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 Hbα(64-76)/I-Ek 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 Set69 peptide now being the agonist and Hbα(64-76) being the APL. In this report, we show that the constitutive level of endogenous Hbα(64-76)/I-Ek complexes presented by APCs in vivo is too low to affect the response of Set69 reactive T cells. However, by increasing the number of Hbα(64-76)/I-Ek 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 Hbα(64-76)/I-Ek 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), energy (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, Hbα and Hbδ, that differ by 12 amino acids, two of which are found in the immunogenic (64-76) peptide sequence. Mice expressing the Hbα allele (CE/J and B10.BR, H-2b) generate a strong T cell response to the Hbδ allelic
protein. From this response we have developed a large panel of T cell clones and hybridomas, all of which recognize the immunodominant Hb(64-76)/I-Ek determinant. As shown by stimulation of these hybridomas with normal APCs from CBA/J (Hb(64-76)/I-Ek) mice, the minimal Hb(64-76)/I-Ek epitope is constitutively processed and presented from endogenous Hb(64-76) protein (17). We have generated a TCR transgenic (G2-Tg) mouse in which the transgenic β chain derived from a Hb(64-76)/I-Ek-specific T cell clone pairs with endogenous α chains, leading to a serendipitous primary reactivity to the Ser69-peptide, an APL of Hb(64-76) (15). In this system, we have previously demonstrated that synthetic Hb(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(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 antigen 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(64-76)/I-Ek complexes. Our findings reveal that endogenous levels of Hb(64-76)/I-Ek 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(64-76) and Hb(64-76)/G2-Tg 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-2k, Hb(64-76)/I-Ek) and B10.BR (H-2k, Hb(64-76)) female mice, 5–10 wk old, were purchased from the National Cancer Institute and The Jackson Laboratory (Bar Harbor, ME), respectively.

Antigens. The Hb(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 C18 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(64-76) = GKKVITALFNEGLK, Ser69 = GKKVISAENFNEGLK, and MCC(91-103) = RADLIAYLKQK-ATK. The Hb and Hb(64-76) proteins were prepared as previously described (17). Briefly, a hemolysate was prepared from CBA/J (Hb(64-76)) or B10.BR (Hb(64-76)) 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 (10⁶ 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/10⁶ 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 cytometer using Cell Quest software (Becton-Dickinson, Mountain View, CA).

T Cell Hybridomas. The generation and characterization of BT9, a Ser69-I-Ek-specific T cell hybridoma derived from Hb(64-76)/I-Ek transgenic mice were previously described (15). BT9-CD4neg and BT9-CD4pos 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(64-76) G2-Tg mice were activated in vitro for 4 d with Ser69 peptide (50 μM), and fused with the BW5147 TCRα-β 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(64-76)/I-Ek (17), 2B4, specific for cytochrome c/I-Ek (20), and H6.1, specific for hen egg-white lysozyme (HEL) (64-96)/I-Ek (21) have been previously described. T cell hybridomas were cultured at 37°C in 5% CO₂, 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 x 10⁻³ M 2-ME (referred to as complete media).

APC Preparation. The CH27 B cell lymphoma (22) and un-fractionated, 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 x 10⁵/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, CTL2-2 (ATCC), as described (24). TCR antagonism assays of T cell hybridomas were performed as described (24). Briefly, CH27 were preincubated with 20 μM of Ser69 peptide for 2 h at 37°C. The cells were washed and plated (5 x 10⁴/well) in flat-bottomed 96-well microtiter plates with 5 x 10⁴ T hybridoma cells in the presence of indicated concentrations of Hb(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% CO₂, 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 Hb⁶⁺/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-1-µ AK (10.3.6.2), anti-1-EK (14.4.4S) (ATCC) and guinea pig and rabbit complement (GIBCO BRL) to remove any remaining class II-positive cells. Purified TAPCs. Proliferation of the T cells was measured as [³H]TdR. (0.4 TCR. Tg mice were obtained by passing a single-cell suspension of spleen cells (5 × 10⁵/well) were cultured in the presence of Ser69 peptide (0-100 µM), mitomycin c (Sigma Chem. Co., St. Louis, MO) treated CH27 cells (5 × 10⁴/well), or irradiated (2,000 rads) CBA/J or B10.BR spleen cells (5 × 10⁵/well) were used as APCs. Proliferation of the T cells was measured as [³H]TdR (0.4 µCi/well) incorporation over 24 h, as described (25).

Generation of the CH27mHEL/Hb and CH27mHEL Cell Lines. To express the Hb⁶⁺(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 Hb⁶⁺(64-76) epitope was inserted. This plasmid was constructed from pCMV2-mHEL, which contains the HEL coding region from a cDNA clone inserted. This plasmid was constructed from pCMV2-mHEL, contains a hygromycin B-resistance gene and the transfectants contain the HEL coding region (lower case) by 20-23 bp. A StuI site was introduced between the endogenous Hba/I-E k ligand and the synthetic Hba(64-76) peptide in either length, register, or conformation bound to the I-Ek molecule, which could translate into differences in antigenic binding ability.

The presentation of Ser69 peptide by Hb⁶⁺-expressing cells was measured using a TCR transgenic mouse. The Ser69 response of BT9 was apparent due to an insufficient number of complexes expressed on each APC. Antibody response. To determine the effect of endogenous APLs on the processing and presentation of the Hbd(64-76) epitope, the Hbd(64-76)/I-E k complexes was only observed at low agonist concentration (0.25 to 0.5 µM). Thus, the failure of endogenously processed Hb⁶⁺(64-76), to antagonize the T9 response to Ser69 was observed when Ser69 was presented by peritoneal macrophages from CBA/J (Hb⁶⁺) or B10.BR (Hb⁻), which had been activated in vivo to induce MHC class II expression (Fig. 1A). Identical results were obtained with splenocytes (data not shown). Thus, no antagonism of the BT9 response to Ser69 was observed by endogenous Hb⁶⁺(64-76)/I-Ek complexes expressed on B cells or activated macrophages. The failure of endogenously processed Hb⁶⁺(64-76), to antagonize the BT9 response to Ser69 was apparently due to an inefficient number of complexes expressed on each APC. Statistical Analysis. The presentation of Ser69 peptide by Hb⁶⁺-expressing APCs versus Hb⁻-APCs was compared using Student's t test analysis (StatView II; Abacus, Berkeley, CA).

Results

Endogenous Hb⁶⁺/I-Ek 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 Hb⁶⁺(64-76) peptide (15). However, the Ser69 response of BT9 could be antagonized in a specific and dose-dependent manner by the Hb⁶⁺(64-76) peptide when given exogenously to the APCs (15). Thus, this unique experimental system, in which Ser69 acts as an agonist and Hb⁶⁺(64-76) as an APL, allowed us to study the effect of endogenous Hb⁶⁺(64-76)/I-Ek complexes on the BT9 response. To assay for TCR antagonism by endogenous Hb⁶⁺(64-76)/I-Ek complexes, we compared the Ser69 responses of BT9 using normal APCs from Hb⁻ mice (CBA/J) which express the minimal Hb⁶⁺(64-76)/I-Ek epitope, and APCs from Hb⁻ mice (B10.BR) which do not. If endogenous Hb⁶⁺(64-76)/I-Ek complexes could antagonize BT9 cells, one would expect a depressed response to Ser69 when this peptide is presented by APCs from Hb⁻ mice (CBA/J) compared to APCs from Hb⁻ mice (B10.BR). The responses of BT9 to Ser69 were identical when the peptide was presented by peritoneal macrophages from CBA/J (Hb⁻) or B10.BR (Hb⁻), which had been activated in vivo to induce MHC class II expression (Fig. 1A). Identical results were obtained with splenocytes (data not shown). Thus, no antagonism of the BT9 response to Ser69 was observed by endogenous Hb⁶⁺(64-76)/I-Ek complexes expressed on B cells or activated macrophages. The failure of endogenously processed Hb⁶⁺(64-76), to antagonize the BT9 response to Ser69 was apparently due to an inefficient number of complexes expressed on each APC.

Generation and Characterization of an APC Line Expressing Membrane HEL/Hb Protein. The antigenic effect seen by addition of exogenous Hb⁻ protein was most likely due to the processing and presentation of the Hb⁶⁺(64-76) epitope. To formally demonstrate this, we generated an APC line which presents high levels of Hb⁻(64-76)/I-Ek 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),
which limits the usefulness of expressing the Hb(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(64-76) determinant engineered as part of a membrane protein expressed in the APCs. We generated a chimeric membrane protein by inserting Hb(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(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(64-76), and one line of transfectants, CH27mHEL/Hb, was chosen for its high level expression of mHEL/Hb protein.

The CH27mHEL/Hb cells were then tested for their ability to stimulate both HEL- and Hb(64-76)-specific T cell hybridomas. The CH27mHEL/Hb cells strongly stimulated H6.1, a hybridoma specific for HEL(84-96)/I-Ek determinants (data not shown) and YO1.6, a sensitive T cell hybridoma (EC50 = 0.001–0.01 μM) specific for Hb(64-76)/I-Ek complexes (Fig. 2 A). These results indicate that HEL as well as Hb(64-76) determinants were efficiently processed and presented in association with I-Ek molecules by the transfected cells. The cell surface expression of I-Ek by CH27mHEL/Hb was comparable to that of untransfected CH27 cells (data not shown). The level of expression of Hb(64-76)/I-Ek complexes by the CH27mHEL/Hb cells was estimated by determining what concentration of exogenous Hb(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(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(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(64-76)/I-Ek complexes.

**Estimation of the Number of Endogenous Hb/Ek Complexes In Vivo.** To estimate the level of endogenous Hb/Ek complexes expressed by normal APCs, the activation of YO1.6 by either normal splenocytes or in vivo-activated macrophages from CBA/J mice (Hb-, H-2a) was compared to activation by CH27mHEL/Hb (Fig. 2 A). Indeed, the processing and presentation of the Hb(64-76) epitope in mHEL/Hb protein more closely resembles the situation in vivo than adding synthetic Hb(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(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(64-76) peptide presented by CH27 cells (15).

**High Levels of Endogenously Synthesized Hb/Ek Complexes Can Antagonize T Cells.** With the CH27mHEL/Hb cells expressing much higher levels of Hb(64-76)/I-Ek 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...
shifted 10-fold compared to untransfected CH27, indicating that endogenous Hb\(^d\)(64-76)/I-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-E\(^k\) were equally effective in activating the cytochrome c/I-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 \(\mu\)M 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 \(\mu\)M of exogenous Hb\(^d\)(64-76) peptide presented by untransfected CH27. These findings strongly suggest that Hb\(^d\)(64-76)/I-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-E\(^k\) complexes expressed on normal APCs to act as APLs is due to the number of complexes being below a critical threshold.

**Primary T Cells Derived from Hb\(^d\)/I-E\(^k\) G2-Tg Mice Can Be Antagonized by High Levels of Hb\(^d\)/I-E\(^k\) Complexes.** The last question that we addressed was whether endogenous Hb\(^d\)/I-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\(^d\)/G2-Tg mice could be specifically an-

![Figure 2.](image)
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\(^{5}\)/sample) were incubated with the anti-CD4 mAb GK1.5 followed by a FITC-conjugated goat anti-rat IgG Ab and analyzed by FACSscan (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 \times 10\(^{4}\)/well) to indicated concentrations of Ser69 (\(\square\)) or Hb\(^{d}(64-76)\) (\(\bigcirc\)) presented by CH27 cells (5 \times 10\(^{4}\)/well). T cell activation was measured as described in Fig. 1. Values are means \(\pm\) SD. The results are representative of four experiments.

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

| 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\(^{d/s}\) or Hb\(^{s/s}\) 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.
low levels of CD4 can be antagonized by Hbβ(64-76)/I-Ek complexes, and if these cells have higher CD4 expression, Hbβ(64-76)/I-Ek complexes are recognized as an agonist. The effect of the Hbβ(64-76)/I-Ek 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 Hbβ 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 Hbβ(64-76) acts now as an APL. This system permitted us to examine the ability of endogenous Hbβ(64-76)/I-Ek 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 Hbβ/ I-Ek complexes expressed on normal APCs; however, TCR antagonism was observed after increasing the number of endogenous Hbβ(64-76)/I-Ek complexes by raising the Hbβ antigen concentration or by expressing the Hbβ(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(64-76) epitope derived from endogenous, naturally processed Hb protein is efficiently and constitutively presented by I-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(64-76)/I-E^k complexes expressed on APCs appears not to be saturating, being equivalent to 0.01-0.001 M of exogenously added Hb(64-76) peptide. In a study by Marrack and her colleagues (32), no Hb(64-76) peptide was observed among the abundant peptides eluted from I-E^k molecules from C3H mice, however, the endogenous Hb(64-76)/I-E^k complexes have been previously shown to strongly stimulate Hb(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(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(64-76) system, a peptide containing as few as one amino acid in common with Hb(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(64-76)/I-E^k complexes to agonize 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 in 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 co-stimulator 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\(^{ck}\) 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 inductive 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-\(\beta\) 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-\(\beta\) administration to mice can ameliorate EAE disease severity, and that suppression of EAE is associated with the generation of T cells that secrete TGF-\(\beta\) (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 co-injected 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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