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Pharmacology Biochemistry and Behavior, Vol. 53, No. 1, pp. 141–146, 1996

0091-3057/96 $15.00 + .00

0091-3057(95)02006-U

Norepinephrine in Mouse Spleen Shows Minor Strain Differences and No Diurnal Variation

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Received 19 August 1994

KELLEY, S. P., L. J. GROTA, S. Y. FELTEN, K. S. MADDEN AND D. L. FELTEN. *Norepinephrine in mouse spleen shows minor strain differences and no diurnal variation.* PHARMACOL BIOCHEM BEHAV **53**(1) 141–146, 1996. – Strain differences have been invoked to explain differing results when studying neural-immune interactions in laboratory animals. We investigated the splenic norepinephrine (NE) content and concentration in three strains of male mice (BALB/C, C57BL/6, and DBA/2), as well as possible diurnal variability in this innervation. Diurnal plasma corticosterone levels served as a positive control. Mice were housed on a 12 h on/12 h off light/dark cycle for 3 weeks, then sacrificed at one of six times during the 24 h cycle. Spleen NE total content and concentrations were determined using high performance liquid chromatography with electrochemical detection. We found small but significant differences between strains in total resting spleen NE content (BALB/C > C57BL/6 > DBA/2) and in resting NE concentration (C57BL/6 > BALB/C > DBA/2). This may reflect differences in spleen weight (BALB/C > DBA/2 > C57BL/6). The expected diurnal pattern of plasma corticosterone was seen in all strains, but no diurnal differences were found in NE content or concentration.

Catecholamines Circadian rhythm Mouse spleen Norepinephrine

SYMPATHETIC postganglionic noradrenergic nerves innervate lymphoid organs, including bone marrow, thymus, spleen, and lymph nodes (8–10). Studies investigating the complex role of the sympathetic nervous system in the modulation of the immune system have been conducted for the past decade (1) and currently continue (5,22,23). One approach to investigating sympathetic neural influences on immune function has been to remove these nerves and investigate individual cell functions or collective interactions of cells in the immune system. Chemical sympathectomy with the neurotoxin 6-hydroxydopamine (6-OHDA), to transiently destroy sympathetic nerve terminals in adult rodents, has been reported to be associated with (a) enhanced proliferation, altered cellularity (increased B cells, decreased T cells), an immunoglobin isotype switch (from IgM to IgG), and altered lymphocyte trafficking in lymph nodes of nonimmune mice; (b) diminished Con A-induced proliferation, cytotoxic T lymphocyte responses, IL-2 production, and delayed-type hypersensitivity responses in mice; (c) diminished or unaltered primary antibody responses to T-dependent antigen challenge, and enhanced responses to T-independent antigen challenge (12,16,18,21–24). Chemical sympathectomy in neonatal rodents (which is associated with a more long-term depletion of lymphoid organ norepinephrine content and has central nervous system effects) and surgical sympathectomy in adult rodents (which damages additional neuropeptide systems) are associated with enhancement of antibody responses (6,36).

Recently, characteristics such as strain, gender, age, and diurnal variability have been raised as possible variables influencing the effects of sympathetic noradrenergic signaling of lymphoid cells. For example, Livnat et al. (18) found that the C57BL/6 strain of mice showed a decrease in primary antibody response following sympathectomy with 6-OHDA, but only when they received higher 6-OHDA doses than the three other strains being tested; this effect appeared to depend on the dose of 6-OHDA needed to successfully denervate the spleen, not on the responsiveness of cells of the immune system to denervation. Lyte et al. (19) recently reported that chemical sympathectomy with 6-OHDA was associated with a change in mitogen-induced T cell proliferation in DBA/2 mice

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but not in C57BL/6 mice. They also reported a 6-fold difference in baseline splenic norepinephrine (NE) concentration between these two strains of mice; they hypothesized that strain differences in sympathetic innervation may be a reason for apparent conflicting results after chemical sympathectomy.

We sought to confirm this rather striking report of strain differences in splenic NE concentration. Such differences between strains might indicate a variable role for this neural mediator depending on the extent of innervation. It is also possible that circadian differences in splenic NE content may exist, so that sampling only once during 24 h could either mask a true difference between strains or conversely, exaggerate a difference that merely reflects a measurement at a rapidly changing phase of a diurnal variation.

Although we are unaware of any data in the literature regarding circadian rhythms of NE content in mouse spleen, several laboratories have reported circadian rhythms for NE in various peripheral organs of other rodent species. For example, in the rat, a circadian rhythm of NE was observed in the salivary, thyroid, and pineal glands (3,25,37). The golden hamster was reported to show a circadian variation of NE content in the spleen and heart (17). In mouse brain, Huie et al. (13) found evidence of a diurnal rhythm of NE concentration in the hypothalamus and cortex, with sampling at six different times during the 24 h cycle.

Basal splenic NE content and concentration in three strains of mice at six different time points over a 24 h cycle were measured to determine (a) whether there is a diurnal rhythm in splenic NE content or concentration, and (b) whether there is a variation in splenic NE content or concentration among these mouse strains. Plasma corticosterone, a hormone known to follow a diurnal rhythm and also modulate some aspects of neural-immune signalling (28), was measured as a positive control.

METHODS

Animals

A total of 144 male mice of three strains, BALB/c, C57BL/6, and DBA/2 (n = 48 of each strain), were obtained from Jackson Labs at age 4–5 weeks and housed two per cage. Cages were placed on shelves in large, ventilated, enclosed cabinets, each with its own set of fluorescent lights on a timer. All mice were housed 12L : 12D. Half the animals had lights on from 18:00 to 06:00; the other half had lights on from 08:00 to 20:00. Animals were allowed food and water ad lib.

The animals were housed for 2 weeks, then subsets of each strain were taken from the cabinets at six times over the 24-h cycle: 2, 6, and 10 h into the light cycle, and 2, 6, and 10 h into the dark cycle. We examined a total of eight mice per strain per time point. On four successive days, two mice of each strain were removed from their cabinets, and killed by quick cervical dislocation at 08:00, 10:00, 12:00, 14:00, 16:00, and 18:00 h. Blood was removed by cardiac puncture; spleens and lymph nodes were removed, weighed, and frozen on dry ice. These procedures took approximately 1 h. Those sampled in their dark cycle were handled and killed under dim red light.

Plasma Corticosterone

Blood was obtained immediately after sacrifice by heart puncture into a heparinized syringe, and spun at 3000 rpm for 10 min; plasma was immediately frozen at −70°C until assayed. A commercial murine corticosterone radioimmunoassay kit was used to determine plasma corticosterone (ICN Biomedicals). Each sample was analyzed in duplicate; results are reported in nanograms per milliliter.

Splenic Norepinephrine

Spleens were removed, weighed, frozen on dry ice, and stored at −70°C until assay. Spleens were thawed and homogenized using a sonic dismembrator (Fisher Scientific, NY) in 0.1 M perchloric acid (1 : 10, weight : volume) containing the internal standard 3,4-dihydroxybenzylamine (DHBA; 25 mM). The homogenates were centrifuged at 13,000 × g for 10 min and the pellets were saved for protein assays (Bio-Rad protein kit, Rockville Center, NY). The resultant supernatants were placed randomly in autosampler tubes, loaded into the autosampler, and high-performance liquid chromatography

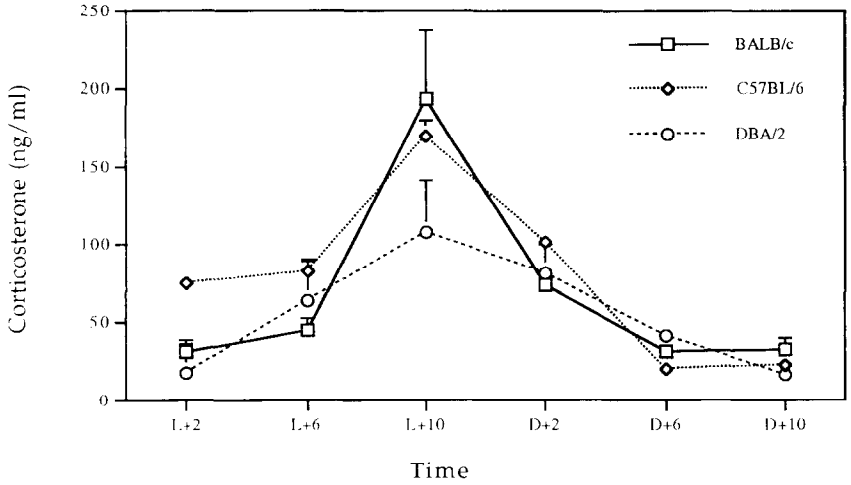

FIG. 1. Plasma corticosterone in BALB/c, C57BL/6, and DBA/2 mice at six time points over 24 h. X-axis labels of L + 2, +6, and +10 represent time points of 2, 6, and 10 h after lights come on; D + 2, +6, and +10 represent 2, 6, and 10 h after lights off. Each data point represents the mean for seven to eight mice. Vertical lines are SEM.

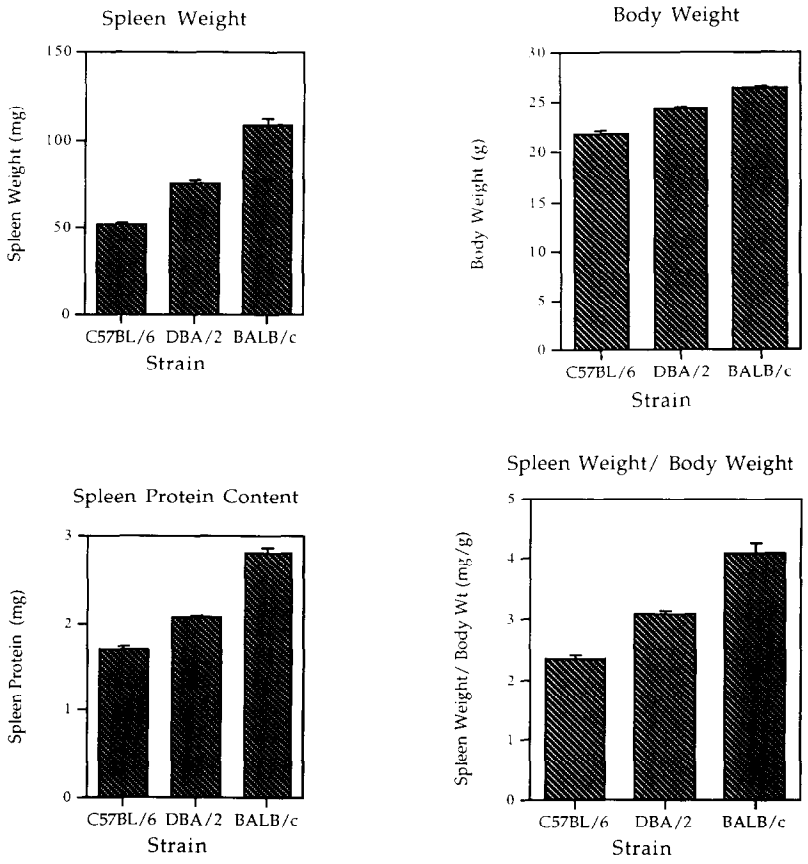

FIG. 2. Mean body weight, spleen weight, spleen weight/body weight, and spleen protein in three strains of mice. Each column represents the mean of 45–48 mice. Vertical lines are SEM.

with electrochemical detection was carried out to determine NE levels.

Samples were analyzed for NE using a Waters Model 510 solvent delivery system operated at a flow rate of 1.0 ml/min, a Waters 710B WISP automatic sample injector, an Econosphere 5 mM C18 reverse phase cartridge (Altech), and a glassy carbon thin-layer electrochemical detector (TL-5, Bioanalytical Systems). The detector potential was set at 0.85 V (vs. an Ag-AgCl reference electrode) using a LC-4B amperometric controller (Bioanalytical Systems). The buffer was 0.1 M citrate-0.1 M phosphate (pH 4.5), with sodium octyl sulfate (1.0–1.2 mM) used as the ion pairing agent, and 17–22% methanol. The signal from the detector was recorded, peak heights and areas were determined, and data analysis was done using a Waters Model 840 data and chromatograph control station. Recovery was determined using DHBA as an internal standard. Samples corrected for recovery were compared with external standards to determine NE concentration, expressed as picomoles NE per gram wet weight, per mg protein, and per total spleen.

Statistics

Significant differences between means for strain, time, and strain by time interactions were determined by use of analysis of variance (ANOVA). If a significant main event was identified ($p < .05$), posthoc analysis by Newman-Keuls was used to compare means. Significant differences are defined as $p < .05$.

RESULTS

Plasma Corticosterone

ANOVA of plasma corticosterone revealed no effect of strain, but a significant effect of time [$F(5,124) = 8.793, p < .0001$]. Posthoc analysis revealed that the sampling time at 10 h after lights on was significantly different from all other time points. There was no strain by time interaction (Fig. 1). Thus, as expected, a diurnal rhythm of plasma corticosterone was evident with the peak level occurring just prior to the animals' active cycle.

Body Weight, Spleen Weight, Spleen Protein Concentration

A significant effect of strain on body weight [$F(2,126) = 53.8, p < .0001$], spleen weight [$F(2,126) = 110.7, p < .0001$], spleen weight per body weight [$F(2,126) = 72.8, p < .0001$], and spleen protein content [$F(2,123) = 219.3, p < .0001$] was found. Posthoc analysis revealed that each strain was significantly different from all other strains. BALB/c mice were highest in body weight, spleen weight, spleen weight per body weight, and spleen protein content, followed by

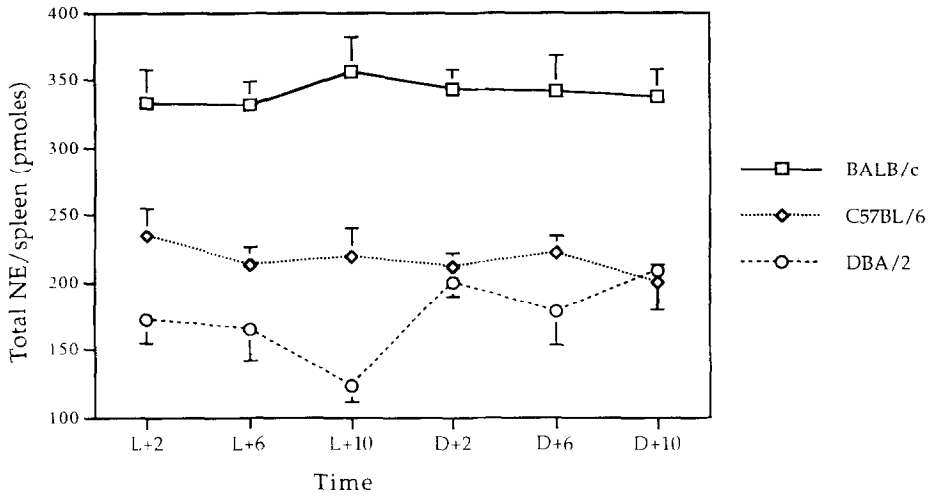

FIG. 3. Total norepinephrine per spleen in three mouse strains. A main effect for strain was found, but no effect of time of day. X-axis labels of L + 2, +6, and +10 represent time points of 2, 6, and 10 h after lights come on; D + 2, +6, and +10 represent 2, 6, and 10 h after lights off. Each data point represents the mean for eight mice. Vertical bars are SEM.

DBA/2 and C57BL/6. There was no effect of time and no strain by time interaction in any of these parameters (Fig. 2).

Norepinephrine Content and Concentration

No significant effect of time and no strain by time interaction for total NE/spleen, NE/mg spleen weight, or NE/mg protein was found. There was a significant difference between strains for total NE/spleen [$F(2,122) = 116.5, p < .001$] and posthoc analysis showed that each strain was significantly different from the other two strains, with BALB/c having the greatest NE/spleen (Fig. 3).

When NE is expressed as NE per mg spleen weight, we again found a main effect of strain [$F(2,122) = 32.6, p < .0001$]. Posthoc analysis showed that each strain was significantly different from the other two strains. The mice with the smallest spleen weight (C57BL/6) have the greatest NE/mg spleen weight (Fig. 4).

Similarly, one can also express the results as NE/mg spleen protein. We found a main effect of strain [$F(2,120) = 22.8$, $p < .0001$] for NE/mg protein. Posthoc analysis revealed that this measurement in DBA/2 was significantly smaller than in BALB/c and C57BL/6, but the latter two were not significantly different from each other (Table 1).

DISCUSSION

Splenic NE content does not vary as a function of time of day under basal conditions in young male BALB/c, C57BL/6, or DBA/2 mice. It should be noted that pulsatile increases in NE could have occurred between our sampling times. However, our method of sampling, consistent with many previous circadian studies (3,13,17,25,37), has not detected any striking circadian variability in the spleen in any strain. Resting plasma corticosterone did vary depending on time of day, as expected; peak levels occurred just prior to the beginning of the animals' active period. This finding is in agreement with previous reports from the literature (2,11,14).

We found only minor variations in basal spleen NE content and concentration between these three strains. NE/mg spleen weight in C57BL/6 male mice is twice that in DBA/2 mice. This finding agrees directionally with results reported in the literature (19); however, the magnitude of difference that we found was considerably smaller than the 6-fold difference reported by Lyte et al. (19). This discrepancy may be related to sample size differences (Lyte et al. studied an n of 4–5), vivarium or environmental conditions that could provoke stress-related alterations in cellularity of the spleen, or differences in experimental procedure, because the mice in our experiment had no prior handling, whereas it appears that the mice investigated by Lyte et al. received IP saline injections prior to sacrifice. The magnitude of these strain differences is unlikely to alter 6-OHDA effectiveness as has been suggested (18,19).

The lack of diurnal variation in splenic NE in all three

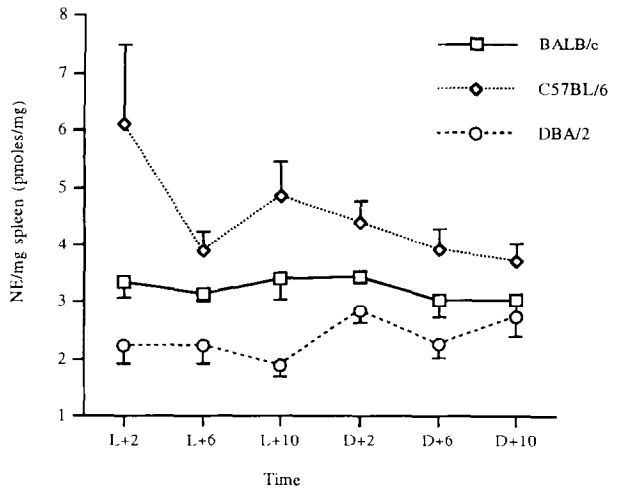

FIG. 4. Norepinephrine per mg spleen weight in three mouse strains. A main effect for strain was found, but no effect of time of day. Each data point represents the mean for eight mice. Vertical bars are SEM.

strains of mice suggests that the preganglionic autonomic neurons that regulate sympathetic outflow to the spleen in these mice are not under the influence of the suprachiasmatic nucleus of the hypothalamus, as are the neurons that regulate the outflow of other mediators that do show diurnal variability, such as the CRF-ACTH-glucocorticoid output from the HPA axis. It appears that circadian influences over sympathetic outflow are topographic, perhaps differentially driving central outflow to the superior cervical ganglion but not central outflow to the superior mesenteric/coeliac ganglionic complex, that innervates the spleen (4,30). Whether NE from sympathetic nerves to the thymus, derived mainly from the superior cervical ganglion (29), shows a diurnal variability remains to be determined.

Catecholamines, including NE, appear to be involved in the regulation of immune responses; in particular, they can enhance the initiative phase of an immune response (31,32). In agreement with these studies, denervation of secondary lymphoid organs results in diminished cellular immune responses in mice (18,20-23). Consistent with these findings in vitro and in vivo are recent data demonstrating the expression of β-adrenoceptors on resting TH1 cells (which produce cytokines that favor cellular immunity) but not on TH2 cells (which produce cytokines that favor humoral immunity) (26, 33). C57BL/6 mice demonstrate a predominant TH1 response, whereas BALB/c mice demonstrate a predominant TH2 response to a number of immunological challenges under a variety of environmental conditions (7,15,27,34,35). For example, individually housed BALB/c mice increase spleen cell production of IL-4 (a cytokine secreted by TH2 cells) compared with group-housed BALB/c mice, whereas individually housed C57BL/6 mice increase spleen cell production of IL-2 (a cytokine secreted by TH1 cells) compared with group-housed C67BL/6 mice (15). If it could be demonstrated that C57BL/6 mice, compared with BALB/c mice, have a much greater sympathetic noradrenergic innervation of lymphoid organs such as spleen, one might hypothesize that the presence of a greater amount of NE stimulates TH1 cells. One might thereby explain the propensity for TH1 responses in this strain because TH1 cells but not TH2 cells have beta-adrenergic receptors. However, because we found evidence for only a relatively minor difference (less than 2-fold) in such innervation in C57BL/6 compared with BALB/c mice, we suggest that strain differences in sympathetic nerves supplying the spleen may not be a prime signal in the determination of TH1 vs. TH2 predominance, although we do not rule out some contribution of neural NE in this TH1 predominance. Clearly, a comprehensive turnover study and a morphometric analysis of splenic compartments would be necessary to obtain greater certainty about the relative contributions of these parameters.

TABLE 1

NOREPINEPHRINE CONTENT AND CONCENTRATION IN THREE MOUSE STRAINS*

Strain	Total NE/Spleen (*p*moles)	NE/mg Spleen wt (*p*moles/mg)	NE/mg Prot (*p*moles/mg)
BALB/c	341 ± 8 (47)†	3.3 ± 0.1 (47)†	121 ± 3 (45)
C57BL/6	216 ± 6 (48)†	4.5 ± 0.3 (48)†	133 ± 8 (48)
DBA/2	175 ± 9 (45)†	2.4 ± 0.1 (45)†	85 ± 4 (45)†

*Mean ± SEM and (*n*) are presented.

†Significantly different from each of the other two strains, $p < 0.05$.

We consider the minor variability in NE content and concentration to be of little functional significance, as noted above. However, even minor differences between strains in content or concentration of NE could reflect differences in stored NE, extent of innervation, or utilization. The largest spleens (BALB/c, DBA/2) showed the lowest concentrations of NE, whereas the smallest spleens (C57BL/6) showed the highest concentration. It is our impression that the minor differences detected in the present study reflect mainly the difference in spleen size. The findings of a 2-fold or less difference among the three mouse strains and lack of diurnal variability does not support a hypothesis that altered 6-OHDA effectiveness among the strains is due to differences in splenic NE.

ACKNOWLEDGEMENTS

This research was supported by R37 MH42076 and a Lucille P. Markey Charitable Trust award. The authors thank Charles Richardson and John Housel for excellent technical assistance.

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