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

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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 thyroitogenic, 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*11011 (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/2)F1 × 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 H2Aβ0 strain (16) by mating the B10.M-DRB1*0301 line with the B10.Abβ 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-H2Aβ (19); 28-14-8S, anti-H2Dβ (20); 14-4-4S, anti-H2E (21); 3F-12, anti-H2Aα (22).

DRB1*1502.Abβ (HLA-DR2, H2Aβ) transgenic mice were
Acquired Susceptibility to EAT Induction after Insertion of HLA-DRB1*0302 (DR3) Transgene into Resistant B10.M Mice

| Antigen | DR3 expression | Number of mice with percent thyroid involvement | Incidence |
|---------|----------------|---------------------------------------------|-----------|
|         |                | 0 >0-10 >10-20 >20-40 | Positive/total % |
| MTg     | +              | 1 | 4 | 4/5 | 80 |
|         |                | 2 | 2 | 2/4 | 50 |
| HTg     | +              | 4 | 2 | 5/5 | 100 |
|         |                | 3 | 1 | 0/4 | 0 |

*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.
| Antigen | Transgene expression | Tg antibody (OD 1:800) | Number of mice with percent thyroid involvement | Incidence |
|---------|----------------------|------------------------|------------------------------------------------|-----------|
|         |                      |                        | 0 >0-10 >10-20 >20-40 >40-80 |           |
| MTg     | None                  | <0.2                   | 3 - - - - | 0/3 | 0 |
|         | DR3+E+                | 0.58 ± 0.07            | - - - - 3 2 | 5/5 | 100 |
|         | DR3+E-                | <0.2                   | 2 - - - - | 0/2 | 0 |
|         | DR3+E-                | 0.73 ± 0.05            | - - - 1 - 5 | 6/6 | 100 |
| HTg     | DR3+E-                | ND                     | 1 - 2 - 2 - 1 | 5/6 | 83 |
|         | DR3+E-                | ND                     | 6 - - - - | 0/6 | 0 |

*See Table 1 for experimental protocol.

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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+ Ab0 Mice Are Susceptible to EAT.** To define the extent of DR3 influence in EAT susceptibility, the DR3 transgene was introduced into H2Ab0 mice, the class II-negative strain, by mating with B10.M-DRB1*0301 mice. The Eαk transgene (Ab0,Eαk) was introduced into some mice to compete with the pairing between the DRα molecule and the endogenous Eβ molecules 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,Ab0 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. 2A). The presence of the Eβ molecule in DR3− mice did not result in thyroiditis (see also Table 3). The DR3 transgene in the E−Ab0 mice also responded to HTg immunization with 83% incidence of thyroid inflammation.

![Figure 2.](image-url) 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 H2Ab0 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

| Transgene expression | MTg antibody (OD 1:800) | Number of mice with percent thyroid involvement | Incidence |
|----------------------|-------------------------|-----------------------------------------------|-----------|
|                      |                         | 0     | >0–10 | >10–20 | >20–40 | >40–80 | Positive/Total | %         |
| DR3⁺E⁺               | 0.74 ± 0.16             | 1     | —     | —     | —     | —     | 9/10          | 90        |
| DR3⁻E⁺               | <0.2                    | 7     | —     | —     | —     | —     | 0/7           | 0         |
| DR2⁺E⁺               | <0.2                    | 7     | —     | —     | —     | —     | 0/7           | 0         |
| DR2⁻E⁺               | <0.2                    | 13    | —     | —     | —     | —     | 0/13          | 0         |

*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, DR2⁻E⁻ mice were uniformly unresponsive (data not shown).

H2E⁺ and H2Eα Genes Do Not Play a Role. Because DRα is highly homologous to Eα (25), in the DR3 transgenic mouse, 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⁺ mice confirms that pairing of DRαDRβ is preferred. In Fig. 3 B, Ag presentation was not blocked by anti-Dβ, a control anti-class I 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-
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