Antigen Feeding Increases Frequency and Antigen-specific Proliferation Ability of Intraepithelial CD4<sup>+</sup> T Cells in αβ T Cell Receptor Transgenic Mice

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Received October 8, 2002; Accepted February 22, 2003

To study how intestinal intraepithelial lymphocytes (IEL) are affected by orally ingested antigen, the phenotypes and responses of the IEL in mice expressing a transgenic T cell receptor αβ (TCR αβ) specific for ovalbumin (OVA) were analyzed after feeding OVA. In the OVA-fed mice, the abundance of αβ-IEL as a proportion of the total IEL population increased and the frequency of CD4<sup>+</sup> cells increased within the TCR αβ<sup>+</sup> IEL population. CD4<sup>+</sup> IEL from OVA-fed transgenic mice proliferated in vitro more markedly in response to antigen stimulation than IEL from mice fed the control diet. These results indicate that antigen-specific proliferation of CD4<sup>+</sup> IEL was amplified as a result of oral administration of antigen.

Key words: intestinal intraepithelial lymphocytes (IEL); transgenic mice; ovalbumin; food antigen

The intestinal epithelium is a region in contact with the outside world, and exposed to the intestinal bacterial microflora and food antigens (Ags). Intestinal intraepithelial lymphocytes (IEL) represent a very large population of T lymphocytes buried in the epithelial layer of villi in the intestinal lumen. In mice, about half of these cells express the T cell receptor (TCR) αβ (αβ-IEL), while the other half express TCRγδ (γδ-IEL). The majority of αβ-IEL are CD8<sup>+</sup>, half of them expressing the CD8αβ heterodimer.1,2) In contrast, peripheral CD8<sup>+</sup> T cells which have developed in the thymus generally express the CD8αβ heterodimer. In the thymus, CD4<sup>+</sup> CD8<sup>+</sup> cells are immature, while there are mature CD4<sup>+</sup> CD8<sup>+</sup> cells among the IEL. These observations demonstrate that the IEL are composed of populations distinct from peripheral T cells in the systemic immune system, although the molecular contexts of their recognition of Ags and their exact function are unknown. The αβ-IEL population also contains a significant number of CD4<sup>+</sup> T cells. Although some studies have shown that CD4<sup>+</sup> IEL can respond to intestinal Ags,3,4) the antigenic recognition and effector functions of the CD4<sup>+</sup> IEL are still unclear.

Several studies have shown that IEL are affected by orally administered Ag. Stimulation was indirectly demonstrated in the case of celiac disease. The percentage of proliferating IEL increased in untreated patients.5) Another experimental study indicated that CD4<sup>+</sup> IEL in mice orally primed with sheep red blood cells were capable of providing a helper function for the Ag-specific B cell response.3) Based on these data, we assumed that these effects of ingested Ag on IEL were due to some specific response of IEL to this Ag, not a non-immunological interaction with the Ag.

In this study we analyzed the IEL in OVA23-3 mice expressing a transgenic TCR αβ specific for OVA...
Antigen-specific proliferation of CD4^+ T cells was observed in the OVA-specific CD4^+ TCR monoclonal antibodies used in this study. It may be easier to observe an Ag-specific response of the IEL in this transgenic mouse, which has more CD4^+ T cells capable of responding to OVA, compared with a wild-type (WT) mouse. We fed the transgenic mice a diet containing OVA and detected a change in the population and activation state of IEL specific to the Ag fed, a change which has been rarely observed in WT mice. This study clearly shows that antigen-specific proliferation of CD4^+ αβ-IEL is upregulated in response to orally administered Ag.

Materials and Methods

Mice. This experiment was done in accordance with the Guidelines for Animal Experiments of the Faculty of Agriculture, The University of Tokyo, and the law (no. 105) and notification (no. 6) of the Japanese Government.

The establishment of the OVA23-3 transgenic mouse line used has been described.7 These mice carry genes encoding TCR αβ (Vα3.1/Vβ15) derived from an OVA-specific CD4^+ T cell clone, 7-3-7. The animals were housed and bred in our animal facility at The University of Tokyo. We obtained WT and transgenic mice from the F1 generation of a cross of BALB/c and heterozygous transgenic mice. IEL were analyzed from transgenic mice of both sexes at 8–24 wk of age. Six to eight wk-old BALB/c mice were purchased from Clea Japan, Inc. (Tokyo, Japan).

Antibodies and reagents for FACS analysis. The monoclonal antibodies used in this study were the following: anti-pan TCR β chain (H57-597) conjugated to biotin was purchased from PharMingen (San Diego, CA). FITC-anti-CD4 (YTS191.1.2) and R-phycoerythrin (R-PE)-anti-CD8a (53.6.7) were obtained from Gibco, BRL (Gaithersburg, MD); anti-CD8β (Y8.77)-FITC was from Seikagaku Corp. (Tokyo, Japan). Streptavidin-R-PE and FITC were products of Gibco, BRL. Streptavidin-PerCP was from Becton Dickinson Immunocytometry Systems (San Jose, CA). Anti-Vα3.1 mAb (1H9) conjugated to biotin was prepared as previously described.8

Isolation of IEL. IEL were prepared as previously described.9 In brief, the inverted intestine was cut into four segments and these segments were transferred to a 50-ml conical tube containing 45 ml of 5% fetal bovine serum (FCS) (Cansera International, Rexdale, Canada) in Hanks’ balanced salt solution. The tube was shaken at 150 rpm in the horizontal position in an orbital shaker for 45 min at 37°C. The cells were collected and passed through a glass-wool column to remove cell debris and adherent cells (the crude cell preparation). Subsequently, the cells were suspended in 30% Percoll (Pharmacia Biotech, Uppsala, Sweden) solution and centrifuged at 400 × g for 20 min. Cells pelleted at the bottom of the tube were then put through Percoll discontinuous gradient centrifugation, and the IEL were recovered from the interface of 44% and 70% Percoll solutions (>95% were CD3-positive).

Preparation of Peyer’s patch cells and splenocytes. Peyer’s patches were removed surgically from mouse intestine, and single-cell suspensions were prepared without enzymes by smashing the organ with the end of a syringe, and passing each cell suspension through a polyester mesh. Single-cell suspensions of splenocytes were prepared in a similar manner.

Oral administration of OVA. For a period of 3–10 days, the mice were allowed free access to a diet containing 20% egg-white protein (egg-white diet; ED) or a control diet containing 20% bovine casein (control casein diet; CCD), both of which had the same components except for egg white and casein (Table 1) (Funabashi Farms, Funabashi, Japan). The daily intake of OVA in the course of ED-feeding was approximately 250 mg.

Flow cytometry. Cells (2 × 10^5/sample) were washed with Hanks’ balanced salt solution containing 5% FCS and 0.2% NaN₃ (the flow cytometry buffer) and centrifuged at 4°C, 400 × g for 5 min. The cells were stained with each antibody on ice for 20 min. The samples were washed again, and flow cytometry buffer was added for analysis. Flow cytometry was done using a FACSort machine (Becton Dickinson Immunocytometry Systems). Aggregated cells were excluded by gating forward and side scatter for analysis. The frequencies of the subsets of IEL in the CCD-fed mice and ED-fed mice were compared using Student’s two-tailed t test.

Magnetic cell sorting. IEL (1 × 10^7–2 × 10^7) were

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<td>Soybean oil</td>
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<td>Vitamin mixture^b</td>
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^a%.
^bAIN-76% composition.

peptide 323-339 (OVA323-339) restricted to MHC class II I-A^d. A majority of the αβ-IEL in this transgenic mouse should express a single type of TCR specific for this OVA peptide. This shows that antigen-specific proliferation of CD4^+ αβ-IEL is upregulated in response to orally administered Ag.
were composed of CD4+ cells. As a result of ED feeding, CD4+ cell counts increased (60%)[11].

Western blotting was performed using a two-site sandwich enzyme-linked immunosorbent assay (ELISA) as described previously.[11]

Cell proliferation and cytokine assay. The whole splenocytes were isolated from BALB/c mice that were irradiated with 3000 R and used as antigen-presenting cells (APC). IEL were plated in 96-well plates at 1 or 2×10^5 cells/well together with Ag and APC at 4×10^5 cells/well in a total volume of 200 μl. The medium for cell culture was RPMI 1640 (Nissui Pharmaceutical) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^-5 M 2-mercaptoethanol, and 10% FCS. After 24 or 48 h, 0.5 μCi of [3H] thymidine was added to each well. The cells were harvested 20 h later and [3H] thymidine incorporation was measured by scintillation counting. Cytokines in culture supernatants were measured using a two-site sandwich enzyme-linked immunosorbent assay (ELISA) as described previously.[11]

Results

Changes in the population of IEL in response to orally administered Ag

OVA23-3 mice were fed ED (containing OVA) or CCD (lacking OVA). We compared the subsets of IEL in ED-fed mice with those in CCD-fed mice by analyzing surface molecule expression. In CCD-fed mice, 57±5% of purified IEL was αβ TCR+ and 44±6% IEL was positive for Vα3.1, which is the α-chain of transgenic TCR specific for OVA23-339. Therefore, it was evident that most of the αβ-IEL in these transgenic mice expressed Vα3.1 (Fig. 1). Compared with CCD-fed mice, transgenic IEL from ED-fed mice had an increased frequency (70±5%) of αβ-IEL (p<0.05) (Fig. 1). The frequency of Vα3.1+ cells also increased as a result of ED feeding (60±5%, p<0.05) (Fig. 1).

Tri-color staining showed that the TCRαβ+ cells were composed of CD4+CD8+, CD4+CD8-, CD4+ CD8+, and CD4-CD8+ subsets (including CD8αα+ and CD8αβ+ subsets). The frequency of CD4+CD8- IEL in ED-fed mice was greater than that in CCD-fed mice (p<0.01), while the frequency of CD8αα+ IEL was lower (p<0.01) (Figs. 2 and 3). IEL phenotypes were also analyzed in transgenic mice that had been immunized i.p. with OVA. The analysis showed no difference in IEL phenotypes between immunized and control mice (data not shown).

Changes in Ag-specific reactivity of IEL in response to orally administered Ag

IEL from ED-fed or CCD-fed mice (ED-IEL or CCD-IEL, respectively) were cultured with OVA in the presence of irradiated BALB/c splenocytes for an assay of cell proliferation. IEL from the ED-fed mice showed a stronger proliferative response to OVA than those from the CCD-fed mice (Fig. 4). IEL from mice fed a commercial diet (CE-2; Clea Japan) showed a proliferative response similar to that of IEL from CCD-fed mice (data not shown). However, ELISA of culture supernatants showed little difference in interferon γ production between CCD-IEL and ED-IEL (data not shown). Interleukin (IL)-4, IL-5, and IL-10 were not detected in the case of either ED-IEL or CCD-IEL (data not shown).

Proliferative response of CD4+ and CD4- IEL sorted by MACS

CD4+ and CD4- cell fractions were obtained from IEL by MACS, and Ag-specific proliferation of the IEL from ED-fed and CCD-fed mice was compared. The results (Fig. 5A) showed that CD4+ ED-IEL proliferated in response to OVA more strongly than CD4+ CCD-IEL, while proliferation of CD4- IEL was drastically weaker in the case of both CCD- and ED-IEL, compared with unfraccionated IEL. On the other hand, little difference was found in the proliferation of CD4+ splenocytes and Peyer’s patch cells from CCD-fed and ED-fed mice (Fig. 5B).
1. Effects of Orally Administered Ag on the Frequency of CD4⁺CD8⁻ and CD8α⁺ TCR β⁺ IEL.

In order to investigate OVA-specific αβ-IEL particularly, cells were gated for positive staining with biotinylated anti-TCR β, followed by streptavidin-PerCP, as shown in A (IEL from CCD-fed mice) and D (IEL from ED-fed mice). αβ-IEL from CCD-fed Tg mice (B and C) and ED-fed Tg mice (E and F) were each stained with FITC-coupled anti-CD4 and R-PE-coupled anti-CD8α (B and E), R-PE-coupled anti-CD8α and fluorescein isothiocyanate-coupled anti-CD8β (C and F). Typical FACS profiles are presented.

2. Effects of Orally Administered Ag on the Frequency of CD4⁺CD8⁻ and CD8α⁺ TCR β⁺ IEL.

Data are the mean values for eight mice per group (five mice for CD8α and CD8αβ) from three independent experiments. Statistical comparisons were done using Student’s two-tailed t test. A difference was significant at p<0.01 (indicated by *) when CD4⁺CD8⁻ IEL and CD8α⁺ TCR β⁺ IEL of ED-fed mice are compared to those of CCD-fed mice.

3. Effects of Orally Administered Ag on the Ag-Specific Proliferation of Transgenic IEL.

IEL (2×10⁵ cells/well) were incubated with 50 μg intact OVA and APC (4×10⁵ cells/well) for 24 h. Proliferation data represent the mean values for duplicate cultures. Data shown are a representative of five independent experiments.

Discussion

We analyzed IEL and their responsiveness in TCR-transgenic OVA23-3 mice fed the Ag for which this TCR is specific. The purpose of this study was to find which population of IEL is immunologically affected by orally administered Ag, i.e. OVA protein. We found that feeding ED increased the frequency of
Orally Administered Antigen Affects CD4⁺ IEL

**Fig. 5.** Effects of Orally Administered Ag on the Ag-Specific Proliferation of CD4⁺ or CD4⁺ IEL (A), CD4⁺ Splenocytes or CD4⁺ Peyer’s Patch Cells (B).

(A) Orally administered Ag increased the proliferative response of the CD4⁺ fraction but not that of the CD4⁺ fraction of IEL. IEL (1 × 10⁵ cells/well) were incubated with 2 or 50 μM intact OVA and APC (4 × 10⁵ cells/well) for 48 h. Data shown represent the mean values for duplicate cultures. (B) Orally administered Ag did not affect the proliferation of CD4⁺ splenocytes (SC) or CD4⁺ Peyer’s patch cells (PP). SC and PP (1 × 10⁵ cells/well) were incubated with 2 or 50 μM intact OVA and APC (4 × 10⁵ cells/well) for 48 h. Proliferation data represent the mean values for duplicate cultures. Similar results were obtained in two experiments.

TCR αβ⁺ cells and Vα3.1⁺ cells compared with IEL from CCD-fed mice (Fig. 1). Furthermore, the frequency of CD4⁺ cells within the TCR αβ⁺ IEL population increased as a result of feeding ED (Figs. 2 and 3). From the data obtained, it is demonstrable that the increase in frequency of TCR αβ⁺ cells was due to an increase in size of the transgenic TCR⁺ population. These changes were not observed in the case of the IEL of i.p. immunized transgenic mice (data not shown). The results suggest that administration of Ag through the intestinal mucosa has unique effects on Ag-specific CD4⁺ αβ⁺ IEL (discussed in detail below).

It has been often observed that IEL do not proliferate strongly in response to stimuli that are very effective for inducing proliferation of other systemic T cell populations in spleens or lymph nodes. The weak proliferating activity of the IEL was considered to reflect a state of low responsiveness due to having been exposed to a large quantity of intestinal Ag. However, our study showed that in vitro proliferation of IEL in response to OVA increased as a result of feeding ED to OVA23-3 mice (Fig. 4). Our results are consistent with those by Sydora et al. who suggested that previous contact with Ag is not likely to be the cause of the relatively weak response of IEL. CD4⁺ IEL from ED-fed mice proliferated more markedly than IEL from CCD-fed mice, while IEL depleted of CD4⁺ cells failed to proliferate in response to Ag (Fig. 5A). It is unlikely that the failure of CD4⁺ IEL to proliferate was due to the presence of contaminating intestinal epithelial cells (IEC) which might be able to inhibit the proliferation of lymphocytes. This is supported by these results indicating that although the unfractionated IEL population as well as the CD4-depleted population might have contained a similar number of contaminating IEC, this unfractionated population was fully capable of proliferation. Although the IEC is a candidate cell population that may present antigen to IEL in vivo (discussed later), studies that have examined antigen presentation of IEC have shown that these cells inhibit T cell proliferation in vitro. Furthermore, in the case of murine cells, there are no appropriate intestinal epithelial cell lines that can be used in antigen presentation studies, and it is difficult to culture primary IEC with sufficient purity and viability. Therefore in this study we considered that it was inadequate to use IEC as APC to examine the transition of the IEL proliferative response, and we studied its capacity to proliferate to antigenic stimulation using splenic APC.

A few other reports have shown that CD4⁺ IEL respond to intestinal Ag, although a proliferative response has not been observed. MHC class II-restricted CD4⁺ IEL from mice orally administered sheep red blood cells provided a Th2-like function for B cell responses. In addition, it was reported that CD4⁺ IEL in immunized mice prevented infection by Cryptosporidium muris, which induces a mucosal disease. These data indicate that intestinal antigen can prime CD4⁺ IEL specifically. Our results extend these observations and indicate that Ag-specific proliferation of CD4⁺ IEL in vitro is upregulated by oral administration of Ag.

In contrast to these observations concerning CD4⁺ IEL, the Ag-specific proliferation of splenic and Peyer’s patch CD4⁺ T cells was unaffected by OVA feeding for a relatively short period (Fig. 5B). This is consistent with the results by another group, showing that the Ag-specific proliferative activity of splenic T cells in TCR transgenic mice is not upregulated after administration of Ag, even if administration is done by immunogenic procedures. From these observations, two possibilities were considered with respect to the upregulation of the proliferative activity of CD4⁺ IEL by orally ad-
have shown proliferation of CD4+ IEL were primed or stimulated by Ag in the intestinal epithelium, and their reactivity was upregulated. Although IEC expressing class II molecules are known to inhibit T cell proliferation in vitro, some studies have shown proliferation of CD4+ T cells in response to antigen presentation by IEC. In our experiments, CD4+ IEL may be primed in vivo or stimulated through interaction with OVA-derived Ag bound to I-A4 molecules expressed on IEC, which are the predominant class II+ cells close to IEL. The upregulation of IEL proliferative capacity was found only in the case of mice administered Ag orally, but did not occur in the case of i.p. immunized mice (data not shown). These results are compatible with the view that Ag primes or stimulates IEL through the intestinal mucosa. However, we still have another possibility, that orally administered Ag primes or stimulates CD4+ T cells at a site different from the intestinal epithelia and that these cells migrated into the epithelium. The Ag-specific proliferative activity of systemic CD4+ T cells in the spleen and Peyer’s patches was not changed by orally administered Ag (Fig. 5B). Nevertheless, their reactivity was much greater than that of the IEL, independent upon those cells from non-treated or Ag-fed Tg mice. Thus, if CD4+ T cells activated by orally administered Ag outside the epithelium were recruited and infiltrated the epithelium, the proliferative activity of these cells would be greater than that of CD4+ IEL of non-treated mice. In the case of CD8+ T cells, it has been reported that activated systemic T cells migrate into the epithelium. We need to study the location for priming and/or stimulation of CD4+ IEL. We also need to show whether CD4+ IEL before Ag feeding are memory or naive cells.

The upregulation of proliferative ability suggests the possibility that CD4+ IEL may proliferate in vivo in response to orally administered Ag. Further, if CD4+ T cells had migrated to the epithelium, this would result in an increase of cell number. Concerning the total number of CD4+ IEL, this tended to increase as a result of feeding OVA, as shown by calculations based on the total cell number and frequency (3.87 ± 1.39 × 10^5 CD4+ IEL in CCD-fed mice as compared to 4.87 ± 3.00 × 10^5 CD4+ IEL in ED-fed mice). However, this increase was slight and not statistically significant. It is possible that even if CD4+ T cells expanded and/or migrated into the epithelium, some of these cells may migrate out and/or undergo apoptosis, resulting in no significant increase in the total cell number. Added to these possibilities, it is possible that number of CD8+ IEL decreased. The increase in the frequency of CD4+ IEL may be in part due to the decrease in the CD8+ IEL population.

In this study using a TCR-transgenic model, we have demonstrated that orally administered Ag elicited an increase in the proliferative response and frequency of MHC class II-restricted CD4+ IEL. These results suggest that the intestinal cellular system has a mechanism by which intestinal exogenous Ags directly prime and/or stimulate CD4+ IEL or localize activated CD4+ T cells in the intestinal epithelium.

ACKNOWLEDGMENTS

We thank Masaaki Hashiguchi and Yoshihiro Ueda (The University of Tokyo) for technical assistance and Dr. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology) for critical reading of this manuscript. We also thank the faculty and staff members of the Biotechnology Research Center, The University of Tokyo, for use of the FACSort flow cytometer.

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