1997

The immunomodulatory effects of 2-deoxy-D-glucose (2-DG) and the neuroendocrine mediating mechanisms

Shiu-Huey Chou
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The immunomodulatory effects of 2-deoxy-D-glucose (2-DG) and the neuroendocrine mediating mechanisms

by

Shiu-Huey Chou

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Immunobiology
Major Professor: Joan E. Cunnick

Iowa State University
Ames, Iowa
1997
This is to certify that the doctoral dissertation of

Shiu-Huey Chou

has met the dissertation requirements of Iowa State University

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For the Major Program

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For the Graduate College
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ABSTRACT

Studies of the effect of stress on the immune system provide a way to evaluate the coordination of the immune system with other systems in the maintenance of homeostasis. The objectives of the present study were to characterize the immunomodulatory effect of 2-DG, a metabolic stressor, on both primary and secondary immune organs and to examine the possible neuroendocrine mechanism(s) mediating 2-DG-induced immunomodulation using male, Lewis rats.

The first study demonstrated that acute and chronic 2-DG administration induced a decrease total leukocyte counts, organ weight, IFN-γ production, and mitogen response of lymphocytes in both spleen and whole blood. The ratio of CD4^+/CD8^+ in the spleen was decreased due to a significant increase in CD8^+ T-cell subpopulation. In contrast, there was a significant increase in nitrite production in cultures of spleen and blood leukocytes from 2-DG-injected rats compared with saline-injected controls. Production of IL-1 and IL-2 by cells from these tissue was significantly reduced in the blood, but not in the spleen. Moreover, the kinetics of 2-DG-induced immunomodulatory effects in the spleen and blood were different. Hence, acute and repeated 2-DG exposure induced alteration of immune function in both spleen and blood.

The objectives of the second and third projects were to examine the role of peripheral catecholamines and adrenal hormones in 2-DG-induced immune alteration. Results indicated that the β-adrenergic receptor antagonist nadolol, attenuated 2-DG-induced suppression in the spleen, but not in the blood. Adrenalectomy attenuated 2-DG-induced alteration of immune parameters in the blood, but did not attenuate altered immune function in the spleen. These
data suggest that the sympathetic release of catecholamines is responsible for splenic immune alterations, whereas adrenal hormones are responsible for immune alterations observed in blood leukocytes. Results from the second and third projects also indicate that common neuroendocrine pathways exist for several types of stressors which induce immunomodulation.

The characterization of 2-DG modulation of thymic parameters was established in the fourth study. Repeated 2-DG administration induced a significant reduction of thymus weight, total thymocytes, mitogen responsiveness of T-lymphocytes, and nitric oxide production by thymic stromal cells. Additionally, the numbers of CD4⁺ and CD4⁺CD8⁺ double positive T-cells decreased in the thymus of rats which received multiple 2-DG injections, but the numbers of CD8⁺ T-cells increased. Moreover, 2-DG-induced an elevation of adrenal hormones which initiates a long lasting process of increased apoptosis in the thymus. Thus, 2-DG-induced immunomodulation of the thymus may result in the changes of immune function in the spleen and blood.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with an abstract and is followed by a general introduction, four papers, and a general conclusion. The abstract provides a summary of the general objectives and conclusions. The general introduction includes the dissertation organization, an introduction, and a review of the literature. The literature review includes sections covering the historical discovery of the effects of stress on health, the definition of stress, the stress-induced physiological response, the evidence of stress effects on immune function, 2-DG and metabolic stress, effect of 2-DG on the immune system, the mechanism of stress effect on the immune system, organization of the immune system, and apoptosis.

The four papers are written in journal article form for publication. The first paper entitled "Characterization of the effect of 2-deoxy-D-glucose (2-DG) on the immune system" has been accepted for publication in *Brain, Behavior, and Immunity*. The second paper entitled "Evidence for the involvement of catecholamines in the 2-DG-induced immunomodulatory effect in the spleen" has been submitted for publication in *Brain, Behavior, and Immunity*. The third paper entitled "Adrenal hormones are responsible for 2-DG-induced immunomodulatory effects in the blood" and the fourth paper entitled "Alteration of thymic T cell subset profiles and apoptosis by a metabolic stress" will be submitted for publication.

The last chapter of the dissertation is a general discussion summarizing the results presented in the four papers and discussing conclusions drawn from them. A list of references cited is included at the end of each chapter.
Introduction

The term "stress" can be defined as a perturbation of the normal homeostasis of the body (Kort and Weijma, 1991). Stress can be caused by psychological processes (e.g. conditioned fear) or physical means (e.g. electrical shock and drug injection). Emerging evidence demonstrates that different forms of stress have modulatory effects on immunological function (for review see Hillhouse et al., 1991). For example, stressful events such as bereavement (Irwin et al., 1987), academic stress (Kiecolt-Glaser et al., 1986; Bonneau et al., 1990), electric shock (Cunnick et al., 1990; Sonnenfeld et al., 1992), restraint (Berry et al., 1991; Zwilling et al., 1993), trauma (Wood et al., 1984), and surgery (Weissman, 1990) can alter parameters of immune cell function in animals and humans.

In addition, studies indicate that stress alters the function of immune cells as evidenced by decreased concentrations of circulating antibodies (Solomon, 1969), suppression of the reactivity of lymphocytes to mitogen (Keller et al., 1981), antigenic stimulation (Jasod and McKenzie, 1976), and also decreased natural killer cell (NK) activity and increased susceptibility to tumor challenge (Shavit et al., 1984). Hence, the consequences of stress-induced alteration of immune cell function may increase an animal's susceptibility to microbial and neoplastic diseases. There also exists evidence that stress can alter health outcomes in humans (Kiecolt-Glaser, 1984; Shoenfeld and Isenberg, 1989; Cohen and Williamson, 1991). Stress is correlated with the onset or exacerbation of a variety of human diseases involving the immune system such as rheumatoid arthritis, diabetes, cancer, and repeated viral infections (Fitzpatrick et al., 1988; Eaton et al., 1979; Jemmott et al., 1984; Baker et al., 1981). Therefore, investigating the significance of stress-induced changes of
immunity in various psychologic or physiologic diseases should be considered to be of particular importance in current biomedical research.

When the effects of stress on the capacity of the immune system are examined, the mechanisms by which stress modulates immune function must be defined. It is not clear by which mechanisms the immune system responds to stress exposure. However, it is generally accepted that during exposure to stressful events, the neurological and endocrine systems play a fundamental role in mediating the changes in immunological function resulting from exposure to the stressor (Ader et al., 1991). Communication between the neuroendocrine and immune systems has been suggested to occur through neuroendocrine peptides, hormones and cytokines (Blalock, 1994; Savino and Dardenne, 1995). Specifically, research by Keller and colleagues (1988) using shock as a stressor has identified a role for the hypothalamic-pituitary-adrenal (HPA) axis in suppression of mitogen-stimulated lymphocyte proliferation in the peripheral blood. Additionally, Cunnick (1990) and Irwin (1991) have reported that the peripheral release of catecholamines is responsible for suppression of spleen mitogenic responses due to shock or central administration of corticotropic releasing hormone (CRH). Hence, two possible pathways of immune regulation can be via the sympathetic nervous system pathway or via the hypothalamic-pituitary-adrenal axis. However, there have been few in vivo studies of the mechanisms responsible for the stress-induced changes of immune response.

In addressing these issues, appropriate animal models can be of substantial benefit. In certain experimental models of stress, such as those which entail restraining rodents in a suspension harness, the animals lost a significant amount of body weight (Miller et al., 1994),
and use of mild electric shock in animals increases locomotor activity and escape behavior. Hence, it becomes difficult to separate the effects on the immune system induced by factors such as nutrient deprivation or increased locomotor activity from the immunomodulatory effects induced directly by the stress response. Since many illnesses are exacerbated by improper dietary intake, drugs of abuse, or disorders of metabolic function, there has been an increased interest in the influence of metabolic disorders on immune function such as protein calorie malnutrition (Chandra, 1983) or heavy alcohol use (Watson et al., 1984).

One model system for studying metabolic stress uses 2-deoxy-D-glucose (2-DG). 2-DG is a glucose analogue, which prevents cellular uptake and utilization of glucose (Brown, 1962). 2-DG produces metabolic changes which include acute intracellular glucoprivation and extracellular hyperglycemia (Smythe et al., 1989). The 2-DG-induced metabolic stress invokes changes in the immune, nervous, and endocrine system (Smith and Root, 1969; Ritter and Neville, 1976; Pascoe et al., 1989; Cato et al., 1990). These responses are similar to those that occur in humans following exposure to a number of physical stressors. Moreover, the neural and endocrine response to 2-DG are similar to electric shock, but induce behaviors that are distinct from the physical stress of shock. Animals which receive 2-DG has less motor activity than animals that experience shock (Smith and Epstein, 1969). In addition, 2-DG administration does not produce a significant nutrient deprivation (Miller et al., 1994). Therefore, the rodent model of 2-DG-induced metabolic stress which is examined here may provide a useful model for studying the influences of stress on immune cell function. In addition, it has been shown that 2-DG-induced metabolic stress has the capacity to modulate the function of lymphocytes (Lysle et al., 1988; Miller et al., 1994).
Nevertheless, the characterization of effects of metabolic stress on immune function are not well documented, and the possible mechanism(s) underlying 2-DG-induced immune modulation is not defined. Therefore, the purpose of this research was to more fully characterize the alterations of immune function on a primary immune organ (thymus) and secondary immune organs (spleen and blood) due to the metabolic stress of 2-DG administration using rats. Moreover, two of the in vivo studies were designed to evaluate the mechanisms underlying the capacity of 2-DG-induced metabolic stress to influence immune function.

The concentrations of plasma corticosterone and endogenous glucose were measured as indicators of a stress response. To evaluate the cellular immune function, this dissertation included a measure of the proliferative response of lymphocytes to various T and B cell mitogens, the T-cell subset distribution, nitric oxide production from mitogen stimulated cell cultures, and production of the cytokines interleukin-1, interleukin-2, interferon-γ, and tumor necrosis factor in the spleen and peripheral blood. In addition to the spleen and blood, immune parameters of the thymus such as T-cell subset distribution, lymphocyte proliferation, and apoptosis were also examined. The results from this study indicated that the metabolic stressor, 2-DG, significantly alters cellular immune function and can influence the immune system via neuroendocrine pathways common to physical and psychological stressors.
Literature Review

Historical discovery of stress effects on health

The postulated relationships between stress exposure and disease states have had a long and colorful history. The historical basis for studying the influence of stress on the immune response was derived from clinical observations regarding individuals who became sick following stressful situations. The pioneer of investigations on the relation between emotion and disease was Ishigami (1919) who studied opsonization of Tubercle bacilli among chronic tuberculous patients during active and inactive phases of the disease. He found that phagocytic activity decreased during episodes of emotional distress and proposed that a stressful life led to immune depression and consequently increases in susceptibility to tuberculosis. Later, the work of Cannon and Selye greatly advanced comprehension of the role of stress in homeostasis and adaptation. Cannon (1914) provided his theory which is called “emergency theory of emotion”, and coined the term “fight-or-flight” response in which the autonomic nervous system regulates the body for defense against stress. His observations provided evidence on the body’s physiologic responses to external stimuli but this theory did not explain the consequence of stress exposure and body response. Later, the coordinated roles of the autonomic nervous system, endocrine system, and immune system in the response to stress were greatly clarified by the work of Selye (1936, 1956). His work has been reviewed by Munck et al., (1984), who proposed that the most important physiologic effect of glucocorticoid secretion is to suppress immunity, thereby interrupting the immune response.
After the work of Ishigami, Cannon, and Selye, the scientific understanding of stress and immunity has progressed slowly over several decades. In that time, much of the knowledge on stress was developed from animal studies by Rasmussen (1969) and Solomon (1969), which produced evidence for the relationship between stress and decreased immunocompetence. These findings led others to investigate the role of stress-induced depression of immunity in various psychologic or physiologic diseases. The work of Rasmussen and Solomon led current scientists to recognize the importance of stress-related immune suppression. Also, there was evidence that such a relationship was possible in man. Later, a monumental work that investigated the relation between behavior and immune suppression was reported by Ader and Cohen (1975). This work brought about the birth of the field of Psychoneuroimmunology (PNI).

A review examining the effects of stress exposure on medical illnesses was reported by Rahe and Arthur (1978). The review demonstrated that significant stress exposure precedes the onset or exacerbation of a variety of medical conditions, including allergic, autoimmune, neoplastic, and infectious processes. Hence, the scientific interest in the influence of stress on immune function has recently resulted in a rapid accumulation of information concerning behavioral immunology, immunophysiology, and neuroendocrine-immunology. However, the methodology on the quantitation of stress and the association of stress with the onset of illness has been challenged. Despite methodological difficulties which slowed down the progress of investigations of the association of stress with disease, the impact of stress on susceptibility to several illnesses is widely accepted.
The definition of stress

Stress, like the air, is a pervasive factor of life, that affects the pathophysiology of nearly all psychiatric illnesses and a range of medical illnesses. The term “stress” is defined as “the reactions of the body to forces of a deleterious nature, infections, and various abnormal states that tend to disturb its normal physiological equilibrium (homeostasis)” (Burgess, 1987; Sklar and Anisman, 1981; Stott, 1981; Riley, 1981; Kort and Weijma, 1991). In addition, the stimulus that causes such a disruption is called “a stressor.”

Stress has been divided into a number of categories according to the nature and the severity of the stress. As such, exteroceptive and interoceptive stress, acute and chronic stress have been distinguished. In addition, these categories have been further divided into numbers of subcategory, e.g., unavoidable vs. avoidable, severe vs. mild, and physical vs. psychological. Furthermore, it is logical that many types of physical stress will have direct metabolic consequences (e.g., cold exposure), which implies that stress is very specific in the nature of its effect on the individual’s physiology (Kort and Weijma, 1991). Thus, it appears difficult to compare one stressor with any other type of stressor.

Generally, we can divide stress into psychological stress and physiological stress (physical stress) (Hillhouse et al., 1991). Psychological stress can be differentiated from physical stress in that it is subject to cognitive appraisal (Paterson and Neufeld, 1989). Psychological stressors have been conceptualized as interactions among external threats, internal evaluations of threats, personal resources available to deal with the threat, and potential physical and psychological outcomes (Lazarus and Folkman, 1984). These interactions are thought to result in emotional responses such as anxiety, fear, anger,
excitement, or depression. Physiological stress can generally be categorized under the rubric of arousal. Arousal would include such physical alterations as elevated heart rate, blood pressure, and respiration, as well as the endocrine changes associated with stress. Physiological stress is typically interrelated with emotional presentation, therefore, it is difficult to evaluate the outcomes which arise directly from the physiological stress or from emotional responses which are induced by physiological arousal.

In addition, the same "stressful" event affects individuals differently and their stress responses can range from extreme to mild to absent. For example, giving a seminar is dreaded by some people, but enjoyed by others. Stress is often considered bad and has been linked to a variety of stress-related diseases. However, stress is not necessarily bad all the time. News of passing Ph.D. defense can markedly affect our homeostasis which modulates by elevation of epinephrine concentration, but the stress response is experienced as joy. Stress can also be helpful; the stress of winning an event at the Olympic Games helps an Olympic runner to practice hard and win future campaigns. Conversely, lack of stress can be bad: no competition or punishment causes school failure and crime rate elevation. Stress is usually bad for the organism if the experience is unpleasant or intense or chronic; in these cases, illness is likely to follow. However, disease only develops in some, not all subjects; some species are susceptible to an infectious agent while others are resistant to the pathological effects of stress. Such health consequences can include hypertension, cardiac arrhythmia, headaches, ulcers, depression, alcoholism, or other stress-related diseases (Vogel and Bower, 1991).
In conclusion, the influences of stress are complicated and diverse. The consequences of stress on the individuals are based on specific genetic vulnerabilities, individual experiences, and environmental circumstances (Vogel and Bower, 1991). However, stress fluctuates and changes as the person interacts with and responds to the stressor. It is this type of change and flux that should be kept in mind when attempting to understand or describe the endocrine and immune consequences of stress.

**The stress-induced physiological response**

Common indicators of stress include changes in 1) biochemical parameters such as serum norepinephrine, epinephrine, and adrenal steroids, 2) physiological parameters such as heart rate and blood pressure, and 3) behavioral effects such as anxiety, fear, and tension (Vogel and Bower, 1991). The endocrine reaction to stress is very complex and only partially understood. Studies have shown that the final common pathways of neuroendocrine response to stress are stimulation of adrenocortical secretion, with consequent increases in serum glucocorticoids and activation of the sympathetic nervous system, followed by a release of catecholamines. The HPA axis appears to play a central role in coordinating the endocrine, autonomic, and behavioral responses to stress. Exposure to stress leads to increased activity of the HPA axis on the immune system through the effects of the adrenal hormones such as glucocorticoids (Axelrod and Reisine, 1984). An elevated concentration of glucocorticoid in plasma is an indicator of stress. The release of catecholamines (norepinephrine and epinephrine) constitutes an initial response to stressors, and is controlled via regional activation of sympathetic neurons as well as a discharge from the adrenal medulla (Kopin et al., 1988).
Other hormone systems are also affected by stress. Stress induces secretion of a wide variety of hormones such as adrenocorticotropin (ACTH), corticotrophin-releasing factor (CRF), vasopressin, β-endorphin, and oxytocin (Hillhouse et al., 1991). In addition, growth hormone and prolactin secretion is increased by physical or psychological stress (Rivier and Vale, 1985; Munck et al., 1987). Release of the endogenous opiates has also been shown to be elevated during stress conditions. Finally, Wartofsky and Burman (1982) have reported thyroid dysfunction during chronic stress exposure. The regulation of the endocrine response to stress involves numerous feedback loops. This complicated regulation interacts with the immune system to maintain homeostasis in the body.

Since stress responses induce changes of hormone concentrations in the body, stress can be measured by determining adrenal hormone and catecholamine concentrations in central and peripheral circulation. Stress and increased corticosteroid levels are very closely related, and events are sometimes not classified as stressful until an increase in corticosterone is detected. Other parameters that are directly or indirectly associated with adrenal activity and are directly related to the category of stress can also be used as indicators of a stress response. For example, body weight and blood glucose values should be determined.

The evidence of stress effects on immune function

Emerging evidence on the immunomodulating properties associated with stress exposure suggests that the cellular arm of the immune system is principally affected by the experience of stress. Thus, the degree of stress may influence susceptibility to illness and prognosis for diseases. For example, the onset and exacerbation of several autoimmune diseases has been associated with major life stressors of marriage, divorce, business loss, or
death of a family member. Correlational studies of patients with rheumatoid arthritis and their female siblings found that the patient was more tense, worried, and moody than the healthy sibling (Fitzpatrick et al., 1988). In the last thirty years, a large number of studies that examined the relation of stress and immunity were completed in humans and animals. It is well recognized that stress can produce alterations in the responsiveness of the immune system. The evidence that various stressors can alter immune function in humans and animals is briefly summarized below.

**Human studies**

In humans several emotional stressors have been shown to affect various aspects of immune function. For example, studies of bereavement resulting from the death of a spouse have been shown to decrease mitogen responses in the peripheral blood (Bartrop et al., 1977; Schleifer et al., 1983) and women who are separated or divorced have higher levels of antibodies to Epstein-Barr virus than married individuals, an indication of lowered T-cell function (Kiecolt-Glaser et al., 1987). In addition, the chronic stress of caring for patients with Alzheimer's disease shows a decreased percentage of total T-lymphocytes, T-helper lymphocytes, T-cytotoxic lymphocytes, and a reduction in the T-helper : T-cytotoxic cell ratio (Kiecolt-Glaser et al., 1987). In addition, the academic stress of examinations in medical students is correlated with a decrease in mitogen responsiveness, interleukin-2 (IL-2) production, natural killer cell (NK) function, mRNA content of c-myc and c-myb, the glucocorticoid receptor and interferon-γ in peripheral blood lymphocytes (PBL) (Kiecolt-Glaser et al., 1984; Glaser et al., 1993).
In addition, a number of studies have examined the relationship between depression and immune function. For example, Schleifer and colleagues (1984) reported that depressed subjects had lower total T- and B- lymphocyte numbers, as well as lower mitogen-induced lymphocyte stimulation. However, these results were not replicated in a follow-up study (Schleifer et al., 1985).

The results of human studies demonstrated a large amount of variability in immunological outcomes. The variability seen in human studies may be due to several external factors that also affect immune function, such as stress induced changes in sleeping, eating, exercising, and other health related habits. Because of the various confounding variables in human studies, it has become important to examine the effects of stress using well controlled animal models.

**Animal studies**

Murine and rat models have been used extensively to study the stress-immune function relationship. Psychoneuroimmunologic studies of stress in rats and mice have focused on alterations of lymphocytes in the spleen and blood. Those studies have used various physical and psychological stressors, such as surgery (Pollock et al., 1987), electric shock (Keller et al., 1981; Laudenslager et al., 1983), rotation (Esterling and Rabin, 1987), noise (Monjan and Collector, 1977), conditioned fear (Lysle et al., 1988a), and 2-DG (Lysle et al., 1988b). Results of these studies provide evidence to support the concept that stress affects immune parameters and subsequently influences the outcomes of health. For example, presentations of electric shock to rats decreased the responsiveness of lymphocytes.
to stimulation by mitogens (Lysle, et al., 1987), decreased natural killer cell activity (Shavit et al., 1984), and increased susceptibility to tumor challenge (Lewis et al., 1984).

In addition, other physical stressors such as surgery and restraint have induced a reduction in NK cell function, depressed delayed type hypersensitivity, and reduced phagocytic activity in affected rats (Pollock et al., 1987; Okimura et al., 1986). Furthermore, social isolation of young domestic fowl produced a time-dependent decrease in the T-lymphocyte mitogen proliferation, a significant increase in B-lymphocyte mitogen proliferation at 30 min, which decreased with increased duration of stress, and a significant increase in the production of an IL-1-like factor by splenic macrophages from animals isolated for 30 min, which decreased in a time-dependent manner to return to baseline by 90 min (Cunnick et al., 1994). Taken together this series of studies demonstrates that immune function can be altered by various stress conditions.

2-deoxy-D-glucose (2-DG) and metabolic stress

2-DG and structure mechanism of action

The compound 2-deoxy-D-glucose (2-DG) is a nonmetabolizable structural analog of glucose (review see Brown, 1962). The difference between 2-DG and glucose is a substitution of hydrogen (-H) on the second carbon rather than hydroxyion. The action of 2-DG was first noted by Cramer and Woodward (1952) who demonstrated the inhibition of metabolic blocking agent by competitive inhibition of glucose utilization, and produces a anaerobic fermentation of glucose by yeast with the addition of 2-DG. 2-DG acts as central neuroglucopenia and a peripheral hyperglycemia. 2-DG competitively inhibits the isomerase reaction and a membrane transport system that is shared by glucose and mannose. Sols and
Crane (1954) demonstrated that the glucose configuration at the second carbon was essential for the inhibitory properties of 2-DG (Figure 1).

Hence, 2-DG produces intracellular hypoglycemia by: (1) acting as a competitive inhibitor of the hexokinase and hexoisomerase enzymes in the cell membrane essential for the transport of glucose into the cell, and (2) inhibiting glycolysis when 2-DG enters inside the cell. 2-DG is converted to the phosphate ester form. Since the phosphorylated production of 2-DG is not a substrate for phosphohexoisomerase or glucose-6-phosphate dehydrogenase, glycolysis can not continue through the subsequent steps and glucose metabolism is blocked.

In addition, the physiological responses to the 2-DG blockage of glucose metabolism are activation of the adrenal medulla with release of medullary hormones resulting in hyperglycemia and fatty acid mobilization. This results in a increased blood glucose level following 2-DG administration and has been reported in the rat, dog, and humans (Landau. et al., 1958). There are some reports that the growth of experimental tumors in an animal host is retarded by 2-DG administration, but large amounts at frequent intervals are required, and side effects limit the usefulness of 2-DG for this purpose (Sokoloff, et al., 1955; Ball, et al., 1957).
Advantages of 2-DG-induced metabolic stress as a model system

Metabolic stress is an interoceptive physical stress in which the stimuli come from internal homeostatic changes. Recently, it has been reported that many diseases are exacerbated by metabolic changes including improper dietary intake, drugs of abuse, or disorders of metabolic function. For example, roughly 50% of all cases of liver cirrhosis in Western societies is related to alcohol abuse and occur in conjunction with viral or metabolic liver diseases (Schuppan et al., 1995). In addition, alcohol consumption causes a marked suppression of NK activity in vivo and then promotes tumor metastasis (Ben-Eliyahu et al., 1996). These reports suggest that metabolic disorders induce immune alterations and should be considered an important issue in current biomedical research. As described above, 2-DG is a glucose analog that competitively inhibits intracellular glucose metabolism. Because neurons are critically dependent on glucose for metabolic activity, the central nervous system is especially sensitive to the effects of 2-DG-induced glucoprivation. Therefore, 2-DG is a useful tool with which to study the nervous system control of blood glucose homeostasis. 2-DG-induced glucoprivic effects include strong activation of the HPA axis (Brodows et al., 1973) with increased adrenomedullary activity (Welle et al., 1980), and stimulation of appetite centers (Muller et al., 1972; Miselis and Epstein, 1970). Thus, 2-DG administration has been an attractive paradigm to address a wide variety of physiological research questions.

2-DG has been studied as a metabolic stressor due to its well characterized physiological response, which produces all the hallmarks of a physical stressor. 2-DG produces stress effects via an acute glucoprivation. The indicators of 2-DG-induced metabolic stress include increased glucocorticoid secretion (Smith and Root, 1969),
sympathetic discharge (Brown, 1962; Smith and Epstein, 1969), and increased turnover of norepinephrine in the brain (Ritter and Neville, 1976) and periphery (Pascoe et al., 1989). In addition, these neural and endocrine responses to stress are similar to electric shock, but induce behavior that is distinct from the physical stress of shock. Whereas animals that experience shock demonstrate increased locomotor activity, animals that receive 2-DG experience a decrease in motor activity (Smith and Epstein, 1969). Thus, use of 2-DG induces all the neuroendocrine hallmarks of a physical stressor without the confounds of increased locomotor activity. Thus, alteration in immune function due to the neuroendocrine changes induced by stress should be consistent across stressors; while changes due to increased or decreased locomotor activity will differ. Hence, use of 2-DG treatment for studying the effect of metabolic stress on the immune system may provide an ideal model to examine the relationship of stress on immune function.

**Effects of 2-DG on the immune system**

There are many studies about the immune function changes with psychological or physiologic stress. However, few studies have examined metabolic stress-induced changes of immune responses. In humans, intravenous administration of 2-DG (50 mg/kg) results in a rapid suppression of concanavalin A (Con A) and phytohemagglutinin (PHA) stimulated T-cell proliferation (Breier et al., 1987). In rodent studies (Lysle et al. 1987; 1988b), 2-DG (500 mg/kg, IP) inhibited splenic and blood T-cell proliferation to Con A and PHA in Sprague-Dawley rats. However, the suppressed reactivity of the splenic lymphocytes attenuates with repeated injections, while the reactivity of blood lymphocytes does not. Their finding suggests that different immune compartments respond to stress are different. In
addition, mitogen stimulation of thymocytes from 2-DG treated rats is reduced after repeated injections of 2-DG. Mitogen stimulation of mesenteric lymph nodes from 2-DG-treated rats does not differ from saline treated controls.

Antelman et al. (1990) reported that exposure of rats to a single 2-DG injection 12 days after immobilization significantly augmented the immunosuppressive effect of 2-DG on the response of splenic lymphocytes to mitogen when compared to groups receiving 2-DG alone and immobilization one hour before 2-DG. Hence, the immunosuppressive effects of an acute stressor can be sensitized with the passage of time. Recently, Miller and colleagues (1993, 1994) have shown that one, two, or three injections of 2-DG (500 mg/kg, tail vein) into Swiss-Webster mice resulted in depressed T-cell proliferative responses and increased the production of interleukins-1, -3 and -6. An attenuation of these immune changes occurs following the fourth or fifth injections. These results indicate that the metabolic stress of 2-DG can alter immune function. However, little work has been done to characterize the effects of metabolic stress on innate immunity, such as the function of natural killer cells and macrophages.

**The mechanism of stress effects on the immune system**

It is becoming increasingly clear that the immune system is very complex in its mechanisms of intercellular molecular communication, and that the neuroendocrine systems that impinge upon and regulate the immune system are equally complex in their hormonal influences. One purpose of current study is to identify the neuroendocrine mediated mechanisms by which metabolic stress affects the immune system. However, there have been few *in vivo* studies of the mechanisms responsible for stress-induced changes of
immune response. An interaction between the immune, nervous and endocrine system is most commonly postulated as the cause of the pronounced effects of stress on immune function. Communication between the neuroendocrine and immune systems has been suggested to occur through neuroendocrine peptides, hormones, and cytokines (Blalock, 1994; Savino and Dardenne, 1995). In general, two possible neuroendocrine pathways of immune regulation are the HPA axis and the sympathetic nervous system pathway.

**Hypothalamic-pituitary-adrenal axis**

As a consequence of its pivotal role in maintenance of physiological homeostasis, the HPA axis serves as a prototype for the coordination of neural information into physiological responses. It also is a key player in stress responses. The hypothalamus is functionally associated with the pituitary gland and the endocrine system. The pituitary gland lies in the sella turcica at the base of the brain, and is connected to the hypothalamus through the pituitary or hypophyseal stalk. The anterior pituitary comprises at least five different types of secretory cells, each of which contains a particular hormone whose release is directed by one or more hypothalamic peptide cell releasing factors or hormones. After release, the pituitary hormone, in turn, stimulates a particular target organ whose products provide feedback inhibition for the pituitary hormone and/or hypothalamic peptide (Bateman et al., 1989).

Products of the activated HPA axis such as, ACTH, β-endorphin, glucocorticoid, and a number of other peptides, may act as immunomodulators. When the immunosuppressive and anti-inflammatory effects of the glucocorticoids were discovered, the HPA axis was widely considered the primary neuroendocrine-mediated mechanism underlying stress-induced immune suppression. Adrenocorticotropic hormone (ACTH) is an immediate
product of activating the HPA axis and is also correlated with stress-induced immune suppression. Stress induces the elevation of glucocorticoids in plasma (Selye, 1956). Glucocorticoids are the final products of HPA axis and are crucial mediators of the endocrine-immune interaction. The effects of glucocorticoids on the immune system have been reviewed (Munck et al., 1984).

**Glucocorticoids**

Glucocorticoids are adrenal steroid hormones which are made in the adrenal cortex. The plasma concentration of glucocorticoid is regulated in a negative feedback loop by ACTH from the pituitary, whose release is itself regulated by corticotropin-releasing factor (CRF) from the hypothalamus. These steroids exhibit a marked circadian rhythm of concentration which peak in the range of $0.5 \times 10^{-6}$ M at about the time of wakening. Stress will increase CRF release and thus raises glucocorticoid level (Cohen, 1992).

Glucocorticoids enter the cell by diffusion through the plasma membrane and bind to a glucocorticoid receptor which is located in the cytoplasm. The effects of glucocorticoid on the cells of the immune system are mediated through both soluble cytosolic and nuclear corticosteroid receptors. There are two types of glucocorticoid receptors which have been characterized by receptor binding studies (Reul and De Kloet, 1985; Miller et al., 1990). Type I adrenal steroid receptors (mineralocorticoid receptors) have a high affinity for the aldosterone (a mineralocorticoid) and cortisol and corticosterone (the glucocorticoids), but a lower affinity for dexamethasone (a synthetic glucocorticoid). Type II adrenal receptors (glucocorticoid receptors) have a high affinity for dexamethasone, but a lower affinity for cortisol, corticosterone, and aldosterone. Glucocorticoids bound to these receptors form a
hormone-receptor complex that can bind to a specific site of DNA to inhibit transcription of specific genes. The hormone-receptor complex has been shown to bind directly to portions of DNA that overlap cyclic AMP-responsive sites, thereby preventing binding and activation of these transcription factors and subsequent gene activation (Akerblom et al., 1988).

The effects of glucocorticoids on the immune system are diverse. Glucocorticoids inhibit immune or inflammatory responses by inhibiting the release of proinflammatory substances, such as complement components, prostaglandins, histamine, and bradykinins (Munck et al., 1984). They inhibit antigen and mitogen-induced lymphocyte proliferation, accessory cell antigen presentation, and accessory cell amplification of the antigenic signal through inhibition of interleukin-1 production (Lee et al., 1988). Corticosteroids depress amplification of lymphocyte proliferation by inhibiting lymphocytic IL-2 production and receptor expression. They suppress effector function of lymphocytes at a cellular level by inhibiting the generation of helper T lymphocytes and cytotoxic lymphocytes, and they restrict mature helper and cytotoxic cell function (Kelso and Munck, 1984). Glucocorticoids inhibit the production of several cytokines including interleukin-1 (IL-1), interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor (TNF), but enhance interleukin-4 (IL-4) production (Daynes and Araneo, 1989). In addition to downregulation of cellular function, glucocorticoids cause an increase in circulating neutrophils, resulting from polymorphonuclear leukocyte egress from the bone marrow stores (Dale et al., 1975).

Glucocorticoid-induced immune suppression depends on the species, lymphocyte type and receptors expressed in the cells. For example, cortisone-resistant species include the human, monkey, guinea pig and cortisone-sensitive species include the rat, mouse, hamster,
and rabbit. Differential expression of glucocorticoid receptor subtypes in immune organs has been reported. Peripheral blood lymphocytes exhibit both Type I and II glucocorticoid receptor binding; the spleen has type II glucocorticoid receptors and relatively few type I receptors; thymic lymphocytes exhibit Type II binding only (Miller et al., 1990). This indicates that different immune compartments differentially respond to glucocorticoids. Differential expression of receptors supports the theory that different mechanisms mediate stress effects in different immune organs. Specifically Keller and colleagues (1988) have identified a role for the HPA in suppression of the blood mitogen response. This work concurs with recent work by Cunnick et al. (1990) in which adrenalectomy attenuates the stress-induced suppression of the blood mitogen responses, but catecholamine antagonists attenuate the stress-induced suppression of splenic mitogen responses. This work indicates that various mechanisms are involved in stress induced modulation of immune function and are dependent upon the immune organ.

The action of glucocorticoids on stress-induced changes of physiological function has been revised several times. Munck et al. (1984) proposed that the physiological function of stress-induced increases in glucocorticoid concentration serves as a mediator to maintain physiological homeostasis. The physiological role of glucocorticoids is to increase organic metabolism, protein catabolism, gluconeogenesis, liver uptake of amino acids, vascular activity and to inhibit glucose uptake and oxidation by many body cells. Glucocorticoids may produce their protective effects by inhibiting defense reactions such as the release of insulin, lymphokines, and prostaglandins. Thus, they prevent undesirable hypoglycemia,
excessive lymphocyte activation, and uncontrolled inflammatory responses (Hillhouse et al., 1991).

**Sympathetic nervous system pathway**

Stressors activate the sympathetic nervous system along with the HPA axis. For example, Smythe et al. (1984, 1989) found that the sympathetic system plays an important integrative role in control of peripheral glucose metabolism during neuroglycopenia induced by 2-DG. This suggests that 2-DG-induced alterations of immune function also may be modulated by the sympathetic nervous system.

Numerous studies indicate that the sympathetic nervous system plays an important role in the modulation of the immune system. The sympathetic nervous system communicates with target organs via a two-neuron chain. Preganglionic neuron cell bodies are located in the thoracic, lumbar, and sacral spinal cord, where they receive a signal from the brain. These preganglionic neurons send cholinergic myelinated axons to ganglia such as the paravertebral, prevertebral, and terminal ganglia. The prevertebral ganglia consist of the celiac, superior mesenteric, aorticorenal, and inferior mesenteric ganglia. Postganglionic axons distribute to cardiac muscle, smooth muscle, secretory glands, and organs of the immune system. Cell bodies of axons involved in postganglionic innervation of the spleen are located in the celiac ganglia. The nerve terminals of the postganglionic axons release catecholamines, such as norepinephrine, which can interact with cells immediately adjacent to, or even distant from the sites of release.

There is anatomical evidence that the nervous system and specific subsets of lymphocytes have direct connections via physical contact between sympathetic nerve endings
and T-lymphocytes in the spleen of rats (Felten et al., 1987a, b). Expression of β-adrenergic receptors, which bind catecholamines, on T and B lymphocytes and macrophages have been reported to further support the interaction of immune and sympathetic nervous system (William et al., 1976; Abrass et al., 1985; Fuchs et al., 1988). Additionally, several early studies have shown that the blastogenic responses of lymphocytes to T and B cell mitogens, IL-2-induced proliferation of lymphocytes, lytic activity of cytotoxic T-cells and T-cell-dependent antibody production are inhibited by the stimulation of β-adrenergic receptors in vitro (review see Madden and Livnat, 1991). The anatomical evidence and presence of β-adrenergic receptors emphasize the important role of catecholamines in modulation of immune function. This interaction is particularly important in the relationship between stress exposure and immune modulation in the spleen.

_Catecholamines_

Catecholamines include the substances of dopamine, epinephrine, and norepinephrine. This group of monoamines contain a catechol ring and an NH₂ (amine) group and thus, they are called catecholamines. Catecholamines are the hormones secreted by the sympathetic nervous system. In peripheral nerves, norepinephrine is a locally produced and secreted neurotransmitter; in the adrenal medulla, epinephrine is the major secretion product. Catecholamines are stored within granules with ATP and a specific protein, such as chromogranin. Catecholamines are released during vesicular exocytosis. Catecholamines are catabolized by two enzymes, catecholamine-O-methyltransferase (COMT) and monoamine oxidase (MAO). The secretion of catecholamines is regulated by a feed back system to prevent further synthesis of catecholamines (Hadley, 1992).
The actions of catecholamines are mediated via $\alpha$ and $\beta$-adrenergic receptors on the effector tissues (Ahlquist, 1948). Four subtypes of catecholamine receptors, the $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$ adrenergic receptors, have been identified on the basis of their pharmacological properties and physiological effects. Both $\alpha$ and $\beta$-adrenergic receptors had different affinities for catecholamines and that could be identified by the use of specific agonists and antagonists. A number of peripheral organs possess both kinds of receptors and they produce opposite effects. However, the effects of $\alpha$ and $\beta$-adrenergic receptor activation are not always antagonistic.

Binding of catecholamine to $\alpha$ or $\beta$-adrenergic receptors leads to a complex sequence of physiological events through modulation of G proteins. It was reported that antibody responses, proliferation, and lytic activity could be reduced by $\beta$-adrenergic stimulation via elevation of intracellular cyclic adenosine monophosphate (cAMP) and in some cases enhanced by $\alpha$-adrenergic stimulation via elevation of cyclic guanosine monophosphate (cGMP) concentration (review see Madden and Felten, 1995). However, no simplistic pattern has emerged, and it is accepted that catecholamines can differentially affect numerous cell types and the function of internal organs. The total effect of catecholamines on cellular response will depend on the timing of its release and the integration of enhancing and inhibiting events in the reaction.

$\beta$-adrenergic receptors

As mentioned previously, murine lymphoid organs such as the spleen, thymus, and lymph nodes receive adrenergic innervation from the sympathetic systems. Furthermore, murine lymphocytes possess $\beta$-adrenergic receptors. Pharmacologic disruption of this
innervation or directly cutting the nerve which connects to the immune organ leads to altered immune responsiveness (Madden and Livnat, 1991). These results have led some investigators to postulate that the immune system may be modulated in vivo by the sympathetic nervous system via β-adrenergic receptors (Livnat et al., 1985; Sanders and Munson, 1984).

Ahlquist (1948) initially proposed a subdivision of catecholamine receptors into α and β receptors in order to distinguish different physiological responses elicited by various organ systems after exposure to catecholamines. β-adrenergic receptors are found in a large variety of mammalian peripheral tissues; most of these are innervated, but some, such as lymphocytes, must be stimulated by circulating catecholamines. Most of the early studies of these peripheral β-adrenergic receptors were pharmacological. The rank order of potency for a series of catecholamines in different tissues and the selectivities of various agonists and antagonist drugs leads to the subdivision of β-adrenergic receptors into β₁ and β₂ subtypes.

Occupation of the β-adrenergic receptor by an agonist or hormone leads to a complex sequence of events, including an increase of cAMP. The hormone-receptor complex becomes coupled to the G-protein which then binds (GTP). Binding of GTP converts adenylate cyclase from the inactive to the active form. The activated adenylate cyclase converts ATP to cAMP, then cAMP binds to the regulatory subunit of protein kinase A, which phosphorylates a variety of proteins, including an ion channel, an ion pump, vesicle proteins, and enzymes (Fillenz, 1990).
Nadolol

Nadolol is 2,3-cis-1,2,3,4-tetrahydro-5-(2-hydroxy-3-tert-butylamino) proproxy-2,3-naphthalene diol, one of a series of compounds synthesized for its β-blocking properties. Nadolol is a β-adrenergic receptor antagonist which has the following characteristics: a long half-life (22 hr); non-selective for β1 or β2 adrenergic receptors, with no partial agonist or membrane-stabilizing activity; does not reduce renal function. As nadolol can reduce heart rate, diastolic blood pressure, and myocardial oxygen consumption by antagonizing sympathetic activation of the heart, nadolol can be used in the treatment of hypertension and angina pectoris (Heel et al., 1980). Moreover, the action of nadolol has been attributed to inhibition of catecholamine-enhanced irregularities of the heart rate (Gibson et al., 1977).

Maximum serum concentrations of nadolol occur 3-4 h after oral administration and the serum half-life range is 14-24 h. Minimum steady-state serum concentrations of nadolol are linearly related to the dose. Nadolol displays low lipid solubility and does not readily cross the blood-brain barrier (Antonaccio and DeForrest, 1981). In the current study, administration of nadolol is used to determine the role of peripheral β-adrenergic receptors in stress-induced immune changes.

In addition to neuropeptides released by the HPA axis or autonomic nervous system, endogenous opioids mediate immune function. Several investigators have demonstrated that both endogenous opioids and exogenous opiates suppress NK cell activity. For example, Shavit and colleagues (1984) demonstrated that opiates are involved in the stress-induced suppression of NK activity by administration of naloxone. Although opiates are involved in the shock-induced suppression of splenic NK activity, they are not involved in the
suppression of the mitogenic responsiveness of T lymphocytes in the blood or spleen, as pretreatment of rats with naltrexone did not prevent shock-induced immune suppression (Cunnick et al., 1988). Recently, Irwin and colleagues (1991) identified a sympathetic release of norepinephrine in the regulation of NK function.

**Organization of the immune system**

In animals and humans, the immune system is diffuse and involves many diverse organs and cells throughout the body. Two major groups of immune organs are the primary lymphoid organs and secondary lymphoid organs. Each immune organ consists of structurally and functionally diverse immune cells including lymphocytes, granulocytes, mononuclear cells, mast cells, and dendritic cells. Primary immune organs consist of the thymus and bone marrow and the avian bursa of Fabricius. The function of the primary immune organs is to provide an appropriate microenvironment for immature lymphocyte differentiation and education. The secondary immune organs include the lymph nodes, the spleen, blood, and mucosal or gut associated lymphoid tissue (MALT and GALT) (Kuby, 1997).

The main cells of acquired immunity are lymphocytes. Lymphocytes are derived from one lineage of hematopoietic stem cells which originate in the bone marrow. The hematopoietic stem cell is pluripotent. It differentiates into either a lymphoid stem cell or a myeloid stem cell based on the existence of soluble factors in the bone marrow such as interleukin-3 or granulocyte-macrophage colony-stimulating factors. The lymphoid stem cells can differentiate into either T or B progenitor lymphocytes. The progenitor lymphocytes do not interact with antigens but become immature and then mature lymphocytes. The
process of maturation in the thymus and bone marrow provides the immunocompetent T- and B-lymphocytes. After maturation, T- and B-lymphocytes migrate into secondary immune organs to respond to pathogens. The mature lymphocytes are in the Go phase of the cell cycle. Naive mature lymphocytes start to proliferate into lymphoblasts in the presence of antigen specific signals and certain cytokines. Lymphoblasts proliferate and finally differentiate into effector cells or into memory cells. In general, lymphocytes constitute of 20%-40% of the total white blood cells and there are estimated at $10^{10}$-$10^{12}$ lymphocytes in the human body.

The maturation of B lymphocytes is a continuous process in the bone marrow. A mature B-cell is characterized by membrane-bound immunoglobulin (antibody) and other surface molecules such as B220, MHC class II, B7, CD21, CD40, CD19, and CD32. After maturation, B cells leave the bone marrow and migrate into secondary immune organs via the circulation. Depending on the interaction of membrane antibody and antigen, the functional importance of B-cells is to induce an antigen-specific proliferation of B-cell clones which can produce antigen-specific plasma cells. Plasma cells can secrete specific antibodies which serve as the effectors of humoral immunity by neutralizing or eliminating invading organisms.

Unlike B-lymphocytes which mature in the bone marrow, the maturation of progenitor T-lymphocytes occurs in the thymus. The developmental pathway generates a functionally immunocompetent mature T lymphocyte which is self-tolerant and self MHC (major histocompatibility complex) restricted. The diversity of the T-cell repertoire is derived from T-cell receptor (TCR)-gene rearrangement, positive and negative selection.
Positive selection ensures that a T-cell which recognizes self-MHC molecules survive. MHC molecules are cell-surface proteins encoded by a complex of genes that are required for antigen presentation and T-cell maturation. A deficiency of MHC I and II molecules disturbs the intrathymic maturation of T-cells and functional performance of T-cells, and can lead to increased susceptibility to viral or microbial infections. Negative selection destroys the immature T-cell which bears a high affinity receptor for self-MHC alone or self-antigen associated with self-MHC molecules. If a T-cell which can react with self-antigen associated with self-MHC survives, it may lead to autoimmune diseases. About 98% of the immature thymocytes in the thymus die by apoptosis due to failed use of TCR gene rearrangement, positive selection, or negative selection during T-cell development. The surviving T-cells mature and differentiate into immunocompetent cells.

Mature T cells express a distinctive array of membrane molecules such as TCR, CD4, CD8, Thy-1, CD3 CD28, and CD45 on the cell membrane. Hence, the different developmental stages of T-cells can be described based on the changes in the expression of various membrane molecules. Generally, mature T-cells consist of two major functional subpopulations: CD4⁺ and CD8⁺ T cells. CD4⁺ T-cells are MHC class II restricted and function as T helper cells which play an important role in both humoral and cell-mediated immunity. CD8⁺ T-cells are class I MHC restricted and function as T cytotoxic cells which differentiate into cytotoxic T-lymphocytes following interaction with tumor or virus-infected cells. The ratio of CD4⁺ and CD8⁺ lymphocytes is approximately 2:1 in normal human peripheral blood. This ratio may be altered in certain diseases such as immunodeficiency disease, autoimmune disease, and other disorders (Kuby, 1997). After maturation, mature T-
cells leave the thymus and migrate into peripheral immune organs such as the spleen and peripheral blood. In secondary immune organs, mature T-cells can be activated to proliferate and produce several cytokines. To this end, the function of T-cells is to serve as an effector of cellular immunity to directly or indirectly eliminate altered self cells.

**Apoptosis**

Apoptosis is defined as programmed cell death by which cells die following activation of specific genes. The process of apoptosis acts as a fundamental component of many physiological processes, including embryogenesis, morphogenesis, cell-mediated immunity, T- and B-cell maturation, hormone-induced tissue atrophy and tumor regression (Walker et al., 1988). Apoptosis can be characterized based on morphological changes including plasma and nuclear membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation ("ladders") (Cohen, 1993) and biochemical events such as endogenous endonuclease and protease activation (Wyllie, 1980).

Apoptosis can occur in response to an enormous range of physiological and toxicological signals such as anti-CD3/TCR antibody, superantigens, or viral infection (Smith et al., 1898; Jenkinson et al., 1989; Acha-Orbea et al., 1991; Sen, 1992). One model of apoptosis demonstrates the induction of programmed cell death in immature thymocytes by exogenously administrated glucocorticoids (Compton and Cidlowski, 1992). Conversely, mature thymocytes are resistant to apoptosis induced by glucocorticoids.

The existence of cell death by apoptosis in *in vivo* systems is still controversial. However, organisms need a strategy such as apoptosis to regulate normal development and maintain biological homeostasis. Furthermore, hypo-and hyper-regulation of apoptosis could
lead to degenerative diseases, cancer, and autoimmune disease. Recently, a variety of anti-cancer drugs have been shown to induce extensive apoptosis in rapidly proliferating cell populations such as lymphoid tissues and tumors. Hence, triggering tumor cells to undergo apoptosis may provide a means of therapeutic interventions.

In summary, as evidenced by this review, the relation of stress exposure, neuroendocrine regulation, and immune regulation is complex. The current study provides a series of coherent reproducible experiments documenting 2-DG-induced changes in immune function and the mechanisms mediating those effects.

References


CHAPTER 2. CHARACTERIZATION OF THE EFFECT OF 2-DEOXY-D-GLUCOSE (2DG) ON THE IMMUNE SYSTEM

A paper published in *Brain, Behavior, and Immunity*

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Abstract

This study was designed to characterize the effects of the metabolic stress of administration of 2-deoxy-D-glucose (2-DG, 500 mg/kg) on immune function. Male Lewis rats were exposed to one or five injections (one every 48 hours) of 2-DG. Control rats received saline injections. Administration of 2-DG induced a reduction of total leukocytes in the spleen, thymus, and blood. The reduction was most prominent in animals that received five injections of 2-DG. The ratio of CD4+/CD8+ in the spleen was decreased due to a significant increase of CD8+ T-cell subpopulation. Additionally, 2-DG induced a suppression of mitogenic responsiveness and IFN-γ production in both whole blood and spleen lymphocytes. The production of IL-1 and IL-2 was significantly reduced in the blood, but not in the spleen. Conversely, there was a significant increase in nitric oxide production in cultures of Con A, PHA, and LPS-stimulated splenocytes from 2-DG-injected animals.

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compared with saline-injected controls. In blood cultures stimulated with Con A and PHA, the nitric oxide production of the group that received five injections of 2-DG was significantly higher than in the group that received one injection of 2-DG or saline. These results demonstrated that the metabolic stress 2-DG induced a down regulation of Th 1 cellular immune function in a manner similar to physical and psychological stressors. Additionally, the use of 2-DG in rats provided an important model with which to study metabolic stress.

**Introduction**

Stress is a pervasive factor of life that contributes to the pathophysiology of nearly all psychiatric illnesses and a range of medical illness (Breier, et al., 1991). Stress caused by both physical and psychological processes is capable of producing similar and consistent changes in physiologic systems. Correlational studies indicate that stress is linked to many diseases involving the skeletal system, endocrine system, metabolic system, and immune system, such as rheumatoid arthritis, diabetes, cancer, and repeated viral infections (Amkraut, et al., 1971; Fitzpatrick et al., 1988; Eaton et al., 1979; Jemmott et al., 1984; Baker et al., 1981).

Many illnesses are exacerbated by improper dietary intake, drugs of abuse, or disorders of metabolic function. It is suggested that metabolic disorders can alter the function of the immune system and subsequently decrease immune defenses, for example, roughly 50% of all cases of liver cirrhosis in Western societies are related to alcohol abuse and occur in conjunction with viral or metabolic liver diseases (Schuppan et al., 1995).
Additionally, administration of opioid compounds specifically alters macrophage, T-cell, and B-cell functions (for review see Sharp et al., 1995).

Metabolic dysfunction (metabolic stress) can lead to interoceptive stress originating from internal signals (e.g. drug injection). One model system for studying metabolic stress uses 2-deoxy-D-glucose (2-DG). 2-DG is a glucose analogue which can prevent cellular uptake and utilization of glucose (Brown, 1962). 2-DG produces metabolic changes which include acute intracellular glucoprivation and extracellular hyperglycemia (Smythe et al., 1989; Pascoe et al., 1989). In addition, 2-DG-induced stress involves changes in the nervous and endocrine systems such as, increased levels of oxytocin (Cato et al., 1990), increased production of norepinephrine in the brain (Ritter & Neville, 1976) and periphery (Pascoe et al., 1989), and increased glucocorticoid secretion (Smith & Root, 1969). These responses are similar to those that occur in humans following exposure to a number of physical stressors. Thus, the neural and endocrine response to stressors are similar, but the stressors may induce behaviors which are distinct from each other. Whereas animals that experience an exteroceptive stress demonstrate increased locomotor activity, animals which experience interoceptive stress of 2-DG exhibit a decrease in motor activity (Smith & Epstein, 1969).

There are several reasons why 2-DG administration is an interesting stress paradigm to address the interaction of metabolic stress and the immune system. First, 2-DG induces a well characterized physiological response, which produces all the hallmarks of a physical stressor. Second, the neural and endocrine response to 2-DG are similar to electric shock, but induce behavior which is distinct from the physical stress of shock. Third, neurons are
critically dependent on glucose for metabolic activity, the central nervous system is especially sensitive to the effects of 2-DG-induced glucoprivation. Fourth, many syndromes of illnesses induce responses that are similar to those caused by interoceptive stressors which affect dietary intake and metabolic functions. However, there are few models which can be used to study the effect of metabolic stress on the immune system. Therefore, use of 2-DG treatment for studying the effect of metabolic stress on the immune system may provide a means to examine the physiological responses induced by microbial or viral infections.

Previously it has been reported that 2-DG produced suppression of T-cell proliferation to Con A and PHA mitogens in humans and rats (Breier, et al., 1987; Lysle et al., 1988) and increased production of interleukin-1, 3, and 6 in blood from LPS treated mice (Miller et al., 1993). However, little work has been done to characterize the effects of interoceptive or metabolic forms of stress on NK cells, macrophages, or the production of cytokines.

Hence, the purpose of this research was to characterize the effect of the metabolic stressor 2-DG on multiple measures of immune function using rats. To evaluate immune function, this study included a measure of the proliferative response of lymphocytes to various T and B cell mitogens, splenic natural killer cell activity, nitric oxide production from mitogen-stimulated cell cultures, interleukin-1, interleukin-2 and interferon-γ production in the spleen and peripheral blood. Additionally, the spleen was examined for T-cell subsets. Measurement of endogenous glucose, 2-DG, and corticosterone concentrations in peripheral blood were used as indicators of a stress response. The results from this study suggested two
major points: 1) metabolic stressors can influence the immune system in a manner common to exteroceptive physical and psychological stressors; 2) 2-DG administration causes a profound effect on the expression of T helper 1 cell-mediated immune responses.

Materials and Methods

Animals

Inbred male rats of the Lewis strain (55-60 days old; 220-250 g), were purchased from Harlan Sprague Dawley laboratories. Upon arrival, the subjects were individually caged in a colony room where a 12-h reversed light-dark cycle was maintained through artificial illumination. They received free access to both food and water throughout the experiment except as noted, and a 2-week acclimation period prior to the experimental manipulation. Additionally, animals were handled on a bi-daily basis to prevent the hyperreactivity that occurs when they are left untouched for a prolonged period. All animal manipulations were approved by the ISU Committee on Animal Use.

Reagents and Chemicals

The culture medium used for all assays were two kinds: "supplemented media" was used to designate RPMI-1640, supplemented with L-glutamine (50 mM), gentamicin (50 μg/ml), and Hepes (200 mM)(Gibco Lab., Grand Island, NY), and "complete media" was used to designate "supplemented media" containing 10% Fetal Bovine Serum (FBS; JHR Biosciences, Lenexa, KS). The mitogens Concanavalin A (Con A), and Lipopolysaccharide (LPS)-from E.coli were purchased from Sigma (St.Louis, MO); and Phytohemagglutinin (PHA) was purchased from Burroughs Wellcome (England). Heparin 100 units/ml
(ELKINS-SINN, NJ) and 2-DG (Sigma) were diluted in Hanks' Balanced Saline Solution (HBSS)(Gibco Lab). All cultures were prepared in flat-bottomed tissue culture plates (Costar, Cambridge, MA). \( ^3 \)H-thymidine (specific activity 6.7 Ci/mmol), and \( ^51 \)Chromium (specific activity 16.45 GBq/mg(Cr)) from Dupont-New England Nuclear (Wilmington, DE) were used as specified below.

**Experimental Paradigm**

Rats were assigned to one control or two experimental groups (n=8/group). Each control and experimental animal was injected a total of five times, and subjects were manipulated so that groups of rats differed only in the content of the inoculum. Injections were performed once per 48 hours, 2 hrs after lights off. For each injection, a total inoculum of 0.28 ml/rat/injection was administered subcutaneously. Control rats received a total of five injections of sterile Hanks' Balanced Saline Solution (HBSS). The experimental groups of animals received a total of five injections comprised of either 4 HBSS plus one 2-DG (acute) or five (repeated) exposures to 2-DG (500 mg/kg body wt) dissolved in sterile HBSS. Thus, 2 groups of rats received 2-DG on the final test day. For 1 hour following each injection of 2-DG or HBSS, all animals were deprived of food and water. One hour following the last injection, each subject was rapidly sacrificed by cervical dislocation with a clamp.

After sacrifice, the animal was placed on its back and a mid-abdominal incision was made to expose the abdominal aorta. Blood (5 ml) was collected from all subjects into heparinized (10 units/ml blood) syringes through 21-gauge needles. One ml was aliquoted to
a microcentrifuge tube to separate plasma for the glucose assay and another one-ml for
corticosterone assay. The spleen and thymus were removed and placed in preweighed
polypropylene tubes containing 8 ml of complete media. Each organ was weighed to the
nearest mg immediately after removal from the body. Each subject's spleen or thymus was
dissociated into a single-cell suspension by grinding the thymus and the spleen between the
frosted ends of two glass slides. The single-cell suspension was collected after 10 min of unit
gavity sedimentation to remove cell debris and clumps. Subsequently, splenocyte and
thymocyte suspensions were counted using a Celltrak 2 (NOVA Biomedical. Waltham, MA)
cell-analyzer to determine the total leukocyte count per organ. Blood leukocyte count/ml was
also determined by using the Celltrak-2.

Plasma Glucose Assay

The heparinized blood obtained from the abdominal aorta was centrifuged at 12,000
rpm for 5 minutes. The level of glucose present in the plasma was measured by two
methods: one was based upon the glucose oxidase (trinder) reaction which detects
endogenous glucose and 2-DG and; a second was based upon the hexokinase (HK) reaction,
which detects only endogenous glucose (both kits from Sigma). For all subjects, plasma was
obtained from whole blood by centrifugation and stored at -70°C until assayed. On the assay
day, glucose (Trinder and HK) reagents were prepared according to the instructions. The
samples were tested in duplicate. The samples were read at 505 nm for trinder and 340 nm
for HK assay with a spectrophotometer (UVIKON 930, Kontron Instruments). A glucose
standard curve was performed with each assay, and sample glucose concentrations were calculated based upon linear regression analysis of the standard.

**Plasma Corticosterone Assay**

Plasma (0.5 ml) was collected as describe above. The level of corticosterone present in the plasma was determined by a competitive radioimmunoassay sensitive to 0.2 μg/dl corticosterone (Murphy 1967; Lysle et al., 1990a).

**Mitogen Stimulation Assay**

The mitogen assay was performed with splenic and blood leukocytes to assess lymphocyte proliferation. Con A and PHA were used as T-lymphocyte mitogens, and LPS as a B-lymphocyte mitogen as described previously (Cunnick et al., 1990).

**Natural Killer-Cell Assay**

Natural killer-cell activity was assayed in the splenocyte suspensions using an NK sensitive tumor target YAC-1 as previously described (Cunnick, et al., 1990). Radioactivity in supernates was determined using a gamma counter (Gamma Trac 1191; TM Analytic, Inc. IL). Lytic units (LU) were calculated by using a computer program based on the equations of Pross and Maroun (1984). The percent cytotoxicity at all effector:target ratios was utilized to determine LU. Lytic units were based on the number of leukocytes per $10^7$ effectors necessary to lyse 20% of targets.

**Nitric Oxide Assay**

Supernatants were harvested from cultures of mitogen-stimulated lymphocytes prior to pulsing with $^3$H thymidine. The nitric oxide concentrations were determined as nitrite by a
microplate assay (Stuehr and Nathan, 1989). Fifty µl-sample aliquots, were mixed with 100 µl of Griess reagent (0.5% sulfanilamide / 0.05% naphthylethylene diamine dihydrochloride / 2.5% H$_3$PO$_4$) and incubated at room temperature for 10 min. The optical density was measured in an ELISA microtiter plate reader (BIO Kinetics Reader, BIO-TEK instruments) at 550 nm against PBS as a blank. A sodium nitrite (NaNO$_2$) standard curve (0-100 µM) was generated in parallel. Results are expressed as the mean of nitrite concentrations in the supernatants of triplicate wells and analyzed as repeated-measures ANOVA by using the no-mitogen control and all concentrations of a given mitogen.

**Interferon-γ Induction and Assay**

The level of interferon-γ in the crude supernatants of Con-A-stimulated spleen and peripheral blood lymphocytes was measured. Whole spleen leukocytes (2.5 x 10$^6$/ml) were incubated in complete media and Con A 3 µg/ml for 48 hours and whole blood leukocytes (1:20 dilution) were incubated in supplemented media and Con A 10 µg/ml for 72 hr at 37°C in 5% CO$_2$. Culture supernatants were harvested and stored at -70°C until the assay (Johnson et al., 1981). The interferon was determined by means of a commercial ELISA assay for rat interferon-γ (Amgen Biologicals, Life Technologies). Titers were determined by comparison to an interferon-γ standard provided in the assay which is calibrated to an international reference standard (Sonnenfeld et al., 1992).

**Interleukin-2 Induction and Assay**

Interleukin-2 (IL-2) is a T-cell growth factor produced by stimulated T-lymphocytes. To test for the functional ability of T-cells to produce IL-2, 2.5 x 10$^6$ splenocytes / ml of
media were incubated with 3 μg/ml Con A for 48 hr in a 12-well microculture plate. After incubation, the culture supernatants were centrifuged (600 xg, 10 min, 4°C). The conditioned media was stored at -70°C until assayed for IL-2 activity. Supernatants obtained from nonstimulated cultures served as "media control." On the assay day, the supernatants and a positive IL-2 control were serially diluted 1:2 (total of five dilutions) in duplicate in a 96-well microtiter plate by using a digital multichannel pipette (Titertek-Plus). The IL-2-dependent cell line, CTLL-2, was washed to removed any exogenous IL-2 and was then diluted in complete media to 1 x 10⁵/ml. Then 100 μl of the CTLL-2 cell suspension was plated in each well and in control wells containing no IL-2. The plates were incubated for 24 hr at 37 °C in a humidified atmosphere of 5% CO₂. Each well was pulsed with 1 μCi of ³H-thymidine (50 μl) for the last 5 hr of the incubation. The cultures were harvested (Skatron Harvester, Model #11028, Sterling, VA), and the thymidine incorporation was determined by using a liquid scintillation counter (1217 Rackbeta, LKB-Wallac, Finland).

Thymocyte Co-Mitogenesis Assay for Interleukin-1

The ability of biological fluids containing IL-1 to enhance the proliferative response of mouse thymocytes stimulated with mitogen was the basis for the bioassay for quantitation of IL-1 activity. Whole spleen leukocytes (2.5 x 10⁶/ml) were incubated in complete media and LPS 5 μg/ml for 48 hours while whole blood leukocytes (1:20 dilution) were incubated in supplemented media and LPS 5μg/ml for 72 hours at 37°C in 5% CO₂. Culture supernatants were harvested and stored at -70°C until the assay. On the assay day, the supernatants were serially diluted 1:2 in duplicate in a 96- well microtiter plate. Fresh
mouse-thymocyte suspension \((10^6 \text{ cells / 100\mu l})\), prepared from 5-6 weeks old C3H/HeJ
mice (Jackson Laboratories), mixed with PHA, was added to the LPS-stimulated or control
supernatants (final concentration of PHA 2.5 \mu g/ml). The plates were incubated for 72 hours
at 37 °C in a humidified atmosphere of 5 % CO₂. Each well was pulsed with 1 \mu Ci of \(^{3}H-
thymidine for the last 16-18 hours of the incubation. The cultures were harvested using a
Skatron Harvester. Thymidine incorporation was determined by using a scintillation counter.

*Flow Cytometric Analysis of Lymphocyte Subsets*

The positive percentages of CD4⁺ and CD8⁺ lymphocyte subsets were studied by
fluorescent-cell-surface staining with monoclonal antibodies and analysis on an EPICS
PROFILE-1 flow cytometer. Monoclonal mouse antibodies to lymphocyte populations were
purchased from PharMingen or Bioproducts for Science. The anti-CD4 antibody (W3/25)
was conjugated to fluorescein iso-thiocyanate (FITC), and the anti-CD8 antibody (OX-8)
biotinylated for conjugation to avidin phycoerythrin (PE) (Vector Labs) to be used for double
staining. The FITC or Biotin-conjugated mouse IgG1,κ-isotype standard antibodies
(PharMingen, San Diego, CA) were used as control antibodies. Spleen suspension diluted in
HBSS supplemented with 6 % FBS (25 \mu l) were suspended in 100 \mu l of cold PBS/Azide
(0.2%) and incubated in the dark at 4°C for 20 min with 5-10 \mu l of the monoclonal antibodies
or isotypic control antibodies. The erythrocytes were lysed with 2 ml fresh lysing solution
(ammonium chloride 8.02 g, sodium bicarbonate 0.84 g, and EDTA disodium 0.37 g in 1 liter
distilled water) for 10 min at room temperature. The remaining lymphocytes were washed
twice with PBS/Azide. The avidin-PE solution (300 \mu l) was incubated with the cells for 20
min (4°C). The cells were washed 2 more times with PBS/Azide and placed in 2% paraformaldehyde prior to analysis.

**Statistical Treatment of Data**

A computerized program for analysis of variance (ANOVA; Statistix, NH Analytical Software) was used to assess differences among experimental and control groups. For the present experiment to characterize the effects of 2-DG, an ANOVA to compare 3 stress-levels (saline injection/non-stress, 1X 2-DG injection/acute stress, and 5X 2-DG injection/chronic stress) was used to assess significance of the various measures. The level of significance for the F test was set at $P < 0.05$.

**Results**

**Plasma Corticosterone Level**

An elevated level of corticosterone in plasma provided an indicator of stress. The results of the assay for plasma corticosterone (Table 1) demonstrated a pronounced stress effect in the 2-DG treated groups. An analysis of variance demonstrated a significant increase in corticosterone due to overall treatment effect, $F(2,18) = 76.00, p < 0.0001$. Orthogonal contrasts indicated that the groups that received 2-DG injection had significantly elevated levels of plasma corticosterone relative to the saline-injected control group, $F(1,18) = 149.08, p < 0.0001$. In addition, the plasma corticosterone levels were inversely correlated with the number of lymphocytes in thymus ($r = -0.61, p < 0.01$) and blood ($r = -0.567, p < 0.01$). However, the corticosterone level of the group that received five 2-DG injections was not significantly different from that of the group receiving one 2-DG injection.
Endogenous Plasma Glucose and Plasma 2-DG Levels

The glucose oxidase method was used to measure total plasma glucose (endogenous glucose and 2-DG), while the hexokinase reaction provided a measure of endogenous glucose alone. The difference between the assays gave us levels of plasma 2-DG. Levels of plasma glucose (Table 1) were directly related to the levels of corticosterone in circulation ($r = 0.85, p < 0.0001$). Injection of 2-DG resulted in nearly a threefold increase in plasma glucose level. As can be see in Table 1, all groups that received one or five injections of 2-DG had a marked increase of endogenous plasma glucose in comparison with the control groups. Analysis of plasma glucose levels showed a significant effect of treatment, $F (2,18) = 284.81, p < 0.0001$. Orthogonal contrasts indicated a significant drug effect, $F (1,18) = 550.37, p < 0.0001$, and also indicated that the elevation of plasma glucose in the five-injection group was significantly lower than in the one-injection group, $F (1,18) = 19.10, p < 0.0001$.

The plasma levels of 2-DG (Table 1) were 30-50 mg/dl one hour after injection. Furthermore, the 2-DG levels in the animals that received five injections of 2-DG were significantly less than for the animals which received one injection, $F (1,18) = 6.76, p < 0.02$.

Quantification of Immune Organ Weight and Leukocyte Content

Organ weight, body weight, and adrenal gland weight. The body weight, organ weight of spleen, thymus, and adrenal gland were determined (Table 1). Treatment did not significantly affect total body weight (Table 1). The overall average body weight was 290.33 mg / per rat. Additionally, the treatments did not significantly affect adrenal gland weight
among the groups. For the spleen, analysis of variance indicated a significant effect due to treatment, $F(2, 18) = 3.91, p < 0.04$. Contrasts showed the groups that received 2-DG injections had marginally decreased spleen weight in comparison with the saline control group, $F(1, 18) = 3.69, p < 0.08$. However, the decrease of spleen weight for the group that received five injections of 2-DG was significantly different than the group that received saline injections, $F(1, 18) = 7.18, p < 0.02$.

A significant main effect of treatment existed for thymus weight, $F(2, 18) = 19.83, p < 0.0001$. Orthogonal contrasts indicated that 2-DG induced a reduction in thymus weight in comparison with the saline treated control, $F(1, 18) = 11.76, p < 0.003$. Moreover, the thymus weight for the group that received five injections of 2-DG was significantly decreased compared with the group that received one injection of 2-DG, $F(1, 18) = 27.98, p < 0.0001$. An ancillary contrast showed no significant difference in thymus weight between the group that received one injection of 2-DG and the group that received saline injections.

**Leukocyte counts.** The leukocytes in spleen, thymus, and peripheral blood were enumerated (Table 1). In the spleen there was a main effect of treatment, $F(2, 18) = 4.00, p < 0.04$. This effect was due to a significant decrease of leukocyte number in the five-injection group in comparison with the groups that received saline injection, $F(1, 18) = 4.71, p < 0.05$. There also was no significant difference between the group that received one injection of 2-DG and the saline-injection group.

There was a significant main effect of treatment on the number of leukocytes in the thymus, $F(2, 18) = 17.81, p < 0.0001$. Orthogonal contrasts indicated that one and five
injections 2-DG induced a reduction of thymic leukocytes/organ in comparison with the
saline injected group. $F (1, 18) = 17.81, p < 0.001$. The reduction of leukocytes/thymus was
greatest in the group that received five injections of 2-DG compared with the group that
received one injection of 2-DG, $F (1,18) = 17.81, p < 0.001$.

There was a main effect of treatment on the number of leukocytes/ml of blood. $F (2, 18) = 7.16, p < 0.006$. The results showed a significant decrease in blood-leukocyte number in the groups that received one or five 2-DG injections in comparison with the saline-treated group, $F (1.18) = 12.96, p < 0.003$. However, no significant difference was found in the group that received five injections of 2-DG and one injection of 2-DG. In addition, the glucose levels were inversely correlated with the number of lymphocytes in the thymus ($r = -0.4377, p < 0.04$) and blood ($r = -0.5821, p < 0.01$).

Response to Mitogen Stimulation

Spleen assay. The proliferative response of splenic leukocytes to various mitogens was measured as an indicator of functional potential. The mitogen-stimulation assay showed comparable effects across all concentrations of Con A, PHA, and LPS. Analysis of variance of the optimal concentrations of mitogen (Con A 3 µg/ml; PHA 5 µg/ml; and LPS 2.5 µg/ml; Table 2) showed significant drug effects, $F (2, 20) > 3.80, p < 0.04$. These results indicated that the one injection group showed significant suppression in comparison with saline-injection group, $F (1, 20) > 5.60, p < 0.03$. Polynomial contrasts showed a significant linear effect, indicating that repeated 2-DG injections induced an attenuation of suppression. An additional contrast showed that the difference between 5 X 2-DG and saline was not
significant when stimulated with Con A, F (1, 20) < 1, but was significantly different with PHA and LPS stimulation, F (1, 20) > 7.00, p < 0.02.

*Whole-blood assay.* The proliferative response of blood leukocytes to various mitogens was measured as an indicator of functional potential. The results of the whole-blood assay showed comparable effects across all concentrations of Con A and PHA. Analysis of variance of these optimal concentrations of mitogen (Con A 10 μg/ml and PHA 5 μg/ml: Table 2) showed a significant treatment effect for Con A and PHA, F (2,19) > 39.04, p < 0.0001. Orthogonal contrasts indicated that there was a significant suppressive effect of 2-DG injection, F (1, 19) > 76.00, P < 0.0001. There was no significant difference between groups receiving five 2-DG injections and one 2-DG injection. Moreover, there was no attenuation of suppression of the peripheral blood lymphocyte response to mitogen in the group that received five injections of 2-DG.

*Response of Nitric Oxide (NO) Production*

In conjunction with mitogen induced blastogenesis, the production of nitric oxide (NO) was measured in the same culture prior to the addition of ^3H-thymidine. The nitric oxide production of mononuclear cells in spleen, and blood was measured as an indicator of macrophage function. As nitric oxide was rapidly reduced to the stable products nitrite and nitrate, nitric oxide production was quantified from the concentration of nitrite.

*NO assay-Spleen.* Nitrite production from spleen mononuclear cells stimulated with the optimal concentration of mitogen Con A (3 μg/ml), PHA (5 μg/ml), and LPS (2.5 μg/ml) were analyzed and indicated a significant effect of treatment, F (2, 20) > 3.70, p < 0.05
Orthogonal contrasts indicated a significant increase in nitrite in Con A and PHA-stimulated cultures of splenocytes from 2-DG injected animals in comparison to saline injected animals, $F(1, 20) > 4.28, p < 0.05$. Additionally, there was more nitrite in cultures of Con A and PHA-stimulated splenocytes from acutely stressed (1 X 2-DG) animals than from chronically stressed (5 X 2-DG) groups, $F(1, 20) > 7.23, p < 0.02$. However, the nitrite production in spleen cultures stimulated with LPS indicated that the groups that received five injections of 2-DG had less nitrite than either the group receiving one injection of 2-DG or the saline-injection group, $F(1, 20) > 7.29, p < 0.02$.

**NO assay- Blood.** The production of nitrite from whole blood mononuclear cells stimulated with the optimal concentration of Con A (10 μg/ml) and PHA (5 μg/ml) were analyzed. The optimal concentration of mitogens showed a marginal treatment effect, $F(2, 20) > 2.80, p < 0.08$. There was no difference between animals treated with one injection of 2-DG and saline-treated animals, $F(1, 20) < 1$. However, the nitrite production of the group that received five 2-DG injections was greater than the groups that received one injection of 2-DG and saline injections, $F(1, 20) > 5.70, p < 0.03$.

**Natural Killer Cell Assay**

Natural killer-cell function of splenic leukocytes was measured using four effector to target ratios and analyzed as Lytic Units (LU). The results of the natural killer-cell assay are displayed in Figure 1. Analysis of LU showed a significant treatment effect among the groups, $F(2, 18) = 9.64, p < 0.006$. Orthogonal contrasts indicated that the groups that received 2-DG injections demonstrated significantly suppressed natural-killer cell activity.
relative to the saline-treated control group, $F(1, 11) = 19.00, p < 0.002$. However, comparison of both one- and five-injection 2-DG treated groups indicated no significant difference between the groups, $F(1, 11) < 1$. Thus, one 2-DG injection suppressed splenic natural killer-cell activity, and this suppression showed no evidence of increase or attenuation after repeated 2-DG injection. Hence, a metabolic stressor induces substantial suppression of splenic natural killer-cell activity.

**Effects of 2-DG on Cytokine Production**

*Interferon-γ production.* Con-A-stimulated supernatants of splenocytes and blood leukocytes from stressed rats and nonstressed rats were tested for their ability to produce interferon-γ (IFN-γ). The induction of IFN-γ by 3 μg/ml Con A for spleen and 10 μg/ml for blood was evaluated by testing the supernatant fluids of the cultures from the same cell sources as those used in the IL-2 assay. In the spleen (Fig. 2A), there was a marginal effect of treatment, $F(2, 17) = 3.85, p < 0.06$. Orthogonal contrasts showed that the groups which received 2-DG injections had significantly decreased splenic IFN-γ production in comparison with the saline control group. $F(1, 17) = 19.27, P < 0.001$. A comparison between the group that received five injections of 2-DG and the group that received one injection of 2-DG demonstrated no significant difference in IFN-γ production, $F(1, 17) < 1$.

Production of IFN-γ by blood leukocytes (Fig. 3A) demonstrated a significant effect due to treatment, $F(2, 17) = 9.66, p < 0.004$. Orthogonal contrasts indicated that 2-DG induced a significant decrease in interferon-γ production of blood, $F(1, 17) = 19.27, P < 0.001$. in comparison with the saline treated controls. However, there was no difference
between those groups that received five injections and those that received one injection of 2-DG, F (1, 17) < 1. These results indicate that the metabolic stress of 2-DG induced suppression of Con A stimulated IFN-γ production in splenocytes and blood leukocytes.

*Interleukin-2 (IL-2) production.* Production of IL-2 was induced in cultures of lymphocytes in response to the T-cell mitogen, Con A. IL-2 was measured by using the IL-2-dependent cell line, CTLL-2. Overall analysis of variance of IL-2 production by splenic supernatants did not demonstrate any significant alteration due to treatment (Fig. 2B).

Analysis of variance of IL-2 production using the optimal dilution (1/4) of supernatant from blood leukocyte cultures showed a marginally significant main effect of treatment. F (2, 17) = 3.39, p < 0.06 (Fig. 3B). Orthogonal contrasts demonstrated that 2-DG induced a significant reduction of IL-2 production in comparison with the saline-treated control. F (1, 17) = 4.45, p = 0.05. Also, there was a significant difference between group which received one injections and saline injection of 2-DG, F (1, 17) = 6.71, p < 0.02. However, there is no difference between group which received five injections and one injection of 2-DG.

These results indicated that the stress of 2-DG induced a suppression of IL-2 production in the blood, but not in the spleen. This pattern differs from that observed for the production of IFN-γ.

*Interleukin-1 (IL-1) production.* The production of IL-1 was used to measure the functional activity of macrophages. LPS was used to induce IL-1 production in cultures of leukocytes. IL-1 production was measured using the thymocyte costimulation bioassay.
Analysis of macrophage production of IL-1 in splenic supernatants did not indicate any significant change due to treatment (Fig. 2C).

Analysis of variance of IL-1 production using the optimal dilution (1/4) of supernatant from blood leukocyte cultures showed a significant treatment effect, \( F(2.23) = 38.40, p < 0.0001 \) (Fig. 3C). Orthogonal contrasts demonstrated that 2-DG induced a pronounced suppression of IL-1 production in comparison to the saline treated control. \( F(1.23) = 75.00, p < 0.0001 \). However, there was no significant difference in suppression between animals that received one and those that received five injection(s) of 2-DG. These results indicated that the metabolic stress of 2-DG induced a suppression of IL-1 production in the blood, but not in the spleen. This pattern was consistent with the results of IL-2 production. Also, the outcome of cytokines production (IFN-\( \gamma \), IL-2, IL-1) was in agreement with the hypothesis that different compartments of the immune system are differentially affected by a stressor.

**Flow Cytometric Analysis of Lymphocyte Subsets**

In order to identify changes in CD4 and CD8 positive T-cell subsets, aliquots of splenocytes were stained with the monoclonal antibodies W3/25 (anti-CD4) and OX-8 (anti-CD8). Analysis of variance of the percentage of CD4\(^+\) and CD8\(^+\) population demonstrated a significant treatment effect, \( F(2, 18) > 4.20, p < 0.03 \). Orthogonal contrasts indicated no significant difference in CD4\(^+\) population between the groups that received 2-DG and saline injection (Fig. 4A). However, 2-DG injection induced a significant increase in CD8\(^+\) population, \( F(1, 18) = 8.47, p < 0.01 \) (Fig. 4B). As there was a decrease in the number of
leukocytes in spleens from animals receiving five injections of 2-DG, we also analyzed the data as number of CD4 and CD8 cells per spleen. These analysis were similar to those described for the percent CD4 and CD8 positive data and demonstrated no significant change in the number of CD4 cells per spleen (grand mean: 226.93 x 106) and a significant increase in the number of CD8 cells per spleen in rats which received 1X2DG and 5X2DG compared to saline treated controls (means: 45.1, 50.7, 37.5 x 106, respectively), F(1,18) = 6.55, p < 0.02.

The ratio of CD4⁺: CD8⁻ cells demonstrated a significant treatment effect, F (2, 18) > 4.20, p < 0.03, which was due to the increase in CD8⁺ lymphocytes. Animals receiving 2-DG had significantly lower ratio than saline treated animals, F (1, 18) = 8.04, p < 0.01 (Fig. 4C). The percent of CD8⁻ population and the ratio of CD4⁺ to CD8⁺ between groups that received one or five injections of 2-DG did not differ.

Discussion

The present study was designed to characterize the alterations of immune function due to the metabolic stress of 2-DG on both innate and adaptive immunity. Results indicated that metabolic stress has a profound effect on the expression of immunological parameters that play a major role in the expression of cell-mediated immune responses.

This study showed that a reduction of lymphocyte content was induced in the spleen, thymus, and blood of rats that received repeated injections of 2-DG. Similar patterns were also observed in measurement of organ weight in spleen and thymus. As expected, 2-DG induced increased secretion of corticosterone and exaggerated plasma hyperglycemia. Data
analysis indicated that the increased plasma corticosterone concentration was positively correlated with an increase in endogenous glucose but was inversely correlated with the decrease of blood lymphocytes. Therefore, the effects of 2-DG on lymphocyte number could be explained because of the production of stress hormones (e.g., corticosterone and catecholamine). Synthetic glucocorticoids have well-known immunosuppressive effects in vitro and in vivo, whereas the immunologic influence of endogenous glucocorticoids at physiological levels is less clear (Munck, et al., 1984). Recently, studies by Dhabhar and his colleagues (1995) suggest that corticosterone released during physiologically relevant conditions plays an important role in regulating the trafficking of leukocytes between the blood and other immune compartments and this redistribution can affect an immune response to potential or ongoing immune challenges.

Beside of the reduction of splenocytes, splenic T-cell subpopulations also changed in animals that received one or five 2-DG injection. Interestingly, the percentage of CD4^ T-cell subpopulation did not change, in contrast, the percentage of CD8^ T-cell subpopulation was increased in 2-DG treated animals. The present study did not examine the mechanism of increase of CD8^ T-cell population or its role in long term immune suppression / regulation. However, the role of CD8^ T suppressor remains controversial.

2-DG-induced metabolic stress altered the function of innate immune mechanisms. Natural killer-cell (NK) activity, was significantly suppressed in the spleen after 2-DG administration, implicating the influence of 2-DG-induced metabolic stress on the capacity of resistance to pathogenic microorganisms and neoplastic diseases. The functional level of
macrophages were examined through the production of nitric oxide (NO) and IL-1. Results indicated more nitrite in cultures of spleen and blood lymphocytes from 2-DG-stressed animals than from saline-treated animals. Although macrophage secreted more NO in 2-DG-stressed rats than saline-treated rats, IL-1 production in peripheral blood was decreased after 2-DG administration. This shift towards increased NO production may be an evolutionarily adaptive response for increased bactericidal activity, if wounds became infected. Contrary to our results, Miller et al (1993) reported that administration of 2-DG in mice could enhance the IL-1 activity in blood plasma. However, mice have been shown to respond differently than rats to physical stressors (Lysle et al., 1990b). Moreover, Miller and colleagues (1993) examined IL-1 activity in the plasma of 2-DG and LPS treated mice, whereas our study measured IL-1 production in vitro.

Previous research reports that 2-DG administration suppresses lymphocyte blastogenesis (Lysle et al., 1988). The mitogen proliferation data obtained from the current study replicates the previous work demonstrating the ability of 2-DG to suppress T-cell mitogenic responses in blood and spleen to Con A and PHA. That work is extended in that this study showing 2-DG also induced suppression of the splenic B-cell mitogen response to LPS. Suppression of LPS mitogen suggested that humoral responses can be suppressed by 2-DG. The present study demonstrated an inverse correlation between blastogenesis and nitrite production in the spleen and blood. Excess NO production may have contributed to the inhibited lymphocyte proliferation. Previous work shows that murine macrophages act as suppressor cells to down-regulate lymphocyte-dependent immune events (Mills, 1991). In
addition, a nitric oxide synthase (NOS)-dependent suppressor activity on mitogen-induced lymphoid proliferation responses in splenocytes has been reported in Fisher rat (Mills, 1991; Albina et al., 1991), and spontaneously hypertensive rats (Pascual et al., 1993). Recently, Lysle and colleagues (1995) provided evidence that morphine and an aversive Palovian conditioned stimulus induce an increase in macrophage-derived NO which is directly involved in the suppressed proliferative response of splenic lymphocytes to mitogen. Those studies provide a reference for the present results and suggest that the increased NO production in 2-DG-treated rats induced the decrease of splenic lymphocyte proliferation. However, the relationship of NO production to blood lymphocyte proliferation needs further study.

Consistent with other stress studies, 2-DG not only inhibited T lymphocyte proliferation, but also influenced the production of T-cell-derived cytokines. Studies using electric shock in rats (Sonnenfeld et al., 1992) and examination stress in humans (Glaser et al., 1986) have shown a decrease of IL-2 and IFN-γ. The results of this study indicated that IFN-γ production by spleen and blood lymphocytes of rats is inhibited after the metabolic stress of 2-DG administration. Alternatively, data also indicated a reduction in the level of IL-2 production by peripheral blood lymphocytes from 2-DG treated rats, but not by splenic lymphocytes. This pattern is similar to effects seen with other stressors (Cunnick et al., 1991), but differs from that observed for the production of IFN-γ. The reduction of IL-2 and IFN-γ in 2-DG-treated animals suggests metabolic stress has a profound effect on the T-helper 1 cell population.
In the current study, 2-DG produced a suppression of splenic T-cell function with a single exposure and a habituation of splenic suppression with multiple exposures. In contrast, the suppressive effect for the whole-blood lymphocytes did not show any attenuation of suppression over repeated administration. Moreover, 2-DG induced depression of IL-1 and IL-2 production in cultures of blood lymphocytes, but not in cultures of splenic lymphocytes. These results are consistent with other research showing that stress differentially affects various compartments of the immune system, such as the spleen and blood. (Lysle et. al., 1988; Cunnick et al., 1990). The reason for the differential effects across compartments of the immune system is not readily apparent from this study. However, Cunnick and colleagues (1990) provided evidence that catecholamines are responsible for splenic immune suppression and that adrenal hormones are responsible for blood lymphocyte suppression in a model of physical stress. Our laboratory is in the process of investigating the mechanisms involved in the 2-DG-induced immune changes (paper in preparation).

In summary, these studies demonstrate that the metabolic stressor, 2-DG, can alter cellular immune function which are T-helper 1 cell predominant. In addition, the immune changes induced by 2-DG are consistent with other stress studies using the physical stress of shock and aversive conditioning. In the past, use of mild electric shock to study immunological effects of stress in animals has shown increased locomotor activity which has made conclusions complicated and often controversial. Conversely, animals which experience interoceptive stress of 2-DG exhibited a decrease in motor activity (Smith &
Epstein, 1969). Hence, the use of 2-DG on rats provides a ideal model for the study of metabolic stress. It also provides insight about immune function during pathogenic conditions which alter metabolism.

References


Table 1. Effects of 1 and 5 injections of 2-DG (500 mg/kg) and saline injection(s) on the plasma corticosterone, endogenous glucose, 2-DG, leukocytes number, total body weight, and organs weight.

<table>
<thead>
<tr>
<th>Injection Manipulation</th>
<th>1X 2-DG</th>
<th>5X 2-DG</th>
<th>Saline</th>
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</thead>
<tbody>
<tr>
<td><strong>Plasma level</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Corticosterone (μg/dl)</td>
<td>41.26 ± 3.45</td>
<td>48.02 ± 6.19</td>
<td>2.74 ± 1.99 a</td>
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<tr>
<td>Endogenous glucose (mg/dl)</td>
<td>319.5 ± 8.12</td>
<td>283.6 ± 8.21</td>
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<td>2-DG (mg/dl)</td>
<td>52.04 ± 5.34</td>
<td>33.90 ± 5.83</td>
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<tr>
<td><strong>Leukocyte number</strong></td>
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<td>Spleen (x 10^-6 /organ)</td>
<td>420.3 ± 13.06</td>
<td>373.8 ± 14.6</td>
<td>411.7 ± 13.48</td>
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<tr>
<td>Thymus (x 10^-6 /organ)</td>
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<td>478.2 ± 14.72</td>
<td>591.2 ± 24.06</td>
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<td>Blood (x 10^-6 /ml)</td>
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<td>3.038 ± 0.39</td>
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<tr>
<td><strong>Total body weight (gm)</strong></td>
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<td>279 ± 6.94</td>
<td>296.5 ± 5.64</td>
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<td><strong>Organ weight (mg)</strong></td>
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<td>562.6 ± 14.3</td>
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<td>Thymus</td>
<td>455.4 ± 18</td>
<td>337.2 ± 17</td>
<td>462.6 ± 17</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>63.75 ± 1.4</td>
<td>69.75 ± 3.2</td>
<td>66 ± 1.8</td>
</tr>
</tbody>
</table>

a. Data are expressed as the mean ± SE; n = 8/grp
Table 2. Effects of 1 and 5 injections of 2-DG (500 mg/kg) and saline injection(s) on the mitogen responsiveness and nitric oxide production of splenic and peripheral blood leukocytes.

<table>
<thead>
<tr>
<th>Injection Manipulation</th>
<th>1 X 2-DG</th>
<th>5X 2-DG</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mitogen response (cpm x 10(^{-3}))</strong>(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>197.5 ± 32.93</td>
<td>239.9 ± 23.76</td>
<td>279.3 ± 7.88(^d)</td>
</tr>
<tr>
<td>PHA</td>
<td>43.81 ± 17.12</td>
<td>89.95 ± 25.53</td>
<td>169.2 ± 23.23</td>
</tr>
<tr>
<td>LPS</td>
<td>27.33 ± 2.127</td>
<td>30.69 ± 1.74</td>
<td>39.09 ± 2.45</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>4.71 ± 1.41</td>
<td>4.92 ± 1.63</td>
<td>44.06 ± 5.68</td>
</tr>
<tr>
<td>PHA</td>
<td>0.90 ± 0.12</td>
<td>0.865 ± 0.16</td>
<td>10.41 ± 1.43</td>
</tr>
<tr>
<td><strong>Nitrite production (µmole/ml)</strong>(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>8.32 ± 0.807</td>
<td>7.57 ± 1.01</td>
<td>6.35 ± 1.14(^c)</td>
</tr>
<tr>
<td>PHA</td>
<td>11.80 ± 1.53</td>
<td>10.24 ± 1.61</td>
<td>9.44 ± 1.74</td>
</tr>
<tr>
<td>LPS</td>
<td>7.84 ± 1.31</td>
<td>6.01 ± 1.12</td>
<td>7.852 ± 1.65</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>5.33 ± 2.72</td>
<td>10.60 ± 3.67</td>
<td>6.08 ± 1.61</td>
</tr>
<tr>
<td>PHA</td>
<td>4.51 ± 1.49</td>
<td>10.01 ± 3.81</td>
<td>5.10 ± 0.71</td>
</tr>
</tbody>
</table>

\(a\) Mitogen stimulation was induced in cultured splenocytes and blood leukocytes with the optimal concentration of Con A (3µg/ml on spleen, 10µg/ml on blood), PHA (5µg/ml), and LPS (2.5µg/ml).

\(b\) Results are expressed as mean / 5 x 10\(^5\) leukocytes.

\(c\) Production of nitric oxide by splenocyte and blood leukocyte cultures incubated with 3 µg/ml or 10 µg/ml ConA, 5 µg/ml PHA, or 2.5 µg/ml LPS. Nitric oxide was measured as nitrite in supernatant by use of the Griess reagent in the supernatant of blastogenesis cultures.

\(d\) Data are expressed as the mean ± SE of average triplicate counts per minute; n = 8 /grp.

\(e\) Nitrite are expressed as mean ± SE of average triplicate µmole/ml; n = 8 /grp.
**Figure 1.** Splenic NK activity expressed as (A): percent cytotoxicity and (B): lytic units (LU) for rats receiving 1 or 5 injections of 2-DG (500 mg/kg) or saline injections.

Results are expressed as the mean (± SE); n = 4 / grp.
A. NK Cytotoxicity

B. NK Lytic units (LU)
Figure 2. Effect of 1 or 5 injections of 2-DG (500 mg/ml) and saline injections on the IFN-γ (A), IL-2 (B), and IL-1 (C) production of splenocytes. IFN-γ and IL-2 production were induced in cultured splenocytes with 3 mg/ml Con A and IL-1 production was induced with 2.5 μg/ml LPS. Results of IFN-γ production were expressed as the mean (± SE) of units/ml (1 unit = 250 pg); n = 6 / grp. Results of IL-2 and IL-1 production were expressed as the mean (± SE) of the average duplicate counts per minute; n = 8 / grp.
Figure 3. Effect of 1 or 5 injections of 2-DG (500 mg/ml) and saline injections on the IFN-γ (A), L-2 (B), and IL-1 (C) production of blood leukocytes. IFN-γ and IL-2 production were induced in cultured blood leukocytes with 10 mg/ml Con A and IL-1 production was induced with 2.5 μg/ml LPS. Results of IFN-γ production was expressed as the mean (± SE) of units/ml (1 unit = 250 pg); n = 6 / grp. Results of IL-2 and IL-1 production were expressed as the mean (± SE) of the average duplicate counts per minute; n = 8 / grp.
Figure 4. Effect of 1 and 5 injections of 2-DG (500 mg/kg) and saline injections on T-cell subpopulations in spleen, expressed as the percent positive cells staining with antibody to A: CD4 (W3/25), B: CD8 (OX8), or C: % CD4$^+$ / % CD8$^+$. Results are expressed as the mean (± SE); n = 8 / grp.
CHAPTER 3. EVIDENCE FOR CATECHOLAMINES INVOLVEMENT IN THE 2-DG-INDUCED IMMUNOMODULATORY EFFECTS IN SPLEEN

A paper submitted to *Brain, Behavior, and Immunity*

Shiu-Huey Chou, Ljiljana D. Kojic, and Joan E. Cunnick

**Abstract**

The role of catecholamines in immune changes associated with the metabolic stress of 2-deoxy-D-glucose (2-DG) was examined in this study. Male Lewis rats were pretreated with the nonselective β-adrenergic receptor antagonist, nadolol (0-0.5 mg/kg), and then received either a saline or 2-DG (500 mg/kg) injection. Nadolol attenuated the 2-DG-induced suppression of splenic T-cell mitogenic response and interferon-γ production and increased nitric oxide production by macrophages in a dose-dependent manner. Conversely, nadolol did not attenuate the 2-DG-induced changes in immune parameters in peripheral blood leukocytes. These results suggest that the peripheral release of catecholamines is responsible for 2-DG-induced splenic immune alterations, whereas the peripheral release of catecholamine is not responsible for 2-DG-induced blood immune alterations. Furthermore, the neuroendocrine mechanisms responsible for splenic immune changes induced by the metabolic stress of 2-DG administration were the same as those involved in immune changes induced by physical and psychological stress. Thus, this study suggests that common neuroendocrine pathways exist for several types of stress-induced immunomodulations.
Introduction

The glucose analogue 2-DG induces metabolic stress by preventing cellular uptake and utilization of glucose. In rodent studies, acute administration of 2-DG inhibited splenic and blood T-cell proliferation induced by Concanavalin A (Con A) and Phytohemagglutinin (PHA) mitogens in Sprague-Dawley rats (Lysle et al., 1988). Similar results were also observed in Swiss-Webster mice (Miller et al., 1994).

Although, the administration of 2-DG to Lewis rats produced suppression of T-cell proliferation after one injection, after multiple (5) injections the immune suppression of splenic T-cell responses was attenuated, but not the suppression of blood T-cell responses. In addition, production of IL-1 and IL-2 in peripheral blood cultures was significantly decreased, but not in spleen (Chou et al., 1996). This finding suggests that different immune compartments are uniquely affected by stress, and the regulation of the immune response in spleen and blood is mediated by different mechanisms.

The mechanisms by which 2-DG exerts the immune suppressive effects is still unclear. Although 2-DG can affect intracellular glucose metabolism, it is unlikely that changes in immune function are a direct effect of 2-DG. In vitro incubation of naive T-cells with varying concentrations of 2-DG did not result in a reduction in cell number or viability (Miller et al., 1994).

An interaction between the immune, nervous, and endocrine system is most commonly postulated as the cause of the pronounced effects of stress on immune function (for review, see Madden & Felten, 1995). As a metabolic stressor, 2-DG administration has
been shown to alter turnover of norepinephrine in the brain (Ritter & Neville, 1976) and peripheral blood (Pascoe et al., 1989) and increase glucocorticoid production (Smith & Root, 1969). Hence, neuroendocrine regulation may provide a possible mechanism for 2-DG-mediated immune modulation. In a rat model of physical stress, catecholamines are responsible for splenic immune suppression, whereas adrenal hormones are responsible for blood lymphocyte suppression (Cunnick et al., 1990). In mice, administration of propranolol, a β-adrenergic receptor antagonist, does not attenuate splenic mitogen suppression induced by 2-DG treatment. However, inhibition of splenic T-cell proliferation is attenuated in hypophysectomized and adrenalectomized mice (Miller et al., 1994). Therefore, the mechanism by which stress modulates the interaction between the immune and neuroendocrine system may vary depending upon species or stressor.

Catecholamines have an important role in the modulation of the immune system (for review, see Madden & Livnat, 1991). T-lymphocytes are in direct physical contact with sympathetic nerve endings in the spleen of rats (Felten et al., 1985; Felton & Olschowka, 1987). Also, β-adrenergic receptors are expressed on T- and B-lymphocytes, macrophages, and neutrophils (Williams et al., 1976; Fuchs et al., 1988; Abrass et al., 1985; Galant et al., 1978). Additionally, several studies show that the blastogenic responses of lymphocytes to mitogens, IL-2-induced proliferation of lymphocytes, the lytic activity of cytotoxic T-cells, and T-cell-dependent antibody production are modulated by the stimulation of β-adrenergic receptor in vitro (for review, see Madden & Livnat, 1991). Moreover, numerous studies provide evidence for the involvement of β-adrenergic receptors in the immunomodulatory
effects of physical and psychological stress (Luecken & Lysle, 1992; Fecho et al., 1993). Thus, there is strong evidence to suggest a role for catecholamines in 2-DG-induced immunomodulation.

The purpose of the current research is to examine the role of the sympathetic release of catecholamines in 2-DG-induced immune alteration, as measured by mitogenic responses, natural killer-cell activity, mononuclear cell-derived nitric oxide production, and interferon-γ (IFN-γ) production in spleen and blood. The nonselective β-adrenergic receptor antagonist nadolol was used to block the binding of catecholamines to their receptors and to determine whether the antagonist could prevent 2-DG-induced immune alterations. This study suggests that catecholamines are the mechanism underlying the capacity of 2-DG-induced metabolic stress to influence immune cell potential in the spleen, but not in the blood, of rats.

Materials and Methods

Animals

Inbred male rats of the Lewis strain (55-60 days old, 220-250 g) were purchased from Harlan-Sprague-Dawley laboratories (Indianapolis, Indiana). Upon arrival, the subjects were individually caged in a colony room where a 12-h reversed light-dark cycle was maintained through artificial illumination. They received free access to both food and water throughout the experiment, except as noted, and a 2-week acclimation period before the experimental manipulation. Additionally, animals were handled every two days to prevent the hyper-reactivity that occurs when they are left untouched for a prolonged period. All animal manipulations were approved by the Iowa State University Committee on Animal Use.
Reagents and Chemicals

The assays employed two kinds of culture media: "supplemented medium" refers to RPMI-1640 supplemented with L-glutamine (2 mM), gentamicin (50 μg/ml), and Hepes (10 mM)(Gibco Lab., Grand Island, NY), and "complete medium" refers to "supplemented medium" containing 10% Fetal Bovine Serum (FBS) (JHR Biosciences, Lenexa, KS). The mitogens Con A, and Lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma (St. Louis, MO), and PHA was purchased from Burroughs Wellcome (England). Heparin (100 units/ml, ELKINS-SINN, NJ) and 2-DG (Sigma) was diluted in Hanks' Balanced Saline Solution (HBSS)(Gibco Lab.). All cultures were prepared in flat-bottomed 96-wells tissue culture plates (Costar, Cambridge, MA). $[^3]H$thymidine (specific activity 6.7 Ci/mmol), and $^{51}$Chromium (specific activity 16.45 GBq/mg(Cr)) from Dupont-New England Nuclear (Wilmington, DE) were used as specified below.

Experimental Paradigm: Nadolol and 2-D Treatments

Animals were treated with nadolol, a β-adrenergic receptor antagonist 2 hr into the dark phase of light-dark cycle. Rats were randomly assigned to one of four nadolol treatment groups (6 rats/group), which received subcutaneous (SC) nadolol (0, 0.05, 0.1, or 0.5 mg/kg body wt) (The SQUIBB Institute, Princeton, NJ). Nadolol, a form of propranolol (cis-5[3-[(1,1-dimethylethyl) amino]-2 hydroxy-propoxy]-1,2,3,4-tetrahydro-2,3-naphthalenediol) that does not cross the blood-brain barrier, was dissolved in dimethyl sulfoxide (DMSO), diluted in HBSS and administered. The vehicle control (0 mg/kg nadolol) for the receptor antagonists was an equal volume of HBSS/1% DMSO.
2-DG (500 mg/kg, SC) or saline (HBSS, equivolume) was administered thirty minutes after the nadolol injection to subgroups of each nadolol treatment. A non-injected control group consisted of animals without nadolol, saline, or 2-DG injections. Animals were sacrificed 60 min after their last injection, as previously described (Chou et al., 1996). The sixty minute time course was chosen to enable a comparison of stressor induced changes with the physical stress model of shock (16 shocks in 64-min session) (Lysle et al., 1987).

**Tissue Collection and Preparation of Leukocytes**

**Blood and Plasma** Blood (5 ml) was collected from the abdominal aorta through 21-gauge needles into heparinized (100 units/ml blood) syringes. One milliliter was aliquoted to a microcentrifuge tube to separate plasma for the glucose assay, and another one milliliter was aliquoted for the corticosterone assay. The remaining blood was diluted to a final dilution of 1:20 with supplemented media for use in mitogen stimulation assay, nitric oxide assay, and IFN-γ induction. The leukocyte count/ml was determined by using the Celltrak-2 (NOVA Biomedical, Waltham, MA) prior to blood dilution.

**Spleen** The spleens were removed and placed in preweighed polypropylene tubes containing 8 ml of complete medium. Immediately after removal from the body, each organ was weighed to the nearest mg. Each subject's spleen was dissociated into a single-cell suspension by grinding the spleen between the frosted ends of two glass slides. The single-cell suspension was collected after 10 min of unit gravity sedimentation to remove cell debris and clumps. Subsequently, the splenocyte suspensions were enumerated by using a Celltrak 2 cell-analyzer to determine the total leukocyte count per organ.
**Plasma Glucose Assay**

The level of glucose present in the plasma was measured by two methods: one was based upon the glucose oxidase (Trinder) reaction, which detects endogenous glucose and 2-DG, and the second was based upon the hexokinase (HK) reaction, which detects only endogenous glucose (Sigma, St. Louis, MO). For all subjects, plasma was obtained from whole blood by centrifugation (12,000 rpm/5 min) and stored at -70°C until assayed. The samples were tested in duplicate and read at 505 nm for the Trinder and 340 nm for the HK assay with a spectrophotometer (UVIKON 930, Kontron Instruments AG, Switzerland). A glucose standard curve was performed with each assay, and sample glucose concentrations were calculated based upon linear regression analysis of the standard.

**Plasma Corticosterone Assay**

Plasma (0.5 ml) was collected as described previously. The level of corticosterone present in the plasma was determined by a competitive radioimmunoassay sensitive to 0.2 μg/dl corticosterone (Murphy 1967; Lysle et al., 1990).

**Mitogen Stimulation Assay**

The mitogen assay was performed with splenic and blood leukocytes to assess lymphocyte proliferation. Con A and PHA were used as T-lymphocyte mitogens, and LPS as a B-lymphocyte mitogen, as described previously (Cunnick et al., 1990).

**Natural Killer-Cell Assay**

Natural killer-cell activity was assayed in the splenocyte suspensions using the NK sensitive tumor target YAC-1, as previously described (Cunnick et al., 1990). Radioactivity
in supernatants was determined by using a gamma counter (Gamma Trac 1191, TM Analytic, ElkGrove Village, IL). Lytic units (LU) were calculated by using a computer program based on the equations of Pross and Maroun (1984). The percent cytotoxicity at all effector:target ratios was utilized to determine LU. Lytic units were based on the number of leukocytes per 10^7 effectors necessary to lyse 20% of targets.

**Nitric Oxide Assay**

Supernatants were harvested from cultures of mitogen-stimulated lymphocytes before pulsing with 3H thymidine. The nitric oxide concentrations were determined by measuring nitrite in a microplate assay (Stuehr and Nathan, 1989). Sample aliquots (50 μl) were mixed with 100 μl of Griess reagent (0.5% sulfanilamide/0.05% naphthylethylenediamine dihydrochloride/2.5% H₃PO₄) and incubated at room temperature for 10 min. The optical density was measured in an ELISA microtiter plate reader (BIO Kinetics Reader, BIO-TEK Instruments, Winooski, VT) at 550 nm against PBS as a blank. A sodium nitrite (NaNO₂) standard curve (0-100 μM) was generated in parallel. Results are expressed as the mean of nitrite concentrations in the supernatants of triplicate wells and analyzed as repeated-measures analysis of variance (ANOVA) by using the no-mitogen control and all concentrations of a given mitogen.

**IFN-γ Induction and Assay**

The level of IFN-γ in the crude supernatants of Con-A-stimulated spleen and peripheral blood lymphocytes was measured. Whole spleen leukocytes (2.5 x 10⁶/ml) were incubated in complete medium and Con A (3 μg/ml) for 48 hours, and whole blood
leukocytes (1:20 dilution) were incubated in supplemented medium and Con A (10 μg/ml) for 72 hr; both were at 37°C in 5% CO₂. Culture supernatants were harvested and stored at -70°C until the assay (Johnson et al., 1981). IFN-γ was determined by means of a commercial ELISA assay for rat IFN-γ (GIBCO BRL, Life Technologies, Gaithersburg, MD). Titers were determined by comparison to a provided IFN-γ standard, which is calibrated to an international reference standard (Sonnenfeld et al., 1992).

**Statistical Treatment of Data**

A computerized program for ANOVA (Statistix, NH Analytical Software) was used to assess differences among experimental and control groups. For the present experiment to assess the effects of nadolol administration on attenuation of acute stress effects of 2-DG, a 1 x 9 ANOVA was used to determine the overall effects of treatments and means square error (M.S.E.) for all data. Contrasts were designed to provide a 2 (stress) x 4 (nadolol dose) + 1 (no injection control vs. saline injection control) of the 9 groups. There were two levels of stress (2-DG injection and saline injection) and four levels of nadolol (0, 0.05, 0.1, and 0.5 mg/kg), except where noted. The level of significance for the F test was set at $P < 0.05$.

**Results**

**Plasma Corticosterone Level**

An elevated level of plasma corticosterone provided an indicator of stress (Table 1). Overall ANOVA indicated a significant effect of the treatments, $F(8, 41) = 36.40, p < 0.0001$. All groups that received 2-DG had a marked increase of plasma corticosterone (mean = 47.31 ± 0.287 mg/dl across all doses of nadolol) in comparison with the saline injection
groups, $F(1, 41) = 273.90, p < 0.0001$. There were no other significant differences among the groups. These findings indicate that the $\beta$-adrenergic receptor antagonist, nadolol, does not modulate the production of corticosterone induced by stress.

**Endogenous Plasma Glucose and Plasma 2-DG Levels**

The glucose oxidase method was used to measure total plasma glucose (endogenous glucose and 2-DG), whereas the hexokinase reaction provided a measure of endogenous glucose alone. The difference between the assays indicates the level of plasma 2-DG. The mean plasma level of 2-DG was 35.41 mg/dl one hour after injection for all 2-DG-treated groups and did not differ significantly among groups which received 2-DG. All groups that received 2-DG (500 mg/kg) injections had a significant increase in endogenous plasma glucose in comparison with the saline treated group (Table 1). Analysis of plasma glucose levels showed a significant difference among the groups, $F(8, 41) = 124.90, p < 0.0001$. Orthogonal contrasts revealed a significant increase due to the 2-DG treatment, $F(1, 41) = 859.66, p < 0.0001$, and a significant interaction between 2-DG and nadolol treatments, $F(3, 41) = 3.74, p < 0.02$. The interaction had a significant linear effect, $F(1, 41) = 6.86, p < 0.02$, with the highest glucose at the 0 mg/kg dose of nadolol. However, comparison of the no-injection control group with the saline/nadolol (0-0.5 mg/kg) control group showed no significant effect of the injection procedure.

**Leukocyte Counts**

The number of leukocytes in spleen and peripheral blood were determined, and the results are shown in Table 2. Enumeration and analysis of total splenic leukocytes showed
no significant difference between 2-DG-treated and control rats (grand mean = 4.10 x 10^8 leukocytes/spleen). Overall analysis of blood leukocytes showed a significant difference among the groups, F(8, 41) = 12.07, p < 0.0001. Orthogonal contrasts indicated that the stress of 2-DG injection reduced blood leukocytes in comparison with the saline treatment, F(1, 41) = 76.21, p < 0.0001. There was no dose effect of nadolol on blood leukocyte number. There was, however, a marginal interaction between 2-DG and nadolol treatments, F(3, 41) = 2.656, p < 0.07. The disparity of changes in leukocyte numbers in the spleen and blood further emphasizes the differences in stress-induced mechanisms of change in these two lymphoid organs.

**Natural Killer-Cell Activity**

The natural killer-cell function of splenic leukocytes was measured by using four effector to target ratios and was analyzed as Lytic Units (LU) with the results displayed in Table 2. Although there was no significant overall effect of treatment, orthogonal contrasts of the pre-planned 2 x 4 analysis showed that 2-DG treatment significantly decreased the natural killer activity in comparison with the saline treated groups, F(1, 41) = 12.460, p < 0.001. There was no significant difference between the no-injection control group and saline/nadolol control group. In addition, there was, no main effect for nadolol, nor was there a significant interaction of 2-DG with nadolol. Thus, the administration of nadolol did not attenuate the 2-DG-induced suppression of natural-killer cell activity.
Mitogen Stimulation

Spleen assay. The mitogen stimulation assay using splenic lymphocytes showed comparable effects for all concentrations of Con A, PHA, and LPS. Only the results of the spleen mitogen assay using the optimal concentration of Con A (3 \( \mu \)g/ml) and LPS (2.5 \( \mu \)g/ml) have been displayed in Figure 1 (A & B) as representative data. Except where noted, the PHA data (not shown) was similar to that for Con A stimulation as both are T-cell mitogens. Overall ANOVA of optimum concentrations showed a significant difference among the groups, \( F(8, 41) > 2.60, p < 0.03 \). Comparison of the no-injection control group with the nonstressed control animals, injected with saline/nadolol (0-0.5 mg/kg), showed no significant effect of the injection procedure except in LPS, \( F(1, 41) = 12.67, p < 0.002 \). There was a significant 2-DG-induced suppression of mitogen proliferation, \( F(1, 41) > 7.80, p < 0.01 \). Contrasts demonstrated a significant quadratic dose effect of nadolol for Con A \( F(1, 41) = 4.161, p < 0.05 \), whereas contrasts demonstrated a significant linear effect of nadolol dose for PHA, \( F(1, 41) = 19.71, p < 0.0001 \), which resulted in increased mitogen proliferation with increased nadolol dose. Additionally, the mitogen assay for PHA showed a significant interaction between 2-DG treatment and nadolol injection, \( F(3, 41) = 3.87, p < 0.02 \), whereas the assay for Con A showed a marginal interaction of 2-DG treatment and nadolol injection, \( F(3, 41) = 2.32, p < 0.09 \). These interactions were due to a significant amount of suppression caused by 2-DG in the 0 mg/kg dose of nadolol, which was attenuated in the 0.1 mg/kg dose of nadolol. However, assay for LPS stimulation showed no dose-dependent effect of nadolol and no interaction between 2-DG and nadolol.
Whole-blood assay. The effect of nadolol on the proliferation of blood leukocytes in response to Con A and PHA was examined. The results of the blood mitogen assay using the optimal concentration of Con A (10 μg/ml) is displayed in Figure 1C. Overall ANOVA showed a significant difference among the groups, $F(8, 41) > 19.40, p < 0.0001$. A contrast of the no-injection control group with the saline/nadolol group showed no significant effect of the injection procedure. The analysis revealed a significant suppressive effect of the 2-DG treatment on the lymphocyte response to Con A, $F(1, 41) = 264.71, p < 0.0001$, and to PHA, $F(1, 41) = 120.30, p < 0.0001$. However, there was no attenuation of the suppression by nadolol treatment nor an interaction between 2-DG and nadolol.

Nitric Oxide (NO) Production

Spleen. NO production was measured by converting NO to the more easily detectable form nitrite. The results of nitrite assays of supernatants from spleen mononuclear cells stimulated with the optimal concentration of mitogen Con A (3 μg/ml) or LPS (2.5 μg/ml) are displayed in Figure 2, panels A & B, respectively. Nitrite production by PHA (5 μg/ml) stimulated splenocyte cultures was also examined (data not shown) and provided results similar to those for Con A. Overall ANOVA of nitrite induced by all mitogens showed significant differences among the groups, $F(8, 41) > 3.60, P < 0.003$. Comparison of the no-injection control group with the nonstressed control group animals that received saline/nadolol (0-0.5 mg/kg) treatment showed no significant effect of the injection procedure.
Analysis of orthogonal contrasts revealed that the groups that received 2-DG/nadolol (0-0.5 mg/kg) had significantly increased nitrite production compared with the saline/nadolol (0-0.5 mg/kg) control groups. F(1, 41) > 6.20, p < 0.02. The analysis also showed a linear effect of nadolol injection, F(3, 41) > 5.32, p < 0.004, in which nitrite production was decreased in a dose-dependent manner by nadolol. F(1, 41) > 12.67, p < 0.001. In addition, there was a significant interaction between 2-DG and nadolol in Con-A-stimulated splenocyte cultures. F(3, 41) > 2.84, p < 0.05, which indicates that nadolol reversed the 2-DG-induced increase in nitrite production.

Whole blood. Nitrite assays of supernatants from whole blood cultures stimulated with the optimal concentration of mitogen Con A (10 mg/ml) (Fig. 2C) and PHA (5 μg/ml) (data not shown) demonstrated similar results. Overall ANOVA of nitrite production showed a marginal difference among the groups. F(8, 41) > 2.07, p < 0.07, for Con A and PHA. Comparison of the no-injection control group with the saline/nadolol (0-0.5 mg/kg) nonstressed animals showed no significant effect of the injection procedure. Analysis revealed that 2-DG treatment significantly increased the nitrite production in comparison with saline injection treatment. F(1, 41) = 6.76, p < 0.02. However, there was no effect of nadolol nor significant interaction between 2-DG and nadolol.

IFN-γ Production

The results of IFN-γ assays of spleen and blood are displayed in Figure 3, panel A & B, respectively. Since 2-DG-induced changes in splenic mitogen responses and mononuclear cell-derived nitric oxide production were attenuated equally by 0.1 and 0.5 mg/kg nadolol.
effects of three levels of nadolol (0, 0.05, 0.1 mg/kg) on IFN-\(\gamma\) production were examined in the spleen. Overall statistical analysis of IFN-\(\gamma\) production by splenocytes (Fig. 3A) demonstrated a marginally significant effect among the groups, \(F(8, 21) = 2.53, p < 0.06\). Comparison of the no-injection control group with the nonstressed control group that received saline/nadolol treatment showed no significant effect of the injection procedure.

The analysis revealed a significant decrease in IFN-\(\gamma\) production in 2-DG/nadolol-(0-0.1 mg/kg) injected animals in comparison with saline/nadolol-injected animals, \(F(1.21) = 10.63, p < 0.004\). Analysis of 2-DG and saline groups at each concentration of nadolol showed a significant decrease in IFN-\(\gamma\) production in groups injected with 2-DG + 0 mg/kg nadolol, \(F(1.21) = 8.64, p < 0.01\), a marginal decrease in IFN-\(\gamma\) in groups injected with 2-DG + 0.05 mg/kg nadolol, \(F(1.21) = 3.61, p < 0.08\), and no significant effect in groups injected with 2-DG + 0.1 mg/kg nadolol. These results demonstrate that nadolol attenuated the 2-DG-induced suppression of IFN-\(\gamma\) production. There was no significant interaction between 2-DG and nadolol.

Analysis of the production of IFN-\(\gamma\) by peripheral blood lymphocytes (Fig. 3B) showed a significant difference among the groups, \(F(8, 27) = 10.45, p < 0.0001\). There was no significant effect of the injection procedure as there was no difference between the no-injection control group and the nonstressed, saline/nadolol control group. Moreover, the analysis indicates a significant decrease in IFN-\(\gamma\) production in 2-DG-injected animals in comparison with saline-injected animals, \(F(1.27) = 68.89, p < 0.0001\). There was no effect of nadolol, nor an interaction between stress and nadolol injection.
Discussion

The present research examined the mechanism underlying the immunomodulatory effects induced by an acute injection of 2-DG. It had been argued that the inhibition of T-cell proliferation is caused by 2-DG-induced cytolytic and cytotoxic activity. Previously, Michl et al. (1976) had shown that 2-DG is not toxic to leukocytes, such as macrophages and phagocytes. Miller et al. (1994) also provided evidence that in vitro incubation of murine T-cells with varying concentrations of 2-DG does not result in a reduction in cell number or viability. As mentioned previously, 2-DG may have an inhibitory effect on mitogenic stimulation simply by blocking the lymphocyte's utilization of glucose. However, results from the present study demonstrated that the suppression of mitogenesis is not due to a blockage of lymphocyte glucose utilization because the suppression of the splenic lymphocyte response to Con A, PHA, and LPS mitogens could be prevented by subcutaneous administration of nadolol, a nonselective β-adrenergic receptor antagonist. In addition, nadolol prevented 2-DG-induced suppression of splenic IFN-γ production and increase in NO production. Because nadolol does not cross the blood-brain barrier (Antonaccio & DeForrest, 1981), these findings indicate that 2-DG-induced immune suppression of splenic lymphocytes is due to catecholamines released from peripheral sympathetic neurons.

Enhancement of splenic NO production is found in other stress models (Fecho et al., 1994; Lysle et al., 1995). In addition, NO production has been causally linked to the suppression of splenic mitogen responses (Mills, 1991; Lysle et al., 1995). Thus, in the spleen, it is not surprising to find a mechanism that attenuates suppressed mitogen responses
and increased NO production. As IFN-γ can up-regulate NO production, it is interesting that increased NO suppresses T-cell production of IFN-γ as negative feedback control.

Acute 2-DG administration also induced changes in peripheral blood blastogenesis. IFN-γ production, and NO production. However, nadolol administration was unable to attenuate these effects. Although splenic and peripheral blood leukocytes (PBL) possess β-adrenergic receptors, it is not surprising to find that catecholamines released during stress do not affect PBL responses. The concentration of catecholamines is expected to be much higher in the area surrounding the neuronal terminus, and re-uptake mechanisms are active for unbound catecholamines. Moreover, the concentration of catecholamines in plasma decreases rapidly owing to the plasma volume of blood.

In the current study, the suppression of splenic natural killer (NK) cell activity was not prevented by nadolol administration. This outcome suggests that 2-DG-induced suppression of NK cell activity is not modulated by a sympathetic pathway. Another study also reported that morphine-induced suppression of NK cell activity was not attenuated by nadolol (Fecho et al., 1993). Together, these studies support work indicating that NK function is altered via opioid binding (Shavit et al., 1984). However, this is in contrast to work by Irwin et al. (1991) in humans and rats, which indicates a regulation of NK function by norepinephrine. Therefore, the regulation of NK function may occur through multiple immunomodulatory mechanisms that are dependent on the animal species or type of stress.

It has been reported that administering the β-adrenergic receptor antagonist propranolol did not reverse the inhibited T-cell proliferation of splenocytes in 2-DG-treated
mice (Miller et al., 1994). The difference in results between this mouse study and the current study could be explained by the different route of 2-DG administration used (subcutaneous in the current study and intravenous in the mouse study), the different β-adrenergic receptor antagonists used, and/or dosages of β-blocker used (multiple doses of nadolol were evaluated in the current study and a single dose of propanolol was used in the mouse study). In addition, the responses of IFN-γ production in rats and mice exposed to physical stress differ (Berry et al., 1991; Rose et al., 1984). Hence, there may exist differences in the way that rats and mice respond to this stressor.

Current results also indicate that acute 2-DG injections induced a reduction in total blood leukocytes. This change was not attenuated by nadolol. These findings suggest that β-adrenergic receptors are not involved in the altered trafficking of blood leukocytes. It has been reported that adrenal glucocorticoids released during stress may play a key role in regulating redistribution of leukocytes between the blood and other immune organs (Dhabhar et al., 1995). Nevertheless, the relationship between adrenal hormones and 2-DG-induced immunomodulation in the blood was not examined in the present paper.

Although the mechanism of 2-DG-induced immune alterations of blood lymphocytes is probably not via β-receptors or by catecholamine release, some reports suggest a possible relation between immune suppression and adrenal cortical hormones (Cupps & Fauci, 1982; Kelso & Munck, 1984). Additionally, Cunnick et al. (1990) have reported that adrenal hormones are responsible for shock-induced suppression of blood mitogenic responses. Alternatively, the peripheral blood system is enriched with several circulating hormones that
are produced during periods of metabolic stress (Ritter & Neville, 1976; Pascoe et al., 1989; Smith & Root, 1969) and have the capacity to modulate the activity of peripheral blood lymphocytes (Byron, 1972). Therefore, our laboratory is currently investigating the involvement of adrenal hormones in the 2-DG-induced immunomodulatory effects in the blood.

In conclusion, the results of the present study clearly demonstrate that peripheral β-adrenergic receptors and catecholamines are involved in 2-DG-induced effects on the proliferative responses of leukocytes to mitogens, on nitric oxide production, and on IFN-γ production in the spleen, but are not involved in 2-DG-induced changes in leukocyte function in the blood. These findings indicate that immune suppression in blood and splenic lymphocytes are mediated by separate pathways. Hence, the current study, associated with previous work, supports evidence that different mechanisms are operating in stress-induced immune changes. In addition, these findings suggest that the metabolic stress of 2-DG administration induces immune modulation of splenic lymphocytes via mechanisms similar to physical, psychological, and morphine-induced stress (Cunnick et al., 1990; Luecken & Lysle, 1992). That similitude is important because it implicates a concept that different types of stressors can induce similar outcomes of immune modulation and there may exist a common neuroendocrine pathway to respond to stress exposure. Many illness are considered stressors and induce metabolic changes that are stressful; but it is difficult to remove the immunomodulatory effects of infection from those of the metabolic stress of illness. This 2-
DG model of metabolic stress presents a means to examine the metabolic stress of illness and provides reproducible results for comparison with other types of stressors.

References


Table 1. Effect of nadolol dosage on plasma corticosterone, endogenous glucose, and 2-DG levels in rats receiving 2-DG, saline, or noinjections.

<table>
<thead>
<tr>
<th>Nadolol (mg/kg)</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone (µg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>9.6±2.7</td>
<td>5.3±2.6</td>
<td>14.2±2.7</td>
<td>12.2±3.2</td>
</tr>
<tr>
<td>2-DG</td>
<td>47.8±2.6</td>
<td>46.3±5.0</td>
<td>48.4±3.3</td>
<td>46.7±2.5</td>
</tr>
<tr>
<td>Noinjection</td>
<td>17.0±4.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Endogenous glucose (mg/dl) |             |              |              |              |
| Saline                    | 127.1±4.2   | 133.0±5.2    | 122.4±3.6    | 130.9±6.5    |
| 2-DG                      | 295.2±8.5   | 267.0±7.6    | 271.7±6.8    | 255.5±12.8   |
| Noinjection               | 119.3±2.4   |              |              |              |

| 2-DG (mg/dl) |             |              |              |              |
| Saline       | 0            | 0            | 0            | 0            |
| 2-DG         | 41.7±10.2    | 24.4±12.5    | 33.1±5.9     | 42.4±11.2    |
| Noinjection  | 0            |              |              |              |

a. Nadolol or nadolol vehicle (HBSS/2% DMSO) was injected subcutaneously 30 min prior to 2-DG exposure.
b. Rats received 500 mg/kg of 2-DG (stress group) or of saline (control group).
c. The no-injection group served as a baseline control.
d. Results are expressed as the mean ± SE; n = 4-6/group.
Table 2. Effect of nadolol dosage on total leukocyte counts for the spleen, leukocytes per milliliter of blood, and splenic natural killer-cell activity for animals receiving 2-DG, saline, or no injections.

<table>
<thead>
<tr>
<th>Nadolol (mg/kg)</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte counts/spleen ($\times 10^6$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>442.3±29.9 d</td>
<td>428.9±17.3</td>
<td>391.3±19.9</td>
<td>414.6±31.6</td>
</tr>
<tr>
<td>2-DG</td>
<td>445.7±20.6</td>
<td>403.9±20.6</td>
<td>384.0±29.1</td>
<td>379.5±32.7</td>
</tr>
<tr>
<td>Noinjection</td>
<td>396.3±34.5 c</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Leukocyte counts/ml blood ($\times 10^6$) |    |      |     |     |
| Saline         | 5.0±0.2    | 5.6±0.4 | 4.7±0.3 | 5.0±0.3 |
| 2-DG           | 2.5±0.3    | 2.7±0.3 | 3.4±0.3 | 3.4±0.4 |
| Noinjection    | 5.2±0.5    |      |     |     |

| Splenic natural killer-cell activity |    |      |     |     |
| Saline         | 24.9±0.6  | 27.7±6.9 | 23.2±9.8 | 19.8±4.7 |
| 2-DG           | 9.3±0.4   | 9.6±0.7 | 12.4±1.2 | 8.7±0.5  |
| Noinjection    | 17.2±2.3  |      |     |     |

a. Nadolol or nadolol vehicle (HBSS/2% DMSO) was injected subcutaneously 30 min prior to 2-DG exposure.
b. Rats received 500 mg/kg of 2-DG (stress group) or of saline (control group).
c. The no-injection group served as a baseline control.
d. Results are expressed as the mean ± SE; n = 4-6/group.
e. Results are expressed as the mean (± SE) of LU (20%)/10^7; n = 4-6/group
Figure 1. Proliferative responses of splenic leukocytes and peripheral blood leukocytes to the optimal concentrations of mitogens Con A and LPS as noted in panels A-C. Rats were injected subcutaneously (SC) with the vehicle (HBSS/2% DMSO,) or with 0.05, 0.1, or 0.5 mg/kg nadolol 30 min. before stress manipulation. Each stress rat received a 500 mg/kg 2-DG injection and nonstressed controls received saline injections. The no-injection group remained in the home cage. Results are expressed as the mean (± SE) of count per min; n=4-6 grp.
A: Spleen-Con A (3 µg/ml)

B: Spleen-LPS (2.5 µg/ml)

C: Blood-Con A (10 µg/ml)

- No injection control
- 2DG (500 mg/kg)
- Saline
Figure 2. Production of nitric oxide by splenocyte cultures and peripheral blood leukocyte cultures incubated with the optimal concentration of mitogens Con A and LPS as indicated in panels A-C. Nitrite was measured as an indicator of nitric oxide production in the supernatant of the mitogen induced blastogenesis cultures (Fig. 1). Rats were injected subcutaneously (SC) with the vehicle (HBSS/2% DMSO) or with 0.05, 0.1, or 0.5 mg/kg nadolol 30 min before stress manipulation. Each stress rat received a 500 mg/kg 2-DG injection and nonstressed controls received saline injections. The no-injection group remained in the home cage. Results are expressed as the mean (± SE) of count per min; n=4-6/grp.
A: Spleen-Con A (3 μg/ml)

B: Spleen-LPS (2.5 μg/ml)

C: Blood-Con A (10 μg/ml)

<table>
<thead>
<tr>
<th>NADOLOL mg/kg</th>
<th>NO INJ</th>
<th>0.00</th>
<th>0.05</th>
<th>0.10</th>
<th>0.50</th>
</tr>
</thead>
</table>

- ▲ No injection control
- □ 2DG (500 mg/kg)
- ● Saline
Figure 3. Interferon-γ (IFN-γ) production by splenocytes and peripheral blood leukocytes stimulated with the mitogen Con A (3 μg/ml for spleen and 10 μg/ml for blood). Rats were injected subcutaneously (SC) with the vehicle (HBSS/2% DMSO) or with 0.05, 0.1, or 0.5 mg/kg nadolol 30 min before stress manipulation. Each stress rat received a 500 mg/kg 2-DG injection and nonstressed controls received saline injections. The no-injection group remained in the home cage. Results are expressed as the mean (± SE) of units/ml (1 unit = 250 pg); n=4-6/group.
A: Spleen-IFN-γ Production

B: Blood-IFN-γ Production
CHAPTER 4. ADRENAL HORMONES ARE RESPONSIBLE FOR 2-DG-INDUCED IMMUNOMODULATORY EFFECTS IN THE BLOOD

A paper submitted to Journal of Neuroimmunology

Shiu-Huey Chou and Joan E. Cunnick

Abstract

Administration of 2-DG induces the secretion of several hormones including glucocorticoids and catecholamines. Although peripheral release of catecholamines is involved in 2-DG-induced immune modulation of splenocytes, they are not responsible for 2-DG-induced immune alterations of peripheral blood leukocytes. In this study, 2-DG administration to adrenalectomized, sham operated, and unoperated rats demonstrated that adrenalectomy prevented the 2-DG-induced leukopenia and suppression of the mitogenic responses of peripheral blood leukocytes, but did not attenuate the changes of splenic lymphocytes. Furthermore, adrenalectomy attenuated the 2-DG-induced suppression of IL-2, IFN-γ, and TNF production by Con A-stimulated peripheral blood leukocytes (PBL). Conversely, the TNF production by stimulated splenocytes was enhanced in 2-DG treated rats, and this enhancement was exacerbated in ADX rats. These results indicated that adrenal hormones mediate 2-DG-induced immunomodulation PBL. In addition, it is likely that the stress-induced release of adrenal hormones serves to prevent an exaggeration of immune responses.
1. Introduction

The immune system is regulated not only by cytokines and leukocytes, it communicates bidirectionally with the nervous system and endocrine system throughout the body, which leads to complex networks of systemic regulation (Blalock, 1994; Savino and Dardenne, 1995). In addition, stress-induced alterations of immune parameters provide strong evidence for an interaction between the immune and central nervous system. In general, the sympathetic nervous system and hypothalamic-pituitary-adrenal pathway are the two major mediating components of neuroendocrine-immune interaction. This is supported by ample anatomic and pharmacological evidence (for review see Madden and Felten, 1995).

Maintenance of homeostasis within the immune system is a function of both pathways.

The hypothalamic-pituitary-adrenal (HPA) axis is a primary pathway underlying stress-induced immunomodulation. Exposure to stress leads to an increased output of the hypothalamic-pituitary-adrenal axis (HPA) and changes in the immune system through the effects of glucocorticoids (Axelrod and Reisine, 1984). Furthermore, an elevated glucocorticoid concentration in the plasma is an indicator of stress. Glucocorticoids are involved in regulating many physiological functions including glucose metabolism, water balance, hepatic nutrition, neurological activity, and cardiovascular circulation. The effects of glucocorticoids on the immune system are very diverse and have been extensively reviewed (Munck et al., 1984; Bateman et al., 1989, Madden and Felten, 1995). In the past, glucocorticoids were recognized as immune suppressors more than immune regulators. For example, glucocorticoids cause leukopenia and neutrophilia, reduce the production of several
cytokines, inhibit NK cell activity, and suppress T-cell proliferation (Munck et al., 1984). Moreover, glucocorticoids have been identified as key mediators in certain inflammatory and autoimmune diseases (Sternberg, 1992; Wick, et al., 1993). Recently, the action of glucocorticoids in immune regulation has been re-evaluated. In fact, glucocorticoids may act in both positive and negative ways in immune regulation (Munck and Naray-Fejes-Toth, 1994). Although many actions of glucocorticoids are deemed "immune suppression," in actuality glucocorticoids may serve to shift an immune response from cellular (TH1) to a humoral (TH2) response (Derijk and Sternberg, 1994; Brinkmann and Kristofic, 1995; Ramirez et al., 1996). Hence, stress-induced elevation of glucocorticoids may serve as a regulator to maintain the homeostasis of the immune system.

Administration of 2-deoxy-D-glucose (2-DG), a metabolic stressor, has been reported to alter immune parameters in mice, rats, and humans (Breier et al., 1987; Lysle et al., 1988; Miller et al., 1993, 1994; Chou et al., 1996). Administration of 2-DG to rats induces changes in immune response that are kinetically different in the spleen and blood. For example, IL-1 and IL-2 production were suppressed in the blood, but not in the spleen. Additionally, the suppression of mitogen-stimulated lymphocyte proliferation is attenuated after repeated 2-DG injections in the spleen, but not in the peripheral blood. This suggests that more than one mechanism is responsible for 2-DG-induced immune changes.

Administration of 2-DG induces all the hallmarks of a stressor (Smythe et al., 1989) including increased epinephrine, norepinephrine, and glucocorticoid production (Ritter and Neville, 1976; Pascoe et al., 1989; Smith and Root, 1969). Extended investigations in our
laboratory indicate that the peripheral release of catecholamines is responsible for 2-DG-induced immunomodulation in the spleen. Subcutaneous administration of nadolol, a nonselective β-adrenergic receptor antagonist prevents 2-DG-induced changes in splenic immune function, however, nadolol does not attenuate the 2-DG-induced changes in immune parameters in peripheral blood (Chou et al., submitted). These findings indicate that another mechanism such as glucocorticoid release, is involved in 2-DG-induced immunomodulation of PBL function. Administration of exogenous glucocorticoids induces lymphopenia and alteration in cell trafficking (Hall, 1986), also seen with 2-DG administration (Chou et al., 1996). In addition, adrenal hormones are involved in suppression of PBL function induced by electric shock (Cunnick et al., 1990). Stress-induced lymphopenia is prevented in adrenalectomized rats and reduced by a glucocorticoid synthesis inhibitor (Keller et al., 1983; Dhabhar et al., 1995, 1996).

As nadolol administration prevented epinephrine and norepinephrine from binding to β-adrenergic receptor, but did not alter 2-DG effects on PBL function, we postulated that glucocorticoids were responsible for the observed changes. The present study was designed to test whether adrenal hormones are involved in 2-DG-induced immunomodulation in the blood. Adrenalectomy prevents the release of baseline and stress-induced glucocorticoids and interrupts the interaction between the HPA axis and the immune system. If the immune changes due to 2-DG injection do not occur in adrenalectomized group, then a mechanistic role for adrenal hormones is supported.
2. Materials and methods

Animals

Inbred male rats of the Lewis strain (62 days old, 220-270 g) were purchased from Harlan Sprague Dawley laboratories (Indianapolis, Indiana). Animals were adrenalectomized or sham-adrenalectomized by Harlan Sprague Dawley laboratories and shipped one week after surgery along with unoperated control animals of the same age. Upon arrival, the subjects were individually caged in a colony room in which a 12-h reversed light-dark cycle was maintained through artificial illumination. They received free access to both food and water (0.9% saline for the adrenalectomized rats) throughout the experiment except as noted, and a 3-week acclimation period before the experimental manipulation. Additionally, animals were handled every two days to prevent the hyper-reactivity that occurs when they are left untouched for a prolonged period.

Reagents and Chemicals

The culture medium used for all assays were two kinds: "supplemented media" was used to designate RPMI-1640, supplemented with L-glutamine (2 mM), gentamicin (50 μg/ml), and Hepes (10 mM)(Gibco Lab., Grand Island, NY), and "complete media" was used to designate "supplemented media" containing 10% Fetal Bovine Serum (FBS) (JHR Biosciences, Lenexa, KS). The mitogens Concanavalin A (Con A), and Lipopolysaccharide (LPS)-from E.coli were purchased from Sigma Chemical. (St. Louis, MO); Phytohemagglutinin (PHA) was purchased from Burroughs Wellcome (England). Heparin 100 units/ml (ELKINS-SINN, NJ) and 2-DG (Sigma Chemistry) were diluted in Hanks'
Balanced Saline Solution (HBSS)(Gibco Lab). All cultures were prepared in flat-bottomed tissue culture plates (Costar, Cambridge, MA). $^3$H-thymidine (specific activity 6.7 Ci/mmol) and $^{51}$Chromium (specific activity 16.45 GBq/mg(Cr)) from Dupont-New England Nuclear (Wilmington, DE) were used as specified in the following section.

**Experimental Paradigm**

To determine the role of glucocorticoids in 2-DG-induced immune alteration, adrenalectomized (ADX), sham-adrenalectomized (SHAM), or unoperated (UNOP) male rats were evaluated. The experiment was performed in two replications. The animals in the surgery-treatment groups were assigned to two subgroups (n = 6). One subgroup received a single injection of 2-DG, 500 mg/kg body weight, dissolved in sterile HBSS. The other group received a single injection of sterile HBSS as controls. Injection was administered subcutaneously and performed 2-hr into the dark phase. For 60 min following each injection of 2-DG or HBSS, animals were deprived of food and water. Sixty min following the injection, each subject was rapidly sacrificed by cervical dislocation with a clamp.

After sacrifice, blood was collected via the abdominal aorta into heparinized syringes. The spleen was removed, placed in preweighed polypropylene tubes containing 8 ml of complete media, weighed and dissociated into a single-cell suspension. Spleen and blood leukocytes were enumerated (Celltrak 2, NOVA Biomedical, Waltham, MA). Splenocytes were diluted to $5 \times 10^6 / \text{ml}$ with complete media and blood was diluted 1:10 with supplemented media. Diluted splenocytes and blood were subsequently used for specific functional assays.
Plasma Glucose Assay

The concentration of glucose present in the plasma was measured by two methods: one was based upon the glucose oxidase (Trinder) reaction, which detects endogenous glucose and 2-DG, and a second was based upon the hexokinase (HK) reaction, which detects only endogenous glucose (Sigma, St. Louis, MO). For all subjects, plasma was obtained from 1 ml whole blood by centrifugation (12,000 rpm/5 min) and stored at -70°C until assayed. The samples were tested in duplicate and read at 505 nm for Trinder and 340 nm for HK assay with a spectrophotometer (UVIKON 930, Kontron Instruments AG, Switzerland). A glucose standard curve was performed with each assay, and the glucose concentration of experimental samples was calculated based upon linear regression analysis of the standard.

Plasma Corticosterone Assay

To test for the completeness of adrenalectomy and effectiveness of stress, plasma samples were assayed for corticosterone. Plasma samples were obtained by centrifugation of whole blood (1 ml) and were stored at -70°C. Plasma corticosterone concentration was determined by using a standard radioimmunoassay kit for rat corticosterone (ICN, Biomedical, Inc., CA). The error for the corticosterone radioassay is 0.2 µg/dl and values below this level, as in the ADX groups, can be interpreted as lack of corticosterone.

Mitogen Stimulation Assay

The mitogen assay was performed with splenic and blood leukocytes to assess lymphocyte proliferation. Con A and PHA were used as T-lymphocyte mitogens and LPS as
a B-lymphocyte mitogen as described previously (Cunnick et al., 1990).

**Natural Killer-Cell Assay**

Natural killer-cell activity was assayed in the splenocyte suspensions by using an NK sensitive tumor target YAC-1 as previously described (Cunnick, et al., 1990). Radioactivity (Cr $^{51}$) in supernatants was determined by using a gamma counter (Gamma Trac 1191; TM Analytic, INC., IL). Lytic units (LU) were calculated by using a computer program based on the equations of Pross and Maroun (1984). The percentage cytotoxicity at all effector:target ratios was utilized to determine LU. Lytic units were based on the number of leukocytes per $10^7$ effectors necessary to lyse 20% of targets.

**Nitric Oxide Assay**

Supernatants were collected from cultures of mitogen-stimulated lymphocytes before pulsing with $^3$H thymidine to determine nitric oxide production. Nitrite concentrations were determined by a microplate assay as a determination of nitric oxide production (Stuehr and Nathan, 1989). Sample aliquots (50 µl) were mixed with 100 µl of Griess reagent (0.5% sulfanilamide / 0.05% naphthylethylene diamine dihydrochloride / 2.5% H$_3$PO$_4$) and incubated at room temperature for 10 min. The optical density was measured in an ELISA microtiter plate reader (BIO Kinetics Reader, BIO-TEK Instruments, Winooski, VT) at 550 nm against PBS as a blank. A sodium nitrite (NaNO$_2$) standard curve (0-100 µM) was generated in parallel. Results are expressed as the mean of nitrite concentrations in the supernatants of triplicate wells and analyzed as repeated-measures ANOVA by using the no-mitogen control and all concentrations of a given mitogen.
Interferon-γ (IFN-γ) Induction and Assay

The concentration of interferon in the supernatants of Con-A-stimulated spleen and peripheral blood lymphocytes was measured. Whole spleen leukocytes (2.5 x 10^6 /ml) were incubated in complete media and Con A (3 μg/ml) for 48-h and whole blood leukocytes (1:20 dilution) were incubated in supplemented media and Con A (10 μg/ml) for 72-h; both at 37°C in 5% CO₂. Culture supernatants were harvested and stored at -70°C until the assay (Johnson et al., 1981). IFN-γ was determined by means of a commercial ELISA assay for rat IFN-γ (GIBCO BRL Life Technologies, Gaithersburg, MD). Titers were determined by comparison to an IFN-γ standard supplied with the kit. Data is reported as units/ml (1 unit = 250 pg./ml)

Interleukin-2 Induction and Assay

Interleukin-2 (IL-2) is a T-cell growth factor produced by stimulated T-lymphocytes. The culture supernatants for measuring IL-2 production by splenocyte and blood leukocytes were obtained as for the IFN-γ assay. Supernatants obtained from nonstimulated cultures served as "media control." On the assay day, the supernatants and a positive IL-2 control were serially diluted 1:2 (total of five dilutions) in duplicate in a 96-well microtiter plate by using a digital multichannel pipette (Titertek-Plus). The IL-2-dependent cell line, CTLL-2, was washed to remove any exogenous IL-2 and was then diluted in complete media to 1 x 10^5 /ml. The CTLL-2 cell suspension (100 μl) was plated in each well and in control wells containing no IL-2. The plates were incubated for 24 hr at 37°C in a humidified atmosphere of 5% CO₂. Each well was pulsed with 1 μCi of ^3H-thymidine (50 μl) for the last 5 hr of
the incubation. The cultures were harvested (Skatron Harvester, Model #11028, Sterling, VA), and the thymidine incorporation was determined using a liquid scintillation counter (1217 Rackbeta, LKB-Wallac, Finland). Half-max. units were calculated with software from Lilly Research Laboratories.

**Tumor Necrosis Factor (TNF) Assay**

TNF production by stimulated splenic or blood leukocytes was measured in supernatants as for the IL-2 and IFN-γ assay. The ability of splenocytes or blood leukocytes to produce TNF was determined by using a TNF-sensitive, Actinomycin D-treated murine L929 fibroblasts cell line. This assay, however, cannot distinguish between the α and β forms of TNF.

Briefly, L929 cells were collected and washed once in complete media. The cells were diluted to $2.5 \times 10^5$ /ml and 100 μl of suspension added to a 96 wells plate. The plate were incubated 18-24 hr. At this time, the growth of L929 cells were stopped by adding 10 μl of Actinomycin-D(60 μg/ml, Sigma). To test for the presence of TNF, 100 μl of diluted supernatants were add to wells in triplicate and incubated for 24 hr at 37 °C in a CO₂ (5 %) incubator. The plate was washed twice with phosphate-buffered saline (PBS) and stained with 0.2% crystal violet / 2% formalin in PBS. The fixed cells were washed several times with tap water and allowed to dry overnight at room temperature. Ethanol (50%) in PBS (100 μl) was added to each well of the plates to dissolve the stain-fixed cells. The optical density was measured in an ELISA microtiter plate reader at 550-595 nm. A TNF standard
curve (0-100 units/ml) was generated in parallel on each plate. The TNF content of each sample was calculated from the standard curve and reported as units/ml.

**Statistical Treatment of Data**

A computerized program for analysis of variance (ANOVA; Statistix, NH Analytical Software) was used to assess differences among experimental and control groups. For the present experiment to assess the effects of adrenalectomy on attenuation of acute stress effects of 2-DG, a two (treatment) x three (surgery) analysis was used. One factor was treatment, a 2-DG injection or saline injection. The second factor was surgery, adrenalectomy (ADX), sham operation or no operation. The significance level for all analyses was set at a probability of less than or equal to 0.05.

3. Results

**Plasma Corticosterone and Endogenous Glucose**

The results of the assay for plasma corticosterone are presented in Table 1. An analysis of variance demonstrated a significant increase in corticosterone due to 2-DG treatment, $F(1, 35) = 306.49, p < 0.0001$ and a significant main effect of surgery, $F(2, 35) = 126.46, p < 0.0001$. Orthogonal contrasts of surgery demonstrated that the plasma corticosterone concentration of the sham-operated group was significantly elevated in comparison with the unoperated group, $F(1, 35) = 4.33, p < 0.05$. In contrast, the corticosterone concentration in the adrenalectomy (ADX) group was significantly suppressed in comparison with the sham-operated groups and the unoperated groups, $F(1, 35) = 248.69, p < 0.0001$. The analysis also showed a significant interaction of 2-DG treatment and
surgery, $F(2, 35) = 76.47, p < 0.0001$. Contrasts demonstrated a highly significant interaction of 2-DG treatment and surgery between the ADX group and the unoperated and the sham-operated groups, $F(1, 35) > 103.43, p < 0.0001$. But, there was no interaction of 2-DG and surgery between the sham-operated group and the unoperated group. Thus, the adrenalectomy operations were highly effective in eliminating baseline levels of corticosterone and preventing the normal stress-induced rise in corticosterone levels.

Adrenalectomy also affected the level of plasma glucose induced by stress (Table 1). Analysis of plasma glucose levels showed a significant increase in endogenous glucose due to 2-DG injection, $F(1, 35) = 1440.27, p < 0.0001$. In addition, there was a significant main effect of surgery, $F(2, 35) = 230.92, p < 0.0001$. Orthogonal contrasts indicated that the plasma glucose concentration of the ADX group was significantly lower than sham-operated and unoperated groups, $F(1, 35) = 459.67, p < 0.0001$. There was no difference in plasma glucose between the sham-operated group and unoperated group. Moreover, ANOVA indicated a significant interaction of surgery and 2-DG treatment, $F(2, 35) = 196.78, p < 0.0001$. Contrasts demonstrated that there was significant of 2-DG and surgery interaction between the unoperated and sham-operated groups and the ADX group, $F(1, 35) = 393.23, p < 0.0001$. Therefore, rats lacking adrenal glands failed to display the exaggerated concentration of glucose during 2-DG administration.

**Leukocyte Enumeration**

The leukocytes in the spleen and peripheral blood were enumerated (Table 2). There was no significant main effect of surgery or 2-DG injection on the number of leukocytes in
the spleen. But, there was a significant decrease in the number of peripheral blood leukocytes (PBL) from rats that received 2-DG, \( F(1, 35) = 12.32, p < 0.002 \). In contrast, there was no main effect of surgery on peripheral blood leukocyte counts or a significant interaction between surgery and 2-DG treatment. Ancillary contrasts, however, demonstrate that the decreased number of PBL's was significant in the stressed SHAM-operated and unoperated groups, but was not significant in the stressed ADX-operated group when compared with their respective saline treated controls. Thus, adrenalectomy blunted the 2-DG-induced leukopenia normally seen in unoperated rats.

**Response to Mitogen Stimulation**

**Blood.** The blood leukocyte responses to the optimal concentrations of Con A (5 \( \mu \text{g/ml} \)) and PHA (5 \( \mu \text{g/ml} \)) are summarized in Figure 1, panels A and B, respectively. The analysis of both Con A and PHA stimulated cultures demonstrated a significant main effect of surgery, \( F(2, 35) > 8.9, p < 0.002 \). Orthogonal contrasts of surgery revealed no difference between the unoperated and sham-operated groups; however, these two groups were significantly lower than the ADX groups, \( F(1, 35) > 27.98, p < 0.0001 \). Analysis of variance of the blood leukocyte responses to the optimal concentration of Con A and PHA also showed a significant main effect for 2-DG treatment (\( F(1, 35) = 28.10, p < 0.0001 \) for Con A and \( F(1, 35) = 4.20, p < 0.05 \) for PHA). Orthogonal contrast indicated that animals that received 2-DG injection in both sham-operated and unoperated groups showed a suppression comparison to saline injection. \( F(1, 35) > 6.67, p < 0.02 \). Conversely, there was no significant 2-DG effect in 2-DG treated and saline treated of the ADX group. Most
importantly, there was a significant interaction of 2-DG treatment and surgery for Con A stimulated PBL, F(2, 35) = 5.43, p < 0.02. Orthogonal contrasts revealed that the interaction of the unoperated and sham-operated groups was not significant, but the interaction of these groups and the ADX group was significant, F(1, 35) = 10.69, p < 0.004. Therefore, these data demonstrate an attenuation of 2-DG-induced immune suppression in rats lacking adrenal glands.

*Spleen.* The response of the spleen lymphocytes to Con A, PHA, and LPS were summarized in Table 3. The analysis of variance of the spleen leukocyte responses to the optimal concentration of mitogen (Con A, 1.5 µg/ml; PHA, 0.5 µg/ml; and LPS, 2.5 µg/ml) showed a significant main effect of 2-DG treatment, F(1, 35) > 12.83, p < 0.002. This demonstrates that 2-DG can induce a pronounced decrease in the mitogenic response of splenic lymphocytes. In addition, there was a significant main effect of surgery in splenocyte responses to Con A, F(2, 35) = 4.07, p < 0.04, but there was no main effect of surgery in responses to PHA and LPS. Orthogonal contrasts of the Con A response revealed that the sham-operated group was significantly lower than the unoperated group, F(1, 35) = 5.38, p < 0.03; however, the ADX group was not significantly different from the unoperated group. In addition, there was no interaction of surgery and 2-DG treatment. These results indicate that adrenalectomy did not attenuate the 2-DG-induced immune suppression of splenic lymphocytes. Furthermore, the sham operation procedure exacerbated the 2-DG-induced suppression of the Con A mitogenic response of splenic lymphocyte.
Natural Killer Cell Activity

Natural killer cell function of splenic leukocytes was measured by using four effector to target ratios and analyzed as Lytic Units (LU) (Figure 2). Overall, ANOVA showed that 2-DG injection induced a significant decrease of natural killer cell activity, $F(1, 35) = 26.82$, $p < 0.0001$, and there was a significant main effect of surgery, $F(2, 35) = 36.98$, $p < 0.0001$. Orthogonal contrasts of surgery revealed that the ADX group had significantly lower natural killer cell activity than the sham-operated and unoperated groups; however, there was no significant difference between the sham-operated and unoperated groups. In addition, there was no significant interaction between the surgery and 2-DG treatment.

Cytokine Production

Interferon-γ (IFN-γ) and IL-2

Blood. The results of IFN-γ and IL-2 production by blood lymphocytes are displayed in Figure 3. Administration of 2-DG significantly suppressed IFN-γ production from cultures of blood lymphocytes, $F(1, 35) = 25.84$, $p < 0.0001$ (Fig. 3A). Analysis of variance demonstrated a significant main effect of surgery, $F(2, 35) = 5.45$, $p < 0.02$. Orthogonal contrasts indicated that there was no difference between the unoperated and sham-operated groups; however, these groups were significantly lower than the ADX group, $F(1, 35) = 8.58$, $p < 0.008$. Interestingly, the interaction of 2-DG treatment and surgery was significant for IFN-γ production, $F(2, 35) = 3.32$, $p < 0.05$. Orthogonal contrasts revealed that the interaction of the unoperated and sham-operated groups was not significant, but the interaction of these groups and the ADX groups was significant, $F(1, 35) = 5.02$, $p < 0.04$. 
Thus, ADX-operation attenuated 2-DG-induced suppression of IFN-γ production by Con A-stimulated blood lymphocytes.

Overall ANOVA revealed no significant main effect of 2-DG treatment on con A-stimulated IL-2 production in cultures of blood lymphocytes (Fig. 3B), but there was a significant main effect of surgery, \( F(2, 35) = 10.83, p < 0.0005 \). Importantly, there was a significant interaction between surgery and 2-DG treatment, \( F(2,35) = 5.74, p < 0.01 \). Orthogonal contrasts of surgery revealed that the ADX group showed higher IL-2 production than the sham-operated and unoperated groups, \( F(1, 35) = 21.07, p < 0.0002 \). There was no significant difference between sham-operated groups and unoperated groups. The significant interaction was due to a significant suppression of IL-2 production in both sham-operated and unoperated groups \( F(1, 35) = 7.40, P < 0.02 \), whereas IL-2 production was significantly enhanced by 2-DG in the ADX group \( F(1, 35) = 21.07, P < 0.0002 \). Therefore, the 2-DG induced suppression of IL-2 production by Con A-stimulated blood lymphocytes is induced by adrenal hormones. Furthermore, without adrenal hormones, the production of IL-2 was exacerbated in response to stress.

**Spleen.** The results of IFN-γ and IL-2 production by spleen lymphocytes were displayed in Table 3. The cultures of splenocytes from rats that received a 2-DG injection contained significantly less IFN-γ \( (F(1, 35) = 4.74, p<0.05) \) and IL-2 \( (F(1, 35) = 11.48, p < 0.003) \) compared with those from rats injected with saline. But, there was no main effect of surgery and no significant interaction between surgery and 2-DG treatment for splenic IFN-γ
and IL-2 production. These data demonstrate that adrenalectomy did not attenuate the 2-DG-induced reduction of IFN-γ and IL-2 production by Con A stimulated splenic lymphocytes.

**Tumor Necrosis Factors (TNF)**

*Blood.* The result of TNF production from Con A-stimulated blood leukocytes is summarized in Figure 3C. The induction of TNF was evaluated by testing the supernatant fluids of the cultures from the same cell source as those used in the IL-2 and IFN-γ assay. ANOVA indicated that the production of TNF by Con A-stimulated blood leukocytes was decreased because of 2-DG administration, $F(1, 35) = 3.04, p < 0.09$ and there was a significant main effect of surgery, $F(2, 35) = 18.71, p < 0.0001$. Orthogonal contrasts indicated that there was no difference between sham-operated and unoperated groups; however, PBL from the ADX group produced more TNF than the sham-operated and unoperated groups, $F(1, 35) = 37.45, p < 0.0001$. Importantly, there was a significant interaction between 2-DG treatment and surgery, $F(2, 35) = 4.28, p < 0.03$. The production of TNF by leukocytes from the ADX group was not significantly altered by 2-DG administration. Hence, these data indicated that 2-DG-induced changes of TNF production in Con A-stimulated cultures of blood leukocytes is mediated by adrenal hormones.

*Spleen.* The result of TNF production from Con A stimulated splenocytes was summarized Table 3. Analysis of variance demonstrated a significant increase in TNF production due to 2-DG treatment, $F(1, 33) = 5.49, p < 0.03$ and a significant main effect of the surgery, $F(2, 33) = 7.57, p < 0.004$. Orthogonal contrasts of surgery indicated that the TNF concentration of the sham-operated group was significantly elevated in comparison with
the unoperated group, $F(1, 33) = 4.97, p < 0.04$. The ADX group also showed a significantly higher production of TNF than unoperated groups, $F(1, 33) = 15.05, p < 0.001$; however, there was no significant difference between the ADX and sham-operated groups. In addition, there was no significant interaction between 2-DG treatment and surgery. Hence, 2-DG administration enhanced the production of TNF in Con A-stimulated splenocytes and the stress of surgery exacerbated the 2-DG-induced stress response in a manner similar to a sensitization response.

4. Discussion

The cells in the blood are bathed by a variety of hormones including glucocorticoids and catecholamines. Our previous studies suggested that 2-DG-induced immunomodulation in the blood was not due to a peripheral release of catecholamines (Chou et al., submitted). Glucocorticoids are profoundly elevated after 2-DG administration (Smith and Root, 1969; Lysle et al., 1988; Chou et al., 1996). Generally, glucocorticoids, the end products of hypothalamic-pituitary-adrenal axis activation, are widely considered as the primary mediators underlying stress-induced immune changes. It is well known that physiologic and pharmacologic doses of corticosterone have a profound effect on a variety of immune parameters (Cupps and Fauci, 1982; Munch et al., 1984). The current study clearly demonstrated that the 2-DG-induced changes of peripheral blood immune cell function were attenuated in adrenalectomized rats. Adrenalectomy did not attenuate the 2-DG-induced changes of immune parameters of splenocytes. These findings are consistent with our previous investigation that 2-DG-induced immunomodulation in the spleen was mediated by
the peripheral release of catecholamines. Furthermore, it supports our hypothesis that the changes of blood leukocyte function are mediated by an adrenal hormone such as corticosterone.

In the present study, the 2-DG-induced elevation of endogenous glucose was blunted by adrenalectomy because 2-DG treated ADX rats did not display the same increase of endogenous glucose as saline injected ADX rats. This difference can be partly explained because glucocorticoids are the major, but not sole, hormone to regulate glucose metabolism. Norepinephrine or other sympathetic neuropeptides are also involved in glucose homeostasis (Smythe et al., 1989; Pascoe et al., 1989).

A change in the number of peripheral blood leukocytes is a common stress response (Naliboff et al., 1991; Dhabhar et al., 1994). Our laboratory reported that the number of leukocytes in blood was reduced in rats receiving one or multiple injections of 2-DG (Chou et al., 1996). The cause of the leukopenia is not due to 2-DG cytotoxicity of T-cell (Miller et al., 1994) or noradrenergic activity (Chou et al., submitted). The stress-induced changes in blood leukocytes is probably due to cell redistribution mediated by glucocorticoids released by the adrenal gland (Dhabhar et al., 1995, 1996). The present study supports the theory of glucocorticoid-induced redistribution of PBL, as adrenalectomy attenuated the 2-DG-induced reduction of leukocytes.

Most importantly, the present study demonstrated that the 2-DG-induced changes in blood leukocyte function are also adrenal-dependent. Adrenalectomy prevented the suppression of PBL proliferation to Con A and PHA; however, adrenalectomy did not
attenuate the suppression of the splenic lymphocyte responses to Con A, PHA, and LPS. These data emphasize the mechanistic differences that mediate stress-induced change in the two lymphoid compartments. Observations from this study also indicated that the response of blood lymphocytes to T-cell mitogens in the ADX group were higher than in the sham-operated and unoperated groups, and in some instances, 2-DG exacerbated the increased T-cell responses. Thus, glucocorticoids play an important role in maintaining a balanced immune response, without these hormones, stress may exacerbate physiological responses.

The stress of 2-DG administration inhibited IL-2 in the blood and IFN-γ in both spleen and blood as seen previously (Chou et al., 1996). The suppression of IFN-γ production by splenocytes from 2-DG treated animals is mediated by peripheral release of catecholamines (Chou et al., submitted). In the present study, adrenalectomy prevented 2-DG-induced reduction of IL-2 and IFN-γ production by peripheral blood leukocytes, implicating adrenal hormones in homeostasis of blood lymphocyte responsiveness.

Glucocorticoids affect T-cell growth and differentiation by inhibiting IL-2 secretion and IL-2 receptor expression (Gillis et al., 1979). Therefore, administration of 2-DG elevated glucocorticoid levels in peripheral blood, which directly or indirectly affected IL-2 secretion and T-cell proliferation. By down-regulating T-cell growth, IFN-γ production was also influenced by glucocorticoids. This concurs with the research of others showing that exogenous glucocorticoids decrease the production of IL-2, IFN-γ, and TNF at physiological and pharmacological concentration in vivo and in vitro (Gillis et al., 1979; Kelso and Munck, 1984; Dupont et al., 1985; Perretti et al., 1993). Furthermore, studies examining stress
(Glaser et al., 1986), electric shock (Sonnenfeld et al., 1992), and microgravity (Berry et al., 1991) demonstrate that stress can inhibit cytokine production.

We extend our previous work by showing that 2-DG induced suppression of TNF production by blood leukocytes, whereas the production of TNF in the spleen was enhanced after 2-DG administration. The present study demonstrates that the change of TNF production by blood lymphocytes was also adrenal-dependent. TNF is an inflammatory and antiviral cytokine. It is not surprising that 2-DG induced stress also disturbs the balance of TNF production. It is well accepted that the actions of glucocorticoids are antiinflammatory and induce immune suppression. Removal of the adrenal glands has been reported to exacerbate many aspects of acute inflammation and increase susceptibility to endotoxic shock (Perretti et al., 1993). Additionally, adrenalectomy enhances the sensitivity of animals to the toxic action of these cytokines (Bertini et al., 1988). Recently, the role of glucocorticoids as essential in mediating physiological function has been revised (Munck and Naray-Fejes-Toth, 1994; Derijk and Sternberg, 1994). They suggest that the immune suppression induced by glucocorticoids may serve as a buffer to optimize host responses during external stimuli such as stress. Without this mediator, the balance of physiologic activity may be interrupted and subsequently cause unexpected tissue damages. The present study supports this theory because IL-2 and TNF production was significantly increased in 2-DG treated rats lacking adrenal glands, demonstrating the importance of glucocorticoids in homeostasis.

As expected, 2-DG induced a suppression of splenic NK cell activity. This suppression of NK activity was not attenuated in adrenalectomized rats. Moreover, the baseline NK activity in ADX rats was significantly lower than in the sham-operated and
unoperated groups. This may indicate that glucocorticoids are important mediators in recovering from surgical treatment and reestablishing homeostatic immune function.

Conversely, it could be interpreted that the ADX rats were under a different and consistent metabolic stress since surgery, and this activated pathways that suppress NK activity. It has been reported that NK cell activity is modulated via opioids (Shavit et al., 1984) or norepinephrine (Irwin, 1991); however, we previously reported that 2-DG-induced suppression of splenic NK activity was not blocked by the administration of nadolol, a β-adrenergic receptor antagonist. This was intriguing because other splenic immune functions altered by 2-DG are prevented by nadolol (Chou et al. submitted).

The present study supports our hypothesis that adrenal hormones are responsible for 2-DG-induced immunomodulation in the blood. These data also point out that the action of glucocorticoids on immune function are involved in more than immune suppression. Combined with the results of previous research, this study supports the general concept that communication exists between the immune system and the neuroendocrine system.

Acknowledgments

We thank Dr. Eugenia Farrar for her critical comment and technical support. We also thank Kim Downard and Valerie Rosenwald for their excellent technical assistance.

References


Table 1. Plasma corticosterone and endogenous glucose levels in adrenalectomized (ADX), sham-operated (SHAM) or unoperated (UNOP) rats that received 2-DG (500 mg/kg) or saline injection.

<table>
<thead>
<tr>
<th></th>
<th>2-DG</th>
<th>SALINE</th>
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<tbody>
<tr>
<td><strong>Corticosterone (μg/dl)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>2.0 ± 0.7 *</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>SHAM</td>
<td>66.1 ± 4.3</td>
<td>13.5 ± 2.3</td>
</tr>
<tr>
<td>UNOP</td>
<td>63.4 ± 4.2</td>
<td>5.5 ± 2.2</td>
</tr>
<tr>
<td><strong>Endogenous Glucose (mg/dl)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>152.6 ± 5.1</td>
<td>110.8 ± 6.7</td>
</tr>
<tr>
<td>SHAM</td>
<td>339.4 ± 8.1</td>
<td>123.2 ± 7.8</td>
</tr>
<tr>
<td>UNOP</td>
<td>335.0 ± 9.2</td>
<td>112.8 ± 5.6</td>
</tr>
</tbody>
</table>

* Mean ± SE
Table 2. Total leukocyte counts for the spleen and total leukocyte counts per milliliter of blood in adrenalectomized (ADX), sham-operated (SHAM) or unoperated (UNOP) rats that received 2-DG (500 mg/kg) or saline injection.

<table>
<thead>
<tr>
<th></th>
<th>2-DG</th>
<th>SALINE</th>
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<tbody>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>386.9 ± 48.8 **</td>
<td>443.6 ± 15.6</td>
</tr>
<tr>
<td>SHAM</td>
<td>427.7 ± 21.9</td>
<td>453.2 ± 15.3</td>
</tr>
<tr>
<td>UNOP</td>
<td>428.4 ± 15.4</td>
<td>449.3 ± 21.8</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>3.7 ± 0.5</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>SHAM</td>
<td>2.7 ± 0.2</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>UNOP</td>
<td>2.3 ± 0.3</td>
<td>4.3 ± 0.6</td>
</tr>
</tbody>
</table>

* number of $10^6$ leukocytes / spleen
** Mean (± SE )
*** number x $10^6$ leukocytes / ml blood
Table 3. Effects of 2-DG injection on the proliferative response of splenocytes to the mitogens Con A, PHA, and LPS and the production of IFN-γ, IL-2, and TNF by Con A stimulated splenocytes from adrenalectomized (ADX), sham-operated (SHAM), and unoperated (UNOP) rats.

<table>
<thead>
<tr>
<th>Mitogen Response (cpm x 10^3)</th>
<th>2-DG</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A (1.5 μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>117.0 ± 18.4*</td>
<td>162.0 ± 13.8</td>
</tr>
<tr>
<td>SHAM</td>
<td>64.2 ± 18.9</td>
<td>141.0 ± 14.4</td>
</tr>
<tr>
<td>UNOP</td>
<td>122.0 ± 21.0</td>
<td>149.0 ± 17.9</td>
</tr>
<tr>
<td>PHA (0.5 μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>2.7 ± 0.8</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>SHAM</td>
<td>4.1 ± 0.6</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>UNOP</td>
<td>3.8 ± 0.4</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>LPS (2.5 μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>9.7 ± 2.4</td>
<td>16.6 ± 1.3</td>
</tr>
<tr>
<td>SHAM</td>
<td>9.4 ± 1.7</td>
<td>16.1 ± 1.8</td>
</tr>
<tr>
<td>UNOP</td>
<td>11.6 ± 0.5</td>
<td>16.9 ± 1.2</td>
</tr>
<tr>
<td>Cytokin Production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ (Units/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>143.0 ± 9.7</td>
<td>161.5 ± 7.2</td>
</tr>
<tr>
<td>SHAM</td>
<td>118.3 ± 33.6</td>
<td>149.8 ± 9.8</td>
</tr>
<tr>
<td>UNOP</td>
<td>148.3 ± 6.8</td>
<td>159.2 ± 7.0</td>
</tr>
<tr>
<td>IL-2 (1/2 MAX)</td>
<td></td>
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<tr>
<td>ADX</td>
<td>19.1 ± 3.0</td>
<td>22.8 ± 5.9</td>
</tr>
<tr>
<td>SHAM</td>
<td>18.22 ± 3.96</td>
<td>24.3 ± 6.8</td>
</tr>
<tr>
<td>UNOP</td>
<td>15.2 ± 3.6</td>
<td>24.3 ± 4.1</td>
</tr>
<tr>
<td>TNF (Units/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX</td>
<td>286.5 ± 27.5</td>
<td>205.1 ± 20.8</td>
</tr>
<tr>
<td>SHAM</td>
<td>219.0 ± 47.3</td>
<td>198.3 ± 19.1</td>
</tr>
<tr>
<td>UNOP</td>
<td>172.4 ± 13.4</td>
<td>142.6 ± 16.1</td>
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</table>

* Mean ± SE, n = 5-6 / grp
Figure 1. Proliferative response of whole blood lymphocytes to (A) Con A 5 μg/ml and (B) PHA 5 μg/ml from adrenalectomized (ADX), sham-operated (SHAM), and unoperated (UNOP) rats. Stressed rats received 2-DG, 500 mg/kg subcutaneously, and control rats received an equal volume of saline. Results are expressed as the mean (± SE) of counts per min (cpm); n = 6/group.
BLOOD BLASTOGENESIS

A. Con A = 5 μg/ml

B. PHA = 5 μg/ml

2-DG
SALINE
Figure 2. Splenic NK cell activity expressed as lytic units (LU) from adrenalectomized (ADX), sham-operated (SHAM), and unoperated (UNOP) rats. Stressed rats received 2-DG, 500 mg/kg subcutaneously, and control rats received an equal volume of saline. Results are expressed as the mean (± SE); n = 6/group.
SPLEEN

NATURAL KILLER CELL ACTIVITY

![Graph showing natural killer cell activity in spleen with data for ADX, SHAM, and UNOP groups for 2-DG and SALINE treatments.](image)
Figure 3. Effects of 2-DG injection on the (A) IFN-γ, (B) IL-2, and (C) TNF production by Con A stimulated whole blood leukocytes from adrenalectomized (ADX), sham-operated (SHAM), and unoperated (UNOP) rats. Stressed rats received 2-DG, 500 mg/kg subcutaneously, and control rats received an equal volume of saline. Results of IFN-γ and TNF production were expressed as the mean (± SE) of units / ml (1 unit = 250 pg.). Results of IL-2 were expressed as the mean (± SE) of half Max Units.; n = 6/group.
BLOOD

A. IFN-γ

B. IL-2

C. TNF

2-DG
SALINE

Unit/mL

1/2 MAX

Units/mL

ADX SHAM UNOP
CHAPTER 5. ALTERATION OF THYMIC T CELL SUBSET PROFILES AND APOPTOSIS BY A METABOLIC STRESS

A paper to be submitted to Cellular Immunology

Shiu-Huey Chou, Kirsten L. Gruis, and Joan E. Cunnick

Abstract

The proper development and function of mature T-lymphocytes depends upon the interaction of immature T-lymphocytes with the microenvironment of the thymus. In adults, the thymus seems involuted but maintains the ability to educate and reseed mature T-lymphocytes into the periphery. In the present study, 2-deoxy-D-glucose (2-DG, 500 mg/kg) was administered to male, Lewis rats to examine the effects of metabolic stress on adult thymocytes. The thymic parameters of weight, profile of T-lymphocyte subsets, sensitivity to corticosterone-induced apoptosis, nitrite production by thymic macrophages and mitogen-induced blastogenesis of thymocytes were measured. Results indicated that responsiveness to Con A, PHA, and LPS and nitrite production of thymocytes from rats exposed to chronic stress (5 injections 2-DG) was decreased. 2-DG administration (1, 4, or 5 injections) increased thymocyte apoptosis in the presence of a basal concentrations of corticosterone (0.1 μM). The increase in apoptosis was correlated with a decrease in total thymocytes and thymus size in the 4 and 5 injection groups. Adrenalectomy attenuated both the baseline rate and 2-DG-induced increase in the rate of apoptosis in thymus. As expected, 2-DG-induced thymic involution occurred with a significant decrease in the CD4+ and CD4+CD8+ T-cell
subsets in the thymus of rats receiving 4 or 5 injections. Interestingly, the numbers of CD8−
thymocytes increased in response to multiple injections of 2-DG. These differences could be
due to stress-induced changes that alter CD4 and CD8 subset maturation. This study
demonstrates that chronic stress initiates a process of increased apoptosis in the thymus that
alters the cellular profile of thymocyte subsets resulting in an increase of the CD8+ cell subset.
These changes can be seen up to 48 hr after 2-DG injection or 2 weeks after sham-surgery.

**Introduction**

It is well accepted that stress can affect health by modifying the function of
lymphocytes from secondary immune compartments such as the spleen and blood. Relatively
few studies, however, have examined the extent of stress effects on primary immune
compartments such as thymus. Exposure to stress is known to accelerate thymic involution
as evidenced by a decrease in the number of thymocytes and thymus weight (Andersen, 1935;
Selye, 1936, 1950; Dougherty, 1952). Immobilization stress in C3H/H mice has been
reported to change the ratio of the T-cell subsets in the thymus, specifically by inducing an
increase of immature T-cells (CD4−CD8+) (Teshima et al., 1991). In addition, moderate
exercise in rats induces an increase in the percentage of CD4+ and CD8+ (single positive) T-
cells and a decrease in the percentage of double positive CD4+CD8+ thymocytes (Ferry, et al.,

The thymus is a primary immune organ involved in the establishment, maintenance,
and regulation of host immunity. In adults, the thymus seems involuted but still plays an
important role in restoring the pool of competent T-cells and maintaining the ability to
respond to antigens. Stress-induced changes in the thymus may result in a prolonged alteration of the functional capability of T-cells. Because thymic T-cells migrate to peripheral immune organs, these changes may alter T-cell interactions and responses in secondary immune organs. Therefore, an evaluation of stress-induced modulation of thymocytes can provide an explanation for long-term changes observed in secondary immune organs.

Our laboratory previously reported that administration of 2-deoxy-D-glucose (2-DG), a metabolic stressor, induced a decrease in the number of leukocytes in the spleen, blood, and thymus (Chou et al., 1996). In addition, repeated 2-DG exposure changed the profile of T-cell subpopulations in the spleen, resulting in an increase of CD8^{+} T-cells and a decrease in the ratio of CD4^{+} : CD8^{+} T-cells. Lysle et al. (1988) reported that the chronic stress of repeated administration of 2-DG was able to suppress the response of thymocytes to various mitogens. Both acute and repeated 2-DG administration induced alterations in spleen and blood lymphocyte function, such as a suppression of T-cell mitogen responses, a decrease of natural killer cell activity, and a reduction of cytokine (IL-2, IFN-γ) production (Chou et al., 1996). Therefore, the question arises: Are the outcomes of immunomodulation in the secondary immune organs consistent with observed functional changes in a primary immune organ such as the thymus? In addition, how does stress affect apoptosis, a naturally occurring process in the thymus by which cells die through activation of specific genes that turn on endonuclease activity? In the thymus, apoptosis has a critical function in positive and negative selection which deletes unneeded and self-reactive T-cells. Thus, an increased
propensity to alteration of the undergo apoptotic cell death in the thymus may have important consequences when coupled with functional changes during stress.

Adrenal hormones such as glucocorticoids are involved in thymopoiesis (Cohen, 1989). Primarily, about 90-95% cortical lymphocytes within the thymus are induced to enter programmed cell death followings administration of physiological concentrations of glucocorticoids (Cohen, 1992; Compton and Cidlowski, 1986; Compton et al., 1991). Stress elevates endogenous glucocorticoid concentrations. Thus, it is not surprising that physical stress (forced exercise) induces glucocorticoid-mediated apoptosis of rat thymocytes (Concordet and Ferry, 1993), implicating adrenal hormones in stress-induced apoptosis. Increased rates of apoptosis may reduce the number of functional T-cells that can migrate to peripheral organs. Hence, identification of the ways in which stress influences T-cell function remains an interesting subject for further study.

The purpose of the current research was to characterize the alteration of thymic cellular parameters and function after acute and chronic administration of 2-DG in rats. To evaluate the long-term consequences of stress-induced immune alteration, the sensitivity of thymocytes to corticosterone-induced apoptosis at basal and stress-induced concentrations of corticosterone was measured. We also examined whether adrenal hormones mediate the 2-DG-induced changes in thymus parameters. The results from this study indicate that 2-DG-induced stress affects thymocytes, resulting in altered numbers of functional thymocytes after initially altering T-cell responses in peripheral immune organs.
Materials and Methods

Animals

Inbred, male rats of the Lewis strain (55-60 days old, 220-250 g) were purchased from Harlan-Sprague-Dawley Laboratories (Indianapolis, Indiana). Upon arrival, the subjects were individually caged in a colony room in which a 12-h reversed light-dark cycle was maintained through artificial illumination. They received free access to both food and water (0.9% saline for the adrenalectomized rats) throughout the experiment, except as noted, and a 2-week acclimation period before the experimental manipulation. Additionally, animals were handled every two days to prevent the hyperactivity that occurs when they are left untouched for a prolonged period. All animal manipulations were approved by the Iowa State University Committee on Animal Use.

Reagents and Chemicals

The assays employed two kinds of culture media: "supplemented medium" refers to RPMI-1640 supplemented with L-glutamine (2 mM), gentamicin (50 µg/ml), and Hepes (10 mM)(Gibco Lab., Grand Island, NY), and "complete medium" refers to "supplemented medium" containing 10% Fetal Bovine Serum (FBS) (JHR Biosciences, Lenexa, KS). The mitogens concanavalin A (Con A) and Lipopolysaccharide (LPS) from Escherichia coli were purchased from Sigma (St. Louis, MO), and phytohemagglutinin (PHA) was purchased from Burroughs Wellcome (England). Heparin (100 units/ml, ELKINS-SINN, NJ) and 2-DG (Sigma) were diluted in Hanks' Balanced Salt Solution (HBSS)(Gibco Lab.). $[^3H]$thymidine (specific activity 6.7 Ci/mmol) from Dupont-New England Nuclear (Wilmington, DE) was
used as specified below. All cultures were prepared in flat-bottomed 96-well tissue culture plates (Costar, Cambridge, MA).

Experimental Paradigm 1: Acute and Repeated 2-DG Exposure

Rats were assigned to one control or one of two experimental groups (n=8/group). Each control or experimental animal was injected a total of five times, and subjects were manipulated so that groups of rats differed only in the content of the inoculum. Injections were performed once per 48 hours, 2 hr. after lights off. For each injection, a total inoculum of 0.28 ml/rat was administered subcutaneously. Control rats received a total of five injections of sterile HBSS. The experimental groups of animals received a total of five injections composed of either 4 HBSS plus one 2-DG (acute) or five (repeated) exposures to 2-DG (500 mg/kg body wt) dissolved in sterile HBSS. Thus, 2 groups of rats received 2-DG on the final test day. For 1 hour following each injection of 2-DG or HBSS, all animals were deprived of food and water. One hour following the last injection, each subject was rapidly sacrificed by cervical dislocation.

Experimental Paradigm 2: Duration of Stress Effects on the Thymus

Lewis rats were pretreated with four injections of saline or four injections of 2-DG (500 mg/kg; subcutaneously; one every 48 hours). On the test day, rats were assigned to four groups (n = 4 / group): half of the HBSS pretreatment group received saline (0-2-DG) or 2-DG (1X-2-DG), and half of the 2-DG pretreatment group received no injection (4X-2-DG) or 2-DG (5X-2-DG). On the test day, saline was not administered to the 4X-2-DG to prevent any conditioned response to the injection procedure. This group was included to examine the
duration of thymic changes induced by repeated 2-DG exposure. All animals were deprived of food and water for 1 hour following each injection of 2-DG or saline.

Experimental Paradigm 3: Adrenalectomy Manipulation

Adrenalectomized (ADX), sham-adrenalectomized (SHAM), or unoperated (UNOP) male rats were used to determine the role of adrenal hormones in 2-DG-induced immune alteration. The experiment was performed as two replicates. Each animal of the surgery-treated groups was assigned to one of two subgroups (n=6). One subgroup (stress) received a single injection of 2-DG, 500 mg/kg body weight dissolved in sterile HBSS. The other group (control) received a single injection of sterile HBSS. The injection was administered subcutaneously and performed 2-hour into the dark phase. For one hour following each injection of 2-DG or HBSS, all animals were deprived of food and water. One hour following injection, each subject was rapidly sacrificed by cervical dislocation with a clamp.

Quantification of Thymus Weight and Thymocytes Content

After sacrifice, blood collected via the abdominal aorta into heparinized syringes was used as a source of plasma to determine endogenous corticosterone and glucose concentrations. Each thymus was removed and placed in a preweighed polypropylene tube containing 8 ml of complete media. Each organ was weighed to the nearest mg immediately after removal from the body. Each subject's thymus was dissociated into a single-cell suspension by grinding the thymus between the frosted ends of two glass slides. The single-cell suspension was collected after 10 min. of unit gravity sedimentation to remove cell debris and clumps. Subsequently, the thymocyte suspensions were counted by using a
Celltrak 2 (NOVA Biomedical, Waltham, MA) cell-analyzer to determine the total leukocyte count per organ.

**Plasma Glucose Assay**

The level of glucose present in the plasma was measured by two methods: one was based upon the glucose oxidase (Trinder) reaction, which detects endogenous glucose and 2-DG, and the second was based upon the hexokinase (HK) reaction, which detects only endogenous glucose (Sigma, St. Louis, MO). For all subjects, plasma was obtained from whole blood by centrifugation (12,000 rpm/5 min.) and stored at -70 °C until assayed. The samples were tested in duplicate and read at 505 nm for the Trinder and 340 nm for the HK assay using a spectrophotometer (UVIKON 930, Kontron Instruments AG, Switzerland). A glucose standard curve was performed with each assay, and sample glucose concentrations were calculated based upon linear regression analysis of the standard.

**Plasma Corticosterone Assay**

To test for the completeness of adrenalectomy and the stress effect of 2-DG administration, plasma samples were assayed for corticosterone. Plasma corticosterone levels were determined by using a standard radioimmunoassay kit for rat corticosterone (ICN Biomedical, Inc., CA). The error for the corticosterone radioassay is 0.2 µg/dl and values below this level, as in the ADX groups, can be interpreted as lack of corticosterone.

**Mitogen Stimulation Assay**

The mitogen stimulation assay was performed with thymocytes to assess lymphocyte proliferation. Con A and PHA were used as T-lymphocyte mitogens and LPS as a B-
lymphocyte mitogen as described previously (Cunnick et al., 1990).

**Nitric Oxide Assay**

Supernatants were harvested from cultures of mitogen-stimulated lymphocytes before pulsing with $^3$H thymidine. The nitric oxide concentrations were determined by measuring nitrite in a microplate assay (Stuehr and Nathan, 1989). Sample aliquots (50 µl) were mixed with 100 µl of Griess reagent (0.5% sulfanilamide/0.05% naphthylethylenediamine dihydrochloride/2.5% H$_3$PO$_4$) and incubated at room temperature for 10 min. The optical density was measured using a microtiter plate reader (BIO Kinetics Reader, BIO-TEK Instruments, Winooski, VT) at 550 nm against PBS as a blank. A sodium nitrite (NaNO$_2$) standard curve (0-100 µM) was generated in parallel. Results are expressed as the mean of nitrite concentrations in the supernatants of triplicate wells.

**Flow Cytometric Analysis of Lymphocyte Subsets**

The percentages of CD$^+$ and CD$^8^+$ lymphocyte subsets were studied by fluorescent-cell-surface staining with monoclonal antibodies and analysis on an EPICS PROFILE-1 flow cytometer. Monoclonal mouse antibodies to lymphocyte populations were purchased from PharMingen or Bioproducts for Science. The anti-CD4 antibody (W3/25) was conjugated to fluorescein iso-thiocyanate (FITC), and the anti-CD8 antibody (OX-8) biotinylated (PharMingen) for conjugation to avidin phycoerythrin (PE) (Vector Labs) to be used for double staining. The FITC or Biotin-conjugated mouse IgG1.k-isotype standard antibodies (PharMingen, San Diego, CA) were used as control antibodies. Thymus suspensions ($\sim$1x10$^6$) diluted in HBSS supplemented with 6 % FBS were diluted in 100 µl of cold
PBS/Azide (0.2%) and incubated in the dark at 4°C for 20 min. with ~1.5 μg of the monoclonal antibodies or isotypic control antibodies. The erythrocytes were lysed with freshly diluted lysing solution (ammonium chloride [150 mM], sodium bicarbonate [1 mM], and EDTA disodium [1 mM]) for 10 min. at room temperature. The remaining lymphocytes were washed twice with PBS/Azide. An avidin-PE solution (Vecta stain) was incubated with the cells for 20 min. (4°C) according to the instructions. The cells were washed twice with PBS/Azide and placed in 2% paraformaldehyde before analysis.

*Corticosterone-Induced Apoptosis Assay*

Apoptotic cells were quantified by flow cytometry to measure the incorporation of Hoechst 33342 (Molecular Probes, INC) fluorescent dye in thymocytes (Sun et al., 1992). A single cell suspension of thymocytes (2 x 10^6) were incubated with or without corticosterone (0, 0.1, & 1.0 μM) for four hours and stained for 10 min. with 1 μg/ml Hoechst 33342 at 37°C. The cells were rapidly centrifuged for 5 min. at 1500 rpm, and all media and Hoechst dye were removed. The thymocytes were resuspended in propidium iodide (PI, 5 μg/ml; Sigma) in PBS and analyzed on a flow cytometer (EPICS, Coulter Co.). The cells staining positive for PI were excluded from the analysis because they constitute dead cells. The apoptotic cell was identified by a decrease in mean size and bright blue staining with Hoechst dye. Data was reported as percentage apoptotic cells.

*Statistical Treatment of Data*

A computerized program for analysis of variance (ANOVA; Statistix, NH Analytical Software) was used to assess differences among experimental and control groups. For the
first experiment to characterize the effects of acute (1X) and chronic (5X) 2-DG administration, an ANOVA to compare 3 stress-levels (saline injection, 1X 2-DG injection, and 5X 2-DG injection) was used to assess significance of the various measures. For the second experiment to examine the duration of the 2-DG effect on the thymus, an ANOVA to compare 4 levels of stress (0-2-DG injection, 1X-2-DG injection, 4X-2-DG injections, and 5X-2-DG injections) was used to determine differences in T-cell parameters between the groups. For the ADX experiment to assess the effects of adrenalectomy on attenuation of acute stress effects of 2-DG, a 2 x 3 analysis was used. One factor was treatment, a 2-DG injection, or saline injection. The second factor was surgery, adrenalectomy (ADX), sham operated or unoperated. To analyze data from apoptosis experiments a 4 x 3 analysis was used: One factor was 2-DG treatment (0-2-DG injection, 1X-2-DG injection, 4X-2-DG injections, and 5X-2-DG injections), and the second factor was a dose of corticosterone (0.0, 0.1, 1.0 μM). The level of significance for the F test was set at $P \leq 0.05$.

**Results**

*Experiment 1: Acute and Repeated 2-DG Exposure*

2-DG administration elevated plasma endogenous glucose and corticosterone levels

An elevated level of endogenous glucose and corticosterone in plasma provided an indicator of the 2-DG-induced stress response. In the current study, analysis of variance indicated that rats that received 2-DG administration on the test day had a significant increase of endogenous plasma glucose (mean = 319.37 ± 8.41 mg/dl) and corticosterone (mean =
54.69 ± 4.51 μg/dl) in comparison to rats that received a HBSS injection (glucose = 123.65 ± 4.61 mg/dl; corticosterone = 7.27 ± 2.18 μg/dl).

**Repeated 2-DG administration reduced thymus weight and thymocytes number**

The number of thymocytes were enumerated and the ratio of thymus weight / body weight was calculated (Table 1). Analysis of variance indicated a significant effect of 2-DG on thymocytes and thymus weight, F(2, 23) > 17.00, p < 0.0002. Orthogonal contrasts indicated that repeated administration (5X) of 2-DG induced a significant reduction in thymus weight, F(1, 23) = 41.60, p < 0.0001 in comparison with the HBSS and 1 X 2-DG injection groups. Also, the number of thymocytes was significantly decreased in rats that received 5X 2-DG injections, F(1, 23) = 33.29, p < 0.0001. But, there was no difference between the animals that received one injection of 2-DG or HBSS.

**Repeated exposure to 2-DG suppressed thymus mitogenic responses and nitrite production**

The proliferative response of thymic leukocytes to mitogen stimulation was measured as an indicator of functional potential. The assay results showed comparable effects across all concentrations of Con A, PHA, and LPS. The results of stimulation of thymic leukocytes with the optimal concentration of the mitogens are shown in Table 1. Analysis of variance of the optimal concentration of Con A and LPS indicated a significant effect of treatment for Con A, F(2, 22) = 5.91, p < 0.01 and a marginal effect of treatment for LPS, F (2, 22) > 3.17, p < 0.07, however, there was no significant treatment effect for PHA, F (2, 22) > 1. Orthogonal contrasts indicated that the responses to Con A and LPS were significantly depressed in the 2-DG injected groups (1X and 5X) in comparison with the saline injected
group, F (1, 22) > 6.25, p < 0.02, but the effect of 2-DG on the response to PHA was
marginally suppressed, F (1, 22) = 3.27, p < 0.09. Furthermore, contrasts indicated that there
was no difference between the groups that received 5X 2-DG and 1X 2-DG except for Con
A, F(1, 22) = 4.45, p < 0.05. Thus, the 2-DG-induced suppression of the Con A mitogen
response was more pronounced in rats that received 5X 2-DG than 1X 2-DG.

The results of nitrite production from thymic stromal cells stimulated with the optimal
concentration of Con A (3 mg/ml), PHA (5 mg/ml), and LPS (2.5 mg/ml) are also presented
in Table 1. Overall analysis of data from all three mitogens indicated a significant effect due
to treatment, F(2, 23) > 9.84, p < 0.002. Orthogonal contrasts indicated that the 2-DG treated
groups showed a significant decrease of nitrite production relative to the saline-treated group.
F (1, 23) > 12.80, p < 0.002. Additionally, there was less nitrite in cultures of thymocytes
from chronically-stressed (5 X 2-DG) animals than from acutely stressed (1 X 2-DG)
animals, F (1, 23) > 19.5, p < 0.001. This finding indicated that 2-DG not only changed the
number of thymocytes, but also changed the function of thymic stromal cells.

Experiment 2: Duration of Stress Effect on the Thymus

Plasma endogenous glucose and corticosterone concentration

Rats that received 2-DG injection on the test day (1X and 5X 2-DG) produced a
significant increase of plasma glucose (mean = 276 ± 8.32 mg/dl) and corticosterone (mean =
34.44 ± 6.62). In contrast, the 4X 2-DG group that did not receive a 2-DG injection on the
test day showed a basal concentration of plasma glucose (104.75 ± 2.80 mg/dl) and
corticosterone (6.44 ± 2.80 μg/dl). Saline injected rats had a mean plasma glucose
concentration of 130.11 ± 4.47 mg/dl and a mean corticosterone concentration of 1.57 ± 0.39 μg/ml.

2-DG induced a decrease of CD4$^+$ population, but an increase of CD8$^+$ T-cell population

The results of CD4 and CD8 surface-molecule staining of thymocytes are displayed in Figure 1 and Figure 2, panel A-F, respectively. The percentage positive for single CD4$^+$ or CD8$^+$ and double positive CD4$^+$/CD8$^+$ populations is displayed in Figure 1. These data were then transformed to the actual number of cells for each subpopulation per thymus (Figure 2). Analysis of variance of CD4$^+$ and CD8$^+$ populations indicated a significant treatment effect, $F(3, 15) > 5.10, p < 0.02$. Orthogonal contrasts demonstrated that rats that received repeated 2-DG administration (4X 2-DG; 5X 2-DG) showed a marked decrease in the percent of CD4$^+$ cells, $F(1, 15) = 16.16, p < 0.002$ (Fig. 1A), whereas there was an increase in the percent CD8$^+$ population, $F(1, 15) = 14.36, p < 0.003$ (Fig. 1B). There was no significant change in the percentage of double positive (Fig. 1C) or double negative (mean = 2.6%; data not shown) cell populations. The increase in the percentage of CD8$^+$ cells translated into a real increase in the actual number of CD8$^+$ cells in the thymus from stressed rats, $F(1, 15) = 5.29, p < 0.04$ (Fig. 2E). Because of a decrease in the total number of thymocytes/thymus, the number of CD4$^+$, CD4$^+$/CD8$^-$, and null populations were significantly decreased in rats that received multiple injections of 2-DG (4X 2-DG; 5X 2-DG), $F(1, 15) > 6.80, p < 0.02$ (Fig. 2). Thus, even though the thymus was decreased in size, the CD8$^+$ population increased.
2-DG increases the rate of apoptosis in the thymus in vitro

The results of corticosterone induced apoptosis in the thymus were displayed in Fig. 3. For this assay, thymocytes were exposed to various concentrations of corticosterone [0.0.1, or 1.0 μM] for 4 hours. The 0.1 μM dose of corticosterone represented a basal concentration found in resting rats (3.4 μg/dl), and the 1.0 μM corticosterone represents a concentration obtained (and often exceeded) during a stress response. The analysis of variance of the percentage of apoptosis demonstrated a significant treatment effect. F(3, 47) = 7.97, p < 0.004. The results indicated that 2-DG-induced stress increased thymocyte sensitivity to corticosterone-induced apoptosis in animals that had received a single injection or multiple injections of 2-DG, F(1, 43) = 21.34, p < 0.0002. There was no difference between groups in which rats received 4 injections of 2-DG and 5 injections of 2-DG. Note that the 4x 2-DG group did not receive 2-DG on the test day, but had received the last injection 2 days prior to the test day and had a basal concentration of corticosterone. Thus, the rate of corticosterone induced apoptosis remained elevated for up to 2-days poststress, and the increased sensitivity to corticosterone was not due to elevated serum corticosterone concentration in the animal when the thymocytes were harvested.

Experiment 3: Adrenalectomy Manipulation

Plasma corticosterone and endogenous glucose concentrations

As observed in experiment 1 and 2, administration of 2-DG induced a significant increase in the plasma concentration of corticosterone (64.74 ± 4.20 μg/dl) and endogenous glucose (337.19 ± 8.66) in both the sham-operated and unoperated groups; however.
adrenalectomy attenuated the 2-DG-induced increase of endogenous glucose (152.55 ± 5.06 mg/dl) and corticosterone (1.93 ± 0.53 µg/dl). Saline injected rats had mean plasma glucose concentrations of 110.77 ± 6.66 mg/dl and mean corticosterone concentrations of 9.54 ± 2.7 µg/dl (SHAM and UNOP) or 1.85 ± 0.35 µg/dl (ADX).

Adrenal hormones affect thymus weight and cellularity

To determine the role of adrenal hormones as mediators in 2-DG-induced changes of the thymus, the stress effect of 2-DG administration on thymus weight and thymocyte number in adrenalectomized, sham-operated, and unoperated rats was examined (Table 2). Analysis of variance demonstrated a significant effect of surgery on thymus weight and thymocyte number, F(2, 35) > 19.62, p < 0.0001. Contrasts indicated that the weight of the thymus gland and the number of thymocytes were greater in the ADX group than in sham-operated and unoperated groups, F(1, 35) > 37.08, p < 0.0001; however, there was no difference between the sham-operated and unoperated groups. Additionally, there was no significant effect of treatment or a significant interaction. These results indicate that adrenal hormones regulate thymus growth.

Adrenal hormones are involved in regulation of mitogen-stimulated lymphocyte response

Results of 2-DG administration on the mitogen response in adrenalectomized, sham-operated, and unoperated rats were examined (Table 2). Overall analysis of variance of the optimal concentration of Con A, PHA, and LPS indicated a significant main effect of surgery, F (2, 35) > 6.43, p < 0.006; however, there was no significant effect of 2-DG treatment or interaction of surgery with 2-DG. Orthogonal contrasts revealed that adrenalectomized rats
had a pronounced suppression of proliferation in response to mitogens in comparison with sham-operated and unoperated rats, $F(1, 35) > 7.9, p < 0.01$.

**Adrenalectomy attenuated corticosterone-induced apoptosis in the thymus**

Thymocytes from adrenalectomized, sham-operated, and unoperated rats injected with saline or 2-DG were incubated with corticosterone to induce apoptosis (Table 3). The ANOVA demonstrated a significant effect of surgery, $F(2, 105) = 56.36, p < 0.0001$ and a significant effect of corticosterone concentration, $F(2, 105) = 1280.78, p < 0.0001$. Orthogonal contrasts of the surgery effect on thymocyte apoptosis showed that thymocytes from the ADX group had less apoptosis than cells obtained from sham-operated and unoperated groups, $F(1, 105) > 14.50, p < 0.001$. The adrenalectomized group also contained a higher percentage of live cells from the thymus after incubation with corticosterone. $F(1, 105) > 12.50, p < 0.001$. Although ANOVA did not indicate a main effect of 2-DG treatment, there was a marginal interaction of surgery and stress, $F(2, 105) = 2.41, p = 0.095$. A contrast comparing apoptosis in cells from unoperated and sham-operated rats that received 2-DG and saline injections demonstrated a significant elevation of apoptosis in 2-DG injected rats $F(1, 105) = 5.11, p < 0.03$. The 2-DG-induced elevation of apoptosis was more marked in the unoperated group, $F(1, 105) = 6.35, p < 0.02$. The elevation in apoptosis could be clearly observed at 0 and 0.1 $\mu$M corticosterone for the unoperated group, but was only evident at 0 $\mu$M corticosterone for the sham-operated group. Interestingly, the 2-DG-induced increase in percentage apoptosis for the unoperated group was equivalent to the baseline rate of apoptosis for the sham-operated group injected with saline. This indicates that surgery stress
can increase the rate of apoptosis in rats with intact adrenal glands. In addition, the stress of sham-surgery increased the rate of apoptosis for a period of time greater than two weeks. These results support the hypothesis that adrenal hormones are involved in basal rates of apoptosis in the thymus. Because the stress of 2-DG administration elevated the concentration of plasma corticosterone, the endogenous corticosterone may have exacerbated the sensitivity to corticosterone-induced apoptosis \textit{in vitro} in the unoperated rats. But apoptosis was not prevented 100\% by adrenalectomy, therefore, adrenal hormones are not solely responsible for apoptosis in the thymus.

\textbf{Discussion}

The present study demonstrated that the cells of the thymus are profoundly influenced by 2-DG injection; however, changes of thymic parameters were most evident in the groups what received multiple injections of 2-DG. Hence, the 2-DG-induced modulation of thymic parameters was more resistant to stress-induced changes than cells from the spleen and blood. Our previous work has shown that the alteration of function of lymphocytes from blood and spleen can be observed after ether an acute (1X) or repeated (5X) 2-DG injection animals.

The profile of T-lymphocyte subpopulations was altered in the thymus of 2-DG-injected rats. There was a marked decrease in the percentage of CD4$^+$ cells, an increase of the CD8$^+$ cells, and no change in the percentage of double positive and double negative cells. As thymic size decreased with repeated stress, the actual numbers of each cell type in the thymus were calculated. Thus, the actual number of CD4$^+$, CD4$^+$CD8$^+$, and double negative cells were decreased due to 2-DG stress. Conversely, the increase in the percentage of CD8$^+$
cells represented a real increase in the total number of CD8\(^+\) cells in the thymus. Previously, other researchers have reported that metabolic stress decreases in only the double positive CD4\(^+\)CD8\(^+\) cells by using chronic morphine treatment (Freier and Fuchs, 1993), dexamethasone injection (Jondal and McConkey, 1993), or glucocorticoid administration (Ferry et al., 1993). The difference between these results may be due to a difference in the temporal pattern of 2-DG administration or a species difference (rats vs. mice).

The 2-DG-induced decrease of CD4\(^+\) and CD4\(^+\)CD8\(^+\) populations in the thymus was correlated with an increased rate of apoptosis. The percentage of apoptosis was higher in 2-DG treated animals than in control animals. Administration of 2-DG like other stressors induced a marked increase of the concentration of plasma corticosterone. In addition, animals lacking adrenal glands, are unable to produce baseline or stress levels of corticosterone, and had lower rates of apoptosis in comparison to sham-operated and intact animals; this supports a causal role for corticosterone in apoptosis. Note that in experiment 2 the 4X 2-DG group had received their last injection 2 days before the test day and had baseline concentrations of plasma corticosterone. In addition, the SHAM-operated group in experiment 3 had experienced the stress of surgery more than two weeks before the experiment. Both of these groups demonstrated elevated rates of apoptosis without high concentrations of plasma corticosterone in the animals. Thus, the increase of corticosterone-induced apoptosis remained elevated for a long time post-stress and was not due solely to increased plasma corticosterone levels in the animals on the day the thymocytes were harvested.
The current study did not identify whether the CD4^+ or CD4^-CD8^+ populations were preferentially induced to undergo apoptosis more than the CD8^+ cell population. Although stress induced a decrease in thymic size, the CD8^+ population was protected or induced to differentiate. The CD8^+ T lymphocytes express both cytotoxic and regulatory functions. An increase of T suppressor cells may be responsible for some or all aspects of decreased function observed in the thymus or secondary immune organs. A shift to greater production of CD8^+ T-lymphocytes could have profound effects on the health of immunocompromised animals or animals prone to autoimmune diseases (Ackerman, et al., 1996).

Maturation and differentiation of developing T-lymphocytes depends upon the interaction of these cells with the microenvironment of the thymus and thymic hormones. The stress of 2-DG administration also affected the function of cells responsible for the microenvironment. Experiment 1 indicated that there was less nitrite in cultures of thymocytes from the chronically-stressed group (5X 2-DG) than from the saline-treated and acutely-stressed (1X 2-DG) groups. Nitrite production reflects one component of macrophage function and indicates that the macrophages in the thymus were changed in stressed animals. Alternatively, there may be fewer macrophages present. In either instance, the microenvironment for the thymocytes is changed and should result in changes in thymocyte differentiation such as those found in repeatedly stressed rats. In our study, adrenalectomy prevented thymic involution. Additionally, Con A stimulated responsiveness was decreased in adrenalectomized rats. Hence, adrenal hormones may affect the interaction of thymocytes and macrophages and change their functional capacity.
In conclusion, the primary role of the thymus is to educate and produce the T-cell repertoire, which can migrate into secondary immune compartments to perform their function. Stress-induced alterations of thymic development can have long-term implications for immune function in secondary immune organs. In the current study, 2-DG altered immune parameters of the thymus suggesting that the effect of stress on thymocytes could result in altered numbers of functional T-lymphocytes that can migrate to secondary immune organs resulting in an alteration of the T-cell responses and interactions in those organs. In support of this hypothesis, our previous paper demonstrated an increase in splenic CD8+ T-cells after both acute and chronic stress (Chou et al., 1996). Additionally, the stress-induced increase of apoptosis may selectively alter the profile of the T-cell subpopulations in the thymus.

There are many neuroendocrine pathways that may influence thymic development. Stress-induced corticosterone is a known modulator of thymic involution. This study supports that hypothesis. Recently, the noradrenergic pathway, cholinergic pathway, and opioid system have also been linked to modulation of thymic function (Teshima, et al., 1991; Madden and Felten, 1995; Dabrowski and Dabrowska-Bernstein, 1990). The regulation of the thymus is complex and needs further study.

Acknowledgment
We thank Chi-Lei Chin and Valerie Rosenwald for their excellent technical assistance.

References
Table 1. Effects of one or five injections of 2-DG (500 mg/kg) or saline injection(s) on the thymus weight, thymocyte number, the mitogen responsiveness and nitric oxide production of thymocytes.

<table>
<thead>
<tr>
<th>Injection Manipulation</th>
<th>1 X 2-DG</th>
<th>5X 2-DG</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of Thymus Weight / Body Weight (mg/gm)</td>
<td>1.53 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.21 ± 0.05</td>
<td>1.56 ± 0.05</td>
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<tr>
<td>Thymocytes Number (x 10&lt;sup&gt;6&lt;/sup&gt; /organ)</td>
<td>561.00 ± 19.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>478.20 ± 14.72</td>
<td>591.20 ± 24.06</td>
</tr>
<tr>
<td>Mitogen response (cpm x 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A (3 µg/ml)</td>
<td>223.10 ± 33.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>173.20 ± 37.23</td>
<td>253.80 ± 24.85</td>
</tr>
<tr>
<td>PHA (5 µg/ml)</td>
<td>75.12 ± 5.52</td>
<td>62.89 ± 2.76</td>
<td>83.76 ± 8.71</td>
</tr>
<tr>
<td>LPS (2.5 µg/ml)</td>
<td>2.87 ± 0.21</td>
<td>2.59 ± 0.09</td>
<td>4.68 ± 1.03</td>
</tr>
<tr>
<td>Nitrite production (µmole/ml)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A (3 µg/ml)</td>
<td>4.38 ± 0.53&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.37 ± 0.58</td>
<td>5.07 ± 0.76</td>
</tr>
<tr>
<td>PHA (5 µg/ml)</td>
<td>2.95 ± 1.03</td>
<td>2.74 ± 1.01</td>
<td>3.52 ± 0.96</td>
</tr>
<tr>
<td>LPS (2.5 µg/ml)</td>
<td>3.48 ± 1.07</td>
<td>3.18 ± 1.09</td>
<td>3.97 ± 0.94</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are expressed as the mean (± SE), n = 8 / grp.

<sup>b</sup> Data are expressed as the mean ± SE of triplicate wells; n = 8 /grp.

<sup>c</sup> Nitric oxide was measured as nitrite in the supernatant of mitogen-stimulated cultures using Griess reagent.

<sup>d</sup> Nitrite concentrations are expressed as the mean ± SE of triplicate well; n = 8 /grp.
Table 2. Thymus weight, thymocyte number, and the mitogen response in adrenalectomized (ADX), sham-operated (SHAM), and unoperated (UNOP) rats.

<table>
<thead>
<tr>
<th>Surgery Manipulation</th>
<th>ADX</th>
<th>SHAM</th>
<th>UNOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus Weight / Body Weight (mg/gm)</td>
<td>2.19 ± 0.13°</td>
<td>1.59 ± 0.04</td>
<td>1.74 ± 0.07</td>
</tr>
<tr>
<td>Thymocytes Number (x 10^6/organ)</td>
<td>828.13 ± 48.77°</td>
<td>464.87 ± 51.05</td>
<td>465.40 ± 53.37</td>
</tr>
<tr>
<td>Mitogen Response (cpm x 10^3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A (3 μg/ml)</td>
<td>66.5 ± 6.43°</td>
<td>92.3 ± 11.32</td>
<td>108 ± 8.42</td>
</tr>
<tr>
<td>PHA (5 μg/ml)</td>
<td>1.46 ± 0.11</td>
<td>1.926 ± 0.14</td>
<td>1.68 ± 0.11</td>
</tr>
<tr>
<td>LPS (2.5 μg/ml)</td>
<td>0.983 ± 0.18</td>
<td>1.24 ± 0.24</td>
<td>1.08 ± 0.23</td>
</tr>
</tbody>
</table>

° Results are expressed as the mean (± SE), n = 12 / grp.

° Data are expressed as the mean ± SE of average triplicate wells; n = 12 / grp.
Table 3. Effect of corticosterone-induced apoptosis in adrenalectomized (ADX), sham-operated (SHAM), and unoperated (UNOP) rats. Thymocytes from each rat were exposed to corticosterone (0, 0.1, 1.0 μM) for 4 hours. Results are expressed as percentage of live cells positive for apoptosis.

<table>
<thead>
<tr>
<th>Surgery Manipulation</th>
<th>Corticosterone Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM</td>
</tr>
<tr>
<td>ADX 2-DG a</td>
<td>13.78 ± 0.69 c</td>
</tr>
<tr>
<td>Saline b</td>
<td>14.67 ± 1.10</td>
</tr>
<tr>
<td>SHAM 2-DG</td>
<td>17.68 ± 1.0</td>
</tr>
<tr>
<td>Saline</td>
<td>16.43 ± 0.67</td>
</tr>
<tr>
<td>UNOP 2-DG</td>
<td>16.90 ± 0.71</td>
</tr>
<tr>
<td>Saline</td>
<td>15.02 ± 0.54</td>
</tr>
</tbody>
</table>

a Stress rats received 2-DG, 500 mg/kg subcutaneous injection.
b Nonstress rat received an equal volume of saline injection.
c Results are expressed as the mean (± SE) of the percentage of apoptosis, n = 6 / grp.
Figure 1 Effect of one, four, or five injections of 2-DG (500 mg/kg) or saline injections on T-cell subpopulations in the thymus, expressed as the percentage of positive cells staining with antibody to (A) CD4 (W3/25), (B) CD8 (OX8), or (C) CD4 and CD8 (double positive). Results are expressed as the mean (± SE); n = 4 / group.
INJECTIONS OF 2 x DC

C. CD4+ / CD8+

B. CD8+

A. CD4+

(Percent)
Figure 2 Effect of one, four, or five injections of 2-DG (500 mg/kg) or saline injections on T-cell subpopulations in the thymus, expressed as the actual number of positive cells staining with antibody to (D) CD4 (W3/25), (E) CD8 (OX8), or (F) CD4 and CD8 (double positive). Results are expressed as the mean (± SE); n = 4 / group.
(NUMBER)

D. CD4^+

![Bar graph showing CD4^+ number over different injections of 2-DG.]

E. CD8^+

![Bar graph showing CD8^+ number over different injections of 2-DG.]

F. CD4^+/CD8^+

![Bar graph showing CD4^+/CD8^+ number over different injections of 2-DG.]

INJECTIONS OF 2-DG
Figure 3 Effect of one, four, or five injections of 2-DG (500 mg/kg) or saline injections on corticosterone-induced apoptosis in the thymus. Thymocytes from each rat were exposed to corticosterone (0, 0.1, or 1.0 μM) for 4 hours. Percentage of apoptotic cells were calculated based on the results of flow cytometric analysis. Results are expressed as the mean (± SE); n = 4 / group.
THYMOCYTE APOPTOSIS IN VITRO

![Graph showing the effect of corticosterone on thymocyte apoptosis.](image)

- **SALINE**
- **1X 2DG**
- **4X 2DG**
- **5X 2DG**
CHAPTER 6. GENERAL CONCLUSION

General Discussion

The progression of illness is aggravated if an animal experiences a stress state (Plotnikoff et al., 1991). Metabolic disorders represent stress situations that can be caused by improper diet, alcohol abuse, or drugs of abuse. Metabolic disorders suppress immune defenses of an animal and enhance its susceptibility to invading pathogens (Sharp et al., 1995; Schuppan et al., 1995). Therefore, studies of metabolic stressors as immunomodulators may provide a means to understand the consequences of enhanced microbial or viral infections. To this end, administration of 2-DG, a metabolic stressor, in rats provides a model system used in this dissertation for studying the effects of metabolic stress on the immune system.

The advantages of using 2-DG are based on several criteria. Administration of 2-DG induces well characterized physiological responses, decreased escape behavior, lowers locomotor activity, and significant neurological activity. These criteria for stress can be used to monitor animals during experimental processes. Also, the neural responses to 2-DG provide a useful tool for investigating the relationship between the immune system and the neuroendocrine system. The objectives of this study were to characterize the changes in selected immune parameters subsequent to treatment with 2-DG; and to examine possible mechanisms of neuroendocrine mediation by 2-DG-induced immunomodulation.

The first set of experiments was designed to characterize the effects of 2-DG stress on secondary immune compartments: Spleen and peripheral blood. Elevated concentrations of
plasma corticosterone and exaggerated endogenous glucose confirm a stress response and effective 2-DG treatment. These measurements were made in all subsequent experiments. The 2-DG-induced metabolic stress altered the immune cellular function of both innate and adaptive immunity in the spleen and blood. The concentration of leukocytes in the spleen and blood and the organ weight of the spleen were markedly reduced in rats which received repeated 2-DG injections. The splenic T-lymphocytes subpopulations showed a decreased ratio of CD4+/CD8+ cells due to a significant increase of the CD8+ T-cell subpopulation. In addition, the innate immunity of splenic natural killer cell activity, nitrite production of spleen and blood leukocytes, and IL-1 production of blood macrophages were changed due to 2-DG administration.

Adaptive immune mechanisms were suppressed by 2-DG including mitogenic responsiveness and IFN-γ production in both whole blood and splenic lymphocytes. Additionally, this study showed that the 2-DG-induced suppression of splenic T-cell mitogen response was habituated in the group which received five 2-DG injections. Conversely, the suppressive effect of 2-DG on the whole blood lymphocytes was not attenuated after repeated administration. A difference between splenic and blood responses also was observed in the results of IL-1 production. The production of IL-1 by macrophages and IL-2 by T-lymphocytes was significantly reduced in the blood, but not in the spleen. This difference is consistent with other studies showing that stress differentially affects various compartments of the immune system (Lysle et al., 1988; Cunnick et al., 1990). To this end, identification of the mechanism(s) responsible for the differences in kinetics of 2-DG-induced
immunomodulation in spleen and whole blood was a major goal in the second and third sets of experiments.

Numerous experiments provide anatomic and pharmacological evidence that catecolamines are involved in stress-induced immunomodulation in the spleen, while adrenal hormones mediated hypothalamic-pituitary-adrenal pathway are responsible for stress-induced alteration of blood (Madden & Felten, 1995). We followed this track in designing experiments to determine mechanisms of 2-DG-induced immune modulation.

The purpose of the second set of experiments was to examine the role of catecholamines in 2-DG-induced immune alteration. Rats were pretreated with a nonselective β-adrenergic receptor antagonist, nadolol, and then received either a saline or 2-DG injection. As expected, nadolol prevented 2-DG-induced suppression of splenic T-cell mitogenic responses, decreased of IFN-γ production, and increased of nitrite production by macrophages in a dose-dependent manner. In contrast, nadolol did not attenuate the 2-DG-induced changes in immune parameters of peripheral blood. These results illustrated that the peripheral release of catecholamines is responsible for 2-DG-induced splenic immune alterations, but not for those of the peripheral blood.

The evidence for adrenal hormone involvement in 2-DG-induced alteration of immune parameters in the blood was established in third set of experiments. 2-DG-induced changes in leukocyte concentration, mitogenic response of lymphocytes, and cytokine production (IL-2, IFN-γ, TNF) of blood lymphocytes were attenuated in rats lacking adrenal glands, implicating adrenal hormones as mediators of 2-DG-induced immunomodulation of
blood. Interestingly, removal of adrenal glands exacerbated the production of IL-2 and TNF by blood lymphocytes from 2-DG stressed animals. This suggests that stress-induced release of adrenal hormones may serve to maintain immune homeostasis.

The objective of fourth study was to characterize the alteration of thymic parameters and apoptosis after acute and chronic administration of 2-DG in rats. This study demonstrated that 2-DG-induced alteration of thymic parameters are prolonged (48hr). Repeated 2-DG administration induced a marked reduction of total thymocyte numbers, thymus weight, mitogen response of thymocytes, and nitrite production of thymic macrophages. The profile of T lymphocyte subpopulations was changed after 2-DG administration. A significant decrease in the percentage and actual number of CD4+ and CD4+CD8+ and null population was observed. The actual numbers of CD8+ T-cells in thymus increased in response to multiple injections of 2-DG and may explain the observed increase of CD8+ T-lymphocytes in the spleen. Since apoptosis is involved in the maintenance and establishment of the T-cell repertoire, an increased rate of corticosterone-induced apoptosis in the thymus after acute and repeated 2-DG administration may have important consequences when coupled with thymus modulation during stress. However, adrenalectomy attenuated the 2-DG-induced increase of thymocyte apoptosis, suggesting adrenal hormones are responsible for mediating apoptosis. These results suggest that adrenal hormones produced during stress differentially increased apoptosis in the thymus resulting in altered distribution of thymocyte subset and changing the concentration of functional T-
lymphocytes which can migrate to peripheral immune organs. Thus, changes in T-
lymphocyte maturation can alter the peripheral T-cell responses.

Modulation of immune function by stressful events is a well accepted concept clearly
supported by the results from this dissertation. Studies such as these provide important
information for understanding the disruption of immune homeostasis by stress, and can also
provide a way to study the interaction of the immune system with other physiological
systems.

References

induced immune suppression is mediated by adrenal hormones and peripheral β-
D-glucose modulation of T-lymphocyte reactivity: Differential effects on lymphoid
Press Inc, Florida.
ACKNOWLEDGMENTS

There are numerous peoples contributed to my research to make this dissertation complete. Without their endless patient and encouragement, I may not achieve my Ph.D. degree. I express my sincere gratitude to Dr. Joan E. Cunnick for her tremendous encouragement, guidance, and support throughout my graduated studies. She was not only invaluable in developing my research projects and providing insightful suggestions for the improvement of these manuscripts, but also she provided totally trust and meticulously carefulness on my life. When I struggled and frustrated in English speaking and writing, she provided an immense help for them improvement. She is my mentor, teacher, and my friend. Without her professional and moral support, I would not have grow independently and professionally to accomplish this dissertation. Joan, thanks a lot.

I would like to thank the other members of my graduate committee: Dr. Michael Wannemuehler, Dr. Ronald Griffith, Dr. Richard Hughes, and Dr. Eugenia Farrar. Their unending encouragement, critical review, and valuable comments on my work and manuscripts greatly facilitated both my research and dissertation.

I would especially like to thank Dr. Ljiljana D. Kojic for her countless hours in working with me through the first and second year of graduate study. Without her advice, technical assistant, and moral support, I may not have smoothly beginning and willing continuos studing to the end.

In addition, my colleagues, Kelly Nordyke-Messingham and Kim Downard have been a tremendous help for my projects and paper writing. I would also like to thank Ginger
Iseminger and Veena Rajaraman for your endless moral support and advise. All of you made my study life smoothly and happy.

Finally, but most importantly, I genuine acknowledge to my parents, sisters, and brothers for their endless love and heartfelt support in pursuing my Ph.D. degree. Without their regretless contribution and willing sacrifice some of their dreams, I would not have been able to make my dream come true.