmacologic conditioning began with Ader and Cohen (1975). While studying conditioned taste aversion, these investigators found that the pairing of saccharin and cyclophosphamide (CY), an immunosuppressive and nausea inducing drug, resulted in not only the development of an expected conditioned taste aversion to the flavor of saccharin, but also an unexpected increase in mortality following CS re-exposure. Briefly, Ader and Cohen's paradigm consisted of the single presentation of a taste cue followed by the intraperitoneal (IP) administration of CY. Three days following conditioning the animals were immunized with sheep red blood cells and six days following immunization hemagglutinating antibody titres were measured. Comparisons were made between groups that were re-exposed to the CS either on the day of immunization or three days later and groups that controlled for the effects of prior conditioning per se, residual effects of CY, systemic or unconditioned effects of CY, handling effects (i.e., placebo treated animals), and effects of the CS (i.e.,

saccharin). It was found that conditioned animals re-exposed to the CS exhibited an attenuated anti-sheep red blood cell response in relation to animals that had been conditioned and not re-exposed to the CS as well as all nonconditioned control groups (cf. Ader & Cohen 1985; 1991).

This immunosupressive conditioning effect with CY has been replicated by Ader and Cohen (1985) and others (e.g., Rogers, Reich, Strom, & Carpenter, 1976; Wayner, Flannery, & Singer, 1978) and has also been observed under a variety of experimental manipulations with mice as well as rats (e.g., Schulze, Benson, Paule, & Roberts, 1988). The dose of CY and the CS solution have also been manipulated (Ader & Cohen, 1981; Wayner et al, 1978). The taste aversion paradigm has also been used to condition other humoral and cellular immune responses including plaque forming cells (Gorczynski, Macrae, & Kennedy, 1984), graft versus host responses (Bovbjerg, Ader, & Cohen, 1982; 1984), NK cell activity (O'Reilly & Exon, 1986), and arthritic inflammation (Klosterhalfen & Klosterhalfen, 1983; see also Ader & Cohen, 1991 for a review). These latter studies suggest that conditioning may be useful in treating dysregulated immunological processes.

The potential clinical significance of taste aversion conditioning with CY has been most dramatically illustrated by studies conducted with lupus-prone (NZB) mice (Ader & Cohen, 1982; Ader, Grota, & Cohen, 1987; Grota, Ader, & Cohen, 1987). Systemic lupus

erythematosus is a fatal autoimmune disease in these animals. Ader and colleagues have shown that the paired presentation of the immunosuppressive drug CY with saccharin, using dosages of CY which normally elicit a taste aversion in other species of mice such as C57BL/6, failed to do so in the NZB mice or in congeneic strains of lupus-prone mice that had manifest disease symptoms (Grota et al., 1987). Although deficits in acquiring an active avoidance response to electric shock in NZB mice has been reported (e.g., Nandy, Lal, Bennett, & Bennett, 1983), the failure of the mice in this study to develop a taste aversion per se (a passive avoidance response) may reflect a performance deficit in this conditioning protocol since comparisons between the two strains showed no difference in the development of an aversion to a novel flavor if lithium chloride, an aversive but immunologically inert compound, was used as the UCS. It was hypothesized that the failure of the lupus-prone mice to develop an avoidance response to a flavor paired with CY, a drug which enhanced their survival by suppressing immune function, reflects an adaptive ability of these animals to "recognize" their immune imbalance which is ameliorated by the effects of CY (cf. Ader & Cohen, 1991). Further evidence of this adaptive response was demonstrated with the early lupus-prone MRL lpr/lpr and late lupus prone (or lupus resistant) MRL +/+ strains (Ader et al., 1987; Grota et al., 1987). Prior to the onset of

lupus symptoms both strains of mice displayed an equal propensity to develop a taste aversion to a novel flavor paired with CY. After the onset of disease however, only those with manifest symptoms failed to acquire a taste aversion.

These investigators reasoned that if the absence of a taste aversion in these animals represented an immunorestorative or adaptive response to rectify hemeostatic imbalance (therapeutic effects), then perhaps animals with disease symptoms would voluntarily consume a flavored solution containing CY. It was found that while male animals with symptoms of disease drank more of the CY solution than male animals without symptoms, there was no significant difference for females. However, among animals voluntarily drinking the CY-laced solution, sufficient quantities were consumed to attenuate symptoms of the disease regardless of gender. Taken together, these authors (cf. Ader & Cohen, 1991) propose that lupus-prone animals have the ability to associate a distinctive flavor with the therapeutic effects of CY and are therefore acting to restore homeostatic balance.

Conditioned Enhancement

Although the vast majority of conditioned alterations of immune responses have been immunosuppressive, there have been several reports of conditioned enhancement and compensatory responses as

well (cf. Ader & Cohen, 1991). An enhanced immune CR has been observed with a delayed type hypersensitivity (DTH) response. A DTH response occurs during graft rejection. Following an initial graft a subsequent graft bearing the same antigenic markers (i.e., from the same donor) would be rejected immediately due to the host organism's previous sensitization by the first graft. Temporal manipulation of the UCS presentation has impacted on the dynamics of the response. For example when a particular dose of CY is injected at the time of sensitization, the DTH response is enhanced to subsequent antigenic challenge (Turk & Parker, 1982). On the other hand, if sensitized animals are presented with CY just before the second antigenic challenge then a suppressive DTH response is elicited (e.g., Rodinone, Giovaniello, Barrios, & Nota, 1983). Other manipulations of the temporal arrangement of the CS with sensitization and antigenic challenge have resulted in mixed enhancing and suppressive DTH responses (Bovbjerg, Cohen, & Ader, 1987). The enhanced DTH response to cues paired with CY may reflect an abrogated suppressor cell function to CY alone (e.g., Shand & Liew, 1980) and thus the enhanced response demonstrated by Bovbjerg et al. (1987) may be a result of a conditioned suppression of a subset of T-cells. Elevated T-helper:suppressor cell ratios have been conditioned in a taste aversion paradigm and evidence suggests that this result reflects the depletion of

the cytotoxic/suppressor subset (Husband, King, & Brown, 1986).

In another demonstration of an enhanced cellular response, Solvason and colleagues (Solvason, Ghanta, & Hiromoto, 1988) have conditioned elevations of NK activity to a compound CS (involving saccharin and LiCl) or odor cues which were previously paired with poly I:C, an NK cell stimulating drug, or interferon-ß. These results are controversial due to replication difficulties encountered by other investigators (cf. Ader and Cohen, 1991).

Of particular clinical interest in the area of conditioned immunoenhancement is a study by Gorczynski, Macrae, and Kennedy (1982) in which an antigen rather than an immunomodulatory drug was used as the UCS. These authors paired environmental cues consisting of the surgical procedure (CS) involved in repeated skin grafting (UCS). Upon presentation of the cue alone (i.e., shaving, anesthetizing, sham grafting, and bandaging) approximately 50-60% of conditioned mice demonstrated a conditional elevation of cytotoxic T-lymphocyte precursors. More traditional Pavlovian conditioning procedures (i.e., repeated CS-UCS pairings) have also been used to demonstrate conditioned elevations of histamine, a product of a variety of cells including mast cells, (Russell et al., 1984) as well as rat mast cell protease II, an enzyme involved in the mediation of mucosal mast cell function (MacQueen, Marshall, Perdue, Siegel, & Bienenstock, 1989).

In all of the studies summarized thus far the conditioned response has resembled the effects elicited by the immunopharmacological UCS. In the next section we describe a contrasting pattern of results which suggest that the conditioned immune response may sometimes be opposite in direction to the UCR.

Compensatory Response Conditioning

The most common result in Pavlovian conditioning studies is that the CR resembles the UCR. However, this result is not inevitable. It has been reported that the CR may oppose the UCR in some situations, including when pharmacological UCSs are used. The best example of this so-called "compensatory response conditioning" is to be found in the morphine conditioning literature. A number of studies by Siegel (1976; 1977) have shown that a stimulus previously paired repeatedly with morphine elicits a reaction which opposes the analgesic and thermic properties of the drug and contributes to the phenomenon of tolerance. Conditioned compensatory immune responses have also been reported with both immunosuppressive drugs such as CY and immunostimulatory drugs such as poly I:C. In an instance of the first type of study, Krank and MacQueen (1988) investigated the effect of environmental cues or a combination of environmental cues and a taste stimulus paired with CY on the development of a conditioned antibody

response. It was reported that antibody levels in animals re-exposed to the CS were not different from saline controls but were higher than animals not re-exposed to the cues as well as animals receiving unpaired presentation of the CS and UCS in training. Since the conditioned and re-exposed group did not differ from baseline, these results do not provide direct evidence of compensatory conditioning (i.e., conditioned immunoenhancement). However, indirect support for a compensatory mechanism can be made on the basis of the relatively elevated antibody titres of the conditioned and re-exposed group relative to not re-exposed and unpaired groups. In a similar study, MacQueen and Siegel (1989) reported that conditioned rats which were re-exposed to a taste CS previously paired with CY displayed an enhanced antibody response to sheep red blood cells in relation to other conditioning controls but not in relation to a negative control. As in the previous report by Krank and MacQueen (1988) these results provide only indirect support for a compensatory conditioning analysis.

The aforementioned observations are puzzling. First, they are inconsistent with a number of studies showing immunosuppression using a very similar conditioning protocol (cf. Ader & Cohen, 1991). Secondly, insufficient data has been presented to explain the inference of a compensatory response, particularly in light of the failure to demonstrate differences between conditioned and re-exposed animals in

relation to negative controls. It is evident that further research is necessary to develop an understanding of the mechanisms involved in this response and thus clarify how a compensatory conditioning response could develop after a single CS-UCS exposure (e.g., MacQueen & Siegel, 1989).

Ader and Cohen (1991) conclude that the most direct and internally consistent evidence for compensatory immune response conditioning are the studies showing tolerance of poly I:C induced NK cell activation by Dyck, Greenberg, and colleagues. Modeled on reports of conditioned tolerance to various effects of morphine (e.g., Siegel, 1976; 1977), Dyck et al. (1986) demonstrated that repeated paired exposure to environmental cues and poly I:C resulted in a tolerance effect as reflected by suppressed stimulation of NK cell activity to cued presentation of the drug. This effect was shown to be reversible by extinction and latent inhibition. Further, extinction of NK cell tolerance to poly I:C was found to be specific to the environmental stimulus that had been paired with the drug during acquisition (Dyck et al., 1987).

The aforementioned results are consistent with the analysis of conditioned tolerance developed by Siegel (e.g., 1983). According to this analysis, associative factors can contribute to drug tolerance through the development of an underlying drug-compensatory CR which opposes the unconditional effects of the drug. Applied to the results of Dyck,

Greenberg and colleagues, NK tolerance to poly I:C. reflects the development of drug-compensatory CRs that minimize the immunostimulatory effects of the drug.

Conclusion

It is implicit in all studies demonstrating conditioned immune responses that efferent CNS signals can affect immunity based on previously acquired associations. Although the mechanisms remain obscure, through the process of association, previously immunologically neutral environmental cues can alter peripheral immunity. It is important to note that most conditioned responses reported have been immunosuppressive in nature (cf. Ader & Cohen, 1991). It therefore seems prudent to investigate potential mechanisms mediating conditioned immunosuppression with the goals of identifying the CNS-IS pathways involved in immunoregulation and host resistance to disease.

MECHANISM OF IMMUNE SYSTEM CONDITIONING

Introduction

The mechanisms involved in the conditioned modification of immune responses are at present not well understood. Central to a conditioning analysis are three assumptions. First, it is assumed that conditioning is a CNS phenomenon involving the integration and association of perceptual and interoceptive signals with physiologically salient events. Second, it is assumed that interoceptive messengers derived from the immune system must be capable of signaling the CNS and third, that the CNS activity can modify peripheral immune responses. On this line of reasoning, a conditioned immune response requires CNS integration of the perceptual signal (CS) and association of this signal with the CNS-signaling properties of an immunoactive stimulus (UCS) such that, upon re-exposure to the CS, peripheral immune function is altered by the CNS (CR) in anticipation of the CNS-signaling effects of the UCS (cf. Dyck & Greenberg, 1991). Assumption one is definitional and below we consider the empirical evidence for assumptions two and three.

Central Nervous System Signaling by the Immune System

Perhaps the earliest evidence that immune activation can result

in CNS signaling was provided by Besedovsky, Sorkin, Felix, and Haas (1977). These authors demonstrated increased neuronal firing rates and decreased norepinephrine levels (Besedovsky et al., 1983) in the hypothalamus during the peak antibody response to immunization. They also found that soluble products called cytokines released from activated immune cells can induce a number of neurochemical and neurohormonal changes. One such peptide, IL-1, has been proposed to act as an important immune-derived afferent messenger to the CNS (Sapolsky et al., 1987; Berkenbosch, van Oers, del Rey, Tilders, & Besedovsky, 1987). Evidence in support of this proposal is derived from the observation that supernatant fluid from homogenates of virally infected mouse spleen cells or human peripheral blood leukocytes, stimulated the pituitary adrenal response. Further, the pituitary adrenal response was prevented by the immuno-neutralization of this supernatant fluid with an antibody specific to IL-1. Finally, it was also observed that the supernatant-induced pituitary adrenal response was mimicked by IP injections of small doses of purified IL-1ß or recombinant IL-1ß (Besedovsky et al., 1986; Besedovsky & del Rey, 1987). It is believed that one of IL-1's central effects is the stimulation of CRF release from the hypothalamus which in turn elicits the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. Since ACTH induces adrenal steroidogenesis resulting in increased levels of

the immunosuppressive glucocorticoid corticosterone (e.g., Besedovsky et al., 1986), a neurohormonal bi-directional feedback mechanism between the CNS and IS is supported (cf. Berkenbosch, de Rijk, del Rey, & Besedovsky, 1990).

In addition to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, IL-1 also induces a broad range of neurochemical changes affecting central NE, dopamine, and seretonin secretion (Dunn, 1992; Zalcman et al., 1992). As shown in the next section, there is now evidence that immunosuppression can result from CNS output through sympathetic fibers which innervate a variety of lymphatic organs as well as lymphocytes, mast cells, and macrophages in immune organs (Felten et al., 1987).

Immune System Signaling by the Central Nervous System

In addition to the neuroendocrine pathway described above, anatomical studies by Felten and colleagues (e.g., Felten & Felten, 1991) indicate that the spleen is sympathetically innervated which suggests a possible neural pathway involved in immunoregulation. Functional studies in our own laboratory (Brown et al., 1989; 1991; Vriend et al., 1993) as well as studies by Sundar and colleagues (Sundar et al., 1989; 1990), have corroborated these anatomical results. In an examination of the immunoregulatory pathways that may be important for the

IL-1-induced CNS regulation of macrophage function, Brown et al. (1989; 1991) observed that splenic macrophage IL-1 secretion was suppressed by ICV infusion of 5 to 100 ng of IL-1 and was coincidental with increases in the secretion of ACTH and corticosterone. Adrenalectomy completely reversed the suppressive effect of central IL-1 and cutting the splenic nerve was as effective as adrenalectomy. Combined nerve section and adrenalectomy resulted in enhanced IL-1 secretion from macrophages stimulated by lipopolysaccharide (LPS). Of further interest, nerve section alone enhanced LPS responses in spite of the presence of high levels of serum corticosterone. More recent work by Vriend et al. (1993) indicates that ICV IL-1 increases norepinephrine turnover in the spleen during the period in which IL-1-induced immunosuppression is known to occur. These experiments confirm and extend earlier work by Sundar et al. (1989) who identified an immunosuppressive effect on NK cell activity, phytohemagglutinin responsiveness, and interleukin-2 (IL-2) production in animals given ICV IL-1 and LPS. This effect was abrogated by α-MSH, an IL-1 antagonist which blocks many of its biological activities, but was only partially reversed by adrenalectomy.

In anticipation of the results from the above studies, Greenberg, Dyck and colleagues (e.g., Dyck & Greenberg, 1991) proposed that Pavlovian conditioning of immunity is mediated by immunomodulatory

CNS signals, such as those induced by IL-1, cued by environmental events previously paired with the immunomodulation. The pathways utilized by cued signals were hypothesized to be the same as those used by IL-1. Therefore, it was postulated that alterations in specific brain areas and neural pathways by unconditioned stimuli during immune conditioning are reinstated by cues inducing conditioned immune responses. Given the central role of IL-1 in immunoregulation, the next section provides an overview of the structure, sources, and functions of this cytokine.

Effects of IL-1

Interleukin-1 is a 17kd peptide existing in α and β forms. While sharing only 26-30% homology, these types bind to many of the same receptor sites but their affinities for a particular receptor and their systemic effects differ substantially (cf. Berkenbosch et al., 1990). Primary sources of IL-1 include blood monocytes, phagocytic cells of the liver and spleen, and various other mononuclear cells related to macrophage. IL-1-producing cells such as astrocytes and microgila are also found in the CNS. Endogenous IL-1 production and release is stimulated early in an immune response to trauma or infective agents such as bacterial endotoxin. IL-1 has a primary role in the enhancement of T-cell responses, induction of IL-2, and synthesis of

acute phase proteins. Further, injections of IL-1 in experimental animals results in hypozincemia, hypoferremia, increases in circulating interleukin-6 (IL-6) and colony stimulating factors, and anorexia. (cf. Berkenbosch et al., 1990; Dinarello, 1984; 1991 for reviews). That IL-1 acts centrally is demonstrated by its ability to induce fever and slow wave sleep, alter hypothalamic electrical activity and NE turnover, and stimulate CRF and pituitary ACTH release (Sapolsky et al., 1987; Tobler, Borbley, Schwyzer, & Fontana, 1984; Berkenbosch et al., 1987; Besedovsky et al., 1986; Kabiersch, del Rey, Honegger, & Besedovsky, 1988). In particular, the hypothalamic paraventricular nucleus appears to mediate CRF release. It is also a target for IL-1 and antigen induced alterations in NE metabolism and electrical activity (Saphier, 1989; Carlson, Felten, Livnat, & Felten, 1987; Kabiersch et al., 1988). Many of these responses are mimicked during an endogenously generated immune response (Besedovsky, del Rey, Da Prada, & Keller, 1979; Besedovsky et al., 1977). Genetic evidence indicates that IL-1 and LPS can induce the expression of mRNA for the proto-oncogene proteins c-jun and c-fos in T-cells (Muegge, Williams, & Kent, 1989) and c-fos expression in the periventricular nucleus (Wan, Janz et al., 1993; Rivest, Torres, & Rivier, 1991).

Pavlovian Conditioning of IL-1 Mediated Responses

The idea that associatively mediated immunosuppression can be a result of IL-1 induced corticosterone production is supported by the finding that IP administration of IL-1, when paired with various cues (taste, odor and pharmacological), leads to conditioned elevations in corticosterone production (Dyck et al., 1990). In a subsequent study, however, ICV infusion of IL-1 paired with saccharin did not result in conditioned elevations in corticosterone despite behavioral evidence of a conditioned taste aversion (Janz et al., 1991). While procedural (forced exposure to saccharin versus choice of drinking fluid in the test) and species (mouse versus rat) differences in these respective conditioning studies could have been responsible for the differences observed in these experimental outcomes, it is also possible that the route of delivery is a critical variable in the mechanism mediating IL-1 induced signals to the CNS and the conditioning effects. It is a reasonable hypothesis that peripherally administered IL-1 may produce a more salient sensory signal which is more readily conditioned than central administration. Evidence consistent with this hypothesis indicates that prostaglandin E_2 (PGE_2), a pain-producing agent, may mediate some of the central effects of peripherally administered IL-1 (Katsuura, Gottschall, Dahl, & Arimura, 1988). Moreover the paucity of reports concerning pharmacological conditioning effects involving centrally administered UCSs may be indicative of the difficulty inherent in this procedure.

Another strategy to investigate IL-1-mediated responses is the use of drugs known to induce the production of IL-1. Since LPS stimulates IL-1 and has been used as a UCS to condition responses purportedly mediated by IL-1, a brief discussion of this drug follows.

LPS-induced Responses

LPS, or endotoxin, a major component of the outer membrane of gram-negative bacteria such as E. coli, is a pleiotropic stimulus for immune cells and has been implicated in the clinical syndrome of bacterial septic shock (reviewed in Lynn & Golenbeck, 1992). It has been shown to contribute to bacterially induced anorexia; an effect that may be mediated by IL-1 (Langhans, Harlacher, Balkowski, & Scharrer, 1990). Endotoxin is a potent activator of macrophages and induces the release of inflammatory mediators and cytokines such as IL-1, IL-6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β; reviewed in Andersson et al., 1992). Central effects of peripherally administered LPS include the induction of slow wave sleep, fever and the stimulation of the hypothalamic-pituitary axis. LPS also elevates catecholamine metabolism in the hypothalamus (Dunn, 1992) and induces central IL-1 and IL-1 mRNA (Ban, Hauor, & Lenstra, 1992). LPS injected centrally mimicks the ability of IL-1 to suppress peripheral NK cell activity and IL-2 production as well as stimulate corticosterone

release (Sundar et al., 1989). Since LPS induces the release of IL-1 and other cytokines with potent CNS signaling properties, it should therefore be a salient UCS for conditioning. In this regard, it is already known that IP LPS will support a conditioned taste aversion in rats (Tazi, Dantzer, Crestani, & Le Moal, 1988) as well as conditioned thermoregulatory reactions (Bull, King, Pfister, & Singer, 1990; Bull, Brown, King, & Husband, 1991)

THE PRESENT STUDY

It has been shown that LPS, an IL-1 inducing compound, can suppress IL-2 production (Sundar et al., 1989), stimulate corticosterone release and alter norepinephrine turnover in the spleen (Pardini, Jones, & Filkins, 1983). The present study was therefore designed to test the hypothesis that LPS-induced alterations in IL-2 production can be conditioned using a taste aversion paradigm, and that these alterations are accompanied by concurrent changes in adrenocortical and sympathetic nervous system activity. Although a conditioned taste aversion (e.g., Tazi et al., 1988) and a conditioned thermoregulatory response (e.g., Bull et al., 1991) to IP administration of LPS have previously been reported, no studies have attempted to condition IL-2 production to this UCS. Nor has there been any attempt to concurrently measure conditioned alterations of corticosterone and splenic norepinephrine content, which may mediate LPS induced immunosuppression.

This study examined the effect of pairing a distinctive gustatory cue (saccharin in the drinking water) with IP administration of LPS on the development of conditioned corticosterone, NE, and IL-2 responses using the one-bottle test procedure that was previously successful in conditioning corticosterone responses in mice (Dyck et al., 1990). In the

training phase, two groups of rats received saccharin followed by the drug. In the test phase, one of the groups was re-exposed (Re-exposed) to the cue and the other not (Not Re-exposed). A third group received the drug and cue in an unpaired manner and was tested in the presence of saccharin (Unpaired). An additional group controlled for water deprivation, injection ritual, and cue presentation (Negative Control). Finally, a group was included to asses the unconditioned effects of LPS in conjunction with water deprivation (UCS). Group Re-exposed was run to evaluate the potential of a conditioned elevation of plasma corticosterone levels and suppressed splenic NE and IL-2 levels in relation to the Unpaired, Not Re-exposed, and CS Groups. The Unpaired and Not Re-exposed Groups were not expected to show any difference in corticosterone, IL-2, or NE levels relative to the CS Group which received saline injections and cue exposure in the training and test trials. It was hypothesized that relative to controls, the group receiving paired presentation of saccharin and LPS would display conditioned elevations of plasma corticosterone and suppressed splenic NE and IL-2 levels when re-exposed to the cue. It was also predicted that conditioned animals would show an aversion to the saccharin flavor as evidenced by suppressed fluid consumption in comparison to groups receiving saccharin paired with saline or unpaired with the drug.

METHOD

Subjects

The subjects were 47 experimentally naive, male, 300-350g Sprague Dawley rats acquired from the Charles River breeding facility. The animals were housed individually in stainless steel, wire mesh cages (20 cm wide x 25 cm long x 18 cm high) and maintained on a 12-hr light cycle beginning at 6:00 a.m. Food and water was available *ad libitum* until 3 days before the experiment, at which time fluid was available each day for a 30 min period between 7:00 and 8:30 a.m.

Apparatus

The conditioned stimulus was 0.15% w/v saccharin in the drinking water. A one-bottle watering procedure described previously (Dyck et al., 1990) was used. The bottles were graduated at 2 ml intervals and equipped with bent glass spouts designed to minimize spillage. Fluid was available throughout the entire 30 min drinking period during habituation and throughout the entire experiment. The unconditioned stimulus consisted of IP injections of 50 μg LPS (E. coli 055:B5 Sigma, St Louis, MO) in 200 μl lactated Ringer's solution (saline). Preliminary dose-response experiments indicated that this dose elevated plasma corticosterone (depicted in Figure 1), suppressed splenic

lymphocyte IL-2 production (depicted in Figure 2), and splenic NE content (depicted in Figure 3). Additional time course experiments indicated that IL-2 (Figure 2) and corticosterone levels (Depicted in Figure 4) returned to baseline measures within 72 h. Therefore a 4 day inter-stimulus interval was established between the cue and drug presentation for the Unpaired Group, and a 5 day interval between training and test for conditioning groups. Since associations in TA learning are known to develop over considerable CS-UCS intervals (Hiramoto et al., 1992) the separation of the stimuli by 4 days was designed to minimize this possibility.

Procedure

Upon arrival to the laboratory the animals were randomly assigned to one of the five groups. Each group was subdivided into three equal sub-groups with the exception of one sub-group in each of Groups Re-exposed and Unpaired which had an additional animal. Thus three replicates, with all groups represented in each replicate, were formed and run on consecutive days. The experimental manipulations were conducted on each day in the order depicted in Table 1. Habituation to water deprivation began seven days following arrival and lasted for three days (days -3, -2, and -1). During this time, and throughout the entire experiment, each animal received fluid for 30 min between 7:00

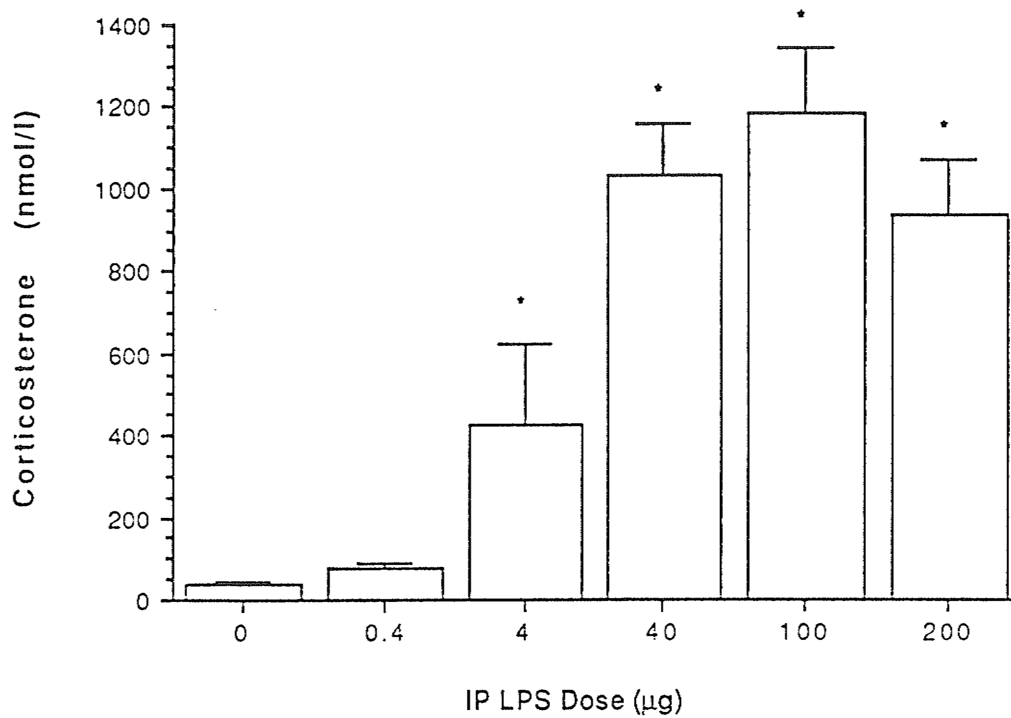

Figure 1. Dose response relationship between IP injection of LPS and plasma corticosterone levels three hours post injection. Values are illustrated as means (± SEM) and the number of animals per group was 5-6. Based on this relationship a 50 µg dose was selected. *Denotes significantly different from 0 (saline) and 0.4 µg doses (*p* <.05).

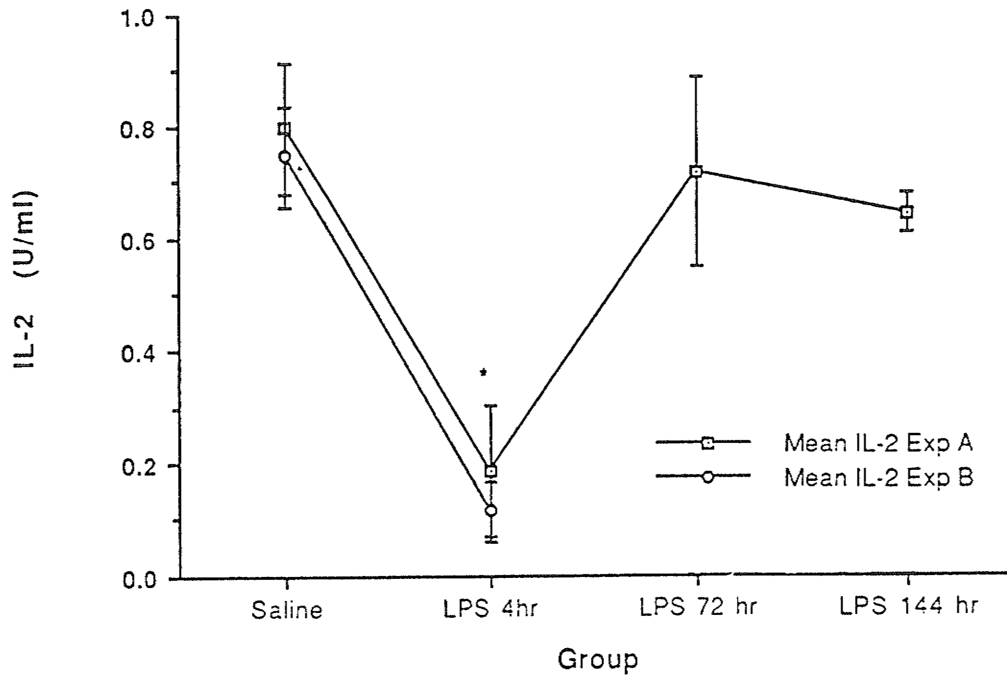

Figure 2. Time course of whole population splenocyte IL-2 secretion to IP administration of 50 μg of LPS per rat in experiment A. Experiment B replicated the 4 hr time point. Values are illustrated as means (± SEM) with 4-5 animals per group. Based on this evidence, a four hour time point was selected as the interval between cue exposure and sacrifice in the test trial. *Indicates significantly different from saline in Experiment A (p <.05).

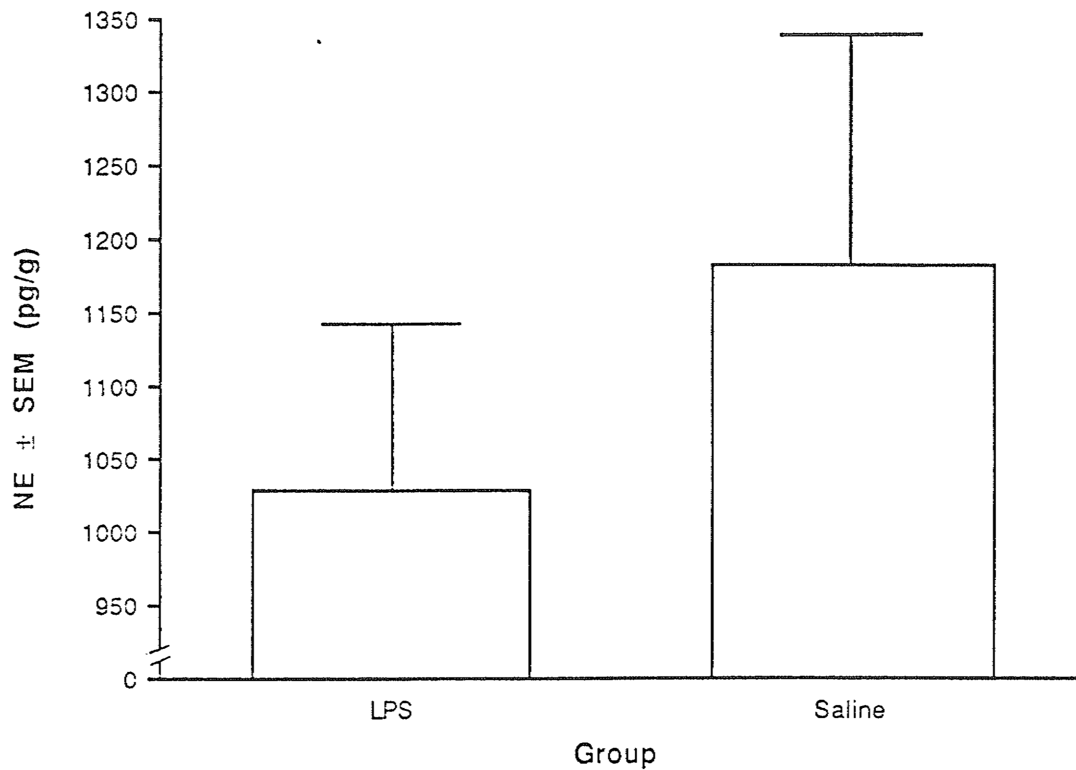

Figure 3. Splenic NE content following IP LPS (50 µg/rat) expressed as mean concentrations (± SEM) of NE per gram wet tissue weight with 4-5 animals per group. Spleens were collected 4 hr post injection. Differences are not significant.

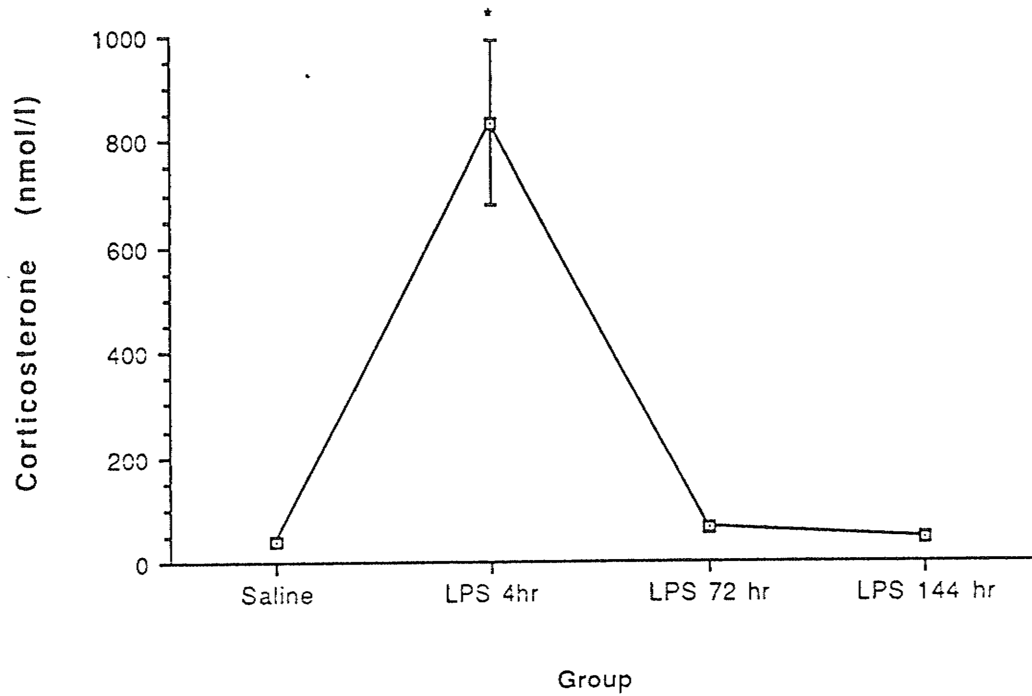

Figure 4. Time-course relationship between plasma corticosterone levels and post injection interval following 50 μg IP LPS per rat. Values are expressed as means (± SEM) with 4-5 animals per group. *Denotes significant difference from all other points (p <.05).

and 8:30 a.m. After the graduated bottles had been removed they were visually inspected to determine the amount of fluid consumed and the data recorded. During habituation all animals were handled following fluid exposure in a manner mimicking experimental manipulations.

On the fourth day (day 0), the conditioning trial was conducted. Groups receiving cue exposure had a 0.15% w/v saccharin solution available for the entire 30 min drinking period while animals not receiving CS exposure were given plain water. On conditioning day, after bottle removal and recording of fluid consumption, all animals except those in the UCS Group were injected IP with either saline or LPS. Following injection the animals were replaced in their cages and returned to the shelf. Non-injected animals (Group UCS) simply had their bottles removed and consumption recorded after fluid exposure.

As shown in Table 1, the conditioned and re-exposed group (Re-exposed) as well as the conditioned and not re-exposed group (Not Re-exposed) received saccharin on the conditioning day (Day 0) followed by an LPS injection. The unpaired group (Unpaired) received plain water followed by an LPS injection. The injection, cue, and water deprivation control group (Negative Control) received saccharin followed by a saline injection while the UCS Group simply received plain water. On days 1-4 plain water was available during the 30 min drinking period

TABLE 1

Conditioning Protocol

Group	n	Treatment (day) Habituation (-3 to -1)	Train (0)	NoTreat[a] (+1 to +3)	Unpair[b] (+4)	Test (+5)
Re-exposed	10	H_2O[c]	Sac/LPS[d]	H_2O	H_2O	Sac/Sal[e]
Unpaired	10	H_2O	H_2O/LPS	H_2O	Sac	Sac/Sal
Not Re-exposed	9	H_2O	Sac/LPS	H_2O	H_2O	H_2O/Sal
UCS	9	H_2O	H_2O/nil[f]	H_2O	H_2O	H_2O/LPS
Negative Control	9	H_2O	Sac/Sal	H_2O	H_2O	Sac/Sal

[a]Animals were left undisturbed in their home cages between training and test. [b]Unpaired Group received saccharin instead of H_2O. [c]30 min plain water. [d]30 min Saccharin exposure followed by IP LPS injection. [e]Saccharin followed by an IP saline injection. [f]No injection following 30 min water exposure.

with the exception of the Unpaired Group which received saccharin on day 4. On day 5 the test trial was conducted. Groups Unpaired, Re-exposed, and Negative Control received saccharin while the other groups received plain water. The UCS Group received an injection of LPS after water exposure while all other groups were injected with saline.

Four hours following test-trial injections the rats were decapitated and trunk blood collected in 10 ml tubes containing 200 μl 0.5 *M* EDTA (pH 8.0). The blood was centrifuged at 3000 RPM for 10 min and the supernatant removed and frozen until the corticosterone assay. Spleens were aseptically removed immediately following blood collection and one half placed in sterile RPMI 1640 on ice until the initial step in the IL-2 assay. The remaining half was rapidly frozen in liquid nitrogen and then maintained at -80° C for future NE analysis.

Corticosterone Radioimmunoassay. Vials of supernatants and standards were thawed and 10 μl added to 0.5 ml assay buffer (in distilled water, 0.82% anhydrous sodium acetate, 0.01% sodium azide, and pH adjusted to 5.2 with acetic acid. After pH adjustment 0.1% bovine serum albumin was added). Duplicate 100 μl volumes of unknown and standard dilutions were placed in glass tubes and 100 μl of antiserum was added (diluted 1:4 with physiological saline containing 0.1% sodium

azide then further diluted 1:100 with assay buffer). Following gentle mixing 100 µl of ^3H corticosterone (New England Nuclear, No. NET399; 250 µCi in 7.5 ml re-distilled ETOH then diluted with assay buffer so that 100 µl yielded approximately 25,000 cpm) was added to all tubes, mixed, and incubated at 4° C for 90 min. Following incubation, 1 ml of charcoal/dextran (0.025% dextran T-70 and 0.25% Norit A charcoal in assay buffer) was added to all tubes, mixed well, incubated at 4° C for 10 min, and then centrifuged at 3000 rpm for 15 min in a 4° C centrifuge. Supernatants were decanted into scintillation vials and 5 ml Universol (ICN Biomedicals, Irvine, CA) added. Vials were mixed vigorously for 1 min, allowed to stand for 2 hr at room temperature and then counted in a Beckman ß scintillation counter for 10 min or 10^4 counts.

NE Assay. The frozen spleens were weighed and homogenized in 0.1 *N* perchloric acid containing 0.1 m*M* EDTA to yield a final tissue concentration of 20 mg/ml. Homogenates were centrifuged at 1500 rpm for 10 min, and 1.0 ml of supernatant was taken for alumina extraction using the ESA plasma catecholamine methodology (Bedford, MA) with an internal standard. Samples were separated by HPLC using ESA Model 5700 solvent delivery system with a CSC-S ODS2 5µm column. Analysis and quantitation was accomplished with a ESA Coulochem 5100 A electrochemical detector and Shimadzu CR601 Chromatopac

integrator. Raw data were expressed as ng NE per gram wet tissue weight.

IL-2 Bioassay. Immediately after removal, spleens were disaggregated through stainless steel wire mesh and washed in RPMI 1640. Red cells were removed by NH4Cl lysis and remaining cells were washed, resuspended in RPMI 1640 supplemented with 10% fetal calf serum, 5 x 10-5 *M* 2-ß-mercaptoethanol, 1 x 10- 3 *M* sodium pyruvate, and 2 x 10- 3 *M* glutamine, and plated into 96-well plates at a concentration of 1 x 10^6 splenocytes/ml, 0.1 ml per well. Five μl of R73 antibody was immediately added at a concentration of 5 μg/ml . This monoclonal antibody, which recognizes a constant determinant of the rat T-cell antigen receptor and induces T-cell activation (Hunig, Wallney, Hartley, Lawetzky, & Tiefenthaler, 1989), is produced by a hybridoma cell line (supplied by T. Hunig, Munchen, Germany) and purified by ammonium sulphate precipitation. Following a 24 hr incubation at 37° C in 5% CO2 supernatants were removed and frozen at -80° C pending bioassay.

For the IL-2 bioassay the CTLL assay system was employed (Conlon, 1983). Briefly, CTLL-2 cells were maintained in RPMI 1640 with10% fetal calf serum and 3.5% IL-2 (Pharmacia, Columbia, MD). Prior to the assay the cells were washed four times to remove any trace

of IL-2 present in the growth medium and resuspended in RPMI at 10^5 cells/ml. Thawed supernatents and standards, which were prepared from IL-2 at known concentrations, were placed in 96-well plates at 100 µl/well, 100 µl of cells added and incubated at 37° C, 5% CO_2 for 24 hr.

Four hours prior to harvest, wells were pulsed with 0.5 µCi [^{3}H] thymidine and cells harvested onto glass fibre paper, dried, placed into scintillation vials and 5 ml Betamax (ICN Biomedicals, Irvine, CA) added. Incorporation was determined by counting disks in a Beckman ß scintillation counter. IL-2 concentration of the supernatant was determined by comparing the [^{3}H] TdR incorporation of the supernatant samples with the IL-2 standards and then converted to U/ml.

RESULTS

Taste Aversion

Suppressed saccharin consumption was observed in the group that received paired presentation of saccharin and LPS in training and was re-exposed to the cue in the test trial (i.e., Group Re-exposed relative to Groups Unpaired and Negative Control). A 3 (Groups) X 2 (Trials), mixed design with repeated measures ANOVA confirmed the presence of a significant Groups effect, $F(2,26) = 49.54$, $p < .0001$, Trials effect, $F(1,26) = 151.29$, $p < .0001$, and a significant interaction, $F(2,26) = 102.956$, $p < .0001$. The interaction, broken down using Dunn's procedure to control for error rate, revealed no significant differences in fluid consumption on the training day. However, on the test day the Re-exposed Group drank significantly less saccharin than the Unpaired and Negative Control Groups, respectively ($ps < .01$). In addition, Group Re-exposed drank significantly less saccharin in the test than in training ($p < .01$) while the reverse was true for the Unpaired and the Negative Control Groups ($ps < .01$ respectively) which were comparable to each other. The results clearly indicate that the conditioning protocol resulted in the development of a robust taste aversion. The saccharin consumption volumes in training and test (mean values ± SEM) are shown in Figure 5.

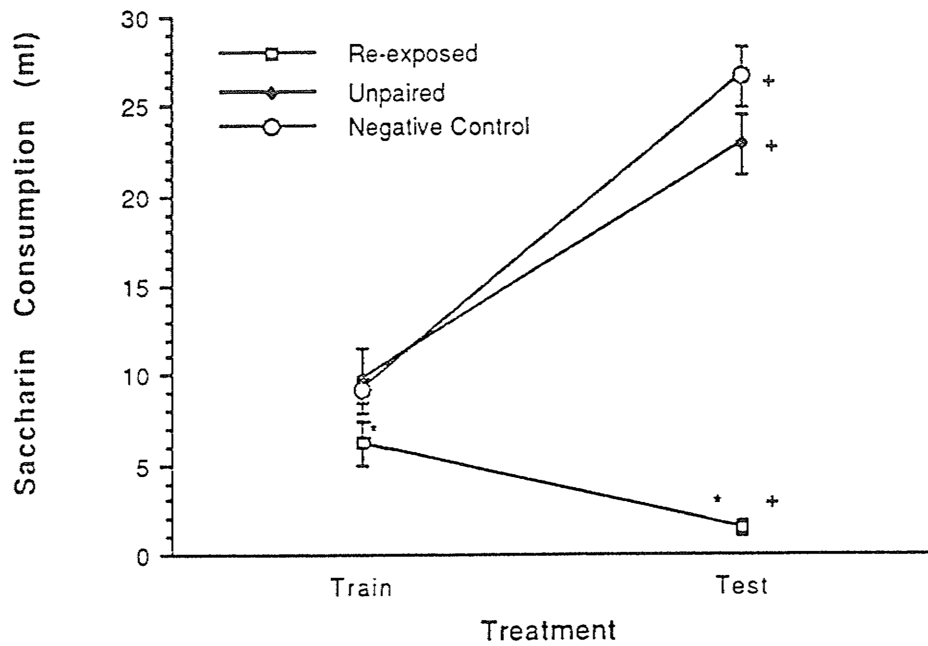

Figure 5. Effects of taste aversion conditioning protocol on saccharin consumption. The Re-exposed Group drank significantly less saccharin on the test trial in comparison with the training trial (*$p < .01$) as well as on the test trial in comparison with the Unpaired and Negative Control Groups (+ $ps < .01$).

Conditioning of Physiological Responses

The data on each of the physiological responses was analysed by initially conducting ANOVAs on all five groups. Following this, an ANOVA with only the three control groups (i.e., Not Re-exposed, Unpaired, and CS) was performed (IL-2; $F(2,24) = 1.49, p>.05$; Corticosterone; $F(2,25) = .29, p>.05$; NE; $F(2,22) = 1.33, p>.05$). Since the latter analyses did not indicate any significant differences between the control groups on any of the measures, they were combined and subsequently included in a three group ANOVA which compared the Re-exposed Group, Combined Control, and the UCS Group.[2] Combining the controls increased the statistical power for critical comparisons involved in the determination of conditioning effects (i.e., Re-exposed versus Combined Controls). Significant ANOVAs were followed by pairwise comparisons using Dunn's procedure ($\alpha<.05$).

IL-2 Production

In contrast to preliminary studies, LPS did not significantly suppress IL-2 production. While a one way ANOVA on all five groups revealed a significant groups effect, $F(4,38) = 2.87, p <.05$, a pairwise comparison indicated that IL-2 production in Group UCS did not differ

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2 Since there were some values that were unusable in each of the physiological assays due to assay difficulties, ns are marginally different for each of the analyses.

from Group CS. Furthermore, each pairwise comparison between the Re-exposed group and the individual control groups involved in demonstrating conditioned suppression of IL-2 levels approached but did not obtain significance ($.05 < ps < .10$). To further investigate this trend, an analysis of the Combined Control, Re-exposed and UCS Groups was conducted. This latter three group ANOVA again revealed a significant groups effect $F(2,40) = 4.01$, $p<.05$. This time, pairwise comparisons indicated that IL-2 levels in the Re-exposed Group were lower than in the UCS Group ($p < .05$) and marginally lower than the Combined Control Group ($p=.07$). Reasoning that the most relevant comparison to demonstrate the presence of a conditioned response is that involving the Combined Control and the Re-exposed Groups, and taking into account the directional nature of the hypothesis, a one tailed *t*-test was conducted. This comparison indicated a modest but significant suppression of IL-2 levels in the Re-exposed Group relative to the Combined Controls, $t(33) = 1.92$, $p < .05$, one-tailed. The IL-2 results comparing Group Re-exposed, Combined Controls and UCS are shown in Figure 6.

Corticosterone Production

An initial ANOVA on all five groups revealed significant differences in plasma corticosterone production, $F(4,40) = 132.334$, $p<.0001$. Subsequent comparisons revealed that LPS unconditionally

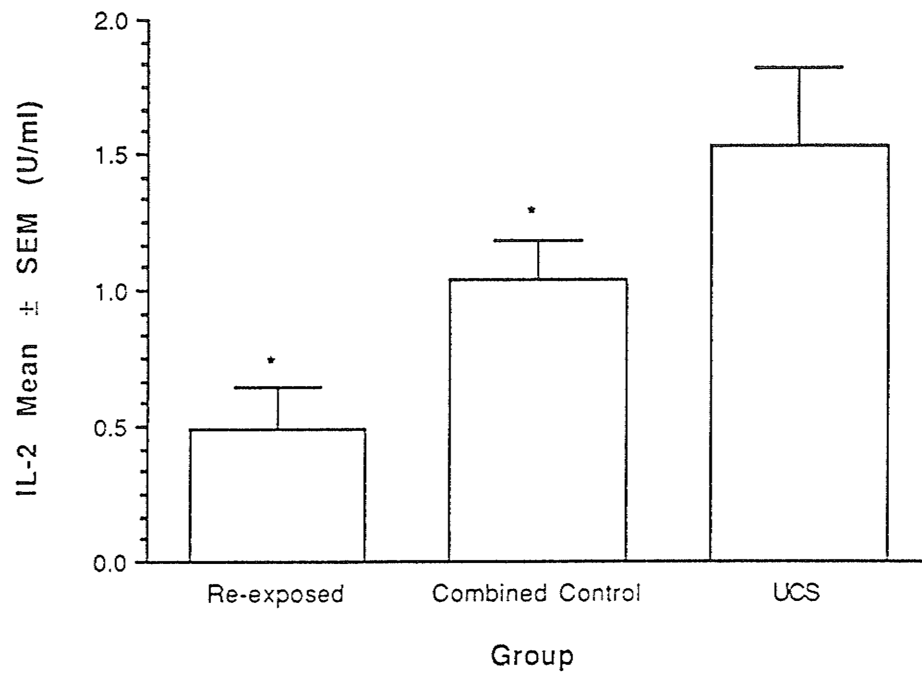

Figure 6. Effect of the conditioning protocol on suppression of splenocyte IL-2 production expressed as means (± SEM) in the Re-exposed Group in relation to the Combined Control Group, *denotes significant difference ($p < .05$, one-tailed t-test).

elevated corticosterone ($p < .01$), that each of the three control groups did not differ (Groups Unpaired, Not Re-exposed, and CS), and that each of the critical comparisons necessary to demonstrate conditioning approached but did not reach significance ($.05 < ps < .10$). A subsequent three group analysis (Group Re-exposed, Combined Controls and UCS) again resulted in a significant group effect $F(2,42) = 276.738$, $p < .0001$. More importantly, a subsequent comparison between the Re-exposed and the Combined Control Groups was signficant. Specifically, the Re-exposed Group exhibited significantly higher levels of corticosterone than did the Combined Controls ($p < .05$). Figure 7 shows mean corticosterone production for the Re-exposed, Combined Controls, and UCS Groups.

Splenic NE Content

In order to assess group differences in splenic NE content, a one-way ANOVA including all five groups was initially conducted. This analysis approached but did not reach significance, $F(4,36) = 2.50$, $p < .06$. However, significance was reached in the subsequent three group analysis conducted with the Re-exposed, Combined Control, and UCS Groups, $F(2,38)=3.48$, $p<.05$. Subsequent comparisons showed that NE content was significantly lower in the Re-exposed Group relative to the Combined Controls ($p < .05$). On the other hand, the UCS Group did not

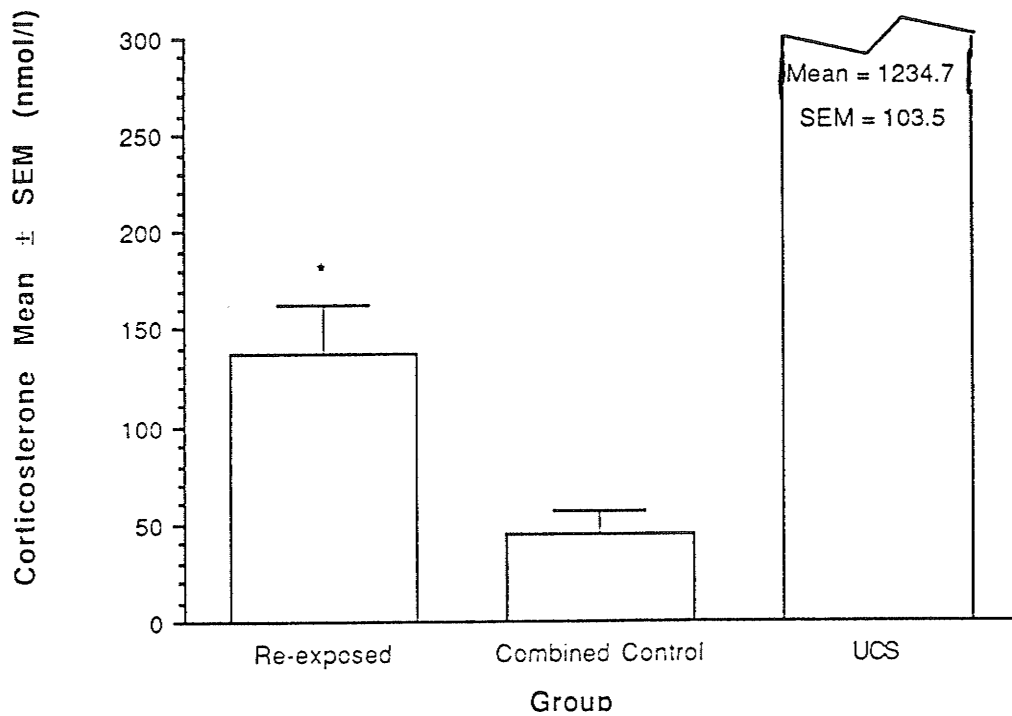

Figure 7. Effect of conditioning protocol on serum corticosterone levels 4 hr following re-exposure to the CS. Rats in the Re-exposed Group had significantly higher corticosterone levels than the Combined Control Group. *Denotes significantly different from the remaining groups (p <.05).

differ from the Re-exposed Group and only approached significance in the comparison with the Combined Controls (p=.07). As shown in Figure 8, while in the UCS Group, LPS only marginally reduced splenic NE content, the conditioning protocol in Group Re-exposed led to significant suppression. As with IL-2 production, the NE conditioning effect was more robust than the unconditioned effect.

Additional Analyses

A number of correlational analyses were conducted to evaluate whether IL-2 production in the conditioning group was significantly associated with either splenic NE content, corticosterone levels, or fluid consumption. However, in large part due to small sample size, no significant correlations were observed in this group. The single significant correlation occurred between IL-2 and corticosterone in the Not Re-exposed Group ($p < .05$, Fishers r to z). The lack of consistency in correlations across groups for these measures renders interpretation of this lone significant correlation difficult. For descriptive purposes these correlations are presented. Although some significant correlations were found for the sample as a whole, the confounding effect of different treatment groups precluded meaningful interpretation.

Finally, an analysis was conducted on changes in daily water consumption comparing animals that received LPS versus those that

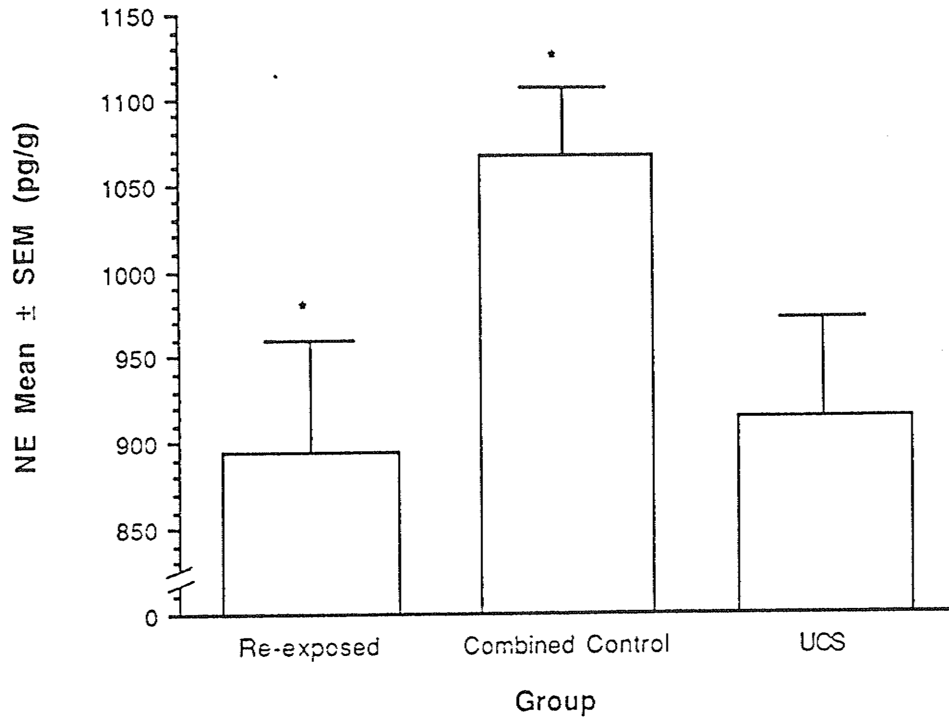

Figure 8. Conditioning protocol induced a significant suppression (denoted by *) of splenic NE content in the Re-exposed Group relative to the Combined Control Group (p <.05). Values are illustrated as group means (± SEM).

TABLE 2

Dependent Variable Correlations

	GROUP				
Correlations	Re-exposed	Unpaired	Not Re-exposed	UCS	CS
IL-2/Corticosterone	-.08	-.47	.84[a]	.05	-.06
IL-2/NE	-.60	-.18	.20	-.44	.21
Corticosterone/NE	.19	.15	.02	<.01	.10
Saccharin/IL-2	-.05	.24	—[b]	—	.51
Saccharin/Corticosterone	-.38	-.14	—	—	.02
Saccharin/NE	.14	-.16	—	—	-.37

[a]P<.05, Fisher's r to z all others p>.05. [b]Animals were not exposed to saccharin in the test trial.

did not on days where no experimental manipulations were performed (i.e., saccharin exposure or injection). Although no systematic changes were anticipated, a one-way ANOVA revealed a significant suppression of fluid consumption on the first day (Day +1) following training in animals that had received an LPS injection on training day (Groups Re-exposed, Unpaired, and Not Re-exposed) in comparison with animals that did not (Groups CS and UCS) $F(1,43) = 8.40, p<.01$. Since there were differences in the rate of acquisition of asymptotic drinking volumes during the habituation period between these two groups, an ANCOVA was subsequently conducted. In spite of a significant covariate on the first day of habituation (day -3; $F(1,43) = 9.61, p<.01$), the suppressed water consumption by the animals that received LPS in relation to those that did not on the day following injection remained significant. These results indicate that LPS reduced water intake on the day following injection regardless of whether or not it was paired with saccharin or water. However, this generalized suppression of water intake by LPS animals cannot account for any of the observed differences in saccharin consumption or physiological conditioning effects. Figure 9 illustrates water consumption on days where no manipulations took place.

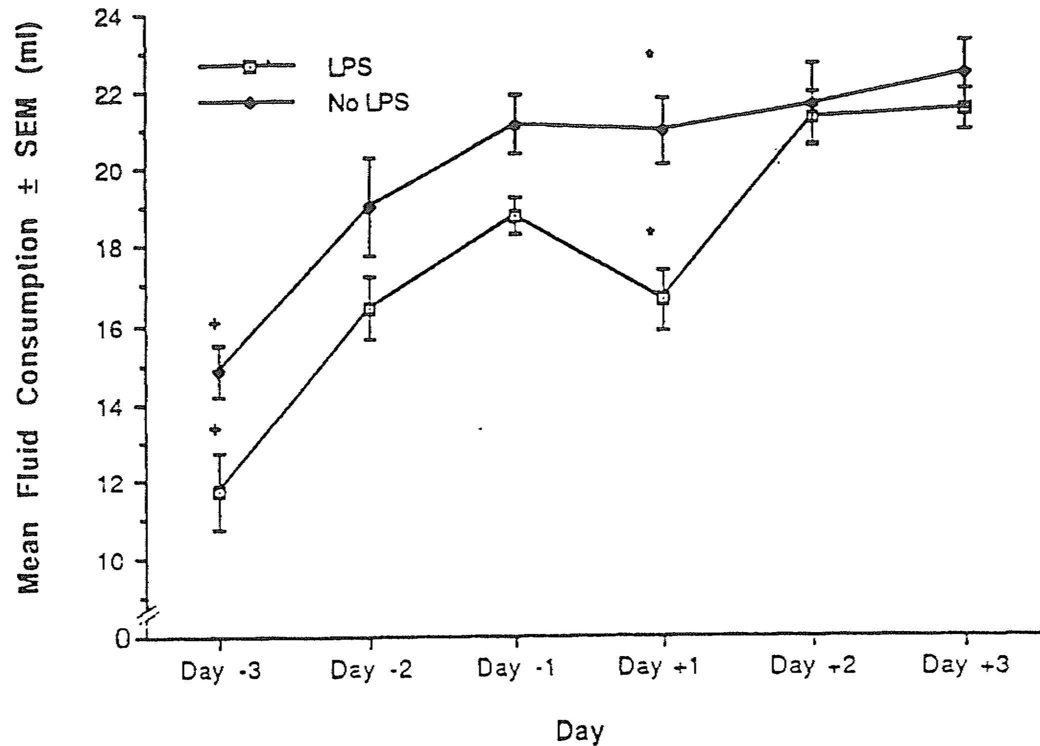

Figure 9. Mean water consumption (± SEM) during habituation and intervening days between trial and test. Animals that had received LPS in training (LPS) drank significantly less than those that did not (No LPS) on Day +1 following injection (p <.05 denoted by *). This result remained significant in spite of different rates of acquiring asympototic fluid consumption resulting in a significant covariate on Day -3 (p <.05 denoted by +).

DISCUSSION

Previous behavioral conditioning studies have indicated that LPS supports the development of taste aversion learning (Tazi et al., 1988) and fever response conditioning (Bull et al., 1990; 1991). The results of the present study add to the growing list of LPS induced responses which are sensitive to Pavlovian conditioning. Specifically, in addition to the development of a taste aversion, modest but consistent evidence was found for a conditioned alteration in glucocorticoid release, splenic NE content, and IL-2 production. Although the small sample size did not allow for statistically significant associations between these various responses, the results are consistent with the idea that conditioned suppression of IL-2 production was paralleled by similar conditioning of corticosterone production and sympathetic activity as reflected by altered splenic NE content.

A highly significant taste aversion was observed in the conditioned animals as a result of a single pairing of a novel flavor with an IP injection of LPS. These results support previous taste aversion studies with IP LPS (Bull et al., 1990;1991) and ICV IL-1 (Janz et al., 1991). The absence of a taste aversion in the unpaired group indicates that the interval of 4 days betweeen UCS and CS presentation was sufficient to prevent development of a conditioned taste aversion.

In addition to behavioral evidence for conditioning, the present results support a significant conditioned elevation of corticosterone to a taste cue previously paired with LPS. While conditioned elevations of corticosterone have been previously reported to cyclosphamide, lithium chloride (Ader, 1976), and IP IL-1 (Dyck et al., 1990), this is the first evidence for glucocorticoid conditioning to LPS. Since LPS-induced glucocorticoid responses are likely IL-1 mediated, (e.g., Berkenbosch et al., 1990; Dunn, 1992) the present results support and extend our previous glucocorticoid conditioning studies with mice where IP IL-1 and a combination of odor, taste, and pharmacological cues were used as CSs (Dyck et al., 1990).

While the corticosterone data support previous conditioning work with IP IL-1, it is noteworthy that another study from our laboratory which used ICV IL-1, and a two-bottle procedure did not find enhanced corticosterone production despite the observation of taste aversion learning (Janz et al., 1991). This suggests that the pituitary-adrenal response to the taste paired with LPS or IL-1 may, in part, reflect the conflict imposed by the one bottle test procedure used, argued previously by Smotherman, Hennessy, and Levine (1976). However, it is unlikely that the non-specific stress effects associated with the one-bottle test procedure can account for the conditioned alteration of immune responses observed here and elsewhere. For example, there are

numerous studies which have reported conditioned immunosuppression with CY using two-bottle procedures (cf. Ader & Cohen, 1991 for a review). Further, in many instances, a dissociation between conditioned immunological and overt behavioral responses has been observed (cf. Ader & Cohen 1991), regardless of whether a two or a one-bottle test is used. Taken together, these results argue against the notion that the stressful effects of the test procedure are sufficient to account for the observations of conditioned immunomodulation.

In addition to procedural variations in testing animals, differences in glucocorticoid conditioning effects may also be influenced by the route of administration (IP versus ICV). ICV administration was used in the Janz et al. study (1991) which did not result in a conditioned adrenal response. Differences in route of administration may be due to the involvement of different signalling pathways. Since studies have failed to demonstrate that peripheral IL-1 can cross the blood-brain barrier (Dinarello, 1991), the HPA conditioning effects to IP IL-1 and LPS may reflect the action of an unidentified intermediary such as PGE_2. The potential existence of such an intermediary is indicated by the demonstration that HPA activation to both IV and ICV IL-1 was blocked by the PGE_2 blocker indomethacin, but the plasma ACTH response to immobilization stress was unaltered (Katsuura et al., 1988). Thus there appears to be alternative mechanisms capable of inducing

HPA activity.

LPS has been shown to be able to induce IL-1 secretion both in vitro and *in vivo* (Morrison & Ryan, 1987; Bone, 1991), as well as a number of responses typically attributed to IL-1 such as fever, slow wave sleep, and associated components of the acute phase response (cf. Andersson et al., 1992; Chensue, Terebuh, Remick, Scales & Kunkel, 1991). Because of the conditioned and unconditioned ability of IL-1 to activate the HPA axis (Dyck et al., 1990), the preferred interpretation of the conditioned elevation of corticosterone to cues paired with LPS found in the present study is that such effects were likely mediated by IL-1 acting as an afferent signal to the CNS. This interpretation however, does not preclude the involvement of other factors.

As noted above, it is not clearly established whether or not IL-1 enters the brain. It has however been suggested that IL-1 can, either directly or indirectly through the action of intermediaries (e.g., PGE_2; Katsuura et al., 1988), promote the release of hypothalamic CRF (e.g., Berkenbosch et al., 1987) and pituitary ACTH resulting in adrenal steroidogenesis (Besedovsky et al., 1986). Moreover, peripherally administered LPS has recently been demonstrated to induce both the expression of IL-1 mRNA in discrete nuclei within the brain (Ban et al., 1992) and immunoreactive IL-1ß in central macrophage and microglia (van Dam, Brouns, Louisse, & Berkenbosch, 1992). Thus it appears

likely that the initiation of the HPA cascade may result from IL-1 signaling the brain either through intermediaries or by stimulating the synthesis of IL-1 within the CNS. In addition, the possibility exists that other neuroactive products released after endotoxin challenge (e.g., IL-6 and TNF-α) may also be involved in this response. TNF-α is pyrogenic *in vivo* (Dinarello et al., 1986) mimicking an effect of IL-1 and is found in the cerebrospinal fluid during bacterial meningitis infection (reviewed in Manogue, van Deventer, & Cerami, 1991). IL-6, like IL-1, regulates part of the acute-phase reaction and has been shown to stimulate ACTH through the induction of CRF (Naitoh et al., 1988; also reviewed in Hirano, 1991). Further study is necessary to clarify the role of primary and secondary contributors in the development of a conditioned corticosterone response.

While the involvement of cytokines other than IL-1 are not ruled out in the present study, the observed suppression of fluid consumption following LPS is consistent with the notion of a central role of IL-1. It is well established that IL-1 induces anorexia (cf. Dinarello, 1984). This effect has been extended to reports of suppressed fluid intake following IP and ICV IL-1 as well (Massotto et al., 1992). Anorexia and suppressed fluid consumption have also been reported following IP LPS (Langhans et al., 1990). Although other cytokines such as TNF have been implicated in hypophagia (e.g., Patton et al., 1987) it is likely that

some of the effects reported here are mediated, at least in part, by IL-1.

The present data also suggest the presence of a conditioned suppression of splenic NE content which mimics the effect of drug administration alone. Although modest in magnitude, this effect parallels previous unpublished findings from our laboratory of small decreases in splenic NE content to IP LPS at the post injection time point employed here. We have previously reported similar decreases in splenic NE content to IP IL-1 two hours post injection: A time point that was coincident with suppression of splenic macrophage IL-1 secretion (Vriend et al., 1991). Moreover, the suppressive effect of ICV IL-1 on splenic macrophage cytokine production two hours post injection was abrogated by splenic nerve section (Brown et al., 1991). In contrast, an elevation of splenic NE content has been documented to ICV LPS at 3 hr post injection (Vriend et al., 1992; Wan, Janz et al., 1993) which coincided with the induction of c-fos protein in neurons in the paraventricular nucleus of the hypothalamus. These apparently contradictory findings are typical of studies on splenic NE activity where NE content has been measured. Madden and Livnat (1991) have commented on the difficulty in accommodating conflicting experimental observations and have emphasized the necessity of detailed NE turnover studies to clarify the dynamics of NE release and disposition in lymphoid organs during an immune response in order to clarify the role of NE in

immunoregulation. For example, an initial suppression of sympathetic activity may be necessary in systems where sympathetic activity is suppressive to allow for the immune system to facilitate its response in reaction to invasion or trauma (e.g., splenic macrophage secretion of IL-1; Brown et al., 1991). At a later time point, adequate signals from the lymphoid environment provide feedback through which the nervous system detects immunological activation and responds accordingly. Thus the content of NE in a lymphoid organ may change dramatically as a function of time as it is influenced during different stages of immune activation. Although alterations in splenic NE content are indicative of changes in sympathetic activity at a particular time point, this method does not address the dynamics of NE mediated sympathetic signaling. It does however, provide initial evidence of sympathetic involvement.

Finally, the conditioning protocol employed produced a modest suppression of IL-2 secretion by splenocytes stimulated with the R73 monoclonal antibody. Although, the evidence for conditioning is not unequivocal, particularly in the absence of an unconditioned effect of LPS on IL-2 in the experiment proper, the Re-exposed group did exhibit lower IL-2 levels than the combined controls. Although LPS is widely regarded as a pleiotropic stimulus for immune cells both *in vivo* and *in vitro* (Andersson et al., 1992), reports on the effects of LPS on IL-2 levels

are mixed. For example, Sundar et al. (1989) reported that IL-2 production was suppressed in splenic lymphocytes following administration of ICV LPS or IL-1. Similarly, prior unpublished results from our laboratory indicated that following administration of IP LPS, IL-2 production was suppressed relative to controls at various time points in both conditioning and systemic studies including the 4 hr post injection time point employed here. On the other hand, Andersson et al., (1992) and others (Gillis, Fern, Ou, & Smith, 1978) report no detectable IL-2 from monocytes and lymphocytes stimulated *in vitro* with LPS. In summary, it is not clear why the suppressive effect was seen in the conditioning group when a parallel unconditioned effect that had been observed previously was not detectable.

Although the mechanisms underlying the conditioned alteration of IL-2 production require further elucidation, the alteration of glucocorticoid levels and splenic NE activity represent likely mediators. Glucocorticoids have been shown to suppress IL-2 production at the mRNA level (Grabstein, Dower, Gillis, Urdal, & Larsen, 1986), although most of the evidence which supports glucocorticoid-suppressive effects on cytokine production is based on *in vitro* evidence (cf. Munck & Guyre, 1991). However, Sundar et al. (1989; 1990) reported that IL-1, and IL-1-like activity, induced by ICV LPS suppressed IL-2 levels and that this effect was partially abrogated by adrenalectomy. Thus this

suppressive effect was, at least in part, dependent on adrenal hormones.

In addition to the evidence which supports the influence of adrenal hormones on IL-2 production, it is well known that sympathetic fibers are associated with lymphocytes, mast cells, and macrophages in immune organs (Felten et al., 1987), and that these cells express receptors for the sympathetic transmitter norepinephrine (Roszman & Carlson, 1991). Other recent studies indicate that central administration of IL-1 is associated with increased sympathetic outflow to the spleen during the period when IL-1 induces immunosuppression (Vriend et al., 1993) and that splenic nerve cuts block the immunosuppression that is otherwise observed (Brown et al., 1991). Further, suppression of NK cell activity and splenic T-cell mitogen responsiveness induced by IL-1 is blocked by administration of a sympathetic ganglion blocker (Sundar et al., 1989). This result corroborates observations of decreased IL-2 synthesis in both a human T-cell line to ß-adrenoceptor stimulation and lymphocytes from chemically sympathectomized mice (Madden & Livnat, 1991). Although the dynamics of NE activity are not well understood the depleted NE content is likely indicative of increased sympathetic activity. Thus, products of sympathetic as well as adrenal activation may have contributed to the conditioned suppression of IL-2 production.

This study is a further step in the elucidation of mechanisms

involved in conditioned immunosuppression. The ability of IL-1 to act as a UCS was hypothesized in an early conditioning study by Dyck, Greenberg, and colleagues (Dyck et al., 1986). These investigators observed suppressed NK cell activity to cued administration of the macrophage-activating drug, poly I:C. Following Besedovsky and colleagues (e.g., Besedovsky et al., 1986), Dyck et al. hypothesized that IL-1 from poly I:C-stimulated macrophages might be an important immune-derived afferent signal particularly in light of its ability to induce NK-suppressive corticosterone release. More direct tests of this model demonstrated associatively mediated pyrexia (a well known effect of IL-1) to poly I:C (Dyck et al., 1989) as well as corticosterone release to IP IL-1 (Dyck et al., 1990). This evidence is consistent with the hypothesized associatively sensitive immunosuppressive pathway in which IL-1 acts as a afferent signal.

Bull and colleagues (Bull et al., 1990; 1991) provide further conditioning evidence congruent with the results and interpretation of the present and aforementioned studies. Using a taste aversion paradigm these investigators demonstrated conditioned alterations in body temperature to taste cues paired with LPS; a result which they suggest is mediated by IL-1. An additional study, in corroboration of this suggestion, demonstrated that an LPS-induced fever was suppressed to cues previously paired with the IL-1 blocker α–MSH.

Taken together, these results support the existence of an associatively sensitive bi-directional feedback loop where IL-1 from a stimulated immune system influences the brain to induce immunosuppression (cf. Besedovsky et al., 1986).

As noted previously, future research is required to more clearly characterize the mechanisms suggested here. The role of IL-1 in central signaling might be investigated further in conditioning studies where IL-1 bioactivity, or the activity of potential mediators such as PGE_2 could be blocked in conditioning trials involving LPS or IL-1 administration. Thus, a clearer picture of the afferent signaling capabilities of IL-1 or its mediators would be afforded. Similarly, the involvement of centrally produced IL-1 could be investigated by the central administration of the IL-1 receptor antagonist in conjunction with drugs known to stimulate central IL-1 production such as LPS (Ban et al., 1992). This latter stragety could be implemented during training or test phases to elucidate the role of centrally produced IL-1 in signalling the brain or in the generation of a CR. The suppressive influence of steroids and sympathetic activity on splenocyte IL-1 and IL-2 production (Brown et al., 1991; Sundar et al., 1989; 1990) is suggestive of another strategy. Further conditioning studies involving denervation of the spleen and adrenalectomy prior to or after conditioning trials may clarify the role of sympathetic and steroid

involvement in the suppression of IL-2 observed here.

In conclusion, previous work suggests that IL-1 can induce corticosterone production as well as alter splenic NE content and turnover. Further, these products of CNS activity can suppress IL-2 production and interruption of both adrenal and sympathetic pathways abrogate this suppression. Since the conditioned suppression of IL-2 in the present study was paralled by conditioned alterations in corticosterone and NE content, a plausible interpretation is that these products may have mediated the conditioned alteration in IL-2 production. However, more definitive support in the form of additional studies which manipulate these mediators are needed to confirm this hypothesis. In view of the rather weak and inconsistent effects by LPS on both NE and IL-2 and the absence of significant associations between the putative mediators (NE and corticosterone production) and IL-2 production, replication studies with a larger sample size are needed. However, the present study has, for the first time, provided evidence for conditioned alterations of an immune response and concurrent alterations of plausible mediators of that response.

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APPENDIX

List of Abbreviations		*Introduced on page*
ACTH	adrenocorticotropic hormone	20
CNS	central nervous system	3
CR	conditioned response	7
CS	conditioned stimulus	7
CRF	corticotropin releasing factor	4
CY	cyclophosphamide	5
DTH	delayed-type hypersensitivity	12
HPA	hypothalamic-pituitary-adrenal	21
IS	immune system	3
IL-1	interleukin-1	1,3
IL-2	interleukin-2	1,22
IL-6	interleukin-6	24
ICV	intracerebroventricular	1,4
IP	intraperitoneal	1,9
LPS	Lipopolysaccharide	1,4
α-MSH	alpha-melanocyte stimulating hormone	3
NK	natural killer	4
NE	norepinephrine	1,4
Poly I:C	polyinosinic polycytidylic acid	5

PGE_2	prostaglandin E_2	25
TGF-β	transforming growth factor-beta	26
TNF-α	tumor necrosis factor-alpha	26
UCR	unconditioned response	7
UCS	unconditioned stimulus	7