Antigen-specific inhibition of CD4⁺ T-cell responses to β -lactoglobulin by its single amino acid-substituted mutant form through T-cell receptor antagonism

Mamoru Totsuka, Shigetada Furukawa, Eiichiro Sato, Akio Ametani & Shuichi Kaminogawa
Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

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Abstract

T-cell responses can be antagonized by some single amino acid-substituted analogs of a peptide ligand for T-cell receptors (TCR), and these are called TCR antagonists. In this study, we addressed the question of whether TCR antagonism can be elicited by a whole protein antigen carrying a mutated T-cell determinant region corresponding to a TCR antagonist peptide. To clarify this, we examined the ability of a single amino acid-substituted mutant form of bovine β -lactoglobulin (β -Lg) to inhibit three CD4⁺ T-cell clones recognizing a peptide corresponding to an immunodominant determinant region 119–133 of β -Lg (p119–133). First, we identified pD129A, an analog of p119–133 with a substitution of Ala for ¹²⁹Asp, as an antagonist which can inhibit the response of two of the three T-cell clones. Then, using a yeast expression system, we prepared a mutant β -Lg (mutD129A) with the same substitution of Ala for ¹²⁹Asp as that in pD129A. This mutant protein could inhibit the proliferation of the two T-cell clones in a manner similar to the effect of pD129A. From these results we can demonstrate that TCR antagonism can be elicited by peptides naturally processed from a single-substituted mutant protein as well as by the corresponding peptides added exogenously.

Abbreviations: APC – antigen-presenting cells; β -Lg – β -lactoglobulin; ELISA – enzyme-linked immunosorbent assay; mAb – monoclonal antibody; MHC – major histocompatibility complex; PBS – phosphatebuffered saline; RCM β -Lg – Reduced and carboxymethylated β -lactoglobulin; TCGF – T-cell growth factor; TCR – T-cell receptor

Introduction

T cells are activated after recognizing peptide fragments in the form of a complex with a major histocompatibility complex (MHC) molecule, via their surface antigen receptors (T-cell receptor; TCR). Recent studies have revealed that T-cell responses are altered by stimulation with analogs of peptide ligands for their TCRs, which are called altered peptide ligands (Sloan-Lancaster and Allen, 1996; Kersh and Allen, 1996). Altered peptide ligands partially activate the T cells of both CD4⁺ (Evavold and Allen, 1991; Evavold *et al.*, 1993; Sloan *et al.*, 1993; Racioppi *et al.*, 1993; Sloan *et al.*, 1994) and CD8⁺ (Cao *et al.*, 1995) phenotypes, or induce antigen-specific inhibition of T-cell responses, a

phenomenon called TCR antagonism (De Magistris *et al.*, 1992). By analogy with the concept of antagonism in pharmacology, a TCR antagonist peptide in complex with a MHC molecule was originally considered to competitively bind to TCR without inducing any signals (De Magistris *et al.*, 1992). This hypothesis was challenged by the evidence that antagonist peptides induce a partial phosphorylation of CD3 ζ chains, which play a key role in the signaling through TCR (Reis e Sousa *et al.*, 1996; La Face *et al.*, 1997). Two hypotheses have been proposed for operative mechanisms of partial activation and TCR antagonism; the kinetic model that a different response results from quantitatively less interaction between TCR and an altered peptide ligand than that between TCR and an

agonist ligand (Jameson and Bevan, 1995; McKeithan, 1995), and the conformational model that TCR signal transduction involves some type of conformational change and this change is qualitatively different when the TCR recognizes an altered peptide ligand as compared to an agonist (Janeway, 1995). Although there have been several reports supporting either of these models (Lyons *et al.*, 1996; Yoon *et al.*, 1994), the molecular mechanisms underlying TCR antagonism are not yet fully clarified.

There have been several reports implying that TCR antagonism may be of physiological importance. TCR antagonist peptides added to a thymus organ culture can positively and/or negatively select T cells, suggesting that thymic selection of T cells is influenced by self peptides functioning as TCR antagonists (Hogquist *et al.*, 1994; Ashton-Rickardt *et al.*, 1994). The *in vivo* effects of TCR antagonists have also been demonstrated with respect to inhibition of the onset of several experimental autoimmune diseases caused by pathogenic T-cell responses, such as experimental allergic encephalomyelitis (Kuchroo *et al.*, 1994; Karin *et al.*, 1994; Nicholson *et al.*, 1997) and experimental autoimmune uveitis (Singh *et al.*, 1994). In infections by some viruses, naturally occurring variants of the determinants recognized by CD8⁺ cytotoxic T cells may act as antagonists *in vivo* to avoid recognition and elimination by host T cells, because the corresponding peptides specifically prevent the T-cell response directed against the virus *in vitro* (Allen and Zinkernagel, 1994; Klenerman *et al.*, 1994; Bertoletti *et al.*, 1994). It is important to note that these variant determinants, which are restricted to MHC class I molecules, can act as antagonists not only in the form of exogenous peptides but also when expressed as proteins endogenously by recombinant viruses. However, as for class II-restricted T-cell responses, TCR antagonism has been demonstrated only using exogenously added peptides. Therefore, it is not obvious whether mutant proteins incorporated or generated in the organism may function as TCR antagonists for CD4⁺ T cells, because (i) antigen processing pathways involving class I and class II MHC molecules are totally different from each other (reviewed in Germain and Margulies, 1993) and (ii) peptide and protein antigens reportedly require distinct antigen-presenting cell (APC) subsets for the priming of CD4⁺ T cells (Constant *et al.*, 1995). Considering that natural immune responses *in vivo* are elicited primarily by protein antigens and not by peptides, it is worth examining whether mutant proteins with a single substitution can antagonize specific CD4⁺ T cells.

In the present study, we investigated the TCR antagonistic activity of a mutant protein using a panel of T-cell clones specific for region 119-133 of bovine β -lactoglobulin (β -Lg), a major allergen in cow's milk (Taylor, 1986). Single-substituted analogs of peptide 119-133 (p119-133) were prepared and examined for their TCR antagonist activity. pD129A, an analog of p119-133 with a substitution of Ala for residue ¹²⁹Asp, was shown to have a TCR antagonistic activity for the p119-133-specific clones. A mutant of β -Lg with the same substitution as that in pD129A produced using a yeast expression system (Totsuka *et al.*, 1990; Katakura *et al.*, 1994) inhibited the proliferative responses of these clones in an antigen-specific manner. Our data demonstrate that TCR antagonism can be elicited by mutant proteins with a single substitution, and suggest the natural occurrence of TCR antagonism mediated by mutant proteins generated *in vivo* by a genetic mutation.

Materials and methods

Animals

C57BL/6 (B6) mice (H-2^b: female, 8-week old) were purchased from Charles River Japan Inc. (Yokohama, Japan).

Proteins and peptides

Bovine β -lactoglobulin (β -Lg; genetic variant A) was prepared from raw milk of a cow of the genotype AA for β -Lg as described previously (Kaminogawa *et al.*, 1989). Reduced and carboxymethylated (RCM) β -Lg was prepared by carboxymethylation of reduced β -Lg according to the method of McKenzie (McKenzie *et al.*, 1972). A peptide corresponding to region 119-133 in bovine β -Lg (p119-133; CQCLVVRTPEVDDEAL), its analog peptide having a substitution of Ala for ¹²⁹Asp, pD129A, and a peptide corresponding to region 136-155 of bovine α_{s1} -casein (P10; IGVNQELAYFYPELFRQFYQ) were synthesized using a peptide synthesizer (Model 430A; Perkin-Elmer, Norwalk, CT, U. S. A.) and purified by reversed-phase HPLC. Single-substituted analogs of p119-133 used for identifying TCR antagonists (Table 1) were purchased from Chiron Mimotopes (Clayton, Australia).

Table 1. Single-substituted analogs of p119-133 used in this study

Peptide	Amino acid sequence ^a														
	119					125					130			133	
p119-133	Cys	Gln	Cys	Leu	Val	Arg	The	Pro	Glu	Val	Asp	Asp	Glu	Ala	Leu
pL122D	—	—	—	Asp	—	—	—	—	—	—	—	—	—	—	—
pV123D	—	—	—	—	Asp	—	—	—	—	—	—	—	—	—	—
pR124A	—	—	—	—	—	Ala	—	—	—	—	—	—	—	—	—
pT125A	—	—	—	—	—	—	Ala	—	—	—	—	—	—	—	—
pE127A	—	—	—	—	—	—	—	—	Ala	—	—	—	—	—	—
pD129A	—	—	—	—	—	—	—	—	—	—	Ala	—	—	—	—
pD130A	—	—	—	—	—	—	—	—	—	—	—	Ala	—	—	—

^a Dashes indicate identity with the p119-133 sequence.

T-cell clones

CD4⁺ T-cell clones specific for β -Lg, G1.19, F2.8, and F12.5, were established as described elsewhere (submitted for publication). Briefly, B6 mice were immunized subcutaneously with bovine β -Lg emulsified with CFA-H37Ra. Lymph-node cells prepared from the mice 7 days after immunization were stimulated every week with 5 μ M β -Lg, and 10% T-cell growth factor (TCGF; supernatant from ConA-activated rat splenocytes) prepared as described previously (Rosenberg *et al.*, 1978), in the presence of 3000-rad irradiated syngeneic splenocytes as antigen-presenting cells (APCs). The medium used for the cell culture was RPMI1640 medium supplemented with 10% fetal calf serum (FCS), 50 μ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin. After a four-month culture period, the cells were cloned by the limiting dilution method. The cloned cells were stimulated with 2.5 μ M β -Lg, 10% TCGF and irradiated syngeneic splenocytes once a week for 4–5 months. The resulting T-cell clones, G1.19, F2.8, and F12.5, were established from different T-cell lines each derived from different mice. The characteristics of the T-cell clones used in this study are summarized in Table 2.

T-cell clones were cultured for the proliferation assay at 4×10^4 cells/well in 96-well plates together with irradiated syngeneic splenocytes at 2×10^5 cells/well in the presence or absence of antigen. After 52 h, 1 μ Ci of [³H]thymidine was added to each well. The cells were harvested 20 h later, and the uptake of [³H]thymidine was assessed by liquid scintillation counting.

Pre-pulse assay for identifying T-cell receptor antagonist peptides

Irradiated syngeneic splenocytes as APC were incubated with a suboptimal concentration of p119-133 (0.3 μ M) for 2 h at 37 °C (pre-pulse). After removal of the unbound peptides by washing, the antigen-pulsed cells were cultured in 96-well plates at 2×10^5 cells/well with cloned T cells (4×10^4 cells/well) and varying concentrations of the analog peptides. Proliferation of the cells was measured as described above.

Construction of the expression plasmid for a mutant β -Lg

Bovine β -Lg cDNA isolation and expression in yeast were described previously (Totsuka *et al.*, 1990). To facilitate construction of an expression plasmid in yeast, a *Sac* I/*Sma* I fragment of β -Lg cDNA containing the whole coding region was ligated with a *Sal* I linker at each end, and inserted into the *Sal* I site of pUC118. Then, the cDNA fragment was modified in the region around the initiation codon to give the sequence, 5'-CTAAAAAATGAAG-3', instead of 5'-CTGCAGCCATGAAG-3', and the resulting plasmid was designated pBAML. This upstream sequence is common to mRNAs for proteins expressed at high levels in yeast, and is expected to increase the expression level in yeast (Hamilton *et al.*, 1987).

Site-directed mutagenesis of the coding region of β -Lg cDNA was performed by means of the polymerase chain reaction (PCR). To introduce a mutation resulting in the substitution of Ala for ¹²⁹Asp, two mutagenic oligonucleotide primers, D129A-back primer (5'-CCCCGGAGGTGGCTGACGAGGCCCTG-

Table 2. Characteristics of β -Lg-specific CD4⁺ T-cell clones used in this study

T-cell clone	Antigen specificity /MHC restriction	Cytokine production			Th subset
		IFN- γ	IL-4	IL-10	
G1.19	p119-133 ¹ /I-A ^b	+	–	–	Th1
F2.8	p119-133/I-A ^b	+	–	–	Th1
F12.5	p119-133/I-A ^b	+	+	–	Th0

¹ A peptide corresponding to region 119-133 of bovine β -lactoglobulin.

3') and D129A-forward primer (5'-CAGGGCCTCGT-CAGCCACCTCCGGGG-3') were used as well as M13-universal primer (5'-GTAAAACGACGGCCAGT-3') and M13-reverse primer (5'-CAGGAAACAGCTA-TGAC-3'). These primers were synthesized with a DNA synthesizer (Model Cyclone Plus; Millipore, Bedford, MA, U.S.A.). For the first round of PCR amplification, a set of the D129A-back primer and the M13-universal primer (for PCR-A), or a set of the D129A-forward primer and the M13-reverse primer (for PCR-B) was used. A 50- μ l reaction mixture was prepared containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 10 pmol of each primer, 0.1 pmol of pYBAML as template DNAs, 250 μ M dNTP and 0.5 U of *Taq* DNA polymerase and then overlaid with mineral oil. The reaction was allowed to proceed for 30 cycles (30 s, 94 °C; 20 s, 55 °C; 2 min, 72 °C). The second PCR amplification was performed with the M13-universal primer and the M13-reverse primer in a 50- μ l mixture containing 3 μ l each of the products of PCR-A and PCR-B. After fractionating the reaction products according to size on a 1.5% agarose gel, the appropriate band was excised and purified using a Gene Clean Kit II (Bio 101, La Jolla, CA, U.S.A.). The PCR product was digested with *Sal* I and ligated into pUC118. The sequence of the PCR product was confirmed by DNA sequencing using a PRISM kit (Perkin-Elmer) and a DNA sequencer (Model 370A; Perkin-Elmer). The *Sal* I fragment of the mutated β -Lg cDNA was excised and ligated into the *Sal* I site of pYG100, an expression plasmid vector for yeast (Kumagai and Miura, 1989). The resulting plasmid for expression of the mutant β -Lg was designated pYD129A.

Expression and purification of recombinant proteins

The expression plasmid, pYD129A, was introduced into *Saccharomyces cerevisiae* strain AH22 (*Mat a*, *leu2-3*, *leu2-112*, *his4-519*, *can1*) by the lithium acetate method (Ito *et al.*, 1983). Yeast culture and

purification of recombinant proteins from the yeast-culture supernatant were performed as described previously (Katakura *et al.*, 1994; Katakura *et al.*, 1997). Briefly, transformed yeast cells were grown at 30 °C for 4 days in a modified synthetic minimal medium (Ichikawa *et al.*, 1989). The culture supernatant was concentrated 160-fold by ultrafiltration and diluted eight-fold with 0.05 M imidazole-HCl buffer (pH 6.7). Proteins in the concentrated sample were purified by sequential chromatography on a DEAE-Sephacel column (Pharmacia Biotech, Uppsala, Sweden) and a Superose 12 gel-filtration column (Pharmacia Biotech). At each purification step, mutant β -Lgs were detected by sandwich ELISA using anti- β -Lg mAb 62A6 and alkaline phosphatase-labeled anti- β -Lg mAb 61C1 as described previously (Totsuka *et al.*, 1990).

SDS-polyacrylamide gel electrophoresis

An SDS-polyacrylamide gel containing 15% acrylamide was used as the running gel and electrophoresis was performed under reducing conditions by the method of Laemmli (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R-250.

Competitive enzyme linked immuno-sorbent assay (ELISA)

To examine the conformational integrity of purified mutD129A, competitive ELISA was performed using anti-bovine β -Lg monoclonal antibodies (mAbs) as probes, as employed in our previous studies (Kaminogawa *et al.*, 1989; Hattori *et al.*, 1993; Katakura *et al.*, 1994). Anti- β -Lg mAbs from the B-cell hybridoma clones 21B3, 31A4, and 61C1 were purified from ascites as described previously (Kaminogawa *et al.*, 1989). Microtiter plate wells were incubated with 100 μ l of phosphate-buffered saline (PBS; 0.11 M phosphate buffer at pH 7.2 with 0.04 M NaCl and 0.02% NaN₃) containing 0.01% bovine β -Lg for 2 h at

room temperature. After washed with PBS containing 0.05% Tween-20 (PBS-Tween), the wells were blocked with 125 μ l of 1% ovalbumin in PBS. After incubating and washing the wells, solutions containing mAbs and varying concentrations of native β -Lg or mutD129A were added. mAbs bound to β -Lg immobilized in the wells were detected by adding alkaline-phosphatase-labeled goat anti-mouse immunoglobulin (Organon Teknika, Durham, NC, U. S. A.) and subsequently by developing with the substrate, *p*-nitrophenylphosphate. The absorbance was measured at 405 nm.

Results

Identification of a TCR antagonist peptide effective for p119-133-specific T-cell clones

In our previous study, we identified an immunodominant T-cell determinant of bovine β -Lg in region 119-133 using B6 mice and revealed that residues of region 122-130 are essential for the determinant to be recognized by T cells (Totsuka *et al.*, in press). Further, residues 126 Pro and 128 Val were identified to play a critical role for p119-133 to bind to MHC class II I-A^b molecules from B6 mice (manuscript in preparation). From these findings, we assumed that among the residues in the region 122-130 other than 126 Pro and 128 Val there are critical TCR contacting residues and we prepared a series of single-substituted peptides of p119-133 with a substitution at each of these residues (Table 1), since most of TCR antagonist peptides previously identified have a substitution at a TCR contacting residue. First, we examined the ability of the analog peptides to induce proliferation of G1.19, a CD4⁺ T-cell clone specific for p119-133 (Table 2), because a TCR antagonist induces no proliferation of relevant T cells by itself (De Magistris *et al.*, 1992). As shown in Figure 1, pL122D, pV123D, pT125A, and pD130A at 50 μ M each elicited a positive response (stimulation index > 2) in the proliferation assay using G1.19 cells. These peptides were not tested further in an antagonist assay.

Next, we examined whether pR124A, pE127A, and pD129A which induced no response of G1.19 were effective to inhibit the proliferative response of G1.19 to p119-133 (Figure 2). To avoid competition between the analog peptide and p119-133 in binding to I-A^b molecules, APCs were incubated with p119-133 prior to the incubation with G1.19 cells and the analog peptide. In preliminary experiments, G1.19 showed

a proliferative response to APC that had been previously incubated with p119-133 at concentrations of 0.1 μ M or more, and the intensity of the response was dependent on the dose of p119-133 at the time of pre-incubation (data not shown). Based on these findings, we chose to employ 0.3 μ M as the concentration of p119-133 for the pre-incubation, at which a sub-optimal but significant proliferative response of G1.19 was induced. As shown in Figure 2, only pD129A inhibited the proliferative response of G1.19 to p119-133. The response of G1.19 was not inhibited in the presence of a control peptide, P10, which is known to bind to I-A^b molecules and contains a determinant of bovine α_{s1} -casein recognized as an immunodominant T-cell determinant in B6 mice (unpublished observation).

We next examined the ability of pD129A to function as a TCR antagonist for two other p119-133-specific clones, F2.8 and F12.5 (Table 2). As shown in Figure 3, the proliferative response of F12.5 to p119-133-pre-pulsed APC was inhibited by pD129A as well as that of G1.19, confirming the TCR antagonist activity of pD129A for this clone. However, pD129A enhanced the proliferation of F2.8, demonstrating that F2.8 recognizes pD129A as an agonist. In these experiments, P10 added to the culture instead of pD129A did not inhibit the responses (data not shown).

Preparation of mutD129A, a mutant β -Lg with the same substitution as that in pD129A which is a TCR antagonist for p119-133-specific T-cell clones

We prepared a mutant of β -Lg with the same single substitution as that in pD129A using the yeast expression system previously established (Totsuka *et al.*, 1990). The mutated β -Lg cDNA fragment was expressed in yeast under control of the promoter and terminator of the yeast glyceraldehyde triphosphate dehydrogenase gene in the yeast expression plasmid pYG100 (Kumagai and Miura, 1989). The mutant protein was secreted into the culture medium, from which the recombinant protein was purified by two sequential steps of column chromatography (Katakura *et al.*, 1997). The purified protein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue. As shown in Figure 4, mutD129A was purified to more than 90% purity and showed the same mobility as β -Lg.

To examine the conformational integrity of the recombinant protein, competitive ELISA was performed using anti- β -Lg mAbs, 21B3, 31A4 and 61C1,

Figure 1. Proliferative response of G1.19, a p119-133-specific T-cell clone, to single-substituted analogs of p119-133. G1.19 cells were cultured at 4×10^4 cells/well in the presence of each analog peptide at $50 \mu\text{M}$ and irradiated syngeneic splenocytes at 2×10^5 cells/well in 96-well microtiter plates. The data are shown as the ratio of average cpm for each peptide to that of background cpm (stimulation index). The average background value ($\text{cpm} \pm \text{SD}$) was 6488 ± 430 . The values shown are representative of data from three independent experiments.

Figure 2. Identification of a TCR antagonist peptide effective for G1.19. G1.19 cells were cultured in the presence of each analog peptide at $3 \mu\text{M}$ (hatched bars) or $30 \mu\text{M}$ (closed bars) and irradiated syngeneic splenocytes as APCs that had been previously incubated with p119-133 at $0.3 \mu\text{M}$ for 2 h at room temperature. P10 was used as an irrelevant I-A^b binding peptide control. An open bar represents the value in the absence of analog peptides. The background value for G1.19 cells cultured with non pre-pulsed APCs alone was 8561 ± 1093 ($\text{cpm} \pm \text{SD}$). The data are shown as the average cpm of triplicate wells with the background cpm subtracted. The values shown are representative of data from three independent experiments.

Figure 3. Effect of pD129A on the proliferative response of p119-133-specific T-cell clones to p119-133-pre-pulsed antigen-presenting cells. p119-133-specific T-cell clones, G1.19 (A), F12.5 (B) and F2.8 (C), were cultured in the presence of the indicated concentrations of pD129A and APCs pre-pulsed with p119-133 at 0.3 μM. The data are shown as the average cpm of triplicate wells. The values shown are representative of data from three independent experiments.

which were shown to be useful as probes for examining conformational changes occurring in β -Lg (Kaminogawa *et al.*, 1989; Hattori *et al.*, 1993; Katakura *et al.*, 1994). Other anti- β -Lg mAbs employed in previous studies, 62A6 and 61B4, that recognize the region 125-135 (Kaminogawa *et al.*, 1989), could not be used for evaluating the conformation of mutD129A, because the reactivity of these mAbs was impaired by the substitution at residue ¹²⁹Asp (data not shown). In contrast, the mAbs used in this study recognize regions irrelevant to the substitution (Kaminogawa *et al.*, 1989), and thus are not considered to be affected directly by the

difference in amino acid sequence between the mutant and the wild-type. We compared the binding curves of each mAb to native β -Lg immobilized in plate wells in the presence of various concentrations of competitive antigen added together with mAbs in the aqueous phase. Reduced and carboxymethylated (RCM) β -Lg was used as a denatured- β -Lg control. As shown in Figure 5, anti- β -Lg mAbs 21B3 and 31A4, recognizing regions 15-29 and 8-19 of β -Lg (Kaminogawa *et al.*, 1989), respectively, bind to mutD129A with almost the same binding affinities as those to native β -Lg. mAb 61C1 which is specific for region 15-29 (Kaminogawa

Figure 4. SDS-polyacrylamide gel electrophoresis pattern of the purified mutD129A. SDS-PAGE was performed using a 15% gel according to the method of Laemmli (Laemmli, 1970), and subsequently subjected to staining with Coomassie Brilliant Blue. Lane 1; molecular weight markers (from top, bovine serum albumin, ovalbumin, and hen egg-white lysozyme), lane 2; purified mutD129A (10 μ g), lane 3; native β -Lg (10 μ g).

et al., 1989) showed almost the same binding curves for native β -Lg and mutD129A, although the difference in its binding to native β -Lg and RCM β -Lg is not so obvious. These results suggest that mutD129A retains almost the same overall conformation as that of native β -Lg.

TCR antagonist activity of mutD129A

To examine whether a mutant protein can mediate TCR antagonism, we examined the ability of mutD129A to inhibit the proliferative responses of the p119-133-specific T-cell clones. The T-cell clones, G1.19, F2.8 and F12.5, were cultured with native β -Lg at 0.3 μ M and various concentrations of mutD129A in the presence of APCs. As shown in Figure 6, the proliferation of G1.19 and F12.5, for which pD129A functions as a TCR antagonist, were inhibited in a dose-dependent manner by mutD129A added to the culture. It is worth noting that 1 μ M mutD129A, a dose only about threefold that of native β -Lg, could inhibit the proliferative response of F12.5 cells by 50%. In contrast,

F2.8 showed increased proliferation in the presence of mutD129A. These results indicate that the inhibition of T-cell responses by mutD129A is antigen-specific, and demonstrate that TCR antagonism for CD4⁺ T cells can be mediated by mutant proteins.

Discussion

In the present study, we identified a TCR antagonist peptide effective for CD4⁺ T-cell clones, and demonstrate that a mutant form of a protein antigen with the same single substitution as the TCR antagonist peptide can inhibit the activation of the CD4⁺ T-cell clones displayed in response to the wild-type protein. This illustrates that TCR antagonism of class II MHC-restricted CD4⁺ T cells can be mediated by peptides naturally processed from a mutant protein as well as by the corresponding peptides added extracellularly. It is of importance to note that a point mutation can provide a protein with a potent TCR antagonist activity. Some of the variants of a protein antigen resulting from genetic mutations may be able to mediate TCR antagonism *in vivo*. This is comparable with the finding that class I MHC-restricted CD8⁺ T cells can be inhibited by antagonist epitopes expressed as proteins endogenously by variant viruses (Allen and Zinkernagel, 1994; Klennerman *et al.*, 1994; Bertoletti *et al.*, 1994). Antagonistic activity by these protein antigens may explain the mechanisms of self tolerance, the poor immune response to tumor cells, and the escape of infectious microbes and viruses from immune surveillance.

We employed three β -Lg-specific CD4⁺ T-cell clones (Table 2) each recognizing the same single peptide, p119-133, to evaluate the inhibitory effect of mutD129A prepared from the yeast culture supernatant. T-cell clones G1.19 and F12.5 were inhibited by pD129A through TCR antagonism (Figure 3). In contrast, the TCR of F2.8 cells recognized pD129A as an agonist and the proliferative response of this clone was increased by the addition of pD129A. Similarly, mutD129A having the same substitution as pD129A inhibited both G1.19 and F12.5 but not F2.8, indicating the same specificity of inhibition as pD129A (Figure 6). The inhibition by mutD129A is not likely due to nonspecific inhibitors which may be included as impurities in the preparation of the mutant protein, because F2.8 cells showed strong proliferation in the presence of mutD129A at 10 μ M which inhibited almost completely the proliferation of G1.19 and F12.5 cells.

As shown above, pD129A and mutD129A could

Figure 5. Competitive inhibition of binding of anti- β -Lg mAbs 21B3, 31A4, and 61C1 to immobilized native β -Lg by mutD129A, native β -Lg, and RCM β -Lg in solution. Microtiter plate wells were coated with 0.01% native bovine β -Lg in PBS and blocked with 1% ovalbumin in PBS. Then, solutions containing either anti- β -Lg mAbs 21B3 (A), 31A4 (B), or 61C1 (C) and varying concentrations of competitor antigen were added to the wells. mAbs bound to β -Lg immobilized in the wells were detected by adding alkaline-phosphatase-labeled goat anti-mouse immunoglobulin and subsequently by developing with the substrate, *p*-nitrophenylphosphate. B and B_0 are the values of absorbance at 405 nm at the last step of the competitive ELISA in the presence or absence of the competitor antigen, respectively. The competitive antigens used were mutD129A (Δ), native β -Lg ($\circ$), and reduced carboxymethylated (RCM) β -Lg ($\bullet$). RCM β -Lg was employed as a denatured β -Lg control. The values shown are representative of data from three independent experiments.

not inhibit all T-cell clones specific for p119-133. Analog peptides acting as TCR antagonists reportedly differ in effectiveness towards T-cell clones specific for the same determinant, as recognition is probably dependent on TCR sequences. When we consider the practical application of TCR antagonists to inhibit deleterious T-cell responses, it is not necessary to employ a TCR antagonist that can inhibit all specific T cells. A TCR antagonist with the ability to inhibit most of the relevant T cells may effectively inhibit T cell responses *in vivo*. In fact, a single TCR antagonist peptide could efficiently inhibit experimental autoimmune diseases mediated by a diverse T-cell repertoire (Kuchroo *et al.*, 1994; Singh *et al.*, 1994).

A MHC class II molecule has a peptide-binding groove that is open at each end (Brown *et al.*, 1993;

Stern *et al.*, 1994). Therefore, it is possible that a denatured protein molecule with a long extended shape binds to a MHC class II molecule (Sette *et al.*, 1989; Lee *et al.*, 1988). Moreover, denatured protein molecules may be susceptible to attack by enzymatic digestion in the culture supernatant of T cells, resulting in the formation of peptide fragments which can bind to MHC class II molecules displayed on APC. If mutD129A has a denatured conformation, these possibilities should be considered in evaluating the inhibitory effect of the mutant protein. From the results of binding analyses using our set of mAbs (Figure 5), we can rationally demonstrate that mutD129A has an overall conformation almost equal to that of native β -Lg. Therefore, mutD129A can be considered to be processed in APC and displayed on MHC molecules in the same way

Figure 6. mutD129A inhibits the proliferative response of T-cell clones, G1.19 and F12.5, but not F2.8, to native β -Lg. p119-133-specific T-cell clones, G1.19 (A), F12.5 (B) and F2.8 (C), were cultured in the presence of native β -Lg at 0.3 μ M and the indicated concentrations of mutD129A and irradiated syngeneic splenocytes as APCs. The background values ($\text{cpm} \times 10^{-3} \pm \text{SD}$) of G1.19, F12.5, and F2.8 cultured with non pre-pulsed APCs alone were 44.95 ± 3.84 , 3.08 ± 0.62 , and 2.89 ± 0.69 , respectively. The data are shown as the average cpm of triplicate wells with the background cpm subtracted. The values shown are representative of data from three independent experiments.

as native β -Lg, excluding the possibilities mentioned above.

The possibility that competitive exchange occurs between antigenic peptides bound to MHC class II molecules and TCR antagonist peptides cannot be completely excluded even in the pre-pulse assay where antigenic peptides were incubated with APC in advance to decrease the competition to as little as possible. The finding that mutD129A has TCR antagonist activity clearly shows that the competition between peptides for MHC binding at the cell surface is totally irrelevant to TCR antagonism, because mutD129A needs to be processed in APC in order to exhibit this activity. Moreover, competition for binding to class II MHC molecules in endocytic vesicles between peptides derived from proteins added extracellularly is not likely to influence the T-cell response, considering that the corresponding doses of hen egg-white lysozyme added to the culture instead of mutD129A showed no effect on proliferation of the T cells (data not shown). Thus, inhibition of T-cell response by mutD129A was mediated by TCR antagonism, and not by antigenic competition.

mutD129A inhibited 50% of the response of F12.5 cells at 1 μ M, approximately a 3-fold higher concentration than that of β -Lg added to the culture, and mutD129A almost completely inhibited the response

at 10 μ M (Figure 6). G1.19 was also inhibited thoroughly by mutD129A at 10 μ M. In the pre-pulse assay, pD129A at 10 μ M showed only 30 to 50% inhibition of the proliferative response of G1.19 or F12.5 cells to APC pre-incubated with p119-133 at 0.3 μ M (Figure 3). The same results were obtained in an assay where p119-133 and pD129A were added to the culture simultaneously (data not shown). It is possible that mutD129A is more efficient than pD129A in inhibiting the response of the T-cell clones specific for p119-133, although it cannot be definitely ascertained from only these results. Legge *et al.* have recently shown that immunoglobulin (Ig) molecules displaying the sequence of a proteolipidprotein (PLP) TCR antagonist peptide can abrogate more effectively than the peptide antagonist the activation of T cells stimulated with the original PLP peptide (Legge *et al.*, 1997). They attributed the effectiveness of the Ig antagonist to its efficient endocytic processing leading to the generation of TCR antagonist peptides. In a situation in which immune responses have already been elicited by a protein antigen *in vivo*, a TCR antagonist mutant form of the protein with a single substitution may have greater potential for inhibiting the T-cell responses as compared to an analog peptide with the same substitution. B cells can present peptides from a protein that can bind to their surface immunoglobulin $\sim 10^4$ times

more efficiently than those from non-specific proteins (Lanzavecchia, 1985). A protein molecule bound by specific antibodies is also efficiently captured by APC through their surface Fc receptors and presented to T cells. When immune responses to a protein antigen have been established, a larger number of B cells and antibodies bind specifically to a mutant form of that protein than to an analog peptide which is only a fragment of the mutant protein. Therefore, the antagonistic mutant protein is considered to be presented more effectively as compared to the analog peptides, resulting in more potent inhibition of T-cell activation. In addition, a mutant protein may have a longer half-life *in vivo* than an analog peptide because of its higher resistance to enzymatic digestion. The use of artificial genes encoding a mutant TCR antagonist protein to be expressed *in vivo* may be applicable as a means of gene therapy for abrogating pathogenic T-cell responses and ameliorating T-cell mediated autoimmune diseases, such as multiple sclerosis, type I diabetes, and rheumatoid arthritis.

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Address for correspondence: Mamoru Totsuka, Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan. E-mail: atotuka@hongo.ecc.U-tokyo.ac.jp