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Leptin and Cellular and Innate Immunity in Abstinent Alcoholics and Controls

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Background: Basic studies indicate that in vitro and in vivo doses of leptin modulate cellular immune responses. Given evidence that concentrations of leptin are altered in alcoholics who also show immune abnormalities, this study examined the relationships between circulating levels of leptin and markers of cellular and innate immunity.

Methods: Circulating levels of leptin, natural killer cell (NK) activity, interleukin-2 (IL-2)–stimulated NK activity, and concanavalin A–stimulated production of IL-2, IL-6, IL-10, and IL-12 were compared between abstinent DSM-IV alcohol-dependent men ($n = 27$) and age- and gender-matched controls ($n = 34$).

Results: As compared with controls, alcoholics showed lower NK activity ($p < 0.01$) and a trend for lower levels of leptin ($p = 0.055$). In the total sample, leptin predicted NK activity ($\beta = 0.33$; $p < 0.05$) after controlling for the confounding influence of body mass index, alcohol intake, and smoking. Leptin was not correlated with any of the cytokine measures. To examine whether the effects of leptin were mediated by its direct action on NK, additional studies examined in vitro effects of leptin on NK activity in healthy volunteers ($n = 10$); leptin doses (0.1, 1, and 10 nM) yielded levels of NK activity comparable to those with media alone.

Conclusions: These data show that circulating levels of leptin are associated with NK activity in humans and suggest that abnormal in vivo concentrations of leptin may contribute to the declines of NK activity in alcoholics who are at risk for infectious diseases.

Key Words: Alcoholism, Leptin, Immunity, Natural Killer Cell Activity.

ALCOHOL DEPENDENCE IS associated with greater susceptibility to infectious diseases such as pneumonia (Nelson and Kolls, 2002), tuberculosis (Bucholz et al., 1994), hepatitis C (Balasekaran et al., 1999), and, possibly, human immunodeficiency virus (Crum et al., 1996), due in part to impairments of host defense mechanisms. In alcoholics recovering from surgery, immunological alterations were found to predict risk of infectious disease complications (Sander et al., 2002). Weakened host defense in alcoholics is characterized most prominently by impaired natural killer cell (NK) activity and interleukin-2 (IL-2)–stimulated killer cell activity, as well as decreased ex vivo production of IL-6 and a shift from T helper 1 to T helper 2 cytokine expression: these characteristics persist after weeks of abstinence (Irwin and Miller, 2000; Redwine et al., 2003).

Determinants of impaired host defense in alcoholics likely involve a number of factors, including alcohol intake, nutritional status, cigarette smoking, and neuroendocrine function. Circulating levels of the neurohormone leptin are altered in alcoholics, although both increases and decreases have been found that are due in part to differences in alcohol use, alcohol withdrawal, nutritional status, and possibly liver disease function. Given evidence that nutritional status and body fat correlate with leptin (Donahue et al., 1999; Faggioni et al., 2001; Friedman and Halaas, 1998; Prolo et al., 1998), the combination of alcohol intake and undernourishment in alcoholics likely contributes to altered leptin levels in this population. However, because of the heterogeneity of alcoholic populations (e.g., abstinent versus active users, malnourished versus nonmalnourished, and presence versus absence of liver disease), adequate control of these factors is essential in determining whether abnormal leptin levels are found in association with alcohol dependence.

Basic studies indicate that leptin has a role in the regulation of immunity. Physiologic concentrations of leptin in vitro stimulate T-cell activation and proliferation, macrophage phagocytosis, and cytokine production (Lord et al.,

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1998), whereas animals that are deficient in leptin or leptin receptor show diminished NK numbers in the blood, spleen, and liver (Tian et al., 2002). Human T and NK cells both express the leptin receptor (Lord et al., 1998; Zhao et al., 2003), suggesting a possible direct pathway by which leptin could influence these cell populations. In addition, murine T cells and macrophages have been found to secrete leptin (Sanna et al., 2003). Despite these basic observations, studies in humans are limited, and no study has examined the relationships between leptin and measures of immunity in abstinent alcoholics who have impairments of immunity and show abnormal expression of leptin.

METHODS

Subjects

Sixty-one men were included in this study: 27 abstinent alcoholic subjects and 34 healthy control subjects. The dataset was drawn from a sample whose immunological findings have been reported previously as part of a study examining sleep and immunity in alcoholics (Irwin and Miller, 2000). Alcoholic subjects were inpatients in an alcohol and drug treatment program at the Veterans Affairs San Diego Healthcare System. Nonpatient controls were recruited from the general community in San Diego through flyers and advertisements in local newspapers. In addition, we used a direct mailing to the San Diego County population of veterans to target controls age-matched (±5 years) to alcoholic patients and with similar sociodemographic characteristics.

Research diagnoses of controls and alcoholic patients were based on results of a semistructured interview developed by the multisite Collaborative Study on the Genetics of Alcoholism (Bucholz et al., 1994). This interview assessed alcohol, as well as use of cigarettes and other substances, and assessed diagnostic criterion data to evaluate the presence of a lifetime history of a psychiatric and substance dependence disorder. Alcoholic subjects fulfilled DSM-IV (American Psychiatric Association, 1994) criteria for alcohol dependence in the absence of major preexisting psychiatric disorders; in other words, alcoholics had primary alcohol dependence. Control subjects were required to meet "never mentally ill" status based on the DSM-IV.

Subjects included in the study were free of physical illness, as assessed by physician examination. All subjects were free of infections for at least 2 weeks and tested negative for hepatitis B and C. Alcoholics were excluded if they had histories of overt alcohol-related liver disease such as jaundice and esophageal varices. Subjects taking medications known to alter the immune system were excluded from the study.

All alcoholics maintained abstinence, as confirmed by nursing observations and random urine toxicology screens, during their 2-week inpatient stay in the Alcohol and Drug Treatment Center. Liver function tests were performed by previously described methods (Irwin et al., 1990). Alcohol consumption history is reported in Table 1. As expected, alcoholics reported significantly more days in which they drank alcohol per month than controls and also reported drinking more alcohol per day than control subjects. These reports are consistent with previous studies with alcoholics conducted in our laboratory (Irwin et al., 2002; Thompson et al., 1995). Detoxification and withdrawal routinely occurred before inpatient admission. Because withdrawal from alcohol can affect immune function (Laso et al., 1997), alcoholics were studied after acute and subacute withdrawal symptoms resolved, with the exception of sleep disturbance. Three alcoholic patients reported use of diazepam for withdrawal symptoms in the 30 days before admission; all other alcoholic patients denied use of such medications before admission. All subjects were free of psychotropic medications at least 2 weeks before immune assessment. During their inpatient stay, alcoholic patients participated in an inpatient sober treatment milieu that involved Alcoholics Anonymous, education, and group and individual counseling.

Table 1. Age, Education, Ethnicity, Severity of Depressive Symptoms, Alcohol Consumption Histories, and Liver Toxicity in Control and Alcoholic Groups

Variable	Controls (n = 34)	Alcoholics (n = 27)	Alcoholism effect	
			t	p
Age (years)	44.1 ± 11.7	43.4 ± 9.3	0.22	0.83
Education (years)	15.4 ± 1.9	13.2 ± 1.4	5.15	**<0.001**
Ethnicity (European American/ African American)	22/12	14/13		
HDRS scores	0.8 ± 1.2	2.6 ± 3.0	−3.03	**0.005**
Alcohol use (last 3 months)				
Drinking days/month	6.4 ± 6.9	26.1 ± 6.3	−11.46	**<0.001**
Drinks/day	1.5 ± 1.0	12.5 ± 10.4	−5.47	**<0.001**
Days since last drink	66.2 ± 133.9	22.0 ± 11.2	1.92	0.06
ALT (units/liter)	25.8 ± 8.0	31.8 ± 23.7	−1.26	0.22
AST (units/liter)	28.8 ± 12.6	40.7 ± 32.8	−1.78	0.09
Bilirubin, total (mol/liter)	1.0 ± 1.2	0.7 ± 0.5	1.35	0.18
Bilirubin, direct (mol/liter)	0.10 ± 0.04	0.14 ± 0.1	−1.94	0.06
Alkaline phosphatase (units/liter)	66.5 ± 17.0	68.6 ± 19.0	−0.46	0.65

HRSD, Hamilton Rating Scale for Depression; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Boldface refers to significant p values. Values in group columns reflect mean ± SD.

Procedures

After at least a 2-week period of abstinence during their inpatient stay, subjects entered the study protocol. Subjects stayed at the University of California at San Diego General Clinical Research Center for five nights as part of a sleep protocol (Irwin et al., 2002). Immunological variables (NK activity, proinflammatory cytokines, and IL-2–stimulated NK activity) were measured from blood collected between 0600 and 0700 hr on the third day. Leptin was measured from blood samples collected between 2300 and 0000 hr later that night. Blood sampling for leptin and for immunity was obtained at different times to coincide with differences in the circadian peaks in immunity and leptin, respectively. For example, NK activity follows a circadian pattern and peaks in the morning waking hours (Bourin et al., 1993), whereas leptin peaks from midnight to 0200 hr (Mantzoros et al., 2001).

The effects of blood-sampling stress on immunoregulatory cell number and function were minimized by placement of an intravenous catheter 30 min before blood sampling. We have previously demonstrated that NK activity reaches a stable, basal level within 15 min after placement of the intravenous catheter (Irwin et al., 1991; Pike et al., 1997). Blood was drawn into tubes containing heparin.

Immunocellular Assays. As previously described (Irwin and Miller, 2000), immediately after collection, peripheral blood mononuclear cells (PBMC) were isolated by using Ficoll-Hypaque (Pharmacia, Piscataway, NJ), washed twice with phosphate-buffered saline (Gibco Life Technologies, Grand Island, NY), and resuspended at a final concentration of 7×10^5 cells per milliliter in a 1:1 mixture of RPMI 1640 and Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum (Hyclone, Logan, UT; inactivated for 1 hr in a 56°C water bath), 4 mM glutamine, 20 mM HEPES, 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), and 50 μg/ml gentamicin, along with an optimal dose of concanavalin A (10 μg/ml; Sigma). Cultures were incubated for 24, 72, or 96 hr before supernatant collection. Supernatants were taken off carefully under sterile conditions, divided into Eppendorf tubes, and frozen immediately at −70°C until thawed for assay of IL-12 (24-hr samples only), IL-2, IL-6, and IL-10. In one pair of alcoholic and control subjects, cytokine supernatants were not frozen immediately, and these data are omitted from the analyses. Cytokines were quantified by means of enzyme-linked immunosorbent assay methods (R&D Systems, Minneapolis, MN). All samples from controls and alcoholics were assayed at the same time, in a single run, with a single lot number of reagents, and with consumables used by a single operator. Values were log-transformed for statistical analyses.

For NK activity, all assays were begun immediately after separation of

mononuclear cells (Irwin and Miller, 2000). Three effector/target (E/T) cell ratios (40:1, 20:1, and 10:1) were performed to accurately assess the dose-response relationship between target lysis and the number of effector cells present. After a 3-hr incubation at 37°C, plates were again spun at 200 × *g* for 5 min, and killed targets were quantitated by measuring the amount of chromium-51 released into the supernatant, by using a gamma counter. Each assay involved control wells to evaluate spontaneous release and maximal release, and results were expressed as percentage cytotoxicity. For IL-2–stimulated NK activity, PBMCs were isolated and cultured with 50 units/ml of natural human IL-2 (Boehringer, Indianapolis, IN) for 18 to 24 hr at 37°C in a 5% CO_2 incubator (Irwin et al., 1996). NK-resistant Daudi cell targets were used in a standard chromium-release assay because effector cells are not cytotoxic against these cells unless stimulated.

In Vitro Effects of Leptin on NK Activity. For in vitro analysis of leptin and NK activity, blood from age-matched healthy, nonalcoholic volunteer men ($n = 10$; mean age, 45.2 years) was used. Isolated PBMCs were cultured with human leptin (R&D Systems) at concentrations of 0 (media only), 0.1, 1, and 10 nM for 16 hr. NK activity assessment began immediately after separation of mononuclear cells, as described previously.

Leptin. Heparinized blood for leptin assays was centrifuged at 4°C immediately after blood draw, and plasma was stored at −80°C until assay. Leptin was measured via radioimmunoassay kit HL-81 K for human leptin (Linco Res, Inc., St. Charles, MO); the assay has been used and validated in previous studies (Ma et al., 1996; Maffei et al., 1995). The lower limit of detection was 0.5 ng/ml. Coefficients of variation were <10%.

Statistical Analyses

All data were entered into and analyzed with SPSS (SPSS Inc., Chicago, IL). Independent-sample *t* tests were used to test for group differences whenever applicable. For the measures examining group differences in NK activity and IL-2–stimulated NK activity, repeated-measures ANOVAs were undertaken with the E/T ratios as dependent variables and group status as the independent variable. A series of multiple regression analyses were also executed by using immunological variables as dependent variables and leptin as the predictor variable while controlling for body mass index (BMI), cigarette use, and alcohol intake (defined as drinks per month). For NK activity and IL-2–stimulated NK activity, composite scores were used in the regression analyses. The composite scores were generated as follows: values at each E/T ratio were transformed to a *z* score, and *z* scores for each E/T ratio were averaged together, yielding a single composite score for each subject. For stimulated cytokines, raw data were log-transformed, and univariate ANOVAs were run. For correlation analyses, all correlations were Pearson's correlations.

RESULTS

Table 1 summarizes basic information about our sample, including age, depressive symptoms, alcohol consumption, and liver toxicity. The groups did not differ in age, but alcoholics reported fewer years of education than control subjects. The alcoholic group had higher scores on the Hamilton Rating Scale for Depression than the control group; although statistically higher than the control group mean, the mean score of 2.6 does not indicate depression. As expected, the alcoholic group reported greater alcohol consumption over the past 3-month period, with significantly more drinking days per month and more drinks per day. The groups did not differ on the number of days since the last drink, likely because the alcoholic subjects had been abstinent for 2 weeks. The vast majority of alcoholic subjects reported having smoked a pack of cigarettes for greater than 30 days (93%), as compared with healthy control subjects (50%). In terms of liver toxicity, the alcoholic group represented a medically healthy, detoxified group—they did not significantly differ from controls on measures of alanine aminotransferase, aspartate aminotransferase, bilirubin, or alkaline phosphatase. Levels were within normal limits for both groups.

Table 2. Leptin, Height, Weight, and Body Mass Index in Control and Alcoholic Groups

Variable	Controls ($n = 34$)	Alcoholics ($n = 27$)	t	p
Leptin (ng/ml)	6.8 ± 4.0	4.9 ± 3.1	1.95	0.055
BMI	27.0 ± 3.2	24.8 ± 2.6	2.85	**0.006**
Height (inches)	69.8 ± 2.9	70.1 ± 2.7	−0.52	0.6
Weight (lb)	186.9 ± 28.3	173.7 ± 22.4	1.98	0.052
Leptin/BMI	0.2 ± 0.1	0.2 ± 0.1	1.63	0.1

Body mass index (BMI) = (weight × 703)/(height^2).
Boldface refers to significant *p* values.
Values in group columns reflect mean ± SD.

Table 2 shows group differences in body weight, BMI, and plasma concentrations of leptin. Leptin levels in alcoholics and controls were similar to levels previously reported in adults (Buettner et al., 2002; Kiefer et al., 2001; Nicolas et al., 2001). Alcoholics showed lower BMI because they weighed less than controls. Regarding leptin, the groups were nearly significantly different ($p = 0.055$), with alcoholics having lower leptin levels, but this slight difference was abrogated when leptin values were adjusted for BMI. Consistent with previous reports, leptin significantly correlated with BMI ($r = 0.64$; $p < 0.001$) and with body weight ($r = 0.48$; $p < 0.001$) in the total sample ($n = 61$). Leptin was also marginally correlated with daily alcohol intake (number of drinks per day; $r = -0.24$; $p = 0.06$), but not with any of the liver function measures. Because we have previously reported interactions between African American ethnicity and alcohol status for measures of sleep, IL-6, and IL-10 (Irwin and Miller, 2000), further analyses evaluated whether ethnic group differences in leptin were present in our sample. Analyses of variance indicated that leptin levels were similar among European and African Americans for both controls and alcoholics. These findings are consistent with larger epidemiological studies that have not found differences in leptin when comparing European and African Americans (Rice et al., 2002).

Findings for immunological parameters are shown in Table 3. For NK activity, results from repeated-measures analysis demonstrated that alcoholics had significantly lower levels than controls. However, the groups did not differ on IL-2–stimulated NK activity or any of the cytokine measures.

To determine whether leptin was associated with any of the immune measures in the total sample, a series of multiple regression analyses were performed by using NK activity, IL-2–stimulated NK activity, and cytokines as dependent variables and leptin as the predictor variable. Three additional predictors were also included in the model to determine whether leptin was associated with immune measures over and above the effects of BMI, alcohol intake, and cigarette use. For NK activity, a composite score

Table 3. Immunological Parameters in Control and Alcoholic Groups

Variable	Controls		Alcoholics		Statistic	*p*
	n	Mean ± SD	*n*	Mean ± SD		
NK 40:1	32	20.3 ± 11.8	27	12.7 ± 10.5	$F = 8.27$[a]	**0.006**
NK 20:1	32	13.2 ± 8.0	27	7.7 ± 5.9		
NK 10:1	32	7.5 ± 5.6	27	3.8 ± 3.8		
IL-2 NK 40:1	22	31.7 ± 28.3	27	24.3 ± 24.4	$F = 1.09$[a]	0.30
IL-2 NK 20:1	22	22.64 ± 26.1	27	17.52 ± 18.9		
IL-2 NK 10:1	22	15.8 ± 22.5	27	8.81 ± 11.8		
IL-10 (pg/ml)[b]	26	2370.4 ± 1840.3	25	3279.8 ± 2490.4	$t = -1.80$	0.08
IL-12 (pg/ml)[b]	15	7.9 ± 13.9	22	6.7 ± 9.0	$t = 0.59$	0.56
IL-6 (pg/ml)[b]	25	1839.4 ± 352.6	25	1731.0 ± 449.0	$t = 0.44$	0.66
IL-2 (pg/ml)[b]	25	271.1 ± 504.1	26	526.8 ± 607.0	$t = -1.15$	0.26

NK, natural killer cell activity; IL, interleukin; IL-2 NK activity, IL-2–stimulated NK activity; both expressed as percentage cytotoxicity.
Boldface refers to significant *p* values. Values in group columns reflect SD.
[a] *F* statistic reflects repeated-measures ANOVA with three E/T ratios.
[b] Indicates raw values shown, but log-transformed values were used in *t* tests.

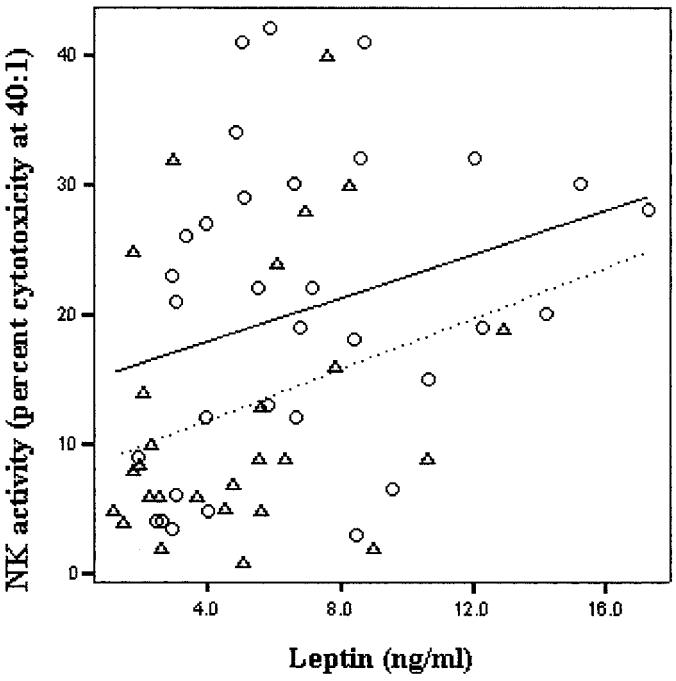

Fig. 1. Scatterplot of leptin and NK activity at a 40:1 effector/target ratio. (○) controls; (△) alcoholics. The solid line represents the regression line for controls; the dotted line represents that for alcoholics. Scatterplots for leptin and NK activity at 20:1 and 10:1 were comparable.

of the three E/T ratios was generated and used as the dependent variable. The overall regression model was significant [$F(4,54) = 3.13$; $p < 0.05$], and leptin was significantly associated with the NK composite score ($\beta = 0.33$; $p < 0.05$). Neither cigarette use nor alcohol intake nor BMI was a significant predictor of the NK composite score. Leptin did not predict IL-2–stimulated NK activity or cytokines. In Fig. 1, a scatterplot of leptin and NK activity at 40:1 illustrates that the relationship between leptin and NK activity was similar for both groups. The slopes of the lines illustrated do not take into account the full regression model that included alcohol intake, BMI, and cigarette use. Inclusion of these covariates seemed to strengthen the relationship between leptin and NK activity.

Because leptin was significantly associated with NK activity in our total sample, a follow-up analysis was conducted in which a series of in vitro studies were undertaken with a separate sample ($n = 10$) of healthy male volunteers (mean age, 45.2 years) to test whether leptin exerts direct effects on NK activity. Either media alone or leptin was preincubated with PBMCs; three concentrations of leptin were used: 0.1, 1.0, and 10.0 nM. After this, PBMCs were washed, and the NK activity assay was undertaken by following the same protocol as used in the in vivo study. NK activity composite scores were generated for each subject. NK composite scores did not significantly differ on the basis of leptin incubation or media alone.

DISCUSSION

Abstinent alcoholics showed diminished NK activity compared with controls. Previous reports have found similar numbers of NK cells in alcoholics and controls (Cook et al., 1997; Irwin and Miller, 2000; Kronfol et al., 1993; Sacanella et al., 1998; Schleifer et al., 1999), suggesting that the decline of NK activity is due to a decrease of cytotoxic activity rather than a change in NK numbers in alcoholics. In addition, in the total sample, leptin was positively associated with NK activity independent of alcohol consumption, smoking, and BMI. This was a novel finding; to date, no study has examined in vivo relationships between leptin and NK activity in humans.

Basic studies provide support for the role of leptin in modulating NK function. The leptin receptor is a member of the class I cytokine receptor family and is expressed on NKs (Zhao et al., 2003). Leptin receptor–deficient mice show lower levels of NK in the liver, spleen, lung, and peripheral blood and lower levels of stimulated NK activation (Tian et al., 2002). In the present study, in vitro analyses did not show an effect for physiologic doses of leptin on NK activity, suggesting that leptin may not influence NK via a direct pathway. Another possibility is that the leptin used in our in vitro study was not biologically active. Although we did not specifically test the bioactivity of our recombinant human leptin, previous studies have used recombinant leptin from the same source and demonstrated

biological activity at similar concentrations across similar time intervals (Lord et al., 1998; Martin-Romero et al., 2000). Together, these studies suggest that the concentrations of leptin used in the current study were biologically active over the incubation interval.

Alternatively, leptin may influence NK activity in the presence of alcohol; however, this pathway has not been adequately studied. As of yet, no studies have reported in vitro effects of leptin on NK activity in the presence of alcohol. Finally, it may be that leptin exerts immunological effects through neuroendocrine mediators. These relationships were not tested because neither adrenocorticotropic hormone nor cortisol was measured in this study. However, leptin inversely correlates with both adrenocorticotropic hormone and cortisol (Prolo et al., 1998), and cortisol has been found to be associated with decreased NK cytotoxicity (Jabaaij et al., 1993).

In active alcoholics, leptin levels have been found to be either increased or decreased as compared with healthy controls (Nicolas et al., 2001; Santolaria et al., 2003). In this study, alcoholic subjects reflected a relatively homogeneous group who had already experienced withdrawal, were abstinent for at least 2 weeks, and were receiving a standardized caloric diet during the 2-week period before the study. Thus, this sample showed a trend for leptin levels to be lower in alcoholics, although this difference was primarily accounted for by decreases in BMI in alcoholics.

There are several limitations to this study. Alcoholics are a heterogeneous group; our sample was a small but relatively homogeneous sample of abstinent alcoholics. However, conclusions about the effects of alcohol intake on leptin cannot be made, because actively drinking alcoholics were not in the sample. Furthermore, the cross-sectional design of the study prevents analysis of within-subject variations in leptin levels over time during active and abstinent periods. Because only men were evaluated in the study, the generalizability of these findings to women is not known. Finally, our groups differed on BMI, a variable that is highly correlated with leptin, although alcoholics had a mean BMI of 24.8, which does not suggest wasting or undernutrition. Future studies may benefit from matching subjects on BMI to determine whether abstinent alcoholics differ on measures of leptin.

Despite these limitations, these data show the association between leptin and NK activity and suggest that abnormal in vivo concentrations of leptin may contribute to declines in NK activity in alcoholics who are at risk for infectious diseases.

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