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An Unique CD4$^+$CD8$^+$ Intestinal Intraepithelial Lymphocyte Specific for DnaK (*Escherichia coli* HSP70) may be Selected by Intestinal Microflora of Rats

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Abstract

We have previously shown an age-associated increase in unique CD4$^+$CD8$^+$ intestinal intraepithelial lymphocytes (i-IEL) in rats. To elucidate the potential causes of the increase in CD4$^+$CD8$^+$ i-IEL with age, we analyzed the specificity of the CD4$^+$CD8$^+$ i-IEL and influence of intestinal microflora on the increase in this subset in aged rats. The purified CD4$^+$CD8$^+$ i-IEL proliferated in response to DnaK [*Escherichia coli (E. coli)* HSP70] in the presence of mitomycin-c (MMC)-treated syngeneic spleen cells. The proportion of CD4$^+$CD8$^+$ T cells in whole i-IEL were significantly increased in aged rats fed commercial (CL-2) diet but not in those fed home-made (purified) diet under conventional condition. No CD4$^+$CD8$^+$ i-IEL were detected in aged rats under germfree condition, irrespective of diet feeding. A larger number of *Enterobacteriaceae*, especially *E. coli*, were detected in the intestinal contents and feces from aged rats with CD4$^+$CD8$^+$ i-IEL compared with those from aged rats fed without CD4$^+$CD8$^+$ i-IEL. The unique CD4$^+$CD8$^+$ i-IEL population specific for *E. coli* HSP may be associated with long term exposure to intestinal *E. coli* in aged rats.

Introduction

Although the peripheral T cell repertoire is generated primarily in the thymus, a significant fraction of T cells in intestinal intraepithelial lymphocytes (i-IEL) is

Abbreviations: i-IEL = intestinal intraepithelial lymphocytes; *HSP70* = heat shock protein 70; *MMC* = mitomycin-c; *APC* = antigen presenting cells; *FCM* = flowcytometric; *GALT* = gut-associated tissues; *TCR* = T cell receptor; *PP* = Peyer's patch; *E. coli* = Escherichia coli; *PBS* = phosphate-buffered saline; *MHC* = major histocompatibility complex; *LN* = lymph nodes; *PPD* = purified protein derivative; *DAB* = diaminobenzine; *mAb* = monoclonal antibody; *FBS* = fetal bovine serum.

known to develop along extrathymic pathways, resulting in the generation of an additional T cell repertoire outside the thymus (1–9). Furthermore, environmental antigens including intestinal microflora and food antigens are also responsible for the skewed expansion of the T cell repertoire in the periphery, especially in the gut-associated tissues (GALT) (1).

GALT are composed of non-aggregated components such as i-IEL and lymphocytes in lamina propria, and aggregated components such as Peyer's patch (PP) (10). The mouse i-IEL consist of heterogeneous populations expressing $Thy1^+$ or $Thy1^-$, CD8αα or CD8αβ and TCRαβ or TCRγδ (5, 11–13), whereas the rat i-IEL consist mainly of $CD8^+$ T cells bearing TCRαβ (14, 15). In both mice and rats, i-IEL also contain the unique $CD4^+CD8^+$ IEL bearing TCRαβ, which increase in number with age (15, 16).

i-IEL show non-MHC-restricted cytotoxic activity in a redirected killing assay (17) and help IgA production by B cells through cytokine production (18), suggesting that these cells may play an important role in host defense against microorganisms as invaders through the intestinal wall (17, 19–24). BEAGLEY et al. reported that i-IEL of mice responded to mycobacterial HSP70 but not HSP60 (25). We previously reported that the proliferation of i-IEL of aged rats significantly proliferated in response to irradiated-syngeneic splenocytes, but those of i-IEL of young rats did not (15). In a recent report, a proliferative response of whole i-IEL of aged rats was enhanced by bacterial HSP70 in the presence of irradiated-syngeneic splenocytes (26). These results suggest that a significant fraction of i-IEL in aged rats may be associated with microbial stimulation with highly conserved antigens in the intestines.

In this study, we examined the specificity of unique $CD4^+CD8^+$ i-IEL increasing in aged rats under conventional condition and the roles of intestinal microflora for the expansion of this subset in i-IEL. The unique $CD4^+CD8^+$ i-IEL bearing TCRαβ were found to respond to HSP70 derived from *Escherichia coli (E. coli)* in the presence of syngeneic APC. The $CD4^+CD8^+$ population expanded in the i-IEL of aged rats in association with the increased number of *Enterobacteriaceae,* especially *E. coli*, in the contents of small intestine and feces. The implications of our findings for the influence of the intestinal microflora on the expansion of unique T cell population in i-IEL were discussed.

Materials and Methods

Animals

Germfree Wistar male rats and conventional Wistar male rats (supplied by Wistar Inst. PA, USA) were maintained in our laboratory and fed commercial diet (CL-2) obtained from Crea Japan (Tokyo, Japan) or home-made diet which were made in our laboratory (27). The composition of the diets are shown in Tables 1 and 2. The germfree rats had been produced by our own technique and maintained for more than 40 generations in this laboratory (28). They were maintained in stainless isolators. Their germfree status was checked as described by WAGNER et al. (29).

Table 1. Composition of home-made diet.

Ingredient	Amount
	g/kg
Casein	230
Wheat Starch	277
Rice flour	277
Rice bran	20
Non-nutritive fiber	30
Sesame oil	70
Yeast extract	30
Desiccated liver	30
AIN-76 mineral mixture[1]	30
Vitamin mixture	10

[1] AIN-76 mineral mixture (AIN 1977).

Table 2. Difference in composition of amino acid and vitamin between home-made and CL-2 diets.

	Home-made diet	CL-2 diet[1]
Amino acid	g/100 g	
Lysine	51.6	14.2
Histidine	16.9	5.3
Arginine	25.3	13.8
Aspartic acid	65.8	18.4
Threonine	30.2	9.4
Serine	44.8	37.0
Proline	71.7	11.9
Glycine	17.9	12.4
Alanine	25.9	11.9
Valine	33.5	11.6
DL-Methionine	30.2	5.9
Iso-leucine	23.0	10.0
Leucine	72.5	19.1
Tyrosine	38.9	7.8
Phenylalanine	37.1	10.4
Vitamin		
Vitamin A (IU/100 g)	15,000	20,000
Vitamin D (IU/100 g)	1,000	4,000
Vitamin E (mg/100 g)	3,500	418
Thiamine (mg/100 g)	120	38
Riboflavin (mg/100 g)	30	18
Vitamin B6 (mg/100 g)	40	20
Vitamin B12 (mg/100 g)	250	415
Vitamin C (mg/100 g)	2,000	0
Panthothen acid (mg/100 g)	300	35
Nicotinic acid (mg/100 g)	120	131
Folic acid (mg/100 g)	10	5
Chorine chloride (mg/100 g)	2,500	10
Biotin (mg/100 g)	10,000	152
Inositol (mg/100 g)	1,000	245

[1] CL-2 diet was commercially supplied by Crea Japan (Tokyo).

Antibodies

The following monoclonal antibodies (mAbs) were used in this work: PE-conjugated W3/25 (anti-rat CD4 mAb), FITC-conjugated OX-8 (anti-rat CD8 mAb), FITC-conjugated OX-19 (anti-rat CD5 mAb), and FITC-conjugated OX-12 (anti-rat κ chain mAb) were purchased from Serotec (Oxford, UK). R73 (anti-rat TCRαβ mAb) was kindly provided by Dr. T. HÜNIG (Universität Würzburg) (30) and was conjugated with FITC or biotin according to standard procedure. Biotin-conjugated 1F4 (anti-rat CD3 mAb) was kindly provided by Dr. T. TANAKA (the Tokyo Metropolitan Institute of Medical Science) (31, 32). Secondary antibodies were PE-conjugated anti-mouse IgM, biotinylated anti-mouse IgM and biotinylated anti-mouse IgG purchased from Caltag Laboratories (San Francisco, CA, USA). Peroxidase-conjugated goat anti-mouse (F(ab')2 fragments of IgG was obtained from Amersham International plc. (Amersham, UK). Streptavidin-PE and streptavidin-DuoCHROM was obtained from Becton Dickinson (San Jose, CA, USA). Streptavidin-RED613 was purchased from Gibco BRL (Gaithersburg, MD, USA).

Preparation of lymphocytes

i-IEL were prepared according to a modification of a method of CERF-BENSUSSAN et al. (33). Briefly, small intestine was flushed with 50 ml of sterile phosphate-buffered saline (PBS), fat and PPs were removed. The intestine was opened longitudinally, cut into 5 mm-long pieces, and then washed in PBS. The pieces were stirred in 199 medium (GIBCO, Gland Island, NY, USA) supplemented with 20% Nu serum (Collaborative Research Inc., Bedford, MA, USA) and 1 mmol/L dithioerythritol (Sigma Chemical, Co., St. Louis, MO, USA) at 37 °C for 30 min and then centrifuged [Kubota model 5700 (Tokyo, Japan), × 150 g, 4 °C, 5 min]. The pieces were resuspended in RPMI 1640 (GIBCO) supplemented with 20% Nu serum and agitated at 37 °C for 20 min, and then debris were removed. The resultant soup was then filtered through a glass wool column. This filtration procedure removed a large proportion of the dead cells and debris. This glass wool column-passed cell suspension was centrifuged, resuspended in 8 ml of 44% Percoll (Sigma), and layered on 5 ml of 67.5% of Percoll. The gradient was centrifuged at × 700 g at 20 °C for 20 min (Kubota model 5700). Lymphocytes at the interface were harvested, and washed twice with Hanks-balanced salt solution (HBSS). The $CD4^-CD8^+$ or $CD4^+CD8^+$ i-IEL were sorted with EPICS® Elite ESP (COULTER Corp., Miami, FL, USA) after stained with FITC-conjugated OX-8 and PE-conjugated W3/25. The purity of $CD4^-CD8^+$ i-IEL or $CD4^+CD8^+$ i-IEL was 99.3% or 97.5%, respectively as accessed by two color flowcytometric (FCM) analysis. The syngeneic spleen was homogenized with HBSS using glass slides and then filtered by cotton gauze to remove cell debris.

Flowcytometric (FCM) analysis

For two-color FCM analysis, cells were stained with FITC-conjugated mAb and PE-conjugated mAb or biotinylated mAb followed by streptavidin-PE. For three color FCM analysis, cells were stained with FITC-conjugated mAb, PE-conjugated mAb and biotinylated mAb followed by streptavidin-DuoCHROM or streptavidin-RED613. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Proliferation assay

i-IEL were cultured according to NAKAMURA et al. with minor modification (26, 34). Briefly, these cells (2×10^5) were suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (CSL Limited, Victoria, Australia), 10 mmol/L HEPES (Dojin chemical institute, Japan), 5×10^{-5} mol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin and cultured with mitomycon-c (MMC)-treated syngeneic splenocytes (2×10^5) in the presence or absence of 10 μg/ml *E. coli* HSP70 (DnaK) (StressGen Biotechnologies Corp., Victoria, B. C., Canada). Five or triplicate cultures of i-IEL were incubated in 200 μl of medium in 96-well flat

bottom plates (FALCON, Lincoln. NJ, USA) for 3 days. For the last 6 h, cells were pulse-labeled with [^{3}H] thymidine (Amersham, Buckinghamshire, UK) (1 μCi/well) before harvesting on glass-fiber filters.

Immunohistochemistry

Immunohistochemical analysis was performed by a peroxidase staining technique as previously described (13). Briefly, intestine was promptly fixed in periodate-lysine 4% paraformaldehyde for 6 h, frozen in OCT compound (Miles Pharmaceutical, Naperville, IL, USA) and sectioned at 6 mm on a cryostat. For single staining, the sections were incubated with anti-CD4 mAb or anti-CD8 mAb washed and incubated with peroxidase-conjugated goat anti-mouse F(ab')2 fragments of IgG and then reacted with 0.25% diaminobenzine (DAB) containing 0.5% hydrogen peroxide. For double staining with anti-CD4 and anti-CD8 mAb, the sections were incubated with anti-CD4 mAb followed by peroxidase-conjugated anti-mouse IgG, washed and reacted with 0.02% 4-chloro-1-naphtol containing 0.5% hydrogen peroxide. The stain sections were washed with 0.1 mol/L glycine (pH 2.2). The sections were incubated with anti-CD8 mAb, washed and stained with DAB.

Bacterial counts in the small intestine and feces

The contents in the small intestine and feces were collected from rats fed CL-2 diet or home-made diet. They were homogenized in 10 ml of sterile PBS. The homogenates were diluted serial with PBS and spread on agar medium plates (Eiken Chemical Co., Ltd., Tokyo, Japan) for *Enterobacteriaceae,* and on to LBS agar medium plates (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 8 g/L Lab Lemco powder (Oxoid Ltd., Basingstoke, UK), 1.5 g/L sodium acetate trihydrate and 3.7 g/L acetate for *Lactobacilli.* The agar plates were incubated at 37 °C for 24 h, and the LBS agar plates were incubated anaerobically with an anaerobic system (GasPak Anaerobic System; BBL Microbiology Systems) at 37 °C for 48 h. The colonies on the plates were finally enumerated (35).

Statistical analysis

Two-way ANOVA (36) was used to analyze the main effects (diet, breeding condition) and the interaction (diet × breeding condition). The analyses were performed by using a statistics package (Mascot; Yukkum, Tokyo, Japan). Data were analyzed by Student's *t* test. Differences of $p < 0.05$ were considered to be significant. All experiments were performed at least twice.

Results

Proliferative responses of CD4$^+$CD8$^+$ i-IEL from aged rats to DnaK (*E. coli* HSP70)

We have previously reported that i-IEL from aged F344 rats but not from young rats respond to syngeneic spleen cells (15), and NAKAMURA et al. has recently reported that the proliferation of the i-IEL from aged Lewis rats was enhanced by mycobacterial HSPs (26). To confirm these earlier findings, we first examined the proliferative responses of whole i-IEL from 6 month-old Wistar rats against *E. coli* HSP70 (DnaK) in the presence of syngeneic antigen presenting cells (APC). Consistent with our previous report with F344 rats (15), the i-IEL from the aged Wistar rats showed proliferative response in the presence of MMC-treated syngeneic splenocytes, whereas such response was not detected in the i-IEL of young (8 week-old) Wistar rats (Fig. 1a). The proliferative response of

the aged rats i-IEL was significantly enhanced ($p < 0.05$) in response *E. coli* HSP70 (Fig. 1b), but those of the young rats did not respond to the HSP70. To further determine which cell population in the i-IEL from aged rats was able to respond to the bacterial HSP70, we purified $CD4^-CD8^+$ i-IEL and $CD4^+CD8^+$ i-IEL from aged rats by using EPICS® Elite ESP of COULTER cell sorting system. The purity of each subset was 99.3% for $CD4^-CD8^+$ i-IEL or 97.5% for $CD4^+CD8^+$ i-IEL (Fig. 2a). As shown in Figure 2b, the purified $CD4^+CD8^+$ i-IEL responded to syngeneic spleen cells and much more vigorously to HSP70 in the presence of syngeneic spleen cells, whereas $CD4^-CD8^+$ i-IEL showed

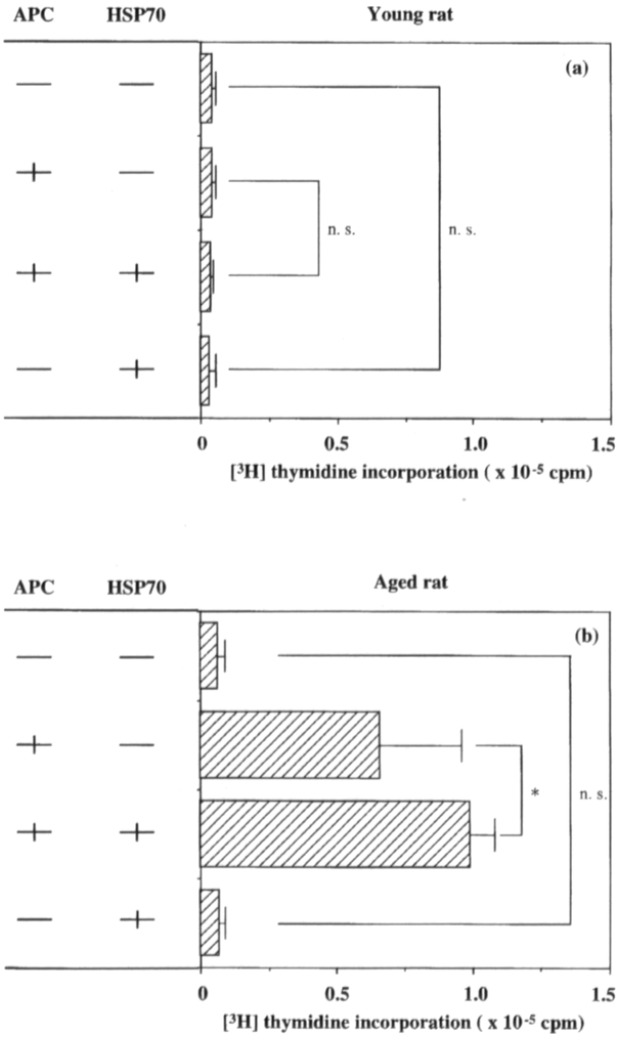

Figure 1. Proliferative responses of i-IEL of young (8 week-old) (a) or aged (6 month-old) (b) Wistar rats fed on a CL-2 diet under conventional condition. Proliferation was assayed after 3-day incubation of primary culture with HSP70 (10 μg/ml) or not in the presence or absence of MMC-treated syngeneic splenocytes. *$p < 0.05$. n. s., not significant.

little, if any, proliferation in response to HSP70 with syngeneic spleen cells. These results indicate that $CD4^+CD8^+$ i-IEL of aged rats can respond to *E. coli* HSP70 in the presence of syngeneic APC.

Comparison of appearance of $CD4^+CD8^+$ i-IEL among aged rats fed CL-2 diet or home-made diet under conventional conditions or germfree conditions

i-IEL were recovered from 6 month-old rats bred under conventional conditions, which were fed commercial CL-2 diet or home-made diet. After staining with various mAbs, the i-IEL were examined by FCM analysis. A representative FCM profile from 5 rats of each group was shown in Figure 3 and the data were

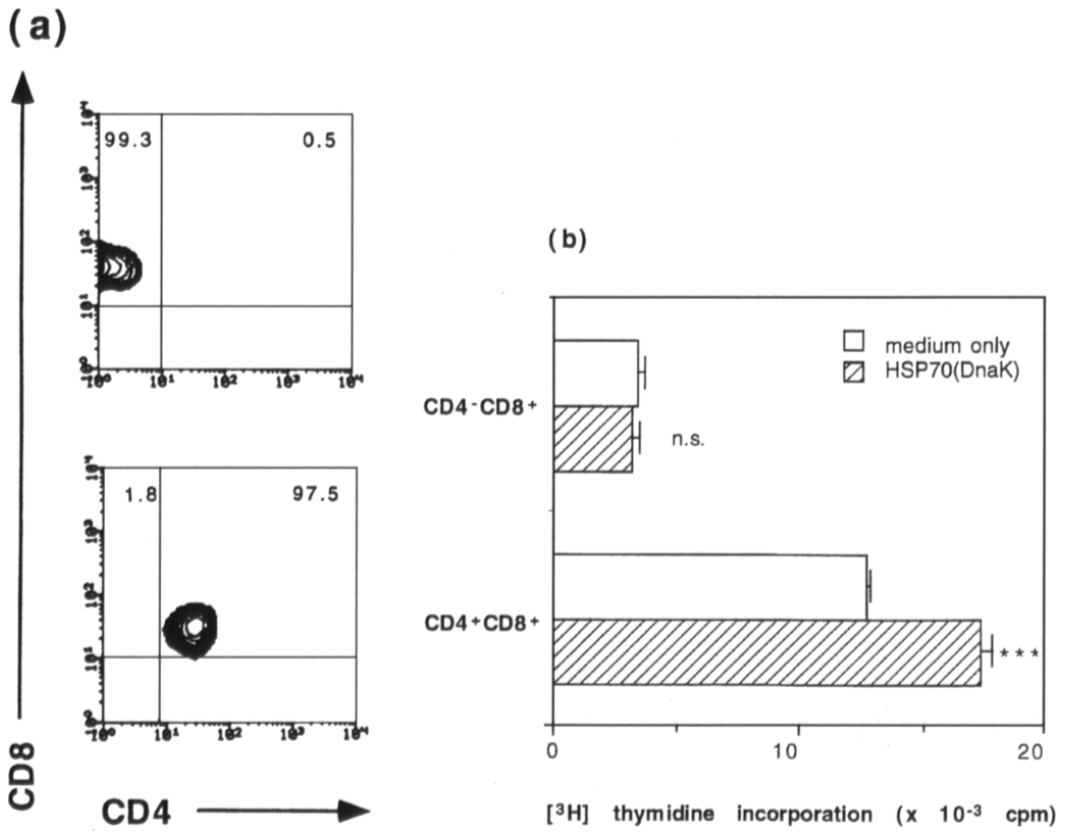

Figure 2. Proliferative responses of purified $CD4^-CD8^+$ i-IEL and $CD4^+CD8^+$ i-IEL to HSP70 (DnaK). $CD4^-CD8^+$ cells and $CD4^+CD8^+$ cells from i-IEL of aged rats fed CL-2 diet were purified with EPICS® Elite ESP of COULTER cell sorting system. Sorted cells before culture were analyzed by two-color flow cytometry. y-axis for both panels presents FITC fluorescence for CD8 and x-axis for both panels represents PE fluorescence for CD4. The purity of enriched $CD4^-CD8^+$ i-IEL and $CD4^+CD8^+$ i-IEL were 99.3% and 97.5%, respectively. Proliferation of these cells (2 × 104 cells/well) was assayed after 3-day incubation of primary culture with or without HSP70 (10 μg/ml) in the presence of MMC-treated syngeneic splenocytes. $^{***}p < 0.001$. n. s., not significant.

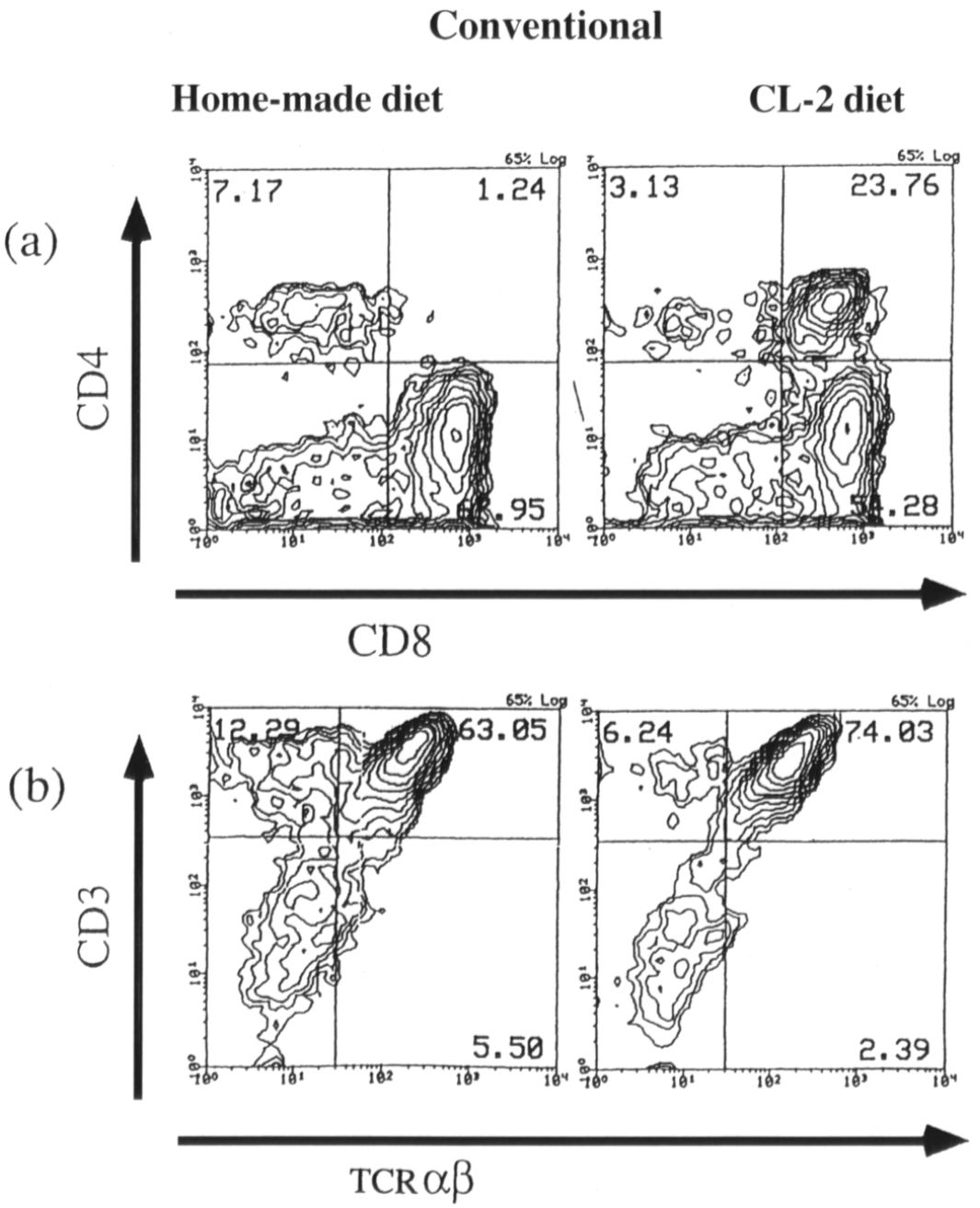

Figure 3. Flowcytometric analysis on i-IEL from 6-month old rats fed home-made or CL-2 diet under conventional conditions. (a) Expression of CD4 and CD8 on i-IEL. i-IEL were stained with PE-conjugated W3/25 (anti-CD4 mAb) and FITC-conjugated OX-8 (anti-CD8 mAb), and analyzed on a FACScan flow cytometer. (b) Expression of CD3 and TCRαβ. i-IEL were stained with biotin-conjugated 1F4 (anti-CD3 mAb) and FITC-conjugated R73 (anti-TCRαβ mAb). Streptavidin-PE was added before analysis. Data were presented as two-dimensional contour maps.

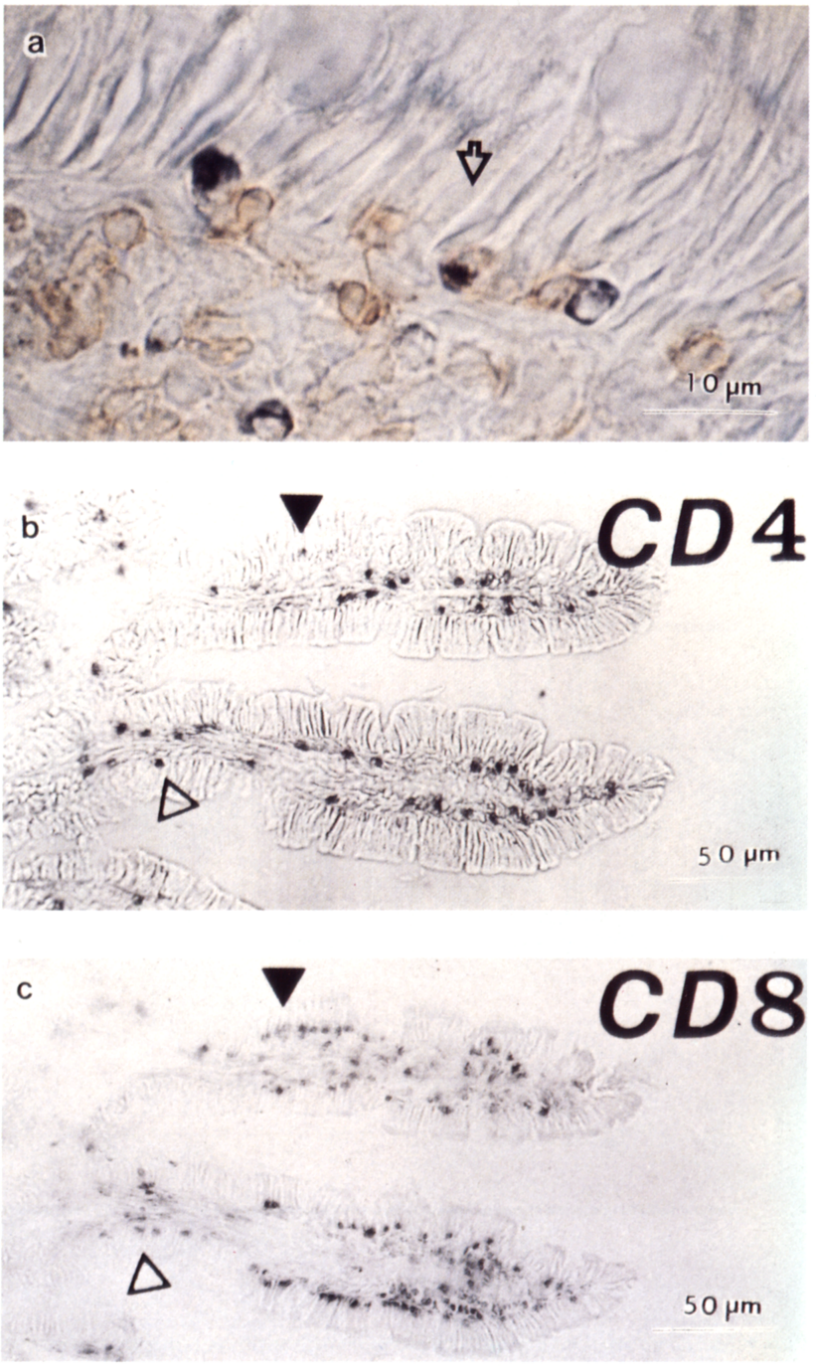

Figure 4. The lower part of the small intestine from aged rats fed CL-2 diet under conventional conditions was sectioned and fixed in periodate-lysine paraformaldehyde. Each section was incubated with monoclonal antibodies and stained with diaminobenzine (DAB) and/or 4-chloro-1-naphtol. (a) IEL was incubated with W3/25 (anti-CD4 mAb) and OX8 (anti-CD8 mAb).

Table 3. Proportion of T cell subset in IEL of aged rats fed home-made or CL-2 diet under conventional or germfree conditions.

Breeding condition	Diet	% of total IEL[1]		
		$CD4^+CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^+$
Conventional	Home-made	6.73 ± 0.52	1.38 ± 0.22	68.04 ± 3.57
	CL-2	2.70 ± 0.21	24.40 ± 2.33**	55.40 ± 3.85
Germfree	Home-made	2.49 ± 0.12	1.35 ± 0.55	76.80 ± 6.20
	CL-2	8.33 ± 2.83	2.04 ± 0.33	73.15 ± 7.74

[1] The percentage of each subset was calculated by FCM analysis after staining i-IEL with anti-CD4 and anti-CD8 mAb. Values are means of five rats ± SEM.
** Significant difference from value for rats fed home-made diet under conventional conditions ($p < 0.005$).

summarized in Table 3. In 6-month old rats bred under conventional conditions, which were fed CL-2, a much larger proportion of $CD4^+CD8^+$ i-IEL were detected (Fig. 3a, Table 2, $p < 0.005$). Although most of the i-IEL expressed TCRαβ in these rats, an appreciable number of $CD3^+$ TCRαβ$^-$ i-IEL corresponding to TCRγδ i-IEL were detected in the i-IEL (Fig. 3b). The $CD3^-$ TCRαβ$^-$ i-IEL were also detected at an appreciable level in the rats. The $CD3^-$ TCRαβ$^-$ $CD4^-CD8^-$ i-IEL did not belong to B cells because they did not express surface Ig as accessed by staining with anti-Igκ chain mAb (data not shown).

The $CD4^+CD8^+$ cells were reported to be detected, albeit only a few, in the GALT including PP and lamina propria in mice (10). To confirm that the $CD4^+CD8^+$ i-IEL were really located within the epithelium, we carried out immunohistochemical analysis of the small intestine of aged conventional rats. Two color staining using naphtol and DAB revealed that $CD4^+$ i-IEL coexpressed CD8 in these rats (Fig. 4a). The $CD4^+CD8^+$ i-IEL were detected at the intraepithelium and preferentially located at the lateral surface in the longitudinal sections of villi from the terminal ileum, whereas these $CD4^+CD8^+$ i-IEL were rare in the crypts or dome epithelium overlying PP (Fig. 4b, 4c, and data not shown).

To further characterize the $CD4^+CD8^+$ i-IEL, three color staining analysis with anti-CD4 mAb, anti-CD8 mAb, and anti-CD5 mAb was carried out for lymph nodes (LN) and IEL from aged rats fed CL-2 diet under conventional conditions. As shown in Figure 5, only a few $CD4^-CD8^+$ i-IEL expressed CD5, which is a pan T cell marker, but all $CD4^+CD8^+$ i-IEL expressed much the same intensity of CD5 as the peripheral mature T cells, suggesting that the lineage of $CD4^+CD8^+$ i-IEL may differ from that of $CD4^-CD8^+$ i-IEL.

◀ CD4 was stained with 4-chloro-1-naphtol and CD8 with DAB. (b) CD4 was stained with DAB. $CD4^+$ i-IEL were detected in the lower part of villi. (c) $CD8^+$ i-IEL were stained with DAB. $CD8^+$ i-IEL were detected in the middle lower part of villi. The arrow points at $CD4^+CD8^+$ i-IEL.

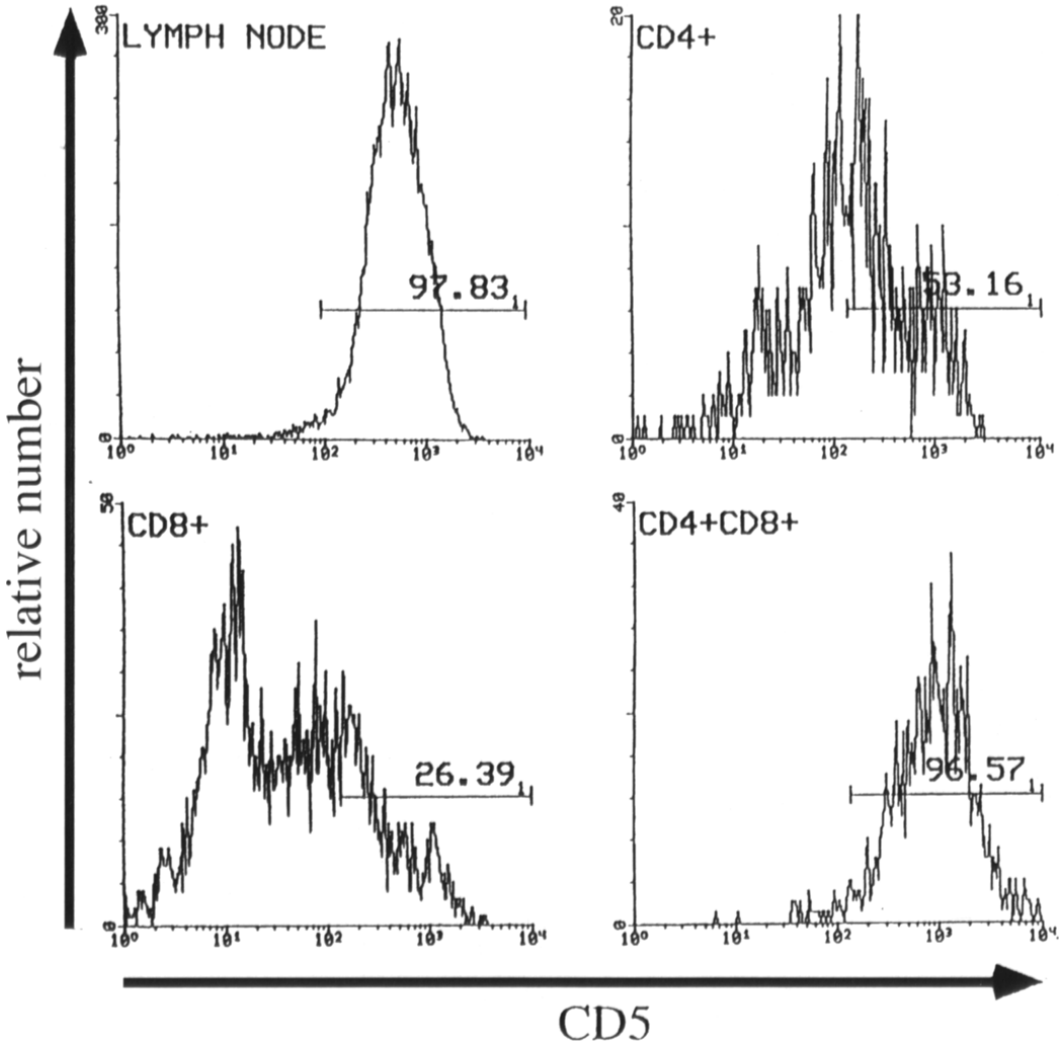

Figure 5. A relative number of $CD5^+$ lymphocytes of LN and i-IEL from aged rats fed CL-2 under conventional conditions were stained with FITC-conjugated OX-19 (anti-CD5 mAb), PE-conjugated W3/25 (anti-CD4 mAb) and biotin-conjugated OX8 (anti-CD8 mAb). RED613 was added before analysis. i-IEL were subdivided into the $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^+CD8^+$ populations.

The phenotype of i-IEL from aged rats fed home-made diet under conventional conditions were found to be quite different from those fed CL-2. The i-IEL consisted of a large number of $CD4^-CD8^+$ cells and appreciable numbers of $CD4^-CD8^-$ cells and $CD4^+CD8^-$ cells but very few, if any, $CD4^+CD8^+$ cells (Fig. 3a). Thus, these results suggest that the composition of a diet may deeply influence the increase in the $CD4^+CD8^+$ i-IEL with age.

To elucidate whether diets affect directly or indirectly the expansion of $CD4^+CD8^+$ i-IEL, we examined cell surface markers on i-IEL from aged rats

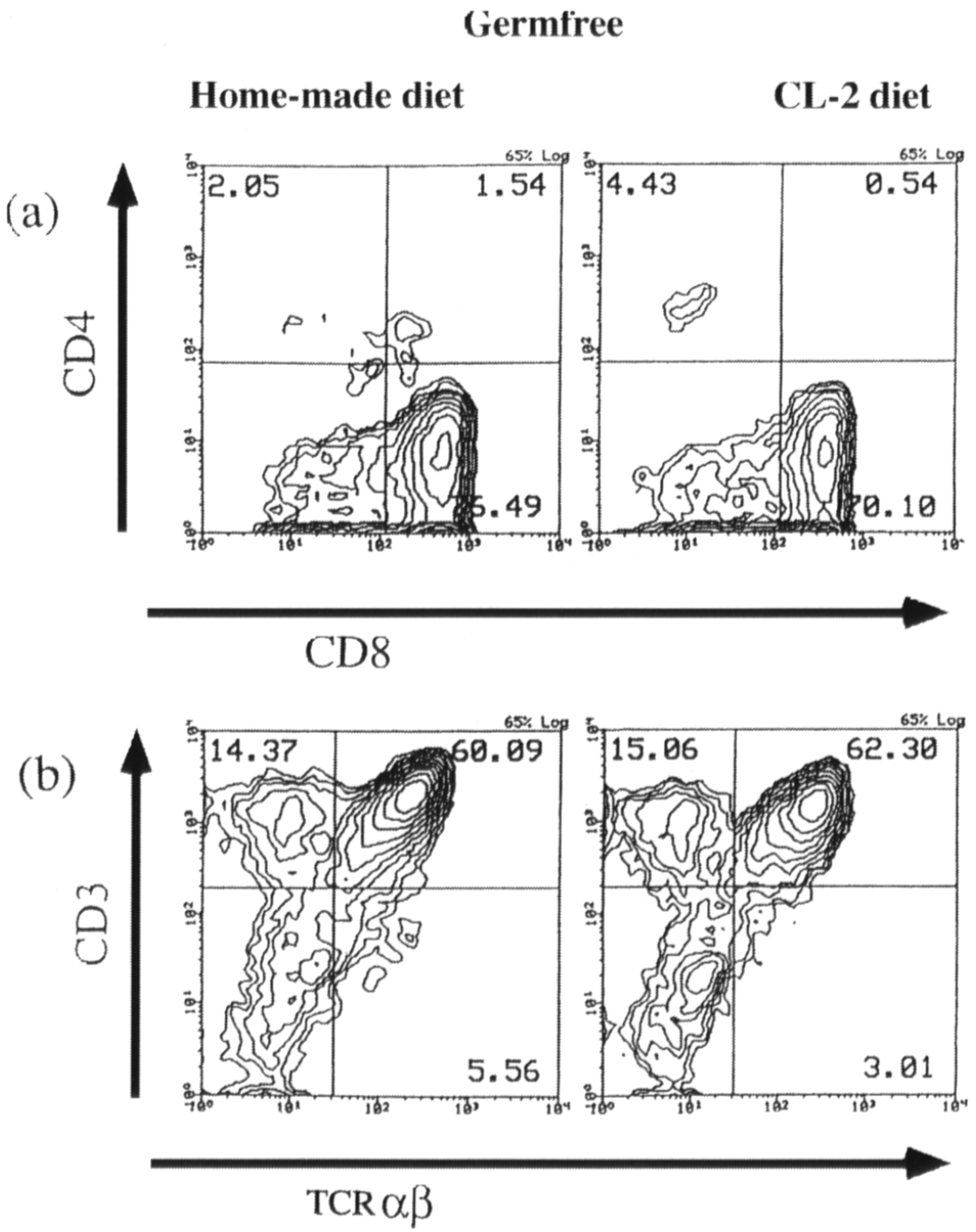

Figure 6. Flowcytometric analysis on i-IEL from 6-month old rats fed on home-made or CL-2 diet under germfree conditions. (a) Expression of CD4 and CD8 on i-IEL. i-IEL were stained with PE-conjugated W3/25 (anti-CD4 mAb) and FITC-conjugated OX-8 (anti-CD8 mAb), and analyzed on a FACScan slow cytometer. (b) Expression of CD3 and TCRαβ i-IEL were stained with biotin-conjugated 1F4 (anti-CD3 mAb) and FITC-conjugated R73 (anti-TCRαβ mAb). Streptavidin-PE was added before analysis. Data were presented as two-dimensional contour maps.

fed CL-2 diet or home-made diet which were bred under germfree conditions. Representative FCM profile from data of five rats of each groups was shown is Figure 6 and the data were summarized in Table 3. i-IEL in aged germfree rats, irrespective of home-made or CL-2 diet feeding, consisted of a large number of $CD4^-CD8^+$ cells, $CD4^-CD8^-$ cells and only a few $CD4^+$ cells but no $CD4^+CD8^+$ cells. These results suggest that intestinal microflora play important roles in expansion of $CD4^+CD8^+$ i-IEL in aged rats fed CL-2 and exclude the possibility for direct stimulation of antigens in CL-2 diet to expand the $CD4^+CD8^+$ i-IEL.

Comparison of intestinal microflora between aged rats fed CL-2 diet and those fed home-made diet

To determine the effects of diets on microflora, bacterial counts of the contents in the small intestine and feces were examined in three aged rats fed CL-2 diet or home-made diet under conventional conditions. There was no significant difference in total bacterial counts both in the contents and feces between aged rats fed CL-2 diet and those fed home-made diet. However, the number of *Enterobacteriaceae*, especially *E. coli*, in contents and feces of rats fed CL-2 diet were approximately 100-fold higher than those in rats fed home-made diet ($p < 0.05$), while fewer H2S-producing bacteria were detected in rats fed CL-2 diet ($p < 0.05$, Table 4). The counts of the other bacterial group present in the rats fed CL-2 diet were much the same as those in rats fed home-made diet.

Table 4. Difference in composition of intestinal microflora between aged rats fed home-made diet and those fed CL-2 diet under conventional conditions[1].

	Home-made		CL-2	
	Small intestine	Colon	Small intestine	Colon
		logN/g		
Total bacteria	7.77	9.77	7.86	9.60
Bacteroides	<4.76	9.14	<4.60	9.36
Bifidobacterium	<4.76	<5.43	<4.60	<5.33
Clostridium (Lecithinase+)	<0.76	<1.43	<0.60	<1.33
Enterobactericeae	3.65	3.88	5.58*	6.50*
E. coli	<1.76	<2.43	5.54*	6.49*
$H2S^+$	2.06*	3.13*	<1.60	<2.33
others	3.64	3.79	4.51	5.03
Enterococcus	3.93	6.34	5.00	6.52
Lactobacillus	7.77	8.98	7.73	9.32
Staphylococcus	4.88	5.55	4.83	6.05
Bacillus	<1.76	3.88	<1.60	<2.33
Candida	<1.76	<2.43	<1.60	<2.33

[1] The contents in the small intestine and feces were collected from 6-month old rats fed home-made or CL-2 diet under conventional conditions. Values are means of three rats of each group. SEM are less than 1log in all samples.

* Significant difference from values for rats fed home-made or CL-2 diet ($p < 0.05$).

Discussion

We have obtained evidence that the unique $CD4^+CD8^+$ i-IEL from aged Wistar rats are able to respond to *E. coli* HSP70 in the presence of syngeneic APC. This extends the scope of an earlier finding of NAKAMURA et al. that whole i-IEL from aged Lewis rats recognize purified protein derivative (PPD) including mycobacterial HSP70 presented by syngeneic APC (26). Our present data suggest that the responders to the bacterial HSP are a $CD4^+CD8^+$ i-IEL subset, which are increased in aged rats of various strains. Furthermore, this unique subset is able to respond to HSP70 derived from *E. coli*, which is one of major members in intestinal microflora. $CD4^+CD8^+$ i-IEL may expand with age through long term exposure to the bacterial HSPs.

We also demonstrate in the present study that diets influence the selection of $CD4^+CD8^+$ i-IEL in aged rats under conventional conditions. The $CD4^+CD8^+$ i-IEL were detected in aged rats fed CL-2 diet but hardly detected in aged conventional rats fed home-made diet. Two possibilities can be brought forward to explain the difference between aged rats fed CL-2 diet and those fed home-made diet. The first possibility is that CL-2 may contain antigens capable of directly stimulating $CD4^+CD8^+$ i-IEL. The second possibility is that the different diets may cause differences in the composition of the microflora, which may affect the selection of $CD4^+CD8^+$ i-IEL. Our results with aged germfree rats, fed a CL-2 diet or home-made diets suggest that stimulation with intestinal microflora, rather than antigens present in a CL-2 diet, is important for the expansion of the $CD4^+CD8^+$ i-IEL. Interestingly, aged rats fed on a CL-2 diet contained a large number of *E. coli* in microflora, which may express DnaK *E. coli* HSP70. Taken together, these results suggest that an unique $CD4^+CD8^+$ i-IEL population may be selected by HSP70 through long term exposure to intestinal *E. coli*. So far, we do not know why rats who fed home-made contained fewer *Enterobacteriaceae* but a larger number of H2S-producing bacteria in their microflora as compared with those fed on a CL-2 diet. Major differences in the composition of these two diets were the amount of vitamins and amino acids. The differences in various compositions of diets and the mutual interaction of microflora may be responsible for the different compositions of the microflora between rats fed on a CL-2 diet and those fed home-made diet.

The origin of the unique $CD4^+CD8^+$ i-IEL is unknown. Immature $CD8^+$ T cell precursors in the thymus are reported to acquire CD4 during differentiation *in vivo* and *in vitro* (37, 38). Recent studies, which were examined with athymic nude mice, scid mice, and RAG gene knock-out mice repopulated with T cell-depleted bone marrow cells, have revealed that a significant fraction of i-IEL can differentiate independently of the thymus (3, 39). It is assumed that the $CD8^+$ i-IEL may differentiate to $CD4^+CD8^+$ i-IEL at the local site of intestine under the influence of stimulation with intestinal microflora. On the other hand, there is evidence that activation and culturing of human $CD4^+$ T cell clones in IL-4 resulted in the acquisition of CD8 due to its *de novo* synthesis (40). Therefore, it is alternatively possible that $CD4^+CD8^+$ i-IEL may originate from $CD4^+$ cells. IL-4 is known to have an ability to induce expression of Ia on B cells and

macrophages. CERF-BENSUSSAN et al. (33) reported that IFN-γ but not IL-2 appear to contribute to induction of Ia on intestinal epithelium. Stimulation with intestinal microflora including *E. coli* HSP70 may activate the i-IEL to produce both Ia-inducing and CD8-inducing cytokines, resulting in appearance of $CD4^+CD8^+$ i-IEL and Ia intestinal epithelium in aged conventional rats fed CL-2 diet.

CD4 molecules are known to play an important role as accessory molecules for T cell recognition to MHC class II molecules (41). $CD4^+CD8^+$ i-IEL may be involved in recognizing antigens in the context of MHC class II expressed on intestinal epithelium. NAKAMURA et al. have suggested that the proliferative response by $CD4^+CD8^+$ i-IEL to PPD is also involved in CD8 and MHC class I molecules (26). Therefore, $CD4^+CD8^+$ i-IEL may contain cells responding to the bacterial antigens in a MHC class I-restricted CD8-dependent manner. The cytolytic activity of i-IEL is known to be regulated by externally derived stimuli via a specific functional interaction between i-IEL and gut-associated antigens (21). Expression of CD8 is reported to be important for anti-CD3 directed cytolytic activity by the $CD4^+CD8^+$ T cell clones (37). The $CD4^+CD8^+$ i-IEL have been recently reported to display a high level of cytolytic activity upon TCR triggering (23). Taken together, the $CD4^+CD8^+$ i-IEL may play important roles in the first line of defense against various pathogens in epithelium through recognizing them. And they may be presented by MHC class II in a CD4-dependent manner and through their cytolytic activities against MHC class I-restricted antigens. We need further functional analysis for elucidation of the roles of the $CD4^+CD8^+$ i-IEL in the mucosal immunity.

In the present study, we demonstrated that purified $CD4^+CD8^+$ i-IEL of aged rats proliferated in response to *E. coli* HSP70 in the presence of APC and an increase number of *E. coli* were detected in the intestinal content and feces of aged rats. In conclusion, $CD4^+CD8^+$ i-IEL specific for *E. coli* HSP70 are expanding during exposure to microbial flora, especially *E. coli* that has been in the intestines for a long period during aging.

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