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Brief Report

CIRCADIAN RHYTHM OF CYTOKINE SECRETION FOLLOWING THERMAL INJURY IN MICE: IMPLICATIONS FOR BURN AND TRAUMA RESEARCH

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ABSTRACT—Although there are many reports of circadian variation in hormone secretion, there are only a few reports on the relationship between circadian rhythm and cytokine production. The aim of the present studies was to investigate whether there is a circadian effect on cytokine production of splenic lymphocytes and adherent splenocytes in mice after burn or sham injury. We selected day 7 after injury for our determinations because we have previously shown day 7 is the time of maximal suppression of T cell IL-2 and IFNγ production and maximal increase in adherent cell proinflammatory cytokine secretion in this model. IL-2 and TNFα were chosen as reference cytokines since the former is known to be produced by T cells and the latter by adherent cells of the innate immune system. The results showed that seven days after sham or thermal injury both T cell IL-2 and adherent cell TNFα production were altered by time of injury or time of cell harvest. IL-2 secretion was significantly decreased in burn compared to sham animals when splenocytes were harvested in the morning; the decrease was non-significant when splenocytes were harvested in the afternoon. TNFα secretion was significantly increased in burn vs. sham adherent cells only when injury took place in the morning. The observed circadian variations in cytokine production could have a significant effect on cytokine levels measured in clinical and animal studies of injury and may explain some of the reported discrepancies among these studies.

KEYWORDS—IL-2, TNFα, burns

INTRODUCTION

Although there are many reports of circadian variation in hormones, which may regulate the immune response, there are only a few reports on circadian rhythm and cytokine secretion. It was previously reported from our laboratory that there is a circadian rhythm of IL-2 production by peripheral blood mononuclear cells (PBMC) from normal volunteers with higher phytohemagglutinin (PHA) induced IL-2 secretion at 12 PM compared with cells obtained from the same volunteers at 8AM (1). Petrovsky and Harrison (2) reviewed work that has been reported regarding the chronobiology of cytokine secretion in human blood, noting that maximal production of proinflammatory cytokines IFNγ, TNFα, IL-1 and IL-12 occurs during the night and early morning when circulating cortisol is lowest and circulating white cells, in particular $CD4^+$ T cells, are at their peak. They showed further that there is a diurnal rhythm in T helper-1 (Th1)/Th2 cell balance with alternating periods of cellular or humoral responses within any 24 h period (3). Colombo et al. observed circadian effects on IFNγ secretion in a study of the effect of melatonin on cytokines in mice (4).

None of the above studies addressed the question of circadian changes in cytokine production after injury. Therefore the present study was undertaken to determine whether or not there was circadian regulation of production of cytokines by macrophages of the innate and splenic lymphocytes of the adaptive immune system in mice after burn or sham burn injury. We believed that if significant circadian effects on cytokine production after injury were found, this information would be helpful to other investigators working in this field.

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MATERIALS AND METHODS

Thermal Injury

As previously described (5) 8-week-old male A/J mice received either sham burn or burn injury. A standard 25% full thickness scald burn to the dorsum was administered by inducing general anesthesia with intraperitoneal (ip) pentobarbital (60 mg/kg diluted in about 0.75 mL sterile non-pyrogenic 0.9% saline), shaving the dorsum of the mouse, placing the animal in a plastic template and immersing for 9 seconds in 90EC water. Animals were dried and resuscitated with 1 mL of sterile 0.9% saline ip after burn. Sham burn animals underwent the same procedure, but were immersed in thermoneutral water. All procedures were carried out after review and with permission of the Harvard Medical School's Standing Committee on Animal Research using NIH guidelines.

Cytokine determination

Mice were sacrificed and splenocytes isolated and cultured as previously described (5, 6). Total splenocytes were cultured at 2×10^6/well in 96 well plates with or without concanavalin A (Con A, 250 ng/well; Sigma Chemical Co. St. Louis, MO;) for IL-2 synthesis. Adherent cells were selected by culturing splenocytes (200ul/well) at a concentration of 1×10^7/mL in similar plates for 1 hour at 37°C in complete medium. Plates were then washed three times with 200 μL/well of serum free medium to remove non-adherent cells and adherent cells were cultured in 200 μL serum-free medium/well with or without LPS (*E.coli* 026:B6, 25 ng/well Difco Laboratories Detroit, MI). The concentration of ConA and LPS used in these studies were those previously found to induce optimal cytokine production in this model. After 24 h in culture, supernatants were harvested, pooling similar wells and frozen at −20°C until assay. Supernatants from all mice were assayed individually and all supernatants from each experimental group were measured in the same cytokine assays. IL-2 was measured by ELISA (Endogen, Boston, MA). TNFα was determined by ELISA as previously described (6). Based on multiple prior studies (5–7) IL-2 was selected as a representative T cell cytokine and TNFα as a typical proinflammatory product of adherent cells of the innate immune system.

Statistical analysis.—Results of cytokine production were compared using the Mann-Whitney test for non-parametric data and one way analysis of variance (1 way ANOVA with Student Newman-Keuls post hoc analysis) using the statistics program GraphPad InStat for Windows 95 (GraphPad Software, San Diego, CA). Data are indicated as different if $p < 0.05$ and all results are given as mean ± standard error of the mean (SEM)

RESULTS

Circadian rhythm of cytokine secretion after sham or burn injury

The experimental protocol is shown in Figure 1. We looked at the time of day of sham/burn injury as well as the time of harvest of splenocytes, 7 days post injury. As noted above, IL-2 and TNFα were chosen as reference cytokines for Con A stimulated T lymphocytes and LPS stimulated adherent cells, respectively. Mice were given either a sham or thermal injury at either 8AM or 2 PM, 14 mice had a thermal injury procedure performed at 8 AM (AM), 14 mice served as sham controls. At 2 PM (PM) 14 mice underwent thermal injury and 14 mice served as sham controls. At day 7 after thermal injury 7 AM sham and 7 AM burn mice were sacrificed at either 8 AM (AM/AM) or 2 PM (AM/PM) and 7 PM sham and 7 PM burn mice were sacrificed at either 8AM (PM/AM) or 2 PM (PM/PM) and splenectomy was performed for splenocyte isolation and for T cell (IL-2) and adherent cell (TNFα) cytokine determination. All supernatants were tested in the same cytokine assays.

Effect on IL-2 secretion

Decreased IL-2 secretion by Con A stimulated splenocytes 7 days post injury is a hallmark of this burn model (5, 7). IL-2 secretion by Con A stimulated splenocytes (Fig. 2) was significantly decreased 7 days following thermal injury when splenocytes were harvested in the morning (AM/AM or PM/AM; $P = 0.0041$ and $P = 0.0006$ respectively sham vs. burn), regardless of time of sham or burn injury. IL-2 secretion was highest when mice were injured or sham injured in the morning and splenocytes harvested in the AM. When splenocytes were harvested in the afternoon, whether sham/burn had occurred in the AM or PM, there was no significant difference between burn and sham injured mice ($P > 0.1$ sham vs. burn AM/PM or PM/PM). When sham groups were compared by one way ANOVA all other sham groups were significantly different from the AM/AM group ($P < 0.05$). When burn groups were compared all other groups were significantly different from the burn AM/AM group ($P < 0.05$)

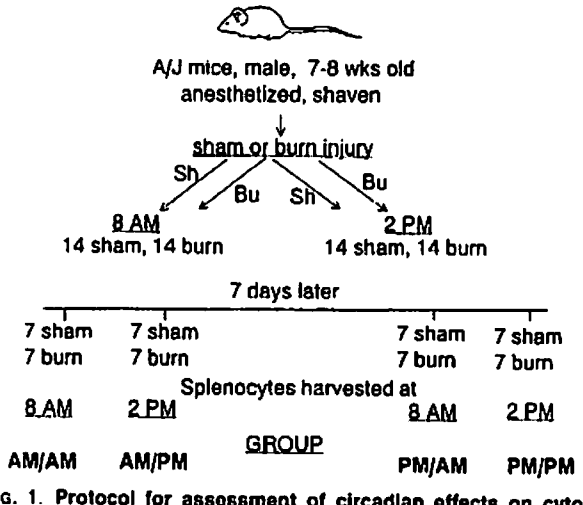

FIG. 1. Protocol for assessment of circadian effects on cytokine secretion in mice given either sham or burn injury in the morning at 8 AM or the afternoon at 2 PM and splenic lymphocytes and adherent cells harvested and cultured in the morning or afternoon, respectively, 7 days later. Eight different groups of 7 mice resulted from this protocol: injury/harvest- AM/AM, AM/PM, PM/AM or PM/PM for sham and burn groups.

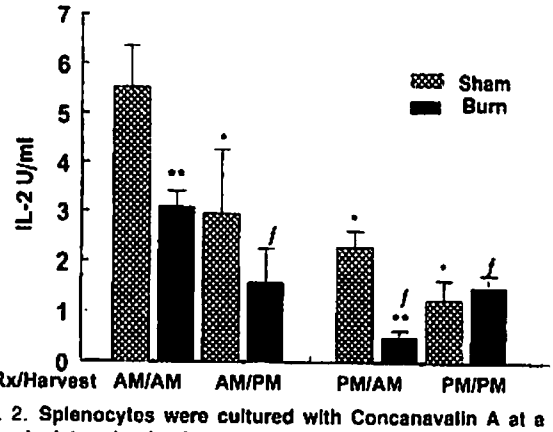

FIG. 2. Splenocytes were cultured with Concanavalin A at a dose previously determined to induce optimal IL-2 production in 24 h following sham or burn injury in the AM or PM and cells harvested in the AM or PM as in Fig 1. ** = $P < 0.05$ sham versus burn by Mann Whitney test. *$P < 0.05$ comparing sham groups by one way ANOVA and post hoc Newman-Keuls, ƒ $P < 0.05$ comparing burn groups in a similar manner.

Effect on TNFα secretion

TNFα secretion by LPS-stimulated adherent splenocytes was measured as well and the results (Fig. 3) showed, as has been observed repeatedly in this model (6), that TNFα secretion was increased in cells harvested from mice 7 days following thermal injury compared with shams. However this difference was only statistically significant ($P < 0.01$ sham vs. burn) when mice were injured in the morning (AM/AM and AM/PM). The time of day of harvest of the cells did not appear to be as much a factor as the time of injury in revealing the expected increase of proinflammatory cytokine secretion after injury. However when sham groups were compared by one

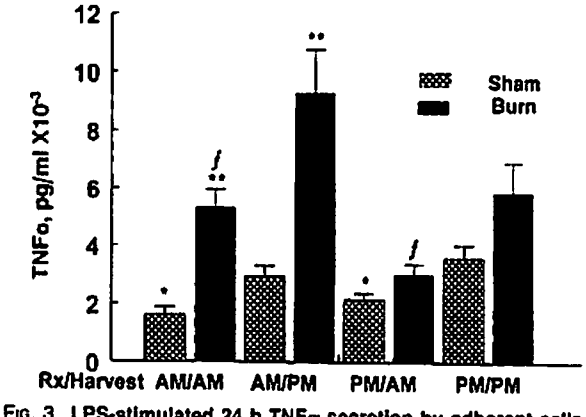

FIG. 3. LPS-stimulated 24 h TNFα secretion by adherent cells from mice following sham or burn injury in the AM or PM and cells harvested in the AM or PM as in Fig. 1. **= $P < 0.05$ sham versus burn by Mann-Whitney test. *$P < 0.05$ comparing sham groups to sham AM/PM (maximal TNFα) by one way ANOVA and post hoc Newman-Keuls test; ƒ $P < 0.05$ comparing burn groups in a similar manner.

way ANOVA adherent cells harvested in the PM produced more TNFα than in those taken in the AM (AM/AM vs. PM/PM $P < 0.01$, AM/AM vs. AM/PM and PM/AM vs. PM/PM, both $P < 0.05$). When burn groups were compared there was also a significant increase in TNFα secretion by cells harvested in the PM (AM/PM vs. AM/AM, AM/PM vs. PM/AM: $P < 0.05$ for both).

DISCUSSION

These results show that measurement of T cell or adherent cell cytokine production after sham or burn injury can be significantly affected by circadian rhythm. Importantly the time of injury and time of cell harvest significantly affected previously identified differences between cytokine production by burn vs. sham splenic lymphocytes and adherent splenocytes in this model (5–7).

The molecular basis for the circadian release of cytokines is beyond the scope of this brief report and remains to be elucidated. However, it is known that cytokines cooperate with prostaglandins and hormones in the orchestration of the immune response. Brown & Poyser (8) detected elevated PGE_2 production in normal rats at 6AM and 10PM. The endogenous production of prostaglandins could clearly influence cytokine, particularly IL-2, production. For several years it has been known that cortisol production occurs with a circadian rhythm (9). Normal healthy subjects showed a maximum cortisol production at 7AM which gradually fell through the afternoon. Endogenous cortisol production may serve as a down regulator for IL-1, TNFα, PGE2 and IL-2 production (9, 10).

In a recent report by Muret et al. (11) it was noted that *ex vivo* T cell derived cytokine production in patients with the systemic inflammatory response syndrome (SIRS) was influenced by experimental procedures. They cited divergent results reported in SIRS patients by other investigators, which showed that IL-4 and IL-10 were either reduced, unmodified or enhanced. Similar differences were also noted for these cytokines in studies using mouse or rat models. They proposed that these differences may have been due to the type of insult, the nature of the cell populations studied, e.g., splenocytes vs. isolated CD4+ cells, differing therapeutic regimens, and the type of cell stimulant used. The results presented herein suggest that in an animal model of injury the time of day cells are obtained or experimental procedures are carried out also may have a very important influence on results.

On a more positive note, significant differences in production of cytokines by sham versus burn animals appeared to be readily demonstrable when both injury and cell harvest were performed in the morning, a schedule that would be convenient for most laboratories engaged in trauma and burn research.

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