Generation of Humanized Mice Susceptible to Peptide-Induced Inflammatory Heart Disease

Kurt Bachmaier, MD; Nikolaus Neu, MD; Rae S.M. Yeung, MD; Tak W. Mak, PhD; Peter Liu, MD; Josef M. Penninger, MD

Background—Dilated cardiomyopathy (DCM) is a major cause of sudden cardiac death. In certain mouse major histocompatibility complex (MHC) backgrounds, myocarditis and inflammatory cardiomyopathy can be triggered by immunization with heart muscle–specific proteins. Similarly, chronic heart disease in humans has been linked to certain HLA alleles, such as HLA-DQ6. However, there is no experimental evidence showing that human MHC class II molecules and peptides derived from human proteins are involved in the pathogenesis of myocarditis and DCM.

Methods and Results—We generated double CD4- and CD8-deficient mice transgenic for human CD4 (hCD4) and human HLA-DQ6 to specifically reconstitute the human CD4/DQ6 arm of the immune system in mice. Transgenic hCD4 and HLA-DQ6 expression rendered genetically resistant C57BL/6 mice susceptible to the induction of autoimmune myocarditis induced by immunization with cardiac myosin. Moreover, we identified heart-specific peptides derived from both mouse and human α-myosin heavy chains capable of inducing inflammatory heart disease in hCD4 and HLA-DQ6 double transgenic mice but not in hCD4 single transgenic littermates. The autoimmune inflammatory heart disease induced by the human heart muscle–specific peptide in hCD4 and HLA-DQ6 double transgenic mice shared functional and phenotypic features with the disease occurring in disease-susceptible nontransgenic mice.

Conclusions—Our data provide the first genetic and functional evidence that human MHC class II molecules and a human α-myosin heavy chain–derived peptide can cause inflammatory heart disease and suggest that human inflammatory cardiomyopathy can be caused by organ-specific autoimmunity. The humanized mice generated in this study will be an ideal animal model to further elucidate the pathogenesis of inflammatory heart disease and facilitate the development of rational treatment strategies. (Circulation. 1999;99:1885-1891.)

Key Words: cardiomyopathy ■ molecular biology ■ myocarditis ■ genes

Cardiovascular disease remains the most frequent cause of death in the industrialized world. A common cause of progressive heart disease, heart failure, and sudden death is dilated cardiomyopathy (DCM).1–4 DCM is a myocardial disease of heterogeneous pathogenesis defined by enlargement of the cardiac chambers and impaired myocardial contractility.5,6 DCM is the principal condition necessitating heart transplantation.7 Viral myocarditis frequently precedes the development of DCM,8–10 and clinical and experimental studies suggest that the chronic stages of myocarditis and DCM are mediated by autoimmune responses to cardiac autoantigens exposed to the immune system after cardiomyocyte damage.11,12 Myocarditis and DCM can be experimentally induced in susceptible mouse strains by immunization with purified cardiac myosin.13 In this model, upregulation of major histocompatibility complex (MHC) class II molecules and their presentation of heart-specific antigens to autogressive CD4+ T cells are crucial prerequisites for the induction of disease.14–17

Genetic predisposition is a characteristic feature of organ-specific autoimmune diseases. Specific HLA alleles have been epidemiologically linked to susceptibility or resistance to inflammatory heart disease and chronic DCM.18–23 However, there is no experimental evidence showing that human MHC class II molecules and peptides derived from human proteins are involved in the pathogenesis of myocarditis and DCM. Since HLA-DQ6 has been implicated in the pathogenesis of human DCM,18 we generated mice transgenic for human CD4 (hCD4) and human HLA-DQ6 to specifically reconstitute the human CD4/DQ6 arm of the immune system. We report here that the HLA-DQ6 transgene renders mice susceptible to inflammatory heart disease. A dominant, auto-
aggressive peptide was mapped to the human α-myosin heavy chain molecule. These results indicate that human inflammatory heart disease can be caused by organ-specific autoimmunity.

Methods

Mice

Human CD4 and human DQ6 double transgenic mice in a murine CD4 (L3T4) and CD8\(^+\) (Lyt2, CD8\(\delta\)) double knockout background (termed hCD4/DQ6 TG throughout this report) have been described previously.\(^{24-27}\) If not otherwise stated, all mice used were of the C57BL/6 background (8 backcrosses), which is resistant to autoimmune myocarditis.\(^{13}\) Human CD4 transgene expression was under the control of the human CD2 expression cassette, which confers lymphocyte-specific, copy number–dependent, integration site–independent expression of human CD4.\(^{28,29}\) To generate human MHC class II DQ6 single transgenic mice, genomic DQ6 α- and β-genomic DNA fragments under the control of the endogenous promoters were coinjected.\(^{30}\) To obtain control littersmates, mice heterozygous for both hCD4 and DQ6 transgenes were intercrossed to obtain hCD4 single TG and hCD4/DQ6 double TG mice. If not otherwise stated, all hCD4 single TG and hCD4/DQ6 double TG mice in this study were on an endogenous CD4 and CD8 gene–deficient background. Control C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). All mouse strains were phenotyped with the use of fluorescence-activated cell sorter immunostaining and genotyped with Southern blotting. Care of animals was in accordance with guidelines of the Medical Research Council of Canada.

Immunization

Cardiac myosin was purified from mice as described previously.\(^{31}\) The polypeptides derived from either murine or human cardiac-speciﬁc α-myosin heavy chains were synthesized by Fmoc (fluorenylmethoxycarbonyl)/t-butyl–based, solid-phase peptide chemistry as described.\(^{32}\) Eight-week-old mice were immunized twice subcutaneously at 0 and 7 days with 100 \(\mu\)g of cardiac myosin emulsified in Freund’s complete adjuvant (FCA) or with FCA alone.\(^{13}\) Peptides were as follows: mM7A\(\alpha\), acetyl-SKLKLMATLFSYTASA; hM7A\(\alpha\), acetyl-SKLKLMATLFSYYTAT. Peptides were dissolved in FCA at 1 mg/mL and emulsified in a 1:1 dilution with PBS. Emulsified peptide (100 \(\mu\)L) was injected into mice by use of the same protocol as for purified cardiac myosin. Twenty-one days after the first immunization, mice were euthanized and processed for histological and immunohistochemical analysis of heart muscle inflammation. For histological analysis, hearts were ﬁxed in formaldehyde and processed for hematoxylin and eosin staining.

Immunocytochemistry

To determine the phenotypic characteristics of cells infiltrating the heart and the induction of MHC class II on cardiac interstitial cells, hearts were processed for cryosections. For immunoperoxidase staining, cryosection sections were ﬁxed in acetone and the endogenous peroxidase activity was blocked with NaN\(_3\) and \(H_2O_2.\)\(^{33}\) Sections were incubated with anti-CD3ε (PharMingen) to detect inﬁltrating T cells, anti-CD11b (clone Mac1) (PharMingen) to detect macrophages/dendritic cells, anti-I–A\(^\text{d}\) (PharMingen) to detect murine MHC class II expression, and anti-HLA DQ (Leu10; Becton Dickinson & Co) to detect expression of human DQ6. Anti–nitrotyrosine staining (Upstate Biotechnologies) to detect inﬂammation-dependent nitrosylation of heart muscle proteins was performed on paraformaldehyde–ﬁxed and parafﬁn–embedded sections as described previously.\(^{34}\) Antibody binding was visualized with the use of alkaline phosphatase–labeled streptavidin or peroxidase-conjugated secondary antibodies. Reactions were developed with the use of fast red or DAB tablets (Sigma), and sections were counterstained with hematoxylin.

Results

Human MHC Class II Allele DQ6 Confers Susceptibility to Autoimmune Heart Disease

The pathogenesis of murine autoimmune heart disease depends on the recognition of heart-specific autoantigens in association with MHC class II molecules by CD4\(^+\) autoreactive T cells.\(^{14-17}\) However, it is not known whether a human MHC class II molecule can present heart-speciﬁc autoantigens to T cells in vivo and thus whether the development of inflammatory autoimmune myocarditis and DCM in humans resembles the murine model. To address this question, we generated mice (designated hCD4/DQ6 TG) that were transgenic for the human class II allele DQ6 and human CD4 in a murine CD4 and CD8 double knockout background. This strategy eliminated potentially confounding effects caused by endogenous murine CD4\(^+\) and CD8\(^+\) T cells. Subsequently, hCD4/DQ6 TG mice were backcrossed into mouse strain C57BL/6, which is genetically resistant to autoimmune myocarditis induced by immunization with cardiac myosin.\(^{13}\)

hCD4/DQ6 TG mice and littersmates carrying only the hCD4 transgene were immunized with puriﬁed cardiac myosin emulsiﬁed in FCA.\(^{13}\) Within 21 days after the initial injection, 65% of mice carrying both transgenes developed autoimmune myocarditis, as assessed by the presence of inﬂammatory inﬁltration (Table 1 and Figure 1A). In contrast, hCD4 single TG littermate mice immunized with cardiac myosin developed only very mild inﬂammation at a signiﬁcantly lower frequency (Table 1 and Figure 1B), and hCD4/DQ6 TG mice immunized with FCA alone, inﬂammation failed to develop in the heart (Table 1 and Figure 1C). The fact that mild autoimmune heart disease still developed in mice lacking DQ6 molecules suggests that cardiac myosin–derived epitopes with minor pathogenicity can be present–ed by the endogenous MHC class II I–A\(^\text{d} \). These results demonstrate that the induction of autoimmune heart disease in hCD4/DQ6 TG mice is dependent on the autoantigen cardiac myosin and that transgenic expression of the human

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Immunization</th>
<th>Prevalence (Positive Mice/Total)</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCD4TG</td>
<td>Cardiac myosin + FCA</td>
<td>2/16 (13%)</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>hCD4/DQ6TG</td>
<td>Cardiac myosin + FCA</td>
<td>15/23 (65%)</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>hCD4/DQ6TG</td>
<td>FCA only</td>
<td>0/10 (0%)</td>
<td>...</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Murine M7A(\alpha) + FCA</td>
<td>0/10 (0%)</td>
<td>...</td>
</tr>
<tr>
<td>hCD4/DQ6TG</td>
<td>Murine M7A(\alpha) + FCA</td>
<td>8/12 (67%)</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>hCD4TG</td>
<td>Murine M7A(\alpha) + FCA</td>
<td>0/10 (0%)</td>
<td>...</td>
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</table>

Mice were immunized with murine cardiac myosin or with M7A\(\alpha\) peptide, derived from the murine cardiac-specific α-myosin heavy chain, as described in Methods. Prevalence and disease severity were scored histologically on hematoxylin and eosin–stained heart sections. Histological grading of severity was 0 = no infiltration in heart muscle; 1 = up to 5% of histological cross section is infiltrated; 2 = 6% to 10%; 3 = 11% to 20%; and 4 = > 20%. Mean values of disease severity ± SD are indicated. One result representative of 3 independent experiments is shown.
DQ6 molecule renders mice susceptible to cardiac myosin–induced autoimmune heart disease.

**Peptide Derived From Murine Cardiac-Specific α-Myosin Heavy Chain Induces Autoimmune Heart Disease in hCD4/DQ6 TG Mice**

We and others have recently mapped pathogenic epitopes for the induction of autoimmune heart disease within the murine cardiac α-myosin molecule. A peptide designated mM7Aα derived from the murine α-myosin heavy chain is a potent inducer of autoimmune myocarditis in susceptible BALB/c mice but not in resistant C57BL/6 mice (Table 1). Peptide mM7Aα was therefore a good candidate to evaluate autoimmunity pathogenicity in hCD4/DQ6 TG mice. Immunization of hCD4/DQ6 TG mice with mM7Aα peptide induced autoimmune heart disease in hCD4/DQ6 TG mice with a prevalence and severity similar to that induced in mice immunized with mM7Aα (Table 3 and Figure 1E). Myocarditis did not develop in single hCD4 TG littermates immunized with hM7Aα (Figure 1F) or in hCD4/DQ6 TG mice immunized with FCA alone (Table 3). These data provide the first experimental evidence that a human autoantigen, the cardiac α-myosin heavy chain–derived peptide hM7Aα, can induce autoimmune heart disease.

**Phenotypic and Functional Characteristics of Autoimmune Heart Disease in hCD4/DQ6 TG Mice**

The expression of MHC class II molecules is strongly upregulated during the course of autoimmune heart disease and, in fact, is a prerequisite for the induction of autoimmune heart disease in mice. To confirm that the human DQ6 molecule was expressed in the inflamed heart, we analyzed the expression of DQ6 and endogenous I-Aβ class II molecules in immunized and unimmunized hCD4/DQ6 TG mice (Table 4). Immunostaining with antibodies specific for HLA-DQ showed that the expression of HLA-DQ molecules was present in the inflamed heart.

**Identification of Peptide Derived From Human α-Myosin Heavy Chain That Induces Autoimmune Heart Disease in hCD4/DQ6 TG Mice**

Human and murine cardiac-specific α-myosin heavy chains share extensive sequence homology. Comparison of the human and murine α-myosin heavy chain protein sequences showed that the mM7Aα peptide differs in only 2 amino acids from the corresponding region of the human peptide, designated hM7Aα (Table 2). Because mM7Aα is a potent inducer of autoimmune myocarditis, we analyzed the pathogenicity of its human homologue. The hM7Aα peptide induced autoimmune heart disease in hCD4/DQ6 TG mice with a prevalence and severity similar to that induced in mice immunized with mM7Aα (Table 3 and Figure 1E). Myocarditis did not develop in single hCD4 TG littermates immunized with hM7Aα (Figure 1F) or in hCD4/DQ6 TG mice immunized with FCA alone (Table 3). These data provide the first experimental evidence that a human autoantigen, the cardiac α-myosin heavy chain–derived peptide hM7Aα, can induce autoimmune heart disease.
TABLE 4. Phenotype of Inflammatory Cells in Hearts of Humanized Mice Immunized With Purified Cardiac Myosin and Human Cardiac-Specific \( \alpha \)-Myosin Heavy Chain–Derived Peptide hM7A\( \alpha \)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Immunization</th>
<th>HLA-DQ( ^{+} )</th>
<th>I-A( ^{+} )</th>
<th>CD3( ^{+} )</th>
<th>CD11b( ^{+} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCD4/DQ6TG</td>
<td>None</td>
<td>2.2±1.0</td>
<td>1.2±1.2</td>
<td>2.2±1.3</td>
<td>2.0±1.0</td>
</tr>
<tr>
<td>hCD4/DQ6TG</td>
<td>Cardiac myosin</td>
<td>123.0±7.9</td>
<td>93.2±9.6</td>
<td>254.7±27.6</td>
<td>126.7±7.7</td>
</tr>
<tr>
<td>hCD4/DQ6TG</td>
<td>Human M7A( \alpha )</td>
<td>107.3±9.3</td>
<td>101.3±9.3</td>
<td>234.7±14.2</td>
<td>116.3±10.1</td>
</tr>
</tbody>
</table>

Frequency of positive cells in heart sections from hCD4/DQ6TG mice immunized with mouse cardiac myosin or the human cardiac-specific \( \alpha \)-myosin heavy chain–derived peptide M7A\( \alpha \) in FCA or from unimmunized control littermates. Numbers of positive cells (±SD) were counted per visual field at magnification of \( \times 160 \) on cryostat sections from hearts of at least 2 animals. A minimum of 10 different visual fields was evaluated per heart. Cryostat sections were incubated with either anti–HLA-DQ (human MHC class II), anti–I-A\( \beta \) (mouse MHC class II), anti-CD11b (to detect macrophages), or anti-CD3\( \epsilon \) (to detect T cells) antibodies. Visualization of antibody binding and immunization of mice were as described in Methods. One result representative of 3 independent experiments is shown.

was strongly upregulated within the heart after immunization with either the complete human cardiac myosin protein or peptide hM7A\( \alpha \) (Figure 2A). Endogenous I-A\( \beta \) was also upregulated on inflammatory cells. Minimal DQ6 and I-A\( \beta \) expression was detected in the hearts of unimmunized hCD4/DQ6 TG control mice (Table 4, Figure 2B). The human DQ6 transgene is expressed under the control of its own promoter\(^{30}\); therefore these results suggest that the expression of human HLA-DQ and mouse I-A molecules is regulated by similar pathways. More importantly, these data show that myocardial inflammation leads to upregulation of both transgenic human and endogenous murine MHC class II molecules. Moreover, the inflammatory infiltrate in hCD4/DQ6 TG mice immunized with either complete cardiac myosin or hM7A\( \alpha \) was similar to the infiltrate in disease-susceptible nontransgenic mouse strains. This infiltrate consisted primarily of CD11b\( ^{+} \) (Mac1) macrophages and CD3\( ^{+} \) T cells (Table 4).\(^{15,33}\)

In murine autoimmune heart disease, inducible nitric oxide synthase (iNOS) is highly expressed in macrophages and heart muscle cells. The increased expression of iNOS in hearts is accompanied by the formation of the NO reaction product nitrotyrosine.\(^{34}\) Nitrosylation of heart muscle proteins is indicative of iNOS activation and nitric oxide production and is considered a diagnostic marker for inflammatory heart disease. Nitrosylation of heart muscle proteins depends entirely on the presence of an inflammatory infiltrate within the heart.\(^{34}\) To analyze whether autoimmune heart disease in hCD4/DQ6 TG mice was accompanied by nitrosylation of tyrosine residues within the heart, we stained for nitrotyrosine in heart sections in situ. Figures 2C and 2D show that inflammatory cells and heart muscle cells were positive for nitrotyrosine. Mice immunized with FCA alone did not show nitrotyrosine formation in the heart muscle (Figure 2E). These data demonstrate that inflammatory autoimmune heart disease in hCD4/DQ6 TG mice shares phenotypic and functional characteristics with the inflammatory disease that occurs in disease-susceptible nontransgenic mice.\(^{13,15,16,32–34,37}\)

**Discussion**

Organ-specific autoimmune diseases arise because of the disruption of the balance between surveillance and tolerance. Autoantigens unique to the affected organ are recognized by autoreactive T cells in the context of MHC molecules.\(^{13,16,19}\) Our study provides the first genetic and functional evidence in vivo that human MHC class II molecules can confer susceptibility to inflammatory heart disease. Homologous murine and human cardiac-specific \( \alpha \)-myosin heavy chain–derived peptides were identified as immunodominant antigens that induce inflammatory heart disease in mice carrying human MHC class II molecules. Inflammatory heart disease in hCD4/DQ6 TG mice exhibited phenotypic and functional...
features characteristic of autoimmune myocarditis in non-transgenic susceptible mice.

Genetic predisposition to various cardiomyopathies has been associated with specific HLA alleles, and a subgroup of patients with idiopathic DCM have in their sera autoantibodies specifically directed against heart proteins. As well, genetically determined immune response factors associated with HLA loci have been implicated in the pathogenesis of this disorder. Antigen-induced autoimmune myocarditis and the development of DCM have also been linked to certain MHC class II alleles in mice, and the MHC class II haplotype is the single most important genetic factor associated with disease susceptibility. In both mice and humans, expression of MHC class II molecules is strongly upregulated during the course of the disease. In this study, immunostaining with antibodies specific for HLA-DQ and endogenous I-Aβ showed that expression of both HLA-DQ and I-Aβ within the heart is strongly upregulated on immunization with either complete cardiac myosin or the α-myosin heavy chain–derived peptides hM7Aα and mM7Aα.

It has previously been shown that both hCD4 and DQ6 are functional in mice and that the introduction of hCD4 and human DQ6 molecules into mouse mutants lacking both CD4 and CD8 reconstitutes this limb of the human immune system. The expression of both hCD4 and DQ6 is regulated in a tissue-specific manner and confers normal thymocyte development and selection of CD4+ T cells; that is, these generate a functional immune system that is restricted to DQ6 and the endogenous I-Aβ molecules. Although these transgenic mice express both HLA-DQ6 and I-Aβ, they do not form hybrid human/mouse class II molecules. These animals have thus provided an ideal model system for the study of the role of MHC class II in the induction of autoaggressive inflammatory heart disease. In this report, we have shown that the expression of hCD4 and DQ6 combined with immunization with hM7Aα or mM7Aα peptide was sufficient to induce myocarditis in mice in a genetically resistant background. That the experimental disease mirrors the in vivo situation was shown by the analysis of the cellular composition of the inflammatory infiltrate in affected hearts. Not only were the same cell types observed at a similar frequency as is observed in natural inflammatory autoimmune disease occurring in susceptible non-TG mice, but significant nitrosylation of heart muscle proteins was also detected.

The similarities between this murine model and the human disease lead to the speculation that in humans, professional antigen-presenting cells in the heart and peripheral lymphoid organs present cardiac-derived peptides in context with MHC class II molecules to autoreactive CD4+ T cells and thus initiate and/or maintain organ-specific autoimmune disease. In this study, homologous M7Aα peptides derived from the human or mouse α-myosin heavy chains were identified as potent autoantigens that induced inflammatory heart disease in humanized mice derived peptide can trigger inflammation. Interestingly, in this study, hDQ6 transgenic mice exhibited significantly increased susceptibility (tissue damage and viral replication) to CVB3 infections (J.M.P.; unpublished observations), indicating that the hDQ6 molecule confers susceptibility to both CVB3 infections and peptide-induced inflammatory heart disease. DCM and inflammatory cardiomyopathy in humans obviously represent heterogeneous diseases with diverse causes. However, if cardiac autoantigens are processed by professional antigen-presenting cells within the heart after acute cardiomyocyte damage, a pathogenic process similar to that demonstrated in our humanized murine model might indeed be initiated.

Abnormalities in cellular and humoral immunomodulation have long been recognized as factors in both human myocarditis and DCM. Whether these immune alterations are the causes or consequences of these pathological conditions has been controversial. Our data provide the first experimental evidence that human MHC class II molecules and a human α-myosin heavy chain–derived peptide can trigger inflammatory heart disease. Furthermore, we have shown that human MHC class II molecules and human heart muscle–specific autoantigens can cause organ-specific autoimmune heart disease. The humanized mice generated in this study will be an ideal animal model to further elucidate the pathogenesis of inflammatory heart disease and facilitate the development of rational treatment strategies.

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References


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