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Circadian Variations of Bone Marrow Engraftability

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Circadian rhythms exist for hematopoiesis, but little is known about circadian variation of bone marrow engraftability and host ''acceptability''. Using a B6.SJL to C57BL/6J congenic transplant model, we chose 3-times with light on: Hours After Light Onset (HALO) 4, 8, and 12 and 3-times with light off: HALO 16, 20, and 24. The mice were conditioned on a 12-h light/dark cycles. Recipient mice (100 cGy) received 40 million cells. We demonstrated a significant variation of bone marrow engraftability into bone marrow, spleen, and thymus when donor animals were subjected to changes in their light/dark cycles. Two statistically significant nadirs in all three organs were observed at HALO 8 and 24 in experiments carried out in July, while an identical set of experiments in February analyzing engraftment in marrow and spleen showed nadirs at HALO 8, but not at HALO 24. Marrow progenitors from the July experiments showed nadirs at HALO 12 and 24. The percentage of progenitors in S phase peaked at HALO 8 and 24. Interestingly, there were no changes in the ability of host to accept grafts with changes in the light/dark cycles of host animals. Circadian variations of bone marrow engraftability are important and should be considered in bone marrow transplant strategies. J. Cell. Physiol. 200: 63–70, 2004. © 2004 Wiley-Liss, Inc.

The phenotype of the engraftable stem cell is still a work in progress. Recent studies have shown that the cell shows a fluctuating phenotype as it transits cell cycle under cytokine stimulation (Habibian et al., 1998). When highly purified murine lymphohematopoietic stem cells are exposed to a cytokine cocktail, these relatively dormant cells progress to S phase in approximately 16–18 h, and through the first mitoses by 36–38 h. Thereafter, population doubling occurs every 12 h for at least five doublings (Reddy et al., 1997). The kinetics of cell cycle progression may be more rapid after in vivo engraftment, as studies using in vivo hydroxyurea treatment have indicated entrance into S phase by at least 12 h (Nilsson et al., 1997). In addition, 5-flurouracil (5-FU) treated bone marrow displayed an engraftment defect probably induced by induction of stem cells to enter the cell cycle (D'Hondt et al., 2001). When the engraftment capacity of unseparated marrow cells, cultured under identical condition was evaluated in normal or irradiated hosts, striking fluctuations were observed. Engraftment was lost in late S/early G2 and regained in the next G1 phase (Habibian et al., 1998). Time course studies showed that engraftment, as measured at 8 weeks and 6 months, fluctuated dramatically over 2–4 h intervals. Recent data indicated that alterations in engraftment maybe secondary to defects in immediate stem cell homing (Cerny et al., 2002). This, in turn, appears to be secondary to alteration in cell surface adhesion protein expression (Becker et al., 1999; Berrios et al., 2001). While these studies were from in vitro cultures in which marrow cells were exposed to cytokines, they raised the question of whether circadian rhythms might have a possible influence on the observed fluctuating stem cell phenotype.

The existence of circadian rhythms within the hematopoietic system has been recognized since the late 1940s (Goldeck, 1948). Differentiated cell types and different progenitor classes, including day-8 CFU-S, CFU-GM, BFU-E, CFU-E, and CFU-GEMM have been shown to exhibit distinct circadian rhythms and to show seasonal variations (Aardal and Laerum, 1983; Stevold et al., 1988; Smaaland et al., 1992). In addition, bone marrow susceptibly to cytotoxic drugs has been shown to vary according to circadian and seasonal rhythms (Levy

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et al., 1988). This provided evidence for use of chronomodulated chemotherapy administration. Little is known about circadian variation of bone marrow engraftability and about circadian modulation of the "host acceptability" to bone marrow. We have investigated these two topics in vivo using a B6.SJL (Ly-5.1) to C57BL/6J (Ly-5.2) congenic transplant model. To provide a quantitative endogenous competitive transplant model our recipient mice were sublethally irradiated (100 cGy) and infused with high levels of marrow, 40 million unseparated murine marrow cells. These in vivo engraftment analyses were complemented by determination of circadian variation of bone marrow lineage type, progenitor numbers, and cell cycle status.

MATERIALS AND METHODS

Mice

Male B6.SJL (Ly-5.1) and male C57BL/6J (Ly-5.2) mice were purchased from Jackson Laboratory (Bar Harbour, ME) 6- to 8-weeks-old and maintained under virus-free conditions. They were housed at least 1 week before experimental use and were given food and acidified water ad libitum. All experiments were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Housing cabinets

We constructed light/dark cabinets covered in black contact paper (Flexcon) to ensure no light penetration. Black nylon curtains covered the door-side of cabinets. Each cabinet was composed of three shelves. There was a light fixture internally for each shelf and a fan for each cabinet. Each cabinet was controlled and programmed for light/dark by a chrontrol XT timer. The space in one cabinet was adequate to house nine mouse cages. We used five cabinets in all.

TRANSPLANTATION MODEL

In our initial experiments, mice were on a 12-h light and 12-h dark (L:D::12:12) cycle. Male mice were used in order to avoid effects of estrus. Bone marrow collection time was the only variable. There were six bone marrow collection timepoints: HALO (Hours After Light Onset) 4, 8, 12, 16, 20, and 24 (Table 1). Mice were entrained in their light/dark cabinet for 2 weeks prior to marrow harvest. The actual harvest times were at 10:00 AM, 02:00 PM, and 06:00 PM. At each HALO, a light and dark group were harvested and transplanted. Each cabinet had a different light or dark onset time, which made this possible. Donor mice: B6.SJL (Ly-5.1) were sacrificed 1 h before transplant. The time of injection for the hosts was consistently at HALO 9 (Fig. 1). Recipients C57BL/6J (Ly-5.2) received, 100 cGy whole body irradiation. Radiation was given at a rate of 92–94 cGy/min in one fraction using a cesium 137 gamma source (gamma cell 40; Nordian, Kanata, Ontario, Canada) at least 2 h prior to transplant. They were injected via tail vein with 40×10^6 B6.SJL cells within 60 min of marrow harvest. Volume of injection was 0.5 ml. Percent engraftment was analyzed in bone marrow, spleen, and thymus 10 weeks after transplant using fluorescence-activated cell sorter (FACS) analysis and cell staining with Ly-5.1 and Ly-5.2 monoclonal antibodies.

In separate experiments, we determined whether there were circadian rhythms for the ability of a 100 cGy irradiated host to accept a donor marrow cell graft. In these experiments we essentially reversed the groups used for our previous experiments on circadian rhythms of donor engraftable stem cells. Here recipients were staggered in light/dark cabinets and donor mice were harvested all at the same circadian time: HALO 9. Donor mice were B6.SJL (Ly-5.1) and recipients were 100 cGy irradiated C57BL/6J (Ly-5.2) mice. Forty million donor cells per recipient were injected via tail vein. Volume of injection was 0.5 ml. Percent engraftment was determined in bone marrow, spleen, and thymus 10 weeks post transplant by FACS analysis.

CHIMERISM DETERMINATION

Bone marrow from two tibiae and two femurs per recipient was flushed using Hank's balanced salt solution (HBSS)/1% heat inactivated fetal bovine serum (HI FBS) (Hyclone, Logan, UT). Spleen and thymus were crushed between two frosted slides and rinsed with 5 ml HBSS/1% HI FBS. Two million cells were washed one time in 3 ml HBSS/1% HI FBS. Cells were

TABLE 1. Circadian time

Hours After Light Onset (HALO)	Clock time
4	10:00 AM
8	02:00 PM
12	06:00 PM
16	10:00 PM
20	02:00 AM
24	06:00 AM

Lights were on a 12-h light/dark cycle.
Time points investigated in our studies. There were three time points with light on (HALO 4, 8, and 12) corresponding to the resting period of mice and three time points with light off (HALO 16, 20, and 24) corresponding to the activity period of mice. Lights were on a 12-h light/dark cycle. The corresponding clock times are presented in the last column.

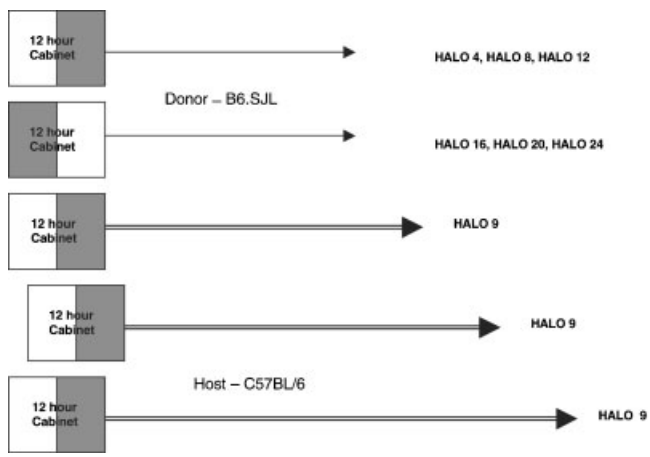

Fig. 1. Conditioning and transplant model. Schematic representation light/dark schedule. The mice were housed in five cabinets, which were on a 12-h light/dark schedule. Conditions were kept constant for all groups of mice. The first two cabinets were on exactly opposite light/dark schedule and housed donor mice. The last three cabinets were used for recipients. Donor marrow was harvested at Hours After Light Onset (HALO) 4, 8, 12, 16, 20, and 24. The actual harvest times were at 10:00 AM, 2:00 PM, and 6:00 PM. At each HALO, a light and a dark group were harvested and transplanted. The recipients were all injected at HALO 9.

resuspended in 200 μl HBSS/1% HI FBS. Cells were incubated with fluorescein isothiocynate (FITC)-conjugated rat anti-mouse Ly-5.2 antibody (1.0 μg/10^6 cells; PharMingen, San Diego, CA) and biotinylated rat anti-mouse Ly-5.1 antibody (1.0 μg/10^6 cells; PharMingen). Cells were incubated 30 min at 4°C in the dark. Cells were then washed in 3 ml HBSS/1% HI FBS. They were resuspended in 200 μl HBSS/HI FBS. Cells were then incubated 30 min with streptavidin-allophycocyanin (0.75 μg/10^6 cells; Molecular Probes, Eugene, OR) at 4°C protected from light. After washing and resuspension in 200 μl HBSS/HI FBS, 0.1% propidium iodide (Sigma Chemical Co, St Louis, MO) was added. Cells were then analyzed by FACS (MoFlo; Cytomation, Inc., Fort Collins, CO). The percentage of engraftment in recipients was calculated using the formula: number of Ly-5.1 cells/number of Ly-5.1 cells + number of Ly-5.2 cells.

Lineage analysis of donor bone marrow cells

A sample of bone marrow cells from each HALO group was obtained. Two million cells were washed in HBSS/1% HI FBS and resuspended in 200 μl of the same solution. Antibodies were added at a concentration of 0.25 μg per million cells. We used CD45R (B220) and Ly-6G (Gr-1) antibodies (PharMingen). Samples were incubated for 30 min at 4°C protected from light. Cells were washed in 3 ml HBSS/1% HI FBS and resuspended in 200 μl of the same solution. Cells were then incubated for 30 min with streptavidin-allophycocyanin (0.75 μg/10^6 cells) at 4°C protected from light. After washing and resuspension in 200 μl HBSS/HI FBS, 0.1% propidium iodide was added. Cells were then analyzed by FACS.

Colony assays

Donor bone marrow samples from each HALO group was collected. Colony-assays were performed on each sample as previously described (D'Hondt et al., 2001). Cells were counted and cultured at a cell density of 20,000 per plate in the double-layer nutrient agar system using plastic tissue culture dishes. Underlays were α-minimal essential medium (α-MEM) with 20% FBS supplemented with vitamins and L-glutamine. Seven growth factors (colony-stimulating factor 1 [CSF-1] = 7,500 U/dish, granulocyte-macrophage colony-stimulating factor [GM-CSF] = 3.75 ng/dish, granulocyte colony-stimulating factor [G-CSF] = 7.5 ng/dish, IL-1α = 375 U/dish, IL-3 = 150 U/dish, recombinant rat stem cell factor [rrSCF] = 150 ng/dish, and basic fibroblast growth factor [bFGF] = 7.5 ng/dish) were included in an 1 ml underlayer with a final agar concentration of 0.5%. Cells were included in a 0.5 ml overlays with a final agar concentration of 0.3%. Culture dishes were incubated at 37°C for 14 days in presence of 5% O_2, 10% CO_2, and 85% N_2. Five plates were set up for each group. Plates were scored on a dissecting microscope for high-proliferative potential colony-forming cells (HPP-CFC, highly dense colonies > 0.5 mm in diameter) and colony-forming unit-culture cells (CFU-c, all cell clusters with more than 50 cells and not fulfilling the HPP-CFC criteria).

Thymidine suicide assay

Donor bone marrow samples from each HALO group were collected. The in vitro ^{3}H-thymidine suicide technique was used as previously described by Quesenberry and Stanley (1980). Concentrations of 1×10^6 marrow cells/ml were incubated at 37°C for 30 min in HBSS containing 200 μCi/ml ^{3}H-TdR (Dupont New England Nuclear; NET-027X, specific activity = 20.0 Ci/mM) or a concentration of unlabeled "cold" thymidine (Sigma Chemical Co, T-9250) equivalent to that in the ^{3}H-TdR incubation, in the presence of 5% CO_2. Following this incubation, the cell suspension was diluted by the addition of 30 ml of cold HBSS containing 10% HI FBS and 100 μg/ml of unlabeled thymidine. Cells were then centrifuged and resuspended in 10 ml of cold HBSS containing 10% HI FBS and 100 μg/ml of unlabeled thymidine for a second wash. Cells were resuspended in 0.5 ml of cold HBSS containing 10% HI FBS and cultured at an equivalent cell density of 20,000 per dish (based on the original count). Cells were plated in the double-layer nutrient agar system using plastic tissue culture dishes as described above. Five cultures with ^{3}H-TdR or unlabeled thymidine were set up for each group in an experiment. The number of cells in cycle was determined by percent reduction of CFU-c or HPP-CFC induced by ^{3}H-TdR exposure.

Statistical analysis

For the analysis of engraftment data, percent engraftment was calculated taking a C57BL/6J (Ly-5.1) male control as 100% and a B6.SJL (Ly-5.2) male control as 0%. Pair-wise comparisons were performed and the level of statistical significance set at 0.05. For the analysis of colony assay, HPP-CFC and CFU-c are presented as the number of colonies per dish. Samples from each time point were compared together. For the analysis of in vitro ^{3}H-TdR suicide assay, results are presented as the percentage of kill induced by ^{3}H-TdR. Pair-wise comparisons were made and the level of statistical significance set at 0.05. Graphs display the mean and the standard error of the mean. The non-parametric Wilcoxon rank-sum test was used for comparison. All *P*-values are two-sided. These statistical results were confirmed and extended using a one-way ANOVA and Bonferroni multiple comparison statistics.

RESULTS

Variation of bone marrow engraftabilty

In the summer (July) three sets of experiments were performed in which engraftment was determined at HALO 4, 8, 12, 16, 20, and 24. In each individual experiment, marrow from 5 B6.SJL mice was harvested at each HALO and then 10 week engraftment in marrow, spleen, and thymus determined in 5 C57BL/6J mice. Thus, there were a total of three experiments analyzing a total of 15 mice at each individual HALO. In the three experiments there was a reproducible nadir at HALO 8 and 24 (Fig. 2). The combined results are presented in Figure 3. HALO 8 was significantly less than the immediately preceding HALO 4 ($P = 0.0004$) and HALO 24 was significantly less than the preceding HALO 16 ($P = 0.00001$). Pooled results from the engraftment results for spleen and thymus were similar to those for marrow with biphasic diurnal nadirs at HALO 8 and 24 and with significant differences from preceding HALO of 4 and 16 ($P \leq 0.0003$) (Figs. 4 and 5).

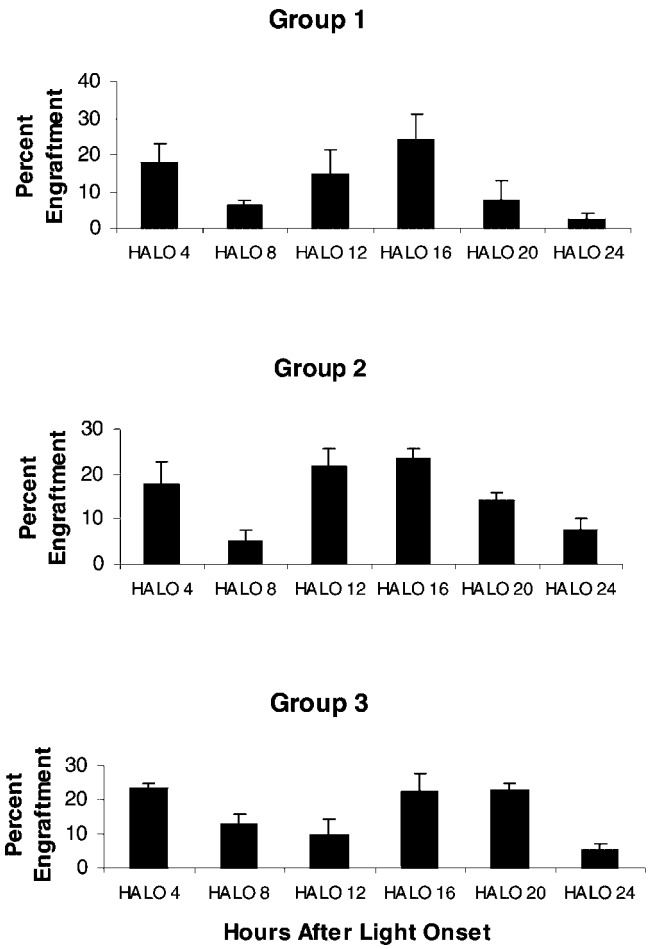

Fig. 2. Percent engraftment of three individual experiments carried out in July. The Ly-5.1/Ly-5.2 congenic transplant model was utilized. The mice were entrained in light/dark boxes for 2 weeks prior to study. Donor marrow (B6.SJL) was harvested at different time points in light/dark cycle (HALO 4, 8, 12, 16, 20, and 24). Host mice (C57BL/6) were irradiated with 100 cGy and were injected with 40.0×10^6 whole bone marrow cells at HALO 9. Same sex male transplants were carried out. Percent engraftment was determined 10 weeks post transplant by fluorescence-activated cell sorter (FACS) analysis determining the ratio of Ly-5.1/(Ly-5.1 + Ly-5.2). Each HALO in each group represented pooled cells from five individual mice.

These data were also analyzed using a one way ANOVA and the Bonferroni multiple comparisons test. Significant differences were seen in marrow engraftment with comparisons of HALO 4 vs. 8, 4 vs. 24, 8 vs. 16, 12 vs. 24, and 16 vs. 24. Spleen engraftment showed significant differences for HALO 4 vs. 8, 4 vs. 24, 8 vs. 16, 12 vs. 24, and 16 vs. 24. Similarly, there were significant differences in thymus engraftment with HALO 4 vs. 8, 4 vs. 24, 8 vs. 16, 12 vs. 24, and 16 vs. 24.

A similar number of experiments and mice were analyzed in a different season, February. Here marrow engraftment was studied. These results were different from those seen in July, in that the only definite nadir observed was seen at HALO 8 which when analyzed by a one-way ANOVA was significantly different from HALO 4 and 20; the difference between HALO 8 engraftment

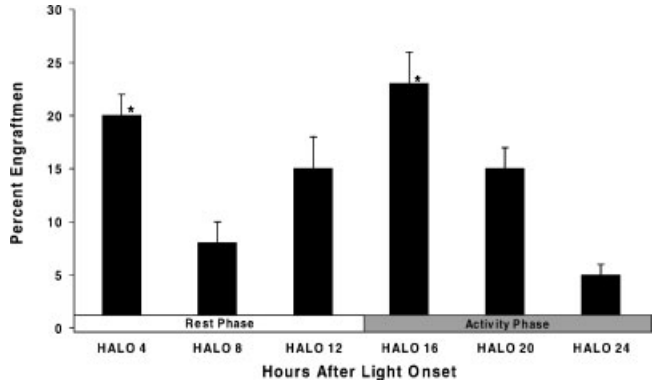

Fig. 3. Mean percent engraftment of bone marrow from three experiments carried out in July. The mean percent engraftment from the experiments presented in Figure 2. These data represent three different experiments with a total of 15 donor mice per time point. *$P \leq 0.0004$ from HALO 8 and 24.

and other HALO was less dramatic (Fig. 6). Thus, a uniphasic diurnal rhythm was present.

Variation of host engraftability

Two consecutive studies were carried out. They used the same design as the previous three except that here we analyzed circadian rhythms of recipient mice. All bone marrow were collected at the same circadian time: HALO 9, but injections into hosts were done at different host HALO; HALO 4, 8, 12, 16, 20, or 24. Each experiment included 15 mice per HALO studied. We found no statistically significant difference between groups, suggesting the absence of circadian variation of host acceptability to infused bone marrow. Results are presented in Figure 7A,B.

Lineage analysis of donor bone marrow

In two experiments we also assessed donor cell representation in different lineages in pre-engraftment marrow tissue. These data are presented in Figure 8. Results are expressed as the percentage of bone marrow cells positive for a given lineage antibody. There was relatively little circadian variation with the infused

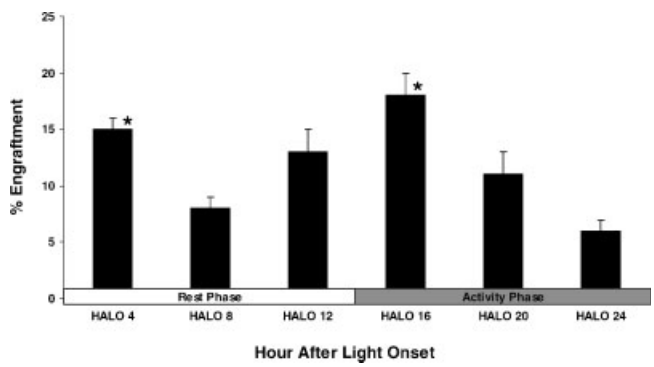

Fig. 4. Mean percent engraftment of spleen from three experiments carried out in July. The assessment of spleen cell engraftment in the same experiments presented in Figure 2. *$P \leq 0.00002$ from HALO 8 and 24.

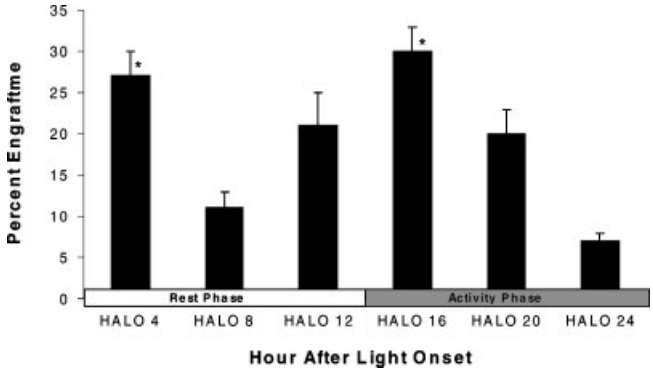

Fig. 5. Mean percent engraftment of thymus from three experiments carried out in July. The assessment of thymus cell engraftment in the same experiments presented in Figure 2. $^*P \leq 0.0003$ from HALO 8 and 24.

marrow. B cells (B220 antibody) appearing decreased at HALO 12 and 24 while granulocytes (Gr-1 antibody) appeared increased at HALO 12 and 24.

Clonogenic and thymidine suicide tests. HPP-CFC and total colonies (seven hematopoietic growth-factor-responsive) showed a rhythm. However, the nadir in the rest phase was HALO 12 contrasting with the nadir of engraftment. A second statistically significant nadir was observed during the end of the activity period (HALO 24). This one coincided with the engraftment nadir (Fig. 9).

To assess the cell cycle status (% cell in S phase) of progenitors we performed thymidine suicide tests. The cycling status of HPP-CFC and total colonies is shown in Figure 10. At time of engrafting nadir (HALO 8 and 24) there was a high-proportion of progenitor cells in S phase.

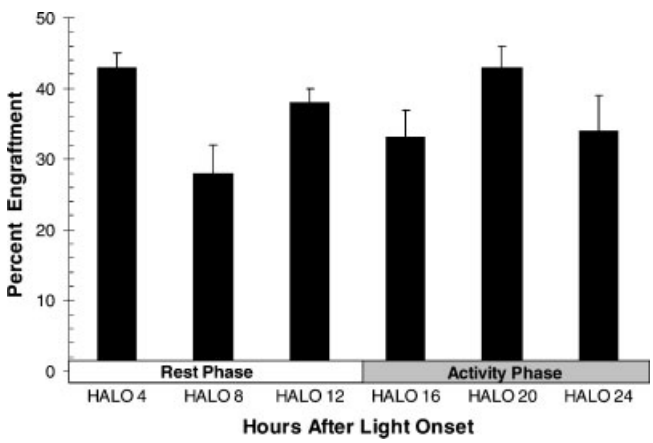

Fig. 6. Percent marrow engraftment from an experiment carried out in February. B6.SJL (Ly-5.1) and C57BL/6 (Ly-5.2) mice were entrained in light/dark boxes for 2 weeks prior to the transplant. The marrow was harvested at different time points in light/dark cycles (HALO 4, 8, 12, 16, 20, and 24). Host male mice (B6.SJL) were irradiated with 100 cGy and were injected at HALO 9 with 40.0×10^6 C57BL/6 whole bone marrow cells. Percent engraftment into marrow was determined 10 weeks post transplant by FACS analysis determining the ratio of Ly-5.1/(Ly-5.1 + Ly-5.2). Each HALO represents pooled cells from 15 individual mice.

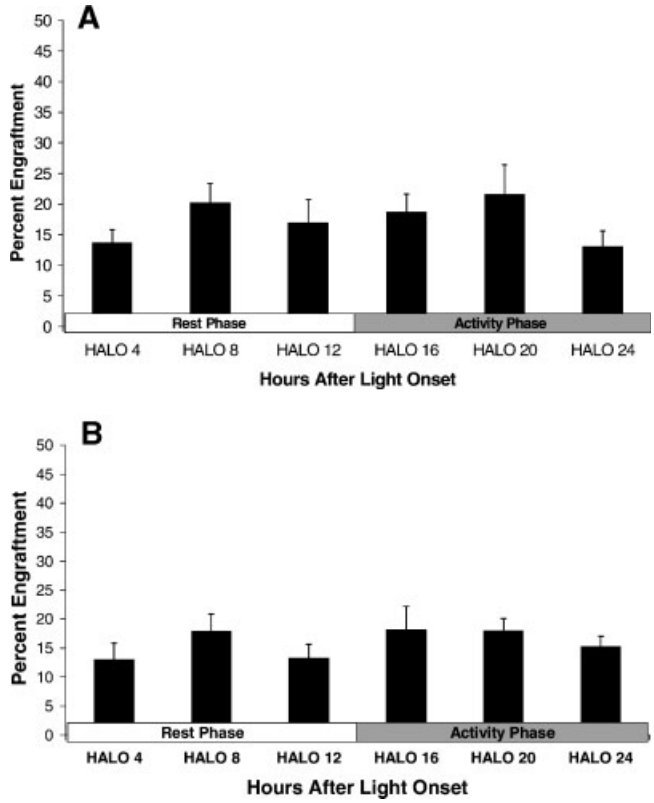

Fig. 7. Host engraftability. In these experiments we essentially reversed the groups used for our previous experiments on circadian rhythms of donor engraftable stem cells. Here the recipients were staggered in light-dark boxes and the donor mice were harvested all at the same circadian time, 9 (or HALO 9). Donor mice were male B6.SJL mice (40 million injected cells) and recipients were 100 cGy treated male C57BL/J mice. Percent engraftment was determined at 10 weeks post-transplant by FACS analysis determining the percent Ly-5.1 cells. **A**: The mean percent engraftment of 6 different times (15 host mice per HALO) in 3 separate experiments are presented. **B**: The mean percent engraftment of three additional experiments.

DISCUSSION

Daily rhythms of biologic activities of plants and animals are universal phenomena (Takahashi and Zatz, 1982). Ordinarily, the alteration of environmental light and darkness synchronizes these rhythms to the natural day-night cycle. However, even in the absence of periodic environmental timing cues, many rhythms continue to oscillate with periods slightly different from that of the day-night cycle to which they were previously entrained. That is they "free run" with approximate 24 h (circadian) periods. The persistence and properties of such environmentally-independent, self-sustaining rhythms suggests the existence of an innate time-keeping mechanism, i.e., a "biological clock". Such a clock consists of three functional components: an input (afferent) pathway for entrainment to light-dark or other cycles, a circadian pacemaker that actually generates the oscillation, and an output (efferent) pathway that regulates the expression of measurable rhythms. The dominant circadian pacemaker is located in the suprachiasmatic nucleus of the hypothalamus (Meijer and Rietveld, 1989). The existence of circadian rhythms

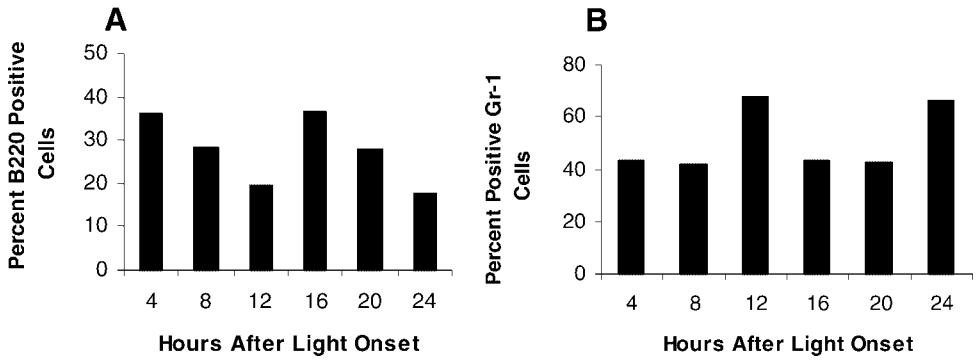

Fig. 8. Lineages in pre-engraftment marrow. Pre-engraftment BM donor cell expression of different lineages markers. Whole bone marrow from each HALO group was incubated with two different monoclonal antibodies to investigate the different cell populations. We used CD45R (B220) and Ly-6G (Gr-1). Cells were analyzed by FACS. **A**: Cells were decreased at HALO 12 and 24 **B**: Granulocytes were increased at HALO 12 and 24.

within the hematopoietic system was recognized a long time ago. Differentiated cell types and different progenitor classes, including day-8 CFU-S, CFU-GM, BFU-E, CFU-E, and CFU-GEMM have been shown to exhibit distinct circadian rhythms and to show seasonal variations (Smaaland, 1997; Wood et al., 1998; Kolaczkowska et al., 2001). This was demonstrated by measurement of DNA synthesis, mitotic index, or clonogenic tests. Results from different studies have not always been consistent, since other factors may play an important role: interstrain variations (Kolaczkowska et al., 2001); influence of sex, age, and season may all be important. It seems, however, that the DNA synthesis is the highest during the activity (dark) period in mice. The mitotic index is at its highest approximately 12 h later; the second half of the activity period. Wood et al. (1998) showed that erythroid and myeloid bone marrow subpopulations had different circadian patterns with respect to the percentage of cells incorporating BrdU. The acrophase for colony-forming unit was found to be in the late activity or early rest phase of mice (Smaaland, 1997). In addition, Wood et al. (1998) showed that multipotent and early colony numbers each exhibit 24-h rhythms, while later progenitor colony numbers exhibit two peaks per day. In healthy humans the number and the proliferative activity of $CD34^{+}$ cells in bone marrow has been shown to vary along the circadian time scale (Abrahamsen et al., 1998). In addition bone marrow susceptibility to cytotoxic drugs has been shown to vary according to circadian and seasonal rhythms providing

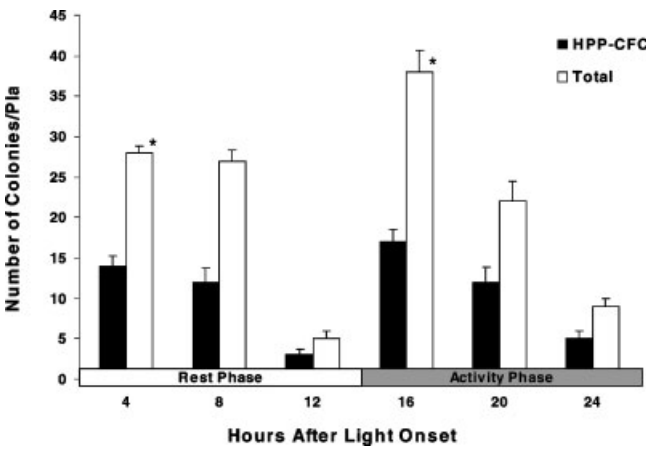

Fig. 9. HPP-CFC and total colonies. Circadian variations of the number of HPP-CFC and total number colonies. Five plates were set up for each group. Whole bone marrow cells from donor mice at each HALO (4, 8, 12, 16, 20, and 24) were plated in a double layer agar system at 20,000 cells per dish (5 plates per HALO). Seven-factor responsive HPP-CFC were counted and analyzed at 14 days. HPP-CFC were considered highly dense colonies = 0.5 mm in diameter. CFU-c were counted as colonies with >50 cells. Total progenitors were combination HPP & CFU-c counts. These data were based on three individual experiments. Two statistically significant nadirs were observed at HALO 12 and 24 for both HPP-CFC and the total number of colonies *($P \leq 0.004$) from HALO 4 and 16, respectively.

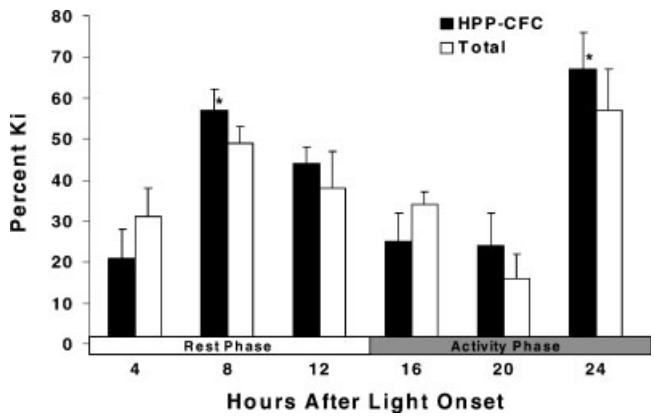

Fig. 10. Cell cycle analysis. Percent kill (Ki) of HPP-CFC by ^{3}H-thymidine according to HALO time of BM harvest. Whole bone marrow was collected at each HALO time. Five plates for cold and for ^{3}H-thymidine were set up for each HALO. The percentage of cells in S phase was determined by the number of cells killed by ^{3}H-thymidine. Plates were scored on a dissecting microscope after an incubation of 14 days. These data were based on three individual experiments. A high proportion of cells in S phase was observed at HALO 8 and 24 for both HPP-CFC and the total number of colonies. *$P \leq 0.002$ from HALO 4 and 16.

rational for the use of chronotherapy (Levy et al., 1988). The same pattern of response was observed for stimulatory drugs. Ohdo et al. (1998) reported bone marrow circadian response to G-CSF in mice as did Wood et al. (1998) for Epo. Recent studies have shown that bone marrow expresses *mPer1* and *mPer2*, known regulators of the clock system. In addition, the expression pattern of these two genes exhibited two peaks over a 24-h period (Chen et al., 2000). Other authors found that melatonin, an important hormone for the circadian regulation, was present in bone marrow at high concentration. They also showed that bone marrow was able to synthesize melatonin (Conti et al., 2000).

Little is known about circadian variation of bone marrow engraftability and about circadian modulation of the host engraftability. We have investigated these two topics in vivo using a B6.SJL (Ly-5.1) to C57BL/6J (Ly-5.2) congenic transplant model. To increase global levels of engraftment our recipient mice were sublethally irradiated (100 cGy) essentially giving a competitive transplant assay in which donor cells are competed against residual host marrow cells (Stewart et al., 1998). We also evaluated circadian variation of bone marrow clonogenicity and cell cycle status. We showed a clear diurnal rhythm for 10 week engraftment into marrow, spleen, and thymus when mice were studied in the summer. Nadirs of engraftment were seen at HALO 8 and 24 with peaks of engraftment at HALO 4 and 16. The rhythm of engraftable stem cells was different from that for progenitors (HPP-CFC and CFU-c). Progenitors also show a diurnal rhythm, but significant nadirs were seen at HALO 12 and 24 rather than at HALO 8 and 24. Peaks remain at HALO 4 and 16. The cell cycle status of primitive (HPP-CFC) and total progenitors were also determined. There were significantly increased numbers of progenitors in S phase as determined by tritiated thymidine suicide at HALO 8 and 24, the same time points as observed for the engraftment nadirs. Others have reported diurnal circadian patterns for progenitors (Stevold et al., 1988) and for mPer1 and mPer2 marrow expression (Chen et al., 2000). Presumptively periodic diurnal expression of clock genes could underlie the circadian alterations in engraftable stem cells and progenitors seen here.

We have previously reported that stem cell engraftment varied reversibly with cell cycle transit (Habibian et al., 1998). More recently we have found reversible changes in progenitor cell numbers and a major change in stem cell gene expression when engraftment is suppressed during cell cycle transit (Lambert et al., 2003). These data have formed a basis for the hypothesis that stem cells exist on a cell cycle continuum in which the phenotype is constantly changing in a reversible fashion (Quesenberry et al., 2002). The interplay of circadian rhythm with the cell cycle-related phenotype changes remains to be elucidated.

We also assessed whether there was an intrinsic rhythm to the capacity of a host's marrow cavity to accept incoming stem cells, Our studies in this regard revealed no evidence of an engraftability rhythm.

We also observed a shift in the engraftment circadian rhythm when engraftment was evaluated in February rather than in July. Here there was a significant, but less impressive, nadir of marrow engraftment at HALO 8 but no nadir at HALO 24. Seasonal fluctuations in circadian rhythms have been clearly described for CFU-GM from February to June to November (Stevold et al., 1988). Our data shows that there is a clear seasonal change from February to July, the only seasonal periods evaluated.

One might speculate that the suprachiasmatic nucleus, which determines cytokine rhythms, might underlie these stem/progenitor cell rhythms, but there is as yet no data on this point. The initial data with the Per-1 luc transgenic indicates that intrinsic cellular clocks may be critical, although this, of course, could be influenced by rhythmic cytokine exposure.

Circadian rhythms represent fundamental life processes and clearly impact on virtually all biologic characteristics. The present study shows a double rhythm for marrow cell engraftment and indicates that such a rhythm needs to be considered in experimental studies on stem cell engraftment and probably should be factored into strategies for clinical stem cell engraftment.

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