Disordered Sleep, Nocturnal Cytokines, and Immunity in Alcoholics

LAURA REDWINE, PHD, JEFF DANG, MPH, MARTICA HALL, PHD, AND MICHAEL IRWIN, MD

Objective: Alcoholics who are at risk for infectious disease show profound disturbances of sleep along with decrements of cellular immunity. This study examined the relationships between sleep, nocturnal expression of immunoregulatory cytokines, and natural killer (NK) cell activity in alcoholic patients as compared with control subjects. **Methods:** Alcoholic patients ($N = 24$) and comparison control subjects ($N = 23$) underwent all-night polysomnography and serial blood sampling at 23:00, 03:00, and 06:30 hours. Stimulated expression of T_H1 (interferon gamma, IFN-γ), anti-inflammatory/T_H2 (interleukin 10, IL-10), and proinflammatory cytokines (IL-6) was measured along with NK cell activity across the night. **Results:** Alcoholic patients showed lower levels of IL-6 production, suppression of the IL-6/IL-10 ratio, and a reduction of NK cell activity, coupled with losses of delta sleep and increases of rapid eye movement sleep, as compared with control subjects. In addition, alcoholics showed a persistent low ratio of IFN-γ/IL-10 and reduced levels of NK cell activity, whereas controls had increases of these two immune measures across the night. IL-6 also differentially changed in the two groups; alcoholics showed increases and controls had decreases of IL-6 from 03:00 hours to 06:30 hours. At 06:30 hours, rapid eye movement sleep predicted increases of IL-6 and decreases of NK cell activity independent of the relative contribution of age and chronic alcohol consumption. At 23:00 hours before sleep onset, levels of IL-10 predicted subsequent amounts of delta sleep. **Conclusions:** These data further implicate sleep in the regulation of immune function and suggest that disordered sleep contributes to immune alterations in patients with chronic alcoholism. Moreover, the association between awake levels of the anti-inflammatory/T_H2 cytokine IL-10 and subsequent amounts of delta sleep support the notion of a bidirectional interplay between cytokines and sleep in humans. **Key words:** sleep, immunity, cytokines, natural killer cell activity, alcoholism.

ANOVA = analysis of variance; DSM-IV = *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition; EEG = electroencephalogram; E:T = effector to target (ratio); IFN-γ = interferon gamma; IL = interleukin; NK = natural killer; REM = rapid eye movement; T_H = T helper cell.

INTRODUCTION

Alcoholics show profound alterations of sleep intensity and sleep depth with decreases in total sleep time, frequent awakenings, loss of delta sleep, and increased amounts of rapid eye movement (REM) sleep (1–3). Recent data further implicate sleep disturbance as a predictor of risk for relapse and poor outcome (4, 5). However, despite the salience of disordered sleep in this population, limited effort has been made to evaluate the health consequences of poor sleep in alcoholics. Given the role of sleep in the homeostatic regulation of immune cell functioning (6) and the risk of infectious disease among alcoholics (7–10), the impact of disordered sleep on natural and cellular immune function in alcoholics deserves attention.

Animal studies provide compelling evidence that sleep is closely intertwined with three classes of cytokines: T_H1 (eg, IFN-γ), ant-inflammatory/T_H2 cytokines (eg, IL-10), and proinflammatory cytokines (IL-6) (11). IL-6 is essential in differentiation, maturation, and proliferation of T and B cells (12), whereas T_H1/T_H2 cytokines show an intraservice rivalry in the regulation of cellular and humoral immune response (13, 14). Less is known about the relationships between sleep and cytokines in humans. However, it seems that normal sleep onset is associated with increases of circulating levels of IL-6 (15–17) independent of circadian-dependent mechanisms (18) and that the amount and depth of sleep negatively correlate with daytime levels of this proinflammatory cytokine (19, 20). Sleep also affects T_H1 cytokine expression; nocturnal levels of the T_H1 cytokines, IL-2 and IFN-γ, are reported to increase during sleep with declines in the production of these T_H1 cytokines after sleep loss (16). The effect of disordered sleep or sleep loss on the expression of T_H2 cytokines or the ratio of T_H1 to T_H2 cytokines in humans is not known.

In addition to the effects of sleep on cytokine expression, basic observations suggest a bidirectional interplay between sleep and cytokines. For example, proinflammatory, T_H1, and T_H2 cytokines have all been found to have a role in the regulation of sleep with both somnogenic and inhibitory effects depending on the cytokine, dose, and circadian phase (11, 21).

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Proinflammatory cytokines such as IL-1 and tumor necrosis factor increase delta sleep (22, 23) with similar somnogenic effects reported for the T_H1 cytokine IFN-γ. In contrast, the anti-inflammatory/T_H2 cytokine, IL-10, inhibits slow-wave sleep (24). Translation of these basic studies to the clinical setting has not yet advanced, although one study in humans found that peripheral doses of IL-6 reduce delta sleep in the first half of the night when circulating levels of IL-6 are elevated (25).

The objective of this study was to evaluate the relationships between sleep and nocturnal cytokine expression in alcoholic patients as compared with control subjects. Expression of IFN-γ, IL-10, and IL-6 was measured in view of the hypothesized bidirectional relationship between these cytokines and sleep (11). In addition, the relative balance of IFN-γ/IL-10 and of IL-6/IL-10 was determined because decreases in the ratio of these cytokines are associated with increased infectious disease risk in alcoholics, partly through alteration of cellular immune responses and natural killer (NK) cell activity (14, 26, 27). Blood sampling with assessment of immune functioning was performed repeatedly during the nocturnal period before sleep onset and during sleep to evaluate whether alterations of cytokines predict abnormalities of sleep architecture and whether disordered sleep is associated with changes in late night or early morning immune measures in alcoholics.

METHODS

Subjects

African American alcoholics show more severe disturbances of sleep and immune alterations than European American alcoholics (3, 28). Thus, the present study focused on African Americans in its comparison of control subjects ($N = 23$) and alcoholic patients ($N = 24$) on nocturnal measures of NK cell activity and cytokine production; EEG results showing abnormal sleep for a subsample of this population ($N = 31$) were previously reported (3).

Alcoholic patients were clinically hospitalized for 2 weeks in the Alcohol and Drug Treatment Program at the Veterans Affairs San Diego Healthcare System before testing in this study. Nonpatient control subjects were identified though a standardized search strategy of the San Diego area involving the placement of flyers and advertisements in local newspapers. In addition, a direct mailing to the San Diego County veterans population was used to target control subjects who were age-matched (±5 years) and had sociodemographic characteristics similar to alcoholic patients.

Administration of a semistructured interview developed for the multisite Collaborative Study on the Genetics of Alcoholism (29) yielded research diagnoses of control and alcoholic patients. This interview assessed consumption and associated problem histories for cigarettes, alcohol, and other substances and assessed diagnostic criterion data to evaluate the presence of a lifetime history of a psychiatric and substance dependence disorder. Alcoholic patients fulfilled DSM-IV criteria for alcohol dependence that had occurred in the absence of major preexisting psychiatric disorders (primary alcohol dependence) (30, 31). Control subjects fulfilled DSM-IV criteria for "never mentally ill" (30).

All subjects were free of physical illness by examination; none had a history of recent (<2 weeks) viral infection or disease (eg, autoimmunity or cancer) that would influence immune function. Alcoholics were excluded if they had a history of overt alcohol-related liver disease such as jaundice and esophageal varices. Subjects were negative for hepatitis B and C. Before entry into the study, a medication history was obtained; subjects who were taking medications known to alter the immune system were excluded.

Withdrawal from alcohol can affect immune function (32). Thus, the present sample of alcoholics was studied after all acute and subacute withdrawal symptoms except sleep disturbance had resolved. Detoxification and withdrawal routinely occurred before admission to the Alcoholism and Drug Treatment Program. Five alcoholic patients reported use of diazepam for withdrawal symptoms in the 30 days before admission, but none had used diazepam in the last 2 weeks. All other alcoholic patients denied use of such medications before admission. During the 2-week interval between admission to the treatment program and immune testing, alcoholic subjects participated in an inpatient sober treatment milieu that involved Alcoholics Anonymous, education, and group and individual counseling. All alcoholics maintained abstinence, as confirmed by nursing observations and random urine toxin screens. The average number of days since the last drink is shown in Table 1.

TABLE 1. Age, Education, Severity of Depressive Symptoms, and Alcohol Consumption History in Control and Alcoholic Groups

	African American Control Subjects ($N = 23$)		African American Alcoholic Subjects ($N = 24$)		Group Effect	
	Mean	SD	Mean	SD	*F*	*p*
Age, y	41.3	8.4	42.6	6.8	0.38	.54
Education (y)	14.3	1.5	13.2	1.3	6.90	.01*
Depressive symptoms[a]	0.8	2.5	0.9	2.8	0.03	.87
Alcohol consumption (last 3 months)						
Drinking days/month	6.3	9.8	26.3	5.8	73.06	.001*
Drinks/day	1.4	1.0	14.4	11.8	27.81	.001*
Days since last drink (365 days maximum)	96.0	149.4	23.1	11.7	5.68	.02*

[a] Scores on Hamilton Rating Scale for Depression.
* Significant results.

Procedures

On the day immediately before nocturnal assessment of sleep and immunity, severity of depressive symptoms was evaluated using the 17-item Hamilton Rating Scale for Depression (33, 34). This evaluation was performed by a trained interviewer; test-retest reliability on total scores was 0.94.

The sleep protocol involved five nights. On the first night, participants adapted to the conditions of the laboratory and underwent recordings of oxygen desaturation and tibial myoclonus to exclude subjects with either sleep apnea or nocturnal myoclonus. On the second night, sleep was monitored by EEG as previously reported (3). On the third night, sleep was again monitored by EEG; in addition, nocturnal blood sampling was performed before sleep and throughout the night (described below). On the fourth and fifth nights, subjects underwent partial night sleep deprivation and recovery sleep; these data are to be reported separately. Thus, the data in this report represent sleep on night 3 of the studies.

For each experimental night, subjects arrived at the laboratory between 20:00 and 21:00 hours. On night 3 between 20:30 and 21:30 hours, an intravenous catheter was inserted into a forearm vein. Subjects then prepared for sleep and had electrodes placed for EEG, electro-oculography, and submental electromyography; sleep EEG measures were obtained during continuous recordings between 22:00 and 06:30 hours. Subjects then assumed a supine horizontal position and maintained this position for at least 30 minutes before the first blood sample was taken. Lights were turned off at 23:00 hours, and subjects were on average asleep by 23:30 hours. After a night of uninterrupted sleep, subjects were awakened after the last blood draw at 06:30 hours if they were not already awake. All subjects included in the present analyses remained supine throughout the entire nocturnal period; a bedside urinal was used if subjects needed to urinate during the night.

For blood sampling, the intravenous catheter was connected to a long, thin plastic tube, which enabled blood collection from an adjacent room without disturbance of sleep. Between blood samplings, continuous heparinized isotonic saline was infused, totaling about 1000 ml across the nocturnal period. Blood was sampled at three time points (23:00, 03:00, and 06:30 hours) for assay of stimulated cytokine production and NK cell activity. Immediately after blood was obtained, the sample was put into tubes containing ethylenediaminetetraacetic acid and kept at room temperature until assay.

Sleep records were visually scored according to the criteria of Rechtschaffen and Kales (35). Sleep onset was defined as the first minute of stage 2 or REM sleep followed by at least 8 minutes of sleep in the next 9 minutes. A REM period was defined by not less than 3 consecutive minutes of REM sleep. Sleep architecture was defined as the duration and relative percentage of time spent asleep in non-REM, stage 1, stage 2, and delta sleep, as well as the duration of REM sleep. Sleep research technicians show high scoring reliability: sleep latency ($r = 0.96$), REM latency ($r = 0.99$), REM density ($r = 0.91$), amounts of stage 3 and 4 sleep ($r = 0.85$), and total sleep time ($r = 0.99$).

Assays

Liver function tests. Values of liver function tests were obtained and measured by previously described methods (34).

Peripheral blood mononuclear cell preparation. Samples were maintained at room temperature, and peripheral blood mononuclear cells were sedimented on Ficoll-Hypaque (Pharmacia, Piscataway, NJ), washed twice with phosphate-buffered saline (Gibco Life Technologies, Grand Island, NY), and resuspended in a 1:1 mixture of RPMI 1640 and Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT; inactivated 1 hour in 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.

Stimulated cytokine production. As previously described (28), peripheral blood mononuclear cells were resuspended at a final concentration 7×10^5 cells/ml in the above-described media along with an optimal dose of concanavalin-A (Con-A, 10 μg/ml; Sigma). Cultures were incubated for 72 hours before supernatant harvest. Supernatants were taken off carefully under sterile conditions, divided into Eppendorf tubes, and frozen immediately at −70°C until thawed for assay of IL-6, IFN-γ, and IL-10. Cytokines were quantified by means of enzyme-linked immunosorbent assay methods (R & D Systems, Minneapolis, MN). All samples from control and alcoholic subjects were assayed at the same time, in a single run with a single lot number of reagents and consumables used by a single operator. The intraassay coefficient of variation for all variables was <5%.

NK cell activity. As previously described (28), assays of NK cell activity were started immediately after separation of mononuclear cells. To remove adherent cells, mononuclear cells were incubated on plastic Petri dishes, and nonadherent, effector cells were then obtained and resuspended to a concentration of 2×10^6 cells/ml.

The target cells used in the assay of NK cell activity were K562, an erythroleukemia cell line; cells were in the log phase of growth on the day of assay for NK cell activity. After isolation of nonadherent cells, a constant number (5×10^3) of chromium 51 (New England Nuclear, Boston, MA) K562 target cells were mixed with graded numbers of effector cells in triplicate. Three effector:target (E:T) cell ratios (40:1, 20:1, and 10:1) were used. Killed targets were quantitated by measuring the amount of chromium 51 released into the supernatant using a gamma counter. Each assay involved control wells to evaluate spontaneous release and maximal release. Results of the NK cell assay for each E:T ratio were expressed as the percentage of specific lysis, rather than in lytic units, consistent with prior studies of NK cell activity in alcoholics (34, 36–39). Daily variability in the assessment of NK cell activity was minimized by a number of quality-control procedures described previously (28).

Statistical Analyses

To test for differences between the two groups for the dependent variables, including age, alcohol consumption, depressive symptoms, liver function tests, and sleep EEG measures, one-way ANOVAs were performed. To evaluate change of stimulated cytokine production across the nocturnal period in the two groups, 2×3 (group: alcoholic, control; time: 23:00, 03:00, 06:30 hours) repeated-measures ANOVAs were used, covarying for age. To correct for the skewed distribution of IFN-γ, values were $\log_{10}$-transformed before analysis. For NK cell activity, a $2 \times 3 \times 3$ (group by time by E:T cell ratios) repeated-measures ANOVA was used. In the analyses where significant time effects were found, post hoc tests were calculated on the basis of estimated marginal means in which age was a covariate. Differences between time points within each group were tested in pairwise comparisons following the overall mixed-design ANOVA adjusting for multiple comparisons. Bonferroni-adjusted multiple comparisons with differences of $p < .05$ are reported as significant. Before the onset of this study, effect sizes were estimated using data generated in the comparison of stimulated cytokine production and NK cell activity in alcoholic vs. control subjects (28). In the instance of NK cell activity, a sample size of 10 subjects per group yields 0.85 power to detect a significant ($p < .01$) group by E:T ratio interaction. Of the total sample ($N = 47$), repeated assessment of NK cell activity was available on 19 control and 22 alcoholic subjects, whereas

repeated assessment of all three cytokines was available for 10 control and 14 alcoholic subjects.

To evaluate the central hypothesis that the severity of disordered sleep is associated with alterations of cytokine production and NK cell activity, a series of correlational analyses were conducted. Zero-order correlations were performed between EEG sleep variables and immune measures taken at the three time points. To reduce the likelihood of Type II error, the correlational analyses were restricted to the EEG sleep variables that differed between alcoholics and controls. Because of the large number of EEG variables, only a restricted number of correlations were performed using selected sleep variables that differed between the groups (eg, percentage and amounts of REM and delta sleep). Given evidence that the amounts of sleep loss and REM sleep are associated with altered levels of IL-6 and NK cell activity (18, 40, 41), a priori hypotheses were generated to guide the correlational analyses that examined the consequences of disordered sleep on immune parameters measured at 06:30 hours. REM sleep was hypothesized to correlate positively with 06:30 hours levels of IL-6 and to be associated with lower levels of NK cell activity at 6:30 hours. Correlations between REM and delta sleep and the two other individual cytokines (IFN-γ, IL-10) were also tested. Correlations were first performed in the total sample and then in the separate groups of alcoholic and control subjects. For correlations that were statistically significant, regression models were used to test the relationship between disordered sleep and immunity after controlling for the relative contribution of alcohol consumption and age. In regression models that tested the effects of sleep measures on morning level of cytokines or NK cell activity (E:T ratio: 40:1 or 20:1), baseline (23:00 hours) levels of immune function was also controlled.

RESULTS

Table 1 summarizes the demographic characteristics, severity of depressive symptoms, and alcohol consumption histories in the control and alcoholic groups. Although the alcoholic and control groups were similar in age, the control subjects reported more years of education than the alcoholic subjects. Severity of depressive symptoms was not significantly different between the two groups. Alcohol consumption during the last 3 months showed expected between-group differences, with alcoholic subjects reporting more drinking days per month, more drinks per drinking day, and more recent use than control subjects. However, on average the alcoholics had not used alcohol for more than 3 weeks before testing. None of the alcoholics fulfilled criteria for primary substance dependence, although five alcoholics reported marijuana use and nine alcoholics reported psychostimulant use in the last 3 months. No subject had used these substances in the last 2 weeks. All control subjects denied use of marijuana, stimulants, or other substances in the last year.

In this sample of medically healthy, detoxified alcoholics, values of alanine aminotransferase (serum glutamic-oxaloacetic transaminase), bilirubin, and alkaline phosphatase were statistically indistinguishable between the two groups. Although serum glutamic-pyruvic transaminase was higher in the alcoholic than in the control subjects, values were within the clinical laboratory normal range (data not shown).

Differences in EEG sleep measures between the two groups are shown in Table 2. Compared with controls, alcoholics showed prolonged sleep latency along with loss of stage 3 and delta sleep amounts. In addition, alcoholics showed profound increases in REM sleep amounts and reduced REM latency as compared with controls. There were no other significant effects.

The T_H1 (IFN-γ), anti-inflammatory/T_H2 (IL-10), and proinflammatory (IL-6) cytokines were analyzed as individual cytokines and as the ratio of IFN-γ/IL-10 and IL-6/IL-10, controlling for age. The ratio of IFN-γ/IL-10 reflects the relative balance of T_H1/T_H2 cytokines with effects on the regulation of cellular vs. humoral immune responses. The ratio of IL-6/IL-10 is reportedly lower in individuals with chronic alcoholism, in which a suppressed ratio predicts increased rates of postoperative infection after surgery (42).

Analysis of the nocturnal expression of the T_H1 cytokine IFN-γ revealed no significant effects. The anti-inflammatory/T_H2 cytokine, IL-10, showed a significant time effect but no other effects (Fig. 1). The time effect was due to significant increases of IL-10 from 23:00 to 03:00 hours in both groups; levels at 03:00 and 06:30 hours were similar.

For the ratio of IFN/IL-10, the two groups showed a differential change across the nocturnal period with a significant group by time interaction (Fig. 2). In control subjects, the ratio of T_H1/T_H2 cytokines was unchanged from 23:00 to 03:00 hours but increased from 03:00 to 06:30 hours. In contrast, within the alcoholic group the IFN/IL-10 ratio decreased from 23:00 to 03:00 and remained low from 03:00 to 06:30 hours.

The production of the proinflammatory cytokine IL-6 also changed differentially across the night in the two groups (Fig. 3). For IL-6 production, there was a significant group effect, a significant time effect, and a significant group by time interaction. Within the control group, IL-6 levels were similar between 23:00 and 03:00 hours and decreased from 03:00 to 06:30 hours. In contrast, within the alcoholic group, expression of IL-6 was similar between 23:00 and 03:00 hours and increased from 03:00 to 06:30 hours.

Evaluation of the ratio of IL-6/IL-10 showed a group effect in which alcoholics had a suppressed ratio as compared with controls, consistent with previous findings (42). There were no other significant effects.

For NK cell activity, alcoholic subjects had lower levels of cytotoxicity as compared with control subjects. In addition, there was a time effect; NK cell activity showed an increase from 23:00 to 06:30 hours

TABLE 2. Sleep Continuity, Sleep Architecture, and REM Sleep Measures in Control and Alcoholic Groups

	African American Control Subjects at Baseline (N = 23)		African American Alcoholic Subjects at Baseline (N = 24)		Group Effect	
	Mean	SD	Mean	SD	F	p
Sleep continuity						
Total sleep time (min)	345.1	65.9	347.7	46.0	0.03	0.88
Sleep efficiency (%)	80.8	11.8	80.8	9.2	0.001	0.98
Sleep latency (min)	10.9	10.2	22.0	26.1	3.62	0.06
Sleep architecture						
Stage 1						
Minutes	26.8	21.8	31.4	12.8	0.76	0.39
%	7.9	5.9	9.1	3.6	0.74	0.39
Stage 2						
Minutes	236.2	52.0	223.8	33.9	0.92	0.34
%	68.7	8.5	64.7	7.6	2.78	0.10
Stage 3						
Minutes	17.9	18.2	8.2	9.5	5.31	0.03*
%	5.4	6.0	2.3	2.5	5.70	0.02*
Stage 4						
Minutes	4.3	7.7	1.8	8.6	0.99	0.33
%	1.2	2.2	0.5	2.3	1.25	0.27
Delta						
Minutes	22.2	24.9	10.0	15.2	4.05	0.05*
%	6.7	7.8	2.7	4.0	4.67	0.04*
REM						
Minutes	59.8	26.6	82.6	26.4	8.25	0.006*
%	17.6	6.0	23.5	6.0	10.69	0.002*
REM measures						
Latency (corrected)	76.8	33.6	51.5	30.3	7.05	0.01*
Density	1.3	0.9	1.6	0.9	1.59	0.22
Duration (first period)	14.9	8.5	18.1	9.4	1.44	0.24

* Significant results.

in the control group but not in the alcoholic group (Fig. 4). However, there was no group by time interaction.

IFN-γ is a potent inducer of NK cell activity, and increases of IFN-γ and/or the ratio of IFN-γ/IL-10 are thought to lead to increases of NK cell activity. The relationships between IFN-γ, IFN-γ/IL-10 ratio, and NK cell activity were tested in the present sample; the pattern of change for NK cell activity was similar to that found for the ratio of IFN-γ/IL-10. Levels of IFN-γ at 06:30 hours were positively correlated with NK cell activity at 06:30 hours ($\rho = 0.54$, $p < .001$, $N = 24$), whereas levels of IFN-γ at other time points (eg, 23:00 and 03:00 hours) were not associated with NK cell activity. No correlation was found between IL-6 and NK cell activity or between IL-10 and NK cell activity at individual time points.

Nocturnal levels of cytokines and NK cell activity in the alcoholics vs. controls could be due to group differences in severity of sleep disturbance, effects of alcohol consumption, or a combination of these two factors. To evaluate whether abnormalities of sleep architecture predicted measures of nocturnal immunity independent of the effects of chronic alcohol consumption, a series of additional analyses were conducted. In the first set of analyses, we examined the consequences of disordered sleep by focusing on immune measures obtained during sleep (03:00 hours) and at the end of nocturnal sleep (06:30 hours).

In the total sample, the amount and percentage of REM sleep were positively correlated with expression of IL-6 at 06:30 hours (Spearman $\rho = 0.45$, $p < .05$; Spearman $\rho = 0.36$, $p = .08$; $N = 24$) (Fig. 5). The level of correlation between amount and percentage of REM sleep and IL-6 production at 06:30 hours was similar in the alcoholics and controls. No correlation between REM measures and IL-6 levels at 23:00 hours or 03:00 hours was found, and amounts of delta sleep were not associated with IL-6 in this sample.

To further test the effects of REM sleep amounts on morning levels of IL-6 separate from the relative contribution of age, alcohol consumption histories, and baseline (23:00 hours) levels of IL-6, regression analyses in which each predictor variable was fit sequentially into the model were used. Together the model

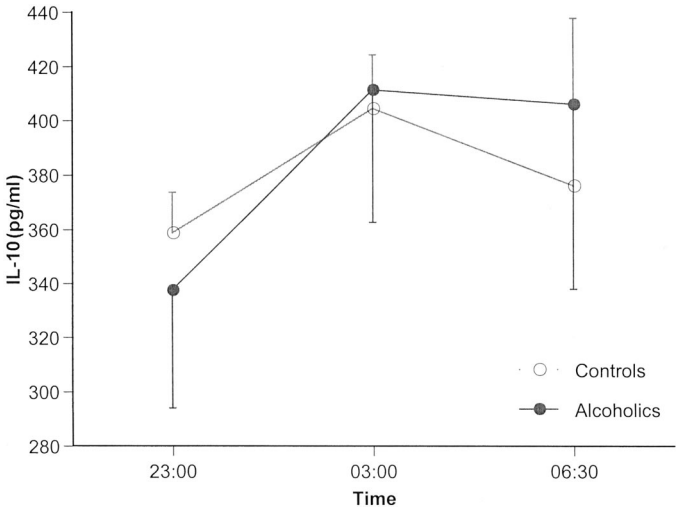

Fig. 1. Concanavalin-A–stimulated production of IL-10 by peripheral blood mononuclear cells in control (○) and alcoholic (●) African American subjects. Data are presented as mean ± SEM. Blood was drawn at 23:00, 03:00, and 06:30 hours. There was a time effect ($F(1,21) = 3.2$, $p = .05$) but no group effect or group by time interaction. Post hoc comparisons revealed significant ($p < .05$) increases of IL-10 from 23:00 to 03:00 hours in both groups; levels at 03:00 and 06:30 hours were similar.

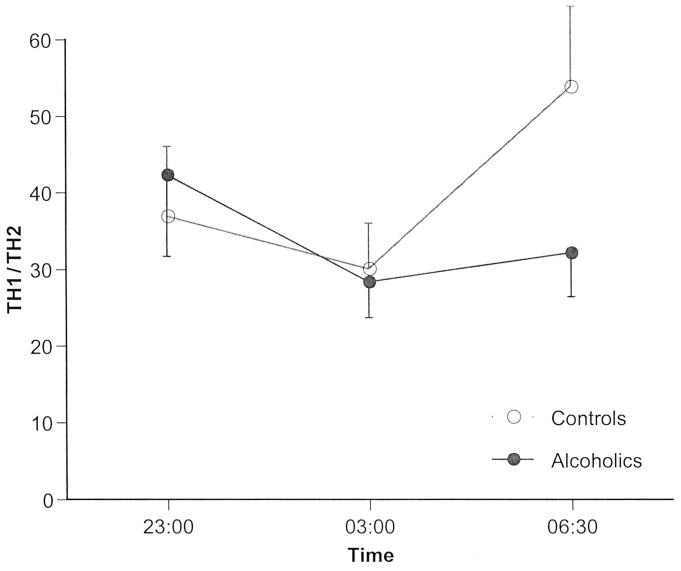

Fig. 2. Concanavalin-A–stimulated production of T_H1 and T_H2 cytokines by peripheral blood mononuclear cells in control (○) and alcoholic (●) African American subjects. The ratio of the T_H1 to T_H2 was determined by dividing the T_H1 cytokine IFN-γ by the T_H2 cytokine IL-10. A significant group by time interaction was found ($F(2,42) = 4.1$, $p < .02$). The T_H1/T_H2 ratio increased from 03:00 to 06:30 hours ($p < .01$) in the controls, whereas the IFN-γ/IL-10 ratio decreased from 23:00 to 03:00 hours ($p < .05$) and remained low from 03:00 to 06:30 hours in the alcoholics.

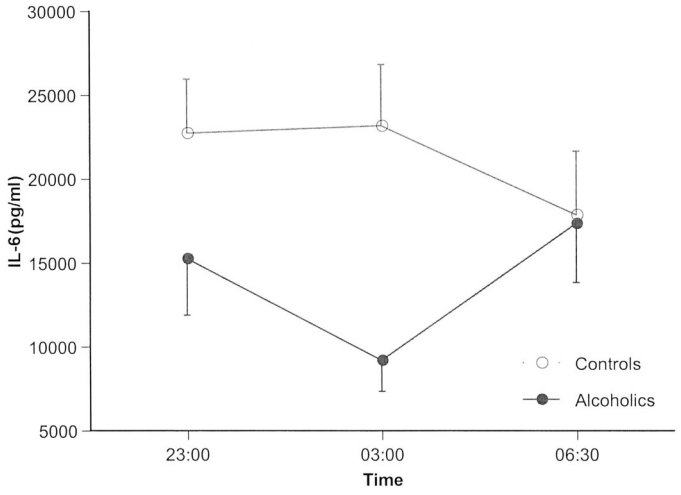

Fig. 3. Concanavalin-A–stimulated production of IL-6 by peripheral blood mononuclear cells in control (○) and alcoholic (●) African American subjects. Data are presented as mean ± SEM. Blood was drawn at 23:00, 03:00, and 06:30 hours. There was a significant group effect ($F(1,21) = 6.9$, $p < .01$), a significant time effect ($F(2,42) = 5.0$, $p < .01$), and a significant group by time interaction ($F(2,42) = 5.7$, $p < .01$). IL-6 levels decreased from 03:00 to 06:30 hours ($p < .05$) in the controls, whereas IL-6 increased from 03:00 to 06:30 hours ($p < .01$) in alcoholics.

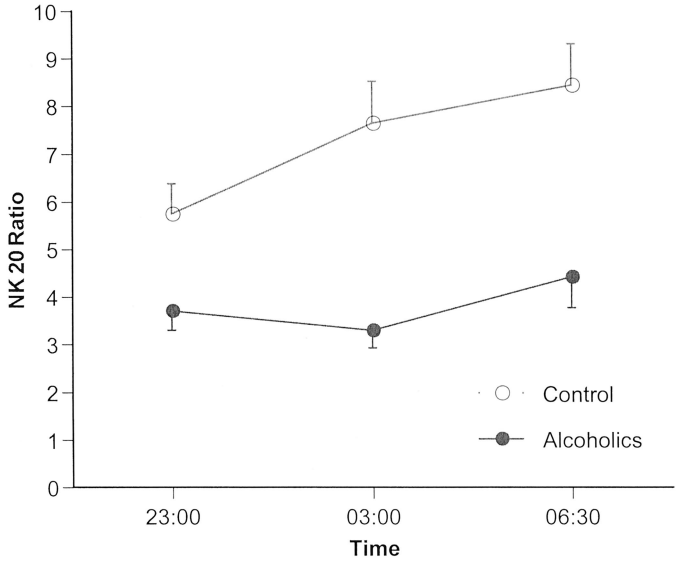

Fig. 4. NK cell activity in African American controls (○) and alcoholics (●). Data are presented as mean ± SEM percentage of specific cytotoxicity at the 20:1 E:T ratio. For NK cell activity, there was a group effect ($F(1,39) = 5.6$, $p < .02$) and a time effect ($F(2,78) = 3.0$, $p < .05$), but no group by time interaction. In the controls, there was a significant increase of NK cell activity from 23:00 to 06:30 hours ($p < .05$); the alcoholics showed no change across the nocturnal period.

predicted 23% of the variance of IL-6 production at 06:30 hours; IL-6 levels were significantly predicted by REM sleep amounts ($\beta = 0.43$, $p = .05$) but not by age

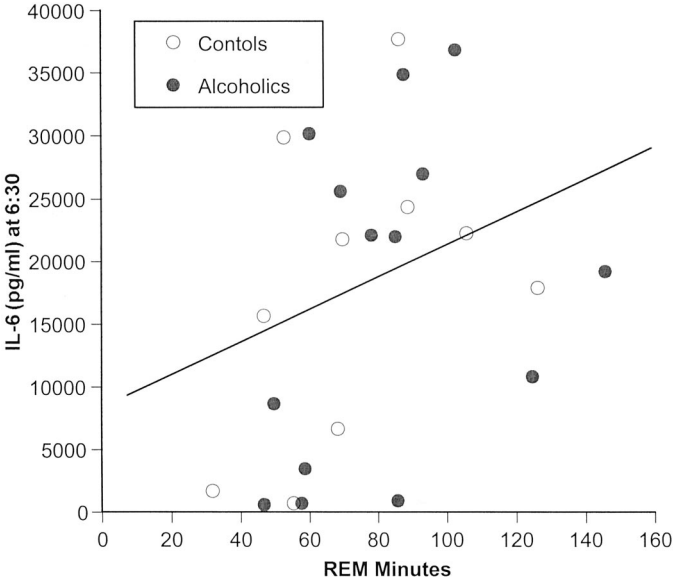

Fig. 5. Relation between REM sleep (minutes) and concanavalin-A–stimulated production of IL-6 by peripheral blood mononuclear cells at 6:30 hours in control (○) and alcoholic (●) African American subjects. In the total sample, amount of REM sleep was positively correlated with expression of IL-6 at 06:30 hours (Spearman $\rho = 0.45$, $p < .05$).

($\beta = -0.19$, $p = .5$), alcohol consumption (average drinks per day; $\beta = 0.11$, $p = .67$), or IL-6 at 23:00 hours ($\beta = 0.24$, $p = .31$).

A similar series of analyses was performed to test the hypothesized relationships between disordered sleep and morning levels of NK cell activity. Consistent with our predictions, the amount and percentage of REM sleep were negatively correlated with NK cell activity at 06:30 hours (Spearman $\rho = -0.44$, $p < .01$; Spearman $\rho = -0.49$, $p < .01$; $N = 41$) in the total sample. An identical level of correlation was found in the alcoholics and controls. A regression model was again used to evaluate the relative contribution of REM sleep percentage on NK cell activity, controlling for age, alcohol consumption, and baseline (23:00 hours) levels of NK cell activity. The overall model predicted 76% of the variance in NK cell activity at 6:30 hours; morning levels of NK cell activity were negatively predicted by percentage of REM sleep ($\beta = -0.19$, $p < .05$) and were positively associated with baseline levels (23:00 hours) of NK cell activity ($\beta = 0.76$, $p < .01$). Neither age ($\beta = -0.01$, $p = .46$) nor alcohol consumption (average drinks per day; $\beta = -0.12$, $p = .47$) contributed to morning levels of NK cell activity. There was no correlation between REM sleep and NK cell activity at 03:00 hours or between delta sleep and NK cell activity at 03:00 hours or 06:30 hours. Finally, there were no correlations between REM or delta sleep and measures of IFN-γ or IL-10 obtained at 03:00 hours or 06:30 hours.

In addition to evaluating the consequences of disordered sleep on late-night and morning levels of immunity, the repeated-measures experimental design also included measurement of cytokines before the onset of sleep. Thus, further analyses were conducted to examine whether levels of IFN-γ, IL-10, or IL-6 at 23:00 hours predicted subsequent differences in amounts of delta or REM sleep in the two groups. Again a conservative approach was used with the selection of only REM and delta sleep in the correlational analyses. The results showed that expression of IL-10 at 23:00 hours was positively correlated with delta sleep amounts and percentage (Spearman $\rho = 0.46$, $p < .05$; Spearman $\rho = 0.46$, $p < .05$; $N = 24$) in the total sample (Fig. 6). Similar correlations between IL-10 and delta sleep amounts were found in alcoholics and controls. The effect of IL-10 at 23:00 hours on delta sleep was independent of age and alcohol consumption. In a regression model that included IL-10 at 23:00 hours, age, and alcohol consumption, 33% of the variance in delta sleep was predicted. Only IL-10 reached statistical significance ($\beta = 0.47$, $p < .01$), and neither age ($\beta = -0.35$, $p < .12$) nor alcohol consumption ($\beta = 0.10$, $p = .64$) was associated with delta sleep amounts. Indeed, IL-10 levels before sleep onset predicted 23% of the variance in delta sleep. It is also important to note that IL-10 expression increased from 23:00 to 03:00 hours; delta sleep predominantly occurs during the first half of the night. Levels of IL-10 at 23:00 hours

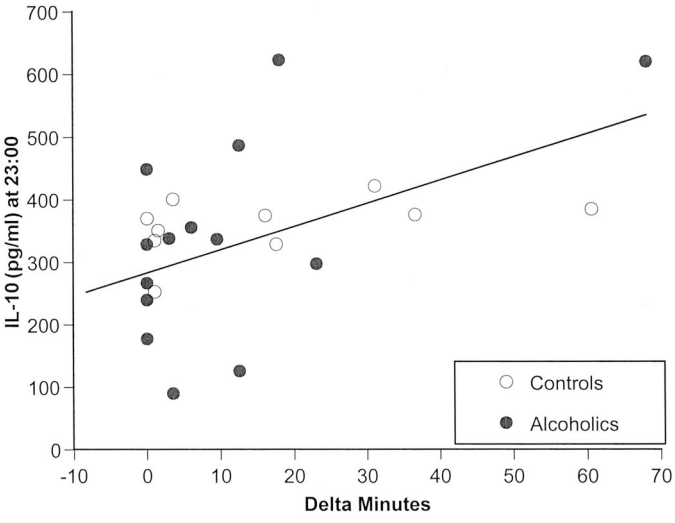

Fig. 6. Relation between delta sleep (minutes) and concanavalin-A–stimulated production of IL-10 by peripheral blood mononuclear cells at 23:00 hours in control (○) and alcoholic (●) African American subjects. In the total sample, expression of IL-10 at 23:00 hours was positively correlated with delta sleep amounts (Spearman $\rho = 0.46$, $p < .05$).

were not correlated with REM sleep, and there were no correlations between IFN-γ or IL-6 at 23:00 hours and REM or delta sleep measures.

DISCUSSION

Alcoholics show disordered sleep with losses of delta sleep and increases of REM sleep (1, 3, 43). Coupled with these abnormalities of sleep architecture, the nocturnal production of cytokines and activity of NK cells is altered in alcoholic subjects as compared with control subjects. First, the nocturnal expression of IFN-γ/IL-10 changes differentially in alcoholics as compared with controls. In alcoholics, there is a shift toward a T_H2 cytokine response after the onset of sleep, and this predominant T_H2 response persists throughout the night. In contrast, controls show an increase in the relative expression of T_H1 cytokines during the later half of the nocturnal period. Second, IL-6 production is lower in alcoholics as compared with controls during the early part of the night, but it then increases in alcoholics and decreases in controls during the second half of the night. Third, the ratio of IL-6/IL-10 is suppressed throughout the nocturnal period in alcoholics. Finally, alcoholics show low levels of NK cell activity across the night as compared with controls.

Amount of REM sleep is a unique correlate of morning levels of IL-6 production independent of the effects of chronic alcohol consumption and age. Given further evidence that IL-6 is elevated during REM as compared with delta sleep (18), it is possible that greater amounts of REM sleep during the late part of the night contribute to the rise of IL-6 in alcoholics. Daytime elevations of IL-6 are also reported to occur in association with sleep loss (19, 44) and to correlate with fatigue (20). Thus, morning increases of IL-6 may have implications for daytime fatigue in alcoholics, although it is important to note that levels of IL-6 at awakening did not differ between the groups. Evaluation of the contribution of cytokine mechanisms to daytime fatigue will require sampling of IL-6 during the day and simultaneous behavioral assessments.

The lower production of IL-6 in the alcoholics during the early part of the night is consistent with the notion that sleep has a role in the regulation of IL-6 expression. Alcoholics show prolonged sleep latency, and loss of sleep likely impairs the nocturnal expression of IL-6. Sleep deprivation delays the nocturnal increases in circulating levels of IL-6, independent of circadian-dependent processes (18).

Sleep is hypothesized to have a restorative function on the immune system. In the present study, controls showed increases in the relative expression of the T_H1 cytokine IFN-γ that was correlated with the activity of NK cells. In contrast, alcoholics with disordered sleep have low T_H1 cytokine and NK cell responses across the nocturnal period. In prior studies, sleep deprivation induced decreases of NK cell activity and production of the T cell cytokine IL-2 (16, 40, 45, 46). Furthermore, amounts of REM sleep negatively predicted daytime levels of NK cytotoxicity in a sample of depressed patients (47), similar to the present findings.

Another major finding of the present study concerns the possible action of cytokines on sleep (48). In the present study, the anti-inflammatory/T_H2 cytokine IL-10 was positively correlated with amounts of delta sleep, and expression of IL-10 increased across the first part of night, during which time delta sleep is predominantly found. In comparison, the proinflammatory cytokine IL-6 inhibits delta sleep in humans (25). However, the present data contrast with basic findings; acute doses of IL-10 inhibit slow wave sleep in rats and rabbits (24, 49), whereas proinflammatory cytokines generally enhance delta sleep in rodents (11). Although further experimental studies are needed to determine whether anti-inflammatory cytokines augment delta sleep and proinflammatory cytokines inhibit delta sleep in humans, these data suggest that cytokines have a role in the regulation of normal sleep and that abnormalities of cytokines levels may contribute to disordered sleep in clinical populations such as alcoholics.

The clinical significance of the immune alterations found in the alcoholics is not known. However, decreases of NK cell activity and/or abnormal cytokine expression in alcoholics are thought to contribute to increased susceptibility to infectious diseases in alcoholics (7), including an increase in the incidence and severity of tuberculosis (10), hepatitis C (8), and possibly HIV infection (50). The present relationships between disordered sleep and altered cellular and natural immunity in alcoholics suggest that behavioral interventions that target sleep may have potential effects on the recovery of immune functioning in abstinent alcoholics.

There are several limitations to the present study. First, an African American population was exclusively studied because substantial evidence suggests that African American alcoholics, as compared with European Americans, have increased morbidity, including infectious disease risk. Thus, it is not known whether differences of sleep and immunity generalize to European Americans. Second, the generalizability of the findings to active alcoholics in the community is limited because the present sample was studied after withdrawal from alcohol and during maintenance of abstinence in a controlled clinical setting. Neverthe-

less, it is important to note that by the use of this experimental design, the differences in sleep and immunity cannot be ascribed to a direct action of ethanol or to withdrawal effects. Third, the potential effects of other substance use on sleep and immune measures in this alcoholic population requires consideration. Although none of the alcoholics met criteria for primary substance dependence, more than half of this group reported a lifetime history of psychostimulant use. Nevertheless, none of the subjects used psychostimulants or other substances in the 2 weeks before study, and stratification of the sample by substance use history did not reveal differences of sleep or immune measures. Fourth, control subjects were more educated and more likely to be employed than alcoholic subjects, with effects of immunity through diet or other health behaviors. Alcoholics were hospitalized for 2 weeks before testing with receipt of nutritionally balanced meals. Fifth, the alcoholic group was limited to male veterans; conclusions about differences in alcohol-dependent women or community groups cannot be made. Finally, placement of an intravenous catheter has been found to produce elevations in IL-6 (51); there may be a differential response to a catheterization and serial blood sampling.

Considerable evidence has shown that various measures of immune function, including IL-6 expression and NK cell activity, follow a circadian rhythm (16, 17, 52), and the contribution of altered circadian rhythms to these immune changes cannot be determined from these data because assessments of immunity were limited to the nocturnal period. However, abstinent alcoholics seem to show only a modest circadian phase shift, and immune differences were identified over an interval of 4 hours. Moreover, sleep, but not circadian, processes drive the nocturnal release of IL-6 (18).

The neurobiological mechanisms that account for the associations between disordered sleep and alterations of nocturnal measures of immunity are not known. However, disordered sleep and loss of sleep are associated with nocturnal elevations of sympathetic outflow with increases of norepinephrine and epinephrine (53–55). Although no data are available regarding nocturnal sympathetic activation in alcoholics, the influence of sympathetic mechanisms on T_H1/T_H2 cytokine expression and NK cell activity are well known. Animal and human studies have shown that stress and the release of sympathetic neurotransmitters shifts the expression of T_H cytokines toward a T_H2 pattern (56) and reduces NK cell activity (57), immune changes similar to those found in our alcoholic population. It is also possible that alterations of the hypothalamic-pituitary-adrenal axis underlie immune differences in alcoholics, although alcoholics who are abstinent for at least 3 weeks show blunted cortisol responses to stress (58).

In conclusion, disordered sleep in alcoholics has consequences for cytokine expression and natural cytotoxicity obtained during the morning. During the late night interval, alcoholics show increases of IL-6 production and have persistently low levels of IFN-γ/IL-10 ratio, IL-6/IL-10 ratio, and NK cell activity as compared with controls. Increases of REM sleep are found in alcoholics, and REM sleep predicts morning increases of IL-6 and decreases of NK cell activity. These data have implications for understanding the role of disordered sleep on increased infectious disease risk in alcoholics. Moreover, the relationship between awake levels of IL-10 taken before sleep and depth of sleep suggest a bidirectional interaction between sleep and cytokines in which abnormalities of cytokines may exacerbate disordered sleep in alcoholics.

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