Brief Definitive Report

HLA-DRB1 Polymorphism Determines Susceptibility to Autoimmune Thyroiditis in Transgenic Mice: Definitive Association with HLA-DRB1*0301 (DR3) Gene

By Yi-chi M. Kong,* Lesley C. Lomo,* Reinhard W. Motte,‡
Alvaro A. Giraldo,‡ Jean Baisch,§ Gudrun Strauss,‖
Gunter J. Hämmerling,‖ and Chella S. Davids§

From the *Department of Immunology and Microbiology, Wayne State University, Detroit, Michigan 48201; the ‡Division of Immunopathology, St. John Hospital, Detroit, Michigan 48230; the §Department of Immunology, Mayo Medical School, Rochester, Minnesota 55905; and the ‖Tumor Immunology Program, German Cancer Research Center, 69120 Heidelberg, Germany

Summary

Familial clustering of autoimmune thyroid diseases has led to studies of their association with human major histocompatibility complex (MHC) class II genes. One such gene implicated in Hashimoto's thyroiditis (HT) is HLA-DR3, but the association is weak and is contradicted by other reports. On the other hand, murine experimental autoimmune thyroiditis (EAT), a model for HT, presents a clear linkage with MHC class II. Moreover, it is inducible with thyroglobulin (Tg), the common autoantigen in either species. Immunization of HLA-DRB1*0301 (DR3) transgenic mice with mouse or human Tg resulted in severe thyroiditis. In contrast, transgenic mice expressing the HLA-DRB1*1502 (DR2) gene were resistant to EAT. Our studies show that HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis and implicate Tg as an important autoantigen.

A well known model for Hashimoto's thyroiditis (HT) is murine experimental autoimmune thyroiditis (EAT). Susceptibility to this T cell-mediated disease is linked to H2A class II molecules of the murine major histocompatibility complex (MHC), which can present thyrotoxicogenic, highly conserved T cell epitopes on thyroglobulin (Tg) from both the mouse (M) and human (H) (1, 2). In contrast with studies on EAT, patient studies over the past 15 yr have not revealed a clear HLA association with HT despite improved typing techniques, although it is clear that ethnic variations exist. Several studies in Caucasians have implicated both DRB1*0301 (DR3) and DRB1*1101 (DR5) (3–8). However, a negative association with DR3 (9) or lack of any DR region association has also been reported (10–12). Recently, certain HLA-DQ alleles have also been implicated, even though the associations are complicated by linkage disequilibrium with DR loci. For instance, while the DQB1*0201 (DQw2) gene has been implicated in HT, its involvement is questionable owing to linkage with DR3 (6, 8, 13). Using HLA-DR and HLA-DQ transgenic mice, we can address the specific role of each HLA class II gene in human thyroid disease. We report here that EAT is induced in HLA-DR3 (DRB1*0301) transgenic mice immunized with either MTg or HTg, an autoantigen also in the human. In contrast, DRB1*1502 (DR2) transgenic mice were unresponsive to MTg. Thus, DRB1 polymorphism is a determining factor in susceptibility to autoimmune thyroiditis.

Materials and Methods

Tg and Adjuvant. MTg and HTg from frozen thyroids were fractionated on a Sephadex G-200 column (Pharmacia Biotech Inc., Piscataway, NJ) and checked for purity by immunoelectrophoresis as detailed previously (14). Salmonella enteritidis LPS was prepared by TCA precipitation.

Generation of HLA-DRB1 Transgenic Mice. The generation of DRB1*0301 (DR3) transgenic mice by coinjection of an HLA-DRα genomic fragment and a DRB1*0301β gene fragment into C57BL/6 × DBA/2F1 × C57BL/6 embryos and backcross to B10 mice was detailed previously (15). In the specific pathogen-free facility at Mayo Clinic, the DR3 transgene was first introduced into B10.M mice by repeated backcrossing. Subsequently, the DR3 gene was introduced into the class II-negative H2Ab− strain (16) by mating the B10.M-DRB1*0301 line with the B10.Ab− line, similar to the strategy detailed recently for HLA-DQ transgenic mice (17). PBLs were typed for expression and segregation during breeding by flow cytometry using the following monoclonal Abs: L227, anti-DRB1 (18); AF6-120, anti-H2Aα (19); 28-14-8S, anti-H2Dβ (20); 14-4-4S, anti-H2E (21); 3F-12, anti-H2Aβ (22).

DRB1*1502.Ab− (HLA-DR2, H2Ab−) transgenic mice were
generated as detailed elsewhere (23). In brief, DRB1*1502-positive founder mice were obtained by microinjecting a linearized 34 kb DNA fragment containing the entire DRB1*1502 gene, isolated from the HLA-homozygous B cell line AKIBA (DR2, Dw12, DOw6) (24), into (SWR × B10.M)F1 embryos and identified by PCR using the primers 5'-C(CT)TAAGAGGGAGT-GTCAAT'TTCTC3' and 5'-TGTCAGCTCTC(AC)3'-CAACCCC-3' in the second DRB1 exon. The DRB1*1502 transgene was then introduced into class II-negative Ab+ mice by mating. To get expression of the DRB1*1502 molecule, the mice were also mated with Ab+Eot transgenic mice. The high homology between DRα and Eo chains enables the pairing of the DRB1*1502 chain with either Tg or HTg.

**Induction and Assay of EAT.** Mice were housed in a pathogen-free facility on acidified, chlorinated water upon arrival and used at 8–16 wk of age. They were immunized intravenously on days 0 and 7 with 40 μg of MTg or 100 μg of HTg, followed by 20 μg of LPS 3 h later. On day 28, sera and thyroids were collected; there were no discernible gender influences in results. As previously described, anti-MTg and anti-HTg titers were determined either by passive hemagglutination with MTg- or HTg-coated human group O RBCs (14) or by ELISA (2). In brief, reagents for ELISA were 96-well plates (Immulon II; Dynatech Laboratories, Inc., Chantilly, VA) coated with 1 μg per well of MTg or HTg, PBS/Tween 20 for washing, PBS/1% BSA for blocking, and alkaline phosphatase-conjugated goat anti-mouse Ig H and L chains (Southern Biotechnology Association, Inc., Birmingham, AL) as the second Ab. Serum dilutions were tested at 1:100, 1:800, and 1:3,200 with standard immune sera and normal serum as controls.

Thyroid inflammation was determined histologically from 30–60 sections (7–10 step levels) containing both thyroid lobes, and the percentage of mononuclear cell infiltration and destruction in individual mice was recorded (26). Thyroids with >10% involvement showed definite follicular destruction accompanying focal areas of infiltration.

**In Vitro Proliferation.** Cellular proliferation was carried out as detailed previously (26, 27). In brief, on day 28 after immunization with either MTg or HTg, spleen cells (SCs) were cultured for 4 or 5 d in flat-bottomed, 96-well plates (6 × 10^5 cells per well) with 20 μg/ml of MTg or HTg, PBS/Tween 20 for washing, PBS/1% BSA for blocking, and alkaline phosphatase–conjugated goat anti–mouse Ig H and L chains (Southern Biotechnology Association, Inc., Birmingham, AL) as the second Ab. Serum dilutions were tested at 1:100, 1:800, and 1:3,200 with standard immune sera and normal serum as controls.

Thyroiditis was determined histologically from 30–60 sections (7–10 step levels) containing both thyroid lobes, and the percentage of mononuclear cell infiltration and destruction in individual mice was recorded (26). Thyroids with >10% involvement showed definite follicular destruction accompanying focal areas of infiltration.

**Results and Discussion.**

**B10.M-DR3+ Transgenic Mice Are Susceptible to EAT.** Initial DR3 gene transfer experiments were conducted in EAT-resistant B10.M mice after mating with DRB1*0301 transgenic mice and backcrossing to B10.M mice. Immunization with either MTg or HTg resulted in thyroid inflammation in the B10.M-DRB1*0301 mice (Table 1). Marked infiltration of mononuclear cells and destruction involving up to 40% of both thyroid lobes were present in 80% of MTg-immunized DR3+ mice, compared with none in the

**Table 1. Acquired Susceptibility to EAT Induction after Insertion of HLA-DRB1*0301 (DR3) Transgene into Resistant B10.M Mice**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>DR3 expression</th>
<th>Number of mice with percent thyroid involvement</th>
<th>Incidence</th>
<th>Positive/total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTg</td>
<td>+</td>
<td>0 &gt;0–10</td>
<td>0 &gt;10–20</td>
<td>&gt;20–40</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>HTg</td>
<td>+</td>
<td>0 &gt;0–10</td>
<td>0 &gt;10–20</td>
<td>&gt;20–40</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/4</td>
<td>50</td>
</tr>
</tbody>
</table>

* Mice were immunized with 40 μg of MTg or 100 μg of HTg and 20 μg of LPS intravenously 3 h later on days 0 and 7 and were killed on day 28.
Table 2. Presentation of Thyroiditogenic Epitopes on Both Mouse and Human Tg by HLA-DRB1*0301 (DR3) Molecules in DR3.Ab⁻ Mice

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Transgene expression</th>
<th>Tg antibody (OD 1:800)</th>
<th>Number of mice with percent thyroid involvement</th>
<th>Incidence</th>
<th>Positive/Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTg</td>
<td>None</td>
<td>&lt;0.2</td>
<td>3</td>
<td>0</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>DR3⁺E⁺</td>
<td>0.58 ± 0.07</td>
<td>1</td>
<td>3</td>
<td>2/5 100</td>
</tr>
<tr>
<td></td>
<td>DR3⁻E⁺</td>
<td>&lt;0.2</td>
<td>2</td>
<td>0/2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>DR3⁺E⁻</td>
<td>0.73 ± 0.05</td>
<td>1</td>
<td>5</td>
<td>6/6 100</td>
</tr>
<tr>
<td></td>
<td>DR3⁻E⁻</td>
<td>&lt;0.2</td>
<td>5</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>HTg</td>
<td>DR3⁺E⁻</td>
<td>ND</td>
<td>1</td>
<td>5/6</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>DR3⁻E⁻</td>
<td>ND</td>
<td>6</td>
<td>0/6</td>
<td>0</td>
</tr>
</tbody>
</table>

*See Table 1 for experimental protocol.

DR3⁺ sibs, which remained resistant. In HTg-immunized mice, all the DR3⁺ mice displayed similarly typical inflammation of >10–40%. The DR3⁻ mice were essentially negative; a focal perivascular infiltration in two mice could be due to a low response mediated by endogenous H2A molecules. In addition to conserved epitopes shared between MTg and HTg, HTg contains foreign epitopes that are known to stimulate T and B cells of both EAT-susceptible and EAT-resistant mice (26, 27). Thus, in Fig. 1, both DR3⁺ and DR3⁻ mice responded strongly and comparably to HTg in both in vitro proliferative response and anti-HTg production, in contrast with the differential response to MTg. These data show that the DR3 transgene renders a resistant strain susceptible to EAT induction by either MTg or HTg.

DR3⁺Ab⁻ Mice Are Susceptible to EAT. To define the extent of DR3 influence in EAT susceptibility, the DR3 transgene was introduced into H2Ab⁻ mice, the class II-negative strain, by mating with B10.M-DRB1*0301 mice. The Eα⁺ transgene (Ab⁺,Eα⁺) was introduced into some mice to compete with the pairing between the DRα molecule and the endogenous Eβ⁺ molecule and to determine a role for Eα and Eβ chains. These mice do not express any H2A molecules. Initial experiments on EAT induction with MTg not only showed that EAT was induced in DRB1*0301,Ab⁻ mice, but also that expression of the Eβ⁺ molecules played no role. Table 2 presents typical data from such MTg-immunized groups. Severe thyroiditis involving up to 80% of the gland was observed in all the animals from both DR3⁺ groups regardless of Eβ⁺ expression (Fig. 2 A). The presence of the Eβ⁺ molecule in DR3⁻ mice did not result in thyroiditis (see also Table 3). The DR3 transgene in the E⁺Ab⁻ mice also responded to HTg immunization with 83% incidence of thyroid inflammation.

Figure 2. Thyroid inflammation with typical mononuclear cell infiltrates involving ~40% of the gland in MTg-immunized (A) or HTg-immunized (B) HLA-DR3 transgenic, class II-negative H2Ab⁻ mice (originally 100X).
Table 3. Induction of EAT with Mouse Tg Is Specific for HLA-DRB1*0301 (DR3) and Not HLA-DRB1*1502 (DR2) Gene in H2Ab+ Mice

<table>
<thead>
<tr>
<th>Transgene expression</th>
<th>MTg antibody (OD 1:800)</th>
<th>Number of mice with percent thyroid involvement</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>&gt;0-10</td>
</tr>
<tr>
<td>DR3+E+</td>
<td>0.74 ± 0.16</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>DR3+E-</td>
<td>&lt;0.2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>DR2+E+</td>
<td>&lt;0.2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>DR2+E-</td>
<td>&lt;0.2</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

*See Table 1 for experimental protocol.

averaging 30% of the gland (Fig. 2 B). In both MTg- and HTg-immunized mice, high anti-MTg Ab titers (detectable at 1:800 dilution) were observed only in DR3+ mice (anti-HTg titers tested separately).

DR2. Ab+ Mice Are Resistant to EAT. The role of HLA-DRB1 polymorphism in susceptibility to thyroiditis was tested with HLA-DR2 transgenic mice. The DRB1*1502. Ab+ (DR2Dw12) mice were generated by mating positive founder mice with class II-negative Ab- mice as well as Eot transgenic mice. After MTg immunization, all DR2+ mice displayed resistance to EAT, in contrast with DR3+ mice, which exhibited marked to severe thyroid inflammation (Table 3). A repeat experiment with DR2+E+ (seven mice) and DR2+E- (six mice) groups immunized with MTg also revealed no significant thyroid involvement, compared with all nine DR3+ mice with thyroiditis (data not shown). In both experiments, the anti-MTg titers in DR2+ mice were undetectable at 1:800 dilution. Upon retesting, only two mice had detectable OD values between 0.2 and 0.5 at 1:100. Furthermore, as in MTg-immunized DR3-E+ mice, DR3-E+ mice were uniformly unresponsive (data not shown).

H2Eβ and H2Eα Genes Do Not Play a Role. Because DRα is highly homologous to Eα (25), in the DR3 transgenic mice, four combinations of class II molecules could be generated: DRαDRβ, EαEβ, DRαEβ, and EαEβ. Some mice could express all four forms, while others, only the cis-pairing. In mice lacking the Eα gene, only the DRαDRβ and DRαEβ combinations are possible, with some mice expressing only the cis-pairing. Susceptibility to EAT clearly required the expression of the DRB1*0301 gene. The resistance of all other mice negated a role for the H2Eβ molecule. This is further confirmed by the resistance of DR2+E+ transgenic mice to induction with MTg.

Lymphocyte Proliferation to Tg is CD4+ T Cell-mediated and DR3-restricted. The function of DR3 molecules in vivo as classic Ag presenters during EAT induction was verified by in vitro blocking studies with mAbs to DRα and DRB1 and appropriate control mAbs. Fig. 3 shows that the proliferative responses to MTg of primed SCs were blocked by both mAbs, reducing the response to near background levels of cells plus only mAb in the absence of MTg. In Fig. 3 A, a rat mAb to mouse CD4 served as positive control for blocking MTg proliferation (28). The abrogation of T cell proliferative response by anti-DRβ in DR3+E+ mice confirms that pairing of DRαDRβ is preferred. In Fig. 3 B, Ag presentation was not blocked by anti-Dβ, a control anti-class 1 mAb. More importantly, proliferation was not affected by anti-Eβ, indicating that DRαEβ pairing was minimal and not involved in MTg-priming. Similarly, the proliferative response to HTg of SCs from HTg-immunized DR3+ mice was inhibited by mAbs to either DRα or DRB1 (data not shown).

In conclusion, our findings demonstrate an important role for the HLA-DR3 gene in susceptibility to HT. The conflicting reports on HT and DR3 association mentioned earlier are complicated by low relative risk (2.2-3.5), link-
age disequilibrium with other genes such as DQw2 and HLA-B8 (3, 5, 8), unknown modifying background genes, and different typing techniques. On the other hand, our transgenic mice express uniform background genes with specific HLA class II genes. Our evidence associating DR3 and dissociating DR2 from EAT underscores the usefulness of this DRB1 transgenic model to pinpoint the involvement of particular HLA genes in HT. The DR3 association in autoimmune thyroiditis induced by either HTg or MTg also demonstrates the importance of Tg as a thyroid antigen, possibly involved in human pathogenesis rather than just as a diagnostic tool to be replaced by thyroperoxidase, the microsomal Ag. Our data suggest that the long-time usage of Tg as a model antigen in murine EAT has real relevance. The use of a humanized model should identify potential Tg epitopes involved in human disease. As more HLA transgenic mice become available, the importance of other class II genes, such as DQ, and other thyroid Ags may be learned.

The authors gratefully acknowledge P. Zhou and S. Savarirayan for producing DR2 transgenic mice, J. Hansen and M. Smart for technical assistance, C.D. Jeffries for providing LPS, H. Waldmann for YTS 177.9, M. Duhaime and N. Maples-Vollhardt for histology sections.

This work was supported by Grant DK-45960 and a grant from St. John Hospital (Y.M. Kong); the production of transgenic mice was funded by grant AI-14764 (C.S. David). All animal care was in accordance with institutional guidelines.

Address correspondence to Yi-chi M. Kong, Ph.D., Professor of Immunology and Microbiology, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, MI 48201.

Received for publication 2 May 1996 and in revised form 13 June 1996.

References


123:15-18.


