HLA-DQ–Regulated T-Cell Responses to Islet Cell Autoantigens Insulin and GAD65

Timothy I.M. Tree,1 Gaby Duinkerken,2 Sabine Willemen,2 René R.P. de Vries,2 and Bart O. Roep2

HLA-DQ is strongly associated with genetic predisposition to type 1 diabetes. It is assumed that HLA-DQ molecules exert their effects on the disease via the presentation of peptides from islet autoantigens to CD4+ T-cells, but little information regarding HLA-DQ–restricted, islet antigen–specific, autoreactive T-cells is available. To investigate the role of HLA-DQ in the immune response to islet autoantigens, we measured T-cell proliferation to insulin and GAD65 in the presence and absence of monoclonal antibodies that block HLA-DQ–mediated antigen presentation in recent-onset type 1 diabetic patients and their siblings. Positive proliferative T-cell responses to GAD65 were observed in 60% of type 1 diabetic patients and 52% of siblings. This proliferation was significantly reduced in the presence of anti-DQ antibody, demonstrating the presence of primed, effector HLA-DQ–restricted T-cell responses to GAD65. Positive proliferative responses to insulin were observed in 25% of type 1 diabetic patients and 10% of siblings. However, blocking HLA-DQ–restricted T-cell responses led to a significant increase in proliferation to insulin, implying the presence of primed suppressive HLA-DQ–restricted T-cell responses to insulin. These results indicate that HLA-DQ acts as a restriction element for both proliferative and suppressor cells, with the relative balance of these cells dependent on the nature of the autoantigen. Diabetes 27: 1692–1699, 2004

Type 1 diabetes is caused by the T-cell–dependent, immune-mediated destruction of the insulin-producing pancreatic β-cells (1,2). CD4 and CD8 T-cells, which recognize islet autoantigens, are believed to play a pivotal role in this process. Indeed, T-cell responses to the major islet autoantigens, insulin, the islet tyrosine phosphatase (insulinoma-associated protein 2 [IA-2]), and GAD65, have been observed (3–8) in patients with type 1 diabetes. Studies have also reported responses to these autoantigens from nondiabetic siblings and nondiabetic control subjects (3–5,7,9); however, there is evidence (10) that the quality of response in these individuals is different from that seen in diabetic patients.

Development of type 1 diabetes is strongly associated with major histocompatibility complex (MHC)-region genes, and a large number of studies (11,12), encompassing different populations, have identified MHC class II genes (particularly encoding HLA-DRB1, HLA-DQA1, and HLA-DQB1 molecules) that are associated with disease. In particular, genes encoding specific HLA-DQ hetrodimeric molecules are strongly associated with susceptibility to (e.g., HLA-DQA1*0301/DQB1*0302) and dominant protection from (e.g., HLA-DQA1*0102/DQB1*0602) type 1 diabetes.

The primary function of MHC class II molecules is the presentation of antigen-derived peptides to CD4+ T-cells. It is therefore believed that HLA-DQ molecules exert their dominant effect on islet autoimmunity via the thymic selection and/or peripheral activation of autoreactive T-cells. It has been suggested (13,14) that certain HLA-DQ molecules may be poor at deleting autoreactive thymocytes, thus permitting the presence of potentially pathogenic T-cells in the periphery. However, the simple hypothesis of HLA-DQ restriction of autoreactive T-cells discords with the observation that all autoreactive T-cell lines and clones obtained from individuals with type 1 diabetes have been restricted by HLA-DR or HLA-DP but not HLA-DQ. Conversely, it has been suggested (15,16) that HLA-DQ may have a role in the selection of regulatory cells. These alternatives are clearly not exclusive, and it is possible that the degrees of susceptibility conferred by different HLA-DQ molecules could represent a combination of their ability to delete pathogenic and recruit regulatory T-cells. However, little information is available on the nature of HLA-DQ–restricted, islet antigen–specific T-cells in type 1 diabetes.

To investigate the role of HLA-DQ in the T-cell response to islet autoantigens, we have measured T-cell responses in fresh blood samples, from 20 individuals with newly diagnosed type 1 diabetes and 23 of their siblings with no evidence of type 1 diabetes, to the diabetes-associated autoantigens insulin and GAD65 in the presence of monoclonal antibodies designed to interfere with HLA-DQ–mediated T-cell activation or an irrelevant antibody.

RESEARCH DESIGN AND METHODS

Type 1 diabetic patients and their siblings were recruited from the Kobilbie cohort of juvenile-onset type 1 diabetes. Following informed consent, peripheral blood was drawn from 20 patients (5 girls, mean age 8.2 ± 4.2 years, range 1.1–15.1) within 2 weeks after the clinical manifestation of type 1 diabetes. The blood of unaffected first-degree family members was drawn afterward, HLA typed, and tested for the presence of islet autoantibodies. None of the
siblings in the present study (n = 23; 7 girls; mean age 8.6 ± 3.1 years, range 3.0–13.5) were seropositive for these antibodies.

**T-cell proliferation assays.** A T-cell proliferation assay was performed as described before (17) on freshly isolated peripheral blood mononuclear cells in autologous serum. Briefly, 1.6 × 10^6 cells in culture medium (Iscove’s modified Dulbecco’s medium [Gibco, Paisley, U.K.] with 10% autologous heat-inactivated serum) were seeded per well in 96-well round-bottomed plates (Costar, Cambridge, MA) and cultured for 6 days at 37°C in 5% CO₂ in a humidified atmosphere, and in the absence or presence of various stimuli. Recombinant interleukin-2 (25 units/ml; Genzyme, Cambridge, MA) was added in separate wells to check viability of the T-cells. Proliferative responses were measured against insulin (25 μg/ml; Sigma, Zwijndrecht, the Netherlands) and GAD65 (5 μg/ml; Diamyd Med, Stockholm, Sweden) in the absence and presence of a blocking monoclonal antibody against HLA-DQ (SPV-L3; 10 μg/ml). This antibody has repeatedly been shown to block HLA-DQ–restricted T-cell proliferation (18) and is not selective for HLA-DQ polymorphism because it is directed against the conserved HLA-DQ backbone (19). Proliferative responses to the antibody alone were 1.24 ± 0.74 and 1.44 ± 0.81 times higher than the response in medium alone in patients and siblings, respectively, and similar to those to the isotype control antibody B8.11.2 directed against the isotype control antibody B8.11.2.

The normality of the distributions of lymphocyte proliferative responses was determined using the Kolmogorov-Smirnov goodness-of-fit test. Proliferation in the presence of irrelevant or anti–HLA-DQ antibody were compared using the paired Student’s t test and differences in responses between patients with type 1 diabetes and nondiabetic control subjects compared using the unpaired Student’s t test or Mann-Whitney U test as appropriate. Differences between the HLA-DQ ratio obtained for GAD65 and insulin were compared using the paired and unpaired Student’s t test. Differences in the proportion of positive responses or proportion of high-risk HLA types were examined using Fisher’s exact test. Relationships between proliferative responses in each individual were examined by calculation of the Spearman’s correlation coefficient. P values <0.05 were considered significant.

All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

**RESULTS**

**Proliferation in the presence of media alone and tetanus toxoid.** Background proliferation (in the absence of specific stimulation) was similar in siblings and type 1 diabetic patients ([mean ± SD] 1,469 ± 1,648 cpm and 1,538 ± 1,306, P = 0.88, respectively) (data not shown). As a positive control, responses to the recall antigen tetanus toxoid were measured. A vigorous proliferative response to tetanus toxoid was observed in both siblings (median SI 21.3; range 2.8–362.6; n = 20) and type 1 diabetic patients (19.8; 3.1–552.6; n = 19) (Fig. 1). No significant difference in either the percentage of individuals with a positive response, defined as an SI ≥3, (sibling 95% and patient 100%, P = 1.0) or magnitude of response (P = 0.41) was observed between siblings and patients.

**T-cell proliferative response to insulin and GAD65.** T-cell proliferative responses to insulin and GAD65 are shown in Fig. 2. Proliferation in response to insulin was low in both siblings and patients with no difference in either the number of positive responses (SI ≥3; 2 of 20 siblings and 4 of 19 type 1 diabetes) or the mean SI (1.7 ± 1.3 and 2.0 ± 1.8, respectively) between the two groups. In contrast, a positive proliferative response to GAD65 was observed in 12 of 23 (60%) siblings and 12 of 20 (52%) patients. Although there was no difference in the number of individuals with a positive response or the overall mean SI between siblings and patients (13.9 ± 15.1 and 7.3 ± 6.9, respectively; P = 0.08), the SI of individuals with a positive response to GAD65 was significantly higher in siblings than in patients (25.0 ± 13.1 and 11.1 ± 6.5, respectively; P = 0.0035).

**Effect of anti–HLA-DQ blocking antibodies on proliferation to GAD65.** Proliferative responses to GAD65 in the presence or absence of an anti–HLA-DQ blocking...
antibody are shown in Fig. 3. Blocking HLA-DQ led to a significant reduction in the proliferation to GAD65 in siblings \((P = 0.013)\), with a reduction in mean SI from 13.9 to 8.34 (Fig. 3A). In the type 1 diabetic group, blocking HLA-DQ also led to a reduction in mean SI (7.1 to 4.9), but this did not reach statistical significance (Fig. 3B). Although blocking lead to a reduction in the mean SI in both siblings and patients, the proportion of individuals with a positive response remained similar in unblocked and blocked cultures (siblings 12 of 23 vs. 12 of 23 and type 1 diabetes 12 of 20 vs. 11 of 20).

In addition to analyzing the sibling population as a whole, it was also divided into individuals who had a positive response in unblocked cultures (Fig. 3C) and those who did not (Fig. 3D). In individuals with a positive response, blocking HLA-DQ led to a marked reduction in proliferation (mean SI 23.3 to 11.4; \(P = 0.0002\)). Conversely, in individuals with an initially negative response, blocking HLA-DQ led to an increase in proliferation (1.5 to 4.4), although this did not reach statistical significance \((P = 0.06)\).

**Effect of anti-HLA-DQ blocking antibodies on the proliferation to insulin.** Blocking HLA-DQ led to a marked increase in proliferation to insulin in the sibling group (mean SI 1.7 to 4.8; \(P = 0.0013\)) (Fig. 4A). This led to a significant increase in the proportion of siblings with a positive response from 2 of 20 to 11 of 20 \((P = 0.006)\). In the type 1 diabetic group, blocking HLA-DQ led to an increase in both the mean SI (2.0 to 3.9) and the proportion of positive responses (4 of 19 to 7 of 19); however, the
increases were not statistically significant ($P = 0.06$ and $P = 0.24$, respectively).

**Relation between the effect of blocking HLA-DQ on the response to GAD65 and insulin.** The relationship between the effect of blocking HLA-DQ antibody on the responses to GAD65 and insulin within an individual were investigated using an HLA-DQ ratio (Fig. 5). This was calculated as follows: (SI in the presence of anti–HLA-DQ antibody/SI in the absence of anti–HLA-DQ antibody) × 100. Thus, a ratio > 1 indicates that blocking HLA-DQ led to an increase in proliferation, whereas a ratio < 1 indicates a decrease in proliferation. This figure clearly demonstrates that within an individual, blocking HLA-DQ can exert a markedly different (and often inverse) effect on proliferation, depending on the antigen used to stimulate the cells. Furthermore, the HLA-DQ ratio for insulin was significantly higher (i.e., DQ-restricted suppression) than that of GAD65 when paired results from all individuals were analyzed ($P = 0.0035$). However, this difference was more marked in the type 1 diabetic patients ($P = 0.007$) than in the siblings ($P = 0.06$).

**Correlation between responses.** Individual responses to GAD and insulin in the absence or presence of anti–HLA-DQ antibodies were examined using Pearson’s correlation coefficient (Fig. 6). A highly significant positive correlation was observed between unblocked and anti-
HLA-DQ–blocked responses to GAD65 for siblings ($r^2 = 0.67; P < 0.0001$) and type 1 diabetic patients ($r^2 = 0.39; P = 0.003$) (Fig. 6A). A weak positive correlation was also observed between unblocked and anti-HLA-DQ–blocked responses to insulin for siblings ($r^2 = 0.2; P = 0.036$) (Fig. 6B). No significant correlation was observed between responses to GAD65 and insulin in either group of individuals (Fig. 6C). However, a highly significant positive correlation was observed between the unblocked GAD65 and HLA-DQ–blocked insulin response in siblings ($r^2 = 0.61; P < 0.0001$) (Fig. 6D).

**Relation between HLA type and proliferation.** Alleles present at the HLA-DRB1, HLA-DQA1, and HLA-DQB1 loci were determined. The percentage of individuals expressing either of the main HLA-DQ–predisposing alleles (DQA1*0301/DQB1*0302 or DQA1*0501/DQB1*0201) was significantly higher in the type 1 diabetic compared with the sibling group (93 and 63%, respectively, $P = 0.037$). The relation between proliferative responses and HLA status were also examined, but no significant correlations were observed.

**DISCUSSION**

Given the strong influence of HLA-DQ alleles on the development of type 1 diabetes, a T-cell–mediated disease, there is a surprising paucity of information relating to HLA-DQ–restricted autoreactive T-cells. Indeed, to date, there is only one published report (20) of islet antigen–specific T-cell clones restricted by HLA-DQ. This may simply reflect the observation that T-cell clones obtained from human peripheral blood are typically predominantly restricted by HLA-DR. On the other hand, it may be that the lack of knowledge regarding HLA-DQ–restricted autoreactive T-cells is in itself a key observation, indicating that HLA-DQ molecules do not frequently act as a restricting element for these cells. However, this would present a paradox, wherein HLA-DQ exerts a strong effect on the development of type 1 diabetes but that same effect does not operate through its role as a major restricting element for islet antigen–specific autoreactive T-cells.

To unravel these possibilities, we investigated the role of HLA-DQ–restricted T-cells in the response to the diabetes-associated autoantigens GAD65 and insulin in type 1 diabetic patients and their siblings. Current technology only allowed us to do this indirectly, by negating HLA-DQ effects through the use of monoclonal antibodies that block its function. The results presented demonstrate that HLA-DQ predominantly acts as a restricting element for a T-cell proliferative response to GAD and as a restricting element for the suppression of insulin-specific responses.

Consistent with other studies (10,21,22) demonstrating the presence of GAD65–specific T-cells in the peripheral blood of newly diagnosed type 1 diabetic patients, a positive proliferative response (SI >3) to GAD65 was
observed in 60% of patients and in a similar proportion (52%) of siblings. The sibling group represents a population at increased risk of autoimmunity compared with unrelated individuals. Indeed, many of the abnormalities observed in the immunophenotype of T-cells from individuals with type 1 diabetes, compared with unrelated non-diabetic individuals, are also present in their siblings (23–25). The magnitude of positive responses (SI >3) to GAD65 was significantly higher in the sibling group. This observation appears to be GAD65 specific and does not represent a generalized state of hyperresponsiveness in the sibling group, as demonstrated by the similar levels of proliferation in response to the recall antigen tetanus toxoid and insulin. The lower response in the type 1 diabetic patients may, however, be a consequence of chronic stimulation of these cells during the preclinical phase of diabetes, leading to replicative senescence, or may reflect differences in the availability of GAD65-specific T-cells in the peripheral circulation due to homing of these T-cells to the pancreas in individuals with type 1 diabetes.

Blocking HLA-DQ–restricted responses led to a reduction in proliferation in siblings and to a lesser degree in type 1 diabetic patients, demonstrating the presence of HLA-DQ–restricted, GAD65-specific T-cells with proliferative capacity. Although no additional functional information is available regarding the phenotype of the proliferating T-cells, the fact that these cells proliferate directly ex vivo in response to an antigen in a 5-day assay suggests that they are primed effector T-helper cells. These studies demonstrate that HLA-DQ does indeed act as a restricting element for GAD65-specific T-cells. The lack of published data on T-cell clones restricted by HLA-DQ may be due to a number of factors, including the preferential expansion of HLA-DR–restricted cells by HLA-DQ may be due to a number of factors, including the preferential expansion of HLA-DR–restricted cells in response to repeated in vitro stimulation, which is perhaps due to the relatively low level of expression of HLA-DQ relative to HLA-DR on antigen-presenting cells (26), or a cloning artifact due to differences in the stimulation and culture requirements of HLA-DQ–restricted cells.

Although blocking HLA-DQ reduced proliferation, it did not completely ablate the response, perhaps indicating the presence of additional GAD65-specific cells restricted by other class II alleles or by HLA class I.

Further analysis of the unaffected sibling group revealed that some individuals with an initially low response to GAD65 had T-cells that proliferated more vigorously when HLA-DQ–restricted responses were blocked (Fig. 3D). Although the difference between unblocked and HLA-DQ–blocked responses did not reach significance in this group (P = 0.06), the results suggest that in this group of siblings there may be an HLA-DQ–restricted suppression of proliferation to GAD65.

In contrast with the vigorous proliferation observed to GAD65, responses to insulin were present at a lower frequency and magnitude in both patients and siblings. However, blocking HLA-DQ led to a marked increase in proliferation to insulin in siblings, demonstrating the presence of a potent HLA-DQ–restricted suppression of the response to insulin in these individuals. Whereas blocking HLA-DQ in the type 1 diabetic group also revealed an increase in proliferation to insulin, this increase was of a slightly lesser magnitude and frequency and not statistically significant. In this study, the presence of specific suppressor cells is based on responsiveness when the action of the suppressor is blocked; this clearly relies on the presence of a response to be regulated. Thus, while the observation that there is a significant increase in proliferation to insulin when HLA-DQ is blocked in the sibling but not in the type 1 diabetic group warrants further investigation. It may not simply equate to a deficiency in suppressor function in the type 1 diabetic group, but also relates to the balance of suppressor and responder cells. As discussed above, responsiveness to GAD65 and insulin in the type 1 diabetic group could be affected by a number of factors, including replicative senescence, availability of T-cells in the peripheral circulation, or the effects of exogenous insulin administration. However, the significant positive correlation between the unblocked GAD65 and the HLA-DQ–blocked insulin response (but not unblocked GAD65 and unblocked insulin responses) in siblings may indicate a group of individuals who are predisposed to islet-specific autoimmunity, in whom the underlying response to insulin is suppressed by HLA-DQ–restricted T-cells. In vivo, it is unlikely that antigen-presenting cells acting at the site of β-cell damage would present a single antigen to T-cells but would rather present a number of antigens, including GAD65 and insulin. It is therefore possible that a suppressive response to insulin may also be able to suppress the response of GAD65-specific T-cells, and a form of infectious tolerance to islet cell antigens may be established (27). It is tempting to speculate that such mechanisms may be involved in protecting individuals who are prone to autoimmunity from disease. Further experiments, including mixed-antigen cultures, will be needed to investigate this further.

The immune response is naturally regulated by various mechanisms to prevent autoimmune disease. These mechanisms include populations of T-cells capable of suppressing or regulating the function of other immune effectors (28,29). A number of studies (30–32) have suggested that these regulatory mechanisms may be impaired in individuals with type 1 diabetes. However, to date, there is only one published study in human diabetes demonstrating the presence of islet antigen–specific regulatory T-cells. This study (33) identifies a population of T-cells (based on expression of CD45RA) that are capable of suppressing the response to insulin in patients with type 1 diabetes. It is unknown whether there is any relationship between these CD45RA-expressing suppressor cells and the HLA-DQ–restricted suppression of responses to insulin we observed in this study.

It has previously been suggested (15,16) that HLA-DQ may play a key role in the generation of suppressor cells. In light of recent findings demonstrating the thymic selection of self-antigen–specific suppressor T-cells (34), it is noteworthy that, whereas peripheral antigen-presenting cells all have a defined hierarchy of expression of class II alleles (HLA-DR–DP–DQ) (26), expression of HLA-DQ is much higher in the thymic medulla (35,36), a region demonstrated to contain cells expressing self-antigens such as insulin (37,38). It is possible that these thymic cell types may be involved in the education of HLA-DQ–restricted T-cells with an antigen-specific suppressor phe-
notype. To speculate further, it is plausible that different levels (or locations) of thymic autoimmune expression may explain why HLA-DQ can act as a restriction element for both effector and suppressor cells within an individual, acting predominantly as a restriction element for proliferative responses to GAD65 and as a restriction element for the suppression of responses to insulin. For example, it has been reported (37) that insulin may be expressed at higher levels than GAD65 in the thymus and may therefore be more available for the generation of insulin-specific regulatory cells.

Our data address a fundamental role of DQ-restricted T-cell responses to particular islet autoantigens in general (and less in the relationship with type 1 diabetes) because the responses of type 1 diabetic patients are quite similar to those of their unaffected siblings. This is in line with previous studies (23–25,31) indicating that the degree of T-cell autoimmunity and proportions of lymphocyte subpopulations are quite similar between patients and their siblings but different from unrelated unaffected subjects. Furthermore, preliminary data on the role of HLA-DQ on T-cell reactivity against tetanus toxoid provide no evidence for regulated immunity against this vaccine recall antigen, suggesting that the phenomenon of DQ-associated suppression could be more specifically confined to autoantigens.

As the present study investigated individuals with type 1 diabetes and their unaffected siblings, the prevalence of the major diabetes HLA-DQ-predisposing molecules was high. Although these alleles were more prevalent in the type 1 diabetic group, there were no significant correlations between proliferative responses and HLA type. As discussed earlier, genes encoding HLA-DQ molecules are associated with both susceptibility to and dominant protection from type 1 diabetes, and it is possible that susceptibility may be equated to a lack of effective protection. Clearly, it will be important to examine responses to islet autoantigens in individuals expressing a range of HLA-DQ molecules, especially those encoded by both susceptibility and protective HLA-DQ molecules, to determine the relation between restriction element and the phenotype of the T-cell response.

In summary, we have demonstrated that HLA-DQ acts as a restriction element for both proliferative and suppressive responses to islet autoantigens in an antigen-specific manner. Furthermore, the effective HLA-DQ-mediated suppression of responses seen in siblings may be involved in the protection of these individuals from autoimmune disease.

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REFERENCES


