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# Strain and Age-Related Changes in the Localization of Intestinal CD161$^+$ Natural Killer Cells and CD8$^+$ Intraepithelial Lymphocytes along the Longitudinal Crypt Axis in Inbred Rats

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**Using a cell position approach, this study indicates that the frequency of CD161$^+$ natural killer (NK) cells in the epithelia of DA rats was greater than that of WKAH and F344 rats. We further divided the epithelia into proliferating and differentiated regions according to the localization of BrdU-incorporated cells. Comparison between the different regions indicates that a majority of CD161$^+$ NK cells were located in the proliferating region. With age, a decline in the number of CD161$^+$ NK cells and CD8$^+$ intraepithelial lymphocytes (IELs) was observed in the distal colon, especially in the proliferating region of all three strains. Taken together with our previous report that DA rats have far stronger resistance in the colon to preneoplastic lesion than do other strains, these results indicate that CD161$^+$ NK cells play an important role in immune-surveillance at the bottom of the crypt.**

**Key words:** intraepithelial lymphocytes; CD161$^+$ NK cells; ageing; intestine

A previous study in our laboratory found that formation of aberrant crypts varies among inbred rat strains (WKAH, DA, and F344), regardless of the manner of DNA damage (chemical carcinogen or gamma-irradiation).[1)] DA rats had fewer preneoplastic lesions in the colorectum than other strains. It has been reported that DA rats are susceptible to the development of 4-nitroquinoline-1-oxide-induced squamous cell carcinomas of the tongue with high proliferating response of the tongue epithelium,[2)] collagen-induced arthritis,[3)] and autoimmune thyroiditis.[4)] This strain appears to show a characteristic autoimmune response.

It is widely acknowledged 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 are known to contain unusual proportions of T cells[6)] and to possess cytotoxity[6,7)] and cytokine-producing activity.[8,9)] Recently, the CD3$^-$NKR-P1 (CD161)$^+$ subset was found in the isolated IEL fraction from the rat small intestine, and CD161$^+$ cells have strong natural killer (NK) activity.[10,11)] Despite many reports on the impact of various conditions on the characteristics of IELs isolated from the intestine,[12–15)] there have been few histological analyses of the localization of IELs and NK cells in the intestinal crypt. From a histochemical point of view, the intestinal epithelium is a highly hierarchal organ in which stem cell positions are well defined in terms of the spatial arrangement within the crypt.[16)] Selecting longitudinally sectioned crypts and recording information on a cell positional basis has the potential to provide useful insights into cell function. In this way, we evaluated the strain and age-related changes in the histological localization of NK cells and IELs along the longitudinal crypt axis in the rat intestine.

## Materials and Methods

*Animals and diets.* Three strains of young (5 weeks old) or older (about 6 months old) male rats (WKAH/HKmSlc, DA/Slc, and F344/NSlc, purchased from Japan SLC, Hamamatsu, Japan) were housed in individual cages in a temperature controlled ($23 \pm 2\,^\circ$C) room under a 12:12-h light:dark photoperiod (light: 08:00–20:00). Rats were allowed free access to a standard diet and drinking water throughout the experimental period. The composition of the diet was as follows (%): casein, 25; corn oil, 5; mineral mixture (AIN-93G),[17)] 3.5; vitamin mixture (AIN-93),[17)] 1; choline bitartrate, 0.25; sucrose 65.25. This study was approved by the Hokkaido University Animal Use Committee, and the animals were maintained under the guidelines for the care and use of laboratory animals of Hokkaido University. After 7 d, all rats were injected with a bromodeoxyuridine (BrdU, 15 mg/kg body weight) solution containing 15 mg BrdU (Sigma Chemical, Steinheim, Germany) and 1.5 mg fluorodeoxyurin-

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*Abbreviations*: IELs, intraepithelial lymphocytes; NK cells, natural killer cells; BrdU, bromodeoxyuridine

dine (Sigma Chemical) per 1 ml saline. The rats were then killed in the morning (10:00–11:30) by exsanguination under anesthesia with sodium pentobarbital at 1 h post-injection. Segments of the small intestine, cecum, and distal colon were flushed with saline, embedded in OCT compound (Sakura Finetecnical, Tokyo), rapidly frozen in liquid nitrogen, and stored at −80°C.

*Immunohistochemical staining for BrdU-incorporated cells.* Frozen sections from these samples were prepared and stained with anti-BrdU monoclonal antibody (clone OS94.6, Calbiochem, Cambridge, MA). Briefly, the frozen sections were fixed in 10% formalin in phosphate buffered saline. They were then immersed in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, treated with a pepsin (Wako Pure Chemical Industries, Osaka, Japan) solution (0.4 mg/ml 0.1 N HCl), and then with 10% normal rabbit serum to reduce nonspecific binding. After incubation with the primary antibody as mentioned above, they were incubated with biotinylated rabbit anti-mouse IgG+A+M (H+L; Zymed Laboratories, San Francisco, CA). Then they were incubated with peroxidase-conjugated streptavidin (Cosmo Bio, Tokyo). 3,3′-diaminobenzidine tetrahydrochloride was used as the chromogen. After BrdU staining, these sections were counterstained with hematoxylin.

*Immunohistochemical staining for $CD4^+$, $CD8^+$, and $CD161^+$ cells.* Other frozen sections were fixed at 4 °C with Zamboni's fixative for CD4 and CD8 staining and periodate-lysine-paraformaldehyde for CD161 staining. After being fixed, these sections were immersed in 3% hydrogen peroxide in methanol to reduce endogenous peroxidase activity. They were incubated for 1 h with 10% normal rabbit serum to block nonspecific binding. As primary antibodies, mouse anti-rat CD4 (clone W3/25, Serotec), mouse anti-rat CD8 (clone OX-8, Cedarlane), or mouse anti-rat CD161 (NKR-P1, clone 10-78, Cedarlane) were used respectively. After incubation with the primary antibody, sections were incubated with biotin-conjugated rabbit anti-mouse IgG. Then they were incubated with peroxidase-conjugated streptavidin. 3,3′-diaminobenzidine tetrahydrochloride was used as the chromogen. After staining, the specimens were counterstained with hematoxylin.

*Immunohistochemical scoring.* The numbers of BrdU-incorporated epithelial cells, $CD161^+$ NK cells, and $CD8^+$ and $CD4^+$ IELs in the epithelial-layer were scored in every cell position from bottom to top along the longitudinal half crypt axis of the cecum and distal colon, according to the method of Ijiri and Potten.[18] In sections of the small intestine, cells were scored from the bottom to position 20 along the longitudinal axis, since in the histological view the section above position 20 usually appears to be villus.[16] Fifty half-crypts were scored in each segment of each individual rat. Frequencies of $BrdU^+$ epithelial cells, $CD161^+$ NK cells, $CD8^+$ IELs, and $CD4^+$ IELs against 10,000 epithelial-lining cells were calculated, and the percentage of BrdU-incorporated cells was expressed. Further, the large intestinal crypt was divided into proliferating and differentiated regions according to the distribution of BrdU-incorporated cells to evaluate the localization of the immune cells. The bottom region of crypts in which more than 95% of BrdU-incorporated cells was located was defined as the proliferating region (see Table 3 for details). Correspondingly, the upper region was defined as the differentiated region.

*Statistical analyses.* All results are expressed as mean ± SEM, $n = 6$. Statistical comparisons among the three rat strains were performed using Tukey-Kramer's test, and those between young and older groups or between proliferating and differentiated regions were performed using Student's-*t* test. A $P$ value less than 0.05 was considered significant. All statistics were calculated using JMP software (SAS Institute, Cary, NC).

## Results

Table 1 shows clear differences in the growth parameters among the inbred rat strains. The initial body weight, body weight gain, and food intake of DA rats were significantly lower than those of WKAH rats, regardless of age. The initial body weight of young F344 rats and the body weight gain of older F344 rats were similar to those of DA rats. The food intake of young F344 rats and the initial body weight of older F344 rats were at similar levels to those of WKAH rats. The body weight gain of young F344 rats and the food intake of older F344 rats were between those of the other strains.

The frequency of BrdU-incorporated epithelial cells in the cecal crypt was highest in young DA rats. When the rats got older, it began to decrease from the small intestine to the distal part of the large intestine in all strains. Especially in the distal colon, the age-related changes were significant in each strain (Table 2).

**Table 1.** Initial Body Weight, Body Weight Gain, and Food Intake

| | Initial body wt. (g) | Body wt. gain (g/7 d) | Food intake (g/7 d) |
|---|---|---|---|
| Young rats | | | |
| WKAH | $92.4 \pm 1.6^{A}$ | $42.5 \pm 0.9^{A}$ | $85.8 \pm 1.8^{A}$ |
| DA | $77.1 \pm 1.9^{B}$ | $26.3 \pm 0.9^{C}$ | $70.4 \pm 1.7^{B}$ |
| F344 | $76.9 \pm 0.5^{B}$ | $31.4 \pm 1.0^{B}$ | $81.6 \pm 0.9^{A}$ |
| Older rats | | | |
| WKAH | $427.3 \pm 8.7^{a}$ | $43.2 \pm 3.1^{a}$ | $158.1 \pm 2.3^{a}$ |
| DA | $264.3 \pm 8.7^{b}$ | $18.3 \pm 2.2^{b}$ | $99.2 \pm 4.9^{c}$ |
| F344 | $403.1 \pm 5.1^{a}$ | $21.8 \pm 1.4^{b}$ | $130.4 \pm 3.4^{b}$ |

Values are expressed as mean ± SEM, $n = 6$.
Values with different capital superscript letters show significant differences in mean values among young strains. Values with different small superscript letters show significant differences in mean values among older strains ($P < 0.05$).

**Table 2.** Numbers of BrdU$^+$ Cells per 10,000 Epithelial-Lining Cells in the Small Intestine, Cecum, and Distal Colon of Young and Older WKAH, DA, and F344 Rats

| | Young rats | Older rats |
|---|---|---|
| Small intestine | | |
| WKAH | $5213 \pm 150$ | $4818 \pm 143^{ab}$ |
| DA | $4967 \pm 147$ | $4477 \pm 119^{b*}$ |
| F344 | $5235 \pm 187$ | $5082 \pm 213^{a}$ |
| Cecum | | |
| WKAH | $1574 \pm 40^{B}$ | $1449 \pm 72^{a}$ |
| DA | $1966 \pm 85^{A}$ | $1357 \pm 52^{a,b*}$ |
| F344 | $1718 \pm 50^{B}$ | $1227 \pm 50^{b*}$ |
| Distal colon | | |
| WKAH | $1391 \pm 94$ | $821 \pm 43^{b*}$ |
| DA | $1721 \pm 119$ | $1135 \pm 63^{a*}$ |
| F344 | $1536 \pm 110$ | $1040 \pm 82^{a,b*}$ |

Values are expressed as mean ± SEM, n = 6.
Values with different capital superscript letters show significant differences in mean values among young strains. Values with different small superscript letters show significant differences in mean values among older strains ($P < 0.05$). *Significantly different from the corresponding young group ($P < 0.05$).

**Table 3.** Cell Positions for the Proliferating Region of Crypts*

| | Cecum | Distal colon |
|---|---|---|
| Young rats | | |
| WKAH | ≦17 | ≦23 |
| DA | ≦18 | ≦27 |
| F344 | ≦18 | ≦22 |
| Older rats | | |
| WKAH | ≦16 | ≦17 |
| DA | ≦19 | ≦24 |
| F344 | ≦17 | ≦22 |

*Cell position was ascertained according to the standard that the proliferating region contained ≧95% BrdU-incorporated cells.

We defined the bottom region of crypts in which more than 95% of BrdU-incorporated cells was located as the proliferating region. The detailed cell position for each strain, each age, and each segment is shown in Table 3. The proliferating regions of DA rats were longer than those of WKAH rats regardless of age and segment. The distal colon had longer proliferating region than the cecum as well.

Figure 1 shows representative photographs of BrdU-incorporated epithelial cells, CD161$^+$ NK cells, CD8$^+$ IELs and CD4$^+$ IELs in the large intestinal crypt of rats. Many BrdU$^+$ epithelial cells were observed in the mucosa (Fig. 1A). CD161$^+$ NK cells appeared to locate in the lamina propria and near the crypt bottom, and some NK clusters were observed (Fig. 1B). In contrast, CD8$^+$ IELs mostly located in the upper region of the lamina propria and the intraepithelial region (Fig. 1C). The number of CD4$^+$ IELs was very small, although some CD4$^+$ lymphocytes were detected in the lamina propria (Fig. 1D).

We evaluated the frequency of CD161$^+$ NK cells, CD8$^+$ IELs, and CD4$^+$ IELs in the crypt (Fig. 2). The frequency of CD161$^+$ NK cells in the large intestinal epithelium of DA rats was the highest among the three rat strains. In the small intestine, young DA rats had more CD161$^+$ NK cells than did WKAH and F344 rats, but there were no significant differences among the three older strains. On the other hand, the frequency of CD8$^+$

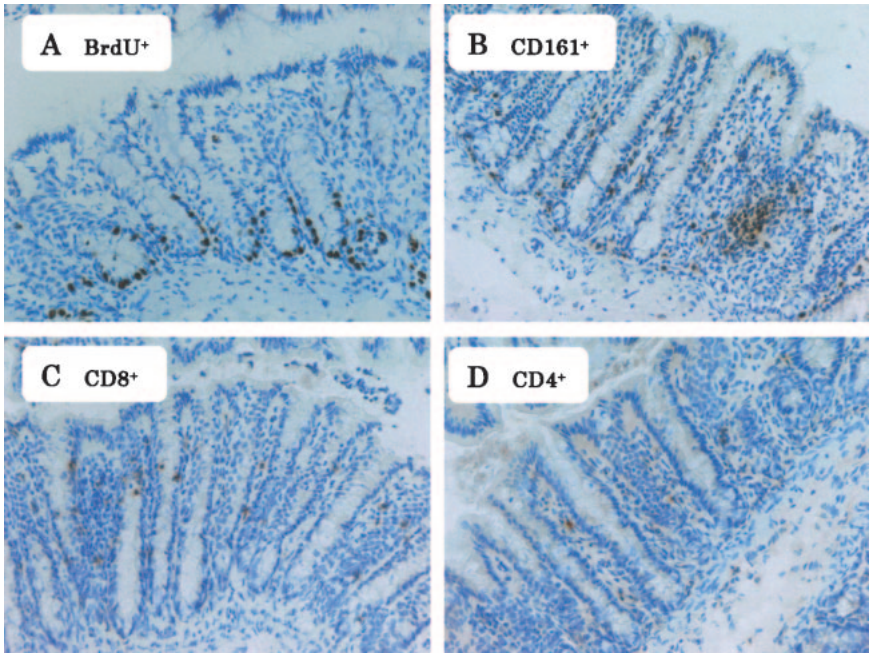


**Fig. 1.** Photomicrographs of Immunohistochemical Stainings in Rat Large Intestinal Crypts.
Immunohistochemical stainings were performed as described in "Materials and Methods". In the epithelium of the large intestine, (A) the localization of BrdU-incorporated epithelial cells showed that the bottom of crypts is the proliferating zone; (B) most CD161$^+$ NK cells located in the bottom of crypts; (C) the number of CD8$^+$ IELs in the bottom of crypts was not predominant; (D) few CD4$^+$ IELs were detected in crypts.

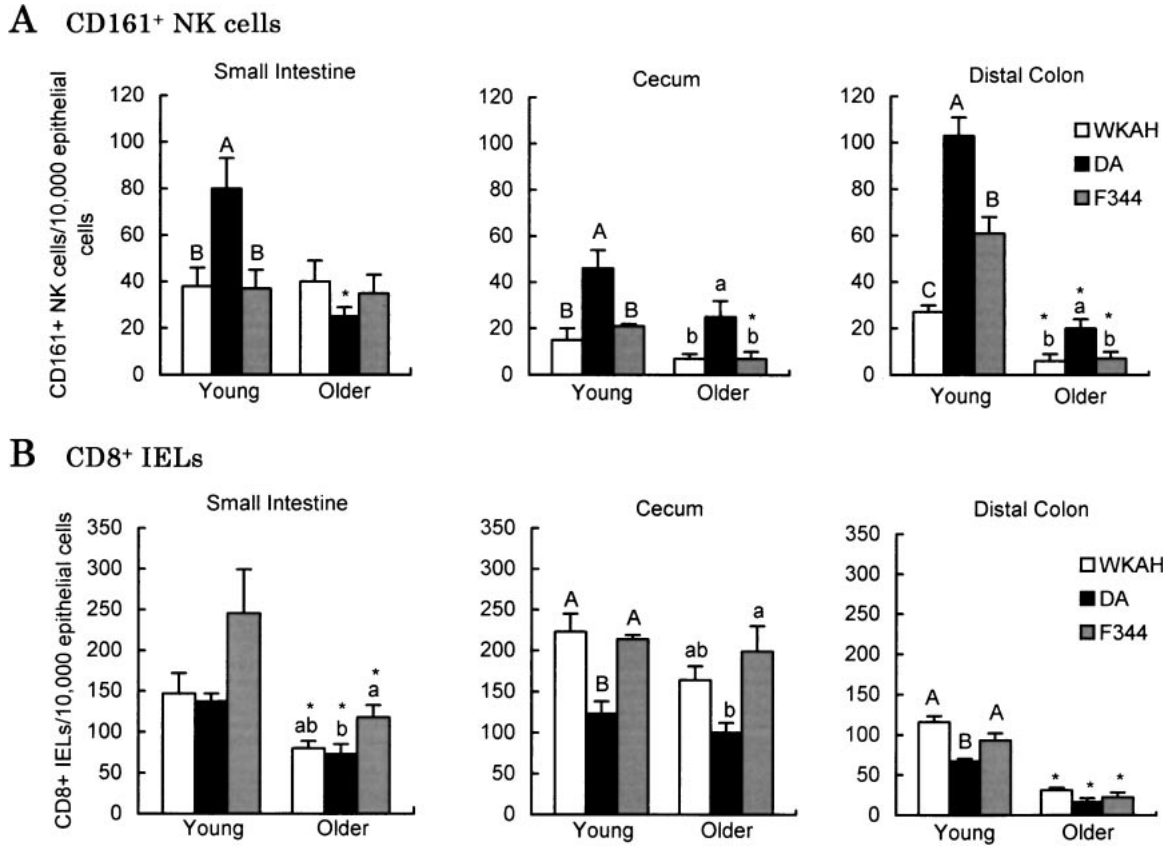


**Fig. 2.** The Number of (A) CD161$^+$ NK Cells and (B) CD8$^+$ IELs per 10,000 Epithelial-Lining Cells in the Small Intestine, Cecum, and Distal Colon of Young and Older WKAH, DA, and F344 Rats.

Values are expressed as mean ± SEM, n = 6. Bars with different capital superscript letters show significant differences in mean values among young strains. Values with different small superscript letters show significant differences in mean values among older strains ($P < 0.05$). *Significantly different from the corresponding young group ($P < 0.05$).

IELs in DA rats tended to be the lowest among the three strains. There were no statistical differences in the frequency of CD4$^+$ IELs in the small intestine (WKAH, DA, and F344: $25 \pm 11$, $20 \pm 4$, $18 \pm 7$ respectively), cecum (WKAH, DA, and F344: $7 \pm 3$, $15 \pm 8$, $10 \pm 5$ respectively), or distal colon ($5 \pm 2$, $15 \pm 5$, $6 \pm 4$ respectively) of the three strains of young rats. Age-related decline of CD161$^+$ NK cells was observed in the distal colon. CD8$^+$ IELs in the small intestine and distal colon also significantly decreased with age. No CD4$^+$ IELs were detected in intestinal crypts of any of the older rats.

As Fig. 3 indicates, the crypt bottom of each intestinal segment was the proliferating zone in all strains. We further analyzed the frequency of CD161$^+$ NK cells and CD8$^+$ IELs in the proliferating and differentiated regions of the large intestinal crypt. The frequency of CD161$^+$ NK cells in the large intestinal crypt of young rats tended to be greater in the proliferating region than in the differentiated region, but no significant regional differences were observed in older rats. In both the proliferating and differentiated regions, DA rats had more CD161$^+$ NK cells in the distal colon crypt than did WKAH or F344 rats. With increasing age, the number of CD161$^+$ NK cells appeared to decrease in each intestinal segment (Fig. 4).

In older rats, the distribution of CD8$^+$ IELs tented to be located mostly in the differentiated region. However, in young rats, except that more CD8$^+$ IELs were found in the differentiated region of the cecal crypt in WKAH rats, there were no significant regional differences in either the cecum or the distal colon. DA rats had fewer CD8$^+$ IELs in the cecal crypt than did WKAH rats regardless of region. The number of CD8$^+$ IELs in the distal colon crypt declined with age (Fig. 5).

## Discussion

Using a cell positional approach, we analyzed the localization of CD161$^+$ NK cells, CD8$^+$ IELs, and CD4$^+$ IELs in young and older WKAH, DA, and F344 rats. It is well known that NK cells are one of the types of cells involved in immune surveillance and that they are able to kill certain tumor cells and virus-infected cells.[19] CD161 is a C-type lectin molecule and a surface marker expressed on most NK cells.[20,21] The observation that there were a great many CD161$^+$ NK cells in the intestinal epithelium of DA rats (Fig. 2A) might provide an explanation why DA rats have stronger resistance in the colon to 1,2-dimethylhydrazine or

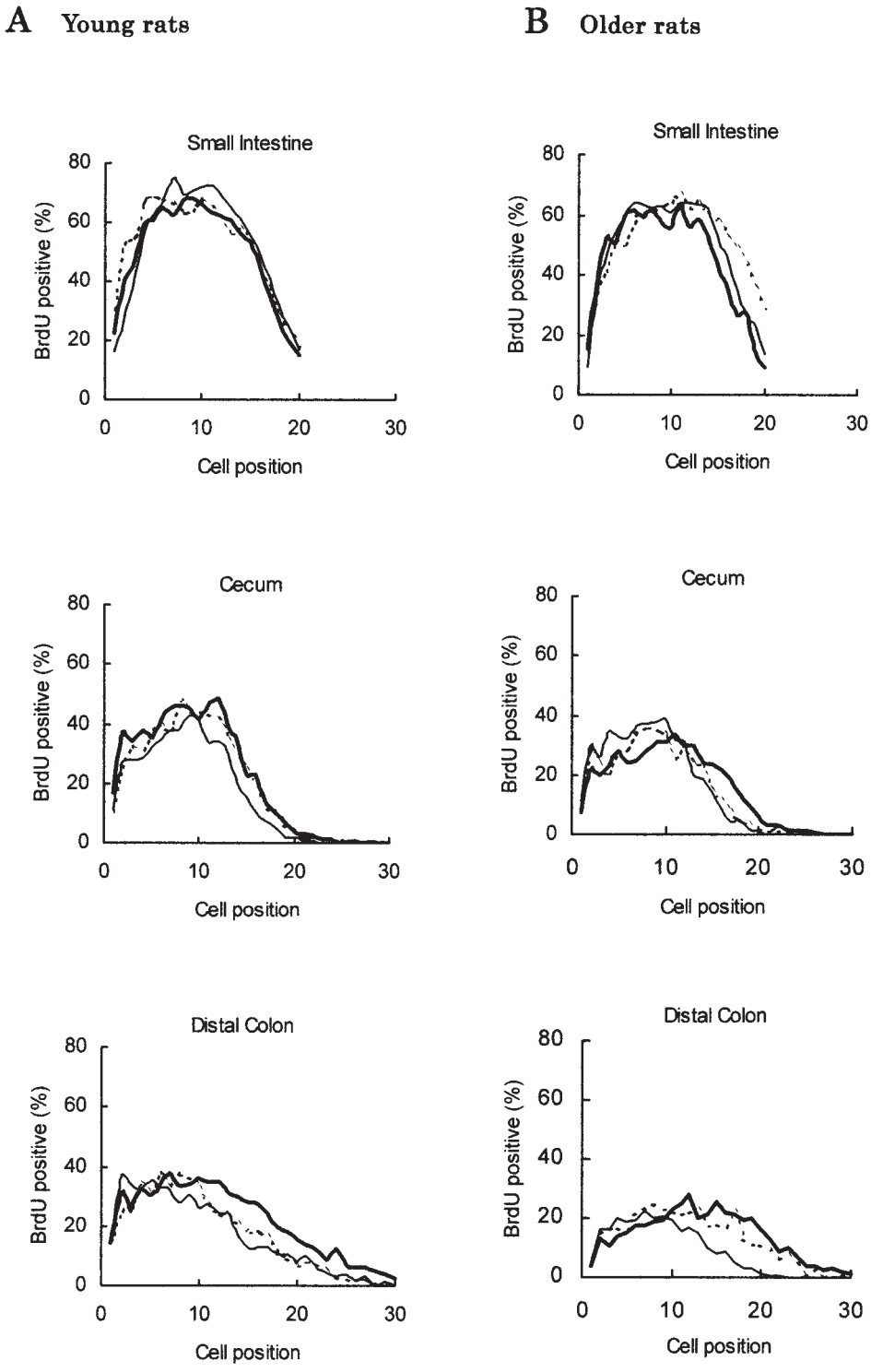


**Fig. 3.** Cell-Positional Profiles of BrdU-Incorporated Epithelial Cells in the Small Intestine, Cecum, and Distal Colon of (A) Young and (B) Older WKAH (solid line), DA (bold line), F344 (broken line) Rats.
Cell positions were measured from the bottom of the crypt.

gamma-ray-induced aberrant crypts than do WKAH or F344 rats, even though DA rats have a faster response in the onset of $G_1$ arrest and more rapid termination of $G_2$/M arrest and apoptosis of the colonic epithelia.[1] Previous studies have indicated that dietary fiber is able to modify the proportion of $CD8^+$ IELs in the differentiated region and of NK cells in the proliferating region of the cecal crypts in WKAH rats.[22,23] Comparison in this study indicates that differences in $CD8^+$ IEL frequency in the intestinal epithelium also existed among the different strains (Fig. 2), tending to be lower in DA rats than in WKAH or F344 rats. But only a few

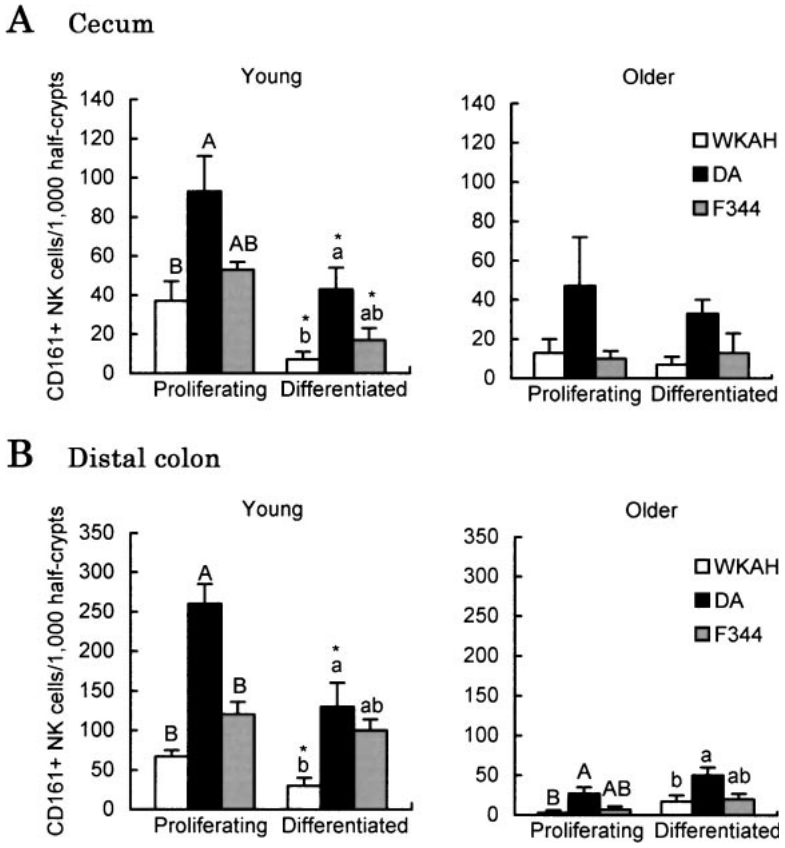


**Fig. 4.** The Number of CD161$^+$ NK Cells per 1,000 Half-Crypts in the Proliferating and Differentiated Regions of (A) the Cecum and (B) the Distal Colon Crypts of Young and Older WKAH, DA, and F344 Rats.

Values are expressed as mean ± SEM, n = 6. Bars with different capital superscript letters show significant differences in mean values in the proliferating region among three strains. Values with different small superscript letters show significant differences in mean values in the differentiated region among three strains ($P < 0.05$). *Significantly different from the corresponding proliferating region ($P < 0.05$).

CD4$^+$ IELs were observed in the intestinal crypts, and there were no significant differences among the three strains. Thus there might be a balance between CD161$^+$ NK cells and CD8$^+$ IELs in the mucosa.

The intestinal epithelium has a highly organized system for the maintenance of its epithelial cell layer, with stem cells in the base of the crypt and many clonogenic cells in the intermediate region and differentiated cells at higher cell positions in the crypt.[16,24] The possibility that there is a difference in the ability of stem and differentiated epithelial cells to attract immune cells to the epithelial layer merits investigation. Such information might be useful for the prevention or treatment of diseases related to epithelial homeostasis. Hence, we divided the longitudinally sectioned crypt into proliferating and differentiated epithelial regions according to the localization of BrdU-incorporated epithelial cells. As Fig. 3 indicates, there were no significant differences in the distribution of BrdU-incorporated epithelial cells among WKAH, DA, and F344 rats in any of the three segments of the intestine. This indicates that the conditions of cell proliferation in healthy crypts were similar in the three inbred strains. Obviously, the crypt bottom is the proliferating zone in which the stem cells are located (Figs. 1A and 3). Hence, the bottom region of crypts was defined as the proliferating region in the present study. Correspondingly, the upper region was defined as the differentiated region. Subsequently, we analyzed the localization of CD161$^+$ NK cells and CD8$^+$ IELs in the proliferating and differentiated regions respectively.

Interestingly, a majority of CD161$^+$ NK cells was found to be located in the proliferating region of the cecum and the distal colon in all young rats (Fig. 4). DA rats had a predominant number of CD161$^+$ NK cells in both the proliferating and differentiated crypt regions as compared with the WKAH and F344 rats (Fig. 4). This finding suggests that stem cells have a stronger ability to attract CD161$^+$ NK cells to the epithelial layer than do differentiated cells. But regional differences in the localization of CD161$^+$ NK cells were only slight in older strains (Fig. 4). This might be due to a decrease in the total number of CD161$^+$ NK cells and the number of proliferating cells with age. In contrast to the localization of CD161$^+$ NK cells, the number of CD8$^+$ IELs in the proliferating epithelial region was not predominant (Fig. 5). In the inbred rats used in this study, the number of CD8$^+$ IELs was lower in DA rats in both the proliferating and the differentiated region (Fig. 5). Taken together, the specific localization of CD161$^+$

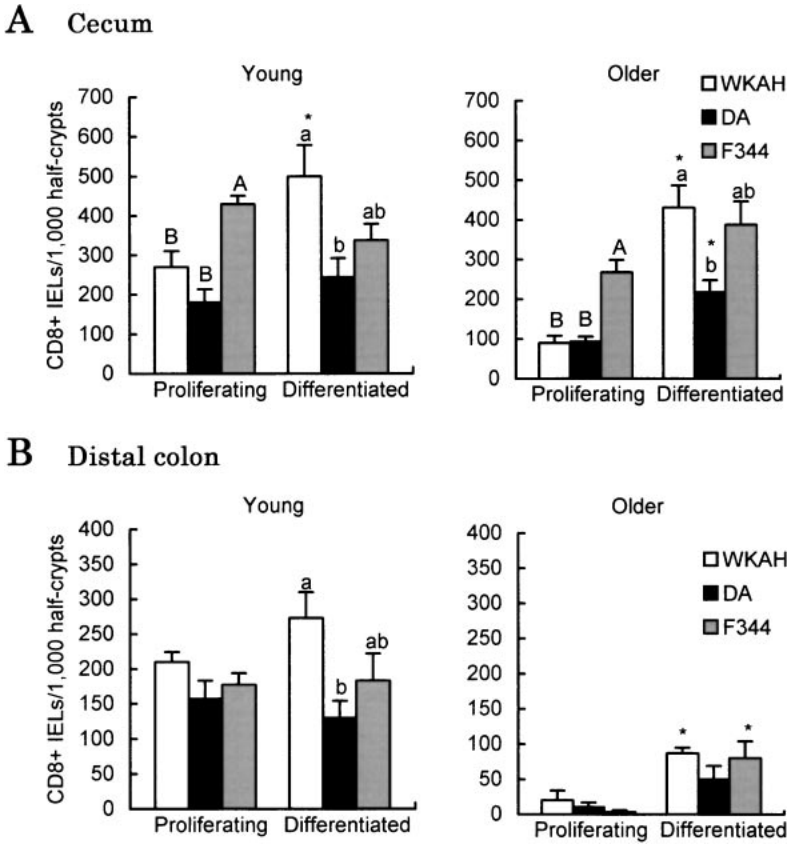


**Fig. 5.** The Number of $CD8^+$ IELs per 1,000 Half-Crypts in the Proliferating and Differentiated Regions of (A) the Cecum and (B) the Distal Colon Crypts of Young and Older WKAH, DA, and F344 Rats.

Values are expressed as mean ± SEM, n = 6. Bars with different capital superscript letters show significant differences in mean values in the proliferating region among three strains. Values with different small superscript letters show significant differences in mean values in the differentiated region among three strains ($P < 0.05$). *Significantly different from the corresponding proliferating region ($P < 0.05$).

NK cells and $CD8^+$ IELs in the large intestinal crypts might be of strategic importance in resisting antigens and in maintaining epithelial homeostasis.

The functional capacity of many organs within the body declines with age, probably due to intrinsic changes at the cellular level. Martin *et al.* have reported that after high doses of irradiation, the surviving crypts in old mice were both smaller and fewer in number than in young mice, and there was a growth delay of between about one half and one day in the older mice.[25] It is also well established that the immune system changes with age, including a decline in both cell-mediated and humoral immune response in mice, rats, and humans.[26,27] Consistently with these reports, in the present study we found that the frequencies of $CD161^+$ NK cells, $CD8^+$ IELs, and $CD4^+$ IELs as well as BrdU-incorporated epithelial cells were lower in older rats compared to young rats, especially in the distal region of the large intestine (Fig. 2, Table 2). These alterations are likely to be the result of progressive accumulation of damage occurring with age which can affect regulation of proliferation, migration, and differentiation of epithelial cells, and their interactions with immune cells and the cellular microenvironment. It is worth noting that the age-related changes were observed to be different between two crypt regions. As Figs. 4 and 5 indicate, the numbers of $CD161^+$ NK cells and $CD8^+$ IELs located in the proliferating epithelial region declined more rapidly with age than in the differentiated region. This suggests that there might be a physiological relationship between the distribution of immune cells and the proliferation of epithelial cells in the intestine.

In conclusion, our study found significant strain and age-related changes in the localization of intestinal $CD161^+$ NK cells and $CD8^+$ IELs along the longitudinal crypt axis in inbred rats. DA rats have a predominant number of $CD161^+$ NK cells, but fewer $CD8^+$ IELs compared to WKAH or F344 rats in the large intestinal epithelium. Furthermore, intestinal stem cells appear to have a stronger capacity to attract $CD161^+$ NK cells than do differentiated cells, resulting in a majority of $CD161^+$ NK cells located in the proliferating region of the crypts. Taken together with the previous report that DA rats have far stronger resistance in the colon to preneoplastic lesion than do other strains,[1] $CD161^+$ NK cells appear to play an important role in immune-surveillance at the bottom of intestinal crypts, where abnormal cells might be generated.

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