FULL PAPER *Immunology*

Regional Variations in the Distribution of Small Intestinal Intraepithelial Lymphocytes in Three Inbred Strains of Mice

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ABSTRACT. The regional variation in the intraepithelial lymphocytes (IELs) in the small intestine was examined in BALB/c male and female mice and C3H/He and C57BL/6 male mice. The small intestines were taken from 11 to 12-week-old mice and divided equally into 3 parts (the proximal, middle and distal parts). IELs were isolated from each part of the intestine and analyzed with flow cytometer. The number of IELs was highest in the proximal part and lowest in the distal part. The distribution of IEL subsets was markedly different between the proximal and the distal parts, and that in the middle part showed the intermediate pattern. The percentage of $\alpha\beta$ T cells were higher in the distal part. In $\alpha\beta$ T cell subset, the percentage of CD8$\alpha\alpha$ T cells was higher in the proximal part, whereas those of CD4 and CD4CD8$\alpha\alpha$ double positive T cells were higher in the distal part. In $\gamma\delta$ T cell subset, no regional variations were found. The regional variations in the number and subsets of IELs showed almost the same patterns between male and female BALB/c mice and similar patterns among three strains of mice. This strongly suggests that the regional variations in the small intestinal IELs are common to mouse species.

KEY WORDS: intraepithelial lymphocyte (IEL), IEL subset, mouse, regional variation, small intestine.

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It is widely approved that a unique immune system exists in the mucosal tissues. It differs from the systemic immune system and is called the mucosal immune system [5]. Intraepithelial lymphocytes (IELs) are one of the components of the mucosal immune system and known to contain unusual proportions of T cells [12] and possess cytotoxicity [12, 18] and cytokine-producing activity [7, 8].

The small intestine consists of three parts, i.e. duodenum, jejunum and ileum. It is reported that they show the regional variations in activities of enzymes [6, 31], abilities of absorption [9], intestinal flora [26], the expression of MHC class II molecules on the enterocytes [25] and the number of IgA-producing cells in the lamina propria [1] as well as in the histological features, histologically [14, 16].

Recently, we clarified in BALB/c mice that the number of IELs in the distal part was significantly fewer than those in the proximal and middle parts and the composition of IEL subsets was significantly different between the proximal and the distal parts of the small intestine [27].

The aim of this study is to examine the regional variations in the small intestinal IELs in three inbred strains of mice in order to certify that such variations are fundamentally common to mouse species.

MATERIALS AND METHODS

Specific-pathogen-free (SPF) C3H/He and C57BL/6 male mice and BALB/c male and female mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were adapted for at least 2 weeks in our SPF animal facility until used. The room temperature, relative humidity, ventilation and lighting were 23 ± 2°C, 55 ± 5%, 15 times an hour and 14 hrs light (08:00–22:00)-10 hrs dark (22:00–08:00) cycle, respectively. Mice were kept in metal cages and fed commercial pellets (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. The present study was approved by the Laboratory Animal Use and Care Committee of Faculty of Agriculture, the University of Tokyo.

Five mice were used in each strain of mice and sampling of the small intestine was performed between 13:00 and 14:00 to avoid diurnal variation [28]. Mice were used at 11 to 12 weeks old. The mice were sacrificed under ether anesthesia, then the small intestine was taken from each mouse and the length of the intestine was measured. The intestine was then divided into three parts with the same length (the proximal, middle and distal parts). IEL samples were prepared from each part separately according to the method previously described by us with a minor modification [27, 28]. Briefly, Peyer's patches and mesentery were carefully removed from the intestine and each part of the small intestine was first cut longitudinally and then into small pieces. The pieces were incubated in 15 m*l* of Joklik-modified minimum essential medium (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.) (JMM-EDTA) with shaking at 37°C for 20 min in 50 m*l* centrifuge tube to detach the epithelial layer. The supernatant was removed and new 15 m*l* of JMM-EDTA was added and the incubation step was repeated again. The supernatant thus obtained was passed through cotton gauze column and then centrifugated and resuspended with RPMI 1640 medium (Nissui Co., Tokyo, Japan) containing 5% FBS, 0.05 mg/m*l* DNase I (Boehringer Mannheim, Tokyo, Japan) and 0.5 mg/m*l* collagenase (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Then the suspension was incubated at 37°C for 5 min in water bath shaker to prepare single-cell suspensions. After washing with RPMI

1640 medium containing 5% FBS, the number of IELs in the resultant cell suspensions was counted by hemocytometer. The suspension was subjected to Percoll (Pharmacia, Tokyo, Japan) density-gradient centrifugation to separate IELs from epithelial cells, and the sediment of cells separating at the 40% Percoll gradient were IELs.

IELs were then stained with the following antibodies. Namely, FITC-conjugated anti-mouse CD3ε (145-2C11), PE-conjugated anti-mouse CD19 (1D3), Cy-Chrome-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 β T cell receptor (TCR) (H57-597), PE-conjugated anti-mouse γδ TCR (GL3) and FITC-conjugated anti-mouse CD90 (Thy-1.2) (53–2.1) monoclonal antibodies (mAb) were purchased from PharMingen (San Diego, CA, U.S.A.). Anti-mouse CD16/32 (FcγIII/II receptor) mAb purified from tissue culture supernatant of the hybridoma clone, 2.4G2 (American Type Culture Collection, HB-197) was used to inhibit nonspecific binding of the fluorescence-conjugated mAbs. After staining, the cells were fixed with 5% formalin-added phosphate buffered saline. Analysis was performed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, U.S.A.).

Statistical analysis was carried out by unpaired *t*-test between the percentages of each subset of three parts of the small intestine in each strain of mice. $P<0.05$ was judged to be significant and $P<0.01$ to be highly significant, respectively.

RESULTS

The numbers of viable IELs from three parts of the small intestine are shown in Fig. 1. Although the total number of IELs in the entire small intestine was different among the strains, the number of IELs in the distal part was significantly fewer than those in the proximal and middle parts in all strains of mice ($P<0.01$). The number of IELs in the proximal part

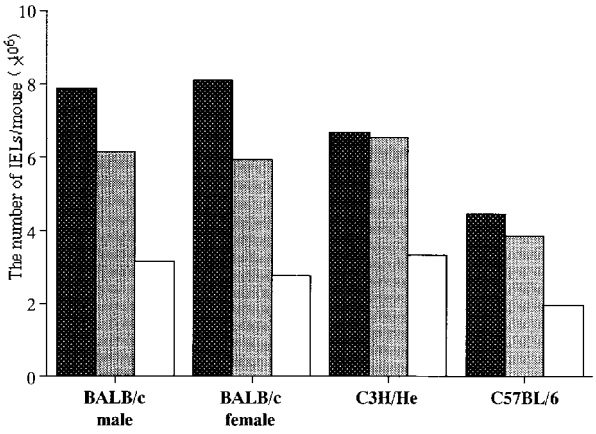

Fig. 1. The number of IELs in each part of the intestine. ■ Proximal. ■ Middle. □ Distal.

was significantly larger than those in the middle part in BALB/c male and female mice ($P<0.01$) and C57BL/6 mice ($P<0.05$). The percentages of IELs among three parts to total IELs showed significant differences (proximal>middle>distal) except that there was no difference between the proximal and middle parts in C3H/He mice.

The percentage of non-T non-B cells ($CD3\varepsilon^{-}CD19^{-}$) to total IELs was higher in the proximal and middle parts, whereas the percentage of T cells ($CD3\varepsilon^{+}CD19^{-}$) to total IELs was higher in the distal part in BALB/c male ($P<0.01$ between the proximal and the distal parts, and $P<0.05$ between the middle and the distal parts) and female mice ($P<0.05$ between the proximal and the distal parts, and $P<0.01$ between the middle and the distal parts), although no differences were found in C3H/He and C57BL/6 mice (Fig. 2).

In all strains of mice, the percentage of αβ T cells to all T cells was significantly higher in the distal part than in the prox-

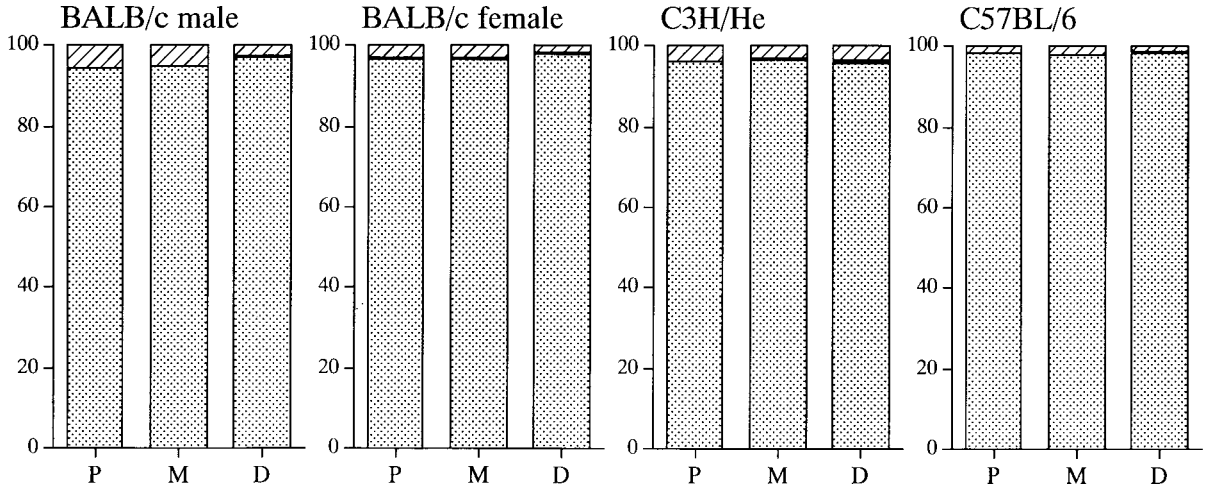

Fig. 2. The percentages of each IEL subset to total IELs in each part of the intestine. P: proximal, M: middle, D: distal. ■ T cells. □ B cells. □ non-T non-B cells.

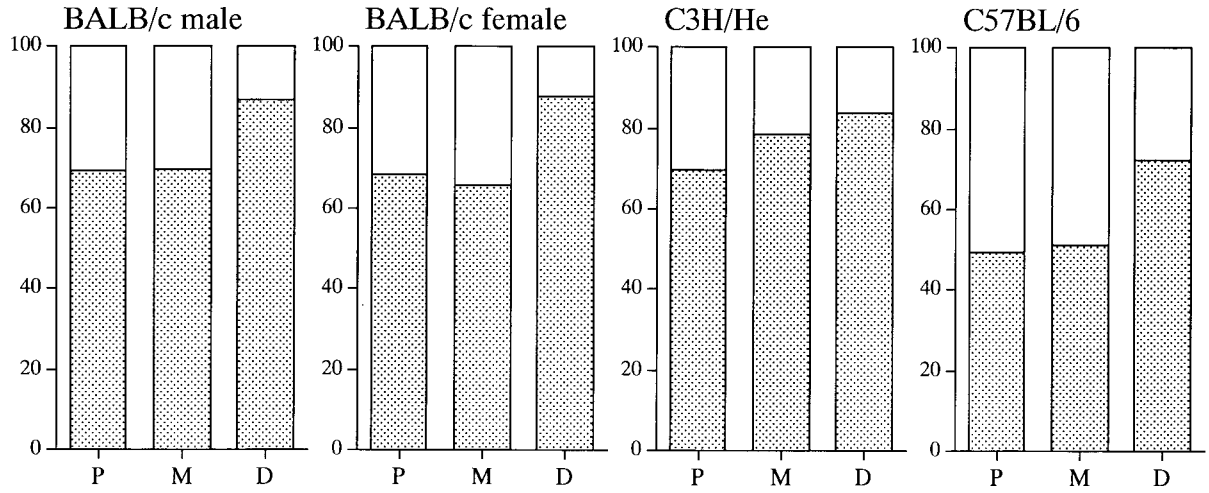

Fig. 3. The percentages of αβ T and γδ T cells to all T cells in each part of the intestine. P: proximal, M: middle, D: distal. □ αβ T cells. □ γδ T cells.

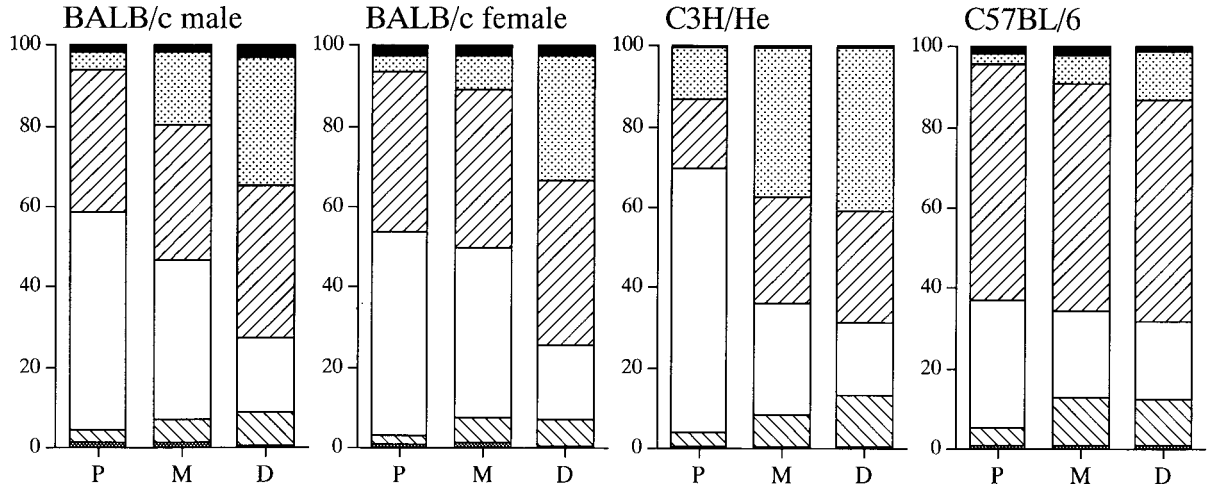

Fig. 4. The percentages of each subset in αβ T cells to all αβ T cells in each part of the intestine. P: proximal, M: middle, D: distal. ■ double negative T cells. □ CD4 T cells. □ CD8αα T cells. □ CD8αβ T cells. □ CD4CD8αα double positive T cells. ■ CD4CD8αβ double positive T cells.

imal and middle parts, and the percentage of γδ T cells to all T cells was significantly lower in the distal part ($P<0.01$ between the proximal and the distal parts and between the middle and the distal parts in BALB/c male and female and C57BL/6 mice, and $P<0.05$ between the proximal and the middle parts and between the middle and the distal parts, and $P<0.01$ between the proximal and the distal parts in C3H/He mice) (Fig. 3).

The proportions of each subset in αβ T cells showed almost similar patterns in all strains of mice (Fig. 4). The percentage of CD4 T cells ($P<0.05$ between the proximal and the middle parts, and $P<0.01$ between the proximal and the distal parts in BALB/c male mice, and $P<0.01$ between the proximal and the middle parts and between the proximal and the distal parts in BALB/c female mice, and $P<0.01$ among the three parts in C3H/He mice, and $P<0.01$ between the proximal and the middle parts and between the proximal and the distal parts in C57BL/6 mice) and CD4CD8αα double positive (DP) T cells ($P<0.05$ between the proximal and the middle parts, and $P<0.01$ between the proximal and the distal parts in BALB/c male mice, and $P<0.05$ between the proximal and the middle parts, and $P<0.01$ between the proximal and the distal parts and between the middle and the distal parts in BALB/c female mice, and $P<0.05$ between the proximal and the middle parts and between the proximal and the distal parts in C3H/He mice, and $P<0.05$ between the proximal and the middle parts and between the middle and the distal parts, and $P<0.01$ between the proximal and the distal parts in C57BL/6 mice) to all αβ T

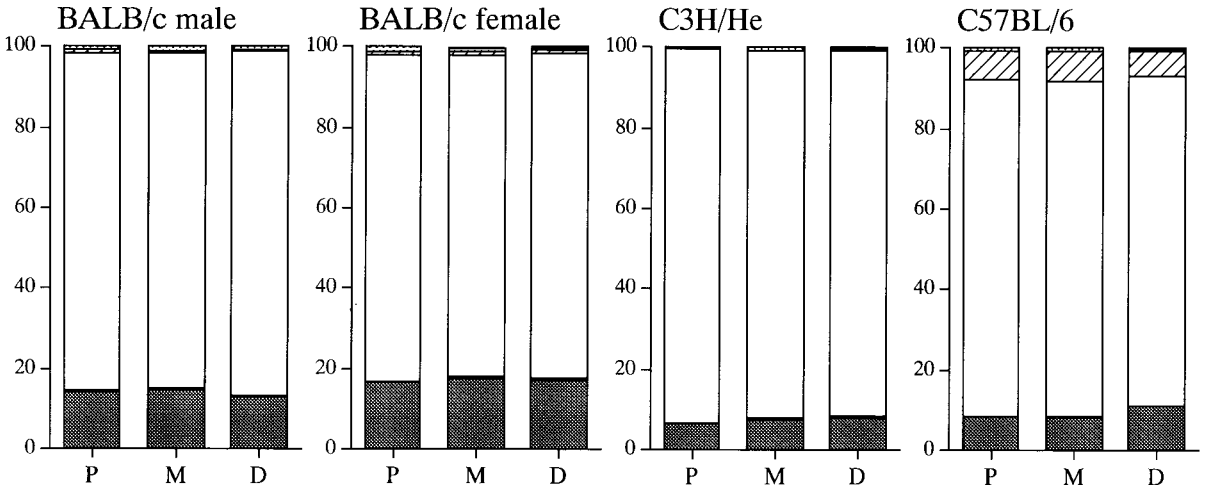

Fig. 5. The percentages of each subset in γδ T cells to all γδ T cells in each part of the intestine. P: proximal, M: middle, D: distal. ■double negative T cells. □CD4 T cells. □CD8αα T cells. □CD8αβ T cells. □CD4CD8αα double positive T cells. ■CD4CD8αβ double positive T cells.

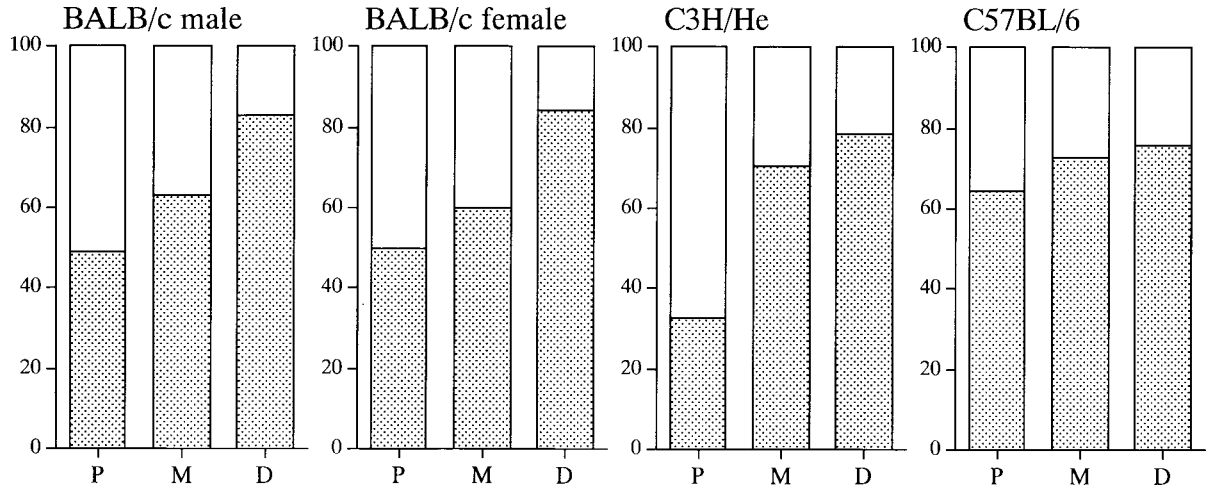

Fig. 6. The percentages of Thy-1.2 expression on αβ T cells in each part of the intestine. P: proximal, M: middle, D: distal. □ Thy-1.2^{+} αβ T cells. □Thy-1.2^{-} αβ T cells.

cells were higher in the distal part than in the proximal part. In contrast, the percentage of CD8αα T cells ($P<0.01$ among the three parts in BALB/c male mice, and $P<0.05$ between the proximal and the middle parts, and $P<0.01$ between the proximal and the distal parts and between the middle and the distal parts in BALB/c female mice, and $P<0.01$ between the proximal and the middle parts and between the proximal and the distal parts in C3H/He mice, and $P<0.05$ between the proximal and the middle parts and between the proximal and the distal parts in C57BL/6 mice) to all αβ T cells was highest in the proximal part and lowest in the distal part. The percentage of double negative (DN) T cells to all αβ T cells was significantly lower in the distal part than in the proximal and middle parts only in BALB/c male and female mice ($P<0.01$ between the proximal and the middle parts and between the proximal and the distal parts), while DN αβ T cells were a negligible population (<1%) in C3H/He and C57BL/6 mice.

On the other hand, the proportions of each subset in γδ T cells showed no significant differences in all strains of mice (Fig. 5).

The percentage of Thy-1.2 expression on αβ T cells to all αβ T cells was highest in the distal part and lowest in the proximal part in all strains of mice ($P<0.01$ among the three parts in BALB/c male mice, and $P<0.01$ between the proximal and the distal parts and between the middle and the distal parts in BALB/c female mice, and $P<0.01$ between the proximal and the middle parts and between the proximal and the distal parts in C3H/He mice, and $P<0.05$ between the proximal and the distal parts in C57BL/6 mice) (Fig. 6). The percentage of Thy-1.2 expression on γδ T cells to all γδ T cells was also

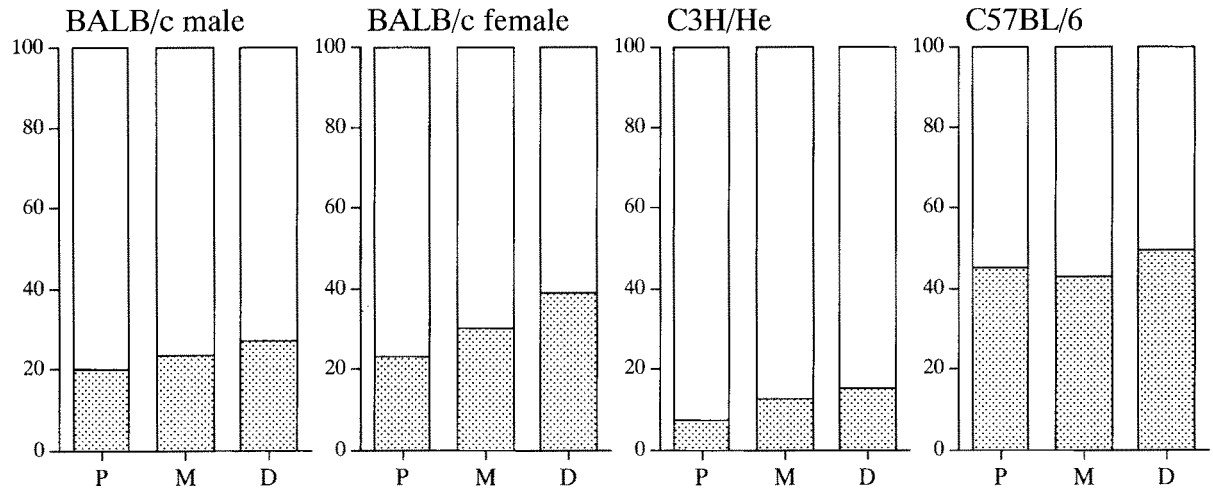

Fig. 7. The percentages of Thy-1.2 expression on γδ T cells in each part of the intestine. P: proximal, M: middle, D: distal. ▨Thy-1.2$^+$ γδ T cells. □ Thy-1.2$^-$ γδ T cells.

highest in the distal part and lowest in the proximal part in BALB/c male ($P<0.01$ between the proximal and the distal parts) and female ($P<0.05$ between the middle and the distal parts, and $P<0.01$ between the proximal and the distal parts) and C3H/He mice ($P<0.05$ between the proximal and the middle parts and between the proximal and the distal parts) (Fig. 7).

DISCUSSION

Regional variations in the number and subsets of IELs were examined in the small intestine of three strains of mice. The numbers and percentages of IELs distributed in each part of the small intestine showed almost similar patterns in three strains of mice. The number and percentages of IELs were highest in the proximal part and lowest in the distal part. Such regional variation in the number of IELs is considered to correlate with the regional difference in the length of villi which is longer in the proximal part than in the distal part [14, 16].

The percentage of non-T non-B cells, which are supposed to be immature T cells because of bearing intracellular CD3ε$^+$ (data not shown), was higher in the proximal and middle parts whereas that of T cells was higher in the distal part in BALB/c male and female mice. Such differences were not found in C3H/He and C57BL/6 mice. The low percentages of B cells, less than 1%, suggest negligible contamination of the isolated IELs preparation with lamina propria cells, because lamina propria cells include a relatively large number of B cells [22].

In all strains of mice, the percentage of αβ T cells was significantly higher in the distal part than in the proximal and middle parts, while there was no significant difference between the proximal and middle parts. The number of αβ T cells is reported to increase significantly after the conventionalization of germ-free mice [30], and the number of intestinal bacteria in the distal part is said to be about 100 times as many as that in the proximal part [26]. These findings suggest that αβ T cells may recognize intestinal bacteria and expand locally, especially in the distal part of the intestine.

In αβ T cell population, the percentage of CD8αα T cells was significantly higher in the proximal part, whereas the percentages of CD4 and CD4CD8αα DP T cells were higher in the distal part in all strains of mice. CD4 and CD4CD8αα DP T cells belong to the same lineage, and CD4 T cells migrate from the thymus to the intestine, express CD8αα homodimers in response to the intestinal bacteria and become CD4CD8αα DP T cells [21, 29]. Therefore, in the distal part of the small intestine, the higher number of intestinal bacteria may be responsible for the higher percentage of CD4CD8$_{AA}$ DP T cells. Higher percentage of CD4 and CD4CD8αα DP T cell lineage in the distal part may also indicate regional variations in homing capacity of T cells from the thymus, and therefore regional variations in the expression of cell adhesion molecules will be examined in the next study. On the other hand, CD8αβ T cells which also migrate from the thymus to the intestine [11, 12, 24] did not show regional variations. This suggests that CD4 T cells and CD8αβ T cells may use different homing receptors. CD8αα T cells are said to be extrathymically-developed because they are largely bearing self-reactive forbidden TCRs [24], employ FcRγ chains instead of CD3ζ chains in their TCR-CD3 complex [13, 23] and mature in IL-2Rβ-dependent manner [23]. Recently, it is reported that the sites for extrathymic development of IELs exist beneath the intestinal crypts and are called cryptopatches [17].

In γδ T cell population which consists of DN and CD8αα T cells, no regional variations were detected in all strains of mice. These suggest that there may be no regional variations in the migration and extrathymic-development of γδ T cells.

The percentage of Thy-1.2 expression on αβ T cells and γδ T cells was highest in the distal part and lowest in the proximal part in all strains of mice. The roles of Thy-1 antigens on IELs were controversial up to the present [3, 4, 10, 15, 20]. In αβ T cells, thymus-derived subsets (e.g. CD4, CD8αβ and

CD4CD8αα T cells) were known to express higher level of Thy-1 [11] and this is likely to reflect the regional differences in Thy-1 expression on αβ T cells. Thy-1 expression on both αβ and γδ T cells increased in the SPF mice compared with GF mice [2, 19, 20], suggesting a role of intestinal bacterial antigens in the induction of Thy-1 expression. As mentioned above, the number of intestinal bacteria in the distal part is larger than that in the proximal part [26]. Therefore, increased expression of Thy-1 may reflect the number of intestinal bacteria.

In conclusion, the regional variations in the number and subsets of IELs showed almost the same patterns between male and female BALB/c mice and similar patterns among three strains of mice. This strongly suggests that the regional variations in the small intestinal IELs are common to mouse species.

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