Diurnal Changes in Intraepithelial Lymphocytes (IELs) in the Small Intestine of Mice

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**Abstract:** Diurnal changes in small intestinal intraepithelial lymphocytes (IELs) were examined in 8- to 10-week-old BALB/cA male mice. The ratio of T cell subsets expressing CD8α homodimer/CD8αβ heterodimer was found to be higher in the dark period than that in the light period. Increased expression of Thy-1.2 on γδ T cells was also observed in the light period. No significant changes were found in other subsets. This is the first report to document diurnal changes in the small intestinal IELs in mice.

**Key words:** intraepithelial lymphocyte (IEL), diurnal change, small intestine

Circadian rhythms exist in the various phenomena of organisms [2, 11]. In the immune system, circadian rhythms were reported to influence the numbers of leukocytes and lymphocytes in peripheral blood [1, 13] and the functional activities of lymphocytes, natural killer cells and macrophages in the spleen and lymph nodes [4, 8] in humans, mice and rats, but there are few reports on circadian rhythms of leukocytes in the mucosal immune system, such as in the intestines. This study was therefore done by flow cytometrical analysis to examine the diurnal changes in the subpopulation of mouse small intestinal intraepithelial lymphocytes (IELs). The present study was approved by the Laboratory Animal Use and Care Committee of the Faculty of Agriculture, the University of Tokyo.

Six-week-old specific pathogen free (SPF) BALB/cA male mice were purchased from CLEA Japan Inc. (Tokyo, Japan), and adapted in our SPF animal facility for at least 2 weeks. The room temperature, relative humidity, ventilation and lightening were 23 ± 2°C, 55 ± 5%, 15 times an hour and 14 hr light (8:00–22:00) and 10 hr dark (22:00–8:00) cycle, respectively. The mice were kept in metal cages and fed commercial pellets (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

The mice were used at 8 to 10 weeks old and each group consisted of 5 mice. Each sampling was performed within 90 min at 2:15–3:45 and 14:15–15:45, respectively. IELs were prepared according to the method described previously [10] with a minor modification. Briefly, the small intestines were taken from ether-anesthetized mice and visible Peyer’s patches and mesenteric fat tissues were removed. The small intestines were dissected into pieces, and then incubated at

(Received 28 August 1998; Accepted 16 November 1998)

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37°C in Joklik-modified minimum essential medium (JMM; GIBCO BRL, Grand Island, N.Y., U.S.A.) containing 1mM EDTA-4Na and 2% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, U.S.A.) with shaking in a water bath. The resultant cell suspension was passed through a cotton gauze column and resuspended with RPMI 1640 medium (Nissui Co., Tokyo, Japan) containing 5% FBS, 0.05 mg/ml DNase I (Boehringer Mannheim, Tokyo, Japan) and 0.5 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). After the incubation at 37°C for 5 min in a water bath shaker, the cell suspension was washed with RPMI 1640 medium containing 5% FBS, and then subjected to Percoll density-gradient centrifugation. IELs were collected from the sediment of 30% Percoll gradient and stained with the following monoclonal antibodies (mAb): FITC-conjugated anti-mouse CD3ε (145-2C11), PE-conjugated anti-mouse CD19 (1D3), PE-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-mouse CD8α (53-6,7), PE-conjugated anti-mouse CD8β (53-5,8). Cy-Chrome-conjugated anti-mouse βTCR (H57-597), PE-conjugated anti-mouse γδTCR (GL3) and FITC-conjugated anti-mouse CD90 (Thy-1.2) (53-2,1) mAbs, all purchased from Pharmingen (San Diego, CA, U.S.A.). FITC-conjugated rat IgG2a, κ (R35-95; Pharmingen, San Diego, CA, U.S.A.) was used as an isotype control antibody for FITC-conjugated anti-mouse Thy-1.2 mAb (53-2,1). Anti-mouse CD16/32 mAb pu-
The ratios of CD8αα (CD8αα/CD8ββ) to that expressing CD8αβ (CD8αα/CD8ββ) are shown in Fig. 2. P<0.05 was judged to be significant.

The representative FACS profiles of IELs in the light and dark periods are shown in Fig. 1. No significant differences were found between the percentages of T cells (CD3εε), B cells (CD19εε) and non-T non-B cells (CD3εε/CD19εε) in the light and dark periods (data not shown). The percentages of B cells from each mouse were less than 1 %. This suggests negligible contamination by lamina propria cells in the isolated IELs preparation, because lamina propria cells have been reported to include a relatively large number of B cells. The percentages of double negative (DN) cells, CD4+CD8αε cells, CD4−CD8αε cells and double positive (DP) cells showed no significant differences in the light and dark periods (data not shown). The ratio of CD4+ cells to CD8εε cells was not also significantly different (data not shown). On the other hand, the ratio of T cell subsets expressing CD8αεε homodimer/CD8αβε heterodimer was significantly higher in the dark period than that in the light period (P<0.030, Fig. 2). CD8αεε and CD8αβε are known to be expressed on CD4+CD8αε cells and DP cells. The ratio of αβTCR+ T (αβ T) cells to γδTCR+ T (γδ T) cells was not significantly different (data not shown). The percentages of Thy-1.2 positive-cells in the γδ T cell population were significantly higher in the light period than that in the dark period (P<0.041, Fig. 3), but the percentages of Thy-1.2 positive-cells in αβ T cell population were not significantly different.
In the present study, the ratio of cells expressing CD8αα/CD8αβ (Fig. 2) and the percentage of Thy-1.2-positive γδ T cells (Fig. 3) were significantly different in the light and dark periods. Although CD8αα homodimer is expressed on both αβ T cells and γδ T cells, it is also known to be a specific marker of extrathymically developed T cells [15], but the cause of the observed difference between the CD8αα/CD8αβ ratios in the dark and the light periods remains undetermined. In addition, Thy-1.2 antigen is thought to be an activation-associated antigen for IELs [3, 5]. It has been reported that increasing expression of Thy-1.2 was found in conventionalized germ-free mice [5, 12, 14] and that Thy-1.2 was more highly expressed in conventional mice than in germ-free mice [9, 12]. αβ T cells are generally thought to respond to foreign antigens, and γδ T cells in particular respond to self antigens, respectively [7, 12, 14]. Especially, γδ T cells are thought to play a role in the turnover of intestinal epithelial cells [7]. Although the reason remains unknown, γδ T cells might be activated in association with the accelerating turnover (migration) of intestinal epithelial cells in the light period [6].

Circadian rhythms of lymphocytes are controlled by many factors, such as hormones, neurotransmitters, cytokines and the turnover and migration of lymphocytes [1, 4, 8, 13]. This is the first report to document the diurnal changes in subpopulations of small intestinal IELs in mice. More sampling points and functional analysis will be needed to clarify the mechanism involved in the diurnal changes in IELs subpopulation.

References