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Development and cytolytic function of intestinal intraepithelial T lymphocytes in antigen-minimized mice

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SUMMARY

Intraepithelial T lymphocytes in the small intestine (IEL) consist of $\alpha\beta$ T-cell receptor (TCR)-bearing T cells ($\alpha\beta$-IEL) and $\gamma\delta$ TCR-bearing T cells ($\gamma\delta$-IEL). Development and cytolytic activation of $\alpha\beta$-IEL sharply attenuate in germ-free (GF) mice fed a natural diet (Nat-GF), but the number and cytotoxicity of $\gamma\delta$-IEL are comparable between conventional (CV) and Nat-GF mice. In this report, we compared the properties of IEL in Nat-GF mice and GF mice fed antigen-minimized diet (AgM-GF mice) of C57BL/6 strain to evaluate an influence of gut antigenic load on IEL development. Numbers of $\alpha\beta$-IEL and $\gamma\delta$-IEL in AgM-GF mice were less by 1·9- and 1·4-fold than those in Nat-GF mice, respectively. Significant decreases in the proportions of $CD4^+8^-$, $CD4^-8\alpha\beta^+$, and $CD4^+8^+$ subsets and a resultant increase in the ratio of $CD4^-8\alpha\alpha^+$ subset were evident in $\alpha\beta$-IEL of Nat-GF mice compared with CV mice, but the subset constitution of $\alpha\beta$-IEL was similar between Nat-GF and AgM-GF mice. In contrast, relative composition of $\gamma\delta$-IEL was not different between CV, Nat-GF, and AgM-GF mice. $\alpha\beta$-IEL displayed low cytolytic activity in Nat-GF mice and were almost deprived of their cytotoxicity under the antigen-minimized condition. While $\gamma\delta$-IEL were strongly cytolytic in Nat-GF mice their cytolytic activity was remarkably reduced in AgM-GF mice. These results indicate that $\gamma\delta$-IEL are activated independently of microbial colonization in the gastrointestinal tract but their activation occurs in response to the exogenous antigenic substances other than live micro-organisms.

INTRODUCTION

Most T cells in the peripheral lymphoid organs differentiate in the thymus and express $\alpha\beta$ T-cell antigen receptor (TCR) together with either CD4 or CD8 molecules. On the contrary, abundant T cells located in the intestinal epithelial layer (intraepithelial T lymphocytes; IEL) are composed of $\alpha\beta$ TCR^+ and $\gamma\delta$ TCR^+ cells ($\alpha\beta$-IEL and $\gamma\delta$-IEL, respectively) and the major population of IEL expresses CD8$\alpha\alpha$ homodimer. A sizeable number of $CD8\alpha\alpha^+$ $\gamma\delta$-IEL are present in the small intestine of athymic nude mice and all subsets of $\alpha\beta$-IEL are generated in thymectomized and irradiated mice reconstituted by fetal liver cells, demonstrating that IEL differentiate in the absence of thymus.[1–3] $CD8\alpha\alpha^+$ $\gamma\delta$-IEL also develop independently of major histocompatibility complex (MHC) class I expression, although the development of $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ $\alpha\beta$-IEL is almost blocked in β_2-microglobulin- and/or TAP1-deficient mice.[4,5] These findings suggest that the developmental pathway and selection mechanism of IEL are distinct from those of peripheral T cells.

Physiologic function of IEL remains unclear, but taking into consideration that IEL are in close contact with intestinal epithelial cells (IEC) and kill target cells when bridged by anti-TCR antibodies, it has been widely accepted that IEL may recognize and remove infected or transformed enterocytes.[6,7] The fact that the cytolytic activity of $\alpha\beta$-IEL sharply attenuates in germ-free (GF) mice supports the idea that $\alpha\beta$-IEL play a crucial role against infection of enteric pathogens.[8–10] Actually, reovirus-specific, cytolytic $\alpha\beta$ TCR^+ cells are induced between IEC of mice which have been exposed to reovirus orally.[11] In contrast, $\gamma\delta$-IEL in GF mice can kill target cells as effectively as those in conventional (CV) mice, suggesting other functions of $\gamma\delta$-IEL than anti-infectious activity.[9,10] Recently, we found by examining the intestinal epithelia of mice deficient in $\alpha\beta$ TCR^+ cells or $\gamma\delta$ TCR^+ cells (TCRβ- or TCRδ-gene-targeted mice) that the absence of $\gamma\delta$ TCR^+ cells in the intestine is associated with reduction of the proliferative rate of IEC, but no such change is detected in TCRβ-deficient mice.[12] These results

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Abbreviations: AgM-GF, antigen-minimized diet-fed germ-free; CV, conventional; IEC, intestinal epithelial cells; IEL, intraepithelial T lymphocytes; Nat-GF, natural diet-fed germ-free.

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indicate that γδ-IEL, but not αβ-IEL, may regulate the generation of IEC.

In this report, we compared the composition and cytolytic activity of IEL from CV C57BL/6 mice and GF C57BL/6 mice fed natural or antigen-minimized diets. Our results show that the development and cytolytic activation of γδ-IEL are independent of live micro-organisms but they become cytolytic in response to the exogenous antigenic substances other than live micro-organisms in the gut.

MATERIALS AND METHODS

Mice

Conventional (CV) C57BL/6 mice were purchased from the Shizuoka Laboratory Animal Centre (Hamamatsu, Japan) and kept in our animal facility before use. Germ-free (GF) C57BL/6 mice were produced in the animal facility of Yakult Central Institute and separated into two groups. One group was given natural diet (Nat-GF mice) and another group was fed chemically defined, antigen-minimized diet (AgM-GF mice) whose composition is described in Table 1.[13] Mice of both sexes were used between 7 to 25 weeks of age.

Measurement of immunoglobulins and albumin in sera

Concentrations of immunoglobulin M (IgM), IgG and IgA in sera were measured by a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, each well of a 96-well polystyrene immunoplate (NUNC, Roskilde, Denmark) was coated by adding 100 μl of anti-mouse IgM, anti-mouse IgG, or anti-mouse IgA (all from Cappel, West Chester, PA) dissolved in sodium carbonate buffer (pH 9·6) and incubating at 37° for 2 hr. After wells were washed with phosphate-buffered saline (PBS) containing 0·05% Triton-X-100 (wash solution), they were blocked by adding 110 μl of 1% bovine serum albumin (BSA) solution in sodium carbonate buffer and incubating at 37° for 1·5 hr. After washing wells, serially diluted serum samples were added at 90 μl/well and incubated at 37° for 1 hr. Peroxidase (PO)-conjugated anti-mouse IgM (Cappel), PO-conjugated anti-mouse IgG (Cappel), or PO-conjugated anti-mouse IgA (Zymed, San Francisco, CA) were added at 100 μl/well after washing wells and the incubation at 37° for 1 hr was followed. After removing free antibodies, 100 μl of 0·04% *o*-phenylenediamine/0·02% H_2O_2/citrate buffer (pH 5·0) was added, wells were incubated at 37° for 10 min, and then 50 μl of 2·5 M sulphuric acid was added. Immunoglobulin concentrations were calculated from the absorbance determined by a Titertek Multiscan (Flow, McLean, VA).

Concentration of serum albumin was measured using the bromocresolgreen (BCG) method. Albumin level in each sample was automatically calculated by a 7170 Automatic Analyzer (Hitachi, Japan).

Isolation of IEL

Intestinal intraepithelial T lymphocytes (IEL) were prepared as described elsewhere.[14] Briefly, a small intestine free of the lumen content was turned inside-out with the aid of polyethylene tubing. An inverted intestine was cut into three segments, soaked in 200 ml of Hank's balanced salt solution (HBSS) supplemented with 2% fetal calf serum (FCS) and 10 mM HEPES (pH 7·3), and incubated at 37° for 45 min with constant shaking (180 r.p.m.). Cell suspensions were collected and filtered through a gauze. The resultant cells were applied to a Percoll density gradient (30%). The pellet was suspended and applied on a Percoll density gradient (44%/70%), and then cells at the interface were recovered as IEL.

Immunofluorescence analysis

IEL were stained by the following antibodies and analysed by an EPICS ELITE flowcytometer: fluorescein isothiocyanate (FITC)-conjugated or biotinylated anti-αβ TCR monoclonal antibody (mAb) H57–597, FITC-conjugated anti-γδ TCR mAb GL-3, phycoerythrin (PE)-conjugated anti-CD4 mAb RM4–5, biotinylated anti-CD8α mAb 53–6·7, and PE-conjugated anti-CD8β mAb 53–5·8 (all from PharMingen, San Diego, CA). Biotinylated antibodies were detected by incubating the cells with PE-conjugated streptavidin (Caltag, Burlingame, CA) or with allophycocyanin (APC)-conjugated streptavidin (Molecular Probes, Eugene, OR).

Redirected cytotoxicity assay

IEL and ^{51}Cr-labelled P815 cells were incubated in the presence of anti-αβ TCR mAb (0·2 μg/ml) or anti-γδ TCR mAb (1 μg/ml) at 37° for 6 hr.[10] After the incubation, the radioactivity released into supernatants was counted and the per cent specific lysis was calculated as 100 × [(c.p.m. released from target cells with effector cells − c.p.m. released from target cells alone)/(c.p.m. released from target cells by detergent − c.p.m. released from target cells alone)].

Statistical analysis

All the data obtained from different groups of mice were compared by unpaired Student's *t*-test.

RESULTS

Establishment of antigen-minimized GF mice

Our previous reports had shown that first, cytolytic activities of αβ-IEL from CV mice are uniformly high but sharply attenuate in the GF condition, and second, cytolytic activities of γδ-IEL are strain-dependent and not weakened by microbial deprivation.[10,13] As the cytolytic activity of γδ-IEL was strongest in C57BL/6 mice among the inbred mouse strains tested, we decided to compare the constitution and cytolytic activity of IEL freshly isolated from GF C57BL/6 mice fed a natural diet (Nat-GF mice) or antigen-minimized diet (AgM-GF mice) to evaluate an influence of gut antigenic load on the development and cytolytic activation of γδ-IEL.

First, we tried to give antigen-minimized diet to Nat-GF mice and subject them to mating several times, but it was not successful. Therefore, Nat-GF mice at the age of 2–10 weeks were fed antigen-minimized diet during the next 4–6 weeks or during the next 12–14 weeks and used as AgM-GF mice. We have already confirmed that the antigen-minimized diet, the composition of which is described in Table 1, can maintain GF outbred ICR mice and GF inbred C3H/He mice.[13,15] AgM-GF C57BL/6 mice grew normally and appeared healthy by visual inspection.

Immunoglobulin and albumin concentrations in sera of CV, Nat-GF and AgM-GF mice

Immunological condition of our AgM-GF mice was evaluated

Table 1. Composition of antigen-free diet

Solution 1*

Leucine†, 4·32 g; Phenylalanine, 1·80 g; Isoleucine, 2·34 g; Methionine, 2·34 g;Tryptophan, 0·90 g; Valine, 1·80 g; Asparagine, 2·52 g; Arginine HCl, 1·98 g; Threonine, 1·80 g; Lysine HCl, 4·32 g; Histidine HCl · H_2O, 1·80 g; Glycine, 0·72 g; Proline, 3·60 g; Serine, 3·24 g; Alanine, 1·44 g; Sodium Glutamate, 8·28 g; Ethyl Tyrosine HCl, 1·80 g; Ferrous glucuronate, 0·129 g; Calcium glycerophosphate, 8·01 g; $CaCl_2 \cdot 2H_2O$, 0·99 g; Magnesium glycerophosphate, 3·63 g; NaCl + KI‡, 0·21 g; Choline Cl, 0·75 g; $K(CH_3COO)$, 3·18 g; $Mn(acetate)_2 \cdot 4H_2O$, 135 mg; $ZnSO_4 \cdot H_2O$, 33 mg; $Cu(acetate)_2 \cdot H_2O$, 9 mg; $Cr(acetate)_3 \cdot H_2O$, 6 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0·9 mg; Na_2SeO_3, 0·054 mg; $Co(acetate)_2 \cdot 4H_2O$, 0·27 mg; NaF, 1·5 mg; Thiamin HCl, 2·25 mg; Pyridoxine HCl, 2·84 mg; Biotin, 0·45 mg; Folic acid, 0·68 mg; Vitamin B_{12}, 0·27 mg; Riboflavin, 3·38 mg; Niacin, 16·88 mg; i-inositol, 112·5 mg; Calcium pantothenate, 22·5 mg; *P*-aminobenzoic acid, 135 mg.

Water sufficient to make 500 ml was added and the mixture was filter-sterilized.

Solution 2*

Anhydrous α-D-glucose, 229·95 g.

Water sufficient to make 500 ml was added and the mixture was autoclaved.

Solution 3§

Corn oil, 10 g; Vitamine A palmitate, 0·195 mg; Vitamin K_1, 2·18 mg; DL-α-tocopheryl acetate, 0·2 g; DL-α-tocopherol, 0·1 g; Vitamin D_3, 0·873 μg.

Mixture was filter-sterilized.

* Mixtures of equal amounts of solutions 1 and 2 were fed *ad libitum*.

† L-form amino acids were used.

‡ This contains 1·65 mg KI.

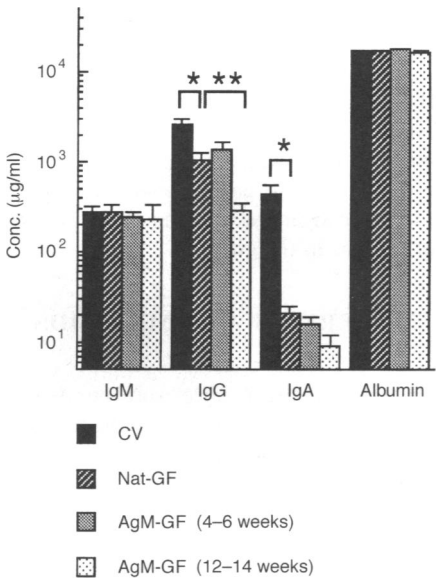

Figure 1. Serum immunoglobulin and albumin levels of CV, Nat-GF and AgM-GF mice. Numbers of serum samples examined were as follows: CV mice, $n = 17$; Nat-GF mice, $n = 16$; AgM-GF mice fed antigen-minimized diet for 4–6 weeks, $n = 10$; AgM-GF mice fed antigen-minimized diet for 12–14 weeks, $n = 4$. Significant differences between CV and Nat-GF mice, or Nat-GF and AgM-GF (12–14 weeks) mice are shown with asterisks; *, $P < 0{\cdot}01$; ** $P < 0{\cdot}001$.

by measuring the concentrations of serum immunoglobulins. IgM levels in sera were similar between CV, Nat-GF and AgM-GF mice. In contrast, Nat-GF mice contained significantly reduced amounts of IgG and IgA in sera compared with CV mice. Serum IgG and IgA levels were comparable between Nat-GF mice and AgM-GF mice fed antigen-minimized diet for 4–6 weeks, but antigen deprivation from food for 12–14 weeks caused a further threefold reduction of IgG and twofold reduction of IgA in comparison with Nat-GF mice (Fig. 1). In order to check whether the decrease in serum immunoglobulins in AgM-GF mice is due to the deterioration of nutritional conditions, the concentration of serum albumin was examined. As shown in Fig. 1, the levels of serum albumin of CV, Nat-GF and AgM-GF mice were not significantly different, suggesting that AgM-GF mice are nutritionally normal.

Constitution of IEL in CV, Nat-GF and AgM-GF mice

Next, we compared the constitution of IEL in Nat-GF and AgM-GF mice. Number of αβ-IEL in Nat-GF mice was half of that in CV mice. Short-term antigen deprivation (4–6 weeks) did not affect the number of αβ-IEL, but GF mice fed antigen-minimized diet for 12–14 weeks had less αβ-IEL by 1·9-fold than Nat-GF mice (Table 2). In contrast, numbers of γδ-IEL in CV and Nat-GF mice were within the same range, but when GF mice were fed antigen-minimized diet for 12–14 weeks, the number of γδ-IEL was reduced by 1·3-fold, although the difference between Nat-GF and AgM-GF mice was not statistically significant (Table 2).

The αβ-IEL are made up of five discernibly different subsets in terms of CD4/CD8 molecule expression ($CD4^-8^-$, $CD4^+8^-$, $CD4^-8\alpha\alpha^+$, $CD4^-8\alpha\beta^+$, $CD4^+8^+$). The proportion of $CD4^+8^-$, $CD4^-8\alpha\beta^+$, and $CD4^+8^+$ subsets in αβ-IEL decreased with a resultant increase in the ratio of the $CD4^-8\alpha\alpha^+$ αβ-IEL subset in Nat-GF mice when compared with CV mice (Fig. 2), but the relative composition of αβ-IEL did not differ between Nat-GF and AgM-GF mice excepting that the ratio of $CD4^+8^+$ subset was slightly lower in AgM-GF

Table 2. Constitution of IEL in CV, Nat-GF or AgM-GF mice

Mice	No. of cells ($\times 10^6$)	
	αβ-IEL	γδ-IEL
CV ($n = 14$)	2·89 ± 1·24	1·88 ± 0·81
Nat-GF ($n = 14$)	1·27 ± 0·56	1·89 ± 0·87
AgM-GF (4–6 weeks; $n = 10$)	1·17 ± 0·79	1·64 ± 0·82
AgM-GF (12–14 weeks; $n = 4$)	0·68 ± 0·23	1·38 ± 0·25

Absolute numbers of IEL subsets were calculated by multiplying total cell number by % of each subset. Significant differences were detected in numbers of αβ-IEL between CV and Nat-GF mice ($P < 0{\cdot}001$), and between Nat-GF and AgM-GF (12–14 weeks) mice ($P < 0{\cdot}01$). Numbers of γδ-IEL were not statistically different between each group.

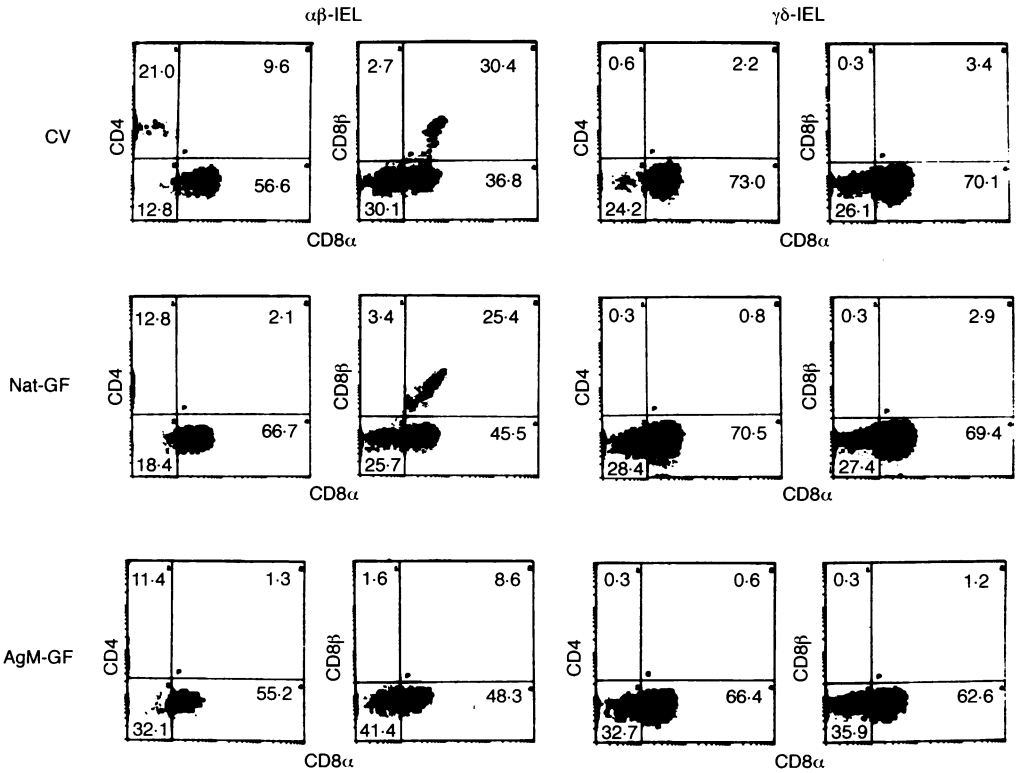

Figure 2. Surface phenotype of IEL in CV, Nat-GF and AgM-GF mice. Stained cells were analysed with an EPICS ELITE flow cytometer after setting the gates on $\alpha\beta$ TCR$^+$ or $\gamma\delta$ TCR$^+$ cells. A representative result from CV, Nat-GF and AgM-GF mice is shown and all the data are summarized in Table 3.

mice (Table 3). Major subsets of $\gamma\delta$-IEL were CD4$^-$8$^-$ and CD4$^-$8$\alpha\alpha^+$ cells, and there was no discernible variation in the constitution of $\gamma\delta$-IEL between CV, Nat-GF and AgM-GF mice while a small difference in the proportion of CD4$^+$8$^+$ subset was detected between CV and Nat-GF mice (Fig. 2, Table 3).

Cytolytic activity of IEL in CV, Nat-GF and AgM-GF mice

IEL in CV mice are constitutively cytolytic when assayed using Fc receptor-bearing P815 cells as the target in the presence of anti-$\alpha\beta$ TCR or anti-$\gamma\delta$ TCR monoclonal antibodies.[6,7] The cytolytic activity of $\alpha\beta$-IEL sharply attenuated in Nat-GF mice

Table 3. Subset composition of $\alpha\beta$-IEL and $\gamma\delta$-IEL in CV, Nat-GF and AgM-GF mice

	% of each subset				
Mice	CD4$^-$8$^-$	CD4$^+$8$^-$	CD4$^-$8$\alpha\alpha$	CD4$^-$8$\alpha\beta$	CD4$^+$8$^+$
$\alpha\beta$-IEL					
CV (n = 11)	12·3 ± 3·8	19·6 ± 5·8	30·8 ± 7·6	30·2 ± 5·4	7·1 ± 3·8
Nat-GF (n = 11)	17·1 ± 7·1	11·5 ± 3·6	51·3 ± 8·1	17·4 ± 5·2	2·7 ± 1·1
AgM-GF (4–6 weeks; n = 7)	25·3 ± 7·5	13·1 ± 5·2	47·5 ± 10·9	12·2 ± 5·7	1·9 ± 1·1
AgM-GF (12–14 weeks; n = 2)	19·9, 34·5	6·2, 11·3	56·7, 44·2	15·8, 8·9	1·3, 1·1
$\gamma\delta$-IEL					
CV (n = 11)	19·0 ± 6·0	0·7 ± 0·5	70·6 ± 8·5	6·9 ± 5·9	2·8 ± 1·3
Nat-GF (n = 11)	18·0 ± 6·4	0·2 ± 0·1	79·1 ± 6·9	1·9 ± 0·8	0·7 ± 0·2
AgM-GF (4–6 weeks; n = 7)	26·6 ± 10·7	0·4 ± 0·1	71·0 ± 10·5	1·3 ± 0·3	0·7 ± 0·3
AgM-GF (12–14 weeks; n = 2)	23·5, 37·1	0·2, 0·4	72·3, 60·6	3·4, 1·3	0·5, 0·6

Significant differences were detected in the percentage of the following subsets: CD4$^+$8$^-$ $\alpha\beta$-IEL, $P < 0.01$ between CV and Nat-GF mice; CD4$^-$8$\alpha\alpha^+$ $\alpha\beta$-IEL, $P < 0.001$ between CV and Nat-GF mice; CD4$^-$8$\alpha\beta^+$ $\alpha\beta$-IEL, $P < 0.001$ between CV and Nat-GF mice; CD4$^+$8$^+$ $\alpha\beta$-IEL, $P < 0.01$ between CV and Nat-GF mice, and $P < 0.01$ between Nat-GF and AgM-GF (12–14 weeks) mice; CD4$^+$8$^+$ $\gamma\delta$-IEL, $P < 0.001$ between CV and Nat-GF mice.

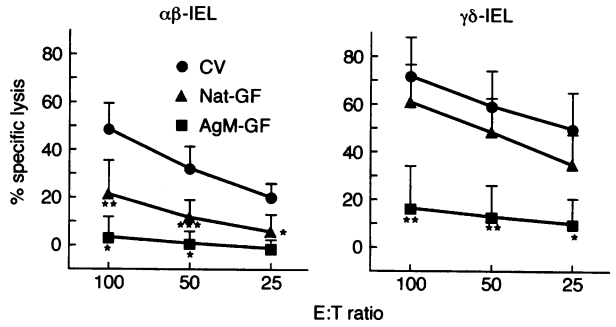

Figure 3. Cytolytic activity of IEL in CV, Nat-GF and AgM-GF mice. IEL and ^{51}Cr-labelled P815 cells were incubated at the effector to target (E:T) ratio = 100, 50 and 25 in the presence of anti-$\alpha\beta$ TCR or anti-$\gamma\delta$ TCR monoclonal antibodies. Data are shown as means ± SD of results obtained from nine CV mice, seven Nat-GF mice, and four AgM-GF mice fed antigen-minimized diet for 12–14 weeks. Cytolytic activities of $\alpha\beta$-IEL and $\gamma\delta$-IEL of AgM-GF mice fed antigen-minimized diet for 4–6 weeks were not significantly different from those of Nat-GF mice (data not shown). Percentages of specific lysis at each E:T ratio were compared between CV and Nat-GF mice, or between Nat-GF and AgM-GF mice. Asterisks under symbols show significant difference from their above symbols: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

as described previously [10] and $\alpha\beta$-IEL of AgM-GF mice were almost deprived of the cytolytic activity (Fig. 3). By contrast, the cytolytic activity of $\gamma\delta$-IEL in Nat-GF mice was comparable to that in CV mice. However, when GF mice were fed antigen-minimized diet for 12–14 weeks, their cytolytic activity was drastically reduced (Fig. 3).

DISCUSSION

The intestinal surfaces of CV mice always make contact with the constant flow of exogenous antigens derived from food and micro-organisms. IEL develop and are activated under such a gut environment so that they acquire cytolytic activity and produce various lymphokines.[6,7,16] Although the localization of $\alpha\beta$-IEL is stimulated by microbial colonization in the intestine, $\gamma\delta$-IEL develop and are activated similarly under either CV or GF conditions,[10,17] indicating that $\alpha\beta$-IEL and $\gamma\delta$-IEL may respond to the different antigens. The former recognizes microbial antigens and the latter does non-bacterial antigens or self-molecules. In this report, we established antigen-minimized mice by feeding GF mice antigen-minimized diet to see an influence of exogenous antigens on the development of IEL. Serum IgG and IgA concentrations of Nat-GF mice were significantly less than those of CV mice, and antigen deprivation for 12–14 weeks induced the further diminution of serum IgG level. These results were in good accordance with those obtained using ICR mice.[15] Even in AgM-GF mice fed antigen-minimized diet for 12–14 weeks, serum albumin level was not different from that in CV mice. Therefore, we judged that AgM-GF mice used in this experiment receive the least antigenic stimulation but are nutritionally normal.

Comparative analysis of IEL in CV, Nat-GF and AgM-GF mice of C57BL/6 strain revealed the following findings. First, microbial deprivation drastically reduced the number of $\alpha\beta$-IEL and antigen deprivation induced the further reduction of $\alpha\beta$-IEL, while the pool size of $\gamma\delta$-IEL was not affected by microbial and antigenic conditions of the gut. Second, the ratios of $CD4^+8^-$, $CD4^-8\alpha\beta^+$ and $CD4^+8^+$ subsets among $\alpha\beta$-IEL were significantly reduced in the Nat-GF condition, but feeding antigen-minimized diet for 12–14 weeks did not induce the further change in the relative composition of $\alpha\beta$-IEL. Subset constitution of $\gamma\delta$-IEL was virtually the same between CV, Nat-GF and AgM-GF mice. Third, cytolytic activities of $\alpha\beta$-IEL were sharply attenuated in Nat-GF mice and were almost deprived in AgM-GF mice. In contrast, $\gamma\delta$-IEL in Nat-GF mice showed cytolytic activity similar to those of CV mice, but their cytotoxicity remarkably fell in AgM-GF mice. These results demonstrate that while the activation of $\alpha\beta$-IEL is stimulated by microbial colonization, the cytolytic activity of $\gamma\delta$-IEL is induced by the existence of exogenous antigens other than live micro-organisms. Recently, Penney *et al.* have reported that $\gamma\delta$-IEL proliferate more rapidly in duodenum, where food antigens flow in large quantities, than in ileum, supporting the idea that $\gamma\delta$-IEL may respond to food antigens in the gut.[18] It is also possible that $\gamma\delta$-IEL are activated by self-molecules expressed on IEC, because some $\gamma\delta$-T cells have been found to recognize non-classical MHC molecules and TL molecules are expressed on IEC of GF mice as well as CV mice.[19,20] At present, it is far from resolution whether exogenous antigenic substances in the gut might directly activate $\gamma\delta$-IEL or induce self-molecules necessary for activation of $\gamma\delta$-IEL.

Anatomical location of IEL indicates that they play a pivotal role as the first line of defence. There is so far no direct evidence showing that freshly isolated IEL recognize and kill infected or transformed enterocytes. However, the finding that reovirus-specific cytolytic T cells bearing $\alpha\beta$ TCR are induced after oral infection of reovirus suggests an anti-infectious activity of $\alpha\beta$-IEL.[11] The previous observation that cytolytic activity of $\alpha\beta$-IEL is enhanced by microbial colonization in the gastrointestinal tract is also consistent with the hypothesis that $\alpha\beta$-IEL are involved in the defence against infection.[10] As oral administration of *Eimeria vermiformis* elicits the drastic increase in $V\gamma1^+$ IEL, a part of $\gamma\delta$-IEL may also play a regulatory role against infection.[21] In contrast, a novel function of $\gamma\delta$-IEL has been proposed. Boismenu and Havran observed that $\gamma\delta$-IEL, but not $\alpha\beta$-IEL, secrete keratinocyte growth factor when triggered by immobilized anti-TCR monoclonal antibodies.[22] We found that the absence of $\gamma\delta$-T cells is associated with the reduction of proliferative rate of IEC but such change is not visualized in $\alpha\beta$-T-cell-deficient mice.[12] These results point out the possibility that $\gamma\delta$-IEL may modulate the proliferation of IEC by producing epithelial cell growth factors. It is well known that turn-over rate of IEC is slower in GF mice than CV mice.[23] As described here, the number and cytotoxicity of $\gamma\delta$-IEL were left unchanged even after sterilization of the gut. Thus, it remains to be clarified how $\gamma\delta$-IEL could regulate the homeostasis of intestinal epithelia.

IEL are considered indispensable for maintaining the normal function of intestine. In view of their unique properties and developmental pathway, IEL may have the different functions from peripheral T lymphocytes. Identification of the ligands recognized by IEL will lead to understanding their physiologic functions.

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