

Clinical assessment of blood leukocytes, serum cytokines, and serum immunoglobulins as responses to sleep deprivation in laboratory rats

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Submitted 11 January 2005; accepted in final form 30 May 2005

Everson, Carol A. Clinical assessment of blood leukocytes, serum cytokines, and serum immunoglobulins as responses to sleep deprivation in laboratory rats. *Am J Physiol Regul Integr Comp Physiol* 289: R1054–R1063, 2005. First published June 9, 2005; doi:10.1152/ajpregu.00021.2005.—The specific systems and mechanisms affected by sleep deprivation that may perpetuate disease processes in humans still are speculative. In laboratory rats, prolonged sleep deprivation induces a state marked by abnormal control over indigenous bacteria that results in transient infections of internal tissues and eventual lethal septicemia. The present studies investigated changes in blood, serum, and bone marrow parameters that may provide diagnostic clues to immunopathology. Prolonged sleep deprivation was produced in rats by the disk-over-water method, a well-established and selective means that does not interfere with normal waking behaviors. Measurements included bone and blood differential white blood cell counts, multiple serum cytokines and chemokines, several major Ig classes and subclasses, and serum endotoxin concentrations. The results indicated mild, regenerative neutrophilia in sleep-deprived rats, initially accompanied by immature neutrophils and later by monocytosis. The corresponding serum cytokine profile revealed an evolving proinflammatory state, particularly by high incidence of interleukin-1 β , implicating mononuclear phagocytes and resident tissue cells as main intermediary sources. In addition, multiple serum Ig classes were increased by sleep deprivation without experimental administration of an exogenous antigen. Despite this immune activation, there was failure to eradicate invading bacteria and toxins, suggesting competing anti-inflammatory processes or interference with immune effector functions during sleep deprivation. Nearly all of the immune-related events that emerged as responses to sleep deprivation have been implicated as etiological or provocative factors in other disease processes and may provide means by which sleep deprivation as a risk factor in disease may become understood.

interleukin-1 β ; inflammation; endotoxin

CHRONIC SLEEP IMPAIRMENTS in humans are believed to promote or compound disease processes. Conversely, sleep is believed to restore body and brain functions that sustain fatigue during sleep deprivation or during illness (58). Despite this intuitive understanding that sleep is critical for health and life, the actual physiological underpinnings that may account for health impairment by sleep deprivation or health restoration by sleep are poorly elucidated. Comparative research in animals is providing evidence that immune-related factors are key to advancing an understanding of the physiological effects of sleep deprivation. In our animal model, impairment of host defenses is chief among sleep deprivation outcomes, as evidenced by strikingly poor control over indigenous microorganisms despite an outwardly healthy appearance and robust appetite (21). In the

general population, sleep deprivation expresses comorbidity with disease states in which dysregulation of immune variables is considered a principal etiological or contributory factor, e.g., cardiovascular disease and autoimmune disease (43, 58), hinting that answers to comorbidity may rest with immune-related factors.

Findings from several other avenues of research have provided evidence for a linkage between sleep and immune-related factors, particularly with regard to the regulation of sleep. For example, experimental challenge tests have shown that bacterial products and particular immunomodulators can alter the amount of sleep and its stages (reviewed in Refs. 31, 40, 44, 50). Changes in sleep patterns in response to infectious disease suggest sleep has a functional impact on recovery (reviewed in Ref. 47). During infection, animals that have robust sleep responses have a better prognosis for survival than those that do not (62). Detection of immune cell signaling factors in the circulation, such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α or its receptor, and/or IL-6, have been reported in patients with narcolepsy and sleep apnea who are affected by excessive daytime sleepiness (67) and in subjects deprived of sleep under research protocols (1, 57). However, as discussed later, the results of sleep deprivation studies are divergent and have not permitted a unifying interpretation. Several comprehensive reviews discuss the outcomes of manipulation of sleep by administration of immune-related factors, and vice versa, in greater detail (e.g., Refs. 30, 35, 47).

Within the first 5 days of sleep deprivation in the animal model employed in this study, live opportunistic pathogens are detected in internal tissues that normally are sterile (21). The first 5 days are considered short term relative to the expected survival time of 21 days (13, 15, 45). Despite these transient infections and the chronic antigenic challenge they represent, fever does not occur, and inflammatory reactions are virtually absent as shown by light microscopy visualization (13). Throughout nearly the entire course of survival, food intake typically increases dramatically, yet body weight is lost (15, 22). The negative energy balance does not appear to be due to malabsorption of calories, diabetes, or behavioral activity (2, 15, 22) but may be a response to infectious processes (51). Eventually, an acute phase of advanced morbidity develops, and bacteria infect the bloodstream (13). Such abnormal control of opportunistic microorganisms is diagnostic of clinical immune suppression. The first suspected mediators of immune suppression, corticosteroids, remain unchanged or decrease in sleep-deprived rats (16, 19) and humans (1, 28, 29, 39, 42, 56, 65) in studies that are controlled for extraneous variables that may elicit behavioral distress.

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The purpose of the present investigation was to clinically assess immune-related factors in laboratory rats under conditions of prolonged sleep deprivation as a first step toward understanding their compromised state. This study addresses an overall goal of discovering how sleep deprivation alters resistance to disease or affects progression of comorbidities. In this animal model, sleep deprivation is prolonged to allow subclinical signs to evolve and become manifested as clinical signs, thereby permitting study. Often these signs are progressive, which can lend significance to less robust changes that occur earlier but that seem fairly innocuous. First, blood leukocyte differentials were performed to determine the cell types responsible for a consistent finding of increased circulating white blood cell counts (15, 22). Second, leukocyte differentials in bone marrow were performed to reveal evidence of altered demands for particular cell types. Third, serum was analyzed for concentrations of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2), which are important in leukocyte migration from bone marrow to the circulation. Fourth, serum was analyzed for the presence of the following cytokines: IL-1 β , -2, -4, -6, -10, and -12, TNF- α , and interferon (IFN)- γ . Because cytokines are local cell-cell signaling mechanisms, concentrations in serum usually are below detection level. Therefore, detection in serum can potentially be linked to underlying cell sources, as well as to potential disease processes and to cell damage (52). Fifth, individual immunoglobulin (Ig) classes were quantified because of a known, marked increase in total serum proteins in sleep-deprived animals despite hypoalbuminemia (22). And, last, serum endotoxin was quantified to determine whether sleep-deprived animals cleared the toxins associated with gram-negative bacteria that were often cultured from their tissues. Endotoxin exposure can result in damage to tissues via stimulation of proinflammatory cytokines (8).

The main results of the present studies indicate that although the clinical outcome in the sleep-deprived animal reflects immune suppression, the clinical immune parameters measured suggest activation of mechanisms associated with innate immunity and responses by B lymphocytes consistent with polyclonal activation. These responses are insufficient to control microbial invasion and endotoxin levels, and they are expected to deliver their own secondary consequences. These are the types of changes believed to contribute to diverse biological effects in many disease states.

MATERIALS AND METHODS

Protocols for animal care and use were approved by institutional animal care and use committees at The University of Chicago, the National Institute of Mental Health, and The University of Tennessee (Memphis, TN), where the live animal experiments were conducted. The specimens were collected across several studies that used the same procedure for producing sleep deprivation described below. Surgery was performed to implant electrodes for recording cortical electroencephalographic (EEG) and theta activity and electromyographic activity (collectively, the EEG). EEG recordings were used to determine wakefulness and specific sleep stages and to deprive sleep by computer-assisted detection of sleep onset, as described by Bergmann et al. (3). All subjects were permitted at least 7 days of recovery from surgery before study. Animals were kept in the apparatus described below under conditions of constant light to diminish the amplitude of the circadian rhythm in all groups for factors that could be measured only once. This was done because sleep deprivation

affects the phase and amplitude of the circadian rhythm (38, 63). Procedures to obtain such data were conducted at the same time of day.

Procedure for producing sleep-deprived and yoked animals. Sleep deprivation was produced by the method of Bergmann and Rechtschaffen, which is described and illustrated elsewhere in detail (3, 22). The method has been extensively validated for its selectivity of sleep deprivation and produces reliable outcomes across studies (2, 3, 15, 22). The method features a benign arousal stimulus, which is brief movement of the cage flooring for 6 s, triggered by the onset of sleep in the sleep-deprived rat. Two rats are housed in an apparatus under identical experimental conditions on either side of a large, round horizontal platform (46-cm diameter) divided by a Plexiglas wall. At a distance of 2 cm beneath, and extending outward from, the platform is shallow water 2.5 cm deep. After initial exploration under freely moving conditions, the animals nearly always stay fully on the platform where they eat, sleep, groom, and ambulate normally. A long recording cable attached to the head electrode assembly of each animal is connected to a 360° commutator and counterbalanced boom to permit continuous polygraphic and digital capture of EEG signals under freely moving conditions. During a 7-day period of basal measurements, the platform is rotated once per hour for 6 s to acquaint the rats with platform movement. Under baseline conditions, rats spend 46% of time awake and 48 and 6% of time in non-rapid eye movement sleep (NREM) and paradoxical sleep (also known as rapid eye movement sleep), respectively (13, 15, 20, 22).

During the sleep deprivation phase, amplitude changes in the EEG recordings that meet behavioral and polygraphic criteria for sleep in the sleep-deprived rat trigger 6 s of rotation of the housing platform, resulting in ambulation by both rats to remain comfortably on the platform. The platform remains stationary when the sleep-deprived animal is engaged in behaviors other than trying to sleep. The average daily rotation time of the platform is typically 21% of total time, which does not increase in duration despite cumulative sleep loss in the sleep-deprived rat (15, 22). These procedures sustain wakefulness to >90% of total time in the sleep-deprived rats, with the remaining 10% of time composed mostly of transitional sleep. Less than 1% of total time is occupied by paradoxical sleep. Yoked animals are kept awake 58% of the time and obtain NREM sleep and paradoxical sleep during 38 and 3% of total time, respectively (13, 15, 20, 22). Not only is the amount of total sleep of yoked rats reduced, it also is highly fragmented because of frequent platform rotations triggered by sleep onset in the totally sleep-deprived animal. The yoked rat therefore is partially sleep deprived. Yoked rats typically develop signs that are qualitatively similar to those of sleep-deprived rats but that usually are less robust and are not associated with marked morbidity (reviewed in Ref. 12). Operationally, findings in totally sleep-deprived animals may lend significance to findings in the yoked animals (i.e., partially sleep deprived).

Animals and determination of leukocyte cell differentials in blood and bone marrow. Differential white blood cell counts were determined in five sleep-deprived and four yoked animals in blood sampled by means of indwelling venous catheters implanted during the survival surgery for polysomnographic recordings (3). In a fifth yoked animal, catheter patency had failed. The subjects were male Sprague-Dawley rats, 22.4 (SD 5.6) wk old and 448 (SD 29) g in weight at the time of surgery. Other data from these animals on energy expenditure and physiological signs across the survival course of sleep deprivation have been reported (2, 15, 17). Differential leukocyte counts were performed on blood smears once during the baseline period and one to three times during each of the first and second halves of the survival period; i.e., between *days 2 and 10* and between *days 12 and 22* in four sleep-deprived and four yoked animals, and on *day 24* for a fifth sleep-deprived animal, because of nonpatency of the indwelling catheter on *day 22*. In addition, two of the sleep-deprived animals were permitted sleep after 16–18 days as part of an extended study (17), and blood leukocyte differentials were completed for postdeprivation *days 2 to 8* and *days 12 to 15*. Smears were stained with Wright's

stain, and leukocytes were counted by a qualified clinical pathologist (Clinical Hematology Laboratory, University of Chicago, Chicago, IL) for numbers and proportions of polymorphonuclear neutrophils, band neutrophils, monocytes, lymphocytes, and atypical (reactive) lymphocytes. The values for the sleep-deprived rat studied on *day 24* were later excluded from analyses because of the animal's leukopenia and documented impending mortality.

Marrow aspirates from the femur were prepared during necropsy procedures for five sleep-deprived and five yoked animals studied for 16–23 days of sleep deprivation to determine nutritional and metabolic consequences, as previously reported (22). These animals were male Sprague-Dawley rats, 21.5 (SD 4.7) wk old and 471 (SD 112) g in weight at the time of surgery. Cell composition of bone marrow specimens was determined by a qualified veterinary clinical pathologist (Mid Atlantic Regional Laboratory, Rockville, MD). Differential cell counts included lymphocytes, prorubricytes, basophilic normoblasts, polychromatophils, metarubicytes, and total erythrocytes, granulocytes (myeloblasts, progranulocytes, myelocytes, metamyelocytes, bands, and segmented), eosinophils (myelocytes, bands, and segmented), and the myeloid-to-erythroid ratio.

Animals in which concentrations of serum cytokines, chemokines, Igs, and endotoxin were determined. Sixty-two male Sprague-Dawley rats that were 22.8 (SD 1.9) wk old and weighed 437 (SD 32) g at the time of surgery were studied. Fifty-four of these animals were in the study that determined the time course and distribution of live microorganisms in body tissues (21), and eight animals were studied subsequently. None were implanted with indwelling catheters. The duration of study was predetermined at 0, 5, 10, 15, or 20 days of sleep deprivation or yoked conditions, after which the animals were deeply anesthetized and exsanguinated by cardiac puncture. Two yoked rats and one baseline control rat were excluded from further study because of death during anesthesia just before necropsy, detachment of a recording head plug assembly, or unexplained poor health. Blood was collected into sterile tubes containing either liquid EDTA (endotoxin determinations) or serum separator medium (cytokine and Ig determinations) and centrifuged for 10 min at 4°C, and the serum was frozen at –80°C. For cytokine, chemokine, and Ig determinations, five to eight animals in each group were studied for each duration of sleep deprivation. Five baseline control animals (i.e., operated animals housed under the same experimental conditions but without sleep deprivation) also were studied. Endotoxin determinations were completed for 50 animals.

Quantification of serum cytokines and chemokines. Determinations of specific rat serum cytokines and chemokines were made using solid-phase sandwich ELISAs. Concentrations for each variable were plotted from corresponding standard curves. Rat IL-1 β and IFN- γ were assayed using kits from R&D Systems (Minneapolis, MN). The lower limits of detection were <5 and <7 pg/ml, respectively. Rat IL-2, -4, -6, -10, and -12, TNF- α , MCP-1, and MIP-2 were assayed using kits from Biosource International (Camarillo, CA). The lower limits of detection were <2.5 pg/ml for IL-4, IL-12, and TNF- α ; <5 pg/ml for IL-2 and MIP-2; and <8 pg/ml for IL-6, IL-10, and MCP-1. Results did not approach the upper limits of these assays.

Quantification of Ig classes and IgG subtypes. A screening for abnormal serum proteins was performed using zone electrophoresis on serum collected from two sleep-deprived and two yoked animals after 9 and 15 days of sleep deprivation (Carlson Animal Facility, Chicago, IL). Comparisons included specimens collected during the baseline period and from unoperated colony animals. The results did not indicate monoclonal proteins but, rather, a polyclonal electrophoretic pattern involving α_1 , α_2 , β_1 , β_2 , and γ . The method of quantification therefore was switched to directly measure the quantities of Ig classes IgM, IgG, and IgA and IgG subclasses IgG1, IgG2a, IgG2b, and IgG2c. Ig quantification was performed by conventional radial immunodiffusion using commercial kits for rat sera (ICN Biomedicals, Aurora, OH). Measurements were based on an 18-h timed diffusion for IgG and end-point determinations for other Igs.

Endotoxin detection in serum. Serum was diluted 1:10 in endotoxin-free water, vortexed, and incubated at 75°C for 12 min to inactivate interfering substances. Four dilutions of each test sample were assayed on endotoxin-free plates (Corning Costar, Corning, NY), in triplicate, with and without positive controls (0.5 EU/ml) and with corresponding control and standard samples. Each plate was incubated for 10 min at 37°C in a plate reader before addition of reagent (Kinetic-QCL; Bio-Whittaker, Walkersville, MD). The absorbance at 405 nm was measured every 60 s for at least 85 min. The results were plotted on a log-log standard curve of concentration in endotoxin units (EU) vs. reaction time, based on the amount of time required for the absorbance to increase 0.2 optical density (OD) units. For a result to be considered valid, the following criteria were met: >50% detected concentration of the positive controls, dilution not less than 1:90 because of interfering substances, and agreement evident in the replicates. Final results were corrected for differences in dilution factors. Values for one sleep-deprived rat and one yoked rat on *day 5* were excluded from study because of likely contamination of the specimens. Values for three animals were excluded because of poor recovery of the internal standard, which renders the values invalid.

Data analysis. Analyses of serum cytokines, chemokines, endotoxin, and Igs compared each sleep-deprived and yoked group at 5, 10, 15, or 20 experimental days with a baseline control group. The values first were tested for statistically significant treatment effects by one-way analysis of variance (ANOVA) and then tested by a series of conditional planned comparisons. Significance was set at $P < 0.05$ for comparisons by ANOVA. The family-wise error for planned comparisons and post hoc comparisons was set at $P = 0.025$, based on four a priori comparisons (i.e., 5, 10, 15, or 20 days of sleep deprivation vs. baseline) and 90% confidence. In cases in which a statistically significant difference was not observed between sleep-deprived animals at 5 or 10 days, or at 15 and 20 days, the two treatment groups were combined to create a pooled “first half” and a “second half” of the experimental period for comparison with baseline. Contingency tables were constructed for individual animals to compare the proportion of subjects per group that were positive for detection of a variable of interest (e.g., cytokines and endotoxin) that is not normally detected. Significance for incidence was determined using Fisher's exact two-tailed tests and $P < 0.05$. In similar manner, differential counts of leukocytes were tested by means of a series of planned and post hoc comparisons. The family-wise error was held constant at $P = 0.03$, based on three a priori comparisons of interest (i.e., baseline values, first half and second half of the sleep deprivation period) and 90% confidence. For bone marrow, tests of individual cell parameters were compared between yoked and sleep-deprived groups by means of paired t -tests. Significance was assigned at $P < 0.025$ in lieu of correcting for the total number of paired t -tests, which was considered too conservative for a survey of bone marrow. Values are expressed as means \pm SE unless designated otherwise as means (SD).

RESULTS

Blood leukocyte differentials. The proportion of total blood leukocytes composed of neutrophils showed strong effects of experimental treatment ($F_{6,21} = 4.95$, $P = 0.003$) (Fig. 1). During the sleep deprivation period, total leukocytes increased in both yoked and sleep-deprived animals to 15.7 (SD 4.0) and 16.1 (SD 4.2) $\times 10^3$ cells/ μ l, respectively, compared with their baseline means of 10.6 (SD 2.1) and 10.2 (SD 3.6) $\times 10^3$ cells/ μ l. During the sleep deprivation period, neutrophils as a proportion of total leukocytes increased from 46% during the baseline period to 61% during the first half of the experimental period ($P < 0.005$) and increased further to 70% during the second half of the experimental period ($P < 0.001$) in sleep-deprived animals. Yoked animals during the second half of the experimental period showed a lesser trend of 51% of total

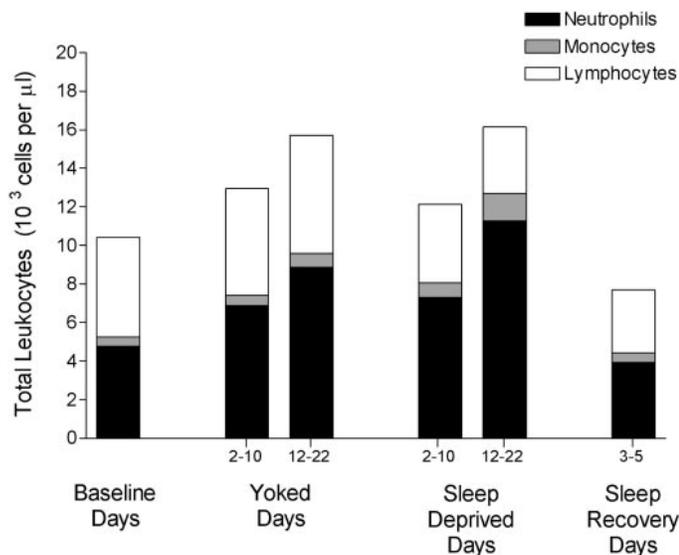


Fig. 1. Populations of neutrophils, monocytes, and lymphocytes expressed as proportions of total blood leukocytes in animals during a baseline period of ad libitum sleep ($n = 9$) and subsequently during *days 2-10* and *days 12-22* of yoked or total sleep deprivation conditions ($n = 4$ or 5 animals per group). Also shown are the same cell populations and their proportions in 2 animals that were permitted sleep for 3–5 days after 16–18 days of sleep deprivation (sleep recovery).

leukocytes identified as neutrophils. Among neutrophils, the number and proportion of bands (i.e., immature neutrophils) were significantly increased in sleep-deprived animals during the first half of the experimental period (both comparisons, $P < 0.01$) (Fig. 2A). Monocyte number tripled during the second half of the experimental period in totally sleep-deprived rats compared with baseline ($P < 0.005$) (Fig. 2B). The proportion of blood leukocytes composed of lymphocytes showed strong effects of experimental treatment ($F_{6,21} = 4.56$, $P = 0.004$). As a proportion of total leukocytes, lymphocytes were significantly decreased from 47% during baseline to 33% and then to 22% during the first and second halves of the experimental period in totally sleep-deprived rats ($P < 0.005$ and $P < 0.001$, respectively) (Fig. 1). The absolute number of blood lymphocytes in sleep-deprived rats showed strong downward trends that did not reach statistical significance (Fig. 2C). Recovery sleep after prolonged sleep deprivation was associated with a substantially reduced proportion and/or number of neutrophils, band neutrophils, and monocytes from deprivation levels to near baseline levels in the two animals studied (Fig. 2). Also, the proportionality of lymphocytes to other leukocytes appeared reestablished (Fig. 1).

Leukocyte differentials in bone. Sleep-deprived and yoked animals did not differ in the number of granulocytes or eosinophils in different stages of development or in the number of lymphocytes, prorubricytes, or basophilic normoblasts. In terms of erythrocyte processes, sleep-deprived rats had fewer polychromatophils but more metarubricytes than did yoked animals ($t_4 = 4.12$, $P = 0.007$ and $t_4 = 2.76$, $P = 0.025$, respectively). The development of metarubricytes is the final step in the maturation process of the erythrocyte, and group differences were not detected for total erythrocytes. Overall cellularity was considered normal. The myeloid-to-erythroid ratio did not differ between sleep-deprived and yoked animals;

however, the ratios of 1.56 and 1.64, respectively, are higher than published norms of 1.16–1.36 for rats (as cited in Ref. 55).

Serum cytokines and chemokines. IL-1 β in serum showed strong effects of treatment on concentration ($F_{8,50} = 3.00$, $P < 0.008$) and frequency of detection ($P < 0.001$). The incidence of IL-1 β detection in serum was marked for sleep-deprived rats throughout the experimental period and for yoked rats later in the experimental period (Fig. 3). The incidence of IL-1 β in sleep-deprived animals compared with baseline control animals was increased significantly in sleep-deprived animals by *day 5* ($P = 0.03$), showed a strong tendency on *day 10*, and again was significantly increased on *days 15* ($P = 0.007$) and *20* ($P = 0.015$). The incidence of IL-1 β in yoked animals compared with baseline control animals was significant during the second half of the experimental period on *days 15* and *20* ($P < 0.007$ and $P < 0.05$, respectively). Planned comparisons revealed that group averages for concentrations of IL-1 β , including subjects in which no IL-1 β was detected, were significantly higher in the sleep-deprived groups than in the baseline control and yoked groups during *days 15* and *20* ($P < 0.005$ or better for each comparison).

Serum IL-2 showed an effect of treatment ($F_{8,50} = 3.25$, $P < 0.005$) due to a *day 10* incidence of positive values in four of six yoked and three of six sleep-deprived animals [mean positive value: yoked = 31.1 (SD 9.2), sleep-deprived = 27.5 (SD 14.2)] compared with no detection on *day 5* (*days 5 vs. 10*: yoked, $P < 0.001$; deprived, $P < 0.01$). IL-2 otherwise was infrequently detected: twice in baseline controls, once in yoked animals on each of the *days 15* and *20*, and once in a sleep-deprived animal on *day 15* (all values < 37 pg/ml). IFN- γ in serum showed an effect of treatment ($F_{8,50} = 2.35$, $P < 0.03$) due to detection solely in sleep-deprived animals (35% frequency) during the period of *days 15* to *20* (sleep deprived vs. baseline, $P < 0.0125$; vs. yoked at 15–20 days, $P < 0.005$). The average concentration of IFN- γ when present in sleep-deprived animals was 20 (SD 14) pg/ml.

Other cytokines did not show statistically significant treatment effects, but the data nonetheless may have clinical significance. IL-4 was not detected in any animal. IL-6 and IL-10 were detected only on *day 15* and only in one or two sleep-deprived animals. IL-12 was detected on *day 15* in 38% of yoked and sleep-deprived animals. Otherwise, IL-12 was detected once in a baseline animal and once in each yoked and sleep-deprived group on *days 5* and *20*. TNF- α was detected only in one to two sleep-deprived animals on *days 15* and *20* and in one yoked animal on *day 5*. As may be apparent, the likelihood that one or more cytokines will be detectable in the serum if the subject is partially or totally sleep deprived is very high ($P < 0.004$). The overall incidences for detection of any cytokine under study were 2 of 5 baseline controls, 20 of 26 animals that were partially sleep deprived (yoked), and 27 of 28 animals that were totally sleep deprived.

The chemokines MCP-1 and MIP-2 showed different responses to sleep deprivation. MCP-1 was strongly affected by treatment ($F_{8,50} = 2.84$; $P < 0.01$). Individual values are shown in Fig. 4. Comparisons revealed a near doubling of MCP-1 concentrations in sleep-deprived animals on *day 20* [mean = 136 (SD 57) pg/ml] compared with baseline controls [mean = 70 (SD 29) pg/ml, $P < 0.001$]. MIP-2 did not show an effect of treatment. MIP-2 was detected in three sleep-

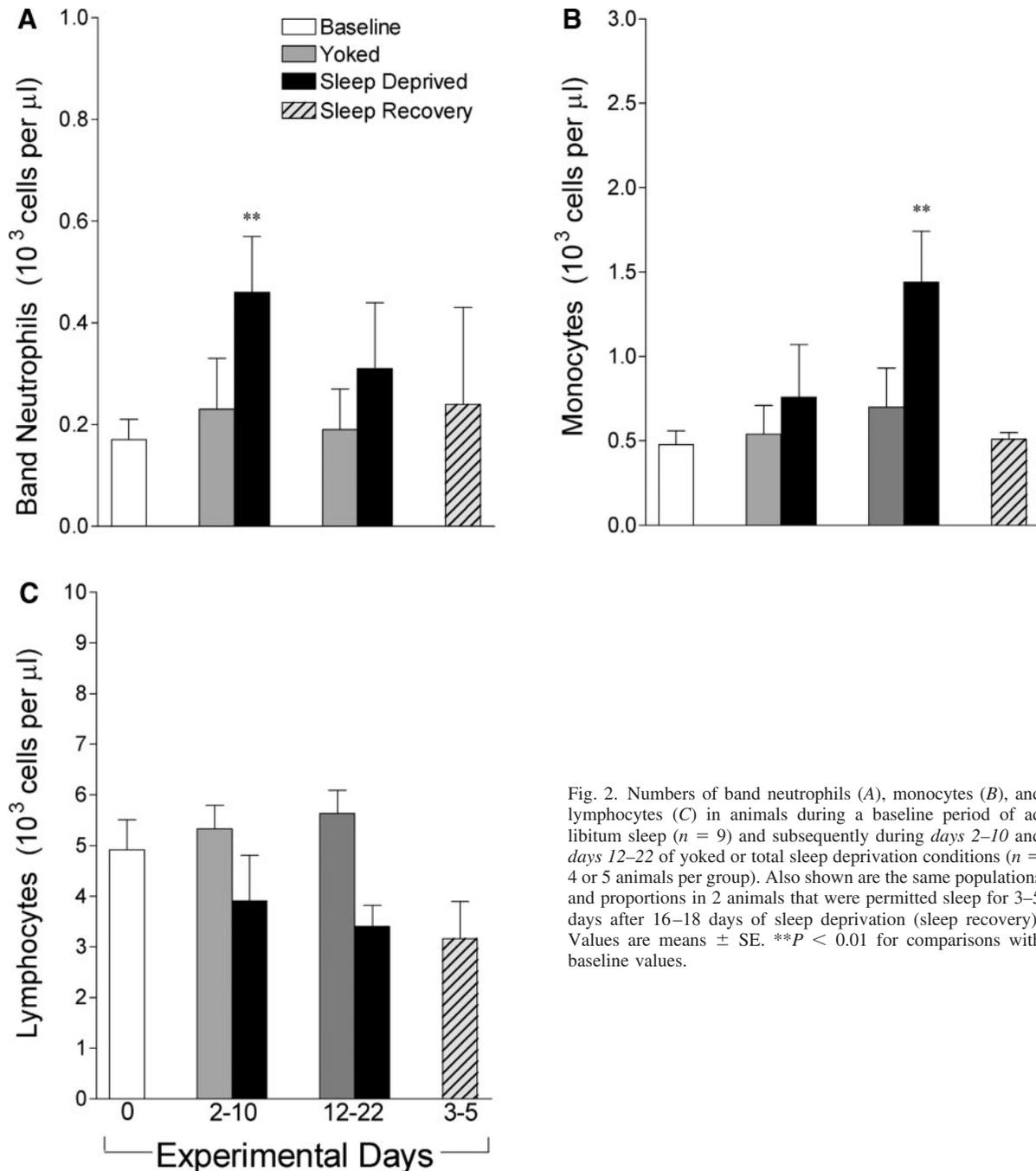


Fig. 2. Numbers of band neutrophils (A), monocytes (B), and lymphocytes (C) in animals during a baseline period of ad libitum sleep ($n = 9$) and subsequently during *days 2–10* and *days 12–22* of yoked or total sleep deprivation conditions ($n = 4$ or 5 animals per group). Also shown are the same populations and proportions in 2 animals that were permitted sleep for 3–5 days after 16–18 days of sleep deprivation (sleep recovery). Values are means \pm SE. ** $P < 0.01$ for comparisons with baseline values.

deprived animals during the inclusive period of *days 15 to 20* and one yoked animal on *day 5*.

Serum Ig concentrations. Serum IgM, IgG, and IgA concentrations each showed a strong effect of treatment (IgM: $F_{8,49} = 2.49$, $P = 0.02$; IgG: $F_{8,49} = 4.24$, $P = 0.001$; IgA: $F_{8,49} = 3.38$, $P = 0.004$). During the first half of the experimental period, during *days 5 to 10*, IgM was significantly increased in sleep-deprived animals compared with baseline control values ($P < 0.025$). This increase was progressive and became more pronounced by *days 15 and 20* (Fig. 5). IgM values in yoked animals were significantly different from baseline controls only after 15 experimental days ($P < 0.005$) and otherwise showed a nonsignificant upward trend. Changes in IgG began with a

strong increase from 5 to 10 days in sleep-deprived animals ($P < 0.025$), and progressive increases at 15 and 20 days were statistically different from values observed in baseline controls ($P < 0.01$ and $P < 0.001$, respectively). Values in yoked animals also were increased significantly by *days 15 and 20* compared with baseline values (each $P < 0.01$), but the magnitude of the increase was not quite as great as for totally sleep-deprived animals. Increases in IgA from 5 to 10 days of sleep deprivation were significant in totally sleep-deprived animals ($P < 0.025$). IgA increased further during *days 15 and 20*, and these changes were statistically different from values observed in baseline controls ($P < 0.025$ and $P < 0.005$, respectively). IgA values in yoked control animals did not

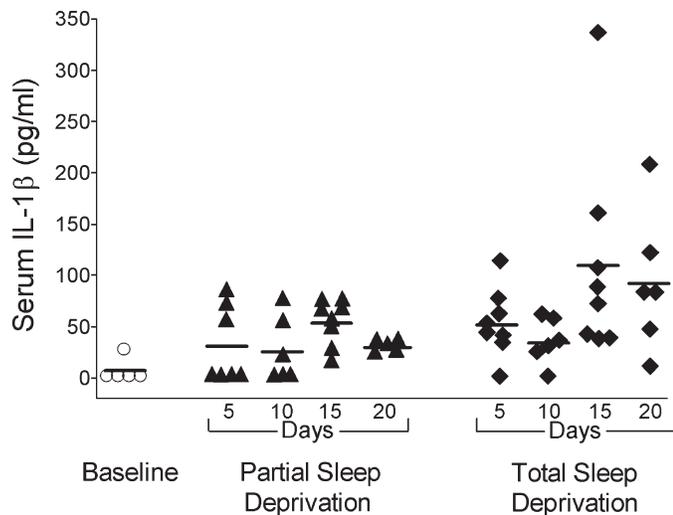


Fig. 3. Interleukin (IL)-1 β concentrations in serum of individual animals in a baseline control group (\circ) and in partially [i.e., yoked (\blacktriangle)] and totally sleep-deprived (\blacklozenge) groups after 5, 10, 15, and 20 experimental days. The incidence of IL-1 β in serum was high for sleep-deprived animals at most time points studied, and the concentration was statistically different from baseline controls and yoked animals on *days* 15 and 20. Horizontal bars indicate group means for each treatment condition.

change enough to achieve statistical significance, but observations show an upward trend very late in the experimental period.

The results for the Ig subtypes are given in Table 1. Increases in IgG2a were progressive and were statistically different from baseline values in both sleep-deprived and yoked rats on *day* 20 ($P < 0.025$ and $P < 0.005$, respectively). Values for IgG1, IgG2b, and IgG2c all showed late upward trends in sleep-deprived rats on *day* 20, but these did not reach statistical significance. IgG2b and IgG2c also tended upward in yoked rats on *day* 20.

Endotoxin concentrations in serum. Endotoxin concentration in serum showed a strong effect of experimental treatment ($F_{8,39} = 2.21$; $P < 0.05$). Individual values are provided in Fig. 6. Comparisons revealed that sleep-deprived rats had higher concentrations of endotoxin in their bloodstream than did yoked animals during each the first ($P < 0.025$) and second ($P < 0.001$) halves of the experimental period.

DISCUSSION

The major findings of the present study are that prolonged sleep deprivation in rats altered all major classes of leukocytes in the blood and stimulated production of serum antibodies without experimental administration of antigen. Sleep deprivation increased the incidence and concentration of certain cytokines and chemokines, particularly IL-1 β and MCP-1, associated with innate immunity, and later, IL-2 and IFN- γ , generally associated with cellular immunity. The blood cytokine profile indicates a proinflammatory state in sleep-deprived rats despite the fact that inflammatory reactions in tissues are considered poor relative to those typically found in infectious disease states (13). Endotoxemia, evident in many sleep-deprived animals, as shown in this study, along with the detection of bacteria in internal organs, shown previously (21), are stimuli that logically would induce production of these factors. At the

same time, the host response is inadequate for controlling these foreign antigenic stimuli.

The cellular reserve of bone marrow is known to be vast, and few changes in bone marrow from sleep-deprived animals were found. The finding of significantly fewer erythrocyte precursors in sleep-deprived rats is consistent with previous observations of mild anemia and increased numbers of circulating immature red blood cells (14), perhaps hinting at weak erythropoiesis. A relatively high ratio of myeloid to erythroid cells compared with published norms (55) suggests that myeloid cell production is favored. The altered leukocyte populations in the blood during sleep deprivation are characterized by a mild but progressive leukocytosis attributable to neutrophilia and monocytosis, and also by lymphopenia. The appearance of a significant number of circulating band neutrophils during the first half of the experimental period denotes an ongoing influx of immature granulocytes from the marrow (i.e., a left shift). Neutrophilia with a left shift is considered the hallmark of inflammatory processes (reviewed in Ref. 64), whether those processes are acute or chronic, infectious or immune-related; e.g., tissue damage, poisoning, metabolic abnormalities (reviewed in Ref. 9). Monocytosis during neutrophilia without a left shift, which occurred during the second half of the experimental period, typically occurs when the tissue demand for phagocytosis of macromolecule particles is increased or in conditions in which cellular immunity is potentiated (reviewed in Ref. 34). It implies the presence of a chronic antigenic challenge, from either microorganisms or autoantigens. The suggestion of a return toward normal numbers or proportions of neutrophils, monocytes, and lymphocytes during the first several days of recovery sleep after prolonged sleep deprivation in two animals studied implies that chronic catheterization was not a confounding factor in this study and hints that leukocyte parameters may be quickly normalized by sleep.

Leukocytosis induced by sleep deprivation has been a consistent finding in human sleep deprivation studies since at least 1925, sometimes composing the only physiological finding

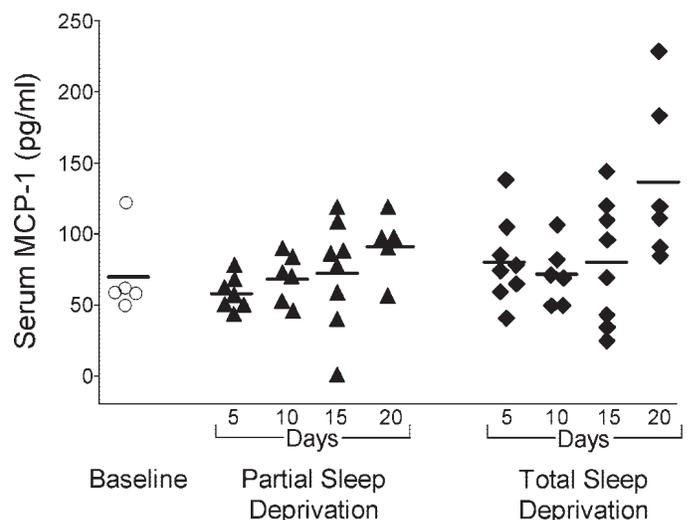


Fig. 4. Macrophage chemotactic protein-1 (MCP-1) concentrations in serum of individual animals in a baseline control group (\circ) and in partially [i.e., yoked (\blacktriangle)] and totally sleep-deprived groups (\blacklozenge) after 5, 10, 15, and 20 experimental days. Horizontal bars indicate group means for each treatment condition.

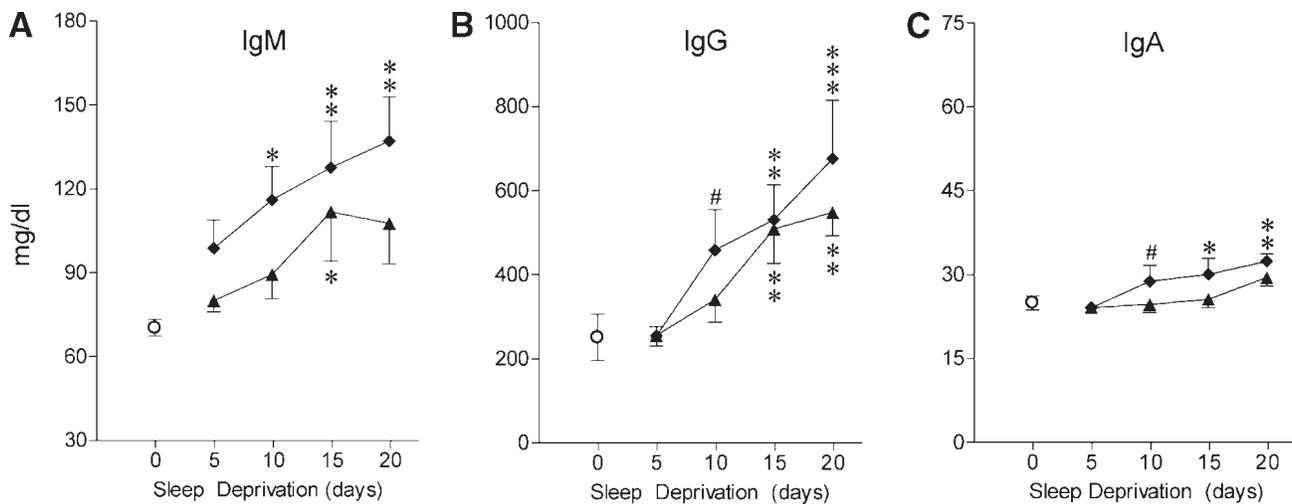


Fig. 5. Major immunoglobulin classes of IgM (A), IgG (B), and IgA (C) in baseline control animals (time 0, \circ) and in partially [i.e., yoked (\blacktriangle)] and totally sleep-deprived (\blacklozenge) groups during 5, 10, 15, and 20 experimental days. * $P < 0.025$; ** $P < 0.01$; *** $P < 0.001$ for comparisons with baseline values. # $P < 0.025$ for comparison with day 5 values of the same group.

(cited in Ref. 27). This leukocytosis has been classified as either neutrophilia (32) or neutrophilia with monocytosis (11), consistent with the present findings. Furthermore, decreased total lymphocytes have been reported in human subjects deprived of sleep in excess of 76 h (Ref. 32; cited in Ref. 27). Whether immature forms of granulocytes appear in the circulation of human sleep-deprived subjects has not, to our knowledge, been evaluated (e.g., Ref. 24). Sympathetic activation observed in both sleep-deprived humans and laboratory animals (reviewed in Ref. 12) is one candidate mechanism that may contribute to changes in granulocyte storage pools. In contrast, serum corticosteroids are not increased by sleep deprivation in rats (16, 19) or humans (1, 28, 29, 39, 42, 56, 65), suggesting that glucocorticoid excess is not a leading mediator. Whether more neutrophils are moving from the circulation into marginal pools is not known, but preliminary evidence from our laboratory indicates high concentrations of myeloperoxidase, an enzyme considered virtually exclusive for neutrophils, in major organs of sleep-deprived rats.

Cytokines and chemokines considered proinflammatory were prevalent in the serum in sleep-deprived animals, concomitant to progressive leukocytosis. The order of their appearance was sequential, giving the impression that effector responses by different cell types were evolving. IL-1 β was

detected earliest, in the serum of seven of eight totally sleep-deprived rats on day 5. IL-1 β is a well-known and potent proinflammatory marker that is produced by innate immune mechanisms without regard to antigen specificity. IL-1 β is produced by many cell types, including granular lymphocytes, endothelium, epithelia, and fibroblasts. However, the principal immune system source for IL-1 β is the macrophage. Increased IL-1 β would be expected to stimulate macrophages and innate immunity and to activate B cells and humoral immunity. IL-1 secretion and other cytokine effects would be expected to lead to activation of helper T cells and the secretion of IL-2 by T cells, as well as to other inflammatory and immune responses. IL-2 was detected in serum on day 10 in a majority of yoked and sleep-deprived animals. IL-2 is important in the proliferation and differentiation of Th1 (cell-mediated immunity) and Th2 (humoral) cells, as well as the activation of macrophages and cytotoxic lymphocytes and the enhancement of immunoglobulin secretion. The transient increases in IL-2 in serum were followed by increases in IFN- γ in serum in a subgroup of totally sleep-deprived animals during days 15 and 20, along with occasional observations of IL-6, IL-12, or TNF- α . Concentrations of IL-1 β and MCP-1 became higher in the totally sleep-deprived group during days 15 and 20 than in the yoked animals that were partially sleep deprived. IL-1 β is a major

Table 1. Immunoglobulin subtypes in sera of totally and partially sleep-deprived animals, compared with baseline control animals permitted sleep ad libitum

Group	Days of Sleep Loss	IgG1, mg/l	IgG2a, mg/l	IgG2b, mg/l	IgG2c, mg/l
Baseline control	0	623 (175)	1,996 (807)	1,276 (407)	353 (104)
Yoked	5	613 (144)	2,091 (880)	1,086 (477)	398 (70)
	10	568 (85)	2,100 (609)	948 (344)	336 (65)
	15	472 (259)	2,696 (1123)	1,036 (363)	326 (119)
	20	470 (206)	3,644 (645) \dagger	1,760 (592)	423 (161)
	Sleep deprived	5	683 (464)	2,263 (1096)	1,042 (262)
Sleep deprived	10	815 (289)	2,592 (947)	1,263 (387)	363 (167)
	15	584 (214)	2,469 (828)	1,158 (522)	320 (54)
	20	809 (347)	3,271 (1110)*	1,808 (751)	448 (117)

Values are means (SD). Planned and post hoc comparisons with baseline values: * $P < 0.025$; $\dagger P < 0.005$.

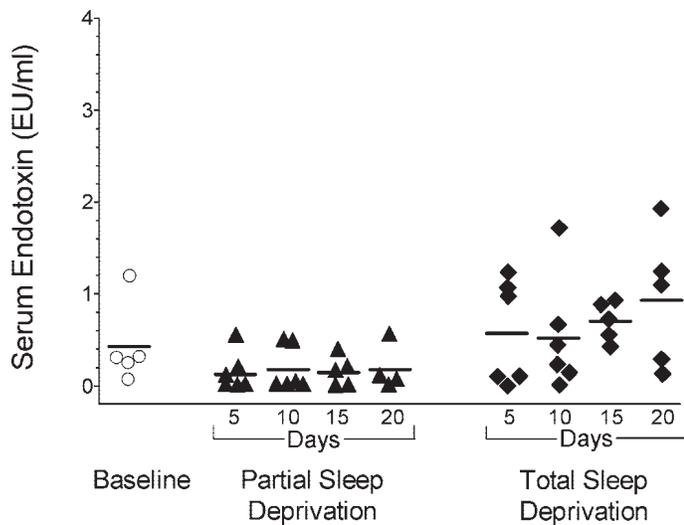


Fig. 6. Endotoxin concentrations in serum of individual animals in a baseline control group (○) and in partially [i.e., yoked (▲)] and totally sleep-deprived (◆) groups after 5, 10, 15, and 20 experimental days. Horizontal bars indicate group means for each treatment condition.

inducer of MCP. Eventual high levels of MCP-1, produced by macrophages and many other host cell types, infer production of ligands for receptors on macrophages, T cells, B cells, and NK cells, thereby influencing effector responses (reviewed in Ref. 41). MIP-2, which attracts and activates neutrophils (66), was infrequently detected in the circulation of sleep-deprived animals.

Cytokine detection in the serum or production by peripheral blood lymphocytes *ex vivo* in sleep-deprived humans has been variously reported. In 1989, Moldofsky et al. (39) reported that 40 h of sleep deprivation enhanced plasma IL-1-like and IL-2-like activities. Altemus et al. (1) reported that 42 h of sleep deprivation enhanced plasma IL-1 β and TNF- α concentrations in women. One night of sleep deprivation has been reported to increase the nighttime sleep levels of IL-1 β and TNF- α and decrease those of IL-2 in whole blood stimulated with mitogens (4). Detection of the receptor for TNF- α and an eventual elevation of IL-6 in subjects deprived of sleep for 2–4 days were reported by Shearer et al. (57). Increased serum IL-6 also has been reported in subjects whose sleep was reduced to 6 h for 1 wk (68). It should be noted, however, that findings on serum IL-6 detection have been called into question on the basis of evidence that local cytokine production in response to indwelling venous catheters creates false-positive findings (23). Increased production of IFN by peripheral blood lymphocytes measured by means of an *in vitro* anti-viral assay has been reported for women during and after 77 h of sleep deprivation combined with military activity (49). Marked elevations in C-reactive protein, thought to reflect the combined activity of IL-1, TNF- α , and IL-6, are progressive during 88 h of sleep deprivation in humans (37). In mice, Hu et al. (25) reported elevated serum levels of IL-1 β , IL-6, TNF- α , and the IL-1 receptor under conditions of 36 h of sleep deprivation without increased corticosterone. In rabbits, a short 4-h sleep deprivation period resulted in an average increase in the concentrations of plasma IL-1 β when detected (46). To date, these observations in the human and animal studies have not been linked with clinical significance.

Increased production of serum Igs was progressive throughout the experimental period in sleep-deprived animals, pointing to a very early activation of production before increased serum levels would be observable. The sequence of Ig changes was reasonably consistent with a primary immune response to novel antigens during which IgM is the first to increase and is observable within the first 3 to 5 days, followed by an increase in IgG, usually after 5 or more days. Circulating IgA also achieved a statistically significant increase. Late changes in sleep-deprived animals included upward trends in all IgG subclasses by day 20. This Ig profile is consistent with antibody production by a thymus-independent (TI) pathway, characteristics of which include the low magnitude of the Ig increases, IgM concentrations that did not appear to peak, and the multiple Igs produced. TI antigens are mainly components of bacterial cell walls that stimulate responses by certain B cell populations without linkage to T cells. Some TI antigens induce cytokine production by macrophages. TI antigens also stimulate T cell help that can, in turn, regulate antibody production and class switching to IgG and contribute to overall execution of the response (reviewed in Ref. 26). The detection of endotoxin and the invasion of body tissues by indigenous bacteria point to logical sources of potentially identifiable TI antigens in sleep-deprived rats.

In humans, Dinges et al. (11) reported a significant and linear increase in the proportion of lymphocytes in the DNA synthesis (S) phase as sleep loss progressed during 64 h, but not a clear change in the end point of lymphocyte proliferation. Their data hint that processes that would culminate in proliferation were activated by sleep deprivation, and, furthermore, T cell linkage was not required to initiate these first steps toward proliferation. In other human studies, sleep deprivation is either relatively short term (e.g., Ref. 48) or combined with exercise and caloric deficiency, whereby outcomes cannot be related to a sole intervention (e.g., Ref. 6). In mouse models, short-term sleep deprivation of 6–14 h has resulted in suppression (reviewed in Ref. 7), no change (61), and enhancement (53, 54) of anti-viral antibody titers.

The presence of endotoxin in the circulation and opportunistic microorganisms in the tissues of sleep-deprived animals are stimuli that would logically promote the proinflammatory state observed in this study. Importantly, however, increases in circulating phagocytes, cytokines, chemokines, and Igs during sleep deprivation are insufficiently effective in controlling microbial invasion and eliminating foreign antigens. Complications with infectious agents may arise because the proinflammatory changes are suppressed or because they may be responses to noninfectious causes, such as tissue injury. For example, recent evidence has shown unlocalized cell injury and oxidative stress in sleep-deprived rats (18), which would be expected to produce metabolic and immune-related burdens. The mononuclear phagocyte system is responsible not only for clearance of foreign virulence factors but also removal of macromolecules and apoptotic cells, which may signal a suppressed inflammatory response. Sleep-deprived rats also have marked reductions in anabolic hormones and failed negative feedback to hypothalamic mechanisms (16) that may be expected to have immunosuppressant consequences. There exists an apparent interference with IL-1 β effector functions, suggested by the fact that high IL-1 β incidence in sleep-deprived rats was not associated with fever, aphagia, or signs of malaise.

Quite the opposite, sleep-deprived animals typically double their food intake and do not show aphagia or other sickness behaviors before becoming moribund (13). Some of the possible sites and mechanisms of interference with IL-1 β include reduced interaction of IL-1 β with the capillary network in the hypothalamus, augmented actions of anti-inflammatory cytokines and receptors, production of central antipyretics, and/or altered type and level of stimulation of second signals (reviewed in Ref. 10). Decreased cytokine availability also can occur through increased binding of cytokines in the periphery, as is known to occur in chronic disease states. The causal directions among these possibilities, their mediation, and their contribution to systemic immune compromise are under study.

The present results indicate an array of immune-related mechanisms appear activated by sleep deprivation and in a manner that does not necessarily rely on immunologic specificity or memory. The present results are congruous with the weight of evidence from human studies on the composition of the circulating leukocyte pool and detection of cytokines and chemokines considered proinflammatory. The changes in the amount of cells and molecules circulating in the bloodstream of sleep-deprived subjects are not expected to be inert or benign. For example, increases in the circulating pool of white blood cells imply changes in adhesion characteristics. In humans, a high white blood cell count can be a risk factor for myocardial infarction in certain populations and is correlated with, for example, changes in coagulation factors and measures of glucose metabolism (5). Overactivation of cytokine systems, as indicated by their detection in serum, is understood to result in systemic effects that may cause tissue damage (reviewed in Ref. 60). Furthermore, broad-based polyclonal patterns of B cell responses accompany adverse systemic reactions in a variety of conditions, such as persistent antigenic stimulation and inflammation, neoplasia, pulmonary disorders, and autoimmune and other immune-mediated disorders (reviewed in Ref. 36). Production of TI antibodies, which awaits confirmation through further study, are well recognized for a polyreactive nature and potential to react maladaptively against native antigens. TI antibodies are known to interfere with vaccination and, if increased by sleep deprivation, provide a potential explanation for recent reports of poor antibody production after vaccination in individuals whose sleep was restricted (33, 59). Such potential far-reaching physical implications resulting from alterations in immune status may explain why sleep deprivation effects are risk factors for disease and yet are not well defined or specifically localized.

ACKNOWLEDGMENTS

Technical assistance was provided by Christa D. Laatsch. Appreciation is expressed to Linda A. Toth, Ph.D., D.V.M., and Jeannine A. Majde, Ph.D., for review of a preliminary version of the manuscript. A portion of this work was presented at a joint meeting of the American Academy of Sleep Medicine, the Sleep Research Society, and the 2003 World Federation of Sleep Research Societies in Chicago, IL.

GRANTS

Research support was provided by National Heart, Lung, and Blood Institute Grant HL-59271 and National Institute of Neurological Disorders and Stroke Grant NS-38733. Additional support was provided by the University of Chicago Sleep Research Laboratory, the National Institute of Mental Health Intramural Research Program, The University of Tennessee Health Science Center, The Medical College of Wisconsin, and Veterans Affairs.

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