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REVIEW ARTICLE

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Molecular and cellular analyses of *HLA class II*-associated susceptibility to autoimmune diseases in the Japanese population

Abstract It is well known that individuals who are positive for particular *HLA class II* alleles show a high risk of developing autoimmune diseases. HLA class II molecules expressed on antigen-presenting cells present antigenic peptides to $CD4^+$ T cells. Their extensive polymorphism affects the structures of peptides bound to HLA class II molecules to create individual differences in immune responses to antigenic peptides. In order to gain a better understanding of mechanisms of the association between *HLA class II* alleles and susceptibility to autoimmune diseases, it is important to identify self-peptides presented by disease-susceptible HLA class II molecules and triggering disease-causative T cells. Many of the autoimmune diseases are observed in all ethnic groups, whereas the incidence of diseases, clinical manifestations and disease-susceptible *HLA class II* alleles are different among various ethnic groups for some autoimmune diseases. These phenomena suggest that differences in autoimmune self-peptide(s) in the context of disease-susceptible HLA class II molecules may cause these differences. Therefore, comparisons among disease-susceptible *HLA class II* alleles, autoantigenic peptides, and clinical manifestations of autoimmune diseases in different ethnic groups would be helpful in elucidating the pathogenesis of the diseases. In this review, we describe our recent findings on (1) the uniqueness of both clinical manifestations and the *HLA*-linked genetic background of Asian-type (opticospinal form) multiple sclerosis, (2) the characteristics of glutamic acid decarboxylase 65 (GAD65) or β_2-glycoprotein I (β_2-GPI) autoreactive T cells in Japanese patients with insulin-dependent diabetes mellitus (IDDM) or anti-β_2-GPI antibody-associated autoimmunity, respectively, and (3) the generation of an efficient delivery system of peptides to the HLA class II-restricted antigen presentation pathway by utilizing a class II-associated invariant chain peptide (CLIP)-substituted invariant chain, which may be applicable to an evaluation of the "molecular mimicry hypothesis" for the activation of autoreactive T cells.

Key words Autoantigenic peptide · Autoimmune disease · Autoreactive T cell · Disease susceptibility · HLA class II molecule

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Introduction

The human histocompatibility leukocyte antigen class-II (HLA-II) molecule is a highly polymorphic heterodimeric membrane protein consisting of α and β chains and is expressed on B cells, antigen-presenting cells (APC), and activated T cells. As shown in Fig. 1A, the HLA-II molecule has a peptide binding groove on top of the molecule and binds antigenic peptides processed by APC to present them to $CD4^+$ T cells.[1] Even in the presence of exogenous nonself antigens, the majority of HLA-II molecules bind self-peptides processed mainly from self-membrane or secretory proteins. If the density of HLA-II plus self-peptides expressed on the surface of cells is large, most $CD4^+$ T cells autoreactive to them are deleted in the thymus or become anergic in the periphery. If the density of HLA-II plus self-peptides is small enough not to activate T cells in the pe-

riphery, T cells do not need to acquire tolerance to such complexes and ignore them. Thereby, $CD4^+$ T cells do not respond to these self-peptides in the context of self HLA-II molecules, except in autoimmune states.

The structural requirements for HLA-II binding peptides have been determined in many combinations of HLA-II molecules and peptides.[2–4] They revealed that 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-II molecules. On the other hand, side chains of amino acid residues flanking anchor residues were the main recognition sites for T cell receptors (TCR). This view was clearly established by crystallographic analyses of the DR molecules bound by either self[5] or nonself peptides.[6] At least five DR anchor residues on the DR1-binding peptide and five corresponding independent pockets in the peptide-binding groove of the DR1 molecule which accommodate side chains of the DR anchor residues of the peptide were identified (Fig. 1B). Sixty-five percent of the peptide surface made contact with the DR molecule and the remaining portion was accessible to solvents, and was thus recognized by TCR. Many polymorphic residues of HLA-II molecules locate in the peptide-binding groove and toward bound peptides. Thereby, the polymorphism of HLA-II molecules determines differences in the structures of peptides bound to HLA-II molecules. In other words, the polymorphism of HLA-II molecules determines individual differences in T cell response to a given antigenic peptide.

The frequencies of particular *HLA-II* allele(s) or haplotype(s) are increased in patients with several autoimmune diseases,[7] including rheumatoid arthritis (RA), insulin-dependent diabetes mellitus (IDDM), myasthenia gravis (MG), and multiple sclerosis (MS), compared with those observed in healthy controls. It is also well known that disease-associated *HLA-II* genes differ among different ethnic groups in some diseases.[8] There are two possible mechanisms for this statistical association between the *HLA-II* alleles and diseases. (1) Disease-associated HLA-II molecules themselves determine susceptibility to the disease by controlling the immune responsiveness of $CD4^+$ T cells to the self- or nonself antigenic peptides which trigger the disease. (2) Non-HLA gene(s) in strong linkage disequilibria with the disease-associated *HLA-II* alleles control susceptibility to the disease. At this stage, we cannot distinguish between these two possibilities. To test the former hypothesis, it is essential to identify the antigenic peptide(s) bound to disease-susceptible HLA-II molecules that activate the $CD4^+$ T cells responsible for the development of the disease.

It was hypothesized that human T cells do not acquire tolerance to some disease-associated self-peptide(s) in the context of disease-susceptible HLA-II molecules in patients with autoimmune diseases. Some autoimmune diseases are caused by the production of autoantibodies, which is stimulated by autoreactive Th2 (helper T) cells, and others are caused by tissue destruction induced by immune responses of autoreactive Th1 (inflammatory T) cells.[9] In our previous studies, we clarified a mechanism for HLA-associated susceptibility to insulin autoimmune syndrome (Hirata disease), which is unique to Asians[3] to which susceptibility is extraordinarily strongly associated with HLA-DR4 (DRB1*0406) in the Japanese population.[10] In this disease, HLA-DR4 (DRB1*0406) presents a reduced form of insulin α-chain-derived peptide to autoreactive T cells to promote the production of autoantibodies reactive to insulin. This happens because HLA-DR4 (DRB1*0406) has an unique binding peptide motif to bind insulin α-chain peptides, and because a reduction of insulin molecules can be induced in patients by the administration of drugs having a reducing capacity through their sulfhydryl (SH) group.

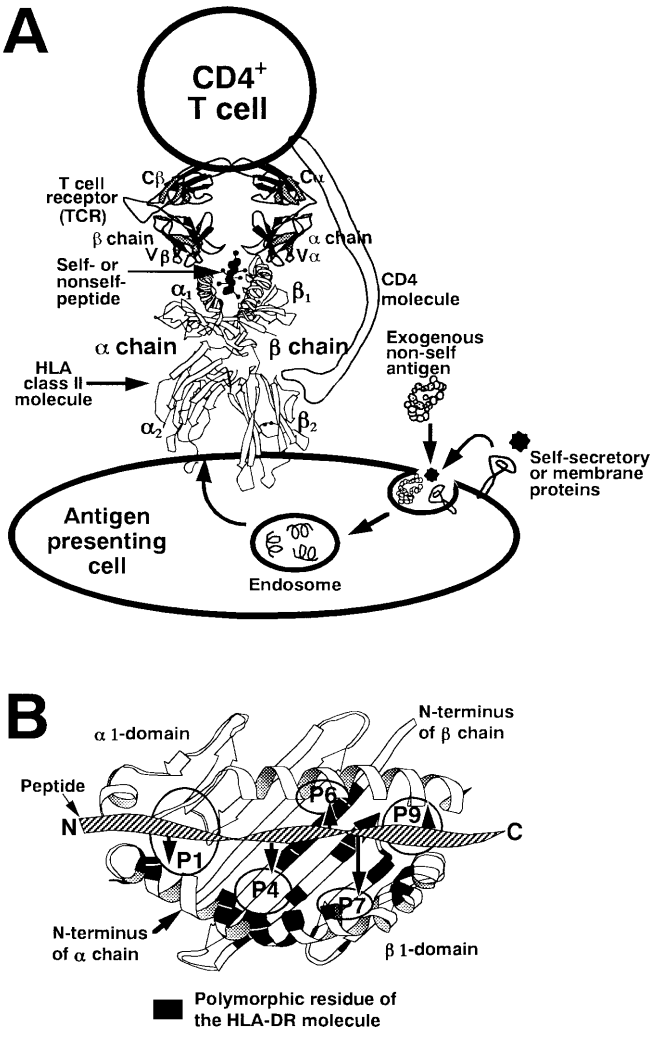

Fig. 1. The structure and function of HLA class II molecules. **A** The presentation of peptides derived from extracellular or membrane nonself or self proteins to $CD4^+$ T cells by HLA class II molecules expressed on the surface of an antigen presenting cell. α_1, α_2, β_1, and β_2 indicate the extracellular domains of α and β chains of HLA class II molecules. V_α, V_β, C_α, and C_β indicate the variable and constant regions of T cell receptor α or β chains, respectively. **B** The structure of a peptide-binding groove of an HLA class II molecule. The top of the α_1 and β_1 domains create a groove-like structure consisting of a β-sheet floor and two side walls made of two antiparallel α-helices. There are five pocket-like structures, which are indicated by *circles* in the groove, and the side-chains of P1 (the most N-terminal 1st DR anchor position), P4, P6, P7, and P9 anchor residues of the binding peptide are accomodated in these pockets, as shown in this figure. *Black* residues are polymorphic in HLA-DR molecules.[1,6]

Furthermore, we identified motifs of peptides bound to HLA class II molecules which are susceptible to autoimmune diseases in the Japanese population. These HLA class II molecules include DR4 (DRB1*0406), which is susceptible to insulin autoimmune syndrome,[3] DR4 (DRB1*0405),[3] and DQ4,[11] which are susceptible to RA and IDDM, and DR9,[12] which is susceptible to IDDM and the anti-β_2-glycoprotein I (β_2-GPI) antibody-associated autoimmunity observed in patients with systemic lupus erythematosus (SLE). Motifs of peptides bound to DQ8[13] susceptible to IDDM in Caucasians, and I-A^{g7}[14] susceptible to IDDM in mice were also identified. An analysis of a T-cell clone autoreactive to acetylcholine receptor α-chain peptides in the context of the disease-susceptible HLA-DQ6 unique to Asians, and established from a patient with infant-onset MG, again unique to Asians, has also been published.[15] Reviews summarizing these studies have been published elsewhere.[16–18]

In order to provide helpful information for further analyses of *HLA-II*-associated susceptibility to autoimmune diseases in the Japanese population, we here summarize our recent molecular and cellular analyses on *HLA-II*-associated susceptibility to MS,[19,20] IDDM,[21] and anti-β_2-GPI antibody-associated autoimmunity.[22] The method of efficient delivery of a given peptide to the HLA-II pathway by utilizing a class II-associated invariant chain peptide (CLIP)-substituted invariant chain is also described. This method will be applicable to the identification of nonself peptides derived from microorganisms which mimick autoantigenic peptides to cross-activate autoreactive T cells through infection.[23]

Existence of a clinically and immunogenetically unique subtype of multiple sclerosis (MS) in the Japanese population

MS is thought to be an autoimmune disease mainly caused by autoreactive Th1 (inflammatory T) cells specific to myelin-derived proteins produced by oligodendrocytes. This hypothesis comes from investigations of an experimental autoimmune encephalomyelitis in mice and rats, and human patients with MS.[24] It was noticed by many neurologists that there was a unique clinical subtype (Asian-type or opticospinal form) of MS in the Asian population. Patients with Asian-type MS show a relapsing–remitting disease course and have severe lesions in the spinal cord and optic nerves, and a relatively small number of brain lesions.[25] We recently found that Asian-type MS is distinct from Western-type MS not only in its clinical manifestations, but also in an *HLA*-linked genetic background.

It has been well documented that susceptibility to MS is associated with an *HLA-DRB1*1501 (DR2 subtype)-DQ6* haplotype in Caucasians.[26] Moreover, HLA-DRB1*1501 or DQ6-restricted autoreactive T cells specific to encephalitogenic myelin antigens such as myelin basic protein (MBP) and proteolipid protein (PLP) were predominantly evidenced in MS patients carrying the *DRB1*1501* allele.[27,28] In past studies of *HLA* in Japanese patients with MS, the association between *HLA-class II* alleles and MS was controversial, and was reported for *DP4*,[29] *DR8* and *DR52*, and *DR2* and *DQB1*0602*.[30] Moreover, the strong association of *DRB1*1501* with MS which was observed in Caucasians has never been proven in Asian populations (including Japanese) by *HLA* typing at the DNA level. None of the previous studies on *HLA* in Japanese patients with MS have drawn researcher's attention to distinct subtypes of MS. Since the clinical characterization of Asian-type MS was done before the introduction of magnetic resonance imaging (MRI), we performed both *HLA-II* allele typing at the DNA level and a MRI study on the clinically distinct subtypes of MS in the Japanese population in order to obtain a better definition of the MS subtypes in Asians.[19,20]

According to the clinical findings, 44 patients who showed a relapsing–remitting disease course, as well as a selective involvement of the optic nerve and spinal cord with minimal brainstem signs, were classified as having Asian-type MS. Another 46 patients with involvement of multiple sites in the central nervous system were classified as having Western-type MS. Asian-type MS showed a significantly lower number of brain lesions on MRI than did Western-type MS, while Asian-type MS showed a significantly higher frequency of gadolinium-enhanced lesions on spinal cord MRI than did Western-type MS. As shown in Table 1, among the DRβ chain genes examined, only the frequency of the *DR2*-associated *DRB1*1501-DRB5*0101* haplotype was found to be significantly higher in Western-type MS ($N = 46$, 37.0%, corrected $P < 0.05$, Corrected P value was calculated by multiplying P value with number of *HLA* alleles investigated.) than in either Asian-type MS ($N = 40$, 7.5%) or healthy controls ($N = 113$, 14.2%).[19] On the other hand, the frequency of the *HLA-DPB1*0501* allele was significantly increased in Asian-type MS ($N = 44$, 88.6%, corrected $P < 0.05$) but not in Western-type MS ($N = 46$, 71.7%) as compared with that in healthy controls ($N = 92$, 63.0%).[20] No association was observed between *DPA1* alleles and either MS subtype. This heterogeneity in the immunogenetic background and in the MRI features between the two subtypes of MS suggests the presence of two etiologically distinct types of MS in the Japanese.

It is interesting and important to ask whether the Asian-type MS is also associated with autoimmunity to some protein(s) specifically expressed in the myelin sheath, as is the case in Western-type MS.[27,28] If this is the case, there may be autoantigen(s) unique to Asian-type MS, because the tissue distribution of the inflammatory lesions is apparently different between the two subtypes. We started to investigate the frequencies of autoreactive T cells specific to MBP, PLP, and other myelin-derived proteins among Asian-type MS, Western-type MS, and healthy controls. If T cells autoreactive to these proteins are evidenced in higher frequencies in Asian-type MS as compared with Western-type MS and healthy controls, it will be interesting to investigate whether autoreactive T cells are preferentially restricted by disease-susceptible HLA-DPB1*0501 or not.

Table 1. Association between *HLA class II* alleles and two subtypes of MS observed in the Japanese population

HLA	Antigen frequency		of *HLA* alleles	
	Asian-type MS ($n = 40$)	Western-type MS ($n = 46$)	Total MS ($n = 86$)	Control ($n = 113$)
DR2 (DRB1*1501)-DR51 (DRB5*0101)	7.5%*	37.0%**	23.3%	14.2%
	Asian-type MS ($n = 44$)	Western-type MS ($n = 46$)	Total MS ($n = 90$)	Control ($n = 92$)
DP5 (DPB1*0501)	88.6%**	71.7%	80.0%	63.0%

*The corrected P value (calculated by multiplying P value with number of *HLA* alleles investigated) is statistically significant for a difference between two MS subtypes ($P_c < 0.05$)
**The corrected P value is statistically significant for a difference between the subtype of MS indicated and the control ($P_c < 0.05$)
MS, multiple sclerosis

Characterization of self-glutamic acid decarboxylase 65 (GAD65)-reactive CD4$^+$ T-cell clones established from Japanese patients with insulin-dependent diabetes mellitus

Insulin-dependent diabetes mellitus (IDDM, type 1 diabetes) is considered to be a consequence of T-cell-mediated autoimmune destruction of pancreatic islet β-cells. Among the self-antigens of islet cells, GAD65 was recently reported to be a key autoantigen which initiated autoimmunity in nonobese diabetic (NOD) mice.[31,32] These reports suggested that CD4$^+$ T cells specific to GAD65 played a critical role in the initiation of the diabetogenic process in NOD mice. In humans, anti-GAD65 autoantibody was detected not only in patients with IDDM, but also in healthy subjects at risk of IDDM.[33] It was also reported that the proliferative response of T cells to GAD65 protein in newly diagnosed IDDM patients was significantly higher than that in nondiabetic healthy subjects.[34] These reports collectively indicate that GAD65-specific T cells with helper activity are activated in the initial stages of IDDM. The Fas–FasL-mediated etiology of IDDM has been shown by investigators[35] using mouse models. Thus, NOD mice lacking the functional expression of Fas do not develop overt diabetes.

Susceptibility to IDDM in Caucasians is in strong association with *DQB1* alleles carrying nonaspartic acid at DQβ residue 57, or with several combinations of *DQA1* and *DQB1* alleles.[36] Regarding the mechanisms of association between *HLA* and IDDM, an attractive hypothesis is that disease-causative autoreactive T cells recognize autoantigenic peptides in the context of HLA class II molecules encoded for by the IDDM-susceptible *HLA* alleles. However, there have been few reports concerning DQ-mediated presentation to autoreactive T cells of islet β-cell antigens such as GAD65, insulin, β-membrane antigen, etc.[37,38] Another important aspect is that the susceptibility to IDDM associated with DQβ57 nonaspartic acid is not applicable to Japanese patients because the DQ4 (DQA1*0302-DQB1*0401) and DQ9 (DQA1*0302-DQB1*0303) molecules encoded for by IDDM-susceptible *HLA-DRB1*0405-DQ4* and *DRB1*0901-DQ9* haplotypes,[39,40] respectively, have aspartic acid at DQβ57.

To investigate autoimmunity to GAD65 in Japanese patients with IDDM, we established seven CD4$^+$ T-cell clones by stimulating peripheral blood mononuclear cells (PBMC) of six IDDM patients using a mixture of overlapping human GAD65 peptides.[21] No GAD65 autoreactive T-cell clones were evidenced in four healthy controls. As shown in Table 2, the specificity of T-cell clones was as follows: (a) two clones specific to GAD65 p111–131 (residue 111–131) + DR53 (DRB4*0103); (b) one clone specific to GAD65 p413–433 + DR1 (DRB1*0101); (c) two clones specific to GAD65 p200–217 + either DR9 (DRB1*0901) or DR8 (DRB1*0802); (d) two clones specific to GAD65 p368–388 + DP2 (DPA1*01 or 0201-DPB1*0201). Two DR53-restricted and one DR1-restricted T-cell clones responded to a recombinant human GAD65 protein and showed cytotoxicity against B lymphoblastoid cell lines pre-pulsed with the peptides. Six T-cell clones exhibited the Th1-like phenotype. Interestingly, two DR53-restricted T-cell clones killed a Fas-deficient B lymphoblastoid cell line, thereby indicating that cytotoxicity was not completely dependent on a Fas–Fas ligand interaction. Thus, the T-cell epitopes were mapped in a limited portion of the GAD65 protein, with a tendency to be restricted by disease-associated HLA-DR but not DQ molecules. It has been well documented in mice that epitope spreading occurs in autoreactive T cells during the development of autoimmune diseases.[31,32] Therefore, it may be due to epitope spreading that patients have autoreactive T cells restricted not only by disease-susceptible HLA-II molecules, but also by other HLA-II molecules even when they have disease-susceptible *HLA-II* alleles.

Analysis of T-cell responses to the β_2-glycoprotein I (β_2-GPI)-derived peptide library in patients with anti-β_2-GPI antibody-associated autoimmunity

Antiphospholipid syndrome (APS) exhibits a variety of clinical manifestations, including arterial/venous thrombo-

Table 2. Summary of Japanese patients with IDDM investigated, and the characteristics of the GAD65-autoreactive T-cell clones established

Patient	Gender	Age[a] (years months)	*HLA-DRB1**[b]	T-cell clone	Restriction molecule[b,c]	GAD65 epitopes	Response to r-hGAD65	IFNγ IL-4	Anti-GAD65 autoantibody (U/ml)[d]
MS[e]	F	15 y 8 m (1 y 10 m)	*0405/0803*	MS15.2	DP2 (*DPA1*0201-DPB1*0201*)	p368–388	NT[f]	NT[f]	5.8
SA	F	20 y 11 m (2 y 1 m)	*0405/0802*	SA32.5	DR53 (*DRB4*0103*)	p116–127	Yes	51	168.0
				SA21.4	DR8 (*DRB1*0802*)	p200–217	No	23	
NT[g]	F	25 y 7 m (13 y 7 m)	*0901/0901*	NT22.19	DR9 (*DRB1*0901*)	p202–216	No	51	25.0
SY	M	13 y 10 m (1 y 2 m)	*0901/0901*	SY11.4	DP2 (*DPA1*01-DPB1*0201*)	p370–386	No	219	94.5
MK	F	17 y 1 m (8 y 7 m)	*0301/0407*	MK20.2	DR53 (*DRB4*0103*)	p116–126	Yes	45	43.0
YK[h]	F	12 y 9 m (7 m)	*0101/0701*	YK11.3	DR1 (*DRB1*0101*)	p417–429	Yes	388	24.3

[a] Duration from the onset is indicated in parentheses
[b] IDDM-susceptible HLA alleles [30, 31] are underlined
[c] HLA alleles are indicated in parentheses
[d] The normal value is less than 5 U/ml
[e] Grandmother has diabetes mellitus
[f] NT, not tested
[g] Complicated with hyperthyroidism from 9 to 11 years old
[h] Complicated with chronic thyroiditis
IDDM, insulin-dependent diabetes mellitus

sis, recurrent spontaneous abortion/intrauterine fetal death, and thrombocytopenia with the presence of antiphospholipid antibodies. Anticardiolipin antibodies (aCL), one of the antiphospholipid antibodies, have frequently been detected in sera from patients with APS, systemic lupus erythematosus (SLE), and related collagen diseases. Recent studies have shown that aCL does not directly recognize the cardiolipin structure, but does recognize β_2GPI interacting with a variety of negatively charged molecules such as anionic phospholipids and oxidized polystyrene surfaces (plates).[41] β_2GPI is the most common and best-characterized antigenic target for aCL. It has been proposed that anti-β_2GPI antibody recognizes a cryptic epitope expressed by a conformational change occurring when β_2GPI interacts with negatively charged molecules. Alternatively, it has been suggested that the binding of β_2GPI to anionic structures increases the density of β_2GPI to promote the binding of autoantibodies to β_2GPI. Because anti-β_2GPI antibodies bind to β_2GPI with low affinity in the fluid phase, antibodies require an increased density of β_2GPI to bind efficiently in vitro.[42]

β_2GPI is a plasma protein consisting of 326 amino acids, with a molecular mass of 50 kDa, and is heavily glycosylated.[43] β_2GPI is present in plasma at 200 μg/ml or less, and possesses several in vitro properties which qualify it as an anticoagulant. It has been shown that β_2GPI inhibits the contact-phase activation system of blood coagulation, prothrombinase activity, and ADP-induced platelet aggregation.[44] It was suggested that anti-β_2GPI antibodies bind to protein C via β_2GPI and interfere with the anticoagulant activity of protein C. Anti-β_2GPI antibody of autoimmune patients can be distinguished from aCL found in patients with infectious diseases which reacts with cardiolipin in the absence of β_2GPI. However, it was reported that aCL in patients with acute human parvovirus B19 infection exceptionally shows specificity to β_2GPI, thus sharing a characteristic of aCL found in SLE patients.[45] It was also reported that the presence of anti-β_2GPI IgG and anti-β_2GPI IgA are associated with thrombosis in patients with SLE, and the presence of anti-β_2GPI IgM correlates well with a history of pregnancy loss.[46]

Various *HLA class II* alleles are reportedly associated with patients carrying anti-β_2GPI antibodies.[47] One of the *HLA-DR4* haplotypes (*HLA-DR4-DQB1*0302*) was associated with Mexican–American patients who were positive for anti-β_2GPI antibodies and, to a lesser extent, with white American patients. The frequency of *HLA-DR53* (*DRB4*0101*) was significantly increased in Mexican–American patients having anti-β_2GPI antibodies. The frequency of an *HLA-DR13* haplotype (*DRB1*1302-DQB1*0604/5*) was also significantly increased in anti-β_2GPI antibody-positive black American patients. In the Japanese population, SLE patients positive for anti-β_2GPI IgG were associated with *DRB1*0901*, as compared with those who were negative for anti-β_2GPI IgG, although the corrected *P* value was not significant.[48] An association between *HLA-DR5* (possibly *DRB1*1201*) and Mexican patients with the aCL-positive primary APS was also reported.[49] Shoenfeld's group reported that treatment of

Table 3. Data on Japanese subjects investigated, and characteristics of the β_2GPI peptide reactive T cell lines established

Donor	Age (years) and gender	Disease	*HLA-DRB1**[a]	T-cell line (clone)	Restriction HLA class II molecule	Epitope (core)	Response to purified β_2GPI	IFN-γ[b] IL-4	Serum anti-β_2GPI IgG[c] (U/ml)
AK	41 F	APS[d] secondary to SLE	*0403/0901*	AK2 (AK2.2.9)	DR4 (DRB1*0403)	p244–264 (p248–261)	No	1.54	>125
NT	39 F	SLE	*0405/0405*	NT5 (NT5.3.1)	DR53 (DRB4*0103)	p244–264 (p247–262 (261))	No	1.39	11.9
KM	52 F	SLE	*0901/1502*	KM27	DP9 (DPA1*0201 + DPB1*0901)[e]	p244–264 (p246–260)	No	12.4	28.8
OAA	36 F	APS[f] secondary to SLE	*0901/1401*	OAA	ND[g]	p154–174	No	2.96	67.7
ITO	32 M	Healthy	*0901/1502*	IT090 (IT090.3.1)	DR2 (DRB1*1502)	p226–246	No	77.8	<1.2
SM	23 F	Healthy	*0901/1201*	SM2[h] SM21[h]	DR52 (DRB3*0101) DR53 (DRB4*0103)	p64–83 p244–264 (p247 (248)–261)	No No	36.1 336	<1.2 <1.2

[a] *HLA-DRB1*0901* allele susceptible to β_2GPI autoimmunity is underlined
[b] Ratio of cytokines produced in response to a specific peptide
[c] The normal value of serum anti-β_2GPI IgG is less than 3.5 U/ml
[d] AK has a history of cerebral infarction
[e] DPA1*0201-DPB1*0901 complex was speculated based on their linkage disequilibrium and T-cell proliferation assay using allogeneic peripheral blood mononuclear cells (PBMC) as antigen-presenting cells (APC)
[f] OA has a history of recurrent spontaneous abortion
[g] Restriction molecule of OAA T-cell line could not be identified
[h] SM2 and SM21 were established from the same subject
APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus

BALB/c mice with anti-CD4$^+$ antibodies suppressed the development of experimental APS. They also demonstrated that whole bone marrow cells were able to transfer experimental APS in BALB/c mice, whereas T cell-depleted bone marrow cells could not do so.[50] These studies indicate an essential role for CD4$^+$ T cells in the development of APS, and HLA class II-restricted CD4$^+$ T cells responding to β_2GPI in PBMC of patients with APS have been reported.[51]

To investigate T-cell responses to β_2GPI, we previously stimulated PBMC of anti-β_2GPI IgG-positive SLE patients using β_2GPI purified from human plasma, but we could not establish β_2GPI-reactive T cell lines. Therefore we stimulated PBMC of 18 APS or SLE patients carrying anti-β_2GPI autoantibodies and 10 healthy controls using a peptide library covering the whole β_2GPI sequence. We established and analyzed seven CD4$^+$ T cell lines reactive to β_2GPI peptide.[22] As shown in Table 3, three of four epitopes for T cell lines derived from patients were p244–264, whereas one T cell line from a control subject also recognized p244–264. Furthermore, there was no tendency for particular HLA class II molecules to present β_2GPI peptides. However, cytokine-producing patterns were significantly different between T cell lines from patients and those from healthy individuals ($P = 0.028$); patients' T cells tended to exhibit higher IL-4 and lower IFN-γ productions. These T cell lines did not react to β_2GPI purified from human plasma. These results indicate that β_2GPI-reactive CD4$^+$ T cells of APS/SLE patients mainly recognize cryptic p244–264 in the context of various HLA class II molecules, and exhibit Th0–Th2-type responses. Our findings may provide a clue to the pathogenesis of APS. Because we have not established any HLA-DR9-restricted T cell lines autoreactive to β_2GPI peptides to date, we are not convinced that DR9-associated production of anti-β_2GPI autoantibodies is due to the existence of DR9-restricted autoreactive T cells. This problem must be further investigated by examining greater numbers of patients.

Development of an efficient system for the delivery of peptides to the HLA class II-restricted antigen presentation pathway

In autoimmune diseases, the activation of autoreactive CD4$^+$ T cells, which are inactive under normal conditions, is considered to be a crucial step in the development of the disease. Cross-recognition of foreign antigens by autoreactive T cells, the so-called "molecular mimicry between nonself and self-antigenic peptides," is one of the possible mechanisms involved in this activation.[52–55] In fact, many cases were noticed to develop autoimmune diseases after having had infectious diseases.[56,57] It is now widely accepted that T cells can be stimulated by a variety of antigenic peptides. In other words, recognition of antigenic peptides by TCR is unexpectedly degenerate.[58–60] The identification of epitopes recognized by certain CD4$^+$ T cells and information about the variation of epitopes cross-recognized is

important in understanding the etiology of autoimmune diseases, and for the development of antigen-specific immunomodulation therapy. Elucidation of the structural requirements for peptides to be recognized by certain T cells also has implications related to immunity to infectious microorganisms and tumors.

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 potent vaccine and a library of cells expressing a diverse array of peptides in the context of HLA class II molecules. This library system could be used to investigate degeneracy in peptides recognized by a T-cell clone. However, except for membrane and secretory proteins, many proteins endogenously synthesized by gene introduction as well as viral infection are degraded into peptide fragments by proteasomes in the cytoplasm, which are transported into the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP). The peptides then bind to MHC class I molecules and are presented to $CD8^+$ T cells.[61] The use of antigenic molecules with targeting signals to endosomes is a pertinent strategy for antigen presentation to $CD4^+$ T cells via a class II pathway by gene introduction. Several systems have been reported to use various molecules with targeting signals, including cathepsin D, lysosomal-associated membrane protein LAMP-1, and the invariant chain (Ii).[62–64]

The Ii is a type II integral membrane glycoprotein. The p33 and p35 forms of Ii, both encoded by eight exons, differ by 16 N-terminal residues as a result of alternative translational initiation. The p41 Ii, encoded for by nine exons, is generated by alternative splicing of a common pre-mRNA. Theoretically, an additional p43 form could be expected, but it has not yet been unequivocally detected in immunoprecipitates from human cell lysates. Among Ii isoforms, the p33 form is predominant in the human Ii. Ii has two known important functions with regard to antigen presentation by MHC class II molecules. First, Ii associates with newly synthesized MHC class II α and β dimers in the endoplasmic reticulum to form a complex and directly prevents peptides in the endoplasmic reticulum from binding to MHC class II molecules, using a domain termed class II-associated Ii peptide (CLIP) that occupies the peptide-binding groove of class II αβ dimers.[65] This αβ-Ii complex is composed of one Ii trimer and three αβ dimers, and is transported via the Golgi apparatus to the endosomal pathway by the targeting signal, di-leucine motif in the cytoplasmic domain of Ii.[66,67] In this pathway, Ii is proteolytically degraded and the αβ-CLIP complex is generated. In specialized endocytic organelles enriched in MHC class II molecules and designated MHC class II compartments (MIIC) or class II vesicles (CIIV),[68] HLA-DM catalyzes the dissociation of CLIP from class II αβ dimer and the binding of other peptides.[69]

Thus Ii contains not only the targeting signal to the endosomal pathway, but also CLIP bound in the peptide-binding groove of MHC class II molecules. We reasoned that a CLIP-substituted Ii vector, encoding the Ii chain containing an antigenic peptide replacing CLIP, might efficiently load antigenic peptides onto MHC class II molecules. Thereby we developed a new system for the delivery of an antigenic peptide to the MHC class II pathway, using Ii.[14] We designed a mutated human p33-form Ii, CLIP-substituted Ii in which streptococcal M12p55–68 (RDLEQAYNELSGEA) was substituted for CLIP. We examined the peptide presenting function of this construct in comparison with the previously reported C-terminal fused Ii, in which a cathepsin cleavage site and M12p54–68 was ligated-to the C-terminus of Ii (Fig. 2A). Mouse L cell transfectants expressing either of these two mutated Ii along with HLA-DR4 could process and present M12p55–68 to the peptide-specific and DR4-restricted $CD4^+$ T-cell clone. CLIP-substituted Ii was much more efficient at antigen presentation than was the C-terminal fused Ii, and this capacity is increased in proportion to the expression level of CLIP-substituted Ii (Fig. 2B,C). As with the wild-type Ii, the CLIP-substituted Ii was associated intracellularly with DR4 molecules. These results indicate that the peptide substituted for CLIP of Ii p33 bound to the groove of DR molecules in the same manner as CLIP, and it was preferentially presented to the $CD4^+$ T-cell clone in the absence of HLA-DM molecules. This system may prove useful for immunotherapy with DNA vaccines, or for the construction of a library of antigen-presenting cells expressing a diverse array of peptides in the context of HLA-II molecules.

As shown in Fig. 3, we used this vector to develop a new expression cloning system to identify $CD4^+$ T-cell epitopes. We inserted double-stranded oligo-DNAs of randomized sequences into this vector, and prepared an epitope-presenting library which loads randomized 13-mer peptides onto HLA class II molecules co-expressed in COS-7 cells. By using this library, we are trying to identify cross-reactive epitopes recognized by an HLA-DR53-restricted and GAD-65 autoreactive T-cell clone, as described above. Our system may be applicable not only for the identification of cross-reactive epitopes recognized by $CD4^+$ T cells of known specificity, but also for the identification of epitopes stimulating a $CD4^+$ T cell clone of which the epitope is unknown.

Conclusions

We have described the results of our recent analyses of HLA class II associated susceptibility to autoimmune diseases, including MS, IDDM, and anti-β_2-GPI antibody-associated autoimmunity in the Japanese population. Susceptibility to all of these diseases is associated with particular *HLA class II* alleles unique to, or common in, Asian populations, and clinical manifestations of some of the diseases are different in Caucasians and Asians. We clearly indicated that Asian-type MS is distinct from Western-type MS not only in its clinical manifestations, but also in its HLA-linked genetic background. Thus Asian-type MS affects not only the brain, but also the spinal cord and optic nerves, and susceptibility is associated with *HLA-DPB1*0501*, whereas Western-type MS affects the brain alone, and susceptibility is associated with *HLA-*

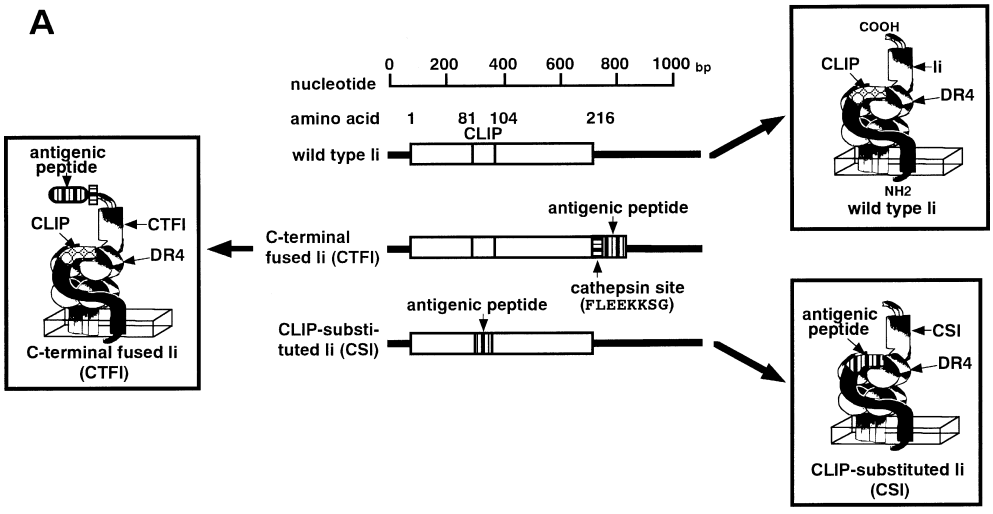

Fig. 2. Comparison of a class II-associated Ii peptide (CLIP)-substituted invariant chain (Ii) and C-terminal fused Ii for their capacity to stimulate a specific T-cell clone. **A** Schematic illustrations of the genes encoding for CLIP/antigenic peptide-substituted Ii (CSI) and Ii fused with antigenic peptide at the C-terminus (CTFI), and those for interactions between their gene products and HLA-II. **B** L-cell transfectants, including those expressing CSI with different expression levels of intracellular Ii (LDR4-CSI^{hi} and LDR4-CSI^{lo}) or CTFI (LDR4-CTFI), were stained intracellularly with an anti-Ii monoclonal antibody M-B741 (*thick lines*) or isotype-matched monoclonal antibody as negative controls (*dotted line*) and ana-lyzed by flow cytometry. **C** Proliferative response of an HLA-DR4-restricted and streptococcal M12p54-68 peptide-reactive CD4$^+$ T cell clone, YN5-32 co-cultured with LDR4-CSI^{hi}, LDR4-CSI^{lo}, LDR4-CTFI, or LDR4 in the absence or presence of various doses of an antigenic peptide, M12p54–68. YN5-32 was cultured with L-cell transfectants prepulsed for 16 h with the indicated dose of the peptide M12p54–68. Results are expressed as means of triplicate determination ± standard errors

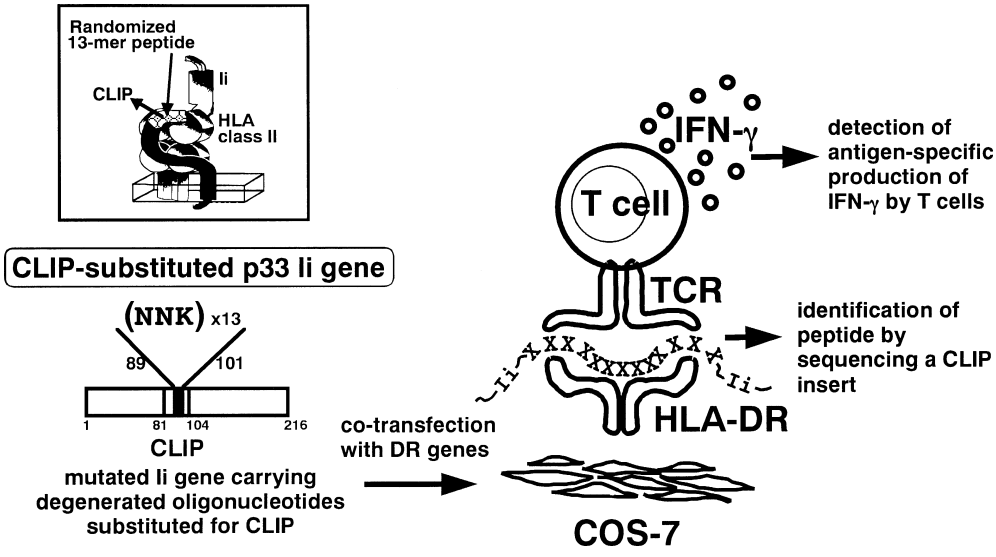

Fig. 3. Identification of diverse T cell receptor (*TCR*) ligands using a library of COS-7 cell transfectants expressing both HLA-DR and CLIP-substituted Ii genes. Double-stranded oligo-DNAs of randomized sequences (NNK×13, where N means any nucleotide and K means G or T.) were inserted into the CLIP region of the expression vector for the Ii gene and prepared an epitope-presenting library which loads randomized 13-mer peptides onto HLA class II molecules co-expressed in COS-7 cells. By detecting IFN-γ produced by a T-cell clone in recognition of agonistic peptide ligands, COS-7 transfectants expressing CLIP/agonistic peptide-substituted Ii genes can be identified. Subsequent DNA sequencing of CLIP inserts reveals an amino acid sequence of agonistic peptide. This system may be applicable not only for the identification of cross-reactive epitopes recognized by a CD4$^+$ T cell clone of known specificity, but also for the detection of epitopes stimulating a CD4$^+$ T cell clone of which the epitope is unknown

*DRB1*1501-DQ6*. In IDDM, it seems that GAD65-autoreactive T cells are restricted by HLA-DR or DP but not by DQ, suggesting that HLA-DQ may not contribute to a genetic predisposition for a development of IDDM at least in the Japanese. In the case of anti-phospholipid syndrome, β_2GPI-autoreactive T cells generated by stimulating the patient's PBMC with β_2GPI peptides did not responded to β_2GPI protein. Therefore the significance of these β_2GPI-autoreactive T cells in the pathogenesis of the disease is still unclear, although it might be possible that β_2GPI protein can be processed into peptides which activate pathogenic autoreactive T cells in an environment unique to each patient. Our studies have not yet clarified the mechanisms of disease-susceptibility control by particular *HLA-II* alleles, but further analyses of T cells autoreactive to disease-associated autoantigens will provide us with important information. Finally, the expression library of a CLIP-substituted Ii gene has been proposed to identify T-cell epitopes other than the original agonistic epitope peptide for a given T-cell clone. This library will provide a good way to evaluate the "molecular mimicry hypothesis," in which autoreactive T cells are activated to trigger autoimmune diseases in recognition of mimicked nonself peptides derived from infectious microorganisms.

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