Endogenous Altered Peptide Ligands Can Affect Peripheral T Cell Responses

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Summary

T cells potentially encounter a large number of endogenous self-peptide/MHC ligands in the thymus and the periphery. These endogenous ligands are critical to both positive and negative selection in the thymus; however, their effect on peripheral T cells has not been directly ascertained. Using the murine allelic $Hb^d(64\text{-}76)/I\text{-}E^k$ self-antigen model, we have previously identified altered peptide ligands (APLs) which are able to stimulate some but not all TCR-mediated effector functions. To determine directly the effect of endogenously synthesized APL/MHC complexes on peripheral T cells, we used a TCR transgenic mouse which had reversed our normal antigen system, with Ser69 peptide now being the agonist and $Hb^d(64\text{-}76)$ being the APL. In this report, we show that the constitutive level of endogenous $Hb^d(64\text{-}76)/I\text{-}E^k$ complexes presented by APCs in vivo is too low to affect the response of Ser69 reactive T cells. However, by increasing the number of $Hb^d(64\text{-}76)/I\text{-}E^k$ complexes expressed by the APCs, TCR antagonism is observed for both primary T cells and T cell hybridomas. In addition, the level of the CD4 coreceptor expressed on T cells changes the response pattern to endogenously presented $Hb^d(64\text{-}76)/I\text{-}E^k$ ligand. These findings demonstrate that T cells are selected to ignore the constitutive levels of endogenous complexes they encounter in the periphery. T cell responses can be affected by endogenous APLs in the periphery under limited but attainable circumstances which change the efficacy of the TCR/ligand interaction. Thus, endogenous APLs can play a role in both the selection of T cells in the thymus and the responses of peripheral T cells.

A series of recent studies has definitively shown that a T cell can productively interact through its TCR with less-than-optimal ligands, resulting in partial T cell activation. These ligands range from peptides with a single amino acid substitution to peptides with little amino acid similarity to the full agonist (1, 2). These peptides bind to MHC molecules with the same affinity as the immunogenic peptide, but are perceived by the TCR in a subtly different way. Recognition of these altered peptide ligands (APLs)[1] by mature T cells results in the stimulation of some, but not all, TCR-mediated effector functions (3). For example, APLs can induce cytokine production without proliferation (1), changes in the profile of cytokines produced (4), anergy (5), or TCR antagonism (6–8). We and others have viewed the recognition of APLs by T cells as part of a continuum of activation events, encompassed by the term partial T cell activation. Therefore, the several different functional assays used to describe the effect of APLs, such as anergy induction or TCR antagonism, should be viewed as part of the same overall phenomenon. In the thymus, APLs could play a major role in both positive and negative selection (9–12). The establishment of the ability of T cells to engage in partial T cell activation, including anergy induction, raised the critical issue of why peripheral T cells are not all continually anergized. Recent studies have shown that APLs do occur naturally, being generated either from self-antigens and from pathogens (2, 13–16), but their effect on peripheral T cells has not been demonstrated.

In this study, we wanted to investigate the ability of endogenously synthesized self-peptide/MHC complexes to induce partial T cell activation in peripheral T cell responses. To accomplish this, we used our well-established self-antigen model based on the murine β-minor chain of hemoglobin (Hb). The Hb β chain exists in two allelic forms, Hb^s and Hb^d, that differ by 12 amino acids, two of which are found in the immunogenic (64-76) peptide sequence. Mice expressing the Hb^s allele (CE/J and B10.BR, $H\text{-}2^k$) generate a strong T cell response to the Hb^d allelic

[1] *Abbreviations used in the paper:* APL, altered peptide ligand; Hb, hemoglobin; HEL, hen egg-white lysozyme; PECs, peritoneal exudate cells; Tg, transgenic.

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protein. From this response we have developed a large panel of T cell clones and hybridomas, all of which recognize the immunodominant $Hb^d(64-76)/I-E^k$ determinant. As shown by stimulation of these hybridomas with normal APCs from CBA/J (Hb^d, $H-2^k$) mice, the minimal $Hb^d(64-76)/I-E^k$ epitope is constitutively processed and presented from endogenous Hb^d protein (17). We have generated a TCR transgenic (G2-Tg) mouse in which the transgenic β chain derived from a $Hb^d(64-76)/I-E^k$-specific T cell clone pairs with endogenous α chains, leading to a serendipitous primary reactivity to the Ser69-peptide, an APL of $Hb^d(64-76)$ (15). In this system, we have previously demonstrated that synthetic $Hb^d(64-76)$ peptide antagonizes Ser69 reactive T cells in a specific and dose-dependent manner. Thus, this transgenic model has reversed our normal antigen system, with Ser69 being the agonist and $Hb^d(64-76)$ now being an APL. In addition, this transgenic model has allowed us to examine the effect of APLs on T cell activation and in vivo T cell development. In this study, we use antagonism of T cells as our assay for APL-induced partial T cell activation. We determine whether the Ser69 response of purified T cells or T cell hybridomas derived from the G2-Tg mouse can be antagonized by endogenous $Hb^d/I-E^k$ complexes. Our findings reveal that endogenous levels of $Hb^d/I-E^k$ complexes are too low to antagonize the T cells. However, when higher levels of complexes are expressed by the APCs, TCR antagonism is observed. We also report that changing the level of coreceptor expression directly affects the response of the T cells to the endogenous ligand. These results suggest that peripheral T cell responses can be affected by endogenous APLs under limited circumstances which influence the efficacy of the TCR/ligand interaction, such as increased number of antagonistic peptide/MHC complexes on the APCs or changes in CD4 expression on T cells.

Materials and Methods

Animals. The generation and characterization of the $Hb^{s/s}$ and $Hb^{d/s}$ G2-TCR transgenic mice have been previously described (15). The G2-Tg mice express a functional Vβ1-Dβ1-Jβ2.4 transgene that is expressed on >95% of the T cells. CBA/J ($H-2^k$, Hb^d) and B10.BR ($H-2^k$, Hb^s) female mice, 5–10 wk old, were purchased from the National Cancer Institute and The Jackson Laboratory (Bar Harbor, ME), respectively.

Antigens. The $Hb^d(64-76)$ peptide and the analogue APLs were synthesized on either an Applied Biosystems Model 432 (Foster City, CA) or a Rainin Symphony Multiplex synthesizer (Woburn, MA) and purified by HPLC using a C_{18} column. The composition, purity, and concentration of the peptides was determined by amino acid analysis using an amino acid analyzer (model 6300; Beckman Instrs., Inc., Fullerton, CA) and by mass spectrometry at the Washington University Mass Spectrometry Facility. The amino acid sequences of the peptides using the one letter code are: $Hb^d(64-76)$ = GKKVITAFNEGLK, Ser69 = GKKVISAFNEGLK, and MCC(91-103) = RADLIAYLKQATK. The Hb^s and Hb^d proteins were prepared as previously described (17). Briefly, a hemolysate was prepared from CBA/J (Hb^d) or B10.BR (Hb^s) blood by pelleting the erythrocytes, washing them twice in saline buffer, and then lysing in one volume of distilled water. Insoluble material was removed by centrifugation at 10,000 *g* for 10 min, and the resulting preparation contained >95% hemoglobin. The Hb concentration was determined by the absorbence at 280 nm.

Flow Cytometry. Single cell suspensions were stained in PBS supplemented with 0.5% BSA and 0.1% sodium azide. The anti-mouse CD4 mAb GK1.5 (American Type Culture Collection [ATCC], Rockville, MD) was used as primary reagent, followed by a fluorescein (FITC)-conjugated goat anti–rat IgG(H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells (10^6 per sample) were incubated on ice for 30 min with the primary mAb (neat culture supernatant), washed twice, and labeled with FITC-conjugated secondary Ab (1 μg/10^6 cells) for 30 min. The negative control consisted of cells stained with the secondary Ab alone. Cells were washed twice and fixed in 1% paraformaldehyde and analyzed on a FACScan® flow cytofluorometer using Cell Quest software (Becton-Dickinson, Mountain View, CA).

T Cell Hybridomas. The generation and characterization of BT9, a $Ser69/I-E^k$-specific T cell hybridoma derived from Hb^s G2-TCR transgenic mice were previously described (15). BT9-$CD4^{low}$ and BT9-$CD4^{high}$ are subclones of BT9 cells expressing low and high cell surface CD4 molecule, respectively. They were derived from the BT9 hybrid after staining for CD4 and cell sorting using a FACS®-Vantage (Becton-Dickinson) and a single-cell cloning step. For the generation of the A11.F3 hybridoma, unprimed spleen cells from $Hb^{d/s}$ G2-Tg mice were activated in vitro for 4 d with Ser69 peptide (50 μM), and fused with the BW5147 $TCR\alpha^-\beta^-$ thymoma (ATCC) according to a standard protocol (18). The clonal hybridomas were tested for antigen specificity using the B cell line CH27 as APCs (19), and were subcloned for low and high CD4 expression as described above. Three additional T cell hybridomas, YO1.6, specific for $Hb^d(64-76)/I-E^k$ (17), 2B4, specific for cytochrome $c/I-E^k$ (20) and H6.1, specific for hen egg-white lysozyme (HEL) $(84-96)/I-E^k$ (21) have been previously described. T cell hybridomas were cultured at 37°C in 5% CO_2, in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) medium supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT), 2 mM glutamax (GIBCO BRL), 50 μg/ml gentamicin, and 2×10^{-5} M 2-ME (referred to as complete media).

APC Preparation. The CH27 B cell lymphoma (22) and unfractionated, irradiated splenocytes (2,000 rads) were used as APCs where indicated. Adherent peritoneal exudate cells (PECs) were used as a macrophage-enriched population. PECs were obtained by peritoneal lavage of mice injected with ConA (60 μg/ml at day −7) and peptone (at day −3) (23). PECs (2×10^5/well) were plated in 96-well microtiter plates in complete media. After 2 h at 37°C, the non-adherent cells were removed, and complete media was added to adherent cells.

T Cell Hybridoma Activation and TCR Antagonism. T cell hybridomas were stimulated by various amounts of peptide and indicated numbers of APCs following an established protocol (19). The stimulation of the T cell hybridomas was ascertained by determining the level of IL-2 released using the IL-2–dependent cell line, CTLL-2 (ATCC), as described (24). TCR antagonism assays of T cell hybridomas were performed as described (24). Briefly, CH27 were prepulsed with 20 μM of Ser69 peptide for 2 h at 37°C. The cells were washed and plated (5×10^4/well) in flat-bottomed 96-well microtiter plates with 5×10^4 T hybridoma cells in the presence of indicated concentrations of $Hb^d(64-76)$ peptide. After 24 h, T cell hybridoma stimulation was determined by IL-2 assay as described above.

Primary T Cell Proliferation. Proliferation assays of primary T

cells were performed at 37°C in 5% CO_2, in flat-bottomed 96-well microtiter plates containing 200 μl/well of RPMI-1640 medium supplemented with 10% heat-inactivated FCS (Hyclone), 10 mM Hepes (GIBCO BRL), 2 mM glutamax, 50 μg/ml gentamicin, and 2×10^{-5} M 2-ME. Purified T cells from $Hb^{d/s}$ G2-TCR Tg mice were obtained by passing a single-cell suspension of spleen cells over a nylon wool column, followed by treatment of the non-adherent cells with anti-I-A^k (10.3.6.2), anti-I-E^k (14.4.4S) (ATCC) and guinea pig and rabbit complement (GIBCO BRL) to remove any remaining class II-positive cells. Purified T cells (5×10^5/well) were cultured in the presence of Ser69 peptide (0–100 μM), mitomycin c (Sigma Chem. Co., St. Louis, MO) treated CH27 cells (5×10^4/well), or irradiated (2,000 rads) CBA/J or B10.BR spleen cells (5×10^5/well) were used as APCs. Proliferation of the T cells was measured as [^{3}H]TdR (0.4 μCi/well) incorporation over 24 h, as described (25).

Generation of the CH27mHEL/Hb and CH27mHEL Cell Lines. To express the Hb^d(64-76) epitope on the surface of an APC, CH27 cells were transfected with a plasmid containing a chimeric form of membrane HEL into which the Hb^d(64-76) epitope was inserted. This plasmid was constructed from pCMV2-mHEL, which contains the HEL coding region from a cDNA clone joined to the 3′ end of the L^d gene (C. Nelson, manuscript in preparation). Following the work of Bodmer et al. (26), the sequence of the Hb^d(64-76) epitope was introduced between amino acids 43-44 of the HEL coding region using PCR with the following oligonucleotides: GCCTGAAAaaccgtaacaccgatgggag (coding), and CTTCGTTAAAGGCAGTTATCACCTTTTTGCCtgtagcctgggtgttgaagttac (non-coding). These oligonucleotides were designed to be directly adjacent to one another, and overlap the HEL coding region (lower case) by 20–23 bp. A StuI site was introduced at the junction between the oligonucleotides to aid in screening recombinants, and did not alter the amino acid sequence of the Hb^d(64-76) epitope. CH27 cells (5×10^6) were co-transfected by electroporation with 65 μg of pCMV2-mHEL/Hb and 3.25 μg of a plasmid bearing puromycin resistance, followed by one pulse of 960 μFarads at 225 V using a Gene pulser (Bio-Rad, Hercules, CA). Cells were seeded into flat-bottom 96-well plates and stable transfectants were selected in 1 μg/ml of puromycin (Sigma). Drug-resistant colonies were analyzed by direct surface staining using a FITC-conjugated polyclonal rabbit anti-HEL Ab (kindly provided by Emil Unanue, Washington University). A puromycin-resistant clone that expressed high levels of the recombinant protein was selected and designated CH27mHEL/Hb. A control cell line, expressing the unmodified mHEL protein, termed CH27mHEL, was generated by transfection of CH27 with 50 μg of pCMV2-mHEL in a similar manner. This plasmid contains a hygromycin B-resistance gene and the transfectants were selected using 450 μg/ml of hygromycin B (Calbiochem Novabiochem, La Jolla, CA).

Statistical Analysis. The presentation of Ser69 peptide by Hb^d-expressing APCs versus Hb^s-APCs was compared using Student's *t* test analysis (StatView II; Abacus, Berkeley, CA).

Results

Endogenous Hb^d/I-E^k Complexes Do Not Antagonize Mature T Cells. To determine the effect of endogenous APLs on peripheral T cell responses, we studied TCR antagonism of the T cell hybridoma BT9 derived from unprimed lymph node cells of G2-Tg mice (15). Unlike the bulk population of T cells derived from G2-Tg mice, BT9 responds only to Ser69, but not to Hb^d(64-76) peptide (15). However, the Ser69 response of BT9 could be antagonized in a specific and dose-dependent manner by the Hb^d(64-76) peptide when given exogenously to the APCs (15). Thus, this unique experimental system, in which Ser69 acts as an agonist and Hb^d(64-76) as an APL, allowed us to study the effect of endogenous Hb^d(64-76)/I-E^k complexes on the BT9 response. To assay for TCR antagonism by endogenous Hb^d(64-76)/I-E^k complexes, we compared the Ser69 responses of BT9 using normal APCs from Hb^d mice (CBA/J) which express the minimal Hb^d(64-76)/I-E^k epitope, and APCs from Hb^s mice (B10.BR) which do not. If endogenous Hb^d(64-76)/I-E^k complexes could antagonize BT9 cells, one would expect a depressed response to Ser69 when this peptide is presented by APCs from Hb^d mice (CBA/J) compared to APCs from Hb^s mice (B10.BR). The responses of BT9 to Ser69 were identical when the peptide was presented by peritoneal macrophages from CBA/J (Hb^d) or B10.BR (Hb^s), which had been activated in vivo to induce MHC class II expression (Fig. 1 *A*). Identical results were obtained with splenocytes (data not shown). Thus, no antagonism of the BT9 response to Ser69 was observed by endogenous Hb^d(64-76)/I-E^k complexes expressed on B cells or activated macrophages. The failure of endogenously processed Hb^d(64-76), to antagonize the BT9 response could be that the level of endogenous complexes is too low. Another explanation is that there are differences between the endogenous Hb^d/I-E^k ligand and the synthetic peptide in either length, register, or conformation bound to the I-E^k molecule, which could translate into differences in antagonist ability.

TCR Antagonism by Hb^d/I-E^k Complexes Can Be Detected by Increasing the Amount of Hb^d Protein Processed and Presented by APCs. To test the hypothesis that the level of endogenous Hb^d(64-76)/I-E^k complexes is too low to observe an antagonist effect, we added exogenous Hb^d protein to the APCs in vitro. Macrophages were used as APCs because of their high endocytic capacity. As shown in Fig. 1 *B*, significant inhibition of the Ser69 response was observed when macrophages from CBA/J mice were prepulsed with 0.5 to 1 mg/ml of Hb^d protein, as compared to presentation by Hb^s-prepulsed B10.BR macrophages. Consistent with the activity of other TCR antagonists, the antagonism by Hb^d/I-E^k complexes was only observed at low agonist concentrations (0.25 to 0.5 μM). Thus, the failure of the endogenous Hb^d(64-76)/I-E^k complexes to antagonize the Ser69 responses of BT9 was apparently due to an insufficient number of complexes expressed on each APC.

Generation and Characterization of an APC Line Expressing Membrane HEL/Hb Protein. The antagonistic effect seen by addition of exogenous Hb^d protein was most likely due to the processing and presentation of the Hb^d(64-76) epitope. To formally demonstrate this, we generated an APC line which presents high levels of Hb^d(64-76)/I-E^k complexes derived from the processing of endogenously synthesized protein.

Hemoglobin is a tetrameric protein composed of two α and two β chains. The free chains are highly unstable (27),

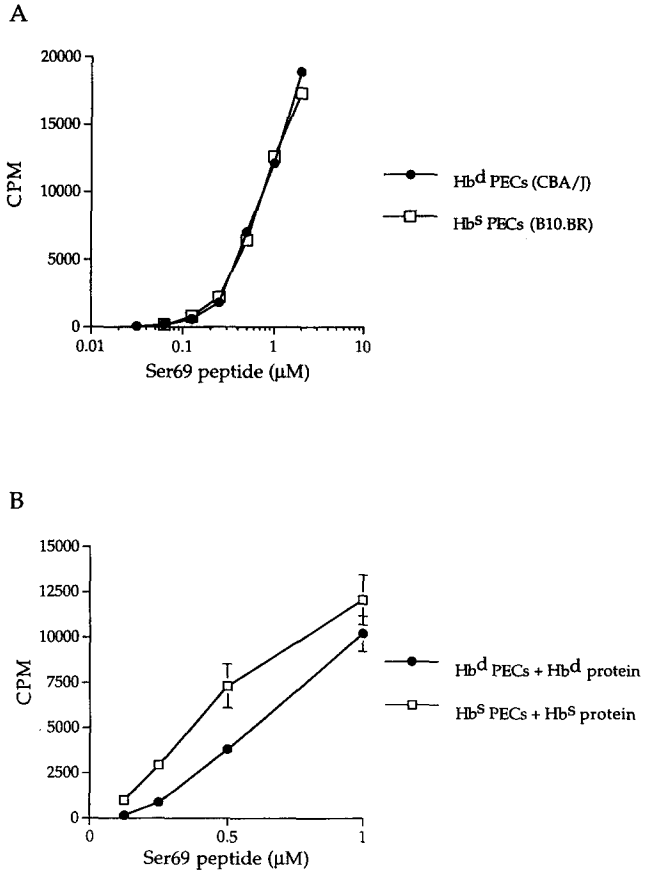

Figure 1. The ability of endogenous $Hb^d/I\text{-}E^k$ complexes to influence T cell responses depends on the level of expression. (*A*) BT9 T cells (5×10^4/well) were stimulated for 24 h with the indicated concentrations of Ser69 peptide presented by freshly isolated adherent peritoneal macrophages (PECs) (2×10^5/well) from Hb^d (CBA/J) (●) or Hb^s (B10.BR) (□) mice. (*B*) The PECs (2×10^5/well) were pulsed for 2 h with a saturating amount of Hb^d (●) or Hb^s (□) protein (0.5 to 1 mg/ml), washed and used to present the indicated concentrations of Ser69 peptide to BT9 cells (5×10^4/well). T cell hybridoma activation was measured by IL-2 production after 24 h of culture using the CTLL-2 cell line. The values represent the mean of triplicate of [^{3}H]TdR incorporation by the CTLL-2 cells. In *A*, the SD were <20% of the mean, and the data are representative of five independent experiments. In *B*, the values represent the mean ± SE of five experiments and are statistically different at the 0.5, 0.25, and 0.125 μM Ser69 concentration ($P = 0.0242$, $P < 0.001$ and $P < 0.002$, respectively).

which limits the usefulness of expressing the Hb^d(64-76) determinant in the form of free β chains. Since membrane proteins expressed on the surface of APCs are efficiently processed and presented via the class II processing pathway (28, 29), we examined the presentation of the Hb^d(64-76) determinant engineered as part of a membrane protein expressed in the APCs. We generated a chimeric membrane protein by inserting Hb^d(64-76) into a transmembrane form of the HEL protein, between amino acids 43 and 44, as originally described by Bodmer et al. (26). The presence of the Hb^d(64-76) sequence in HEL in this chimera did not appear to have a major effect on the HEL molecule itself, because conformational-dependent Abs still recognized the soluble HEL/Hb molecule, and because the chimeric molecule retained HEL enzymatic activity (C. Williams, unpublished observation). CH27 B lymphoma cells were stably transfected with an expression plasmid encoding the hybrid protein, mHEL/Hb^d(64-76), and one line of transfectants, CH27mHEL/Hb, was chosen for its high level expression of the mHEL/Hb protein.

The CH27mHEL/Hb cells were then tested for their ability to stimulate both HEL- and Hb^d(64-76)-specific T cell hybridomas. The CH27mHEL/Hb cells strongly stimulated H6.1, a hybridoma specific for HEL(84-96)/$I\text{-}E^k$ determinants (data not shown) and YO1.6, a sensitive T cell hybridoma (EC_{50} = 0.001–0.01 μM) specific for Hb^d(64-76)/$I\text{-}E^k$ complexes (Fig. 2 *A*). These results indicate that HEL as well as Hb^d(64-76) determinants were efficiently processed and presented in association with $I\text{-}E^k$ molecules by the transfected cells. The cell surface expression of $I\text{-}E^k$ by CH27mHEL/Hb was comparable to that of untransfected CH27 cells (data not shown). The level of expression of Hb^d(64-76)/$I\text{-}E^k$ complexes by the CH27mHEL/Hb cells was estimated by determining what concentration of exogenous Hb^d(64-76) peptide achieved equivalent stimulation of YO1.6. As shown in Fig. 2 *A*, the level of stimulation of YO1.6 by CH27mHEL/Hb was similar to that obtained by untransfected CH27 presenting 10 μM of exogenous Hb^d(64-76) peptide. On an individual cell basis, the CH27mHEL/Hb cells were excellent APCs, with as few as 30 cells giving a detectable stimulation of the YO1.6 cells. Thus, by expressing the Hb^d(64-76) determinant in a membrane protein, the APCs efficiently process and present the chimeric mHEL/Hb protein and stably present high levels of Hb^d(64-76)/$I\text{-}E^k$ complexes.

Estimation of the Number of Endogenous $Hb^d/I\text{-}E^k$ Complexes In Vivo. To estimate the level of endogenous $Hb^d/I\text{-}E^k$ complexes expressed by normal APCs, the activation of YO1.6 by either normal splenocytes or in vivo-activated macrophages from CBA/J mice (Hb^d, $H\text{-}2^k$) was compared to activation by CH27mHEL/Hb (Fig. 2 *A*). Indeed, the processing and presentation of the Hb^d(64-76) epitope in mHEL/Hb protein more closely resembles the situation in vivo than adding synthetic Hb^d(64-76) peptide. On an individual cell basis, splenocytes and macrophages were ~10- and 100-fold less efficient, respectively, than CH27mHEL/Hb in stimulating YO1.6 (Fig. 2 *A*). The level of stimulation of YO1.6 by the splenocytes was equivalent to the addition of 0.001 μM Hb^d(64-76) peptide to CH27 cells. These findings are consistent with the idea that no antagonism of the BT9 response was observed with endogenous complexes because they were expressed at insufficient number. This hypothesis is supported by the fact that no antagonism was observed with 0.1 μM of exogenous Hb^d(64-76) peptide presented by CH27 cells (15).

High Levels of Endogenously Synthesized $Hb^d/I\text{-}E^k$ Complexes Can Antagonize T Cells. With the CH27mHEL/Hb cells expressing much higher levels of Hb^d(64-76)/$I\text{-}E^k$ complexes than endogenous APCs, one would predict that they would be more efficient at antagonizing the BT9 response to Ser69. The presentation of Ser69 by CH27mHEL/Hb was

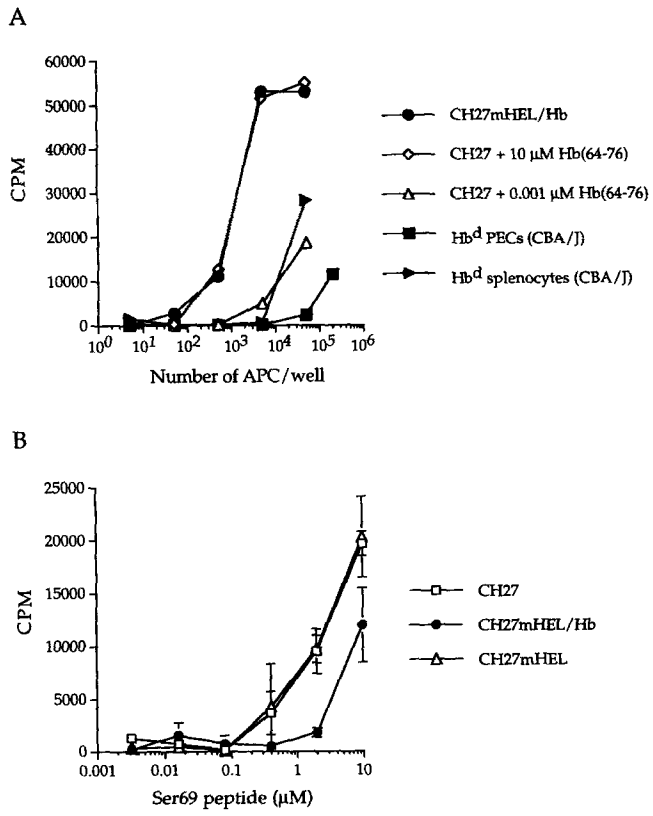

Figure 2. High expression of endogenously synthesized $Hb^d(64-76)$/$I\text{-}E^k$ complexes can specifically antagonize T cells. (*A*) Graded numbers of CH27mHEL/Hb (●) were tested for their ability to activate YO1.6 (5×10^4/well), a $Hb^d(64-76)/I\text{-}E^k$-specific T cell hybridoma. The activation of YO1.6 by CH27mHEL/Hb was compared to that induced by graded numbers of either splenocytes (▶) or PEC (■) derived from CBA/J mice. As controls, graded numbers of untransfected CH27 cells cultured in the presence of the indicated concentrations of $Hb^d(64-76)$ peptide (*open symbols*) were added. (*B*) BT9 cells (10^5/well) were activated by the indicated amounts of Ser69 peptide presented by either untransfected CH27 cells (□), CH27mHEL/Hb (●) or CH27mHEL transfected cells (△) (5×10^4/well). T cell activation was measured as described in Fig. 1. In *A*, SD were ≤20% of the mean and in *B*, values are the mean ± SD. The results are representative of five experiments.

shifted 10-fold compared to untransfected CH27, indicating that endogenous $Hb^d(64-76)/I\text{-}E^k$ complexes presented by CH27mHEL/Hb were able to antagonize activation of BT9 (Fig. 2 *B*). This antagonist effect was specific for the $Hb^d(64-76)$ determinant because CH27 cells transfected with mHEL alone did not affect the Ser69 response of BT9. Moreover, both untransfected and transfected CH27 cells which expressed similar levels of $I\text{-}E^k$ were equally effective in activating the cytochrome c/$I\text{-}E^k$-specific 2B4 T cell hybridoma (data not shown). CH27mHEL/Hb transfectants antagonized the Ser69 response of BT9 as effectively as untransfected CH27 with 10 μM of $Hb^d(64-76)$ synthetic peptide (data not shown). This latter observation confirms our estimation of the number of complexes expressed by CH27mHEL/Hb as equivalent to those obtained by 10 μM of exogenous $Hb^d(64-76)$ peptide presented by untransfected CH27. These findings strongly suggest that $Hb^d(64-76)/I\text{-}E^k$ complexes that are endogenously processed and presented do not differ functionally from complexes generated by the addition of exogenous synthetic peptides in their ability to induce partial T cell activation. Our results also support the idea that the failure of endogenous $Hb^d/I\text{-}E^k$ complexes expressed on normal APCs to act as APLs is due to the number of complexes being below a critical threshold.

CD4 Interaction with the Peptide/MHC Complexes Can Determine Whether a Ligand Is Perceived as an Agonist or an Antagonist by Mature T Cells. Recent studies have shown that T cells can limit their response to self-ligands by decreasing the expression of coreceptors (30, 31). To determine the role of coreceptor levels in TCR antagonism, we sorted BT9 cells into $CD4^{high}$ and $CD4^{low}$ populations and then isolated subclones stably expressing different levels of CD4, but similar levels of CD3. The BT9-$CD4^{low}$ and BT9-$CD4^{high}$ lines are representative subclones which expressed dramatically different levels of CD4 (Fig. 3, *top panels*). CD4 expression on the BT9-$CD4^{low}$ cells was similar to that of unsorted BT9 cells, whereas the BT9-$CD4^{high}$ cells expressed significantly higher levels. Both BT9 subclones were stimulated by Ser69 and, consistent with the different levels of CD4 expression, the BT9-$CD4^{high}$ T cells were more reactive to Ser69 than the BT9-$CD4^{low}$ T cells (Fig. 3, *bottom panels*). Interestingly, BT9-$CD4^{low}$ cells were stimulated only by Ser69, whereas BT9-$CD4^{high}$ cells were stimulated by both Ser69 and $Hb^d(64-76)$ peptides. The response of the BT9-$CD4^{low}$ cells was identical to that of unsorted BT9 cells, with $Hb^d(64-76)$ acting as an antagonist (data not shown, reference 15). In contrast, $Hb^d(64-76)$ was a weak agonist for BT9-$CD4^{high}$ cells (Fig. 3, *bottom right*). Interestingly, BT9-$CD4^{high}$ cells were also weakly stimulated by endogenous $Hb^d(64-76)/I\text{-}E^k$ complexes expressed on Hb^d splenocytes from CBA/J mice and on CH27mHEL/Hb cells (data not shown, Table 1). To establish that CD4 was directly responsible for the $Hb^d(64-76)$ reactivity gained by the BT9-$CD4^{high}$ cells, we determined the effect of anti-CD4 antibodies (Fig. 4). The addition of anti-CD4 mAb profoundly inhibited the response of BT9-$CD4^{high}$ cells to $Hb^d(64-76)$ peptide (Fig. 4 *A*). Moreover, treatment with anti-CD4 mAb converted $Hb^d(64-76)$ from a weak agonist to a potent antagonist for BT9-$CD4^{high}$ cells (Fig. 4 *B*). These results demonstrate that the level of CD4 surface expression on T cells can directly affect whether a ligand is perceived as an agonist or an antagonist.

Primary T Cells Derived from $Hb^{d/s}$ G2-Tg Mice Can Be Antagonized by High Levels of $Hb^d/I\text{-}E^k$ Complexes. The last question that we addressed was whether endogenous $Hb^d/I\text{-}E^k$ complexes would act as APLs for peripheral T cells that were developed in the presence of the antagonist Hb^d ligand. We have shown that the presence of the Hb^d allele in G2-Tg mice resulted in the complete elimination of the $Hb^d(64-76)$ reactive T cells and the higher-avidity Ser69-reactive T cells, while leaving low-avidity Ser69-reactive T cells (15). Thus, we were able to study the Ser69 response of naive T cells, and we have shown that purified T cells from $Hb^{d/s}$ G2-Tg mice could be specifically an-

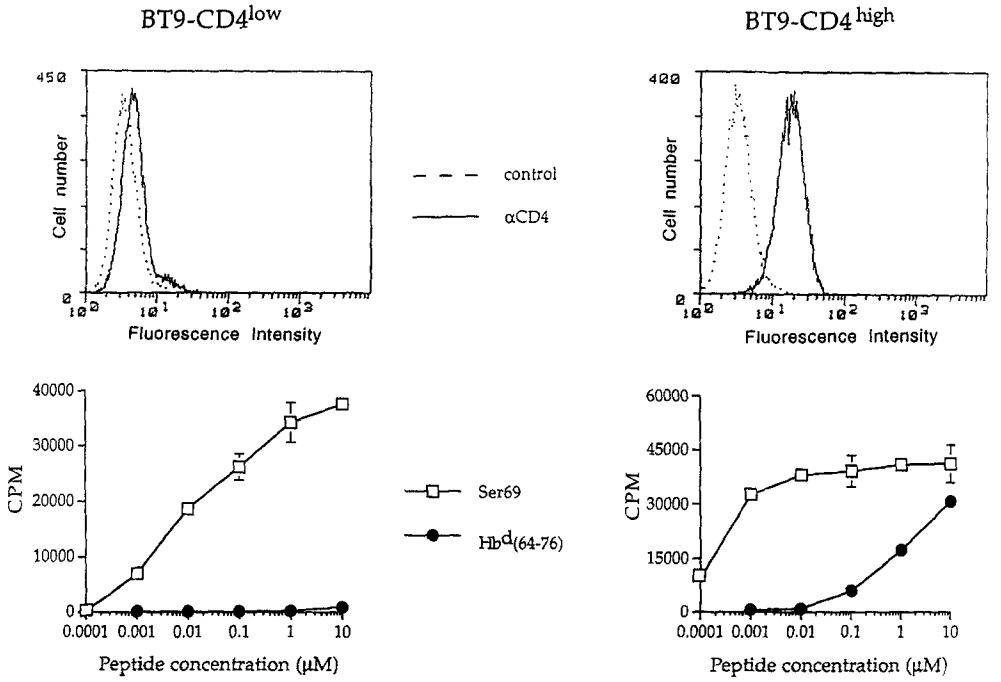

Figure 3. Increased CD4 levels convert the $Hb^d(64-76)$ peptide from an antagonist to an agonist. The top panels show cell surface expression of CD4 by two subclones, BT9-$CD4^{low}$ (*left*) and BT9-$CD4^{high}$ (*right*) obtained as described in Materials and Methods. T cells (10^6/sample) were incubated with the anti-CD4 mAb GK1.5 followed by a FITC-conjugated goat anti-rat IgG Ab and analyzed by FACScan (*solid line*). Cell staining with the secondary Ab alone is shown by the dotted line. The bottom panels show the response of BT9-$CD4^{low}$ (*left*) and BT9-$CD4^{high}$ (*right*) (5×10^4/well) to indicated concentrations of Ser69 (□) or $Hb^d(64-76)$ (●) presented by CH27 cells (5×10^4/well). T cell activation was measured as described in Fig. 1. Values are means ± SD. The results are representative of four experiments.

tagonized by exogenous $Hb^d(64-76)$ peptide (15). As shown in Fig. 5 *A*, the Ser69 response of primary T cells derived from $Hb^{d/s}$ G2-Tg mice was not antagonized by endogenous Hb^d/I-E^k complexes expressed on CBA/J splenocytes. However, these primary T cells were antagonized by high levels of $Hb^d(64-76)$/I-E^k complexes presented by CH27mHEL/Hb cells (Fig. 5 *B*). These results are consistent with the findings using T cell hybridomas derived from $Hb^{s/s}$ G2-Tg mice (Fig. 1). To analyze the effect of CD4 expression on the ability of these T cells to be antagonized, we used a Ser69 reactive T cell hybridoma, A11.F3, generated from $Hb^{d/s}$ G2-Tg mice and sorted into $CD4^{low}$ and $CD4^{high}$ populations. Neither A11.F3-$CD4^{low}$ T cells, nor A11.F3-$CD4^{high}$ T cells were reactive to $Hb^d(64-76)$/I-E^k complexes (data not shown). However, only the Ser69 response of A11.F3-$CD4^{high}$ T cells was specifically antagonized by $Hb^d(64-76)$/I-E^k complexes presented on CH27mHEL/Hb (Table 1). Thus, low-avidity T cells from $Hb^{d/s}$ T mice that express low level of CD4 do not productively interact with $Hb^d(64-76)$/I-E^k complexes, whereas if they express higher levels of CD4, they can perceive this ligand as an APL. Furthermore, T cells from $Hb^{s/s}$ Tg-mice that express

Table 1. *Summary of the Phenotype of $Hb^d(64-76)$I-E^k Complexes on Ser69-Reactive T Cells*

		Effect of Hb^d (64-76)/I-E^k complexes formed by‡			
T cells*	Hb genotype	Endogenous in vivo level	Addition of Hb^d protein	mHEL/Hb protein	Hb^d (64-76) peptide
BT9	s/s	–	APL	APL	APL
BT9-$CD4^{low}$	s/s	–	APL	APL	APL
BT9-$CD4^{high}$	s/s	Weak agonist	ND	Agonist	Agonist
Primary T cells	d/s	–	ND	APL	APL
A11-F3-$CD4^{low}$	d/s	–	ND	–	–
A11-F3-$CD4^{high}$	d/s	–	ND	APL	APL

*The primary T cells and the T cell hybridomas listed were derived from mice expressing either the $Hb^{s/s}$ or $Hb^{d/s}$ allele, and all recognized the Ser69 peptide as an agonist.

‡The Hb^d (64-76)/I-E^k complexes were formed either by endogenous processing in vivo, addition of exogenous Hb protein in vitro, expression of Hb^d (64-76) determinant in a chimeric membrane protein on CH27 cells or addition of exogenous synthetic peptide to the APCs as described in Materials and Methods. The complexes were either not recognized by the T cell hybridomas (–) or recognized as an antagonist (APL) or as an agonist. The rank order of the phenotype of $Hb^d(64-76)$/I-E^k complexes is: – < APL < weak agonist < agonist.

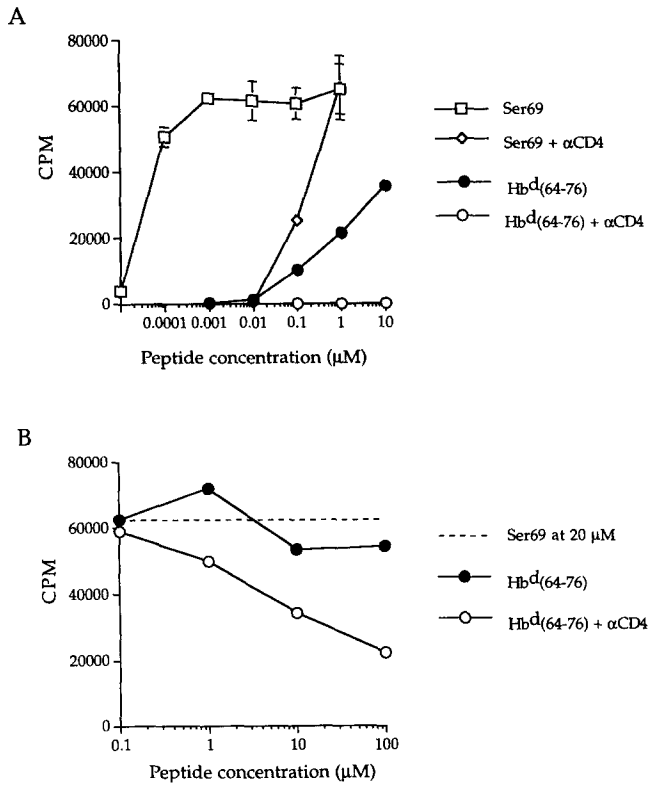

Figure 4. Effect of anti-CD4 mAb on the reactivity of BT9-CD4^{high} cells. (*A*) BT9-CD4^{high} cells (5×10^4/well) were activated by the indicated amounts of Ser69 (□, ◇) and Hb^d(64-76) (●, ○) peptides presented by CH27 cells (5×10^4/well), in the absence (□, ●) or presence (◇, ○) of the anti-CD4 mAb GK1.5. (*B*) CH27 cells were prepulsed for 2 h with 20 μM of agonist Ser69 peptide, before addition of the indicated amounts of Hb^d(64-76) in the absence (●) or presence (○) of GK1.5 mAb. Activation in the absence of added antagonist is depicted as a dashed line. T cell activation was measured as described in Fig. 1. In *A*, values represent the mean ± SD and in *B*, SD were less than 15% of the mean. These results are representative of three experiments.

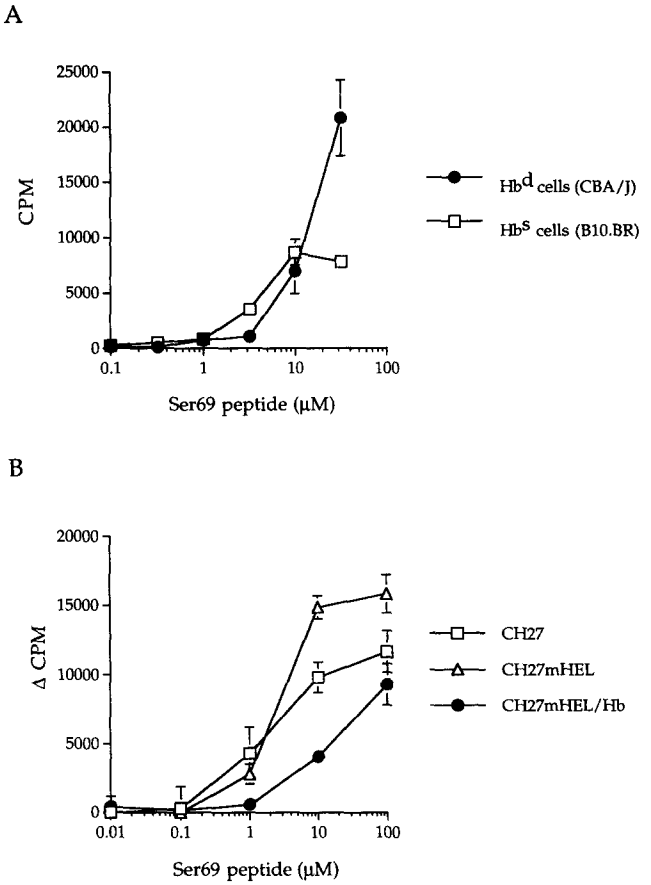

Figure 5. Primary T cells derived from Hb$^{d/s}$ G2-Tg mice are only antagonized by high expression of Hb^d(64-76)/I-E^k complexes. (*A*) Nylon wool-purified T cells (2×10^5/well) derived from unprimed Hb$^{d/s}$ G2-Tg mice were incubated with the indicated concentrations of Ser69 peptide presented by splenocytes (5×10^5/well) from either Hb^d (●) or Hb^s (□) mice. (*B*) Primary T cells were activated by the indicated amounts of Ser69 peptide presented by either untransfected CH27 cells (□), CH27mHEL/Hb (●) or CH27mHEL transfected cells (△) (5×10^4/well). The cultures were incubated for 4 d and proliferation was measured by incorporation of [^{3}H]TdR (0.4 μCi/well) over the last 20 h. In *A*, each data point represents the mean of triplicate cultures ± SD. In *B*, a representative of six separate experiments is shown. Results are expressed as Δcpm obtained by subtracting the mean background proliferation from the mean proliferation at each antigen concentration. Background proliferation were: CH27, 4655 cpm; CH27mHEL, 7016 cpm; and CH27mHEL/Hb, 1530 cpm.

low levels of CD4 can be antagonized by Hb^d(64-76)/I-E^k complexes, and if these cells have higher CD4 expression, Hb^d(64-76)/I-E^k complexes are recognized as an agonist. The effect of the Hb^d(64-76)/I-E^k complexes observed T cell responses are summarized in Table 1. Taken together, these results indicate that the efficacy of the interaction between a T cell and an APC directly influences the activity of an APL. Moreover, these data suggest that T cells that have developed in the presence of the antagonist Hb^d ligand are selected to ignore the constitutive levels of endogenous complexes they encounter in the periphery.

Discussion

APLs derived from both exogenous as well as endogenous antigens can productively interact with T cells resulting in partial T cell activation. To investigate whether endogenously synthesized APLs are able to affect peripheral T cell responses, we studied the response of purified T cells and T cell hybridomas from a TCR-Tg mouse in which our normal antigen system has been reversed: the Ser69 peptide is an agonist and Hb^d(64-76) acts now as an APL. This system permitted us to examine the ability of endogenous Hb^d(64-76)/I-E^k complexes to induce partial T cell activation, as detected by their ability to be TCR antagonists. We observed no detectable antagonism of either primary T cells or T cell hybridoma responses to Ser69 by endogenous Hb^d/I-E^k complexes expressed on normal APCs; however, TCR antagonism was observed after increasing the number of endogenous Hb^d(64-76)/I-E^k complexes by raising the Hb^d antigen concentration or by expressing the Hb^d(64-76) determinant in a membrane protein on APCs. In addition, by

changing CD4 expression on the T cells, the activity of an APL could be increased (i.e., an antagonist becomes a weak agonist). Thus, we contend that in the periphery endogenous APLs can affect peripheral T cells under limited but attainable circumstances, such as when the number of APL complexes on the APCs increases, and/or when a T cell increases its sensitivity to the ligand.

The $Hb^d(64-76)$ epitope derived from endogenous, naturally processed Hb protein is efficiently and constitutively presented by $I\text{-}E^k$ molecules on most APCs in the mouse (17). Hb is an abundant self-protein, existing predominantly inside erythrocytes, with a minor component being bound to haptoglobin (27). Even with its abundance, however, the number of $Hb^d(64-76)/I\text{-}E^k$ complexes expressed on APCs appears not to be saturating, being equivalent to 0.01–0.001 μM of exogenously added $Hb^d(64-76)$ peptide. In a study by Marrack and her colleagues (32), no $Hb^d(64-76)$ peptide was observed among the abundant peptides eluted from $I\text{-}E^k$ molecules from C3H mice; however, the endogenous $Hb^d(64-76)/I\text{-}E^k$ complexes have been previously shown to strongly stimulate $Hb^d(64-76)$-reactive T cells (17). Circulating self-antigens, such as HEL, C5 of the complement system and α1-anti-trypsin, have been shown to be endogenously processed and presented via the class II pathway in vivo (25, 33, 34). The concentration of the self-antigen was directly related to the number of endogenous complexes detected (25, 34). The number of complexes generated from HEL even at 30 ng/ml was sufficient to stimulate specific T cells (25), but most likely these complexes would be difficult to detect by current biochemical techniques. In support of this, it has been shown that the majority of self-peptides bound to class II molecules on the surface of an APC are derived from membrane proteins, with the others being derived from circulating antigens (reviewed in reference 35). Thus, membrane proteins on APCs are efficiently processed and presented via the class II pathway (28, 29). This was dramatically shown in this study when the $Hb^d(64-76)$ epitope was expressed as a chimeric mHEL/Hb protein, resulting in a 1,000-fold increase in the apparent number of endogenous complexes. Therefore, many self-antigens can be processed and presented, and have the potential to act as APLs, with the membrane proteins on an APC being the most highly represented.

Several studies have now clearly shown flexibility of the TCR in the recognition of antigen. Unrelated peptides can stimulate the same T cell, a finding that directly relates to the existence of endogenous APLs. The phenomenon of cross-reactivity has been observed for T cells specific for myelin basic protein (36) and human α1-anti-trypsin (34), but the molecular basis was not established. In addition, a recent study has shown that a TCR can recognize at least five different overlapping peptides, highlighting the flexibility in the TCR recognition of antigen (37). Moreover, two other studies, which used the protein sequence databases, have identified peptides from endogenous proteins (2) and from pathogens (38), which share a minimal degree of sequence homology to the natural immunogenic peptide, but are able to stimulate the same T cells. In the $Hb^d(64-76)$ system, a peptide containing as few as one amino acid in common with $Hb^d(64-76)$, could stimulate a specific T cell response. From a search of the SwissProt database, two endogenous ligands, an agonist and a partial agonist, were also readily identified (2). In addition, Wucherpfennig and colleagues identified several bacterial and viral peptides capable of stimulating T cell clones which were derived from multiple sclerosis patients and which recognized the MBP(84-102)/HLA-DR2b determinant (38). Importantly, a stimulating peptide from EBV was shown to be processed naturally in an EBV-transformed B cell line and was able to activate the T cells. These cross-reactive peptides did not bear a major degree of sequence homology to the natural agonist ligand. Taken together, these studies strongly support the idea that one TCR can interact productively with multiple endogenous ligands, and provide evidence that such ligands exist in vivo.

Our results support the idea that the failure of endogenous $Hb^d(64-76)/I\text{-}E^k$ complexes to antagonize T cell responses is due to a limiting number of complexes expressed by normal APCs in vivo, and the fact that T cells have been selected to ignore these endogenous levels. However, the lack of reactivity of peripheral T cells to endogenous APLs is not absolute and several different situations can be envisioned that would result in productive interactions occurring. In our studies, we showed that an increase in the number of APL/MHC complexes on an APC resulted in increased activity of the APL. An increase in the expression of endogenous APL/MHC complexes could easily occur in vivo, if the availability of the antigen increases. This might occur by upregulation of the synthesis of a self-protein or increased uptake via endocytosis or receptor-mediated events. For example, during an inflammatory response, the synthesis of acute phase reactive proteins is induced, and when cells become activated, the expression of some cell surface molecules is upregulated. In addition, the effective concentration of a self-protein could be increased by upregulating MHC class II expression on the APCs. The effect of increasing MHC expression may only be related to certain self-antigens, such as membrane proteins, because in our studies the up-regulation of class II expression on macrophages by IFN-γ did not raise the level of endogenous complexes enough for them to act as APLs. Productive interactions between the TCR and APLs could also occur by changes in the T cell activation threshold, which might result from production of cytokines locally, and/or by changing the expression level of coreceptors (CD4, CD8), adhesion molecules (e.g., LFA-1, ICAM-1), or costimulator ligands (e.g., CD28, CTLA-4).

The coreceptors CD4 and CD8 are intimately involved in the T cell recognition of antigen, contributing both to cell–cell adhesion and to intracellular signaling (39). Our results indicate that the effect of an APL is directly influenced by the level of expression of CD4 on the responding T cells and support two previous studies which showed that alterations in CD4 expression could change the fine specificity of a TCR (40, 41). Moreover, consistent with our findings, recent studies have shown that decreasing cell sur-

face CD8 or CD4 expression levels on MHC class I- or class II-restricted T cells converts an agonist ligand into an antagonist (30, 31). Thus the coreceptor's interaction with the peptide/MHC complexes can apparently determine whether a peptide antigen is perceived as an agonist or an APL. The mechanism by which increased CD4 levels would change the activity of a peptide antigen is not known. It could simply be due to an enhanced or prolonged T cell/APC interaction and/or could involve an enhanced signaling by the localization of more $p56^{lck}$ into the TCR complex (42, 43). In any case, the increased CD4 levels would result in an enhanced efficacy of the T cell/APC interaction.

Our findings support the view of Jameson and his colleagues (30), that there is a major difference between the effect of endogenous APLs on developing T cells in the thymus and on peripheral T cells. Using fetal thymic organ cultures, they showed that during positive selection the CD8 levels of developing thymocytes were lowered to avoid reactivity of the mature T cells with the positive selecting ligand. In the thymus, endogenous APLs could play a major role in both positive and negative selection (9–12). In contrast, the present studies clearly show that peripheral T cells do not react with the endogenous levels of APLs. A possible mechanism by which the peripheral T cells ignore the endogenous APLs might first involve the selection of the proper avidity of T cell/APC interactions by matching the TCR affinity and coreceptor expression. Second, it has been shown that a lower receptor avidity is required for T cell deletion in the thymus than for effector T-cell function (44), supporting the idea that the activation threshold of mature resting T cells might be higher than that of developing thymocytes (45). Taken together, these processes would prevent T cells from reacting with their positively selecting ligands in the periphery. Thus, because the process of positive selection involves the recognition of self peptides, the immune system has developed mechanisms by which mature T cells do not normally recognize the endogenous APLs, including the positive selecting ligands themselves.

Our data suggest that self-proteins are constitutively processed and presented at levels that do not affect T cell responses, which is likely to be an important feature of the maintenance of self-tolerance. On the other hand, an inducible expression of endogenous APLs potentially could be involved in regulating an immune response. For example, in a pathological situation, where many inflammatory mediators and cytokines enhance antigen presentation, endogenous APLs may be capable of down regulating specific T cells against agonist ligands. Moreover, endogenous APLs might influence the responses activated against foreign antigens, e.g., by antagonism, anergy induction or by influencing the phenotype of the T cell population being activated. Thus, the repertoire of endogenous self-peptides may influence susceptibility to disease, and also play a significant role in the shaping of immune responses to all foreign challenges.

What are the possible effects of natural APLs on peripheral T cells? First, APLs can activate different signals from those stimulated by agonist ligands. This results in a dramatic change in the functional phenotype of the T cells. For example, in the absence of T cell proliferation, the engagement of APLs by a TCR can initiate a biological response by inducing production of certain cytokines, such as IL-4, by providing B cell help (1), or by lysis of target cells (46). A recent study demonstrated that engagement of APLs induced production of TGF-β from the T cells, a cytokine which was not produced upon stimulation by the agonist peptide (4). The biological significance of this as a possible way of regulating specific T cell immune responses is highlighted by the observations that TGF-β administration to mice can ameliorate EAE disease severity, and that suppression of EAE is associated with the generation of T cells that secrete TGF-β (47, 48). Second, the presence of endogenous APLs could give the immune system an added layer of control of potentially autoreactive T cells. For example, if a T cell expressing a TCR reactive to a self-peptide escapes deletion in the thymus and arrives in the periphery where the agonist peptide is present, or if the processing of a foreign antigen gives rise to some T cell epitopes which are cross-reactive with self-peptides, one can imagine that the risk of autoimmune responses could be blocked in the periphery by the presence of endogenous APLs. This idea is supported by recent studies demonstrating that APLs that could inhibit specific T cells in vitro were potent inhibitors of EAE in vivo, and could even downregulate disease when coinjected with the agonist peptide (49, 50). However, the interaction of peripheral T cells with APL's might also have negative consequences. For example, during the processing of a foreign antigen, some T cell epitopes might be cross-reactive with endogenous APLs. The recognition of some self-peptides in this way could misdirect T cell responses, thus creating a risk of autoimmune responses. In addition, the endogenous partial agonist could induce disease by being a molecular mimic of a TCR specific for a certain pathogen. Several recent papers, using the examples of hepatitis B virus and human immunodeficiency virus, have documented that APLs of immunogenic epitopes made by mutations in pathogen proteins can enhance a chronic viral infection (13, 14, 16). Thus, partial agonists and antagonists can be exploited by pathogens as a method of evading or misdirecting the immune system (51, 52). Finally, the role of specific antigen in the maintenance of T cell memory has remained a controversial issue in the literature (reviewed in reference 53). It is possible that memory T cells are maintained in the periphery in the absence of foreign antigen by constant engagement of weak ligands. Thus, some cross-reactive ligands, which have lower affinity for the TCR than the antigenic peptide, could partially stimulate memory T cells. Overall, these studies indicate that naturally occurring APLs may play a significant role in the biology of T cells.

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