1992

The Effects of Exercise Stress on Equine Immune Function.

Tammie Lee Keadle
Louisiana State University and Agricultural & Mechanical College

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The effects of exercise stress on equine immune function

Keadle, Tammie Lee, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1992
THE EFFECTS OF EXERCISE STRESS ON EQUINE IMMUNE FUNCTION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
(Option Veterinary Microbiology and Parasitology)

by
Tammie L. Keadle
D.V.M., Louisiana State University, 1982
December, 1992
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<td>Adrenocorticotropic Hormone</td>
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<td>ADCC</td>
<td>Antibody Dependent Cell-Mediated Cytotoxicity</td>
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<td>ANOVA</td>
<td>Analysis of Variance (one way)</td>
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<td>AP</td>
<td>Alpha Fentanyl</td>
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<td>C</td>
<td>Cortisol</td>
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<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<td>CBC</td>
<td>Complete Blood Count</td>
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<td>CBG</td>
<td>Corticosteroid Binding Globulin</td>
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<td>Con A</td>
<td>Concanavalin A</td>
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<td>CMF-PBS</td>
<td>Calcium and Magnesium Free Phosphate Buffered Saline</td>
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<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
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<td>CS</td>
<td>Corticosteroids</td>
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<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<td>E</td>
<td>Epinephrine</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal Axis</td>
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<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<td>I</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
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<td>LAK</td>
<td>Lymphokine Activated Killer</td>
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<td>LGL</td>
<td>Large Granular Lymphocyte</td>
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<td>Methoxamine</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>Mixed Lymphocyte Reaction</td>
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<td>NE</td>
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<td>NK</td>
<td>Natural Killer</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<td>PCV</td>
<td>Packed Cell Volume</td>
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<td>Plaque Forming Cells</td>
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<td>PHA</td>
<td>Phytohemagglutinin</td>
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<td>PMN</td>
<td>Polymorphonuclear</td>
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<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<td>PPD</td>
<td>Purified Protein Derivative</td>
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<td>Pokeweed Mitogen</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>RPFC</td>
<td>Reverse Plaque Forming Cell</td>
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<td>sRBC</td>
<td>Sheep Red Blood Cells</td>
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<td>S</td>
<td>Stallion</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>TP</td>
<td>Total Plasma Protein</td>
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Abstract

The physiologic demands of exercise constitute a form of stress which is accompanied by modulation of immune responses. However, numerous studies in many different model systems have failed to clearly define the effects of exercise on immunity. Furthermore, the role of the neuroendocrine system in exercise-induced immunomodulation has been largely unexplored. To address these issues, we evaluated the effect of acute exercise stress on 3 indices of immune function in horses, before and after completion of a 12 week treadmill-based physical conditioning program. In addition, we determined the effects of corticosteroids, catecholamines, beta endorphin, and adrenocorticotropic hormone on these same immune parameters. In this work, we found that an acute severe exercise challenge of unconditioned horses induced immediate and significant post-exercise decreases in pokeweed mitogen (PWM) and equine influenza virus type 2 lymphoproliferative responses. Lymphokine activated killer (LAK) cell activity was also significantly elevated. After training, basal immune responses of resting horses were unchanged compared to those of untrained animals. Further, acute exercise stress no
longer produced significant elevations of equine LAK activity, and depressions in PWM responses were delayed and enhanced. Of the neuroendocrine compounds tested for their effects on immune responses, most were capable, at one or more of their tested concentrations, of causing changes in PWM, influenza, or LAK responses similar to those observed after exercise. Physical conditioning attenuated their effects. Surprisingly, in vitro corticosteroids augmented influenza, LAK, and PWM responses depending upon concentration. However, intravenous cortisol injection did not mimic immediate post-exercise changes in the hemogram or immune responses. We conclude that acute exercise stress of horses may be temporarily immunosuppressive, but the observed decrease in lymphoproliferative responses is partially offset by increases in LAK cell-mediated cytotoxicity. While providing no enhancement of basal immune responses, physical conditioning may serve to increase the severity of immune suppression following exercise, possibly contributing to increased morbidity of equine athletes experiencing repeated episodes of exercise stress. Finally, direct effects of corticosteroids, catecholamines and neuropeptides on immune cell function may contribute to exercise effects on immunity in unfit horses and to a lessor extent in fit horses.
Chapter I

Introduction

Rationale for the Present Study

Numerous studies in many different model systems have failed to clearly define the effects of acute exercise stress and exercise conditioning on immune function (Caren, 1991; Fitzgerald, 1988; Fitzgerald, 1991; Hickson and Boone, 1991; Keast, et al., 1988; Simon, 1984). In addition, the role of neuroendocrine peptides and hormones in exercise-induced immunomodulation is largely unexplored.

The equine model of exercise stress offers a unique opportunity for studies of exercise and immunity. First, the horse is a willing runner and need not be coerced into exercising as is the case for rats and mice. It is also less subject to the added psychological stress of competition seen in many human studies. Second, exercise physiology in the horse has been well characterized and thus exercise intensity can easily be quantitated. Third, horses provide a ready source of large quantities of sample material.
By utilizing an exercise model free of extraneous stressors, quantitating the degree of exercise stress, and examining overlapping indices of immune function, it is hoped that the work performed in this study will more closely describe the relationship between exercise and immunity in the horse, while information gained from studies of the effects of corticosteroids, amines, and neuropeptides on equine immune function may provide the causal link. Such knowledge will have broader application to an understanding of how immune cell activation and proliferation are controlled.

Specific Aims

1. To determine the effects of acute exercise of varying intensity on 3 parameters of equine immune function: pokeweed mitogen and equine influenza virus type 2-stimulated lymphoproliferation and lymphokine activated killer cell activity.

2. To assess the effects of physical conditioning on resting and post exercise pokeweed mitogen, equine influenza virus type 2, and lymphokine activated killer cell responses.
3. To characterize the effects of cortisol, epinephrine, norepinephrine, adrenergic agonists, beta endorphin, and adrenocorticotropic hormone on equine pokeweed mitogen, equine influenza virus type 2, and lymphokine activated killer cell responses throughout the course of an exercise conditioning program.

4. To integrate findings of these studies into a model for how exercise impacts immune function in the horse.
Literature Review

Exercise and Immune Function

Part of the athlete's lore is that exercise somehow promotes resistance to infections. The scientific reality in this regard is, however, controversial. There is some evidence to support the belief that exercise can boost the immune system. Accordingly, a program of moderate exercise improved immune responses and morbidity in a group of elderly people (Chandra, 1989). Likewise, laboratory animals (rats and mice) had increased antibody responses to antigens, increased resistance to chemically induced mammary tumors, and a delay in tumor growth following injection with tumor cells, as a result of regular exercise (Cohen, et al., 1988; Goode and Fernandes, 1981; Liu and Wang, 1987). In epidemiologic studies, job-related physical activity was found to correlate with a reduction in colon cancer (Gerhardson, et al., 1986) and former female college athletes experienced fewer breast and reproductive system cancers (Frisch, et al., 1985).

On the other hand, there is ample data suggesting that too much exercise can be immunosuppressive. Reports of infectious disease outbreaks among athletes emphasize their increased susceptibility to infections. For
example, in an outbreak of polio at a school, the only persons affected were those participating in strenuous sports (Weinstein, 1973). In a college outbreak of infectious hepatitis, the majority of the members of the football team were affected while other students and staff were not (Morse, et al., 1972). In an outbreak of aseptic meningitis at a high school, the incidence of illness was highest and the symptoms were more severe among members of the football team. Finally, in a study of 150 marathon finalists, respiratory symptoms in the days following the race were significantly more common in the fastest runners with the toughest training regimens (Peters and Bateman, 1983).

Much of the confusion about the relationship between exercise and immunity arises from a lack of distinction between acute exercise and physical training effects. This is particularly true in the case of elite athletes in which the effects of repetitive severe exercise bouts are superimposed upon training-related changes in immune function. Because a separate set of immunologic alterations have been described for short term exercise and physical conditioning, separate consideration of each is given.
Studies of acute exercise stress have employed exercise regimens of variable intensity and duration sometimes making interpretation difficult (reviewed in Hickson and Boone, 1991; Keast, et al., 1988). Nonetheless, generalizations can be made. Thus short duration exercise (5-25 minutes) results in leukocytosis consisting of increases in the absolute levels of neutrophils, total lymphocyte, total T cells, T-helper cells (CD4+), T-cytotoxic/suppressor cells (CD8+), B cells, and natural killer (NK) cells. On a relative basis, there is an increase in neutrophils and total lymphocytes, a decrease in total T cells and T-helper cells, no changes in T-cytotoxic/suppressor cells (resulting in a decrease in the CD4+:CD8+ ratio), an increase in the percentage of B cells (no change or decreases have also been reported), and an increase in NK cells. Both increases and decreases in eosinophils or monocytes have been associated with exercise. Exercise of longer duration (118-240 minutes) may produce no changes in total lymphocyte number, the number of total T cells or CD4+:CD8+ ratios, and decreases in B cell numbers. Exercise intensities greater than 50% VO₂ max (a measure of aerobic capacity) produce hematologic changes similar to those described for short exercise sessions. Hematologic parameters return to baseline levels in 15
minutes to 24 hours dependent upon the cell subtype affected and the length of the exercise period (Caren, 1991; Hickson and Boone, 1991).

Acute exercise episodes modulate immune function usually within the subsequent 3 hour period. Effects are generally short lived but may last up to 48 hours (Hickson and Boone, 1991). Specific examples include 1) depressions in proliferative responses to mitogens, salivary IgA and IgM, serum immunoglobulins, in vitro production of IgA, IgM, and IgG, neutrophil adherence and bacteriocidal activity, and in vitro IL-2 production; 2) elevations in plasma IL-1, IL-6, and TNF alpha, IL-1 and IL-6 production by blood mononuclear cells in vitro, IL-2 receptor (TAC antigen) expression, and IL-2 stimulated proliferation; and 3) modulation of plasma IL-2 levels and of NK cell numbers and activity (Caren, 1991; Esperson, et al., 1990; Fitzgerald, 1988; Fitzgerald, 1991; Keast, et al., 1988; Pedersen, 1991). Further discussion of exercise effects on mitogen, NK and antigen specific responses follow.

A number of different immunologically active mitogens have been studied relative to exercise stress. Phytohemagglutinin (PHA) and concanavalin A (Con A) are polyclonal T lymphocyte mitogens, while pokeweeds mitogen
(PWM) and bacterial proteins such as purified protein derivative (PPD) are B lymphocyte mitogens. Suppression of mitogen responses following short term exercise protocols is a commonly reported phenomenon (Caren, 1991; Fitzgerald, 1988; Fitzgerald, 1991; Keast, et al., 1988; Simon, 1984). For instance, responses of peripheral blood leukocytes to PHA, Con A and PPD were decreased 30 minutes after completing a 2.5 hour marathon, but had returned to normal within 24 hours (Eskola, et al., 1978). Likewise, bicycling reduced the mitogen response to PHA (Oshida, et al., 1988), PWM (Hedfors, et al., 1976), and Con A (MacNeil, et al., 1991). In addition, depressed Con A blastogenesis was found in rat spleen cells following exhaustive swimming, and decreased PHA and Con A responses occurred 5 and 30 minutes after treadmill exercise of steers (Blecha and Minocha, 1983; Mahan and Young, 1989).

Acute episodes of intense exercise generally result in an increase in the percent of NK cells in peripheral blood as well as an increase in NK cell activity (Fiatarone, et al., 1988; Hickson and Boone, 1991; Mackinnon, 1989; Oshida, et al., 1988; Pedersen, et al., 1988; Targan, et al., 1981). Although a correlation between the latter two effects has been demonstrated in some cases (Brahmi, et al., 1985), other studies reveal
additional influences at work. One group of researchers showed that the beta endorphin inhibitor, naloxone, could negate the exercise associated rise in NK activity although it had no effect on the rise in NK cell numbers (Fiatarone, et al., 1988). Other scientists found that, during exercise, IL-2 augmented NK activity significantly more than before exercise (Pedersen, et al., 1988). They suggested that NK cells with a high IL-2 response capacity are recruited to the peripheral blood during exercise. In a different study, there were no increases in NK activity immediately post exercise and a decrease in NK activity at one hour post exercise was documented (Mackinnon, et al., 1988a). However, when cytotoxic activity was expressed on a per cell basis, in order to account for measured decreases in NK cell number, cytotoxic activity actually increased 40% at both times after exercise. Using a single cell cytotoxicity assay, different researchers were able to show that even moderate exercise could enhance NK activity by increasing the proportion of NK cells that both bind to and kill their targets (Targan, et al., 1981). Interferon-induced increases in NK cell cytotoxic recycling during and after exercise was proposed to further enhance NK activity.
Unfortunately, there has been little direct testing of the relationship between antigen-driven immune responses and exercise of any kind. One group has shown that moderate exercise conditioning enhances the murine antibody response to injected *Salmonella typhi* (Liu and Wang, 1987). Another researcher found that antibody responses to tetanus toxoid were normal, despite the fact that antigen was administered immediately after maximal physical exertion concurrent with marked decreases in lymphocyte responses to Con A, PHA, and PPD (Eskola, et al., 1978). In addition, examination of proliferation in mixed-lymphocyte cultures (MLC) before and after 10 minutes of exercise on a bicycle ergometer revealed higher levels of MLC proliferation using responder cells from resting individuals compared to those from exercising individuals (Hedfors, et al., 1983).

Like studies of acute exercise effects, studies of physical conditioning effects on immunity are heterogenous. Thus a number of different training protocols, sampling times, and training subjects have been utilized with sometimes divergent results. Nevertheless, the data indicate that physical conditioning affects both resting and post exercise immune parameters. Accordingly,
trained individuals, at rest, may exhibit increased type II major histocompatibility complex (MHC) antigen expression on lymphoid cells (Esperson, et al., 1990), natural killer cell percentages and activity (Pedersen, et al., 1989), IL-1 levels (Caren, 1991), lymphoproliferative responses to mitogens (Soppi, et al., 1982; Watson, et al., 1986), and antibody response to immunization (Liu and Wang, 1987). Conversely, fit individuals may experience decreased resting complement levels (Nieman, et al., 1989), blood lymphocyte numbers (Green, et al., 1981), NK cytotoxicity (Watson, et al., 1986), salivary IgA and serum IgG (Fitzgerald, 1988; Tomasi, et al., 1982), and neutrophil adherence (Lewicki, et al., 1987) compared to controls. Following exercise, athletes display smaller increases in blood lymphocytes (Ferry, et al., 1990; Soppi, et al., 1982), greater or smaller decreases in mitogen responses (MacNeil, et al., 1991; Mahan and Young, 1989; Soppi, et al., 1982), greater increases in the percentage of NK cells (Oshida, et al., 1988), lower complement levels (Neiman, et al., 1989), decreased salivary IgA (Tomasi, et al., 1982), and depressed neutrophil adherence and bacteriocidal activity (Lewicki, et al., 1987) relative to untrained individuals.
Collectively then, data regarding mitogen responses of physically conditioned animals are inconsistent. Thus, following progressive cycling to exhaustion, participants in a six week naval training program experienced a milder post exercise decline in their proliferative response to PHA and Con A than prior to training (Soppi, et al., 1982). At the same time, their resting levels of mitogen-induced blastogenesis were increased. Similarly, when rats were swim trained for 10 weeks, the reduction, after exercise, in splenic mitogenesis using Con A was decreased compared to untrained rats (Mahan and Young, 1989). To the contrary, cycle ergometry (120 minutes at 65% VO$_{2}\text{max}$) produced greater reductions in Con A-induced lymphoproliferation in highly fit individuals compared to people with only low levels of physical training (MacNeil, et al., 1991) and trained mice had a reduced response to Con A compared to untrained animals after a single bout of exhaustive exercise (Hoffman-Goetz, et al., 1986). After a heavy training period, another researcher found mitogen responsiveness to be suppressed following an acute exercise episode, whereas before the training period, acute exercise had no effect on mitogenesis (Verde, 1990). Finally, there are instances wherein there is no change in resting or post exercise responses to mitogens following exercise training. In particular, moderate exercise
conditioning of young pigs produced no effect on basal response to PWM and PHA, and trained people had the same PHA responses as untrained people after cycle ergometry (120 minutes at 60% VO₂ max) (Jensen, 1989; Oshida, et al., 1988).

Data regarding the effects of physical training on NK activity are likewise equivocal. Hence, in one study the percentage of NK cells and NK cytotoxicity at rest were higher in highly trained cyclists than in untrained individuals, while in another investigation, people who had completed a walk/jog/run training program had lower resting values for NK-mediated target cell lysis than those who had not (Pedersen, et al., 1989; Watson, et al., 1986). Furthermore, exhaustive exercise has been shown to increase or have no effect on the percentage of NK cells within the circulation of athletes compared to non-athletes (Brahmi, et al., 1985; Oshida, et al., 1988).

It should be noted that most data on physically conditioned subjects has been obtained from studies of highly trained competitive athletes. Such individuals experience the psychological stress of competition in addition to repeated episodes of acute exercise stress. Because psychological stress itself may be
immunosuppressive and several researchers have suggested that negative effects of acute exercise stress may be cumulative, a careful interpretation of information regarding the effects of physical conditioning on immunity is warranted (Mackinnon, et al., 1988b; MacNeil, et al., 1991).

For the equine model of exercise stress used in the present work, there is a limited amount of information relating exercise to modulation of immune function. What is known is summarized here. Immediately following acute maximal exercise (galloping), the equine hemogram registers leukocytosis, lymphocytosis, and a decrease in the neutrophil to lymphocyte (N/L) ratio (Rossdale, et al., 1982; Snow, et al., 1983). Two to three hours later, lymphocytosis declines toward lymphopenia, neutrophilia develops, and the N/L ratio is increased. These early and late changes in the hemogram of horses after exercise probably reflect the influence of epinephrine and corticosteroids, respectively, at these times (Hickson and Boone, 1991; Rose and Allen, 1985). No significant changes in the total or differential leukocyte count have been found in response to training (Rose and Allen, 1985).
Regarding specific immunologic responses, several studies indicate that exercise temporarily depresses phagocytic cell function in the horse (Huston, et al., 1987; Wong, et al., 1990; Wong, et al., 1992). Thus after vigorous running on a treadmill (11 meters/sec top speed, 6% incline), intracellular oxidative metabolism and phagocytic activity of alveolar macrophages was depressed for up to 3 days (Huston, et al., 1987; Wong, et al., 1990). In addition, similar exercise stress resulted in impaired blood neutrophil chemotaxis and oxidative metabolism 1 day after exercise (Wong, et al., 1992).

Where responses to mitogens have been examined in relation to exercise in the horse, there have been conflicting results. One study has reported that significant suppression of the blastogenic response to Con A and PHA occurs 30 minutes after exercising to the point of fatigue on a treadmill (Kurcz, et al., 1988). On the other hand, different researchers observed no change in PHA, Con A, or PWM responses of mononuclear cells obtained from horses 30 minutes after an exercise challenge (Wong, et al., 1992). These same researchers found no exercised-induced changes in serum IgG, IgM, and IgA levels.
Physical exertion is known to produce elevated plasma levels of cortisol, catecholamines, and neuropeptides. In the horse, post exercise blood levels reach $10^{-7}$, $10^{-9}$, $10^{-10}$, and $10^{-11}$M for cortisol, epinephrine/norepinephrine, beta endorphin, and ACTH, respectively (Church, et al., 1987; Hamra, 1990a; Snow, 1979; Snow and Rose, 1981). Concentrations of these substances within lymphoid tissues may be further elevated by activation of sympathetic nerves and local production of neuropeptides (Heijnen, et al., 1991; Felton, et al., 1987). Relevant to understanding the role of these neuroendocrine compounds in modulating immune function after exercise or physical conditioning, a review of each is presented below.

**Corticosteroids and Immune Function**

Corticosteroids (CS) play a critical role in the maintenance of homeostasis and the adaptive responses to stress. As such their effects are far reaching and include regulation of carbohydrate, protein, and fat metabolism; modulation of responses to other hormones and growth factors; control of tissue differentiation and development; and moderation of inflammatory and other immune responses (Feldman, 1989; McDonald, 1988).
Cortisol, the primary biologically relevant corticosteroid of humans and horses, is a cholesterol derivative produced by the adrenal cortex, under the direction of adrenocorticotropic hormone (ACTH) from the pituitary (McDonald, 1988). In turn, ACTH release is controlled by hypothalamic corticotropin releasing hormones (CRH), which increase in response to stressors or central signals controlling basal cortisol levels. This hypothalamic-pituitary-adrenal (HPA) axis is negatively regulated by high levels of corticosteroids.Interestingly, production of CRH, ACTH, and thus corticosteroids is promoted by cytokines such as IL-1, IL-6, IL-2, interferon (IFN) alpha and beta, and tumor necrosis factor (TNF), (Reviewed in Blalock, 1989) which could account for increased cortisol levels present at the time of peak immune responses.

The influence of CS on cell functions depends upon plasma cortisol concentration and receptor density on susceptible cells. These parameters are likewise subject to modulation. Accordingly, stressors may elevate blood CS levels which then decline to pre-stress levels with a half life of 1 to 1.5 hours in the horse (Thornton, 1985). Additionally, a normal diurnal variation of blood cortisol levels occurs. In horses, daily cortisol levels are
greatest from 6 to 8 AM with concentrations reaching the $10^{-7} M$ range and lowest from 4 to 10 PM with levels in the $10^{-8} M$ range (Hamra, 1990b; James, et al., 1970; reviewed in Thornton, 1985).

Several factors are known to increase or decrease target cell CS receptor numbers. These include the cell cycle, circadian rhythms, prior hormone exposure, and cell type. Thus entry of cells into S-phase of the cell cycle is associated with increased CS receptor expression (Crabtree, et al., 1980). CS receptor expression can therefore be induced by activating signals such as mitogens or antigens (Lacroix, et al., 1984). In humans, basal CS receptor expression by leukocytes parallels but is independent of the diurnal variation in plasma cortisol with high morning and low evening values (Xu, et al., 1991). Homologous down-regulation of CS receptors by their ligand has been reported (Lacroix, et al., 1984) and may be achieved by CS-mediated decreases in receptor gene transcription or receptor half life (Feldman, 1989). However, long term CS exposure may be required to attain these effects (Cidlowski and Cidlowski, 1981; Lacroix, et al., 1984). Recycling would be expected to play a part in receptor modulation following short term CS elevations of exercise. Finally, cell populations may differ in the
number of CS receptors they express. For example, large granular lymphocytes (LGL), monocytes, and polymorphonuclear (PMN) leukocytes have significantly more CS receptor sites/cell than T and B cells (Katz, et al., 1985).

Net corticosteroid effects are also influenced by extraneous factors such as the presence of corticosteroid binding globulin (CBG), interleukins, and other hormones. CBG levels determine the amount of cortisol which is free in the plasma and thus biologically active (Feldman, 1989; McDonald, 1988). Furthermore, IL-1 and IFN gamma have been shown to assist CS in increasing beta endorphin production and NK activity respectively, in cell culture (Heijnen, et al., 1991; Holbrook, et al., 1983). Last, CS have permissive effects on the activity of hormones such as catecholamines (Tepperman and Tepperman, 1987) and may be themselves naturally opposed by hormones such as progesterone, androgens, and dehydroepiandrosterone (Baulieu and Mester, 1989; Blauer, et al., 1991).

Regardless of the cell type affected, the basic sequence of subcellular events leading to CS effects is thought to be the same. That is 1) CS binding to its receptor within the cytoplasm, 2) movement of the
CS-receptor complex to the nucleus, 3) binding of the complex to particular DNA sequences (glucocorticoid responsive elements) within the regulatory regions of genes, 4) stimulation/blockade of specific gene transcription and 5) protein synthesis from induced mRNA with resulting effects on cell activation, proliferation, or differentiation (Baulieu and Mester, 1989; Feldman, 1989). Since many cells including lymphocytes, and monocytes, contain CS receptors, it is thought that tissue specific or developmental control of CS actions is mediated by specific cellular transcription factors, chromatin structure, and degradative enzymes (Bowen and Fauci, 1988).

Other possible modes of action for CS include:
1) induction of antiphospholipase proteins or lipocortins,
2) increases in cAMP, 3) changes in membrane fluidity, and
4) alterations in ion channels (Bowen and Fauci, 1988).

CS are known to be potent anti-inflammatory and immunosuppressive agents. The basis of their actions may lie in their effects on leukocyte circulatory kinetics and the functional capability of leukocyte effector cells. Thus, within 2 to 3 hours following CS administration in vivo, researchers have routinely observed leukocytosis,
neutrophilia, lymphopenia, monocytopenia eosinopenia, increased percentage of NK cells, and decreased percentages of CD4+ T cells (reviewed in Katz, 1984; Parillo and Fauci, 1978; Tonnessen, et al., 1987). Therefore with regard to exercise, CS may mediate delayed changes (2 hours or greater) in the post exercise hemogram (Hickson and Boone, 1991; Pedersen, 1991; Rossdale, et al., 1982).

Corticosteroids also have dramatic effects on immune cell function. For example, CS inhibit synthesis of lymphokines and monokines such as IL-1, IL-2, IL-3, IL-6, interferon (IFN) gamma, granulocyte-monocyte-colony stimulating factor, and tumor necrosis factor (TNF) alpha and beta (Wu, et al., 1991), although the actual effect may depend on the activation signal for cytokine production (Hurme, et al., 1991). Likewise, the production or release of lymphotoxin, monocyte chemotactic factors, vasoactive amines, prostaglandins, and plasminogen activator is diminished in the presence of CS (Katz, 1984). Of note, IL-4 production has been reported to be either enhanced or depressed by CS (Daynes and Araneo, 1989; Wu, et al., 1991). In addition to their effects on cytokines, CS may decrease MHC II and IL-2
receptor expression while increasing IL-1 receptor expression (Dupont, 1988).

Predictably, CS effects on cell trafficking and leukocyte function translate into altered immune responses in vitro. Hence, after CS administration, PHA-induced proliferative responses and lectin mediated cytotoxicity were reduced (Fauci, 1976), while ADCC and NK activity was increased (Parillo and Fauci, 1978) coincident with peak changes in blood mononuclear cell proportions (Katz et al., 1983). Direct CS effects on in vitro lymphoid cell function have likewise been reported, presumedly resulting from down regulation of lymphokine production and activity and/or antigen presenting cell function. Thus a variety of research has demonstrated CS related decreases in mitogen responses (Almawi, et al., 1991; Deitch and Bridges, 1987; Goodwin, et al., 1979; Magnuson, et al., 1978; Reed, et al., 1986; Van Vorhies, et al., 1989), LAK activity (Grimm, et al., 1985; McVicar, et al., 1989; Papa, et al., 1986), CTL function (Grimm, et al., 1985), NK-mediated cytotoxicity (Fauci and Parillo, 1978; Holbrook, et al., 1983; Masera, et al., 1989), and lymphocyte proliferative responses to specific antigens (Almawi, et al., 1991; Katz and Fauci, 1979). Because there is a large amount of data regarding
immunosuppressive CS effects, the latter summary serves only to highlight altered immune responses relevant to the present work.

Notwithstanding the above reports, the relationship between CS and the immune system is complex and may be obscured by factors such as steroid dose or preparation and species sensitivity to CS. Studies that demonstrate CS-associated immune suppression in vitro often utilize synthetic preparations and/or non-physiologic concentrations of the steroid. Synthetic hormones such as dexamethasone or prednisolone may have a longer half life in circulation, bind less strongly to CBG, and have a higher affinity for the CS receptor (Feldman, 1989). As a consequence, synthetic CS are more potent than cortisol. Pharmacologic concentrations of cortisol and its analogues are almost invariably immunosuppressive and not particularly relevant to the normal in vivo situation. Finally, it should be noted that most studies on the effects of CS have been performed on CS-sensitive species such as rats and mice. In these animals, relatively small amounts of CS have dramatic suppressive effects on immune function including thymic involution and lymphoid cell death. In corticosteroid-resistant species such as the horse, immune suppression by CS may be absent or else
require high doses and prolonged exposure to CS for expression (Claman, 1972; Magnuson, et al., 1978). Clearly, the effects of CS on the immune system should be evaluated with these caveats in mind.

Accordingly, evidence suggests that the range and degree of CS-mediated immunosuppression has been overstated. A well documented example is the case of stress (electric shock)-induced suppression of mitogen-triggered lymphocyte proliferation and NK activity. For many years it was believed that this effect was mediated by CS. However, recent studies have documented that these same immunosuppressive effects occur in adrenalectomized animals exposed to stress, and the real culprit may be CRH-related catecholamine release (Jain, et al., 1991).

In fact, in vivo and in vitro studies support the concept that, under certain conditions, CS may enhance immune function. In this regard, replacement CS administered to adrenalectomized animals have been shown to protect against infections (Jefferies, 1990). Additionally, examination of the literature reveals instances of CS-induced augmentation of immune reactivity. Thus increases in mitogen responses (3 x 10⁶M cortisol) (Magnuson, et al., 1978; McVicar, et al.,
1989), LAK activity (10^7 M cortisone acetate) (Papa, et al., 1986), CTL activity (10^-6 M cortisol) (Grimm, et al., 1985), and antibody production (Bowen and Fauci, 1988; Cupps, et al., 1982; Jefferies, 1990) (10^-4 - 10^-5 M cortisol) have been reported. Moreover, high in vivo cortisol levels are compatible with high NK and PHA responsiveness (Kappel, et al., 1991; McGlone, et al., 1991), and the lack of effect of CS-induced lymphopenia on mitogen and antigen-specific proliferation suggests that CS might boost these responses on a per cell basis while decreasing the number of responding cells (Katz, et al., 1983; Magnusson, et al., 1978; Tavadia, et al., 1975).

The enhancing effect of CS on antibody production has been relatively well examined. In these studies, CS appear to amplify comparatively weak co-stimulatory signals resulting in an increased number of plaque forming cells. Thus spontaneous immunoglobulin production, polyclonally activated antigen-specific direct PFC responses to sRBC, and MLR-associated antibody production were elevated by 10^-4 - 10^-5 M cortisol (Bowen and Fauci, 1988; Cupps, et al., 1982). Brief in vitro exposure of B cells to CS was also demonstrated to result in increased spontaneous antibody production. Significantly, the authors noted that fetal bovine serum in the media could
provide the necessary auxiliary signal(s), but its capacity to do so varied with the lot of serum.

Catecholamines and Immune Function

Biologically active catecholamines such as epinephrine (E) and norepinephrine (NE), are derivatives of tyrosine produced by sympathetic nerve endings and the adrenal medulla. They mediate a multitude of physiologic changes which broadly compose the "fight or flight" response to stressors. Thus, catecholamines, and in particular epinephrine, stimulate a large number of the mechanisms required for vigorous exercise (Campese and DeQuattro, 1989). They favor the conversion of muscle glycogen to glucose phosphate, activate tissue lipases, inhibit insulin release, stimulate the heart, increase cardiac output and promote redirection of blood flow to muscles from organs not involved in exercise, facilitate neuromuscular transmission in skeletal muscle, stimulate contractile processes in fast twitch fibers, relax bronchioles, and stimulate respiration. Unlike steroid hormones, catecholamines exert their effects within minutes.

Four types of receptors (alpha\textsubscript{1}, alpha\textsubscript{2}, beta\textsubscript{1}, beta\textsubscript{2}) are known to interact with natural and synthetic
adrenergic agonists. They differ in their affinity for agonists and associated second messengers. The alpha receptors are most responsive to NE and E and least responsive to isoproterenol (I), a synthetic agent (Campese and DeQuattro, 1989). Activation of alpha1 adrenergic receptors precipitates signal transduction via the breakdown products of inositol phosphates, while amine stimulation of alpha2 receptors initiates their coupling to an inhibitory guanine nucleotide binding protein that ultimately decreases cellular cyclic adenosine monophosphate (cAMP) levels (Exton, 1985). In contrast to alpha receptors, beta receptors are more responsive to I than to E and NE. Both beta receptors are mated to stimulatory guanine nucleotide binding proteins that cause intracellular cAMP accumulation (Campese and DeQuattro, 1989). Elevated intracellular cAMP is thought to decrease lymphocyte proliferation, promote differentiation of immature lymphocytes, and/or inhibit mature differentiated lymphocyte function (Coffey and Hadden, 1985).

Just as the individual receptor types have defined affinities for agonists, adrenergic agonists themselves display characteristic patterns of interaction with different receptor types. Accordingly, E is considered a mixed alpha/beta agonist while NE has predominantly alpha
effects (Tepperman and Tepperman, 1987). Methoxamine (M) and I are artificially derived agonists possessing alpha_1 and beta_1/beta_2 stimulating activity respectively.

The outcome of any interaction between the immune system and catecholamines depends critically upon the type and number of adrenoceptors present on immune effector and/or auxiliary cells. Accordingly, studies have confirmed the existence of beta_2 receptors on peripheral blood lymphocytes but the presence of alpha receptors on these same cells requires further definition (Coffey, et al., 1975; McPherson and Summers, 1982). Beta adrenergic receptors are non-randomly distributed on immune cells and their subsets as reflected by differences in the magnitude of I induced increases in cellular cAMP (Khan, et al., 1985a; Khan, et al., 1986). Direct measurement of beta adrenergic receptors has confirmed the latter findings by demonstrating that NK cells possess the highest density of receptors with macrophages, B, and T suppressor cells trailing. T helper cells were found to have the fewest receptors (Maisel, et al., 1990).

Several factors may determine the number of adrenoceptors present on immune cells. For example, beta_2 receptors on PBL have been show to increase after short
term intense exercise (Frey, et al., 1989; Landman, et al., 1988; Maisel, et al., 1990). This increase may be achieved by both recruitment into the blood of cells with high beta receptor density (NK and T suppressor) and an elevation in per cell receptor numbers (Frey, et al., 1989; Landman, et al., 1988; Maisel, et al., 1990). On the other hand, physical conditioning is reported to decrease sympathetic nervous system responsiveness and is associated with a decline in beta receptor density on lymphocytes (Butler, et al., 1982; Ohman, et al., 1987). Related to physical conditioning is the phenomenon of beta adrenoceptor desensitization. In such cases, recurrent or continuous exposure to adrenergic agonists results in a depression in adenylate cyclase responsiveness and, at high doses, a decrease in the total number of receptors (Landman, et al., 1988). Studies of in vitro treatment of lymphocytes with beta agonists and prolonged exercise bouts further support this concept (Ohman, et al., 1987; Dailey, et al., 1988). Finally beta adrenoceptor numbers on lymphoid cells are influenced by the activation state of the cell (Daily, et al., 1988). Similar to transferrin, insulin, and IL-2 receptors, the beta\textsubscript{2} receptor of lymphocytes is up regulated by G\textsubscript{0} to G\textsubscript{1} progression in the cell cycle (Strom and Carpenter, 1980).
The stimulation of adrenergic receptors by their hormone ligands and subsequent intracellular signaling is subject to modulation by other receptor-mediated interactions. Glucocorticoids, for example, have striking effects on the responsiveness of many tissues to beta adrenergic stimulation (Tepperman and Tepperman, 1987). In some cases, glucocorticoid treatment increases receptor density and agonist affinity. Enhanced adrenergic receptor-G protein coupling and adenylate cyclase activity in the presence of corticosteroids has also been described. In general, adrenalectomy has the opposite effects. In addition, recovery from desensitization is impaired in the absence of CS and stimulated when the deficiency is corrected. Despite these observations, no unifying explanation for the permissive action of steroids on adrenergic function has been offered.

Cytokines such as IL-2 may likewise interfere with the catecholamine-mediated rise in cAMP in PBL (Beckner and Farrar, 1988), probably through an inhibition of adenylate cyclase activity by protein kinase C. The potential for other instances of cross regulation of the 2 primary intracellular signaling pathways, and thus for modulation of adrenergic actions, exists (Roszman and Brooks, 1988).
During physical exertion, catecholamines exert their effects on immune responses by direct and indirect means. An example of the latter case is the redistribution of subpopulations that occur during exercise. Indeed, epinephrine infusion into healthy volunteers mimics the leucocytosis, lymphocytosis, neutrophilia, decreases in CD4:CD8 ratio, and increases in NK cell numbers that accompany physical challenges (Crary, et al., 1983; Tonnesen, et al., 1987). Therefore, with regard to exercise, catecholamines may mediate acute changes in the post exercise hemogram especially apparent after short term physical exertion. A change in in vivo cell subtype proportions translates into similar in vitro changes that positively or negatively affect immune function. Accordingly, a decreased CD4:CD8 ratio and enhanced NK cell presence in peripheral blood could account for depressions in mitogen/antigen responsiveness (decreased CD4+ T helpers or increased CD8+ T suppressors) and elevations in NK cell activity, respectively, as measured in vitro.

Catecholamines may directly influence 3 general types of immune responses: antigen specific immune responses, mitogen-stimulated lymphocyte blastogenesis, and natural cytotoxicity. A number of studies have examined the
effects of adrenergic receptor activating substances on antigen-specific in vitro immune responses. Among these, the primary antibody response of murine splenocytes exposed to sRBC has been particularly well studied (Sanders and Munson, 1984; Sanders and Munson, 1985a; Sanders and Munson, 1985b). Adrenergic agonists have been observed to both suppress and enhance this response (Sanders and Munson, 1985a; Besedovsky, et al., 1979). In one in depth study, NE at $10^{-5}$ to $10^{-7}$M caused an increase in the number of IgM secreting cells when added to cultures at the same time as antigen. Synthetic adrenergic agonists were then used to define the relative contribution of adrenoceptors to the final NE effect. Thus stimulation by I ($10^{-4} - 10^{-6}$ and $10^{-9} - 10^{-10}$M) revealed a pervasive beta, enhancing effect and methoxamine treatment ($10^{-4} - 10^{-6}$) uncovered a similar, though subordinate alpha, enhancing effect. Alpha, agonists were suppressive (Sanders and Munson, 1984; Sanders and Munson, 1985a; Sanders and Munson, 1985b). While beta agonists increased the number of IgM PFC when added early to cultures, their presence in the PFC assay itself was inhibitory (Melmon, et al., 1974).

In an in vitro system generating alloantigen specific cell mediated cytotoxicity, addition of E ($10^{-6} - 10^{-7}$M) or
NE (10^{-4} - 10^{-3} \text{M}) at culture initiation increased the number of lytic units produced compared to controls (Hatfield, et al., 1986). A second similar study found that 10^{-6} - 10^{-3} \text{M} E and NE could enhance cytotoxicity in mixed lymphocyte cultures (Felton, et al., 1987). Addition of I (10^{-6} - 10^{-7} \text{M}) and other agonists and antagonists indicated that the response was primarily beta_2 adrenoceptor mediated. Interestingly, methoxamine was also stimulatory, while alpha_2 agonists were inhibitory. Again, beta agonists added to effector:target mixtures depressed the ability of CTL to kill appropriate target cells (Strom and Carpenter, 1990). Thus, while adrenergic receptor activating drugs appear to enhance the generation of antibody forming and CTL cells, they have the opposite effect on the function of the mature effector cells elicited. In fact, beta adrenergic agonists have been shown by other researchers to inhibit release phenomena associated with immune responses including the cytolytic activity of sensitized CTL and antibody production by plasma cells (Bourne, et al., 1974).

Aside from their effects on plasma cell and CTL development and function, adrenergic agents influence other aspects of antigen specific immunity. For example, both E and NE inhibit gamma interferon activation of
murine macrophages to become cytotoxic for herpes simplex virus (HSV) infected targets (Koff and Dunnegan, 1986). Another study revealed elevations of specific antigen-induced T cell proliferation by NE ($10^{-5} - 10^{-7}$M) along with an inhibition of the same response by I ($10^{-5} - 10^{-7}$M) (Winchurch and Mardiney, 1977). Lastly, I at $10^{-6}$M was found to depress tritiated thymidine incorporation in a one way mixed lymphocyte reaction (Feldman, et al., 1987).

Catecholamines modulate mitogen-stimulated lymphoproliferation and their final effects are concentration, time, and cell subset dependent. Thus high concentrations ($10^{-4}$M or greater) of these drugs are known to inhibit mitogen responses (Smith, et al., 1971; Johnson, et al., 1981; Hadden, et al., 1970). More physiologic levels, however, produce a range of outcomes. For example, in an early study, human PBMC were examined for their ability to take up tritiated thymidine following simultaneous stimulation by the plant lectin PHA and exposure to adrenergic agents (Hadden, et al., 1970). Under these circumstances, NE ($10^{-7} - 10^{-9}$M) caused significant enhancement of the PHA response. At the same time, I ($10^{-7}$M) produced a significant depression. E had no significant effect. Through the use of selective
antagonists, the increased response was attributed to alpha adrenoceptor stimulation, while the decreased responses were found to be mediated by beta adrenoceptor stimulation. Interestingly, a late pulse with NE or I produced an elevation in PHA-stimulated proliferation apparently associated with both alpha and beta receptor activation. As might be predicted from the variation in cell receptor expression, recent studies have demonstrated that cell subsets respond differently to adrenergic agonists. In particular, (Khan, et al., 1985b) the PHA responsiveness of Leu3+ 8+ T helper cells was enhanced in the presence of 10^5M I while that of Leu3+ 8− T helper cells was not.

A few studies have focused on adrenergic control of natural cytotoxicity. Natural killer and LAK cells are members of this group of immune effectors. As in the case of CTL and plasma cells, NK cell activity is enhanced by early pulsing with E (10^7 - 10^9M) and inhibited by the direct addition of E (10^6M) to lymphocyte target cell mixtures (Hellstrand, et al., 1985). Again, the stimulatory effect was associated with beta adrenoceptor function. What is known concerning adrenergic effects on LAK activity primarily relates to their modulation of IL-2 proliferative responses. Two studies indicate that I
(10^4 - 10^6M) inhibits IL-2-stimulated proliferation of human T cell lines (Beckner and Farrar, 1988; Feldman, et al., 1987). Concomitant decreases in IL-2 production and TAC antigen expression of treated cultures were reported in one paper (Feldman, et al., 1987). However, to elicit a comparable TAC suppression, E and NE had to be added at pharmacologic levels (10^2 -10^3M). The significance of the latter findings with regard to LAK cell generation in vivo is questionable in that 1) high doses of natural catecholamines are required and 2) expression of high affinity IL-2 receptors was not affected.

Neuropeptides and Immune Function

In addition to corticosteroids and catecholamines, the neuropeptides, beta endorphin (BE) and adrenocorticotropic hormone (ACTH), are elevated during acute exercise stress and may mediate exercise effects on immune function. Beta endorphin and ACTH are initially produced as a larger precursor molecule called pro-opiomelanocortin (POMC). After removal of the N-terminal sequences, appropriate processing of the precursor molecule gives rise to a number of peptide hormones including ACTH and BE (Eiper and Mains, 1980).
The main source of POMC peptides is the pituitary, but recent evidence indicates that B cells, T cells, and monocytes can be stimulated to secrete BE and ACTH (Heijnen, et al., 1991). Immune cell-derived POMC peptides appear identical to their pituitary counterparts at the amino acid level and bind to classical BE and ACTH receptors (Blalock, 1989). However, depending upon the inciting stimulus, different post-translational processing pathways are activated (Blalock, 1989; Heijnen, et al., 1991).

As was the case for pituitary secretion of ACTH (and BE), immune cell production of these compounds is positively and negatively regulated by other hormones and interleukins. Thus beta adrenergic agonists, CRH (acting through IL-1), and interleukins 1,2, and 6 up regulated the number of beta endorphin secreting cells measured by reverse plaque forming cell (RPFC) assay (Blalock, 1989; Heijnen, et al., 1991; Kavalaars, et al., 1990). Mitogens also enhance BE/ACTH production in culture. Down regulation of POMC production by immune cells is achieved by corticosteroids apparently through inhibition of CRH effects. Interestingly, when IL-1 was added to BE RPFC cultures, instead of CRH, corticosteroids were able to stimulate BE/ACTH secretion, presumably via increasing

Neuropeptides interact with cellular opioid and non-opioid receptors (Blalock, 1989). Stimulation of the ACTH receptor by its ligand results in increased cAMP while BE receptor activation has the opposite effect (Blalock, 1989; Johnson, et al., 1988). Like catecholamine receptors, neuropeptide receptor expression and activity is modulated by exercise and conditioning. Thus, in the horse, acute exercise bouts produce both a decrease in the number and an increase in the affinity of BE receptors (Hamra, 1990c). After physical conditioning, BE receptor affinity declines while per cell receptor numbers remain the same.

Beta endorphin and ACTH affect a number of neurophysiologic processes, including learning, memory, feeding behavior, body temperature, and pain perception (reviewed in Griffin, 1989). In addition, there is growing recognition that neuropeptides play a role in control of immune responses. Both humoral and cellular immunity have been shown to be affected. Accordingly, ACTH and BE have each been shown to regulate in vitro antibody production. Whereas high concentrations of ACTH
(10^{-6}M) inhibited the antibody response to a T independent antigen (DNP-ficoll), lower concentrations (10^{-7}M) were sufficient to depress antigen-specific (sRBC) antibody production, suggesting that T cell function was more sensitive to blockade by this substance than was B cell function (Johnson, et al., 1982). Beta endorphin exerted biphasic control over antibody production in that higher doses suppressed and low doses enhanced PFC responses (Blalock, 1989; Harbour and Smith 1990; Williamson, et al., 1988). The latter point was particularly well illustrated by studies of production of specific anti-herpes virus antibodies (Williamson, et al., 1988) in which BE concentrations of 10^{-14}M or below enhanced antibody production while higher concentrations suppressed antibody responses.

The role of BE and ACTH in modulation of cellular immunity is complex. Hence, in contrast to its suppressive effects on antibody production, ACTH (10^{-6}M) may enhance activated B cell proliferation and differentiation in the presence of IL-2 (Alvarez-Mon, 1985; Brooks, 1990). The apparent disparity in results between the antibody and proliferation effects was attributed to hormone concentration and differing cell subpopulations. T cell proliferation, on the other hand,
may be increased \((10^{-13}\text{M})\), decreased \((10^{-13}\text{M})\), or unchanged \((10^2 - 10^9\text{M})\) by ACTH, dependent upon the cell donor (Alvarz-Mon, et al., 1985; Heijnen, et al., 1987). In other work, \(10^{-6}\text{M}\) ACTH blocked IFN gamma production and activity (Johnson, et al., 1984; Koff and Dunegan, 1985). Notwithstanding the latter findings, ACTH \((10^{-8} - 10^{-11}\text{M})\) had no effect on NK and IL-2 activated NK cytotoxicity (McGlone, et al., 1991).

A good deal of information, albeit controversial, is available regarding BE effects on cell mediated immunity. Thus some investigators found that BE enhanced PHA and Con A-induced proliferation (overall effective range of \(10^{-6}\text{M} - 10^{-14}\text{M}\)) (Fontana, et al., 1987; Gilman, et al., 1982; Gilmore and Weiner, 1989; Heijnen, et al., 1987; Kusnecov, et al., 1987; Van den Bergh, et al., 1991), while others showed that BE \((10^{-7} - 10^{-9}\text{M})\) inhibited lymphocyte proliferative responses to PHA and Con A (McCain, et al., 1982; McCain, et al., 1986; Heijnen, et al., 1987). When peripheral blood mononuclear cells (PBMC) from conditioned horses were examined, BE depressed "suboptimal" PHA, Con A, and PWM-induced proliferation at \(10^{-7} - 10^{-11}\text{M}\) (Hamra, 1990d). Finally, studies of antigen-induced lymphoproliferation indicated that physiologic concentrations \((10^{-8} - 10^{-16}\text{M})\) of BE augmented an autologous
but not an allogeneic MLR (Froelich, 1987). Beta endorphin had no effect on herpes simplex virus-specific lymphoproliferation (Williamson, et al., 1988). In all of the above studies, the final proliferative response in the presence of BE seemed to depend upon the mitogen, the species, and once again, the peptide concentration with low doses increasing and high doses decreasing the response (McCain, et al., 1987; Heijnen, et al., 1987).

Cell-mediated cytotoxicity is also influenced by BE. Consequently, BE has been reported, by a number of investigators, to augment NK cytotoxicity (reviewed in Blalock, 1989; Harbour and Smith, 1990; Sibinga and Goldstein, 1988). Concomitant induction of NK-suppressor-inducer cells at higher BE concentrations has been postulated to be responsible for the biphasic effects of BE on antibody and proliferative responses (Williamson, et al., 1988). Beta endorphin may, additionally, enhance the generation of CTL ($10^8 - 10^{10}$M) (Carr and Klimpel, 1986) and LAK cells ($10^8$M) (Beckner and Farrar, 1988), consistent with BE induced increases in IL-2 and interferon production and IL-2 receptor expression (Brown and Van Epps, 1986; Gilmore and Weiner, 1988; Mandler, et al., 1986; Van den Bergh, et al., 1991). However, the relationship between BE, IFN, and IL-2 in enhancing
cytotoxicity may be more complicated than suspected, as binding of one of these ligands to its respective receptor modulates subsequent binding/activity of the other factors (Kay, et al., 1990).

In addition to the effects already discussed, BE may modulate other cellular immune responses. Among these are monocyte chemotaxis (increased) and T cell surface antigen modulation (increased HLA-Dr, decreased T3/T4) (Blalock, 1989; Harbour and Smith, 1990; Puppo, et al., 1985).
Chapter II

Materials and Methods

Horses in the Study

Twenty different thoroughbred horses were utilized in the course of these studies. A description of these horses and a summary of their use with regard to exercise tests, exercise conditioning, and immunologic assays is provided in Table 2.1.

Six of these horses (#20, 56, 58, 102, 108, 135) were entered into a physical training program (see below). Two months prior to the start of the program, the selected horses were placed on a nutritional conditioning program and were vaccinated with a commercially available influenza virus vaccine (Fort Dodge, Fort Dodge, IA). Each horse received 2 doses, 14 days apart, per label instructions. Horses were housed in open-air stalls and were fed 10 quarts of Omalene (Purina Mills, St. Louis, MO) at 8:00 AM and 4:00 PM except on the day of exercise stress tests when food was withheld until the stress test was completed.
### TABLE 2.1. Horses Used in the Studies.

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Sex</th>
<th>Age (yrs)</th>
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<td>9</td>
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<tr>
<td>16</td>
<td>M</td>
<td>8</td>
<td>ST-A,C,CP,I,AP,IV,H</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>21</td>
<td>C</td>
</tr>
<tr>
<td>20</td>
<td>G</td>
<td>11</td>
<td>ST-AB,CO,C,CP,I,AP,IV,F,NE</td>
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</tr>
<tr>
<td>58</td>
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</tr>
<tr>
<td>59</td>
<td>M</td>
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</tr>
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<td>83</td>
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<td>G</td>
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<td>C,I</td>
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<td>102</td>
<td>G</td>
<td>11</td>
<td>ST-AB,CO,C,CP,I,AP,IV,F,NE</td>
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<tr>
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<td>9</td>
<td>C,I</td>
</tr>
<tr>
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<tr>
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<td>ST-AB,CO,C,CP,I,AP,IV,F,NE</td>
</tr>
<tr>
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</tr>
<tr>
<td>Sly</td>
<td>S</td>
<td>3</td>
<td>ST-A,C,CP,H</td>
</tr>
</tbody>
</table>

a. M = Mare; S = Stallion; G = Gelding.

b. AP = LAK assay at 8:00 AM and 8:00 PM; C = LAK assays in the presence of cortisol; CO = Physical conditioning program; CP = LAK assay after in vitro pulse with cortisol; F = FACS analysis; H = hemogram data; I = IL-2 titration of LAK activity in the presence of cortisol; IV = PWM, influenza, and LAK assays after intravenous injection of cortisol or vehicle; NE = examination of PWM, influenza, and LAK response in the presence of neuropeptides and catecholamines; ST = stress test A or B including PWM, influenza and LAK assays.
A different group of five unconditioned horses (#16, 59, 83, 138, SLY) underwent an exercise stress test but were not subsequently trained. Pre-test handling of these animals was the same as that for the first group.

Exercise

Exercise Conditioning

A Mustang 2200 High Performance equine treadmill (Kagra Intl. Inc., Fahrwangen, Switz.) was used for both exercise conditioning and stress tests. After an adjustment period during which the horses were walked and trotted on the treadmill and an initial exercise stress test (protocol A—see below), a 12 week physical training program was initiated (Table 2.2). Each horse was exercised five days a week. The program consisted of graded increases in the duration, intensity, and speed of treadmill sessions culminating with speeds of 8.0 m/s for 7 minutes on a 7% incline. Horses were maintained in the conditioned state by continuation of the week 12 training protocol for 6 additional weeks.

Exercise Stress Tests

Prior to, and at the end of the exercise conditioning period, an exercise stress test utilizing protocol A
TABLE 2.2. The Treadmill Exercise Schedule.

<table>
<thead>
<tr>
<th>WEEK 1</th>
<th>WEEK 2</th>
<th>WEEK 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warmup</td>
<td>Warmup</td>
<td>Warmup</td>
</tr>
<tr>
<td>2 min. @ 1.5 m/s</td>
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<td>2 min. @ 1.5 m/s</td>
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<tr>
<td>9 min. @ 4.0 m/s</td>
<td>10 min. @ 4.0 m/s</td>
<td>12 min. @ 4.0 m/s</td>
</tr>
<tr>
<td>1 min. @ 1.5 m/s</td>
<td>1 min. @ 1.5 m/s</td>
<td>1 min. @ 1.5 m/s</td>
</tr>
<tr>
<td>Cooldown</td>
<td>Cooldown</td>
<td>Cooldown</td>
</tr>
</tbody>
</table>

<table>
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<tr>
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<td>1 min. @ 1.5 m/s</td>
<td>1 min. @ 1.5 m/s</td>
</tr>
<tr>
<td>2 min. @ 3.5 m/s</td>
<td>2 min. @ 3.5 m/s</td>
<td>2 min. @ 3.5 m/s</td>
</tr>
<tr>
<td>5 min. @ 7.6 m/s</td>
<td>7 min. @ 7.6 m/s</td>
<td>7 min. @ 8.0 m/s</td>
</tr>
<tr>
<td>3 min. @ 3.5 m/s</td>
<td>3 min. @ 3.5 m/s</td>
<td>3 min. @ 3.5 m/s</td>
</tr>
<tr>
<td>Cooldown</td>
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<table>
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<tr>
<th>WEEK 7</th>
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<tr>
<td>Warmup</td>
<td>Warmup</td>
<td>Warmup</td>
</tr>
<tr>
<td>1 min. @ 1.5 m/s</td>
<td>1 min. @ 1.5 m/s</td>
<td>1 min. @ 1.5 m/s</td>
</tr>
<tr>
<td>2 min. @ 3.5 m/s</td>
<td>2 min. @ 3.5 m/s</td>
<td>2 min. @ 3.5 m/s</td>
</tr>
<tr>
<td>8 min. @ 8.0 m/s</td>
<td>10 min. @ 8.0 m/s</td>
<td>9 min. @ 8.5 m/s</td>
</tr>
<tr>
<td>3 min. @ 3.5 m/s</td>
<td>3 min. @ 3.5 m/s</td>
<td>3 min. @ 3.5 m/s</td>
</tr>
<tr>
<td>Cooldown</td>
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</table>

<table>
<thead>
<tr>
<th>WEEK 10</th>
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<td>1 min. @ 1.5 m/s</td>
<td>1 min. @ 1.5 m/s</td>
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<tr>
<td>2 min. @ 3.5 m/s</td>
<td>2 min. @ 3.5 m/s</td>
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</tr>
<tr>
<td>9 min. @ 9.0 m/s</td>
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<td>3 min. @ 3.5 m/s</td>
</tr>
<tr>
<td>Cooldown</td>
<td>Cooldown</td>
<td>Cooldown</td>
</tr>
</tbody>
</table>

Warmup = Cooldown = 3 min. at 1.0 m/s. Horses were exercised five days a week.

* Performed on a 7% incline.
(exercise stress test A) was performed. The test was initiated at 6:00 AM and consisted of walking the horses for 2 minutes at 1.5 m/s, trotting for 3 minutes at 3.5 m/s, galloping for 3 minutes at 8 m/s, galloping for 5 minutes at 8 m/s on a 7% incline at and finally, trotting again for 2 minutes at 3.5 m/s. The end of the second trot was designated as the end of the stress test or T₀. Heart rate was assessed throughout the test using an on board heart rate monitor (Hippocard PEH2000, Bioengineering, Zurich, Switz.).

A second exercise stress test utilizing protocol B (exercise stress test B) was performed at the end of the maintenance period. The test was initiated at 6:00 AM and consisted of walking the horses for 2.0 minutes at 1.5 m/s, trotting for 3 minutes at 3.5 m/s, galloping for 3 minutes at 10 m/s, galloping for 5 minutes at 10 m/s on a 7% incline, and trotting for 2 minutes at 3.5 m/s.

Immunologic Assays

Preparation of Cells

Blood for determination of immunologic responses was collected on two occasions before any exercise tests or training program began. The first study consisted of a
determination of PWM, influenza, and LAK responses of cells collected from horses at 7:00 AM. The second study was conducted two weeks before exercise test A of unconditioned animals and consisted of blood collection from resting horses at times approximating the exercise test collections. These results comprised the sham test. On the day of an exercise stress test, blood was obtained 60 minutes before ($T_{-60}$), concurrent with ($T_0$), and 20 ($T_{+20}$) and 120 ($T_{+120}$) minutes after the end of test. Blood for immunologic assays not associated with exercise tests was collected between 6 and 9 AM.

Peripheral blood samples were obtained from the jugular vein (via an indwelling catheter during exercise tests) and aseptically collected into heparinized (10U/ml blood) vacutainer tubes. Buffy coat cells were obtained by centrifugation (750 x G, 10 min) and resuspended in calcium and magnesium free phosphate buffered saline (CMF-PBS). Buffy coats were underlayered with Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) and centrifuged (600 x G, 30 min). Subsequently, peripheral blood mononuclear cells (PBMC) were extracted from the interface, washed 3 times with CMF-PBS (150 x G, 10 min), and counted.
**Pokeweed Mitogen (PWM)-Induced Lymphoproliferation**

A bulk culture system was utilized for PWM assays during exercise stress tests. The assay consisted of incubating $1 \times 10^6$ cells/ml in 5 ml of media in 25 cm$^2$ flasks with 2 ug PWM (GIBCO/BRL, Gaithersburg, MD) per ml of culture at $39^\circ$ C, 5% CO$_2$ for 9 days. Media consisted of RPMI 1640 (GIBCO/BRL, Gaithersburg, MD) supplemented with 5% horse serum (v/v) (Whittaker Biosciences, Walkersville, MD), glutamine (GIBCO/BRL, Gaithersburg, MD), antibiotics (Pfizer, New York, NY), 20mM HEPES (Sigma, St. Louis, MO), and $2 \times 10^{-7}$ M 2-mercaptoethanol (Sigma, St. Louis, MO). On incubation days 1,3,5,7, and 9 triplicate 100 ul aliquots of cells were removed, pulsed with 0.5 uCi $^3$H-thymidine (New England Nuclear, Boston, MA) for 4 hours, and then harvested onto glass fiber filter paper and counted in a liquid scintillation counter. Corrected CPM was calculated as experimental CPM minus unstimulated control CPM. Statistical analysis was performed on data from peak proliferation days.

A plate culture system for PWM assays was used in studies of the in vitro effects of neuroendocrine hormones. These assays consisted of incubating $1 \times 10^5$ cells/well in quadruplicate on 96 well round bottomed plates (Corning, Corning, NY) with 2 ug PWM per ml of
culture, with or without 3 different concentrations of added test compounds, at 39° C, 5% CO₂ for 5 days. Final well volume with added media or test compounds was 0.2 ml. A control for determining the level (maximal or submaximal) of proliferation was included wherein cells were cultured with PWM and 10 U/ml IL-2 (Boehringer Mannheim, Indianapolis, IN; Cellular Products, Buffalo, NY). Media consisted of RPMI 1640 supplemented with 5% horse serum (v/v), antibiotics, 20 mM HEPES, 2 x 10⁻⁷ M 2-mercaptoethanol, and 0.06 mM 1-ascorbic acid (Sigma, St. Louis, MO) to minimize oxidation of catecholamines. On the fifth incubation day, wells were pulsed with 0.5 uCi ³H-thymidine for 4 hours, and then harvested onto glass fiber filter paper and counted in a liquid scintillation counter.

Equine Influenza Virus-Specific Lymphoproliferation

Antigen-specific lymphoproliferative responses to equine influenza virus were assessed during exercise stress tests using a bulk culture system. Equine Influenza virus type II was propagated in embryonated hen's eggs, and harvested allantoic fluid was stored at -70° C until use. 5 x 10⁶ PBMC were infected with 3 or 30 ul of influenza virus stock (HA titer = 160) for 20 minutes in 25 cm² tissue culture flasks in 1 ml of media.
Media was the same as that for the PWM studies. A sham infected control was included for each horse. The volume was subsequently increased to 5 ml using media, and cells were incubated at 39° C and 5% CO₂. On days 1, 3, 5, 7, and 9 of culture, triplicate 100 ul aliquots from each flask were removed, pulsed with ³H-thymidine, and counted as before. The virus dose and pulse day were optimized for each horse using corrected CPM.

In studies of the in vitro effects of cortisol on equine influenza virus-specific lymphoproliferation, a plate assay system was used. In these assays 12 x 10⁶ PBMC in 1 ml of media were infected with 30 ul (HA titer = 160) per 10⁷ cells equine influenza virus stock for 20 minutes. A sham infected control was included for each horse. The cell concentration was subsequently adjusted to 1 x 10⁶ cells/ml with media, and 0.1 ml aliquots were dispensed into 4 replicate wells of 96 well round bottomed plates. Final well volume with added media or cortisol (3 different concentrations) was 0.2 ml. Plates were incubated at 39° C and 5% CO₂ for five days. An IL-2 control was included as in the PWM assays. On day 5 of culture, plates were pulsed with ³H-thymidine, and counted as before.
Lymphokine Activated Killer (LAK) Cell Activity

As we reported previously, LAK cell activity in horses is demonstrated by incubating PBMC with high doses of IL-2 for 3 days and assaying these cells for cytolytic activity against an equine tumor cell line (Hormanski, et al., 1992). Initial IL-2 titration studies had determined that 250 U per ml human recombinant IL-2 (Boehringer Mannheim, Indianapolis, IN; Cellular Products, Buffalo, NY) was optimal for most horses. Accordingly, 2 x 10^5 equine PBMC in 100 ul volumes were placed into each of 4 replicate wells of 96 well round bottomed plates along with 250 U IL-2 per ml of culture or a matching amount of media (unstimulated controls) for a total well volume of 200 ul. In assays of the effects of neuroendocrine hormones on LAK activity, 2 x 10^5 PBMC in 50 ul volumes along with IL-2 at 250 U/ml of culture were placed into 4 replicate wells of 96 well round bottomed plates with or without 0.1 ml volume of 3 different concentrations of test compounds. Final well volume was 0.2 ml. In certain experiments (i.e., the titration of LAK activity in the presence of varying concentrations of IL-2 and cortisol -chapter 5), the concentration of IL-2 varied between 0 and 250 U/ml of culture for a given concentration of cortisol. Media consisted of RPMI 1640, 5% fetal bovine serum (v/v) (Hyclone Laboratories, Inc., Logan, UT), antibiotics, 20
mM HEPES, and $2 \times 10^{-7}$M 2-mercaptoethanol. Assays with neuroendocrine hormones utilized the above media with added l-ascorbic acid as before. Following a 72 hour incubation at 39° C and 5% CO$_2$, a standard $^{51}$Cr-release cytotoxicity assay was performed. An equine anaplastoid cell line, EqT8888, which was developed at Louisiana State University, was used as the target. EqT8888 cells were graciously provided by Dr. Robert Truax. Target cells were labeled for 2 hours with 100 uCi sodium chromate/10$^6$ cells and then washed and counted. Various numbers of LAK cells were incubated with 2000 $^{51}$Cr-labeled EqT8888 cells for 4 hours. Lysis of the EqT8888 cells was measured by quantitating the release of the radioisotope into the culture supernate. Percent specific lysis was calculated by the formula: (experimental release - spontaneous release)/(total release - spontaneous release).

Preparation and Use of Cortisol, Catecholamines and Neuropeptides

Corticosteroids, catecholamines and related agonists, and neuropeptides were evaluated for their effects on immune function. They included: (+) epinephrine, norepinephrine (-arterenol), methoxamine HCl,
(-) isoproterenol, and cortisol (hydrocortisone) (Sigma, St. Louis, MO); as well as beta endorphin and adrenocorticotropic hormone (Bachem, Inc., Torrance, CA). Concentrations used in studies were $10^{-6}$, $10^{-8}$, $10^{-10}$M (cortisol, beta endorphin, and adrenocorticotropic hormone); $10^{-6}$, $10^{-9}$, $10^{-12}$M (epinephrine, norepinephrine); and $10^{-4}$, $10^{-6}$, $10^{-8}$M (methoxamine, isoproterenol). Human beta endorphin and ACTH$_{1-39}$ were diluted in CMF-PBS, aliquoted, lyophilized, and maintained in air tight containers at $-70^\circ$ C until dilutions were prepared. Cortisol (.002 grams) was first dissolved in 500 ul absolute ethanol and then diluted 1:5500 with media to produce a $2 \times 10^{-6}$M solution. Further dilutions were made from this concentration. Ethanol by itself in the media had no effect on immune assays. All other compounds were directly dissolved in media to produce a solution with two times the highest desired final concentration and further diluted to the next lower concentrations. Different concentrations of test compounds were each aliquoted (0.1 ml) into 4 replicate wells of 96 well plates round bottomed plates, and frozen at $-70^\circ$C until use. Preliminary tests showed no significant mitogenic activity of compounds on unstimulated cells. In addition compounds had no significant effect on cytotoxicity of cells incubated without IL-2.
For the experiment in which cells were pulsed with cortisol in vitro, cortisol was dissolved in ethanol as before. Three stock concentrations (10⁻³, 10⁻⁵, and 10⁻⁷ grams/ml) of cortisol were subsequently produced by diluting the ethanol solution with CMF-PBS. No more than 7 ul of stock solution (minimum final ethanol dilution of 1:11,500) was added to individual 20 ml vacutainer tubes containing heparinized whole blood to produce the correct final cortisol concentration within the tubes, i.e., 10⁻⁶, 10⁻⁸, or 10⁻¹⁰M. Seven microliters of CMF-PBS/ethanol was added to a control tube for each horse. Tubes were mixed 10 times by gentle inversion and allowed to stand at room temperature for 1 hour. At the end of an hour, blood was processed as described previously.

Studies of in vivo cortisol effects were initiated at 6:30 AM and included 3 (# 16, 83, 135) cortisol and 4 (#20, 59, 108, 138) vehicle-treated horses. Blood for plasma cortisol determinations was collected 60 minutes before injection and 5, 10, 15, 30, 45, 60, and 120 minutes after injection. Blood for immunologic assays (PWM, influenza, and LAK) was collected 60 minutes before injection and 20 and 120 minutes after injection. Cortisol-treated horses received 150 ug cortisol.
intravenously, while control horses received an equal volume of vehicle.

FACS Analysis

During exercise test A and B of conditioned horses, PBMC were evaluated for expression of a pan T cell and a CD8-like antigen reactive with EqT3 and HT14A antibodies (VMRD, Inc., Pullman, WA), respectively, 60 minutes before \(T_{60}\) and 20 minutes after \(T_{20}\) the test. Approximately, \(10^6\) PBMC were resuspended in CMF-PBS with 10% goat serum and incubated on ice for 30 minutes with the EqT3 (1:30 dilution) or the HT14A (1:1000 dilution) monoclonal antibody. The labeled cells were washed with ice-cold CMF-PBS (3 ml, 200 x G, 10 min, 4° C) and incubated an additional 30 minutes with a 1:400 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Sigma, St. Louis, MO). Optimal working dilutions of EqT3, HT14A, and FITC were determined prior to use. The cells were again washed with CMF-PBS. One percent paraformaldehyde solution was added prior to storage of cells overnight at 4° C. The next morning, percent positive staining cells was determined using a Becton Dickinson model 440 flow cytometer.
Hematology

During exercise test A of unconditioned horses, samples for complete blood count (CBC) determinations were collected 60 minutes before \( (T_{-60}) \) and at the end \( (T_0) \) of the test for each horse. Accordingly, Vacutainer tubes containing EDTA were used for blood collection and CBCs were performed by the Veterinary Diagnostic Laboratory at Louisiana State University School of Veterinary Medicine. Blood for CBC determinations was also collected 60 minutes before and 20 and 120 minutes after intravenous cortisol/vehicle injection.

Measurement of Plasma Cortisol and Lactate

For plasma cortisol determinations during exercise stress tests, heparinized blood was obtained at \( T_{-60}, T_0, T_{+20}, \) and \( T_{+120} \) and centrifuged. Collected plasma was stored at \(-70\degree C\) until assayed. Concentrations of cortisol were measured by RIA using duplicate 10 ul aliquots of plasma after extraction with acetone, as previously described (Thompson, et al., 1988). Plasma cortisol assays were performed by the laboratory of Dr. Patricia A. Melrose.
Blood for lactate determinations was drawn before (0 minutes), and 5, 10, 20, 30, 45, 60, 90, and 120 minutes after the exercise stress test \( (T_0) \). Samples were drawn from a pre-placed jugular catheter and were collected into vacuum sealed tubes containing oxalate, for its anticoagulant properties, and fluoride, to stop further glucose metabolism and production of lactate. The tubes were immediately centrifuged and the plasma was separated for storage at \(-20^\circ\) C. Subsequent analysis utilized a lactate analyzer (model 23L, YSI Inc., Yellow Springs, OH). Lactate assays were performed by the laboratory of Dr. Steven G. Kamerling.

Statistical Analysis

Six horses were included in exercise stress test A of unconditioned horses. Six and five, respectively, of these same horses were included in exercise stress test A and B of conditioned horses. PWM and influenza virus proliferation data were expressed as corrected CPM. LAK data was calculated as percent specific lysis and analyzed as such at the 75:1 effector to target ratio. Where specified, lytic units were used. Time point effects were established using one way Analysis of Variance (ANOVA) and Least Significant Difference (LSD) comparisons (Blossom
Stat-Pack for LOTUS 1-2-3; Brian Cole, DMB, DCRT, National Institutes of Health) on PWM, influenza, and LAK data from T_{-60}, T_0, T_{+20}, and T_{+120} time points. To examine conditioning effects on baseline LAK, PWM, and influenza responses, data from the T_{-60} time point of exercise test A of unconditioned horses and from the same time point of exercise test B of conditioned horses were compared using a T test.

Proliferation and LAK studies in the presence or absence of cortisol, catecholamines, and neuropeptides were performed 3 times (T_{-60} of exercise test A and sham test, initial screening) on unconditioned horses and 2 times (T_{-60} of exercise test A and B) on conditioned horses. Where analysis of variance indicated that compound effects were equivalent, data were pooled and a consensus effect was derived by comparison of control data with those for 3 different tested concentrations for each compound.
Chapter III

Acute Exercise Stress Modulates Equine Immune Function

Introduction

Exercise stress, in various forms, has been shown to alter immune function. Accordingly, a spectrum of changes in immune reactivity is produced, dependent upon the physical fitness of the research subject and the intensity and chronicity of the exercise regimen. Thus, while a single strenuous exercise bout transiently suppresses certain immune parameters, moderate exercise conditioning may enhance immune responses (Fitzgerald, 1988; Fitzgerald, 1991). Progressive intensification of training appears to cause long term immunosuppression and accompanying susceptibility to infection.

Most research investigating exercise effects on the immune system has focused on those changes which occur following a single episode of intensive exercise. Studies performed primarily on human athletes and laboratory animals indicate that acute exhaustive exercise may temporarily depress the in vitro response of lymphocytes to mitogens, decrease CD4:CD8 ratios in the
presence of a leukocytosis, reduce levels of salivary IgA and IgM, modulate natural killer (NK) cell numbers and function, alter interleukin production patterns, and diminish neutrophil adherence and bacteriocidal activity (Caren, 1991; Fitzgerald, 1988; Fitzgerald, 1991; Mackinnon, 1989).

Despite large amounts of accrued data, experiments relating acute exercise stress to immune function are difficult to interpret and compare. This is due, in part, to differences in methodology. However, other factors contribute significantly to the confusion. For example, psychological stress is often superimposed upon physical stress as in the case of competing human athletes and laboratory rodents that are coerced into exercising. In addition, most studies have focused on an isolated aspect of humoral or cellular immunity, thereby obscuring functional cellular relationships.

In order to circumvent such difficulties, we evaluated the effect of acute exercise stress on 3 indices of immune function in the horse. An equine model of exercise stress was chosen because of its several advantages. First, exercise physiology in the horse is well understood, as is the quantitation of equine fitness
and exercise performance (Persson, 1985a). Second, the horse is a willing exerciser and, as such, experiences minimal extraneous stress. Third, the neuroendocrine system of the horse has been extensively studied and the hormones associated with stress responses have been identified and characterized (Alexander, et al., 1987; Church, et al., 1987; Irwin, et al., 1990). Finally, the peripheral blood of the horse is a readily available source of large quantities of sample material.

We examined aspects of both antigen-specific and non-specific immunity for the affects of exercise stress on immune function. These included pokeweed mitogen (PWM)-induced blastogenesis, influenza-specific proliferation, and lymphokine activated killer (LAK) cell activity of equine peripheral blood mononuclear cells (PBMC). PWM stimulates proliferation of B cells via a T cell dependent pathway, thus providing general information on immune status and T cell/B cell communication. Influenza virus-triggered immunoproliferation is an element of acquired immunity that involves relatively small numbers of T and B cells. Consequently, it may more accurately reflect in vivo circumstances. Equine LAK cells, as we have recently demonstrated, are derived from T cell subpopulations and are capable of lysing allogeneic tumor targets (Hormanski,
et al., 1992). Since their ability to develop into cytotoxic effectors hinges upon IL-2 responsiveness, LAK cells may serve as a barometer of lymphocyte sensitivity to this cytokine.

By using these 3 partially overlapping immunologic indicators along with the equine exercise stress model, we were able to investigate the relationship between uncomplicated exercise stress and a broad profile of immune function. In so doing, we have accumulated new data regarding the relationship between immune status and physical activity in the horse.

Results

Generation and Quantitation of Exercise Stress

In order to examine the effects of acute exercise stress on equine immune function, 6 unconditioned thoroughbred horses were subjected to a severe treadmill-based exercise challenge (exercise stress test A - Materials and Methods). Since the quantitation of exercise stress is key to interpretation of associated changes in immune function, 3 separate measures of physiologic strain were assessed: heart rate, plasma lactate, and plasma cortisol levels. Figure 3.1 relates heart rate data from
the exercise test to the exercise regimen itself. It demonstrates the progressive increase in heart rate in association with increasing physical demands. Peak heart rate values were obtained during the gallop on a 7% incline with values exceeding 200 beats per minute.

Blood lactate, as shown in Figure 3.2, was determined before (0 minutes) and 5, 10, 20, 30, 45, 60, 90, and 120 minutes after the exercise stress test. A plasma lactate level of 4mM/L marks the onset of exercise-induced plasma lactate accumulation (anaerobic threshold) in both humans and horses (Persson, 1985a). We noted a maximum lactate reading of 9.3 mMol/L 5 minutes post exercise which declined rapidly over the next 40 minutes.

Increases in cortisol are a normal part of the endocrine response to exercise. In addition, glucocorticoids have often been demonstrated to have immunomodulatory effects. Consequently, plasma cortisol was determined 60 minutes before (T_{50}), concurrent with (T_0), and 20 (T_{+20}) and 120 (T_{+120}) minutes after the end of the exercise test. As Figure 3.3 indicates, peak cortisol levels of approximately 60 ng/ml were attained at T_0 and T_{+20} with values 76% higher than at T_{50}. 
Figure 3.1. The effect of exercise on the heart rate of unconditioned horses. Unconditioned thoroughbred horses were exercised on an equine treadmill according to exercise protocol A (see Materials and Methods). Heart rates were determined by means of an on board electronic heart monitor. Speed (.....) and heart rate (____) values represent the mean for 6 horses.
Figure 3.2. Plasma lactate levels of unconditioned horses before and after exercise. Unconditioned thoroughbred horses were exercised on an equine treadmill according to exercise protocol A (see Materials and Methods). Blood for lactate determinations was collected before (0 minutes) and 5, 10, 20, 30, 45, 60, 90, and 120 minutes after the test. Data points represent the mean for six horses.
Figure 3.3. The effect of exercise on plasma cortisol levels of unconditioned horses. Unconditioned thoroughbred horses were exercised on an equine treadmill according to exercise protocol A (see Materials and Methods). Blood for plasma cortisol determinations was collected 60 minutes before (T_{-60}), concurrent with (T_{0}), and 20 and 120 minutes after (T_{+20} and T_{+120}) the end of the exercise test. Values represent the mean for 6 horses ± SEM.
Effects of Exercise Stress on Immune Function

To gain information on the range of alterations in immune function that occur following acute exercise stress, we examined PWM-induced blastogenesis, equine influenza virus type II-specific lymphoproliferation, and LAK activity of equine PBMC at T₆₀, T₀, T₂₀, and T₁₂₀. Figure 3.4 relates the findings for PWM (black bars) and equine influenza virus (open bars) assays during a sham and an exercise stress test of unconditioned horses. Results for PWM studies indicate no significant differences in corrected CPM between any of the time points during the sham test. On the contrary, a significant (P < .05) decrease in corrected CPM occurred at T₀ relative to T₆₀ of the exercise stress test. Likewise, a significant (P < .05) reduction in influenza virus-stimulated proliferation was noted at T₀ of the exercise stress test only.

In contrast to our findings for PWM and influenza virus-associated lymphoproliferation, equine LAK activity underwent a post exercise enhancement. Figure 3.5 is a side by side comparison of the cytotoxic activity of LAK cells generated from the blood of sham or exercised horses. After acute exercise stress, LAK activity was significantly (P < .05) increased at T₀ and T₂₀, and
Figure 3.4. Pokeweed mitogen (PWM) and equine influenza virus-induced lymphoproliferative responses before and after a sham and an exercise stress test of unconditioned horses. Six unconditioned thoroughbred horses were exercised on an equine treadmill according to exercise protocol A (see Materials and Methods). Blood for lymphoproliferation studies was collected 60 minutes before (-60), concurrent with (0), and 20 and 120 minutes after the end of the exercise test. For the sham test, blood was collected at the corresponding times from resting horses. Values for PWM (solid bars) and influenza (open bars) responses were derived from corrected CPM expressed as a proportion of the -60 time point.

* Significantly different from the -60 time point using ANOVA; (P < .05, n = 6).
Figure 3.5. Lymphokine activated killer (LAK) cell activity of unconditioned horses during a sham and an exercise stress test. Six unconditioned thoroughbred horses were exercised on an equine treadmill according to exercise protocol A (see Materials and Methods). Blood for LAK studies was collected 60 minutes before (T$_{60}$), concurrent with (T$_0$), and 20 and 120 minutes after (T$_{+20}$ and T$_{+120}$) the end of the exercise test. For the sham test, blood was collected at the corresponding times from resting horses. Percent specific lysis values obtained with 250 U/ml IL-2 (solid circles) or without IL-2 (open circles) represent the mean for 6 horses. A significant increase (P < .05, ANOVA at each effector to target ratio) in LAK activity relative to the T$_{60}$ time point was observed at T$_0$ and T$_{+20}$ of the exercise test only.
non-significantly increased at T_{+120} relative to the T_{40} time point. No significant changes in LAK activity were produced by bleeding the horses as in the sham stress test. No significant cytolytic activity was exhibited by control cells that did not receive IL-2 (open circles).

In order to assess blood cell subset redistribution patterns occurring after exercise, we collected blood for complete blood counts (CBC) at T_{40} and T_{0} from a different group of horses (4M, 1S) undergoing a comparable exercise challenge. Results are presented in Table 3.1. Major findings were leukocytosis, relative and absolute neutropenia and lymphocytosis, a decreased neutrophil to lymphocyte (N/L) ratio, and an increase in the packed cell volume (PCV) and total serum protein (TP) immediately following exercise. Exercise-induced physiologic and immunologic changes (data not shown) were similar to those found after exercise with the first group of horses.
TABLE 3.1. Hematologic Data from an Acute Exercise Stress Test of Unconditioned Horses.

<table>
<thead>
<tr>
<th>Time After Exercise* (minutes)</th>
<th>Value</th>
<th>-60</th>
<th>+0</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCb</td>
<td></td>
<td>6780</td>
<td>7020</td>
</tr>
<tr>
<td>Segs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rel. c</td>
<td></td>
<td>59.8</td>
<td>54.2</td>
</tr>
<tr>
<td>abs. d</td>
<td></td>
<td>4.1</td>
<td>3.8</td>
</tr>
<tr>
<td>Lymphs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rel.</td>
<td></td>
<td>34.2</td>
<td>42.0</td>
</tr>
<tr>
<td>abs.</td>
<td></td>
<td>2.3</td>
<td>2.9</td>
</tr>
<tr>
<td>N/L</td>
<td></td>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>PCVf</td>
<td></td>
<td>38.2</td>
<td>49.8</td>
</tr>
<tr>
<td>TPg</td>
<td></td>
<td>6.6</td>
<td>7.1</td>
</tr>
</tbody>
</table>

a. Five unconditioned thoroughbred horses (4M, 1S) were subjected to a treadmill-based exercise stress test (protocol A - Materials and Methods) and blood for complete blood counts was collected 60 minutes before (-60) and immediately after (+0) exercise. Results represent the average for the five horses.


c. Relative number of cells expressed as a percentage.

d. Absolute number of cells ( X 10³/ul).

e. Neutrophil to Lymphocyte ratio.

f. Packed Cell Volume expressed as a percentage.

g. Total Protein in grams/dl.
Discussion

Our data demonstrate that acute strenuous exercise both depresses and enhances aspects of immune function in the horse. The intense nature of the stress that we observed is validated by comparison to the results of other researchers. Thus, just as in our studies, other researchers reported average heart rates in excess of 200 bpm in horses exhaustively exercised on a treadmill (Kurcz, 1988). Cortisol levels 30 minutes post exercise were increased 35 and 100% in 2 studies of horses undergoing acute treadmill-based exercise stress, while we documented a 76% increase in cortisol levels 0 and 20 minutes post exercise (Church, et al., 1987; Kurcz, et al., 1988). In horses, lactate levels do not exceed 4mM/L after submaximal exercise but increase 20 to 25 fold over baseline after maximal exercise (Persson, 1985a). Accordingly, we noted a 23 fold increase in lactate levels 5 minutes after the end of the exercise test as compared to levels in resting horses.

Like exercise stress intensity, detected changes in immune reactivity require interpretation with regard to existing experimental data. A number of different immunologically active mitogens have been studied relative
to exercise stress. Our finding of depressed PWM responses in the horse immediately after exercise is not unique when compared to other species. Thus suppression of mitogen responsiveness following various short term exercise protocols has been demonstrated in humans (Fitzgerald, 1988; Fitzgerald, 1991; Keast, et al., 1988; Simon, 1984). For example, it has been reported that responses of peripheral blood leukocytes to phytohemagglutinin (PHA), concanavalin A (Con A), and purified protein derivative (PPD) were decreased 30 minutes after completing a 2.5 hour marathon, but had returned to normal within 24 hours (Eskola, et al., 1988).

Recently, in a study of unconditioned horses experiencing an exercise challenge, no significant change in blastogenic response to the mitogens PHA, Con A, or PWM was observed after exercise (Wong, et al., 1992). These results are in contrast to our own and to those of another group of researchers investigating equine exercise stress (Kurcz, et al., 1988). The latter group reported a significant decline in PHA and Con A responsiveness 30 minutes subsequent to exhaustive exercise. However, a nonsignificant decrease in mitogen-induced proliferation occurred 15 minutes after exercise. The nonsignificant
decrease in the mitogen response at this time point could be explained by the refractoriness to change of high proliferation rates produced by the optimal mitogen doses used in the study. Therefore, their findings support our own in that early suppression of mitogenic responses followed acute severe exercise. The absence of significant findings by other researchers working with horses is puzzling, but may be related to differences in exercise intensity or methodology (Wong, et al., 1992). Of note, mitogen responses immediately after exercise were not examined, and the peak heart rate, cortisol, or lactate levels were not presented in the study making comparison of exercise stress levels difficult.

Because response to mitogens is an index of the polyclonal proliferative capacity of lymphoid cells, it provides an incomplete answer to the question of whether acute exercise stress affects susceptibility to specific disease. Mitogenic response may not accurately predict B and T cell reactivity to specific antigens (Eskola, et al., 1978). Unfortunately, there has been little direct testing of the relationship between antigen-driven immune responses and exercise of any kind (Eskola, et al., 1978; Hedfors, 1983; Liu and Wang, 1987). Our own observations indicate that, in the horse, secondary in vitro immune
responses to influenza virus are strongly inhibited following strenuous exercise. An implication of the latter is that exposure to virulent virus during or after exercise could result in increased susceptibility to viral infection. In fact, epidemiological studies of illness in human athletes support this contention (Peters and Bateman, 1983). Likewise, post exercise immunosuppression may be responsible for increased vaccine failure and susceptibility to respiratory infection seen in athletic horses during racing season (Bayly, et al., 1987; Laigreid, et al., 1988). Regardless, the data relating acute exercise stress to antigen specific immune function is scant and any conclusions in this area would be premature.

A fair amount of research has focused on the effects of exercise stress on natural killer (NK) cell activity. Natural Killer cells possess the ability to lyse certain tumor and virally infected cells in a genetically unrestricted fashion. Acute episodes of intense exercise generally result in an increase in the percent of NK cells in peripheral blood as well as an increase in NK cell activity (Mackinnon, 1989). Although a correlation between the latter two effects has been demonstrated in some cases, other studies reveal that an increase in NK
IL-2 responsiveness may also occur (Pedersen, et al., 1988). Until recently, detection of NK cell activity in normal adult horses has been difficult and hence data regarding exercise and NK function in the equine are lacking (Chong, et al., 1992; Magnuson, et al., 1987).

In contrast to NK cells, little is known about exercise induced modulation of related lymphokine activated killer cell activity. LAK cells exhibit enhanced cytotoxicity as a result of incubation with IL-2. The fact that our LAK assay measures T-cell- rather than NK cell-derived LAK activity makes our results even more unique. Nonetheless, our data was surprisingly similar to previous studies of NK activity in that we found LAK activity to be maximally elevated within 20 minutes of the exercise stress with a decline toward normalcy by 120 minutes post exercise.

The mechanism whereby PWM and influenza virus-triggered lymphoproliferation is suppressed while LAK activity is enhanced is not known. However, a variety of immunomodulatory substances are released into the blood during exercise (Keast, et al., 1988). Prominent examples of such compounds include cortisol, beta endorphin, epinephrine, interferon, IL-1, and IL-2. Their likely
modes of action are through alteration of blood cell trafficking and direct effects on lymphocyte function. Accordingly, the post exercise leukogram in humans includes transient leucocytosis, lymphocytosis involving increased B cell numbers and decreased CD4+ T cell numbers, neutrophilia, and an increase in the percentage of cells in peripheral blood expressing NK surface markers (Caren, 1991; Keast, et al., 1988; Simon, 1984). In the horse, early increases in leukocyte and lymphocyte counts and decreases in the N/L ratio, followed by late (2 hours post) increases in the N/L ratio have been noted after exercise (Rose and Allen, 1985; Rossdale, et al., 1982). Although we did not perform CBCs on blood collected at T+2 h, our results are comparable to those of others for the immediate post exercise period. Changes in PCV and TP are compatible with splenic release of red blood cells and hemoconcentration known to occur during exercise in the horse. Both corticosteroids and epinephrine are capable of producing part or all of this white blood cell profile.

Changes in peripheral blood cell distribution patterns provide an easy explanation for some of our experimental results. If CD4+ cells are decreased after exercise in the horse, then there will be a relative lack of the T helper cells that are necessary to support
influenza virus- and PWM-stimulated proliferation. Conversely, an increased proportion of CD8+ T cells (cytotoxic/suppressor subset) could be providing direct suppression through suppressor cell activity. Further investigation of cell surface antigens on equine T cell-derived LAK cells are required before a full explanation of post exercise increases in LAK activity can be offered.

Apart from their influence on PBMC compartmentalization, exercise stress-related compounds may have more direct effects on immune cells. Alterations in cell function could also account for some of the post exercise changes in immunity observed in this work. However, little information on this subject area is available in the equine.

Whatever the mediator or the mechanism, the significance of transient exercise stress-induced changes in immune parameters remains obscure. Are these changes a harmless consequence of strenuous physical exertion or a risk factor for disease? Epidemiological studies in humans as well as clinical observations by equine veterinarians suggest that alone, or in combination with additional physical, mental or immunologic stressors, exercise stress may precipitate or exacerbate certain
infections. On the contrary, one researcher has found that antibody responses to a test antigen were normal, despite the fact that the antigen was administered immediately after maximal physical exertion concurrent with marked depressions in lymphocyte responses to mitogens (Eskola, et al., 1978). The response to a live infectious agent, however, was not examined and might have yielded different results. The equine model of exercise stress offers a unique opportunity for future studies of the relationship between exercise and immune function.
Chapter IV

Physical Conditioning Modifies Exercise Effects on Equine Immune Function

Introduction

In humans, exercise conditioning is associated with multiple health-related benefits. Among these are improved cardiovascular function, training dependent changes in muscle metabolism, increased aerobic capacity, an enhanced sense of well-being, and possibly, increased longevity (Hickson and Boone, 1991).

The effects of physical conditioning on the immune system are not however, as clear cut. At rest, trained individuals may exhibit increases in MHC II expression of lymphoid cells (Esperson, et al., 1990), natural killer (NK) cell activity (Pedersen, et al., 1989), interleukin 1 levels (Caren, 1991), and lymphoproliferative responses to mitogens (Soppi, et al., 1982; Watson et al, 1986). Such training-induced immune enhancement could prove to be the basis for observations of elevated antibody response to antigens and delayed tumor growth in conditioned mice when
compared to unconditioned animals (Good and Fernandes, 1981; Liu and Wang, 1987). Likewise, the association between job-related physical activity and lower colon cancer rates in people may reflect positive immune function changes related to exercise conditioning (Gerhardsson, et al., 1986).

Negative effects of training on immune function have also been noted. They include depressed resting salivary IgA, serum IgG, complement levels, total lymphocyte counts, and neutrophil adherence (Fitzgerald, 1988; Lewicki, et al., 1987; Nieman, et al., 1989; Tomasi, et al., 1982). Again, these immune adaptations may have correlates in disease outbreaks that appear to preferentially target or be most severe in conditioned athletes (Fitzgerald, 1988).

Seemingly antithetic effects of exercise conditioning on immune responses probably reflect differences in methodology. In particular, many human studies utilize elite athletes who, by definition, experience regular intense exercise bouts. Acute exhaustive exercise, by itself, produces a variety of immune alterations ranging from temporary depressions in lymphocyte responses to mitogens, decreased CD4:CD8 ratios, and lymphocytosis to
reduced salivary IgA and IgM levels, altered NK numbers and function, and diminished neutrophil adherence (Caren, 1991; Fitzgerald, 1988; Mackinnon, 1989; Pedersen, 1991). At least one author has suggested that the suppressive effects of intense exercise may be cumulative over certain time intervals making the distinction between acute exercise effects and conditioning effects more difficult when professional athletes are used (Mackinon, et al., 1988b). The situation becomes even more complex if training-related changes in acute exercise effects on immune function are considered.

In order to clarify the relationship between exercise conditioning, acute exercise stress, and immune reactivity, we entered six thoroughbred horses into a physical training program. Concurrently, we evaluated their immune status before and after exercise of different intensities. Our experimental strategy afforded 2 advantages. First, it allowed for pre- and post-conditioning comparisons of the same group of animals. Second, it utilized an animal that is a willing exerciser and is less subject to the additional stress associated with coercion. We have obtained results indicating that, while physical conditioning did not cause profound changes in baseline values of immune parameters, it did
significantly alter the immunologic response of the horse to acute exercise.

Results

Physical Conditioning and Exercise Intensity Determine the Physiologic Response to Exercise

In order to determine the effects of physical conditioning on immunologic function, we subjected 6 thoroughbred horses to a 12 week exercise conditioning program and 2 separate exercise stress protocols (A and B). Table 4.1 displays the physiologic data obtained from the exercise stress tests of trained and untrained animals. As expected, both the severity of the stressor and the physical conditioning of the horse affects the physiologic response to exercise. When trained horses were exercised at 2 different intensities (maximum speeds of 8 m/s vs 10 m/s), maximum heart rate, plasma lactate, and cortisol levels were relatively increased in the more severely stressed animals (Table 4.1). Likewise, when trained and untrained horses experienced the same exercise challenge (maximum speed of 8 m/s), untrained horses responded with greater elevations in heart rate and plasma lactate, and equivalent increases in plasma cortisol (Table 4.1). Untrained horses undergoing stress test A
and trained horses undergoing stress test B exhibited similar peak heart rate and lactate levels while the exercise-induced plasma cortisol increase was greater in trained animals.
### TABLE 4.1. Physiologic Data from Exercise Stress Tests of Trained and Untrained Horses.

#### Peak Response

<table>
<thead>
<tr>
<th>Horses</th>
<th>Test^a</th>
<th>Heart Rate^b (bpm)</th>
<th>Lactate^b (mM)</th>
<th>Cortisol (% increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>untrained</td>
<td>A</td>
<td>205 ± 17</td>
<td>9.3 ± 2.8</td>
<td>76</td>
</tr>
<tr>
<td>trained</td>
<td>A</td>
<td>180 ± 11</td>
<td>1.5 ± 0.1</td>
<td>78</td>
</tr>
<tr>
<td>trained</td>
<td>B</td>
<td>210 ± 6</td>
<td>12.2 ± 1.0</td>
<td>139</td>
</tr>
</tbody>
</table>

^a. Horses were exercised on a treadmill according to the protocol given in Materials and Methods for each exercise test. Six horses were included in exercise test A of trained and untrained horses, while five horses were included in exercise test B of trained horses.

^b. Mean ± SEM
Physical Conditioning Modulates Post Exercise Immune Function

During the course of these studies, three immunologic parameters were assessed before and after each exercise test. Heparinized blood samples for immunologic testing were collected 60 minutes before (T-60), concurrent with (T0), and 20 (T+20) and 120 (T+120) minutes after the end of a given stress test. Table 4.2 presents average proliferation data for PWM and influenza responses, as well as average percent specific lysis data (75:1 effector to target ratio) for LAK responses of cells obtained from resting horses prior to (T-60) exercise stress test A of untrained and exercise stress test B of trained horses. No conditioning effect on basal immune parameters was detected (P > .05).

On the other hand, post exercise immune responses were modulated by both exercise intensity and conditioning. Accordingly, significant reductions (data not shown) in mean PWM responses relative to the T-60 time point were found at T0 (21% decrease, P<.01) and T+20 (44% decrease, P < .05) for untrained and trained horses, respectively, experiencing stress test A. A significant decrease in PWM responses (62% decrease, P < .01) occurred at T+120 in trained horses undergoing stress test B.
TABLE 4.2. Basal Immune Responses of Trained and Untrained Horses.

<table>
<thead>
<tr>
<th>Immune Response</th>
<th>Untrained</th>
<th>Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68266 ± 20829</td>
<td>68066 ± 5428</td>
</tr>
<tr>
<td>Influenza&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7358 ± 4264</td>
<td>8480 ± 1494</td>
</tr>
<tr>
<td>LAK&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.2 ± 1.8</td>
<td>12.8 ± 3.1</td>
</tr>
</tbody>
</table>

a. Blood for determination of basal immune responses was drawn from horses prior to (T<sub>40</sub>) exercise stress test A of untrained and exercise stress test B of trained horses. PWM, influenza, and LAK assays were performed as in Materials and Methods. Six untrained and five trained horses were included in the study. No significant changes in immune function were found to occur as a result of training (P > .05; T test).

b. Mean corrected CPM ± SEM.

c. Mean % specific lysis at the 75/1 effector to target ratio ± SEM.
Figure 4.1 shows the individual lymphoproliferative responses of horses to equine influenza virus type II at T₀ for the same fitness and exercise stress levels indicated in Table 4.1. Results are presented as percentage of the T₅₀ time point in order to graphically compare exercise stress tests performed on different days or horses which differ in baseline immune function. The variation in responses of trained horses to exercise protocol A is illustrated, and consequently, a significant decrease (P<.01) in T₀ average corrected CPM was observed only for stress test A of untrained and stress test B of trained horses.

The effects of exercise on LAK cell function in trained and untrained horses at the 75:1 effector to target ratio were analyzed (Figure 4.2). Exercise protocol A produced a significant elevation (P < .01) in LAK cell-mediated cytotoxicity in untrained horses at T₀ and T₂₀ with % lysis values approximately 3 times greater than at T₅₀. Trained horses did not register comparable LAK activity increases under any exercise conditions.

In order to examine changes in lymphocyte subpopulations occurring after exercise of different
Figure 4.1. Equine influenza virus-stimulated lymphoproliferation of equine PBMC immediately after exercise (T₀) for 3 training/exercise stress conditions. Horses were exercised on an equine treadmill according to exercise protocol A (trained and untrained horses) or B (trained horses). Blood for proliferation assays was collected 60 minutes before (T₉₀), concurrent with (T₀), and 20 and 120 minutes (T₊₂₀ and T₊₁₂₀) after the end of each exercise test. Since significant changes in the influenza response did not occur at other times, only the T₀ time point is presented here. Data points are the mean corrected CPM for each horse expressed as percentage of the T₉₀ time point. A significant decrease (P < .01; ANOVA) in T₀ average corrected CPM was observed for exercise stress test A of untrained (n = 6) and exercise stress test B of trained horses (n = 5).
Figure 4.2. Lymphokine activated killer (LAK) cell activity of equine PBMC before and after exercise for 3 training/exercise conditions. Horses were exercised on an equine treadmill according to exercise protocol A (trained and untrained horses) or B (trained horses). Blood for LAK assays was collected 60 minutes before (T-60), concurrent with (T0), and 20 and 120 minutes (T+20 and T+120) after the end of each exercise test. Values are the mean percent specific lysis ± SEM at the 75:1 effector to target ratio expressed as percentage of the T-60 time point. A significant increase (P < .01; ANOVA) in LAK activity was only observed at T0 and T+20 for exercise stress test A of untrained (n = 6) horses.
intensities, we labelled PBMC obtained at T_{40} and T_{420} for exercise stress tests A and B of trained horses with antibodies recognizing equine T cells (EqT3) or a CD8-like cell subpopulation (HT14A) (Table 4.3) and determined the percent positive cells using a fluorescence activated cell sorter (FACS). A moderate but significant (P<.05) increase in the percentage of CD8+ cells was observed only after severe exercise stress (B) of trained horses. No change in the percentage of equine T cells occurred following either exercise protocol.
TABLE 4.3. Percent Positive Staining of Equine PBMC Before and After Two Different Exercise Stress Tests of Trained Horses.

<table>
<thead>
<tr>
<th>REACTIVITY WITH ANTIBODY*</th>
<th>EqT3</th>
<th>HT14A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise Test</td>
<td>T-60</td>
<td>T+20</td>
</tr>
<tr>
<td>A</td>
<td>59.1 ± 2.8</td>
<td>57.6 ± 2.8</td>
</tr>
<tr>
<td>B</td>
<td>50.9 ± 5.2</td>
<td>45.9 ± 3.9</td>
</tr>
</tbody>
</table>

a. Equine PBMC collected before (T-60) and 20 minutes after (T+20) exercise were stained with monoclonal antibodies recognizing T cells (EqT3) or CD8+ cells (HT14A) and subjected to FACS analysis. This procedure was performed for exercise stress tests A (n = 6) and B (n = 5) of trained horses. Results are presented as average percent staining ± SEM.

* Significantly different from T-60 (P < .05; T test).
Discussion

The equine athlete, like its human counterpart, undergoes a series of physiologic adaptations upon conditioning. Changes in skeletal muscle fiber type composition and enzymatic activity have been described along with an increased aerobic capacity and anaerobic threshold (Persson, 1985a; Rose, 1985). Physical approximations of the latter two findings are V200 and VLA4 (Persson, 1985a). V200 refers to the treadmill speed that produces a heart rate of 200 bpm, while VLA4 is the treadmill velocity required to produce blood lactate levels of 4mM/L. The onset of blood lactate accumulation, or the anaerobic threshold, is considered to occur at 4mM/L of lactic acid in horses. Both V200 and VLA4 are expected to increase as training proceeds (Persson, 1985b).

Our results indicate, by three measures, that physical conditioning of the subject horses was achieved. First, a comparison of stress test A of untrained and trained horses reveals differences in V200. Whereas a treadmill speed of 8.0 m/s was sufficient to cause heart rates exceeding 200 bpm in unconditioned animals, it did not have the same effect on trained animals. In fact,
speeds of 10 m/s (stress test B) were required to produce heart rates above 200 bpm in these animals. Similarly, VLA4 was increased, as treadmill speeds of 8.0 m/s were unable to cause plasma lactate accumulation of 4mM/L or greater in conditioned horses compared to unconditioned horses. Consistent with reports of enhanced plasma cortisol levels in conditioned horses following exercise, we found a larger percent increase in blood cortisol in trained compared to untrained horses after physiologically equivalent exercise stress (stress test A of untrained and stress test B of trained horses) (Snow and Mackenzie, 1977).

Regardless of the state of conditioning, an estimate of the degree of exercise stress can be obtained by examining heart rate, plasma lactate, and cortisol levels following the exercise challenge. The severity of stress test B of conditioned horses is confirmed by comparison to the work of other researchers. Thus, just as in our studies, other researchers recorded average heart rates exceeding 200 bpm in horses exhaustively exercised on a treadmill (Kurcz et al., 1988). Plasma cortisol levels 30 minutes after exercise were increased 35% and 100% in 2 studies of horses undergoing intense treadmill-based exercise stress (Church, et al., 1987; Kurcz, et al.,
1988), while we observed a 139% increase in cortisol levels within 20 minutes after exercise. In horses, lactate levels may increase 20 to 25 fold over baseline after maximal exercise (Persson, 1985a). Accordingly, we noted a 30 fold increase in lactate levels by 5 minutes after the end of the stress test as compared to levels in resting horses. Similarly, stress test A of unfit horses produced maximal heart rates above 200 bpm, and 23 fold and 76% increases in plasma lactate and cortisol, respectively. These same parameters identify exercise stress test A of conditioned horses as being of only moderate intensity. Thus the average heart rate values peaked at 180 bpm, while maximal lactate and percent cortisol increases were only 1.5 mMol/l and 78%.

Data from FACS analysis (Table 4.3) reveal differences in the hematologic response to moderate and severe exercise. Thus the post exercise increase in percentage of CD8-like cells was significant for stress test B but not stress test A of conditioned animals. Nevertheless, 4 out of 6 conditioned horses experienced an increase in the percentage of CD8-like cells after exercise stress test A. An increased proportion of CD8+ cells might account for observed decreases in mitogenic responses through an increased T suppressor cell presence.
or a relative decrease in the number of T helper cells. Unlike what has been reported in other species, we did not detect a decrease in the percentage of circulating T lymphocytes after exercise (Hickson and Boone, 1991).

With regard to the effects of exercise conditioning on immunity, we did not observe any changes in the responses of cells from resting horses to PWM, equine influenza virus, or IL-2 (LAK activity) over the course of the training program (Table 4.2). These findings are consistent with those of researchers studying pigs undergoing a moderate treadmill-based exercise conditioning program (Jensen, 1989). However, in human studies, elevated resting lymphocyte mitogenesis and natural killer cell cytotoxicity have been reported following training (Pedersen, et al., 1989; Soppi, et al., 1982; Watson, et al., 1986).

Conditioning effects on post exercise immune function were detected by our studies, particularly with regard to the T₀ time point of exercise stress tests. In untrained horses a significant reduction in PWM-induced blastogenesis was observed at T₀. This decrease in mitogen response is not found, however, at the corresponding time in trained horses undergoing a
submaximal or a comparable severe exercise challenge. Findings such as these are in agreement with research in humans and rats showing that conditioning decreases the suppressive effect of exercise stress on mitogen reactivity (Mahan and Young, 1989; Soppi, et al., 1982). Nevertheless, at later post stress time points, a definite downward trend in mitogen responsiveness occurs in conditioned horses and reaches significance at \( T_{+20} \) and \( T_{+120} \) following moderate and severe stress, respectively. In accordance with our results, depressed responses to selected mitogens have been described by other researchers at a variety of exercise intensities (Hickson and Boone 1991; Kurcz, et al., 1988). Additionally, it is possible that the delays in stress effects on PWM proliferation relative to our earlier study are training related.

There have been few reports on the effects of exercise or conditioning on antigen-specific immune responses (Eskola, et al., 1978; Hedfors, et al., 1983; Liu and Wang, 1987). We show here that secondary in vitro immune responses to equine influenza virus are inhibited following strenuous exercise in both fit and unfit horses. Moderate exercise of conditioned horses does not have the same effect. Therefore moderate exercise
may not be sufficient to profoundly lower influenza-specific proliferation in conditioned horses. Indeed, in an earlier submaximal stress test of the same group of horses before training (data not shown), no significant decrease in influenza response was detected. Thus, the horse to horse variation in influenza virus-specific immune responses following stress test A of trained animals could be a normal consequence of moderate exercise stress, but further studies are required.

In our studies of the effect of intense exercise on equine LAK activity in unconditioned horses, we found significant elevations in lytic ability. Equivalent increases in LAK activity of conditioned horses were not demonstrable under any stress condition, including severe exercise stress, although a general upward trend in LAK cell mediated cytotoxicity did occur. The latter finding indicates that there is a training effect on post exercise LAK responses. In contrast to our findings with LAK cells, a study of human cyclists found double the percentage of natural killer cells (and presumably an increase in NK cytotoxicity) in the circulation of athletes, compared to non-athletes after exercise stress (Oshida, et al., 1988). However, since equine LAK cells are primarily of T cell origin, the applicability of such
data to our research is questionable (Hormanski, et al., 1992).

The principle effect of training on the immune reactivity of horses subjected to comparable severe exercise stress, is a modulation of post exercise PWM and LAK responses. Hence significant depression of PWM induced lymphoproliferation is delayed and deepened and a substantial increase in LAK activity is absent following maximal exercise in fit horses. We did not, however, detect any significant differences in baseline (T\(_{40}\)) PWM, influenza, LAK, or NK activity (0 IL-2 control) during the exercise conditioning program. We can speculate then, that training dependent changes in immune responses following exercise in horses may arise from training dependent changes in the physiologic response to exercise and not, necessarily, changes in resting lymphocyte capabilities. Specifically in this regard, the levels of several stress related hormones may be modified by training. Cortisol, for example, with its myriad immunomodulatory effects, may reach higher post exercise levels in trained horses and return more quickly to resting values (Snow and Mackenzie, 1977). Conversely, catecholamines, which also exert direct and indirect
control over immune function, increase less in trained subjects following exercise of comparable intensity (Irvine, 1983).

Regardless of any training effects, both fit and unfit horses experience depressed PWM and influenza virus-specific proliferation after a maximal exercise challenge. An implication of the latter is that exposure to virulent virus during or after exercise could result in an increased incidence or severity of disease particularly in elite athletes. Epidemiological studies of illness in human athletes support this contention (Peters and Bateman 1983). Likewise, post exercise immunosuppression may be a factor in increased vaccination failure and susceptibility to respiratory infection observed in competing horses during racing season (Bayly, et al., 1987; Laegreid, et al., 1988). In addition, the absence of a significant rise in post exercise LAK activity in conditioned horses compared to unconditioned horses, might enhance vulnerability to infection and disease during periods of repeated physical stress. Future studies employing coincident vaccination/infection and exercise stress will be required to further define the clinical relevance of our work.
Chapter V

Cortisol and Equine Immune Function

Introduction

The immunosuppressive effects of corticosteroids (CS) have been well documented. Thus, CS treatment of immune cells depresses mitogen and antigen specific proliferation (Almawi, et al., 1991; Katz and Fauci, 1979; Van Voorhies, 1989), and natural killer (NK) cell (Holbrook, et al., 1983; Masera, et al., 1989; Parillo and Fauci, 1978), cytotoxic T Lymphocyte (CTL) (Grimm, et al., 1985), and lymphokine activated killer (LAK) cell activity (Grimm, et al., 1985; McVicar, et al., 1989; Papa, et al., 1986). They are thought to mediate their effects through down regulation of cytokine production (IL-1, IL-2, IL-3, IL-6, IFN gamma, TNF alpha and beta, etc.) and/or cellular receptors (e.g. IL-2 receptors) and recognition antigens (MHC II) (Dupont, 1988; Wu, et al., 1991). In addition CS may alter blood cell subset proportions with the typical response being leukocytosis, neutrophilia, lymphopenia, and increased percentages of NK and CD4+ cells (Katz, 1984). Presumably, the latter effects would disrupt in vitro intercellular interactions involved in responses to
mitogens, antigens, or interleukins, thereby changing the immune response measured.

Less well touted is the possibility of enhancing effects of CS on the immune system in vivo and in vitro. For example, it has long been known that replacement CS therapy increases disease resistance in adrenalectomized humans and animals (Jefferies, 1990). Furthermore, diurnal variation in peak blood CS levels coincide with similar peaks in NK and phytohemagglutinin (PHA) responses (McGlone, et al., 1991, Tavadia, et al., 1975). Corticosteroids increase antibody production (Cupps, et al., 1982; Jefferies, 1990) and there are scattered reports of CS-induced elevations in mitogen, LAK, and CTL activity (Grimm, et al., 1985; McVicar, et al., 1989; Papa, et al., 1986). Finally, immunosuppressive CS effects are often manifested in experimental systems utilizing steroid sensitive species (rats, mice), pharmacologic doses, and/or synthetic CS with greater potency than the natural substance (Claman, 1972).

In earlier work we documented an exercise-induced decrease in pokeweed mitogen (PWM) and equine influenza-
stimulated lymphoproliferation, and an increase in LAK cell-mediated cytotoxicity (see chapter 3 and 4). These changes occurred immediately after the cessation of exercise in unconditioned horses and were delayed (PWM) or decreased (LAK activity) in conditioned horses experiencing comparable exercise stress. Within twenty minutes of completion of the exercise protocol, in vivo cortisol concentrations reached their highest post exercise level ($10^{-7}$M range). Conditioned horses experienced a greater percent increase in plasma cortisol levels after exercise than unconditioned horses. In view of the known immunomodulatory actions of CS, and in order to investigate the cause(s) of the immune function modulation accompanying exercise, we examined the effects of cortisol, the biologically predominant CS of horses, on equine immune function. Our results indicate that while cortisol can influence immunity in a positive or negative manner, its interactions in vivo are complex and multifactorial.

Results

In Vitro Effects of Cortisol on Immune Function

We began our examination of cortisol effects on immunity with a set of in vitro assays. In experiments
performed on 6 horses (4G, 2M) undergoing a treadmill-based exercise conditioning program, we determined the effect of varying concentrations of cortisol on 3 indices of immunity. The parameters measured included PWM and equine influenza virus type II-stimulated lymphoproliferation (corrected CPM) and LAK activity (percent specific lysis at the 75:1 effector to target ratio). All assays were performed on samples obtained from resting horses (T<sub>0</sub> time point of exercise stress tests). Figure 5.1 presents the averaged data for unfit (open circles) and fit (filled circles) horses. Surprisingly, in unfit horses, a significant (P < .01) cortisol enhancement of LAK (10<sup>-8</sup>, 10<sup>-10</sup>M), influenza (10<sup>-10</sup>M), and PWM (10<sup>-10</sup>M) responses occurred. Cortisol-mediated depression of LAK (10<sup>-6</sup>M, P < .01), influenza (10<sup>-6</sup>M, P < .01), and PWM (10<sup>-6</sup>, 10<sup>-8</sup>M, P < .01) activity was observed at higher concentrations. In fit animals, suppression of PWM responses was apparent at high cortisol concentrations (10<sup>-6</sup>M, P < .01), while enhancement of LAK responses (P < .01) was seen with 10<sup>-10</sup>M added cortisol. Interestingly, suppression of PWM responses by 10<sup>-6</sup>M cortisol was significantly less in fit compared to unfit horses. A determination of cortisol effects on influenza specific lymphoproliferation of fit horses was not performed due to technical reasons.
Figure 5.1. The effect of cortisol on PWM, equine influenza virus, and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of cortisol (C) at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with C ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles)- PWM $10^{-6}$ - $10^{-10}$M cortisol, influenza virus $10^{-8}$ and $10^{-10}$M cortisol, LAK $10^{-6}$-$10^{-10}$M cortisol; fit (filled circles)- PWM $10^{-5}$M cortisol, LAK $10^{-10}$M cortisol.
Because of a possible link with post exercise increases in LAK activity, we further investigated cortisol effects on LAK function in vitro. Unfit animals were used in all of the following studies since they were more susceptible to exercise-induced immunomodulation. We first examined the mechanism of cortisol-mediated enhancement of LAK activity by performing an IL-2 titration in the presence or absence of cortisol. Table 5.1 gives the results from a representative horse in which data are presented in lytic units. With this animal, as with 4 out of 4 other horses, cortisol-induced increases in LAK activity (above corresponding untreated control levels) can be observed at supra-optimal IL-2 doses, i.e., IL-2 concentrations greater than that necessary to produce maximum LAK cytotoxicity of otherwise untreated cells. These cortisol-induced increases in LAK activity again occurred at $10^{-8}$ and $10^{-10}$M cortisol concentrations.

Realizing that in vivo cortisol elevations associated with exercise are not equivalent to several days of cortisol treatment in vitro, we determined the effect of a one hour in vitro cortisol pulse on equine LAK function.
TABLE 5.1. Equine LAK Activity in the Presence of Varying Concentrations of Interleukin-2 and Cortisol.

LAK ACTIVITY*  

<table>
<thead>
<tr>
<th>Cortisol Concentration</th>
<th>IL-2c</th>
<th>0</th>
<th>10^-6</th>
<th>10^-8</th>
<th>10^-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>42.9</td>
<td>34.5</td>
<td>43.6</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>158.7</td>
<td>30.2</td>
<td>76.7</td>
<td>144.4</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>162.1</td>
<td>43.9</td>
<td>110.8</td>
<td>198.0**</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>124.4</td>
<td>72.7</td>
<td>168.8</td>
<td>179.1</td>
<td></td>
</tr>
</tbody>
</table>

a. PBMC from an unconditioned horse were incubated with varying concentrations of cortisol and IL-2 for 72 hours. At the end of the culture period, a 4 hour cytotoxicity assay was performed using ^51 chromium labelled EqT8888 targets. Four additional horses in the same experiment displayed similar results. Results are expressed as mean lytic units 10%.

b. Molarity.

c. Units per milliliter of culture media.

** Significantly different from 0 cortisol control for that IL-2 concentration, (P < .01; ANOVA).
Figure 5.2 presents the average results for 8 different horses. PBMC from these horses were either placed into LAK cultures with cortisol for 72 hours (open bars) or pulsed for 1 hour with cortisol (hatched bars) and then put into LAK cultures. The graph shows that 1) brief and continuous exposure to cortisol may produce different effects and 2) a simple cortisol pulse can affect subsequent LAK activity. With regard to the latter point, four of eight horses experienced a significant change (P < .01) in LAK activity following the cortisol pulse.

In Vivo Effects of Cortisol on Immune Function

Finally, we turned our attention to the effects of cortisol on immune function in vivo. It is known that, in horses, plasma cortisol is highest from 6-8 AM (10^−8 - 10^−7M) and lowest from 4-10 PM (10^−8M or less) (reviewed in Thornton, 1985). Therefore, blood was collected at 8:00 AM and 8:00 PM for determination of LAK activity. We detected no significant differences between morning and evening LAK cytotoxicity (mean lytic units 10% at 8:00 AM of 98.2 ± 26 SEM and at 8:00 PM of 117.8 ± 30 SEM, P > .05).

As a more rigorous test of the ability of cortisol to mediate exercise-associated immunologic changes, we
Figure 5.2. Pulse and continuous cortisol exposure affect equine LAK activity. PBMC from 8 unconditioned horses were either placed into LAK cultures with cortisol for 72 hours (open bars) or pulsed for 1 hour with cortisol (hatched bars) and then put into LAK cultures. Results are presented as specific lysis (at the 75:1 effector to target ratio) expressed as percent of the value for control cells which were not treated with cortisol.
injected 150 ug of cortisol intravenously into 3
unconditioned horses (1G,2M). Four unconditioned control
horses (2G,2M) received only vehicle. Tables 5.2 and 5.3
present hematologic and immunologic/plasma cortisol data
from this trial. For comparison, Table 5.4 contains
information from an exercise stress test (protocol A—see
chapter 3) of 5 unconditioned horses. Hemogram data
(Table 5.2) from cortisol-treated horses reveals elements
of a typical response to CS, i.e., leukocytosis, relative
and absolute neutrophilia, relative and absolute
lymphopenia and an increase in the neutrophil to
lymphocyte (N/L) ratio most apparent 2 hours after
cortisol injection. (Rossdale, et al., 1982; Tonnesen, et
al., 1987). Lymphopenia also developed in control horses
but was less severe. Consequently the slight N/L ratio
increase in vehicle-treated horses is due to the decrease
in lymphocytes, whereas the greater increase in N/L ratio
in cortisol-treated horses is due both to an increase in
neutrophils and a decrease in lymphocytes. Exercise
induced changes in the equine hemogram (Table 5.4)
included leukocytosis and lymphocytosis. There was no
significant change in immunologic assays (Table 5.3)
performed concurrent with in vivo cortisol treatment
despite peak increases in plasma cortisol levels of 77%
above pretreatment values at 5-15 minutes after injection.
TABLE 5.2. Hematologic Data from Unconditioned Horses Following Intravenous Cortisol or Vehicle Injection.

<table>
<thead>
<tr>
<th>TREATMENT^a</th>
<th>Cortisol</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>-60</td>
<td>+20</td>
</tr>
<tr>
<td>WBC^c</td>
<td>8425</td>
<td>7700</td>
</tr>
<tr>
<td>Segs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rel. ^d</td>
<td>60.5</td>
<td>63.7</td>
</tr>
<tr>
<td>abs. ^e</td>
<td>5.1</td>
<td>4.9</td>
</tr>
<tr>
<td>Lymphs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rel.</td>
<td>33.0</td>
<td>31.7</td>
</tr>
<tr>
<td>abs.</td>
<td>2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>N/L^f</td>
<td>1.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

a. Seven unconditioned thoroughbred horses (3G, 4M) were injected intravenously with either 150 ug cortisol or an equal volume of vehicle. Blood was collected for a complete blood count at the specified times. Data represent the average for 3 and 4 horses in the cortisol and vehicle treatment groups, respectively.

b. Time after treatment in minutes.

c. Cells/ul.

d. Relative number of cells expressed as a percentage.

e. Absolute number of cells ( x 10^3/ul).

f. Neutrophil to Lymphocyte ratio.
TABLE 5.3. Immune Responses of Unconditioned Horses Following Intravenous Cortisol or Vehicle Injection.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Immune Response</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PWM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Flu&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LAK&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Cortisol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35.3 ± 6.5</td>
<td>4.3 ± 1.1</td>
<td>62.3 ± 14.7</td>
<td></td>
</tr>
<tr>
<td>+20</td>
<td>35.0 ± 3.4</td>
<td>1.9 ± 3.7</td>
<td>53.2 ± 15.2</td>
<td></td>
</tr>
<tr>
<td>+120</td>
<td>33.4 ± 7.9</td>
<td>3.6 ± 1.6</td>
<td>105.9± 38.4</td>
<td></td>
</tr>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-60</td>
<td>28.4 ± 2.6</td>
<td>3.4 ± 2.9</td>
<td>52.9 ± 10.2</td>
<td></td>
</tr>
<tr>
<td>+20</td>
<td>31.5 ± 6.1</td>
<td>2.3 ± 1.9</td>
<td>58.2 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>+120</td>
<td>35.7 ± 12.7</td>
<td>4.1 ± 1.9</td>
<td>86.5 ± 24.5</td>
<td></td>
</tr>
</tbody>
</table>

a. Seven unconditioned thoroughbred horses (3G, 4M) were injected intravenously with either 150 ug cortisol or an equal volume of vehicle. Blood was collected for determination of immune responses at the specified times. Data represent the average for 3 and 4 horses in the cortisol and vehicle treatment groups, respectively. No significant change in PWM, Flu, or LAK responses occurred over time in either treatment group (P > .05; ANOVA).

b. Mean corrected CPM ± SEM (x 10<sup>3</sup>).

c. Mean lytic units 10% ± SEM.

d. Time after treatment in minutes.
TABLE 5.4. Acute Exercise Stress Produces Hematologic Changes in Unconditioned Horses.

<table>
<thead>
<tr>
<th>Time After Exercise&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WBC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Segs</th>
<th>Lymphs</th>
<th>N/L&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(minutes)</td>
<td>(cell/ul)</td>
<td>rel.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>abs.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>rel.</td>
</tr>
<tr>
<td>-60</td>
<td>6780</td>
<td>59.8</td>
<td>4.1</td>
<td>34.2</td>
</tr>
<tr>
<td>+0</td>
<td>7020</td>
<td>54.2</td>
<td>3.8</td>
<td>42.0</td>
</tr>
</tbody>
</table>

a. Five unconditioned thoroughbred horses (4M, 1S) were subjected to a treadmill-based exercise stress test (protocol A - Chapter 3) and blood for complete blood counts was collected at the specified times. Results represent the average for the five horses.


c. Relative number of cells expressed as a percentage.

d. Absolute number of cells (X 10<sup>3</sup>/ul).

e. Neutrophil to Lymphocyte ratio.
Our studies demonstrate that cortisol is not always immunosuppressive and may, under certain conditions, be immunoenhancing. Thus we found that low in vitro concentrations of cortisol can augment some immune parameters. Relevant to our work is the finding by others of enhanced PHA, CTL, and LAK activity in the presence of $10^{-8}$ M cortisol, $10^{-6}$ M cortisol and $10^{-7}$ M cortisone, respectively (Grimm, et al., 1985; McVicar, et al., 1989; Papa, et al., 1986). Researchers studying circadian variations in NK and PHA responses have likewise detected a positive correlation with in vivo cortisol levels (McGlone, et al., 1991; Tavadia, et al., 1975), though LAK activity was unaffected by diurnal changes in plasma cortisol levels in this study.

While reports of CS-mediated augmentation of immune function are scarce, the paucity of such information may simply reflect the overwhelming use of research protocols favoring immunosuppression by CS, i.e., the use of steroid sensitive species, large doses, and synthetic CS analogues. Even so, we observed that $10^{-8}$ and $10^{-10}$ M cortisol did not always augment immune responses (e.g. Fig. 5.2, open bars). In 151 assays of 20 different
horses, significant positive cortisol effects were dependent upon the lot of serum used, the time of day blood was collected, and the horse. Other researchers have confirmed the co-stimulatory role of fetal bovine serum in CS mediated immune function enhancement (Cupps, et al., 1982). Obviously then, in vitro CS effects like in vivo CS effects may depend upon a number of factors, including levels of corticosteroid binding globulin (CBG), other hormones with immunomodulatory effects, and cytokines, as well as CS receptor activity and CS concentrations (Baulieu and Mester, 1989; Blauer, et al., 1991; Deitch and Bridges, 1987; Feldman, 1989; Heijnen, et al., 1991; Holbrook, et al., 1983; Teperman and Tepperman, 1987).

The mechanisms whereby CS suppress immunity are relatively well known. By contrast, one can only speculate as to the means by which cortisol increases PWM, influenza, and LAK responses in horses. Furthermore, multiple mechanisms may be involved. At $10^{-6}$M cortisol concentrations LAK cell-mediated cytotoxicity is increased, equine influenza virus-specific proliferation is slightly enhanced, and PWM responses are suppressed. A differential effect of cortisol on LAK proliferation and/or differentiation is thereby indicated.
Alternatively, cortisol mediated augmentation of proliferation at 10^{-8} M in all assay types could result in preferential cell overgrowth and media exhaustion in PWM cultures due to the higher magnitude of mitogen-induced proliferative responses. In support of this theory, research into in vitro antibody production systems indicates that submaximal stimulatory signals are subject to enhancement by CS (Bowen and Fauci, 1988; Cupps, et al., 1982). In the case of enhancement of LAK activity, cortisol may act by reducing the suppressive effects of supraoptimal concentrations of IL-2, possibly by decreasing IL-2 receptor expression, suppressor cell activity, or prostaglandin release.

While our in vitro results indicate that direct cortisol actions on immune cells may be responsible for the changes in immune function associated with exercise in horses, data gathered from in vivo treatment of unconditioned horses with CS does not support this contention. Accordingly, cortisol injection produced different early effects (+20 vs +0) on the hemogram than exercise i.e., leukopenia, lymphopenia, and increased N/L ratio vs leukocytosis, lymphocytosis, and decreased N/L ratio, respectively. It is possible however that, had we examined the CBC of horses 120 minutes after exercise, we
would have found a profile similar to that of horses treated in vivo with cortisol (slight leukocytosis, neutrophilia, lymphopenia, and increased N/L ratio). The latter is consistent with the findings of others studying in vivo cortisol and exercise effects (Rose and Allen, 1985; Rossdale, et al., 1982) and reflects the delayed course of action of cortisol on blood cell subsets. Further evidence against a solitary role for cortisol in exercise-induced immunomodulation is provided by the immunologic data itself. Thus, after cortisol injection, peak percent increases in blood cortisol levels approximated post exercise increases (exercise test A of unconditioned horses), but no significant change in the immunoassays was detected at the 3 time points examined. This is in contrast to what occurred after exercise stress test A of unconditioned horses wherein PWM and influenza responses were reduced at T₀ and LAK responses were enhanced at T₀ and Tₚᵢ. Given that a number of other factors (hormone, peptides, interleukins, etc.) accumulate in the blood as a result of exercise, the latter result is not surprising. Indeed, differences between the hematologic data gathered early after in vivo cortisol injection (+20) and exercise (+0) may document the contribution of other exercise related hormones (e.g. epinephrine) to the overall response to exercise.
While we may conclude that the contribution of cortisol to the specific changes in immune function detected following exercise in unfit horses is most likely indirect or else in concert with other hormones or factors, we nonetheless found that regular exercise can have an effect on the response of immune cells to cortisol. Consistent with a training-related change in cortisol receptor activity, we noted a refractoriness of cells from conditioned horses to suppressive cortisol effects. Thus higher post exercise cortisol levels in conditioned horses may be matched by a decreased response of their immune cells to cortisol, leaving unexplained the relative increase in the severity of immune suppression following exercise in these animals.
Chapter VI

Neuropeptides and Amines Modulate Immune Function in Fit and Unfit Horses

Introduction

Unconditioned horses subjected to an acute exercise challenge exhibit a decrease in lymphoproliferation along with an increase in lymphokine activated killer (LAK) activity (Chapter 3). In exercise-conditioned horses, by contrast, there is a significant modulation of these exercise-induced changes in the immune responses (Chapter 4). Specific alterations include a delay in the suppression of pokeweed mitogen (PWM) -induced proliferation and an attenuation of the positive effects on LAK activity.

The mechanism whereby acute exercise produces and physical conditioning modulates these effects on the immune system is multifactorial. Likely mediators include those amines and neuropeptides whose plasma levels fluctuate in response to acute physical exertion and conditioning (Butler, et al., 1982; Church, et al., 1987; Hamra, 1990a; Howlett, 1987; Irvine, 1983; Luger, et al.,
1987; Ohman, et al., 1987; Snow, 1979: Snow and Rose, 1981). In the horse, post exercise blood concentrations reach $10^{-9}$, $10^{-10}$, and $10^{-11}$M for the catecholamines epinephrine/norepinephrine and the neuropeptides beta endorphin and adrenocorticotropic hormone (ACTH), respectively (Church, et al., 1987; Hamra, 1990a; Snow, 1979; Snow and Rose, 1981). These compounds have been shown to affect lymphocyte function and distribution patterns at these concentrations (Pedersen, 1991; Keast, et al., 1988). Furthermore, concentrations of these substances within lymph nodes, spleen, thymus, and bone marrow may be further elevated through activation of sympathetic nerves within lymphoid tissues and local production of neuropeptides (Felton, et al., 1987; Heijnen, et al., 1991). Physical conditioning, likewise may result in changes in basal plasma hormone levels and/or hormone receptor activity on susceptible immune cells (Butler, et al., 1982; Hamra, 1990c; Howlett, 1987; Luger, et al., 1987; Ohman, et al., 1987).

In order to better define the relationship between exercise, fitness and the immunomodulatory effects of neuroendocrine compounds, we tested a group of such substances and their synthetic analogues for in vitro effects on 2 immune parameters in unfit and fit horses.
Specifically, we evaluated the in vitro effects of epinephrine, norepinephrine, methoxamine, isoproterenol, beta endorphin, and ACTH on lymphokine activated killer cell induction and PWM-induced proliferation. Results of the present study indicate that exercise-induced changes in immune function may be mediated by neuroendocrine compounds and that physical conditioning can alter the sensitivity of the lymphocytes to these compound effects.

Results

Catecholamines and Related Agonists Modify Immune Function

The effects of catecholamines and related agonists on PWM and LAK responses of equine cells are depicted in Figures 6.1-6.4. Presented data are the result of 3 and 2 separate determinations performed on peripheral blood mononuclear cells (PBMC) of unconditioned and conditioned horses, respectively. In unfit horses (Fig. 6.1), the addition of epinephrine at culture initiation significantly (P < .01) depressed PWM-induced blastogenesis (10^6M) and increased (P < .01) LAK-mediated cytotoxicity (10^6 - 10^{12}M). In fit animals, suppression of PWM responses was lost while enhancement (P < .01) of LAK activity was maintained at all concentrations.
When norepinephrine was added to cultures of cells from unfit horses (Fig. 6.2), a similar depression \((P < .05)\) in PWM responses \((10^{-6} \text{M})\) and enhancement \((P < .01)\) of LAK cytotoxicity \((10^{-6} - 10^{-9} \text{M})\) was noted. In fit horses the decrease in PWM response was absent and LAK activity was increased \((P < .01)\) only at the 2 lower concentrations of norepinephrine. At \(10^{-9} \text{M}\) norepinephrine, the enhancement of LAK activity in conditioned horses was significantly less \((P < .05)\) that that in unconditioned horses.

We also conducted studies of the in vitro effects of the synthetic alpha, agonist methoxamine, and the beta, beta agonist isoproterenol. Figure 6.3 depicts the data from methoxamine assays. Again, after addition of methoxamine to cultures, significantly depressed \((P < .01)\) PWM responses \((10^{-4} \text{M})\) and enhanced \((P < .01)\) LAK \((10^{-4} - 10^{-8} \text{M})\) responses in unfit horses gave way to nonsignificant changes in PWM-induced lymphoproliferation, and limited increases \((P < .01)\) in LAK function \((10^{-4} - 10^{-6} \text{M})\) in fit horses. Isoproterenol (Fig. 6.4) at \(10^{-4}\) and \(10^{-6} \text{M}\) significantly \((P < .01)\) diminished PWM stimulated proliferation of PBMC from unconditioned horses. In addition, isoproterenol caused both a depression \((10^{-4} \text{M})\) and an enhancement \((10^{-6} \text{M})\) of LAK responses \((P < .01)\) in
these animals. In fit horses, significant decreases (P < .01) in PWM responses and LAK activity of cells treated with $10^4$M isoproterenol were evident along with increases (P < .01) in PWM and LAK responses at lower doses of the drug ($10^{-6}$ - $10^4$M). Because $10^4$M isoproterenol consistently caused a reduction in the size of the cell pellet within each well, media discoloration, and background level readings for immunoassays, a direct toxic effect on cells was suspected.

The Neuropeptides, Beta Endorphin and ACTH, Modify Immune Function

We next performed the immunologic assays in the presence of beta endorphin or ACTH. Similar to the catecholamines, addition of beta endorphin to cultures of cells from unfit horses caused a decline (P < .01) in PWM responses at $10^4$M and enhancement of LAK activity at $10^{-6}$ - $10^4$M (Fig. 6.5). By contrast, cells from fit horses experienced an increase (P < .05) in PWM responses when beta endorphin was added at $10^{-6}$ - $10^{10}$M. LAK activity in treated cultures was unchanged from that of unfit animals. When ACTH was placed into cultures with PBMC from unfit horses (Fig. 6.6), suppression (P < .01) of PWM-triggered lymphoproliferation occurred at all concentrations while augmentation (P < .01) of LAK was apparent at $10^4$M. In
assays using cells from fit horses, ACTH no longer affected PWM responses, but significantly (P < .01) increased LAK activity when present at $10^{-10}M$. 
Figure 6.1. The effect of epinephrine on PWM and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of epinephrine (E) at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with E ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles)- PWM $10^{-6}$ M E, LAK $10^{-6}$-$10^{-12}$ M E; fit (filled circles)- LAK $10^{-6}$ - $10^{-12}$ M E.
Figure 6.2. The effect of norepinephrine on PWM and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of norepinephrine (NE). at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with NE ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles)- PWM $10^{-6}$M NE, LAK $10^{-9}$-10^{-12}$M NE; fit (filled circles)- LAK $10^{-9}$ and $10^{-12}$M NE.
Figure 6.3. The effect of methoxamine on PWM and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of methoxamine (M) at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with M ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles) - PWM 10^{-4}M, LAK 10^{-4}-10^{-8}M; fit (filled circles) - LAK 10^{-4} and 10^{-6}M.
Figure 6.4. The effect of isoproterenol on PWM and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of isoproterenol (I) at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with I ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles)—PWM $10^{-4}$ and $10^{-6}$M I, LAK $10^{-4}$ and $10^{-8}$M I; fit (filled circles)—PWM $10^{-4}$ and $10^{-6}$M I, LAK $10^{-4}$ to $10^{-8}$M I.
Figure 6.5. The effect of beta endorphin on PWM and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of beta endorphin (BE) at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with BE ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles) - PWM $10^{-6}$M BE, LAK $10^{-6}$ and $10^{-8}$M BE; fit (filled circles) - PWM $10^{-6}$ - $10^{-10}$M BE, LAK $10^{-6}$ and $10^{-8}$M BE.
Figure 6.6. The effect of adrenocorticotropic hormone on PWM and LAK responses of cells collected from resting fit and unfit horses. Immunoassays were performed as in Materials and Methods in the presence or absence of adrenocorticotropic hormone (ACTH) at the indicated concentrations. Results are expressed as a percentage of the response of cells that were not treated with ACTH ± SEM. Significant differences (ANOVA) from untreated control values occurred at: unfit (open circles)- PWM $10^{-6}$ - $10^{-10}$M ACTH, LAK $10^{-8}$M ACTH; fit (filled circles)- LAK $10^{-10}$M ACTH.
Discussion

Physical exertion is known to alter various parameters of immune function and to produce elevated plasma levels of catecholamines and neuropeptides. In an attempt to assess the role of exercise stress-related substances in post exercise immune modulation, we have examined the direct effects of varying doses of catecholamines and peptides on immunoreactivity of PBMC collected from conditioned and unconditioned horses.

In our studies, catecholamines and related artificial agonists had similar effects. Cultures containing cells from unfit horses were susceptible to suppression of PWM responses in the presence of high concentrations (10^{-6}M) of all compounds. Other research workers have, likewise, observed depressed mitogen responses of cells treated with comparable levels of epinephrine, norepinephrine, and isoproterenol (Feldman, et al., 1987; Hadden, et al., 1970). Consistent with our findings with 10^{-4}M isoproterenol, other studies have demonstrated a decrease in proliferation of IL-2 responsive T cells in the presence of 10^{-4} - 10^{-6}M isoproterenol (Beckner and Farrar, 1988; Feldman, et al., 1987). In our study, as in others (Hatfield, et al., 1986), direct toxic effects of
isoproterenol at high concentrations may play a part in the suppression of responses.

With regard to LAK activity, all of the adrenoceptor binding compounds, with the exception of $10^{-4}$ M isoproterenol, were stimulatory. While our use of LAK cell function in these assays is novel, there are corroborating data from other systems measuring cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activity. In accordance with our results, an immunoenhancing role of catecholamines in the generation of CTL and NK effectors has been found by others. Hence, epinephrine ($10^{-8}$ M) increased natural killer cell activity after an initial pulse (Hellstrand, et al., 1985) and isoproterenol ($10^{-7}$ M), epinephrine ($10^{-6}$ M), and norepinephrine ($10^{-4}$ M) enhanced the number of lytic units produced in mixed lymphocyte cultures (MLC) through a beta-adrenoceptor-mediated effect (Hatfield, et al., 1986). A second MLC study found that, along with $10^{-6}$ - $10^{-9}$ M epinephrine or norepinephrine, methoxamine could enhance cytotoxicity (Felton, et al., 1987). The data from the CTL system may be most applicable to our work in that equine LAK cells are of T cell origin (Hormanski, et al., 1992).
The augmentation of LAK activity at the same concentration of compounds that decrease PWM proliferative responses suggests that neuroendocrine hormones exert a proliferation-independent effect upon LAK generation. It remains possible, however, that the compounds may cause the induction of additional LAK precursor cells from the T lymphocyte pool.

In general, catecholamines and related agonists had less prominent effects on cells from conditioned horses than on cells from unconditioned horses. Thus, in fit horses there was an absence (epinephrine, norepinephrine, methoxamine) or reversal ($10^{-6}$M isoproterenol) of the suppression of PWM responses of treated cells seen in unfit horses. In addition, positive compound effects on LAK activity were lost ($10^{-4}$M methoxamine) or significantly lessened ($P < .05$, $10^{-5}$M norepinephrine) in fit compared to unfit animals. In this regard, fit individuals have been shown to express lower numbers of lymphocyte beta adrenergic receptors than unfit individuals (Butler, et al., 1982, Ohman, et al., 1987).

In examining catecholamine effects on immune parameters of fit and unfit horses, we noted no distinctive differences between alpha and beta receptor
agonists effects on treated cells. Such differences have at times been described by other researchers (Hadden, et al., 1970; Sanders and Munson, 1985a), and indeed, might be expected to occur based on variable cellular expression of different adrenergic receptor types. Our results may be explained therefore, by the heterogeneity of the cell population under study (T cells, B cells, monocytes), multiple receptor affinities of some of the agonists (norepinephrine, epinephrine, and isoproterenol), and the possibility of similar alpha/beta effects on the immune functions assayed (Hadden, et al., 1970; Hatfield, et al., 1986).

When we examined neuropeptide effects on immune function of unfit horses, we found that ACTH suppressed PWM responses at all concentrations, but was able to increase LAK responses at $10^{-8} \text{M}$. Since there is little data concerning in vitro effects of ACTH on immune parameters, especially in the horse, our findings are unique. Clues to interpreting our PWM results may be found, however, in the suppressive effect of ACTH ($10^{-7} \text{M}$) on plaque forming cell (PFC) response to sRBC (Johnson, et al., 1982) and the stimulatory effect of ACTH ($10^{-6} \text{M}$) on proliferation and differentiation of purified B cells in the presence of IL-2, IL-4, or IL-5 (Alvarez-Mon, et al., 1985; Brooks,
1990). If the number of antibody producing cells can be used as an indirect measure of B cell proliferation, then our PWM results, which embody T cell-assisted B cell proliferation, are in agreement with the findings of others. A greater sensitivity of T cell or antigen presenting cell function to ACTH has been suggested (Johnson, et al., 1982) and may explain depressed equine PWM responses at lower doses wherein enhancement of the proliferation of purified B cells occurred in other studies. Alternatively, if the ACTH effect on antigen specific PFC numbers represents abrogation of some T cell dependent B cell differentiative event, then ACTH-induced enhancement of purified B cell responses becomes important to interpretation of our data. Thus ACTH might increase PWM-induced proliferation and could result in media exhaustion and suppression of PWM responses as measured on day 5 of culture. Relevant to this explanation, stimulatory effects of ACTH on purified B cell proliferation occurred at concentrations up to $10^{-6}$M which covers the range used in our studies.

Our demonstration of enhanced LAK activity of PBMC from unconditioned animals in the presence of ACTH is unprecedented. For example, ACTH ($10^{-8} - 10^{-11}$M) added to cultures had no direct influence on porcine NK or
IL-2-stimulated NK cytotoxicity (McGlone, et al., 1991). In addition, ACTH ($10^{-7} - 10^{-9}$M) had no effect on IL-2-stimulated proliferation of Con A activated T cell (Alvarez-Mon, et al., 1985). These results, therefore, await corroborating studies of NK/LAK function in other systems.

Information gained from this study indicates that beta endorphin has inhibitory (unfit horses) and stimulatory (fit horses) effects on mitogen responses. Indeed, other researchers have demonstrated similar beta endorphin effects on mitogen activity dependent upon the concentration of beta endorphin, the mitogen, the lymphoid tissue, or the species examined (Fontana, et al., 1987; Gilman, et al., 1982; Gilmore and Weiner, 1989; Kusnekov, et al., 1987; McCain, et al., 1982; Puppo, et al., 1985; Van Den Bergh, et al., 1991). More to the point, beta endorphin has been shown to either suppress or enhance PWM, concanavalin A (Con A), or phytohemagglutinin (PHA) responses of conditioned thoroughbreds contingent upon the mitogen dose (Hamra, 1990d). Specifically, the proliferative response produced by "optimal" doses of PWM and Con A was enhanced by $10^{-11}$M beta endorphin while proliferation of lymphocytes to "suboptimal" PHA, PWM, and Con A doses was suppressed by $10^{-7} - 10^{-9}$M beta endorphin.
The results of these researchers with regard to beta endorphin effects on suboptimal PWM-induced lymphoproliferation of cells from conditioned horses are, therefore, in direct contrast to our own. The source of the disparity may lie in the definition of "optimal". We propose, based on the ability of beta endorphin to enhance the response, that the latter researcher's "optimal" mitogen dose actually represents submaximal proliferation as in our studies. Consequently, results from this researcher may be a manifestation of the biphasic action of beta endorphin on mitogen responses, i.e., enhancement of proliferation at low concentrations of beta endorphin and suppression of proliferation at high concentrations of beta endorphin (McCain, et al., 1987; Heijnen, et al., 1987). Our own results with fit and unfit horses follow the same pattern, if it is assumed that post conditioning decreases in beta endorphin receptor affinity shifts the dose response curve so that the effective concentration of beta endorphin is decreased in fit animals (Hamra, 1990c).

In contrast to ACTH, beta endorphin effects on natural cytotoxicity have been well documented. Accordingly, beta endorphin has been reported, by a number of investigators, to augment NK cytotoxicity (reviewed in
Blalock, 1989; Harbour and Smith, 1990; Sibingha and Goldstein, 1988). In addition, this neuropeptide may enhance CTL (10^-8 - 10^-4 M) and LAK cell (10^-8 M) generation (Beckner and Farrar, 1988; Carr and Klimpel, 1986), possibly in conjunction with its ability to increase IL-2 and interferon gamma production (Gilmore and Weiner, 1988; Mandler, et al., 1986). Our findings in conditioned and unconditioned horses are in agreement with these data.

In conclusion, our results indicate that the direct actions of neuroendocrine hormones and peptides on immune cells may be partly responsible for the post exercise changes in immune function in unfit and fit horses. Thus unconditioned horses experienced a decrease in PWM responses and an increase in LAK activity following acute severe exercise (Chapter 3) which was mimicked in vitro by the addition of various adrenergic agonists or neuropeptides to PWM and LAK cultures containing cells obtained from the same horses before exercise. Additionally, post exercise augmentation of LAK cell mediated cytotoxicity was reduced in fit horses (Chapter 4), while at the same time cells from non-exercising fit horses became somewhat refractory to the LAK-enhancing effects of test compounds. Direct effects of catecholamines and neuropeptides may, nevertheless, play a
smaller role in the immunologic changes associated with acute exercise stress in fit horses compared to unfit horses, in that these compounds are unable to depress mitogen response of cells from physically conditioned animals.
Chapter VII

Conclusions

Stress has been defined as the response of the body to any stimulus that disturbs its normal physiologic equilibrium or homeostasis (Khansari, et al., 1990). In this respect, physical exertion or exercise may be classified as a stressor. Thus in order to meet the energy needs of contracting muscle without impairing fuel availability to other vital organs such as the brain, a series of hormonal and neurogenic changes take place. The net result of this neuroendocrine activity is stimulation of the hypothalamic-pituitary-adrenal axis with consequent increases in serum adrenocorticotropic hormone (ACTH), beta endorphin, and corticosteroids, and activation of the sympathetic nervous system, followed by release of catecholamines. These exercise-associated changes in hormone levels mediate the physiologic response to acute physical stress. Likewise, repetitive exercise stress (i.e. physical training) elicits compensatory changes in resting plasma hormone levels and cellular hormone receptor activity.
Immunologic alterations are also part of the response to exercise stress. Accordingly, studies performed primarily on human athletes and laboratory animals indicate that short term exhaustive exercise may depress the in vitro response of lymphocytes to mitogens, decrease CD4:CD8 ratios in the presence of leukocytosis, reduce salivary IgA and IgM, modulate natural killer (NK) cell numbers and function, alter interleukin production patterns, and diminish neutrophil and macrophage bacteriocidal activity (reviewed in Caren, 1991; Fitzgerald, 1988; Fitzgerald, 1991; Keast, et al., 1988). Physical conditioning, on the other hand, produces positive and negative effects on resting immune function including: 1) increased MHC II expression of immune cells, NK cell activity, IL-1 levels and lymphoproliferative responses to mitogens and 2) decreased salivary IgA and serum IgG, complement levels, total lymphocyte counts, and neutrophil adherence and bacteriocidal activity (Caren, 1991; Fitzgerald, 1988; Fitzgerald, 1991; Keast, et al., 1988). The effects of physical conditioning on post exercise immune responses are similarly dichotomous (reviewed in Hickson and Boone, 1991).

Despite large amounts of accrued data, experiments relating exercise to immune function are difficult to
interpret and compare. This is due, in large part, to differences in methodology, particularly the quantitation of exercise stress and conditioning. However, other factors contribute significantly to the confusion. For example, psychological stress is often superimposed upon physical stress as in the case of competing human athletes and laboratory rodents that are coerced into exercising. In addition, most studies have focused on isolated aspects of humoral or cellular immunity, thereby overlooking functional cellular relationships.

To circumvent these difficulties, we evaluated the effect of acute exercise stress on 3 indices of immune function in horses, before and after completion of a 12 week treadmill-based physical conditioning program. This research plan offered several advantages. First, the horse is a willing exerciser and experiences minimal extraneous stress. Second, because exercise physiology in the horse is well understood, we were able to utilize peak heart rate, plasma cortisol, and lactate levels in the assessment of relative physical stress. Third, the treadmill system permitted reliable quantitation and control of the exercise protocol. Fourth, pre- and post-conditioning comparisons of the same group of animals could be made. Finally, concurrent examination of
mitogen, antigen, and IL-2 responses allowed for a broader evaluation of the impact of exercise on immune function.

In this work, we found that an acute severe exercise challenge of unconditioned horses induces immediate and significant post exercise decreases in pokeweed mitogen (PWM) and equine influenza virus-stimulated proliferative responses. Lymphokine activated killer (LAK) cell activity, at the same time, is significantly elevated. Therefore, we have confirmed the findings of others regarding exercise-induced depression of mitogen responses, and demonstrated that exercise can suppress elements of specific immunity in the horse. Additionally, these are the first reports of the effects of exercise on equine LAK activity or natural cytotoxicity of any sort.

Nonetheless, questions remain as to the proximate cause of these immunologic alterations. We have obtained preliminary evidence regarding blood cell subset redistribution following exercise. Thus, leukocytosis, and lymphocytosis were observed immediately after exercise in unconditioned animals and a moderate increase in the percentage of CD8-like cells was observed 20 minutes after exercise in conditioned horses experiencing a comparable intense physical challenge. A rise in the
proportion of CD8+ cells could potentially be responsible for the immunologic changes we observed, i.e., a relative decrease in CD4+ T helper cells or increase in CD8+ T suppressor cells could decrease proliferation in test cultures, while increased CD8+ LAK cells would elevate LAK responses in vitro. Nevertheless, CD8 typing of cells obtained after exercise in unfit horses remains to be accomplished as does that of equine LAK cells themselves. It would also be of interest to determine the percentage of peripheral blood mononuclear cells (PBMC) expressing the EqT12 antigen, a possible marker for equine natural killer cells, relative to exercise (Magnuson, et al., 1987). Further studies of cell subset changes associated with physical activity in horses await appropriate defining antibodies.

Apart from cell subset alterations, there may be changes in immune cell functions/interactions that accompany exercise stress. This is particularly true with regard to LAK cell mediated cytotoxicity in which the response measured (cytotoxicity) is the end result of a series of immunologic steps (activation by IL-2, proliferation, differentiation, and mature effector function). Accordingly, further studies are needed in this area and might include pre- and post-exercise
1) limiting dilution analysis of LAK precursors, 2) IL-2
dose response studies of LAK cytotoxicity, 3) examination
of proliferation within LAK cultures, and 4) cold target
inhibition studies of LAK cytotoxicity.

Related to this same subject area are two more
intriguing questions. How do changes in immune cell
subsets and capabilities within the blood relate to events
in lymph nodes, lung, spleen, and skin etc., where a large
portion of immune responses take place, and do temporary
changes in in vitro immunologic parameters translate into
increased or decreased susceptibility to disease in vivo?
Presumably, the percentage and functional capacity of
immune cells within the blood would be a determinant of
immune cell percentages and function at the site of active
immune responses within the tissues. Pertinent to the
latter question, the net effect of acute exercise stress
on disease susceptibility may depend upon a) the ability
of post exercise elevations of natural cytotoxicity (LAK
and/or NK) to offset depressions in other aspects of
natural immunity (neutrophil and macrophage functions-
Wong, et al., 1990; Wong, et al., 1992) and relatively
late developing antigen-specific immunity (influenza-
specific lymphoproliferation), and b) the rate of return
of immune cell proportions/functions to pre-exercise
levels. The relative importance of either of these occurrences would in turn depend upon the virulence of the pathogen and the route and time course of the infection. Answers to these questions might be found in experiments in which horses are challenged with a given pathogen, such as equine influenza virus, before or after an exercise challenge and associated clinical and immunologic responses are measured.

In the equine industry, exercise stress has been considered one of the most common insults with potential to impair lung immune defense mechanisms (Bayly, et al., 1987; Laegreid, et al., 1988). Thus, the increased incidence of respiratory infection in racing thoroughbreds may be the result of a "real life" experiment similar to that proposed above but performed with fit animals. Indeed, our own data regarding the effects of exercise on immune function in conditioned horses support the contention that acute exercise stress may be more immunosuppressive in these animals. Hence, while resting values for PWM, influenza, and LAK responses remained unchanged in fit compared to unfit horses, post exercise depressions in PWM responses were delayed and increased, and elevations in LAK activity were no longer significant.
Because race horses routinely experience episodes of exhaustive exercise and the negative effects of acute physical stress may be cumulative (Mackinnon, et al., 1988b; MacNeil, et al., 1991), it would be of interest in future studies to examine the effects of repeated exercise challenges on resting and post exercise immune parameters in fit and unfit horses. Such studies might reveal detrimental changes in basal immune responses similar to those observed in elite human athletes.

Inasmuch as hormones, peptides and amines contribute significantly to the body's response to physical activity, we examined the effects of some of these same substances on in vivo and in vitro immune function of horses. The data from these studies are unique in the equine system. Experiments utilizing cells from resting animals indicate that direct effects of corticosteroids, epinephrine, norepinephrine, beta endorphin, and ACTH each may contribute to the changes in immune response that occur after exercise in unfit horses. In contrast, the effects of neuroendocrine agents on immune reactivity did not correlate as well with post exercise immune responses in trained subjects. Accordingly, epinephrine, cortisol, norepinephrine, beta endorphin and ACTH significantly increased LAK activity at certain concentrations and were
unable (except for cortisol) to significantly depress PWM responses of cells obtained from conditioned horses, while acute exercise stress in these same animals produced no significant changes in LAK activity and strong suppression of mitogen responses. Of the naturally occurring neuroendocrine compounds tested, cortisol alone retained its ability to suppress mitogenic responses of cells from fit horses, but was less effective in doing so. Therefore, direct compound effects may play a greater role in mediating immunologic changes associated with acute exercise stress in unfit horses than in fit horses. Studies aimed at quantitating resting and exercising levels of neuroendocrine hormones, as well as hormone receptor numbers and affinity in horses, are required for further understanding of this phenomenon.

The data regarding immunoenhancing effects of cortisol presented here are relatively unique and require further investigation. Specifically, serum lots with and without co-stimulatory activity could be identified and compared, at the constituent level. Subsequent addition of neuroendocrine agonists and/or antagonists to media might help pinpoint likely co-factors in cortisol-induced augmentation of immune responses. Timed treatment, limiting dilution, cold target inhibition, and
proliferation studies would serve to describe the stage and the means by which cortisol exerts its unusual effects. In light of the inability of in vivo cortisol by itself to mimic exercise effects on immunity, and in order to determine the relative contribution of cortisol to these same immunologic changes, it would also be of interest to determine the in vitro and in vivo effects of the corticosteroid antagonist RU486 on exercise-induced changes in immune function.

Future investigations of the effects of hormones, peptides, and amines on immune function should be carried out with purified cell populations in the presence of agonists and/or specific antagonists. In this manner, we may be able to clarify whether auxiliary or immune effector cells are the target of these substances and how the final effect is mediated. Furthermore, the use of antagonists will determine if the effects of neuroendocrine compounds are initiated directly through interaction with their known receptors or indirectly through interaction with the cell membrane or an alternative receptor.

In summary, the following model of the effects of exercise stress on equine immune function is offered.
Exercising muscles make physiologic demands on the body which are met, in part, by elevated levels of neuroendocrine hormones. These hormones in turn exert direct (changes in cell function) and indirect (changes in blood cell trafficking) effects on immune responses. While epinephrine/norepinephrine act early, corticosteroids exert their influence at later post exercise time points. Thus increased proportions of CD8+ cells in the blood and/or alteration of lymphocyte functions results in depressed mitogen and antigen-specific responses and increased LAK responses following acute exercise bouts. Whether or not short term immunologic alterations result in clinically relevant immunosuppression, depends upon many factors, among which are the balance between positive and negative exercise effects on immunity, the rate of return of immune parameters to pre-exercise levels, the route of exposure and the virulence of the pathogen, and the presence or absence of additional stressors. Physical conditioning modulates resting and post exercise neuroendocrine hormone levels and hormone receptor activity. As a result, post exercise changes in immune responses are modified and include a delay and a deepening of the depression in PWM responses and an attenuation of positive exercise effects on LAK activity. The direct effects of neuroendocrine
compounds may be less important in mediating these changes in conditioned horses than in unconditioned horses. Persistence of this post exercise immunosuppression may account for observed increases in disease susceptibility in equine athletes repetitively experiencing exercise stress.
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Vita

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