Protective Role of Major Histocompatibility Complex Class II Eb^d Transgene on Collagen-induced Arthritis

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Summary

Collagen-induced arthritis (CIA) is an animal model of autoimmune inflammatory polyarthritis that has features similar to rheumatoid arthritis (RA). Much like RA, susceptibility to mouse CIA is influenced by the major histocompatibility complex (MHC), H-2, and restricted to the H-2^q and H-2^r haplotypes. Whereas the role of the H-2A molecule in susceptibility to CIA is well established, little is known about the role of H-2E molecule in the disease. In this study, we analyzed the effect of a transgenic Eβ^d molecule on CIA susceptibility in a recombinant mouse B10.RQB3, which expresses the CIA susceptible A^q genes and an Ea^k gene, but does not produce an E molecule since Eb^q is nonfunctional. In the presence of an Eb^d transgene, a viable E molecule is generated. Whereas B10.RQB3 were susceptible to CIA, B10.RQB3-Eβ^{d+} showed a dramatic reduction in the incidence of arthritis as well as a decrease in the level of anti–mouse and anti–bovine CII antibodies in their serum. No clear cut differences in the expression of T cell receptor (TCR) Vβ was observed between Eβ^{d+} and Eβ^{d-} transgenic mice. Mechanisms underlying the protective effect of Eβ^d transgenic molecule on CIA may shed light on how HLA-DR molecules influence human RA.

Type II collagen (CII) is a sequestered molecule found in joint cartilage (1). Rodents can be induced to generate an autoimmune inflammatory polyarthritis (CIA) by injection of heterologous CII (2). Susceptibility to arthritis in mice is restricted to the MHC haplotypes H-2^q and H-2^r (3). Using recombinant strains, susceptibility in the H-2^q haplotype has been narrowed down to the class II molecule encoded by H-2A loci (4).

Four genes encode the mouse class II molecules; Aa and Ab genes encode for the heavy (H) and light (L) chains of the A molecule respectively whereas Ea and Eb encode for the H and L chains of the E molecule (5). The A molecule is expressed in all mouse haplotypes studied, whereas the E molecule is not (6). Four haplotypes of inbred mouse strains, b, s, q, and f, as well as 19 of 33 mouse strains carrying wild-type MHC haplotypes are E negative. Mice of the f and q haplotypes fail to synthesize both Eα and Eβ chains. Mice of the b and s haplotypes fail to produce Eα chains but do express Eβ molecules which remain in the cytoplasm as partially glycosilated precursors (7).

While the critical role of A molecule in the susceptibility to CIA has been stressed (8), little is known about the role

of E molecule in the disease. The human MHC genes (HLA) are also known to play a critical role in the susceptibility to rheumatoid arthritis (RA) (9). The human class II molecule HLA-DR, which is homologous to mouse H-2E, is clearly important for the predisposition to RA (9). Previous studies have demonstrated a relationship between severity of RA and increasing DR4 or DR1 frequency (10, 11). Recent studies have further located the structural element conferring disease susceptibility to the third hypervariable region of the DRβ₁ chain (12). To determine the role of E molecule in CIA, we analyzed CIA susceptibility in transgenic B10.RQB3-Eβ^d mice which expresses the CIA susceptible A^q genes as well as a viable E molecule. Our results support a protective role of the Eβ^d molecule in CIA susceptibility.

Materials and Methods

gene with 4.1 kb of 5'-flanking regulatory region (13) was microinjected into (SWR × B10.M)F_1 embryos as described (14). Transgene positive mice were identified by Southern blot analysis. The Eb^d gene was introduced into the B10.RQB3 mice by backcrossing. Expression of the $E\beta^d$ molecule was analyzed by flow cytometry as described below (15). The transgenic mice used in these studies were from the N9-N10 backcross population.

Flow Cytometry. Analysis of $E\beta^d$ and $V\beta$ TCR expression on PBL were determined by flow cytometry as previously described (15). Briefly, PBL and were isolated by Ficoll separation, washed, and then incubated with the $E\beta^d$-specific mAb 34-1-4S (16) for 30 min at 4°C. After washing, the cells were incubated with FITC goat anti-mouse IgG (Accurate Chemicals and Scientific Corp., Westbury, NY) for 30 min at 4°C, washed, and then fixed with 1% paraformaldehyde before analysis. Analysis for $V\beta$ TCR was done using the following mAbs: B20.6, anti-$V\beta2$; KT4-10, anti-$V\beta4$; MR9-4, anti-$V\beta5.1$-5.2; 44-22-1. anti-$V\beta6$; TR-310, anti-$V\beta7$; F23.2, anti-$V\beta8.2$; MR10-2, anti-$V\beta9$; KT11, anti-$V\beta11$; and 14.2, anti-$V\beta14$. Second antibodies consisted of FITC-conjugated mouse anti–rat IgG or IgM or goat anti–mouse IgG. T cell subsets were identified by incubation with mAbs specific for mouse CD4 and CD8a conjugated to R-Phycoerythrin and Red 613, respectively (GIBCO BRL, Gaithersburg, MD). Single-color (for $E\beta^d$) and three-color (for $V\beta$ TCR) flow cytometric analysis was performed using a FACS®IV flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Induction and Quantification of Arthritis. Bovine CII (BII), prepared as previously described (3), was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml then emulsified 1:1 with complete Freund's adjuvant H37 Ra (Difco Laboratories, Detroit, MI). Mice received 100 μg of cold emulsion intradermally in the base of the tail. Mice were monitored three times a week from week 3 to 9 post immunization for the onset and development of CIA. The arthritic severity of all four limbs was determined as previously described (3) using a grading system for each paw based on a scale of 0–3 as follows: 0 = normal; 1 = redness and swelling in paws or toes; 2 = deformity in paw; and 3 = ankylosis in the affected joint. The clinical score from each limb was summed thus giving a severity range of 0–12 per mouse. The mean arthritic severity was determined using arthritic animals only.

Measurement of Serum Anti-CII Antibody. Mice were bled via the tail artery on day 35 post immunization and the level of anti–mouse CII (MII) and anti-BII Abs in serum were determined using a standard ELISA technique (17). Sera was tested at 1/100 and 1/400 dilutions and compared with a pooled positive control sera. The data is expressed as mean OD ± SD.

Statistical Analysis. Arthritis incidence between groups was analyzed using chi-square test with Yates' correction. Antibody levels were compared using the Student's *t* test.

Results

Unlike mice carrying the standard H-2^q haplotype (B10.Q, DBA/1), B10.RQB3 recombinant mice synthesize the $E\alpha^k$ molecule. The introduction of a 15.7-kb DNA fragment containing the entire Eb gene from the H-2^d haplotype was sufficient to induce the expression of H-2E molecules in these mice. Flow cytometric analysis showed that the level of surface expression of $E\beta^d$ molecule in the 15.7 transgene positive ($E\beta^{d+}$) mice was comparable to the expression of the same molecule in mice of the H-2^d haplotype (Fig. 1). Expression of $E\beta^d E\alpha^d$ in positive control animals (B10.D2) was 54% whereas the expression of $E\beta^d E\alpha^k$ in 15.7 $E\beta^{d+}$ B10.RQB3 mice was 52%. Negligible staining (<1%) was detected in $E\beta^d$ transgene negative ($E\beta^{d-}$) littermates and B10 control (H-2^b) mice. Tissue distribution of the Eb transgene was comparable to the endogenous molecule in E positive strains of mice (data not shown).

To investigate the effect of H-2E molecule on the susceptibility to CIA, B10.RQB3 15.7 $E\beta^{d+}$ and $E\beta^{d-}$ littermates were immunized with bovine CII in CFA. At 9 wk postimmunization, the incidence of arthritis in $E\beta^{d+}$ mice was significantly lower than in $E\beta^{d-}$ littermates ($p < 0.01$, Table 1). Among the three $E\beta^d 15.7^+$ animals that developed CIA, the extent of arthritis was mild as indicated by arthritic scores from 1 to 2. In contrast, a majority of arthritic $E\beta^{d-}$ littermates displayed severe swelling in at least two limbs, and their arthritic scores ranged from 1 to 8.

The inhibitory effect of the 15.7 Eb^d transgene was also apparent in the development of antibody against CII. Analysis of sera at a 1/100 dilution showed a 32% reduction in mean OD of BII-reactive Ab mice and a 45% decrease in reactivity against homologous MII Ab in $E\beta^{d+}$ vs. $E\beta^{d-}$ littermates ($p < 0.001$, Table 1).

Through flow cytometry, deletion in $V\beta5.1$, 5.2, and 11 bearing T cell populations was found in both CD4 and CD8 subsets for $E\beta^{d+}$ and $E\beta^{d-}$ mice (Table 2). No significant difference in the $V\beta$ TCR expression was evident between the two groups with the exception of CD4$^+$, $V\beta11^+$ T cells, which were further decreased in $E\beta^{d+}$ mice. The deletion of $V\beta5$ and $V\beta11$ T cells was expected in Eb^d transgene negative B10.RQB3 mice since they express an interisotypic $A\beta/E\alpha$ molecule known to delete some TCR $V\beta$-bearing T cell populations (18).

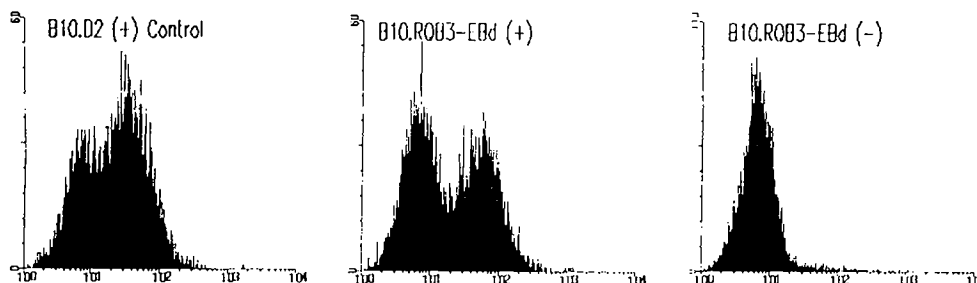To confirm our observations, we used a second line of

Figure 1. Analysis of $E\beta^d$ expression using the 34-1-4S mAb in B10.RQB3 (Eb^d transgene + and -) mice.

Table 1. *Resistance to CIA in B10.RQB3-E_β^d 15.7 Transgenic Mice**

| Mice | CIA[‡] | | Anti-CII Ab[§] | |
	Incidence	Arthritis severity	BII	MII
		mean ± SD		
E_β^d Tg Positive	3/13	1.3 ± 0.6	0.35 ± 0.05	0.12 ± 0.1
	p <0.001		p <0.001	p <0.001
E_β^d Tg Negative	16/20	3.8 ± 2.0	0.51 ± 0.06	0.22 ± 0.08

* Mice were immunized with 100 μg BII in CFA on day 0 and monitored regularly for the onset and development of CIA.
‡ Determined at 9 wk post immunization. The mean severity of arthritis was calculated using arthritic animals only.
§ Animals were bled at 5 wk post immunization and the level of antibody against BII and MII determined by an ELISA. Data is presented as the mean OD at a 1:100 dilution.

Table 2. *Peripheral Expression of Vβ TCR in Transgenic B10.RQB3-E_β^d Positive and B10.RQB3-E_β^d Negative Mice**

| | Percentage of positive T cells | | | |
| | E_β^d positive | | E_β^d negative | |
Vβ	CD4	CD8	CD4	CD8
		mean ± SD		
Vβ2	5.2 ± 1.0	4.9 ± 1.9	5.1 ± 1.1	3.9 ± 2.4
Vβ4	6.5 ± 0.3	5.5 ± 0.4	7.3 ± 0.3	5.8 ± 0.2
Vβ5.1.2	0.4 ± 0.1	5.1 ± 0.3	1.0 ± 0.5	7.4 ± 0.5
Vβ6	7.8 ± 1.9	12.6 ± 1.3	4.6 ± 0.9	13.1 ± 1.3
Vβ7	2.2 ± 0.3	5.9 ± 0.1	3.6 ± 1.1	9.4 ± 3.8
Vβ8.2	14.5 ± 0.6	7.4 ± 0.8	16.4 ± 0.5	7.1 ± 0.6
Vβ9	1.9 ± 0.1	5.2 ± 1.8	1.7 ± 0.1	6.3 ± 1.6
Vβ11	0.6 ± 0.3	1.1 ± 0.8	1.9 ± 1.2	1.9 ± 1.5
Vβ14	7.3 ± 0.5	2.9 ± 0.5	6.9 ± 0.4	2.4 ± 0.2

* Normal transgenic B10.RQB3-E_β^d+ and B10.RQB3-E_β^d- mice were bled and their PBL stained for Vβ TCR expression as detailed in Materials and Methods. The mean percentage was calculated based on four animals per group.

$E\beta^d$ transgenic mice, $E\beta^d$ 10.2, generated in our laboratory. This second Eb^d gene construct contains only 200 bp of 5'-flanking region. The well defined cis-regulatory elements controlling class II gene expression are containing in this flanking region (6, 19). The presence of this 200 bp upstream sequence is sufficient to control expression of the transgene similar to the Eb^d 15.7 transgene and endogenous class II gene (Glimcher, L.H., unpublished data). Flow cytometric analysis also showed that expression of $E\beta^d E\alpha^k$ in B10.RQB3-$E\beta^d$ 10.2+ was comparable to that observed in B10.RQB3-$E\beta^d$ 15.7+ mice (data not shown).

Similar to B10.RQB3-$E\beta^d$ 15.7+ mice, B10.RQB3-$E\beta^d$ 10.2+ mice showed a significant reduction in the incidence and severity of arthritis vs. $E\beta^{d-}$ littermates after immunization with bovine CII (Table 3). Also, among the four $E\beta^d$ 10.2+ mice that developed CIA, the severity of arthritis was mild compared to $E\beta^d$ 10.2- littermates. Finally, like in $E\beta^d$ 15.7 mice, analysis of sera at a 1/100 dilution in $E\beta^d$ 10.2+ animals showed a 37% reduction in mean OD of BII-reactive Ab and a 42% decrease in reactivity against homologous MII Ab vs. $E\beta^{d-}$ littermates.

To examine the suppressive effect of $E\beta^d$ molecules on arthritis susceptibility in heterozygous mice, the development of CIA in (B10.RQB3 × B10.D2)F1 animals was examined. Flow cytometry showed that the level of surface expression

Table 3. *Protection against CIA in B10.RQB3-E_β^d 10.2 Transgenic Mice**

| Mice | CIA[‡] | | Anti-CII Ab[§] | |
	Incidence	Arthritis severity	BII	MII
		mean ± SD		
E_β^d Tg positive	4/14	1.7 ± 1.0	0.22 ± 0.03	0.24 ± 0.1
	p <0.03		p <0.01	p <0.01
E_β^d Tg negative	7/11	3.3 ± 1.7	0.35 ± 0.08	0.41 ± 0.10

* Mice were immunized with 100 μg BII in CFA on day 0 and monitored regularly for the onset and development of CIA.
‡ Determined at 9 wk post immunization. The mean severity of arthritis was calculated using arthritic animals only.
§ Animals were bled at 5 wk post immunization and the level of antibody against BII and MII determined by an ELISA. Data is presented as the mean optical density at a 1:100 dilution.

Table 4. *Effect of Eβ Molecule on CIA in (B10.RQB3 × B10.D2)F1 Mice**

Mice	CIA[‡]	
	Incidence	Arthritis severity
		mean ± SD
(B10.RQB3 × B10.D2)F1	6/15[§]	2.7 ± 1.5
B10.RQB3-Eβ Tg positive	3/12[‖]	2.0 ± 1.0
B10.RQB3-Eβ Tg negative	6/8[§‖]	3.6 ± 1.0

* Mice were immunized with 100 μg BII in CFA on day 0 and monitored regularly for the onset and development of CIA.
‡ Determined at 9 wk post immunization. The mean severity of arthritis was calculated using arthritis animals only.
§, $p < 0.05$
‖, $p < 0.01$

of $E\beta^d$ molecule in (B10.RQB3 × B10.D2)F1 mice was similar to B10.RQB3-$E\beta^{d+}$ and B10.D2 mice (data not shown). The incidence of CIA was compared between (B10.RQB3 × B10.D2)F1 mice and B10.RQB3-$E\beta^{d+}$ and $E\beta^{d-}$ transgenic mice. Like B10.RQB3-$E\beta^{d+}$ animals, (B10.RQB3 × B10.D2)F1 mice showed a significant reduction in the incidence of arthritis versus B10.RQB3-$E\beta^{d-}$ mice (Table 4). However, in (B10.RQB3 × B10.D2)F1 mice, the decrease of CIA appeared intermediate compared with B10.RQB3-$E\beta^{d+}$ animals (40% vs. 25%). This observation shows an inhibitory role of $E\beta^d$ molecule on CIA coded by an endogenous gene as well as a transgene.

Discussion

Here we report for the first time the generation of mice presenting the unique feature of being H-2A^q positive and expressing an H-2E molecule at the same time. As expected, B10.RQB3 mice are CIA susceptible because they express I-A^q (18). In this strain, E molecules are not expressed since Eb^q is nonfunctional (7, 8). By introducing an Eb^d transgene, we induced expression of E molecules in B10.RQB3 mice, resulting in a dramatic reduction in the incidence of CIA as well as the level of anti-BII and anti-MII Ab in the serum of $E\beta^{d+}$ transgenic mice. Analysis of Vβ TCR expression on PBL demonstrated the deletion of Vβ5.1, Vβ5.2, and Vβ11 bearing T cells in both $E\beta^{d+}$ and $E\beta^{d-}$ B10.RQB3 mice. Although the presence of the $E\beta^d$ molecule justifies these results in E positive mice (20, 21), it has been demonstrated that the Eα/Aβ heterodimer expressed in B10.RQB3 ($E\beta^-$) mice plays a similar role to Eα/Eβ dimers in the deletion of several Vβ bearing T cell populations (18).

Previous studies in other mouse models of autoimmune diseases have demonstrated that the expression of a functional E molecule can play a protective role (22). By introducing an Ea transgene into nonobese diabetic (NOD) mice, the restored expression of E molecules led to protection against insulin-dependent diabetes mellitus (23–25). Also, expression

of an Ea^d transgene led to a dramatic reduction in the development of autoimmune glomerulonephritis in BXSB mice (26). There are several distinct differences between our studies and the earlier work in NOD and BXSB mice. First, in the aforementioned studies, expression of E molecules was restored by introduction of a transgene derived for the nonpolymorphic Ea gene. In our studies in CIA, we restored expression of E molecules using a polymorphic Eb gene. Second, Merino et al. (26) reported that resistance to glomerulonephritis in Eα^d transgenic BXSB mice was likely due to an exceedingly high copy number (~50) of Ea^d transgene. Southern blot analysis of our two lines of B10.RQB3-$E\beta^d$ transgenic mice, 15.7 and 10.2, revealed that the copy number of Eb^d transgene was ~4–8 and 5–10, respectively. Finally, expression of E molecules in heterozygous H-2^{b/d} BXSB mice did not protect these animals from the development of autoimmune disease (27). Likewise, Podolin et al. (28) showed that progeny of an (NOD × NOD.H-2^k)F1 cross were not protected from the development of diabetes. In our studies, we observed that (B10.RQB3 × B10.D2)F1 mice showed a significant reduction in the incidence of CIA vs. B10.RQB3 animals (Table 4). Therefore, through both transgenic and conventional genetic techniques, it appears that introduction of an Eb^d gene can alter susceptibility to CIA.

Several nonmutually exclusive mechanisms may account for the protective effect of $E\beta^d$ in CIA. H-2$E\beta^d$ expression could modulate the T cell repertoire by presenting a "tolerogenic" peptide which leads to the deletion or regulation of arthritogenic T cell clones. It is also possible that a Th1 to Th2 switch occurs in the CII response in the context of the $E\beta^d$ molecule. It has been shown that the administration of the Th2-derived cytokine IL-4 could facilitate remission of CIA in DBA/1 (H-2^q) mice (29). Finally, it is possible that H-2A^q mediates the presentation of Eβ peptides derived from the $E\beta^d$ molecule itself which, in turn, modulates the arthritogenic response. Although studies directed towards characterizing naturally processed peptides associated with class II molecules have shown that a portion of peptides bound to H-2A molecules are derived from Eα chains (30), the contribution of Eβ-derived peptides is not clear. Likewise, the scope of self-peptides associated with H-2A^q molecules, which is of interest in our system, remains to be elucidated.

Of importance, B10.RIII (H-2^r) mice are susceptible to CIA despite expression of functional E molecules (31). It is possible that, unlike $E\beta^d$, the $E\beta^r$ molecule does not lead to protection in CIA. Previously, we found that (B10.Q × B10.K)F1 mice are CIA susceptible, suggesting that $E\beta^k$ does not play a protective role. Thus, polymorphism of the Eb gene, in the context of particular haplotypes, may play a role in determining self tolerance or autoimmunity. Indeed, preliminary studies suggest that the protective effect of the $E\beta^d$ molecule in CIA is q haplotype specific; no difference was observed in arthritis susceptibility between transgene positive and negative offspring of a (B10.RQB3-$E\beta^d$ × B10.RIII)F1 cross (our unpublished observations). In B10.RIII mice, H-2E^r would be functionally equivalent to DR1 and DR4 subtypes associated in human with RA. Presentation of DRβ

peptide (Eβ peptide) by DQ molecule (H-2A equivalent) may explain observed differences in the role of DR molecules in RA. Variants of the HLA-DR4 family and HLA-DR1 subtypes may be deficient in mediating protection, analogous to H-2E^r in r mice.

In closing, our data is the first demonstration of a protective role of Eβ molecules in murine CIA. Experiments are currently underway to further delineate the role of H-2Eβ molecules in CIA and understand the mechanisms involved in the protection. These studies will shed light on the possible role of DR molecules in human RA.

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