for the clinical association between microbial infection and the clonal expansion of autoreactive T cells as a cause of autoimmune disease. One mechanism is the activation and expansion of autoreactive T cells by an antigen nonspecific inflammatory stimulus of the innate immune system.[3] Microorganism-induced activation of antigen-presenting cells (APCs) leads to the release of inflammatory cytokines and/or chemokines, thus enhancing antigen-processing, and the up-regulation of peptide–MHC complexes and co-stimulatory molecules on the cell surface (microbial adjuvant effects).[4-8] Local infection causes tissue destruction, and the release of sequestered antigen follows.[9,10] The inflammatory state can also promote the expansion of memory T cell populations (bystander activation).[3,11] Another mechanism is that microbial superantigens activate large numbers of T cells expressing particular Vβ gene segments, and a limited population can cross-react to a self-antigen.[12-14] Cross-recognition by T cells between self-antigens and infectious agents is another important mechanism (molecular mimicry theory).[15,16] In any case, tissue destruction would be expanded by epitope spreading. These concepts are useful to explain the as yet unsolved mechanisms for the etiological linkage between infection and autoimmunity.

The concept of molecular mimicry was broadened by recent insights into T cell recognition. Molecular mimicry phenomena were noted in disease-associated autoreactive CD4+ T cells in autoimmune disorders such as multiple sclerosis (MS), type I diabetes, and Lyme arthritis in humans.[17-20] Lyme disease, a chronic inflammatory joint disease, is caused by infection with the spirochete Borrelia burgdorferi.[19] Susceptibility to Lyme arthritis is associated with HLA-DR4 and HLA-DR1 alleles. It has been reported that synovial fluid T cells from patients with treatment-resistant Lyme disease showed a strong response to outer surface proteins A (OspA) of B. burgdorferi and cross-reacted to self LFA-1. The sequence homology between OspA p165–173 and the αL-chain of hLFA-1 p332–340 was considered to be a basis for molecular mimicry in treatment-resistant Lyme disease.[19] On the other hand, the association between microbial infection and autoimmune disease has also been investigated using a mouse model.[21-24] In a model of herpes simplex virus (HSV) type-I induced herpes stromal keratitis (HSK), which is a T cell-mediated inflammatory disease of the cornea, corneal antigen autoreactive T cells recognized HSV-1 UL-6 protein.[21] However, an HSV-1 point mutant that contains a single amino acid substitution within the putative mimicry epitope impaired its capacity to induce HSK.[22] In this system, two different pathogenic pathways, innate immune mechanisms and molecular mimicry, are involved. In this report, mimicry was considered to be essential for disease induction with a limited number of autoreactive T cells, while innate immune mechanisms are also important to provoke disease with high numbers of autoreactive T cells.

Because of the importance of CD4+ T cells in the development of autoimmunity, efforts have been directed toward the identification of cross-reactive epitopes of microbial antigens recognized by autoreactive CD4+ T cells. For many years, antigen recognition by TCR was considered to be highly specific, and the concept of molecular mimicry had been defined based on the level of primary sequence similarities between self and antigenic determinants of infectious microorganisms.[25] Since the 1990s, studies using peptide analogues with single amino acid substitution or positional scanning synthetic combinatorial peptide libraries (PS-SCLs) have demonstrated that antigen recognition by TCR is highly degenerate and many different peptides can activate an individual T cell.[26-31] Wucherpfennig and Strominger[27] reported that microbial peptides with a relatively limited sequence homology to myelin basic protein (MBP) could activate MBP autoreactive T cell clones. Using PS-SCLs, Hemmer et al.[28] noted differing recognition profiles in individual autoreactive T cell clones from patients with MS, and predicted stimulatory ligands that showed no sequence homology with the known cognate peptide. Therefore, molecular mimicry may be a more frequent event than was generally assumed.
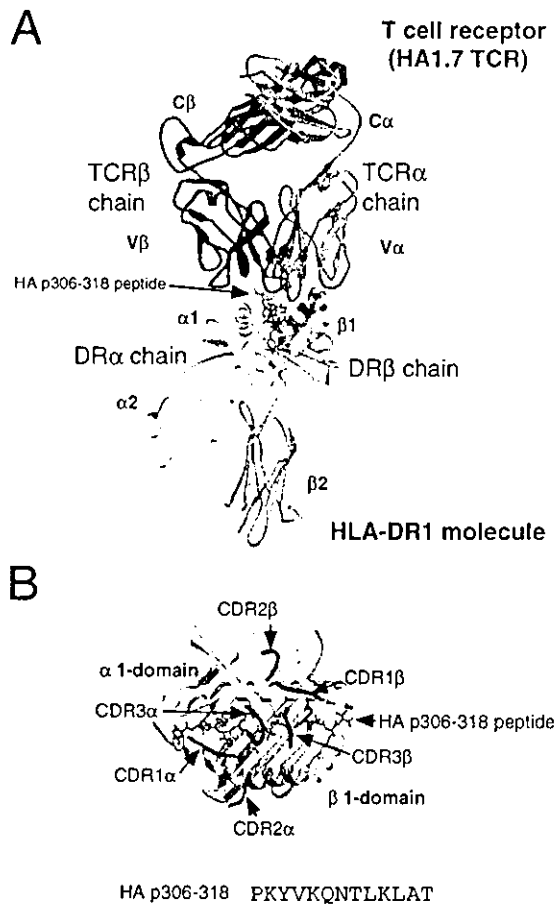
Here, we focus on: (1) degeneracy in antigen recognition by TCR; (2) differences in the physiological outcomes of T cell responses manipulated by altered peptide ligands; (3) the latest methods for the identification of diverse TCR epitopes for CD4+ T cell clones.

Structure of the TCR-peptide/HLA-class II complex

The HLA-class II molecules are heterodimeric membrane glycoproteins consisting of α and β chains. DRα chains are monomorphic, but DPα and DQα chains and β chains are highly polymorphic. The molecule has a peptide-binding groove on the top of the molecule and binds antigenic peptides processed by APCs such as dendritic cells (DCs) or B cells. The structural requirements for HLA-class II binding peptides have been analyzed in detail, and peptide binding motifs specific for various human and mouse class II molecules have been reported.[32] Three to five amino acid residues, separated from each other by one to two intervening residue(s), acted as anchor residue(s), for binding to HLA-class II molecules.[33] On the other hand, side chains of amino acid residues flanking anchor residues were the main recognition sites for TCR. This view was clearly established in crystallographic analyses of the DR molecules bound by either self-[34] or nonself-peptides.[35] 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. Most pockets in the groove of the HLA-class II molecules are shaped by clusters of polymorphic residues, indicating that the class II allelic variant has a major effect on differences in the structures of bound peptides, and determines the individual differences in T cell responses to a given antigenic peptide.

TCR is composed of two membrane-anchored polypeptides, α and β chains, and each chain consists of one constant (C) and one variable (V) domain. The TCR Vα or Vβ regions are composed of V-Jα or V-D-Jβ productively rear-

A

T cell receptor (HA1.7 TCR)

Cβ

Cα

TCRβ chain

TCRα chain

Vβ

Vα

HA p306-318 peptide

α1

β1

DRα chain

DRβ chain

α2

β2

HLA-DR1 molecule

B

CDR2β

α 1-domain

CDR1β

CDR3α

HA p306-318 peptide

CDR1α

CDR3β

β 1-domain

CDR2α

HA p306-318 PKYVKQNTLKLAT

Fig. 1. A Structure of the HA1.7 TCR-HA/DR1 complex[40] with TCR at the top and DR1 at the bottom. The T cell clone HA1.7 is specific to HA p306-318 in the context of HLA-DR1. 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 the variable and constant regions of T cell receptor α or β chains, respectively. **B** The structure of HA p306-318/HLA-DR1 complex and relative orientation of the CDR loops of HA1.7 TCR on top of HA/DR1 complex. CDR loops of TCR Vα and Vβ chains are displayed in tubes. HA p306-318 peptide is shown in a ball-and-stick model. The top of α1 and β1 domains create a groove-like structure consisting of a β-sheet floor and two side walls made of two antiparallel α-helices. The peptide sequences of HA p306-308 are given in a single-letter amino acid code. This figure was produced by BOBSCRIPT[41]

ranged gene products.[36] The complementary determining regions (CDRs) are hypervariable loops at one end of the TCR that recognize the HLA molecule and the antigenic surface derived from the solvent-accessible side chains of amino acid residues flanking HLA anchoring residues.

Recent crystallographic studies of TCR–peptide/MHC complexes provide a structural basis for antigen recognition by αβTCRs.[37] All αβTCRs represent a relatively flat surface bound to the peptide/MHC complex, and represent a similar binding mode. The angle between the peptide direction and the long axis of the class I-restricted TCR interface is between 45 and 70° (diagonal mode).[38] In contrast, the class II-restricted TCR interface is between 70 and 80° (orthogonal mode).[39,40] Figure 1 shows a recent model of the crystallographic structure of the HA1.7 TCR-HA p306–318/DR1

complex. The CDR1 loops of the TCR V-regions contact both peptide and MHC molecules. On the other hand, the CDR2 loops contact prominent α-helices of the MHC molecule. The long CDR3 loops of the TCR V-regions extend down over the center of the antigenic peptide. TCR contacts span only nine residues (P - 1-P8) of the antigenic peptide in both human and murine TCR-peptide/MHC II complexes.

Differences in physiological outcomes of T cell responses manipulated by altered peptide ligands

Altered peptide ligands (APLs) represent a useful tool for studying differential recognition by TCR. It was previously considered that the recognition and response of T cells were apparently an on/off phenomenon. However, findings in mice utilizing peptide analogues with a single residue substitution revealed that T cell clones recognize these APLs and altered T cell responses occur. APLs induced T cell nonresponsiveness through TCR antagonism[42,43] or the induction of anergy as a consequence of partial activation,[44,45] and sometimes induced dissociation between proliferative response and cytokine production.[46,47] Some peptide analogues with antagonistic properties for TCR partially stimulated T cells to induce increases in cell size and expression levels of CD11a (LFA-1) and CD25 (IL-2R) on the T cell surface, but not proliferation.[44] Analyses of the physical interactions of purified TCR with MHC–APL complexes revealed differences in the half-life of receptor–ligand interactions. These phenomena led to differences in the signals transduced by the TCR, resulting in differences in functional outcomes.[48]

We analyzed the responses of human CD4+ T cell clones to a large number of peptide analogues.[29,31] Figure 2 shows a summary of the antagonistic activity of APLs in an acetylcholine receptor (AChR) α subunit autoreactive T cell clone SK2.11, established from a patient with infant-onset myasthenia gravis (MG).[31] Infant-onset MG is unique to Asian populations, and disease susceptibility is strongly associated with DR9 (DRB1*0901)-DQ9 (DQA1*0301-DQB1*0303) and DR13 (DRB1*1302)-DQ6 (DQA1*0102-DQB1*0604) haplotypes that are also unique to Asians. This T cell clone recognizes AchRα p71–91 in the context of disease-susceptible DQ6 (DQA1*0102-DQB1*0604). Although the majority of analogues substituted at residues Phe-77, Leu-80, and Asn-82 stimulated proliferation of the T cell clone (data not shown), the majority of peptide analogues substituted at either Gln-81 or Glu-83, while those which were the most likely TCR contact residues showed antagonistic activity.
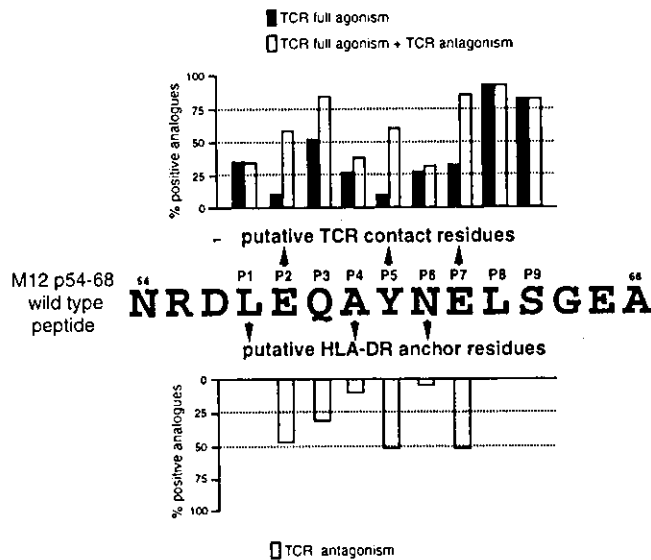
Figure 3 shows a summary of the responses of T cell clone YN5–32 specifically recognizing a streptococcal M12 p54–68 in the context of DR4 (DRB1*0406) to 156 independent peptide analogues with a single residue substitution. P1 (position 1) means the putative most N-terminal DR anchor residue.[29] The residues Leu-57 (P1), Ala-60 (P4), and Asn-62 (P6) were the most likely to be DR-anchor
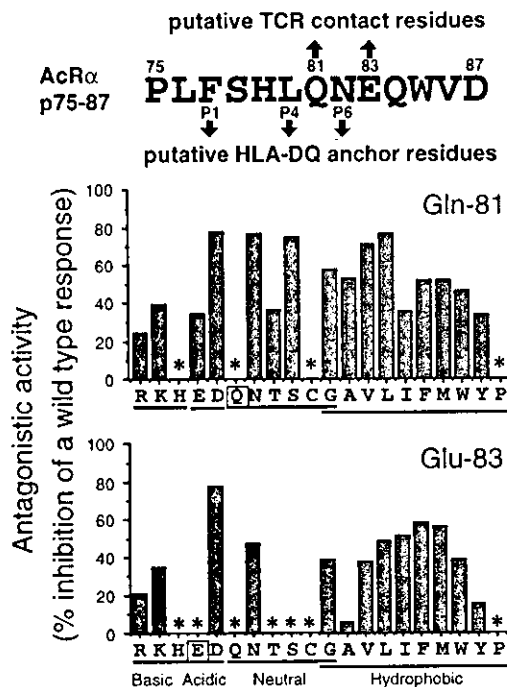
Fig. 2. Summary of antagonistic activities of peptide analogues with single-residue substitutions in human AchRα p75–87 peptide. T cell clone SK2.11 autoreactive to AchRα p75–87 in the context of HLA-DQ6 and established from a patient with infant-onset MG was cultured with irradiated APCs prepulsed with a minimal dose of AchRα p75–87 peptide in the presence of a large number of soluble peptide analogues. P1 (position 1) means the putative most N-terminal anchor residue. Antagonistic activity was expressed by the percentage inhibitory effect of the analogue on proliferative responses of SK2.11 to the wild-type peptide. The response was in the range 7000–10000cpm. Asterisks indicates not tested, because some of these analogues exhibited TCR agonism and because other analogues were not synthesized

Fig. 3. Summary of responses of the HLA-DR4-restricted T cell clone YN5–32 to 156 peptide analogues with single-residue substitutions in a streptococcal M12 p54–68 peptide. From P1–P7 positions, residues were replaced with all other amino acides. Percentages of peptide analogues exhibiting either agonism or TCR antagonism are indicated for positions. Full agonism means stimulation of the proliferative responses of the T cell clone to various degrees. TCR antagonism means that an excess amount of peptide analogues inhibited proliferative responses of the T cell clone to a minimal dose of wild-type peptide, and without inducing T cell anergy. *Black bars*, TCR full agonism; *white bars*, TCR full agonism + TCR antagonism; *gray bars*, TCR antagonism

residues, and 30% (17/57) of peptide analogues substituted at these residues exhibited full agonism to stimulate various magnitudes of proliferative responses in the T cell clone. Only 7.5% (3/40) of not 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, which means that substitutions at these residues frequently abrogate T cell recognition.

Interestingly, as many as 60.4% (29/48) of analogues without fully agonistic properties exhibited TCR antagonism, which inhibited the proliferative response of YN5-32 to the wild-type peptide. Eight (27.6%) of these antagonistic analogues with relatively conservative amino acid substitution exhibited partial agonism, which induced 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 wild-type peptide (but not the partially agonistic APLs) induced down-modulation of CD3 expression and up-regulation of CD54 and CD69 expressions. None of the

APLs with partially agonistic properties stimulated IFN-γ production and induced anergy.

These observations collectively indicate that: (1) different physiological outcomes are induced in the recognition of APLs in human CD4⁺ T cell clones, as noted by other studies of murine T cells; (2) APLs with antagonistic properties were mainly observed by substitution at TCR contact sites, but substitution at the HLA anchoring residues also could contribute to TCR antagonism; (3) many APLs with substitution at HLA anchoring residues exhibit agonistic properties.

A slight alteration in an antigenic peptide or DR molecule, even far from the recognition surface, significantly affects recognition by TCR

Because single amino acid polymorphism at residue 37 of the HLA-DRβ chain (DRβ37) between DRB1*0406 and 0403 markedly influences susceptibility to the insulin autoimmune syndrome,[49] we investigated the effects of DRβ37 polymorphism on the recognition of nonself peptides by YN5-32.[50] As described above, 154 peptide analogues were tested for agonist and TCR antagonist properties. Of these, 46 analogues showed full agonism, 34 analogues exhibited TCR antagonism, and 45 analogues exhibited neither full agonism nor TCR antagonism, irrespective of the presenting molecules DRB1*0406 or

Fig. 4. Summary of distinct responses to peptide analogues of the T cell clone YN5-32 induced by DRβ37 single-residue polymorphism. Data are given as percentage wild-type response in peptide analogues exhibiting agonism, or percentage inhibition of wild-type response in peptide analogues exhibiting TCR antagonism. Open and closed bars indicate responses observed in presentations by DRB1*0406 and DRB1*0403, respectively. A Agonistic proliferative responses of YN5-32. B Antagonistic inhibition of proliferative responses of YN5-32

A Agonistic activity

```
Relative position #        (% wt response)
---123456789---
M12 p54-68 NRDLEQAYNELSGEA  0    50   100
P2(E)  ----Q---------
       ------E---------
P3(Q)  -----I--------
       -----S--------
P4(A)  ------M-------
       ------L-------
P5(Y)  -------M------
P6(N)  -------G-----
       -------L-----
P7(E)  ---------A----
       ----------D----
P8(L)  ----------E---
```

□ DRB1*0406 DRβ37 Ser
■ DRB1*0403 DRβ37 Tyr

B Antagonistic activity

```
Relative position #        (% Inhibition of
---123456789---             wt response)
M12 p54-68 NRDLEQAYNELSGEA  0    50   100
P1(L)  ---K----------
       ---Q----------
P2(E)  ----L---------
       ----Y---------
       ----W---------
       ----S---------
       ----T---------
       ----G---------
P4(A)  ------G--------
       ------M--------  NT
       ------L--------  NT
P5(Y)  -------L-------
       -------I-------
       -------E-------
       -------K-------
       -------M-------  NT
P6(N)  --------F------
       --------H------
       --------W------
P7(E)  ---------N-----
```
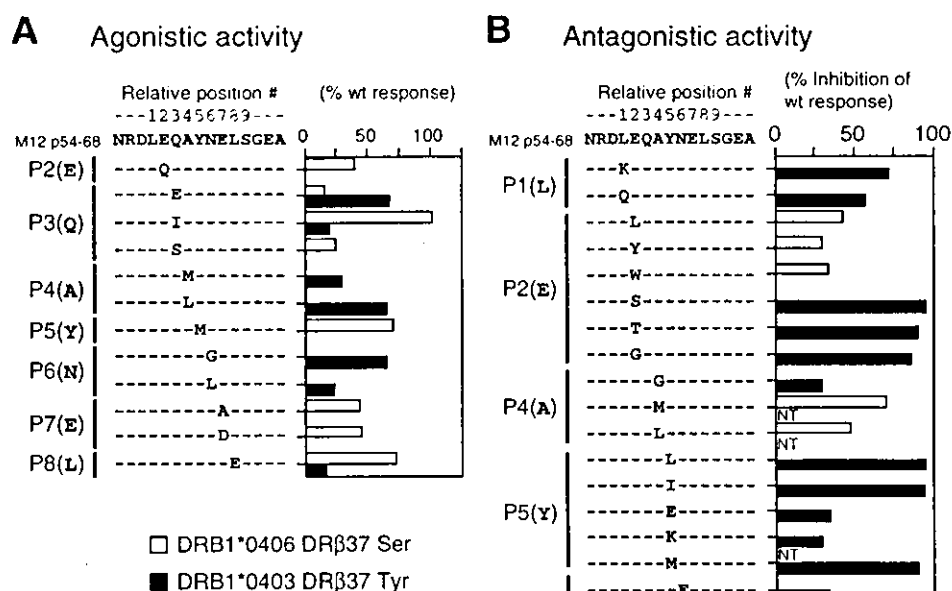
DRB1*0403. On the other hand, 29 analogues substituted at each of residues 57(P1)–63(P7) of M12p54–68 were recognized differently by YN5-32, depending on the presenting HLA–DR molecules. Figure 4 shows a summary of the distinct responses observed to 29 peptide analogues of YN5-32 induced by DRβ37 polymorphism. The agonistic and antagonistic activities of all the 29 analogues with a single substitution spanning the core epitope were clearly diverse. These observations indicate that single amino acid polymorphism (Ser–Tyr) at the DRβ37 residue induced conformational changes in peptides, which can be distinguished by TCR. This can be inferred from the differences in affinity between two DR4 molecules and peptides or between TCR and their ligands. These conformational changes were observed even in APLs with single residue substitutions at residues far from a putative DRβ37 contact site.

In recent studies, Kersh et al.[51,52] showed that TCRs can discriminate between two APLs in which only a single I-E[k] P6 anchor residue was substituted for a chemically conservative one, which does not significantly alter the binding affinity to MHC. Alterations in the main chain conformation in P6–P8 and a slight change in the angle of the P8 TCR-contacted side chain were evident in a crystal analysis. As a result, the physiological response changed from full agonism to antagonism. This observation indicates that recognition by TCR is significantly affected by slight alterations far from the TCR recognition surface, and its physiological reactivity can be markedly changed.

Degeneracy in antigen recognition by TCR is not predictable by an independent contribution model

Much epidemiological evidence related to human autoimmune diseases is almost compatible with the molecular mimicry hypothesis for the development of disease. Indeed, subjects have been seen to develop autoimmune disease after infection. Elucidation of the structural requirements for peptides to be cross-recognized by autoreactive T cells is of great importance in understanding disease processes. To date, mimicry epitopes have been predicted and identified based on primary sequence homology, the data being obtained from single-residue substituted peptide analogues, or PS–SCLs.[53–55] PS–SCLs using synthetic peptide are the main means currently used, and are fundamentally based on the concept that the antigen recognition surface of TCR is relatively flat, and each amino acid in each position on the peptide independently contributes to recognition by TCR (independent contribution model).[56] In such systems, putative mimicry peptides were searched using algorithms designed by combining the amino acids selected for each position by referring to tested data. However, the combinations of amino acids used in these systems did not always function as expected. In fact, artificial peptides composed of the optimal residue for each position selected, and based on analysis with PS–SCLs, do not necessarily show agonistic activity.[57]

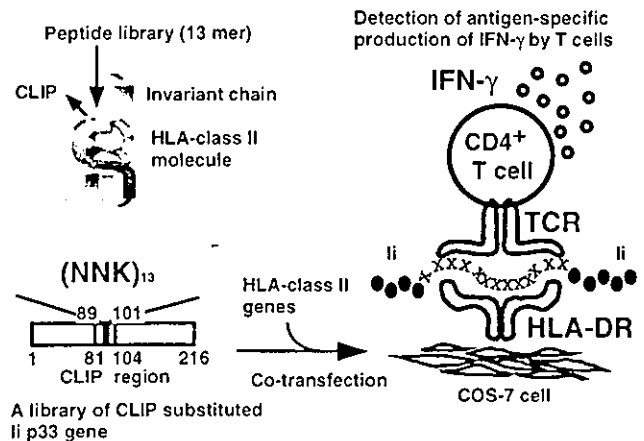Although TCR shows a high degree of degeneracy in recognized peptides, a slight alteration of an antigenic peptide or a DR molecule, even far from the recognition surface, can affect TCRs, which show exquisite specificity. It has been reported that certain TCR recognition is affected by each amino acid adjacent or not adjacent to TCR contact residues, or by each amino acid combination in an antigenic

210

peptide.[58,59] Our extensive analysis of the combinatorial nature of epitopes recognized by TCRs, using a class II-associated invariant chain peptide (CLIP)-substituted epitope expression library, also indicated that recognition by TCR was significantly affected by combinations of amino acids in the antigenic peptide.[60] Therefore, the potency of a peptide to stimulate certain T cells cannot be predicted precisely in approaches based on an independent contribution model.

The use of an epitope expression library for systematic analysis of diverse cross-reactive epitopes

On the basis of these observations, we developed a new strategy to identify epitopes of HLA-class II restricted TCRs from a library of randomized 13-mer peptides.[61] The establishment of an efficient system for the delivery of antigenic peptides to the HLA-class II-restricted antigen presenting pathway should be useful for the development of a library of cells expressing a diverse array of peptides in the context of HLA-class II molecules. We prepared an oligonucleotide library by replacing a gene segment encoding for CLIP (invariant chain p89-101) of invariant chain (Ii) with double-stranded DNAs of randomized sequences, and prepared an epitope-presenting library which loads randomized peptides onto HLA-class II molecules co-expressed in COS-7 cells. The use of an invariant chain with targeting signals to endosomes is a pertinent strategy for antigen presentation to CD4^{+} T cells.[62-65] This approach, by which multiple residues were simultaneously randomized, has the great advantage of producing overall conformation of the peptide/HLA-class II complexes, and increasing the possibility of finding degenerate sequences with agonistic properties.

The screening system is depicted schematically in Fig. 5. Plasmid clones encoding epitopes agonistic to CD4^{+} T cells can be identified by measuring the IFN-γ produced by the stimulated T cells. The sensitivity of the screening was considered to be sufficient to identify unknown epitopes in the presence of more than 1000 irrelevant clones in a single well of a 96-well plate, and it is possible to screen 10[5] clones using one 96-well plate (Fig. 6). As a model system, we searched for a cross-reactive epitope for a T cell clone SA32.5 specific to glutamic acid decarboxylase (GAD) 65, an autoantigen implicated in type I diabetes.[66] Screening a library containing 2 × 10^{5} independent plasmid clones isolated a plasmid clone, designated pCIGm1, that stimulates SA 32.5 (Fig. 7A). We investigated whether the epitope encoded by the inserted DNA in pCIGm1 was stimulatory for SA 32.5 when added as a synthetic peptide. We synthesized four peptides (Gm1.1-1.4) containing all or part of the 13-mer peptide (QLSNQWHVVGATF) substituted for CLIP (Ii p89-101), together with different flanking sequences derived from Ii, and examined the capacity to stimulate SA 32.5. The T cell stimulatory activity of these peptides was tested by proliferation assay in which the T cell clone and DR53-positive irradiated peripheral

Peptide library (13 mer)

Detection of antigen-specific production of IFN-γ by T cells

CLIP — Invariant chain

HLA-class II molecule

(NNK)₁₃

89 101

1 81 104 216
CLIP region

HLA-class II genes

Co-transfection

A library of CLIP substituted Ii p33 gene

IFN-γ

CD4^{+} T cell

TCR

Ii   Ii

HLA-DR

COS-7 cell

Fig. 5. The expression cloning system to identify epitopes for CD4^{+} T cells using the CLIP-substituted Ii-chain-based epitope presenting library. The screening system to identify epitopes from the epitope library and construct the epitope library using the CLIP-substituted Ii p33 gene are presented schematically. Plasmid DNA of the epitope library is introduced along with HLA class II expression vectors into COS-7 cells. Transiently transfected COS-7 cells, which express a diverse array of peptides in the context of class II molecules, are co-cultured with CD4^{+} T cells in 96-well culture plates. The response of stimulated T cells is detected by the production of IFN-γ if agonistic epitopes are expressed in the well. DNA sequencing of CLIP inserts determined the agonistic epitope sequences. N means any nucleotide. K means guanin (G) or (thymine) T, and X means any amino acid

A

GAD65 p116-129

NILLQYVVKSFDRS

89 101

1 81 104 216
CLIP

GAD65/CLIP substituted Ii (pCIG)

B
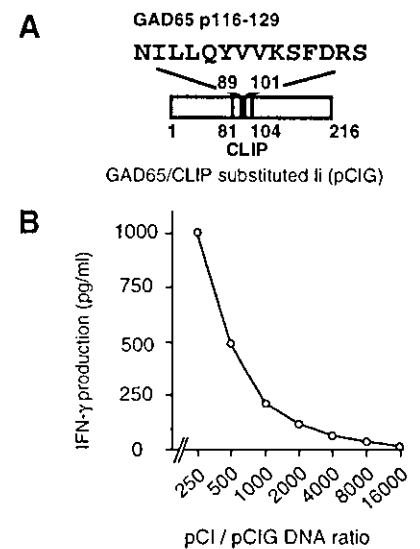
Fig. 6. Determination of the sensitivity of screening for T cell epitopes using the pCI expression vector. A Construct of pCIG, the GAD65-epitope-presenting vector in which human GAD65 p116-129 was inserted to pCI. B pCIG as relevant stimulative plasmid DNA was diluted with pCI as irrelevant plasmid at graded ratios. The mixtures of plasmids (pCI/pCIG) along with HLA-DRA*0101 and HLA-DRB4*0103 genes were transfected into COS-7 cells in a well of the 96-well plate. After overnight culture, GAD65 p116-129 specific T cell clone SA 32.5 (5 × 10⁴ cells/well) were added to COS-7 cells followed by 48 h culture. The amount of IFN-γ in the supernatants was measured using ELISA. Results are expressed as means of triplicate determinations ± SD. Circles indicate IFN-γ produced in a well in which COS-7 cells were transfected with the pCI, pCIG, and DR expression vectors

— 202 —

Ii ▶◀ a peptide substituted for CLIP (Ii p89-101) ▶◀ Ii

A

Library construct	CCCAAACCTGTG	NNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNK	CTGCCTATGGGA
	P K P V	X X X X X X X X X X X X X	L P M G
pCIGm1	CCCAAACCTGTG	CAGTTGTCGAATCAGTGGCATGTTGTTGGTGCTACGTTT	CTGCCTATGGGA
	P K P V	Q L S N Q W H V V G A T F	L P M G

['H] Thymidine incorporation (cpm x 10⁴)

B

Peptide		Amino acid sequence	0 1 2 3 4 5
GAD65 p116-128		NILLQYVVKSFDR	
Gm1.1	PKPV	QLSNQWHVVGA	
Gm1.2	PV	QLSNQWHVVGATF	
Gm1.3		QLSNQWHVVGATF	
Gm1.4		SNQWHVVGATF	LPMG

Ii ▶◀ a peptide substituted for CLIP (Ii p89-101) ▶◀ Ii

Fig. 7. Identification of a cross-reactive epitope for a GAD-65-specific T cell clone SA 32.5. **A** The construct of a CLIP-substituted peptide library and the peptide encoded by the insert DNA of the plasmid clone pCIGm1 identified by screening of the library. *X* indicates a random amino acid encoded by nucleotide triplets NNK, where *N* stands for an equal mixture of the deoxynucleotides G, A, T, and C, and *K* stands for an equal mixture of G and T. **B** Determination of a cross-reactive epitope using synthetic peptides. The amino acid sequence encoded by the nucleotide sequence, substituted for CLIP of the isolated plasmid clone pCIGm1 was QLSNQWHVVGATF. Four overlapping peptides containing all or part of this sequence, together with Ii-derived flanking sequences, were synthesized. To investigate the T cell stimulatory activities of these peptides, a T cell proliferation assay was carried out. The T cell clone SA 32.5 (3 × 10⁴ cells/well) was cultured in the presence of 50-μM tested peptides and irradiated HLA-DR53-positive PBMC (1.5 × 10⁵ cells/well) for 72 h in wells of a 96-well plate, and the proliferative responses were measured by counting [³H]-TdR incorporation. Results are expressed as means of triplicate determinations ± SD

blood mononuclear cells (PBMC) were co-cultured in the presence of 50 μM peptide (Fig. 7B). Of the four peptides, Gm1.1 (PKPVQLSNQWHVVGA) and Gm1.2 (PVQLSNQWHVVGATF), containing all or part of the insert sequence with two or four CLIP-flanking residues at the N-terminus, stimulated SA 32.5, but peptide Gm1.3, with a deletion of two amino acids at the N-terminus of the 13-mer insert, did not do so. Also, the proliferative responses of SA 32.5 to both peptides increased in a peptide dose-dependent manner (data not shown), and the Gm1.2 peptide stimulated comparable responses to those induced by the GAD65 p116-128. Although, the newly identified epitope (PVQLSNQWHVVGATF) was very different from the original epitope, GAD65 p116-128 (NILLQYVVKSFDR), it did have a capacity to stimulate the T cell clone which was comparable to that seen with the original GAD epitope.

Structural mimicry for the future direction of research on molecular mimicry

In the past few years, it has been considered that the application of APLs to down-regulate the responses of pathogenic T cells would be of therapeutic value in treating subjects with autoimmune disease.[67-70] Some TCR antagonists can induce anergy and change the cytokine profiles of T cell clones.[69,70] The secretion of anti-inflammatory cytokines IL-4, IL-10, and TGF-β by autoreactive T cells is associated with potent bystander suppression. APLs allow the in vivo generation of IL-4- IL-10- and TGF-β-secreting autoreactive T cells capable of migrating to the autoimmune site and suppressing local inflammatory responses. The injection of APLs is of clear therapeutic value in treating models of experimental encephalomyelitis (EAE).[68] In this case, the application of APL selectively silences pathogenic T cells, and other nonpathogenic T cells are recruited to the site of the disease as a result of the production of IL-4 and the reduction of TNF-α in the lesion.

However, it is difficult to find APLs of self-antigenic peptides that have no risk of activating autoreactive T cells. Recent clinical trials with an APL of MBP p85-99 for MS patients indicate that a high dose of an APL induced MBP-reactive T cells at a high frequency, resulting in the exacerbation of MRI-detectable lesions. In contrast, a low dose of an APL led to an immune deviation toward increases in IL-4 secretion by MBP-reactive T cells, with no progression in lesions.[71,72] This observation suggests that the engagement of diverse TCRs with a single APL derived from a self-antigen yields a variety of substantial cross-reactivities to a self-antigen. Although monoclonal or oligoclonal expansion of autoreactive T cells has proven to play a central role in the pathogenesis of autoimmunity, T cell populations which recognize certain self-antigens are relatively more diverse. Thus, a completely different sequence that mimics self-antigen may be a key epitope that activates a limited fraction of autoreactive T cells. Our findings indicate that

212

the cross-reactivity of TCR can be triggered by the peptide with, unexpectedly, no resemblance to the epitope of the autoantigen. Therefore, it may be more effective to use TCR antagonists of microbial mimicry epitopes that down-regulate the limited fraction of self-reactive T cells.

Recently, the mode of recognition by a single autoreactive TCR of two independent peptides in the context of two different DR2 molecules was structurally presented.[73] The T cell clone established from an MS patient cross-recognized MBP p85–99 in the context of DRB1*1501 and EBV DNA polymerase p627–641 peptide in the context of DRB5*0101. Both DRB alleles are in a strong linkage disequilibrium, and are associated with susceptibility to MS. This finding is not only important for molecular mimicry involving antigenic peptides, but also supports the structural basis of molecular mimicry generated by peptide–HLA-class II interactions.

Conclusions

Recent elucidation of the three-dimensional structures of the TCR-peptide/HLA-DR1 complex provides a structural basis for antigen recognition by HLA-class II restricted TCR. Flexibility in recognition by MHC-class I restricted TCR could also be structurally explained by large conformational changes of three CDR loops on binding to the ligand.[74] Similar changes in CDR loops of MHC-class II restricted TCR on binding can also be expected. The use of synthetic peptides in analyses of TCR recognition has provided a large number of useful concepts in T cell immunity. Using a set of peptide analogues, it has become clear that many modifications of the antigenic peptide are tolerated. Using extensive PS-SCLs, a high degree of degeneracy in TCR recognition has become evident. Although degeneracy in antigen recognition by TCR becomes an effective protection against infection in a limited T cell repertoire, and also helps maintain an immune system which can cope with a continuously changing antigenic environment, it may eventually cause autoimmunity through molecular mimicry. Previously, conformational changes in peptides on the groove of MHC class II molecules caused by binding could not have been predicted. This was also the case even if PS-SCLs were adapted. We have clearly shown that our epitope expression library system using CLIP-substituted Ii-genes provides an entirely new strategy not only for the identification of the cross-reactive epitopes for CD4⁺ T cells of known specificity, but also for the detection of epitopes which stimulate CD4⁺ T cells, the epitopes of which are unknown. Importantly, in analyzing molecular mimicry, this system deciphers overall conformations within epitopes on TCR recognition. The system also gives the prospect of providing a greater understanding of recognition mode in HLA-class II restricted TCR that could lead to preventive and therapeutic approaches.

References

1. Todd JA, Acha-Orbea H, Bell JI, Chao N, Fronek Z, Jacob CO, et al. A molecular basis for MHC class II-associated autoimmunity. Science 1988;240:1003–9.
2. Goldrath AW, Bevan MJ. Selecting and maintaining a diverse T-cell repertoire. Nature 1999;402:255–62.
3. Horwitz MS, Sarvetnick N. Viruses, host responses, and autoimmunity. Immunol Rev 1999;169:241–53.
4. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194:863–9.
5. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001;106:255–8.
6. Rescigno M, Borrow P. The host-pathogen interaction: new themes from dendritic cell biology. Cell 2001;106:267–70.
7. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. Cell 2001;106:263–6.
8. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106:259–62.
9. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 1992;358:155–7.
10. Miller SD, Vanderlugt CL, Begolka WS, Pao W, Yauch RL, Neville KL, et al. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. Nat Med 1997;3:1133–6.
11. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 1998;4:781–5.
12. Scherer MT, Ignatowicz L, Winslow GM, Kappler JW, Marrack P. Superantigens: bacterial and viral proteins that manipulate the immune system. Annu Rev Cell Biol 1993;9:101–28.
13. Brocke S, Gaur A, Piercy C, Gautam A, Gijbels K, Fathman CG, et al. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. Nature 1993;365:642–4.
14. Dalwadi H, Wei B, Kronenberg M, Sutton CL, Braun J. The Crohn's disease-associated bacterial protein I2 is a novel enteric T cell superantigen. Immunity 2001;15:149–58.
15. Oldstone MB. Molecular mimicry and immune-mediated diseases. FASEB J 1998;12:1255–65.
16. Fujinami RS, Oldstone MB. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. Science 1985;230:1043–5.
17. Hausmann S, Martin M, Gauthier L, Wucherpfennig KW. Structural features of autoreactive TCR that determine the degree of degeneracy in peptide recognition. J Immunol 1999;162:338–44.
18. Hiemstra HS, Schloot NC, van Veelen PA, Willemen SJ, Franken KL, van Rood JJ, et al. Cytomegalovirus in autoimmunity: T cell crossreactivity to viral antigen and autoantigen glutamic acid decarboxylase. Proc Natl Acad Sci USA 2001;98:3988–91.
19. Gross DM, Forsthuber T, Tary-Lehmann M, Etling C, Ito K, Nagy ZA, et al. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis. Science 1998;281:703–6.
20. Sigal LH. Immunologic mechanisms in Lyme neuroborreliosis: the potential role of autoimmunity and molecular mimicry. Semin Neurol 1997;17:63–8.
21. Zhao ZS, Granucci F, Yeh L, Schaffer PA, Cantor H. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. Science 1998;279:1344–7.
22. Panoutsakopoulou V, Sanchirico ME, Huster KM, Jansson M, Granucci F, Shim DJ, et al. Analysis of the relationship between viral infection and autoimmune disease. Immunity 2001;15:137–47.

23. Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM. Chlamydia infections and heart disease linked through antigenic mimicry. Science 1999;283:1335-9.

24. Olson JK, Croxford JL, Calenoff MA, Dal Canto MC, Miller SD. A virus-induced molecular mimicry model of multiple sclerosis. J Clin Invest 2001;108:311-8.

25. Oldstone MB. Molecular mimicry and autoimmune disease. Cell 1987;50:819-20.

26. Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. Immunol Today 1998;19:395-404.

27. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell 1995;80:695-705.

28. Hemmer B, Fleckenstein BT, Vergelli M, Jung G, McFarland H, Martin R, et al. Identification of high-potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. J Exp Med 1997;185:1651-9.

29. Chen YZ, Matsushita S, Nishimura Y. Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. J Immunol 1996;157:3783-90.

30. Maier B, Molinger M, Cope AP, Fugger L, Schneider-Mergener J, Sonderstrup G, et al. Multiple cross-reactive self-ligands for Borrelia burgdorferi-specific HLA-DR4-restricted T cells. Eur J Immunol 2000;30:448-57.

31. Kanai T, Nomura Y, Segawa M, Takagi K, Senju S, Matsushita S, et al. Immuno-suppressive peptides for a human T cell clone autoreactive to a unique acetylcholine receptor alpha subunit peptide presented by the disease-susceptible HLA-DQ6 in infant-onset myasthenia gravis. Hum Immunol 1997;56:28-38.

32. Rammensee HG, Friede T, Stevanoviic S. MHC ligands and peptide motifs: first listing. Immunogenetics 1995;41:178-228.

33. Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, et al. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. Cell 1993;74:197-203.

34. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. Nature 1995;378:457-62.

35. Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature 1994;368:215-21.

36. Arden B, Clark SP, Kabelitz D, Mak TW. Human T-cell receptor variable gene segment families. Immunogenetics 1995;42:455-500.

37. Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. Cell 2001;104:1-4.

38. Ding YH, Baker BM, Garboczi DN, Biddison WE, Wiley DC. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. Immunity 1999;11:45-56.

39. Reinherz EL, Tan K, Tang L, Kern P, Liu J, Xiong Y, et al. The crystal structure of a T cell receptor in complex with peptide and MHC class II. Science 1999;286:1913-21.

40. Hennecke J, Carfi A, Wiley DC. Structure of a covalently stabilized complex of a human alphabeta T-cell receptor, influenza HA peptide and MHC class II molecule, HLA-DR1. EMBO J 2000;19:5611-24.

41. Esnouf RM. An extensively modified version of MolScript that includes greatly enhanced colouring capabilities. J Mol Graph Model 1997;15:132-4.

42. De Magistris MT, Alexander J, Coggeshall M, Altman A, Gaeta FC, Grey HM, et al. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. Cell 1992;68:625-34.

43. Racioppi L, Ronchese F, Matis LA, Germain RN. Peptide-major histocompatibility complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor-dependent intracellular signaling. J Exp Med 1993;177:1047-60.

44. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. Nature 1993;363:156-9.

45. Sloan-Lancaster J, Evavold BD, Allen PM. Th2 cell clonal anergy as a consequence of partial activation. J Exp Med 1994;180:1195-205.

46. Evavold BD, Allen PM. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. Science 1991;252:1308-10.

47. Soloway P, Fish S, Passmore H, Gefter M, Coffee R, Manser T. Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. J Exp Med 1991;174:847-58.

48. Kersh GJ, Kersh EN, Fremont DH, Allen PM. High- and low-potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling. Immunity 1998;9:817-26.

49. Uchigata Y, Omori Y, Nieda M, Kuwata S, Tokunaga K, Juji T. HLA-DR4 genotype and insulin-processing in insulin autoimmune syndrome. Lancet 1992;340.

50. Chen YZ, Matsushita S, Nishimura Y. A single residue polymorphism at DR beta 37 affects recognition of peptides by T cells. Hum Immunol 1997;54:30-9.

51. Kersh GJ, Allen PM. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. J Exp Med 1996;184:1259-68.

52. Kersh GJ, Miley MJ, Nelson CA, Grakoui A, Horvath S, Donermeyer DL, et al. Structural and functional consequences of altering a peptide MHC anchor residue. J Immunol 2001;166:3345-54.

53. Hiemstra HS, van Veelen PA, Schloot NC, Geluk A, van Meijgaarden KE, Willemen SJ, et al. Definition of natural T cell antigens with mimicry epitopes obtained from dedicated synthetic peptide libraries. J Immunol 1998;161:4078-82.

54. Grogan JL, Kramer A, Nogai A, Dong L, Ohde M, Schneider-Mergener J, et al. Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice. J Immunol 1999;163:3764-70.

55. Hemmer B, Gran B, Zhao Y, Marques A, Pascal J, Tzou A, et al. Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease. Nat Med 1999;5:1375-82.

56. Zhao Y, Gran B, Pinilla C, Markovic-Plese S, Hemmer B, Tzou A, et al. Combinatorial peptide libraries and biometric score matrices permit the quantitative analysis of specific and degenerate interactions between clonotypic TCR and MHC peptide ligands. J Immunol 2001;167:2130-41.

57. Tanaka Y, Ohyama H, Ogawa M, Nishimura Y, Matsushita S. Identification of peptide superagonists for a self-K-ras-reactive CD4⁻ T cell clone using combinatorial peptide libraries and mass spectrometry. J Immunol 1999;162:7155-61.

58. Leggatt GR, Hosmalin A, Pendleton CD, Kumar A, Hoffman S, Berzofsky JA. The importance of pairwise interactions between peptide residues in the delineation of TCR specificity. J Immunol 1998;161:4728.

59. Ausubel LJ, Kwan CK, Sette A, Kuchroo V, Hafler DA. Complementary mutations in an antigenic peptide allow for crossreactivity of autoreactive T-cell clones. Proc Natl Acad Sci USA 1996;93:15317-22.

60. Uemura Y, Senju S, Maenaka K, Iwai LK, Fujii S, Tabata H, et al. Systematic analysis of the combinatorial nature of epitopes recognized by TCR leads to identification of mimicry epitopes for GAD65-specific TCRs. J Immunol 2003;170:947-60.

61. Fujii S, Uemura Y, Iwai LK, Ando M, Senju S, Nishimura Y. Establishment of an expression cloning system for CD4⁻ T cell epitopes. Biochem Biophys Res Commun 2001;284:1140-7.

62. van Bergen J, Schoenberger SP, Verreck F, Amons R, Offringa R, Koning F. Efficient loading of HLA-DR with a T helper epitope by genetic exchange of CLIP. Proc Natl Acad Sci USA 1997;94:7499-502.

63. Malcherek G, Wirblich C, Willcox N, Rammensee HG, Trowsdale J, Melms A. MHC class II-associated invariant chain peptide replacement by T cell epitopes: engineered invariant chain as a vehicle for directed and enhanced MHC class II antigen processing and presentation. Eur J Immunol 1998;28:1524-33.

64. Fujii S, Senju S, Chen YZ, Ando M, Matsushita S, Nishimura Y. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. Hum Immunol 1998;59:607-14.

214

65. Senju S, Hirata S, Matsuyosi H, Masuda M, Uemura Y, Araki K, et al. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. Blood 2003;101:3501-8.
66. Tabata H, Kanai T, Yoshizumi H, Nishiyama S, Fujimoto S, Matsuda I, et al. Characterization of self-glutamic acid decarboxylase 65-reactive CD4⁺ T-cell clones established from Japanese patients with insulin-dependent diabetes mellitus. Hum Immunol 1998;59:549-60.
67. Anderton SM, Manickasingham SP, Burkhart C, Luckcuck TA, Holland SJ, Lamont AG, et al. Fine specificity of the myelin-reactive T cell repertoire: implications for TCR antagonism in autoimmunity. J Immunol 1998;161:3357-64.
68. Brocke S, Gijbels K, Allegretta M, Ferber I, Piercy C, Blankenstein T, et al. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. Nature 1996;379:343-6.
69. Windhagen A, Scholz C, Hollsberg P, Fukaura H, Sette A, Hafler DA. Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. Immunity 1995;2:373-80.
70. Ausubel LJ, Krieger JI, Hafler DA. Changes in cytokine secretion induced by altered peptide ligands of myelin basic protein peptide 85-99. J Immunol 1997;159:2502-12.
71. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. Nat Med 2000;6: 1167-75.
72. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. Nat Med 2000;6:1176-82.
73. Lang HL, Jacobsen H, Ikemizu S, Andersson C, Harlos K, Madsen L, et al. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. Nat Immunol 2002;3:940-3.
74. Garcia KC, Degano M, Pease LR, Huang M, Peterson PA, Teyton L, et al. Structural basis of plasticity in T cell receptor recognition of a self-peptide–MHC antigen. Science 1998;279:1166-72.

Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method

Tetsuya Nakatsura[1,2], Satoru Senju[1], Masaaki Ito[2], Yasuharu Nishimura[1] and Kyogo Itoh[2]

[1] Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan
[2] Department of Immunology, Kurume University School of Medicine, Kurume, Fukuoka, Japan

Among a number of human tumor antigens identified using the serological analysis of recombinant cDNA expression libraries (SEREX), only MAGE-1, tyrosinase, and NY-ESO-1 have been reported to be immunogenic tumor antigens that have the potential to elicit both humoral and cellular immunity. In this study, we determined whether our SEREX-defined pancreatic cancer antigens could be recognized by CTL, and report that one SEREX-defined antigen, coactosin-like protein (CLP), encoded cellular epitopes recognized by HLA-A2-restricted and tumor-reactive CTL. Three CLP peptides at positions 15–24, 57–65, and 104–113 possessed the ability to induce HLA-A2-restricted and tumor-reactive CTL from the PBMC of cancer patients. Subsequently, humoral responses to these peptides were investigated. IgG antibodies specific to the CLP 15–24, 57–65, and 104–113 peptides were detected in sera from 12, 0, and 12 of 12 cancer patients tested, and were also found in 5, 0, and 0 of 9 healthy donors, respectively. IgE antibodies specific to these peptides were also detected in sera from certain cancer patients and healthy donors. Since peptide-specific IgE was detected, type-I allergy to these peptides was tested. Unexpectedly the CLP 57–65 peptide, to which IgE was found in only 2 healthy donors, but not the other two peptides, was found to elicit an immediate-type hypersensitivity in all 10 healthy volunteers tested. These results indicate that identical antigenic peptides can be recognized by both cellular and humoral immune systems to a tumor-associated antigen. The CLP 15–24 and 104–113 peptides might be appropriate vaccine candidates for peptide-based immunotherapy of HLA-A2+ cancer patients.

Key words: SEREX / Coactosin-like protein / Cancer antigen / CTL / Antibody

1 Introduction

The recent development of molecular technology for analyzing cellular and humoral immune reactivity to cancer cells at the gene level has led to the identification and characterization of a large number of human tumor antigens recognized by CD8+ T cells and antibodies. Many genes encoding tumor antigens and peptides that are recognized by CTL have been identified by cDNA expression cloning methods [1–6], thereby introducing

[I 22623]

The last two authors contributed equally to this work.

the possibility of a peptide-based cancer immunotherapy. Post-vaccination PBMC became to show CTL activity against tumor cells in the clinical studies of peptide-based cancer immunotherapy, but these immunotherapies have rarely resulted in tumor regression [7, 8]. The failure to obtain tumor regression could be in part due to that immunogenicity of these tumor antigenic peptides was not strong enough to induce adaptive immunity against tumor cells. From this point of view, peptides with the ability to induce both CTL responses and humoral immunity could be better than those with the ability to induce either one. Over 1,500 types of tumor antigens have been identified using the serological analysis of recombinant cDNA expression libraries (SEREX) method [9–11]. However, only three (MAGE-1, tyrosinase, and NY-ESO-1) have been reported to have the ability to elicit both cellular and humoral immune responses to tumor cells [12–15]; CTL responses to the

majority of SEREX-defined tumor antigens remain to be elucidated. Furthermore, there is no report on antigenic epitopes recognized by both cellular and humoral immune system to tumor-associated antigens.

We previously reported 18 SEREX-defined pancreatic cancer antigens [16]. This study has extended these studies and investigated whether five SEREX-defined pancreatic cancer antigens could be recognized by tumor-reactive CTL. We also provide evidence that one of the antigens is able to encode the identical epitopes recognized by both the cellular and the humoral immune system.

2 Results

2.1 Recognition of coactosin-like protein-derived peptides by an HLA-A2-restricted and tumor-reactive CTL line

Five genes coded for SEREX-defined pancreatic cancer antigens were considered from the perspective of whether or not their products could be recognized by an HLA-A2-restricted and tumor-reactive OK-CTL line by co-transfection of these cDNA and HLA-A*0207 cDNA into COS-7 cells. This cell line was established from the tumor-infiltrating lymphocytes (TIL) of a patient (OK) with colon cancer, and responds to tumor cell lines in an HLA-A2-restricted manner, as reported previously [4, 6]. The OK-CTL line produced a significant level of IFN-γ in response to the COS-7 cells transfected with both KM-PA-4 and HLA-A*0207 cDNA (Fig. 1A). Maximum production of IFN-γ was observed when 100 ng KM-PA-4 cDNA was transfected. In contrast, the OK-CTL line failed to produce significant levels of IFN-γ in response to COS-7 cells transfected with both KM-PA-4 cDNA and an irrelevant HLA-A*2402 cDNA, or with one of four kinds of cDNA (KM-PA-5, KM-PA-14, KM-PA-15, and KM-PA-18) together with HLA-A*0207 cDNA. In our previous study [16], the KM-PA-4 was found to encode coactosin-like protein (CLP) consisting of 142 amino acids, as shown. These results indicate that KM-PA-4/ CLP-derived antigens could be recognized by HLA-A2-restricted and tumor-reactive CTL. To determine the antigenic peptides recognized by the OK-CTL, eight different CLP-derived peptides with HLA-A2 binding motifs were prepared, and these peptides were then loaded onto T2 cells at a concentration of 10 μM; samples were tested for the ability to induce IFN-γ production by the OK-CTL (Fig. 1B). Three peptides (CLP 15–24, CLP 57–65, and CLP 104–113) had the ability to induce IFN-γ production by the OK-CTL.
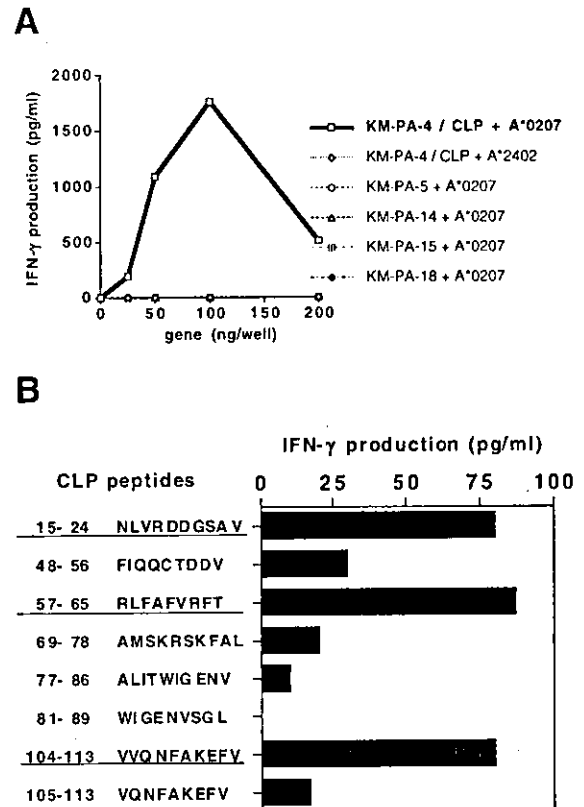
Fig. 1. Recognition of CLP-derived peptides by HLA-A2-restricted and tumor-reactive CTL. One of the highly reproducible results in a triplicate assay is indicated. (A) Different amounts of five SEREX-defined cDNA clones and 100 ng HLA-A*0207 or -A*2402 cDNA were co-transfected into COS-7 cells, followed by a test of the capacity to stimulate IFN-γ production by the HLA-A2-restricted and tumor-reactive OK-CTL. The experimental values, with the background production of IFN-γ release by the CTL in response to untransfected COS-7 cells (less than 100 pg/ml) subtracted, are shown. (B) Eight CLP-derived peptides with HLA-A2 binding motifs were loaded onto T2 cells at a concentration of 10 μM, and were tested for their ability to induce IFN-γ production by the OK-CTL. The experimental values, with the background production of IFN-γ (less than 30 pg/ml) by the CTL in response to T2 cells pre-loaded with the irrelevant HIV peptide subtracted, are shown. Three peptides, which were judged to be positive for this assay, are underlined.

2.2 Induction of tumor-reactive CTL by CLP peptides

We next attempted to generate tumor-reactive CTL from the PBMC of HLA-A2+ patients with pancreatic or colon cancer, or of HLA-A2+ healthy donors. The PBMC were stimulated with either the CLP 15–24, CLP 57–65, or CLP 104–113 peptide. These in vitro-sensitized PBMC

were tested for their cytotoxicity against various kinds of tumor cell lines in a 6-h ^{51}Cr-release assay (Fig. 2). The mRNA expression of the *CLP* gene in these cell lines used as target cells was confirmed by both reverse transcription (RT)-PCR and Northern blot analysis (data not shown). The CLP 15–24 peptide-sensitized PBMC from three of five cancer patients and two of six healthy donors showed significant levels of CTL activity against HLA-A2$^+$ Panc-1 and YPK-3 cells, but not against HLA-A2$^-$ PaCa-2 cells, HLA-A2$^+$ PHA-blasts or an HLA-A2$^+$ EBV-B cell line (Fig. 2). This was also the case with the CLP 57–65 peptide-sensitized PBMC in three of eight cancer patients, and in two of nine healthy donors (Fig. 2). Similarly, the CLP 104–113 peptide-sensitized PBMC from three of five cancer patients and one of seven healthy donors showed HLA-A2 restricted and tumor-reactive CTL activity (Fig. 2). The summary is shown in Table 1. In all cases, the percentage of CD8$^+$ T cells in peptide-stimulated PBMC was more than 80% (data not shown). These PBMC produced IFN-γ in response to T2 cells pre-loaded with a corresponding peptide in a dose-dependent manner (data not shown), and their IFN-γ production in response to HLA-A2$^+$ Panc-1 was inhibited by the addition of anti-HLA-class I, anti-

CD8 or anti-HLA-A2 mAb (data not shown). These results indicate that these cytotoxicities were mediated by the peptide-specific and HLA-A2-restricted CD8$^+$ CTL.

2.3 Detection and quantification of serum IgG and IgE antibodies reactive to the CLP-derived peptides

We determined whether IgG specific to the whole CLP antigen could be detected in sera from pancreatic cancer patients and healthy donors using the SEREX method. IgG antibodies against the CLP antigen were found in the sera from 9 of 10 pancreatic cancer patients, and from 3 of 10 healthy donors (data not shown). We then examined if the three CLP peptides identified above could be recognized by the serum antibodies from cancer patients and healthy donors. ELISA was used to quantify levels of serum IgG and IgE specific to the CLP-derived peptides. Serum samples were judged as positive for peptide-specific antibody when the absorbance (unit) values changed in relation to the dilution of the serum samples; representative results are shown in
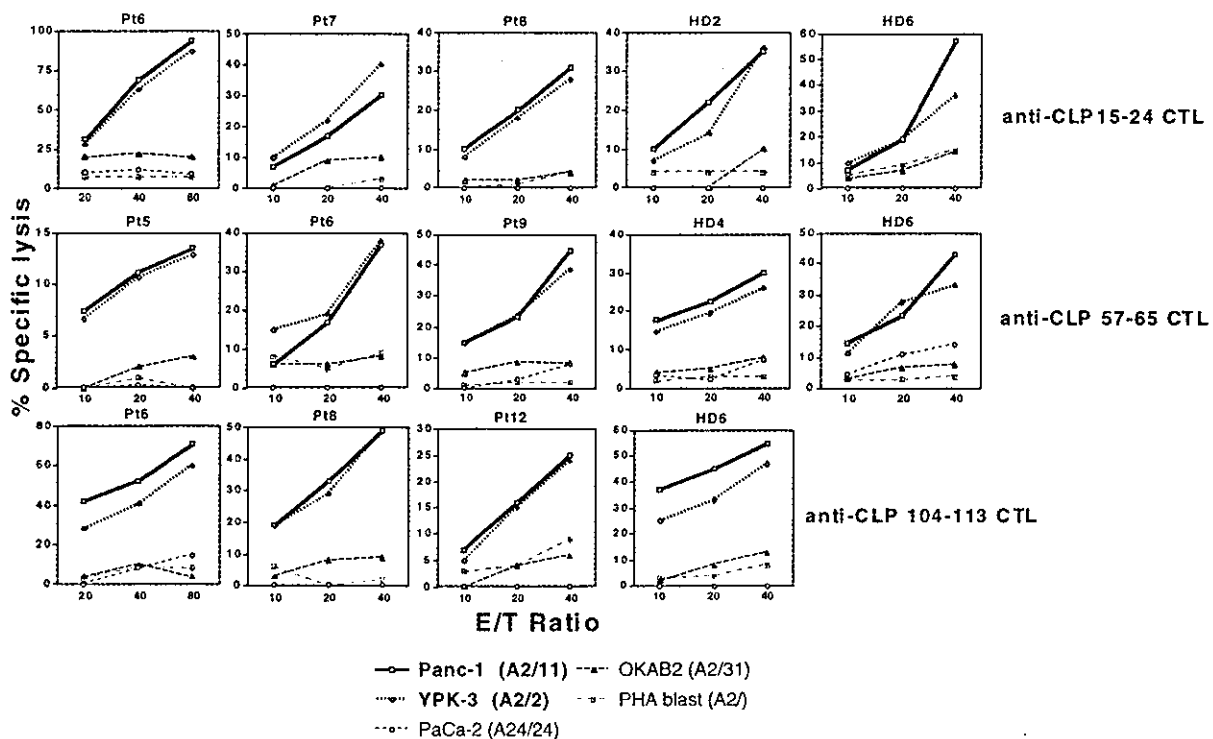

Fig. 2. Cytotoxic activity of PBMC stimulated with the CLP peptides. PBMC from patients with cancer and from healthy donors, listed in Table 1, were stimulated with the indicated peptides, followed by a test of the cytotoxicity against HLA-A2$^+$ Panc-1 and YPK-3, HLA-A2$^-$ PaCa-2 pancreatic cancer cell lines, HLA-A2$^+$ PHA-blasts, and the HLA-A2$^+$ EBV-B cell line, OKAB2, by a 6-h ^{51}Cr-release assay. Values represent the mean of triplicate assays. One of two highly reproducible results is indicated.

Table 1. Serum levels of IgG and IgE specific to the CLP peptides and induction of peptide-specific CTL from PBMC of HLA-A2+ donors

HLA-A2+ Donor	CLP 15-24			CLP 57-65			CLP 104-113		
	CTL induction	IgG	IgE	CTL induction	IgG	IgE	CTL induction	IgG	IgE
Pancreatic Cancer									
Pt1	NT[c]	3.7[d]	0.1	-	<0.1	<0.1	NT	1.0	<0.1
Pt2[a]	NT	4.5	<0.1	NT	<0.1	<0.1	NT	2.3	<0.1
Pt3	NT	6.0	0.4	-	<0.1	<0.1	NT	1.7	0.1
Pt4	NT	2.3	<0.1	-	<0.1	<0.1	NT	1.6	<0.1
Pt5	NT	3.8	1.9	+	<0.1	<0.1	NT	1.5	1.5
Pt6	+[e]	5.2	0.3	+	<0.1	<0.1	+	2.6	<0.1
Pt7	+	3.7	0.4	NT	<0.1	<0.1	-	1.0	1.0
Pt8	+	4.5	<0.1	NT	<0.1	<0.1	+	2.0	<0.1
Colon Cancer									
Pt9 (OK)[b]	-	4.5	0.1	+	<0.1	<0.1	-	2.0	<0.1
Pt10	NT	4.4	0.5	-	<0.1	<0.1	NT	1.9	0.4
Pt11	NT	5.2	<0.1	NT	<0.1	<0.1	NT	2.1	<0.1
Pt12	-	4.3	0.5	-	<0.1	<0.1	+	1.8	1.4
Healthy Donor									
HD1	-	0.3	0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD2	+	1.9	0.4	-	<0.1	0.7	-	<0.1	0.1
HD3	NT	1.8	<0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD4	NT	2.0	0.2	+	<0.1	<0.1	NT	<0.1	1.1
HD5	NT	1.6	<0.1	-	<0.1	<0.1	NT	<0.1	<0.1
HD6	+	<0.1	<0.1	+	<0.1	<0.1	+	<0.1	<0.1
HD7	-	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD8	-	<0.1	0.2	-	<0.1	<0.1	-	<0.1	<0.1
HD9	-	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1

a) The *KM-PA-4/CLP* was identified by the SEREX method using serum from this patient [16].

b) The OK-CTLs were established from this patient [4, 6].

c) NT: not tested.

d) The results of IgG and IgE antibodies binding to either the CLP 15-24, CLP 57-65 or CLP 104-113 peptide are shown by 10X absorbance values (10X OD unit) in ELISA. The experimental values, from which the background absorbance (10X OD unit) without peptide was subtracted, are shown. Serum samples were diluted at x100 for IgG and at x2 for IgE, respectively. Values judged as positive for peptide-specific antibody in ELISA are underlined.

e) The symbol represents successful induction of peptide-specific and HLA-A2+ tumor-reactive CTL. The results are shown in Fig. 2.

Fig. 3A and B. A decrease in the absorbance unit of IgG against both the CLP 15–24 and CLP 104–113 peptides, but not against the CLP 57–65 peptide, was observed in serum dilutions in all 4 patients tested (Fig. 3A). As with IgE, sera from Pt5 and Pt7, and HD4 were evaluated as positive for IgE to both the CLP 15–24 and CLP 104–113 peptides, but not against the CLP 57–65 peptide (Fig. 3B). In the case of HD2, serum dilution resulted in a decrease in the absorbance of IgE against all three peptides (Fig. 3B). A competitive binding inhibition assay was performed to confirm the peptide specificity of IgG or IgE detected in this ELISA system. Detection of IgG or IgE antibody was inhibited by adding a corresponding peptide to the sample serum in a dose-dependent man-

ner, but not by adding an irrelevant peptide (data not shown). These results indicate that an excess of a free peptide in serum samples showed a peptide-specific competition against the coating peptide, validating the peptide specificity of this ELISA system.

IgG and IgE specific to the CLP peptides were measured in sera of 12 HLA-A2+ patients with cancer (Pt1–12) (8 pancreatic cancer and 4 colon cancer) and 9 HLA-A2+ healthy donors (HD1–9), summarized in Table 1. IgG specific to the CLP 15–24 peptide was detected in the sera of all 12 cancer patients, and was also found in 5 of 9 healthy donors, while IgE specific to this peptide was detected in the sera of 8 of 12 cancer patients and of 4 of
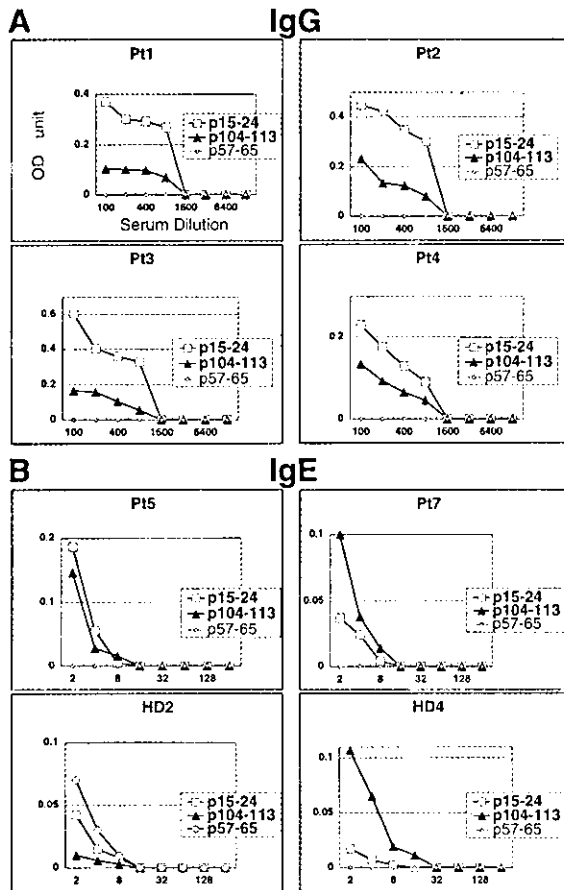
A   **IgG**

B   **IgE**

Fig. 3. Detection and quantification of serum IgG and IgE antibodies specific to the CLP peptides. One representative result of two highly reproducible experiments is shown. Peptide-reactive IgG and IgE in serially diluted serum samples from cancer patients and healthy donors were examined in (A) and (B), respectively.

9 healthy donors. IgG and IgE specific to the CLP 104–113 peptide were detected in the sera of 12 and 5 of 12 cancer patients, and in the sera of 0 and 2 of 9 healthy donors, respectively. In contrast, no IgG reactive to the CLP 57–65 peptide was detected in any of the donors tested. IgE reactive to the CLP 57–65 peptide was detected in only 1 healthy donor (HD2). Similar results were observed in HLA-A2⁻ cancer patients and healthy donors for antibodies reactive to the CLP 15–24 and CLP 104–113 peptides, but IgG specific to the CLP 104–113 peptide was detected in the sera of 4 of 10 HLA-A2⁻ healthy donors (data not shown). There was no significant association between the presence of the CLP 15–24 or CLP 104–113 peptide-specific IgG antibody in sera and successful induction of CTL from PBMC *in vitro* (p=0.546 and 0.222, respectively, evaluated by the χ^2 test). There was no inverse correlation between the exis-

tence of IgE antibody and successful CTL induction. CLP 15–24 or CLP 104–113 peptide-specific CTL were induced from PBMC, regardless of the detection of serum IgE specific to these peptides.

2.4 Type-I allergic reaction to the CLP 57–65 peptide

Type-I allergic reactions to some CTL epitopes were observed in the skin tests of cancer patients in the phase I clinical trial of peptide-based immunotherapy, and those patients who showed type-I allergic reaction against the peptide were not vaccinated with the peptide, because of the risk of systemic anaphylaxis (Gouhara et al., unpublished results). Because serum IgE reactive to the CLP peptides were detected in certain patients and healthy donors (Table 1), we investigated whether these CLP-derived CTL epitope peptides could elicit type-I allergic reactions in five HLA-A2⁺ healthy and five HLA-A2⁻ healthy volunteers. The CLP 57–65 peptide elicited the type-I allergy in all ten healthy volunteers tested, whereas the other two peptides failed to induce the type-I reaction in any donors.

The CLP 57–65 peptide and the analogue peptides in which individual amino acid residues were substituted by glycine (glycine scan) were prepared to gain a better understanding of the molecular basis of the type-I allergy, and the results of the skin test with the CLP 57–65 analogue peptides are listed in Table 2. The glycine substitution at positions 1 and 7 resulted in a decrease of the type-I allergic reaction, suggesting that position 1 (arginine) and position 7 (arginine) were important for the type-I allergic reaction to the CLP 57–65 peptide. On the other hand, substitution at position 6 resulted in an increase of the type-I allergic reaction in all cases tested. The results of IgE binding to analogues of the CLP 57–65 peptide are listed in Table 3. The glycine substitution at position 5, 6, or 9 in HD2 or at position 6, 7, and 9 in HD10 resulted in a complete or a moderate loss of IgE recognition, respectively. There was a discrepancy between important amino acid residues for the type-I allergic reaction and those for IgE antibody binding. These peptide analogues were also tested for the ability to stimulate the CLP 57–65 peptide-stimulated PBMC to produce IFN-γ. In contrast to the results of the type-I allergic reaction and antibody binding, recognition by peptide-specific CTL was lost in the majority of glycine-substituted analogue peptides (data not shown).

Table 2. Results of skin test with the CLP 57-65 analogue peptides [a]

CLP-derived peptides		Donors [b]			
		HD2 (HLA-A2⁺, IgE⁺)	HD6 (HLA-A2⁺, IgE⁻)	HD10 (HLA-A2⁻, IgE⁺)	HD11 (HLA-A2⁻, IgE⁻)
			Area of redness (cm²)		
57-65	RLFAFVRFT	100	100	100	100
R57G	GLFAFVRFT	36.0[c]	17.4	39.1	16.7
L58G	RGFAFVRFT	114	100	156	100
F59G	RFGAFVRFT	114	100	56.3	52.9
A60G	RLFGFVRFT	114	156	66.0	82.6
F61G	RLFAGVRFT	64.0	156	56.3	67.0
V62G	RLFAFGRFT	128	278	352	186
R63G	RLFAFVGFT	16.0	17.4	31.6	13.2
F64G	RLFAFVRGT	114	178	66.0	151
T65G	RLFAFVRFG	100	50.2	100	20.7

a) The results of the skin test with analogues of the CLP 57-65 peptide are shown as relative percentages compared with those with the wild-type peptide.

b) Skin test was tested in 10 healthy volunteers, and the results of 4 donors (HLA-A2⁺ or -A2⁻, positive or negative of serum IgE specific to the CLP 57-65 peptide) are shown in the table.

c) Values showing more than 50% reduction, compared with those with the wild-type peptide, are underlined.

3 Discussion

The SEREX-defined KM-PA-4 gene was highly expressed at the mRNA level in pancreatic cancer cell lines as compared with normal pancreatic tissues in our previous study [16]. The sequence of the *KM-PA-4* gene was identical to that of the *CLP* gene (derived from human placenta) already registered in the GenBank (accession no. L54057). CLP was referred to because CLP protein product (142 amino acids) showed a significant homology to coactosin [17], a filamentous (F)-actin-binding protein from *Dictyostelium discoideum*, with 33.3% identity in amino acid sequence [18]. The mRNA of CLP is 1824 bp long and is expressed predominantly in placenta, lung, liver, and kidney, but not in the heart, brain, skeletal muscle, and pancreas [18]. Smith-Magenis syndrome (SMS), which involves the clinical symptoms of mental retardation, neuro-behavioral abnormalities, sleep disturbances, short stature, minor craniofacial and skeletal anomalies, congenital heart defects, and renal anomalies, is caused by deletion of the short arm of chromosome 17 in band p11.2. *CLP* gene is mapped to the SMS common deletion region [18]. This SMS critical region overlaps with a breakpoint cluster region associated with primitive neuro-ectodermal tumors, suggesting that CLP plays a role in DNA rearrangements of somatic cells [18]. CLP has also been demonstrated to interact directly with 5-

lipoxygenase (5LO), which plays a pivotal role in cellular leukotriene synthesis [19, 20]. 5LO appeared to compete with F-actin for the binding of CLP. Further studies are needed to clarify the biological functions of CLP.

It has been reported that the same immunodominant myelin basic protein peptides were important for antibody binding and Th cell recognition in multiple sclerosis patients [21]. However, there has been no report of antibodies against class I-associated CTL epitopic peptides. In this study, we provided evidence of IgG and IgE antibodies against CTL epitopic peptides. We suppose that IgG and IgE antibodies detected by ELISA were specific to the CLP peptides based on the following: (1) serial dilution of serum resulted in a proportional decrease in antibody binding to the peptides; (2) addition of free peptides inhibited the peptide-specific binding of antibodies in serum to coating peptides, as shown in the competitive binding inhibition assay; and (3) peptide binding was significantly influenced by several analogue peptides with only one amino acid substitution. These results validate the specificity of IgG and IgE against the CLP peptides. On the other hand, IgG and IgE antibodies specific to the CLP-derived peptides were detected in the sera of HLA-A2-negative donors in similar proportion to that of both HLA-A2⁺ cancer patients and healthy donors (data not shown). Determination of class II-associated epitopes of the CLP antigen is critically important to fully understand T cell response to the CLP.

Table 3. Binding of IgE antibodies to the CLP 57-65 analogue peptides[a]

CLP-derived peptides		Donor			
		HD2		HD10	
		IgE bound to analogue peptides (OD unit) [b]			
57-65	RLFAFVRFT	100	(0.067)	100	(0.113)
R57G	GLFAFVRFT	140	(0.094)	77.0	(0.087)
L58G	RGFAFVRFT	139	(0.093)	162	(0.183)
F59G	RFGAFVRFT	118	(0.079)	92.0	(0.104)
A60G	RLFGFVRFT	85.1	(0.057)	85.0	(0.096)
F61G	RLFAGVRFT	0[c]	(0)	85.0	(0.096)
V62G	RLFAFGRFT	0	(0)	15.9	(0.018)
R63G	RLFAFVGFT	85.1	(0.057)	10.6	(0.012)
F64G	RLFAFVRGT	97.0	(0.065)	61.9	(0.070)
T65G	RLFAFVRFG	0	(0)	29.2	(0.033)

a) The levels of IgE antibodies binding to analogues of the CLP 57-65 peptide are shown as relative percentages compared with those of the wild-type peptide.

b) Absorbance values (OD units) are shown in parentheses. The values were subtracted by the background absorbance (OD units) without peptides.

c) Values showing more than 50% reduction, compared with those bound to the wild-type peptide, are underlined.

Three patterns of existence of peptide-specific IgG antibodies were shown in the three CLP peptides. IgG antibodies against the CLP 15–24 peptide were detected in the sera from all of the cancer patients; these antibodies were also found in half of the healthy donors. Antibodies against the CLP 104–113 peptide were detected in the sera from all cancer patients, and they were found in none of the healthy donors. Antibodies against the CLP 57–65 peptide were not detected in the sera from any of the cancer patients or the healthy donors. In the present study, we found that CLP-derived peptide-specific and tumor-reactive CTL could be induced predominantly in cancer patients. In particular, CLP 104–113 peptide-specific and tumor-reactive CTL activity was induced in three of five cancer patients (60.0%), and in one of seven healthy donors (14.3%). Thus, the CLP 104–113 peptide may have the highest potential among the three peptides to induce tumor-specific cellular and humoral immunity in PBMC of cancer patients. On the other hand, we could not induce CTL against various peptides in some of the patients. We think that these variations may be partly due to the heterogeneity of tumor cells and T cell repertoires in each patient. There may be difference in precursor frequency of peptide-specific CTL in each individual. It is also possible that cellular immunity of some cancer patients may be depressed. The number of cancer patients who had CTL precursors reactive to EBV peptide was significantly lower than that of healthy donors (unpublished observation).

The type-I allergic reaction to the CLP 57–65 peptide was not restricted to HLA-A2[+] donors. This type of reaction to some CTL epitopes was also observed in pre-vaccinal skin tests of cancer patients in the phase I clinical trial of peptide-based immunotherapy (Gouhara et al., unpublished results). Allergy is classically defined as an immunological reaction to a foreign antigen [22]. However, anaphylactic shock to a self peptide was recently described in mouse EAE [23]. Injection of a myelin proteolipid protein 139–151 peptide in mice after immunization with the same peptide was reported to cause anaphylactic shock. The type-I allergic reaction to the CLP 57–65 peptide was observed in all donors tested, whereas detectable levels of IgE were only found in sera from two donors (HD2 and 10). The CLP 57–65-specific IgE may be trapped on the surface of mast cells by a high-affinity FcεR-I, and only a small amount of IgE, at levels too low to detect, is present in the circulation.

There was a discrepancy between important amino acid residues for the type-I allergic reaction and for IgE antibody binding. This observation may, in part, explain why IgE reactive to the CLP 57–65 peptide was not detected in the majority of donors who exhibited type-I allergic reaction. Glycine substitution at position 6 (V62G) resulted in an increase of type-I allergic reaction. This could be due to the molecular mimicry between microbial non-self peptides and V62G analogue of the CLP 57–65. *Escherichia coli, Mycobacterium tuberculosis*

and many other microbes have sequences sharing a six-amino acid sequence identity with V62G. On the other hand, the type-I allergic reaction and IgE binding activity was lost when glycine substitution was introduced in relatively localized amino acid residues, whereas CTL recognition was lost in the majority of the glycine-substituted analogue peptides. Both positions 2 and 9 of the peptides are considered to be anchor residues, and residues at other positions are thought to be TCR contact residues. The loss of CTL recognition in analogue peptides carrying a glycine substitution at position 2 or 9 is most likely due to the failure of peptide binding to HLA-A2, because glycine does not have a side chain. The loss of CTL recognition in their analogue peptides might be due to low affinity or to the lack of binding of CTL to TCR, because positions 1, 3, 4, 5, 6, 7, and 8 of the CLP 57–65 peptide have side chains of considerable size.

CLP is a self antigen, and its mRNA is expressed in some normal tissues [16, 19], as well as being overexpressed in pancreatic cancer cell lines, compared to normal pancreatic tissues. CTL induced by stimulation with CLP-derived peptides showed cytotoxicity against cancer cell lines, but not against two kinds of proliferating T and B lymphoblasts (Fig. 2). These results suggest that vaccination with these peptides were not associated with adverse effects on normal cells and tissues. Some self antigens expressed in certain normal tissues prove to be good candidates for cancer immunotherapy. For example, immunizing patients with normal self peptides derived from melanosomal proteins has resulted in dramatic tumor regression with only occasional vitiligo in some patients who had been prescribed immunotherapy with melanoma epitopes together with administration of IL-2 [24]. Further study of immune responses of self-antigen-recognizing and tumor-reactive CTL to normal cells and tissues is required.

NY-ESO-1 protein is thought to be the most immunogenic antigen, since it has been reported to have the ability to elicit both cellular and humoral immune responses to tumor cells. However, there are no reports on tumor-associated antigenic epitopes recognized by both cellular and humoral immune systems. We provide evidence that CLP can encode identical epitopes recognized by both the cellular and humoral immune systems. Further study is needed to clarify whether these peptides have the ability to induce orchestrated anti-tumor immune responses of not only CTL, but also Th cells and antibodies.

In conclusion, we identified three CLP peptides that were capable of propagating HLA-A2-restricted and tumor-reactive CTL from PBMC. One of these peptides, CLP

57–65, elicits a type-I allergic reaction. The HLA-A2 allele is found in 23% of Black Africans, 53% of Chinese, 40% of Japanese, 49% of Northern Caucasians, and 38% of Southern Caucasians [25]. These results indicate that the CLP 15–24 and CLP 104–113 peptides could be appropriate candidates in use for specific immunotherapy for a large number of cancer patients.

4 Materials and methods

4.1 HLA-A2-restricted CTL line

An HLA-A2-restricted and tumor-reactive CTL line, OK-CTL, was used to investigate whether SEREX-defined genes could encode antigens recognized by CTL. This cell line was established from the TIL of a colon cancer patient OK (HLA-A*0207/*3101, -B46/51, -Cw1) by incubation in medium supplemented with IL-2 (100 U/ml) alone for more than 50 days, the details of which have been described elsewhere [4, 6].

4.2 Analysis for antigen recognition by CTL

Eighteen candidate genes encoding pancreatic cancer antigens were identified using serum from Pt2 (HLA-A*0210/*2402, -B*5201/*4006, -DRB1*1502/*09012, -DQB1*0601/03) with pancreatic cancer through SEREX screening of a cDNA library generated from a human pancreatic adenocarcinoma cell line, CFPAC-1 [16]. Among them, five types of full-length cDNA clones encoding either KM-PA-4/CLP, KM-PA-5/HALPHA55, KM-PA-14/CGI55 protein, KM-PA-15/GIF, or KM-PA-18/hsp105, which were packaged in the EcoRI- and XhoI-digested pBluescript vectors, were inserted into the expression vector pCMV-SPORT-2 (Life Technologies, Rockville, MD). The cDNA of HLA-A*0207 or -A*2402 genes was obtained by RT-PCR and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). cDNA (0–200 ng) of the of each of the SEREX-defined genes and 100 ng HLA-A*0207 or -A*2402 cDNA were suspended in 50 µl Opti-MEM (Life Technologies), mixed with Lipofectamine™ reagent (Life Technologies), and samples were cultured at room temperature for 30 min. A 50-µl aliquot of the mixture was then added to the COS-7 cells (5×10^3), which were then incubated for 6 h. Thereafter, the COS-7 cells were cultured for 2 days in RPMI 1640 medium supplemented with 10% FCS, followed by the addition of CTL (5×10^4 cells/well). After an 18-h incubation, 100 µl of the culture supernatant was collected and assayed in triplicate for IFN-γ production by ELISA.

4.3 Peptides

Eight different CLP-derived peptide candidates with the potential to bind to the HLA-A2 molecules [26, 27] were synthesized (Fig. 1B). The peptides and their analogues with a

single amino acid substitution to glycine were synthesized by Fmoc/PyBOP. These peptides were purchased from Sawady Laboratory (Tokyo, Japan), and their purity, estimated by HPLC, was >70%. For the additional studies, three peptides, including the CLP 15–24, 57–65, and 104–113 peptides, with a purity of >95%, were prepared. As a negative control, an HLA-A2-binding HIV-derived peptide (SLYNTYATL) with a purity of 90–98%, kindly provided by Dr. Kanaoka (Sumitomo Pharmaceutical, Osaka, Japan), was used.

4.4 Cancer cell lines

The cancer cell lines used in this study and their HLA-A alleles, shown in parentheses, were as follows: Panc-1 (HLA-A*0201/1101) pancreatic adenocarcinoma, YPK-3 (HLA-A*0201) pancreatic adenocarcinoma, PaCa-2 (HLA-A*2402) pancreatic adenocarcinoma, and RERF-LC-MS (HLA-A*1101) lung adenocarcinoma. HLA-A2⁺ EBV-transformed B cell lines and HLA-A2⁺ PHA-activated T lymphocytes were used as control cells.

4.5 *In vitro* sensitization of PBMC with the peptides

PBMC were isolated from 20 ml of heparinized blood of HLA-A2⁺ cancer patient donors and healthy donors by Ficoll-Conray density gradient centrifugation, as reported previously [5]. Informed consent was obtained from all donors. HLA class I typing was performed on blood lymphocytes using the classical serological method [5]. A simple method was used to generate peptide-specific CTL from PBMC (Hida et al., unpublished results). In brief, PBMC (1×10^5 cells/well) were incubated with 10 μM peptide in 200 μl culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI 1640 medium, 45% AIM-V® medium (Gibco-BRL), 10% FCS, 100 U/ml of recombinant human IL-2, and 0.1 μM MEM nonessential amino acid solution (Gibco-BRL). Half of the medium was removed and replaced with fresh medium containing the corresponding peptide (20 μM) every 3 days for up to 12 days.

4.6 Assays for tumor antigen-reactive T cell responses

Peptide-stimulated PBMC were further expanded in the presence of feeder cells pre-pulsed with the indicated peptide for 21–28 days to obtain a relatively large number of cells. Thereafter, they were tested for their responses against various targets by a 6-h ⁵¹Cr-release assay [5]. The surface phenotypes of the CTL were examined by direct immunofluorescence staining with FITC-conjugated anti-CD3, -CD4, or -CD8 mAb (Nichirei, Tokyo, Japan) [6]. To determine both effector cells and MHC restriction, 20 μg/ml anti-HLA-class I (W6/32, IgG2a), anti-HLA-A2 (BB7.2, IgG2b), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA-DR (H-DR-1,

IgG2a), and anti-CD4 (Nu-Th/i, IgG1) mAb were added at the beginning of the cultures. Anti-CD13 (MCS-2, IgG2a) and anti-CD14 (JML-H14, IgG1) mAb were used as isotype-matched negative controls [6].

4.7 Skin test

Each peptide was dissolved in DMSO at 10 mg/ml, aseptically aliquoted, and stored at –80°C. Stock solutions were diluted with saline just before use. A sterility test was performed according to the method described in Section B-484 of the Japanese Pharmacopoeia 13th edn. Peptide solution (50 μl, 0.2 mg/ml) was injected intradermally into healthy volunteers and, 15 min later, flare and induration were inspected. Informed consent was obtained from all participants.

4.8 Detection of serum IgG reactive to the entire CLP antigen

SEREX-positive clones of CLP were subcloned and re-tested for serum reactivity, according to the following method. To determine the reactivity of other allogeneic sera samples (1:100 dilution) obtained from ten healthy blood donors (50–63 years old, mean 56.7 years, four men and six women) and ten patients with pancreatic ductal adenocarcinoma (43–79 years old, mean 62.1 years, four men and six women) against SEREX-positive clones, plates containing an equal number of sero-positive and sero–negative control clones were similarly processed. The immunoscreening method has been described previously [16].

4.9 Measurement of serum IgG and IgE reactive to the peptides

An ELISA was used to quantify serum levels of IgG and IgE reactive to the CLP-derived peptides. Immobilization of the peptides at their C termini to a 96-well Nunc Covalink flat plate (Fisher Scientific, Pittsburgh, PA) using disuccinimidyl suberate (Pierce, Rockford, IL) was performed according to the manufacturer's instructions. The plate of immobilized peptides (10 μg/well) was blocked with Block Ace (Yukijirushi, Tokyo, Japan), and washed with 0.05% Tween 20-PBS (PBST); 100 μl/well of either serum or plasma samples diluted with 0.05% Tween 20-Block Ace was then added to the plate. The plate was washed with PBST after 2-h incubation at 37°C, and further incubated for 2 h at 37°C with 1:1,000-diluted rabbit anti-human IgG antibodies (Dako, Glostrup, Denmark). The plate was washed nine times, and 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (EnVision, Dako) was added to each well, and the plate was incubated at room temperature for 40 min. After washing, 100 μl/well of tetramethyl-benzidine substrate solution (KPL, Guildford, GB) was added, and the reaction was stopped by the addi-

tion of 1 M H_3PO_4. Absorbance was measured at 450 and 630 nm. As with the IgG-measurement procedure, levels of IgE were measured using 1:1,000-diluted rabbit anti-human IgE (Dako). Analogues of the CLP 57–65 peptide, in which the indicated positions were substituted by glycine, were used to determine amino acid positions crucial for the type-I allergic reaction, antibody binding, and CTL recognition.

References

1 Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T. and Rosenberg, S. A., Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. *Proc. Natl. Acad. Sci. USA* 1994. 91: 6458–6462.

2 Boon, T. and van der Bruggen, P., Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* 1996. 183: 725–729.

3 Shichijo, S., Nakao, M., Imai, Y., Takasu, H., Kawamoto, M., Niiya, F., Yang, D., Toh, Y., Yamana, H. and Itoh, , K., A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. *J. Exp. Med.* 1998. 187: 277–288.

4 Ito, M., Shichijo, S., Miyagi, Y., Kobayashi, T., Tsuda, N., Yamada, A., Saito, N. and Itoh, K., Identification of SART3-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with different HLA-A2 subtypes. *Int. J. Cancer* 2000. 88: 633–639.

5 Harashima, N., Tanaka, K., Sasatomi, T., Shimizu, K., Miyagi, Y., Yamada, A. Tamura, M., Yamana, H., Ito, K. and Shichijo, S., Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. *Eur. J. Immunol.* 2001. 31: 323–332.

6 Ito, M., Shichijo, S., Tsuda, N., Ochi, M., Harashima, N., Saito, N. and Ito, K., Molecular basis of T cell-mediated recognition of pancreatic cancer cells. *Cancer Res.* 2001. 61: 2038–2046.

7 Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Prakhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H. and White, D. E., Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* 1998. 4: 321–327.

8 Wang, F., Bade, E., Kuniyoshi, C., Spears, L., Jeffery, G., Marty, V., Groshen, S. and Weber, J., Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. *Clin. Cancer Res.* 1999. 59: 4056–4060.

9 Sahin, U., Tureci, O., Schmitt, H., Cochlovius, B., Johannes, T., Schmits, R., Stenner, F., Luo, G., Schobert, I. and Pfreundschuh, M., Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc. Natl. Acad. Sci. USA* 1995. 92: 11810–11813.

10 Stockert, E., Jager, E., Chen, Y. T., Scanlan, M. J., Gout, I., Karbach, J., Arand, M., Knuth, A. and Old, L. J., A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J. Exp. Med.* 1998. 187: 1349–1354.

11 Scanlan, M. J., Chen, Y. T., Williamson, B., Gure, A. O., Stockert, E., Gordan, J. D., Tureci, O., Sahin, U., Pfreundschuh, M. and Old, L. J., Characterization of human colon cancer antigens recognized by autologous antibodies. *Int. J. Cancer* 1998. 76: 652–658.

12 van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A. and Boon, T., A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991. 254: 1643–1647.

13 Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P. and Boon, T., The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 1993. 178: 489–495.

14 Jager, E., Chen, Y. T., Drijfhout, J. W., Karbach, J., Ringhoffer, M., Jager, D., Arand, M., Wada, H., Noguchi, Y., Stockert, E., Old, L. and J., Knuth, A., Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J. Exp. Med.* 1998. 187: 265–270.

15 Jager, E., Gnjatic, S., Nagata, Y., Stockert, E., Jager, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., Hoffman, E., Arand, M., Old, L. J. and Knuth, A., Induction of primary NY-ESO-1 immunity: CD8⁺ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1⁺ cancers. *Proc. Natl. Acad. Sci. USA* 2000. 97: 12198–12203.

16 Nakatsura, T., Senju, S., Yamada, K., Jotsuka, T., Ogawa, M. and Nishimura, Y., Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. *Biochem. Biophys. Res. Commun.* 2001. 256: 75–80.

17 de Hostos, E.L., Bradtke, B., Lottspeich, F. and Gerisch, G., Coactosin, a 17 kDa F-actin binding protein from Dictyostelium discoideum. *Cell Motil. Cytoskeleton* 1993. 26: 181–191.

18 Chen, K.S., Manian, P., Koeuth, T., Potocki, L., Zhao, Q., Chinault, C., Lee, C.C. and Lupski, J.R., Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. *Nat. genet.* 1997. 17: 154–163.

19 Provost, P., Samuelsson, B. and Radmark, O., Interaction of 5-lipoxygenese with cellular proteins. *Proc. Natl. Acad. Sci. USA* 1999. 96: 1881–1885.

20 Provost, P., Doucet, J., Hammarberg, T., Gerisch, G., Samuelsson, B. and Radmark, O., 5-Lipoxygenase interacts with coactosin-like protein. *J. Biol. Chem.* 2001. 276: 16520–16527.

21 Ota, K., Matsui, M., Milford, E. L., Mackin, G.A., Weiner, H. L. and Hafler, D. A., T cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* 1990. 346: 183.

22 Galli, S. J. and Lantz, C. S., Allergy. In Paul, W.E. (Ed.) *Fundamental immunology.* Lippincott-Raven Press, Philadelphia 1999, pp 1127–1174.

23 Pedotti, R., Mitchell, D., Wedemeyer, J., Karpuj, M., Chabas, D., Hattab, E. M., Tsai, M., Galli, S. J. and Steinman, L., An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat. Immunol.* 2001. 2: 216–222.

24 Kawakami, Y., Identification of human melanoma antigens recognized by tumor infiltrating T lymphocytes and their use for immunotherapy. In Kikuchi, K., van den Eynde, B. J. and Sato, N. (Eds.) *Recent advances of human tumor immunology and*

immunotherapy. Japan Scientific Societies Press, Tokyo 1999, pp 179–189.

25 **Imanishi, I., Akazawa, T., Kimura, A., Tokunaga, K. and Gojo-bori, T.,** Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In **Tsuji, K., Aizawa, M. and Sasazuki, T.** (Eds.) *Proceedings of the 11th International Histocompatibility Workshop and Conference.* Oxford University Press, Oxford 1992, pp 1065–1220.

26 **Parker, K. C., Bednarek, M. A. and Coligan, J. E.,** Scheme for ranking potential HLA-A2 peptides based on independent binding of individual peptide side-chains. *J. Immunol.* 1994. **152:** 163–175.

27 **Rammensee, H. G., Friede, T. and Stevanoviic, S.,** MHC ligands and peptide motifs: first listing. *Immunogenetics* 1995. **41:** 178–228.

Correspondence: Kyogo Itoh, Department of Immunology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan
Fax: +81-942-31-7699
e-mail: kyogo@med.kurume-u.ac.jp

or Yasuharu Nishimura, Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811, Japan
Fax: +81-96-373-5314
e-mail: mxnishim@gpo.kumamoto-u.ac.jp