Intestinal intraepithelial lymphocytes (i-IEL) expressing CD8α are located in the intestine and may confer protection against invasion of intestinal microflora. We found that mice rendered deficient in CD8α molecules by homologous recombination were susceptible to 5-fluorouracil (5-FU)-induced lethality accompanied by translocation of members of the enterobacteria. The number of i-IEL was greatly reduced on day 6 after 5-FU administration in both CD8α+/+ mice and CD8α−/− mice, whereas the recovery of the level of i-IEL thereafter was significantly impaired in CD8α+/− mice compared with that in CD8α−/− mice. The ability of i-IEL to produce gamma interferon in response to immobilized T-cell receptor (TCR) αβ or TCR γδ monoclonal antibodies was significantly lower in CD8α−/− mice than in CD8α+/− mice. Transfer of CD8+ i-IEL conferred significant protection against 5-FU-induced lethality in CD8α−/− mice. The results suggest that CD8+ i-IEL play an important role in protection against 5-FU-induced lethality with translocation of Enterobacteriaceae.

CD8α-Deficient Mice Are Highly Susceptible to 5-Fluorouracil-Induced Lethality

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Intestinal intraepithelial lymphocytes (i-IEL) are localized to the basolateral surface of intestinal epithelial cells (i-EC), which are continuously exposed to numerous environmental antigens via the intestinal epithelium (18, 24). Murine i-IEL consist of approximately equal amounts of T-cell receptor (TCR) αβ and γδ i-IEL and unique populations bearing CD8 homodimeric α chains in addition to those bearing CD8 heterodimeric α and β chains (6, 15, 17, 27). A significant fraction of i-IEL are thought to differentiate extrathymically presumably at a local site of the intestine such as the crypt patch (29, 36, 37, 39).

i-IEL produce a variety of cytokines, including Th1-type cytokines, Th2-type cytokines, and transforming growth factor beta (TGF-β), and have a helper function for local immunoglobulin A (IgA) response (10). i-IEL are also thought to play important roles in homeostasis of intestinal epithelial cells through production of cytokines such as TGF-β and keratinocyte growth factor (5, 25). i-IEL also exhibit non-major histocompatibility complex (MHC)-restricted cytotoxicity via serine esterase- and Fas/Fas-L-dependent mechanisms that provide surveillance against infected cells, premalignant cells, and effete cells (23, 27, 38).

At least a significant fraction of i-IEL represent a first line of host defense against infections with diverse pathogens in nature. We and others have reported that i-IEL produced gamma interferon (IFN-γ) by per os infection with Listeria monocytogenes, suggesting that i-IEL play a role in host defense against oral bacterial infection (20, 30, 43). On the other hand, TCR-δ-deficient mice showed exaggerated intestinal damage after oral infection with Eimeria vermiciformis (35), suggesting that a significant fraction of i-IEL regulate inflammation caused by infection. Taken together, the findings in previous studies suggest that i-IEL play important roles in mucosal immunity via various functions, including surveillance, maintenance of homeostasis, and differentiation.

5-Fluorouracil (5-FU) is an antimetabolic chemotherapeutic agent with multiple mechanisms of action, including inhibition of the synthesis of thymidine nucleotides and incorporation into RNA (14, 32, 33). Although 5-FU is a widely used antineoplastic agent, the cytotoxicity of 5-FU is not limited to tumor tissue. Hematopoietic cells and normal epithelial cells of the gastrointestinal tract are susceptible to 5-FU-induced cytotoxicity, which produces severe leukopenia and intestinal toxicity, leading to lethal translocation of intestinal microflora (21, 26, 31, 41). We have previously reported that the number of CD8+ i-IEL was severely reduced after 5-FU administration and that a nonapeptide thymic hormone, factor thymic se-rique (FTS), accelerated recovery in a number of CD8+ i-IEL following 5-FU administration and protected mice from 5-FU-induced bacterial translocation (22). These results suggest that CD8+ i-IEL play an important role in protection against bacterial translocation.

In this study, to elucidate the protective roles of CD8+ i-IEL in bacterial location, we examined the susceptibility of mice genetically deficient in CD8α molecules to 5-FU-induced mortality. We found that CD8α-deficient mice were highly susceptible to 5-FU-induced lethality and that adoptive transfer of CD8+ i-IEL conferred significant protection against 5-FU-induced mortality in CD8α-deficient mice. The implications of the present findings are discussed in terms of the protective roles of CD8+ i-IEL in bacterial translocation.

MATERIALS AND METHODS

Mice. CD4-deficient mice, CD8α-deficient mice, and their heterozygous controls of the C57BL/6 background were provided by T. W. Mak (University of Toronto, Toronto, Canada) (12, 34). B6-Ly5.1 mice (H-2b) were kindly provided by Kenji Kishihara (Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). Mice were used in experiments at 8...
weeks of age. Sterile food and water were given ad libitum. All of the mice were bred under specific-pathogen-free conditions.

Treatment of mice with 5-FU. Mice were injected intraperitoneally (i.p.) with 600 or 800 mg of 5-FU (Kyowa Hakko Kogyo, Tokyo, Japan) per kg.

Counting of endogenous bacterial colonies. The livers and spleens were removed and placed separately in homogenizers containing 5 ml of cold phosphate-buffered saline (PBS). The organs were homogenized thoroughly, and the homogenates were serially diluted with PBS. Samples were spread on agar medium plates to detect enterobacteria (MacConkey; Nissui Pharmaceutical, Tokyo, Japan), and colonies were counted after incubation for 24 h at 37°C.

Cell preparation. i-IEL were prepared according to the previously described procedure with some modifications (22). Briefly, the small intestine from the mice was cut into 5-mm pieces and stirred at room temperature for 30 min in medium 199 (Gibco, Grand Island, N.Y.) containing 10% inactivated fetal calf serum and 1 mM dithiothreitol. After shaking, the cells were passed through a 25%–40%–75% discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient at 600 × g at 20°C for 20 min. The i-IEL were obtained at the 40%–75% interface. Splenocytes were obtained by gently crushing the spleens between two slides. The number of viable cells was counted by trypan blue staining.

Flow cytometry. All cell preparations suspended in Hank’s balanced salt solution (HBSS) containing 2.5% Nu-serum (Becton Dickinson) and 0.1% NaN3, were stained with appropriate monoclonal antibodies (MAbs) at 4°C for 30 min. The MAbs used in these experiments were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε (145-2C11) MAb, Cy-chrome-conjugated anti-CD4 (L3T4) MAb and phycoerythrin (PE)-conjugated or biotin-conjugated anti-CD3ε (UC7-13D5, 10 μg/ml) MAb, Cy-chrome-conjugated anti-TCRβ MAb, PE-conjugated anti-TCRγδ MAb, and purified anti-Ly5.1 MAb. The MAbs were all purchased from Pharmingen (San Diego, Calif.). Cy-chrome-conjugated anti-mouse immunoglobulin G (IgG; heavy and light chains) were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Red613-conjugated streptavidin was purchased from Gibco-BRL (Gaithersburg, Md.). Two- or three-color analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). The live lymphocytes were carefully gated by forward and side scattering. The data were analyzed using CellQuest software (Becton Dickinson).

Cytokine production. i-IEL were cultured in 200 μl of a complete culture RPMI medium in 96-well flat-bottomed plates (Falcon; Becton Dickinson Ltd., Oxford, England) at a density of 10⁵ cells/well with anti-TCRαβ (H57-597, 10 μg/ml) that had been immobilized on the plates by prior incubation at 4°C overnight. The cells were cultured for 72 h at 37°C under 5% CO₂ in air and pulsed with 1 μCi of [³H]thymidine (Tdr) 6 h before harvest. [³H]Tdr incorporation was determined by liquid scintillation counting. The supernatant was collected to estimate cytokine production after culturing for 72 h. The cell-free culture supernatants were collected from the 72-h culture of i-IEL, and the cytokine activity in the culture supernatant was assayed by enzyme-linked immunosorbent assay (ELISA).

IgA in fecal samples. Fecal sample (0.1 g) was incubated with 1 ml of PBS at room temperature for 60 min, vortexed, left for 15 min, revortexed until all materials were suspended, and centrifuged at 2,000 × g for 10 min. The supernatants were removed and tested for total IgA by ELISA.

Cell transfer. i-IEL (10⁶) from Ly5.1 congenic mice were adoptively transferred into recipient mice via tail vein inoculation. At 3 days after the adoptive transfer of these cells, mice were challenged with 5-FU.

Statistical analysis. Student’s t test was used to determine the significance of differences in cell number. A P value of less than 0.05 was taken as significant. The statistical significance of the survival rate was determined by the generalized Wilcoxon’s test.

RESULTS

Increased susceptibility of CD8α-deficient mice to lethality caused by 5-FU administration. We first compared the susceptibilities of CD8α- and CD4-deficient mice to 5-FU-induced lethality. All of the CD4⁻/⁻ mice and CD8α⁻/⁻ mice (n = 20 in each group) survived for more than 18 days after administration of 600 mg of 5-FU per kg. Eighty percent of the CD4⁻/⁻ mice survived after 5-FU administration, whereas 80% of the CD8α⁻/⁻ mice died within 12 days after 5-FU administration (P < 0.01, Fig. 1). Similarly, 40% of the CD8α⁺/⁺ mice (n = 10 in each group) survived following administration of 800 mg of 5-FU per kg, whereas all of the CD8α⁻/⁻ mice died within 10 days after administration (P < 0.05). Thus, CD8α⁻/⁻ mice are more susceptible to 5-FU-induced lethality than are CD8α⁺/⁺ mice.

Bacterial translocation in CD8α-deficient mice after 5-FU administration. It has been reported that a high dose of 5-FU often induces cytotoxicity in intestinal tissue, including i-EC and i-IEL, resulting in ulceration, diarrhea, and bacterial translocation (8). To determine whether bacterial translocation occurs in CD8α⁻/⁻ mice following 5-FU administration, we examined the number of enterobacteria in the liver and spleen after administration of 5-FU (600 mg/kg). Large numbers of bacteria were found in the liver and spleen of CD8α⁻/⁻ mice on day 10 after 5-FU administration, whereas few if any bacteria were detected in CD8α⁺/⁺ mice on days 10 and 11 after...
5-FU administration ($P < 0.05$, Fig. 2). These results suggest that CD8α-deficient mice are susceptible to 5-FU-induced mortality accompanied by bacterial translocation from the intestine.

**Impaired recovery of i-IEL in CD8α-deficient mice after 5-FU administration.** We previously reported that the numbers of i-IEL dramatically decreased in the first 6 days after a single injection of 5-FU and thereafter recovered to the initial level by day 10 (22). In the present study, we next compared the kinetics of i-IEL and thymocytes following 5-FU administration in CD8α−/− mice and CD8α+/− mice.

The numbers of i-IEL in CD8α−/− mice and CD8α+/− mice on day 6 were almost the same (Fig. 3A). The level of i-IEL recovered rapidly to the initial level by day 8 in CD8α+/− mice, whereas the recovery was severely impaired in CD8α−/− mice on day 8 ($P < 0.05$) and on day 10 ($P < 0.01$) after 5-FU administration. There was no significant difference between cell numbers in the thymus (Fig. 3B) or the spleen (data not shown) in CD8α−/− and CD8α+/− mice. These results suggest that CD8α molecules play an important role in i-IEL differentiation.

i-IEL consist of unique T-cell subpopulations bearing CD8αα and CD8ββ or TCR αβ and γδ (18, 24). The changes in i-IEL subpopulations were analyzed by flow cytometer before (day 0) and on day 10 after 5-FU administration. Representative results are shown in Fig. 4. The i-IEL in naive CD8α+/− mice consisted of 80% CD4− CD8α+ cells and a few CD4− CD8− T cells and CD4+ CD8+ T cells, whereas most of

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**FIG. 2.** Growth of endogenous bacteria in CD8α-deficient mice administered 5-FU. Numbers of bacteria in the liver and spleen of CD8α+/− and CD8α−/− mice were determined on the indicated days after intraperitoneal administration of 600 mg of 5-FU per kg. Each point and vertical bar represent the mean ± standard deviation (SD) of six animals. *, $P < 0.05$, significant difference compared with CD8α−/− mice.

**FIG. 3.** Kinetics of i-IEL and thymus of CD8α-deficient mice after 5-FU administration. Cell numbers in (A) i-IEL and (B) thymus in CD8α+/− and CD8α−/− mice were determined on the days indicated after intraperitoneal administration of 5-FU (600 mg/kg). Each point and vertical bar represent the mean ± SD of six animals. *, $P < 0.05$; **, $P < 0.01$, significant difference compared with the value in CD8α−/− mice.
the i-IEL in naive CD8α−/− mice were of the CD4− CD8− phenotype. Approximately half of the i-IEL in CD8α+/− mice were TCR γδ cells, whereas more than 80% of i-IEL in CD8α−/− mice were TCR γδ cells. In both groups, the composition of i-IEL on day 10 after 5-FU administration was much the same as before 5-FU administration.

**Impaired IFN-γ production by i-IEL in CD8α-deficient mice after 5-FU administration.** i-IEL produce a variety of cytokines, including Th1-type cytokines, Th2-type cytokines, and TGF-β (9). We next examined cytokine production by i-IEL from CD8α−/− mice and CD8α+/− mice before and after 5-FU administration. i-IEL were incubated with immobilized anti-TCR αβ MAb or anti-TCR γδ MAb for 48 h, and cytokine activity was examined in the culture supernatant by ELISA.

As shown in Fig. 5, i-IEL from naive CD8α−/− mice produced significantly less IFN-γ than those from CD8α+/− mice in response to immobilized anti-TCR αβ MAb or anti-TCR γδ MAb. Similarly, the ability of the i-IEL to produce IFN-γ was impaired in CD8α−/− mice on day 8 after 5-FU administration compared with those from CD8α+/− mice. The level of IL-4 in

**FIG. 4.** Flow cytometric analysis of i-IEL for expression of CD4, CD8α, αβ TCR, and γδ TCR in CD8α-deficient mice before and after 5-FU administration. The i-IEL CD8α+/− and CD8α−/− mice were recovered before (day 0) and 10 days after intraperitoneal administration of 5-FU (600 mg/kg). The i-IEL were stained with PE-anti-CD8α MAb or -anti-TCRγδ MAb and Cy-chrome-anti-CD4 MAb or anti-TCR αβ MAb for FACS analysis. Values represent percentages of subpopulations in selected areas. The FACS analysis results shown are representative of three separate experiments.
the culture supernatants of i-IEL stimulated with anti-TCR MAbs was marginal in both CD8α+/− and CD8α−/− mice, and conclusive results on TGF-β production could not be obtained because the measurements resulted in high medium background (data not shown).

Effect of adoptive transfer of i-IEL from normal mice on protection of CD8α−/− mice from 5-FU-induced lethality. To try to elucidate the role of i-IEL in protection against 5-FU-induced lethality, adoptive transfer experiments were conducted in Ly5.2+/− CD8α−/− mice using i-IEL from naive Ly5.1 congenic mice. We confirmed that adoptive transfer with Ly5.1+ i-IEL reconstituted the i-IEL population in Ly5.2+/− CD8α−/− mice 3 days after transfer (Fig. 6A). As shown in Fig. 6B, the adoptive transfer with i-IEL conferred protection against 5-FU-induced lethality in CD8α−/− mice. No bacteria were detected in surviving CD8−/− mice receiving i-IEL on day 10 after 5-FU administration.

**DISCUSSION**

In the present study, we demonstrated that CD8α-deficient mice were highly susceptible to 5-FU-induced lethality accompanied by translocation of enterobacteria. Transfer of i-IEL conferred resistance to the 5-FU-induced lethality in CD8α-deficient mice. These results suggest that CD8+ i-IEL play an important role in protection against 5-FU-induced lethality with bacterial translocation.

A high dose of 5-FU would destroy the first line of defense, such as intestinal epithelial cells and immunocompetent cells derived from hematopoetic cells, against intestinal microflora, leading to lethal translocation of intestinal microflora (21, 26, 31, 41). CD8+ i-IEL are located at the basolateral surfaces of i-EC, which are continuously exposed to numerous environmental antigens via the intestinal epithelium (18, 24). We showed that the number of i-IEL was severely reduced on day 5 after 5-FU administration in both CD8α+/− mice and CD8α−/− mice, whereas the recovery of the level of i-IEL thereafter was significantly impaired in CD8α−/− mice compared with CD8α+/− mice.

The IEL from CD8α−/− mice before and after 5-FU administration showed an impaired ability to produce IFN-γ, which is important for protection against bacteria. Yamamoto et al. reported that i-IEL produce IFN-γ after per os infection with *Listeria monocytogenes* (43). We have also shown that i-IEL produced IFN-γ at the early stage during oral infection with *L. monocytogenes* (20, 30). Taken together, the finding suggests that IFN-γ produced by CD8+ i-IEL function in host defense against microbial translocation and that the absence of CD8α i-IEL in CD8α-deficient mice hampers the control of intestinal microflora, leading to lethal translocation of intestinal microflora.

i-IEL are thought to produce a variety of cytokines, including not only Th1-type cytokines but also Th2-type cytokines and TGF-β, and have a helper function for local IgA response (5, 25). IgA against intestinal microflora is important for protection against invasion of microbial flora (1, 7). Therefore, it is also possible that IgA production is impaired in CD8α-deficient mice, leading to lethal bacterial translocation. However, there are several lines of evidence that T-cell-mediated immunity is more important for protection against bacterial translocation than IgA (3, 44).

Our preliminary data showed that total IgA in feces did not
differ between CD8α−/− mice and CD8α+/− mice (6.74 ± 2.21 versus 5.85 ± 0.86 μg/mg). The difference between IgA specific for intestinal microflora in CD8α−/− mice and that in CD8α+/− mice may not be responsible for the difference in susceptibilities to 5-FU-induced bacterial translocation. A significant fraction of i-IEL, such as γδ i-IEL, have been reported to play important roles in homeostasis of intestinal epithelial cells through production of cytokines such as keratinocyte growth factor (5, 25). Therefore, it is also possible that turnover of i-EC may be impaired in CD8α−/− mice administered 5-FU, resulting in increased bacterial translocation from the intestine. Furthermore, maturation of the i-EC in CD8α−/− mice may be impaired, making them highly susceptible to 5-FU-induced cytotoxicity. Further analysis of the mode of action by which CD8α i-IEL protect mice from 5-FU-induced lethality is needed.

CD8α-deficient mice had a large number of CD4− CD8− i-IEL and a small number of CD4+ CD8− i-IEL. However, CD8α-deficient mice were found to be more susceptible to 5-FU-induced lethality than normal mice. Thus, CD8+ i-IEL but not CD4+ CD8− or CD4− CD8− i-IEL may be essential for protection against 5-FU-induced lethality.

Mouse CD8+ i-IEL include unique populations bearing CD8 homodimeric α chains besides i-IEL bearing CD8 heterodimeric α and β chains (18, 24). Most of the CD8αβ i-IEL are thought to recognize antigens from intestinal flora presented by MHC class Ia molecules, composed of an α chain associated with β2 microglobulin (β2m), whereas CD8+ i-IEL recognize TAP-independent peptides in the context of MHC class Ib molecules, such as Tla, encoded by the T3 and T18 genes, and CD1 molecules, both of which are expressed by i-EC in association with β2m (4, 11, 13, 19, 40, 42). Beagley et al. reported that the reactivity of i-IEL to syngeneic spleen cells is enhanced by bacterial antigens such as PPD, HSP70, and HSP60 (2). It has also been reported that human γδ i-IEL recognize stress-induced MHC class I-like molecules MICA.
11. Transfer of CD8

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