Co-Expression of HLA DR3 and DQ8 Results in the Development of Spontaneous Insulitis and Loss of Tolerance to GAD65 in Transgenic Mice

Roshini S. Abraham, Yogish C. Kudva, S. Brian Wilson, Jack L. Strominger, and Chella S. David

Specific HLA DQ and DR alleles have been associated with susceptibility to type 1 diabetes. HLA DQ8 and DQ2 have been shown to strongly predispose to disease and to be in linkage disequilibrium with at-risk DR4 and DR3 alleles, respectively. Inheritance of a mixed DR3/DR4 haplotype confers the greatest risk. A double transgenic mouse expressing both DR3 and DQ8 was generated to investigate potential major histocompatibility complex class II interactions. The DR3/DQ8 transgenic mice developed a spontaneous loss of tolerance to GAD65, in which the T-cell response to GAD65 was restricted by HLA DR. Although the mice also showed spontaneous insulitis, they did not progress to overt diabetes. Mice expressing either transgene (DQ8 or DR3) alone showed mild infiltration of their islets, which disappeared when DQ8 or DR3 was co-expressed with a resistant DR2 allele or the neutral DQ6 allele. Therefore, in a fashion analogous to human diabetes, the murine model demonstrated a requirement for a combination of at-risk DR and DQ allotypes for the initiation of spontaneous autoimmunity. *Diabetes* 49:548–554, 2000

Major histocompatibility complex (MHC) genes have been shown to confer the greatest risk for the development of type 1 diabetes. The HLA DR3 and DR4 haplotypes have been shown to be associated with susceptibility, whereas the HLA DR2 haplotype is protective (1,2). Epidemiological studies have shown that HLA DQ8 (DQB1*0302) and DQ2 (DQB1*0201), which occur in linkage with HLA DR4 (DRB1*0401,0402) and DR3 (DRB1*0301), respectively, have the highest relative risk for diabetes (3–5).

The role of HLA DQ as a susceptible (6,7) or protective (8) locus has been a controversial issue. The HLA DQβ gene has been implicated in determining susceptibility or resistance to type 1 diabetes (9,10). The presence of a nonaspartic acid (ASP) residue at position 57 on the DQβ-chain has been positively associated with disease (9). Heterodimers having non-ASP residue at 57 on DQβ and an arginine at position 52 on the DQα-chain are thought to confer the greatest susceptibility (5,7,11). The risk conferred by DQ8 can be modulated by the DR4 allele to which it is linked, with DRB1*0401, 0402, and 0405 conferring little or no protection and with DRB1*0404, 0403, and 0406 providing an increasing degree of protection (12).

Whether HLA DQ or DR plays a primary role in disease association is debatable. Several studies have suggested that DQ plays a more important role and that DR plays a secondary role (13–15). The risk for diabetes is greatest when DR3/DQ2 and DR4/DQ8 haplotypes occur in a heterozygous combination (11,16–19).

There is also evidence to suggest that HLA DQ primarily confers resistance and that HLA DR confers susceptibility to type 1 diabetes (4,8). For example, the expression of the DQA1*0102-DQB1*0602 (DQ6) molecule, which occurs in linkage disequilibrium with DR2, strongly protects against the development of diabetes (1). Taken together, these data suggest that, although DQ alleles play a significant role in determining susceptibility (6,20), the extent of this effect is strongly modulated by the expression of other DR and DQ alleles (3,21).

Given this body of evidence, we used transgenic mice expressing both HLA DQ and DR in the absence of cell surface endogenous class II dimers (Aβo/DR3/DQ8, Aβo/DR2/DQ8, and Aβo/DR3/DQ6) to develop a model that would allow us to define the interactions between HLA DQ and DR molecules in type 1 diabetes.

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. The generation of Aβo/DQ8 mice has been previously described (22–24). The Aβo/DR3 mice were generated from B10M.DR3 (25). Table 1 shows the generation of Aβo/DR3/DQ8 transgenic mice. The HLA DR2 (DRB1*1502) (26) transgenic mice were crossed with the Aβo/DQ8 mice to obtain Aβo/DR2/DQ8 mice. Similarly, Aβo/DQ6 (DQB1*0601) mice (27) were crossed with Aβo/DR3 mice to generate Aβo/DR3/DQ6. The HLA DQ and DR expression was analyzed by flow cytometry and polymerase chain reaction (PCR).

Recombinant human GAD65. Recombinant human (rh) GAD was prepared as previously described (28).

Peptides. GAD peptides were synthesized as described earlier (28).

Fluorescence-activated cell sorter and PCR analysis of transgenic mice. Analysis of HLA expression and absence of endogenous MHC class II by flow cytometry (23) and PCR (29,30) has been described (28). Fluorescein-labeled NK1.1 and anti-CD4 (GK1.5) (Pharmingen, San Diego, CA) were used for the T-cell population analyses.

T-cell proliferation assay. Lymphocytic proliferation assays were performed according to a previously described protocol (28).

From the Department of Immunology (R.S.A., C.S.D.) and the Division of Endocrinology (Y.C.K.), Mayo Clinic, Rochester, Minnesota; the Department of Cancer Immunology and AIDS (S.B.W.), Dana Farber Cancer Institute, Boston; and the Department of Molecular and Cellular Immunology (J.L.S.), Harvard University, Cambridge, Massachusetts.

Address correspondence and reprint requests to Chella S. David, PhD, Department of Immunology, Mayo Clinic, 200 First St. S.W., Rochester, MN 55905. E-mail: david.chella@mayo.edu.

Received for publication 21 May 1999 and accepted in revised form 29 December 1999.

ASP, aspartic acid; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; PCR, polymerase chain reaction; rh, recombinant human.

TABLE 1
Genetic background of the three main strains of the HLA transgenic study mice

Strain	Parental haplotype	Generation	B10 Background (%)
Aβo	B6 × 129/J	F1	0
Aβo/DR3	Aβo × DR3αβ (B10.M/N4)	N1	47
Aβo/DQ8	Aβo × DQ8αβ (B10.M/N5)	N1	48
Aβo/DR3/DQ8	Aβo/DR3 × Aβo/DQ8	N1	47

Antibody estimation by enzyme-linked immunosorbent assay. The double- and single transgenic mice were immunized with GAD65 (50 µg/mouse) subcutaneously at the base of the tail and in the hind foot-pads. They were bled 2 weeks after immunization, and serum was collected. Sera from nonimmunized NOD mice were also collected to serve as a control. Each group consisted of 4–5 mice, and 96-well flat bottom nonsterile polyvinyl chloride microtiter plates were coated with 10 µg/ml of GAD65 in carbonate-bicarbonate coating buffer (pH 9.6) overnight at 37°C. Enzyme-linked immunosorbent assay (ELISA) was performed on the sera samples by a standard protocol. The optical density was read at the wavelength of 450 nm in an ELISA reader. The antibody levels were quantitated using a standard curve of mouse immunoglobulin.

Cytokine analysis. Culture supernatants from in vitro stimulations of naive or immunized Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice with GAD65 were collected after 24 h of culture. These supernatants were tested in a cytokine ELISA for interferon (IFN)-γ and interleukin (IL)-4 (31).

Histopathology and estimation of insulitis. Naive transgenic mice were killed, and the pancreases were collected in 4% neutral buffered formalin solution (Sigma, St. Louis, MO). For each group, 5–12 mice were collected. Paraffin-embedded blocks were made, 4-µm–thick sections were cut at 300-µm intervals transversely throughout the entire length of the tissue, and the intervening tissue was discarded. The sections were stained with hemotoxylin and eosin and mounted on slides. The hemotoxylin- and eosin-stained sections were evaluated for insulitis in a blinded manner. The insulitis score used for grading the islets was as follows: 0 = normal islet, 1 = peri-insulitis, 2 = <50% insulitis, and 3 = >50% insulitis. The insulitis score was estimated by dividing the total score by the number of islets (32).

Blood glucose levels were estimated by placing a drop of venous blood onto glucose detection strips, and the reading was obtained by use of an Elite glucometer (Bayer Diagnostics; Bayer, Elkhart, IN).

Microsatellite analysis. To perform microsatellite analysis, three markers were chosen for aids 1 and 2 on chromosome 7 (D7Mit232, D7Mit245, and D7Mit308), and one was chosen for chromosome 1 (D1Mit22) (33). The haplotype present at these four loci was assessed in 19 Aβo/DR3/DQ8, 5 Aβo/DR3, and 5 Aβo/DQ8 transgenic mice. C57/BL10 and C57/BL6 mice were used as controls.

RESULTS

Genetic background of transgenic mice. The genetic background, in particular non-MHC genes, has been shown to significantly influence the nature of the immune response and the course of autoimmune diseases (28). Table 1 shows the parental haplotype of each of the three primary transgenic mouse strains used in this study.

Spontaneous loss of tolerance to GAD65. The presence of two diabetes-predisposing haplotypes in the Aβo/DR3/DQ8 mice indicated that there might be a loss of peripheral tolerance to islet autoantigens. GAD65, which was identified previously as an early dominant autoantigen, was chosen as the candidate antigen for testing this hypothesis. Single transgenic Aβo/DR3, Aβo/DQ8, and Aβo/DR2/DQ8 mice (1) were used as controls. Lymph node cells from naive animals were stimulated in vitro with GAD65 or 19 individual GAD peptides (Fig. 1). Only the Aβo/DR3/DQ8 mice responded to GAD65 (Fig. 1). A consistent T-cell response was also seen in the naive

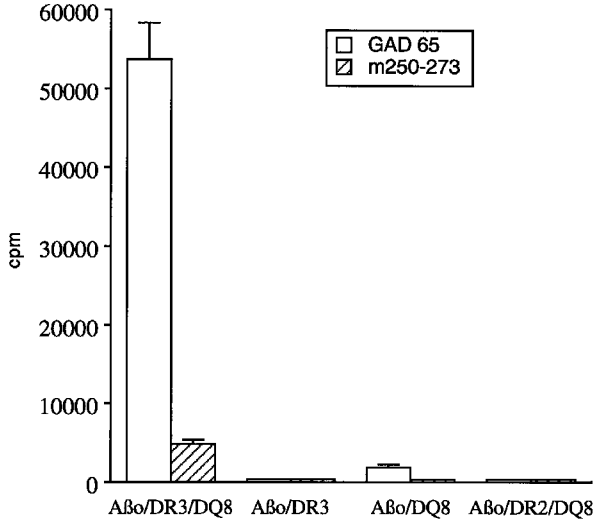

FIG. 1. Lymphoproliferative responses in naive Aβo/DR3/DQ8, Aβo/DR2/DQ8, Aβo/DR3, and Aβo/DQ8 transgenic mice. Lymph nodes (popliteal, inguinal, and para-aortic) were pooled from three mice per group between 8 and 14 weeks of age. Half-log titrating amounts of GAD and peptides were used. Data are shown at the 30 µg/ml concentration for GAD and the 100 µg/ml concentration for the peptides. Only Aβo/DR3/DQ8 mice showed spontaneous proliferation in response to GAD and one peptide, m250-273. Experiments were repeated at least five times, and one representative experiment is shown. Background counts were between 200 and 500 cpm.

double transgenic mice to one peptide, m250-273, but not to the other peptides tested. No proliferative responses to either GAD65 or its peptides were detected in the Aβo/DR3 and Aβo/DQ8 single transgenic and the Aβo/DR2/DQ8 double transgenic mice. Thus, the spontaneous loss of tolerance to GAD65 was restricted to the Aβo/DR3/DQ8 mice.

In vitro antibody-blocking experiments (Fig. 2) that used anti-CD4, anti-DQ, and anti-DR antibodies were performed to characterize the autoreactive GAD65 response. Surprisingly, the GAD response was blocked in naive Aβo/DR3/DQ8 only by the anti-DR antibody (50%) and not by the anti-DQ antibody (0%). The anti-CD4 antibody blocked ~90% of the GAD response (Fig. 2*A*). These data suggest that the DQ8 allotype is permissive for DR3-restricted autoreactivity. Importantly, the anti-DQ antibody blocked GAD65 responses in vitro in GAD-immunized Aβo/DQ8 (Fig. 2*B*).

The amount of MHC class II that was present would be a determining factor in the development of loss of tolerance. In this context, levels of tissue-specific surface expression of DR and DQ were analyzed by flow cytometry in thymi, spleens, and peripheral blood lymphocytes (PBLs) from the various transgenic mice. The levels of DR and DQ were comparable with each other in both the spleen and the PBLs in the Aβo/DR3/DQ8 mice (Fig. 3). The Aβo/DR3 and Aβo/DQ8 mice had levels of DR and DQ expression that were comparable to Aβo/DR3/DQ8 mice (data not shown). Because the anti-GAD65 response was CD4 T-cell–restricted, CD4 populations were compared among the Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice in spleens (Table 2). These transgenic mice had similar numbers of CD4 T-cells (Table 2), which suggests that the spontaneous autoreactivity in the Aβo/DR3/DQ8 mice was not a result of elevated levels of CD4 T-cells. The role of NK1.1 T-cells, as a regulatory cell pop-

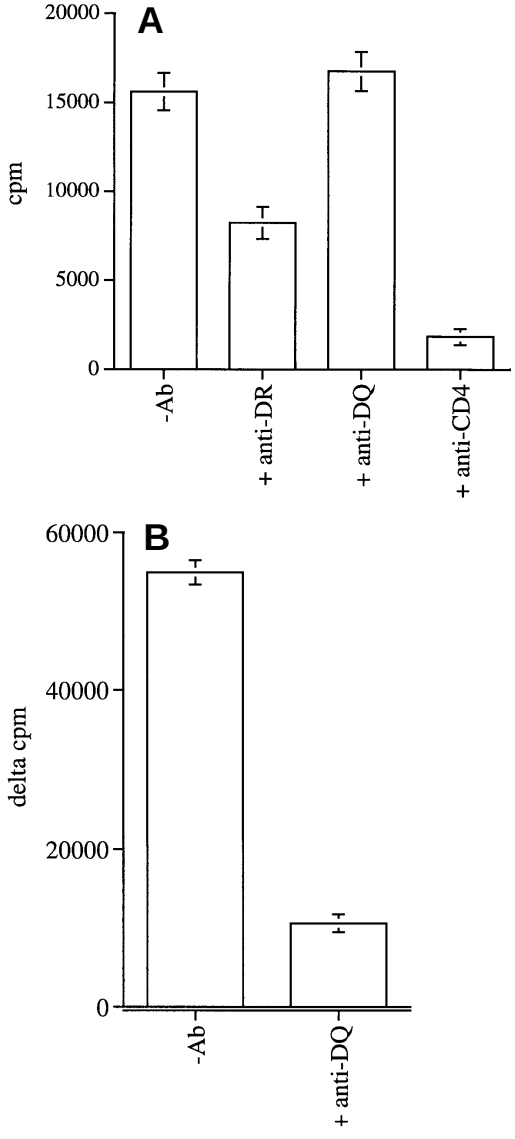

FIG. 2. *A*: Antibody blocking of the T-cell response to GAD in naive Aβo/DR3/DQ8 mice. Anti-DR blocked ~50% of the response, whereas anti-DQ did not block any of the response. Anti-CD4 antibody blocked >90% of the response. *B*: To confirm that the anti-DQ antibody (IVD12) was working, GAD65 recall responses were blocked in Aβo/DQ8 mice immunized with GAD. Anti-DQ blocked the response significantly. Background counts were <1,000 cpm for naive Ao/DR3/DQ8 mice and were between 4,000 and 6,000cpm for Aβo/DQ8 mice immunized with GAD65. All antibodies were used at a concentration of 10 μg/well.

ulation, in the etiology of autoimmune disease has been fairly well documented (34–36). It has been reported that there is a deficiency of NK1.1 T-cells in NOD mice (37). Therefore, we examined the three groups of transgenic mice for the presence of NK1.1 T-cells in the spleen (Table 2). The Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice had similar NK1.1 T-cell numbers as the B6 or B10 control mice (Table2).

Natural processing of GAD65. Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice were immunized with GAD65 subcutaneously to identify epitopes generated by the natural processing of GAD in vivo. (Fig. 4). All three strains responded comparably to whole GAD65 protein. The peptides that stimulated a recall response were h247-266, h250-270, m250-270, and mouse and human sequence identical (mh) 487-507, in both the Aβo/DR3/DQ8 and Aβo/DR3 mice. Aβo/DQ8 mice recognized m487-507 and h507-527. As shown in Fig. 1, peptide m250 is the only peptide among those tested that stimulated a spontaneous response in Aβo/DR3/DQ8 mice. Immunization with GAD65 in these mice, however, allows for the recruitment of T-cells of a variety of specificities, which results in the recognition of more epitopes on the GAD65 molecule. There was a strong proliferative response to h247-266 in the Aβo/DR3 mice, which was suppressed by the introgression of DQ8 (Fig. 4). These data demonstrate that combinations of MHC haplotypes can regulate epitope-specific responses.

Cytokine analysis. Cytokine profiles for the responder T-cells were analyzed to characterize the phenotype of spontaneous autoreactivity. (Fig. 5). Naive Aβo/DR3/DQ8 mice produced IFN-γ in response to administration of GAD65. IL-4 was not detectable in the supernatants. Cells from nonimmunized single transgenic mice (Aβo/DR3 and Aβo/DQ8) did not secrete any detectable cytokines in response to GAD65 administration. After immunization with GAD, cells prepared from all three groups produced only IFN-γ on rechallenge with GAD (Fig. 5).

Antibody responses in Aβo/DR3/DQ8 mice. Because the development of GAD autoantibodies is a hallmark of human diabetes but is unusual in NOD mice (38), sera of immunized and naive Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice were analyzed for the presence of GAD-specific antibodies (Fig. 6). All three groups of mice produced anti-GAD65 antibodies after immunization with GAD, but there was no spontaneous generation of autoantibodies in any group.

Evaluation of disease and pathological changes in islets. The development of a spontaneous loss of tolerance to GAD65 in the Aβo/DR3/DQ8 transgenic mice suggested the presence of pathological changes in the islets of Langerhans. Disease progression in the double (Aβo/DR3/DQ8) and single transgenic mice (Aβo/DR3, Aβo/DQ8) was evaluated by monitoring for hyperglycemia and by histological examination of the pancreas. The double transgenic mice did not develop hyperglycemia for the duration of the 30 weeks of testing. Hemotoxylin- and eosin-stained sections of pancreases from naive Aβo/DR3/DQ8, Aβo/DR2/DQ8, Aβo/DR3/DQ6, Aβo/DR3, Aβo/DQ8, and age-matched NOD mice were prepared (Fig. 7). The Aβo/DR3/DQ8 mice showed intra-islet insulitis with ~5% of islets showing >50% infiltration (Fig. 8). Control Aβo/DR3 mice showed some peri-insulitis with 2–3% of islets showing >50% destruction, whereas Aβo/DQ8 mice showed only mild peri-insulitic changes. The percentage of islets showing severe infiltration in the Aβo/DR3/DQ8 and Aβo/DR3 mice were not significantly different, but the total number of islets showing peri-insulitis and insulitis was greater in the Aβo/DR3/DQ8 mice. However, in both the Aβo/DR3 and Aβo/DQ8 mice, the pathology was completely abrogated when one nonsusceptible allele, either DR2 or DQ6, was present in Aβo/DR2/DQ8 and Aβo/DR3/DQ6 mice (Fig. 8). The islets of Aβo, Aβo/DR2, and Aβo/DQ6 single transgenic mice were completely normal with no evidence of inflammation or infiltration.

DISCUSSION

Genetic studies of human populations have demonstrated that polymorphisms of MHC class II genes are associated with predisposition to, or protection from, the development

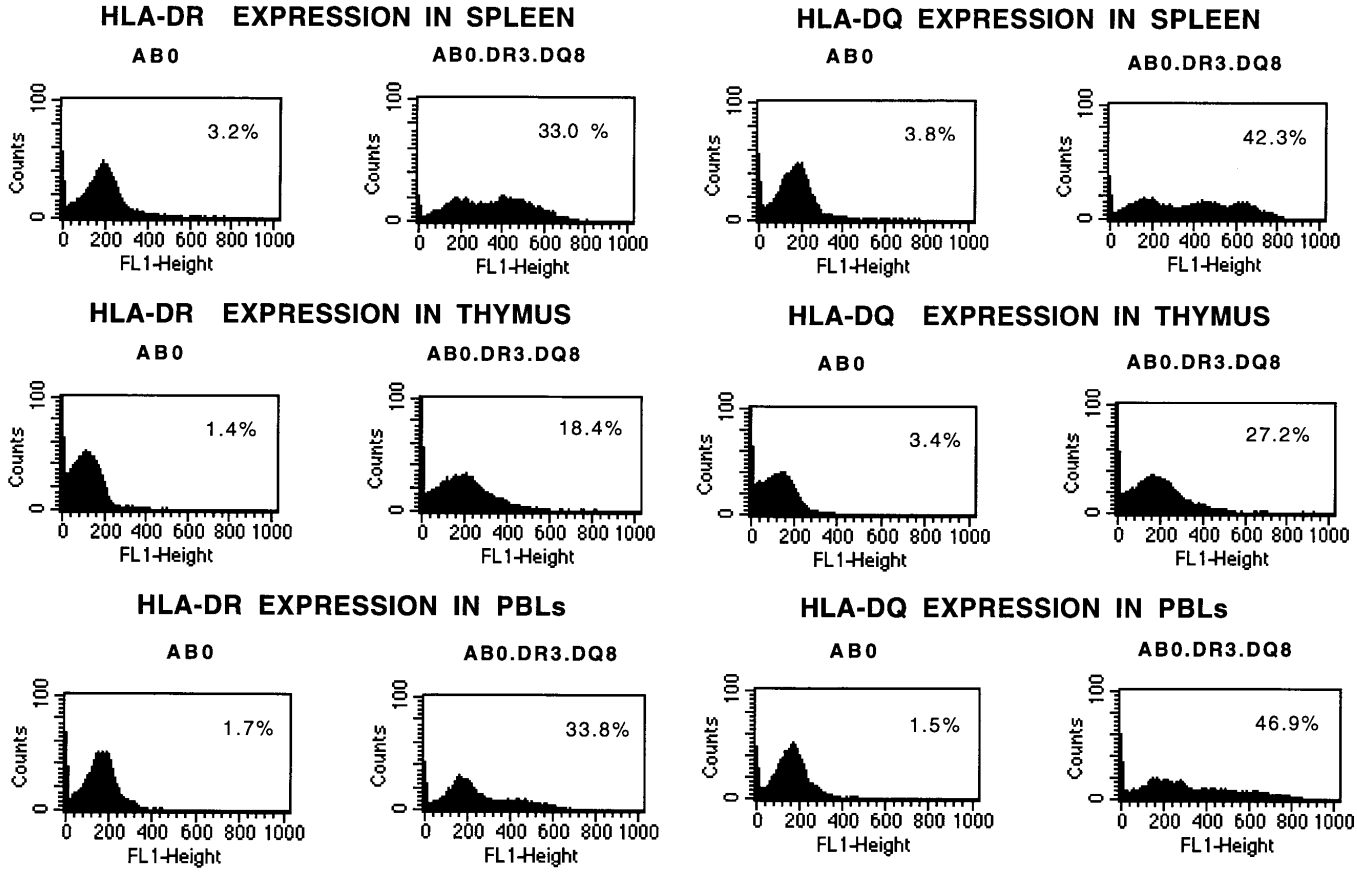

FIG. 3. Flow cytometric histogram showing surface expression levels of HLA DR and DQ in Aβo/DR3/DQ8 transgenic mice. Class II knock-out animals (Aβo) were the negative controls. Class II expression was analyzed in the spleen, thymus, and peripheral blood. DR and DQ expression was comparable in both the spleen and PBLs. In comparison, the thymus showed slightly lower levels of class II. Aβo/DR3 and Aβo/DQ8 mice showed similar profiles (data not shown).

of type 1 diabetes (39). The relationship between the HLA complex and disease can be threefold: *1*) HLA molecules are directly involved in the disease process by modulating the immune response in some fashion; *2*) the association is merely a marker where the particular MHC allele(s) is not directly involved but is inherited in linkage disequilibrium with other genes that are responsible for the disorder; or *3*) the association may be completely artifactual. To the best of our knowledge, this is the first report on type 1 diabetes that demonstrates that the interactions of the MHC molecules are crucial to the development of the earliest manifestations of autoimmunity. The phenomenon of linkage disequilibrium within the MHC limits the study of either the individual contributions of or epistatic interactions between various DR and DQ haplotypes, with respect to diabetes. To study these effects, transgenic mice lacking endogenous MHC class II, but expressing various DR and DQ alleles alone or in combination, were generated.

The Aβo/DR3/DQ8 mice showed strong spontaneous T-cell responses to GAD, whereas the control mice, the Aβo/DR2/DQ8, Aβo/DR3, and Aβo/DQ8 naive transgenic mice, did not. This suggests that both susceptible class II alleles are required for the selection and expansion of autoreactive T-cells, an event that did not occur in either the single transgenic or the Aβo/DR2/DQ8 mice. Antibody-blocking studies revealed that the GAD65 recall response in Aβo/DR3/DQ8 mice was inhibited by anti-DR and anti-CD4. The levels of DR and DQ expression were similar in the three strains of transgenic mice and, additionally, did not show much tissue-specific variation, although levels in the thymus were a little lower than those in the spleen or peripheral blood. The latter finding, though, may merely be a result of lower numbers of antigen-presenting cells in the thymus. Also, there were no differences in the numbers of CD4 or NK1.1 T-cells in the transgenic mice, which indicates that the loss of tolerance to GAD65 in the Aβo/DR3/DQ8 mice could not be attributed to a difference in the number of "effector" CD4 T-cells. Furthermore, in the Aβo/DR3/DQ8

TABLE 2
Analysis of T-cells in spleens of HLA transgenic mice (three mice in each group)

Strain	$CD4^+$ T-cells (%)	NK1.1 T-cells (%)
Transgenic mice		
Aβo/DR3/DQ8	13.89 ± 1.48	3.26 ± 0.67
Aβo/DR3	15.11 ± 3.62	4.33 ± 1.19
Aβo/DQ8	13.13 ± 1.61	3.90 ± 0.44
Control mice		
B10 (positive control)	—	3.55 ± 0.17
B6 (positive control)	—	3.06 ± 0.6
NOD (negative control)	—	0.21 ± 0.07

Data are means ± SD.

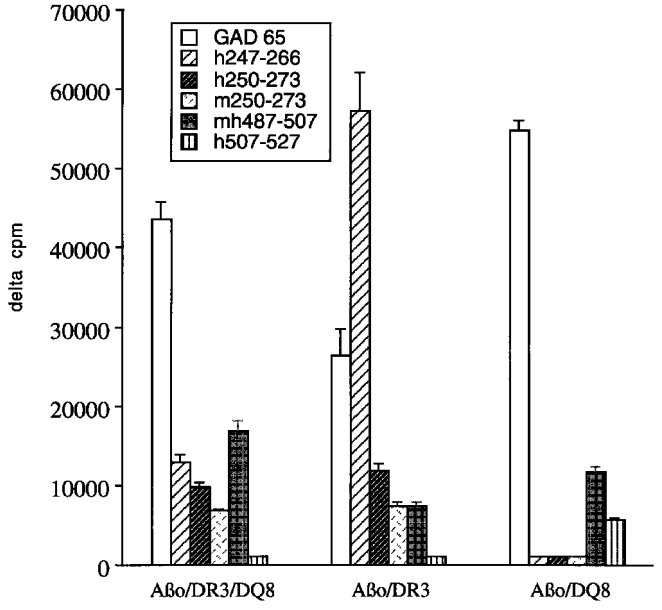

FIG. 4. Identification of immunodominant epitopes on GAD65. Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 transgenic mice (three mice in each group) were immunized with 50 μg of GAD65 that was administered subcutaneously in the hind foot-pad and the base of the tail. On day 10 postimmunization, lymph nodes (popliteal, inguinal, and para-aortic) were harvested and stimulated in vitro with half-log titrating amounts of GAD or peptides. Data are shown at a 30 μg/ml concentration for GAD and a 100 μg/ml concentration for the peptides, with standard deviation for each group. The experiment was repeated thrice, and one representative experiment is shown. Background counts ranged from 2,000 to 6,000 cpm, and data are shown as delta cpm.

mice, co-expression of DQ8 suppressed the recognition of h247-266; in contrast, h247-266 was the dominant epitope recognized in the single DR3 transgenic mice. Similar findings have been reported with *Schistosoma japonicum* infection and cedar pollinosis (40,41). In these studies, a model was proposed (40) in which DQ and DR interact epistatically, and the presence of certain DQ molecules was sufficient for suppression of DR responses. This model seems to fit with the data from the DR3/DQ8 transgenic mice. The DR3 molecule did not affect recognition of the apparent DQ-restricted peptide, mh487-507.

Because MHC class II alleles have been suggested to influence the Th-phenotype of responder T-cells, cytokine profiles in response to GAD were determined. Naive Aβo/DR3/DQ8 mice showed low levels of IFN-γ, but there was no discernible IFN-γ or IL-4 in the single transgenic mice. However, on GAD immunization, all three groups of mice produced large amounts of IFN-γ. Thus, there appears to be a Th1-dominant response to GAD in all of the MHC backgrounds tested, which is in accord with the findings on the loss of tolerance to GAD65 in the NOD mice (42). Therefore, the progression to insulitis in the double transgenic mice cannot be explained solely on the basis of cytokine profiles as determined by responses to GAD65.

Histologic evidence of spontaneous insulitis occurred largely in the double transgenic mice (Aβo/DR3/DQ8). Insulitic infiltration was found in ~17% of islets, 5% of which were either completely destroyed or showed >50% infiltration. The single transgenic groups, Aβo/DR3 and Aβo/DQ8 mice, showed lesser insulitic changes, although the Aβo/DR3 mice did have 2–3% islets with >50% inflammatory destruction, which was not significantly different from that seen in the Aβo/DR3/DQ8. In the double transgenic mice, however, the total number of islets affected to varying degrees by lymphocytic infiltration was greater than that in either of the single transgenic mice. Interestingly, in the Aβo/DR2/DQ8 and Aβo/DR3/DQ6 mice, insulitis was completely abrogated, which clearly demonstrates that the presence of a resistant (DR2) or a neutral allele (DQ6) can

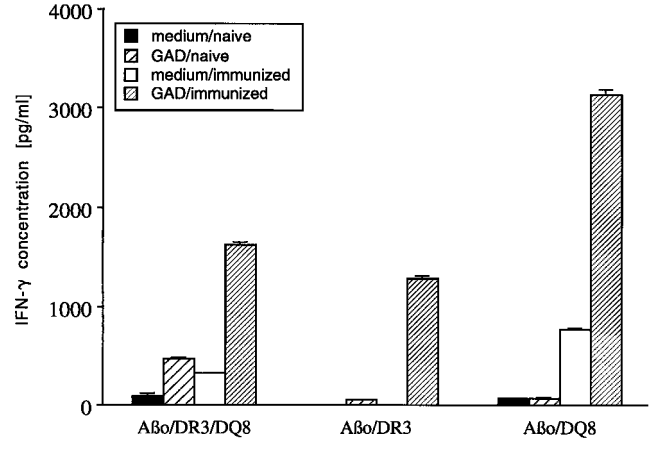

FIG. 5. Cytokine analysis of culture supernatants from naive and immunized mice. Cells from the Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 groups (lymph nodes from three naive or immunized mice pooled in each group) were stimulated in vitro with GAD65 at 30 μg/ml or without antigen (medium control). Culture media from cells stimulated for 24 h were collected, and the levels of IFN-γ and/or IL-4 were determined with an ELISA. There were no detectable levels of IL-4. Small amounts of IFN-γ were seen in naive Aβo/DR3/DQ8 mice. Data are expressed as picograms per milliliter with standard deviation, based on a standard curve run for each experiment. The sensitivity of detection was ~30 pg/ml.

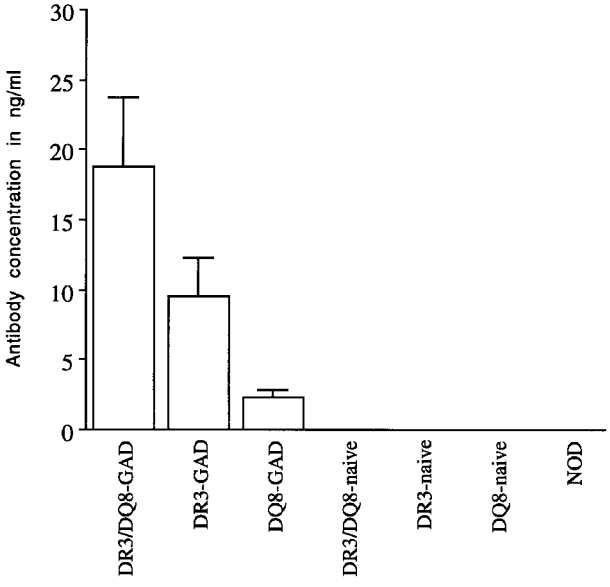

FIG. 6. Anti-GAD antibody estimation. Sera collected from naive and GAD-immunized Aβo/DR3/DQ8, Aβo/DR3, and Aβo/DQ8 mice were tested for the presence of anti-GAD antibodies. The antibody levels were quantitated by use of a mouse immunoglobulin standard curve. Only immunized transgenic mice showed antibodies to GAD. Each group consisted of five mice, and standard deviation is shown for each group. The sensitivity of detection in the assay was 1 pg/ml. Mean antibody values have been shown for each group.

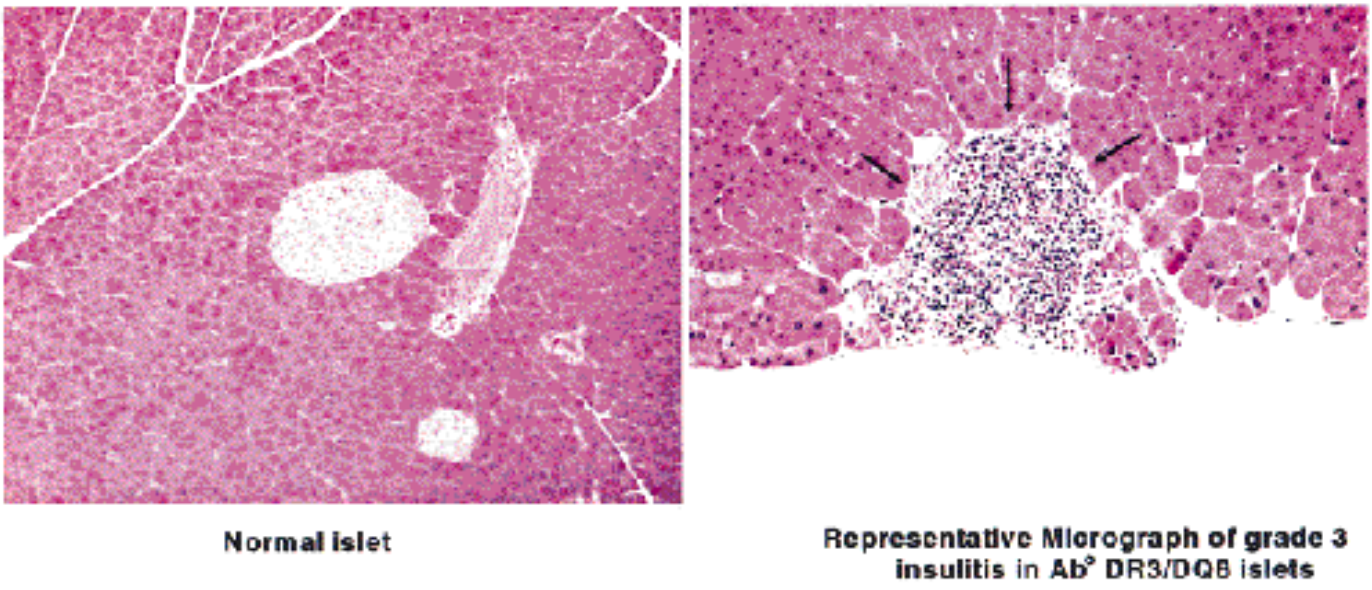

FIG. 7. Hemotoxylin- and eosin-stained sections of pancreas from transgenic mice. Pancreases were harvested from Aβo/DR3/DQ8 (n = 12), Aβo/DR3/DQ6 (n = 5), Aβo/DR2/DQ8 (n = 5), Aβo/DR3 (n = 8), and Aβo/DQ8 (n = 8) mice of both sexes, and paraffin-embedded blocks were made. The mice were 12–16 weeks of age. Before scoring for insulitis, 4-μm–thick sections were stained with hemotoxylin and eosin. A representative photomicrograph of an islet from Aβo/DR3/DQ8 mice with severe infiltration is shown. The Aβo/DR3 and Aβo/DQ8 mice showed either peri-insulitis or <50% infiltration. The control mice and the Aβo/DR2/DQ8, Aβo/DR3/DQ6, Aβo, Aβo/DR2, and Aβo/DQ6 mice had completely normal islets with no signs of abnormal pathology; the section on the left shows one such islet.

override the effect of a predisposing MHC allele. Therefore, the progression of insulitis appeared to require also the epistatic interaction of at-risk MHC class II genes.

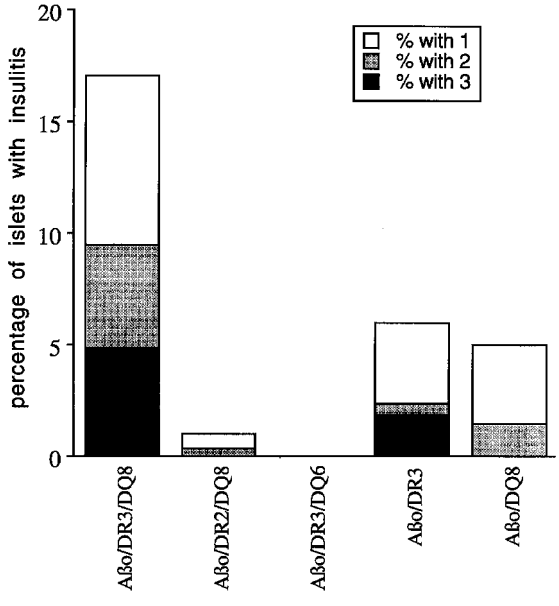

FIG. 8. Insulitis score from pancreatic sections. Based on the histopathological evaluation of pancreata from the HLA transgenic mice, the following scores were assigned to assess the extent and severity of islet infiltration: 1 = peri-insulitis, 2 = <50% insulitis, and 3 = >50% insulitis or completely destroyed islet. The y-axis represents the percentage of total islets that showed infiltration, and each stack on the bar represents the percentage of islets with a particular score. Of the total number of islets examined, 351 were from the Aβo/DR3/DQ8 group, 74 were from the Aβo/DR2/DQ8 group, 82 were from the Aβo/DR3/DQ6 group, 340 were from the Aβo/DR3 group, and 124 were from the Aβo/DQ8 group.

Recently, two areas on the genome, each composed of four loci, have been identified. These two areas are crucial for hastening the progression of insulitis to diabetes in T-cell receptor transgenic B6 (C57BL/6) mice. Homozygosity of at least one of these loci appears to be necessary for disease to develop in this model (33). The strains of mice tested in this study were homozygous for all four B6 susceptibility loci (data not shown).

The structure of the DQ molecule is considered to be important in determining the nature of the autoimmune response in type 1 diabetes. The DQ8 molecule has been shown to be a poor selection element, and this could influence the T-cell repertoire (43). In humans, there appears to be more DR expression in the periphery and less in the thymus, whereas the reverse is true for DQ expression. Therefore, it has been proposed that predisposition to disease may involve thymic repertoire selection by DQ and presentation of self antigens in the periphery by DR (44–46). If such were the case, this phenomenon would support the findings (47) that MHC class II genes can differentially regulate the immune response. The stage would then be set for epistatic interactions between MHC class II molecules, which, as shown in this article, predispose to autoimmunity.

ACKNOWLEDGMENTS

This work was supported by a Juvenile Diabetes Foundation International Fellowship (to R.S.A.), a research grant (to C.S.D.), and a National Institutes of Health grant (AI-14764).

We gratefully acknowledge Michele Smart for the technical assistance in flow cytometry and Julie Hanson for the technical assistance in animal breeding. We would also like to acknowledge the assistance of Dr. Kenneth Batts (Department of Pathology, Mayo Clinic) in evaluating pancreatic histology.

REFERENCES

1. Thomson G: HLA DR antigens and susceptibility to insulin-dependent diabetes mellitus. *Am J Hum Genet* 36:1309–1317, 1984
2. Thomson G, Robinson WP, Kuhner MK, Joe S, MacDonald MJ, Gottschall JL, Barbosa J, Rich SS, Bertrams J, Baur MP, Partanen J, Tait BD, Schober E, Mayr WR, Ludvigsson J, Lindblom B, Farid NR, Thompson C, Deschamps I: Genetic heterogeneity, modes of inheritance and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. *Am J Hum Genet* 43:799–816, 1988
3. Sheehy MJ, Scharf SJ, Rowe JR, Neme de Gimenez MH, Meske LM, Ehrlich HA, Nepom BS: A diabetes-susceptible HLA haplotype is best defined by a combination of HLA DR and DQ alleles. *J Clin Invest* 83:830–835, 1989
4. Kockum I, Wassmuth R, Holmberg E, Michelsen B, Lernmark A: HLA DQ primarily confers protection and HLA DR susceptibility in type 1 diabetes studied in population-based affected families and controls. *Am J Hum Genet* 53:150–167, 1993
5. Khalil I, Deschamps I, Lepage V, Al-Daccak R, Degos L, Hors J: Dose effect of cis-and trans-encoded HLA DQαβ heterodimers in IDDM susceptibility. *Diabetes* 41:378–384, 1992
6. Bohme J, Carlsson B, Wallin J, Moller E, Persson B, Peterson PA, Rask L: Only one DQ-β restriction fragment pattern of each DR specificity is associated with IDDM. *J Immunol* 137:941–947, 1986
7. Khalil I, d'Auriol L, Gobet M, Morin L, Lepage V, Deschamps I, Park MS, Degos L, Gailbert F, Hors J: A combination of HLA DQβ Asp57-negative and HLA DQa Arg52 confers susceptibility to IDDM. *J Clin Invest* 85:1315–1319, 1990
8. Sterkers G, Zeliszewski D, Chausee AM, Deschamps I, Font MP, Freidel C, Hors J, Betuel H, Dausset J, Levy JP: HLA DQ rather than HLA DR region might be involved in dominant non-susceptibility. *Proc Natl Acad Sci U S A* 85:6473–6477, 1988
9. Todd JA, Bell JI, McDevitt HO: HLA DQβ gene contributes to susceptibility and resistance to IDDM. *Nature* 329:599–604, 1987
10. Baisch JM, Weeks T, Giles R, Hoover M, Stastny P, Capra JD: Analysis of HLA DQ genotypes and susceptibility in IDDM. *N Engl J Med* 322:1836–1841, 1990
11. Nepom BS, Schwarz D, Palmer JP, Nepom GT: Transcomplementation of HLA genes in IDDM: HLA DQα- and β-chains produce hybrid molecules in DR3/4 heterozygotes. *Diabetes* 36:114–117, 1987
12. Undlien DE, Friede T, Rammensee HG, Joner G, Dahl-Jorgensen K, Sovik O, Akelsen HA, Knutsen I, Ronningen KS, Thorsby E: HLA-encoded genetic predisposition in IDDM: DR4 subtypes may be associated with different degrees of protection. *Diabetes* 46:143–149, 1997
13. Owerbach D, Lernmark A, Platz P, Ryder LP, Rask L, Peterson PA, Ludvigsson J: HLA-D region β-chain DNA endonuclease fragments differ between HLA-DR identical healthy and insulin-dependent diabetic individuals. *Nature* 303: 815–817, 1983
14. Thorsby E: HLA-associated disease susceptibility: which genes are primarily involved? *Immunologist* 3:51–58, 1995
15. Thorsby E, Ronningen KS: Particular HLA DQ molecules play a dominant role in determining susceptibility or resistance to type 1 diabetes. *Diabetologia* 36:371–377, 1993
16. Cohen-Haguenauer O, Robbins E, Masset C, Busson M, Deschamps I, Hors J, Lalouel J, Dausset JM, Cohen D: A systematic study of HLA class II-β DNA restriction fragments in IDDM. *Proc Natl Acad Sci U S A* 82:3335–3339, 1985
17. Eisenbarth GS, Rewers M: Refining genetic analysis of type 1 diabetes. *J Clin Endocrinol Metab* 80:2564–2566, 1995
18. Tait BD, Boyle AJ: DR4 and susceptibility to type 1 diabetes mellitus: discrimination of high risk and low risk DR4 haplotypes on the basis of TA10 typing. *Tissue Antigens* 28:65–71, 1986
19. Schreuder GM, Tilanus MG, Bontrop RE, Bruining GJ, Giphart MJ, Van Rood JJ, de Vries RR: HLA DQ polymorphism associated with resistance to type 1 diabetes detected with monoclonal antibodies, isoelectric point differences, and restriction fragment length polymorphism. *J Exp Med* 164:938–943, 1986
20. Festenstein H, Awad J, Hitman GA, Cutbush S, Groves AV, Cassell P, Ollier W, Sachs JA: New HLA DNA polymorphisms associated with autoimmune diseases. *Nature* 322:64–67, 1986
21. Yasunaga S, Kimura A, Hamaguchi K, Ronningen KS, Sasazuki T: Different contribution of HLA DR and DQ genes in susceptibility and resistance to IDDM. *Tissue Antigens* 47:37–48, 1996
22. Wei B-Y, Martin J, Savarirayan S, Little R, David CS: Transgenic mice with MHC class II genes: the use in the study of allelic α/β chain pairing and the production of engineered mice with mutant I-A genes. In *Transgenic Mice and Mutants in MHC Research*. Egorov IK, David CS, Eds. Berlin, Springer-Verlag, 1990, p. 237
23. Nabozny GH, Baisch JM, Cheng S, Cosgrove D, Griffiths M, Luthra HS, David CS: HLA DQ8 transgenic mice are highly susceptible to collagen-induced arthritis: a novel model for human polyarthritis. *J Exp Med* 183:27–37, 1996
24. Okada K, Boss JM, Prentice H, Spies T, Mengler R, Auffray C, Lillie J, Grossberger D, Strominger JL: Gene organization of DC and DX subregions of the human MHC. *Proc Natl Acad Sci U S A* 82:3410–3414, 1985
25. Strauss G, Vignali DAA, Schonrich G, Hammerling G: Negative and positive selection by HLA DR3 (DRw17) molecules in transgenic mice. *Immunogenetics* 40:104–108, 1994
26. Gonzalez-Gay MA, Zanelli E, Khare SD, Krco CJ, Zhou P, Griffiths MM, Luthra HS, David CS: HLA DRB1*1502 transgene reduces incidence and severity of arthritis in mice. *Hum Immunol* 50:54–60, 1996
27. Bradley DS, Nabozny GH, Cheng S, Zhou P, Griffiths MM, Luthra HS, David CS: HLA DQB1 polymorphism determines incidence, onset and severity of collagen-induced arthritis in transgenic mice. *J Clin Invest* 100:2227–2234, 1997
28. Abraham RS, Wilson SB, DeSouza NF, Strominger JL, Munn SR, David CS: NOD background genes influence T cell responses to GAD 65 in HLA DQ8 transgenic mice. *Hum Immunol* 60:583–590, 1999
29. Chen S, Evans GA: A simple screening method for transgenic mice using the polymerase chain reaction. *Biotechniques* 8:32–33, 1990
30. Cosgrove D, Gray D, Dierich A, Kaufman J, Lemeur M, Benoist C, Mathis D: Mice lacking MHC class II molecules. *Cell* 66:1051–1066, 1991
31. Chapoval SP, Neeno T, Krco CJ, Marietta EV, Harders J, David CS: HLA DQ6 and HLA DQ8 transgenic mice respond to ragweed allergens and recognize a distinct set of epitopes on short and giant ragweed group 5 antigens. *J Immunol* 161:2032–2037, 1998
32. Kurrer M, Pakala SV, Hanson HL, Katz JD: Beta cell apoptosis in T cell-mediated autoimmune diabetes. *Proc Natl Acad Sci U S A* 94:213–218, 1997
33. Gonzalez A, Katz JD, Mattei M-G, Kikutani H, Benoist C, Mathis D: Genetic control of diabetes progression. *Immunity* 7:873–883, 1997
34. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, Porcelli SP, Schatz DA, Atkinson MA, Balk SP, Strominger JL, Hafler DA: Extreme Th1 bias of invariant Vα24JαQ T cells in type 1 diabetes. *Nature* 391:177–181, 1998
35. Takeda K, Dennert G: The development of autoimmunity in C57Bl/6 *lpr* mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J Exp Med* 177:155–164, 1993
36. Mieza MA, Itoh T, Cui JQ, Makino Y, Kawano T, Tsuchida K, Koike T, Shirai T, Yagita H, Matsuzawa A, Koskei H, Taniguchi M: Selective reduction of Vα14+ NK T cells associated with disease development in autoimmune-prone mice. *J Immunol* 156:4035–4040, 1996
37. Baxter AG, Kinder SJ, Hammond KJL, Scollay R, Godfrey DI: Association between aβTCR$^+$CD4$^-$CD8$^-$ T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes* 46:572–582, 1997
38. Bach J-F, Mathis D: The NOD mouse. *Res Immunol* 148:285–366, 1997
39. Eisenbarth GS: Type 1 diabetes mellitus: a chronic autoimmune disease. *N Engl J Med* 314:1360–1368, 1986
40. Sasazuki T, Kikuchi K, Hirayama K, Matsushita S, Ohta N, Nishimura Y: HLA-linked immune suppression in humans. *ImmunolSuppl* 2:21–24, 1989
41. Matsushita S, Muto M, Suemura M, Saito Y, Sasazuki T: HLA-linked nonresponsiveness to Cryptomeria japonica pollen antigen. I. Nonresponsiveness is mediated by antigen-specific suppressor T cells. *J Immunol* 138:109–115, 1987
42. Tian J, Clare-Salzler M, Herschenfeld A, Middleton B, Neuman D, Mueller R, Arita S, Evans C, Atkinson MA, Mullen Y, Sarvetnick N, Tobin AJ, Lehmann PV, Kaufman DL: Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 2:1348–1353, 1996
43. Nepom GT, Kwok WW: Molecular basis for HLA DQ associations with IDDM. *Diabetes* 47:1177–1184, 1988
44. Charron D: Molecular basis of HLA class II disease associations. *Adv Immunol* 48:107–159, 1990
45. Sansom DM, Bidwell JL, Maddison PJ, Campion G, Klouda PI, Bradley BA: HLA DQ alpha and DQ beta restriction fragment length polymorphisms associated with Felty's syndrome and DR4-positive rheumatoid arthritis. *Hum Immunol* 19:269–278, 1987
46. Lanchbury JSS, Sakkas LI, Marsh SGE, Bodmer JG, Welsh KI, Panayi GS: HLA DQ β 3.1 allele is a determinant of susceptibility to DR4-associated rheumatoid arthritis. *Hum Immunol* 26:59–71, 1989
47. Hirayama K, Matsushita S, Kikuchi I, Iuchi M, Ohta N, Sasazuki T: HLA DQ is epistatic to HLA DR in controlling the immune response to schisostomal antigens in humans. *Nature* 327:426–430, 1987