

et al. 1997, 1998). Moreover, some bifidobacteria increased total IgA production in the intestine (Fukushima et al. 1998). Immunomodulation by *Bifidobacterium in vivo* is expected to be beneficial for human health. However, the molecular and cell biology of the immunoregulatory responses induced in the gut-associated lymphoid tissue by oral administration of *Bifidobacterium* components has not been elucidated.

The unique characteristics of the intestinal immune response are secretory IgA production and oral tolerance. Peyer's patches (PP) which are representative of lymphoid follicles in the intestinal mucosa, are the inductive sites of immune reaction to oral antigens. However, the mechanisms of immunomodulation by probiotic bacteria have not been clarified in detail. In particular, there is no data available as to how antigen-presenting cells (APCs) or CD4⁺ T cells respond to probiotic bacteria in the PP.

In this study, we have investigated the immune responses of PP cells by oral feeding of *Bifidobacterium* immunomodulators (BIM) derived from sonicated *B. pseudocatenulatum* 7041 on mucosal immune responses in the intestine. We found that oral administration of BIM enhanced IgA production by PP cells and observed characteristic responses of CD4⁺ T cells in PP following oral administration of BIM.

Materials and methods

Animals

Female 6–9 weeks old BALB/c mice were obtained from Clea Japan (Tokyo, Japan) and were housed in a room with a 12 h light-dark cycle. The mice were naturalized and given MF diet (Oriental Yeast, Tokyo, Japan) for 3 days before experiments. All mice were kept in accordance with Guideline for the Care and Use of Laboratory Animals by College of Bioresource Sciences, Nihon University.

Preparation of *Bifidobacterium* immunomodulator (BIM) derived from sonicated *B. pseudocatenulatum* 7041, and oral administration of BIM

Sonicated *B. pseudocatenulatum* 7041 was prepared by the method described in previous reports

(Lee et al. 1993; Hosono et al. 2003). Mice were orally administered a dose of 10 mg/day of BIM in saline by using a feeding-tube for 7 consecutive days. The control mice were given saline by the same method of tube feeding. Mice were allowed free access to a pelleted MF diet and sterile deionized water throughout the experimental period.

Preparation of CD4⁺ T cells and Thy1.2⁻ cells from PP, and APC from splenocytes

After 7 days oral administration of BIM, PP cells were obtained from each experimental group by the method described previously (Hosono et al. 2003), and CD4⁺ T cells were isolated by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-mouse CD4-conjugated magnetic microbeads and an LS column following the manufacturer's instructions. Thy1.2⁻ (CD90) negative PP cells from the experimental mice were isolated by MACS with anti-mouse CD90-conjugated magnetic microbeads and LS column. APC were derived from splenocytes of BALB/c mice, which had not been given the experimental diet. The cell suspensions of splenocytes were treated with 50 µg/ml mitomycin C (Sigma, St. Louis, MO) for preparation of APC.

Flow cytometric analysis

Flow cytometric analysis of CD4⁺ cells was performed using a FACScalibur with CellQuest software (BD Biosciences, Mountain View, CA) and staining with anti-TCRαβ-biotin, anti-CD4-FITC, and either PE-, anti-CD45RB, anti-CD44, or anti-CD69. Fc receptors (FcγRIII/II) were blocked using anti-mouse CD16/CD32. The above Abs were purchased from BD PharMingen (San Diego, CA). Isotype-matched negative controls were included in the analysis of the cells suspensions. Purity of CD4⁺ cells from PP was >93% (data not shown). We checked the expression of CD90 (<0.8%) of Thy1.2⁻ PP cells by FACS (data not shown).

Measurement of total IgA

Total IgA in the intestinal ingredients and culture supernatant was measured by sandwich ELISA.

The intestinal tracts from duodenum to rectum were dissected from the experimental mice. The intestinal tracts were homogenized with 2 ml PBS containing 50 mM EDTA and 0.1 mg/ml trypsin inhibitor, and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatants obtained from the intestinal ingredients were diluted appropriately for ELISA. PP cells from the experimental groups were plated onto the wells of a 48-well plate at 2.5×10^6 cells per well, and co-cultured for 5–7 days with 0, 10, or 50 $\mu\text{g/ml}$ BIM in RPMI 1640 medium containing 5% fetal bovine serum. The amounts of total IgA in the intestinal ingredients or culture supernatants were measured by means of a sandwich ELISA method described previously (Hosono et al. 2003).

Measurement of BIM-specific IgA

BIM-specific IgA was measured by ELISA. Plates were coated with 50 μl of 100 $\mu\text{g/ml}$ BIM. Subsequent handling was followed as described above. BIM-specific IgA was qualitatively determined by absorbance at 405 nm.

Culture and cytokine determinants

CD4^+ T cells from PP cells from experimental mice (2×10^6 cells/well) in a total volume of 1 ml were cultured with 0, 10, or 50 $\mu\text{g/ml}$ BIM in the presence of APC derived from splenocytes (8×10^6 cells/well) in RPMI 1640 medium containing 5% FCS in a 48-well plate. Thy1.2^- PP cells (6×10^6 cells/well) in a total volume of 1 ml were cultured with BIM. The culture supernatants were collected after 24 or 72 h and assayed for IL-12 p40 and for IL-5, IL-6, and IFN- γ respectively. The amounts of IL-5, IL-6, and IFN- γ in the supernatants were measured by means of a sandwich ELISA method described previously. IL-12 was measured using an OptEIA mouse IL-12 (p40) set (BD PharMingen).

Statistical analysis

Data are expressed as means \pm SD. Differences were examined by one-way analysis of variance (ANOVA), and significant differences found

between groups were further evaluated by Tukey's test (SPSS Ver. 10.0, Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

Results

Total IgA production and BIM-specific IgA of PP cells after BIM administration

There was no significant increase of body weight gain among the experimental groups (data not shown). The total number of PP and PP cells were not different among the experimental groups. We examined total IgA production by PP cells derived from the experimental mice during a 7-day primary cells culture with different doses of BIM. PP cells had been prepared from the experimental mice. The total IgA production by PP cells of BIM-fed groups was higher than that of control groups for all doses of BIM *in vitro* (Figure 1a). In addition, BIM-specific IgA production by PP cells was enhanced by oral administration of BIM (Figure 1b).

Cytokine production patterns of CD4^+ from PP cells

We examined the cytokine production patterns of CD4^+ T cells derived from PP cells, which were obtained from both the control mice and mice administered BIM. The prepared CD4^+ T cells were cultured with BIM in the presence of APC from naïve mice splenocytes *in vitro*, and the amounts of cytokine in the supernatants were measured (Figure 2). Secretion of both IFN- γ and IL-6 was enhanced by orally administered BIM. However, the secretion of IL-4 and IL-5 was not detected (data not shown).

Increased $\text{CD4}^+ \text{CD45RB}^{\text{high}}$ PP T cells following orally administered BIM

To examine the effects of BIM feeding on the state of CD4^+ PP T cells derived from experimental mice, we analyzed surface cell antigens of purified CD4^+ PP cells by flow cytometric analysis. All results were gated $\text{TCR}\alpha\beta$. As shown in Table 1, the BIM-fed group shows higher expression of

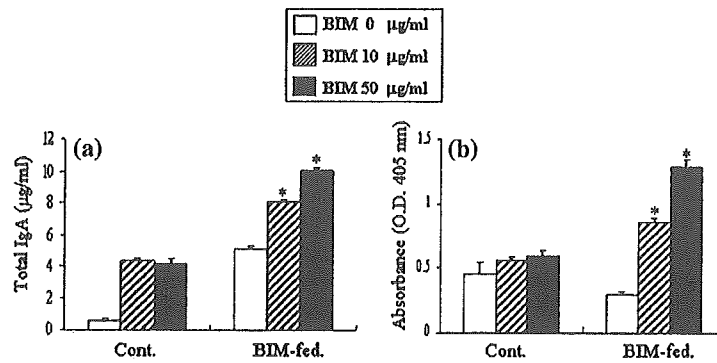


Figure 1. Effects of oral administration of BIM on total IgA production from PP cells. PP cells were obtained and pooled for each experimental group respectively, and then the cells were cultured with 0–50 µg/ml of BIM for 7 days. (a) Total IgA in the culture supernatants was measured by ELISA. (b) BIM-specific IgA in the culture supernatants was qualitatively measured by ELISA. The values are expressed as the mean of triplicate cultures ± S.D. Additional concentrations in *in vitro* culture were BIM 0 µg/ml (white bar), BIM 10 µg/ml (shadow bar), and 50 µg/ml (black bar). *Significant difference from control group of the same BIM dose at $p < 0.05$ by Tukey's test. The results shown are representative of two independent experiments.

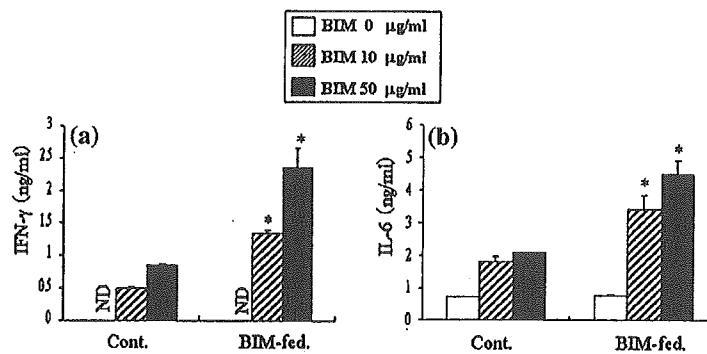


Figure 2. Effects of oral administration of BIM on cytokine production by murine PP CD4⁺ T cells. PP CD4⁺ T cells were obtained and pooled for each experimental group respectively. The cells were then co-cultured with 0–50 µg/ml BIM in the presence of APC for 72 h. The cytokines in the culture supernatants were measured by ELISA. The values are expressed as the mean of triplicate cultures ± S.D. ND, not detected. Additional concentrations in *in vitro* culture were BIM 0 µg/ml (white bar), BIM 10 µg/ml (shadow bar), and 50 µg/ml (black bar). *Significant difference from control group of the same BIM dose at $p < 0.05$ by Tukey's test. The results shown are representative of two independent experiments.

CD4⁺CD45RB^{high} than control. In contrast, activation-markers CD44 or CD69 were not significantly different from control.

Cytokine production patterns of Thy1.2⁻ cells from PP

To examine the effects of BIM administration on responses of APCs in the PP, we measured the cytokine production patterns of Thy1.2⁻ cells prepared by negative sorting of Thy1.2⁺ cells from PP cells obtained from experimental groups. We

observed the level of IL-12 p40 on 24 h-cell cultures from orally administered BIM groups was higher than that of the control group. The secretion of IL-5, IL-6, and IFN-γ was maintained at high levels, but there were no significant differences among these groups (Figure 3).

Oral administration of BIM did not induce total IgA in the intestinal mucosa in vivo

Although we measured the total IgA secretion in the intestinal contents from the ileum to the

Table 1. Effects of oral administration of BIM on the expression of cell surface antigen on CD4⁺ T cells.

	Cont.	BIM-fed.	p value
CD4 ⁺ CD45RB ^{high}	49.6 ± 2.4	57.9 ± 6.1	0.084
CD4 ⁺ CD45RB ^{low}	47.3 ± 2.7	39.0 ± 5.7	0.094
CD4 ⁺ CD44 ^{high}	59.5 ± 9.7	58.9 ± 8.2	0.933
CD4 ⁺ CD44 ^{low}	37.8 ± 9.3	38.2 ± 7.3	0.949
CD4 ⁺ CD69 ^{high}	40.8 ± 4.8	40.7 ± 8.4	0.975
CD4 ⁺ CD69 ^{low}	56.5 ± 3.7	55.9 ± 8.3	0.885

Purified CD4⁺ PP cells derived from experimental mice and analyzed by flow cytometric analysis. All results were gated TCR $\alpha\beta$. Values are means of three independent experiments \pm S.D and expressed as percentage of viable cells.

rectum *in vivo*, no significant increase was observed in these experimental groups (Figure 4).

Discussion

There are some reports showing that probiotic bacteria supplementation induces immunological responses in treated animals. However, it was not clear exactly how probiotic bacteria and their components modulated mucosal immune response in the gut. In particular, it has not been clearly elucidated that how helper T cells and APCs interact to IgA production respectively by oral administration of probiotic bacteria.

Probiotic bacteria enhance humoral immune responses by increasing the number of IgA producing cells (Kaila et al. 1992; Park et al. 2002) and increasing the systemic and mucosal IgA response to dietary antigen (Kaila et al. 1992; Shroff et al. 1995; Takahashi et al. 1998). In addition, *Lactobacillus*-specific IgA in PP cells cultured from conventional mice was significantly increased when compared with that in germfree mice (Ibnou-Zekri et al. 2003). We examined whether sonicated *Bifidobacterium* components that have strong mitogenic activity influenced the ability of PP cells to produce IgA. We showed that BIM administration up-regulated the proliferation activity of PP cells *in vitro* (data not shown). These results indicate that orally administered BIM was taken up by M cells on the PP and then activated PP cells. We demonstrated that BIM-feeding enhanced the production of cytokines, such as IFN- γ , IL-5, IL-6, and IL-12, in the PP cells (data not shown). We also observed that both total IgA and BIM-specific IgA production in the PP derived from BIM-fed animals was higher than that of the control group (Figure 1). Recent studies have shown that a novel cell subset in

the PP secreted IL-5 and increased IgA secretion by B cells (Kuraoka et al. 2004). Previous studies have shown that the induction of CD4⁺ cells in PP is important in the activation of mucosal IgA responses (Kiyono et al. 1992; McGhee, et al. 1992). Our results show that secretion of both IFN- γ and IL-6 by CD4⁺ T cells were increased following BIM administration (Figure 2). On the other hand, IFN- γ , IL-5, and IL-6 secretion by Thy1.2⁻ PP cells from experimental groups showed no significant alteration in response to BIM-feeding (Figure 3). It has been shown that orally administered antigens increase Th2 type cytokine responses in PP (Yoshida et al. 2002), and that IL-5, IL-6, or IL-10 induces the differentiation of B cells to surface IgA⁺ B cells (DeFrance et al. 1992; Xu-Amano et al. 1992). It has also been reported that the oral administration of lactic-acid bacteria induces IFN- γ production in mucosal lymphocytes (Aattour et al. 2002), and that IFN- γ can promote the uptake of antigens in PP, where specific IgA-related cells are generated (Sutas et al. 1997). Our results suggest that BIM-feeding could induce cytokine production in PP cells including CD4⁺ T cells, which enhanced both total IgA and BIM-specific IgA production in the PP.

The level of expression of CD45RB on mouse CD4⁺ T cells has been shown to distinguish naïve (CD45RB^{high}) from activated/memory (CD45RB^{low}) cells (Powrie 1995). It was reported that the cytokine profiles of CD45RB^{high} and CD45RB^{low} cells differ (Ten Hove et al. 2004), and that CD45RB^{high} cells produce more IFN- γ (Horgan et al. 1994). We observed that CD4⁺ PP T cells showed enhanced IFN- γ and IL-6 secretion (Figure 2), but increased naïve marker, CD45RB^{high} expression (Table 1) following oral administration of BIM. Although we have not examined the responses of CD4⁺ T cells in the lamina propria as an effector site, we suspect

that $CD4^+ CD45RB^{high}$ cells regulated total IgA production *in vivo*. When we examined mucosal total IgA production in the intestine *in vivo*, total IgA was not increased following oral administration of BIM (Figure 4). Further studies will be needed to elucidate the reason why the numbers of these naïve cells in the PP increased following feeding with BIM.

Although secretion of IFN- γ , IL-5 and IL-6 by Thy1.2 $^-$ PP cells from each experimental group

was not significantly different, it should be noted that secretion of IL-12 by these cells was enhanced by orally administered BIM (Figure 3). When $CD4^+$ T cells only were cultured with BIM, no proliferative activity was observed (data not shown). In other words, alteration of $CD4^+$ T cells following oral administration of BIM may be mediated by APCs in the PP. Because secretion of IL-12 was enhanced by BIM feeding (Figure 3), this naturally implies that dendritic cell (DC) or

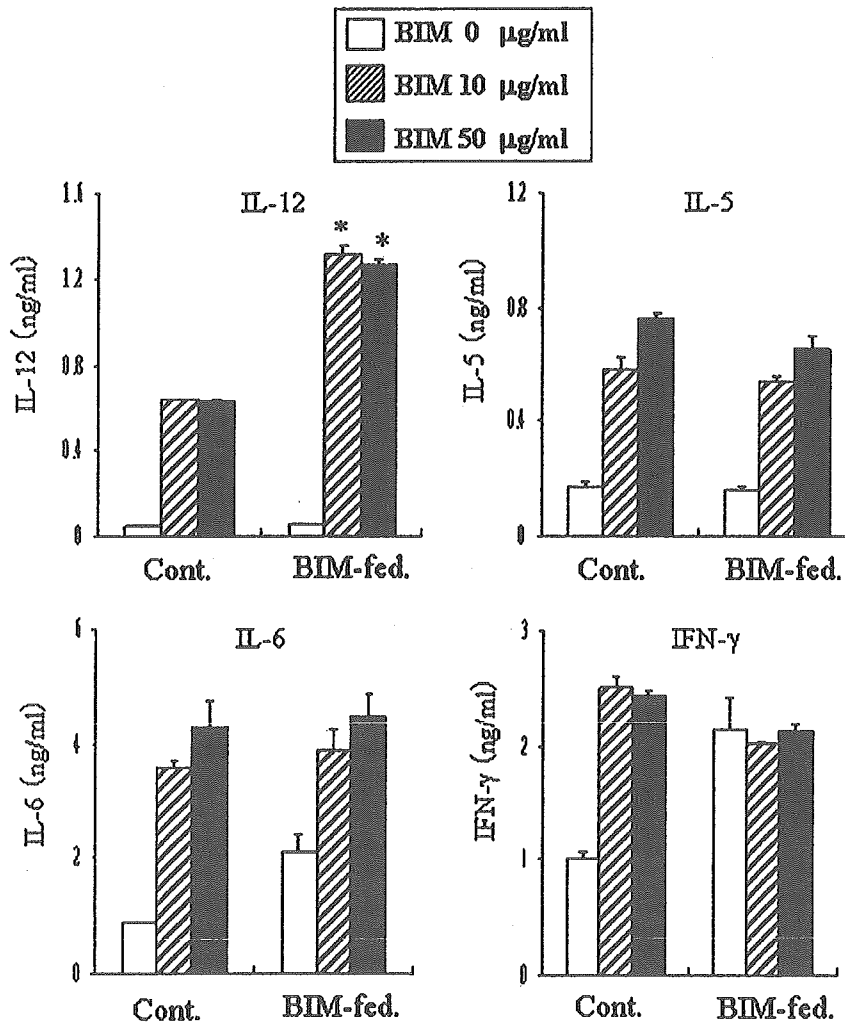


Figure 3. Effects of orally administered BIM on cytokine production from murine Thy1.2 $^-$ PP cells. Thy1.2 $^-$ PP cells were obtained and pooled for each experimental group respectively. The cells were then cultured with 0–50 μ g/ml of BIM for 24 or 72 h. The cytokines in the culture supernatants were measured by ELISA. The values are expressed as the mean of triplicate cultures \pm S.D. Additional concentrations in *in vitro* culture was BIM 0 μ g/ml (white bar), BIM 10 μ g/ml (shadow bar), and 50 μ g/ml (black bar). *Significant difference from control group of the same BIM dose at $p < 0.05$ by Tukey's test. The results shown are representative of two independent experiments.

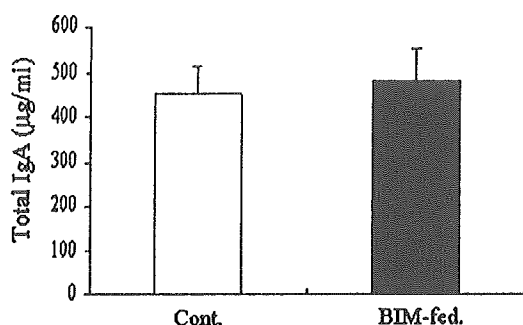


Figure 4. Effects of orally administered BIM on total IgA production in the intestine *in vivo*. After 7 days of oral administration of BIM, total IgA levels in the intestinal tract were measured by ELISA. The intestinal tracts were obtained from experimental group. The values are expressed as the means \pm S.D. ($n = 9$). The results shown are representative of three independent experiments.

macrophage ($M\phi$) in the $Thy1.2^-$ PP cells was activated by BIM in the initial phase. Cells of the innate immune system, such as DC and $M\phi$, use a variety of pathogen-associated molecular pattern (PAMP) recognition receptors to recognize the patterns shared between pathogens (Janeway 1998; Banchereau et al. 2000). PAMPs include lipopolysaccharide from Gram-negative bacteria, lipoteichoic acid, lipoarabunomannan, lipoprotein, and unmethylated DNA with a CpG motif. These cause $M\phi$ and DC to produce a set of proinflammatory cytokines, such as tumor necrosis factor α , IL-1, IL-6 and IL-12. They also induce the maturation of DC which drive $Th1$ cell development leading to the activation of adaptive immunity (Takeuchi and Akira 2002). Recently, Toll-like receptors (TLR) were identified as major recognition receptors for PAMP (Poltorak et al. 1998; Yang et al. 1998; Hemmi et al. 2000). In particular, TLR2 responds to Gram-positive cell wall components (Yoshimura et al. 1999). In the intestinal mucosa, we speculate that DC in the PP recognize BIM, including peptidoglycan from Gram-positive bacteria, via TLR2, and then activate the adaptive immune response.

The PP has been considered to be the major inductive site for the initiation of high affinity secretory IgA immune responses in the gastrointestinal tract. In this study, we showed that orally administered BIM enhanced total IgA production including BIM-specific IgA from PP cells. We believe that BIM can modulate the cellular immune responses in PP via the adjuvant-like activity of

BIM. Some live *Bifidobacterium* as well as this component derived from them have adjuvant activity (Sekine et al. 1994). Meanwhile, the level of total IgA in the intestinal contents did not increase significantly following BIM feeding *in vivo*. This suggests that BIM might not be excluded from the host because BIM was derived from a non-pathogenic bacterium, which was permitted symbiosis. Although, oral feeding of BIM did not induce higher levels of total IgA production in conventional conditions *in vivo*, we speculate that BIM will induce protective immune responses if pathogenic bacteria invade the gut.

In this study, we demonstrated that BIM, acting as a probiotic immunomodulator, induced mucosal IgA secretion in PP, and induced $CD4^+$ PP T cells to change the expression of cell surface antigens and cytokine secretion. In addition, since BIM is not pathogenic and is derived from a bacterium symbiotic with human intestinal commensal bacteria, IgA hyperproduction in the mucosa might be regulated in the healthy host, although the heightened ability for IgA production is maintained in the PP cells. It will be interesting to clarify the role of probiotic bacteria in the modulation of the adaptive immune response via the innate immune response. Further study will be needed to clarify this clear interaction between probiotic bacteria and immune cells.

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Oral antigen induces antigen-specific activation of intraepithelial CD4⁺ lymphocytes but suppresses their activation in spleen

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Abstract

Intraepithelial lymphocytes (IELs) are considered to drive immune surveillance of the epithelial layer to the mucosa, which is initially exposed to exogenous antigens. However, how IELs are activated by orally administered antigens remains unclear. To clarify this mechanism, we fed ovalbumin (OVA) to T cell receptor transgenic (TCR-Tg) mice with OVA-specific MHC class II-restricted TCR and found that the cytotoxic activity of IELs was increased against both NK and LAK target cells, but notably reduced after depleting CD8⁺ IELs. Cytoplasmic staining showed that the production of IFN- γ and IL-2 was increased in mice fed with OVA both in the supernatant of cultured IELs with immobilized anti-CD3 mAb and in fresh CD4⁺ IELs.

In contrast, the cytotoxic activity against NK and LAK target cells and the production of IL-2 and IFN- γ was decreased in splenic T cells from mice fed with OVA. However, when the splenic T cells from these mice were cultured with OVA and IL-2, IFN- γ production recovered. The decreased response demonstrated the clonal anergy of T cells. Furthermore, tumor growth was enhanced in TCR-Tg mice carrying an OVA-transfected counterpart A20 B cell lymphoma (OVA-A20) and fed with OVA.

These results indicate that the oral administration of soluble antigens can activate CD4⁺ IELs in an antigen-specific manner but induces hyporesponsiveness in the spleen. In addition, Th1-type cytokines produced by activated CD4⁺ IEL might provide a bystander effect on the cytotoxic activity of IELs.

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Keywords: Cytotoxic activity; Intraepithelial lymphocytes; Oral tolerance; Transgenic mice

Introduction

The oral ingestion of antigens including food and pathogens induces peripheral immune hyporesponsiveness of T cells in an antigen-specific manner (Mowat, 1994). The induction of this suppressive response, termed oral tolerance, is not apparently unique since clonal deletion (Weiner et al., 1994; Chen et al., 1995a;

Abbreviations: IEL, Intraepithelial lymphocytes; OVA, Ovalbumin; OVA-A20, A20 tumor cells transfected with OVA gene; Tg, Transgenic mice

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Weiner, 1997), clonal anergy (Weiner et al., 1994; Chen et al., 1996; Houten and Blake, 1996) and clonal suppression (Miller et al., 1991; Weiner et al., 1994; Weiner, 1997) can generate oral tolerance. However, the mechanism through which digested dietary antigens induce immune hyporesponsiveness remains obscure. Oral tolerance has mainly been detected in peripheral T cells, but whether oral antigens activate or suppress the T cell response in the intestine where T cells initially confront exogenous antigens is unknown. Lymphocytes are numerous among intestinal epithelial cells (intraepithelial lymphocytes, IELs; Fichtelius, 1968) and they are thought to function as the front line of the immune system because they comprise a phenotypically heterogeneous population of T cells including CD8⁺, CD4⁺8⁺, CD4⁺8⁻ and CD4⁺ cells, as well as cells bearing an NK marker (Petit et al., 1985; Mosley et al., 1990; Lefrancois, 1991; Maloy et al., 1991). However, the functions of these subsets are unclear. The dominant subset of CD8⁺ IELs (approximately 70–80%) has NK activity, spontaneous cytotoxicity and antigen-specific CTL in vivo and in vitro (Klein and Kagonoff, 1984; Offit and Dudzik, 1989; Guy-Grand et al., 1991; Sydora et al., 1993; Gelfanov et al., 1996; Guy-Grand et al., 1996). However, the mechanism that induces cytotoxic activity in these cells has not been defined. Moreover, whether the small population of CD4⁺ IELs produces Th1- and Th2-type cytokines after antigen stimulation is obscure (Fujihashi et al., 1993).

Here, we demonstrated using ovalbumin (OVA)-specific TCR-Tg mice that the oral administration of OVA induces CD4⁺ IEL activation in an antigen-specific manner as well as a subsequent increase in polyclonal cytotoxic T cell activity. Furthermore, the growth of OVA-transfected counterpart A20 B lymphoma cells that were transplanted into TCR-Tg mice fed with OVA was significantly enhanced compared with that of control TCR-Tg mice fed with casein. Our results suggest that the oral administration of antigen enhances the mucosal, but suppresses the systemic immune response.

Materials and methods

Mice

We established and maintained TCR-transgenic (TCR-Tg) mice specific for OVA 323–339 peptide in the context of I-A^d (Sato et al., 1994). BALB/c mice obtained from CLEA (Kawasaki, Japan) were maintained under pathogen-free conditions in our laboratory. Animals were cared for and handled according to our institutional guidelines and our institutional review board approved all procedures associated with the study.

Administration of OVA

The TCR-Tg mice were fed for 14 days with a diet containing 20% autoclaved egg-white protein and about 250 mg OVA per day (OVA mice). Control mice were fed with a diet containing 20% casein.

Isolation of IELs

We isolated IELs as described (Mosley et al., 1990). Briefly, dissected pieces of the small intestine were mechanically shaken at 37 °C for 20 min in medium containing 1 mM dithioerythritol. Supernatants containing IELs were filtrated through nylon wool before centrifugation through a 45%/67.5% Percoll gradient. Cells at the interface of the Percoll gradient were collected for FACS and cytotoxicity analyses.

IEL cell populations were depleted of CD8⁺ IELs (including CD8 α / α ⁺ and CD8 α / β ⁺ cells) by using anti-CD8 α mAb (53-6.7, PharMingen, San Diego, CA) followed by anti-Rat IgG-coated Dynabeads (DynaL AS, Oslo, Norway). The CD8 α / α ⁻ T cells were usually >95% pure according to flow cytometry. In order to obtain a CD8 β ⁺-depleted cell population, IELs were selected by using anti-CD8 β mAb (53-5.8, PharMingen, San Diego, CA) followed by magnetic sorting using Dynabeads coated with anti-Rat IgG antibody (DynaL AS, Oslo, Norway). Also, CD4⁺ IELs were deleted by using Dynabeads coated with anti-CD4 mAb. The CD8 α ⁺ IELs were >95% pure, according to FACScan.

Monoclonal Abs and FACS analysis

Anti-CD3 ϵ (145-2C11), biotin-labeled anti-CD8 α , anti-CD8 α -FITC and anti-CD4-PE were purchased from PharMingen (San Diego, CA). An anti-clonotype Ab (1H9) to transgene TCR-V α 3.1 was prepared in our laboratory (Sato et al., 1996). Avidin-labeled Red-613 and PE were purchased from GIBCO/BRL (Life Technologies, Gaithersburg, MD) and from Biomedica Corp (Foster City, CA), respectively. FITC-labeled anti-hamster IgG was purchased from Southern Biotechnology (Birmingham, AL). IELs stained with mAbs were analyzed using a FACScan flow cytometer and Lysis II software from Becton Dickinson (San Jose, CA). The cytoplasm was stained for cytokines as described (Ferrick et al., 1995; Openshaw et al., 1995). Briefly, after a 4 h incubation at 37 °C in brefeldin A (10 μ g/ml, Sigma Chemical Co., St. Louis, MO), IELs were incubated with anti-Fc γ II receptor (2.4G2) mAb and FITC-conjugated anti-CD4 mAb (PharMingen, San Diego, CA). The IELs were then fixed in 100 μ l of fixation buffer and incubated in 50 μ l permeabilization buffer containing 0.2 μ g/ml of PE-conjugated anti-IL-2

(S4B6) or IFN- γ (XMG1.2) mAb (PharMingen, San Diego, CA).

Cytokine assay

Splenic T cells passed through nylon wool and purified IELs ($1\text{--}2.5 \times 10^5$ /well) were cultured for 30–48 h in 96-well flat-bottomed plates containing the indicated concentrations of purified OVA grade VI (Sigma Chemical Co., St. Louis, MO) or coated with $10 \mu\text{g}$ of anti-CD3 mAb/well. To determine the recovery of cytokine production, splenic T cells were cultured with $100 \mu\text{g}/\text{ml}$ of OVA and $30 \text{ U}/\text{ml}$ of IL-2 for 72 h. Levels of IL-2 and IFN- γ in supernatants obtained from cultured cells were determined by respective ELISA assays (Genzyme, Cambridge, MA) and absorbance was read at 450 nm. All assays were performed in duplicate.

Assessment of cell proliferation

Splenic T cells passed through nylon wool and purified IELs (5×10^4 /well) were cultured as described for the cytokine assays. Proliferation of the cultured IELs was determined by measuring [^3H]-thymidine incorporation in a scintillation counter 16 h after adding $1 \mu\text{Ci}$ of [^3H]-thymidine.

Cytotoxicity assay

We assayed ^{51}Cr -release by IELs and splenic T cells for 4 h at various E/T ratios. A20, OVA-A20, YAC-1 (NK sensitive), RDM4 (LAK sensitive) and the targets were all EL-4 (resistant to NK and LAK) cell lines. The OVA-A20 cell line derived from the mouse A20 B lymphoma cells by transfection with chicken OVA cDNA (Nishimura et al., 1999) was cultured in RPMI 1640 without glutamine supplemented with 5% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate. The medium for OVA-A20 cells also contained G418. Cytotoxicity was defined by the following equation: Specific lysis (%) = [(cpm release by experimental group – cpm spontaneous release)/(cpm 1 N HCl release – cpm spontaneous release)] \times 100. In some experiments, fresh IELs were cultured for 4 days with or without IL-2 and IFN- γ (supplied by Shionogi Pharmaceutical Co. Ltd.).

Tumor transplantation

To determine whether the oral administration of OVA can enhance tumor growth, TCR-Tg mice were fed for 14 days with OVA (OVA mice) or casein (control mice). The TCR-Tg mice were continuously fed with OVA and subcutaneously transplanted with 5×10^6 OVA-transfected A20 B lymphoma cells in one flank and the same

number of A20 cells in the other. Tumor weight and cytotoxic activity of the spleen were measured 14 days later. Furthermore, we weighed tumors and examined the cytotoxic activities of the spleen from TCR-Tg mice that were sensitized with OVA ($100 \mu\text{g}/\text{mouse}$) or PBS, respectively. OVA or PBS was administered intraperitoneally (i.p.) to TCR-Tg mice 7 times at one-day intervals and then tumors were transplanted into the mice, which were continuously sensitized with OVA.

Statistical analysis

Results are expressed as mean \pm s.d. Differences between groups were examined for statistical significance using Student's *t*-test using StatView (SAS, Cary, NC). Differences of $P < 0.05$ were considered statistically significant.

Results

The oral administration of antigens induces immune tolerance in peripheral T cells, but whether the response is the same in IELs, which constitute the first cell type exposed to dietary antigens remains unclear. We investigated how IELs generate an antigen-specific response to oral antigens that elicit peripheral tolerance using TCR-Tg mice with specificity for OVA peptide (323–339) and MHC class II (I-A^d). The major T cell population comprised CD4⁺ cells expressing the TCR transgene (Fig. 1A).

We initially examined the antigen-specific response of splenic T cells from TCR-Tg mice fed with OVA or casein after 14 days. T cell subsets defined by CD4 and CD8 expression (Fig. 1A) and total numbers of IELs (data not shown) did not significantly differ between the two groups of mice. The IELs were mainly composed of CD8⁺ cells (approximately 70%), of which about 95% were CD8 α/α ⁺ and about 5% were CD4⁺. These proportions of T cell subsets are consistent with those of normal mice. Levels of TCR (IH9) expression were about 44%, 67% and 60% on CD8 α/α ⁺, CD8 α/β ⁺ and CD4⁺ IELs, respectively (Fig. 1B).

We examined cytokine activity produced by IELs isolated from TCR-Tg mice fed with either OVA or casein and cultured in plastic plates coated with anti-CD3. The IELs from mice fed with OVA produced more IFN- γ and IL-2 than those from mice given casein (Fig. 2). Proliferation activity was also significantly increased ($P < 0.01$). We then examined cytokine production by IELs in vivo. We stained fresh CD4⁺ IELs isolated by FACStar for IL-2 and IFN- γ . Table 1 shows that the numbers of IL-2⁺CD4⁺ and IFN- γ ⁺CD4⁺ cells were obviously increased in TCR-Tg mice fed with OVA, whereas those of CD8⁺ cells was not (data not

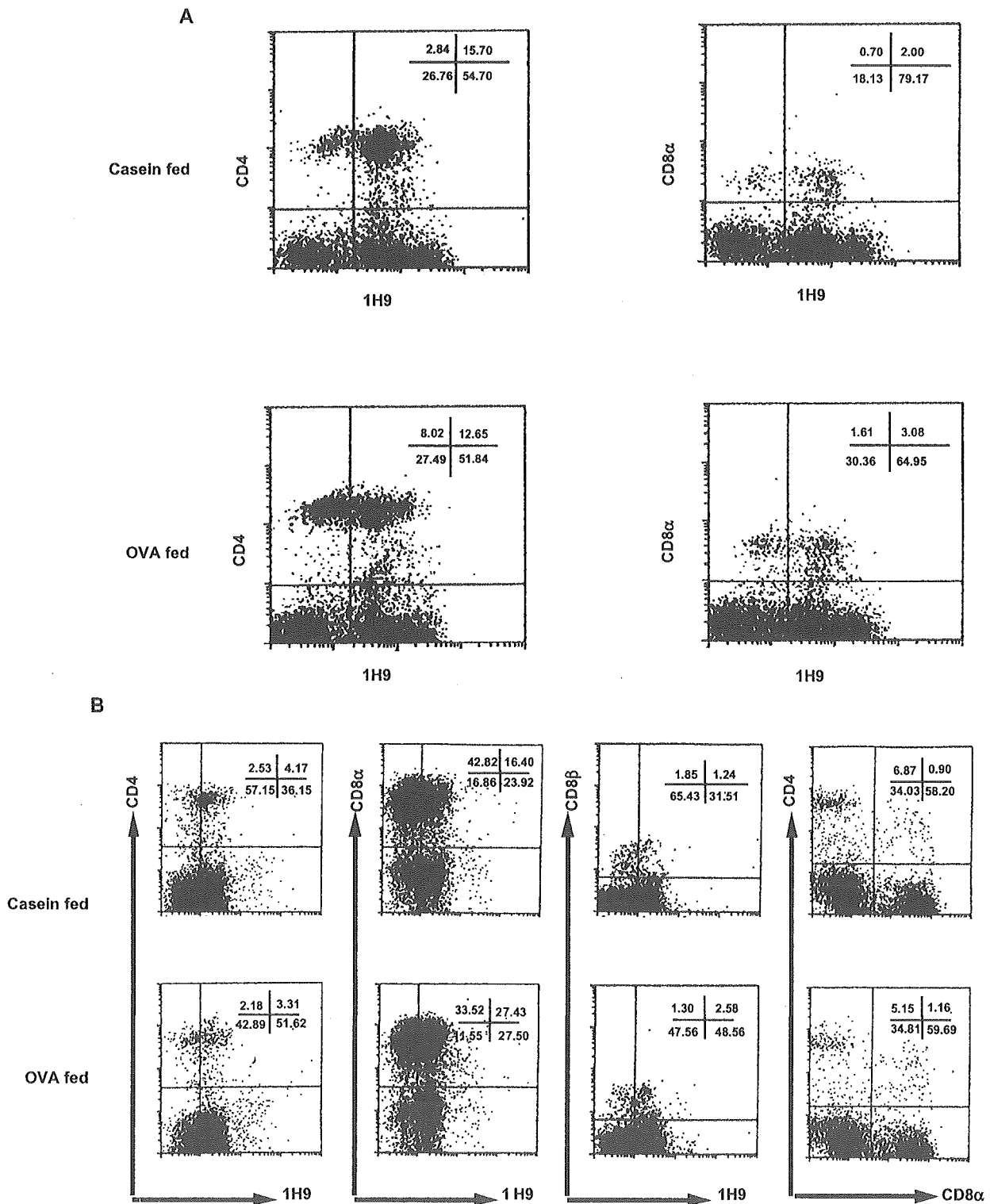


Fig. 1. Surface phenotype of T cells in OVA TCR-Tg mice fed with OVA or casein. TCR-Tg mice ($n = 9$) were fed with a diet containing 20% autoclaved egg-white protein and about 250 mg OVA per day. Control mice ($n = 9$) were fed with a diet containing 20% casein. After 14 days, (A) splenic and (B) IELs T cells from OVA or casein-fed TCR-Tg mice were analyzed by two-color fluorescence flow cytometry using indicated labeled mAb. Monoclonal 1H9 is an mAb against transgenic TCR-V α 3.1. The figure shows one representative of 3 repeated experiments.

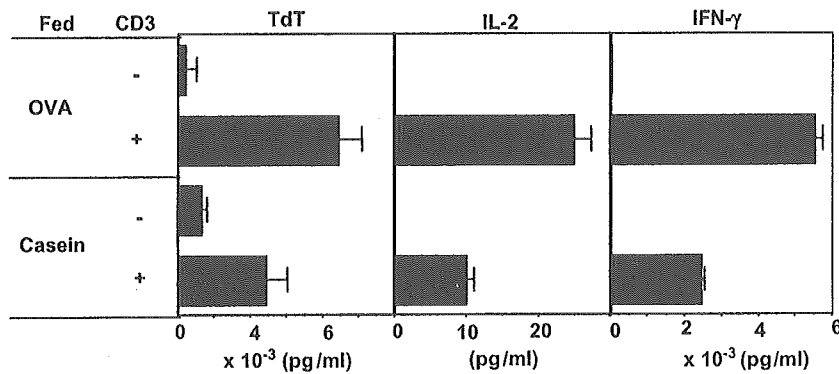


Fig. 2. Potential proliferation and lymphokine production of IELs. Fresh purified IELs from TCR-Tg mice fed with OVA or casein for 14 days were cultured in plastic plates coated with anti-CD3 mAb. After 48 h, cell proliferation was estimated as [³H]-thymidine incorporation. Cytokine production in culture supernatants was measured using ELISA.

Table 1. Frequency of IELs producing cytokines in TCR-Tg mice fed with OVA or casein^a

Fed	% Positive cells (/total CD4 ⁺)	
	IL-2 ⁺ CD4 ⁺	IFN- γ ⁺ CD4 ⁺
Casein	3.2	7.5
OVA	26.6	30.1

^aCell surface of fresh IELs purified from OVA or control TCR-Tg mice after 14 days was stained with anti-CD4-FITC after fixation, followed by anti-IL-2-PE or anti-IFN- γ -PE.

shown). We then examined the production of cytokines by splenic T cells from TCR-Tg mice fed with OVA or casein. The production of IL-2 and IFN- γ by purified splenic T cells cultured with OVA in the presence of splenic APC was increased in casein-fed mice but undetectable in OVA-fed mice, indicating a down-regulation in TCR-Tg mice fed with OVA compared with those given casein (Fig. 3A). The response of splenic T cells was also similarly decreased when the splenic cells were cultured in 96-well plastic plates with immobilized anti-CD3 mAb for 48 h (Fig. 3B). The TCR-mediated suppression of the splenic T cell response in TCR-Tg mice fed with OVA was restored when IL-2 was added to the culture medium (Fig. 3C). In contrast, the amount of IL-4 produced by spleen cells from TCR-Tg mice fed with OVA was not decreased (data not shown) and the numbers of splenic T cells did not differ among TCR-Tg mice regardless of diet. Collectively, these results indicated that Th1-type CD4⁺ IELs are activated by antigens when the response of splenic T cells is suppressed by the continuous oral administration of OVA.

The main constituents of IELs are CD8⁺ cells that mostly comprise CD8 α/α ⁺ and a few CD4⁺ cells. As anticipated from the proportion of IEL subsets, the main function of IELs is cytotoxic activity, but how

these CD8⁺ cells become cytotoxic remains unclear. The proportions of the IEL subsets did not significantly differ in our TCR-Tg mice, since CD8⁺ and CD4⁺ cells accounted for about 70% and 6%, respectively, of the subset (Fig. 1B). Since activated CD4⁺ IELs produced Th1-type cytokines (Table 1 and Fig. 2), we examined the cytotoxic activities of fresh IELs isolated from TCR-Tg mice fed with OVA or casein against various tumor cells. Fig. 4A shows that IELs from TCR-Tg mice fed with OVA were more cytotoxic against YAC-1 (NK cell target) and RDM4 (LAK cell target) than those from TCR-Tg mice fed with casein. However, cytotoxicity against EL-4, which is resistant to LAK and NK activities, was similarly low in both types of mice. We also examined cytotoxic activity against A20 (I-A^d+ B lymphoma) and the A20 transfectant, OVA-A20, which was established by transfection with the OVA gene. Activity was increased in IELs from TCR-Tg mice given OVA, but not casein. Considering the nature of the target tumor cells, IELs probably do not lyse targets in an antigen-specific manner like CTL. Cytotoxic activities in TCR-Tg mice fed with OVA were reduced when CD8 α/α ⁺ (Fig. 4B), but not CD4⁺ or CD8 β ⁺ (data not shown) cells were depleted.

As CD8 α/α ⁺ cells predominate (Fig. 1B), they are mainly responsible for the increased cytotoxicity of CD8⁺ IELs. However, CD4⁺ but not CD8 α/α ⁺ cells were specifically activated by dietary OVA in the TCR-Tg mice, suggesting that most of the cytotoxic activity is induced due to a bystander effect of Th1-type cytokines produced from antigen-specific CD4⁺ IELs.

We investigated the role of Th1-cytokines in tumor immunity in TCR-Tg mice fed with OVA or casein. Tumor growth was more effectively inhibited in TCR-Tg mice given dietary casein than in OVA-fed mice (Fig. 5A). Furthermore, the cytotoxic activity of spleen cells against OVA-A20 and YAC-1 was decreased in TCR-Tg mice fed with OVA but not those fed with casein. These experiments were repeated over 5 times,

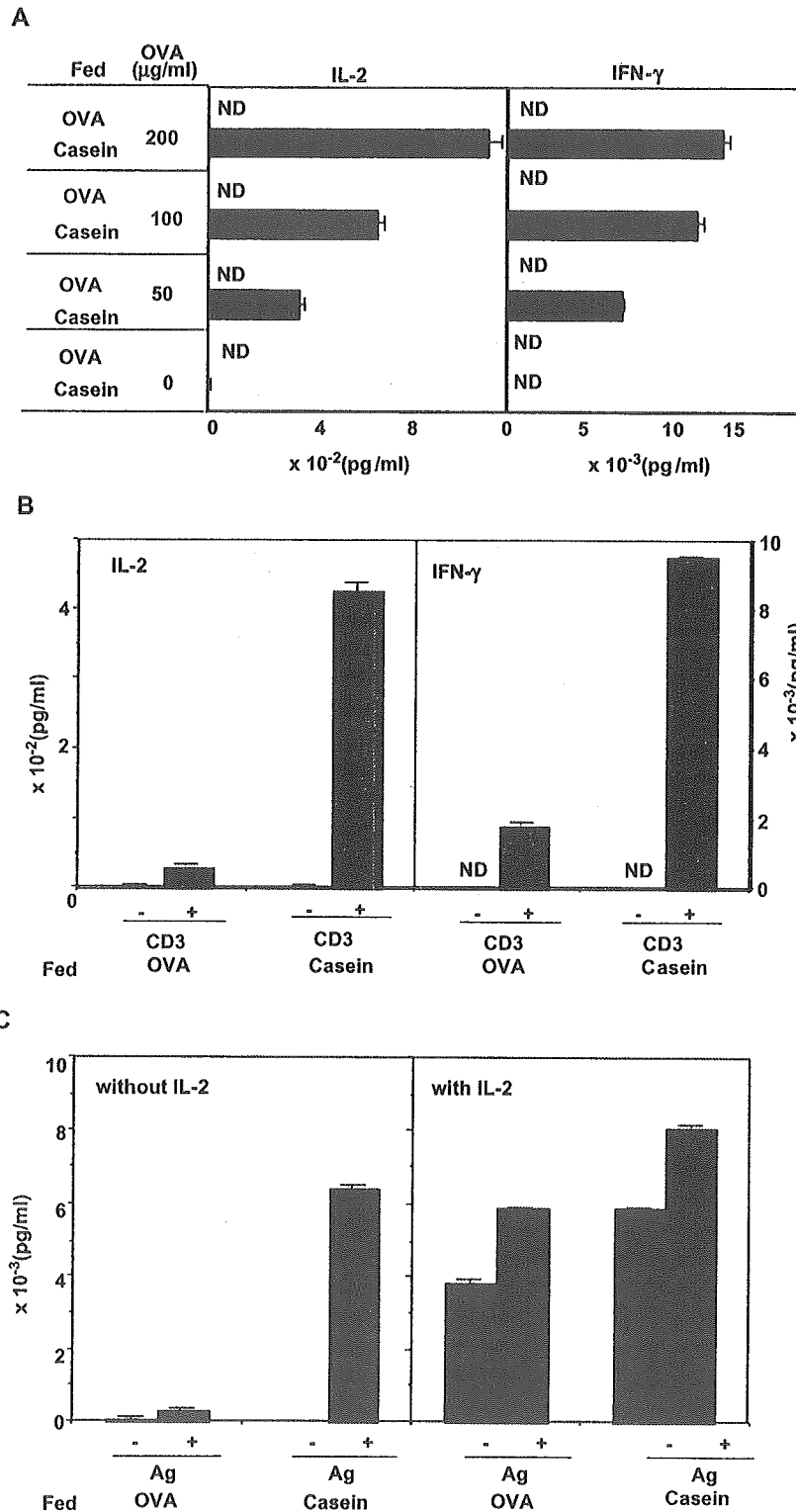


Fig. 3. Continuous oral administration of OVA diminishes the immune response of splenic T cells stimulated by OVA or immobilized anti-CD3 mAb. (A) Splenic T cells from TCR-Tg mice fed with OVA ($n = 20$) or casein ($n = 20$) for 14 days were cultured with indicated concentrations of OVA and irradiated (20 Gy) BALB/c spleen cells. Cytokine production was measured after 48 h (open bars, OVA; closed bars, casein). ND, not detected. (B) Splenic T cells (1×10^5 /well) from TCR-Tg mice fed with OVA or casein for 14 days were passed through nylon and cultured on anti-CD3-coated plastic plates. After 48 h, cytokine production was measured as described in Materials and methods. (C) Reduced production of cytokines by splenic T cells from TCR-Tg fed with OVA was recovered by culture with OVA and IL-2. ND, not detected.

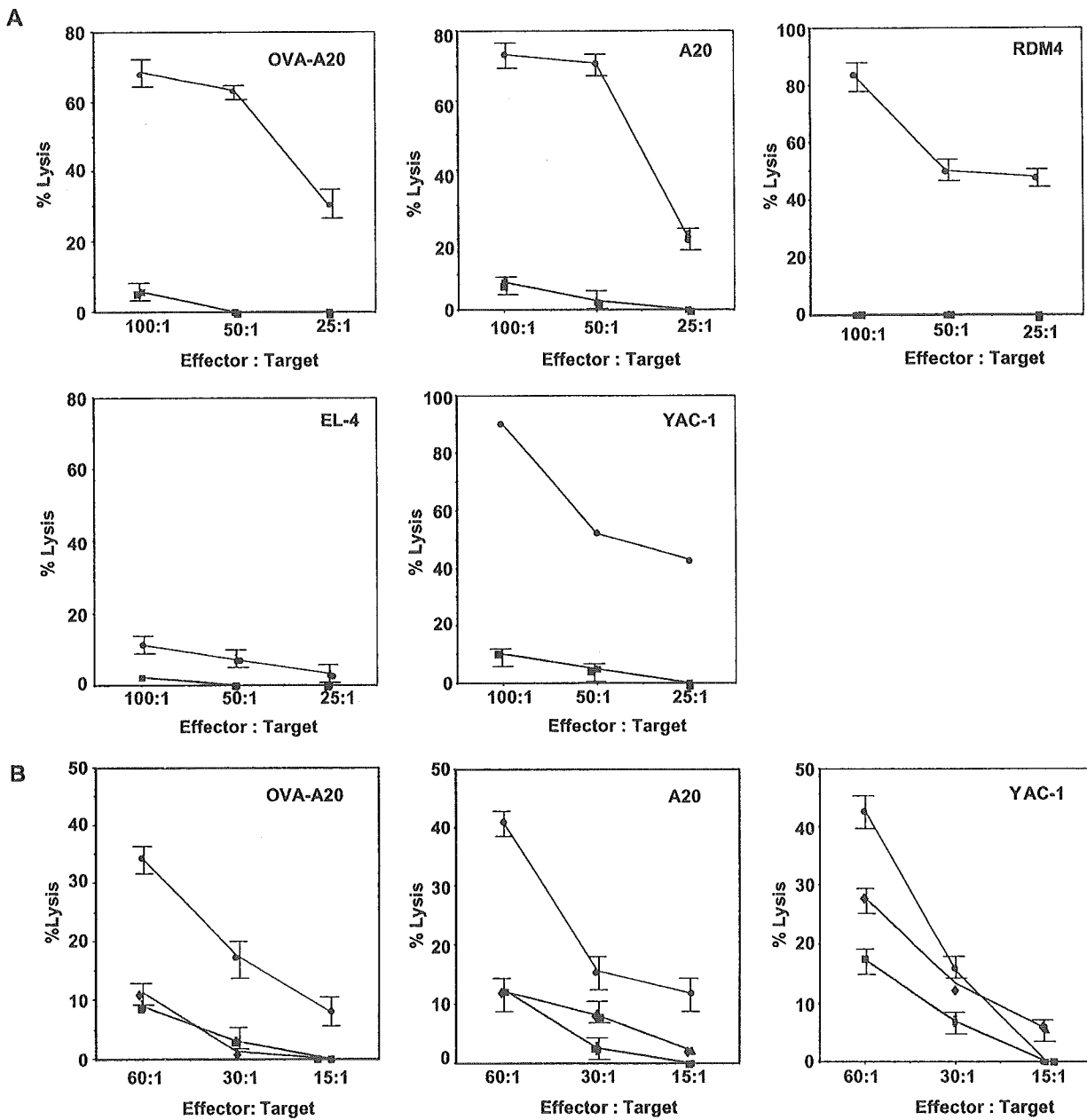


Fig. 4. Oral OVA increases cytotoxic activity of IELs. (A) Cytotoxic activity of gut IEL from TCR-Tg mice fed with OVA ($n = 20$) or casein ($n = 20$) was tested against ^{51}Cr -labeled OVA-A20, A20, YAC-1, RDM4 and EL-4 (closed circles, OVA; closed squares, casein). (B) Cytotoxic activity of CD8^+ -depleted IELs from TCR-Tg mice fed with OVA was measured against OVA-A20, A20 and YAC-1. CD8^+ -depleted (CD8^-) IELs were obtained using antibody-coated Dynabeads as described in Materials and methods (closed circles, OVA; closed squares, casein; closed diamonds, IELs of OVA-fed TCR-Tg mice depleted of CD8^+ cells).

and the results were identical (Fig. 5B and C). We also intraperitoneally (i.p.) injected TCR-Tg mice bearing OVA-20 tumors with OVA soluble antigen every other day for 14 days, assayed cytotoxic activities against OVA-A20, A20 and YAC-1 and measured the tumor mass. The results showed that the cytotoxic activity against OVA-A20 and YCA-1 in spleen cells from TCR-

Tg mice injected i.p. with OVA was enhanced and that the OVA-A20 lymphoma weighed less than that in control TCR-Tg mice injected i.p. with PBS (Fig. 6A). The A20 tumor did not regress in either TCR-Tg mice immunized with OVA or in control TCR-Tg mice (data not shown). However, the spleens from TCR-Tg mice sensitized with OVA were not cytotoxic against the A20

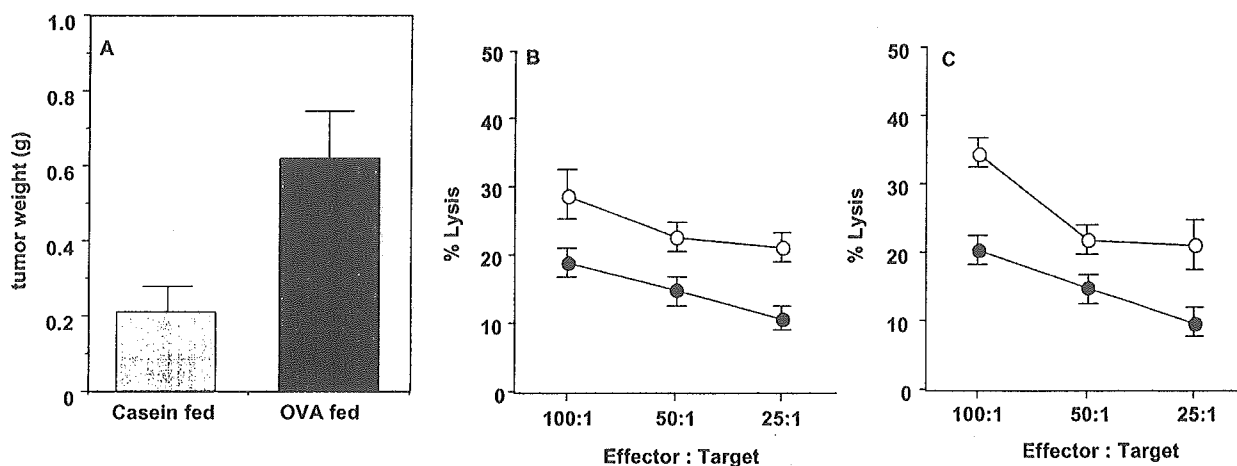


Fig. 5. Enhanced OVA-A20 lymphoma growth and decreased cytotoxic activity in splenic cells from TCR-Tg mice given oral OVA. OVA-A20 lymphoma cells were intradermally inoculated into TCR-Tg mice fed with OVA for 14 days or continuously. (A) Tumors were weighed after 14 days. Cytolytic activities of splenic cells from these TCR-Tg mice against (B) OVA-A20 and (C) YAC-1 were measured as described in Materials and methods. Open and closed circles: spleens from TCR-Tg mice fed with casein ($n = 20$) and OVA ($n = 20$), respectively.

target (Fig. 6B). These findings showed that the cytotoxic activity of spleen cells from TCR-Tg mice sensitized with OVA was antigen-specific (Fig. 6B).

Discussion

A key function of IELs is cytotoxic activity, which might be important as the first immune response to dietary pathogens. However, whether IELs become cytotoxic when orally administered antigens induce systemic tolerance remains unclear. We used TCR-Tg with TCR recognizing an OVA peptide (323–339) in the context of MHC class II (I-A^d) and demonstrated that orally administered OVA induces a split response in T cells among IELs and from the spleen in an antigen-specific manner. Splenic T cells from TCR-Tg mice fed with OVA for 14 days produced less Th1-type cytokines whereas IELs produced more as compared with levels in TCR-Tg mice fed with casein. Activated CD4⁺ cells comprise Th1 and Th2 subsets in the context of cytokine production; Th1 cells produce IFN- γ and IL-2 whereas Th2 cells produce IL-4 and IL-5 (Mosmann and Coffman, 1989). Cytokines are also produced by CD4⁺ IELs. Fresh CD4⁺ IELs isolated from normal mice and maintained in SPF contain Th1 and Th2 cells that secrete cytokines (Fujihashi et al., 1993). However, whether CD4⁺ IELs produce IL-2 and IFN- γ in response to orally administered antigen in TCR-Tg mice with OVA-specific and MHC class II restricted TCR has remained obscure. We demonstrated that CD4⁺ IELs from TCR-Tg mice produced more IL-2 and IFN- γ after feeding with OVA than with casein.

In contrast to the activation of IELs in TCR-Tg fed with OVA, splenic T cells from these mice produced less IL-2 and IFN- γ when cultured with OVA or stimulated on immobilized anti-CD3 antibody. Cytokine production was restored by adding IL-2 to the culture, suggesting that the CD4⁺ cells among splenic T cells can become tolerant to antigens via continuous oral administration. Although the inductive mechanism of systemic immune tolerance by orally administered soluble antigens remains controversial (Weiner et al., 1994; Strobel et al., 1998) some reports suggest that an active suppression mechanism is induced by antigen-specific triggering via suppressive cytokines such as TGF- β , IL-10 and IL-4 (Weiner et al., 1994; Saparov et al., 1997; Strobel et al., 1998). The production of IL-4 by splenic T cells from TCR-Tg mice fed with OVA was increased despite the reduction of Th1-type cytokines (data not shown). These results indicated that IELs are activated in TCR-Tg mice in which Th1 among splenic T cells become hyporesponsive due to the continuous oral administration of OVA. The cytotoxic activities of fresh IELs from TCR-Tg mice fed with OVA were also increased against NK- and LAK-targets but reduced when CD8 α/α ⁺ cells were eliminated. Such cytokines produced by CD4⁺ IELs in mice given OVA can activate cytotoxic activity and might induce non-specific cytotoxicity in CD8⁺ IELs.

IELs are composed of heterogeneous populations that are defined by combinations of various surface markers such TCR- α/β , TCR- γ/δ , CD4, CD8 and NK1.1 (Selby et al., 1981; Parrot et al., 1983; Petit et al., 1985; Mosley et al., 1990; Lefrancois, 1991; Maloy et al., 1991). Most IELs are CD8⁺ cells (approximately 70–80%) (Selby

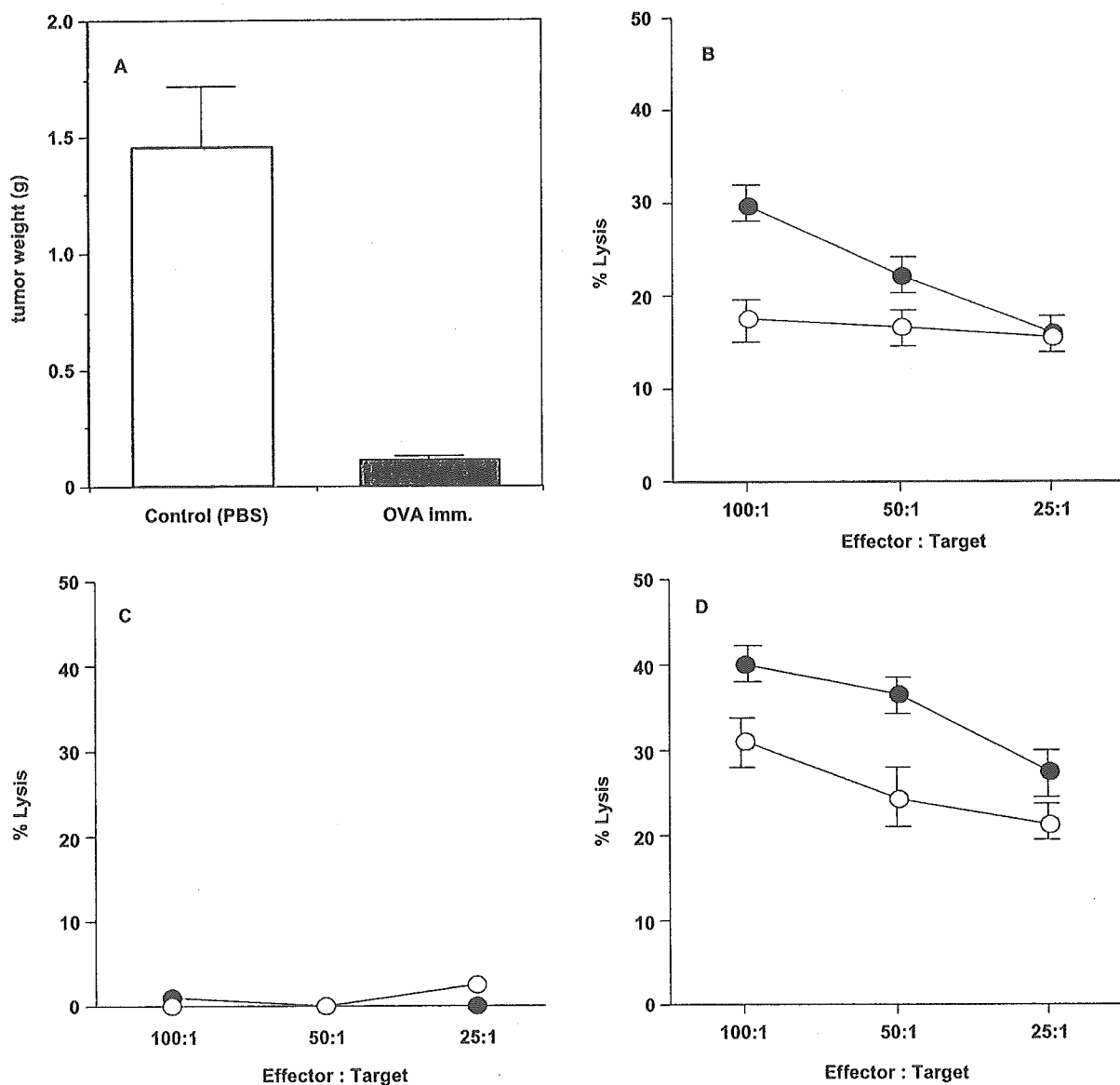


Fig. 6. Induction of OVA-specific cytotoxic activity by OVA-injected i.p. TCR-Tg mice were sensitized (i.p.) with OVA on days 0, 2, 4, 6, 8, 10 and 12, and then intradermally inoculated with OVA-A20 lymphoma cells. The mice were continuously inoculated with OVA for 14 days. (A) OVA-A20 tumors were weighed 14 days after transplantation. Open and dotted bars, weight of transplanted tumors from TCR-Tg mice administered with PBS and with OVA (i.p.), respectively. Cytotoxic activities of spleens from these mice were measured against OVA-A20 target (B), as well as A20 and YAC-1 (C). Open and closed circles: spleens from TCR-Tg mice administered with PBS ($n = 15$) and OVA ($n = 15$), respectively.

et al., 1981; Parrot et al., 1983) and about two-thirds of them are CD8 α/α homodimer-positive (Parrot et al., 1983). However, little is known about how these heterogeneous IELs are induced in vivo as a consequence of antigen exposure in the small intestine when OVA-specific TCR-Tg mice are fed with OVA but not casein. These IELs lysed target cells without antigen specificity (Fig. 4B). Furthermore, CD4⁺ cells possessed antigen specificity to OVA in our TCR-Tg mice (Fig. 1A

and B). Thus, the increased cytotoxic activity of IELs induced by dietary OVA is generated through a bystander effect presumably by cytokines elaborated during the course of an antigen-specific response as speculated by another group (Klein and Kagonoff, 1984). In fact, IELs from TCR-Tg without dietary OVA become cytotoxic when cultured with IL-2 plus IFN- γ (data not shown). In terms of the cytokine effect on the cytotoxic effector capacity of IELs, the function of

CD4⁺ IELs around CD8⁺ IELs should be understood. Since activated CD4⁺ IELs produced Th1-type cytokines (Table 1 and Fig. 2B), we examined the cytotoxic activity of fresh IELs from TCR-Tg mice fed with either OVA or casein against various tumor cells. Fig. 4A shows that IELs from mice given OVA were more cytotoxic against YAC-1 (NK cell target) and RDM4 (LAK cell target) than those from mice given casein. However, cytotoxicity against EL-4, which is resistant to LAK and NK activities, was similarly low in both types of mice. We also examined IEL cytotoxic activities against A20 (I-A^d+ B lymphoma) and the A20 transfectant, OVA-A20, which was established by transfection with the OVA gene. Activity was increased in TCR-Tg mice fed with OVA, but not in casein-fed mice. Considering the nature of the target tumor cells, IELs probably do not lyse targets in an antigen-specific manner like CTL. IEL cytotoxic activities in TCR-Tg mice fed with OVA were reduced when CD8⁺ (Fig. 4B), but not CD4⁺ (data not shown) cells were depleted. As CD8 α / α ⁺ cells predominate (Fig. 3B), they are mainly responsible for the increased cytotoxicity of CD8⁺ IELs. However, CD4⁺ but not CD8 α / α ⁺ cells were specifically activated by dietary OVA in our TCR-Tg mice, suggesting that most of the cytotoxic activity is induced due to a bystander effect of Th1-type cytokines produced from antigen-specific CD4⁺ IELs (Table 1).

Interestingly, oral OVA enhanced the growth of transplanted OVA-A20 tumors in OVA-fed TCR-Tg mice. Several mechanisms have been proposed to explain why the immune system fails to induce an effective anti-tumor response. Tumor cells can escape immune surveillance by down-regulating MHC expression or by the *in vivo* selection of tumor cells that no longer express the target antigen (Chen et al., 1995b; Ferrone and Marincola, 1995; Marincola et al., 1996). In our system, most tumors in OVA-A20-transplanted TCR-Tg mice were regressed by *i.p.* immunization with OVA (Fig. 6A), suggesting that the growth of OVA-A20 tumors after the oral administration of OVA cannot be explained as antigen loss by the tumor cells. We excluded the possibility that the OVA antigen is ignored by the immune system since antigen non-specific effectors were primed after immunization with OVA and anergy was induced on splenic T cells by orally administered OVA (Fig. 3). Tolerance can develop due to ignorance of the antigen (Hermans et al., 1998; Ochsening et al., 1999) or anergy if the antigen is not presented with a sufficient co-stimulatory signal (Mueller et al., 1989; Staveley-O'Carroll et al., 1998). Deletion or suppression of activated cytotoxic lymphocytes (Benacerraf and Germain, 1981; North, 1982; Moskophidis et al., 1993; Groux et al., 1997; Kurts et al., 1997; Bronte et al., 1998) as well as the migration of cytotoxic lymphocytes away from the tumor site can occur (Shrikant and Mescher, 1999). The precursor

frequency of cytotoxic lymphocytes can be too low, causing T cells to lose the competition with the growing tumor, or the tumor cells could produce humoral factors that locally block cytotoxicity (Wojtowicz-Praga, 1997). We excluded the possibility that the OVA antigen is ignored by the immune system since OVA-specific cytotoxic lymphocytes were primed after immunization with OVA protein. Moreover, the cytotoxic lymphocyte response against OVA-A20 after immunization with OVA protein dominates the response to OVA-A20 but does not induce cytotoxic lymphocytes against A20. The A20 tumors did not regress in either OVA-immunized or control TCR-Tg mice (data not shown). The present study did not define which cytokine(s) performed the regulatory function. However, we found that the concentrations of IFN- γ and IL-2 in splenocyte culture supernatants were closely related to the cytotoxic activity of spleen cells against tumor targets and that dietary OVA enhanced the growth of transplanted tumors in TCR-Tg mice. More IFN- γ and IL-2 were produced *in vitro* in splenocyte re-culture supernatants from OVA-*i.p.* immunized TCR-Tg mice than in that from non-immunized TCR-Tg mice (data not shown) and the growth of transplanted tumors was suppressed in OVA-immunized TCR-Tg mice (Fig. 6A). This evidence indicated that tumor growth is closely related to the production of Th1-type cytokines in the periphery. However, the findings were the opposite for IELs in OVA-fed TCR-Tg mice. In casein-fed TCR-Tg mice, the OVA antigen produced from transplanted OVA-A20 activated the peripheral immune system and elicited IFN- γ and IL-2, which subsequently induced the activity of spleen cells and suppressed tumor growth.

A single high dose of antigen induces a state of tolerance characterized by clonal anergy or deletion, whereas multiple low doses induce a state of tolerance characterized by active suppression (Weiner et al., 1994; Chen et al., 1995a). Other groups have noted that oral tolerance is maintained by other regulatory mechanisms and not exclusively through clonal deletion or anergy (Kang et al., 1999). Sakaguchi et al. recently identified CD4⁺CD25⁺ T cells as regulatory cells, which can suppress the immune response to self-antigens and thus maintain peripheral tolerance (Sakaguchi et al., 1995; Shevach, 2000). Furthermore, such subsets of T cells have also been isolated and defined in humans (Dieckmann et al., 2001; Jonuleit et al., 2001; Taylor et al., 2001; Thorstenson and Khoruts, 2001), and they might play a role in the induction and maintenance of oral tolerance (Levings et al., 2001). Haneda et al. (1997) reported that oral tolerance is regulated by TGF- β , whereas others have found that IL-10 is the regulatory cytokine associated with oral tolerance (Gonnella et al., 1998; Adachi et al., 1999). However, the regulatory roles of cytokines in oral tolerance remain controversial. To assume that only one cytokine determines regulatory

function during oral tolerance is naïve, because the inductive events of oral tolerance are highly orchestrated and complex.

In summary, we discovered that the continuous oral administration of OVA to OVA-specific TCR-Tg mice activates CD4⁺ IELs to produce Th1-type cytokines and induces splenic T cells to become antigen-specifically hyporesponsive. Our results also suggested that the cytokines produced from CD4⁺ IELs produce a bystander effect on the non-specific cytotoxic activities of IELs. Because another group demonstrated that the Th1 immune response is highly cytotoxic and eradicated a tumor mass by inducing cellular immunity (Nishimura et al., 1999), the present findings provide an important basis for the development of anti-cancer vaccines and for understanding the pathogenesis of cancer.

Acknowledgments

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