Oral administration of antigen does not influence the proliferation and IFN- γ production of responsive CD8⁺ T cells but enables to establish T cell clones with different lymphokine production profile.

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Abstract

Feeding of a whole casein diet, which abolished the α_{s1} -casein-specific proliferation and IFN- γ productivity of CD4⁺ T cells, did not affect the proliferative response of CD8⁺ T cells with regard to the antigen dose response, cell dose response, kinetics of the proliferation and epitope specificity, as well as IFN- γ production. To assess the characteristics of the CD8⁺ T cells, we established α_{s1} -casein-specific CD8⁺ T cell clones from both casein-fed and control mice. The established clones produced different amount of IFN- γ and IL-10, and one clone derived from the casein-fed mice produced a remarkable amount of IL-10. The clones from casein-fed mice produced considerable amounts of TGF- β , while those from control mice produced only small amounts. The possible role of CD8⁺ T cells in oral tolerance is discussed.

Abbreviations: APC – antigen presenting cell; bm1 – B6.C-H-2^{bm1}; bm3 – B6.C-H-2^{bm3}; 4R – B10.A(4R); CFA – complete Freund's adjuvant; CN – casein; dThd – thymidine; ELISA – enzyme-linked immunosorbent assay; LN – lymph node; MBP – myelin basic protein; t- – trypsin-digested-; TcR – T cell receptor; Th1 – type 1 helper T cells; Th2 – type 2 helper T cells.

Introduction

Most CD8⁺ T cells recognize endogenous antigens in the context of MHC class I, and are involved in the protection against viruses and in the graft-versus-host reaction. In addition, recent findings have revealed that some CD8⁺ T cells are specific to exogenous soluble antigens (Hisatsune *et al.*, 1995; Jensen *et al.*, 1984; Krzych *et al.*, 1985; McMenemy and Holt, 1993; Lipford *et al.*, 1993), indicating that these cells have more importance in immune regulation than previously expected. Furthermore, the contributions of the CD8⁺ T cell population to oral tolerance, in which oral administration of exogenous antigen induces a profound and

long-lasting unresponsive state in both T and B cell populations (Weiner *et al.*, 1994; Enomoto *et al.*, 1993; Kim *et al.*, 1993; Hachimura *et al.*, 1994; Hirahara *et al.*, 1995; Melamed and Freidman, 1993), have been reported: transfer of CD8⁺ T cells from orally tolerated mice has been shown to induce immunological tolerance (Weiner *et al.*, 1994; Richman *et al.*, 1981) and TGF- β produced by CD8⁺ T cells is responsible for oral tolerance in the rat system (Lider *et al.*, 1989; Miller *et al.*, 1991; Miller *et al.*, 1992).

Therefore, it is important to investigate the influence of oral administration of antigens on the immunological features of CD8⁺ T cells, especially to determine whether antigen-specific responsiveness of CD8⁺ T cells is reduced by oral administration of antigen or not. However, the lack of a primary culture system to directly assess their responsiveness in compari-

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son with the regulation of the CD4⁺ T cell population has hindered investigation of this issue.

In addition to a primary culture system, isolation of a clonal population is essential for more precise analysis of their function and antigen recognition. Establishment of CD8⁺ T cell clones from orally tolerized mice, which has not been done because of difficulty in establishing T cell clones from antigen-specific CD8⁺ T cells occurring only at a low frequency, will allow us to elucidate the difference in characteristics of each clone in relation to their function and regulation by antigen.

We have previously reported assessing the responses of CD8⁺ T cells in a primary condition (Hisatsune *et al.*, 1995). Purified CD8⁺ T cells proliferated in response to α_{s1} -casein (α_{s1} -CN) without the help of CD4⁺ T cells. In the present study, we assessed the effects of orally administered CN on the responses of primary CD8⁺ T cells. In addition, we succeeded in establishing CD8⁺ T cell clones and analyzed their features.

Materials and methods

Mice

Female C57BL/6 mice were purchased from Charles River Japan, Tokyo, Japan. B10.A (4R), B6.C-H-2^{bm1} (bm1) and B6.C-H-2^{bm3} (bm3) mice were kindly provided by Dr. T. Tada and Dr. Y. Asano, University of Tokyo, Tokyo, Japan.

Ags and mAbs

Bovine α_{s1} -CN was prepared from fresh raw skim milk, and was purified by anion-exchange chromatography (Hisatsune *et al.*, 1995). Purified α_{s1} -CN was digested with trypsin (Sigma, St. Louis, MO) as previously described (Hisatsune *et al.*, 1995). Synthetic peptides of α_{s1} -CN were synthesized with a peptide synthesizer (430A; Applied Biosystems, Foster City, California), and purified by HPLC (Enomoto *et al.*, 1990; Shon *et al.*, 1991). Anti-IL-10 mAb SXC-1 (rat IgM (Mosmann *et al.*, 1990)) was kindly provided by Dr. K. W. Moore (DNAX, Palo Alto, CA). Anti-IL-10 mAb 2A5 (rat IgG1 (Abrams *et al.*, 1992)) and anti-IFN- γ mAb XMGI.2 (rat IgG1 (Cherwinski *et al.*, 1987)) were kindly provided by Dr. J. S. Abrams (DNAX, Palo Alto, CA). Anti-IFN- γ mAb R4-6A2 (rat IgG1 (Spitalny and Havell, 1984)) was obtained from the American

Type Culture Collection (Rockville, MD). Anti-CD8 mAbs 83-12-5 (Leo *et al.*, 1987a), 53-6-72 (Ledbetter and Herzenberg, 1979), anti-CD4 mAb GK1.5 (Dalynas *et al.*, 1983), anti-CD3 mAb 145-2C11 (Leo *et al.*, 1987b), anti-TcR β chain mAb H57-597 (Kubo *et al.*, 1989), anti-D^b mAb 28-14-8 (Ozato and Sachs, 1981), anti-K^b mAb 28-13-3 (Ozato and Sachs, 1981) and anti-A^b mAb M5/114 (Bhattacharya *et al.*, 1981) were kindly provided by Dr. T. Tada, University of Tokyo, Tokyo, Japan. Anti-V β 2 mAb B20.6 (Gregorie *et al.*, 1991), anti-V β 3 mAb KJ25 (Pullen *et al.*, 1988) and anti-V β 11 mAb RR3-15 (Bill *et al.*, 1989) were kind gifts of Dr. Y. Itoh, Hokkaido University, Sapporo, Japan.

Oral administration of CN

C57BL/6 mice were maintained by feeding a diet containing bovine whole CN as the only protein source for 14 days. The T cell proliferative responses to α_{s1} -CN in these mice were completely reduced (Enomoto *et al.*, 1993; Kim *et al.*, 1993; Hachimura *et al.*, 1994; Hirahara *et al.*, 1995; and this paper). The control mice were fed a commercially available diet (MF, Oriental yeast, Tokyo, Japan) that did not contain any milk component.

Preparation of antigen-primed CD8⁺ T cells

C57BL/6 mice were immunized s.c. at the base of the tails and in the hind footpads with 50 μ g of α_{s1} -CN in complete Freund's adjuvant (CFA; H37Ra, Difco, Detroit, Michigan). Ten days after the immunization, the inguinal and popliteal lymph node (LN) cells were isolated as a single cell suspension as previously described (Hisatsune *et al.*, 1995). CD8⁺ T cells were purified with magnetic beads (Dynal, Oslo, Norway), which were coupled to anti-CD8 mAb 83-12-5. The purity of the resulting population was more than 98% and the extent of contamination by CD4⁺ T cells was less than 0.5% (Hisatsune *et al.*, 1995; and data not shown). The CD4⁺ T cells were purified similarly by means of anti-CD4 (GK1.5)-coupled magnetic beads. The purity of the CD4⁺ T cells was 96%, and less than 0.1% of this population was CD8⁺ T cells.

Assessment of the proliferation of T cells

The proliferative response was assessed by the method previously described (Hisatsune *et al.*, 1995; Hisatsune *et al.*, 1990). Briefly, T cells (4×10^5 purified

primary CD8⁺ T cells or 2×10^5 purified CD4⁺ T cells) were stimulated with 2×10^5 3 Gy-irradiated syngeneic spleen cells (used as APC) together with the antigen in 200 μ l of medium in microtiter plates. The cells were cultured for 4 days and pulsed with 18.5 kBq of ³H-dThd (248 GBq/mmol; New England Nuclear, Boston, MA) during the last 20 h. The results are expressed as the arithmetic mean of the cpm with SD calculated from triplicate cultures. All experiments were performed at least two times to assess the reproducibility.

Assessment of the quantity of lymphokines in the supernatant by ELISA

The cells were cultured in 250 μ l of medium, and 50 μ l of the medium was collected 22 h after culture initiation to assess the amount of lymphokines. To assess the lymphokine production pattern of T cell clones, 2×10^6 T cells of each clone were stimulated with 1×10^6 APC in 1 ml of medium in 48 well plates. The quantity of IFN- γ and IL-10 in the culture supernatant was assessed by two-site ELISA as previously reported (Abrams *et al.*, 1992). The quantity of IL-2 activity in the culture supernatants was measured by a bioassay using IL-2-dependent line CTLL-2. TGF- β was measured by the TGF- β_1 ELISA System (Promega, Madison, WI), which detects biologically active form of TGF- β_1 . Bioactivity of produced TGF- β_1 was confirmed by the bioassay using MV 1 Lu cells (Danielpour *et al.*, 1989).

Establishment of α_{s1} -CN-specific CD8⁺ T cell clones

After oral administration of CN, C57BL/6 mice were immunized with α_{s1} -CN in CFA and CD8⁺ T cells were purified from LN with anti-CD8 coupled magnetic beads. The purified CD8⁺ T cells were stimulated every seven days with APC and antigen (1 μ M of trypsin-digested α_{s1} -CN) in the presence of 50 U/ml of rIL-2 (presented by Takeda Chemical Industries LTD., Osaka, Japan) or 5% T cell growth factor (supernatant of rat spleen cells cultured with Con A). The cells were cloned by limiting dilution. These clones and a CD4⁺ Th1 clone 3D20 (Hisatsune *et al.*, 1992a), which recognized α_{s1} -CN in the context of A^b, were maintained under culture conditions described previously (Hisatsune *et al.*, 1990; Hisatsune *et al.*, 1994). To obtain CD8⁺ T cell clones from non-tolerized mice, the mice fed the control diet were immunized with

α_{s1} -CN and CD8⁺ T cell clones were established in a similar manner from antigen-primed LN.

Results

The proliferative response of the CD8⁺ T cell population was not reduced by oral administration of antigen

We studied the effect of oral administration of antigen on the peripheral T cell responses using draining LN cells. Administration of a whole CN-containing diet to mice for 2 weeks strongly reduced the proliferative response of α_{s1} -CN-primed LN cells (data not shown). The proliferative response induced by intact- or trypsinized- α_{s1} -CN was reduced to a similar extent in each instance. IFN- γ production by these cells was also strongly reduced (not shown). Since the proliferative response of α_{s1} -CN-primed LN cells was effectively blocked by anti-CD4 antibody (data not shown), the response of purified CD4⁺ T cells was examined directly. CD4⁺ T cells highly purified by means of magnetic beads exhibited a vigorous proliferative response to antigenic stimulation, and this response was completely abrogated by the oral administration of CN (Fig. 1A). These results indicate that the CD4⁺ T cell population was not responsive after oral administration of antigen for 2 weeks.

We have previously established an assay to assess the α_{s1} -CN-specific proliferative response of CD8⁺ T cells in a primary condition (Hisatsune *et al.*, 1995). In the present study, we investigated whether the proliferative response of CD8⁺ T cells was reduced by feeding CN. As shown in Fig. 1B, CD8⁺ T cells purified from antigen-primed LN cells exhibited proliferation in response to trypsin-digested α_{s1} -CN in a dose dependent manner. The responses of CD8⁺ T cells from mice fed the whole CN-containing diet and those fed the control diet were similar. Figure 2 further confirmed that the magnitude of α_{s1} -CN-specific proliferation of CD8⁺ T cells was similar in both groups as revealed by cell dose response (A) or time course (B). These results are the first evidences to indicate that the proliferative responsiveness of CD8⁺ T cells is not reduced by oral administration of antigen.

(A) CD4⁺ T cells **(B) CD8⁺ T cells**

Figure 1. The proliferative responsiveness of CD8⁺ T cells was not reduced by oral administration of CN, unlike that of CD4⁺ T cells. (A) Highly purified CD4⁺ T cells (2×10^5) were stimulated in the presence of APC (2×10^5) with graded doses of α_{s1} -CN. (B) The proliferation of highly purified CD8⁺ T cells (4×10^5) was examined similarly as shown in (A) except that trypsinized α_{s1} -CN was substituted for α_{s1} -CN.

CD8⁺ T cells retained the productivity of IFN- γ after oral administration of antigen

We examined whether IFN- γ production by CD8⁺ T cells is reduced by oral administration of antigen. As shown in Fig. 3, CD8⁺ T cells from mice fed the CN-

containing diet and those fed the control diet produced similar amounts of IFN- γ in a similar time course. This result indicates that the α_{s1} -CN-specific CD8⁺ T cell population could produce IFN- γ even after oral administration of CN. Neither IL-2 nor IL-10 was detectable in assays of CD8⁺ T cells from either group of mice

(A) Cell dose response **(B) Time course of the proliferation**

Figure 2. The proliferative response of CD8⁺ T cells was not reduced by oral administration of antigen. (A) Graded doses of highly purified CD8⁺ T cells were stimulated with APC and t-CN (25 μ M), and ³H-thymidine uptake of last 20 h of 4-day culture was assessed. (B) Highly purified CD8⁺ T cells (4×10^5) were stimulated with APC and t-CN (25 μ M) and cultured for indicated period and ³H-thymidine uptake of last 20 h was measured.

under the conditions employed (data not shown). The level of production of TGF- β by CD8⁺ T cells from the control group was 107 pg/ml and the level was reduced to 75 pg/ml following oral administration of CN, how-

ever, an antigen-specific response was not detectable in either group.

Figure 3. CD8⁺ T cells from CN-fed mice produced similar levels of IFN- γ to those from control mice. Purified CD8⁺ T cells (3×10^6) were stimulated with APC (1.5×10^6) and 15 μ M of trypsinized α_{s1} -CN in 1 ml of medium in 48 well plate. After indicated period of culture, the supernatant was harvested and the amount of IFN- γ was assessed.

Oral administration of antigen did not affect the antigenic determinant recognized by the CD8⁺ T cell population

The antigenic determinant recognized by α_{s1} -CN-specific CD8⁺ T cells from orally tolerized mice was examined next. CD8⁺ T cells from control mice proliferated in response to region 136–155 only (Fig. 4A), confirming our previous results (Hisatsune *et al.*, 1995). The antigenic determinant recognized by CD8⁺ T cells from CN-fed mice was similar (Fig. 4B). Thus oral administration of CN as a component of the diet did not affect the determinant specificity of the CD8⁺ T cell population.

Establishment of α_{s1} -CN-specific CD8⁺ T cell clones from mice fed CN

To further characterize the CD8⁺ T cells specific to α_{s1} -CN in CN-fed mice, we established T cell clones. By the limiting dilution method, two T cell clones specific to trypsin-digested α_{s1} -CN, 6B1 and 5F1, were established from α_{s1} -CN-primed LN cells of C57BL/6 mice, which had been fed whole CN. Microfluorometric analysis revealed that these two clones expressed similar levels of CD8, CD3, TcR β chain, D^b and K^b molecules but not CD4, A^b (not shown). 6B1 and 5F1 were stained with V β 2 and V β 11, respectively.

Both clones exhibited significant IFN- γ production in response to stimulation with trypsinized α_{s1} -CN in the presence of APC (see below). This is the first report of the establishment of antigen-specific CD8⁺ T cell clones from orally tolerized mice.

To compare the characteristics of CD8⁺ T cells from CN-fed mice and control mice, we established T cell clones from control C57BL/6 mice in a similar manner. Two clones were established, Uc and Ud, and both clones expressed CD8, CD3, TcR β chain, K^b and D^b (not shown). The expression levels in each instance were similar to those of 6B1 or 5F1. The cells were stained with neither anti-CD4 nor A^b antibody. It was also demonstrated that both clones expressed TcR V β 3, unlike 6B1 or 5F1. Both Uc and Ud produced IFN- γ when stimulated with trypsinized α_{s1} -CN plus APC.

All clones recognized the region 142–149 of α_{s1} -CN in the context of K^b molecules but their specificity of antigen recognition differed slightly

The MHC restriction of the clones was determined. Monoclonal antibody specific to the A^b molecule did not affect the production of IFN- γ by these clones while that of the A^b-restricted CD4⁺ Th1 clone 3D20 was completely inhibited, indicating that all of the established CD8⁺ T cell clones recognize α_{s1} -CN pre-

(A) Control group **(B) Tolerant group**

Figure 4. Determinant specificity of CD8⁺ T cells from CN-fed mice was similar to that of cells from control mice. α_{s1} -CN-primed CD8⁺ T cells (4×10^5) were stimulated with APC and 13 synthetic overlapping peptides of α_{s1} -CN at a concentration of 5 μ M. Indicated numbers correspond to the α_{s1} -CN sequence.

sented by class I molecules (data not shown). The responsiveness of these clones was studied further using congenic mice. As shown in Table 1, all of the clones recognized trypsin-digested α_{s1} -CN presented by APC from C57BL/6 mice but not APC from 4R

mice that express D^b but not K^b. From the results, it was concluded that these CD8⁺ T cell clones recognize α_{s1} -CN presented by K^b. To assess the specificity of antigen recognition of these clones further, their responsiveness in the presence of APC from bml

Table 1. Restriction mapping using congenic mice^a

APC		IFN- γ production (U/ml) from							
Strain	MHC	6B1		5F1		Uc		Ud	
	KAED	-Ag	+Ag	-Ag	+Ag	-Ag	+Ag	-Ag	+Ag
B6	bbbb	<8	64 $\pm$ 3	<8	24 $\pm$ 1	<8	40 $\pm$ 1	<8	72 $\pm$ 2
4R	kkbb	<8	<8	<8	<8	<8	<8	<8	<8
bm1	b ^b bbb	<8	36 $\pm$ 3	<8	<8	<8	<8	<8	<8
bm3	b ^b bbb	<8	140 $\pm$ 4	<8	60 $\pm$ 2	<8	<8	<8	<8
C3H	kkkk	<8	<8	<8	<8	<8	<8	<8	<8

^a Antigen-pulsed spleen cells from each strain of mice were used as APC. T cells (2×10^4) were stimulated with trypsinized- α_{s1} -CN-pulsed APC (8×10^5) for 24 h and IFN- γ was assessed by ELISA.

^b bm1 and bm3 expressed mutated K^b molecules.

and bm3 mice was examined. The K^b molecules of bm1 and bm3 mutant mice have three point mutations (position 152, 155 and 156) and two mutations (position 77 and 89), respectively, which are located inside the groove of K^b molecule involved in binding peptide antigens (Nathenson *et al.*, 1986). As shown in Table 1, none of the clones produced IFN- γ in the absence of trypsin-digested α_{s1} -CN, indicating the complete lack of alloreactivity under the experimental conditions employed. Uc and Ud did not produce IFN- γ in response to trypsinized α_{s1} -CN presented by either type of mutated K^b. On the other hand, when bm3 mice were used as the source of APC, IFN- γ production by clones 6B1 and 5F1 was even higher than that observed when APC from C57BL/6 mice were used. Only 6B1 exhibited weak IFN- γ production in response to trypsinized α_{s1} -CN presented by APC from bm1. From these results, it was concluded that the specificity of antigen recognition of each clone was slightly different.

To determine the epitopes of the CD8⁺ T cell clones, the stimulating activity of a fragment consisting of the region 136–155 of α_{s1} -CN, the only determinant of the primary response, was examined. This peptide induced IFN- γ production by all four of the established clones (Table 2), indicating that the epitope recognized by these clones was contained in this region. To localize the antigenic determinant precisely, we compared this region with the motif sequence of K^b binding peptides postulated by Falk *et al.* (1991). The region 142–149 (Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu) was in complete accordance with the proposed motif sequence (X-X-Tyr-X-**Phe/Tyr**-X-X-**Leu**/Met, in which bold letters indicate anchor residues), thus we next examined the antigenicity of a synthetic peptide corresponding to region 142–149. Fragment 142–149

induced a strong response of IFN- γ production by all four clones (Table 2). These results indicate that all of the clones recognize the region 142–149 of α_{s1} -CN in the context of the K^b molecules. Trypsin-digestion of α_{s1} -CN generates peptide fragment 133–151 (Ametani *et al.*, 1988), which probably stimulated the clones in culture.

CD8⁺ T cell clones from CN-fed mice exhibited a lymphokine production pattern different from that of CD8⁺ T cells from control mice

The lymphokine production patterns of the clones were examined by stimulating the T cells with trypsinized α_{s1} -CN. All of the clones produced a large amount of IFN- γ comparable to that obtained with typical CD4⁺ T cell clones (Hisatsune *et al.*, 1994; Fong and Mosmann, 1990; Nishijima *et al.*, 1994) on antigenic stimulation (Table 3). The amount of IFN- γ produced by 6B1 was greater than that of 5F1 or Uc, while that of Ud was in the intermediate range. An abundant amount of IL-10 was produced by 5F1, while the other clones produced only small amounts. The culture supernatant of the clones did not induce the proliferation of an IL-2-dependent cell line, CTLL-2, indicating that IL-2 was not present.

Next, the amount of biologically active form of TGF- β was assessed by ELISA. Table 4 shows that 6B1 and 5F1 produced TGF- β in the absence of serum. On the other hand, Uc and Ud produced no detectable TGF- β under this condition. However, Uc was capable of TGF- β production, since serum induced the production of TGF- β by this clone. Furthermore, 5F1 and 6B1 produced much larger amounts of TGF- β when compared with Uc and Ud in the presence of serum. The supernatants of 5F1, 6B1 or Uc significantly inhib-

Table 2. Epitope mapping of α_{s1} -casein specific CD8⁺ T cell clones^a

Antigen	IFN- γ production (U/ml) from			
	6B1	5F1	Uc	Ud
none	<8	<8	<8	<8
t-CN	76 $\pm$ 1	76 $\pm$ 2	260 $\pm$ 20	56 $\pm$ 8
136–155	36 $\pm$ 1	40 $\pm$ 1	176 $\pm$ 24	28 $\pm$ 1
142–149	68 $\pm$ 2	56 $\pm$ 1	228 $\pm$ 8	48 $\pm$ 2

^a T cells (2×10^4) of each clone were stimulated with APC (2×10^5) and 1 μ M of antigen (trypsinized casein (t-CN), synthetic peptide fragment 136–155 and synthetic fragment 142–149). IFN- γ was assessed after 24 h of culture.

Table 3. Lymphokine production by the CD8⁺ T cell clones^a

Lymphokine	Lymphokine production (U/ml) from			
	6B1	5F1	Uc	Ud
IFN- γ	770 (310–1600)	180 (50–250)	270 (180–360)	460 (400–490)
IL-10	11 (4–24)	104 (40–260)	25 (4–40)	4 (2–5)
IL-2	<0.5 (<0.5 ^b)	<0.5 (<0.5 ^b)	<0.5 (<0.5 ^b)	<0.5 (<0.5 ^b)

^a T cells (1×10^6) of each clone were stimulated with APC (1×10^6) and trypsinized α_{s1} -casein (1 μ M) in 1 ml of medium in 48 well plates. After 24 h of culture, the amount of each lymphokine was assessed by either ELISA (γ -IFN, IL-10) or bioassay (IL-2). The values indicate the arithmetic means of 2–7 independent experiments, with the ranges shown in parentheses.

^b Not detectable in all experiments.

ited the proliferation of MV 1 Lu cells, confirming that the produced TGF- β was biologically active (data not shown).

Discussion

In this study, we have shown that the antigen-specific proliferative response and IFN- γ production response of the CD8⁺ T cell population are not reduced by oral administration of antigen. In contrast, the responsiveness of the CD4⁺ T cell population was completely reduced by the same treatment. The production of IFN- γ was not affected by the oral administration of antigen, as demonstrated in assays of the primary CD8⁺ T cell population as well as the isolated clones, suggesting that this lymphokine may have some role in orally tolerized mice. The major function of IFN- γ is its role in preventing infections by viruses and other pathogens

by means of its anti-viral activity or its ability to induce inflammation (Farrar and Schreiber, 1993). Maintaining the productivity of IFN- γ is possibly useful against pathogens that have antigens similar to food antigens especially in the absence of the responses of an antigen-specific CD4⁺ T cell population. The enhancing effects on antigen presentation of class I restricted antigen of IFN- γ (Farrar and Schreiber 1993) further support the responses.

Recently, oral tolerance has been used for curing or preventing autoimmune diseases and is effective for some experimental and clinical diseases (Chen *et al.*, 1996; Lider *et al.*, 1989; Gregerson *et al.*, 1993; Trentham *et al.*, 1993; Weiner *et al.*, 1993). Our study, however, suggested that this approach could not be applicable for all immune disorders. We have shown that the proliferative response and IFN- γ production of class I-restricted CD8⁺ T cells were not reduced by preceding feeding of antigen. Oral administration

Table 4. TGF- β production by the CD8⁺ T cell clones^a

Serum in culture	TGF- β production (pg/ml) from			
	6B1	5F1	Uc	Ud
–	49 $\pm$ 3	64 $\pm$ 6	<16	<16
+	440 $\pm$ 4	340 $\pm$ 10	172 $\pm$ 4	<32

^a Each clone (1×10^6) was stimulated with APC (2×10^6) and t-CN ($1 \mu\text{M}$) either in the presence or absence of serum (10%) in 1 ml of medium in 48 well plates. After 22 h of culture, the amount of TGF- β was assessed by ELISA.

of antigen possibly fails to reduce immune responses mediated by these T cells, such as graft rejection and some autoimmune diseases. In contrast, it is reported that oral administration of antigen reduced the responses of CD8⁺ T cells in another situation: feeding relevant antigen reduced the IFN- γ production (Chen *et al.*, 1995) or cytotoxic responses (Garside *et al.*, 1995). This apparent discrepancy is possibly due to the differences in experimental conditions, such as antigen or strain of mice, which determined the magnitude of their dependency on CD4⁺ T cells.

The production of IL-10 and TGF- β might be the specific features of CD8⁺ T cells in orally tolerized mice. Consistent with our results, recent report showed that feeding myelin basic protein (MBP) enhanced the frequency of MBP-specific CD8⁺ T cells that produce IL-10 or TGF- β (Chen *et al.*, 1995). The amount of IL-10 produced was in the range of typical CD8⁺ T cell clones (Kohyama *et al.*, unpublished) except 5F1, which produced extremely high amounts of IL-10. The most clear difference in characteristics of CD8⁺ T cell clones between cells originating from tolerized and non-tolerized mice exist in their productivity of TGF- β . Clones from tolerant mice produced a large amount of TGF- β , but those from control mice produced only marginal amounts of TGF- β , if any. We can deduce the biological role of the CD8⁺ T cells in orally tolerized mice from the lymphokine production pattern. Our results support that CD8⁺ T cells have important role in down-regulating immune responses in oral tolerance, which has been suggested by the fact that the transfer of CD8⁺ T cells from tolerized mice induces immunological tolerance (Weiner *et al.*, 1994; Richman *et al.*, 1981), because IL-10 and TGF- β is known as lymphokines with potent immune-inhibitory activities (Moore *et al.*, 1993; Schull *et al.*, 1992; Hisatsune *et al.*, 1992b). Although the inhibitory activity of CD8⁺ T cells was not required under our experimen-

tal conditions since the responsiveness of the CD4⁺ T cell population was completely reduced, its existence may be still meaningful as a fail-safe system to ensure tolerance. In the event that clonal unresponsiveness was ineffective, inhibition by these T cells may be important such as in the case reported by Roeckman *et al.* wherein infection antagonized unresponsiveness (Roeckman *et al.*, 1992).

We consider that it is better to make use of the function of responsive CD8⁺ T cells rather than suppressing them. Our study suggested that oral administration of antigen is useful to establish CD8⁺ T cell clones with different lymphokine production pattern. Similar observation was reported for CD4⁺ T cell population (Chen *et al.*, 1994). The established clones were stimulated much more effectively by a shorter peptide than a longer one, suggesting that their precise epitopes should be determined to stimulate and expand such T cells effectively. We suggested the way to predict epitope of these cells. We firstly found that the major determinant of α_{s1} -CN (region 136–155) recognized in mice fed the control diet was the same as that recognized in mice fed the CN-containing diet. To determine the epitope that stimulates CD8⁺ T cells in antigen-fed mice, the epitope can be mapped in control mice, and the use of the information of MHC-restriction and motif sequence of MHC-binding peptide is valuable in this process.

In this study, we have shown that the proliferative response and IFN- γ production response of CD8⁺ T cell population were resistant to oral tolerization. This was in clear contrast to the CD4⁺ T cell population, suggesting that the CD8⁺ T cell population plays a unique role in orally tolerized mice and owns potential usefulness. We believe that these findings will be helpful in understanding the nature of oral tolerance and the role of CD8⁺ T cells.

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