Comparative Study of Circadian Variation in Numbers of Peripheral Blood Cells among Mouse Strains: Unique Feature of C3H/HeN Mice

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We examined strain differences in numbers of blood cells and their circadian rhythms in male Jcl:ICR, BALB/cA, C57BL/6J and C3H/HeN mice. The total numbers of circulating white blood cells (WBCs) were increased during subjective day and night, and the peaks in the active period were common to all strains. However, the number of WBCs in C3H/HeN mice remained lower and plasma levels of corticosterone (CS) were slightly higher throughout the day compared with the other strains. The numbers of circulating red blood cells (RBC) also differed according to strain. The numbers of RBCs, hematocrit (HCT) and hemoglobin (HGB) were considerably lower in C3H/HeN mice compared with the other strains, although mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) were highest among the tested strains. We found that serum erythropoietin (EPO) levels were considerably higher in C3H/HeN mice than in the other three strains. The high EPO level might be related to the unique features of RBCs in C3H/HeN mice. The present observations provide basic information about the numbers of peripheral blood cells and their circadian rhythm in mouse models and also demonstrate a unique feature of C3H/HeN mice.

Key words blood cell; circadian; difference; erythropoietin; mouse; strain

Circadian variations in circulating blood cells have been recognized in various species. 1) Periodic changes in the numbers of circulating cells in the peripheral blood might be the result of multiple factors such as the distribution between the circulating and marginal cell compartment among the tissues and organs of the body, influx from storage sites, cell proliferation, release of cells formed de novo into the circulation, as well as cell destruction and removal. 2) The underlying mechanisms of circadian changes in circulating blood cells have not been fully elucidated, but the numbers of monocytes, natural killer (NK) cells, T and B cells, red blood cells fluctuate in a circadian manner. 3—6) Thus, to study the hematology aspect such as immune function, anti-tumor activities and hematopoiesis, circadian rhythms in the numbers of circulating blood cells must be considered.

Animal models are revolutionary tools for chronobiological studies that provide some obvious benefits. Experiments can be conducted in animals that would not be feasible in the humans, and genetic background as well as most environmental factors can be controlled. Such models have served as powerful tools for understanding the circadian rhythms of various biological parameters. However, the effect of the difference of genetic backgrounds on circadian rhythmicity of peripheral blood cells has not been studied in detail.

In most inbred mouse strains except for the C3H, a genetic defect in the rate-limiting enzyme N-acetyl transferase blocks the production of the pineal melatonin. 5) Interestingly, the number of circulating WBCs is sensitive to photoperiod via melatonin-mediated effects. 6) Therefore, the initial goal of the present study was to test the hypothesis that the absence of a circadian melatonin rhythm in the circulation affects the circadian profile of the circulating blood cells.

On the other hand, genetically engineered animals, such as transgenic and knockout mice are indispensable to elucidating the molecular mechanisms of many physiological functions. To breed knockout mice for investigations of these functions, parental or backcrossed strains must be appropriately selected. Although many knockout mouse lines have been generated and many physiological functions have been observed in vivo, the effect of the genetic background of strains has not always been considered. Consequently, a comparison of numbers of blood cells and their circadian rhythms in several strains of mice should provide useful information for experimentation using these mouse models.

In this study, we observed changes in the numbers of circulating WBCs and RBCs in Jcl:ICR, BALB/cA, C57BL/6J and C3H/HeN mice throughout the day to obtain fundamental information about strain differences in the number of peripheral circulating blood cells and their circadian rhythms. We also examined the plasma concentrations of CS and EPO in these strains of mice that related to the numbers of circulating WBSs and RBCs, respectively. The present observations should provide basic information for applying the mouse model to the chronobiological studies.

MATERIALS AND METHODS

Jcl:ICR, C57BL/6J, C3H/HeN and BALB/cA obtained from Japan Clea (Tokyo, Japan) were housed under a 12 h light–12 h dark cycle [LD 12:12; lights on 0:00—12:00 h] with food and water available ad libitum. Before blood sampling, mice were housed under constant darkness for 1 d, and blood sampling was started. Blood samples were taken 3 h intervals (2:00, 5:00, 8:00, 11:00, 14:00, 17:00, 20:00 and 23:00 h) and blood cells were enumerated using an automated counter (Sysmex F-800, Sysmex, Japan). Red blood...
cell (RBC) parameters were determined using an automated blood cell analyzer attached to the cell counter. Serum erythropoietin (EPO) and corticosterone levels were measured using Quantikine mouse immunoassay kits (R&D systems Inc., MN, U.S.A.) and Active Corticosterone EIA (Diagnostic Systems Laboratories, Inc., TX, U.S.A.), respectively.

All values are expressed as the means ± S.E.M. Fluctuations were statistically evaluated using the one-way ANOVA and group differences were tested by two-way ANOVA. Multiple comparisons among group mean differences were checked using the Bonferroni/Dunn test or Student’s t-test.

RESULTS

Figure 1 shows that the number of total WBCs was fluctuated in a circadian manner in BALB/cA (one-way ANOVA; \( F = 14.466, p < 0.0001 \)), Jcl:ICR (\( F = 5.302, p = 0.0009 \)), C3H/HeN (\( F = 14.675, p < 0.0001 \)) and C57BL/6J (\( F = 8.106, p < 0.0001 \)). Significant circadian variations were evident with a peak during the rest period and a trough during the active period. The number of WBCs obviously decreased at a time close to the rest-active transition. These phase relationships were comparable in the four strains of mice. Circadian fluctuation of WBC numbers significantly differed among specific strains (two-way ANOVA); C3H/HeN vs. C57BL/6J, \( F = 22.161, p < 0.0001 \); C3H/HeN vs. BALB/cA, \( F = 16.160, p = 0.0002 \); C3H/HeN vs. Jcl:ICR, \( F = 26.113, p < 0.0001 \) and Jcl:ICR vs. BALB/cA, \( F = 7.729, p = 0.0077 \). However, Jcl:ICR vs. C57BL/6J and C57BL/6J vs. BALB/cA did not significantly differ (\( F = 2.886, p = 0.1552 \) and \( F = 2.444, p = 0.1246 \), respectively). The number of WBCs in C3H/HeN significantly differed compared to other strains.

Figure 2 shows that the concentrations of corticosterone (CS) fluctuated in a circadian manner in BALB/cA (one-way ANOVA; \( F = 13.676, p = 0.0005 \)), Jcl:ICR (\( F = 7.405, p = 0.0046 \)) and C3H/HeN (\( F = 10.999, p = 0.0017 \)) mice. Although CS tended to fluctuate in C57BL/6J similarly to other strains, the difference was not significant (\( F = 1.939, p = 0.2119 \)). The CS level in C3H/HeN at 8:00 was significantly higher \((p < 0.05)\) than those in the other three strains.

Figure 3 shows the circadian variation in the numbers of RBCs, hemoglobin (HGB) level, hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and serum erythropoietin (EPO) levels in four strains of mice. Figure 3A shows that the number of RBCs in all strains slightly but significantly fluctuated in a circadian manner (one-way ANOVA): BALB/cA, \( F = 2.956, p = 0.0242 \); Jcl:ICR, \( F = 3.342, p = 0.0236 \); C3H/HeN, \( F = 3.926, p = 0.0059 \) and C57BL/6J, \( F = 4.710, p = 0.0033 \). The numbers of RBC in C3H/HeN and BALB/cA weakly fluctuated in a two-peaked circadian manner. Strain differences were ob-

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**Fig. 1. Circadian Variations in Peripheral Circulating WBCs in Four Mouse Strains**

Hatched and closed bars: rest and active periods, respectively. Values are shown as means ± S.E.M. \((n = 4)\). \( \Delta \), BALB/cA; \( \square \), C57BL/6J; \( \bigcirc \), Jcl:ICR; \( \bullet \), C3H/HeN. *Significant difference \((p < 0.05)\) observed between C3H/HeN and two or three other strains.

**Fig. 2. Circadian Variations in Serum CS Level in Four Mice Strains**

Hatched and closed bars: rest and active periods, respectively. Values are shown as means ± S.E.M. \((n = 4)\). \( \Delta \), BALB/cA; \( \square \), C57BL/6J; \( \bigcirc \), Jcl:ICR; \( \bullet \), C3H/HeN. *Significant difference \((p < 0.05)\) between C3H/HeN and three other strains.

**Fig. 3. Circadian Variations in Peripheral Circulating RBC (A), HGB (B), HCT (C), MCV (D), MCH (E), and Serum EPO (F) Level in Four Strains of Mice**

Hatched and closed bars: rest and active periods, respectively. Values are shown as means ± S.E.M. \((n = 4)\). \( \Delta \), BALB/cA; \( \square \), C57BL/6J; \( \bigcirc \), Jcl:ICR; \( \bullet \), C3H/HeN. *Significant difference \((p < 0.05)\) between C3H/HeN and three other strains.
vions in the amplitude of the numbers of circulating RBCs (Fig. 3A) between C3H/HeN vs. C57BL/6J (two-way ANOVA; $F = 424.431$, $p < 0.0001$), C3H/HeN vs. BALB/cA ($F = 378.783$, $p < 0.0001$), C3H/HeN vs. Jcl:ICR ($F = 194.667$, $p < 0.0001$), C57BL/6J vs. BALB/cA ($F = 7.657$, $p = 0.0084$), C57BL/6J vs. Jcl:ICR ($F = 11.592$, $p = 0.0017$) and Jcl:ICR vs. BALB/cA ($F = 24.418$, $p < 0.0001$). Compared with the other strains, the number of RBCs in C3H/HeN was extremely low. The amplitudes of hemoglobin (HGB; Fig. 3B) and hematocrit (HCT; Fig. 3C) correlated with those of the numbers of RBCs. These parameters were extremely low in C3H/HeN compared with the other strains, although mean corpuscular volume (MCV; Fig. 3D) and mean corpuscular hemoglobin (MCH; Fig. 3E) and the EPO level in C3H/HeN plasma were significantly higher than those in the other three strains (Fig. 3F).

**DISCUSSION**

The numbers of circulating WBCs involved in the immune defense of organisms are subject to high-amplitude circadian rhythms. In this study, we observed significant circadian variations in WBCs with a rest period peak and an active period trough in four strains of mice. The phase relationships were comparable in all four strains. However, the number of WBCs was slightly but significantly decreased in melatonin-positive C3H/HeN mice compared with the other three strains. The number of circulating WBCs is sensitive to photoperiod via melatonin-mediated effects. Circulating melatonin might be involved in the regulation of the absolute number of circulating WBCs by affecting the trafficking/redistribution of leukocytes between the blood and other immune compartments.

Mouse blood contains more circulating lymphocytes than human blood, a fact that is predominantly responsible for the peak number of circulating WBCs. The circadian rhythm of WBCs in nocturnal mice peaks at the beginning of the light period. On the other hand, the peak of circulating WBCs in diurnally active humans is at the time of the light–dark transition. Thus, the difference of about $180^\circ$ between circadian rhythms in diurnally active humans and nocturnally active rodents is essentially applicable to the rhythm of the numbers of WBC. Since the numbers of WBCs peaked in all strains during the active period, we speculate that the increase in the numbers of WBCs is related to an increase in murine immune function at this time. Studies of the relationship between basal circadian rhythm of WBC numbers and immune rhythms using mouse models might help to establish immunomodulatory therapies on a chronobiological basis.

The circadian fluctuation of plasma corticosteroids is significant both in mice and humans. Corticosteroids are also related to the numbers of plasma lymphocytes by causing some lymphocyte efflux from the vasculature and retention in the lymphatic circulation. The mechanism by which corticosteroids cause such movements may involve the expression of cell adhesion molecules (CAMs). The expression levels of CAMs such as L-selectin, ICAM-1, LFA-1a are highest when plasma cortisol concentration are maximal. Plasma levels of CS were slightly higher in C3H/HeN mice through out the day than in the other three strains. The decrease in the number of lymphocytes number after the CS concentration peaks reflects a decrease in the number of WBCs in mice. Therefore, the increased plasma CS concentration might contribute to the low WBCs count in the C3H/HeN strain. Thus, the CS-induced changes in CAMs expressions in leukocytes or endothelial cells might be involve in the low number of WBCs in C3H/HeN mice.

Circulating RBCs in diurnally active humans undergo obvious circadian fluctuation with the peak at the time of the light–dark transition. We still detected the slight circadian fluctuations of circulating RBCs in all strains with a peak during the light period. In addition to the numbers of RBCs, we compared HCT, HGB, MCV and MCH in four strains of mice. The MCV values reflect the size of the RBCs, whereas MCH and MCHC reflect the amount and concentration of hemoglobin of individual cells, respectively. The present study found considerably less RBCs number in C3H/HeN mice than in the three other strains, whereas the MCH and MCV were the highest among the four strains. The number of RBCs, hematocrit and MCV are increased in mice injected with EPO. We predicted that the EPO concentration would be low in C3H/HeN compared with other strains, whereas in fact it was high. Fewer RBCs which reflects a decreased ability to supply adequate amounts of oxygen in the circulating blood of C3H/HeN mice might be compensated by the higher MCH and MCV values that indicate a high oxygen maintenance capability of RBC. Thus, the high concentration of EPO might in part contribute to the higher MCH and MCV value in C3H/HeN mice.

On the other hand, circulating reticulocytes fluctuate with a circadian rhythm suggesting that these cells are periodically released from the bone marrow in a circadian manner as shown by Haus et al. Serum level of erythropoietin (EPO), which is an essential growth factor that promotes the growth, proliferation, and differentiation of erythroid lineage cells, affects the circadian rhythms of RBCs in humans. Except for C3H/HeN ($p = 0.019$), we did not detect a significant difference in serum EPO concentrations between active period and rest periods, but the levels tended to increase in all strains during the active period.

Circadian changes in the number of WBCs circulating in the peripheral blood might result from several factors. These include the distribution of circulating and marginal cell components or tissues and organs, influx from storage sites, cell proliferation, release of de novo cells into the circulation, cell destruction and removal. Hormonal mechanism regulate the robust circadian rhythms of the number of peripheral circulating WBCs. Glucocorticoids play an important role in regulating the trafficking/redistribution of leukocytes between the blood and other immune compartments. Such redistribution is involved in the circadian fluctuation of the number of WBC. On the contrary, the amplitude of RBC rhythms is very small and though of interest from a physiological viewpoint, it is most likely a consequence of changes in the distribution of circulating RBCs and plasma volume. These differences in the circadian regulation of WBCs and RBCs appear to be reasonable in terms of exerting their functions and to be reflected in their life spans of $<10$, and about $120$ d.

The numbers of circulating WBCs fluctuated in a circadian manner in all strains, and peaked during the active period. The numbers of circulating RBCs differed in amplitude and circadian rhythm number according to strain. The pres-
ent results therefore cannot be extrapolated to humans and thus require confirmation including the phase relationship to environmental synchronizers and to other circadian rhythms whenever feasible. However, considering the time structure of humans and mice, the present observations of four mouse strains is realistic for use in animal studies of chronobiology, for example as models for experimental chemotherapy.

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