

# Unique T Cell Antagonist Properties of the Exact Self-Correlate of a Peptide Antigen Revealed by Self-Substitution of Non-Self-Positions in the Peptide Sequence

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The role of self-peptides in shaping the T cell receptor (TCR) repertoire remains to be established. While TCR reactive to certain self-peptides are thought to be deleted in the thymus, the selection of TCR specificity for foreign peptide reactivity appears to require recognition of self-peptide(s) bound to the groove of thymic major histocompatibility complex (MHC) molecules. This dichotomy suggests that different TCR affinities, accessory signals, and/or different sets of self-peptides dictate the eventual fate of any given TCR-bearing clone. Recently, it has been established for several T cell epitopes that derivatives with substitutions in TCR-contact residues can antagonize the proliferation of T cell clones against the wild-type peptide antigen. Moreover, these altered peptide ligands have demonstrated activity in the positive selection of thymocytes with TCR reactive to the wild-type peptide antigen. We have investigated the specificity of T cell antagonism with step-wise substitution of self-amino acids into each nonconserved position of a 12-amino-acid foreign peptide antigen. Our data demonstrate that the ability to antagonize proliferation without competition for MHC binding is unique to the exact self-derivative, where all five of the self-substitutions are inserted. These properties may specifically allow certain self-peptides to downregulate T cell activation to the foreign ligand and/or provide a source of stimulation for immunologic memory. © 1996 Academic Press, Inc.

## INTRODUCTION

Mature T cells encountering peptide:MHC at sufficient density on peripheral antigen-presenting cells (APC) are usually stimulated to proliferate and differentiate into effector cells. By contrast, when thymocytes of the same TCR specificity encounter antigen in

the thymus they undergo apoptosis (1, 2). Recent studies have shown that single amino acid substitutions in the peptide component of the foreign peptide:MHC ligand can alter this relationship such that the peptide derivative sends a negative signal to the mature T cell and leads to positive selection of thymocytes (3). These altered peptide ligands (APL)<sup>2</sup> function as partial agonists, and/or antagonists, of T cell proliferation against the wild-type foreign peptide (3–7). Aside from their potential role in TCR selection, APL have also been shown to occur naturally in HIV and hepatitis B virus infections and have demonstrated antagonist effects on CTL destruction of infected target cells (8, 9). Clearly, endogenous peptides with limited homology to foreign epitopes exist within the pool of “self-”peptides and may select TCR of appropriate affinity (10, 11). To date, however, the relationship between the sequence of a foreign epitope and its self-correlate with regard to agonist/antagonist effects on T cell activation or TCR selection has not been established.

In this report, we investigate the fine specificity of T cell antagonism with a series of stepwise substitutions (self for non-self) at the nonconserved positions of a minimal antigenic peptide. We examine this set of “self-replacement” analogs for T cell proliferation, competition for MHC binding on the APC surface, and for the ability to antagonize T cell proliferation. Our results demonstrate that the exact self-derivative is unique in its functional activities, i.e., sending a dominant negative signal without significantly decreasing the level of the foreign ligand presented to the TCR. Together, these findings support the notion that TCR are selected on precisely matched versions of peptide immunogens in the thymus to enable the effect of low-density T cell antagonism by such self-peptides within the mature T cell response.

<sup>2</sup> Abbreviations used: APL, altered peptide ligand; hCol IV, type IV human collagen;  $\alpha 2(\text{IV})$ , the  $\alpha 2$  chain of human collagen type IV; SWMb, Sperm whale myoglobin; Hu12, 12-amino-acid minimal peptide from human collagen IV sequence; Mo12, corresponding minimal peptide of the mouse collagen IV sequence.

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## MATERIALS AND METHODS

*Mice*

A.SW/SnJ (H-2<sup>s</sup>, I-A<sup>s</sup>), A.BY/SnJ (H-2<sup>b</sup>, I-A<sup>b</sup>), and C57Bl/6J (H-2<sup>b</sup>, I-A<sup>b</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were used between 8 and 12 weeks of age and experimental groups were age and sex matched for these studies.

*Antigens*

Sets of synthetic peptides corresponding to the biologically active sequences were synthesized by Research Genetics (Huntsville, AL), and all peptide structures were confirmed by mass spectrometry (Mass Spec Lab, University of Kansas, Lawrence, KS). The structures of the peptides are shown in Fig. 1. Peptides were biotinylated, as previously described (12), with a short-chain NHS-biotin (*N*-hydroxyl-succinimido-biotin) (Sigma Chemical, St. Louis, MO). To verify biotinylation and MHC binding, detergent-extracted I-A molecules were incubated with the biotinylated peptide, subject to SDS-PAGE without boiling, transferred to nitrocellulose, and probed with avidin-HRPO as previously described (13). Densitometry of these blots detected bands of appropriate molecular weight corresponding to class II MHC as detected in separate lanes with a biotinylated MAb (clone 10-3-6.2, American Type Culture Collection (ATCC), Rockville, MD) (Murray *et al.*, manuscript in preparation).

*T Cell Cultures*

Mice were immunized subcutaneously with 40 nmol of peptide emulsified 1:2 in CFA (H37RA, Difco Laboratories, Detroit, MI). After 10 days, draining lymph nodes were harvested and made into a single-cell suspension. T cell cultures and clones were generated as described by Hom *et al.* (14). Briefly,  $5 \times 10^6$  lymph node cells were cultured for 4 days in 24-well plates (Corning, Corning, NY) with 10  $\mu$ M Hu12 peptide in Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 50  $\mu$ M  $\beta$ -mercaptoethanol (Bio-Rad, Hercules, CA), containing 5% FBS (Hyclone, Logan, UT). At 4- to 6-day intervals, half of the media were replaced with Click's containing 5 U/ml human recombinant IL-2 (R & D Systems, Minneapolis, MN) and the cultures were expanded into T25 flasks (Corning). Individual T cell clones were isolated by two cycles of limiting dilution cloning and all clones were verified by flow cytometry as CD4<sup>+</sup>CD8<sup>-</sup>, with single V $\beta$  expression analyzed by a panel of V $\beta$ -specific MAb (PharMingen, San Diego, CA). Each clone showed either a single uniform peak of staining with just one of the MAb of the panel or did not react with any of the MAb tested (Table 1). All of the clones reported here have been in culture for 1–2 years and display consistent

cytokine patterns (Th1, Th2, or Th0) as determined by peptide stimulation of IL4, IL2, and IFN- $\gamma$  (see below).

*Cytokine Release Assay for Th Type*

The assay system has been previously reported (12). Briefly, Falcon 96-well polyvinyl chloride plates were coated overnight with capture antibodies at 2.5–4  $\mu$ g/ml in 0.1 M NaHCO<sub>3</sub>, pH 8.2. The next day, plates were blocked with 5% FBS-PBS for 1 hr, followed by washing twice in PBS-Tween 20 (0.05% v/v). Th clone culture supernatants were titrated in triplicate onto these blocked wells and incubated along with corresponding titrations of recombinant standard cytokines for 16 hr at 4°C. Plates were then incubated with biotinylated detection antibodies (0.67  $\mu$ g/ml in 5% FCS-PBS), followed by avidin-HRPO (5  $\mu$ g/ml in 5% FBS-PBS). Finally, the plates were washed six times with PBS-Tween and incubated for 10 min with 0.3 mg/ml ABTS (2,2'-azino-di(3-ethyl-benzthiozoline-sulfonic acid)) substrate (Cat. No. A-1888, Sigma Chemical), 0.03% (v/v) hydrogen peroxide in 0.1 M citrate buffer, pH 4.35. Reaction product was measured as optical density at 405 nm with an Emax automatic plate reader (Molecular Devices, Palo Alto, CA). All antibodies and standard cytokines for the ELISAs were purchased from PharMingen.

*T Cell Proliferation Assay*

The assay system has been previously described (12). Briefly, in 96-well plates (Corning),  $5 \times 10^4$  T cells,  $5 \times 10^5$  irradiated (2000 Rad) syngeneic spleen cells, and peptides were incubated in 200  $\mu$ l Click's medium for 48 hr. Triplicate wells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]-thymidine (ICN Biochemicals, Irvine, CA) for 18 hr and cells harvested onto glass fiber filters with a PHD cell harvester (Cambridge Technology, Watertown, MA). Thymidine incorporation was measured by liquid scintillation in a Beckman LS6000 counter and standard errors were computed.

*T Cell Proliferation Antagonism Assay and Antagonism Index*

Proliferation assays were set up as above; however, the altered peptides were used as inhibitors of proliferation at varying concentrations from 200 to 0.2  $\mu$ M with a constant concentration, 0.1  $\mu$ M, of the Hu12 peptide agonist. Percentage antagonism was calculated as follows: (mean cpm without inhibitor – mean cpm with inhibitor)/(mean cpm without inhibitor). For comparison, an antagonism index was defined as (percentage inhibition of T cell proliferation/percentage inhibition of MHC binding) (see below). Small indices indicate a greater contribution to antagonism by competition for MHC presentation on the APC surface, whereas large indices indicate greater TCR-mediated antagonism.

*Peptide Competition Assay for Cell Surface Ligand Density by Flow Cytometry*

We used a method previously described to determine whether individual members of the set of altered peptides could compete with the Hu12 agonist for MHC binding on live APC (12, 15). APC were prepared from groups of three naive animals, and T cells were depleted with a cocktail of monoclonal antibodies [anti-CD8 (TIB-105, -210, ATCC), anti-CD4 (GK1.5, ATCC), and anti-Thy1 (Y19, Yale University)], followed by C-mediated lysis (LowToxM rabbit complement, Accurate, Westbury, NY). The resulting APC suspension, in HBSS, was then fractionated over lymphocyte separation medium (LSM, Organon Teknika, Rockville, MD) to remove nonviable cells. Viable cells ( $1 \times 10^6$ ) were incubated with biotinylated peptide antigens at 37°C, 5% CO<sub>2</sub> for 16 hr together with unlabeled inhibitor peptides. All binding reactions were done in sterile 0.5% BSA-PBS. After binding and each subsequent staining step, cells were washed three times with 5% FBS-PBS buffer and kept on ice. For staining, avidin-FITC was incubated with the cells on ice for 30 min, followed by biotinylated antiavidin for 1 hr, and then again with avidin-FITC. Avidin and antiavidin reagents were purchased from Vector Laboratories (Burlingame, CA). After washing, the cells were fixed in 1% paraformaldehyde-PBS and analyzed with a FACScan (Becton-Dickinson, Mountain View, CA). Data were acquired on 10,000 cells for each sample and live cells were gated by forward/side scatter; in all samples the live cells analyzed were found in this same gate.

RESULTS

*N- and C-Terminal Nonconserved Positions of the Human Collagen Type IV (hCol IV) Minimal Peptide Antigen Control T Cell Recognition*

We previously mapped the minimal immunodominant peptide of hCol IV, an immunogenic polypeptide that elicits Th1-like immunity in I-A<sup>S</sup> genotype mice and Th2-like immunity in I-A<sup>b,d,k</sup> strains (12). This 12-amino acid peptide, which corresponds to amino acids 675-686 in the α2(IV) chain of the hCol IV molecule (16), was identified as the minimal peptide on the basis of the above biologic activity; that is, the C-truncated 11mer, or the N-truncated 11mer, of this sequence does not elicit the MHC-linked Th1/Th2 response patterns observed with the 12mer minimal peptide and the whole hCol IV molecule (12). When compared to sequences in the data bases, the best match is the corresponding sequence of the mouse α2(IV) chain (amino acids 670-681) (16-18). As shown in Fig. 1, the peptide antigen and the mouse self-correlate share a core motif of seven residues (- - - Q P G C I - G P -) and differ at the N- and C-terminal positions; (E A I . . . . G . . K) for human versus the (V V V . . . . E . . T) mouse

Peptide	Sequence
Hu12	675-E <u>A</u> I <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>K</u> -686
Mo12	670-V <u>V</u> V <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>T</u> -681
T12	E <u>A</u> I <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>T</u>
E9	E <u>A</u> I <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>K</u>
V3	E <u>A</u> V <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>K</u>
V2	E <u>V</u> I <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>K</u>
V1	V <u>A</u> I <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>K</u>
V3T	E <u>A</u> V <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>T</u>
ET	E <u>A</u> I <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>T</u>
V3ET	E <u>A</u> V <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>T</u>
V1V2ET	V <u>V</u> I <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>T</u>
V1V3ET	V <u>A</u> V <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>T</u>
V2V3ET	E <u>V</u> V <u>Q</u> P <u>G</u> C <u>I</u> E <u>G</u> P <u>T</u>
VVV	V <u>V</u> V <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>K</u>
VVVT	V <u>V</u> V <u>Q</u> P <u>G</u> C <u>I</u> G <u>G</u> P <u>T</u>
SWMb	110-A <u>I</u> I <u>H</u> V <u>L</u> H <u>S</u> R <u>H</u> P-120

FIG. 1. Amino acid sequences of self-substituted peptides. All sequences are denoted in single-letter code. Hu12, minimal immunodominant peptide from the α2(IV) chain of human collagen type IV (12, 17). Mo12, corresponding mouse (self-) peptide from the α2(IV) chain of mouse collagen type IV (12, 18). The core group of seven residues is underlined in each peptide, and the other positions have been substituted (mouse-for-human) to create each self-substituted derivative. For example, peptide T12 has the threonine of the mouse sequence inserted at position 12 for the human lysine at that position. Multiple substitutions follow this nomenclature, dropping the number where it is understood; e.g., V3T has valine for isoleucine at position 3 and threonine for lysine at position 12. SWMb, peptide from sperm whale myoglobin.

sequence. Strikingly, when we compared several I-A<sup>S</sup>- versus I-A<sup>b</sup>-derived Th clones for their sensitivity to substitutions between the N- and C-terminal amino acids of these sequences (Fig. 2, Table 1), we found that I-A<sup>b</sup>-restricted clones were not sensitive to the K → T substitution at the peptide's C-terminus, whereas this change created a partial agonist peptide with regard to the I-A<sup>S</sup>-restricted clones (compare clone responses to peptide T12 versus the wild-type Hu12 peptide; Fig. 2, Table 1). A similar I-A genotype-linked difference was observed at the N-terminus, where the E → V substitution rendered the peptide nonreactive with I-A<sup>b</sup> clones, yet had a limited (partial agonist) effect on I-A<sup>S</sup>-restricted Th clones (compare peptide V1 to the wild-type Hu12 peptide; Fig. 2, Table 1).

Interestingly, with multiple self- for-non-self-substitution, both I-A genotype clones displayed reactivity similar to that observed with the mouse peptide, Mo12. For example, T12 and E9 had different effects on clones derived from each I-A genotype; however, the ET peptide with both substitutions was essentially like the Mo12 in that it was nonstimulatory for both sets of Th clones (Fig. 2, Table 1). Therefore, with regard to the induction of proliferation (agonist activities), it appears that single changes (T12, V3, V1, and E9) are still able to induce proliferation at a reduced level, which displays MHC-linked differences in peptide specificity. A slight exception was peptide V2 which exhibited very limited activation with any of the Th clones, thus resembling the Mo12 self-peptide. Note, however, that

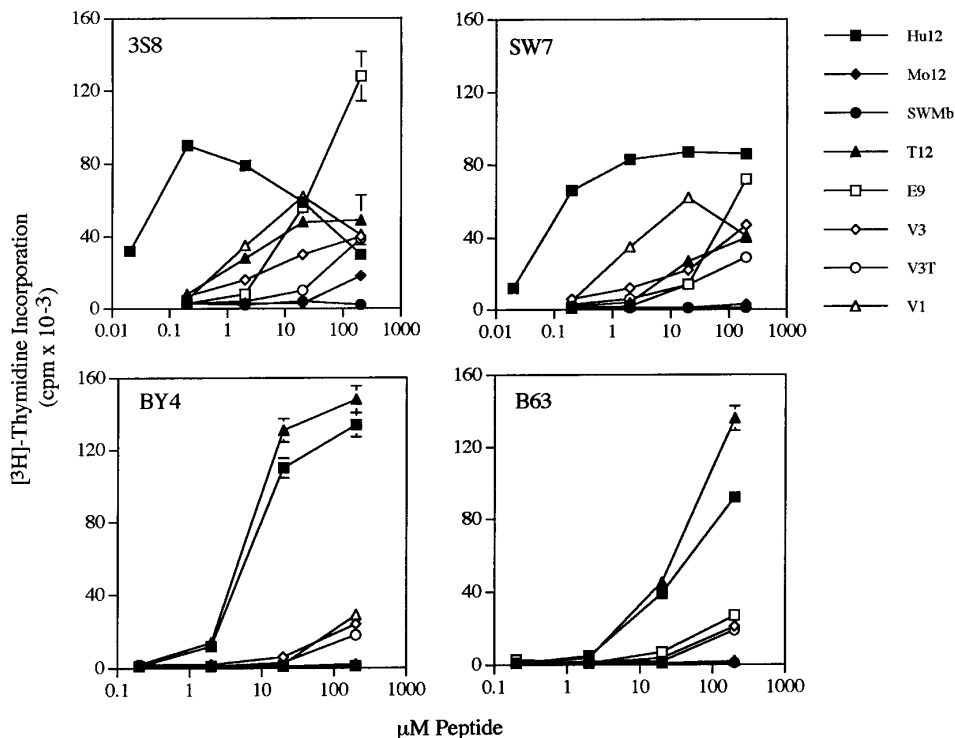


FIG. 2. Proliferation of I-A<sup>S</sup> and I-A<sup>b</sup> CD4<sup>+</sup> T cell clones to self-substituted peptides. Peptides were incubated with  $5 \times 10^4$  T cells and  $5 \times 10^5$  irradiated (2000 Rad) syngeneic spleen cells for 48 hr followed by an 18-hr pulse with  $1 \mu\text{Ci}$  [<sup>3</sup>H]thymidine. Cells were collected and thymidine incorporation was measured by liquid scintillation. Values represent mean cpm of triplicate wells ( $\times 10^{-3}$ ) and error bars represent standard deviations. Clones 3S8 and SW7 are I-A<sup>S</sup>-derived and clones BY4 and B63 are I-A<sup>b</sup>-derived.

peptide V1V3ET, with just a foreign alanine at position 2, did not activate proliferation like Hu12. Thus, it was not possible to dissect the sequence into stepwise changes that explained either the lack of agonist activity by the Mo12 peptide or the induction of proliferation at low peptide doses by the Hu12 antigen. Multiple changes (ET, V3T, V3ET, V1V2ET, etc.) did, however, render the ligand essentially inactive; similar to the mouse correlate, with regard to the stimulation of T cell proliferation (Table 1). From these data, we predicted that the ability to inhibit T cell proliferation to the Hu12 peptide would mirror this inactivation as the peptide was changed to self. However, as described in the next section, we found that the sensitivity for self was more acute with respect to antagonism than it was with respect to Th proliferation.

#### *Self-Substituted Derivatives of the hCol IV Minimal Peptide Show Antagonism of Th1 Proliferation*

Certain partial agonist peptides have been shown to antagonize the responses of Th clones to the wild-type peptide antigen (3–7). Therefore, we co-incubated the various derivatives of the peptide antigen (Fig. 1), at increasing doses with a constant (half-maximal) concentration of the human peptide agonist, Hu12. As illustrated by Fig. 3 and in Table 2, we found that three

peptides of the panel were able to inhibit proliferation at a nominally stimulating dose of the foreign antigenic peptide. Clearly, most members of the panel were not antagonists, and antagonist activity was specific to the Th1 clone, 3S8 (i.e., other clones, like SW7, were not antagonized by these peptides even though several of them were partial agonists of proliferation). This suggested true TCR antagonism involving TCR specificity/affinity of the Th clone, rather than an antigen nonspecific effect of this particular set of peptide derivatives. Importantly, the derivative showing the most antagonist activity (53% inhibition of proliferation) was the mouse peptide correlate (i.e., where all of the five non-conserved positions of the Hu12 peptide had been switched to the corresponding mouse amino acid). Note that this is not a cumulative effect, as peptide T12 and peptide V3, each with just a single substitution, display more inhibition than peptides like VVVT, which differs from the mouse correlate only at position 9 (Fig. 1, Table 2).

#### *The Mo12 Antagonist Peptide Does Not Compete Effectively with the Hu12 Agonist Peptide for Cell Surface Density on the APC*

The relative abilities of the peptide derivatives to compete for MHC binding could explain their relative

TABLE 1  
Relative Th Cell Activation by Self-Substituted Peptides<sup>a</sup>

T cell clone <sup>b,c</sup>	Peptides ( $\mu\text{M}$ at 50% maximal proliferation) <sup>d</sup>														
	Hu12	Mo12	T12	E9	V3	V2	V1	V3T	ET	V3ET	V1V2ET	V1V3ET	V2V3ET	VVV	VVVV
3S8 (Th1 V $\beta$ 14)	0.1	90	20	30	9	90	3	80	100	100	100	100	>200	70	>200
SW4 (Th1)	0.08	>200	30	60	40	100	3	40	100	100	100	90	>200	70	>200
SW7 (Th2 V $\beta$ 12)	0.08	>200	20	80	30	80	2	50	100	90	90	90	>200	50	>200
BY4 (Th2 V $\beta$ 4)	10	>200	8	>200	80	>200	70	80	>200	100	>200	>200	>200	>200	>200
BY5 (Th0)	20	>200	9	>200	70	>200	80	80	>200	100	>200	>200	>200	>200	>200
BY7 (Th1)	20	>200	8	20	70	>200	70	80	>200	100	>200	>200	>200	>200	>200
B63 (Th1 V $\beta$ 2)	40	>200	20	30	20	>200	>200	20	>200	100	>200	>200	>200	>200	>200

<sup>a</sup> Columns represent titration curves run with each synthetic peptide listed in Fig. 1.

<sup>b</sup> Th type determined by clone response to Hu12 peptide with cytokine ELISA (see Materials and Methods).

<sup>c</sup> V $\beta$  expression (where identified) was determined by flow cytometry with V $\beta$ -specific monoclonal antibodies (data not shown).

<sup>d</sup> Values are micromolar concentrations of peptide necessary to induce 50% maximal proliferation of each clone (>200 indicates that no proliferation was observed even at 200  $\mu\text{M}$  peptide).

effects on Th proliferation by a simple competition mechanism (19, 20). In this model, the agonist ligand density is reduced by that of the antagonist (a peptide:MHC complex to which the TCR has lower affinity), in this case the self-antagonist, Mo12 peptide. Strict adherence to this mechanism would predict that the ability of a given derivative to compete for MHC presentation would be of the order Mo12 >> T12  $\cong$  V3 >>> all others. However, as is shown in Fig. 4, it is clear that the Mo12 correlate does not effectively compete with the foreign peptide for binding to the APC. Moreover, peptides like V1, T12, and V3, which were effective MHC competitors, did not as effectively antagonize T cell proliferation.

Therefore, the Mo12 self-peptide appears to be unique in that it does not stimulate proliferation on its own (an expected result), but does most effectively antagonize Th proliferation to the foreign peptide analog of its sequence. Therefore, it follows that the Mo12 derivative must deliver a dominant negative signal, because it does not require significant reduction of the stimulatory ligand density on the APC surface.

#### *TCR-Mediated Antagonism of Th1 Proliferation Shows Fine Specificity for the Exact self-Correlate of the Peptide Antigen*

As a means of comparison between the peptide derivatives, we compiled the percentage inhibition data and MHC competition results of all the derivatives tested (Table 2). We derived a TCR antagonist index (Ai) as the ratio of these two parameters, respectively, where a larger number reflects the ability of the peptide to antagonize proliferation without significantly decreasing the agonist ligand density. From this analysis (Table 2), it can be seen that the Mo12 peptide is unique in its effect (Ai = 3.31 versus Ai = 0.67, for the next highest derivative, V3). Partial agonist peptides like T12 and V3, which compete for MHC presentation, were able to inhibit Th proliferation by 32–33% and these peptides reduced density of the biotinylated agonist peptide by 48–65% in the APC binding assay. However, the inhibition with these peptides was significantly greater than that observed with the SWMb peptide (at 16%) which was a better MHC competitor (at 84%), and SWMb peptide would not likely ligate the TCR on its own. Thus, simple MHC competition does not explain the antagonist effect of partial agonists, but it does suggest that for these peptides the negative signal delivered may result from an ~50% dilution in the engagements of the TCR with the APL (see below). By contrast, the greater antagonist effect of the self-peptide (Ai = 3.31) actually requires little reduction in the density of the wild-type peptide; thus, the endogenous APL sends a signal that dominates T cell recognition of the foreign ligand.

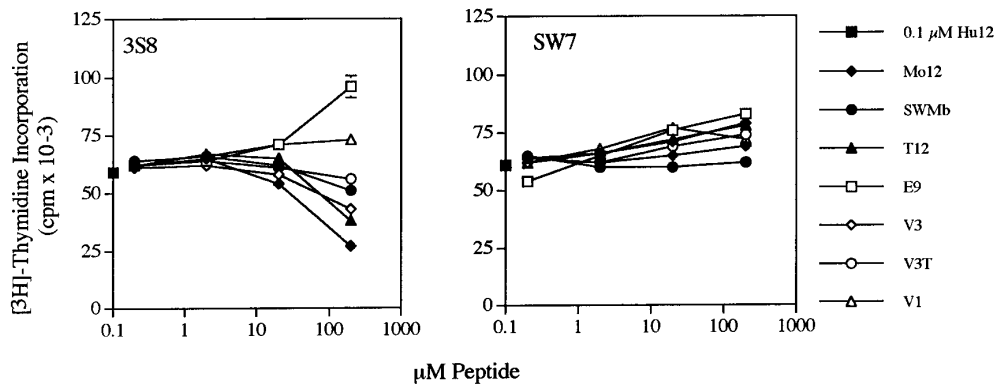


FIG. 3. Antagonism of T cell clones by self-substituted peptides. T cells were incubated with increasing doses of altered peptides as in Fig. 2; however, a dosage of Hu12 peptide sufficient to yield approximately one-half maximal proliferation ( $0.1 \mu\text{M}$ ) was co-incubated in each well. Thymidine incorporation was measured and mean cpm of triplicate wells were calculated with standard deviation.

## DISCUSSION

T cell recognition and development require specific interaction between the TCR  $\alpha/\beta$  and a peptide fragment bound to the groove of a self-encoded MHC molecule. It has been demonstrated that peptides substituted at key amino acid positions generate TCR ligands

that can antagonize the recall proliferation of mature T cell clones against their respective wild-type peptide antigens (3–5). Most interestingly, certain antagonist peptides form ligands that provide the recognition signal for positive selection during T cell development (3, 7, 10). Molecular matching between foreign peptides and self-peptides has been proposed such that reactivity patterns of individual T cell clones may reflect upon previous encounters with the endogenous ligand during T cell development (21). To elucidate this relationship between self and non-self, we have investigated the fine specificity of murine T cell clones for a 12-amino-acid minimal peptide from human collagen type IV. Single and multiple substitutions of the five positions which differ from the mouse correlate of the peptide revealed a striking MHC-linked split within our panel of CD4 T cell clones. Monoclonal T cells derived from I-A<sup>b</sup> mice did not recognize peptide analogs where the N-terminal position (glutamate, E) was substituted with the amino acid present in the mouse sequence (valine, V). Interestingly, these clones were not significantly affected by C-terminal substitution of the position 12 lysine with threonine. By contrast, I-A<sup>S</sup> T cell clones were at least three orders of magnitude less sensitive to the N-terminal substitution, yet they did not react well with derivatives of the peptide substituted at the C-terminal position. Since several clones from each MHC genotype displayed these distinct patterns of peptide recognition, these data may reflect distinct CDR3:peptide contacts with the terminal peptide positions (22). Alternatively, the peptide termini may effect distinct conformations of the ligand (peptide or MHC component) that create a dominant negative influence on diverse CDR3:peptide interactions in all TCR selected within a given MHC genotype (23). In either case, our direct binding and inhibition data demonstrated that the effects of the peptide termini on T cell proliferation did not derive from an influence on the

TABLE 2  
Relative Peptide Antagonism of T Cell Proliferation

Peptide <sup>a</sup>	% inhibition T cell proliferation <sup>b</sup>	% inhibition ligand density <sup>c</sup>	Peptide antagonism index <sup>d</sup>
Antagonists			
Mo12	53	16	3.31
SWMb	16	84	0.19
T12	33	65	0.51
V3	32	48	0.67
Nonantagonists			
E9	0		
V3T	7		
V1	0		
V2	4		
ET	2		
V3ET	2		
V1V2ET	0		
V1V3ET	0		
V2V3ET	0		
VVV	2		
VVVT	0		

<sup>a</sup> Peptide sequences listed in Fig. 1.

<sup>b</sup> Proliferation inhibition calculated as (mean cpm without inhibitor – mean cpm with inhibitor)/(mean cpm without inhibitor)  $\times$  100.

<sup>c</sup> Inhibition of ligand density defined as (percentage of cells in marker 2 without inhibitor – percentage of cells in marker 2 with inhibitor)/(percentage of cells in marker 2 without inhibitor).

<sup>d</sup> Antagonism index defined as (percentage inhibition of T cell proliferation/percentage inhibition of ligand density).

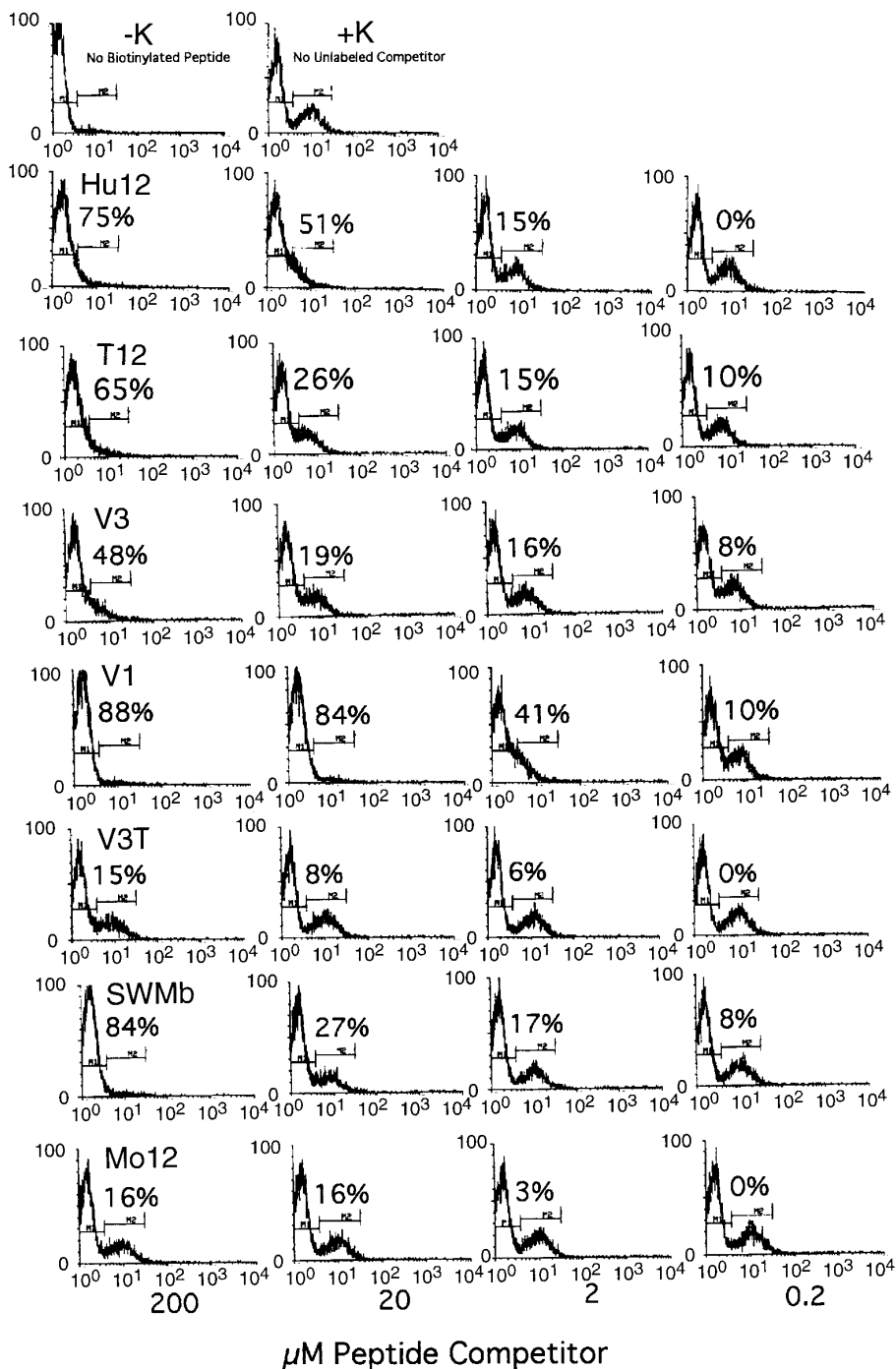


FIG. 4. MHC binding competition of biotinylated Hu12 with unlabeled self-substituted peptides. T-depleted splenocytes were generated as described under Materials and Methods. Viable cells ( $1 \times 10^6$ /well) were incubated separately with increasing concentrations of each unlabeled peptide and  $1 \mu$ M biotinylated Hu12 overnight in 0.5% BSA-PBS at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . Cells were washed and stained with avidin-FITC, then antiavidin, and avidin-FITC again. Bound Hu12 peptide density was examined by flow cytometry on 10,000 events gated by forward/side scatter on live lymphocytes. Percentage binding inhibition (shown) was calculated as (percentage of cells in marker 2 without competitor peptide - percentage of cells in marker 2 with competitor peptide)/(percentage of cells in marker 2 without competitor peptide)  $\times 100$ .

overall presentation densities of the respective peptide:MHC ligands. We have not been able to identify clones from the I-A<sup>b</sup> genotype which show antagonism by the endogenous Mo12 peptide (to date, a total of

10 clones have been examined; Schountz and Murray, unpublished data). Taken together, our results indicate that the ability of the mouse peptide to antagonize the mature T cell response is linked to MHC genotype and

to apparent TCR contacts dictated by the C-terminus of the minimal peptide.

Qualitative differences in the signal delivered by APL have been suggested by the work of Sloan-Lancaster *et al.* (5) and Madrenas *et al.* (6), which provided evidence that altered phosphorylation of the TCR  $\zeta$  chain and activation of the ZAP-70 kinase are linked to the antagonist effect. The crystal structure of the ZAP-70 SH2 domains bound to a phosphorylated  $\zeta$  peptide suggests that distinct signals may be delivered through an allosteric mechanism (24). We have shown that antagonism by an endogenous APL did not require a significant decrease in the presentation density of the Hu12 agonist. Therefore, these data are consistent with a mechanism involving a qualitatively dominant signal delivered by the APL and may require little if any diminution of the activation signal delivered by the agonist peptide (5, 7, 24). Alternatively, Valitutti *et al.* have reported evidence for serial engagement of multiple TCR by the same peptide:MHC complex. By this model, higher TCR affinity could lead to decreased stimulation, because a lower off-rate would tend to decrease critical reengagement of the TCR complex (20). In the present study, the SWMb peptide significantly reduced the level of the Hu12 agonist with little decreased proliferation. This finding supports the idea of a small number of serially engaged Hu12:I-A<sup>S</sup> complexes, because activation was not significantly reduced by strict MHC competition. Most importantly, we found a peptide which does not effectively compete for MHC presentation (the endogenous Mo12 peptide) to be the best antagonist of T cell proliferation. Therefore, it is possible that the Mo12 ligand is preferred by the TCR (i.e., the TCR has a higher affinity for self), which would also explain why a reduction in agonist density is not required for signal dominance. Note that this quantitative mechanism is also consistent with alternative phosphorylation/signaling delivered by the endogenous APL. Finally, distinguishing between qualitative and quantitative mechanisms in this system may have important implications for T cell development, if our self-antagonist functions in the positive selection of thymocytes, as previously demonstrated in TCR-transgenic systems (7, 10).

In summary, we have shown that each position of a foreign epitope, which is not conserved with the endogenous correlate of the sequence, is required for the agonist and MHC binding characteristics of the peptide. Interestingly, we found that the reciprocal of this logic applies to the functional activity of the endogenous self-peptide as well. In this case, effective TCR antagonism required that each foreign position was returned to the self-amino acid placed by evolution in the mouse protein sequence. These results indicate a fine specificity for the mechanism of antagonism by an endogenous

APL. The present challenge is to determine if this effect is an exploitable by-product of the TCR selection process.

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