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# Limit of T Cell Tolerance to Self Proteins by Peptide Presentation

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**Cytotoxic T lymphocytes (CTLs) recognize foreign peptides bound to major histocompatibility complex (MHC) class I molecules. MHC molecules can also bind endogenous self peptides, to which T cells are tolerant. Normal mice contained CTLs specific for self peptides that were from proteins of ubiquitous or tissue-restricted expression. In vivo, these endogenous self peptides are not naturally presented in sufficient density by somatic cells expressing MHC class I molecules. They can, however, be presented if added exogenously. Thus, our data imply that CTLs are only tolerant of those endogenous self peptide sequences that are presented by MHC class I–positive cells in a physiological manner.**

SELF TOLERANCE IN THE IMMUNE system is established by elimination of self-reactive T lymphocytes during thymic differentiation (*1*). The self antigens involved in this negative selection are combinations of self MHC molecules and other self molecules, since T cells specific for combinations of self molecules and foreign MHC are not negatively selected (*2*). These experiments confirmed the hypothesis (*3*) that antigen recognition during establishment of self tolerance follows the same rules as those that apply to the recognition of foreign antigen in the mature immune system. CTLs were found to recognize specific peptide-defined epitopes of cellular proteins, for example, of viral origin, in the context of MHC class I molecules (*4*). Not all possible peptide sequences from a given endogenous cellular protein are actually presented in sufficient density (*5, 6*). Several possible reasons have been put forward to account for this limitation, including specificity of intracellular proteases, problems in peptide-MHC interaction, or self peptides competing for the relevant binding sites. These limitations in peptide presentation have led to the postulate that T cells need not be tolerant of those autologous peptide sequences that are not physiologically presented by MHC-expressing cells in sufficient density if the above hypothesis on establishment of self tolerance is valid (*6, 7*). Thus, nonphysiologic combinations of self MHC and self peptides would not induce negative selection. Our data show this postulate to be correct.

Mouse spleen cells were stimulated with several sources of peptides derived from autologous proteins in a manner that yields primary CTL responses to peptides (*5*). First, C57BL/6 [B6; H-$2^b$, $\beta_2M^b$ ($\beta_2$-microglobulin allele)] or B10.C-*H-3*$^c$ (H-$2^b$, $\beta_2M^a$) spleen cells were stimulated with a synthetic peptide representing amino acids 77 to 89 of $\beta_2M^b$, which is expressed in nearly all somatic cells including those thymic resident cells inducing self tolerance, and in T cells themselves. We consistently obtained CTL lines with specificity for the $\beta_2M^b$ peptide regardless of the $\beta_2M$ allele expressed by responder cells (Fig. 1). Two representative CTL lines, one expressing $\beta_2M^a$, the other $\beta_2M^b$ (Fig. 1, A and B), did not recognize $\beta_2M^b$-expressing EL4 target cells, but they both killed EL4 cells incubated with the stimulating $\beta_2M^b$ 77–89 peptide. The reactivity pattern of both lines was similar when tested on a panel of $\beta_2M$-derived peptides. The $\beta_2M^a$ 77–89 peptide is hardly recognized or not at all. The $\beta_2M^b$ 77–89–specific CTL line 041188-5 ($\beta_2M^b$) can be more easily blocked with CD8-specific antibody than the $\beta_2M^b$-specific CTL line 181B ($\beta_2M^a$) (Fig. 1C), which was generated by in vivo immunization (*8*). This supports the suggestion that in vitro–primed, peptide-specific CTLs are of lower affinity than in vivo–primed CTLs (*5*). The P815 targets (H-$2^d$) incubated with $\beta_2M^b$ 77–89 are not lysed by 041188-5 CTLs (Fig. 1D), indicating MHC class I–restricted recognition, since neither EL4 nor P815 cells express MHC class II. Thus, normal mice contained CTLs specific for a peptide derived from a ubiquitous autologous protein. These CTLs did not recognize target cells that express that protein.

A second source of autologous peptide was prepared by hydrolysis of hemoglobin, as an example of an abundant protein with a specific tissue distribution. Self tolerance to proteins not synthesized in the thymus is presumably established by presentation of imported protein by thymic antigen-presenting cells. Spleen cells from a B10.129-*H-1*$^b$ mouse [H-$2^b$, Hbb$^d$ (hemoglobin allele)] or from a C57BL/10 mouse (H-$2^b$, Hbb$^s$) were stimulated with hydrolyzed hemoglobin of C57BL/10 (Hbb$^s$). The CTL lines that developed did not distinguish between heterologous and autologous hemoglobin fragments, and they recognized autologous hemoglobin fragments in an antigen-specific and MHC class I–restricted fashion (Fig. 2).

A third source of autologous peptides was prepared by tryptic digest of total liver proteins. B6.K1 (H-$2^b$) spleen cells were stimulated with such a pool of self peptides. In a parallel culture, B6.K1 spleen cells were stimulated against a pool of peptides derived by similar treatment of allogeneic BALB.HTG (H-$2^g$) liver proteins. CTL lines derived from these cultures recognized the stimulating peptides in an antigen-specific and MHC class I–restricted fashion (Fig. 3). The line against autologous peptides (709K1-5) cross-reacts to some extent with allogeneic fragments, and vice versa, indicating that some of the determinants recognized by either CTL line are monomorphic between the two mouse strains.

In conclusion, our data show that normal mice contain CTLs reactive with peptide sequences or fragments of ubiquitous or tissue-specific self proteins prepared in a nonphysiologic way, implying that such CTLs are spared from thymic negative selection (*9*). The peptides recognized by our CTL lines may either be not produced at all by normal cells or they may be produced in insufficient quantities to be recognized by

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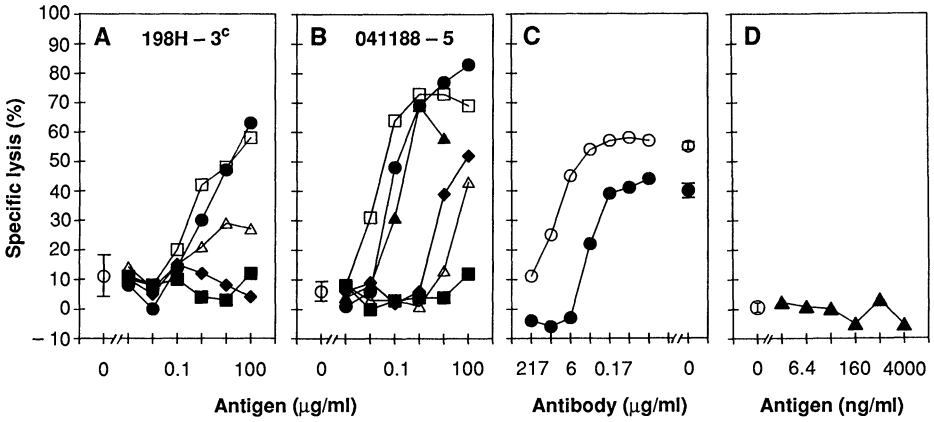


**Fig. 1.** Characterization of $\beta_2$M-specific CTL lines (*15*). In vitro–primed, B10.C-*H-3*$^c$ ($\beta_2M^a$)–derived CTL line 198H-3$^c$ (A), C57BL/6 ($\beta_2M^b$)–derived CTL line 041188-5 (B and D, and ● in C), and in vivo–primed CTL line 181B (*8*) (C, ○) were tested in standard $^{51}$Cr-release assays on targets EL4 (H-2$^b$) (**A** to **C**) or P815 (H-2$^d$) (**D**) incubated in medium (○) or in titrated concentrations of $\beta_2M^b$ 77–89 (▲), $\beta_2M^b$ 77–89* from a second synthesis (●), $\beta_2M^b$ 80–92 ($R^-$) (this peptide lacks an Arg residue at position 81) (△), $\beta_2M^a$ 77–89 (◆), $\beta_2M^b$ 80–92 (□), and $\beta_2M^b$ 74–86 (■) peptides. The effect of CD8-specific antibodies on the lytic activity of the CTL lines 041188-5 (●) and 181-B (○) was compared by incubating EL4 target cells in medium (○) or $\beta_2M^b$ 77–89* (●, 10 μg/ml) before adding monoclonal antibody (MAb) 19/178 (*16*) to the a $^{51}$Cr-release assay (C). Effector:target ratio was 10:1 (A to C) or 18:1 (D). Spontaneous release of target cells was between 10.2 and 26.7%. In vitro–primed CTL lines were produced by culturing $6.0 \times 10^7$ spleen cells from irrelevantly primed B10.C-*H-3*$^c$ (*17*) for 198H-3$^c$ or $4.0 \times 10^7$ spleen cells from unprimed C57BL/6 mice (041188-5) with $\beta_2M^b$ 77–89 peptide (1 μg/ml) for 7 days. Thereafter, responder cells were restimulated weekly with syngeneic feeder cells and peptide (1 μg/ml) in interleukin-2–supplemented medium. CTL assays were carried out as described (*18*). Target cells were tumor cells incubated either in medium alone or with peptides for 90 min at 37°C. The peptide $\beta_2M^b$ 77–89 (TYACRVKHASMAE) was synthesized manually. The $\beta_2M^b$ 77–89*, $\beta_2M^a$ 77–89 (TYACRVKHDSMAE), $\beta_2M^b$ 80–92 (CRVKHASMAEPKT), $\beta_2M^b$ 80–92 ($R^-$) (CVKHASMAEPKT), or $\beta_2M^b$ 74–86 (ETDTYACRVKHAS) (*19*) peptides were synthesized by continuous-flow solid-phase peptide synthesis using a MilliGene 9050 peptide synthesizer. All peptides were purified by high-performance liquid chromatography (HPLC). Purity was greater than 96%. Peptides were controlled by amino acid composition analysis.

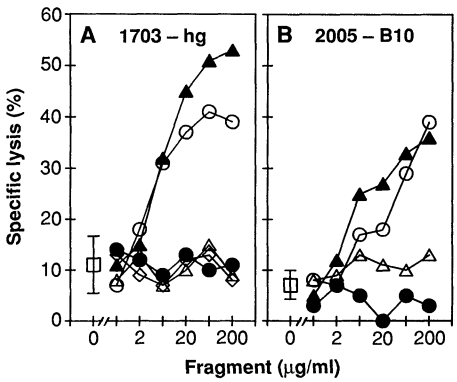


**Fig. 2.** CTL lines 1703-hg or 2005-B10 recognize fragments of autologous and heterologous hemoglobin. The B10.129-*H-1*$^b$ (H-2$^b$, Hbb$^d$)–derived CTL line 1703-hg (**A**) or C57BL/10 (H-2$^b$, Hbb$^s$)–derived CTL line 2005-B10 (**B**) was tested (i) on EL4 target cells incubated with medium (□), which contained hydrolyzed Hbb$^d$ (○), Hbb$^s$ (▲) hemoglobin, native Hbb$^d$ hemoglobin (●), or hydrolyzed ovalbumin (△) or (ii) on P815 target cells incubated with hydrolyzed Hbb$^d$ hemoglobin (◇). The effector:target ratio was 6:1; spontaneous $^{51}$Cr release of target cells ranged between 10.8 and 13.1%. These CTL lines were derived by stimulation of unprimed B10.129-*H-1*$^b$ (for 1703-hg) or C57BL/10 (for 2005-B10) spleen cells with hydrolyzed C57BL/10 hemoglobin (1 mg/ml) by a protocol similar to that described in the legend to Fig. 1. Hemoglobin of either C57BL/10 (H-2$^b$, Hbb$^s$) or 129/Sv (H-2$^b$, Hbb$^d$) mice was prepared according to a standard protocol (*20*). Hemoglobin was hydrolyzed by boiling 10 mg for 2 min in 2 ml of 1*N* NaOH then neutralizing with 1*N* HCl as described (*21*). Reversed-phase HPLC of this preparation revealed several distinct peptide peaks. Tryptic fragments of hemoglobin were also recognized by the CTL lines (*22*).

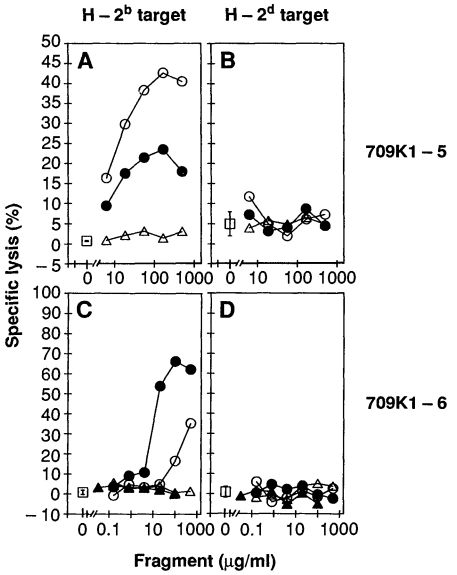


**Fig. 3.** Recognition of syngeneic protein fragments by B6.K1-derived CTL lines 709K1-5 and 709K1-6. The 709K1-5 CTLs (**A** and **B**) were generated with B6.K1 liver protein fragments and 709K1-6 CTLs (**C** and **D**) were generated with BALB.HTG fragments. These CTLs were tested in a $^{51}$Cr-release assay on targets EL4 (A and C), J558 [H-2$^d$] (B), or P815 (D) that had been first incubated either in medium (□) or with titrated concentrations of B6.K1 fragments (○), BALB.HTG fragments (●), ovalbumin fragments (△), or $\beta_2M^b$ 77–89 peptide (▲). Effector:target ratios were 9:1 (A and B) or 19:1 (C and D). Spontaneous release of target cells ranged between 7.5 and 13.6%. The actual concentration of the active fragment in the B6.K1 liver digest is less than 1% of the total, as judged from the amount of semipurified active peptide eluted from a reversed-phase HPLC column. The 709K1-5 and 709K1-6 lines were produced by culture of $8.0 \times 10^7$ spleen cells from unprimed female B6.K1 (H-2$^b$) mice with either B6.K1 709K1-5) (2 mg/ml) or BALB.HTG (709K1-6) (2 mg/ml) liver protein digest in 10 ml of culture medium. Thereafter, responder cells were restimulated weekly with syngeneic feeder cells and initially a concentration of 2 mg/ml, then 0.2 mg/ml, of the respective digest in interleukin-2–supplemented medium. For preparation of protein digests, livers from 15 female B6.K1 or BALB.HTG mice were homogenized in 200 m*M* tris-HCl, 10 m*M* EDTA at *p*H 8.12, sonicated, and stirred for 1 hour in 1.5% (v/v) Triton X-100. Proteins were isolated from the supernatant by acetone precipitation, washed, and dried. The acetone powder (50 mg) was digested with 1 mg of trypsin for 5 hours at 37°C in 20 m*M* tris buffer, *p*H 8.5. As a control antigen preparation, ovalbumin (Sigma) was treated in the same way.

our CTL lines, which are likely to be of low affinity. At present, we cannot distinguish between these two possibilities.

The biological impact of our findings is as follows. First, we have confirmed the hypothesis that nonphysiologic combinations of self MHC and self peptides do not induce thymic negative selection. The presence in normal mice of such CTLs further illustrates how economic the immune system is with its T cell repertoire: it tries to keep the "hole" in this repertoire as small as possible. Previous examples of this tendency are that CTLs specific for combinations of self peptides and foreign MHC are not deleted (*2*), and that polyploid frogs selectively reduce the number of expressed MHC alleles (*10*).

Second, our data may have a bearing on the etiology of autoimmune diseases, although the CTLs described here are not autoreactive because they do not recognize autologous cells. However, pathological conditions may lead to expression of nonphysiologic combinations of MHC and self peptides, for example, if the pathogen activates unusual proteases or if it enhances the expression of self proteins normally kept at low concentrations. Under such conditions, CTLs specific for pathological combinations of self peptides and self MHC may cause

autoimmunity. There is evidence that CTLs recognize a peptide from a self heat shock protein and that these cause tissue destruction during mycobacterial infection (*11*).

Third, one could speculate that the relative ease in the production of CTLs against randomly selected self peptides could be related to positive thymic selection. It is a paradox of T cell development that T cells are positively selected for the recognition of complexes of self MHC and foreign peptide in the absence of foreign peptide (*12*). As a way out of this dilemma, altered MHC molecules or special peptides presented by thymic epithelium have been discussed (*1, 13*). Our data raise a third possibility, that the CTLs specific for virtually nonphysiologic self peptide sequences have been selected for on thymic epithelial cells by presentation of these peptides. A mechanism for production of peptides through a novel type of RNA not under conventional transcription control (*14*) or thymus-specific proteases could initiate the presentation of self peptides on thymic epithelium that would be nonphysiologic elsewhere in the immune system, thereby mimicking a universe of foreign peptides.

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# Endogenous Cholecystokinin Reduces Feeding in Young Rats


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**The hypothesis that endogenous cholecystokinin (CCK) released from the small intestine during feeding causes satiety was tested in rat pups, 9 to 12 days old. Intragastric administration of soybean trypsin inhibitor, a procedure that releases CCK from the small intestine, decreased the subsequent intake of a test meal. This effect was reversed by prior treatment with MK-329, a selective antagonist of CCK at alimentary-type CCK (CCK-A) receptors. Thus, endogenous, small intestinal CCK can cause satiety in the neonatal rat and this effect involves CCK-A receptors.**


CHOLECYSTOKININ (CCK) IS A PEPtide that is found in the periphery, where it acts locally and hormonally, and in the central nervous system, where it acts as a neuromodulator and a neurotransmitter. The peptide has diverse effects, both gastrointestinal (for example, on gastric motility, pancreatic and biliary secretion, and gastric emptying) and behavioral (for example, on feeding and sleeping). During development, CCK exerts trophic effects on the gastrointestinal tract (*1*) and influences learning and stress responsivity (*2, 3*). This report focuses on the hypothesized satiating effect of endogenous CCK released from the small intestine in young (preweanling) rats.

In 1973, we hypothesized that CCK released from the small intestine by food ingested during a meal was part of the mechanism that terminated the meal and elicited postprandial satiety (*4*). Subsequent work has shown that peripherally administered CCK reduces feeding in rats, from birth through adulthood (*5–6*), and in many other species, including humans (*7*). Three observations suggest that the satiating effect of peripherally administered CCK in adult rats is mediated by CCK-A (alimentary) receptors (*8*) outside the blood-brain barrier: (i) peripherally administered CCK does not penetrate the blood-brain barrier (*9*); (ii) the relative potency of CCK-8, desulfated CCK-8, CCK-4, and gastrin for inhibiting food intake (*6, 10*) is similar to the relative potency of these peptides for binding to the CCK-A receptor, but not for binding to the brain-type receptor (CCK-B) (*8*); and (iii) MK-329, a potent and selective antagonist for CCK-A receptors (*11*), administered in low doses decreases the satiating effect of peripherally administered CCK-8 (*12–14*).

We tested this hypothesis further by investigating whether endogenous CCK released from the small intestine can inhibit milk intake in 9- to 12-day-old rats by acting on CCK-A receptors.

Pregnant Sprague-Dawley rats (Taconic Farms, Germantown, New York) were obtained 1 week before delivery, housed indi-


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