Brief Definitive Report

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 thyroiditogenic, 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

Tgs 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)F_1 × 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^0 strain (16) by mating the B10.M-DRB1*0301 line with the B10.Ab^0 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-H2A^b (19); 28-14-8S, anti-H2D^b (20); 14-4-4S, anti-H2E (21); 3F-12, anti-H2A^f (22).

DRB1*1502.Ab^0 (HLA-DR2, H2Ab^0) transgenic mice were

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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, DQw6) (24), into (SWR × B10.M)F_2 embryos and identified by PCR using the primers 5′-C(CT)TAAGAGGGAGT-GTCATTTCTTC-3′ and 5′-TGTCAAGCTCTC(AC)(AC)-CAACCCC-3′ in the second DRB1 exon. The DRB1*1502 transgene was then introduced into class II–negative Ab^0 mice by mating. To get expression of the DRB1*1502 molecule, the mice were also mated with $Ab^0.E\alpha^k$ transgenic mice. The high homology between DRα and Eα chains enables the pairing of the DRB1*1502 chain with Eα (25). DR2 (Dw12) expression was determined with the DRB1-specific L227 mAb by flow cytometry.

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 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 40 μg/ml of either Tg. For blocking of Ag presentation, HLA-specific and control mAbs were added to cells at the concentrations indicated in the legend, with or without Tg. Culture medium consisted of RPMI 1640 medium with 25 mM Hepes buffer, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL, Gaithersburg, MD), 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO), and 1% normal mouse serum. After pulsing with 1.2 μCi of [^{3}H]thymidine (ICN Biomedicals, Inc., Costa Mesa, CA) for the final 18 h, the cells were harvested on glass fiber filters. [^{3}H]Thymidine uptake in triplicate cultures was determined in an LKB-Wallac liquid scintillation counter (LKB-Wallac, Turku, Finland).

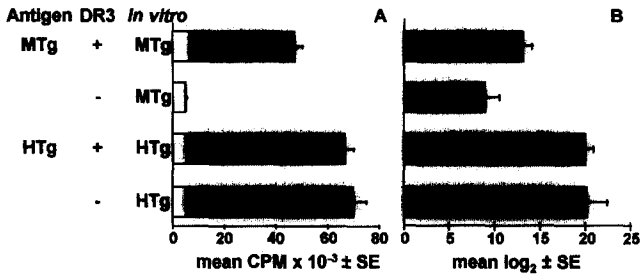

Figure 1. Comparison of T cell proliferative response and Ab titers to MTg and HTg after immunization of DR3 transgenic, EAT-resistant B10.M mice with either Tg. Note that only the T cell proliferative response to self-Ag MTg is correlated with acquired susceptibility to EAT induction as seen in Table 1, since it is not masked by response to foreign epitopes on HTg. (*A*) On day of thyroid removal, SCs from MTg- or HTg-immunized DR3$^+$ and DR3$^-$ B10.M mice were cultured (6 × 10^5 cells per well) for 5 d with (*black bar*) or without (*open bar*) 40 μg/ml MTg or HTg, and [^{3}H]thymidine uptake was measured. (*B*) Anti-MTg or anti-HTg titers were assayed by passive hemagglutination of MTg- or HTg-coupled RBCs and expressed as reciprocal $\log_2$ titers. Significantly higher anti-MTg titers were detected in DR3$^+$ B10.M mice.

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*0302 (DR3) Transgene into Resistant B10.M Mice*

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

*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^0 Mice*

Antigen	Transgene expression	Tg antibody (OD 1:800)	Thyroiditis*						
			Number of mice with percent thyroid involvement					Incidence	
			0	>0–10	>10–20	>20–40	>40–80	Positive/Total	%
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
	DR3$^-$E$^-$	<0.2	5	—	—	—	—	0/5	0
HTg	DR3$^+$E$^-$	ND	1	—	2	2	1	5/6	83
	DR3$^-$E$^-$	ND	6	—	—	—	—	0/6	0

*See Table 1 for experimental protocol.
‡Class II–negative H2Ab^0 control mice.

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^0 Mice Are Susceptible to EAT. To define the extent of DR3 influence in EAT susceptibility, the DR3 transgene was introduced into H2Ab^0 mice, the class II–negative strain, by mating with B10.M-DRB1*0301 mice. The Eα^k transgene (Ab^0.Eα^k) was introduced into some mice to compete with the pairing between the DRα molecule and the endogenous Eβ^b 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^0 mice, but also that expression of the Eβ^b 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^b expression (Fig. 2 *A*). The presence of the Eβ^b molecule in DR3$^-$ mice did not result in thyroiditis (see also Table 3). The DR3 transgene in the E$^-$Ab^0 mice also responded to HTg immunization with 83% incidence of thyroid inflammation

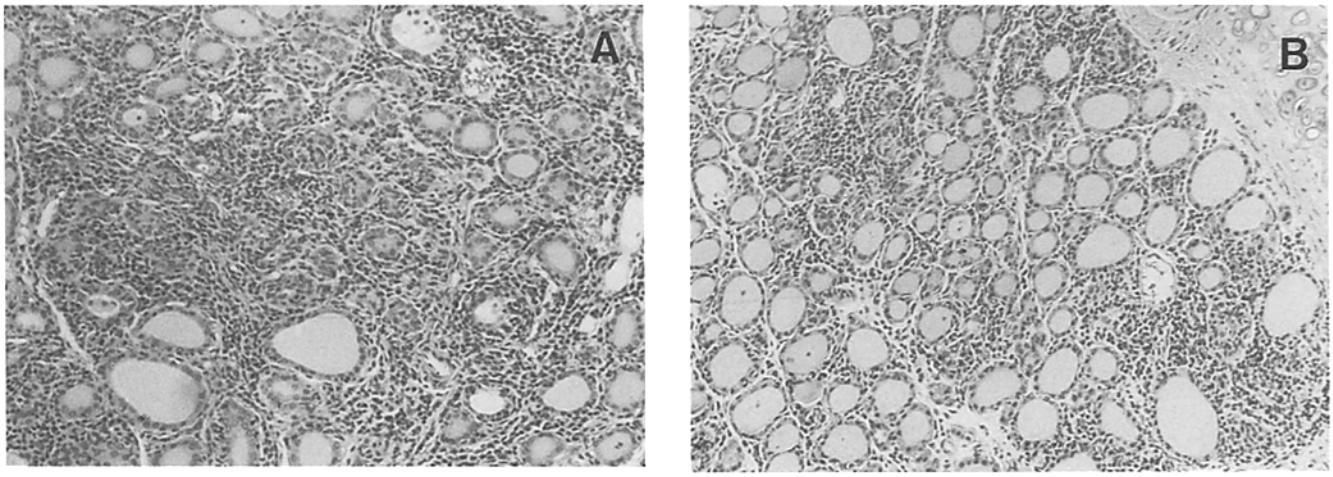

Figure 2. Thyroid inflammation with typical mononuclear cell infiltrates involving ~40% of the gland in MTg-immunized (*A*) or HTg-immunized (*B*) HLA-DR3 transgenic, class II–negative H2Ab^0 mice (originally 100×).

Table 3. *Induction of EAT with Mouse Tg Is Specific for HLA-DRB1*0301 (DR3) and Not HLA-DRB1*1502 (DR2) Gene in H2Ab⁰ Mice*

Transgene expression	MTg antibody (OD 1:800)	Thyroiditis*						
		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	—	4	4	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.Ab⁰ 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.Ab⁰ (DR2Dw12) mice were generated by mating positive founder mice with class II–negative Ab⁰ mice as well as $E\alpha^k$ 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).

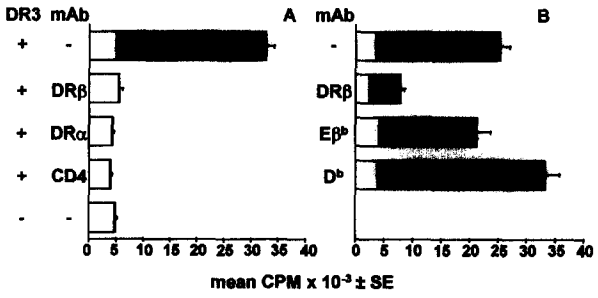

Figure 3. MTg presentation to SC from MTg-immunized $DR3^+$ Ab⁰ mice was specifically blocked by mAb to DRβ (L227; supernatant 1:10) (18), as illustrated in *A* and *B*. (*A*) SCs from $DR3^+E^+$ mice, blocking by mAb to DRα (L243; 50 μg/ml) and to mouse CD4 (YTS 177.9; 25 μg/ml). (*B*) SCs from $DR3^+E^-$ mice, no blocking by mAb to D^b (28-14-8S; supernatant 1:40) or $E\beta^b$ (Y17; supernatant 1:10) (21). [³H]Thymidine uptake was measured after 4 d in culture in the presence of mAb only (background cpm, *open bar*) or 40 μg/ml MTg plus mAb (*black bar*). Control proliferative assay to MTg in $DR3^+$ and $DR3^-$ Ab⁰ mice was performed with (*black bar*) or without (*open bar*) MTg.

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 EαDRβ. 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-D^b, a control anti–class I mAb. More importantly, proliferation was not affected by anti-$E\beta^b$, 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 autoantigen, 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.

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