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DIFFERENTIAL EFFECTS OF GLUCOCORTICOIDS ON HOST RESISTANCE TO MYCOBACTERIAL INFECTION MEDIATED BY NRAMP1

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
David Herbert Brown, B.S.

*****

The Ohio State University
1996

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ABSTRACT

*Mycobacterium tuberculosis* is responsible for more deaths each year than any other single pathogen. Following a relative quiescence of disease, tuberculosis is undergoing a dramatic resurgence in the United States and throughout the world. A better understanding of both the cellular and molecular interactions between mycobacteria and host immune cells could provide new strategies for prevention and treatment of tuberculosis that extend beyond currently available antimicrobial therapy. The purpose of this study was to investigate the effects of glucocorticoids on the regulation of mycobacterial resistance in BCG resistant (*Bcg'*') and BCG-susceptible (*Bcg'*') congenic mice which differ only in the region on chromosome 1 which contains the gene *Bcg* (now *Nrampl*) which confers resistance to mycobacterial growth. Previous studies in our laboratory have shown that glucocorticoids result in the increased susceptibility of *Bcg'* mice to *in vivo* and *in vitro* growth of *Mycobacterium avium*. In contrast, glucocorticoids did not affect the resistance of *Bcg'* mice or their macrophages to *in vivo* or *in vitro* growth of *Mycobacterium avium*. In addition, we have demonstrated that corticosteroids suppressed the production of TNF-α and of reactive nitrogen intermediates by macrophages from both strains of mice without affecting the resistance of macrophages from *Bcg'* mice to mycobacterial growth. Corticosterone has been shown to suppress both the antimycobacterial activity and expression of *Nrampl* by rIFN-γ
and rGM-CSF activated macrophages from Bcg' mice but not from Bcg' mice. Macrophages from Bcg' mice were resistant to the suppressive effects of corticosterone. Finally, our results indicate that intracellular iron levels are associated with mRNA stability and resistance to mycobacterial growth. Levels of intracellular iron detected in splenic macrophages from Bcg' mice were lower as compared to levels detected in Bcg' macrophages. In addition, chelation of iron in Bcg' macrophages results in abrogation of corticosterone induced decay of Nramp1 expression in Bcg' macrophages. Conversely, iron loading of Bcg' macrophages resulted in unstable Nramp1 mRNA following corticosterone treatment. Together these results suggest that Nramp1 may serve as an iron transport protein that results in the stabilization of effector molecule mRNAs that are required for resistance to mycobacterial infection.
To Karen, who has only known me in this capacity.
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To my committee members, Drs. Whitacre, Sheridan, and Coggeshall, thank you for your guidance and enlightenment and for embarassing me time and time again. I think I may miss the latter most of all. My cortisol levels may finally recede after all.

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A special thanks goes to my parents, whom at one time in my not so glistening college career, must have sincerely believed I would never reach this point. You weren’t alone in that thought. Thank you for your love and support in all my good and bad endeavors.

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PUBLICATIONS

1. B. S. Zwilling, D. Brown, R. Christner, M. Faris, M. Hilburger, M. McPeek, C. Van
Epps and B.A. Hartaub. Differential Effect of Restraint Stress on MHC Class II Expression

2. V. Matkovic, A. Balboa, D. Clinchot, C. Whitacre, B. Zwilling, D. Brown,
S.E.Weisbrode, G. Apseloff,N.Gerber. Gallium Prevents Adjuvant Arthritis in Rats and
Interferes with Macrophage/T Cell Function in Immune Response. *Current Therapeutic Res.*

3. B. S. Zwilling, D. Brown, and D. Pearl. Induction of MHC Class II Glycoproteins

4. W. P. Lafuse, D. Brown and B.S. Zwilling. Assignment of the Microtubule-


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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

The study of the effects of neuroendocrine-immune interactions on microbial pathogenesis and immunity during the course of disease has emerged as a new interdisciplinary research area, termed psychoneuroimmunology. Over the last two decades, studies have found that psychological stress and psychiatric illness can compromise immune function. The historical basis for studying the influence of stress on the immune response stems from early clinical observations that individuals became sick following stressful situations. Benjamin Richardson writes, in Diseases of Modern Life (c. 1882), about diseases arising from excessive mental strain or from mental shock that are found mainly in four classes of the community: (1) persons engaged in art, science, or literature; (2) those engaged in political life; (3) those who are occupied in commerce, exchange, and speculation; and (4) in the too laborious scholars or students (Richardson 1882). Richardson goes on to say that "the diseases induced are limited in number, and, physiologically, hang closely together - links, as it were, of one chain. They all depend primarily upon a deficiency of power or paralysis of the organic nervous system, of that part of the nervous organism which sustains the motion of the heart, the stomach, and digestive system, which governs the secretions, and which, in
a word, ministers to the involuntary and instinctive, as distinguished from the voluntary and intellectual life" (Richardson 1882). Although there exists difficulties associated with the quantitation of stress and its ultimate association with the onset of illness, it is widely accepted that stress can have an impact on susceptibility to several infectious diseases, most notably, tuberculosis. (Collins 1989, Wiegeshaus 1989)

**STRESS INDUCED IMMUNOMODULATION**

The concept of stress is not sharply defined. A stressor can be defined as any sort of external or internal challenge, visual, tactile or emotional, that disrupts the physiological equilibrium or homeostasis of an individual (Ramsey, 1982). Regardless of the nature of the stress, the common mammalian response to these stressors results in: (1) the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis producing adrenocortical secretions followed by consequential increases in serum glucocorticoids and (2) the activation of the sympathetic nervous system (SNS) followed by the release of both tissue and plasma catecholamines (Axelrod 1984, Bateman 1989, Berkenbosch 1991, Gustafsson 1987, Sheridan 1994). SNS activation results in the local release of norepinephrine and epinephrine from chromaffin cells of the adrenal medulla (Sheridan 1994). Norepinephrine and epinephrine, through their α and β-adrenergic receptors, mediate known cardiovascular and metabolic effects under conditions of stress (Gehring 1985, Gustaffson 1987). There is increasing evidence that the SNS plays a role in the modulation of the immune response. Lymphoid tissues are known to receive an extensive intraparenchymal innervation. A wide range of neurotransmitters, via receptors on
these lymphoid tissues, influence lymphocyte and monocyte function in vitro (Madden 1991). Treatment of animals with 6-hydroxydopamine, a drug used to ablate the SNS by destruction of noradrenergic neurons causing a temporary, yet functional axotomy, results in a reduction of primary antibody response to T-dependent antigens and suppression of alloantigen-induced cytotoxic T-lymphocyte (CTL) activity (Hall 1982, Livnat 1985).

Neuroendocrine control of a wide range of immune responses occurs via a host of neuropeptides and neurohormones. A direct effect of these neuroendocrine-derived factors on immune function has been suggested based upon the presence of β-adrenergic receptors on immunocompetent cells. Receptors for these neurohormones have been identified and include: ACTH, vasoactive intestinal peptide (VIP), substance P, somatostatin, prolactin, growth hormone, steroid hormones, a number of hormone releasing factors, and catecholamines (norepinephrine and epinephrine) (Abrass 1985, Fuchs 1988, Madden 1991, Livnat 1985). These receptors are expressed on both T and B lymphocytes, macrophages, neutrophils and natural killer (NK) cells (Fuchs 1988, Galant 1978, Loveland 1981, Motulsky 1982). Any cells possessing these receptors could respond to norepinephrine and epinephrine released during the SNS response to a stressor. The interaction of the neuroendocrine factors and their receptors on immunocompetent cells could alter the cellular activity through the activation of second messengers including cAMP and cGMP (Madden 1991). Some of the direct effects of these catecholamines on cellular immune responses include suppression of lymphocyte migration and proliferation (in response to mitogen), suppression or enhancement (dependent on concentration, target cell and immune function) of cytokine production, NK
activity, antibody synthesis, and macrophage activation including the inhibition of the cytokine induced major histocompatibility complex class II antigen expression on antigen presenting cells (Frohman 1988, Fuchs 1988, Livnat 1985, Madden 1991).

Activation of the HPA axis represents the second physiologic response to stress. The hypothalamus is considered to be the efferent arm of the visceral brain. It receives information from the periphery, integrates it with that of the internal milieu and adjusts important functions, such as sympathetic activity and endocrine secretions. Ultimately, the activation of the HPA axis leads to increases in plasma glucocorticoids. Glucocorticoids exert many different effects, including effects on cardiovascular function, metabolism, muscle function, behavior and the immune system (Baxter and Forsham 1972, Baxter and Harris 1975, Chrousos 1988, Fauci 1976). These effects are grouped into two categories defined as permissive and regulatory. "Permissive" effects of glucocorticoids function to "permit" other hormones or immunological factors to accomplish their function at a normal level. These permissive effects are often observed primarily in the resting state of an individual. The permissive role of the glucocorticoids holds and maintains the homeostasis of the individual at a basal state. "Regulatory" effects of glucocorticoids are exerted normally by stress-induced levels of these hormones. These elevated levels of hormone are thought to be necessary to prevent overreaction of the components of the immune system, which, if unchecked, can lead to tissue injury (Ingle 1954, Munck 1986, Rivier 1983).
The production of glucocorticoids from cells of the zona fasciculata of the adrenal cortex is stimulated by pituitary adrenocorticotropic hormone (ACTH) (Axelrod 1984). Hypothalamic control of ACTH secretion is via the paraventricular nuclei which have projections into the posterior pituitary that are ultimately responsible for controlling the secretion of a number of peptide hormones including, vasopressin, oxytocin, and corticotropin-releasing hormone (CRH) (Johnson 1992, Sawchenko 1982). It has been shown that following stress ACTH release can be inhibited by anti-CRH antiserum. CRH secretion, conversely, is controlled by a sequential process involving catecholamines and $\alpha_1$-adrenoreceptors (Rivier 1982, Vale 1981). It has been demonstrated that the hypothalamic injection of acetylcholine increases the secretion of CRH in portal blood which ultimately stimulates increased production of ACTH (Eipper 1980). In addition to catecholamines, other neuropeptide hormones, such as vasopressin and oxytocin, mediate ACTH release (Eipper 1980). The relationship between the HPA axis and the SNS is bridged here by the fact the glucocorticoids have been shown to be necessary in vivo for normal $\beta$-adrenoreceptor function (Brodie 1966). The threshold for catecholamine receptor stimulation increases dramatically in the absence of glucocorticoids. Steroids, or more precisely, lipocortin, has been shown, in several studies, to potentiate the relaxing effects of $\beta_2$-stimulants (Dvorsky-Gebauer 1976, Townley 1970). Although the exact role of opioid peptides derived from proopiomelanocortin gene expression has yet to be elucidated in the stress response, these CRH induced factors including $\beta$-endorphin and methionine-enkephalin have been implicated in pain control responses and in learned behavior (Krieger 1983).
Stress studies on immune function have led many to believe that adrenal glucocorticoids are the only stress induced biological modifiers of immune response. This misinterpretation is in part due to the well known effects of administration of both natural and synthetic analogs of glucocorticoid hormones. Although the majority of stress studies focus on the immunomodulatory effects of the glucocorticoids, other hormones in addition to corticosterone are altered in response to stress. Growth hormone, gonadotropin, and prolactin are just a few examples of hormones that may mediate immunomodulation by altering antibody synthesis, macrophage activation and IL-2 production (Johnson 1992).

The ability of glucocorticoid hormones to effectively modulate an immune response has been and continues to be widely studied. As mentioned earlier, glucocorticoid production and release from the adrenal cortex is stimulated primarily by ACTH, which in turn is controlled by CRH derived from the hypothalamus in conjunction with catecholamines. The glucocorticoids exert negative feedback on both CRH and ACTH production which in turn results in the ultimate regulation of glucocorticoids themselves (Johnson 1992). Circadian rhythms or "episodic" increases and decreases occur during each day. Persistently high levels of glucocorticoids can result in diseases such as Cushing's syndrome, suggesting that relief from glucocorticoids is needed for "normal" bodily function (Gustaffson 1987). Stress overrides these feedback controls and results in elevated levels of glucocorticoids resulting in either an enhancement or suppression of an organism's defense mechanisms.
Glucocorticoid effects are produced when the hormone, which is assumed to freely penetrate the cell, binds to its receptor to form a non-activated complex (Payvar 1981). Once activated (or "transformed"), this complex, characterized by an enhanced affinity for DNA, forms the nuclear-bound complex, which by binding to regulatory elements associated with certain genes, can activate (and sometimes inhibit) transcription of those genes. Glucocorticoids more than likely have primary and secondary targets. Primary targets (cells) are affected directly by glucocorticoids through the binding of the hormone to its receptor, whereas other cells, the secondary targets, are affected by mediators (cytokines) produced by primary target cells and regulated by glucocorticoids (Munck 1990). Mononuclear cells represent one of the best studied primary target cells of glucocorticoids (Crabtree 1980, Munck 1990, Werb 1978). Mononuclear cells possess high affinity receptors (type II) for glucocorticoids (Miesfeld 1990, Munck 1990). Many elements of the cellular immune response are altered by glucocorticoids (Munck 1990). Antigen processing and presentation by macrophages in association with MHC class II expression is inhibited by glucocorticoids (Zwilling 1992). The proinflammatory cytokines IL-1, IL-6 and TNF-α produced by the activated macrophages are blocked at the transcriptional and post-transcriptional level (Kern 1988, Lee 1988, Northop 1992). The down regulation of these potent mediators of inflammation as a result of stress underlies the immunosuppressive and anti-inflammatory actions of the glucocorticoids. Glucocorticoids also inhibit interleukin-2 (IL-2) gene expression by lymphocytes at the transcriptional level (Lee 1988). Inhibition of interleukin-2 (IL-2) receptor expression also occurs (Northop 1992). Glucocorticoids exert anti-inflammatory effects by increasing synthesis of proteins which inhibit phospholipase A₂.
activity which in turn leads to a further inhibition of the arachidonic acid cascade and platelet activating factor (PAF) synthesis (Fraser 1990, Svedmyr 1990). Glucocorticoids have also been shown to alter lymphocyte trafficking and inhibit T-cell binding to endothelial cells by modulating expression of adhesion molecules, e.g. intracellular adhesion molecule-1 (ICAM-1), on these cells (Eguchi 1992).

In addition to the immunomodulatory effects produced by glucocorticoids, a direct effect of ACTH on several types of immunocompetent cells including mononuclear cells has been observed (Bost 1987, Johnson and Smith 1988, Smith 1987). ACTH, interacting with a receptor found on mononuclear cells, suppresses antibody production and enhances proliferation by B lymphocytes. ACTH has also been shown to inhibit T-lymphocyte IFN-γ production and IFN-γ induced macrophage activation and MHC class II expression (Koff 1985, Johnson and Blalock 1984, Zwilling 1992).

Detection of opioid binding sites present on lymphocytes, polymorphonuclear leukocytes, and platelets raises the possibility that opioids are also immunomodulatory (Ausiello 1984, Merishi 1983). However, the effects of the opioids are not purely suppressive. Enhancement of natural killer (NK) cell function has been noted depending on the particular stressor (Irwin 1988, Shavit 1984). In addition, increased synthesis of IL-2 and IFN-γ by lymphocytes as well as NK cells has been observed as a consequence of in vivo stress-induced release of opioid peptides (Brown 1986, Gilmore 1988, Mandler 1986).
STRESS AND MICROBIAL IMMUNITY

One of the first observations that stressful life events affected pathogenesis of disease was reported by Ishigami, who studied the opsonization of tubercle bacilli among chronically ill tuberculous Japanese school children and their teachers during both active and inactive phases of the disease (Ishigami 1919). Ishigami found decreased phagocytic cell activity during periods of emotional distress and therefore postulated that the stressful school environment of these children and their teachers led to their immunodepressed state and consequently to an increased susceptibility to tuberculosis. This observation has been suggested to be the instigator of the now commonly held belief that certain stressful situations can serve as cofactors in the development of active tuberculosis infections. Until recently, very little solid evidence was available that suggested that stress affected the pathogenesis of certain diseases.

Early in vivo studies on the effect of stress on microbial pathogenesis focused primarily on the modulatory effects of the products of HPA axis activation, or glucocorticoids. Unfortunately, a good number of the studies were conducted with little or no controls and without the experimental certainty that the animals used in these studies were actually being stressed to the point that significant activation of the HPA axis occurred. Without that knowledge, easily determined in present day studies, any effect of stress reported in some of these studies must be noted with some reservation.
Many of the early stress studies were limited in their ability to indicate if HPA axis activation was a factor in modulation of certain infectious diseases. Exogenously injected adrenal cortical extracts served as the immunomodulator (stressor). These studies, conducted primarily on rodents, led to the conclusion that the injection of exogenous glucocorticoids resulted in the animal’s increased susceptibility to the particular pathogen being studied (Table 1.1). The availability of synthetic analogs of glucocorticoids, as well as glucocorticoid agonists and antagonists, have led to the identification of the components of the immune system that are affected by products of HPA axis activation (Table 1.1).

While exogenous glucocorticoids served as the "stressor" in many of the early defining stress experiments, various stress paradigms were used in order to modulate the immune response to certain pathogens. Friedman et. al. (Friedman 1973) showed that novel environment stress (crowding) altered the resistance of mice to malarial infections. Crowding stress, in another study, resulted in an increase in susceptibility of mice to infection with *Salmonella typhimurium* (Edwards 1977). Predator stress (a large cat) reduced the resistance of immune mice to subsequent reinfection by the cestode *Hymenolepis nana* (Hamilton 1974). Green reported that cold stress and hypoxia (oxygen deprivation) resulted in the inhibition of clearance of *Staphylococcus albus* and *Proteus mirabilis* from the lungs of mice (Gross 1984).
Stress effects on resistance in farm animals has been reported by several laboratories. Gross et al. showed that social stress (rotating cage mates) increased the susceptibility of chickens to aerosol challenge with *Escherichia coli* and to challenge with *Mycobacterium*

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>EFFECT OF GLUCOCORTICOIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>increased cellular infiltration, increased bacterial growth</td>
</tr>
<tr>
<td>(Follet 1990, Wentink 1988)</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium paratuberculosis</em></td>
<td>increased susceptibility, Increased shedding</td>
</tr>
<tr>
<td>(Follet 1990, Wentink 1988)</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium leprae</em></td>
<td>alters rIFN-γ efficacy of monocytes</td>
</tr>
<tr>
<td>(Vachula 1991)</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium terrae</em></td>
<td>increases resistance by stabilization of drug interaction</td>
</tr>
<tr>
<td>(Petrini 1989)</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>impairs macrophage function</td>
</tr>
<tr>
<td>(Brown and Zwilling, 1993,1994)</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>increases invasive infection, bacteremia</td>
</tr>
<tr>
<td>(Payne 1955)</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>decreases inflammation-does not affect bacterial clearance</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>exacerbates infection</td>
</tr>
<tr>
<td>(Wong 1990)</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>increases susceptibility</td>
</tr>
<tr>
<td>(Baltch 1986, Jones 1991)</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ameliorates infection (when Infected via IP route)</td>
</tr>
<tr>
<td>(Badenoch 1985)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.1 (continued)

**Bordetella pertussis**  
(Parton 1985)  
promotes initial protection, then promotes microbial survival

**Trichnella spiralis**  
(Coker 1956)  
decreases nematode clearance, cellular infiltration

**Listeria monocytogenes**  
(Schaffner 1985, Pung 1984, Wesley 1989)  
increases susceptibility, impairs monocyte function

**Pneumocystis carinii**  
(Walzer 1979)  
increases susceptibility

**Propionibacterium acnes**  
(Gloor 1978)  
exacerbates infection

**Neisseria gonorrhoeae**  
(Arko 1972)  
increases rate of infection

**Streptococcus faecalis**  
(Gross 1991)  
increases resistance

**Plasmodium berghei**  
(Friedman 1973)  
Decreases parasitemia

**Pneumocystis carinii**  
(Walzer 1979)  
Increases susceptibility

Table 1.1: Effect of glucocorticoids on microbial immunity.

*Avium* (Gross 1984, Gross 1988, Gross and Payeur 1989). Controlled food deprivation (fasting) resulted in the increased susceptibility of chickens to *Salmonella enteritidis* infection and exacerbated the infection (Holt 1993, Holt and Porter 1992). Zamri-saad et al. (Zamri-Saad 1991) found that transportation stress in combination with treatment of dexamethasone
was associated with an increased susceptibility of goats to infection with *Pasteurella haemolytica*. Animals treated with dexamethasone alone, but not subjected to transportation stress, displayed no increase in susceptibility to the *Pasteurellae*. The specific *in vivo* stress effects on microbial pathogenesis of these and other studies are summarized in Table 1.2.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>ANIMAL</th>
<th>STRESSOR</th>
<th>EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. avium</em></td>
<td>mouse</td>
<td>restraint</td>
<td>increases susceptibility</td>
</tr>
<tr>
<td>(Brown 1993)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Chicken</td>
<td>social</td>
<td>increases granuloma formation</td>
</tr>
<tr>
<td>(Gross 1984)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Rabbit</td>
<td>tumbling</td>
<td>increases susceptibility</td>
</tr>
<tr>
<td>(Lockard 1970)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>mouse</td>
<td>electric</td>
<td>increases survival</td>
</tr>
<tr>
<td>(Friedman 1973)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>mouse</td>
<td>crowding</td>
<td>increases susceptibility</td>
</tr>
<tr>
<td>(Edwards 1977)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. enteritidis</em></td>
<td>chicken</td>
<td>fasting</td>
<td>decreases Ab response</td>
</tr>
<tr>
<td>(Holt 1992,1993)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. nana</em></td>
<td>mouse</td>
<td>predator</td>
<td>increases reinfection rate</td>
</tr>
<tr>
<td>(Hamilton 1974)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Chicken</td>
<td>cold</td>
<td>increases resistance</td>
</tr>
<tr>
<td>(Gross 1984, 1988)</td>
<td></td>
<td></td>
<td>increases resistance</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>mouse</td>
<td>cold</td>
<td>inhibits clearance and exacerbates infection</td>
</tr>
<tr>
<td>(Green 1965)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. Albus</em></td>
<td>mouse</td>
<td>cold</td>
<td>inhibits clearance</td>
</tr>
<tr>
<td>(Green 1965)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2. (Continued)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Host</th>
<th>Stress Event</th>
<th>Immunological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>rabbit</td>
<td>tumbling</td>
<td>increases susceptibility</td>
</tr>
<tr>
<td>(Lockard 1970)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. Hemolytica</em></td>
<td>goat</td>
<td>transport</td>
<td>increases susceptibility</td>
</tr>
<tr>
<td>(Zamri-Saad 1991)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2: Effects of stress on microbial immunity.

STRESS AND VIRAL INFECTIONS

Although there has been substantial documentation regarding the association of emotional stress and increased rates and duration of various viral infections in humans, the scope of this chapter must be limited to the effects of stress on microbial (bacterial, parasitic) infections in animal models. However, before dismissing stress-induced effects on viral pathogenesis altogether, it must be noted that experimental animal models are used frequently to study the effects of stress on viral infections. There are similarities between studies of stress on viral infection and studies on the effects of stress in microbial infections. For example, farm animals exposed to social (crowding or novel environment) stress demonstrated increased susceptibility to Newcastle disease virus infection (Mohamed 1980). Transportation stress in cattle resulted in increased susceptibility to an initial challenge with bovine herpesvirus-1 and increased incidence of reactivation of latent bovine herpesvirus-1 infection (Filion 1984, Thiry 1987). Most studies conducted on the effects of stress on viral
immunity, have utilized rodent models. A wide variety of stress paradigms including restraint, cold, foot shock, immobilization and isolation have been shown to differentially affect the pathogenesis of a number of viral infections in these animal models (Ben-Nathan 1990, Bonneau 1991, Brown et al. 1993, Chetverikova 1987, Friedman 1970, Kusnecov 1992, Ozherelkov 1990). Interestingly, the ultimate outcome effects of physical restraint stress on viral pathogenesis (influenza virus and HSV) and microbial pathogenesis (*Mycobacterium avium*) in mice are similar. Recent studies have shown that mice must be subjected to restraint stress, resulting in activation of the HPA axis prior to or concurrently with infection in order to detect a stress induced alteration in pathogenesis (Bonneau 1991, Brown et al. 1993). Infection prior to restraint results in no apparent alteration in the course of the mycobacterial infection or the viral infections.

**MYCOBACTERIAL INFECTION**

Humans have been infected with tuberculosis for thousands of years (Daniel 1994). After a four decade period of decline in incidence of tuberculosis cases reported in the United States, a resurgence in TB has emerged. For the years between 1985 and 1992, active cases of tuberculosis in the United States increased by nearly 20% (Bloom 1992). This resurgence is due in part to infection of individuals already infected with the Human Immunodeficiency Virus as well to other cofactors both demographic and socioeconomic in nature. Tuberculosis remains a global health problem. It is estimated that 1.8 billion persons are infected with *Mycobacterium tuberculosis*, that 8 million people display clinical symptoms, that 7 to 9 million new cases develop annually and that by the year 2005, the World Health
Organization has predicted that 4 million people will die annually as a result of infection with tuberculosis. In addition to the significant increase in incidence of disease, it should be noted that recent reports by the Centers for Disease and Control have determined that of all newly diagnosed cases of tuberculosis, 54% are classified as antibiotic drug resistant. Through the better understanding of both the cellular and molecular interactions between mycobacteria and host immune cells, tuberculosis infection will remain largely curable and perhaps preventable in the future.

The pathogenesis of pulmonary tuberculosis is dependent upon the establishment of a primary lesion upon the inhalation of a droplet nucleus generated by a patient with active tuberculosis (Wiegeshaus 1989). The infectious dose of tuberculosis is extremely low, as one droplet nucleus containing three bacilli has been shown to initiate a primary lesion in the lung (Dannenberg 1993). Once inhaled, less than 10 percent of *M. Tuberculosis* organisms reach the respiratory bronchioles and alveoli; most will settle in the upper respiratory epithelium, where they are likely to be expelled by the mucociliary escalator (Nardell 1993). Bacteria that reach the deep lung are phagocytosed by alveolar macrophages. This event alone can result in the killing of the bacilli. In other cases the tubercle bacilli survive to initiate an infection. Over a period of 2 to 3 weeks, the bacilli replicate intracellularly, eventually resulting in the lysis of their host macrophage (Wiegeshaus 1989, Kaplan 1988). The released mycobacteria are then ingested by newly arrived macrophages. As the cycle is repeated, a primary lesion forms and the bacilli are transported into the draining lymph nodes and eventually into the bloodstream. The asymptomatic bacteremia coincides with the appearance of enhanced
reactivity to the purified protein derivatives of *Mycobacterium tuberculosis* and the onset of cell mediated immunity (Wiegeshaus 1989, Taylor 1990). As the tubercle laden macrophages disperse the bacilli into the body, the bacilli are effectively trapped in the tissues and create metastatic foci. These granulomatous focal lesions are composed of macrophage-derived epithelioid giant cells and lymphocytes. T lymphocytes activated by macrophage derived cytokines migrate to the foci resulting in the further local activation of macrophages and lead to caseation necrosis which results in the gradual sterilization of most metastatic lesions. Exceptions to the sterilization process include those lesions in the apical-subapical regions of the lung as well as the bone marrow, kidney and meninges. In these areas, the caseaous necrosis decreases the amount of bacilli to a low steady state level. In most healthy individuals with intact immune function, organisms contained within these foci can remain dormant for decades. The relative strength of the host’s cellular mediated immunity ultimately determines if reactivation of disease will occur. In 5 to 10 percent of infected individuals, a temporary suppression of the cellular arm of the immune system allows the reactivation of the disease and consequently the resumption of an uninhibited multiplication of the bacilli leading to tissue destruction, systemic dissemination of disease and, when left untreated, death.

The role of the macrophage in controlling mycobacterial infection has been widely studied. For the most part, the killing of mycobacteria occurs within the phagolysosomes of the macrophage. Contained within these structures are toxic constituents including lysosomal hydrolases, reactive oxygen intermediates such as hydrogen peroxide and superoxides, and
reactive nitrogen intermediates (RNI) including nitrites, nitrates and nitric oxide (NO). RNI production by murine macrophages is an important effector mechanism against a host of pathogenic organisms. Many of the biostatic and cytostatic activities of murine macrophages are thought to be dependent upon the formation of RNI such as NO derived from the enzymatic oxidation of the guanidino group of L-arginine controlled by an inducible nitric oxide synthase (iNOS) (Green 1991, Nathan and Hibbs 1991). Many of the biological effects of NO are chemically based on direct interactions with iron-containing proteins, such as guanylyl cyclase (heme iron), ribonucleotide reductase (non-heme iron) or aconitase (iron sulfur) (Drapier and Hibbs 1988, Weinberg 1992). Cytokines are powerful modulators of murine macrophage RNI synthesis. rIFN-γ and TNF-α are potent activators of iNOS while IL-4 and IL-10 serve as effective suppressors of iNOS (Gazzinelli 1992, Ozwald 1992). Several studies regarding growth inhibition of mycobacteria by cytokine stimulated murine macrophages correlate with the production of TNF-α dependent RNI (Chan and Bloom 1992, Chan and Bloom 1995, Denis 1991, Flesch and Kaufman 1991). rIFN-γ deficient mice infected with *M. tuberculosis* are unable to restrict the growth of the organisms. These mice develop granulomas but do not produce RNI. Treatment of mice infected with BCG or *M. Tuberculosis* with a neutralizing antibody directed against TNF-α was originally shown to block granuloma formation and enhance bacterial growth (Denis 1991, Kindler 1989). Additional studies demonstrated that transgenic mice lacking the 55-kDa TNF-α receptor were unable to clear an *M. Tuberculosis* infection. The mice were able to produce granulomas, but in a delayed manner. In addition, the granulomas lacked epithelioid cells and contained unusually large numbers of *M. Tuberculosis* (Flynn 1995). Nonetheless, the role
of RNI in resistance to mycobacterial infection remains unclear. Other cytokines in addition to rIFN-γ and TNF-α that have been determined to activate murine macrophages to kill mycobacteria include IL-12 and recombinant granulocyte macrophage-colony stimulating factor (rGM-CSF) both of which fail to induce the production of RNI (Blanchard 1991, Denis and Ghardirian 1991, Gazinelli 1992, Zhang 1994).

It has long been postulated that resistance or susceptibility of a host to Mycobacterial infection is under genetic control. Epidemiological studies reviewed by Buschman (Buschman 1990) first confirmed this as fact. Later studies showed that monozygotic twins exhibited a similar susceptibility to infection (Comstock 1978). Cosegregation of known chromosomal markers associated with the disease in addition to evidence that susceptibility to infection corresponded to racial differences further substantiated these findings (Stead 1990, Crowle 1990). Animal models of mycobacterial infections also reflected the involvement of the genetic makeup of the host in resistance or susceptibility to infection. The first animal studies demonstrating differential responses to identical doses of *M. Tuberculosis* were carried out in inbred rabbit strains (Buschman 1990, Skamene 1989). As opposed to the genetically susceptible rabbits, the genetically resistant rabbits were capable of sequestering tubercle bacilli in the macrophages thus inactivating the pathogens. The results of these early studies served to delineate the two phases of the host response to mycobacterial infections. Inbred strains of mice segregated into two non-overlapping groups upon intravenous infection with $10^4$ CFU of *M. Bovis*. Typically, infection of inbred mouse strains with low dose inoculums of nonpathogenic *M. Bovis* (BCG) progresses in two distinct phases: an early nonimmune
phase (0 to 3 weeks) characterized by either rapid proliferation of the bacteria in
reticuloendothelial organs (liver, spleen) of susceptible strains ($Bcg'$) or absence of bacterial
growth in resistant strains ($Bcg'$), and a late phase (3 to 6 weeks) associated with the
development of specific immunity (Gros 1981, 1983).

Similar strain variations in innate resistance to mycobacterial infections were identified
in mice (Gros 1981) based upon their varied survival time following infection with $M.\ Tuberculosis$ as well. No intermediate variations were observed in the segregation of the
two groups suggesting that resistance to mycobacterial infection was under the control of a
single gene. Extensive Mendelian analyses of crosses between resistant and susceptible strains
revealed that the trait of innate resistance to $Mycobacterium bovis$ infections was controlled
by a single dominant, autosomal gene designated as the $Bcg$ gene (Lynch 1965, Forget
1981). The strain distribution pattern of the $Bcg'$ and the $Bcg'$ alleles segregated with two
isoenzymes, the isocitrate dehydrogenase ($Idh-1$) and the dipeptidase 3 ($Pep-3$) located on
the centromeric portion of murine chromosome 1. Recombination frequencies established the
gene order as $Idh-1, Bcg, Pep-3$ (Gros 1981, Skamene 1982). The creation of congenic
strains of mice that differ only in the defined portion of chromosome 1 that carries the $Bcg$
gene confirmed these results (Potter 1981) and served to further map and determine the gene
order of five cloned genes in the vicinity of the $Bcg$ gene (Schurr 1989a). The $Bcg$ gene is
located in a conserved linkage group consisting mainly of a cluster of genes encoding
structural extracellular matrix and cytoskeletal proteins. Restriction fragment length
polymorphism (RFLP) analysis revealed the presence of a homologous linkage group on the
telomeric end of the long arm of chromosome 2 (2q) in humans (Schurr 1989b, 1990). Therefore, in humans genetic susceptibility to mycobacteria could be controlled by a homolog of the murine \( B_{cg} \) gene (Schurr 1990).

Subsequent mapping of the \( B_{cg} \) gene to the proximal portion of murine chromosome 1 revealed that the same locus is also responsible for the innate resistance to infection by \( Salmonella typhimurium \) and \( Leishmania donovani \) (Plant 1982). This association suggested that the \( B_{cg} \) gene was identical to or closely linked to the \( Ity/Lsh \) gene. The \( B_{cg} \) gene was also found to control resistance to \( Mycobacterium lepraemurium, Mycobacterium intracellulare, \) and \( Mycobacterium smegmatis \) (Skamene 1984, Goto 1989, Buschman 1990).

The cellular source expressing the \( B_{cg}/Ity/Lsh \) gene during the beginning innate stages of infection was identified as mature tissue macrophages (Gros 1983, Lissner 1985). The resistant or susceptible phenotype was expressed \textit{in vivo} in the absence of functional T lymphocytes. Depletion of T cells, B cells and NK cells from resistant mice did not alter their ability to control Mycobacterial infections indicating that innate resistance is not associated with acquired immunity (Gros 1983). The resistance mechanism was suggested to be due to the superior antimicrobial ability of macrophages from \( B_{cg}^{+} \) mice as observed by the ability to inhibit the incorporation of \(^{3}H\)-uracil by intracellular bacilli. Skamene suggested that the superior effector function of macrophages from \( B_{cg}^{+} \) mice was due to the fact that these macrophages are at a more advanced level of activation as compared to macrophages from \( B_{cg}^{-} \) mice. \( B_{cg}^{+} \) macrophages appear superior to \( B_{cg}^{-} \) macrophages in the production of
toxic oxygen, nitrogen radicals, and TNF-α in response to secondary stimuli such as interferon-γ and BCG infection, both in vivo and in vitro, and in the expression of surface markers associated with the state of activation (Ia and the macrophage activation marker, AcM.1) (Buschman 1989). According to this theory, the likely candidate for the Bcg gene product would be a protein involved in the regulation of macrophage priming for activation (Skamene 1989).

Because of numerous functional differences associated with the Bcg locus and in the absence of a known protein product, Skamene et al. used a positional cloning approach to isolate the Bcg gene. With the use of 67 polymorphic markers (cDNA probes) corresponding to known genes, anonymous DNA probes derived from a microdissected chromosome 1 library, and novel DNA markers from the region obtained by chromosome walking, the isolation of the Bcg gene was identified solely on the basis of its chromosomal location. Using the markers D1Mcg105 and D1Mcg136 the genetic interval carrying Bcg was estimated at 0.3 cM. The interval included the two tightly linked genes; cytoskeleton protein villin (Vil) and the microdissected probe D1Mcg165. These markers were used to isolate 400kb of the Bcg gene region in two overlapping YAC clones. The YAC clones were used to create and order a contiguous series of cosmid and bacteriophage genomic clones of the region (20 to 40 kb each). Exon amplification techniques identified full length cDNAs corresponding to transcription units corresponding to candidate genes for Bcg within the cloned domain. This analysis led to the identification of six novel genes (Vidal 1993). One of the genes was found to encode a 2.4 kb mRNA expressed exclusively in the
reticuloendothelial organs (spleen and liver). The expression of this gene was greatly enriched in mature tissue macrophages isolated from spleen and the macrophage cell line J774A.1 (Vidal 1993). This gene was designated as *Nramp* (later *Nramp1*) (Gruenheid 1995) when an associated family was discovered for natural resistance associated macrophage protein. Nucleotide and predicted amino acid sequence analysis revealed that *Nramp1* encodes a novel protein with features associated with integral membrane proteins. Hydropathy plot analysis identified a minimum of 10 and a maximum of 12 putative membrane spanning domains. *Nramp1* shares an ancestral relationship with several prokaryotic periplasmic transport proteins through the presence of a consensus transport motif known as the binding protein dependent inner membrane component transport signature. This motif was also detected in a few eukaryotic membrane proteins, including Crna, a nitrate/nitrite transporter of *Aspergillus nidulans* (Vidal 1993).

Vidal et al. analyzed 27 inbred mouse strains for nucleotide sequence variations within the coding portion of *Nramp1* to detect association between sequence variants of *Nramp1* polypeptide and the *Bcg* allele. Haplotype mapping studies and sequence analysis revealed that a single glycine to aspartic acid non-conservative substitution at position 169 within transmembrane region 4 was associated with susceptibility to mycobacterial infection (Vidal 1993, Malo 1994). Vidal et al demonstrated via a mutant mouse carrying a null allele at the *Nramp1* locus (*Nramp1*') on the *Bcg* genetic background that *Nramp1* and *Bcg* were the same gene (1993).
CHAPTER 2

REGULATION OF MYCOBACTERIAL GROWTH BY THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS: DIFFERENTIAL RESPONSES OF *Mycobacterium bovis* BCG-RESISTANT AND SUSCEPTIBLE MICE

INTRODUCTION

The incidence of Mycobacterial diseases has increased dramatically in the United States during the past 10 years (Bloch 1989, Kochi 1991). An increase in *Mycobacterium avium* infections has occurred largely because of infection by the human immunodeficiency virus (HIV). While approximately 50% of the increased incidence in *Mycobacterium tuberculosis* can also be attributed to HIV infection, infection of individuals who are not infected with HIV, has also increased significantly. The susceptibility of man to Mycobacterial disease is determined, in part, by genetic differences (Crowle 1990, Schurr 1989, Stead 1990, 1992) but is also affected by their environment. Homelessness and malnutrition (Bloom 1992, Jones 1954) are two cofactors that can contribute to the likelihood of Mycobacterial infection. Additional factors such as ageing, chronic alcoholism and stress have also been cited as being associated with activation of Mycobacterial disease (Collins 1989, Feingold 1976, Hodolin 1975, Ishigami 1919, Nagami 1983, Orme 1988, Pincock 1964, Powell 1980, Wiegeshaus 1989).
In mice the genetic resistance to the \textit{in vivo} growth of Mycobacteria has been shown to be controlled by a gene, termed \textit{Bcg}, which maps to chromosome 1 (Schurr 1989). A syntenic group of genes maps to human chromosome 2q in man (Schurr 1990). We have reported that macrophages from congenic \textit{Bcg}^\text{a} and \textit{Bcg}^\text{b} mice express MHC class II glycoproteins differently. Thus, macrophages from \textit{Bcg}^\text{a} mice can be induced to persistently express I-A while macrophages from \textit{Bcg}^\text{b} mice will only transiently express I-A (Johnson 1985, Zwilling 1990). In subsequent studies we reported that I-A expression by macrophages from the \textit{Bcg}^\text{a} mice was suppressed by activation of the hypothalamic-pituitary-adrenal (HPA) axis as a result of a stressor, physical restraint (Zwilling 1991, Zwilling 1992). In contrast I-A expression by macrophages from the \textit{Bcg}^\text{b} mice was not affected by HPA axis activation. Together, these observations suggested that Mycobacterial growth that is controlled by \textit{Bcg} may also be differentially affected by HPA axis activation.

The purpose of this investigation was to explore the role of the HPA axis in regulating the growth of \textit{Mycobacterium avium} in congenic \textit{Bcg}^\text{a} and \textit{Bcg}^\text{b} mice. We found that activation of the HPA axis by restraint increases the susceptibility of \textit{Bcg}^\text{a} mice to Mycobacterial growth but did not affect the ability of \textit{Bcg}^\text{b} mice to limit the growth of the Mycobacteria. The suppressive effects of HPA axis activation also resulted in an increased susceptibility to Mycobacterial growth within macrophages from the \textit{Bcg}^\text{a} mice. The effect of HPA axis activation on the \textit{in vivo} growth of the Mycobacteria was abrogated by adrenalectomy and by treatment of mice with the glucocorticoid receptor antagonist RU486.
Activation of the HPA axis resulted in a suppression of TNF-α and reactive nitrogen intermediates produced by macrophages from both Bcg' and Bcg" mice.

MATERIALS AND METHODS

Animals. Male BALB/c.Bcg' mice were obtained at six weeks of age from Harlan-Sprague-Dawley (Indianapolis, IN). The mice were housed in groups of five in PCS-80 laminar flow air isolation cages (Lab Products, Inc., Maywood, NJ). The mice were given food and water ad libitum and acclimated to their housing environment prior to the initiation of all experiments. Adrenalectomized and sham adrenalectomized mice were also obtained from Harlan-Sprague-Dawley. Adrenalectomized mice were then maintained on 1% saline in drinking water. BALB/c.Bcg' mice were provided by Dr. Michael Potter (NCI) (Potter 1983) and bred in our animal facility. Male mice were used at 6-8 weeks of age.

HPA activation via restraint stress. To activate the HPA axis mice were restrained as described by Zwilling et al. (Zwilling 1992). Briefly, mice were placed individually into 50-ml conical centrifuge tubes which were punctured to allow for ventilation and to prevent hyperthermia. The tubes allow for limited forward and backward movement. The mice were maintained horizontally in tubes for single or multiple 18 hour restraint cycles. Mice were restrained prior to the initiation of the dark (6 p.m.) phase of the 12 hour day/night cycle and removed the following morning at the conclusion of the 18 hour stress period. Following 1 or 5, 18 hr restraint cycles, the mice were returned to their standard housing and then sacrificed 12 days after the initiation of the experiment. The mice that received 10 restraint
cycles were restrained initially for 5 consecutive cycles, returned to their standard housing for 2 days, and then restrained again for another 5 consecutive cycles. The mice were then sacrificed immediately after the last restraint on day 12. Control, transported mice were housed in groups of five and deprived of both food and water during periods in which the experimental mice were being restrained. Conventionally housed mice served as an additional control.

**Administration of RU-486 (Mifepristone).** The glucocorticoid receptor antagonist RU-486 (Philbert 1984) was kindly provided by Roussel-Uclaf (Romainville, France). RU-486 was dissolved in 400 mw polyethylene glycol and injected subcutaneously at a dose of 25 mg/kg, daily, beginning at 2 days prior to the initiation of restraint and continuing throughout the entire restraint period. Control mice were injected with equal volumes of the vehicle.

**Hormone Replacement Therapy.** Time release pellets (Innovative Research of America., Toledo, OH) were aseptically inserted through a 5mm incision on the back of the animal. The incision was treated with topical antibiotics and sutured. To achieve basal hormone levels, pellets containing either 0.001 mg of epinephrine, 5ug of d-aldosterone and 0.5 mg of corticosterone were implanted (Green 1966). To achieve corticosterone levels consistent with those found following HPA axis activation, pellets containing 15 mg of corticosterone were implanted together with those containing basal epinephrine and d-aldosterone. Placebo pellets of cellulose were implanted in control mice. Mice were allowed to recover for 48 hours before infection with *Mycobacterium avium*. 
Plasma Corticosterone and ACTH determination. Plasma samples were obtained at the time of sacrifice from blood anticoagulated with 5% EDTA. The concentration of corticosterone and ACTH was determined on fresh plasma by radioimmunoassay using an assay kit obtained from ICN Biomedical (Irvine, CA) as per the manufacturer’s instructions. The amount of corticosterone or ACTH was calculated from a standard curve and expressed as nanograms (corticosterone) or picograms (ACTH) per ml.

Assessment of in vivo Mycobacterial growth. A clinical isolate of *Mycobacterium avium* (Zwilling 1991) was initially grown on Lowenstein Jensen medium and transferred to Middlebrook 7H9 broth seed cultures. The Mycobacteria were grown to a density of 5.5 X 10⁸ colony forming units (cfu) per ml of Middlebrook 7H9 broth and stored frozen at -70 °C in 1 ml vials until use. The Mycobacteria were diluted to 5 X 10⁴ cfu in 0.2 ml sterile pyrogen free saline and injected intravenously via the tail vein. To determine the number of cfu contained within the spleen and lungs of mice, the organs were aseptically removed and homogenized using tissue sieves (Sigma Chemical Co., St. Louis). Suspensions were serially diluted into sterile pyrogen free saline and plated onto Mycobacteria 7H11 agar supplemented with Middlebrook OADC Enrichment (Difco Laboratories, Detroit, MI). Plates were incubated for 2 weeks at 37°C in an atmosphere containing 10% CO₂ in air. Colony forming units were determined and expressed as cfu/gm tissue/gm body weight.

In vitro anti-Mycobacterial activity. The antimycobacterial activity of macrophages was assessed as described by Flesch and Kaufman (Flesch 1991). Splenic macrophages were
obtained from pooled spleens, passed through tissue sieves into Hanks' Balanced Salt Solution (HBSS) (GIBCO, Grand Island, NY) supplemented with 20% Defined Fetal Bovine Serum (FBS, Hyclone Laboratories, Logan, UT) and subsequently passed through sterile needles with successively decreasing bore size in order to achieve a single cell suspension. The cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO/BRL, Grand Island, NY) supplemented with 10% FBS and glutamine but without antibiotics. The splenic macrophages from this suspension were then enriched by adherence onto 100 X 20 mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) by culturing the cells at 37° C overnight in an atmosphere containing 10% CO₂. Non-adherent cells were removed by gentle washing with HBSS and adherent cells were removed by scraping with a rubber policeman. The cells were cultured again at a concentration of 1 X 10⁵ macrophages per well in a 96 well microtiter plate (Becton Dickinson Labware) and incubated overnight. Cultures were again washed with HBSS to remove remaining non-adherent cells. Purified macrophage cultures were then infected with 4 X 10⁵ cfu of M. avium (4:1 ratio of bacteria to macrophage) suspended in 0.2 ml of IMDM without antibiotics and incubated overnight (16 hours) at 37° C to allow for phagocytosis. Following incubation, the cultures were washed with fresh IMDM to remove any uningested bacteria. The infected macrophage cultures were then incubated in IMDM without serum for a period of 5 days to allow intracellular growth of the ingested bacteria. At the end of this incubation period the cultures were lysed and pulsed with media containing a mixture (1:1) of 7H9 medium and IMDM with [³H]uracil (5uCi per milliliter) (Amersham, Chicago, IL., USA, spec. act. 40-60 Ci/mmol) and 0.1% saponin. The bacteria were incubated overnight and were harvested onto glass fiber filter
strips using a PHD Cell Harvester (Cambridge Technology, Inc., Watertown, MA). Radioactivity incorporated by the released bacteria was quantitated by liquid scintillation spectrometry.

**TNF-α and reactive nitrogen intermediate production.** Purified splenic macrophages at a concentration of $1 \times 10^5$ macrophage per well in 96 well microtiter plates were stimulated with 100 ng of lipopolysaccharide (*E. coli* 0111:B4, Sigma) and 100 U recombinant interferon-γ (GIBCO/BRL) for 72 hours at 37° C. Cell free supernatants were then used to determine TNF-α and reactive nitrogen intermediates. TNF-α production was determined by ELISA (Endogen Inc., Boston, MA) and reactive nitrogen intermediates (nitric oxide as indicated by the presence of the stable intermediate $\left[ NO_2^- \right]$ nitrite) was determined using the Griess reagent (Green 1982). The amount of TNF-α and nitric oxide (nitrite) were calculated using a standard curve and expressed as picograms per ml (TNF-α) or μM (nitric oxide).

**Statistical analysis.** Each observation of the Mycobacterial counts in the *in vivo* experiments was made on an independent animal allowing the data to be analyzed using Analysis of Variance models (generally two-factor models). The analysis was done on a log scale in order to better satisfy the normality and homoscedasticity assumptions and an examination of residual plots did not contradict the validity of the methods used. Finally, reports of the pairwise comparison of treatment groups were made using the Least Significant Difference Post-Hoc test. Plasma ACTH levels in the *in vivo* experiments as well as the nitric oxide and TNF-α measurements on the independent replications in the *in vitro* experiments.
were similarly analyzed except on the absolute scale. Plasma corticosterone levels in the adrenalectomized mice with placebo or low dose coticosterone implants were all lower than the assay limit of 25 ng/ml. These data were conservatively analyzed using the worse-case value (i.e. at worst our declarations of significance are understated).

RESULTS

The susceptibility to Mycobacterial growth of Bcg mice to HPA axis activation by restraint correlated with its duration (Figure 2.1). HPA axis activation for 5 or 10 daily 18 hr cycles of restraint resulted in a significant (p<0.001) increase in Mycobacterial growth (Figure 2.1a). The colony forming units isolated from the spleens of mice restrained for 10 cycles was 138,000 cfu/gm spleen/gm body weight. In contrast, only 72,000 cfu/gm spleen/gm body weight were isolated from control mice. Similar observations were made following enumeration of mycobacterial growth in the lung (Figure 2.1b).

Differential Effects of HPA Axis Activation on Congenic BALB/c.Bcg and BALB/c.Bcg mice. When we compared the effect of HPA axis activation on Mycobacterial growth in Bcg and Bcg mice, we found that restraint resulted in a significant increase (p<0.001) in the number of Mycobacterial cfu isolated from the spleens of Bcg mice but did not affect the growth of the microorganisms in the spleens of Bcg mice (Figure 2.2). Thus, the Mycobacteria isolated from the spleen of Bcg mice increased from 100,000 cfu in control
Figure 2.1: Susceptibility to Mycobacterial Growth is Increased as a Result of HPA Axis Activation. Mice were restrained for 1, 5, or 10, 18 hr cycles after infection with $5 \times 10^4$ cfu of *M. avium*. The number of cfu in the spleen (a) and lungs (b) were determined 12 days after injection of the Mycobacteria. The number of cfu isolated from the spleen and lungs prior to restraint was 13,519 cfu/gm spleen/gm body wt and 584 cfu/gm lung/gm body wt. respectively. The data represent the mean ± SD of 7 animals per group. The differences between 5X and 10X restraint and control was significant by ANOVA, $p<0.001$. 
Figure 2.1
Figure 2.2: Differential Effect of HPA Axis Activation on Mycobacterial Resistance of BALB/c.Bcg' and BALB/c.Bcg Mice. Mice were restrained for 10, 18hr cycles and the cfu of *M. avium* in the spleen determined 12 days after the infection. The number of cfu isolated from the spleen of *Bcg' or Bcg* mice prior to restraint was 19,462 cfu/gm spleen/gm body wt and 18,083 cfu/gm spleen/gm body wt. respectively. The data represent the mean ± SD of 7 animals per group. The difference between the restraint and control groups for *Bcg' mice was significant at p<0.001. The differences between the growth of the mycobacteria in the spleens of *Bcg' vs Bcg* mice was also significant at p<0.001. Similar observations were made concerning difference in the effect of HPA activation for growth of the Mycobacteria in the lungs (data not shown).
Figure 2.2
mice to more than 175,000 cfu following restraint. In contrast, the number of cfu isolated from the spleens of \textit{Bcg}' mice remained at about 40,000. The data in Figure 2.2 also shows that \textit{Bcg}' mice were more permissive for Mycobacterial growth than were \textit{Bcg} mice.

**The Effect of Adrenalectomy on Mycobacterial Growth in BALB/c.\textit{Bcg}' Mice.** In order to more directly evaluate the role of the HPA axis in the restraint mediated increase in susceptibility to Mycobacterial growth, adrenalectomized BALB/c.\textit{Bcg}' mice were used. The results in Figure 2.3 shows that adrenalectomy abrogated the effect of restraint on the \textit{in vivo} growth of the Mycobacteria. An increased number of Mycobacteria were isolated from the spleens and lungs of unoperated and sham adrenalectomized mice. The number of \textit{M. avium} isolated from the spleens and lungs of adrenalectomized mice, restrained mice, receiving basal levels of adrenal hormones or placebo was not greater than that isolated from the spleens of control mice. In contrast, increased numbers of \textit{M. avium} were isolated from the spleens of adrenalectomized mice that received basal level replacement of d-aldosterone and epinephrine but with elevated levels of corticosterone. The results in Figure 2.4a shows that adrenalectomy resulted in decreased levels of corticosterone in the plasma of restrained mice compared to control and sham adrenalectomized mice. In contrast the levels of ACTH were suppressed in the mice with high levels of corticosterone (Figure 2.4b) and were elevated in the plasma of mice with low levels of corticosterone. The use of time release pellets that yielded a high level of corticosterone resulted in low levels of ACTH and increased the susceptibility to Mycobacterial growth (Figure 2.3). Implantation of time release pellets containing high levels of corticosterone into \textit{Bcg}' mice failed to alter the growth pattern of
Figure 2.3: Adrenalectomy Abrogates the Effect of HPA Axis Activation on Mycobacterial Susceptibility of Bcg' Mice. Adrenalectomized mice received time release pellets to provide basal levels of d-aldosterone, epinephrine and basal or activated levels of corticosterone or placebo pellets 2 days prior to initiation of the experiment. The mice were infected with 5x10^4 cfu M. avium and then restrained for 10, 18hr cycles. The number of cfu in the spleen (a) and lung (b) was determined 12 days after initiation of the experiment. The number of cfu isolated from the spleens of adrenalectomized or untreated mice prior to restraint was 32,081 cfu/gm spleen/gm body wt and 33,484 cfu/gm spleen/gm body wt respectively. The number of cfu from the lung of adrenalectomized mice was 782 cfu/gm lung/gm body wt and from the lung of untreated mice was 807 cfu/gm lung/gm body wt. The data represent the mean ± SD of 7 animals per group. The effect of HPA activation was significant at p<0.001; the effect of restraint in adrenalectomized mice was not significant, i.e. adrenalectomy abrogated the effect of restraint; the effect of implantation of time release pellets containing high doses of corticosterone on Mycobacterial growth was significant at p<0.001.
Figure 2.3
Figure 2.4: Effect of Adrenalectomy and Restraint on Plasma Corticosterone and ACTH.

Mice were treated as described for Figure 3. The plasma was obtained from mice at the time of sacrifice and the levels of Corticosterone (A) and of ACTH (B) were determined by radioimmunoassay. Plasma Corticosterone levels are expressed as nanograms/ml and ACTH levels as picograms/ml.
Figure 2.4
the Mycobacteria. Thus, despite the high levels of corticosterone in the plasma of these mice, no difference in the growth of the Mycobacteria between mice receiving pellets containing high levels of corticosterone and those receiving placebo pellets was observed (data not shown).

The Glucocorticoid Receptor Antagonist RU486 Abrogates the Effect of Restraint Mediated HPA Activation. To directly implicate the high levels of corticosterone, that resulted from activation of the HPA axis, mice were injected with the glucocorticoid receptor antagonist RU486. The results in Figure 2.5 show that RU486 abrogated the increased susceptibility to Mycobacterial growth that occurred as a result of HPA axis activation. Thus, while 158,626 cfu were isolated from the spleens of carrier injected mice, only 76,456 cfu were isolated from the spleens of RU486 treated mice. This value did not differ from that obtained from the spleens of control mice.

HPA Axis Activation Increases Susceptibility of Splenic Macrophages to Mycobacterial Growth. The macrophage is the major effector cell that controls the growth of the Mycobacteria. HPA activation also resulted in a differential effect on macrophages from Bcg⁺ and Bcg⁻ mice. The results in Figure 2.6 shows that activation of the HPA axis by restraint resulted in a significant increase (p<0.001) in Mycobacterial growth in macrophages from BALB/c.Bcg⁺ mice. In contrast the growth of M. avium in macrophages from BALB/c.Bcg⁺ mice was not affected by HPA axis activation.
Figure 2.5: The Glucocorticoid Receptor Antagonist RU486 Abolishes the Suppressive Effect of HPA Axis Activation. Mice were treated with 25 mg/kg of RU486 in 400 mw polyethylene glycol or with carrier only, for 2 days prior to HPA axis activation and daily for the duration of the experiment. The mice were infected with $5 \times 10^4$ cfu *M. avium* and restrained for 10, 18hr cycles. The number of cfu in the spleen was determined 12 days after the initiation of the experiment. The cfu in the spleen prior to HPA axis activation was 20,792 cfu/gm spleen/gm body wt. Data represent the mean ± SD of 7 animals per group. The effect of RU486 was significant at p<0.001.
Figure 2.5
Figure 2.6: Differential Effect of HPA Axis Activation of Anti-Mycobacterial Activity of Macrophages from BALB/c \( Bcg' \) and BALB/c \( Bcg' \) Mice. Splenic macrophages were isolated from mice and infected with \( M. avium \). The growth of the Mycobacteria was determined by pulsing cultures with \(^3\)H-uracil after lysis of the macrophages with saponin. Infected cultures were pulsed immediately following a period of phagocytosis in order to determine the numbers of microorganisms taken up initially. Replicate cultures were pulsed after 5 days of growth within macrophages. The cpm of \(^3\)H-uracil taken up by the \( M. avium \) following release from the \( Bcg' \) macrophages immediately after phagocytosis was 574 cpm, while that taken up by \( M. avium \) following release from \( Bcg' \) macrophages was 523 cpm and did not differ as a result of HPA axis activation. The \( M. avium \) released from macrophages immediately after phagocytosis incorporated 45,334 cpm \(^3\)H-uracil when pulsed after 5 days of growth and did not differ between those released from macrophages from \( Bcg' \) or \( Bcg' \) mice. The data represent the cpm \(^3\)H-uracil taken up by the bacteria released from macrophages after 5 days of in vitro culture. The data is from a representative experiment. The effect of HPA activation is significant at \( p<0.001 \).
Figure 2.6
Figure 2.7: HPA Axis Activation Diminishes TNF-α Production by Macrophages from BALB/c.Bcg' and BALB/c.Bcg Mice. Splenic macrophages were obtained from mice following 10, 18hr restraint cycles. The macrophages were stimulated with 100 U rIFN-γ and 100 ng LPS for 72 hrs. The amount of TNF-α produced by the macrophages was determined using cell free supernatant by ELISA. Stimulation with LPS alone resulted in the production of 1838±364 pg/ml of TNF-α by macrophages from Bcg' mice and 1798±26 pg/ml by macrophages from Bcg' mice and was not statistically different. Activation of the HPA axis reduced TNF-α production to 1356±164 pg/ml and 1035±42 pg/ml respectively. Treatment with rIFN-γ induced the production of 110 pg/ml of TNF-α and which did not differ between macrophages from Bcg' or Bcg' mice and was not reduced as a result HPA axis activation. The data represent the mean ± SD of 3 separate determinations. The effect of HPA activation is significant at p<0.002.
Figure 2.7

- **Bcg**: Control vs. 10XRestraint
- **Bcg**: Control vs. 10XRestraint

**X-axis**: TNF-alpha pg/ml

**Y-axis**: Sample types

- Control
- 10XRestraint
Figure 2.8: HPA Axis Activation Suppresses the Production of Reactive Nitrogen Intermediates by Macrophages from BALB/c.Bcg' and BALB/c.Bcg Mice. Supernatants from LPS/rIFN-γ activated macrophages were obtained as described for Figure 7 and the production of reactive nitrogen intermediates determined using the Griess reagent (Green 1982). Stimulation of the cultures with LPS alone or rIFN-γ alone resulted in the production of less than 10 μM of NO₂⁻ which did not differ between macrophage from Bcg' or Bcg mice. The addition of N⁰-monomethyl l-arginine to the cultures completely abolished the production of reactive nitrogen intermediates. The data represent the mean ± SD of 5 separate determinations. The effect of HPA activation was significant at p<0.001.
Figure 2.8
Macrophage TNF-α and NO Production is Suppressed by HPA Activation. An antimicrobial effector pathway that has been shown to be important in the control of Mycobacterial growth in mice is the IFN-γ dependent, TNF-α induced production of reactive nitrogen intermediates (9,18). Activation of the HPA axis by restraint resulted in a suppression of TNF-α production following stimulation of splenic macrophages from restrained Bcg¹ and Bcg² mice with rIFN-γ and LPS (Figure 2.7). TNF-α was reduced from 1798 pg/ml produced by macrophages from Bcg² control mice to 1035 pg/ml produced by the splenic macrophages following activation of the HPA axis (p<0.002). The effect of HPA axis activation on TNF-α production by macrophages from Bcg¹ mice was less than that observed for macrophages from Bcg² mice. NO production was also reduced from 40 uM to 16 uM following restraint (Figure 2.8). HPA axis activation also resulted in the decreased production of TNF-α and of reactive nitrogen intermediates by macrophages from the Bcg¹ mice.

DISCUSSION

The results of this investigation show that activation of the HPA axis can increase the susceptibility of mice to Mycobacterial growth. This effect was limited to the BCG susceptible population. The increased susceptibility in genetically susceptible mice was directly proportional to the duration of HPA axis activation. Thus multiple restraint experiences were required to increase the susceptibility of the Bcg¹ mice. In contrast, we have previously reported that a single restraint experience was sufficient to suppress MHC class II expression by macrophages from the Bcg¹ mice (Zwilling 1992).
The failure of HPA axis activation to increase the susceptibility of $Bcg^t$ mice was not the result of an unresponsiveness of this strain of mice to HPA axis activation. Thus, activation of the HPA axis resulted in a similar increase in plasma corticosterone levels in both strains of mice (Zwilling 1992). Additionally, HPA axis activation suppresses the induction of MHC class II expression by macrophages from $Bcg^t$ mice (Snyder 1982, Zwilling 1987). We also found that HPA activation resulted in a suppressed capacity of macrophages from $Bcg^t$ and $Bcg^s$ mice to produce TNF-α and NO$_2^-$ following stimulation with rIFN-γ and LPS. This observation supports those that have shown that corticosteroids suppress both TNF-α production as well as the production of reactive nitrogen intermediates (Beutler 1986, Di Rosa 1990, Zuckerman 1989).

The suppression of TNF-α and of NO$_2^-$ production by macrophages of both $Bcg^t$ and $Bcg^s$ strains of congenic mice as a result of HPA axis activation appears to indicate that the mechanism(s) of resistance that are controlled by $Bcg$ may not be regulated by glucocorticoid hormones. Our results can also be interpreted as indicating that the production of TNF-α and of NO$_2^-$ may also be independent of $Bcg$ control. Macrophages from both strains of mice produced similar quantities of the cytokine and of reactive nitrogen intermediates. Several reports have also shown that there are no apparent differences between the level of TNF-α released by macrophages from resistant or susceptible mice following stimulation of $M. lepere murium$ infected macrophages with LPS (Ha 1983), or stimulation with lipoarabinomannan (Chatterjee 1992) or Leishmania (Liew 1991, Theodos 1991). In contrast Blackwell et al. (Blackwell 1989) reported that $Lsh^t$ macrophages produced more TNF-α
then did Lsh⁰ macrophages. Our result regarding NO₂⁻ production is similar to that reported by Appleberg and Sarmento (Appelberg 1990) but different than that found by Liew et al. (Liew 1991). Others have reported that macrophages from BCG susceptible mice produce more or less NO₂⁻ than macrophages from BCG resistant mice (Roach 1991). The reasons for the differences reported by the different laboratories is not clear but may relate to the differences in NK cells associated with splenic macrophage preparations. Ramarathinam et al. have reported that macrophages from resistant mice produce a factor that regulates IFN-γ production by NK cells and suggests that a function of the Ity⁰ gene may be to regulate IFN-γ production. Thus, NK cells from resistant mice are stimulated to produce more IFN-γ (Ramarathinam 1993a, 1993b). We have previously reported that an increased stimulus can attenuate the effect of HPA activation (Zwilling 1992). It is possible therefore, that the differences we have observed in TNF-α and in NO₂⁻ production may be the result of differences in IFN-γ produced. Similarly, stimulation of the cultures with rIFN-γ and LPS may have attenuated the effect of restraint on the macrophages from the Bcg⁰ mice. These possibilities are currently being explored by using Mycobacteria to stimulate TNF-α and NO₂⁻ production and by comparing the level of IFN-γ produced following stimulation of spleen cell cultures with antigen.

The role of corticosterone in mediating the effects of HPA axis activation was demonstrated in three ways. First, adrenalectomy abrogated the effect of HPA activation. Secondly, implantation of time release pellets, that released levels of corticosterone attained
during HPA axis activation, resulted in an increased susceptibility of the $Bcg^*$ mice to Mycobacterial growth. Finally, treatment of the $Bcg^*$ mice with the glucocorticoid receptor antagonist RU486 abrogated the effects of HPA axis activation.

The effect of corticosteroids on macrophage function has been the subject of considerable investigation. Glucocorticoids have been shown to inhibit cytokine production by macrophages (Lee 1988, Snyder 1982) and to suppress MHC class II expression (Snyder 1982, Warren 1985) and tumoricidal activity (Hogan 1988). However, glucocorticoid mediated effects can also potentiate some macrophage functions. This includes the potentiation of the uptake of opsonized erythrocytes by IFN-γ stimulated macrophages (Warren 1985) and the expression of cytokine receptors (Strickland 1986). Activation of macrophages also results in the up regulation of corticosteroid receptors in macrophages (Salkowski 1992a, 1992b). However, this appears to be similar in a macrophage cell line derived from BALB/c BCG susceptible mice as well as in macrophages from BCG resistant C3H/OuJ mice. The positive effects of glucocorticoids have been attributed to glucocorticoid response elements which positively regulate gene expression. In contrast, no consensus regulatory sequences have been identified that account for the negative regulatory effects of the glucocorticoids (Beato 1989).

The role of HPA axis activation in the control of the growth of tuberculosis in humans has been the subject of some discussion (Collins 1989, Ishigami 1919, Wiegenshaus 1989). Several reports have shown that injection of glucocorticoids suppress the antimicrobial
activity of macrophages and exacerbate the growth of Mycobacteria (Cox 1989, Schaffner 1985, Stovkis 1992). Our investigation is the first that shows that HPA axis activation increases the susceptibility of mice to Mycobacterial growth. Rook et.al. (Roach 1991) reported that dexamethasone increased the susceptibility of monocytes from some human donors to Mycobacterial growth but not monocytes from other donors. This may be analogous to the observation we have made in mice that increased corticosterone levels, that occurs as a result of HPA activation, increases the susceptibility of 

Bcg<sup>a</sup> mice to Mycobacterial growth but not 

Bcg<sup>b</sup> mice (Zwilling 1990, 1992). Recently North and Izzo (North 1993) have shown that weekly injections of SCID mice with hydrocortisone acetate increased the susceptibility of the mice to the growth of Mycobacterium tuberculosis. In contrast the resistance of isocongenic immunocompetent mice was hydrocortisone resistant, presumably due to the development of specific immunity. Several reports have suggested that the resistance of macrophages to Listeria, Salmonella or Toxoplasma induced by rIFN-γ is glucocorticoid resistant (Masur 1982, Schaffner 1985). Thus, it is possible that the insensitivity of the Mycobacterial resistance mechanism(s), controlled by 

Bcg<sup>a</sup>, to HPA axis activation is corticosteroid insensitive. IFN-γ, perhaps produced by NK cells early after infection, or some other cytokine (Morrissey 1990, Ramarathinam 1993a, 1993b), may induce an anti-Mycobacterial mechanism that is not sensitive to glucocorticoids. Our results also appear to rule out the IFN-γ induced, TNF-α dependent induction of reactive nitrogen intermediate pathway of Mycobacterial resistance (Chan 1992) as the primary pathway of 

Bcg mediated resistance.
Finally, our results suggest that activation of the HPA axis may account for the increased susceptibility to Mycobacterial growth. While we have used restraint to activate the HPA axis, it is possible that during the natural course of the disease, chronic inflammatory events that lead to macrophage activation and the production of IL-1, TNF-α or IL-6 may result in stimulation of the hypothalamus that eventually results in increased levels of cortisol and suppression of glucocorticoid sensitive resistance mechanisms (Breder 1988, Cunningham 1993). This suppression may be particularly apparent in individuals that are innately susceptible to Mycobacterial disease and may account for the increase in the incidence of active tuberculosis among susceptible populations. Other cofactors such as homelessness, malnutrition, chronic alcoholism as well as stressful life events (Bloom 1992, Collins 1989, Feingold 1976, Hodolin 1975, Ishigami 1919, Jones 1954, Kiecolt-Glaser 1991, Nagami 1983, Orme 1988, Pincock 1964, Powell 1980, Wiegeshaus 1989) may also result in HPA axis activation or activation of the sympathetic nervous system (Blalock 1989, Felten 1991, Wan 1993) and compound the effects of the disease processes.
CHAPTER 3

ACTIVATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS DIFFERENTIALLY AFFECTS THE ANTI-MYCOBACTERIAL ACTIVITY OF MACROPHAGES FROM BCG-RESISTANT AND SUSCEPTIBLE MICE

INTRODUCTION

Infection of man with *Mycobacterium tuberculosis* and *Mycobacterium avium* has increased in the United States during the past 10 years (Barnes and Barrows, 1993; Bloch et al., 1989; Bloom, 1992; Kochi, 1991; Pasvol, 1993; Young, 1993). The increased incidence is due in part to infection with the human immunodeficiency virus. The increased spread of tuberculosis in the United States has been attributed to the reactivation of previously existing disease in individuals who have become immunosuppressed as a result of HIV infection. Most healthy individuals, with intact host resistance mechanisms, who are infected by *M. tuberculosis* control the growth of the microorganism (Kaufman, 1993). Resistance mechanisms result in granuloma formation and the establishment of a steady state infection. Once this steady state infection has been established, the numbers of microorganisms will remain constant for years. Infected individuals stand a 10% life time chance of having their disease reactivate. Conventional wisdom has indicated that this reactivation is due to some event that results in immunosuppression. Some of the co-factors that have been historically
associated with reactivation of *M. tuberculosis* growth include immunosenescence as a result of ageing, homelessness, chronic alcoholism and associated protein malnutrition and stress (Bloom 1992, Collins 1989, Feingold 1976, Hodolin 1975, Ishigami 1919, Jones et al. 1954, Nagami and Yoshikawa 1983, Orme 1988, Pincock 1964, Powell and Farer 1980, and Wiegeshaus et al. 1989). The role of these co-factors in reactivation of *M. tuberculosis* has not been tested experimentally. To this end, we have been evaluating the effect of hypothalamic-pituitary-adrenal activation, via restraint stress, in an attempt to determine the effect of one of these co-factors on the reactivation of Mycobacterial growth. To accomplish this we have initiated a series of studies to evaluate the effect of HPA activation on the growth of *Mycobacterium avium* as a prelude to future studies to evaluate the mechanisms of *M. tuberculosis* reactivation.

Previous results from our laboratory have shown that activation of the hypothalamic-pituitary-adrenal axis, by restraint stress, results in an increase in the susceptibility of mice to the growth of *Mycobacterium avium* (Brown et al., 1993). The effect of HPA activation was limited to mice that were innately susceptible to Mycobacterial growth and did not alter the growth in innately resistant mice. The increase in the susceptibility of susceptible mice was primarily mediated by glucocorticoids. We found that the increase in the susceptibility to Mycobacterial growth was prevented by adrenalectomy or by treatment of mice with the glucocorticoid receptor antagonist RU486. The increase in the susceptibility to Mycobacterial growth that resulted from HPA activation may be due to a direct effect of corticosterone on the antimycobacterial mechanisms of macrophages. The purpose of this
investigation was to evaluate the effect of HPA activation by restraint stress and of corticosterone on the anti-Mycobacterial activity of the macrophage. We found that activation of the HPA axis or the addition of corticosterone to cultures of macrophages suppresses the ability of macrophages from susceptible mice to control the growth of *Mycobacterium avium*. In contrast, neither HPA activation nor the addition of corticosterone altered the ability of macrophages from BCG resistant mice to control Mycobacterial growth. We found that the production of TNF-α and of reactive nitrogen intermediates by macrophages from both strains of mice was suppressed by HPA activation and corticosterone. The implications of these findings and their relationship to anti-Mycobacterial resistance mechanisms that may be controlled by *Bcg* are discussed.

**MATERIALS AND METHODS**

**Animals:** Male BCG susceptible, BALB/c.Bcg*, mice were obtained from Charles River when 6 weeks of age. The animals were housed in groups of 5 in micro-isolation cages and given sterile food and water *ad libitum*. Congenic C.D2ldh*-Ity*-Pep-3*, BCG resistant mice (BALB/c Bcg*) were bred by us (Potter et.al., 1983; Brown et.al. 1993). Some experiments used BCG resistant DBA.2 mice which were also obtained from Charles River. The results obtained with the DBA.2 mice were identical to those obtained with the BALB/c.Bcg* congenic mice.

**Mycobacterium avium:** *Mycobacterium avium*, a recent clinical isolate obtained from Ohio State University Hospitals, was grown in Middlebrook 7H9 broth (Difco Laboratories,
Detroit, MI) at 37°C in 10% CO₂ in air until bacterial clumps were clearly discernable upon visual assessment. Bacteria were frozen at -70°C for 48 hours at a concentration range of 5 X 10⁸ colony forming units (cfu)/ml. An aliquot was thawed, vortexed and then sonicated (Heat Systems, Farmingdale NY) using 25, 0.25 second pulses of 75 watts each. To determine the viable cfu/ml of *M. avium* in the thawed samples, serial 10-fold dilutions of the mycobacterial stock were plated onto 7H11 agar and colony counts enumerated after 10 days in culture at 37°C and 10% CO₂.

**HPA Activation via Restraint Stress.** The paradigm used to activate the HPA axis has been previously described (Brown et al., 1993). Mice were placed in well ventilated 50 ml conical centrifuge tubes for 5 daily 18 hr restraint cycles, rested for two days and restrained for an additional 5, 18 hr cycles. During each of the restraint cycles, the mice were deprived of food and water. Control mice, therefore, were also deprived of food and water. No differences in mycobacterial growth nor in macrophage antimycobacterial activity were observed between conventionally housed and food and water deprived mice (Brown et al., 1993). In order to evaluate the effect of glucocorticoids on alterations in macrophage function, mice were treated with the glucocorticoid receptor antagonist RU486 which was kindly provided by Roussel-Uclaf (Romainville, France). The RU486 was dissolved in polyethylene glycol (mw 400) and injected subcutaneously at a dose of 25 mg/kg body weight daily, beginning 2 days prior to the initiation of restraint and continuing throughout the entire restraint period (Brown et. al., 1993).
In Vitro Anti-Mycobacterial Activity: The antimycobacterial activity of splenic macrophages were assessed as initially described by Flesch and Kaufman (1991) and adapted by us (Brown et al., 1993). Splenic adherent cells were obtained from pooled spleens, passed through tissue sieves into Hank's balanced salt solution (BSS) (GIBCO/BRL, Gaithersburg, MD) supplemented with 20% defined fetal bovine serum (FBS) (Hyclone, Logan UT) and subsequently passed through sterile needles with decreasing bore size in order to achieve a single cell suspension. The cells were resuspended to a concentration of 70×10^6 cells in 5 ml in Iscove's modified Dulbecco's medium (IMDM) (GIBCO) supplemented with 10% FBS and glutamine but without antibiotics. The splenic macrophages from this suspension were enriched by adherence onto tissue culture dishes (100x20mm) by culturing the cells overnight at 37°C in an atmosphere containing 10% CO₂. Nonadherent cells were removed by gently washing three times with a stream of Hanks' BSS from a 10 ml pipette. The adherent cells were then removed by scraping with a rubber policeman. This population contained approximately 70% viable cells and the percentage of macrophages was determined by differential staining and by staining for nonspecific esterase. The cell suspension was adjusted to contain sufficient numbers of macrophages based on differential (approximately 50%) and adherence. Thus, cell suspensions typically contained 4×10^5 total viable cells so that a concentration of 10^4 macrophages per well in a 96 well microtiter plates was attained after overnight culture. The cultures were washed again to remove the remaining non-adherent cells. The number of adherent cells was confirmed by counting cells in selected wells using an ocular grid attached to an inverted tissue culture microscope. The purified macrophages were then infected with 4×10^5 cfu *M. avium* suspended in 0.2 ml of IMDM
without antibiotics and incubated overnight (16hrs) at 37° C to allow for phagocytosis. Following incubation, the cultures were washed vigorously to remove any uningested bacteria. The infected macrophage cultures were then incubated in IMDM without serum for a period of 5 days to allow intracellular growth of the bacteria. The presence of serum was bacteriostatic. Previous studies have shown that macrophages from Bcg' and Bcg' mice take up equal numbers of microorganisms (Zwilling et.al., 1992). The initial load of bacteria was determined by lysing selected cultures and pulsing the cultures with 3H-Uracil. The bacteria released by the macrophages from Bcg' and Bcg' mice take up identical amounts of radiolabel. To make sure that the ingested bacteria remain viable, selected cultures were lysed immediately after phagocytosis and the bacteria allowed to grow for 5 days prior to the addition of radiolabel. Visual inspection of selected cultures after 5 days of incubation indicated that the numbers of macrophages remained constant throughout the culture period. These cultures were lysed and pulsed with media containing a mixture (1:1) of 7H9 medium and IMDM with 3H-uracil (5uCi/ml) (Amersham, Chicago, IL; specific activity, 40 to 60 Ci.mmol) and 0.1% saponin. The bacteria were incubated overnight and were harvested onto glass fiber filter strips with a PHD cell harvester (Cambridge Technology, Inc. Watertown, MA). Radioactivity incorporated by the released bacteria was quantitated by liquid scintillation spectrometry. To determine the effect of glucocorticoids on macrophage antitycobacterial activity, corticosterone (Sigma Chemical Company, St. Louis, MO.) at concentrations ranging from 10^-4M to 10^-13M was added to the infected macrophage wells immediately after phagocytosis of the bacteria. RU486, initially dissolved in dimethyl sulfoxide (DMSO) (Sigma) (DMSO final volume: 0.5%v/v) was resuspended in IMDM at a
concentration of 625 μg/ml and 0.1 ml added to the infected macrophage wells immediately prior to the addition of corticosterone. The final concentration of DMSO alone did not affect the antimicrobial activity of the macrophages (data not shown).

**Production of TNF-α and reactive nitrogen intermediates.** Purified splenic macrophages at a concentration of 10⁵ macrophages per well in 96 well microtiter plates were stimulated with 5x10⁵ M. avium together with 1, 10 or 100 U of rIFN-γ for 72 hrs at 37°C. Cell free supernatant fluids were then used to determine the amount of TNF-α and reactive nitrogen intermediates. While TNF-α production reaches maximum within 48 hrs, the cultures were harvested after 72 hrs so that the levels of NO and of TNF-α could be determined from the same culture fluids. Preliminary kinetic studies indicated that TNF-α levels between 48 and 72 hours remained constant. TNF-α production was determined by enzyme linked immunosorbant assay (ELISA) (Endogen Inc., Boston, MA) and the amount of reactive nitrogen intermediates (nitrite) was determined by using the Griess reagent (Green, et.al., 1982). The amounts of TNF-α and NO were calculated from a standard curve and expressed as picograms per ml (TNF-α) or micromolar (NO).

**Statistical Analysis:** Analysis of the data was done using the Systat program. Each observation of Mycobacterial growth or of TNF-α or NO was analyzed using an analysis of variance (two factor ANOVA) done on both log and absolute scale. In most cases the data are expressed as the mean of replicate trials. In some cases representative data are shown.
Pairwise comparisons between dosage and treatment groups were determined using the Bonferroni adjusted correlation. All declarations of significance are listed as worst case values.

RESULTS

HPA activation differentially affects macrophage antimycobacterial activity. HPA activation resulted in an increase in the susceptibility of splenic macrophages from BCG susceptible mice to the growth of *M. avium* (Table 3.1). In contrast, HPA activation did not alter the ability of the macrophages from BCG resistant mice to control Mycobacterial growth. Thus, the Mycobacteria cultured with macrophages from *Bcg*+ mice incorporated 12750±921 cpm which represented a 52% increase over that incorporated by the bacteria cultured with macrophages from unstressed mice. In contrast HPA activation did not result in an increase in the growth of the Mycobacteria in macrophages from *Bcg*+ mice.

Effect of RU486. Treatment of mice with the glucocorticoid receptor antagonist RU486 blocked the increase in the susceptibility of the macrophages to Mycobacterial growth that resulted from HPA activation. The results in Table 3.2 shows that HPA activation or the addition of corticosterone to cultures of macrophages increased the susceptibility of splenic macrophages from *Bcg*+ mice to the growth of *M. avium* but did not affect the ability of macrophages from resistant mice to control the growth of the bacterium. The Mycobacterial growth in macrophages from restrained *Bcg*+ mice was 72% greater then the growth of the microorganisms in macrophages from control mice. Treatment of restrained, susceptible mice
Macrophage source | Treatment | 
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>BCG Resistant</td>
<td>restraint</td>
</tr>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>BCG Susceptible</td>
<td>restraint</td>
</tr>
<tr>
<td></td>
<td>control</td>
</tr>
</tbody>
</table>

* HPA axis was activated by 10, 18 hr restraint periods. The splenic macrophages from congeneric Bcg^a and Bcg^b mice were purified by adherence and added to 96 well microtiter plates so that each monolayer consisted of 10^5 macrophages. The cells were infected with 4X10^5 cfu M. avium overnight and then washed to remove unphagocytized bacteria. Some of the cultures were lysed immediately and pulsed with ^3H-uracil to determine the amount of bacteria phagocytized (BCG resistant: 621±14; BCG susceptible: 593±43). Other cultures were lysed immediately and allowed to grow for 5 days prior to the addition of label in order to insure the viability of the Mycobacteria (BCG resistant: 43,602±287; BCG susceptible: 39,506±713). The remaining cultures were incubated for 5 days prior to lysis. In each case the macrophages were incubated with a lysis buffer containing a 1:1 mixture of 7H9 medium, ^3H-uracil and 0.1% saponin. The released bacteria incorporated the radioactivity which is an indication of the relative number of actively metabolizing bacteria. The data represent 1 of 4 experiments and is expressed as the mean±SD of 4 wells for each determination.

^b The effect of HPA activation on the growth of M. avium in macrophages from BCG susceptible mice is significant as determined by ANOVA (p<0.001).

^c The difference between BCG resistant and BCG susceptible is significant as determined by ANOVA (p<0.002)

Table 3.1: Differential effects of HPA activation on mycobacterial growth in macrophages from BCG-resistant and susceptible mice *.
<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Treatment</th>
<th>$^3$H-Uracil Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>17,348 ± 1221$^b$</td>
</tr>
<tr>
<td></td>
<td>Control + RU 486</td>
<td>19,386 ± 621</td>
</tr>
<tr>
<td></td>
<td>Restraint</td>
<td>29,851 ± 486$^c$</td>
</tr>
<tr>
<td></td>
<td>Restraint + RU 486</td>
<td>20,801 ± 703$^d$</td>
</tr>
<tr>
<td>BCG Susceptible</td>
<td>Control</td>
<td>13,528 ± 434</td>
</tr>
<tr>
<td></td>
<td>Control + RU 486</td>
<td>13,556 ± 458</td>
</tr>
<tr>
<td></td>
<td>Restraint</td>
<td>14,113 ± 78</td>
</tr>
<tr>
<td></td>
<td>Restraint + RU 486</td>
<td>11,373 ± 318</td>
</tr>
</tbody>
</table>

* BALB/c.Bcg$^e$ mice and DBA/2 BCG resistant mice were injected with RU486 (25mg/kg) beginning 2 days prior to the restraint cycle and continued throughout. The macrophages were obtained from the spleen and their ability to control the growth of the Mycobacteria assessed as described in Table 1. The results are representative of 1 of 3 experiments and are expressed as the mean±SD of cpm incorporated by the Mycobacteria.

$^b$ The differences between BCG resistant and susceptible control mice is significant as determined by ANOVA (p≤0.003).

$^c$ The effect of restraint is significant by ANOVA (p≤0.002).

$^d$ The effect of RU486 is significant by ANOVA (p≤0.002).

Table 3.2: RU486 abrogates the effect of HPA activation on the growth of *M. Avium* within macrophages from BCG-susceptible mice.
with RU486 in vivo resulted in a growth pattern of the Mycobacteria that was similar to that observed within splenic macrophages from control mice. Activation of the HPA axis did not alter the ability of macrophages from BCG resistant mice to control Mycobacterial growth nor did treatment of the mice or of macrophage cultures with RU486.

**HPA activation suppresses the production of both TNF-α and of reactive nitrogen intermediates (NO).** The antimicrobial effector pathway that has been attributed to account for resistance to Mycobacterial growth is the TNF-α dependent production of NO. Accordingly, we assessed the effect of HPA activation on the ability of splenic macrophages to produce both TNF-α and NO. The results in Tables 3.3 and 3.4 indicate that HPA activation suppressed the ability of macrophages from both BCG susceptible and BCG resistant mice to produce TNF-α (Table 3.3) and NO (Table 3.4). TNF-α and NO produced by uninfected macrophage cultures, not treated with rIFN-γ was < 50 pg/ml and < 5μM respectively. Several other important observations are also apparent from these experiments. First, macrophages from both Bcg^ and Bcg^ mice produced comparable levels of TNF-α. Secondly, the effect of HPA activation on TNF-α production was attenuated by increasing the concentration of rIFN-γ on macrophages from DBA.2 BCG resistant mice. In contrast, increasing concentration of rIFN-γ did not result in a restoration of TNF-α production by macrophages from BCG susceptible mice. Finally, when stimulated with *M. avium* and IFN-γ macrophages from BCG resistant mice produced significantly more NO then did macrophages from BCG susceptible mice. The results presented in Table 3.5 shows that HPA activation suppressed both TNF-α and NO production and that treatment of mice with
Table 3.3: HPA activation suppresses the capacity of macrophages from both BCG-resistant and susceptible mice to produce TNF-α.

*Macrophages were obtained from the spleen of restrained or control BALB/c.Bcg* and BCG resistant DBA/2 mice. The cells were stimulated with several doses of rIFN-γ and 5×10³ cfu *M. avium* for 72 hrs. The level of TNF-α in the supernatant fluids was determined by ELISA. The results represent the mean±SD of 3 separate experiments.

**The effect of restraint on the production of TNF-α by macrophages from BCG susceptible mice is significant as determined by ANOVA (p<0.001).**

**The effect of rIFN-γ on TNF-α production is significant as determined by ANOVA (p<0.001).**

**The effect of restraint on the production of TNF-α by macrophages from BCG resistant mice is significant (p<0.001).**
Macrophage source | Treatment | Dose of rIFN-γ (units) | μM NO
--- | --- | --- | ---
BCG Susceptible | Restraint | 4±2<sup>b,c</sup> | 10±2<sup>c,d</sup> | 15±3<sup>c,d</sup> | 20±2<sup>c,d</sup>
 | Control | 11±1 | 26±5<sup>d</sup> | 25±2<sup>d</sup> | 31±2<sup>d</sup>
BCG Resistant | Restraint | 8±1 | 10±3<sup>d,e</sup> | 26±5<sup>d,e</sup> | 32±2<sup>d,e</sup>
 | Control | 6±2 | 45±1<sup>d</sup> | 43±3<sup>d</sup> | 45±6<sup>d</sup>

<sup>a</sup> Splenic macrophages were obtained from BCG resistant and susceptible mice and treated with *M. avium* together with rIFN-γ as described for Table 3.3. After 72 hrs the supernatant fluids were removed and the amount of NO determined by Greiss reaction. The data are expressed as the mean±SD for 3 separate experiments.

<sup>b</sup> Data represent NO production by macrophages (μM).

<sup>c</sup> The effect of restraint on NO production is significant as determined by ANOVA (P<0.003).

<sup>d</sup> The effect of rIFN-γ was significant as determined by ANOVA (P<0.004).

<sup>e</sup> The effect of restraint on NO production by macrophages from BCG resistant mice was significant as determined by ANOVA (P<0.003).

Table 3.4: HPA activation suppresses the capacity of macrophages from BCG-resistant and susceptible mice to produce reactive nitrogen intermediates *.
Macrophages were obtained from the spleens of BCG susceptible BALB/c mice or BCG resistant DBA/2 mice treated with RU486 and restrained. The cells were stimulated with riFN-γ and M. avium. After 72 hrs the amount of TNF-α was determined by Elisa and NO by the Greiss reaction. The data represent the mean±SD of 3 separate experiments.

The effect of restraint on TNF-α and NO production was significant as determined by ANOVA (p<0.002).

The effect of RU486 on TNF-α and NO production was significant as determined by ANOVA (p<0.003).

Table 3.5: RU486 abrogates the suppressive effect of HPA activation on the production of TNF-α* and NO*.  

<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Treatment</th>
<th>TNF-α (pg/ml)</th>
<th>NO(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG Susceptible:</td>
<td>Restraint</td>
<td>680 ± 70b</td>
<td>24±3b</td>
</tr>
<tr>
<td></td>
<td>Restraint+RU486</td>
<td>1500 ± 205c</td>
<td>38±1c</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2050 ± 75</td>
<td>41±2</td>
</tr>
<tr>
<td>BCG Resistant:</td>
<td>Restraint</td>
<td>775 ± 155b</td>
<td>27±3b</td>
</tr>
<tr>
<td></td>
<td>Restraint+RU486</td>
<td>1300 ± 215c</td>
<td>41±5c</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2350 ± 195</td>
<td>47±1</td>
</tr>
</tbody>
</table>
Splenic macrophages were infected with 4X10^5 cfu *M. avium* and incubated overnight in the presence of 10^{-8}M corticosterone with or without 62.5 μg of RU486. After 5 days the macrophages were lysed and the metabolically active bacteria radiolabeled as described. The data represents one of 5 experiments and is expressed as the mean±SD of 4 wells for each determination. The effect of corticosterone on the growth of *M. avium* was significant by ANOVA (p<0.003). The effect of RU486 is significant (p<0.003).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[³H]Uracil incorporation a</th>
<th>TNF-α (pg/ml)b</th>
<th>NO (μM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>12,321 +/- 173</td>
<td>1668 +/- 75</td>
<td>18 +/- 2</td>
</tr>
<tr>
<td>Cort+RU486</td>
<td>10,347 +/- 773</td>
<td>2448 +/- 114</td>
<td>36 +/- 2</td>
</tr>
<tr>
<td>Control</td>
<td>9,932 +/- 526</td>
<td>2592 +/- 52</td>
<td>41 +/- 1</td>
</tr>
<tr>
<td>Control+RU486</td>
<td>10,105 +/- 421</td>
<td>2483 +/- 107</td>
<td>40 +/- 3</td>
</tr>
</tbody>
</table>

* Splenic macrophages were stimulated with 100 U of rIFN-γ and 5x10^4 cfu *M. avium* for 72 hrs. Corticosterone, with or without RU486 was added to the test wells. The level of TNF-α and NO production in the cell free supernatants was determined by ELISA or by the Griess reagent. Unstimulated macrophages produced 57±6 pg/ml TNF-α and 6±3 μM NO. The data are expressed as the mean ±SD of 3 separate experiments. The effect of corticosterone and RU486 on TNF-α and NO production is significant by ANOVA (p<0.005).

Table 3.6: Effect of RU486 on corticosterone mediated suppression of macrophage mediated anti-mycobacterial activity, TNF-α and NO production.
RU486 in vivo resulted in a restoration of the ability of the macrophages to produce both TNF-α and NO following stimulation with rIFN-γ and *M. avium*.

Treatment of macrophages with corticosterone *in vitro* mimicked the effect of HPA activation and was also abrogated by the addition of RU486 to the culture media. The results in Table 3.6 show that the addition of corticosterone to cultures of infected macrophages resulted in a 24% increase in susceptibility to Mycobacterial growth. The addition of RU486 to these cultures resulted in a level of Mycobacterial growth that was similar to that of control cultures. The data in Table 3.6 also shows that corticosterone suppressed the production of both TNF-α and NO and that this effect was abrogated by the addition of RU486 to the cultures. The corticosterone mediated suppression of TNF-α and of NO production by macrophages from BCG resistant mice was similarly affected by RU486 and the addition of corticosterone did not alter the growth of the Mycobacteria in these macrophage (data not shown).

**DISCUSSION**

The results of this investigation indicate that activation of the HPA axis results in a suppression of macrophage anti-mycobacterial activity. However, this effect of HPA activation is limited to macrophages from BCG susceptible mice. These differences are not the result of the inability of restraint to activate the HPA axis of BCG resistant mice. The mice respond similarly to HPA activation as judged by the increase in plasma glucocorticoids
(Zwilling et al., 1990). Additionally, HPA activation results in a suppression of the induction of I-A expression by macrophages from both strains of mice (Zwilling et al., 1992) as well as their ability to produce TNF-α and NO.

Our findings support previous observations by Beutler et al. (1986) and by Di Rosa et al. (1990) who found that glucocorticoids suppress the production of TNF-α and of NO by macrophages. We have previously reported that activation of the HPA axis results in the suppression of TNF-α and of NO production by macrophages (Brown et al. 1993). The results of the experiments reported here extend that observation by showing that HPA activation suppresses their production when the cells are also stimulated by IFN-γ and M. avium. This is different than our previous studies that showed that HPA activation suppresses both TNF-α and NO production by rIFN-γ and LPS stimulated macrophages. The suppression of macrophage function is due, in part, to an elevation of glucocorticoids following HPA activation. Treatment of the mice with the glucocorticoid receptor antagonist RU486 ameliorated the effect of HPA activation on TNF-α and NO production. However, RU486 only partially restored the ability of the macrophages to produce TNF-α. This may indicate that other factors, possibly activation of the sympathetic nervous system, may also result in a suppression of TNF-α. This possibility is reinforced by our observation that the effect of HPA activation on Mycobacterial growth cannot be solely accounted for by the treatment of the macrophages with corticosterone.
The results of this investigation support our previous observations (Zwilling et al., 1992) that increasing the intensity of the immunologic stimulus attenuates the effect of HPA activation of macrophage function. We have previously reported that injection of mice with increasing concentrations of Mycobacterium bovis (strain BCG) attenuated the suppressive effect of restraint on the induction of MHC class II expression and that increasing concentrations of rIFN-γ had a similar effect on class II expression. The results of this investigation also show that increasing concentrations of rIFN-γ can attenuate the suppressive effect of HPA activation on the production of TNF-α and on NO. However, this effect was more pronounced on macrophages from BCG resistant mice.

The observation that TNF-α and NO production is suppressed by HPA activation suggests that the mechanism of action of Bcg that controls resistance does not involve the production of TNF-α or of NO as suggested by others (Roach et al. 1989, 1991; Vidal et al. 1993). The macrophages from the resistant mice can control the growth of the Mycobacteria despite the fact that an important antimycobacterial pathway has been suppressed. Several other antibacterial mechanisms can account for resistance to Mycobacterial growth. These include iron availability (Payne 1993), tryptophan metabolism (Carlen et al. 1989) or activation of macrophage defensins (Lehrer et al. 1993). Whether or not these pathways are also regulated by corticosteroids is currently under investigation.

Resistance to Mycobacterium tuberculosis has been shown to involve both innate and acquired defense mechanisms (Dannenberg 1993; Kaufman 1993). The results of this
investigation suggest that HPA activation may be one factor that leads to the suppression of antimycobacterial defense mechanisms. Our results further suggest that innate resistance is not affected by corticosteroids and suggests that Mycobacterial growth, in susceptible individuals, may be further exacerbated by activation of the HPA axis (Stead et. al. 1990; Stead 1992). This could occur either as a result of excessive cytokine production induced during Mycobacterial infection (Rook 1993) or by stressful life events.
CHAPTER 4

CYTOKINE MEDIATED ACTIVATION OF MACROPHAGES FROM 
*Mycobacterium bovis* BCG-RESISTANT AND SUSCEPTIBLE MICE: 
DIFFERENTIAL EFFECTS OF CORTICOSTEROIDE ON 
ANTIMYCOBACTERIAL ACTIVITY AND EXPRESSION OF THE *Bcg* GENE 
(CANDIDATE *Nramp*)

INTRODUCTION

Resistance to Mycobacterial growth is controlled by a gene (*Bcg*) that maps to chromosome 1 in mice (Schurr 1989). The candidate *Bcg* gene, *Nramp*, codes for a product termed natural resistance associated macrophage protein, that has a motif of a transporter protein but the function of *Nramp* is not known (Vidal 1993). It has been suggested that the protein may be involved in the transport of nitrates to the phagolysosome of infected macrophages which accounts for the increased capacity of macrophages from *Bcg* resistant mice to control the growth of Mycobacteria. Others have suggested that the protein may play an important role in nitric oxide (NO) mediated signal transduction resulting from priming/activation of macrophages, thereby accounting for the pleiotrophic effects that have been reported to be under *Bcg* mediated control (Gazzinelli 1993, Schurr 1989).
Studies in our laboratory have shown that corticosteroids increased the susceptibility of $Bcg'$ mice to the growth of $Mycobacterium avium$ (Brown et al. 1993). In contrast, the corticosteroids did not affect resistance of $Bcg'$ mice to Mycobacterial growth. The in vivo effects of corticosterone mimic the effect of the steroid on the capacity of the macrophage to control the growth of $M. avium in vitro$ (Brown et al. 1994). Treatment of macrophages from $Bcg'$ mice with corticosterone increased the permissiveness of the macrophages for Mycobacterial growth. Corticosterone treatment of macrophages from $Bcg'$ mice did not alter their resistance to the growth of $M. avium$. We also showed that corticosteroids suppressed the production of TNF-α and of reactive nitrogen intermediates by macrophages from both strains of mice without affecting the resistance of macrophages from $Bcg'$ mice.

Thus, when the macrophage populations were activated in vitro with rIFN-γ, the function of macrophages from $Bcg'$ and $Bcg$ mice was suppressed by the addition of corticosterone. This raised the possibility that the differential effect of glucocorticoids that we have observed, was limited to innate resistance and not resistance induced by rIFN-γ or other macrophage activating cytokines. The purpose of this investigation, therefore, was to evaluate the anti-mycobacterial activity of macrophages from $Bcg'$ and $Bcg$ mice following stimulation of the cells with rIFN-γ or GM-CSF and to determine the effects of glucocorticoids on the capacity of the macrophages to control the growth of $M. avium$. We found that macrophages from both strains of mice responded equally to recombinant interferon rIFN-γ or granulocyte-macrophage colony stimulating factor (GM-CSF) mediated activation. Corticosteroids suppressed the ability of rIFN-γ or GM-CSF activated
macrophages from BCG susceptible mice but not from BCG resistant mice to suppress Mycobacterial growth. *Nramp* expression, by macrophages from both *Bcg* and *Bcg*′ mice, was up regulated by treatment of the cells with rIFN-γ and by treatment with GM-CSF. Corticosterone suppressed *Nramp* mRNA expression by macrophages from *Bcg*′ mice but did not affect *Nramp* mRNA expression by macrophages from *Bcg*′ mice.

**MATERIALS AND METHODS**

**Animals:** Male BALB/c, BCG-susceptible (BALB/c.*Bcg*′) mice were obtained from Charles River when six weeks of age. The animals were housed in groups of five in micro-isolation cages and given food and water ad libitum. Congenic C.D2*Idh*- *Ity*- *Pep*-3 mice, BCG-resistant (BALB/c.*Bcg*′) were initially obtained from Dr. Michael Potter (NCI) (Potter 1983) and bred by us (Brown *et al.* 1993).

**Mycobacterium:** A recent clinical isolate of *Mycobacterium avium* was obtained from Ohio State University Hospitals (Brown *et al.* 1993), and grown in Middlebrook 7H9 broth supplemented with OADC (Difco Laboratories, Detroit, MI) at 37° C in 10% CO₂ in air until mid log phase. Bacteria were aliquoted in 1 ml amounts and stored frozen at -70°C at a concentration of 3-5 × 10⁸ colony-forming units (cfu) per ml. Prior to use each aliquot was thawed, vortexed, and then sonicated (Heat Systems, Farmingdale, NY) using 25, 0.25-sec pulses of 75 watts each. To confirm the number of bacteria used to infect cultures, serial 10-fold dilutions of the mycobacterial stock were cultured onto 7H11 agar plates (Difco) and colony counts enumerated after 10 days of culture at 37°C and 10% CO₂.
In vitro anti-mycobacterial activity: The anti-mycobacterial activity of splenic macrophages was assessed as initially described by Flesch and Kaufman (Flesch and Kaufman 1991) and adapted by us (Brown et al. 1994). Briefly, splenic adherent cells were enriched by overnight adherence onto 100x20 mm tissue culture dishes (Falcon) using Iscove's modified Dulbecco's medium (IMDM) (GIBCO/BRL, Gaithersburg, MD) supplemented with 20% defined fetal bovine serum (FBS) (Hyclone, Logan, UT) containing less then 0.03 ng/ml of endotoxin. Non-adherent cells were removed by gentle washing with Hank's balanced salt solution (HBSS) (Gibco/BRL) and the splenic macrophages were removed by scraping with cell scrapers. Following differential staining and staining for nonspecific-esterase, the adherent cells were then added, in IMDM without serum, to 96 well microtiter plates so that a concentration of $10^5$ macrophages per well was attained. The purified macrophages (>90% as determined by nonspecific esterase) were then infected with $4 \times 10^5$ cfu M. avium suspended in IMDM without antibiotics and incubated overnight to allow for phagocytosis. The cultures were then washed to remove unphagocytized bacteria and incubated for 5 days to allow the intracellular growth of the bacteria. The macrophages were then lysed and bacteria pulsed overnight by incubating in media containing a mixture (1:1) of 7H9 (Difco) and IMDM with [3H]uracil (5µCi/ml) (Amersham, Chicago, IL; specific activity, 40-60 Ci mmol) and 0.2% saponin. The bacteria were harvested onto glass fiber filter strips with a PHD cell harvester (Cambridge Technology, Inc. Watertown, MA). Radioactivity incorporated by the bacteria was quantitated by liquid scintillation spectrometry.
To induce the anti-mycobacterial activity of the macrophages, the cells were treated with either doses of rIFN-γ (Gibco) or GM-CSF (Boehringer Mannheim, Indianapolis, IN) in IMDM (without antibiotics) for 24 hours prior to infection with *M. avium*. Following removal of unphagocytized bacteria, IMDM containing either rIFN-γ or GM-CSF was added back to the macrophage cultures. To assess the role of NO synthase on *in vitro* antimycobacterial activity, N⁰-Methyl-L-Arginine (NMMA) (Sigma Chemical Company, St. Louis, MO) was added in concert (at a final concentration of 250 μM) with the cytokines prior to infection with *M. avium* and added back to the cultures after washing to remove any unphagocytized bacteria. To determine the effect of glucocorticoids on macrophage antimycobacterial activity, corticosterone (Sigma), at a concentration of 10⁻⁶M, was added to the infected macrophage cultures after phagocytosis of the bacteria.

**Production of Reactive Nitrogen Intermediates:** In order to determine the amount of NO, macrophages were stimulated with 5 X 10⁵ *M. avium* together with rIFN-γ or GM-CSF in the presence or absence of NMMA (250μM) for 72 hours at 37°C. Cell-free supernatants were then used to determine the amount of NO. The amount of NO (via nitrite) was determined using the Griess reagent (Green 1982). The amount of NO was calculated from a standard curve and expressed as micromolar.
**Nramp expression**: To determine the effect of corticosterone on *Nramp* expression, splenic macrophage monolayers, at a concentration of 5 X 10⁶ cells per 35mm culture well, were incubated in complete IMDM medium supplemented with 20% FBS (no antibiotics) in the presence of rIFN-γ (2500 units) or GM-CSF (250 units) for 24 hours at 37°C and 5% CO₂.

The macrophage monolayers were washed with complete IMDM and the cultures were then replenished with complete IMDM with or without 10⁻⁶M corticosterone. After incubation for 24 hrs at 37°C in an atmosphere containing 5% CO₂, the monolayers were washed and total RNA was extracted with 8M guanidine hydrochloride as modified by Evans (Cox 1968, Evans 1990, Jacobson 1985). The isolated RNA (7.5 μg per lane) was size fractionated by gel electrophoresis (1.5% formaldehyde agarose) and RNA transferred by capillary blotting onto Hybond-N+ membranes (Amersham). A separate lane containing a 0.24 - 9.5 K RNA ladder (GIBCO/BRL) was stained with ethidium bromide and used to determine RNA size. Northern hybridization of the mRNA was carried out using the protocol as described by Maenads et al. (Maniatis 1982). Gel purified insert *Nramp* and actin cDNA was radiolabelled with ³²P-dCTP by the random primer method (Amersham). Autoradiographs were quantified by scanning with a model 620 CCD densitometer (Biorad, Richmond, CA).

The *Nramp* cDNA probe was produced by RT-PCR using primers 5'TCATTTGGTTTGCTGGTAGAAGGCCTGAC3',5'TGAGCATCGCTTTCCCTTGACC CGGGGA3' originally provided by Phillip Morressey (Immunex), that amplify nucleotides 1191
to 1214 and 470 to 496 of the published \textit{Nramp} sequence (Vidal 1993). Two \textmu{}g of total RNA from splenic macrophages stimulated with rIFN-\gamma was reverse transcribed in the presence of oligo-dT primer using AMV reverse transcriptase (Promega, Madison, WI). PCR amplification reactions contained 10\% of the reverse transcribed RNA, 2.5 units of Taq polymerase, 20 \textmu{}M dNTPs, 0.40 \textmu{}M of each primer, and PCR buffer containing 3 \textmu{}M MgCl\textsubscript{2}. Amplification was performed for 35 cycles with 45 seconds of denaturation at 95°C, 45 seconds of annealing at 60°C and 2 minutes of extension at 72°C. PCR product was cloned into the pGEM-T vector (Promega) and transformed into DH5\alpha \textit{E. coli} competent cells (Gibco/BRL). A colony containing an insert of the appropriate size was isolated and the presence of \textit{Nramp} cDNA confirmed by dideoxy DNA sequencing using Sequenase (US Biochemical Co. Cleveland, OH). The \textit{Nramp} cDNA insert was excised from the pGEM-T plasmid by digestion with Sst I and Sst II and agarose gel purified for Northern blot analysis.

\textbf{Statistical Analysis:} Analysis of the data was carried out using the Systat statistical program (V. 5.2, SYSTAT Inc., Evanston, IL). Each observation of antimycobacterial activity or NO production was analyzed using an analysis of variance (two factor ANOVA) done on both log and absolute scale for comparison. In most cases, the data are expressed as the mean of replicate trials. In some cases, representative data are shown. Pairwise comparisons between dosage treatment groups were determined using the Bonferroni adjusted correlation. All declarations of significance are listed as worst case values.
RESULTS

Cytokine Induced Activation of Macrophages from \textit{Bcg}' and \textit{Bcg}' Mice: Macrophages from \textit{Bcg}' and \textit{Bcg}' mice responded equally to stimulation with rIFN-\(\gamma\) and to GM-CSF. The results in Figure 4.1 shows that 500 U per well of rIFN-\(\gamma\) resulted in 52\% of the growth of \textit{M. avium} within macrophages from \textit{Bcg}' mice not activated with cytokine. Similarly, \textit{M. avium}, within macrophages from \textit{Bcg}' mice, grew to only 44\% of control levels. Increasing amounts of rIFN-\(\gamma\) resulted in increased growth inhibition. The ability of GM-CSF to activate macrophages from \textit{Bcg}' and \textit{Bcg}' mice was similar to that observed with rIFN-\(\gamma\). Thus, 100 U per well of GM-CSF resulted in 48\% and 52\% of control growth of \textit{M. avium} by macrophages from \textit{Bcg}' and \textit{Bcg}' mice respectively (Figure 4.2).

Differential Effect of Corticosterone on Antimycobacterial Activity of Macrophages:
The results in Figure 4.3 and 4.4 shows that corticosterone treatment of rIFN-\(\gamma\) or GM-CSF activated macrophages increased the permissiveness of cells from \textit{Bcg}' mice for \textit{M. avium} growth. While treatment with rIFN-\(\gamma\) or with GM-CSF resulted in 60\% to 50\% of the growth of control cultures not activated with cytokine, the addition of corticosterone increased Mycobacterial growth to 80\% of control levels. In contrast, corticosterone did not affect the capacity of the activated macrophages from \textit{Bcg}' mice to inhibit the growth of \textit{M. avium}.

Role of Reactive Nitrogen Intermediates: Previously, we have shown that corticosterone suppressed the production of TNF-\(\alpha\) and NO by splenic macrophages from \textit{Bcg}' and \textit{Bcg}'
Figure 4.1: Interferon-gamma Induced Activation of Macrophages from BCG Resistant and Susceptible Mice. Splenic macrophages were treated with rIFN-γ for 24 hrs prior to infection with 4×10⁵ cfu M. avium. After 24 hrs to allow for phagocytosis and following removal of the unphagocytized bacteria the cultures were incubated in the continued presence of rIFN-γ for 5 days prior to lysis of the macrophages and pulsing of the bacteria with ³H-uracil. The data are expressed as the percent of control which represents the amount of radioactivity incorporated by the bacteria after infection of cultures of macrophages treated with rIFN-γ divided by that of cultures not treated with rIFN-γ. The effect of rIFN-γ was significant at p≤0.003 as determined by ANOVA. There was no statistical difference between macrophages from Bcg⁺ and Bcg⁻ mice.
Figure 4.1

rIFN-gamma Activation of Macrophages from BCG Susceptible and Resistant Mice
Figure 4.2: GM-CSF Induced Activation of Macrophages from BCG Resistant and Susceptible Mice. Macrophages were treated as described for Figure 4.1 except the cells were treated with GM-CSF for 24 hrs prior to infection. The effect of GM-CSF was significant at p<0.02 by ANOVA. There was no statistical differences between macrophages from \textit{Bcg}^+ and \textit{Bcg}^-' mice.
Figure 4.2

Mycobacterial Growth (% of Control)

GM-CSF (Units/well)

BCG Susceptible

BCG Resistant
Figure 4.3: Differential Effect of Corticosterone on rIFN-γ Induced Anti-mycobacterial Activity of BCG Resistant and Susceptible Mice. Macrophages were treated and infected as described in the legend for Figure 4.1. After infection, the cultures were treated with $10^{-6}$ M corticosterone for 5 days prior to the lysis of the macrophages and addition of $^3$H-uracil. The effect of corticosterone on the anti-mycobacterial activity of rIFN-γ activated macrophages from $Bcg^r$ mice was significant ($p \leq 0.03$).
Figure 4.3
Figure 4.4: Differential effect of corticosterone on GM-CSF induced anti-Mycobacterial activity of BCG resistant and susceptible mice. The cultures were treated as described for Figure 4.3. The effect of corticosterone on the anti-mycobacterial activity of the GM-CSF activated macrophages was significant ($p \leq 0.03$).
Figure 4.4
Time of Incubation in Macrophages | CPM Incorporated by *M. avium* after incubation within macrophages from: | BCG susceptible | BCG resistant
---|---|---|---
Day 0 | 2,130±185\(^b\) | 1,982±223
Day 5 | 13,280±721\(^c\) | 8,213±198
No treatment | 13,413±939\(^d\) | 8,541±256
+NMMA

\(^a\) Macrophages were infected with *M. avium* by overnight incubation. After washing to remove unphagocytized bacteria the macrophages were lysed immediately and the bacteria pulsed with \(^3\)H-uracil (Day 0) for 24 hrs or the cultures were incubated for an additional 5 days in the absence or presence of 250 \(\mu M\) of NMMA. The macrophages were lysed and the bacteria pulsed with \(^3\)H-uracil.

\(^b\) The differences in the uptake of *M. avium* between macrophages from *Bcg*\(^c\) and *Bcg*\(^d\) mice is not significant.

\(^c\) The growth of *M. avium* in macrophages from *Bcg*\(^c\) and *Bcg*\(^d\) mice is significant (\(p<0.001\))

\(^d\) The effect of NMMA on Mycobacterial growth was not significant.

Table 4.1: Effect of \(N^{0}\)-Monomethyl-l-Arginine (NMMA) on Innate Resistance Mediated by *Bcg* (Candidate Nramp).\(^a\)
mice stimulated \textit{in vitro} with rIFN-\(\gamma\) and \textit{M. avium} without affecting the capacity of macrophages from the resistant mice to control the growth of the Mycobacteria (Brown et al. 1994). This suggested that the anti-mycobacterial activity of the macrophages from \textit{Bcg}\(^{\prime}\) mice was independent of the production of NO. In order to test this we determined the effect of NMMA on innate resistance and on the anti-mycobacterial activity and NO production by rIFN-\(\gamma\) or GM-CSF activated macrophages. The results in Table 4.1 shows that the addition of NMMA to cultures of macrophages did not affect the natural resistance of the macrophages from the \textit{Bcg}\(^{\prime}\) mice.

Figure 4.5a shows that the addition of NMMA ameliorated the anti-mycobacterial activity of macrophages from both \textit{Bcg}\(^{\prime}\) and \textit{Bcg}\(^{\prime}\) mice activated with rIFN-\(\gamma\). In contrast, the addition of NMMA did not affect the anti-mycobacterial activity of macrophages activated by treatment with GM-CSF (Figure 4.6a). Similarly, the results in Figure 4.5b shows that rIFN-\(\gamma\) activated macrophages produced as much as 40 \(\mu\)M of NO which was reduced in the presence of NMMA. However, the addition of GM-CSF, which activated the macrophages to suppress mycobacterial growth, induced less then 10\(\mu\)M of NO (figure 4.6b). Furthermore, the addition of NMMA to these cultures failed to alter the level of NO and did not affect the anti-mycobacterial activity of the cells.

\textbf{rIFN-\(\gamma\) and GM-CSF induce the expression of \textit{Nramp}.} It has been suggested that \textit{Nramp} may be a nitrate transporter or involved in NO mediated signal transduction. The results in Figures 4.7 and 4.8 show that both rIFN-\(\gamma\) and GM-CSF induced the expression of \textit{Nramp}
Figure 4.5: \(\text{N}^\text{O}\)-Monomethyl-l-arginine (NMMA) Suppresses the Anti-Mycobacterial Activity and the Production of Reactive Nitrogen Intermediates (NO) by rIFN-\(\gamma\) Activated Macrophages. A) Antimycobacterial activity: Macrophages were treated with rIFN-\(\gamma\) and infected with \(M.\text{avium}\) as described in the legend for Figure 1 except that the cultures were treated with 250 \(\mu\text{M}\) of NMMA concurrently with the addition of rIFN-\(\gamma\) and throughout the 5 day incubation period prior to the addition of \(^3\text{H}\)-uracil. The effect of NMMA on the anti-mycobacterial activity of macrophages from \(Bcg^\text{p}\) and \(Bcg^\text{r}\) mice was significant (p<0.03). B) Production of NO: Splenic macrophages were cultured in the presence of 250 U of rIFN-\(\gamma\), \(4\times10^3\) cfu \(M.\text{avium}\) with or without 250 \(\mu\text{M}\) of NMMA for 72 hrs. The amount of NO (nitrate) in the cell free supernatant fluids was determined by the Griess reaction. The effect of NMMA on NO production by macrophages from \(Bcg^\text{p}\) and \(Bcg^\text{r}\) mice was significant (p<0.02).
Figure 4.6: Lack of NO production by GM-CSF Activated Macrophages from $Bcg'$ and $Bcg'$ mice. A) Effect of NMMA on Anti-Mycobacterial Activity: Macrophages were stimulated and infected as describe in Figure 4.2 and treated with 250 μM of NMMA. The effect of NMMA was not significant. B) NO Production: Macrophages were treated with 250 U of GM-CSF and $4 \times 10^5 M. avium$ as well as 250 μM NMMA. The effect of GM-CSF on NO production was not significantly different then NO produced by macrophages not treated with GM-CSF. The effect of NMMA was not significant.
Figure 4.6

Reactive Nitrogen Intermediates (uM)

Mycobacterial Growth (% of Control)
Figure 4.7: Differential Effect of Corticosterone on \textit{Nramp} Expression by rIFN-γ Activated Macrophages from \textit{Bcg}$^+$ and \textit{Bcg}$^-$ Mice. Splenic macrophages (2X10$^6$) were treated with 2500 U rIFN-γ for 24 hrs prior to the addition of 10$^{-6}$M corticosterone. After 24 hrs the mRNA was extracted with 8M guanidine hydrochloride and the expression of \textit{Nramp} mRNA determined by Northern analysis.
Figure 4.7

<table>
<thead>
<tr>
<th>Nramp</th>
<th>Unstimulated</th>
<th>rIFN-γ</th>
<th>Unstimulated</th>
<th>rIFN-γ</th>
<th>Cort</th>
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<tr>
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<th>Unstimulated</th>
<th>rIFN-γ</th>
<th>Unstimulated</th>
<th>rIFN-γ</th>
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1 2 3 4 5 6 7 8

Bcgs  Bcgr

< 2.5 kb
Figure 4.8: Differential Effect of Corticosterone on *Nramp* Expression by GM-CSF Activated Macrophages from *Bcg* and *Bcg* Mice. Splenic macrophages were treated as described in Figure 4.7 except with 500 U GM-CSF. The expression of *Nramp* mRNA was determined by Northern analysis.
Figure 4.8

The image shows a gel analysis with two bands labeled "Nramp" and "Actin". The bands are compared under different conditions: Unstimulated, rGM-CSF, and Cort. The gel is labeled with "BcgS" and "Bcgr". There is a marker at 2.5 kb.
by macrophages from both $Bcg^{-}$ and $Bcg^{+}$ mice. However, we found that corticosterone suppressed $Nramp$ expression by macrophages from $Bcg^{-}$ mice but did not suppress $Nramp$ expression by macrophages from $Bcg^{+}$ mice.

**DISCUSSION**

The results of this investigation show that the effects of corticosterone on macrophages from congenic BCG resistant and susceptible mice differ and that the difference appears to be related to the different effects of the hormone on the $Bcg$ gene, (candidate $Nramp$). The results also show that rIFN-$\gamma$ and GM-CSF induce different anti-mycobacterial pathways. The rIFN-$\gamma$ induced pathway involves the production of NO and is inhibited by NMMA while the GM-CSF pathway does not appear to be dependent on NO production and is not inhibited by NMMA. Nevertheless, both the rIFN-$\gamma$ and GM-CSF induced pathways appear to be regulated by $Bcg$ because they are differentially affected by corticosterone.

Our results also appear to rule out a role for NO as an effector molecule in $Bcg$ mediated resistance for two reasons. First, NMMA does not interfere with innate resistance mechanisms. Secondly, corticosterone suppresses the production of NO by macrophages from $Bcg^{-}$ mice but does not affect their capacity to control the growth of $M. avium$ (Brown et al. 1993, 1994).

Our results are similar to those reported by others who have also shown that the capacity of macrophages to limit the growth of Mycobacteria and other intracellular
pathogens, is induced by IFN-γ and is dependent on the production of reactive nitrogen intermediates (Chan et al. 1992, Gazzinelli et al. 1993, Green et al. 1982, Stender et al. 1994). The anti-mycobacterial activity of macrophages from both Bcg' and Bcg' mice was inhibited by the addition of NMMA to the culture media. Our observation also supports that of Barrera et al (Barrera et al. 1994) and Roach et al (Roach 1994) who showed that macrophages from Bcg' and Bcg' mice that are activated in vitro with rIFN-γ, are more bacteriostatic or leishmanicidal than are macrophages not treated with rIFN-γ. The antimicrobial activity in both of those studies was ameliorated by the addition of NMMA. Similarly we also found that NMMA reduced the inhibition of M. avium growth by rIFN-γ activated macrophages. However, NMMA did not affect the innate capacity of macrophages from Bcg' mice to control the growth of M. avium.

Our observation that corticosterone suppressed the production of NO by macrophages from both BCG resistant and susceptible mice while only suppressing the capacity of macrophages from Bcg' mice to control M. avium growth confounds the interpretation of these results. These observations suggest that macrophages from Bcg' mice can control mycobacterial growth by a mechanism that is, in part, independent of the production of NO but controlled by the Bcg gene. This possibility is supported by our observation that NMMA did not affect the innate resistance mediated by resident splenic macrophage from Bcg' mice. Indeed, several recent reports have suggested that interferon-γ activated macrophages control the growth of intracellular parasites, including Mycobacteria, by a mechanism that is
independent of the production of NO as well as the production of reactive oxygen intermediates, depletion of tryptophan or sequestration of iron (Gebran et al. 1994, Nibberning et al. 1994, Polsinelli et al. 1994).

The differential effect of corticosterone on the capacity of rIFN-γ or GM-CSF activated macrophages from $Bcg^e$ and $Bcg^f$ mice is similar to that which we have previously reported for macrophages not activated by cytokines (Brown et al. 1993, 1994). Thus, corticosterone increased the permissiveness of resident splenic macrophages from $Bcg^e$ mice to the growth of $M. avium$ but did not result in an alteration of the growth pattern of the microorganism in macrophages from $Bcg^f$ mice.

GM-CSF has also been reported to increase the resistance of mice to the in vivo growth of $Salmonella typhimurium$ and to activate macrophages in vitro to control the growth of intracellular pathogens including $Mycobacterium avium$ (Appelberg and Orme 1993, Bermudez and Young 1990, Heidenreich et al. 1989, Morrisey et al. 1988, Suzuki et al. 1994, Wang et al. 1989). The in vivo effects of GM-CSF appear to be limited to an effect on macrophages from $Bcg^e$ mice (Nibberning et al. 1994). We have found, however, that the ability of GM-CSF to activate macrophages from both $Bcg^e$ and $Bcg^f$ mice in vitro was comparable.

The capacity of GM-CSF activated macrophages to control the growth of $M. avium$ was independent of the production of NO. This observation is similar to that reported by
Buisman et. al. (Buisman 1994) who found that GM-CSF did not induce the production of NO. Our observations are different, however, then that of Denis (Denis 1991) who found that the inhibition of growth of an avirulent strain of *M. avium* by GM-CSF activated human monocytes, was suppressed by treating the cells with NMMA.

The results of this investigation show, for the first time, that the expression of *Bcg* (candidate *Nramp*) is up-regulated by the addition of either rIFN-γ or GM-CSF. There does not appear to be a difference in the level of expression of *Nramp* mRNA by macrophages from *Bcg*° and *Bcg*° mice. Rather, the differences appear to be related to the effect of corticosterone on *Nramp* mRNA. The differences in the effect of corticosterone could be either on the stability of the mRNA, or on its transcription. In preliminary experiments we have found that other rIFN-γ inducible genes are also differentially regulated by corticosterone (unpublished observations). Since these genes do not map to chromosome 1 it suggests that differential regulation by corticosterone is a general phenomena of IFN-γ induction in macrophages from *Bcg*° and *Bcg*° mice.

Glucocorticoids have been shown to have inhibitory and stimulatory effects on macrophage function (Brown et al. 1993). The effects of glucocorticoids are produced following the binding of the steroid to its receptor which translocates to the nucleus and binds to glucocorticoid response elements (GRE) resulting in regulation of gene expression. Several studies have shown that positive or negative effects of glucocorticoids are altered by a single nucleotide difference in the GRE. It is possible therefore that the differential
regulation of *Nramp* expression by corticosterone, that we have observed, may be due to differences in the GRE located in the promoter region of *Nramp* in *Bcg* and *B cg* mice.
CHAPTER 5

THE DIFFERENTIAL EFFECT OF CORTICOSTERONE ON \textit{Nramp1} EXPRESSION MAPS TO \textit{Vil} BETWEEN \textit{1l8rb} AND \textit{Acrg} ON MOUSE CHROMOSOME 1 AND IS THEREFORE ASSOCIATED WITH \textit{Bcg}

INTRODUCTION

The use of congenic strains of mice which differ in defined chromosomal regions for which they have been differentially selected serve as valuable tools in the study of polygenic traits involved in disease resistance. The congenic strain C.D2-\textit{Idh-1b Ity-1b Rep-1b Pep3b} has been used to examine various anti-bacterial effector mechanisms and response to rIFN-\(\gamma\) including MHC class II expression associated with the natural resistance gene \textit{Ity/Lsh/Bcg} gene which confers resistance to Mycobacterial growth (Gros et al. 1981, Mock et al. 1994, Schurr et al. 1989). Through typing for resistance and susceptibility to BCG among recombinant inbred strains combined with linkage analyses and dissection of a 28-centimorgan segment on murine chromosome 1, the cloning of the cDNA for the \textit{Bcg} gene, designated \textit{Nramp1} (natural resistance associated macrophage protein) was acheived. \textit{Nramp1} contains a 1.4 kb open reading frame encoding a trans-membrane protein with homology to a eukaryotic nitrate transporter (Vidal et al.1993). Resistance typing of \textit{Bcg'+} and \textit{Bcg'} mice indicates that BCG susceptibility is the result of a glycine to asparagine substitution at
position 169 within a predicted transmembrane domain of *Nramp1*. The widely speculated molecular mechanism of how *Nramp1* regulates resistance to selected species of Mycobacteria remains unknown.

Previous studies in our laboratory have demonstrated that glucocorticoids result in the increased susceptibility of *Bcg*′ mice to *in vivo* growth of *Mycobacterium avium* (Brown et al. 1993, 1994). *In vitro* treatment of macrophages with exogenous glucocorticoids coincided with the observed *in vivo* effects in that the macrophages from *Bcg*′ mice were more permissive to the growth of intracellular Mycobacteria. Interestingly, glucocorticoids did not affect the resistance of *Bcg*′ mice or their macrophages to the *in vivo* or *in vitro* growth of *Mycobacterium avium*. Glucocorticoid treatment, however, suppressed the production of both TNF-α and of reactive nitrogen intermediates (NO). This suggests that *Nramp1* does not mediate regulation by a TNF-α or NO mediated pathway in mediating Mycobacterial resistance (Brown et al. 1994).

Based then upon these observations of the differential effects of glucocorticoids on mycobacterial resistance, we sought to extend our observations to include the effects of glucocorticoids on the expression of *Nramp1* by splenic macrophages from congenic *Bcg*′ and *Bcg*′ mice (Brown et al. 1995). We found that corticosterone suppressed the expression of rIFN-γ and rGM-CSF induced *Nramp1* (Brown et al. 1995) as well as other rIFN-γ induced genes by macrophages from *Bcg*′ mice (unpublished observations). Macrophages from *Bcg*′ mice were resistant to the suppressive effects of corticosterone on *Nramp1* expression. The
purpose of this investigation was to determine if the effect of corticosterone on the expression of *Nramp1* by splenic macrophages mapped close to *Nramp1* using C.D2-Vil congenic mice to further delineate the 28 cM region on chromosome 1 controlling this specific response.

Previous studies by Mock et al. (Mock et al. 1988, 1990, 1993) of southern blot analysis of haplotypes of six molecular markers in the 26-cM interval extending from Cryg to CchllA3 on chromosome 1 revealed that the closest marker to the *Ity/Lsh/Bcg* disease resistance gene (*Nramp1*) was found to be the villin gene (*Vil*). Founder mice, each heterologous at the *Vil* locus were chosen for further characterization and introgressive backcrosses and subsequently used to generate the 10 C.D2-Vil congenic strains which were made homozygous for the chromosome markers (Mock et al. 1993, Schurr et al. 1989).

In our study, splenic macrophages from C.D2-Vil strains 1, 4, 5, 6, 7, 8, 9, and 10 (BALB/c.Bcg') as well as from BALB/c (BALB/c.Bcg') were used to determine the effect of glucocorticoid treatment on *Nramp1* expression. The results of this study illustrated that *Nramp1* expression by macrophages from C.D2-Vil (Bcg') mice was unaffected by corticosterone treatment. As previously stated, *Nramp1* expression by macrophages from BALB/c (Bcg') was suppressed by corticosterone treatment. Corticosterone treatment of macrophages did not affect expression of *Nramp1* from any of the C.D2-Vil mice suggesting that the observed differential effects of glucocorticoids are associated (linked) with *Ity/Lsh/Bcg*. 

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MATERIALS AND METHODS

Animals: Male BALB/c, BCG-susceptible (BALB/c.Bcgs) mice were obtained from Charles River when six weeks of age. The animals were housed in groups of five in micro-isolation cages and given food and water ad libitum. Congenic C.D2ldh"-Ity'-Pep" mice, BCG-resistant (BALB/c.Bcg') mice were initially obtained from Dr. Michael Potter (NCI) (Potter et al. 1983) and bred in our facilities (Zwilling et al. 1992). Congenic C.D2-Vil mice were kindly provided by Dr. Beverly Mock (NIH) (Mock et al. 1993).

Reagents: Recombinant interferon gamma (rIFN-γ) was obtained from Gibco BRL (Grand Island, NY). Restriction enzymes and 32-P labelling reagents were obtained from Boehringer Mannheim. α32P-dCTP (sp.act.3,000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). The cDNA probe for Nrampl was produced by RT-PCR as previously described by us (Brown et al. 1995). The Actin cDNA probe was obtained by screening a macrophage cDNA library with an Actin specific oligonucleotide.

RESULTS

Splenic macrophages from Bcg' and Bcg' mice purified as previously described by us (Brown et al. 1993) were added in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco/BRL) containing 20% fetal bovine serum (FBS) (Hyclone, Logan, UT) containing less than 0.3 EU of endotoxin to 35 mm tissue culture dishes at a concentration of 5 X 10^6 macrophages per dish. Macrophage cultures were treated with rIFN-γ (10 u / 10^6 cells) for 24 hours. The macrophage cultures were washed and replaced with media containing 10^-6M corticosterone.
Figure 5.1. Differential effect of corticosterone is linked to \( Bcg \). Splenic macrophages from BALB/c.\( Bcg' \) (C.D2 \( Vil \)) and BALB/c.\( Bcg' \) mice were stimulated with \( rIFN-\gamma \) (10 u /10\(^6\) cells) for 24 hours. The macrophage cultures were then treated with corticosterone (10\(^{-6}\)M). After 20 hours, total RNA was extracted and the effect of corticosterone on \( Nramp1 \) expression was determined by Northern analysis. (1; C.D2-\( Vil1,4 \)), (2; C.D2-\( Vil5 \)), (3; C.D.2-\( Vil6 \)), (4; C.D.2-\( Vil7 \)), (5; C.D2-\( Vil8 \)), (6; C.D2-\( Vil9 \)), (7; C.D2-\( Vil10 \)), (8; C.D2\(^{LD} \),\( Pep^{rb} \)), (9, BALB/c.\( Bcg' \)).
Figure 5.1

Corticosterone treatment

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Figure 5.2  Map of resistance locus (Bcg/Ity/Lsh) on mouse chromosome 1. Haplotype analysis of 15 chromosome 1 markers for the series of recombinant C.D2 congenic strains. Each column represents the chromosome identified in the congenic strain. Open rectangles, BALB/cAnPt allele; solid rectangles, DBA/2Npt allele.
Figure 5.2: Map of \textit{Bcg/Ity/Lsh} resistance locus on mouse chromosome 1

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29.7 cM
After 20 hours, total RNA was extracted and separated by formaldehyde agarose gel electrophoresis and the effect on \textit{Nramp1} expression determined by Northern analysis. Treatment with corticosterone suppressed expression of \textit{Nramp1} only by macrophages from \textit{Bcg}\textsuperscript{*} (BALB/c.\textit{Bcg}) mice. Corticosterone did not affect the expression of \textit{Nramp1} by macrophages from C.D2.\textit{Vil} nor C.D2 \textit{Idh}\textsuperscript{*}-\textit{Ity}\textsuperscript{*}-\textit{Pep}\textsuperscript{3*}mice (Figure 5.1). The use of \textit{Vil} congenic mice, which are most closely associated with the resistance locus (\textit{Bcg}), allows us to map the effect of corticosterone induced suppression to the area on mouse chromosome 1 (Figure 5.2) where the \textit{Bcg} gene is situated. Therefore, our results suggest that the differential effect of corticosterone on \textit{Nramp1} expression by \textit{Bcg}\textsuperscript{*} and \textit{Bcg*} macrophages is linked to \textit{Bcg}, and therefore associated with innate resistance to mycobacterial growth.
CHAPTER 6

THE STABILIZATION OF mRNA: A FUNCTIONAL ROLE FOR \textit{Nrampl} IN CONTROLLING RESISTANCE TO MYCOBACTERIAL GROWTH IN MICE

INTRODUCTION

Resistance to Mycobacterial growth is controlled by a gene (\textit{Bcg}) that maps to mouse chromosome 1 (Schurr 1989). The \textit{Bcg} gene, \textit{Nrampl}, codes for a deduced membrane protein product termed natural resistance associated macrophage protein (Vidal 1993). Mice that are \textit{Bcg}\textsuperscript{+} were found to differ from \textit{Bcg}\textsuperscript{-} mice in a non-conservative Gly to Asp substitution within a predicted transmembrane domain of \textit{Nrampl}. The structural motif of \textit{Nrampl} is that of a transport protein, but the function of \textit{Nrampl} is still unknown. It has been suggested that the protein may be involved in the transport of nitrates to the phagolysosome of infected macrophages which accounts for the increased capacity of macrophages from \textit{Bcg} resistant mice to control the growth of Mycobacteria. Alternatively, it has been suggested that the protein plays a part in nitric oxide mediated signal transduction resulting in priming/activation of macrophages, thereby accounting for the pleiotropic effects that have been reported to be under the control of \textit{Bcg} (Formica 1994, Gazzinelli 1993, Roach 1994, Schurr 1989, Hilburger and Zwilling 1994).
Previous studies in our laboratory have shown that corticosteroids increased the susceptibility of *Bcg* *"* mice to *in vivo* growth of *Mycobacterium avium* (Brown and Zwilling 1993). In contrast, corticosteroids did not affect resistance of *Bcg* *'* mice to Mycobacterial growth. *In vitro* treatment of macrophages with corticosterone paralleled the *in vivo* effects in that treatment of macrophages from *Bcg* *"* mice increased permissiveness of macrophages to mycobacterial growth, while treatment of macrophages from *Bcg* *'* mice did not alter their resistance to growth of *M. avium* (Brown et al. 1994). We have demonstrated that macrophages from both *Bcg* *"* and *Bcg* *'* mice responded equally to recombinant interferon (rIFN-γ) or granulocyte-macrophage colony stimulating factor (rGM-CSF) mediated activation as assessed by their anti-mycobacterial activity. The addition of corticosterone to macrophage cultures suppressed the ability of rIFN-γ and rGM-CSF activated macrophages from congenic *Bcg* *"* mice but not from *Bcg* *'* mice to control mycobacterial growth (Brown et al. 1994). Although rGM-CSF did not induce the production of reactive nitrogen intermediates, rGM-CSF activated splenic macrophages from both *Bcg* *"* and *Bcg* *'* mice were able to control the growth of the mycobacteria *in vitro*. We also found that *Nramp1* mRNA expression by macrophages from both *Bcg* *"* and *Bcg* *'* mice was up-regulated by treatment of these cells with rIFN-γ and rGM-CSF (Brown et al. 1995). However, corticosterone suppressed the expression of *Nramp1* mRNA only by macrophages from *Bcg* *'* mice. The purpose of this study was to characterize the differential effects of corticosterone on the expression of *Nramp1* mRNA by macrophages from *Bcg* *"* and *Bcg* *'* mice. Our results show that corticosterone suppresses the mRNA expression of *Nramp1* and several other rIFN-γ induced genes by macrophages from *Bcg* *"* mice. Macrophages from *Bcg* *'* mice were
resistant to the suppressive effects of corticosterone. Functional \textit{Nramp1} resulted in the more stable expression of mRNA to \textit{Nramp1}, \textit{Mag-1(GBP-1)} (Paulnock et al. 1993), \textit{Mg21(GTP-2)} (Lafuse et al. 1995a) and MHC class II as well as \textit{TNF-\alpha} and \textit{iNOS} in the presence or absence of corticosterone. Since differences in mRNA stability can have dramatic effects on protein levels, the increased mRNA stability in macrophages from \textit{Bcg} mice could result in the sustained production of anti-mycobacterial effector molecules and thus could account for the increased resistance of macrophages from \textit{Bcg} mice.

**MATERIALS AND METHODS**

**Animals:** Male BALB/c, BCG-susceptible (BALB/c.\textit{Bcg} \textsuperscript{+}) mice were obtained from Charles River when six weeks of age. The animals were housed in groups of five in micro-isolation cages and given food and water \textit{ad libitum}. Congenic C.D2\textit{ldh} \textsuperscript{b}\textit{-Ity} \textsuperscript{+}\textit{-Pep} \textsuperscript{3b} mice, BCG-resistant (BALB/c.\textit{Bcg} \textsuperscript{+}) were initially obtained from Dr. Michael Potter (NCI) (Potter et al. 1983) and bred in our facilities.

**Reagents:** Recombinant interferon gamma (rIFN-\gamma) was obtained from Gibco BRL (Grand Island, NY). Recombinant granulocyte macrophage colony stimulating factor was purchased from Boehringer Mannheim (Indianapolis, IN) and corticosterone was obtained from Sigma (St. Louis, MO). Restriction enzymes were obtained from by Boehringer Mannheim. \textsuperscript{32}P-dCTP (SpAct 3,000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Mycobacterium avium was a recent clinical isolate and was prepared as previously described by us (Brown et al. 1993). The cDNA probes for \textit{Nramp1} was produced by RT-PCR as
previously described by us (Brown et al 1994). The cDNA probe for iNOS was produced by RT-PCR using primers obtained from Clontech (Palo Alto, CA). The cDNA clones of Mag-1 (Paulnock et al. 1993), Mg21 (Lafuse et al. 1995a) and MHC class II, I-A\textsubscript{e} (Lafuse et al. 1995b) were isolated from a macrophage cDNA subtrac tion library of rIFN-\(\gamma\) induced genes. The actin cDNA probe was obtained by screening a macrophage cDNA library with an actin specific oligonucleotide. The TNF\(\alpha\) probe is a 1.1 kb cDNA containing the entire coding region (Genosys, The Woodlands, TX).

**Analysis of Interferon-\(\gamma\) induced gene expression in macrophage cultures:** Splenic macrophages from both Bcg\(^{+}\) and Bcg\(^{-}\) mice were enriched by overnight adherence onto 100x20 mm tissue culture dishes (Falcon) using Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco/BRL, Gaithesburg, MD) supplemented with 20% defined fetal bovine serum (FBS) (Hyclone, Logan, UT) containing less than 0.03 ng/ml of endotoxin. Non-adherent cells were removed by gentle washing with Hank’s balanced salt solution (HBSS (Gibco/BRL) and the splenic macrophages were removed by scraping with cell scrapers as previously described by us (Brown et al. 1993). The adherent cells were then added in IMDM containing 20% FBS to 35 mm tissue culture dishes at a concentration of 5 x 10\textsuperscript{6} macrophages per dish. Following a second incubation for 16 hours at 37\(^{\circ}\)C, the purified splenic macrophage (>90% as determined by differential staining and staining for nonspecific esterase) were then washed with HBSS and the medium replenished with IMDM containing rIFN-\(\gamma\) and treated as described. Macrophage cultures were incubated at 37\(^{\circ}\)C in 5% CO\textsubscript{2} for the times indicated in each experiment.
After treatment, the macrophage monolayers were washed with ice-cold phosphate buffered saline (PBS) (Gibco/BRL) and incubated on ice for 20 minutes. The PBS was removed and replaced with lysing buffer containing 8M guanidine hydrochloride, 0.3 M sodium acetate and 10% sarcosyl laural sulfate. Total RNA was extracted as modified by Evans (Evans and Kamdar 1990). The isolated RNA was size fractionated by gel electrophoresis (1.5% formaldehyde agarose) and RNA transferred to Hybond-N+ membrane (Amersham) by capillary blotting. A separate lane containing a 0.24 - 9.5 Kb RNA marker ladder (GIBCO/BRL) was stained following electrophoresis with ethidium bromide and used to determine RNA size. Northern hybridization of the mRNA was carried out using the protocol as described by Maniatis et al. (Maniatis et al. 1982). Gel purified insert cDNA's were radiolabelled with $^{32}$P-dCTP by random priming. Autoradiographs were quantified using UVP ImageStore 5000 gel documentation program (San Gabriel, Ca) and NIH Image version 1.58. To determine if corticosterone transcriptionally regulated Nrampl, a nuclear run-off assay of newly synthesized mRNA was performed as described by Howard and Ortlepp (Howard and Ortlepp 1994).

RESULTS

Interferon-γ induces the expression of Nrampl mRNA by macrophages from both Bcg' and Bcg' mice. The results in Figures 6.1A shows macrophages from both strains of mice responded equally to stimulation with rIFN-γ. Maximal expression of Nrampl mRNA was induced with 10 U of rIFN-γ. Treatment of the macrophages for a minimum of 8 hours with 10 U of IFN-γ per 10⁶ cells was necessary to induce maximal expression of Nrampl mRNA.
Figure 6.1. Interferon-gamma induces the expression of *Nrampl* mRNA by macrophages from both *Bcg* and *Bcg* mice. (Panel A) Purified splenic macrophages from *Bcg* and *Bcg* mice were treated with increasing doses of rIFN-γ for 20 hours to determine dose required for maximal expression of *Nrampl*. (Panel B) Macrophages from *Bcg* mice were stimulated with 10 units per 10⁶ cells of rIFN-γ for increasing periods of time to determine length of rIFN-γ treatment required for maximal induction. Following treatment, total RNA was extracted and interferon-gamma induced expression of *Nrampl* mRNA was determined by Northern analysis. (Data for *Bcg* mice not shown).
Figure 6.1

A

units rIFN-γ / 10⁶ cells

Nramp

0 0.1 1 5 10 100
R S R S R S R S R S R S

Actin

B

Hours
1 2 4 8 12 24

Nramp

1

Actin

R $\rightarrow$ Bcg⁺
S $\rightarrow$ Bcg⁻
in both strains of mice (Figure 6.1B). The results of Figure 6.2 show that the addition of actinomycin-D or cycloheximide at the same time as rIFN-γ suppressed the transcription of *Nramp1* mRNA.

**Corticosterone differentially suppresses *Nramp 1* mRNA expression.** The addition of corticosterone to macrophages from *Bcg* * mice, previously stimulated with rIFN-γ, resulted in the suppression of *Nramp* mRNA in a dose dependent manner (Figure 6.3). *Nramp1* mRNA expression by macrophages from *Bcg* † mice was not affected by corticosterone. Treatment of the rIFN-γ stimulated macrophages from *Bcg* * mice with corticosterone for a minimum of 12 hours resulted in maximal suppression of *Nramp1* mRNA (data not shown). rIFN-γ mediated induction of *Nramp1* mRNA expression by macrophages from both *Bcg* † and *Bcg* * mice was inhibited when corticosterone was added at the same time as rIFN-γ. The results of these experiments indicate that the differential effect of corticosterone occurs following the induction of mRNA expression and appears to be regulated post transcriptionally.

**Corticosterone mediated suppression requires new protein and mRNA synthesis.** The addition of Actinomycin-D together with corticosterone to macrophages from *Bcg* * mice, previously treated with rIFN-γ for 24 hrs, abrogated the inhibitory effects of corticosterone on *Nramp1* mRNA expression (Figure 6.4). Similarly, treatment of the macrophage cultures from *Bcg* † mice with cycloheximide at the same time as corticosterone also prevented the suppression of *Nramp 1* mRNA expression. The expression of *Nramp1* mRNA by macrophages from *Bcg* † mice is not affected by corticosterone. Additionally, mRNA
Figure 6.2. Induction of *Nrampl* mRNA expression is inhibited by Actinomycin D and cycloheximide. Purified splenic macrophages (2 × 10^6) were stimulated with 10 units per 10^6 cells of rIFN-γ for 20 hours in the presence or absence of Actinomycin D (10 ug/2 × 10^6 cells) or cycloheximide (10 ug/2 × 10^6 cells). Following treatment, total RNA was extracted and the effect of Actinomycin D and cycloheximide on induction of *Nrampl* expression was determined by Northern analysis.
Figure 6.2.
Figure 6.3. Corticosterone differentially suppresses *Nramp 1* mRNA expression. Purified splenic macrophages from *Bcg* and *Bcg* mice were treated with increasing doses of corticosterone (10^{-8} M to 10^{-6} M) following 24 hour exposure to 10 units/10^6 cells of rIFN-γ. Additional macrophages were simultaneously treated with rIFN-γ and corticosterone for 24 hours. After 24 hours, total RNA was extracted and the effect of corticosterone on the induction and expression of *Nramp1* mRNA was determined by Northern analysis.
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<td>Cort dose (M) following 24 h rIFN-γ treatment</td>
<td>Cort dose (M) following 24 h rIFN-γ treatment</td>
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**Figure 6.3**
Figure 6.4. Corticosterone mediated suppression of *Nramp1* expression requires new protein synthesis. Purified splenic macrophages were stimulated for 20 hours with 10 units/10^6 cells of rIFN-γ followed by an additional 16 hour treatment of the cells with 10^{-6} M corticosterone in the presence or absence of the transcription inhibitor Actinomycin D (10 μg/ 10^6 cells) or translation inhibitor cycloheximide (10μg /10^6 cells). Following treatment, total RNA was extracted and the characterization of corticosterone induced suppression of *Nramp1* expression was determined by Northern analysis.
IFN-γ
IFN-γ then cort
IFN-γ then cort + Act D
IFN-γ then cort + CHX

Figure 6.4
expression of other rIFN-γ inducible genes, guanine nucleotide binding proteins Mag-1 (Paulnock et al. 1993), 21-1 (Lafuse et al. 1995a) as well as MHC class II (Lafuse et al. 1995b) were differentially suppressed in rIFN-γ stimulated macrophages from \( Bcg' \) mice but not in macrophages from \( Bcg' \) mice (Figure 6.5).

**Corticosterone does not affect \( Nramp \) transcription.** In order to confirm that corticosterone did not affect the transcription of \( Nramp \) mRNA, macrophages were treated with rIFN-γ for 24 hrs prior to the addition of corticosterone. Cells were lysed and nuclei isolated at the times indicated after the addition of corticosterone. The results of the nuclear run-off assays (Figure 6.6) showed that corticosterone treatment did not affect the transcription of \( Nramp \) mRNA by macrophages from \( Bcg' \) mice. In other experiments, we added actinomycin D 8 hours after the addition of corticosterone to cultures of macrophages from \( Bcg' \) mice. Northern analysis showed that \( Nramp \) mRNA levels decreased following the addition of the transcriptional inhibitor (Figure 6.7). The calculated half-life of the \( Nramp \) mRNA from corticosterone treated macrophages from \( Bcg' \) mice was 7 hrs. During this time the expression of \( Nramp \) mRNA by macrophages from \( Bcg' \) mice remained relatively stable with a half-life of 27 hrs. Thus, in the absence of continued mRNA transcription, the level of \( Nramp \) mRNA decreased with time. The results in Figure 6.8 show that the half-life of \( Nramp \) mRNA as well as that of Mag-1 were also different in macrophages from \( Bcg' \) and \( Bcg' \) mice in the absence of corticosterone.
Figure 6.5. Differential effect of corticosterone on rIFN-γ induced genes by macrophages from Bcg' and Bcg' mice. Splenic macrophages from Bcg' and Bcg' mice were stimulated with rIFN-γ (100 U/10^6 cells) for 24 hours followed by treatment with corticosterone (10^{-6} M). After 20 hours, total RNA was extracted and the effect of corticosterone on the expression of Mag-1, 21-1, MHC II, and Nrampl mRNA was determined by Northern Analysis.
Figure 6.6 Corticosterone does not affect \textit{Nramp1} transcription. Splenic macrophages from \textit{Bcg}° mice were treated with rIFN-\gamma (10 U/10^6 cells) for 24 hours prior to the addition of corticosterone (10^{-6} M) for the times given. The cells were lysed and mRNA was extracted from isolated nuclei. To determine if corticosterone affected transcription of \textit{Nramp1} mRNA, the nuclei were radiolabelled and hybridized to membrane bound \textit{Nramp1} cDNA and control \textit{B-Actin} cDNA.
Figure 6.6. Corticosterone Treatment
Figure 6.7. Corticosterone induces accelerated decay of *Nramp1* mRNA expression by macrophages from *Bcg'* mice. Splenic macrophages (3.0 X 10⁶) from *Bcg* and *Bcg* mice were stimulated with 10 units rIFN-γ (10 U/10⁶ cells) followed by treatment with corticosterone (10⁻⁶ M). Actinomycin D (10 ug /10⁶ cells) was added 8 hours after the addition of corticosterone (10⁻⁶ M), total RNA was extracted and the effect of corticosterone on *Nramp1* mRNA half-life was determined by Northern and densitometric analysis. (Even loading of RNA samples was confirmed with β-Actin, not shown here.)
Figure 6.7:
Figure 6.8. The half-lives of *Nramp1* (A) and *Mag-1* (B) mRNA differ between macrophages from *Bcg* and *Bcg* mice. Splenic macrophages from *Bcg* and *Bcg* mice were treated with rIFN-γ (10 u/10^6 cells) as previously described. Actinomycin D (10ug/10^6 cells) was added to the macrophage cultures 12 hours after the addition of rIFN-γ and the resultant half-lives of *Nramp1* and *Mag-1* were determined following Northern and densitometric analysis. The half-life of *Nramp1* expressed by macrophages from *Bcg* mice was determined to be 14 hours as compared to 25 hours by macrophages from *Bcg* mice. The half-life of *Mag-1* expressed by macrophages from *Bcg* was determined to be 10 hours as compared to 15 hours by macrophages from *Bcg* mice. (X axis: hours after Actinomycin D treatment, Y axis: percent mRNA remaining)
Figure 6.8
Resistance to corticosterone mediated suppression requires functional \textit{Nramp1} expression. We have previously shown that rIFN-\(\gamma\) and rGM-CSF induce \textit{Nramp1} mRNA (Brown et al. 1995). However, GM-CSF alone will not induce \textit{iNOS} and only low levels of TNF-\(\alpha\) mRNA. In order to determine if the prior induction of a functional \textit{Nramp1} was required for \textit{iNOS} and TNF-\(\alpha\) mRNA stability, we treated macrophages from \textit{Bcg} and \textit{Bcg'} mice with GM-CSF in order to induce \textit{Nramp1}. The macrophages were then treated with LPS for 8 hours. This resulted in the induction of both TNF-\(\alpha\) and \textit{iNOS} mRNA. The cultures were then treated with corticosterone for 12 hours and the presence of TNF-\(\alpha\) and \textit{iNOS} mRNA determined by Northern analysis. The results in Figure 6.9 showed, as expected, that rGM-CSF did not induce TNF-\(\alpha\) or \textit{iNOS} mRNA; treatment of the primed macrophages with LPS resulted in the induction of both TNF-\(\alpha\) and \textit{iNOS} mRNA. The addition of corticosterone resulted in a decrease in the level of both TNF-\(\alpha\) and \textit{iNOS} mRNA in macrophages from both \textit{Bcg'} and \textit{Bcg} mice not previously treated with rGM-CSF to induce \textit{Nramp1} expression. Thus, in the presence of a functional \textit{Nramp1} product, the TNF-\(\alpha\) and \textit{iNOS} mRNA’s were stable. In contrast when \textit{Nramp1} is not functional, as is the case for \textit{Bcg'} macrophages, the addition of corticosterone accelerated the decay of TNF-\(\alpha\) and \textit{iNOS} mRNA. When we added actinomycin D to cultures stimulated with rGM-CSF and LPS we found that the half lives of TNF-\(\alpha\) and \textit{iNOS} mRNA was 2 hrs in macrophages from \textit{Bcg'} mice and was 4hrs in macrophages from \textit{Bcg'} mice in the absence of corticosterone (Figure 6.10). Thus, the half-life of the mRNA of macrophages from \textit{Bcg'} mice is greater than the half-life of mRNA of macrophages from \textit{Bcg'} mice, even in the absence of corticosterone.
Figure 6.9. Resistance to corticosterone mediated suppression requires functional *Nramp1* expression. Splenic macrophages were stimulated with GM-CSF (250 units/5 X 10^6 cells) for 20 hours to induce *Nramp1* expression. The macrophages were then treated with LPS (100ng) for 8 hours to induce *TNF-*α and *iNOS* mRNA. The cultures were then treated with corticosterone (10^-6 M) for 12 hours. Other macrophage cultures were stimulated with LPS (100 ng/5 X 10^6 cells) only or not treated. The effect of prior induction of *Nramp1* expression on induced stability of *TNF-*α and *iNOS* mRNA was determined by Northern analysis with corresponding cytokine cDNA probes.
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<th>iNOS</th>
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Figure 6.10. The half-lives of TNF-α (A), iNOS (B) and Nramp1 (C) mRNA differ between macrophages from Bcg' and Bcg' mice. Splenic macrophages from Bcg' and Bcg' mice were treated with GM-CSF and LPS as previously described in Figure 6.9. Actinomycin D (10ug/10^6 cells) was added to the macrophage cultures 8 hours after the addition of LPS and the resultant half-lives of TNF-α and iNOS mRNA were determined following Northern and densitometric analysis. The half-life of TNF-α expressed by macrophages from Bcg' and Bcg' mice was 2 and 4 hours, respectively. The half-life of iNOS expressed by macrophages from Bcg' and Bcg' mice was 2 and 4 hours, respectively. The half-life of Nramp1 expressed by macrophages from Bcg' and Bcg' mice was 15 and 26 hours, respectively. (X axis: hours after Actinomycin D treatment, Y axis: percent of mRNA remaining)
Figure 6.10

A. 

B. 

C. 

Figure 6.10
DISCUSSION

The results of this investigation indicate that the differences in resistance to microbial growth mediated by Nramp1 appears to be regulated at the level of mRNA stability. Thus, the addition of corticosterone results in the destabilization of mRNA in macrophages from Bcg' mice. Differences in the decay of mRNA in macrophages was also observed without the addition of corticosterone. The glucocorticoid hormone however resulted in an accelerated decay of macrophage mRNA in macrophages from Bcg' mice.

The differential effect of corticosterone appears to require the expression of a functional Nramp1. Thus, following treatment of macrophages from Bcg' mice with rIFN-γ, expression of Mag-1, 21-1 and MHC Class II as well as Nramp1 mRNA was stable. When TNF-α and iNOS mRNA were induced (LPS only) prior to the induction of Nramp1, their mRNA was unstable and was degraded. In contrast, if TNF-α or iNOS mRNA were induced after the induction of Nramp1 (via GM-CSF) in macrophages from Bcg' mice, then iNOS and TNF-α mRNA were stably expressed. The results of these experiments also show that a low level of Nramp1 mRNA was present following stimulation with LPS. This was likely due to an indirect effect mediated by the production of GM-CSF which is induced following stimulation of macrophages with LPS. The presence of Nramp 1 protein in these macrophages was probably not sufficient to abrogate the effect of corticosterone on TNF-α and iNOS mRNA. Since Nramp1 produced by macrophages from Bcg' mice is not functional, the addition of corticosterone results in the destabilization of the mRNA. The effect of corticosterone required the synthesis of new mRNA and protein. This suggests that
corticosterone may induce the synthesis of a novel RNAse or some other factor that results in RNA destabilization. The induction or function of this protein is prevented by the functional expression of the *Nramp1* gene product.

*Nramp1* is constitutively expressed by macrophages from both *Bcg* and *Bcg* mice and is up-regulated by rIFN-γ. There were no apparent differences in the level of constitutive or induced expression of *Nramp1* mRNA by macrophages from *Bcg* and *Bcg* mice. The amount of rIFN-γ required to induce maximal *Nramp1* mRNA expression by macrophages from both strains of mice was identical. Similarly, we have previously reported that the level of expression of MHC class II glycoproteins by macrophages from both strains of mice was identical immediately following treatment with rIFN-γ. We have also reported that the level of MHC class II glycoprotein expressed by macrophages from *Bcg* mice decreased following the removal of rIFN-γ. However, macrophages from *Bcg* mice continued to express MHC class II glycoproteins (Zwilling et al. 1990). This difference can be accounted for by the differences we have observed in the stability of the mRNA. Similar differences in the functional capacity of macrophages from *Bcg* and *Bcg* mice may also be accounted for by differences in mRNA stability. Thus, differences in the levels of cytokines including TNF-α or of NO produced by the cells may be the result of differences in the stability of the mRNA.

RNA stability has been the subject of considerable investigation. Stability of mRNA is determined by different elements along the message (Hentze et al. 1991, Sachs et al. 1993, Peltz 1991). These mRNA sequence elements include the cap structure, 5' untranslated
secondary structures, premature termination codons, open reading frame sequences, 3' untranslated region sequences, AU rich regions and the poly (A) tail (Peppel et al. 1991). Degradation of mRNA requires translation as well as deadenylation of the polyA tail. Protection from degradation is the result of the binding of mRNA by proteins binding to mRNA sequence elements. This results in masking or activation of these elements that lead to deadenylation. Once deadenylation has occurred the sequence elements of the mRNA may provide binding sites for nucleases that result in the rapid degradation of the mRNA.

Glucocorticoids can stimulate or inhibit macrophage gene expression (Barnes and Barrows 1993, Beato 1989, Karin et al. 1993, Yamamoto 1985). Treatment of macrophages with glucocorticoids can result in the stimulation of MIF production (Calandra et al. 1995) or Fc receptor expression (Vogel et al. 1994). In contrast glucocorticoids inhibit the production of other cytokines such as IL-1 or TNF-α as well as MHC class II glycoprotein expression (Kern et al. 1988, Lee et al. 1988, Northup et al. 1992, Zwilling and Pearl 1992, Johnson and Zwilling 1984, Koff and Dunegan 1985). The mechanism of glucocorticoid mediated effect on gene expression has received much attention recently. Glucocorticoids can affect cell function following binding of the receptor to glucocorticoid response elements, 5' to the transcription start site, and stimulate or suppress the initiation of transcription depending on whether the activated receptor binds to positive or negative GRE's. Alternatively, glucocorticoid receptors can interfere with the binding of other transcriptional activators to their response elements. Both mechanisms result in transcriptional regulation of gene expression. The results of our investigation indicate that the glucocorticoid mediated
destabilization of mRNA is inhibited by inhibitors of mRNA and protein synthesis. Further, the observation that the corticosterone mediated effect does not result in an inhibition of Nramp1 mRNA synthesis indicates that the loss of Nramp1 mRNA is the result of its degradation. The exact mechanism of mRNA destabilization is not known. A recent report by Peppel et al (Peppel et al. 1991) has suggested that glucocorticoids may directly activate a ribonuclease that degrades mRNA containing AU-rich sequences in the 3' UTR. The increase in mRNA turnover was not prevented by cycloheximide indicating that the mechanism of the glucocorticoid mediated effect did not require new protein synthesis. In contrast, the effect mediated by corticosterone in our experiments was inhibited by the addition of cycloheximide. Thus, it appears that glucocorticoids can mediate mRNA instability by at least two different mechanisms.

What is the relationship of Nramp1 expression, glucocorticoid mediated destabilization of mRNA and disease resistance? We believe that the glucocorticoid mediated suppression serves to define a possible role of Nramp1. Corticosterone induces mRNA destabilization when Nramp1 is not functional. Similarly, when functional Nramp1 is not induced, mRNA to TNF-α and to iNOS was not stable. Thus, Nramp1 controls mRNA stability. When the mRNA is stable, activated macrophages can produce anti-mycobacterial effector molecules for prolonged periods accounting for increased resistance. An attractive candidate for mediating mRNA stability is the iron response element-binding protein. Induced under conditions of low intracellular iron levels, IRE-BP binds to an iron response element in the 3' UTR and results in the stabilization of transferrin receptor mRNA. Interestingly, NO
has also been reported to induce mRNA stability via IRE-BP (Drapier et al. 1993, Weiss et al. 1993, 1995). Thus, macrophage activation, induction of functional *Nramp1* and iron metabolism appear to be intimately linked. Does *Nramp1* control iron transport in a way that maintains low levels of intracellular iron, high levels of IRE-BP and stable mRNA? Preliminary studies in our laboratory suggest that this may be the case.
CHAPTER 7

THE ROLE OF INTRACELLULAR IRON IN RESISTANCE TO MYCOBACTERIA: PROPOSED ROLE OF \textit{Nramp1} AS AN IRON IRON TRANSPORT PROTEIN IN MACROPHAGES FROM BCG-RESISTANT MICE

INTRODUCTION

Experimental studies on the genetics of resistance to a variety of infectious agents including Mycobacterial infections has been widely studied. Typing for resistance and susceptibility to \textit{Mycobacterium bovis} strain BCG among recombinant inbred mouse strains combined with linkage analyses and dissection of a 30-centimorgan segment on murine chromosome 1, the cloning of the cDNA for the \textit{Bcg} gene, designated \textit{Nramp1} (natural resistance-associated macrophage protein), has been achieved (Malo et al. 1993a, 1993b, Vidal et al. 1993). Sequence analysis of \textit{Nramp1} reveals a 1.4 kb open reading frame encoding a trans-membrane 484-amino acid protein with structural homology to a eukaryotic nitrate transporter. Resistance typing of \textit{Bcg}\textsuperscript{+} and \textit{Bcg}\textsuperscript{−} mice indicates that BCG susceptibility is the result of a glycine to asparagine substitution at position 105 within a predicted transmembrane domain of \textit{Nramp1}. While it is accepted that the \textit{Bcg}\textsuperscript{+} gene confers resistance to mycobacteria during the early non-immune phase of infection, the molecular mechanism of
exactly how Nrampl regulates resistance and susceptibility to Mycobacterial infection is still unknown. Nrampl mRNA's are preferentially expressed in reticuloendothelial cells, in particular splenic macrophages.

Previous studies in our laboratory have shown that glucocorticoids result in the increased susceptibility of Bcg' mice to in vivo growth of Mycobacterium avium (Brown et al. 1993). In vitro treatment of macrophages with exogenous glucocorticoids coincided with the observed in vivo effects in that the macrophages from Bcg' mice were more permissive to the growth of intracellular Mycobacteria (Brown et al. 1994). In contrast, glucocorticoids did not affect the resistance of Bcg' mice or their macrophages to in vivo or in vitro growth of Mycobacterium avium. In addition, we have demonstrated that corticosteroids suppressed the production of TNF-α and of reactive nitrogen intermediates by macrophages from both strains of mice without affecting the resistance of macrophages from Bcg' mice to mycobacterial growth. Corticosterone has also been shown to suppress the antimycobacterial activity of rIFN-γ and rGM-CSF activated macrophages from congenic Bcg' mice but not from Bcg' mice (Brown et al. 1993).

Based upon the observations of the differential effects of glucocorticoids on mycobacterial resistance on a cellular level, we sought to characterize the differential effects of corticosterone on the expression of Nrampl mRNA by macrophages from Bcg' and Bcg' mice. We found that corticosterone suppressed the expression of rIFN-γ and rGM-CSF induced Nrampl mRNA as well as other IFN-γ induced genes by macrophages from Bcg'
mice (Brown et al. 1995, unpublished observations). Macrophages from $Bcg'$ mice were resistant to the suppressive effects of corticosterone. Further studies have shown that the different effects of corticosterone could be accounted for by differences in the stability of mRNA in macrophages from the congenic strains of mice. Thus mRNA's of $Nramp1$, $Mag-1$, $Mg21$, $TNF-\alpha$ and $iNOS$ were more stable in macrophages from $Bcg'$ mice than in macrophages from $Bcg'$ mice.

The glucocorticoid mediated destabilization of mRNA's of macrophages from $Bcg'$ mice may serve to define a possible role for $Nramp1$. The purpose of this study was to evaluate the relationship of iron and mRNA stability in the resistance to mycobacterial growth by macrophages from $Bcg'$ mice. Our results show that low levels of intracellular iron in the macrophages from $Bcg'$ mice result in stabilized mRNA expression. Levels of intracellular iron detected in splenic macrophages from $Bcg'$ mice were lower as compared to iron levels measured in macrophages from $Bcg'$ mice. Iron chelation with desferoxamine resulted in the abrogation of corticosterone induced decay of $Nramp1$ mRNA expression by macrophages from $Bcg'$ mice. Conversely, iron overloading of macrophages from $Bcg'$ mice resulted in unstable $Nramp1$ mRNA following corticosterone treatment.

**MATERIALS AND METHODS**

**Animals:** Male BALB/c, BCG-susceptible (BALB/c.$Bcg'$) mice were obtained from Charles River at six weeks of age. The animals were housed in groups of five in micro-isolation cages
and given food and water *ad libitum*. Congenic C.D2idh<sup>−/−</sup>-Ity<sup>−/−</sup>-Pep-3<sup>−/−</sup> mice, BCG resistant (BALB/c.<em>Bcg</em>) were initially provided by Dr. Michael Potter (NCI) (Potter et al. 1983) and bred in our facilities (Zwilling et al. 1992).

**Reagents:** Recombinant interferon gamma (rIFN-γ) was obtained from Gibco BRL (Grand Island, NY). Recombinant granulocyte macrophage colony stimulating factor (rGM-CSF) was purchased from Boehringer Mannheim (Indianapolis, IN) and corticosterone was purchased from Sigma (St. Louis, MO). Reagents for the chelation of, loading of, and detection of iron including desferroxamine, ferric ammonium sulfate and ferrozine were purchased from Sigma. The cDNA probe for <em>Nramp1</em> was produced by RT-PCR as previously described by us. The <em>Actin</em> cDNA probe was obtained by screening a macrophage cDNA library with actin specific oligonucleotides (Lafuse et al. 1992).

**Analysis of induced gene expression in macrophage cultures:** Splenic macrophages from both <em>Bcg</em> and <em>Bcg</em> mice were enriched by overnight adherence onto 100 X 15mm tissue culture dishes (Falcon, Franklin Lakes, NJ) using Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco/BRL, Gaithersburg, MD) supplemented with 20% defined fetal bovine serum (FBS) (Hyclone, Logan, UT) containing less than 0.03 EU/ml of endotoxin. Non-adherent cells were removed by gentle washing with Hank’s Balanced Salt Solution (HBSS) (Gibco/BRL) and the splenic macrophages were removed using cell scrapers as previously described by us (Brown et al. 1993). The adherent cells were then added in IMDM containing 20% FBS to 35 mm tissue culture dishes at a concentration of 5 X 10<sup>6</sup>
macrophages per dish. Following a second incubation for 16 hours at 37°C, the purified splenic macrophages (as determined by both differential and nonspecific esterase staining) were then washed with HBSS and the medium replenished with IMDM containing rIFN-g or rGM-CSF and subsequently treated as described. Macrophage cultures were incubated at 37°C in 5% CO₂ for the times indicated in each experiment.

After treatment, the macrophage monolayers were washed with ice-cold phosphate buffered saline (PBS) (Gibco/BRL) and incubated on ice for 20 minutes. The PBS was removed and replaced with lysing buffer containing 8M guanidine hydrochloride, 0.3 M sodium acetate and 10% sarcosyl. Total RNA was extracted as modified by Evans. The isolated RNA was size fractionated by gel electrophoresis (1.5 % formaldehyde agarose) and RNA transferred to Hybond-N+ membrane (Amersham) by capillary blotting. A separate lane containing a 0.24 - 9.5 kb RNA marker ladder (Gibco/BRL) was stained following electrophoresis with ethidium bromide and used to determine RNA size. Northern hybridization was carried out using the protocol as described by Maniatis et al. (Maniatis 1982). Gel purified insert cDNA's were radiolabelled with ³²P-dCTP by random priming. Autoradiographs were quantified using UVP Imagestore 5000 gel documentation program (San Gariel, CA) and NIH Image version 1.58.

**Determination of intracellular iron in macrophages:** The iron content of splenic macrophages was determined by a modification of Carter's method (Carter 1971) using the iron buffer recommended by McGowan (McGowan et al. 1988). Briefly, purified splenic
macrophages from $Bcg'$ and $Bcg'$ mice were resuspended in equal volumes of nuclease free ddH$_2$O (Sigma) and 6N HNO$_3$ (Fisher Scientific, Fairlawn, NJ) and incubated at 80°C for 16 hours to digest the cells and release bound iron. The iron was reduced to Fe'' and complexed to the colorimetric reagent, ferrozine. The ferrous iron concentration was quantitated spectrophotometrically by its characteristic absorbance at 562 nm.

RESULTS

Iron content of splenic macrophages from $Bcg'$ and $Bcg'$ mice is different. The results in Figure 7.1 show that the level of intracellular iron detected in rIFN-γ stimulated macrophages from $Bcg'$ mice averaged 0.83 ng/10$^6$ cells as compared to 1.41 ng/10$^6$ cells in macrophages from $Bcg'$ mice. This represents a 42% lower iron content in macrophages for BCG resistant mice. The iron content of macrophages not treated with rIFN-γ did not differ between the strains.

Chelation of intracellular iron abrogates the suppressive effects of corticosterone on expression of Nrampl by macrophages from $Bcg'$ mice. To determine the relationship between high intracellular iron content and susceptibility of Nrampl mRNA expression to corticosterone induced destabilization, desferroxamine was used to chelate free iron prior to treatment of the rIFN-γ treated macrophages from $Bcg'$ mice with corticosterone. The results of Figure 7.2 show that treatment of macrophages from $Bcg'$ mice with desferroxamine resulted in the abrogation of corticosterone induced suppression of Nrampl
Figure 7.1. Levels of intracellular iron in Macrophages from Bcg' and Bcg' mice are different. Splenic macrophages (5 X 10^6) from Bcg' and Bcg' mice were stimulated with 10 U rIFN-γ (per 10^6 cells). Control cultures were untreated. After 20 hours, the macrophage cultures were heated at 80° C for 24 hours to release protein-associated iron. Ferrous iron concentration was quantitated spectrophotometrically by its characteristic absorbance (562 nm) after being complexed with ferrozine. The concentration of iron (ng / 10^6 cells) was determined by comparison to a standard curve of known iron concentration. Data represent the average of 8 separate determinations.
Figure 7.1: Levels of intracellular iron in macrophages from $Bcg'$ and $Bcg'$ are different.
Figure 7.2. Chelation of iron abrogates the corticosterone induced suppression of \textit{Nramp1} expression by macrophages from \textit{Bcg}’ mice. Splenic macrophages from \textit{Bcg}’ and \textit{Bcg}’’ mice were stimulated with rIFN-\(\gamma\) (10 \(\mu\)l/10^6 cells) for 20 hours. Control cultures were unstimulated. The macrophage cultures were then treated with corticosterone (10^{-6} M) in the presence or absence of 100 \(\mu\)M desferroxamine for an additional 20 hours. Following incubation, the effect of decreased intracellular iron levels on the stability of \textit{Nramp1} mRNA expression following corticosterone treatment was determined by Northern analysis.
Figure 7.2

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- Unstim
- Desferox
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- Cort / Desferox
- IFN-γ
- IFN-γ / Desferox
- IFN-γ / Cort
- IFN-γ / Cort / Desferox
Figure 7.3. Iron overloading of macrophages from Bcg' mice induces unstable Nramp1 mRNA expression. Splenic macrophages from Bcg' and Bcg' mice were stimulated rIFN-γ (10 u/10^6 cells) for 20 hours. The macrophage cultures were then treated with ferric ammonium sulfate (50 μM) in the presence or absence of corticosterone (10^{-6}M) for an additional 20 hours. The effect of increased intracellular iron levels on the stability of Nramp1 mRNA following corticosterone treatment was determined by Northern analysis.
Figure 7.3

Bcg

Bcg

rIFN-γ

rIFN-γ / Cort

rIFN-γ / Fe²⁺

rIFN-γ / Cort / Fe²⁺
mRNA. The effect of desferroxamine was dose dependent (data not shown). Treatment of macrophages from Bcg' mice with desferroxamine did not alter Nramp1 expression in response to corticosterone treatment.

**Iron overloading in macrophages from Bcg' mice results in suppressed expression of Nramp1 following corticosterone treatment.** To determine if excess iron overload of splenic macrophages affects stability of Nramp1 mRNA in response to corticosterone treatment, rIFN-γ stimulated macrophages from Bcg' and Bcg' mice were incubated in the presence or absence of ferric ammonium sulfate prior to treatment with corticosterone. The results of Figure 7.3 shows that increased iron levels in macrophages from Bcg' mice resulted in the instability of Nramp1 mRNA expression following corticosterone treatment. Iron overloading of Bcg' macrophages induced a marked instability of Nramp1 expression.

**DISCUSSION**

The results of this investigation indicate that differences in resistance to mycobacterial growth may be due to Nramp1 mediated control of iron transport in macrophages. The expression of a functional Nramp1 in rIFN-γ activated macrophages from Bcg' mice corresponds with lower intracellular iron levels as compared to rIFN-γ stimulated macrophages from Bcg' mice which express a nonfunctional Nramp1. Chelation of iron in macrophages from Bcg' mice as well as iron overloading in macrophages from Bcg' mice demonstrated that relative iron levels in macrophages can influence mRNA stability. The maintenance of cellular iron homeostasis is a prerequisite for many biological processes and
regulation of immune function (Alford et al. 1991, Lane et al. 1991, Pantopoulos and Hentze 1995, Weiss et al. 1993, 1995). However, the regulatory mechanism that controls iron trafficking within the reticuloendothelial system of vertebrates remains obscure.

The function of *Nrampl* in conferring resistance to mycobacterial infection has been the subject of intensive study. We have previously shown that glucocorticoids differentially affect both macrophage activity and rIFN-γ induced gene expression in macrophages from *Bcg* and *Bcg* mice (unpublished observations). Glucocorticoids suppress the antimycobacterial activity of *Bcg* macrophages as well as *Nrampl* expression while having no effect on either capacity in macrophages from *Bcg* mice. We have also shown that induction of functional *Nrampl* expression in macrophages from *Bcg* mice results in the stabilization of *TNF-α* and *iNOS* mRNA in the presence or absence of corticosterone. The induction of *Nrampl* expression in macrophages from *Bcg* mice does not result in stable mRNAs following corticosterone treatment. Rather, glucocorticoids result in accelerated mRNA degradation. Taken together, these results allow us to use the differential effect of glucocorticoids to postulate the role of *Nrampl* in regulating mycobacterial resistance.

There is increasing evidence concerning the role of iron in cell mediated immunity (Weiss et al. 1993, 1995). Experimental evidence suggests that levels of intracellular iron are important regulators of macrophage antimicrobial mechanisms. The primary mechanism by which cells obtain iron is via the transferrin receptor. Macrophages have been shown to possess both intracellular and cell surface receptors for transferrin. Macrophages regulate
intracellular iron levels by an iron dependent modulation of the transferrin receptor. Macrophages at different levels of activation differ in transferrin receptor expression suggesting that cellular iron levels correlate with the activation state of the cell (Hamilton et al. 1984). The antimicrobial effects of rIFN-γ activated macrophages were increased when intracellular iron levels were reduced with desferoxamine. The hypothesis that macrophages from Bcg<sup>−</sup> mice are primed for activation when compared to macrophages from Bcg<sup>+</sup> mice (Buschman et al. 1989, Gros et al. 1983) suggests that Nrampl expression, iron metabolism and mycobacterial resistance could be closely associated.

An attractive candidate for mediating eukaryotic mRNA stability is the iron regulatory protein, IRP (also called iron response element binding protein, IRE-BP) believed to be a mitochondrial aconitase enzyme (Stuehr and Nathan 1989, Drapier et al. 1993). A classic example of iron homeostasis is illustrated by the interaction of the iron regulatory protein (IRP) with the IRE mRNA stem loop (hair-pin) structures present at the 5' untranslated regions of ferritin mRNA and the 3' untranslated regions of transferrin receptor mRNA. A low concentration of intracellular iron in macrophages induces a high affinity binding of the IRP to its IRE consensus sequence. In the case of transferrin receptor mRNA, this interaction stabilizes transferrin receptor mRNA and represses ferritin mRNA translation thereby reducing iron storage. In contrast, high intracellular levels of iron decrease the IRP-IRE interaction by inducing conformational changes in the aconitase enzyme resulting in unstable transferrin receptor mRNA and increased translation of ferritin mRNA leading to increased iron stores. Nucleotide sequence analysis of Nrampl and TNF-α has not revealed UTR
hairpin (stem loop) structures that are classically associated with ferritin and transferrin receptor mRNA UTRs. Lack of these sequence elements, however, does not preclude that other iron regulated proteins may be involved in stabilization of \textit{Nramp1} as well as other effector molecule mRNAs. The results of our study indicate that \textit{Nramp1}, functionally expressed in macrophages from \textit{Bcg} mice, is associated with reduced intracellular iron levels, thus providing conditions necessary to induce iron regulated proteins associated with mRNA stability. The low intracellular iron content of these macrophages would induce a high affinity attachment of these proteins to sequence elements found in the 3' untranslated regions of \textit{Nramp1}, \textit{TNI}-\alpha and \textit{iNOS} as well as other rIFN-\gamma induced genes thus stabilizing their mRNA and rendering them resistant from Rnase degradation induced by corticosterone. The stabilization of these effector molecules would allow their extended production and therefore provide increased host resistance to mycobacterial infection (Figure 7.4).

The role of iron in the microbicidal activity of macrophages is complex. Defective intracellular killing by monocytes from thalassemia patients and from patients with other transfusion iron overload conditions has been reported (Ballart et al. 1986, Van Asbeck et al. 1984). Additional studies have shown that reducing macrophage intracellular iron levels by incubation with desferroxamine in combination with rIFN-\gamma enhanced their ability to kill intracellular pathogens including \textit{Histoplasma} and \textit{Legionella} species presumably by preventing intracellular multiplication (Byrd and Horwitz 1989, Lane et al. 1991). Alford et al. demonstrated that excess cellular iron decreased the ability of thioglycollate-elicited macrophages to kill \textit{Listeria} without affecting phagocytosis (Alford et al. 1991). On the other
hand, iron may, in some situations, enhance microbicidal activity. For example, the killing of trypanosomes by macrophages was increased when the cells were exposed to Fe-lactoferrin, but not apolactoferrin (Lima and Kierszenbaum 1987). Transferrin still remains the key factor in maintaining iron homeostasis in an individual rather than absolute levels of intracellular iron present in host cells. Iron limitation may result in enhanced immune functions only to the point at which the degree of iron saturation of transferrin is so low that delivery of critical iron to cells and tissues is diminished. Conversely, iron overload will only affect immune function when transferrin becomes fully saturated and non-transferrin bound iron is present in the circulation. Excess free iron could affect immune function by production of damaging levels of oxygen radicals such as superoxide and hydrogen peroxide (Hoepelman et al. 1990, Sambri et al. 1991) or by other mRNA destabilizing mechanisms such as that previously described.

Putative models of Nramp1 strongly suggest that the protein is a transport molecule. The proposed role of Nramp1 as a transporter of nitrates/nitrates seems unlikely in light of our observations (Brown et al., 1993, 1994, 1995, unpublished observations) that show that innate resistance is independent of NO production. However, the role of reactive nitrogen intermediates in a combinatorial role with Nramp1 seems feasible. The role of Nramp1 as an iron transporter rather than a nitrate transporter begins to address the controversial argument of where nitric oxide fits in the scheme of mediation of mycobacterial resistance. Evidence for the existence of eukaryotic iron transport proteins was provided when researchers recently identified a candidate iron transport gene in yeast comprised of a
multicopper oxidase (FET3) and a permease (FTR1) molecular complex responsible for translocating iron from the environment into the cell (Kaplan et al. 1996, Stearman et al. 1996). Examination of the protein data base suggests that homologs for both components exist in *S. cerevisiae* and may function to transport iron out of the cell or across some other organelle. If iron metabolism/homeostasis and mycobacterial resistance are linked, then it is conceivable to envision a scenario in which infection of macrophages with mycobacteria results in the stimulation of the proinflammatory *TNF-α* as well as *Nramp1*. The production of *Nramp1* in resistant macrophages would serve to export iron from the macrophage thus lowering intracellular levels of iron and allowing *TNF-α* dependent iNOS to produce NO. IRP or other iron regulated proteins, which bind the 3' UTR of *Nramp1* and other nucleotide sequence elements of rIFN-γ induced genes that are necessary for antimycobacterial responses, stabilize their mRNAs.

Nitric oxide (NO) has attracted increasing interest in recent years following the demonstration that it is a central component of macrophage-mediated cytotoxicity in mammals. Many of the biological effects of NO are based upon its interactions with iron. NO induced IRP modulates intracellular iron regulation via iron responsive elements (IREs) (Drapier 1988, Steuhr 1989). In this particular model, an autoregulatory system between iron metabolism and the production of *iNOS/NO* in activated macrophages could exist by the stabilization of transferrin receptor mRNA thereby leading to increased uptake of iron into the macrophage resulting in the down regulation of *iNOS*, effectively shutting down the pathway until a new inflammatory stimulus reinitiates the pathway. However, this feedback
Figure 7.4. Role of iron and \textit{Nramp1} in mycobacterial resistance. Under conditions of low intracellular iron (functional \textit{Nramp1} expressed by \textit{Bcg} macrophages), IRP or other iron regulated proteins bind to sequence elements along mRNA message. Bound proteins stabilize mRNA and prevent degradation by corticosterone induced RNAses. Under conditions of high intracellular iron (nonfunctional \textit{Nramp1} expressed by \textit{Bcg} macrophages), iron displaces bound proteins resulting in unstable mRNA susceptible to degradation by corticosterone induced RNAses.
Figure 7.4

Nonfunctional Nramp1
Bcg^s

High Iron
Fe Loading: Bcg^r
Displaced
Unstable mRNA

Functional Nramp1
Bcg^r

Low Iron
Fe Chelation: Bcg^s
Bound
Stable mRNA

Nitric Oxide

IRP

mRNA

RNAse

Act D
CHX
Corticosterone
role is dependent upon upregulation of transferrin receptors as a result of activation. Conflicting reports on whether rIFN-γ induces upregulation or down regulation of transferrin receptor expression in macrophages undermines the ability to elucidate a clear model of feedback in this system (Hamilton et al. 1984, Alford et al. 1993, Weiss et al. 1993, 1995). In addition, based upon our observations of NO independent mechanisms, the role of inducible nitric oxide synthase and nitric oxide in this model may not be absolutely essential. Cytokines such as rIFN-γ, TNF-α and LPS may be able to induce iron regulated proteins independently of iNOS / NO.

The proposed iron transport function of Nramp1 not only serves to stabilize cytokine mRNAs but could, in addition, effectively withdraw essential iron from invading pathogens resulting in the limitation of their growth. Of all of the nutrients required by or available to *Mycobacterium tuberculosis*, only iron poses serious acquisition problems. (Wheller and Ratledge, 1995). It is suggested that bacteria undergo a series of “feasts and famines” as nutrients become available or are withheld for long periods (Koch 1971, Wheeler and Ratledge 1995, Gobin and Horwitz 1996). If iron is suddenly made available to pathogens such as *M. tuberculosis*, the organism would need to acquire (via exochelins) and hold (via mycobactin) the iron obtained from major iron containing molecules of the host (including transferrin and ferritin) until it could initiate synthesis of necessary heme groups and iron-containing proteins.
Macrophages activated with rIFN-γ have been shown to withdraw non-heme iron from fungal, bacterial, protozoan and neoplastic cells (Wheeler and Ratledge 1995). The targeted cells in some cases remain viable but are unable to synthesize DNA or maintain oxidative metabolism. The iron withdrawal factors formed by macrophages consist of mainly, but not exclusively, reactive nitrogen intermediates (Nathan and Hibbs 1991). Human blood monocytes fail to support the intracellular growth of *Legionella pneumophila* when treated with desferroxamine or when activated by rIFN-γ. Similarly, mouse peritoneal macrophages activated by rIFN-γ failed to support intracellular growth of *Histoplasma capsulatum*. The activities of rIFN-γ in these cases are notably independent of reactive nitrogen intermediate production.

In summary, our model suggests that BCG-susceptible macrophages, expressing a non-functional Nrampl with associated high levels of intracellular iron, allow access of iron by the bacilli. The iron is effectively mobilized into the bacterium by exochelin/mycobactin mechanisms where sufficient levels of iron enable growth to occur more or less unchecked. In BCG-resistant macrophages, functional Nrampl lowers levels of intracellular iron and withdraws iron from the mycobacteria. The stabilization and consequent prolonged exposure of cytokine effector molecules resist growth of the mycobacteria to an extent that the pathogen is unable to initiate synthesis of necessary heme groups and iron-containing proteins necessary for iron uptake; resulting in resistance to mycobacterial growth.
The historical basis for studying the influence of stress on the immune response stems from a number of clinical observations that individuals became sick following "stressful" situations. Numerous studies have associated psychological and physical stress with compromised immune function. As a result, a new interdisciplinary research area termed psychoneuroimmunology has emerged. Although there exists difficulties associated with the quantitation of stress and its ultimate association with the onset of illness, it is widely accepted that stress can have an impact on susceptibility to several infectious diseases, most notably, tuberculosis. (Collins 1989, Wiegeshaus et al. 1989)

In mice the genetic resistance to the \textit{in vivo} growth of Mycobacteria has been shown to be controlled by a gene, termed \textit{Bcg}, which maps to chromosome 1 (Schurr et al. 1989, Vidal et al. 1993). A syntenic group of genes maps to human chromosome 2q (Schurr et al. 1990). Macrophages from \textit{Bcg} \textsuperscript{'} and \textit{Bcg} mice express MHC class II glycoproteins differently. Macrophages from \textit{Bcg} \textsuperscript{'} mice can be induced to persistently express I-A while macrophages from \textit{Bcg} \textsuperscript{'} mice will only transiently express I-A (Zwilling et al. 1990).
Together, these observations and subsequent studies in which activation of the hypothalamic-pituitary-adrenal axis was shown to suppress I-A expression by macrophages from \( Bcg' \) mice while not affecting I-A expression by macrophages from \( Bcg' \) mice suggested that Mycobacterial growth that is under \( Bcg \) control may also be differentially affected by HPA axis activation.

These observations became the basis for the next course of study, that being to determine the role of the HPA axis in regulating the growth of \( Mycobacterium avium \) in congenic \( Bcg' \) and \( Bcg' \) mice. We found that activation of the HPA axis increased the susceptibility of \( Bcg' \) mice to mycobacterial growth but did not affect the ability of \( Bcg' \) mice to limit the growth of mycobacteria. HPA axis activation also resulted in an increased permissiveness of macrophages from \( Bcg' \) mice to intracellular growth of \( M. avium \) in vitro while again not affecting resistance of macrophages from \( Bcg' \) mice. The effect of HPA axis activation on the in vivo growth of the mycobacteria was abrogated by adrenalectomy and by treatment of mice with the glucocorticoid receptor antagonist RU486. The failure of HPA axis activation to increase the susceptibility of \( Bcg' \) mice was not due to unresponsiveness of the strain to HPA axis activation as evidenced by equal plasma corticosterone and ACTH levels produced in the two strains of mice in response to restraint. The effect of HPA axis activation on the production of two antimicrobial effector molecules, TNF-\( \alpha \) and reactive nitrogen intermediates (nitric oxide, NO) was assessed as well. We found that HPA axis activation resulted in a suppressed capacity of macrophages from \( Bcg' \) and \( Bcg' \) mice to produce TNF-\( \alpha \) and NO following stimulation with rIFN-\( \gamma \) and LPS. This observation
supports other studies which have shown that corticosteroids suppress both TNF-α production as well as the production of reactive nitrogen intermediates. Macrophages from both strains of mice produced equal amounts of the cytokine and of reactive nitrogen intermediates. There are conflicting reports as to whether or not there are differences in production of these effector molecules between the two strains. Our results could be interpreted as indicating that the production of TNF-α and NO may be independent of Bcg control. The results of this study suggested that HPA axis activation could account for the increased susceptibility of individuals to Mycobacterial growth. The use of restraint stress served as a tool to produce physiological levels of corticosterone which, under natural conditions of infection, could be induced by the stimulation of the HPA axis by proinflammatory cytokines produced as a result of macrophage activation.

Based upon the observations that HPA axis activation differentially affected resistance to in vivo and in vitro growth of Mycobacteria in Bcg⁺ and Bcg⁻ mice, we extended our studies to further evaluate the effect of HPA activation on antimycobacterial mechanisms of rIFN-γ activated macrophages. Since the increase in susceptibility to mycobacterial growth that resulted from HPA axis activation was considered to be due to the direct effect of corticosterone, we chose to evaluate the effect of corticosterone on macrophage antimycobacterial activity as well. HPA axis activation or the exogenous treatment of macrophages with corticosterone suppressed the ability of macrophages from susceptible mice to control the growth of M. avium. In contrast, neither HPA axis activation nor the addition of corticosterone altered the ability of macrophages from BCG resistant mice to control
mycobacterial growth. As before, we found that production of TNF-α and of reactive nitrogen intermediates by macrophages from both strains of mice was suppressed by HPA axis activation or corticosterone treatment. The addition of RU486 to Bcg' macrophage cultures ameliorated the effect of HPA axis activation on antimycobacterial activity. RU486 also partially restored the ability to produce TNF-α and NO from macrophages from both strains following HPA axis activation. This partial restoration of effector function could imply that other factors such as sympathetic nervous system components could be involved in the suppression of TNF-α. Previous studies showed that HPA activation suppressed both TNF-α and NO production by rIFN-γ and LPS stimulated macrophages. This study showed that HPA axis activation or corticosterone suppresses their production when the cells were also stimulated by rIFN-γ and M. avium. We had previously demonstrated that injection of mice with increasing concentrations of Mycobacterium bovis (BCG) attenuated the suppressive effect on the induction of MHC class II expression and that increasing concentrations of rIFN-γ had similar effects on class II expression. This study demonstrated that rIFN-γ attenuated the effect of HPA activation on the production of both TNF-α and NO, with a pronounced effect on macrophages from BCG resistant mice.

When macrophage populations were activated in vitro with rIFN-γ, the function of macrophages from Bcg' and Bcg' mice were suppressed by the addition of corticosterone. In order to address the possibility that the differential effect of glucocorticoids observed was limited to innate resistance and not resistance induced by rIFN-γ or other macrophage activating cytokines, we evaluated the antimycobacterial activity of macrophages from Bcg'
and $Bcg'$ mice following stimulation of the cells with rIFN-γ or rGM-CSF and determined the
effect of glucocorticoids on macrophage antimycobacterial activity. In addition, we sought
to establish a correlation between differential effects of corticosterone on macrophage
antimicrobial activity and expression of the gene, Nramp1 ($Bcg$) by macrophages from both
strains of mice. We found that rIFN-γ and rGM-CSF induced similar levels of activation in
macrophages from both strains of mice and that corticosteroids suppressed the ability of
cytokine activated macrophages from $Bcg'$ mice, while not affecting the resistance of
macrophages from $Bcg''$ mice. The results of this study suggested that rIFN-γ and GM-CSF
induced different anti-mycobacterial pathways. The rIFN-γ pathway involves the production
of NO and is inhibited by NMMA while the GM-CSF induced pathway does not appear to
be dependent on NO production and is therefore not affected by NMMA. Both pathways
appear to be regulated by $Bcg$ because they are differentially affected by corticosterone.

Nramp1 expression was upregulated by both rIFN-γ and GM-CSF. Corticosterone
suppressed Nramp1 expression only by macrophages from $Bcg'$ mice suggesting that the
differential effects of corticosterone on macrophages from congenic BCG resistant and
susceptible mice were related to the different effects of the hormone on the gene, Nramp1.

To determine if the differential effects of corticosterone on Nramp1 expression were
associated with $Bcg$, we utilized congenic mice constructed for functional studies of the
natural resistance locus $Ity/Lsh/Bcg$. The congenic C.D2-Vil strains were constructed from
four recombinant founder mice, all heterozygous at the Vil locus (Villin is the closest marker
to the $Ity/Lsh/Bcg$ gene). Corticosterone treatment of rIFN-γ stimulated macrophages from
C.D2-Vil congenic mice strains did not affect expression of \textit{Nramp1}. This observation allowed us to more closely map the differential effects of corticosterone to the area on mouse chromosome 1 between \textit{Tryp-1} and \textit{Vil}, which encompasses the resistance gene, \textit{Bcg}, thereby associating the effect with resistance to Mycobacterial infection.

Based upon the previous results, we sought to extend and characterize the differential effect of corticosterone on the expression of \textit{Nramp1} mRNA by macrophages from \textit{Bcg}' and \textit{Bcg}' mice. Our results show that corticosterone, in addition to \textit{Nramp1}, suppressed the expression of several IFN-\gamma inducible genes by macrophages from \textit{Bcg}' mice. Macrophages from \textit{Bcg}' mice remained resistant to the effects of corticosterone. The effect of corticosterone induced suppression of \textit{Nramp1} expression in \textit{Bcg}' mice was abrogated with Actinomycin D and cycloheximide. Additionally, results of a nuclear run-off assay provided evidence that corticosterone did not transcriptionally regulate \textit{Nramp1} expression. Together these data suggested that corticosterone may be inducing post-transcriptional modifications of \textit{Nramp1} mRNA in \textit{Bcg}' mice. The exact mechanism of mRNA destabilization is not known but a report by Peppel et al. (Peppel 1991) suggested that glucocorticoids may directly activate ribonucleases that degrade mRNA containing AU-rich sequences in the 3' untranslated regions of mRNA. Prior to this point, we had believed that differences in resistance to mycobacterial infection could be accounted for by differential regulation of \textit{Nramp1} by glucocorticoids. However, based upon the absence of negative glucocorticoid response elements associated with published \textit{Nramp1} sequence as well as no evidence to support differences in glucocorticoid receptor number between \textit{Bcg}' and \textit{Bcg}' mice, we chose
to consider whether Nramp1, in fact, could control the immunosuppressive effects of corticosterone by a yet undefined mechanism. Since differences in mRNA stability can affect protein levels, an increased mRNA stability in macrophages from Bcg\(^{-}\) mice could result in the sustained production of anti-mycobacterial effector molecules and could therefore account for the increased resistance of macrophages from Bcg\(^{-}\) mice to mycobacterial growth. We now believed that glucocorticoid mediated suppression served to define a possible role for Nramp1. Corticosterone induces mRNA destabilization when Nramp1 is not functional (Bcg\(^{-}\) mice). When functional Nramp1 is NOT induced, the mRNA’s of the effector molecules TNF-\(\alpha\) and iNOS were not stable in the presence of corticosterone. However, in the case of Bcg\(^{-}\) mice, functional Nramp1 expression results in the stabilization of effector molecule mRNA thereby leading to prolonged release of these antimycobacterial mediators.

An attractive candidate for mediating mRNA stability is the iron regulatory protein IRP. With increasing evidence concerning the role of iron in cell mediated immunity, we began to evaluate the relationship of iron and mRNA stability in the resistance to mycobacterial growth by macrophages from Bcg\(^{-}\) and Bcg\(^{-}\) mice. Our results suggested that low levels of intracellular iron in the macrophages of Bcg\(^{-}\) mice resulted in stabilization of mRNA expression. Levels of intracellular iron detected in rIFN-\(\gamma\) stimulated splenic macrophages from Bcg\(^{-}\) mice were lower as compared to iron levels measured in macrophages from Bcg\(^{-}\) mice. Iron chelation with desferoxamine resulted in the abrogation of corticosterone induced effects on Nramp1 expression. Additionally, induced iron-overloading of macrophages from Bcg\(^{-}\) mice resulted in unstable Nramp1 mRNA following
corticosterone treatment. From these data and currently published observation of a possible
eukaryotic iron transport protein, we proposed that \textit{Nramp1} could function as an iron
transport protein that serves to regulate the level of intracellular iron in macrophages. When
functional \textit{Nramp1} is induced, low intracellular iron levels result and increased affinity of
iron regulated proteins for sequence elements in the 3' UTR occurs. The presence of bound
proteins inhibits mRNA degradation by glucocorticoid induced nucleases. Non-functional
\textit{Nramp1} is unable to control intracellular iron levels in the macrophage, allowing free non-
ferritin bound iron to decrease the interaction of proteins and sequence elements making
these mRNA susceptible to the degradative effects of corticosterone. Short lived effector
molecules result as well as an associated increased susceptibility to mycobacterial infection.

Further studies in characterizing the role of iron in mycobacterial resistance will
include mobility shift assays and deletion analysis to identify stabilizing or destabilizing
proteins that bind to 3' UTR of \textit{Nramp1} or other rIFN-\gamma induced genes involved in
antimycobacterial activity. In addition, to confirm the role of functional \textit{Nramp1} as an iron
transporter in \textit{Bcg} \textsuperscript{+} macrophages, translocation of iron from macrophages from \textit{Bcg} and
\textit{Bcg} \textsuperscript{-} mice will be investigated. Finally, stable transfection of \textit{Nramp1} into susceptible
macrophage cell lines coupled with functional studies with \textit{Nramp1} antibody will allow
clarification of the role of \textit{Nramp1} in mycobacterial resistance.
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