Leptin suppresses mitogenic stimulation of murine T cells by Con A and anti-CD3 antibody

An Honors Thesis (HONRS 499)

by

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ABSTRACT

The purpose of this study was to investigate the role of leptin, an anti-obesity hormone, in immune responsiveness (i.e. proliferation) of murine T lymphocytes, *in vitro*, to the mitogenic stimulation of concanavalin A and anti-CD3 antibodies.

Splenocytes were obtained from 3 to 6 month old male and female BALB/C mice and were cultured in the presence of media alone, Con A or anti-CD3, both with and without leptin. MTT uptake analysis was used to measure the amount of proliferation.

Several proliferation studies were conducted, both with concanavalin A and with anti-CD3 antibodies. The optimum concentrations of Con A and leptin had been established by previous work in our laboratory, and we established the optimum concentration of anti-CD3 for maximum cellular proliferation. Our findings indicate that leptin alone is slightly stimulatory only in concentrations up to 20 ng/ml. Leptin, upon costimulation with either Con A or anti-CD3 antibodies, was stimulatory only at a concentration of 2 ng/ml. Above this concentration, leptin seems to be an immunosuppressor, at both high and low cell concentrations.
**Introduction/Review of Literature**

Leptin, a 16-kDa nonglycosylated peptide hormone, is synthesized only in adipocyte cells (10). It has a 4 alpha helical structure, similar to that of cytokines. Leptin is a product of the obese gene, ob. The ob gene has been localized to chromosome 7q31.3 in humans, and chromosome 6 in mice. In both humans and mice, the gene consists of three exons. The mouse ob gene product encodes a 4.5 kb mRNA, while the human gene encodes a 3.5 kb mRNA. Despite these differences, the amino acid sequence of the two proteins exhibit 84% homology (6).

The leptin receptor, OB-R, is encoded by the db gene. It belongs to the class I cytokine receptor family (10). Multiple forms of the receptor have been identified, varying in length of their intracytoplasmic tail. The long form (ObRb) is the most abundant and is the most competent in intra-cellular signaling in leptin-treated cells (6).

Once leptin binds to its receptor, it decreases food intake and increases energy dissipation. This is its predominant function. Leptin serves as a peripheral signal to the brain (1) by binding to its receptor located in the hypothalamus (7). Serum levels of leptin correlate to total body fat. A study by Considine, et al., found that the mean serum levels of leptin were $31.3 \pm 24.1$ ng/ml in the obese subjects (body-mass index of $\geq 27.8$) and $7.5 \pm 9.3$ ng/ml in the normal-weight patients. Weight loss in the obese subjects caused a reduction in leptin serum levels (2). Mutant obese mice ($ob/ob$), lack active leptin. When given injections of recombinant leptin, these mutant mice can be returned to normal weight (1). These findings led to much speculation that the same sort of therapy could work with obese humans. Obese humans, however, have elevated levels of leptin. Many studies have shown that obese humans tend to just be “leptin resistant”, in
that they do not generate a signal that corresponds to their elevated serum leptin levels, possibly due to a defective transport of leptin across the blood-brain barrier or a defective ObR (1). A study by Esler, et al., found that “the release of leptin from the brain to the circulation was higher in obese than lean men, suggesting that the rise in plasma leptin concentration in human obesity is not entirely attributable to increased release of leptin from adipose tissue.” These results suggested that leptin release could come from tissues other than adipose tissue, and the primary flaw causing obesity may be an inability of leptin to react with the central nervous system (5).

The leptin receptor is not only expressed in the central nervous system. It is also found on other cells in reproductive and hematopoietic tissues (10). This has led to research into possible roles of leptin in other systems. Indeed, leptin has been linked with fertility. Mutant ob/ob mice never progress to puberty. When leptin was administered to female ob/ob mice for 30 days, pregnancy was possible (6). Leptin has also been shown to accelerate puberty in normal mice. Leptin may be a signal to the body that it has stored up sufficient adipose tissue for reproduction, so that it may enter puberty (6).

Because leptin receptors are found on hematopoietic tissues, it is thought that it probably also plays a regulatory role in hematopoiesis. The structures of both leptin and its receptor are very similar to many of the molecules that are involved in the regulation of hematopoiesis. In possible support of this hypothesis, mice that are deficient in either leptin or its receptor exhibit minor hemopoietic deficiencies (6).

Leptin is a multi-functional protein that seems to affect a variety of systems in the body. One other system that has become a focus of leptin research is the immune system. Leptin has recently been shown to enhance cell-mediated immunity in mice. It can
reverse starvation-induced immunosuppression in mice (9). In Martin-Romero’s study, human recombinant leptin (16 – 1,600 ng/ml) was found to enhance the activation effect of submaximal concentrations of mitogens such as PHA or Con A on human peripheral lymphocytes depleted of monocytes. This indicates that leptin alone may not be sufficient to trigger the activation and proliferation of T lymphocytes, but that it may be able to enhance the effect of other more powerful stimuli (10). It also appears that the effect of leptin is much more pronounced on CD4⁺ CD45RA⁺, or naïve, T cells than on memory T cells. Both of these cells express ObRb mRNA (9). The possible mechanism for this immunostimulatory action is not yet known. One possibility is that falling leptin serum levels act as a signal to the brain that the body is going into starvation. This causes the body to conserve energy. Energy is preserved for vital functions, such as energy metabolism, and less essential systems with high energy demands, such as the reproductive and immune systems, are inhibited (9). Lord found that stimulation of T lymphocytes by leptin was due to a specific effect of leptin receptor signaling. ObRb mRNA was detected in human CD4⁺ T cells, but not in freshly isolated monocytes. These results indicated that leptin enhances the T-cell immune response by binding to its receptor on the T cells, rather than through a direct effect on the antigen presenting stimulatory cell (9).

Surprisingly, in previous work in our laboratory, recombinant murine leptin was found to be inhibitory toward both Con A or LPS stimulated murine splenocytes in vitro (8). In the present study, we attempted to repeat and confirm these findings with maximal levels of Con A or anti-CD3 antibodies which stimulate T cells specifically. In this work, we determined whether or not murine leptin stimulated the proliferation of
murine splenocytes in vitro in the presence of media alone, Concanavalin A, or murine anti-CD3 antibodies. Overall, in an in vitro animal model, leptin appears to be a suppressor of T lymphocyte proliferation. At concentrations of leptin up to 20 ng/ml, it was slightly stimulatory on its own. When used together with either Con A or anti-CD3, leptin was only stimulatory at a concentration of 2 ng/ml.

Materials and Methods

Experimental Animals

Three to six month old male and female BALB/C mice were obtained from the breeding colony maintained at Ball State University. The mice were housed and fed under conditions that meet the approval of the Ball State University Animal Care Committee.

Solutions and Reagents

The murine splenocytes were cultured in RPMI 1640 complete media (with Hepes) with penicillin, streptomycin, and antimycotic, 0.05 mM β-Mercaptoethanol, and 10% fetal calf serum. Recombinant murine leptin, produced in E. Coli, was purchased from Sigma Chemical, and dissolved in 100 ul sterile dd water and 900 ul of HBSS with 0.05% Triton-X to give a 0.1 mg/ml stock. Con A, produced by Canavalia ensiformis type IV, was purchased from Sigma Chemical. A 1 mg/ml stock was made in sterile media. Murine anti-CD3 antibody (0.5 mg/ml) was purchased from PharMingen International. A 40 ug/ml stock solution was made in sterile media.
Protocol for Proliferation Studies

The mice were sacrificed by CO$_2$ asphyxiation, and the spleen was removed. The cells were washed in HBSS and then adjusted to concentrations of $2.5 \times 10^6$ or $5 \times 10^6$ cells/ml. The cells were added to a 96-well microtiter plate and Con A (4 ug/ml) or anti-CD3 (0.2 ug/ml) was added to the cells. Wells containing cells and media alone were used as controls. Leptin was added to the cells in varying concentrations (Exp. 1 (Trials 1 and 2): 2 ng/ml, 20 ng/ml, and 40 ng/ml; Exp. 1 (Trial 3) & Exp. 3: 2 ng/ml, 10 ng/ml, 20 ng/ml, and 40 ng/ml). The cells were incubated in a CO$_2$ incubator at 37° C for approximately 48 hours. After 48 hours, they were pulsed with MTT dye (5 mg/ml) and placed back into the incubator for 4-6 hours. The plate was then removed from the incubator and centrifuged. The supernatant was decanted and the stained cells were resuspended in 100 ul of 0.4% HCl acidified isopropanol. The optical densities of each well were read at 600 nm using a Cambridge Technologies Series 700 Microplate Reader.

Results

*Determinant of optimum Concanavalin A concentrations:* The optimal concentration for Con A was determined to be 4 ug/ml by previous work done in our lab. Thus, 4 ug/ml Con A was used in all work except where noted.

*Experiment 1: Influence of leptin on Concanavalin A mitogenesis of spleen cells.* Con A is often used to stimulate proliferation of T lymphocytes. We examined the effect that leptin would have on this stimulation. As can be seen in Figures 1-6, when the splenocytes were stimulated with Con A, leptin showed a dose-dependent inhibition of
proliferation. Table 1 has a summary of the percent inhibition or stimulation of proliferation by leptin, both with and without Con A. Without Con A stimulation, leptin at low concentrations seems to be slightly stimulatory, particularly at the higher cell concentrations. At higher levels, however, leptin suppressed proliferation of both stimulated and non-stimulated cells. A trial was also done in which two different concentrations of Con A were used (2 ug/ml and 4 ug/ml) in order to determine if one would be more effective (see Figure 7). No significant difference was seen between the immunosuppressive effect of leptin at 2 or 4 ug/ml Con A.

Experiment 2: Determination of optimum anti-CD3 antibody concentrations. In order to determine the maximal concentration of murine anti-CD3 for T cell proliferation, cells were incubated in either media alone or various concentrations of anti-CD3 antibody (.01 ug/ml, .1 ug/ml, and 1 ug/ml). The results are shown in Figure 8. The concentration that yielded maximum proliferation for both high and low cell concentrations was 0.1 ug/ml. In order to ensure that we would have significant cell proliferation, a concentration slightly above this maximal concentration was chosen (0.2 ug/ml).

Experiment 3: Influence of leptin on anti-CD3 mitogenesis of spleen cells. In this experiment, we examined the effect of leptin on a second T cell mitogen, murine anti-CD3 antibody. Only the higher cell concentration, 5 x 10^6 cells/ml, was used for this experiment. The results are shown in Figures 9 and 10 and the percents inhibition or stimulation are summarized in Table 2. Again, leptin suppressed cellular proliferation. Slight stimulation was seen at 2 ng/ml of leptin, but it was not significant. Without
stimulation by anti-CD3, leptin showed stimulation at a concentration of 2 ng/ml, but suppression at all higher concentrations.

Discussion

Our research has shown that leptin is immunosuppressive at physiologically relevant levels when either Con A or anti-CD3 are used to stimulate lymphocytes. Murine recombinant leptin dose-dependently suppressed proliferation of murine T lymphocytes, especially at concentrations of 20 ng/ml and higher. This confirmed the previous research that had been done in our lab. The immunosuppression observed when anti-CD3 was used as the immune stimulant is particularly important as anti-CD3 most closely mimics MHC-Ag stimulation of T lymphocytes. Thus, it appears that excessive amounts of leptin observed in many obese individuals (\( \bar{x} \) of 31.3 ng/ml) may indeed be immunosuppressive, and that giving added leptin to obese individuals to induce weight loss, as has been suggested, could be detrimental. Leptin at low levels may be slightly stimulatory but at levels generally observed in normal weight individuals (~ 10 ng/ml), no stimulation was observed and at 20 ng/ml suppression was observed.

Martin-Romero, et al. have reported that human leptin enhances activation and proliferation of human circulating T lymphocytes. They did not report any immunosuppressive effect of leptin. There are, however, some key differences between our research and the research done by Martin-Romero that could explain the differences in our findings. In their research, they used human lymphocytes and human recombinant leptin, while we used murine sources for both. Also, they used submaximal concentrations of Con A to stimulate their lymphocytes. These were the concentrations
that were used when leptin stimulated proliferation. The concentrations of Con A and anti-CD3 that we used were optimal concentrations. It is possible that, when optimal concentrations of mitogens are used, leptin causes suppression of proliferation, rather than stimulation. In addition, the concentrations of leptin that they used, 16-1,600 ng/ml, is much higher than the concentration used in our research, and is significantly higher than serum levels of leptin found in human or mice. It may be that leptin at physiologically relevant levels serves much more importantly as an immunomodulator enhancing certain functions and activities of subsets of lymphocytes, monocytes and macrophages rather than their proliferation.

There is still much research that needs to be done on leptin and its role in the immune response. It seems to have a multitude of functions, and it is possible that not all of them have been discovered. It would be interesting to conduct these same experiments in vivo and see if the same results were obtained.

Acknowledgments

First of all, I would like to thank Dr. Nancy Behforouz, for putting up with my many mistakes and being a wonderful advisor. Thank you for helping me find my way through four years at Ball State. Also, I want to thank my wonderful family. Without your love and support, I would not have made it to where I am today. I love you all very much. Finally, and most importantly, I thank my Lord and Savior Jesus Christ, without whom I would not even be here.
### Stimulation with Con A (4 µg/ml)

<table>
<thead>
<tr>
<th>Leptin (ng/ml)</th>
<th>Without Con A</th>
<th>With Con A</th>
<th>Without Con A</th>
<th>With Con A</th>
</tr>
</thead>
</table>
| 2              | 21.94%  
(4.52 to 47.6%) | -5.50% 
(3.23 to -11.2%) | 56.73% 
(41.6 to 71.0%) | -2.55% 
(-1.99 to -2.88%) |
| 10             | 61.9%  
(9.03 to -28.1%) | 3.02% | -16.9% | 6.17% |
| 20             | -6.18%  
(10.6 to -40.4%) | -32.0%  
(-26.5 to -36.3%) | 51.55% 
(9.74 to 93.3%) | -27.90% 
(-7.9 to -57.0%) |
| 40             | -8.86%  
(9.03 to -28.1%) | -75.23%  
(-68.7 to -79.7%) | -30.97%  
(-13.9 to -47.7%) | -68.83%  
(-65.2 to -75.9%) |

**Table 1:** Percent stimulation (+) or inhibition (-) with leptin. Percentages shown are mean values of three trials, except for the 10 ng/ml leptin concentration (with the range in parentheses).

### Stimulation with murine anti-CD3 antibody (0.2 µg/ml)

<table>
<thead>
<tr>
<th>Leptin (ng/ml)</th>
<th>Without anti-CD3</th>
<th>With anti-CD3</th>
</tr>
</thead>
</table>
| 2              | 100.1%  
(28.2 to 172%) | 1.3%  
(7.1 to -4.5%) |
| 10             | -2.1%  
(12.8 to -16.9%) | -13.5%  
(-4.0 to -22.9%) |
| 20             | -18.7%  
(-18.2 to -19.1%) | -36.4%  
(-33.5 to -39.3%) |
| 40             | -42.9%  
(-21.3 to -64.5%) | -68.7%  
(-63.4 to -73.9%) |

**Table 2:** Percent stimulation (+) or inhibition (-) with leptin. Percentages shown are mean values of two trials (with the range in parentheses). Cell concentrations for all trials of this experiment were 5 x 10^6 cells/ml.
Experiment 1: Effect of leptin - with and without Con A (Trial 1)

**Figure 1:** O.D. readings shown are mean values + 1 S.D.

**Figure 2:** O.D. readings shown are mean values + 1 S.D.
Experiment 1: Effect of leptin - with and without Con A (Trial 2)

Figure 3: O.D. readings shown are mean values + 1 S.D.

Figure 4: O.D. readings shown are mean values + 1 S.D.
Experiment 1: Effect of leptin with and without Con A (Trial 3)

**Figure 5:** O.D. readings shown are mean values + 1 S.D.

**Figure 6:** O.D. readings shown are mean values + 1 S.D.
Figure 7: Costimulation with 2 ug/ml and 4 ug/ml Con A (O.D. readings shown are mean values ± 1 S.D.)
Stimulation by Anti-CD3

Figure 8: Determination of maximal concentration of anti-CD3
Experiment 3: Effect of leptin on anti-CD3 mitogenesis

Stimulation by Anti-CD3

Figure 9: (Trial 1) O.D. readings shown are mean values + 1 S.D.

Figure 10: (Trial 2) O.D. readings shown are mean values + 1 S.D.
References


