Modification of Human T-Cell Responses by Altered Peptide Ligands: A New Approach to Antigen-specific Modification

Yasuharu Nishimura, Yu-Zhen Chen, Takayuki Kanai, Hiroshi Yokomizo, Takako Matsuoka and Sho Matsushita

Human CD4+ T-cells recognize antigenic peptides in the context of human leukocyte antigen (HLA) class II molecules and produce various lymphokines to proliferate and activate other cells. It was once considered that the T-cell response is an all or nothing type event, but recent studies have clearly indicated that T-cells show many different types of activation in recognition of altered ligands for T-cell receptors (TCR). In this review, we summarize our recent findings on the human CD4+ T-cell response to altered peptide ligands (APL); peptides carrying single residue substitutions in antigenic peptides. We observed the following: 1) TCR antagonism for T-cell clones reactive to non-self or autoantigenic peptides, 2) partial activation (agonism) without cell proliferation, including production of lymphokines and increases in cell size, and in expression levels of several cell surface proteins or survival time in the absence of antigenic stimulus, 3) augmentation in cell proliferation and production of interferon-γ (IFN-γ) and granulocyte monocyte colony stimulating factor (GM-CSF), 4) augmentation of interleukin (IL)-12 production by antigen presenting cell (APC) and the subsequent augmented production of IFN-γ by T-cells. This information provides basic knowledge regarding the characteristics of T-cell recognition of antigens and the subsequent activation, and a novel method for modification of human T-cell responses by altered peptide ligands (APLs), as a possible candidate for antigen-specific immunopotentiating or immunosuppressive therapy against autoimmune diseases, allergies, infectious diseases and malignant tumors.

Key words: human leukocyte antigen (HLA) class II molecule, antigen presentation, CD4+ T-cells, antigenic peptides, altered peptide ligands, T-cell activation

Introduction

The human leukocyte antigen class-II (HLA-II) molecule is a highly polymorphic heterodimeric membrane protein consisting of α and β chains and is expressed on B cells, antigen presenting cells (APC) and activated T-cells. As shown in Fig. 1A, the HLA-II molecule has a peptide binding groove on top of the molecule and binds antigenic peptides processed by APC to present them to CD4+ T-cells [see review 1]. The structural requirements for HLA-II binding peptides were determined based on many combinations of HLA-II molecules and peptides (2–6). Three to five amino acid residues were separated by one to two intervening residue(s) and acted as anchor residue(s) for binding to HLA-II molecules. On the other hand, side chains of amino acid residues flanking anchor residues proved to be the main recognition sites by T-cell receptors (TCR); this was clearly established in crystallographic analyses of the DR molecule complexed with either self (7–9) or non-self peptides (10). At least five DR anchor residues on the DR1 binding peptide and five corresponding independent pockets in the peptide-binding groove of DR1 molecule, which accommodate side chains of DR anchor residues of the peptide, were identified (Fig. 1B). Sixty-five percent of the peptide surface made contact with the DR molecule and the remaining portion was accessible to solvents, thus being recognized by the TCR. Many polymorphic residues of HLA-II molecules are located in the peptide-binding groove and toward the bound peptides. Thereby, polymorphism of HLA-II molecules determines the differences

From Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto University Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811

Internal Medicine Vol. 37, No. 10 (October 1998)
Modification of Human T-Cell Responses by APLs

Figure 1. Structure and function of HLA class II molecules (from references 1 and 10).
A. Presentation of peptides derived from extracellular or membrane non-self or self proteins to CD4+ T cells by HLA class II molecules expressed on an antigen presenting cell. α1, α2, β1 and β2 indicate the extracellular domains of α and β chains of HLA class II molecules. Vα, Vβ, Cα and Cβ indicate variable and constant-regions of T-cell receptor α and β chains, respectively. B. The structure of a peptide-binding groove of the HLA class II molecule. The top of α1 and β1 domains is a groove-like structure consisting of a β-sheet floor and two side walls made of two anti-parallel α-helices. There are five pocket-like structures indicated by circles in the groove and the side-chains of P1 (the most N-terminal 1st DR anchor position), P4, P6, P7 and P9 anchor residues of the binding-peptide are accommodated in each pocket as shown in this figure. Black colored residues are polymorphic in HLA-DR molecules.
in structures of peptides bound to HLA-II molecules. In other words, polymorphism of HLA-II molecules determines individual differences in T-cell responses to a given antigenic peptide.

CD4+ T-cells usually recognize non-self peptides in the context of self HLA-DR molecules. Recognition and responses of T-cells were once considered to be an on/off phenomenon, but recent findings obtained using analogue peptides carrying single residue substitutions in antigenic peptides presented by one major histocompatibility complex (MHC) class II molecule (11–23) or one specific peptide presented by different MHC class II molecules showing a limited polymorphism (13, 24, 25), revealed that altered TCR ligands induce altered T-cell responses. As shown in Fig. 2, altered peptide ligands (APLs) carrying amino acid substitutions in an antigenic peptide, induce T-cell nonresponsiveness, through TCR antagonism (14, 16, 17, 24) or anergy induction as a consequence of partial agonism or incomplete activation (15, 18), and sometimes induce dissociation between proliferative response, cytotoxicity and cytokine production (12, 13). These altered T-cell responses to various altered TCR ligands were evidenced in mice (12, 13, 15, 18, 24) and in humans (11, 14, 16, 17, 19–23, 26).

Figure 2. Altered responses of a CD4+ T-cell clone induced by recognition of APLs carrying residue substitutions in an antigenic peptide. CD4+ T-cells usually recognize non-self antigenic peptides in the context of MHC class II molecules to produce lymphokines and proliferate. These TCR ligands are designated as full agonists. APLs having marked changes in conformation do not stimulate activation in T-cells because of loss of the capacity to bind to MHC molecules or to be recognized by TCR and these APLs are irrelevant for recognition by T-cells. In the presence of excess amounts of some APLs plus MHC class II molecules, T-cell activation induced by a minimal amount of wild type peptide plus MHC molecules is inhibited. These APLs are designated as TCR antagonists and they can make contact with TCRs with a lower affinity and/or a larger off-rate than do wild type TCR ligands. However, they do not stimulate T-cell proliferation so that these APLs antagonize interactions between TCRs and their full agonistic ligands. Some TCR antagonistic APLs partially activate T-cells without stimulating cell proliferation and these APLs are designated as partial agonists. Note that partial agonists are distinct from weak full agonists which stimulate the small proliferative response of T-cells, at high doses. Details of partial activations (agonism) of T-cells by APLs are described in the text.
In mouse T-cell clones, TCR antagonists or partially agonistic APLs induce partial phosphorylation of CD3ε chains leading to the absence of phosphorylation and activation of ZAP70 (26, 27). Studies of calcium signaling activity in mouse T-cells stimulated with APLs indicated that the Ca²⁺ response induced by antagonistic APLs was smaller in amplitude and shorter in duration than that induced by fully agonistic ligands (28–30). Studies on a mouse T-cell clone also showed that the APL-stimulated Ca²⁺ response is initiated and sustained at lower levels than that stimulated by a strong agonistic signal, but it resembles that stimulated by a weaker agonistic stimulus (28, 30).

Recent analyses of physical interactions of purified TCR with MHC-APL complexes revealed differences in binding affinities and kinetics of TCRs with their ligands between MHC-wild type peptide complexes and MHC-APL complexes. Thereby, interactions of TCRs with MHC-wild type peptides had stronger affinities and/or smaller off-rates than did those of TCRs with MHC-APL complexes (31). These differences in characteristics of molecular interactions may cause insufficient engagements of TCR with MHC-APL complexes such that intracellular signals mediated by TCR through recognition of APLs are inadequate for full activation of T-cells to induce cell proliferation. In some cases, inappropriate signals induce unique altered T-cell responses.

**Alteration of the Human T-Cell Response Induced by Single Residue Substitutions of an Antigenic Peptide and/or HLA-DR Molecule**

If the general rule is known for structures of analogue peptides derived from antigenic peptides, which stimulate or inhibit T-cell responses to those wild type antigenic peptides, it would be easier to generate peptides which augment or inhibit responses of human T-cells. We analyzed the responses of a human T-cell clone to a large panel of analogue peptides derived from a non-self antigenic peptide presented by Asian-specific DR4 (DRB 1 *0406), which is associated with susceptibility to the insulin autoimmune syndrome (Hirata's disease) unique to Asian populations (32).

We investigated the frequencies and characteristics of TCR agonism and TCR antagonism, with or without partial activation. The CD4⁺ human T-cell clone YN5-32 recognizes a streptococcal M 12p54-68 pepide in the context of HLA-DR4 (DRA+DRB 1 *0406) and produces a large amount of interferon-γ (IFN-γ) and a small amount of IL-4. We investigated the responses of YN5-32 to 154 independent analogue peptides carrying single residue substitutions in a streptococcal antigenic peptide M12p54-68. From p1 to p7 residues, residues were replaced to all other 19 amino acids. The p8 and p9 residues were replaced with 10 and 11 other amino acids, respectively. Percentages of analogue peptides exhibiting either full agonism (open bars), TCR antagonism (shaded bars) or partial agonism (closed bars) are indicated for each residue. APLs carrying substitutions at putative TCR contact residues, P2, P4, P5 and P7, frequently exhibited TCR antagonism. Some of them, especially APLs substituted at P7, exhibited partial agonism. Because APLs having full agonism or TCR antagonism have to bind to MHC molecules, the frequencies of those peptides indicated by cross hatched bars represent the frequency of peptides having MHC-binding capacity.

![Figure 3. Summary of responses of the T-cell clone YN5-32 to 154 analogue peptides carrying single residue substitutions in a streptococcal antigenic peptide M12p54-68. From p1 to p7 residues, residues were replaced to all other 19 amino acids. The p8 and p9 residues were replaced with 10 and 11 other amino acids, respectively. Percentages of analogue peptides exhibiting either full agonism (open bars), TCR antagonism (shaded bars) or partial agonism (closed bars) are indicated for each residue. APLs carrying substitutions at putative TCR contact residues, P2, P4, P5 and P7, frequently exhibited TCR antagonism. Some of them, especially APLs substituted at P7, exhibited partial agonism. Because APLs having full agonism or TCR antagonism have to bind to MHC molecules, the frequencies of those peptides indicated by cross hatched bars represent the frequency of peptides having MHC-binding capacity.](image-url)
responds in the T-cell clone. Only 7.5% (3/40) of non-fully agonistic peptides exhibited TCR antagonism. On the other hand, residues Glu-58 (P2), Tyr-61 (P5) and Glu-63 (P7) were the most likely to be TCR-recognition sites and only 15.8% (9/57) of analogues stimulated proliferative responses in YN5-32 thereby indicating that substitutions at these residues frequently abrogate T-cell recognition. Interestingly, as many as 60.4% (29/48) of non-fully agonistic analogues exhibited TCR antagonism to inhibit the proliferative response of YN5-32 to the wild type peptide. TCR antagonistic activity of the analogue peptides means a definite binding of those peptides to HLA-DR molecules. Therefore, up to 76.2% of analogues substituted at one of these three residues binds to HLA-DR, and thus, three residues could not be DR-anchor residues. Our studies of DR4 (DRB1*0406)-binding peptide motif (5) also suggested that residues Leu-57(P1), Ala-60(P4) and Asn-62(P6) belong to DR anchor residues.

Eight (27.6%) of these antagonistic analogues carrying relatively conservative amino acid substitutions exhibited partial agonism to induce large increases in cell size and expression levels of CD4, CD11a (LFA-1), CD28, CD49d (VLA-4) and CD95 (Fas), and small increases in CD25 and CD44 expression on the T-cell surface, as compared with responses to the wild type peptide. The most prominent at Glu-63 (p7) where 5 of 10 antagonistic analogues exhibited partial agonism. The wild type peptide (but not the partial agonistic APLs) induced down-modulation of CD3 expression and upregulation of CD54 and CD69 expressions. None of the partially agonistic APLs stimulated IFN-γ production and induced anergy. These observations indicate that many APLs carrying substitutions at TCR recognition sites in the T-cell epitope induce a partial agonism as well as TCR antagonism in a human T-cell clone, as noted by other studies of murine T-cell clones. Differences, such as the absence of anergy induction or little increase in CD25 expression by partially agonistic APLs have been noted in human T-cells.

YN5-32 responds better to M12p54-68 presented by allogeic DRB1*0403 with a single Tyr-substitution at DRβ37-Ser of the DRB1*0406 molecule. Because single amino acid polymorphism at residue 37 of the HLA-DRβ chain (DRβ37) between DRB1*0406 and 0403 markedly influences the susceptibility to the insulin autoimmune syndrome (32), we investigated the effects of DRβ37 polymorphism regarding recognition of non-self peptides by YN5-32 (25). One hundred and fifty-four analogue peptides described above were tested for fully agonistic and TCR antagonist activities. Forty-six peptides showed full agonism, thirty-four analogues exhibited TCR antagonism and forty-five analogues exhibited neither full agonism nor TCR antagonism, irrespective of the presenting molecules DRB1*0406 or DRB1*0403. On the other hand, twenty-nine analogue peptides substituted at each of residues 57(P1)-63(P7) of M12p54-68 were recognized differently by YN5-32, depending on the presenting HLA-DR molecules. These observations indicate that 1) single amino acid polymorphism (Ser-Tyr) at the DRβ37 residue induced conformational changes of which can be distinguished by TCR of the T-cell clone in some but not all peptides; and 2) these conformational changes were observed even in APLs carrying single residue substitutions at residues far from a putative DRβ37 contact site. These findings provide further evidence for altered human T-cell responses induced by minor modifications of TCR ligands.

**Immuno-Suppressive Peptides Acting on a T-Cell Clone Autoreactive to Acetylcholine Receptor (AChR)**

Myasthenia gravis (MG) is an autoimmune disease accompanied by immunoglobulin G (IgG)-class autoantibodies to the AChR which is expressed at the neuromuscular junction. Anti-AChR autoantibodies in MG patients cause accelerated degradation of the AChR triggered by AChR crosslinking or by complement-mediated lysis of the postsynaptic membrane, and block the cholinergic site (33, 34). Because CD4+ helper T-cells are essential for induction of IgG production by B cells, CD4+ T-cells autoreactive to AChR have a crucial role in the development of MG, in experimental autoimmune MG in mice (35).

The distribution of the age at onset of Japanese MG patients shows bimodal peaks: the first and the highest peak is seen before the age of 3 years and the second lower peak occurs in the third decade of life (36). In contrast, in Caucasians, the age at onset of MG generally shows a single peak in adulthood (37), in which susceptibility to MG is associated with HLA-DR3 and HLA-B8 (38, 39). In the Japanese patients in whom the disease developed before the age of 3 years old, the frequencies of HLA class II haplotypes common in the Asian populations, HLA-DR9 (DRB1*0901)-DQ9 (DQA1*0301-DQB1*0303), DR13 (DRB1*1302)-DQ6 (DQA1*0102-DQB1*0604) and their double-heterozygotes were significantly increased (40, 41). Clinical manifestations of myasthenia are also different between infant-onset MG and adult-onset MG. The former has a relatively benign prognosis and affects mainly ocular muscles without causing severe and generalized muscle weakness.

The AChR of mature muscle consists of five subunits in the stoichiometry of two α, one each β, δ and ε subunits (42). The AChRα subunit exists in two isoforms, with or without a peptide encoded for by exon P3A which can be alternatively spliced (43, 44). Their functional difference is unknown. The binding sites of the anti-AChR auto-antibodies locate at an extracellular region of the α subunit, designated as the main immunogenic region (45). The acetylcholine binding sites of AChR consist of α-γε and α-δ complexes, and epitopes recognized by CD4+ αβ autoreactive T-cells have been identified in AChR α, β, γ and δ subunits in MG (46-50).

To elucidate the mechanisms involved in susceptibility to infant-onset MG associated with HLA class II alleles, we established and analyzed an AChRα-autoreactive CD4+ αβ T-cell clone from a Japanese patient with infant-onset MG and we analyzed the effects of analogues peptides derived from an autotaginic peptide, on the immune responses of the autoreactive T-cell clone (51). An AChRα p71–91 peptide-
Modification of Human T-Cell Responses by APLs

Specific autoreactive CD4+ αβ T-cell clone was established by stimulating peripheral blood mononuclear cells from a patient heterozygous for two disease-susceptible HLA-DR9-DQ9 and DR12-DQ6 haplotypes with a mixture of overlapping peptides covering AChRα subunit. The T cell clone recognized the AChRα peptide in the context of the HLA-DQ6 molecule and produced a large amount of IFN-γ and a trace amount of interleukin (IL)-4 showing a Th1-like phenotype. The majority (p75–83) of the core epitope of the autoantigenic peptide (p75–87) is encoded for by exon P3A of the AChRα gene which can be alternatively spliced. In Caucasian MG, T-cells autoreactive to this AChRα epitope are rarely noted (50). The T cell clone responds to the recombinant AChRα protein with a P3A exon product, but not without a P3A exon product.

Because analogues of the antigenic peptides substituted at TCR-recognition residues are good candidates for immunosuppressive peptides with a TCR antagonistic property, we applied this knowledge to determine inhibitory APLs which specifically act on AChRα autoreactive T-cells. For this, we investigated the responses of T-cell clone to 114 analogue peptides carrying single residue substitutions in the core AChRα peptide. The majority of APLs substituted at either residue Phe-77, Leu-80 or Asn-82 stimulated proliferation of the T-cell clone. Conversely, the majority of analogue peptides substituted at either Gln-81 or Glu-83 did not stimulate proliferative responses, which suggested that these two residues may be TCR contact sites. As shown in Fig. 4, APLs exhibited potent or intermediate inhibitory effects on proliferative responses of the T-cell clone to the wild type peptide, probably by TCR antagonism. Thus, an HLA class II allele common in Asians may directly control susceptibility to the Asian-specific clinical subtype of myasthenia gravis. Analogues of the auto-antigenic AChRα peptide may provide new tools for effective immunosuppressive therapy.

**Augmentation of T-Cell Response by an Analogue of the Mutated Ras-derived Peptides:**

**A Possible Approach for Generation of a Potent Anti-Tumor Peptide Vaccine**

A T-cell response to a tumor requires a tumor antigen processed into peptides which can be presented to CD8+ T-cells by MHC class I, and to CD4+ T-cells by MHC class II molecules. While CD8+ cytotoxic T-cells can kill tumor cells directly, some Th1 CD4+ T-cells can mediate cytotoxicity to tumors (52, 53), amplify responses of cytotoxic T-cells, and activate APC, through secretion of lymphokines to augment anti-cancer immunity. Ras is a proto-oncogene that encodes p21-Ras and point mutations in this gene are involved in carcinogenesis of various types of malignancies. Mutation of the transforming p21-Ras gene is apparently limited to codons 12, 13, and 61 (54), and these mutated gene products carry single amino acid substitutions representing one of the cancer-specific proteins. If the proteolytically cleaved moiety of mutant p21 Ras encompassing mutation hot spots can bind to MHC molecules, it would be recognized by T-cells and could serve as a target for antigen-specific tumor immunity. Indeed, human T-cells that recognize mutated Ras protein and/or peptide have been noted in healthy donors (55–58) and in cancer patients (59–63). These T-cells can play a role in host-cancer interactions (60), and adoptive transfer of these T-cells or immunization with mutated p21-Ras derived peptides may be one strategy for the design of cancer immunotherapy. In animal models, immunization with a mutated p21-Ras protein elicited protective immunity against a challenge with tumor cells carrying the corresponding mutation, and therapeutic benefit was achieved in mice bearing tumors expressing the same mutated p21-Ras protein (64).
Precise molecular analysis of the interaction between antigenic peptides, HLA, and T-cells that participate in anti-cancer immune responses would pave the way toward development of an effective immunotherapy. The peptide-binding groove of HLA-DR molecules has several pockets that accommodate side chains of the bound peptides (7–10). Augmentation of peptide antigenicity by substituting the MHC anchor residue has been reported (65). This observation suggests that T-cells mediating immune responses and diseases may possibly be controlled by modification of T-cell responses by APLs. Thus, manipulation of anti-cancer T-cell responses by derivatives of tumor-related peptides may be one rationale for the induction of a more effective anti-cancer immunity. However, to date, human T-cell responses against derivatives of mutated p21 Ras peptides have rarely been documented.

We established a CD4+ T-cell clone that recognizes mutated p21 Ras proteins as well as mutated peptides, in an HLA-DR1 restricted manner. We provided the first evidence for augmentation of responses of this T-cell clone by an analogue peptide carrying a single residue substitution in a mutated P21 Ras peptide (66). Using spleen cells from a gastric cancer patient, we established the CD4+ CD8 Th1-like clone C27 that recognizes wild-type (EYKLVVVGAGGVGKS) and mutated p21 Ras protein molecules and peptides, in an HLA-DR1-restricted manner. C27 responded prominently to mutated Ras peptides carrying Val or Ala at position 12 (designated as Ras p3-17 G12V or A), as compared to wild-type and other mutated peptides. C27 also exhibited a much stronger response to a mutated p21 Ras whole protein molecule carrying Val at position 12, as compared with the wild-type protein. The largest and most hydrophobic peptide-binding pocket of HLA-DR in which the side chain of the first DR anchor residue of the peptide is buried, shows a strong preference for large hydrophobic side chains and appears to be the major determinant of peptides bound to DR molecules (3, 7, 10, 67, 68). As shown in Fig. 5, the proliferative response and production of GM-CSF...
and IFN-γ by C27 were further augmented by replacing the possible first DR anchor Tyr of the mutated Ras peptide with Trp (a peptide designated as Ras p3–17 Y4W-G12V), a more potent anchor residue for the DR1 molecule. Enhancement of peptide antigenicity by substituting the HLA anchor residue of an antigenic peptide recognized by tumor-reactive T-cells may possibly be a novel strategy for antigen-specific cancer immunotherapy.

Furthermore, we recently found that anti-CD29 mAb MAR4 is effective for in vitro expansion of K-ras (residue 3-20)-reactive T-cells from PBMC (69). MAR4 inhibits anti-CD3-induced apoptosis of PBMC and is effective in enhancing anti-CD3- and antigen-induced proliferative responses of PBMC. Therefore, mAb MAR4 increases efficiency in establishing T-cell clones from PBMC, both by suppressing antigen-driven activation-induced cell death and by enhancing T-cell proliferation, but only in the presence of TCR/CD3-mediated stimulation. Use of this type of reagent with T-cell co-stimulatory activity together with immuno-potentiating analogues of tumor antigenic peptides may turn out to be an appropriate form of anti-tumor immunotherapy.

**APL Affects Not Only T-Cell Response but Also APC Response to Increase IL-12 Production: Implication to Peptide Therapy Inducing Th1-Dominance**

In mice, CD4+ T-cell clones can be divided into two categories, depending on the lymphokines produced, T helper-1 (Th1) and T helper-2 (Th2) (see review 70). Th1 produces IL-2, IFN-γ and TNF which activate APC and cytotoxic T-cells to induce inflammatory responses. On the other hand, Th2 produces IL-4, IL-5 and IL-10 to induce proliferation and differentiation of B cells and promotes the production of immunoglobulin. Because IL-4 is essential for immunoglobulin E (IgE) production by B cells (71), activation of Th2 has a crucial role in the development of allergic diseases. Even though many human T-cell clones show both Th1 and Th2-like phenotypes designated as Th0, IL-4 produced by T-cells is important for IgE production (72). In contrast, Th1-cytokine IFN-γ inhibits IgE production by B cells.

T-cell epitopes of several major allergens have been reported (21, 73–78). House dust mite is the most potent allergen in Japan; accounting for over 70% of the bronchial asthma in children. Two mite species of the genus *Dermatophagoides*, *Dermatophagoides farinae* (Der f) and *Dermatophagoides pteronyssinus* (Der p), are often responsible for bronchial asthma, allergic rhinitis, and atopic dermatitis. The group I allergen in *Dermatophagoides farinae* extracts (Der f I) from *Dermatophagoides farinae* as well as Der p I from *Dermatophagoides pteronyssinus* show a significant homology with a group of cysteine proteases, the homology in sequence of cDNA between these two mite allergens being approximately 80%. Epitopes of Der p I recognized by human T-cells were reported in other studies (73), and those of Der f I were identified in our laboratory (78).

We generated human T-cell lines and clones specific to Der f I and investigated the effects of analogue peptides on alterations of T-cell responses (22). Using a panel of overlapping synthetic peptides that cover the entire Der f I molecule, we demonstrated that polyclonal Der f I-specific T-cell lines prepared from the peripheral blood of five atopic patients that cover most of the common HLA haplotypes in the Japanese population, can respond to 13 different epitopes of the molecule. Each donor showed a different pattern of reactivity with the synthetic peptides, with some overlaps. One of these Der f I-specific T-cell clones DTI3.2, established from a patient with bronchial asthma, recognized an antigenic peptide Der f I p18–31 in the context of HLA-DQ6 (DQA1*0102-DQB1*0602) molecules and secreted both interleukin-4 and interferon-γ.

As shown in Fig. 6, analysis of changes in DTI3.2 responses to Der f I p18–31-derived analogue peptides revealed that the substitution of 21 Arg to Lys resulted in a significant increase in interferon-γ production, with no remarkable changes either in proliferative response or interleukin-4 production. Tyrosine phosphorylation induced by the analogue peptide was higher in magnitude than that induced by the wild-type peptide, without qualitative differences. Interestingly, the selective enhancement of IFN-γ by the analogue peptide was accompanied by increased production of IL-12, which was suppressed by anti IL-12 antibody down to the level of IFN-γ production induced by the wild-type peptide. Because IFN-γ stimulates IL-12 production by APC, it would seem that APL primarily augmented IFN-γ production by T-cells to enhance IL-12 production by APC. But this is not the case, because even in the presence of sufficient amounts of anti-IFN-γ and anti-IFN-γ receptor antibodies for neutralization of biological activity of IFN-γ, the augmented production of IL-12 remained unchanged.

These results collectively indicate that some APLs induce certain altered signaling within APC, which may eventually lead to the qualitative changes in T-cell responses. Our observations suggest that the mode of interaction between TCR and MHC-peptide complex may determine Th1-predisposing condition by controlling IL-12 production by APC. Furthermore this kind of Th1-response inducing APLs may provide a peptide therapy for diseases caused by Th2 responses such as allergy.

**A Self-Homologue of a Non-Self Antigenic Peptide Exhibits Partial Agonism to Prolong Clonal Survival of Effector T-Cells in vitro**

We are also investigating the biological significance of human T-cell responses to APLs. A self-homologue of a non-self antigenic peptide may contribute to maintain the survival of T-cells specific to non-self antigens. It was reported that recognition of APLs supports survival and differentiation of thymocytes in the thymus, an event known as positive selection (79, 80). In addition to one-residue substituted analogues of wild-type peptides, non-self-reactive T-cell clones can be fully or partially be activated by minimally homologous self-peptides.
Figure 6. Scheme for augmented T-cell production of IFN-γ induced by IL-12 produced from antigen presenting cells in response to an altered peptide ligand. A Der f I-specific T-cell clone DT13.2, established from a bronchial asthma patient, recognized an antigenic peptide Der f I p18–31 in the context of HLA-DQ6 (DQA1*0102-DQB1*0602) molecules and secreted both interleukin-4 and interferon-γ. Der f I p18–31-derived analogue peptide, R21K having substitution of 21Arg to Lys, induced enhancement of interferon-γ production by DT13.2, with no remarkable changes either in proliferative response or interleukin-4 production. This phenomenon was accompanied by the increased production of IL-12 by APC, and was suppressed by anti-IL-12 antibody down to the level of IFNγ production induced by the wild-type peptide. Augmented production of IL-12 by APC stimulated with R21K was still observed in the presence of anti-IFN-γ and anti-IFN-γ receptor antibodies. These results collectively indicate that some analogue peptides induce a certain altered signaling within APC, which may eventually lead to the qualitative changes in T-cell responses.

T-cell clones and peptides used for the study

We established three independent CD4+ Th0 clones from PBMC of a Bacillus Calmette-Guérin (BCG)-primed individual by stimulating with overlapped synthetic peptides of the BCGa protein (85). Three T-cell clones, BC20.7, BC33.5 and BC42.1 recognize BCGa p84–100 (84EEYLILSARDVLAVVSK100) peptide and it’s truncated derivatives presented by DR14 molecules (DRA+DRB1*1405), in a similar manner and have a distinct TCR-β usage (86). To investigate the responses of these T-cell clones to various APLs, we used: (a) single residue-substituted analogues of BCGa p86–94 peptide which is the core-sequence peptide for full activation of all three T-cell clones; and (b) self peptides having characteristics in structure, polarity and static charges similar to those of BCGa p86–94 peptide, that were identified using human protein sequences registered in the Swiss Protein Database (23).

The wild-type BCGa p84–100 induced proliferation of the T-cell clones at peptide concentrations as low as 100 pM and in a dose-dependent manner. The self homologue, human connexin 26p33-49 (HCp33-49; IMILVVAAKEVWGDEQA) did not induce proliferation of all three T-cell clones, even at 10 μM. However, D93E standing for an analogue peptide carrying Glu(E)-substitution at the 93rd residue Asp(D) of BCGa p84–100 peptide and D93Y peptides did induce proliferation of BC42.1 at 10 μM, thereby indicating that they bind to DR14 molecules. D93Y induces very weak proliferation of BC33.5 at 10 μM but not at 3 μM or 1 μM.

Sustained viability and T-cell proliferation after stimulation with non-fully agonistic peptides

We then asked whether the self homologue and analogue peptides described above would be capable of prolonging T-
Modification of Human T-Cell Responses by APLs

cell survival in vitro at concentrations below the optimal concentration required for induction of proliferation or at 10 μM for peptides that do not induce T-cell proliferation. To investigate the antigen-specific proliferative response of surviving T-cells, T-cells were co-incubated with irradiated autologous PBMC in the presence of various peptides that do not induce T-cell proliferation, followed by the addition of irradiated autologous PBMC pulsed with the wild-type peptide, one to nine days after the initial stimulation. After preincubation with 10 μM HCp33-49, the proliferative responses of BC20.7 were significantly higher upon re-stimulation with the wild-type peptide. A similar observation was noted for BC33.5 preincubated with 1 μM of D93Y (p<0.01 on days 1, 3, 5, and 7). In striking contrast, pre-incubation with 10 pM of the wild-type peptide, below the optimal concentration for the induction of proliferation, did not enhance proliferation of T-cells upon re-stimulation as compared with a medium only control. Preincubation with the other peptides showed no differences from those in the case of medium alone.

Moreover, trypan blue exclusion assay with the same combination of T-cells and peptides exhibited viable cell counts parallel to the results (p<0.05 at days 1, 3, 5, 7, and 9). Flow cytometric analysis confirmed that survival of specific TCR Vβ-bearing T-cells but not feeder cells was enhanced. Because day 5 after the stimulation with D93Y or HCp33-49 was optimal to observe effects on survival, the dose response to these peptides of T-cell survival was tested on day 5. As shown in Fig. 7A, B, and C the effects were dose dependent and clone specific. Thus, T-cell clones pretreated with higher concentrations of appropriate APLs, including self HCp33-49 peptide, exhibited a stronger proliferative response to the wild-type peptide, thereby indicating that survival of competent T-cells was prolonged in a manner dependent on the dose of APL.

Because partially agonistic TCR-agonists reported previously exhibited TCR antagonism, we investigated TCR antagonistic activity of T-cell survival-prolonging peptides, using so-called pre-pulse assay where APCs are pre-pulsed with low-dose wild-type peptide followed by incubation with T-cells in the presence of high-dose soluble APLs (20). D93Y peptide antagonized the response to wild-type peptide of BC20.7 and BC33.5 clones, and HCp33-49 peptide did so in BC20.7 and BC42.1 clones, indicating that these peptides are recognized by TCR, in combinations where prolonged T-cell reactivity is observed.

Certain peptide partial agonists can reportedly induce a state of T-cell anergy (15, 18, 81, 87). However, in our human systems with an M12-reactive T-cell clone (20) and BCGa-reactive T-cell clones (87), BC20.7-D93E was the only combination that induced clonal anergy with viable APC (87). Peptide D93E did not prolong survival of BC20.7; connexin peptide was the only partial agonist that induced survival but not anergy of BC20.7. Thus, judging from the peptide-clone combinations tested in our human systems, anergy and survival do not appear to correlate. Connexin 26 shown in this study is expressed in various cell types, in a cell cycle-dependent manner (88), and can be immunohistologically detected in human lymphoid tissues, such as the tonsil (89). This suggests that connexin protein molecules can be processed by APC. Further studies are necessary to determine if the peptide fragment is produced in a natural setting.

Expression of Bcl proteins after stimulation with survival-inducing APLs

It was reported that some proteins of the Bcl-family protein

![Figure 7. Sustained T-cell proliferation after stimulation with non-fully agonistic peptides. All three T-cell clones BC20.7 (A), BC33.5 (B) and BC42.1 (C) were cultured either with medium (closed circle), 1 μM of D93Y (EYLILSARYVLAVVSK) (open circle), or with 10 μM of HCp33-49 (IMILVVAAKEVWGDEQA) (closed triangle) in the presence of irradiated autologous PBMC. Five days after the initial stimulation with these peptides, irradiated autologous PBMC pre-pulsed with the 5 μM wild-type peptide was added to determine the proliferative response of viable T cells. All data are expressed as the mean value of triplicate determinations ± standard error.](image-url)
Table 1. Various Altered Responses of Non-Self-Specific T-Cells in Recognition of a homologous Self-Peptide or Analogue Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>T-cell clone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BC20.7</td>
</tr>
<tr>
<td>Self connexin 26</td>
<td>prolonged</td>
</tr>
<tr>
<td></td>
<td>survival*</td>
</tr>
<tr>
<td>D93Y</td>
<td>IL-4/IFN-γ</td>
</tr>
<tr>
<td></td>
<td>production*</td>
</tr>
<tr>
<td>D93E</td>
<td>anergy</td>
</tr>
<tr>
<td></td>
<td>production*</td>
</tr>
</tbody>
</table>

*These clone-peptide combinations exhibit partial agonism (TCR antagonism with partial activation without proliferation).

Adoptive transfer experiments revealed that in a population of CD8+ T-cells, memory could persist for a long time in the absence of wild-type antigen (94). Furthermore, analysis of memory in the CD8+ T-cell compartment by utilizing T-cell receptor transgenic mice expressing a transgenic TCR specific for H-Y antigen, showed that memory cells can be derived from activated T-cells and persist in the absence of wild-type antigen (H-Y) for at least 13 weeks, in female nu/nu recipient mice (95). Although it is not clear whether T-cells are stimulated by endogenous partial agonistic APLs in these systems, it is suggested that T-cell memory is maintained in the absence of wild-type ligands. It is also noteworthy that long-term T-cell survival is difficult to maintain, in MHC-deficient mice (96). Our current study suggests that possible involvement of TCR partial agonism induced by self-homologues of non-self peptides presented by self-MHC molecules in the prolongation of T-cell survival and in the maintenance of T-cell memory.

In conclusion, analyses of T-cell responses to APLs has provided pertinent information on the basic immunology of T-cell biology and also on the strategy for new methods for modification of antigen-specific responses of T-cells.

Acknowledgements: We appreciate M. Ohara for providing helpful comments. This work was supported in part by Grants-in-Aid 05272104, 05278118, 06454222, 08557027 nd 09470097 from the Ministry of Education, Science, Sports and Culture, Japan, a Research Grant for Intractable Diseases from the Ministry of Health and Welfare, Japan, and the Ichiro Kanehara Foundation, Terumo Life Science Foundation, Japan Rheumatism Foundation, and Mochida Memorial Foundation, The Cell Science Research Foundation, Suzuken Memorial Foundation and The Naito Foundation.

A note added in the proof: We have recently published a study on modulation of calcium response of the T-cell clone YN5-32 by altered peptide ligands in which we found that intracellular calcium responses were different in calcium source, quantity, quality and signaling pathways involved among stimuli by wild type peptide, partially agonistic and simple antagonistic APLs. [Chen, Y-Z., Lai, Z-F., Nishi, K., and Nishimura, Y. Modulation of calcium response by altered peptide ligands in a human T cell clone. Eur. J. Immunol. in press]

References


19) Fukuyama Y, Hirayama Y, Osawa M. Myasthenia Gravis, Pathogenesis and Treatment, Japan Medical Research Foundation, University of Tokyo Press, Tokyo, 1981.


40) Fukuyama Y, Hirayama Y, Osawa M. Myasthenia Gravis, Pathogenesis and Treatment, Japan Medical Research Foundation, University of Tokyo Press, Tokyo, 1981.


49) Tzartos SJ, Kokla A, Walgrave SL, Conti-Tronconi BM. Localization of
Modification of Human T-Cell Responses by APLs


Takeda S, Rodewald HR, Arakawa H, Bluethmann H, Shimizu T. MHC class II molecules are not required for survival of newly generated CD4+ T cells but affect their long-term life span. Immunity 5: 217–228, 1996.