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Immunology

Cytolytic activity of intestinal intraepithelial lymphocytes in germ-free mice is strain dependent and determined by T cells expressing γδ T-cell antigen receptors

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ABSTRACT **We have compared the cytolytic activities and the cellular compositions of the intestinal intraepithelial lymphocyte (i-IEL) populations in three different combinations of conventional (CV) and germ-free (GF) mice. Cytolytic activity of i-IELs expressing γδ T-cell antigen receptors (TCRs) is strain dependent in CV mice (high vs. low), and this strain-dependent variability is unaltered in the GF condition. Although absolute numbers of γδ i-IELs are slightly decreased, the composition of CD8αα$^+$ and CD4$^-$CD8$^-$ subsets and the usage of TCR γ- and δ-chain variable gene segments by γδ i-IELs remain the same in GF mice. By contrast, cytolytic activity of αβ TCR-expressing i-IELs is uniformly high in CV mice but attenuated sharply in the GF condition. A conspicuous decrease in the total numbers of αβ i-IELs is also noted, and CD8αβ$^+$ and CD4$^+$CD8$^+$ subsets are reduced, whereas the CD8αα$^+$ subset is expanded in GF mice. These results indicate that microbial deprivation preferentially influences the αβ i-IEL population to decrease and become noncytolytic but has little effect on the pool size or characteristics of γδ i-IELs. Consequently, cytolytic activity of freshly isolated i-IELs from GF mice is determined by T cells expressing γδ TCRs and is found to be strain dependent.**

Numerous T cells are found between epithelial cells of the small intestine. In adult mice, this intestinal intraepithelial lymphocyte (i-IEL) pool is almost comparable in size to the total peripheral T-cell pool including spleen and all lymph nodes (1) and consists of both αβ T-cell antigen receptor-positive (TCR$^+$) (αβ i-IELs; 40–70%) and γδ TCR$^+$ (γδ i-IELs; 30–60%) cells. However, i-IELs are distinct from peripheral T cells in that most of the γδ i-IELs and many of the αβ i-IELs express the CD8αα homodimer (1–6) and in that they develop extrathymically (1, 4, 5, 7–10). Furthermore, thymus-independent αβ i-IELs expressing potentially self-reactive TCRs are not deleted (1, 11–13); on the contrary, these cells are frequently overrepresented (1) or positively selected (13). Despite the considerable information that has accumulated during the past few years on i-IELs, the physiological significance of these cells remains to be determined (14–16).

It has been proposed that i-IELs are anatomically positioned to participate in the defensive immune response against microbial invasion. In this respect, the finding that both αβ and γδ i-IELs from conventional (CV) mice constitutively display cytolytic activity (10, 17, 18) supports a notion that i-IELs constitute a first line of cellular defense against microorganisms by destroying infected enterocytes. In fact, Lefrancois and Goodman (18) reported that fresh i-IELs from germ-free (GF) mice had no such cytolytic activity. On the other hand, Guy-Grand *et al.* (10) showed that i-IELs from GF mice were cytolytic. These contradictory conclusions could be caused either by a strain-to-strain variation in the activity of cytolytic i-IEL subpopulations or by different assay methods employed by the researchers, or by both. In any event, an important issue regarding the involvement of intestinal microorganisms in activation of i-IEL-mediated cytotoxicity still remains controversial.

It has recently been demonstrated that cytolytic activity of γδ i-IELs but not αβ i-IELs is strain dependent in CV mice (19). For example, this activity in C57BL/6 mice is high whereas that in BALB/c mice is low or marginal. In the present study, we analyzed the cytolytic activity and phenotype of i-IELs from three different CV and GF strains of mice, including newly produced GF C57BL/6 mice. We conclude that the previous observations (10, 18) are not mutually exclusive, since we found that the sterilization of gut lumen abolishes cytolytic activity of αβ i-IELs leaving that of γδ i-IELs unaffected. Furthermore, it has a far greater effect on αβ i-IELs than on γδ i-IELs in terms of their phenotype and population size.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), BALB/c (B/c), and IQI mice raised in a specific pathogen-free (SPF) condition were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu Japan). They were kept in our clean animal facility without use of isolators for a few weeks before use. Therefore, in the strict sense, we regard them as conventional (CV) mice rather than SPF mice. GF IQI mice were also purchased from the same Animal Center and GF B6 and GF B/c mice were produced in our animal facility. These GF mice were kept under sterile conditions in vinyl isolators until just before sacrifice. Age-matched mice of both sexes, 8–12 weeks of age, were used in the experiments.

Isolation of i-IELs. We isolated i-IELs according to an improved method as described (19). The standard technique employed for the purification of i-IELs was also described elsewhere (6, 17).

Immunofluorescence Analysis. i-IELs were stained by the following monoclonal antibodies (mAbs) and analyzed by EPICS Elite flow cytometer: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb 145-2C11 (Boehringer

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Abbreviations: i-IEL, intestinal intraepithelial lymphocyte; CV, conventional; GF, germ-free; TCR, T-cell antigen receptor; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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Mannheim); FITC-conjugated and biotinylated anti-$\alpha\beta$ TCR mAb H57-597, FITC-conjugated anti-$\gamma\delta$ TCR mAb GL-3, phycoerythrin (PE)-conjugated anti-CD8β mAb, anti-γ-chain variable segment $V_\gamma 4$ mAb, and anti-δ-chain variable segment $V_\delta 4$ mAb (all purchased from PharMingen); anti-Thy-1.2 and biotinylated anti-Thy-1.2 mAbs, PE-conjugated anti-CD4 mAb, and biotinylated anti-CD8α mAb (all purchased from Becton Dickinson); anti-$V_\gamma 1$ mAb (kindly provided by P. Pereira and S. Tonegawa, Massachusetts Institute of Technology); and anti-$V_\gamma 7$ mAb (kindly provided by L. Lefrancois, University of Connecticut Health Center).

Redirected Cytotoxicity Assay. Fresh i-IELs and ^{51}Cr-labeled Fc receptor-positive P815 mastocytoma target cells were incubated without addition of any antibodies or in the presence of anti-CD3 mAb (0.2 μg/ml), anti-$\alpha\beta$ TCR mAb (0.2 μg/ml), or anti-$\gamma\delta$ TCR mAb 3A10 (1 μg/ml; kindly provided by S. Itohara, Institute for Virus Research, Kyoto University) at 37°C for 6 hr. After the incubation, radioactivity released into supernatants was counted and percent specific lysis was calculated as 100 × [(cpm released from target cells with effector cells − cpm released from target cells alone)/(cpm released from target cells by detergent − cpm released from target cells alone)].

RESULTS

$\alpha\beta$ and $\gamma\delta$ i-IELs in CV and GF Mice. We performed flow cytometric analysis on i-IELs isolated from three different combinations of CV and GF mice, using anti-CD3 mAb 145-2C11 (20), anti-$\alpha\beta$ TCR mAb H57-597 (21), and anti-$\gamma\delta$ TCR mAb GL-3 (22) (Fig. 1). In each case, absolute numbers of TCR$^+$ i-IELs recovered from the three different CV mice were larger than those recovered from the corresponding GF mice and the difference was within a range of 2- to 5-fold. Furthermore, microbial deprivation resulted in far greater diminution of $\alpha\beta$ i-IELs (3.0- to 10.8-fold) than of $\gamma\delta$ i-IELs (1.3- to 2.2-fold). Thus, these findings are in line with those reported by Bandeira *et al.* (23).

Cytolytic Activity of $\alpha\beta$ and $\gamma\delta$ i-IELs in CV and GF Mice. We next evaluated cytolytic activities of i-IELs isolated from the CV and GF mice in a redirected cytolysis assay (10, 17, 18). Although i-IELs isolated from conventional B6, B/c, and

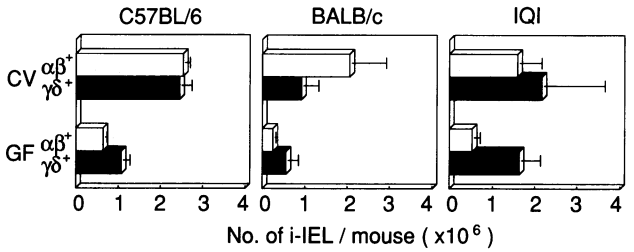

FIG. 1. Numbers of $\alpha\beta$ and $\gamma\delta$ i-IELs recovered from B6, B/c, and IQI mice that had been bred under CV and GF conditions. i-IELs were incubated first with biotinylated anti-$\alpha\beta$ TCR mAb H57-597 and then either with streptavidin-PE (Caltag, South San Francisco, CA) and FITC-conjugated anti-$\gamma\delta$ TCR mAb GL-3 or with streptavidin-PE and FITC-conjugated anti-CD3 mAb 145-2C11. Absolute numbers of $\alpha\beta$ and $\gamma\delta$ i-IELs were calculated by multiplying the ratio of each class of i-IELs by the total number of CD3/TCR-positive cells in the corresponding i-IEL preparation. The results (open bars, $\alpha\beta$ i-IELs; filled bars, $\gamma\delta$ i-IELs) are means ± SD of data obtained from two (B6) and three (B/c and IQI) independent analyses of two to eight mice per group.

IQI mice did not lyse P815 target cells when the cell mixtures were incubated without addition of any mAbs directed against CD3/TCR complexes (Fig. 2*A*), they showed a uniform extent of cytolytic activity in the presence of anti-CD3 mAb (Fig. 2*B*). i-IELs isolated from GF B/c and GF IQI mice showed marginal cytolytic activities when the same anti-CD3 mAb was used (Fig. 2*B*). In contrast, for anti-CD3 mAb-mediated cytolytic activity of B6 i-IELs, no significant difference between CV and GF conditions was discernible (Fig. 2*B*). The reasons for this contradictory observation are as follows. (*i*) Cytolytic activities of $\alpha\beta$ i-IELs from CV mice were uniformly high but sharply attenuated in the GF condition (Fig. 2*C*). Indeed, $\alpha\beta$ i-IELs from GF B/c and GF IQI mice are not cytolytic even in view of the fact that, in these GF mice, the proportion of $\alpha\beta$ i-IELs among TCR$^+$ i-IELs is lower than that among TCR$^+$ i-IELs from the corresponding CV mice (see Fig. 1). (*ii*) Cytolytic activities of $\gamma\delta$ i-IELs from CV mice were high in B6, marginal in B/c, and almost undetectable in IQI (Fig. 2*D*). Notably, these $\gamma\delta$ i-IEL-mediated cytolytic activities were not weakened at all by

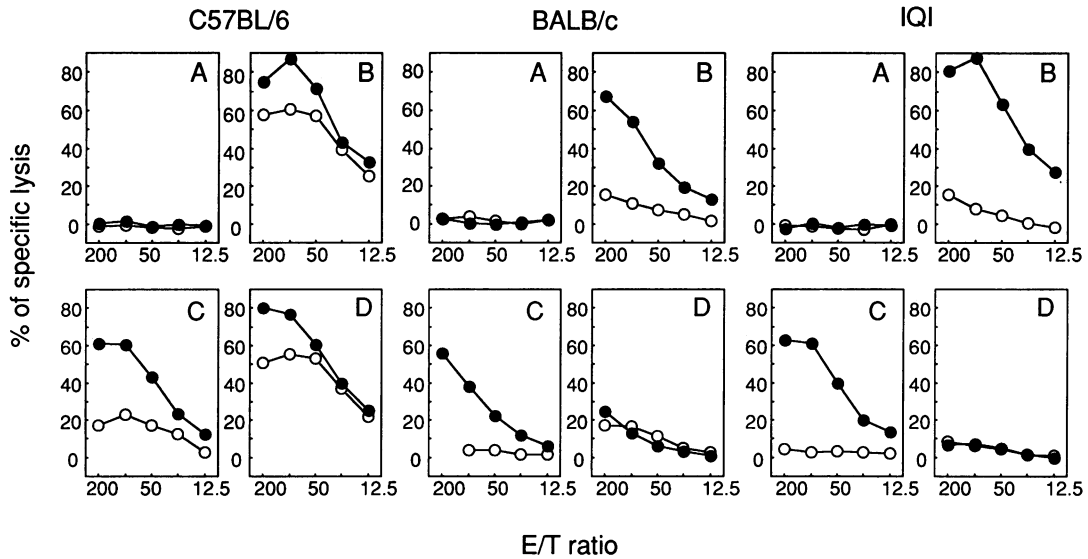

FIG. 2. Cytolytic activity of $\alpha\beta$ and $\gamma\delta$ i-IELs in CV (●) and GF (○) mice. Various numbers of effector i-IELs were incubated with 4 × 10^3 ^{51}Cr-labeled P815 target cells either in the absence of mAb (*A*), or in the presence of anti-CD3 mAb (*B*), anti-$\alpha\beta$ TCR mAb (*C*) or anti-$\gamma\delta$ TCR mAb (*D*) in 75 μl of complete medium [RPMI 1640 containing 10% fetal bovine serum, 10 mM Hepes (pH 7.3), penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml)] for 6 hr at 37°C in each well of 96-well flat bottom microtiter plates. Then, 200 μl of fresh complete medium was added to each well and 100 μl of supernatant was collected after centrifugation for measurement of radioactivity released. E/T, effector/target.

microbial deprivation and the strain-dependent variability was maintained in the GF condition (Fig. 2*D*).

V_γ and V_δ Gene Segment Usage by $\gamma\delta$ i-IELs in CV and GF Mice. The data in Figs. 1 and 2 indicate that colonization by the normal intestinal flora has little effect on not only the numbers but also the cytolytic activity of $\gamma\delta$ i-IELs. In this regard, one would expect that the $\gamma\delta$ TCR repertoire of i-IELs in GF mice does not differ from that in CV mice. In an attempt to verify this proposition, we carried out two-color immunofluorescence analysis on B6 and B/c $\gamma\delta$ i-IELs, using the FITC-conjugated anti-pan $\gamma\delta$ mAb GL-3 and either one of three different V_γ-specific mAbs or anti-V_δ4 mAb. No significant differences in V_γ1, V_γ4, V_γ7, and V_δ4 gene segment use were observed between CV and GF conditions (Fig. 3). Further, in agreement with recent work (19), no obvious relationship between the level of either type of V_γ or V_δ expression and the degree of cytolytic activity of the $\gamma\delta$ i-IELs was detected—i.e., B6, high, vs. B/c, marginal.

Phenotypic Analysis of $\alpha\beta$ and $\gamma\delta$ i-IELs in CV and GF Mice. Bandeira *et al.* (23) showed a marked influence of microbial colonization on the numbers of CD4 and CD8 single-positive $\alpha\beta$ i-IELs, and it was reported by Guy-Grand *et al.* (4) that the i-IEL population in GF mice almost exclusively expresses the homodimer of CD8α chains (CD8$\alpha\alpha^+$). In the present study, we also phenotypically characterized $\alpha\beta$ and $\gamma\delta$ i-IELs isolated from CV and GF mice, and representative data obtained on B6 and B/c i-IELs are presented (Fig. 4). Although absolute numbers of five discriminable $\alpha\beta$ i-IEL subsets were decreased by microbial deprivation, the proportion of single-positive CD8$\alpha\alpha^+$ cells in total $\alpha\beta$ i-IELs was expanded due to the drastic reduction of CD8$\alpha\beta^+$ and CD4$^+$CD8$^+$ T-cell numbers in the GF condition. In contrast to the results of Bandeira *et al.* (23), we could not identify double-negative $\alpha\beta$ T cells as the major subpopulation of $\alpha\beta$ i-IELs in GF mice. Microbial deprivation had little effect on the phenotypic configuration of $\gamma\delta$ i-IELs and the majority of $\gamma\delta$ i-IELs in both CV and GF mice consisted of single-positive CD8$\alpha\alpha^+$ cells. Finally, the ratio of Thy-1$^+$ to Thy-1$^-$ cells either among $\alpha\beta$ i-IELs or among $\gamma\delta$ i-IELs was reduced in the GF condition (data not shown). It appears that our results are compatible with those reported previously (4, 23).

DISCUSSION

Freshly isolated i-IELs from normal mice, bred in CV condition, constitutively display cytolytic activity (10, 17, 18) and kill Fc receptor-positive target cells in the presence of anti-TCR mAb (17, 18, 22). To date, the physiological significance of cytolytic i-IELs in live animals has remained unclear. In view of their anatomical location, however, it has been proposed that cytolytic i-IELs can recognize and destroy intestinal epithelial cells infected with a variety of enteric microorganisms (17, 18, 24). If this argument is correct, i-IELs isolated from GF mice would not display cytolytic activity *in vitro*. Previous reports dealing with this issue described opposing results: Lefrancois and Goodman (18) found that i-IELs from GF mice had no cytolytic activity and that acclimation of GF mice to nonsterile condition resulted in the induction of activity, but Guy-Grand *et al.* (10) found that i-IELs isolated from both CV and GF mice were cytolytic. The results of the present study reconcile the contradictory observations by showing that cytolytic activity of i-IELs from GF mice is strain dependent. This strain-dependent variability was defined on the basis of two criteria. (*i*) Cytolytic activities of $\alpha\beta$ i-IELs from three different CV mice are uniformly high but are severely reduced after acclimation of mice to the GF condition. (*ii*) $\gamma\delta$ i-IEL-mediated cytolytic activity of these CV mice is strain dependent (high vs. low), but this activity is unaltered in the GF condition. Thus, in GF mice, only i-IELs expressing $\gamma\delta$ TCR can display cytolytic activity.

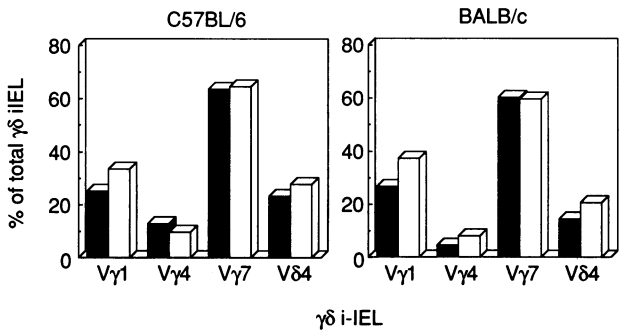

FIG. 3. V_γ and V_δ gene segments used by $\gamma\delta$ i-IELs in CV and GF mice. i-IELs isolated from CV (filled bars) and GF (open bars) mice of B6 and B/c strains were incubated first with anti-V_γ1, anti-V_γ4, anti-V_γ7, or anti-V_δ4 TCR mAb. After washing, the i-IELs were incubated with biotinylated goat anti-hamster IgG and subsequently counterstained with streptavidin-PE and FITC-conjugated anti-pan $\gamma\delta$ TCR mAb GL-3.

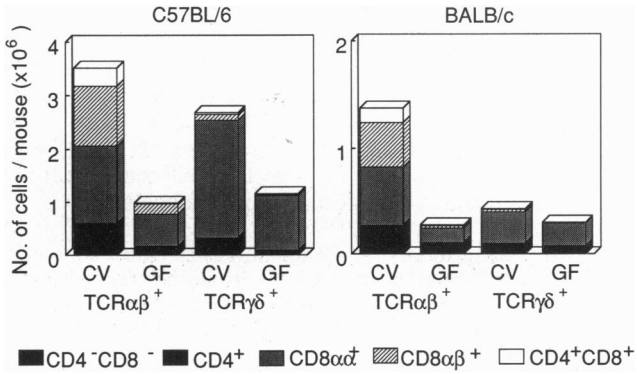

FIG. 4. $\alpha\beta$ and $\gamma\delta$ i-IEL subsets in CV and GF mice. Three-color immunofluorescence analysis was carried out on i-IELs isolated from CV and GF mice of B6 and B/c strains. i-IELs were incubated first with biotinylated anti-CD8α mAb and then with streptavidin-allophycocyanin (Molecular Probes). After washing, the i-IEL were counterstained with four combinations of two PE-conjugated mAbs (anti-CD4 and anti-CD8β mAbs) and two FITC-conjugated mAbs (anti-$\alpha\beta$ and anti-$\gamma\delta$ TCR mAbs), respectively. Absolute numbers of double-negative (CD4$^-$CD8$^-$), single-positive (CD4$^+$, CD8$\alpha\beta^+$, or CD8$\alpha\alpha^+$), and double-positive (CD4$^+$CD8$^+$) subsets in $\alpha\beta$ and $\gamma\delta$ i-IEL populations were calculated on the basis of total numbers of $\alpha\beta$ and $\gamma\delta$ i-IELs, respectively.

Our observations are also in line with a report (23) that microbial colonization has a marked influence on the numbers of CD4$^+$ and CD8$\alpha\beta^+$ $\alpha\beta$ i-IELs but little effect on the pool size or characteristics of $\gamma\delta$ i-IELs. It is conceivable that localization, composition, and functional activation of $\gamma\delta$ i-IELs in the complex microenvironment of the intestinal epithelium are totally independent of the microorganism-induced antigenic load in the gut lumen. In this regard, a slight increase in the pool size of $\gamma\delta$ i-IELs under the CV condition (Fig. 1) could be caused indirectly through secretion of cytokines by activated $\alpha\beta$ i-IELs. Analyses of $\gamma\delta$ i-IELs recovered from $\alpha\beta$ TCR-deficient mice (25) bred in CV and GF conditions will shed light on this issue.

In any event, we cannot formally exclude the possibility that dead bacteria or antigenic materials included in the autoclaved diet are involved in the localization and functional activation of $\gamma\delta$ i-IELs. Although it has been confirmed that absolute numbers of $\gamma\delta$ i-IELs but not of $\alpha\beta$ i-IELs recovered from antigen-free mice are comparable with those recovered from CV mice (A. Bandeira and A. Coutinho, personal

communication), cytolytic activity of γδ i-IELs in the antigen-free mice was not examined. In conclusion, our data reinforce the view that αβ i-IELs have a prominent role in the response to microorganisms of the intestinal mucosa, and thus indicate other roles for γδ i-IELs such as those postulated for T cells expressing γδ TCRs in general (15, 24); e.g., γδ i-IELs may be specific for a certain self-component and monitor the integrity of intestinal epithelial cells.

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