A mutational analysis of the \(\text{A}^\beta/\text{A}^\alpha\) major histocompatibility complex class II molecule that restricts autoreactive \(T\) cells in \((\text{NZB} \times \text{NZW})\text{F1}\) mice. The critical influence of alanine at position 69 in the \(\text{A}^\alpha\) chain

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SUMMARY

Autoimmune symptoms of \((\text{NZB} \times \text{NZW})\text{F1}\) (\(\text{H}-2^\alpha/\beta\)) mice are reported to be critically related to the heterozygosity at the H-2 complex of the murine major histocompatibility complex (MHC). We previously showed that several \(\text{A}^\beta/\text{A}^\alpha\) MHC class II molecule-restricted autoreactive \(T\)-cell clones from B/WF1 mice were pathogenic upon transfer to praeautoimmune B/WF1 mice. In this study, to identify the crucial amino acid residues in \(\text{A}^\beta/\text{A}^\alpha\) molecules for \(T\)-cell activation, we generated a panel of transfectant cell lines. These transfectant cell lines express the \(\text{A}^\beta/\text{A}^\alpha\) MHC molecules with a mutation at each residue \(\text{A}^\alpha_69\text{A}^\alpha_69\) alanine with threonine, valine or serine completely eliminated the ability to stimulate autoreactive \(T\)-cell clones without affecting the ability to present foreign antigen keyhole limpet haemocyanin (KLH) or \(\alpha\)-plastin peptide to specific \(T\)-cell clones. Replacing \(\text{A}^\alpha_69\) alanine with threonine, valine or serine completely eliminated the ability to stimulate autoreactive \(T\)-cell clones without a

INTRODUCTION

NZB \times NZW \times NZW F1 (B/WF1) mice have been studied as an animal model for human systemic lupus erythematosus (SLE) (reviewed in 1–3). At around 6 months of age, these mice start to show elevated serum immunoglobulin G (IgG) anti-DNA antibodies and severe immune-complex glomerulonephritis. Female mice are more susceptible to autoimmune symptoms than are male mice. In addition to the abnormality in both B and T lymphocytes, \(4, 5\) classic genetic studies revealed that the heterozygosity at the H-2 complex of the murine major histocompatibility complex (MHC) is critically involved in the development of B/WF1 autoimmunity. \(6, 7\)

Our previous studies demonstrated the existence of mixed haplotype \(\text{A}^\beta/\text{A}^\alpha\) major histocompatibility complex (MHC) class II molecules on B/WF1 spleen cells and the existence of autoreactive and foreign antigen-reactive \(T\)-cell clones restricted by such \(\text{A}^\beta/\text{A}^\alpha\) molecules. \(8\) Further studies demonstrated that some of these autoreactive \(T\)-cell clones induced anti-DNA antibody of IgG class in cell transfer experiments. \(9\) These studies suggested that the recognition of self-peptide(s) in association with \(\text{A}^\beta/\text{A}^\alpha\) class II molecules by pathogenic autoreactive \(T\)-cells is important for the onset and/or progression of B/WF1 autoimmunity. In an accompanying paper, we examined the characteristics of peptides that bind to \(\text{A}^\beta/\text{A}^\alpha\) class II molecules \(10\) and suggested that charged residues in the peptide sequence affect the binding to the \(\text{A}^\beta/\text{A}^\alpha\) molecules.

In this study, we attempted to clarify whether unique amino acid residue(s) in the \(\text{A}^\beta/\text{A}^\alpha\) class II molecules might exist which are crucial for the activation of autoreactive \(T\)-cells. We generated and analysed a panel of transfectant cell lines that express \(\text{A}^\beta/\text{A}^\alpha\) molecules with single amino acid substitutions at several polymorphic sites on the \(\alpha\) or \(\beta\) chain. Our results suggest that alanine at position 69 of the \(\text{A}^\alpha\) chain is critical for the activation of autoreactive \(\text{A}^\beta/\text{A}^\alpha\)-restricted \(T\)-cell clones.

MATERIALS AND METHODS

Mice

New Zealand Black (NZB; H-2\(^\alpha\)) and New Zealand White (NZW; H-2\(^\beta\)) mice were purchased from Japan SLC Inc.
Site-directed mutagenesis

Site-directed mutagenesis of Aα* or Aβ* cDNA was performed using an Altered Sites II in vitro Mutagenesis System (Promega, Madison, WI) or using a polymerase chain reaction (PCR) mutagenesis.31 Mutagenic oligonucleotides and PCR primers were purchased from Bio-Synthesis, Inc. (Lewisville, TX) and are listed in Table 2. These mutagenic oligonucleotides or primers were named according to their target positions and the site of the wildtype amino acid to the expected mutant amino acid residues by single-letter codes of amino acid. Most of these mutagenic oligonucleotides or PCR primers were designed to introduce or destroy a restriction enzyme site in their sequences, as described in Table 2, to diagnose the expected mutation for screening. The wildtype Aα* cDNA was obtained from Dr. R. Miyazaki (Osaka University) and cloned into pALTER-1 vector (Promega). Mutagenesis was performed according to the manufacturer’s instructions. The wildtype Aβ* cDNA was obtained by reverse transcription (RT)-PCR from RNA of the NZW mouse spleen. Mutagenesis was performed by PCR mutagenesis.22 After confirming the mutagenesis and the absence of unintentional mutations by DNA sequencing (ALExpress DNA Sequencer, Pharmacia Biotech), mutant Aα* or Aβ* cDNA was subcloned into pCEXV vector.22

DNA transfection

For Aα* cDNA transfection, 2 × 10⁷ cell II negative 2C3 cells23 were cotransfected with 20 μg of pCEXVAβ* cDNA and 1 μg of pSV2-bag plasmid (kindly provided by Dr. Itoh, Saga Medical School, Japan) by electroporation. The electroporation was performed three times at 1.0 kV/cm with a capacitance of 25 μF using a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA). 48 hr after transfection, a selection medium containing 300 μg/ml hygromycin (Wako Chemical, Osaka, Japan) was added to the culture. After 2–3 weeks, hygromycin resistant clones were stained with anti-class II mAb (BW 9/13) and were then subcloned by the limiting dilution and stable transfected cell lines were established. In some experiments, a cell-sorting technique by EPICS ELITE (Couler Cytometry, Hialeah, FL) was used to enrich the positive clones before the limiting dilution. For Aβ* cDNA transfection, M12.C3 cells were cotransfected with 20 μg of pCEXVAβ* cDNA and 1 μg of the pSV2-neo plasmid by electroporation. Class II expression by G418-resistant clones was screened using anti-class II mAb (BW 9/13), and stable transfected cell lines were established as above.

Flow cytometry analysis and quantitative immunofluorescence analysis

One million cells were first incubated with 100 μl of appropriately diluted mAbs for 30 min at 4°C. Cells were washed three times with HBBS containing 1% fetal calf serum (FCS) and 0.1% NaN₃ (staining buffer). Then, 20 μl of appropriately diluted fluorescein isothiocyanate (FITC)–protein A (Zymed Lab. Inc., San Francisco, CA) or FITC–Mar 18.5 (mouse anti-tat x chain mAb)24 was added and the mixture was incubated for 30 min at 4°C. This was followed by addition of 10 μl of 50 μg/ml propidium iodide (Sigma, St. Louis, MO) for the final 5 min to gate out dead cells. Cells were then washed three times with cold staining buffer and analysed by fluorescence-activated cell sorting (FACScan®; Becton-Dickinson Immunocytometry Systems, Mountain View, CA). The quantitative immunofluorescence analysis was performed as described by Brausenstein and Germain25 with slight modifications: Briefly, 3 × 10⁷ transfected cells in 100 μl staining buffer were stained with 100 μl of a serial dilution of the K24-199 mAb followed by FITC–protein A. Cells were analysed on a FACScan and the mean fluorescence intensity (MFI) was calculated.
Mutational analysis of MHC class II molecule

Table 1. Amino acid sequences in single-letter codes of the CDR3 region of TCR α and β chains in AβAβ-restricted T-cell clones from B/WF1 mice

<table>
<thead>
<tr>
<th>T-cell clones</th>
<th>TCR sequence</th>
<th>α chain</th>
<th>β chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGU140</td>
<td>AV3S2/3a39 RGDYANKM</td>
<td>BV4S1/Jβ2.6 SQDLSSEYQ</td>
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</tr>
<tr>
<td>AUS-16</td>
<td>AV3S3/3a33 ETGNYKY</td>
<td>BV14S1/Jβ1.6 SRGGRSFL</td>
<td></td>
</tr>
<tr>
<td>KLH150</td>
<td>AV2S9/3a41 SGQGOQNF</td>
<td>BV13S1/Jβ1.1 SHPTEY</td>
<td></td>
</tr>
<tr>
<td>LP207</td>
<td>AV3S1/3a11 TNSGYQ</td>
<td>BV4S1/Jβ1.5 SQDLYNQAP</td>
<td></td>
</tr>
<tr>
<td>LP294</td>
<td>ND5</td>
<td>BV4S1/Jβ2.3 SQEGGLGL</td>
<td></td>
</tr>
<tr>
<td>LP10-3</td>
<td>AV8S1/3a41 SARDTYQ</td>
<td>BV4S1/Jβ2.2 SQDQGQWL</td>
<td></td>
</tr>
</tbody>
</table>

KGU140 and AUS-16 T-cell clones are autoreactive T-cell clones. KLH150 is a keyhole limpet haemocyanin-specific T-cell clone. LP207, LP294 and LP10-3 are λ-plastin 588–605 peptide-specific T-cell clones. The Va/Jα and Vb/Jβ gene usages are indicated on the left side of the sequences. The definition of the CDR3 region is according to Rock et al.20 These sequence data are available from EMBL/GenBank/DDBJ under accession numbers AB015835–AB015845.

*ND: not determined.

Table 2. Mutagenic oligonucleotides and PCR primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Diagnostic enzyme</th>
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<tr>
<td>Oligonucleotides</td>
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<td></td>
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<tr>
<td>n11F-S</td>
<td>CACGTAGGCTcCTATGGTACA</td>
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<tr>
<td>s28H-F</td>
<td>GGCCAGTACACAttcGAATTTGAT</td>
<td>HindIII(+)*</td>
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<tr>
<td>s57L-S</td>
<td>GGCCTATTGAATAcTTCCTGGCAA</td>
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<tr>
<td>s69A-T</td>
<td>AACTGATCGTAGAAAAACAC</td>
<td>PstI(−)==</td>
</tr>
<tr>
<td>s69A-V</td>
<td>AACTGATCGTAGAAAAACAC</td>
<td>PstI(−)==</td>
</tr>
<tr>
<td>n70E-G</td>
<td>CAAACTACGCAGGAAAAACACAC</td>
<td>PstI(−)==</td>
</tr>
<tr>
<td>n76L-V</td>
<td>AACTTGGAAGTCTTGCTAAAG</td>
<td></td>
</tr>
<tr>
<td>PCR primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b11F-L</td>
<td>TTGGTCAGAGb11F-L</td>
<td>SalI(−)</td>
</tr>
<tr>
<td>b13P-M</td>
<td>CAGTCTCAGAGb13P-M</td>
<td>AccI(−)</td>
</tr>
<tr>
<td>j80V-D</td>
<td>GAGACGGAGGj80V-D</td>
<td>BamHI(−)</td>
</tr>
</tbody>
</table>

Diagnostic restriction enzyme sites are underlined. Mismatches with the template are indicated by lowercase letters. The names of the oligonucleotides or primers indicate the target position and the wildtype amino acid to the expected mutant amino acid residues by single-letter codes.

*(+): Introduced enzyme site.
*(−): Destroyed enzyme site.

Peptide binding assay

This was performed according to Malcherek et al.23 with slight modification.24 Briefly, a saturating concentration of 100 μM N-terminally biotinylated λ-plastin 588-605 peptide was incubated with 5 × 10⁴ wildtype or mutant transfectant cell lines in 200 μl culture medium and the mixture was incubated at 37°C for 20 hr in a microtitre plate (Falcon no. 3075, Becton-Dickinson, Lincoln, NJ). The cells were then washed and incubated with FITC-streptavidin (Gibco BRL, Rockville, MD) at 4°C. Propidium iodide was added for the final 5 min to gate out dead cells. Stained cells were analysed on a FACScan. The peptide binding assay was repeated three times with reproducible results.

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cell line) in 0.1 ml complete medium. CT-6 cells were cultured for 48 hr and proliferative responses were assayed by the pulse of 0.5 μCi [3H]-Tdr (ICN Pharmaceuticals Inc., CA) for the final 16 hr, and the uptake of [3H]-Tdr was measured on a Betaplate flat-bed scintillation counter (Pharmacia-Wallac, Gaithersburg, MD). Experiments were performed in triplicate cultures and repeated at least twice, but usually three times, with similar results.

RESULTS

Expression of mutant Aββ/Arβ class II molecules in B lymphoma cells

We selected polymorphic sites in the β and α chain of class II molecules for the target sites of mutagenesis because polymorphic sites in the MHC molecules were shown to be critical for the binding of antigenic peptides (reviewed in 27). Almost all the mutageneses were arbitrarily designed to change from the wildtype to one of the parental (d or z haplotype) amino acid residues. For residue 69 in the α chain, the mutagenesis was also designed to change to representative amino acids in the hydrophobic or hydrophilic side chain group. Each mutated Arβ or Aββ cDNA was transfected together with a higromycin-resistance gene or a neomycin-resistance gene into the class II mutant B lymphoma line 2C3 cells or M12.C3 cells, respectively. After selection, stable transfectant cell lines were analysed for their expression of class II molecules by staining with monomorphic anti-class II mAb (BW/11F-L and TAβ-L) showing that mixed isotype Aββ class II molecules are not expressed in the wildtype transfectant cell lines with high (α, TAβ-H), intermediate (h, TAβ-L) or low (ε, TAβ-L) expression of Aββ/Arβ class II molecules as described in Table 2 and indicated at the right upper corner in each panel. Staining of three independent wildtype transfectant cell lines with high (α, TAβ-H), intermediate (h, TAβ-L) and low (ε, TAβ-L) expression of Aββ/Arβ class II molecules and a class II negative 2C3 cell line (d) for transfection are also shown. Mean fluorescence intensity (MFI) of staining with [3H]-Tdr for each cell line is indicated at the right side of each panel.

Stimulation of Aββ/Arβ-restricted T-cell clones by transfectant cell lines

We examined the ability of these transfectant cell lines to stimulate Aββ/Arβ-restricted autoactive and antigen-reactive T-cell clones. Proliferative responses of T-cell clones against each transfectant APC line were performed in one set of experiments to compare the stimulatory activity of each mutation. Because the amounts of Aββ/Arβ class II molecules on transfectant cells vary for each line, we first assessed whether the amounts of class II molecules affect the stimulatory activity on T-cell clones. As shown in Fig. 2, wildtype transfectant cell lines that express various amounts of Aββ/Arβ (TAβ-H, TAβ-L, TAβ-L) showed a similar degree of stimulation against all the T-cell clones. This indicates that the amounts of Aββ/Arβ molecules do not significantly affect this.

Figure 1. Flow cytometric analysis of expression of the Aββ/Arβ MHC class II molecules on the surface of transfectants. The cells were stained with BW/9 (anti-I-A) mAb followed by FITC-Mac 18.5. Immunofluorescence analysis was performed on FACScan. Transfectant cell lines with the Aββ/Arβ (ε-m) express various amounts of Aββ/Arβ class II molecules. The expected mutations in Aββ/Arβ molecules are as described in Table 2 and indicated at the right upper corner in each panel. Staining of three independent wildtype transfectant cell lines with high (α, TAβ-H), intermediate (h, TAβ-L) or low (ε, TAβ-L) expression of Aββ/Arβ class II molecules and a class II negative 2C3 cell line (d) for transfection are also shown. Mean fluorescence intensity (MFI) of staining with [3H]-Tdr for each cell line is indicated at the right side of each panel.
Mutational analysis of MHC class II molecule

Figure 2. Stimulation of T-cell clones by wildtype or mutant transfectant APC cell lines. 1 × 10⁴ autoreactive T-cell clones KG140 (a) or AU3-16 (b) were cultured with 3 × 10⁴ APC cell lines. 1 × 10⁴ T-cell clones KLH58 (c) were cultured with 1 × 10⁴ APCs in the presence of 250 μg/ml of KLH antigen. 1 × 10⁴ T-cell clones LP207 (d), LP824 (e) and LP10–3 (f) were cultured with 1 × 10⁴ APCs in the presence of 75 μg/ml of plasmin 588–605 peptide. Cytokines released in culture medium were measured by the proliferative responses of IL-2 and IL-4 dependent CT-6 cells. The results were expressed as a mean c.p.m. of triplicate cultures ± SD.

One explanation for the loss of or decreased stimulatory activity of n69 and b86 mutant APC lines is that these mutations resulted in gross conformational changes in the structure of Aββ/Aαα class II molecules. To examine this, we performed quantitative immunofluorescence analysis that allows evaluation of relative affinity of the antibody binding and thus reflects the conformational change of class II molecules by mutagenesis. As shown in Fig. 3(a), n69A-T and n69A-S mutant APC lines showed decreased affinity to anti-Aαα mAb compared to the wildtype TAPβ cells. The data of n69A-V mutant APC lines was difficult to interpret because of the weak intensity of staining. These results indicate that the mutation at position 69 of the αα chain induced slight conformational changes in the Aββ/Aαα molecules. Although weak in staining intensity of the b86V-D mutant cell line, it showed increased affinity to anti-Aαα mAb staining, indicating that this mutation also resulted in a slight conformational change of Aββ/Aαα molecules. Mutations at other positions did not show significant changes in affinity to anti-Aββ and Aαα mAbs. The question of whether these changes contributed to the conformational changes in class II molecules needs further study.

Our previous study revealed that L-plasmin 588–601 peptide as
loss of staining and, (ii) with one exception (the $A^\alpha$-I (···)

Figure 3. Quantitative immunofluorescence analysis of mutant transfectant cell lines. 3 × 10^6 transfectant cell lines were incubated with one batch of serially diluted K24-199 mAb culture supernatants followed by FITC-protein A and analysed on a FACScan. Mean fluorescence intensity (MFI) of each staining is indicated for mutant: $\text{t}69\alpha$-T (•), $\text{t}69\alpha$-V (○), $\text{t}69\alpha$-S (□), $\text{t}69\beta$-V (△) (a); n11F-S (□), n22H-E (●), n57L-S (○), n76I-V (△) (b) cells. MFI of wildtype TAJP-H (●) and TAJP-L (○) transfectant cell lines is also shown.

a naturally eluted peptide from purified $\text{A}^\beta$/$\text{A}^\alpha$ class II molecules and synthetic t-plasmin 588–605 peptide (SMARKIGARVYALPEDLV) bind to $\text{A}^\beta$/$\text{A}^\alpha$. Transfectant cell lines with wildtype or mutant $\text{A}^\beta$/$\text{A}^\alpha$ molecules were incubated with biotinylated t-plasmin 588–605 peptide followed by FITC-streptavidin and analysed by FACScan. Because each transfectant cell line expressed different amounts of $\text{A}^\beta$/$\text{A}^\alpha$ molecules, the efficiency of peptide binding was expressed as the percentage relative peptide binding as described in the legend to Fig. 4. The peptide binding was specific for all the transfectant cell lines because the $\text{A}^\beta$/$\text{A}^\alpha$ negative parental APC line (M12-C3) did not bind the peptide. Also the anti-$\text{A}^\beta$ mAb (10.2.16) partially blocked the peptide binding to each transfectant (data not shown). As shown in Fig. 4, almost all transfectant cell lines with wildtype or mutant $\text{A}^\beta$/$\text{A}^\alpha$ molecules showed similar binding ability to this peptide except $\text{t}69\alpha$-T mutant cell lines. The $\text{t}69\alpha$-T mutation appears to affect the peptide binding to $\text{A}^\beta$/$\text{A}^\alpha$ molecules. Although the results show some variability, especially when class II expression is low, other mutant APC lines in this study may not have altered $\text{A}^\beta$/$\text{A}^\alpha$ conformation for peptide binding.

**DISCUSSION**

The most interesting finding in this study is that substitution of alanine to threonine, valine or serine at position 69 in the $\text{A}^\alpha$ chain of $\text{A}^\beta$/$\text{A}^\alpha$ class II molecules resulted in the complete loss of stimulatory activity against $\text{A}^\beta$/$\text{A}^\alpha$-restricted autoreactive T-cell clones. The mutations affected two independent autoreactive T-cell clones with different TCR $\alpha$ and $\beta$ chains. The $\text{t}69$ mutations, however, did not show a significant effect on the stimulatory activity against antigen-reactive $\text{A}^\beta$/$\text{A}^\alpha$-restricted T-cell clones. The reason for the complete loss of stimulatory activity of $\text{t}69$ mutant APCs was probably not a result of the gross conformational alterations of the $\text{A}^\beta$/$\text{A}^\alpha$ molecules by the mutagenesis, as (i) immunofluorescence staining by haplotype specific anti-class II mAbs did not show loss of staining and, (ii) with one exception (the $\text{t}69\alpha$-T mutant), t-plasmin 588–605 peptide binding studies did not show a substantial difference between mutant and wildtype $\text{A}^\beta$/$\text{A}^\alpha$ lines (Fig. 4). Also, the observation that antigen-reactive T-cell clones showed a similar degree of proliferation against $\text{t}69$ mutant and wildtype APCs supports the idea that gross conformational changes are unlikely.

One explanation for the effect of $\text{t}69$ mutations would be that the peptides recognized by autoreactive T-cell clones have specific affinity to the structure created by alanine at $\text{t}69$. A conservative change of alanine to hydrophobic residue valine or non-conservative changes to hydrophilic residue serine or threonine resulted in the complete loss of stimulation against autoreactive T-cell clones. This may indicate that alanine, by itself or in combination with other surrounding residues around $\text{t}69$, is critical for binding to the target peptides. The small side chain of alanine could exert this effect. Attempts to substitute alanine by the smaller glycine at $\text{t}69$ were unsuccessful in achieving its expression, probably due to the conformational instability of the mutated polypeptide chain.

Because two independent T-cell clones with different TCR sequences and, therefore, two different target peptides, were affected in a similar way, these peptides may have common features for the binding to $\text{A}^\beta$/$\text{A}^\alpha$ and, specifically, to the structure around position $\text{t}69$. It is interesting to note here that our accompanying paper[330] showed that the side chain of binding peptide at relative position 6 (p6) influenced the peptide binding and that residues with large and negatively charged side chains are not tolerated at p6. The position of $\text{t}69$ corresponds to pocket 6 in the class II molecule that accommodates the side chain of residue at relative position 6 of the binding peptide, as speculated by the crystallographic analysis of class II molecules.[32] Another explanation for the loss of stimulatory activity of $\text{t}69$ mutant APCs against auto- but not antigen-reactive T-cell clones is that the responsiveness of these autoreactive T-cell clones, and potentially the autoreactive T-cell clones in general, is sensitive to the slight structural change of class II molecules. As shown in the quantitative immunofluorescence experiments (Fig. 3), substitution of alanine at $\text{t}69$ to other residues
Figure 4. Peptide binding assay of transfectant cell lines. Biotinylated L-plastin 588–605 peptide (100 µM) was incubated with each transfectant cell line followed by FITC–streptavidin and analyzed by FACScan. The same transfectant cell lines were also stained with monomorphic anti-class II mAb (BW 9) followed by FITC–Mar 18·5. Relative systemic lupus erythematosus.

3. The same transfectant cell lines were also stained with monomorphic anti-class II mAb (BW 9) followed by FITC–Mar 18·5. Relative peptide binding to each transfectant cell line was calculated by a decrease in the expression of MHC molecules. Although we were not able to demonstrate a change in peptide binding ability, these findings are essentially consistent with the results reported by several investigators. Substitution of the residue at β86 resulted in very low levels of cell surface expression of class II molecules, decreased peptide binding15,16 and weakened T-cell recognition17,18.

Our previous report suggested that mixed haploype $\text{A}^\beta / \text{A}^\epsilon$ class II molecules play important roles in the pathogenicity of autoimmune in B/WFI mice. It would be interesting to examine whether in vivo expression of mutated $\text{A}^\beta / \text{A}^\epsilon$ class II molecules at position 69 in the β chain can prevent the onset and/or progression of autoimmune symptoms of B/WFI mice. It is also interesting that autoreactive T cells in general are sensitive to mutation at position 69 in the β chain, irrespective of the haploype specificity.

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