

## *Sleep Deprivation and Sleep Extension*

### Are Physiological Effects of Sleep Deprivation in the Rat Mediated by Bacterial Invasion?

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**Summary:** Recent reports have indicated that rats subjected to total sleep deprivation (TSD) by the disk-over-water method and sacrificed when death appeared imminent showed aerobic bacteria in their blood. Yoked control rats did not. Extrapolating from these results, it has been suggested that the late body temperature declines and eventual deaths of TSD rats are caused by septicemia, and that other, earlier-appearing effects of TSD—including weight loss, increased energy expenditure, and regulation of temperature at a higher level—might be mediated by impaired host defenses against bacterial invasion. Three measures of aerobic bacterial invasion were used to evaluate these hypotheses: bacteremia, bacterial colonization in major organs of filtration (liver, kidney, and mesenteric lymph nodes), and adherence of bacteria to the cecal wall. Experiment 1 showed nonsignificant trends toward more bacterial invasion in 4-day TSD rats compared to yoked control rats and no relationship between the bacterial indicators and the early TSD effects. Experiment 2 showed that the elimination of aerobic bacterial infection by antibiotic treatment did not prevent the early TSD effects in 4-day TSD rats. Experiment 3 showed that the elimination of aerobic bacterial invasion in TSD rats did not eliminate the late temperature decline or the progression toward death. The results showed no significant evidence of aerobic bacterial invasion early in TSD and no indication that the major effects of TSD were dependent upon aerobic bacterial invasion. **Key Words:** Sleep deprivation—Bacterial invasion—Host defense—Immune function.

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Rats subjected to total sleep deprivation (TSD) by the disk-over-water method (1) reliably develop the following syndrome: progressive weight loss, increased food intake, and increased energy expenditure (EE) (calculated from the caloric values of weight change and food intake and validated by calorimetry); initially increased waking intraperitoneal temperature ( $T_{ip}$ ), followed by a decline to below baseline; ulcerative and hyperkeratotic skin lesions on the tail and plantar surfaces of the paws; a progressively debilitated appearance; and eventual death after about 2–3 weeks (2–4). Some of these effects have been interpreted as resulting from TSD-induced changes in thermoregulation. The initial waking  $T_{ip}$  increase was attributed to an increased temperature setpoint, the subsequent  $T_{ip}$  decrease was attributed to excessive heat

loss, and the increase in EE was interpreted as supporting the initial temperature increase and compensating for the excessive heat loss. The skin lesions and eventual death remained essentially unexplained (2–4).

Recently, Everson (5) reported aerobic bacteria in the blood of five of six preterminal TSD rats, but not in their yoked controls; subsequently, we confirmed bacteremia in both of two preterminal TSD rats, but not in their yoked controls. These results imply that there was a breakdown in host defense against bacterial invasion. The association of a debilitated condition with the bacteremia suggested to Everson that the cause of death in TSD rats is septicemia and that the hypothermia late in deprivation resulted from bacteremia-induced vasodilation (5). The skin lesions were seen as a cutaneous manifestation of systemic bacterial infection, as well as likely portals of entry for bacteria (5). The lack of inflammation at the sites of skin lesions further suggested that the sepsis produced by sleep deprivation resulted from a weakened immune

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Accepted for publication February 1996.

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response (5). According to Everson (5), challenges to a weakened host defense might produce an elevated temperature setpoint as part of a febrile response (which could explain the early  $T_{ip}$  rise), hypermetabolism secondary to a cytokine response to bacterial assault (which could explain the high EE), and catabolism as a part of cytokine-induced cachexia (which could explain the weight loss). In terms of the earlier studies, the elevated setpoint deduced from the early rise in  $T_{ip}$  during TSD corresponds to the fever; the increased EE corresponds to the hypermetabolism, and the weight loss corresponds to cachexia. Thus, by Everson's (5) model, nearly all of the TSD effects could be explained as the product of a reduced host defense against bacterial invasion, thus supporting the concept that sleep is a part of host defense (6). However, as Everson (5) noted, it is also possible that the bacteremia resulted from end-stage events, as in multiple organ failure syndrome, and that the other TSD effects are independent of impaired host defense.

The present experiments were designed to evaluate the role of impaired host defense against bacterial invasion in the development of TSD effects. As in Everson's (5) work, blood was examined for evidence of bacteria. However, bacteria may enter the bloodstream incidentally and transiently, such as through skin lacerations, without necessarily indicating the severe, progressive impairment of host defense necessary to produce an elevated setpoint, hypermetabolism, and excess catabolism. Therefore, major organs of filtration—the kidneys, liver, and mesenteric lymph nodes—were also examined for bacterial colonization, which would then indicate dissemination of infection and a more generalized impairment of host defense than bacteremia alone. Such dissemination of infection could indicate impairment of acquired (lymphocyte-mediated) immune function. Bacterial invasion can also result from a failure of innate tissue barriers. Therefore, we also evaluated adherence of bacteria to the mucosal epithelium of the cecum as an indication of impaired tissue barriers to infection. Bacterial adherence to the gut wall is significantly correlated with gut permeability both *in vitro* (7) and *in vivo* (8), and it predicts septic complications following injury and infection [see Alverdy et al. (9) for review].

To test whether bacterial invasion was evident early in TSD and whether it was associated with well-established, early appearing effects of TSD (elevated  $T_{ip}$ , elevated EE, weight loss), the three bacterial measures and their relationships to the TSD effects were examined in rats subjected to TSD for 4 days. In a second experiment, rats subjected to 4 days of TSD were concurrently treated with a broad-spectrum antibiotic mixture to determine whether the TSD effects occurred in the absence of bacterial infection. In a third exper-

iment, rats were chronically sleep deprived until the late-appearing TSD effects (primarily lowered  $T_{ip}$ ) were observed; the rats were then treated with the antibiotics to determine whether the late TSD effects, including death, occurred in the absence of bacterial infection.

## METHODS

TSD was accomplished by the disk-over-water method described in detail elsewhere (1). Briefly, a TSD rat and a yoked control (TSC) rat were housed in rectangular Plexiglas cages. A single horizontal 46-cm-diameter disk, which could be rotated in a randomly chosen direction, formed a floor extending 17 cm into each cage. Under the disk and extending to the cage walls was a rectangular tray filled with tap water to a depth of about 2 cm. When sleep onset was detected in the TSD rat, the disk was rotated slowly, forcing both rats to walk in a direction opposite the disk rotation to avoid the water. When the TSD rat was spontaneously awake, the disk was stationary and the TSC rat was able to sleep.

### Animals and surgical procedures

All animal procedures were approved by the University of Chicago Animal Care and Use Committee. Subjects were male Sprague-Dawley rats  $115.4 \pm 6.2$  [mean ( $\bar{x}$ )  $\pm$  standard deviation (SD)] days old at surgery and age-matched for each replication. From their arrival in the laboratory, rats were maintained in constant light to flatten their diurnal sleep and temperature rhythms (10). Food and water were available *ad libitum* during all phases of experimentation. Surgical procedures have been previously described (1). Under pentobarbital anesthesia (55 mg/kg body weight), rats were surgically implanted with electrodes for recording electroencephalogram (EEG) and electromyogram (EMG). Transmitters (Barrows, Inc., Sunnyvale, CA) were implanted in the peritoneum to record  $T_{ip}$ .

### Experimental procedure

After a minimum of 1 week of postoperative recovery, rats were placed in the deprivation apparatus. During adaptation to the apparatus, a removable floor was placed over the disk and trays. After no more than 1 week, the floors were removed, water was added to the trays, and a baseline period was initiated. During baseline, the disk was rotated once per hour for 6 seconds to habituate the rats to rotation and to remove debris from the disk. Food intake and body weight were measured at the same time each day. EE was

calculated daily from caloric values of food intake and weight change, using a formula previously validated by indirect calorimetry (1). The baseline period continued until sleep, food intake, body weight, and temperature had stabilized in both rats ( $11.3 \pm 4.0$  days). Cage air temperature was thermostatically maintained at 29°C. Pan water temperature was 4°C lower than the cage air temperature. In some cases, a third age-matched rat in a standard wire cage in the same room served as a home cage control.

Electroencephalogram and EMG signals from both rats were transmitted sequentially to a polygraph, an analog-to-digital (A/D) converter, and a microcomputer (either an AIM 65; Dynatem, Inc., Irvine, CA or a DAP 2400; Microstar Laboratories, Inc., Redmond, WA). Summed absolute values of the EEG and EMG signals were then passed to an IBM-type personal computer (PC) for storage in 30-second epochs. The  $T_{ip}$  signal was detected by an AM receiver, passed to the microcomputer, and then passed to the PC for storage. Arousal stages were computer scored using the PASS system (11). Sample epochs from the 24-hour paper record were visually checked against the computer scoring for accuracy. Daily  $T_{ip}$  means were calculated as a function of arousal stage. We have previously shown that TSD rats with elevated  $T_{ip}$  engage in warming behavior (12) and increase EE when  $T_{ip}$  falls (12–14), indicating that temperature is below an elevated setpoint. Because TSD also has depressing effects on  $T_{ip}$ , the maximum increase over baseline of daily mean waking  $T_{ip}$  during deprivation ( $\Delta T_{MAX}$ ) was used as an indication of the increase in temperature setpoint produced by TSD.

After 5 days of stable baseline, deprivation was initiated. The microcomputer rotated the disk whenever it detected sleep onset in the TSD rat. Depending on the experiment, deprivation was continued for either 4 days or until the TSD rat showed a fall of  $>1^\circ\text{C}$  below baseline in waking  $T_{ip}$ .

### Sacrifice and microbiology procedures

Rats were deeply anesthetized with pentobarbital (55 mg/kg), the thoracic cavity was opened aseptically, and 5 ml of blood was collected from the heart. Blood culture bottles (tryptic soy broth w/SPS and  $\text{CO}_2$ , DIFCO Blood Culturing System; DIFCO Laboratories, Detroit, MI) were inoculated with 5 ml of blood. The blood culture bottles were vented and incubated for 24 hours at 37°C to detect aerobic bacteria. After 24 hours they were visually examined, subcultured to three media—MacConkey (for gram negative bacilli), CNA (for gram positive cocci), and blood agar plates (TSA with 5% sheep blood for gram positive cocci and gram positive and gram negative bacilli)—and incubated for

another 24 hours at 37°C. Microbes were identified in accordance with standard microbiological techniques using gram stains, the Analytical Profile Index (BioMérieux Vitek, Hazelwood, MD), and other biochemical tests as needed. Negative cultures were reported after 7 days of incubation, per standard procedure. Quantitative cultures were obtained on tissue samples from kidney, liver, mesenteric lymph nodes, and cecum. To preserve only bacteria adhering to the cecum, each cecal sample was vortexed in three successive baths of sterile saline for a total of 13 minutes, at the end of which the bath was clear (15). Each tissue sample was weighed and homogenized into a suspension using a sterile tissue grinder at 1:10 dilution (wt/vol). Tissue suspensions were serially diluted in 10-fold steps from 1:10 to 1:10<sup>6</sup> (1:10<sup>7</sup> for cecal samples) with sterile saline. Blood agar and MacConkey plates were inoculated with 0.1 ml of each dilution. Plates were incubated at 37°C for 24 hours, examined to determine the counts of colony-forming units per gram of tissue (cfu/g), and then incubated again. Final colony counts were compiled after plates had been examined daily for 3 days. Identification of organisms was accomplished as described above for blood cultures. All identification and quantification procedures were performed blind to experimental condition.

### Experiment 1

This experiment was performed to determine whether the early TSD effects (including sleep loss) are correlated with impaired host defense against aerobic bacteria. Because previous long-term sleep deprivation experiments (12–14) showed that increases in EE and decreases in weight were apparent by 4 days of TSD, and the early elevation of  $T_{ip}$  was usually still present, 11 sleep-deprived rats (D-4) and their yoked controls (C-4) were subjected to the experimental procedure for 4 days. Eight additional rats served as home cage controls (H-4) to establish normal levels of bacterial growth for our rat population. At the end of the deprivation period, D-4, C-4, and H-4 rats were sacrificed together. Blood and tissue samples were taken as described above.

### Experiment 2

To determine whether  $T_{ip}$ , EE, and weight loss effects of TSD could be mitigated by preventing bacterial adherence or systemic infection, six rats were treated with a broad-spectrum antibiotic cocktail while deprived of sleep for 4 days (D-4A rats). Yoked (C-4A) and home cage (H-4A) control rats were also given the cocktail. The components of the cocktail were bacitracin, 340

**TABLE 1.** Sleep as a percentage of total time for sleep-deprived (D) and yoked control (C) rats

	a) Experiment 1				b) Experiment 2			c) Experiment 3			
	Baseline		Deprivation		Baseline	Cocktail	Depriva- tion	Baseline		Deprivation	
	D-4	C-4	D-4	C-4				D-LA	C-LA	D-LA	C-LA
Total sleep	56.9%	57.0%	11.6%	34.1%	55.5%	55.6%	11.4%	58.6%	57.8%	10.4%	37.0%
	(3.9)	(4.2)	(2.4)	(5.2)	(2.3)	(2.3)	(3.4)	(4.4)	(5.4)	(3.1)	(6.7)
REM sleep	6.10%	6.22%	0.18%	1.39%	7.64%	7.05%	0.75%	6.60%	6.92%	0.27%	2.80%
	(0.55)	(0.80)	(0.16)	(0.58)	(1.05)	(1.08)	(1.01)	(0.85)	(0.61)	(0.39)	(0.37)

REM, rapid eye movement. Numbers in parentheses are standard deviations. See text for further explanation.

mg/kg/day (for gram-positive bacilli and cocci; Sigma, St. Louis, MO); neomycin sulfate, 500 mg/kg/day (for gram negative bacilli; Pharma-Tek, Huntington, NY); amphotericin B, 3.5 mg/kg/day (for fungal overgrowth; Squibb, Princeton, NJ); and ciprofloxacin, 12.5 mg/kg/day (for enterobacteriaceae; Miles, Inc., West Haven, CT). This composition is similar to others commonly used for gut decontamination (16). The antibiotics were dissolved together in sterile water and administered by gavage in two doses (each feeding was 0.5 ml). Prior to the start of antibiotic treatment, rats were habituated to the gavage procedure by administering tap water. Antibiotic administration was initiated after a stable baseline had been established and 4 days prior to the start of deprivation. All other procedures, including blood and tissue culture, were as described for Experiment 1.

### Experiment 3

This experiment was designed to investigate whether suppressing bacterial infection with a broad-spectrum antibiotic cocktail would block TSD effects and extend survival in long-term TSD rats. Six long-term deprived (D-LA), six yoked-control (C-LA), and four home cage control (H-LA) rats were studied. Deprivation was continued until death appeared imminent in the D-LA rats. Criteria for sacrifice at this point have been described in detail elsewhere (1) and include a  $T_{ip}$  decline of  $>1^{\circ}\text{C}$  below baseline and/or inability to negotiate disk rotation.

A doubled dose of the antibiotic mixture described in Experiment 2 was administered in two daily aliquots to D-LA and C-LA rats during the later portion of deprivation. The criterion for starting antibiotic treatment during sleep deprivation was a decline in mean daily waking  $T_{ip}$  below the baseline mean in the D-LA rat. This point is generally about three-fourths of the way through the survival period for an individual rat. At this point, rats usually recover completely from deprivation-induced effects if allowed to sleep, the critical point for survival being a drop in  $T_{ip} >1^{\circ}\text{C}$  below baseline (17). Antibiotics were also started in the

C-LA and H-LA rats on the same day as in the matched D-LA rat. All other procedures were identical to those described above. Because deprivation periods in D-LA rats were of unequal length, they were divided into quarters for within-deprivation comparisons across replications.

### Data analysis

Data analysis on deprivation categories was performed by analysis of variance (ANOVA), followed by two-tailed, Bonferroni-corrected *t* tests where appropriate. Correspondence between sleep loss, cecal measures, and quantitative measures of sleep deprivation sequelae was tested by multiple regression. Home cage control rats were assumed to undergo no changes in sleep-loss-related variables; degrees of freedom were correspondingly reduced. Blood measures, which were qualitative, were evaluated as a dependent variable by a test for difference in proportions and as an independent variable by ANOVA. Results were considered significant if probability (*p*) was  $<0.05$ .

## RESULTS

### Experiment 1

One (C-4) rat was removed from the experiment when its recording plug became detached after the start of sleep deprivation. Sleep during baseline and deprivation is given for D-4 and C-4 rats in Table 1a. The remaining sleep in D-4 rats was mostly fragmented, relatively low-voltage non-rapid eye movement (NREM) sleep, with probably little functional value (13). Because they were partially sleep-deprived, C-4 rats generally showed TSD effects in the same direction as, but smaller than, those of D-4 rats. (Differences between deprived and yoked control rats generally increase with longer deprivations.)

At the time of sacrifice all rats appeared healthy. None showed the conjunctivitis, chromodacryorhea, hypophagia, diarrhea, or disheveled fur that are signs

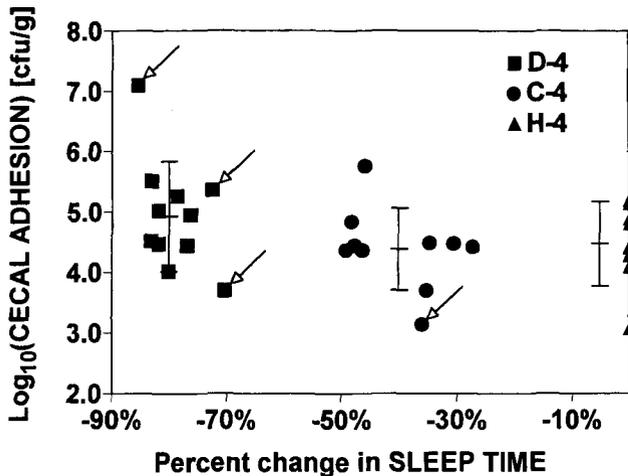


FIG. 1. Adhesion of aerobic bacteria to the cecum as a function of sleep loss in D-4, C-4, and H-4 rats. Error bars indicate mean  $\pm$  SD for log-transformed cecal data in the associated cluster. Arrows indicate rats with bacteremia.

of possible bacterial infection. Ten of 11 D-4 rats showed small inflamed papules on their tails; 6 of 11 had inflammatory foci or small papules on the plantar surfaces of their paws. These papules typically develop into paw and tail lesions in TSD rats.

Bacteremia was found in 3 of 11 D-4 rats (*Staphylococcus aureus*), 1 of 10 C-4 rats (*Streptomyces agalactiae*), and 0 of 8 H-4 rats. The differences between groups did not reach significance ( $z = 1.01$ ,  $p = 0.31$  for D-4 vs. C-4 rats;  $z = 1.61$ ,  $p = 0.11$  for D-4 vs. H-4 rats). ANOVA showed that, not surprisingly, sleep change was highly predicted by rat group (D-4, C-4, H-4;  $F_{2,17} = 400$ ,  $p < 0.00005$ ), but not by bacteremia ( $F_{1,17} = 0.06$ ,  $p = 0.81$ ).

The results from culturing sections from the organs of filtration did not support a diagnosis of systemic infection. Excluding one D-4 rat kidney sample that was contaminated during culturing, all kidney samples were free of bacterial growth. Liver and mesenteric node samples in all rats were either sterile or had low-level colony counts (i.e.  $< 2,500$  cfu/g). It is therefore possible that the bacteremia in the three D-4 rats and the one C-4 rat was transient or artifactual.

As expected, all cecal samples had bacterial growth. Geometric mean cecal growth was  $9.53 \times 10^4$  cfu/g in D-4 rats,  $2.70 \times 10^4$  cfu/g in C-4 rats, and  $3.34 \times 10^4$  cfu/g in H-4 rats (Fig. 1). A total of 34 different organisms were identified. In the ceca from the rats in this experiment, the most frequently identified dominant organisms were *Citrobacter freundii* (D-4), *Bacillus* species (C-4), and *Escherichia coli* (H-4). No species was concurrently present in both cecal and blood samples, nor did bacteremia predict log cecal adhesion ( $F_{1,27} = 0.40$ ,  $p = 0.53$ ), that is, there is no

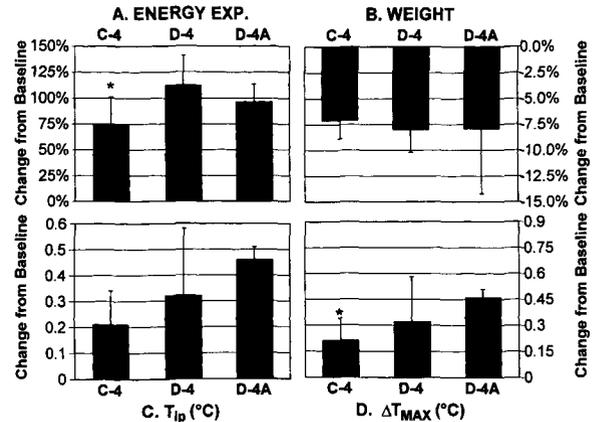


FIG. 2. Change from baseline (mean and SD) of C-4, D-4, and D-4A rats in: A) EE as a percentage; B) body weight as a percentage; C) Waking  $T_{ip}$ ; and D)  $\Delta T_{MAX}$ . The asterisk \* indicates difference from the D-4 mean at  $p < 0.05$ .

indication that bacteria in the blood resulted from translocation from the gut.

Although D-4 rats tended to have higher cecal colony counts, ANOVA on log-transformed cecal colony counts produced no significant difference across experimental groups ( $F_{2,26} = 1.47$ ,  $p = 0.25$ ) (Fig. 1). Similarly, regression of log cecal growth on sleep change produced only a nonsignificant trend ( $t_{19} = -1.65$ ,  $p = 0.11$ ). As can be seen in Fig. 1, much of that trend resulted from one outlying value in the D-4 rats.

Energy expenditure rose in both D-4 and C-4 rats during deprivation (Fig. 2A). ANOVA on percent change from baseline EE yielded a significant effect for experimental group ( $F_{2,17} = 53.4$ ,  $p < 0.00005$ ) and a nonsignificant effect for bacteremia ( $F_{1,17} = 1.5$ ,  $p = 0.24$ ). Regression of change in EE on sleep change and log cecal growth gave a strong inverse relationship between sleep change and EE ( $t_{19} = -9.9$ ,  $p < 0.00005$ ) but no relationship between log cecal growth and EE ( $t_{19} = -0.3$ ,  $p = 0.8$ ).

Analysis of variance on percent change from baseline weight (Fig. 2B) yielded a significant effect for experimental group ( $F_{2,17} = 46.9$ ,  $p < 0.00005$ ) and a nonsignificant effect for bacteremia ( $F_{1,17} = 1.2$ ,  $p = 0.29$ ). In regression analysis, weight change was strongly predicted by sleep change ( $t_{19} = 6.4$ ,  $p < 0.00005$ ) but not by cecal growth ( $t_{19} = -0.2$ ,  $p = 0.8$ ).

The temperature transmitter in one C-4 rat failed during deprivation, so nine C-4 rats were used in the  $T_{ip}$  data analysis. The increase over baseline in waking  $T_{ip}$  usually seen early in deprivation was observed (Fig. 2C). ANOVA on change from baseline waking  $T_{ip}$  yielded a significant effect for experimental group ( $F_{2,16} = 8.9$ ,  $p = 0.002$ ) and a nonsignificant effect for bacteremia ( $F_{1,16} = 0.9$ ,  $p = 0.36$ ). Regression to pre-

dict change in mean waking  $T_{ip}$  gave a strong inverse relationship with sleep change ( $t_{18} = -3.5$ ,  $p = 0.003$ ) and a weak positive relationship with cecal growth ( $t_{18} = 1.8$ ,  $p = 0.09$ ).

Analysis of variance on the maximal daily change from baseline waking  $T_{ip}$ ,  $\Delta T_{MAX}$  (Fig. 2D), yielded a highly significant effect for experimental group ( $F_{2,16} = 33.9$ ,  $p < 0.00005$ ) and a near-significant effect for bacteremia ( $F_{1,16} = 3.7$ ,  $p = 0.07$ ). Regression showed sleep change to be a strong negative predictor of  $\Delta T_{MAX}$  ( $t_{18} = -6.3$ ,  $p < 0.00005$ ), whereas  $\Delta T_{MAX}$  was not predicted by log cecal growth ( $t_{18} = 0.6$ ,  $p = 0.6$ ).

## Experiment 2

In this experiment, rats were treated with the antibiotic cocktail during the last 4 days of baseline and a 4-day deprivation to eliminate bacterial adherence and infection. The antibiotic treatment was completely effective in three of six D-4A rats, in that all blood, cecal, mesenteric node, kidney, and liver samples had no observed bacterial growth. Thus, these three rats were used for data analysis. One of the three D-4A rats was deprived only 3 days because it began to sleep in the water. The deprivation was ended rather than permit excess sleep on the fourth day. The single C-4A rat in which the antibiotic cocktail was completely effective had less sleep loss, lower EE, and a lower  $T_{ip}$  than the D-4A rats. The 8-day antibiotic cocktail regimen also produced sterile blood and tissue cultures in two H-4A rats with no obvious deleterious effects.

Sleep for the three D-4A rats is shown in Table 1b for baseline, the 4 days of antibiotic cocktail treatment, and 4 days of sleep deprivation plus cocktail. Repeated-measures ANOVA on sleep stages over baseline, cocktail, and deprivation plus cocktail was followed by  $t$  tests, which indicated that antibiotic treatment did not significantly change NREM sleep values. Antibiotic treatment did, however, produce a small but significant reduction in rapid eye movement (REM) sleep ( $p = 0.003$ ).

Antibiotic treatment did not affect EE. Mean EE was  $81.0 \pm 7.3$  kcal/day during baseline,  $80.1 \pm 17.8$  kcal/day during antibiotic treatment, and  $157.9 \pm 38.1$  kcal/day during deprivation. All rats had the characteristic EE rise during deprivation. ANOVA on EE repeated over baseline, antibiotic treatment, and deprivation yielded significance ( $F_{2,4} = 21.05$ ,  $p = 0.008$ ), but, due to the small number of rats, the increase between treatment and deprivation was not significant ( $p = 0.09$ ). The mean percentage EE increase during deprivation was  $96.3 \pm 17.2\%$  above treated baseline. This increase was not significantly different from the  $112.1 \pm 28.5\%$  EE increase in the D-4 rats ( $p = 0.41$ ) (Fig. 2A), that is, relative to the untreated

D-4 rats, antibiotic treatment did not affect the rise in EE during the 4-day deprivation period. Weight loss in D-4A rats ( $7.9 \pm 6.3\%$ ) was not significantly different from the 8.0% loss in D-4 rats (Fig. 2B).

The change in mean waking  $T_{ip}$  from baseline to antibiotic treatment was  $-0.03 \pm 0.11^\circ\text{C}$  and from treated baseline to deprivation plus treatment was  $0.46 \pm 0.05^\circ\text{C}$ . ANOVA on waking  $T_{ip}$  repeated over baseline, antibiotic treatment, and deprivation yielded significance ( $F_{2,4} = 70.80$ ,  $p = 0.001$ ). Although, as expected (18), antibiotics did reduce waking  $T_{ip}$  for about 1 hour immediately following the intragastric feedings, the difference between baseline and treated baseline in overall mean waking  $T_{ip}$  was not significant ( $p = 0.71$ ). However, the increase between treated baseline and deprivation was significant ( $p = 0.01$ ), that is, there was a characteristic TSD-induced early  $T_{ip}$  elevation. Both the mean waking  $T_{ip}$  increase and  $\Delta T_{MAX}$  ( $0.59 \pm 0.07^\circ\text{C}$ ) during deprivation were nonsignificantly higher than those of the untreated D-4 rats (Fig. 2C and D;  $p = 0.13$  and  $p = 0.52$ , respectively). Thus, neither absence of bacterial adhesion to the cecum nor absence of bacteria in the blood and organs of filtration affected the mean waking  $T_{ip}$  increase and  $\Delta T_{MAX}$  for the 4-day deprivation period.

Skin pathology in these three rats was also similar to that described in Experiment 1. Two of the three D-4A rats had small papules on their tails, and all three had inflammation or small papules on the plantar surface of their paws.

## Experiment 3

During long-term TSD, rats were given antibiotics beginning on the first day that  $T_{ip}$  in a D-LA rat fell to the baseline mean or below. Although the rats were acclimated to the gavage procedure with tap water for several days prior to the start of antibiotic treatment, two C-LA rats and two H-LA rats did not cooperate and died during a treatment. The remaining six D-LA, four C-LA, and two H-LA rats received antibiotics for a mean of  $4.4 \pm 3.2$  days. The two H-LA rats that did adapt to gavage showed no obvious deleterious effects from administration of antibiotics.

One D-LA rat died in the apparatus at the end of day 15 of deprivation. No meaningful blood or cecal measurements could be obtained from this rat. The other five D-LA rats were sacrificed when they could no longer manage disk rotation and when their  $T_{ip}$  values had fallen well below baseline. Sleep during baseline and the sleep deprivation period is shown in Table 1b. Mean  $T_{ip}$  immediately prior to sacrifice was  $4.27 \pm 1.74^\circ\text{C}$  below baseline. Mean survival of the D-LA rats,  $15.3 \pm 2.6$  days, was not significantly different ( $p = 0.36$ ) from the combined mean survival of oth-

erwise untreated TSD rats in three previous TSD experiments ( $17.1 \pm 7.4$  days) (12,13,19). On average the antibiotic treatment spanned the fourth quarter of survival time.

No bacterial growth was found in the blood, kidney, liver, or mesenteric lymph node samples of any of the rats in this experiment. Thus, by our measures, antibiotic administration was effective in preventing the development of bacteremia and sepsis in these chronic D-LA rats. Minor cecal bacterial adherence ( $<2 \times 10^4$  cfu/g) was present in two of five D-LA rats, two of four C-LA rats, and one of the two H-LA rats.

Repeated measures ANOVA on percent of baseline change in EE over the four quarters of deprivation yielded a significant group-by-quarter interaction ( $F_{3,24} = 5.581$ ,  $p = 0.005$ ). EE in the D-LA rats rose to  $104 \pm 52\%$  over baseline in the last quarter vs.  $35 \pm 22\%$  in the C-LA rats and vs.  $114 \pm 29\%$  in previously reported undosed TSD rats (13). The rate of weight loss during antibiotic administration was  $10.9 \pm 6.0$  g/day for D-LA rats vs.  $-0.03 \pm 11.4$  g/day for C-LA rats and vs.  $6.3 \pm 3.1$  g/day for fourth-quarter undosed TSD rats (13).

Because the temperature transmitter in one D-LA rat failed during deprivation, only five D-LA rats are included in the  $T_{ip}$  data analysis. (In this rat, antibiotics were started on the same day as for another D-LA rat that was being deprived simultaneously. The two D-LA rats had similar EE and, for 7 days prior to the transmitter failure, had similar patterns of temperature change. One survived 16 days, and the other survived 19 days.) D-LA and C-LA rats both showed an increase in mean waking  $T_{ip}$  over baseline in the first quarter, followed by a decrease to below baseline by the third and fourth quarters. ANOVA with repeated measures on group-by-quarter showed a significant interaction ( $F_{3,9} = 7.08$ ,  $p = 0.01$ ), that is, D-LA rats had larger initial increases in  $T_{ip}$  over baseline and/or larger late declines below baseline than the C-LA rats. Overall, the pattern of  $T_{ip}$  changes in D-LA rats was similar to that of other groups of TSD rats (12,14,20). Administration of antibiotics did not halt and may have contributed [by reducing the pyrogenic effects of enteric bacteria (18)] to the continuing decline in  $T_{ip}$ , but the  $T_{ip}$  decline to  $1.45^\circ\text{C}$  below baseline in the fourth quarter in D-LA rats is considerably larger than the  $0.03 \pm 0.11^\circ\text{C}$  produced by antibiotic administration alone in Experiment 2. The rate of temperature drop was unremarkable compared to that of previously tested sleep-deprived rats not given antibiotics (14).

All D-LA rats had characteristic severe lesions (21) on their paws and the dorsal and ventral surfaces of their tails. One C-LA rat developed a severe lesion on one paw. The remaining C-LA rats had smaller, much less severe paw and tail lesions than the D-LA rats.

Although the rats in this experiment were not formally rated for changes in appearance, all of the D-LA rats showed the typical fur deterioration and debilitated appearance previously described (13,21). The lesions showed no improvement after the antibiotic treatment began.

## DISCUSSION

### Are TSD effects mediated by invasion of aerobic bacteria?

The present results provide strong evidence that the major physiological effects of TSD are not mediated by invasion of aerobic bacteria. In the first experiment, sleep loss in 4-day rats strongly predicted EE increase, the early increase in mean and maximal daily waking  $T_{ip}$ , and weight loss, whereas neither adhesion of bacteria to the cecum nor bacteria in the blood significantly predicted any of these effects. Furthermore, in the second experiment, the protection of 4-day TSD rats by antibiotics did not prevent achievement of a  $\Delta T_{MAX}$ , skin pathology, and changes in weight, waking  $T_{ip}$ , and EE like those of unprotected D-4 rats. In the third experiment, antibiotic administration begun late in deprivation (but when recovery is usually possible if sleep is permitted) did not reverse the skin lesions, EE rise, the late temperature decline, nor did it lengthen survival, that is, sleep protects against these effects but antibiotics did not.

The antibiotics did not protect against anaerobic bacteria or viruses. Anaerobes could have replaced the aerobic bacteria and produced similar physiological effects. However, aerobic bacteria are the primary pathogens of septicemia (22), and Everson (5) found no anaerobes in the blood of near-terminal TSD rats. Furthermore, the skin lesions, EE rise, and late temperature decline were already in progress when antibiotic treatment began, so if these signs had been initially induced by aerobic bacteria, they should have been at least temporarily reversed.

The absence of evidence for mediation of TSD effects by bacterial invasion does not necessarily rule out the possibility that some TSD effects may be mediated by cytokines such as tumor necrosis factor or interleukin 1, that produce fever and cachexia, because cytokines may be elevated for other reasons, including inhibition of mechanisms that normally suppress their appearance. However, cytokine-mediated cachexia seems unlikely because it is accompanied by hypophagia [see Plata-Salamán (23) for review], whereas TSD rats in this, as well as previous studies (5,13), have been hyperphagic.

### When was host defense against aerobic bacteria compromised by TSD?

Everson's (5) discovery of bacteremia in five of six near-terminal TSD rats shows that TSD rats were suffering from impaired host defense at the point when death was imminent. What is the evidence for impaired host defense prior to that point? There is equivocal evidence during the first 4 days of TSD. Bacteremia was found more often in D-4 rats than in C-4 and H-4 rats, but the difference was not statistically significant. It was infrequent in both groups, appearing in 3 of 11 D-4 rats and 1 of 10 C-4 rats. Bacterial adherence to the cecum tended to be greater in the D-4 than in the C-4 rats, but again the difference was not significant. Furthermore, the virtual absence of bacteria in the organs of filtration indicates that there was no dissemination of bacterial infection such as might be expected from a generalized impairment of host defense. In fact, the complete absence of any evidence for bacterial dissemination could be taken to suggest that the bacteria that did appear in the blood samples represented minor, transient invasions of the bloodstream or artifact. Nevertheless, given Everson's (5) finding, with appropriate sample sizes we might expect to find a more reliable incidence of bacteremia somewhat later in deprivation.

The failure of antibiotic decontamination to extend survival implies that failure of host defense in TSD rats is a nonessential part of a more general lethal process, such as multiple organ failure syndrome, rather than the culmination of a chronic host defense deficit. The failure of three TSD studies (5,13,24) to reveal severe internal pathogenic foci also argues that infection was not chronically present during deprivation, but fulminant late in deprivation. Furthermore, because the antibiotic regimen was initiated under circumstances (prior to severe temperature drop) where recovery from TSD was still likely if sleep were permitted (17), it is likely that there is a primary deficit in some other vital function, perhaps one that initiates multiple organ failure syndrome, and that the host defense failure in undecontaminated TSD rats is secondary to that failure. We have previously shown that TSD rats still die if hypothermia is prevented (25) or the increase in EE is blunted and skin deterioration is blocked (26). Everson and Wehr (27) have shown that TSD rats still die if weight loss is prevented.

### Where does host defense fail?

Major routes for bacterial invasion are via the skin, lungs, and gut lining. Previous histology (5,13,24) has failed to produce indications of pneumococcal infection. The skin lesions are an obvious candidate route for bacterial invasion. Because allowing rats to sleep

produces obvious signs of recovery from the skin lesions in 2 days (17), the failure of the antibiotic regimen to produce such signs in 4.4 days argues against a role for skin lesions as external manifestations of internal bacterial infection or as foci of such infection. This is consistent with an earlier histological examination of lesion samples that were taken from TSD rats (21) and showed that bacteria could only be observed in clumps at the outer surface of the lesions. This lack of bacterial infiltration argues against the skin as an invasion route. If the lesions were uninfected, the relatively low level of immune response seen in their histology is not remarkable, and it does not contradict other findings (28) that showed no effect of sleep deprivation on several immune parameters, including *in vitro* lymphocyte proliferation responses to mitogens and *in vitro* and *in vivo* plaque-forming responses to antigens in near-terminal rats.

A major route of bacterial invasion in near-terminal conditions such as multiple organ failure syndrome is translocation of bacteria across the gut wall. The 100% success rate of the gut decontamination procedure in preventing bacteremia and sepsis suggests that, in addition to preventing growth of systemic bacteria, the procedure protected the sleep-deprived rat by removing the primary reservoir of invading organisms, enteric bacteria. Translocation as the source of bacteremia is also supported by earlier data (21) showing reduced cell replacement (mitosis) in the gut wall of near-terminal (but not mid-deprivation) TSD rats. However, the major argument for translocation as the source of weakened host defense is the evidence against other candidates.

The results of this study do not support reduced host defense against aerobic bacteria as a mediator of the TSD-induced regulated temperature rise, late temperature decline, increased metabolic rate (EE), weight loss, or skin pathology, or as a necessary mediator of the lethal process. These results, in conjunction with those of earlier studies, support a failure in host defense against bacteria only late in TSD and favor breakdown of the gut wall as a failed defense.

**Acknowledgements:** Support for this research was provided by U.S. Public Health Service grants MH-04151 and MH-18428. We thank Carol Zenko for her assistance in data collection and Ruth Benca, M.D., Ph.D., for her helpful comments on the manuscript.

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