CHAPTER III

EFFECTS OF ACUTE NUTRITIONAL DEPRIVATION ON LYMPHOCYTE SUBSETS AND MEMBRANE FUNCTION IN CATS

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ABSTRACT

Identification of patients with suboptimal nutritional status allows for early treatment intervention. Currently, no definitive test of nutritional status exists. Therefore, this study was conducted to identify possible functional indicators of acute nutritional deprivation. The effects of total nutritional deprivation and subsequent refeeding on lymphocyte functions and subpopulations were examined in 23 healthy cats. Peripheral blood samples were analyzed at various times during fasting and refeeding periods. 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 when 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) when 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. These findings suggest that 7 days starvation had immunosuppressive effects on cats and 7 days refeeding and that these effects were not completely normalized during 7 days refeeding. The use of CD4:CD8 ratio in conjunction with intracellular
calcium flux may be useful as indices of nutritional status.

**INTRODUCTION**

Patients often are malnourished as well as critically ill when admitted to a hospital. Whether a critically ill patient requires surgery or treatment of disease, or is post traumatic or septic, assessing nutritional status is important for identifying nutritional risk (Manning and Shenkin 1995). Incidence of sepsis, prolonged ventilation and increased mortality has been associated with malnutrition (Reinhardt et al. 1980). The primary goal of nutritional status assessment is to identify nutrient deficiencies. In doing so, steps can be taken to improve overall nutritional status which would enhance the body’s capability to fight infection and/or illness. Nutritional assessment can be defined as a systematic method of gathering data, classifying the degree of malnutrition and instituting appropriate treatment and intervention techniques (Gilbride et al. 1984).

Current methods of nutritional status assessment include anthropometric, biochemical, dietary and clinical evaluation. No definitive test of nutritional status exists due to the complexity of the human diet and the multiple effects that nutrients have on various tissues, organs and physiological function (Manning and Shenkin 1995). In addition, nonnutritional aspects of the response to illness such as fever, infections and drugs may also affect assessment tests (Blackburn and Thornton 1979). Tests of immunological functions would be indicative of nutritional status because many are sensitive to overall nutritional status as opposed to deficiencies of individual nutrients (Puri et al. 1985). This is appropriate since it is rare for a patient to be deficient in only one nutrient. The purpose of this study was to identify immune system indicators associated with acute nutrient deprivation in cats. Specifically, the identification and quantification of CD4 and CD8 markers in T lymphocytes, lymphocyte proliferation, and intracellular calcium concentration of mononuclear cell membranes as a measure of monocyte and lymphocyte membrane function were examined in cats before, during and after a 7-day period of acute nutrient deprivation.
METHODS AND MATERIALS

Animals and Experimental Design

Twenty-three (4-7 years of age) healthy, adult, domestic shorthaired, neutered cats were used for this study. These cats were part of an established research colony at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). The study protocol was approved by the Virginia Tech Animal Care Committee. Cats were randomly assigned to one of three groups where each cat acted as its own control. Each group was separately housed in a climate and environmentally controlled room equipped with individual steel cages (24” x 24”). During the 14 day period, food was withheld on days 0-7 and then subsequently fed1 to meet daily energy requirements on days 7-14. Fresh water was available at all times throughout the study period of which intake was monitored.

Six milliliters (ml) of blood was drawn via jugular venipuncture on days 0, 4, 7, 11 and 14 into a 7 ml EDTA Vacutainer® tube and refrigerated until processed. To maintain hydration status and blood volume, subcutaneous fluids were administered (30 ml/lb body weight) immediately following each blood draw. General attitude, health and body condition were monitored each morning and evening by monitoring temperature, respiration, pulse, urination, defecation, capillary refill time, and alertness. Hydration status was determined daily by checking tackiness of mucus membranes and skin elasticity. Body weight was measured and recorded at the same time of day on days 0, 4, 7, 11 and 14. Additionally, total protein, albumin, blood urea nitrogen, hemoglobin and hematocrit were determined (days 0, 4, 7, 11, 14) to further assess the health status of the cats. Peripheral blood differential counts were performed by the clinical pathology lab at VMRCVM on the same days (0, 4, 7, 11, 14).

Cell Isolation. To maintain sterile conditions for lymphocyte (cell culture) proliferation, cell separation was performed under a laminar flow hood. Peripheral blood mononuclear cells (PBMC), consisting of lymphocytes and monocytes, and polymorphonuclear cells (PMNC) were isolated using a double density Ficoll separation

1 Science Diet Feline Maintenance Light, Hill’s Pet Nutrition, Topeka, KS
method (Toth et al., 1992) with centrifugation. Briefly, 3 ml of Histopaque 1.119 (Sigma Chemical Co., St. Louis, MO) were added to a 15 ml conical tube with a 20 gauge needle. Using a separate 20 gauge needle, 3 ml of Histopaque 1.077 (Sigma Chemical Co., St. Louis, MO) were layered over the 1.119 layer. Next, 6 ml of whole blood were layered over the 1.077 layer using an automatic pipette. The conical tube was centrifuged at 700 x g for 20 minutes (min) with no brake at room temperature. After centrifugation, separation of the cells led to two opaque interfaces consisting of PBMC and PMNC. The top layer (PBMC) of each sample was aspirated with a pasteur pipette and transferred to a 50 ml conical centrifuge tube for washing. Polymorphonuclear cells were donated to another study.

**Cell Washing.** The top interface (PBMC) was then washed twice with Hank’s Balanced Salt Solution (HBSS) for cell purification. Twenty-five ml of HBSS (Gibco Laboratories, Burlington, Ontario) were added to 50 ml conical tubes containing the recovered PBMCs and centrifuged at 200 x g for 10 min with the brake on. The supernatant was discarded and the procedure was repeated. The cells were resuspended in 10 ml complete media [RPMI 1640, fetal bovine serum (FBS), L-glutamine, sodium pyruvate, pen-strep, Sigma Chemicals Co., St. Louis, MO] and centrifuged at 400 x g for 20 min with the brake on to remove platelets. The supernatant was discarded and the cells were resuspended in 2-4 ml HBSS for cell counting on a hemocytometer. Cells were adjusted to 1 x 10^6 cells/ml with HBSS for time zero CD4/CD8 quantification, 3 x 10^6 cells/ml for proliferation assay, 2 x 10^6 cells/ml for time 72 hours CD4/CD8 quantification, and 1.0 x 10^6 cells/ml for calcium flux measurement.

**Immunophenotyping.** T lymphocytes were identified by detecting their surface markers utilizing flow cytometric analysis according to the method described by Ackley, et al. (1992). Briefly, 25 microliters (µl) of a 1:25 dilution of primary mouse anti-feline unlabeled purified CD4 or CD8 monoclonal antibody (Southern Biotechnology, Inc., Birmingham, AL) were added to 500 µl of adjusted cells (1 x 10^6 cells/ml). Each tube was incubated for 30 min at 4°C and centrifuged for 10 min at room temperature at 200 x g. The supernatants were discarded and the pellets were resuspended in 500 µl HBSS. Two microliters of Fluorescein Isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (H +
L) secondary antibody (Southern Biotechnology, Inc, Birmingham, AL) were added to each tube and incubated for 30 min at 4°C. Samples were centrifuged at room temperature at 200 x g for 10 minutes. The supernatants were once again discarded and the cell pellets were resuspended in 500 µl of paraformaldehyde (2.0%) and analyzed on the flow cytometer. CD4+ and CD8+ expression of PBMC were calculated by subtracting the value of the negative control (FITC) sample from the value obtained from the sample that had been incubated with both primary (CD4 or CD8) and secondary (FITC) antibodies.

**Lymphocyte proliferation assays.** One hundred microliters of mononuclear cells (3.0 x 10⁶ cells/ml) in complete media were cultured in triplicate in two sterile 96-well round-bottom plates (Corning, NY). One hundred microliters of either complete media alone or concanavalin A (a stimulus for lymphocyte proliferation) (Con-A, 5 µg/ml media; Sigma Chemicals Co., St. Louis) were added. The plates were incubated in a humidified incubator at 37°C at 5% CO₂ for 72 hours.

In one plate, lymphocyte proliferation was determined using the Alamar Blue™ staining methods of Gogal et al. (1997). After 48 hours, 20 µl of Alamar Blue™ (Accumed International, Inc., Westlake, Ohio) was added to all wells in the plate and returned to the incubator. The proliferation of cultures was determined 24 hours later by measuring absorbance at 570nm and 600nm in a microplate reader (Molecular Devices, Menlo Park, CA.). When Alamar Blue™ is added to cell cultures, it is in an oxidized (blue color) form and as the cells proliferate, the dye is reduced (red color). The absorbance at 570nm and 600nm determines optical density (OD) of reduced and oxidized forms of Alamar Blue™. Subtraction of OD at 600nm from OD at 570nm accurately determines the true absorbance (specific absorbance), which reflects proliferation. The specific absorbance of unstimulated cells (media alone) were subtracted from the specific absorbance of the cells incubated with Con-A to yield a Δ-specific absorbance. The mean Δ-specific absorbance of triplicates was calculated.

To determine CD4+/CD8+ quantification of lymphocytes at 72 hours, cells from the other plate were aspirated and placed into 12 x 73 mm tubes and resuspended in 500 µl HBSS. T lymphocyte subsets were quantified using flow cytometric methods as

**Calcium Flux.** Two fluorescent probes, Fluo-3 and SNARF-1 (Molecular Probes, Eugene, OR), were dissolved in dimethylsulphoxide (DMSO) (Sigma Chemical, Co., St. Louis, MO) at concentrations of 1 mM and 2mM. These stock solutions were stored refrigerated at 4°C. A 1/10 dilution of Fluo-3 and SNARF-1 stocks in RPMI 1640 and 1% FBS (Sigma Chemicals, Co., St. Louis, MO) were made and used as working solutions to load the cells for flow cytometric analysis. Fluo-3 is a long wavelength indicator for measurement of intracellular calcium ([Ca$^{2+}$]$_i$), and SNARF-1 is a pH indicator used to calibrate Fluo-3. PBMC cells were resuspended to a concentration of 1.0 x 10$^6$ cells/ml in RPMI 1640 and 1% FBS. Fluo-3 and SNARF-1 were loaded into the cells by the procedure previously described by Rijkers et al. (1990) with modifications. Peripheral blood mononuclear cells were incubated with 10 µl of each Fluo-3 and SNARF-1 working solutions and incubated for 30 min in a 37°C water bath. Cells were washed 2 times in RPMI +1% FBS, centrifuged at 200 x g for 10 minutes and resuspended in calcium-free (Ca$^{2+}$) HBSS at a final concentration of 1 x 10$^6$ cells/ml. Cells were analyzed on a flow cytometer by measuring the Fluo-3/SNARF-1 ratios over time. Cells were allowed to equilibrate for 15 seconds. The Ca$^{2+}$ ionophore, Ionomyocin (Sigma Chemicals Co., St. Louis, MO), was added at a final concentration of 10 µM to the cells. Data was collected for a total of 120 seconds. Intracellular calcium flux was expressed as a mean ratio (Fluo-3 ratio of activated vs. resting population).

**Statistical analysis** of data was carried out using a student’s paired t-test to determine difference between days using SAS Statistical Software. Correlations between variables were determined using Pearson’s correlation coefficients.

**RESULTS**

*Weight and albumin changes*

The cats tolerated the fasting period without incident. Body weight and serum albumin levels are shown in Table 1. All cats lost weight (p < 0.0001) throughout the fasting period and gained weight (p < 0.0001) during the refeeding period. As compared to baseline values, albumin levels (p < 0.05) increased on day 4 of the fasting treatment.
and decreased (p < 0.04) on days 11 and 14 when compared to day 7.

**TABLE 1**

Weight and Serum Albumin During a 7-Day Fast/7-Day Refeeding Study in Cats: Means ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>Refeeding</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 4</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.05 ± 0.03</td>
<td>3.09 ± 0.04*</td>
</tr>
<tr>
<td>(g/dl)</td>
<td>n=22</td>
<td>n=22</td>
</tr>
<tr>
<td>Weight (lbs)</td>
<td>10.12 ± 0.36</td>
<td>9.17 ± 0.07#</td>
</tr>
<tr>
<td>n=23</td>
<td>n=23</td>
<td>n=23</td>
</tr>
</tbody>
</table>

*Significantly different from baseline (p < 0.05); #Significantly different from baseline (p < 0.0001); **Significantly different from day 7 (p < 0.04)

Effects of fasting on peripheral blood leukocytes

Peripheral blood differential counts are shown in Table 2. Blood leukocyte numbers decreased (p < 0.05) on day 4 when compared to baseline. There were no significant changes in blood leukocyte numbers during the refeeding period. The percentage of lymphocytes decreased (p < 0.02) throughout the latter part of the fasting treatment and increased (p < 0.09) throughout the refeeding treatment. Total lymphocyte number decreased during the fasting period and increased (p < 0.03) during the refeeding period when compared to day 7.

**TABLE 2**

Peripheral Blood Leukocyte and Lymphocyte Counts and Percentages During a 7-Day Fast/7-Day Refeeding Study in Cats: Means ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>Refeeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 4</td>
</tr>
<tr>
<td>Leukocytes, x 10³/µl</td>
<td>11.0 ± 1.1</td>
<td>9.2 ± 0.9*</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>30.5 ± 3.0</td>
<td>27.5 ± 2.8</td>
</tr>
<tr>
<td>Lymphocytes - 10³/µl</td>
<td>2.9 ± 0.3</td>
<td>2.3 ± 0.2*</td>
</tr>
</tbody>
</table>

*Significantly different from baseline (p < 0.05); #Significantly different from day 7 (p < 0.03).

Changes in lymphocyte cell membrane surface markers

Figure 1 represents the percent of cells expressing specific lymphocyte membrane markers at time zero. The percentage of CD4+ lymphocytes decreased (p < 0.06) on day 4 of the
fasting period as compared to baseline. An increase (p<0.09 and p < 0.0001, respectively) in CD4+ cells was observed on days 7 and 11 (p < 0.007), and then a decrease by day 14 was observed. An increase (p = 0.16, p < 0.05, and p < 0.04, respectively) in CD8+ cells was observed on days 4, 7 and 11.

![Figure 1](image1.png)

**FIGURE 1.** Percent Expression of CD4+ and CD8+ Lymphocyte Cell Membrane Markers at Time Zero During a 7-Day Fast/7-Day Refeeding Study in Cats: Mean ± SEM.
*CD4+ significantly different from baseline (p < 0.06); #CD8+ significantly different from baseline (p < 0.05); **CD4+ significantly different from day 7 (p < 0.007); ##CD8 significantly different from day 7 (p < 0.04).

Figure 2 represents the percent of cells expressing specific lymphocyte membrane markers after 72-hour *in vitro* stimulation. The percentage of CD4+ cells tended to decrease throughout the starvation treatment and increased (p < 0.01) throughout the refeeding treatment. The percentage of CD8+ cells were increased on day 4 (p <0.002) as compared to baseline and day 14 as compared to day 7.
FIGURE 2. Percent Expression of CD4+ and CD8+ Lymphocyte Cell Membrane Markers at 72 Hours During a 7-Day Fast/7-Day Refeeding Study: Mean ± SEM.
*CD8+ different from baseline (p < 0.003); #CD4+ significantly different from day 7 (p < 0.01)

*Lymphocyte Proliferative Response to Con-A*

Proliferative capacity of lymphocytes in culture tended to decrease during the 7 day fasting period and subsequently increased during refeeding.

**TABLE 3**

Lymphocyte Proliferation During a 7-Day Fast/7-Day Refeeding Study in Cats:
Mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>Refeeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 4</td>
</tr>
<tr>
<td>Lymphocyte proliferation*</td>
<td>0.328 ± 0.06</td>
<td>0.291 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>n=19</td>
<td>n=21</td>
</tr>
</tbody>
</table>

* Δ Absorbance 570-600 nm

Figure 3 represents CD4:CD8 ratios calculated from the percent of cells expressing specific lymphocyte markers at time 0 as well as 72 hours post in vitro stimulation after the initiation of starvation and refeeding treatments. Con-A stimulated CD4:CD8 (72 hours), tended to decrease during the fasting period. The CD4:CD8 ratio at time zero decreased on day 4 (p = 0.003) as compared to baseline. No significant changes were observed in CD4:CD8 ratio at time zero or Con-A stimulated CD4:CD8 ratio during the refeeding period as compared
FIGURE 3. CD4:CD8 Ratios at Time Zero and 72 Hours During a 7-Day Fast/7-Day Refeeding Study In Cats: Mean ± SEM. *CD4:CD8 (0 hours) different from baseline (p < 0.004).

Intracellular Calcium Changes

Figure 4 represents the change in intracellular calcium during starvation and refeeding. An increase (p < 0.02; day7) in [Ca^{2+}] was observed throughout the fasting period and on day 11 (p < 0.08) as compared to day 7 of the refeeding period.
DISCUSSION

Acute starvation and refeeding in this study was defined as 7 days without and with food, respectively. The present investigation demonstrated that there was immunosuppression during periods of acute starvation and refeeding counteracted these effects. These conclusions are based on the decrease in total lymphocyte number, percentage and proliferative capacity, and decrease in CD4:CD8 ratio observed during the starvation period and the subsequent increase or stabilization in these parameters that were seen after refeeding.

In this study, a decrease in percent lymphocytes occurred throughout the fasting treatment and increased during refeeding. Our findings are consistent with other studies (Komaki et al. 1997; Dhaber et al. 1995) which also examined fasting and refeeding treatments. Similar findings were also reported in human subjects consuming a very low energy diet for six weeks followed by a 1-2 week refeeding period (Field et al. 1991). The effects that a decrease in total lymphocytes has on immunodeficiency are still unknown at this time.
Depressed CD4:CD8 ratios are associated with decreased immunity. 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 (~1.0) was below normal feline ratios (1.2 – 2.6) during acute starvation (Dean et al. 1990; Komaki et al. 1997). The change in the CD4:CD8 ratio in our study was attributed to an 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 and therefore, the ratio continued to decrease. The increase in CD8+ cells in our study, was consistent 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 proliferate (Garre et al. 1986). Recirculating cells are limited in number and are short-lived. This along with the fact that acute starvation acts initially upon the proliferation of CD8+ cells in the thymus (Malave 1980) and CD8+ cells further proliferate and differentiate in the spleen, may explain why the proportion of CD8+ cells may remain normal (Chandra 1983) or become elevated in circulating blood.

A decrease in CD4+ cells was seen by day 4, followed by an increase through day 11. CD4+ cells decreased on day 14. CD4+ levels in our study, decreased below the reported normal levels (20 – 33.9%) of CD4+ cells in the cat (Dean et al. 1991; Dah-Sheng Lin 1992) on day 4, which parallels other investigations (Komaki et al.; 1997; Barlough et al. 1991). The development and route of circulation of CD4+ cells may influence their susceptibility to change. When CD4+ cells migrate from the thymus to the lymph nodes, they are more mature than CD8+ cells and therefore have lower proliferation as compared to CD8+ cells. In contrast to CD8+ cells, CD4+ cells recirculate and are long-lived (Garre 1986). During periods of undernutrition, the secondary effects of undernutrition (Garre 1986) affect thymus-derived cells. During the adaptive phase of undernutrition (24-48 hours), differentiation of cells is impaired. This may explain why the proportion of CD4+ cells initially decreased in the circulating blood in our study. After day 4, though, the proportion of CD4+ cells increased, and the total number of lymphocytes decreased throughout the nutrient deprivation period. Therefore, total number of CD4+ cells appeared to decrease.
In humans, many cell types produce interleukin-1 (IL-1) in response to damage, infection or antigens. IL-1 positively influences CD4+ cell proliferation, IL-2 receptor expression and cytokine production (Roitt et al. 1996). Fasting may have stimulated IL-1 production in the cat, which in turn stimulated CD4+ proliferation and the increase in CD4+ cells on day 7. Stimulated CD4+ cells secrete cytokines, which are involved in CMI responses. IL-10 does not appear with initial stimulation of CD4+ cells but does after repeated in vitro stimulation, and this may allow for down-regulation of CD4+ production (Palmer and van Seventer 1997). Secretion of IL-10 by CD4+ cells may have caused the decrease in CD4+ cells observed on day 14.

It is known that malnutrition is associated with increased secretion of corticosterone, a major corticosteroid produced in mammals (Stinet 1983; Watson et al. 1983; Winick and Nobel 1966). Starvation induced stress has been shown to increase corticosteroid production in humans (Komaki et al. 1997) and an inverse correlation between serum cortisol (a corticosteroid) and the proportion of CD4+ cell subsets during fasting in human subjects (Komaki et al. 1997) has been reported. Lymphocytes express receptors for corticosteroid and therefore cortisol affects circulating lymphocytes in humans (Fauci et al. 1976). Serum cortisol can therefore directly influence lymphocyte and lymphocyte proliferation. An 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 resulted from 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 adrenocorticotropic hormone (ACTH), which is a hormone secreted by the pituitary gland that stimulates adrenal production of corticosteroids. Lymphocyte-secreted ACTH in turn induces corticosteroid release (Roitt et al. 1996) and may cause inhibition of lymphocyte proliferation. Increased corticosteroid secretion had a lympholytic effect in response to 72 hours starvation in mice (Wing et al. 1987). We also observed a decrease in total lymphocytes in response to starvation. Although, we did not measure corticosterone secretion, this is a possible mechanism in the cat for alterations in immune
responses. 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 increased during a 7-10 day fast in humans (Komaki et al. 1997). DHEA protects against the immunosuppressive effects of infectious agents on the immune system (Loria et al. 1988), including effects of cortisol (Blauer et al. 1991), and suppression of lymphopoeisis (Risdon et al. 1991). This suggests there is a protective effect against the suppressive action of cortisol on the immune system during fasting. Although DHEA was not measured in our study, it may be a reason for the increase seen 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 have also been observed if DHEA is a feedback mechanism.

Con-A is a plant lectin used to study lymphocyte proliferation (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 numbers of CD4+ cells during fasting and refeeding. Impaired cellular responses have been associated with a variety of immune deficiencies (Lopez-Botet et al. 1982). 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 T-cell subsets stimulated by Con-A are differentially affected in a variety of clinical conditions such as altered nutritional states (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). A decrease in mitogenic
response suggests a decrease in the rate of DNA synthesis, however, mechanisms for the decreased mitogen responses 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; Dowd et al. 1986).

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-\(\gamma\) and IL-2 (Hannigan 1994). In rats fed a protein lacking diet, decreased mitogen responses were accompanied by a dramatic inhibition of IFN-\(\gamma\) and IL-2 secretion as well as mRNAs for IFN-\(\gamma\), IL-2 and IL-2 receptor. IFN-\(\gamma\) and IL-2 are required for lymphocyte proliferation and differentiation. A decrease in DNA transcription would lead to inhibited mRNA synthesis. The effects that they and decreased dietary zinc have on immunosuppression may be an explanation for decreased lymphocyte proliferation.

A decrease in lymphocyte proliferation in our study may have been due to increases in CD8+ suppressor cells. During acute starvation, increased subsets of suppressor cells may contribute to the decrease in lymphocyte proliferation responses (Neuvonin and Salo 1984). A depression in lymphocyte proliferation may also be due to reduction in circulating T cells (Salimonu et al. 1982; Chandra RK 1977) which also occurred in our study. Therefore, decreased lymphocyte proliferation may be explained by decreases in circulating T cells, in addition to decreased cellular zinc concentrations.

Intracellular calcium is determined by stimulating the lymphocytes with ionomycin to release \([\text{Ca}^{2+}]\), stores. The amount of \([\text{Ca}^{2+}]\) is indicative of the activation state of the cell. One of the early responses of T-cell receptor (TCR) stimulation is a rise in \([\text{Ca}^{2+}]\), concentration (Verheugen et al. 1997). In our study, \([\text{Ca}^{2+}]\), 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 starvation. The increased \([\text{Ca}^{2+}]\), may have been due to the heightened activity of the cell for cell
differentiation and proliferation. Increases in $[\text{Ca}^{2+}]_i$ are correlated with the first cellular changes associated with cellular activation such as surface receptor expression, membrane antigen expression, protein turnover and phosphorylation (Ostergaard and Clark 1987). Protein kinase C (PKC), a calcium/phospholipid dependent and diacylglycerol (DAG) triggered enzyme, is involved in cell-mediated immunity (CMI) cascades (Nishizuka 1984; Schuber 1989). Both calcium and PKC are needed for the stimulation of lymphocyte proliferation. PKC phosphorylates surface molecules such as CD28 and TCR, leading to activation of specific genes such as those encoding the cytokine, IL-2 (Alberts et al. 1994).

Interaction with IL-2 and other molecules results in proliferation and maturation of the lymphocytes (Roitt 1996; Ostergaard and Clark 1987). IL-2 is a proposed mechanism correlating the increase during the latter fasting period in CD4+ and CD8+ with the increase in $[\text{Ca}^{2+}]_i$. IL-2 regulates T- lymphocyte proliferation (Cantrell and Smith 1984). IL-2 expression depends on the magnitude of $[\text{Ca}^{2+}]_i$ increase (Negulescu et al. 1994), and the point of action of the increased $[\text{Ca}^{2+}]_i$ may be at the level of IL-2 regulation (Verheugen 1997). Therefore, an accumulation of $[\text{Ca}^{2+}]_i$ is indicative of a cell’s activation state. The ability of T lymphocytes to increase $[\text{Ca}^{2+}]_i$ in response to stimuli is a measure of early signal transduction and thus cellular function. We observed that the cats had increased cellular activity in the T lymphocytes during starvation. Determining calcium flux in different lymphocyte subsets in the future may have a direct bearing on the functional capacity of individual cells in the immune response during starvation.

In conclusion, our data show that immunosuppression can result in cats undergoing 7 days acute starvation. Immunosuppression began to be alleviated with refeeding. The decrease in lymphocyte number, proliferation and CD4:CD8 ratio are all indicative of immunosuppression. Increased $[\text{Ca}^{2+}]_i$ reflects heightened activity within the lymphocytes needed for cell proliferation and differentiation. The use of these variables as functional indicators in conjunction with conventional methods of nutritional status indicators is hopeful. Incidence of sepsis (Chandra 1993), 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 disease. The many complex interactions between starvation and immunity offer a great challenge to researchers. A greater understanding of this area may help decrease patient mortality.

**LITERATURE CITED**


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