Orally tolerant CD4 T cells respond poorly to antigenic stimulation but strongly to direct stimulation of intracellular signaling pathways

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Abstract

The response of splenic CD4 T cells from ovalbumin (OVA)-specific T cell receptor (TCR) transgenic mice after long-term feeding of a diet containing this antigen was examined. These CD4 T cells exhibited a decreased response to OVA peptide stimulation, in terms of proliferation, interleukin-2 secretion, and CD40 ligand expression, compared to those from mice fed a control diet lacking OVA, demonstrating that oral tolerance of T cells had been induced through oral intake of the antigen. We investigated the intracellular signaling pathways, which were Ca/CN cascade and Ras/MAPK cascade, of these tolerant CD4 T cells using phorbol-12-myristate-13-acetate (PMA) and ionomycin, which are known to directly stimulate these pathways. In contrast to the decreased response to TCR stimulation by OVA peptide, it was shown that the response of splenic CD4 T cells to these reagents in the state of oral tolerance was stronger. These results suggest that splenic CD4 T cells in the state of oral tolerance have an impairment in signaling, in which signals are not transmitted from the TCR to downstream signaling pathways, and have impairments in the vicinity of TCR.

Abbreviations: APC – antigen-presenting cell; CD – cluster of differentiation; CN – calcineurin; ELISA – enzyme-linked immunosorbent assay; FITC – fluorescein isothiocyanate; IL – interleukin; MAPK – mitogen-activated protein kinase; MHC – major histocompatibility complex; MMC – mytomicin C; OVA – ovalbumin; PE – phycoerythrin; PMA – phorbol-12-myristate-13-acetate; TCR – T cell receptor; Tg – transgenic.

Introduction

The immune system responds to foreign substances, but antigens orally ingested as components of food, which is important in terms of energy and nutrition for the living body, can induce a state of systemic immunologic unresponsiveness specific to those antigens, which is termed oral tolerance (Mowat, 1987; Weiner et al., 1994; Weiner, 1994; Kaminogawa, 1996). Recently, this method of oral tolerance induction has been used to suppress autoimmune diseases (Nagler-Anderson et al., 1986; Higgins and Weiner, 1988; Nussenblatt et al., 1990) and allergic disease (Hirahara et al., 1998) in animal models and it is currently being tested for the treatment of autoimmune

disorders, such as multiple sclerosis, rheumatoid arthritis, and autoimmune uveoretinitis in humans (Weiner et al., 1993; Trentham et al., 1993). In addition, basic research on prevention of graft rejection (Sayegh et al., 1992) based on oral tolerance has been initiated recently. However, as the mechanisms of oral tolerance remain to be elucidated, further studies are needed. It is hoped that such studies will lead to the establishment of effective means of disease prevention and treatment.

We have previously reported that continuous feeding of a diet containing OVA to OVA23-3 transgenic (Tg) mice, which express a T cell receptor specific to OVA, induced diminished responsiveness of spleen cells as indicated by reduced cytokine secretion after

Figure 1. Activated phenotype of splenic CD4 T cells from OVA23-3 Tg mice fed egg-white diet. OVA23-3 Tg mice were fed a diet containing 20% egg-white or a control diet for 28 days and splenic CD4 T cells were sorted using MACS and analyzed using FACSsort. (A) Splenic CD4 T cells from mice fed the egg-white diet were found to be larger than those from the mice fed the control diet. FSC is indicative of the size of the splenic CD4 T cells. (B) Increased CD69 molecule expression on splenic CD4 T cells from OVA23-3 Tg mice fed the egg-white diet. Data were gated for CD4 expression to obtain histograms for CD69 molecule expression on splenic CD4 T cells. The percentages of positively stained cells present in each area are shown. The straight line indicates CD69 molecule expression and the dotted line indicates background. The results are representative of five experiments.

OVA stimulation *in vitro*, implying that induction of oral tolerance had occurred in these mice (Shida et al., 2000). TCR Tg mice serve as a powerful model for investigating the complicated immune system (Kisielow et al., 1988; Berg et al., 1989). Since most T cells in these transgenic mice show specific reactivity to a known antigen, it is easy to examine the antigen-reactive function of the T cells to the antigen. CD4 T cells are known to play a major role in oral tolerance induction (Hirahara et al., 1995). Therefore, in the present study, we investigated the response of

antigen-specific CD4 T cells in oral tolerance using TCR-Tg mice as a model system. First, we confirmed that the CD4 T cells in this model display diminished responsiveness to the antigen. Then, this suggested that intracellular signaling from the TCR was impaired in these CD4 T cells. At the present time, it is known that two signal transduction pathways from the TCR, the Ca/calcineurin (CN) cascade and the Ras/mitogen-activated protein kinase (MAPK) cascade (Secrist et al., 1991; Weiss and Littman, 1994; Downward et al., 1990) exist. Using reagents known to directly stimu-

Figure 2. Decreased responses of splenic CD4 T cells from OVA23-3 Tg mice fed the egg-white diet when stimulated with OVA peptide *in vitro*. Splenic CD4 T cells (1×10^5 cells/well) were stimulated with APC plus 5 μ M OVA peptide. (A) Decreased proliferative response and decreased cytokine production by splenic CD4 T cells from OVA23-3 Tg mice fed the egg-white diet. [³H]thymidine (1 μ Ci/well) was added 48 hr after the start of the culture period and incubation was continued for another 24 hr. Mean [³H]thymidine incorporation in each culture is shown with the standard deviation. For measurement of IL-2 cytokine concentrations, the supernatants were collected 24 hr after the start of the culture period and assayed by ELISA. (B) Decreased CD40 ligand molecule expression on splenic CD4 T cells from OVA23-3 Tg mice fed the egg-white diet. The cells were collected 18 hr after the start of the culture period, and analyzed using FACSsort. Data were gated for CD4 expression to obtain histograms for CD40 ligand molecule expression on splenic CD4 T cells. The percentages of positively stained cells present in each area are shown. The straight line indicates CD40 ligand molecule expression and the dotted line indicates the isotype control. The results are representative of four experiments.

Figure 3. Increased proliferative response and increased cytokine production of splenic CD4 T cells from OVA23-3 Tg mice fed the egg-white diet, in response to ionomycin and PMA. Splenic CD4 T cells (1×10^5 cells/well) were stimulated with $2.5 \mu\text{g ml}^{-1}$ ionomycin, 10 ng ml^{-1} PMA or ionomycin plus PMA for 24 hr. Then, $[^3\text{H}]$ thymidine ($1 \mu\text{Ci/well}$) was added and incubation was continued for another 24 hr. Mean $[^3\text{H}]$ thymidine incorporation in each culture is shown with the standard deviation. For measurement of IL-2 cytokine concentrations, the supernatants were collected 24 hr after the start of the culture period and assayed by ELISA. Experiments were repeated three times with similar results.

late these two pathways, we tried to obtain information on T cell signaling of oral tolerance.

Materials and methods

Animals

BALB/c mice were purchased from Clea Inc., Tokyo, Japan. OVA323-339-specific TCR Tg (OVA23-3 Tg) mice with the BALB/c genetic background were produced as described previously (Sato et al., 1994). All

Figure 4. Increased CD40 ligand molecule expression on splenic CD4 T cells from OVA23-3 Tg mice fed the egg- white diet, in response to ionomycin. Splenic CD4 T cells (1×10^5 cells/well) were stimulated with $2.5 \mu\text{g ml}^{-1}$ ionomycin, 10 ng ml^{-1} PMA or ionomycin plus PMA for 18 hr, and thereafter the cells were collected and analyzed using FACSsort. Data were gated for CD4 expression to obtain histograms for CD40 ligand molecule expression on spleen CD4 T cells. The percentages of positively stained cells present in each area are shown. The straight line indicates CD40 ligand molecule expression and the dotted line indicates the isotype control. Experiments were repeated three times with similar results.

mice used in this study were maintained in the animal facilities at The University of Tokyo.

Oral administration of OVA

OVA23-3 Tg mice 8–10 weeks old were fed a diet containing 20% egg-white protein (Funabashi Farms, Funabashi, Japan) or a control diet for 28 days. The daily intake of OVA was approximately 250 mg per mouse.

Cell preparation

Splenic CD4 T cells were purified from OVA23-3 Tg mice using a magnetically activated cell sorter (MACS, Miltenyi Biotec, Bergisch, Gladbach, Germany). The purity was checked by flow-cytometry analysis and the percentage of CD4 T cells in the cell preparation was more than 95%.

Immunofluorescent staining and flow-cytometric analysis

Purified splenic CD4 T cells were stained with phycoerythrin (PE)-conjugated anti-CD69 (PharMingen) and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (PharMingen), and analyzed with FACSsort (Becton Dickinson). The cells were gated for CD4 expression in the analysis, to obtain histograms for expression of the CD69 molecule on CD4 positive T cells.

To examine CD40 ligand molecule expression after stimulation, splenic CD4 T cells were stimulated with mitomycin C (MMC)-treated BALB/c spleen cells as antigen presenting cells (APC) plus 5 μ M OVA 323-339 peptide (Ise et al., 2000), ionomycin (2.5 μ g ml⁻¹), PMA (10 ng ml⁻¹) or ionomycin plus PMA, and at the time of initiation of culture, anti-CD40 ligand-biotin (PharMingen) or anti-hamster IgG-biotin (control antibody, PharMingen) was added. Anti-Fc receptor antibody (2.4G2, PharMingen) was added at 10 μ g ml⁻¹ to all samples to reduce non-specific staining (Roy et al., 1995; Jaiswal et al., 1996). After 18 hr, the cells were collected and suspended in Hanks' Balanced Salt Solution (HBSS, Gibco BRL) supplemented with 1% FCS and 0.1% sodium azide, then the cells were stained with FITC-conjugated anti-CD4 followed by RED-670-streptavidin (Gibco BRL), and analysis was performed using FACSsort. The cells were gated for CD4 expression in the analysis, to obtain histograms for expression of CD40 ligand on

CD4 positive T cells. CELLQuest software (Becton Dickinson) was used for collecting and analyzing data.

Proliferation assay

Purified splenic CD4 T cells (1×10^5 /well) were cultured in 200 μ l cultures with 5 μ M of OVA peptide plus APC for 2 days, or with ionomycin (2.5 μ g ml⁻¹), PMA (10 ng ml⁻¹) or ionomycin plus PMA for 24 hr. Then, [³H]thymidine (1 μ Ci/well) was added and the cultures were incubated for another 24 hr. Thereafter, the cells were collected on a glass fiber filter and the amount of incorporated radioactivity was measured by means of a scintillation counter.

Measurement of IL-2 production by ELISA

Splenic CD4 T cells (1×10^5 /well) were cultured with 5 μ M OVA peptide plus APC, ionomycin (2.5 μ g ml⁻¹), PMA (10 ng ml⁻¹) or ionomycin plus PMA and supernatants were collected after 24 hr. The wells of a 96-well plate were coated with purified first antibody (anti-mouse IL-2 monoclonal antibody, clone: JES6-2A12, PharMingen) at 4 °C overnight and then blocked with PBS-containing 3% BSA at room temperature for 2 hr. After overnight incubation with samples at 4 °C, the wells were washed and then biotinylated-second antibody (anti-mouse IL-2 monoclonal antibody, clone: JES6-5H4, PharMingen) was added and the plates were incubated at room temperature for 1 hr. The wells were washed and alkaline phosphatase-conjugated streptavidin (Zymed, San Francisco, CA, U.S.A.) and its substrate, disodium 4-nitrophenylphosphate (Tokyo Kasei Kogyo, Tokyo, Japan), were added for color development. The absorbance was measured at 405 nm using a spectrophotometer. Results were analyzed by means of the Microplate Manager system (Bio-Rad Laboratories, Hercules, CA).

Results and discussion

In this study, we examined the characteristics of T cells in TCR transgenic mice fed an egg-white diet containing the antigen OVA for 28 days. Most of the T cells in these transgenic mice show specific reactivity to OVA, therefore it is possible to examine the antigen-reactive function of the T cells. Splenic CD4 T cells were isolated from the mice by MACS and analyzed. First we investigated the size of the splenic CD4 T cells by flow-cytometry by measuring FSC (forward

scattered light), which is proportional to the surface area or the size of the cell (Figure 1A). Comparing the splenic CD4 T cells from the mice in the control diet group with those from the mice in the egg-white diet group, the CD4 T cells in the mice of the latter group were larger and they resembled activated cells (Figure 1A).

Next, we also investigated the expression of the CD69 molecule, a marker of T cell activation on the T cell surface, by flow-cytometry. Splenic CD4 T cells from the mice in the egg-white diet group showed a higher level of expression of the CD69 molecule as compared to those from the mice in the control diet group (Figure 1B). These findings suggested that splenic CD4 T cells from the OVA23-3 Tg mice fed this antigen were in an activated state, when CD4 T cells sensitized antigen in through the TCR *in vivo* by oral administration of antigen.

Nevertheless, we examined the possibility that these CD4 T cells responded poorly to restimulation by antigen. The splenic CD4 T cells were stimulated with OVA peptide (5 μ M), an antigen known to be specifically recognized by T cells in OVA23-3 Tg mice, and their response in terms of cell proliferation and IL-2 secretion was investigated (Figure 2). As compared with splenic CD4 T cells from mice fed the control diet, the proliferative response and the IL-2 cytokine production displayed by splenic CD4 T cells from the mice in the egg-white diet group were diminished. These findings showed that a hyporesponsiveness state, oral tolerance, was induced in T cells of OVA23-3 Tg mice by feeding the egg-white diet (Figure 2A). We consider that stimulation from the TCR was necessary for the induction of oral tolerance. Then the CD4 T cells acquire an activated phenotype, but afterwards they become hyporesponsive to antigenic stimulation.

Furthermore, by flow-cytometry, we investigated the expression of the CD40 ligand molecule on the T cell surface, which is known to be induced by antigen stimulation (Castle et al., 1993; Roy et al., 1993). This molecule binds to the CD40 molecule expressed on APCs and B cells, and mediates important functions of CD4 T cells (Banchereau et al., 1994). As in the case of the proliferative response and IL-2 cytokine production, CD40 ligand molecule expression was found to be reduced in splenic CD4 T cells from mice fed the egg-white-diet (Figure 2B). This is the first report to demonstrate that CD40 ligand molecule expression is reduced in T cells in the state of oral tolerance.

The results in Figures 1 and 2 show that the splenic CD4 T cells induced by ingested antigen were activated and had increased in size, but the response of these CD4 T cells to specific antigen was decreased and oral tolerance had been induced. We wanted to address the question: how do the splenic CD4 T cells of OVA-fed mice become hyporesponsive to stimulation by specific antigen? Therefore, we examined T cell signal transduction from the TCR in oral tolerance, because there is no information available to date on this aspect. Two major signal transduction pathways from the TCR, the Ca/CN pathway and the Ras/MAPK pathway, are known to exist (Secrist et al., 1991; Weiss et al., 1994; Downward et al., 1990). We investigated these intracellular signaling pathways using two reagents. It is known that ionomycin, a kind of calcium ionophore, activates the Ca/CN pathway (Tsien et al., 1982), and PMA activates PKC (protein kinase C) which acts upstream of the Ras/MAPK pathway (Izquierdo et al., 1992). As these reagents directly activate the intracellular signaling pathways, we used them to obtain information on the status of the T cell signaling pathways in these tolerant T cells. It was important to observe only the response of antigen-specific T cells in the analysis, which are not possible using conventional mice. In the present study, TCR-Tg mice were used, in which most of the T cells were antigen-specific, i.e. specifically reactive to the antigen OVA.

First, the response of splenic CD4 T cells stimulated with ionomycin or PMA was investigated in a proliferation assay and a IL-2 cytokine production assay (Figure 3). The CD4 T cells from the control mice did not proliferate in response to stimulation with ionomycin. Proliferation was induced by stimulation with PMA, and the combination of ionomycin and PMA induced substantial proliferation. IL-2 cytokine production was not detected in CD4 T cells stimulated with ionomycin or PMA independently, but intense IL-2 cytokine production was observed when the cells were stimulated with both reagents (Figure 3). This finding is consistent with reports indicating that both the Ca/CN pathway and the Ras/MAPK pathway are required for IL-2 transcription and secretion (Crabtree, 1989; Karin, 1995). The responses to these reagents that stimulate intracellular signaling pathways were significantly increased in splenic CD4 T cells in the state of oral tolerance induced by the egg-white diet (Figure 3). The CD4 T cells from mice fed the egg-white diet proliferated strongly in response to ionomycin, PMA, or the combination of both. Also, these

cells secreted a substantial amount of IL-2 in response to ionomycin plus PMA.

Next, the expression of the CD40 ligand molecule was investigated in splenic CD4 T cells stimulated with ionomycin or PMA (Figure 4). The splenic CD4 T cells showed elevated CD40 ligand molecule expression following ionomycin stimulation but not after PMA stimulation. These findings show that activation of the Ca/CN pathway was sufficient to induce CD40 ligand molecule expression, whereas activation of the Ras/MAPK pathway was not, which is consistent with previous reports (Nusslein et al., 1996). As shown in Figure 4, splenic CD4 T cells from mice fed the egg-white diet expressed the CD40 ligand molecule more strongly than those from mice fed the control diet. These results show that the T cell response to reagents which stimulate the Ca/CN pathway or the Ras/MAPK pathway is stronger in the case of T cells from OVA-fed mice compared to the controls.

Engagement of the T cell antigen receptor (TCR) by specific antigen-major histocompatibility complex (MHC) complexes results in activation of multiple signal transduction molecules, which in turn activate biochemical pathways such as the Ca/CN pathway and the Ras/MAPK pathway, finally leading to cytokine production and proliferation (Weiss et al., 1994; Wange and Samelson, 1996; Chan and Shaw, 1996). Many important signaling molecules are present in the vicinity of TCR, such as LAT, ZAP-70 and PLC γ -1 (Kane et al., 2000; Myung et al., 2000), which connect TCR stimulation with the Ca/CN and Ras/MAPK pathways. In the present study, it was shown that the response of tolerant splenic CD4 T cells to OVA peptide stimulation or stimulation by reagents that directly activate intracellular signaling pathways was different. The response to OVA peptide stimulation from the TCR was significantly reduced, whereas the responses to stimulation by these reagents which increase Ca²⁺ concentration and activate PKC were not reduced. These results strongly suggest that CD4 T cells in the state of oral tolerance have an impairment in proximal signaling from the TCR, i.e. stimulation signals are not transmitted from the TCR to components downstream in the signaling pathway. However, we were unable to clarify why the orally tolerant CD4 T cells responded more strongly to stimulation by these reagents.

The results in Figure 2 show that CD40 ligand molecule expression, which can be induced by the Ca/CN pathway alone, and IL-2 secretion, which requires activation of both the Ca/CN pathway and the Ras/MAPK pathway, are completely abolished in

T cells in the state of oral tolerance, whereas proliferation, which can partially be induced by activation of the Ras/MAPK pathway, is only partly inhibited. These results suggest the possibility that the Ca/CN pathway is impaired in CD4 T cells in the state of oral tolerance. Thus, signaling molecules acting upstream of the Ca/CN pathway may be impaired.

In this article, we demonstrated that the response of orally tolerant CD4 T cells to signaling through TCR was completely different from that to intracellular signaling using reagents. The response of splenic CD4 T cells to TCR stimulation by OVA peptide decreased, whereas the response of splenic CD4 T cells to ionomycin and PMA are stronger. These results suggest that splenic CD4 T cells in the state of oral tolerance have a defect in proximal signaling from the TCR. Oral tolerance is a very important system for maintaining our bodies. Recently, induction of oral tolerance has been applied to the treatment of autoimmune disease and allergy (Weiner et al., 1993; Trentham et al., 1993; Taudorf, 1992). However, the mechanism of oral tolerance induction has not been fully elucidated, especially, at the molecular level. We are currently extending our T cell signaling studies in an effort to further clarify the mechanisms of oral tolerance.

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