CHAPTER IV
DISCUSSION

Acute starvation and refeeding in this study were defined as 7 days without and 7 days with food, respectively. The present investigation demonstrated that there was immunosupression during periods of acute starvation and these effects can be counteracted by refeeding. These conclusions are based on the decrease in total lymphocyte number, percentage and proliferative capacity, and decrease in CD4:CD8 ratio during the acute fasting period as well as the increased values that were seen after refeeding.

During the fasting period, decreases were observed in leukocyte number (day 4; $p < 0.04$), lymphocyte number ($p < 0.02$), CD4+ cells (day 4; $p < 0.06$), CD4:CD8 ratio (0 hours; $p < 0.004$), and mitogen stimulated CD4:CD8 ratio (72 hours; $p < 0.15$) during the fasting period as compared to baseline. Increases were seen in CD4+ cells (day 7; $p < 0.09$), CD8+ cells (day 7; $p < 0.04$) and intracellular calcium (day 4; $p < 0.02$) as compared to baseline. During the refeeding period increases ($p < 0.05$) were observed in leukocyte number, CD4+ cells, CD8+ cells, lymphocyte proliferation ($p < 0.07$), and lymphocyte number ($p < 0.004$) as compared to day 7. Decreases ($p < 0.001$) were seen in intracellular calcium, CD4+ cells and CD8+ cells by day 14 as compared to day 7.

In this study, a decrease in percent lymphocytes occurred throughout the fasting period and increased during refeeding. Our findings are consistent with the starvation stress-induced reductions in blood lymphocyte numbers, as well as the alterations in leukocyte-subpopulation in humans (Komaki et al. 1997; Dhaber et al. 1995). Similar findings were reported in human subjects consuming a very low energy diet for six weeks followed by a 1-2 week refeeding (Field et al. 1991). The effects a decrease in total lymphocytes has on immunodeficiency is still unknown at this time.

In humans, a CD4:CD8 ratio of <1.5 has been associated with immunosuppression (Chailleux et al. 1985). In our study, the CD4:CD8 ratio significantly fell below normal feline ratios (1.2 - 2.6) during acute starvation, which is similar to findings from human studies (Dean et al. 1990; Komaki et al. 1997). Depressed CD4:CD8 ratios are associated

with decreased immunity (Chailleux et al. 1985). The change in CD4:CD8 ratio in our study was attributed to the increase in CD8+ cells, as well as a decrease in CD4+ cells. Although there was an increase in CD4+ cells on day 7, the increase was not proportional to the increase in CD8+ cells, therefore, the ratio continued to decrease. The increase in CD8+ cells in our study, was compatible with other findings (Nuevonin and Salo 1984; Ogawa et al. 1993). Route of circulation may partially explain an increase in CD8+ cells. CD8+ cells migrate from the thymus to the spleen where non-specific CD8+ cells divide rapidly (Garre et al. 1986). Recirculating cells are limited in number and short-lived. This as well as the fact that acute starvation acts initially upon the proliferation of CD8+ cells (Malave 1980) may explain why the proportion of CD8+ cells may remain normal or become elevated in circulating blood (Chandra 1983).

A decrease in CD4+ cells was seen initially on day 4, followed by an increase through day 11. CD4+ cells decreased on day 14. This same pattern correlated with changes seen in total lymphocyte number. CD4+ levels in our study decreased below the reported normal levels of CD4+ in the cat (20-25% , Dean et al. 1990; 33.9% CD4+, Lin 1992) on day 4, which parallel other investigations (Komaki et al. 1997; Barlough et al. 1991). Cats with short-term infections exhibited a similar decrease in the percentage of CD4+ lymphocytes (Barlough et al. 1991). The changes in CD4+ cells in our study may be attributed to development and route of circulation. When CD4+ cells migrate from the thymus to the lymph nodes, they are more mature than CD8+ cells and hardly divide. In contrast to CD8+ cells, CD4+ cells recirculate in the blood and are long-lived (Garre 1986). During periods of undernutrition, thymus-derived cells are affected by the secondary effects of undernutrition (Garre 1986). During the adaptive phase of undernutrition (24-48 hours), the differentiation of cells is impaired. This may explain why the proportion of CD4+ cells initially decreased in the circulating blood in our study. Despite the fact that the proportion of CD4+ cells increased after day 4, the total number of lymphocytes decreased throughout the nutrient deprivation period; therefore, total number of CD4+ cells may have decreased.

In humans, many cell types, in response to damage, infection or antigens secrete interleukin-1 (IL-1) (Appendix B). The maturation and differentiation of naive human

CD4+ cells is influenced by cytokines and accessory cell-dependent costimulatory signals (Palmer and van Seventer 1997). IL-1 positively influences CD4+ cell proliferation, IL-2 receptor expression and cytokine production (Roitt et al. 1996). An initial decrease in CD4+ in response to fasting may have stimulated IL-1 production in the cat which in turn stimulated CD4+ proliferation and the subsequent increase in CD4+ cells on day 7. Stimulated CD4+ cells are known to secrete cytokines which are involved in CMI responses, such as IL-10. IL-10 does not appear with initial stimulation of CD4+ cells but rather after repeated *in vitro* stimulation, and this may allow for a down-regulation (Palmer and van Seventer 1997) of CD4+ production. IL-10 may not be initially secreted *in vivo*. IL-10 may down-regulate CD4+ production. Therefore, delayed secretion of IL-10 may have caused the decrease in CD4+ on day 14 in our study. Fasting may therefore induce cytokine secretion in lymphocytes which up or down regulates lymphocyte subpopulations.

It is known that malnutrition is associated with increased secretion of corticosterone, a major corticosteroid produced in mammals (Stinet 1983; Watson, Chein and Chung 1983; Winick and Nobel 1966). Starvation induced stress has been shown to increase corticosteroid production in humans (Komaki et al. 1997). There is an inverse correlation between serum cortisol (a corticosteroid) and the proportion of CD4+ cells in fasting human subjects (Komaki et al. 1997). Lymphocytes express receptors for corticosteroid and therefore cortisol affects circulating lymphocytes in humans (Fauci et al. 1976). The increase in serum cortisol during fasting could be responsible for significant alterations in immune suppression (Komaki et al. 1997). Initial decreases in CD4+ cells in our study may have been caused by increased secretion of cortisol by the cat. Corticosteroids act as a major feedback mechanism on immune responses (Roitt 1996).

Lymphocytes can respond to corticotropin releasing factor (CRF) to generate their own ACTH, which is a hormone secreted by the pituitary gland that stimulates adrenal production of corticosteroids. Lymphocyte secreted ACTH induces corticosteroid release (Roitt et al. 1996) and inhibition of lymphocyte proliferation occurs. Increased corticosteroid secretion had a lympholytic effect in response to 72 hours starvation in mice (Wing et al. 1987). In our study, we also observed a decrease in total lymphocytes in

response to starvation. Corticosterone secretion in response to stress induced by starvation may be a possible mechanism in the cat. Corticosteroids may explain the mild reductions in lymphocyte proliferation that occurred during the starvation period.

The level of dehydroepiandrosterone (DHEA), another major adrenal corticol hormone, was measured during a 7-10 day fast in humans (Komaki et al. 1997). DHEA protects the immune system against the immunosuppressive effects of infectious agents (Loria et al. 1988), including effects on cortisol (Blaueret al. 1991) and suppression of lymphopoeisis (Risdon et al. 1991). This suggests that there is a protective effect against the suppressive action of cortisol on the immune system during fasting. DHEA may have been responsible for the increase in lymphocyte number, proliferation of lymphocytes and CD4:CD8 ratio during refeeding as a feedback mechanism. It is also possible that if the starvation period was longer, these responses may also have been observed as well if DHEA is a feedback mechanism.

Con-A is a plant lectin used to study lymphocyte proliferation in cats (Cockerell et al. 1975) as both CD4+ and CD8+ T cell populations respond to this mitogen (Reinherz and Schlossman 1980). In our study, lymphocyte proliferation decreased throughout fasting and increased during refeeding. Lymphocyte proliferative capacity appeared consistent with the observed *in vitro* changes in number of CD4+ cells during fasting and refeeding. The use of Con-A *in vitro* activates T cells which leads to the production of cytokines and cytokine receptors *in vitro*, which together drive the proliferation and maturation of the cells and the production of CD4+ and CD8+ cells (Roitt et al. 1996). The *in vitro* mitogen-response assay detects defects in some aspects of mononuclear and lymphocyte cell function (Field et al. 1991) Con-A is a T-cell activator and it is possible that Con-A stimulated T-cell subsets are differentially affected in a variety of clinical conditions such as altered nutritional status (Dowd et al. 1986). Our study supports these findings. We observed an increase in CD8+ cells when compared to baseline whereas CD4+ cells decreased. Con-A stimulated T-cell subsets may be differentially affected during acute nutritional deprivation.

Altered nutritional states, such as fasting, decrease availability of nutrients needed for cell proliferation. A decrease in mitogen induced lymphocyte proliferation was

observed in rats fed a protein deficient diet (Mengheri et al. 1992). Another example of altered nutritional states decreasing lymphocyte proliferation occurred in hospitalized patients with low serum zinc and transferrin (Dowd et al. 1986). Decreases in mitogen response were observed in obese humans after a 14 day fast (Wing et al. 1983). However, in another study, no change in Con-A response was elicited in fasting humans (Holm et al. 1976). A decrease in mitogenic response suggests a decrease in the rate of DNA synthesis (Field et al. 1991), however the mechanisms for this decrease are unclear (Field et al. 1991). Decreased lymphocyte proliferation may have been a result of depleted nutrients in the cats. Low serum zinc levels have been shown to depress lymphocyte proliferation since it is required for optimal lymphocyte transformation (Messer et al. 1982). A decrease in dietary zinc decreases gene transcription through its interaction with metallothionein (Mt). Metallothionein transcription is influenced by dietary zinc, and therefore, a decrease in dietary zinc down-regulates DNA transcription of Mt. Since zinc regulates transcription, a decrease in dietary zinc through fasting may be responsible for the decrease in lymphocyte proliferation. Zinc also stimulates production of cytokines IFN-γ and IL-2 (Hannigan 1994). In rats fed a protein lacking diet, decreased mitogen responses were accompanied by a dramatic inhibition of IFN-γ and IL-2 secretion as well as mRNAs for IFN-γ, IL-2 and IL-2 receptor. A decrease in DNA transcription would lead to inhibited mRNA synthesis. The effects that these compounds and decreased dietary zinc have on immunosuppression may explain the decrease in lymphocyte proliferation.

A decrease in lymphocyte proliferation in our study may have been influenced by increases in percent CD8+ suppressor cells. It has been suggested that increased suppressor cells during acute starvation may attribute to decreased lymphocyte proliferation (Neuvonin and Salo 1984). Another explanation for the depression in lymphocyte proliferation may also be due to reduction in the amount of circulating T cells (Salimonu et al. 1982; Chandra 1977) which also occurred in our study. Therefore, decreased lymphocyte proliferation may be explained by decreases in circulating T cells, in addition to decreased zinc cellular concentrations.

Increases in CD8+ cells, decreases in CD4+ cells and decreases in lymphocyte proliferation observed during acute nutrient deprivation are indicative of immunosuppression. Incidence of sepsis (Chandra 1983), prolonged ventilation, and increased mortality have been associated with malnutrition in the critically ill (Reinhardt et al. 1980). Patients that are immunosuppressed as a result of malnutrition need to be identified so nutritional intervention can be implemented to enhance the body's capability to fight infection and/or illness.

Intracellular calcium is determined by stimulating lymphocytes with ionomycin to release intracellular calcium ($[Ca^{2+}]_i$) stores. The amount of $[Ca^{2+}]_i$ is indicative of the cells' activation state. One of the early responses of T-cell receptor (TCR) stimulation is a rise of $[Ca^{2+}]_i$ (Verheugen et al. 1997). In our study, $[Ca^{2+}]_i$ increased through day 11 and decreased on day 14. Despite no significant correlation, the augmented calcium response mimics the increased CD4+ and CD8+ response of cells during the latter stages of the starvation period. The increased $[Ca^{2+}]_i$ may have been due to the increased activity of the cell for differentiation. Increases in $[Ca^{2+}]_i$ have been associated with the first cellular changes associated with cellular activation such as surface receptor expression, membrane antigen expression, and protein turnover and phosphorylation (Ostergaard and Clark 1987). Protein kinase C (PKC), a calcium/phospholipid dependent and diacylglycerol (DAG) triggered enzyme, is involved in CMI (cell-mediated immunity) (Nishizuka 1984; Schuber 1989). Both calcium and PKC are responsible for the stimulation of lymphocyte proliferation. PKC phosphorylates surface molecules CD28 and TCR, leading to activation of specific genes such as those encoding the cytokine, IL-2 (Roitt, 1996). T lymphocyte proliferation and maturation is regulated by IL-2 (Cantrell and Smith 1984; Roitt 1996; Ostergaard and Clark 1987), and IL-2 expression depends on the magnitude of $[Ca^{2+}]_i$ (Negulescu et al. 1994). The point of action of the increased $[Ca^{2+}]_i$ may be at the level IL-2 regulation (Verheugen 1997). Increased $[Ca^{2+}]_i$ in our study may be attributed to the heightened activity of the lymphocytes for differentiation and proliferation. The cats had increased activity in the T lymphocytes during starvation as shown by changes in CD4:CD8 ratio. At this time, there are no existing studies which examine the role of $[Ca^{2+}]_i$ during starvation. Determining calcium flux in different

lymphocyte subsets may contribute to the understanding of the functional capacity of individual cells in the immune response.

CONCLUSIONS

Patients who are malnourished are at risk for decreased immune responses which may lead to increased morbidity. Yet, little is known about functional indicators of the immune system that could be used to assess nutritional status. Therefore, this study was conducted to investigate possible functional indicators of nutritional status. Several conclusions can be made from the results of this investigation. First, intracellular calcium needs to be further investigated as a possible functional indicator of lymphocyte activation. There is evidence that measuring intracellular calcium concentrations may be indicative of both the activation state of nutrient deprived lymphocytes, as well as the ability of the nutrient deprived cells to respond to stimuli. In addition, further investigations of intracellular calcium concentrations of different lymphocyte subsets during nutritional deprivation may give more insight into the mechanisms of cellular response during starvation. Measuring the relationship between intracellular calcium concentration and CD4:CD8 may be more indicative of immune responses than current methods of measuring CD4:CD8 ratios alone due to the variability of percent CD4+ and CD8+ within species. Certainly, intracellular calcium is a more sensitive indicator of cellular activation and function. Changes in intracellular calcium were observed on each day of both treatments. No relationship was determined in this study between body weight, CD4+ cells, CD8+ cells, CD4:CD8 or intracellular calcium concentrations, however all variables had significant changes in response to the treatments. Future research should focus on assessing the relationship between starvation, immunity and intracellular calcium concentration and determining its potential for use as a functional indicator of nutritional status in humans.