The frequencies of autoantibodies against glutamic acid decarboxylase 65 (GAD65) and islet cell antigen (ICA) 512/IA-2 (512/IA-2) are functions of the specific human leukocyte antigen (HLA) in type 1 diabetes mellitus (T1D). We investigated the association of HLA class II (DR and DQ) alleles and haplotypes with the presence of GAD and IA-2 autoantibodies in T1D. Autoantibodies were tested in 88 Tunisian T1D patients and 112 age- and gender-matched normoglycemic control subjects by enzyme immunoassay. Among T1D patients, mean anti-GAD antibody titers were higher in the DRB1*030101 allele (P < 0.001), together with the DRBI*030101/DQB1*0201 (P < 0.001) and DRB1*040101/DQB1*0302 (P = 0.002) haplotypes, while lower anti-GAD titers were associated with the DRBI*070101 (P = 0.001) and DRB1*110101 (P < 0.001) alleles and DRB1*070101/DQB1*0201 (P = 0.001) and DRB1*110101/DQB1*030101 (P = 0.001) haplotypes. Mean anti-IA-2 antibody titers were higher in the DRB1*040101 allele (P = 0.007) and DRB1*040101/DQB1*0302 (P = 0.001) haplotypes but were lower in the DRB1*110101 allele (P = 0.010) and the DRB1*110101 (P < 0.001) and DRB1*110101/DQB1*030101 (P = 0.025) haplotypes. Multinomial regression analysis confirmed the positive association of DRB1*030101 and the negative association of DRB1*110101 and DQB1*030101, along with the DRB1*070101/DQB1*0201 and DRB1*110101/DQB1*030101 haplotypes, with anti-GAD levels. In contrast, only the DRB1*040101/DQB1*0302 haplotype was positively associated with altered anti-IA-2 titers. Increased GAD65 and IA-2 antibody positivity is differentially associated with select HLA class II alleles and haplotypes, confirming the heterogeneous nature of T1D.
TABLE 1. HLA-DRB1* and -DQB1* allele distribution

| Allele/haplotype | Frequency (n = 88) | Frequency (n = 112) | p* | p*b |
|------------------|-------------------|---------------------|-----|-----|
| DRB1*030101      | 0.364c             | 0.147               | 3.6 × 10^-4 | 0.006 |
| DRB1*040101      | 0.239               | 0.116               | 7.5 × 10^-3 | 0.120 |
| DRB1*070101      | 0.068               | 0.214               | 1.8 × 10^-4 | 0.0003 |
| DRB1*090101      | 0.028               | 0.000               | 0.023          | 0.362 |
| DRB1*100101      | 0.023               | 0.063               | 0.051          | 0.816 |
| DRB1*110101      | 0.028               | 0.107               | 1.7 × 10^-3 | 0.027 |
| DQB1*0201        | 0.426               | 0.290               | 0.054          | 0.377 |
| DQB1*030101      | 0.097               | 0.201               | 0.001          | 0.007 |
| DQB1*0302        | 0.216               | 0.094               | 0.002          | 0.012 |
| DQB1*060101      | 0.080               | 0.179               | 0.006          | 0.041 |
| DQB1*060201      | 0.287               | 0.065               | <0.001         | <0.001 |
| DQB1*070101      | 0.184               | 0.073               | 0.001          | 0.010 |
| DQB1*070201      | 0.068               | 0.177               | 0.002          | 0.015 |
| DQB1*110101      | 0.023               | 0.097               | 0.005          | 0.036 |

a Determined by Fisher’s exact test; boldface indicates significant differences.

b P is the corrected P value for the number of alleles tested, calculated using the Bonferroni method.

MATERIALS AND METHODS

Subjects. The study subjects comprised 88 unrelated T1D patients (44 males and 44 females; mean age, 16.4 ± 7.7 years). The diagnosis of T1D was based on clinical features and laboratory data. All T1D patients were ketosis prone, lacked endogenous insulin secretion, and were dependent on insulin for controlling hyperglycemia. T1D patients were not obese, were free of any concomitant complications, and were not receiving additional treatment at the time of blood collection. Patients with other forms of diabetes (latent autoimmune diabetes of adults, maturity onset diabetes of the young, or type 2 diabetes) were excluded. Control subjects consisted of 112 university students and healthy children (65 males and 47 females; age, 26.2 ± 5.8 years) who had normal glucose tolerance and no family history of TID or other autoimmune diseases. All patients and control subjects were Tunisian Arabs, were from central Tunisia, and were asked to sign a consent form according to the study protocol, and all institutional ethics requirements were met.

HLA-DRB1 and -DQB1 genotyping. HLA-DRB1- and -DQB1 gene alleles were analyzed using the PCR sequence-specific-primer (SSP) technique, using the MicroTaq SSP Generic HLA Class II (DRB/DQB) DNA Typing kit (lot 05A), according to the manufacturer’s specifications (One Lambda, Thousand Oaks, CA). PCR products were analyzed on ethidium bromide-stained agarose gels. HLA allele nomenclature was as previously reported (15). In total, 16 DRB1 and 7 DQB1 alleles were tested.

Autoantibody screening. IA-2 and GAD-65 autoantibodies were measured at the time of initial diagnosis for T1D patients. Serum samples were obtained from all participants and were stored as small aliquots at or below −40°C. Anti-IA-2 and anti-GAD-65 antibodies were measured on two separate occasions by enzyme-linked immunosorbent assay (ELISA) (Kronus Inc., Boone, ID). Cutoff values for antibody positivity were based on the 99th percentile of antibody levels obtained in nondiabetic controls. The results were expressed as antibody titer (arbitrary units [AU]/ml) or as the percent antibody positive out of the total.

Statistical analysis. Statistical analysis was performed on SPSS v. 17.0 (SPSS Inc., Chicago, IL). Allele frequencies were determined by the gene-counting method, using HLAStat 2000 software, and haplotype frequencies were determined by the maximum-likelihood method. P values were corrected for the number of different alleles or haplotypes tested (Pc) using the Bonferroni inequality method [Pc = 1 − (1 − P)k]. Data were expressed as P values, odds ratios (OR), and 95% confidence intervals (CI) between patients and controls. The Spearman correlation coefficient was used to determine correlation between the level of autoantibodies and HLA alleles and haplotypes. Antibody titers were expressed as mean ± standard deviation (SD); differences between cases and controls were made using a two-tailed Student t test. Logistic regression analysis was performed in order to determine the OR and 95% CI associated with TID risk, taking the controls as the reference group. Statistical significance was set at a P value of <0.05.

RESULTS

HLA allele and haplotype frequencies. Significant DRB1 allelic differences were seen between T1D patients and controls. These comprised DRB1*030101 (Pc = 0.006), which was higher, and DRB1*070101 (Pc = 0.003) and DRB1*110101 (Pc = 0.027), which were lower among patients (Table 1). Similarly, significant DQB1 allelic differences were seen at the DQB1 locus, which comprised DQB1*0302 (Pc = 0.012), which was higher, and DQB1*030101 (Pc = 0.007) and DQB1*060101 (Pc = 0.041), which were lower among patients than among control subjects (Table 1). In addition, the frequencies of DRB1*030101/DQB1*0201 (Pc < 0.001) and DRB1*040101/DQB1*0302 (Pc = 0.010) were higher, while those of DRB1*070101/DQB1*0201 (Pc = 0.015) and DRB1*110101/DQB1*030101 (Pc = 0.036) were lower in T1D patients than in control subjects, thereby conferring TID susceptibility and protection, respectively, on these haplotypes (Table 1).

Correlation studies. We examined the functional attributes of HLA alleles and haplotypes on autoantibody levels among T1D patients. Table 2 summarizes the correlation between the level of autoantibodies and HLA alleles and haplotypes among T1D patients positive for a specific allele and haplotype; patients negative for that allele or haplotype served as controls. Anti-GAD levels were positively correlated with the DRB1*030101 allele (r² = 0.378; P < 0.001) and the DRB1*030101/DQB1*0201 (r² = 0.572; P < 0.001) and DRB1*040101/DQB1*0302 (r² = 0.284; P = 0.001) haplotypes. Anti-GAD titers were negatively associated with the protective DRB1*070101 (r² = −0.322; P = 0.001), DRB1*110101 (r² = −0.345; P < 0.001), and DQB1*030101 (r² = −0.294; P = 0.002) alleles, along with the DRB1*070101/DQB1*0201 (r² = −0.328; P = 0.001) and DRB1*110101/DQB1*030101 (r² = −0.346; P = 0.001) haplotypes. In compar-

TABLE 2. Correlation studies

| Allele/haplotype | Anti-GAD | Anti-I2 |
|------------------|----------|---------|
|                  | p²       | p       | p²     | p     |
| Allelesa         |          |         |        |       |
| DRB1*030101      | 0.378    | 6.5 × 10^-5 | 0.074  | 0.563 |
| DRB1*040101      | 0.122    | 0.211   | 0.219  | 0.084 |
| DRB1*070101      | −0.322   | 0.001   | −0.185 | 0.148 |
| DRB1*110101      | −0.345   | 3.2 × 10^-4 | −0.329 | 0.009 |
| DQB1*030101      | −0.294   | 0.002   | −0.220 | 0.083 |
| DQB1*060101      | −0.129   | 0.189   | −0.009 | 0.945 |
| DQB1*0302        | 0.284    | 0.003   | 0.312  | 0.013 |
| DQB1*030101      | 0.034    | 0.009   | 0.343  | 0.006 |

a Spearman correlation coefficient.
b Only alleles and haplotypes significantly associated with altered risk of TIDM in Tunisians (7).

Boldface indicates statistical significance.
ison, anti-IA-2 levels were positively correlated only with the DRB1*040101/DQB1*0302 (r² = 0.312; P = 0.013) haplotype but were negatively correlated with the DRB1*110101 allele (r² = -0.329; P = 0.009) and the DRB1*110101/DQB1*030101 (r² = -0.343; P = 0.006) haplotype.

**Anti-GAD and anti-IA-2 antibody titers.** The results in Table 3 show that the mean anti-GAD antibody titers were higher in TID patients positive for the DRB1*030101 allele (P < 0.001) and the DRB1*030101/DQB1*0201 (P < 0.001) and DRB1*040101/DQB1*0302 (P = 0.002) haplotypes. Mean anti-GAD antibody titers were lower in carriers of the DRB1*070101 (P = 0.001) and DRB1*110101 (P < 0.001) alleles and in the DRB1*070101/DQB1*0201 (P = 0.001) and DRB1*110101/DQB1*0201 (P = 0.001) haplotypes. In comparison, mean anti-IA-2 antibody titers were higher in TID patients positive for the DRB1*040101 (P = 0.007) allele and the DRB1*040101/DQB1*0302 (P = 0.001) haplotypes but were lower in carriers of the DRB1*110101 (P = 0.010) and DRB1*110101 (P < 0.001) alleles and the DRB1*110101/ DQB1*030101 (P = 0.025) haplotype.

**Regression analysis.** The selective association of DRB1 and DQB1 alleles and haplotypes with altered anti-GAD and anti-IA-2 elevated antibody titers were confirmed by regression analysis after controlling for potential covariates. Multinomial regression analysis confirmed the positive association of the DRB1*030101 allele and the DRB1*030101/DQB1*0201 haplotype and the negative association of the DRB1*110101 and DQB1*030101 alleles and the DRB1*070101/DQB1*0201 and DRB1*110101/DQB1*030101 haplotypes with altered anti-GAD levels. In contrast, only the DRB1*040101/DQB1*0302 haplotype was positively associated with altered anti-IA-2 titers (Table 4).

**DISCUSSION**

TID is an organ-specific autoimmune disease resulting in T cell-mediated destruction of pancreatic β islet cells. TID is also distinguished by the presence of a number of autoantigens (3, 6, 12, 27). GAD and IA-2 are two of the major and best-characterized autoantigens. Several studies have demonstrated that HLA-DQ and -DR alleles influence TID susceptibility (1, 20, 21, 29), and TID-predisposing or -protective HLA-DQ and -DR alleles were identified (1, 29). Apart from the regulation of the autoreactive T cell repertoire by susceptible and protective HLA molecules, presentation of autoantigenic peptides by specific HLA molecules or induction of regulatory T cells (Treg) explains, at least in part, the modulatory capacity of HLA variants on the overall TID risk (8).

This study is the first report on the association of HLA class II alleles and haplotypes and antibody titers with TID in Tunisia. We previously demonstrated that the contribution of HLA haplotypes to T1DM genetic susceptibility among Tuni- sians depends on specific HLA class II haplotypes (29). The results showed that anti-GAD antibody levels were higher in DRB1*030101 allele carriers, in agreement with recent findings in the Saudi population (14) but in partial agreement with an earlier study on Taiwanese subjects, in which anti-GAD positivity was associated with DR3 and DR4 (4). These differences are likely attributable to racial differences in the contribution of HLA class II alleles and haplotypes to TID pathogenesis (2, 8, 11). Our study demonstrated that the prevalence and increased titer of anti-GAD antibodies are associated with

## Table 3. Antibody titers in HLA class II alleles and haplotypes

| Allele/haplotype | TID 30.90 (± 24.52) | Normal 21.89 (± 20.49) | Z | P |
|------------------|---------------------|-------------------------|---|---|
| DRB1*030101      | 73.10 ± 59.51       | 28.16 ± 17.08           | 3.87 | <0.001 |
| DRB1*040101      | 64.83 ± 52.68       | 52.93 ± 40.52           | 1.26 | 0.209 |
| DRB1*070101      | 37.34 ± 21.42       | 60.08 ± 31.45           | -3.30 | 0.001 |
| DRB1*110101      | 11.24 ± 4.35        | 58.62 ± 43.92           | -3.51 | <0.001 |
| DQB1*030101      | 17.22 ± 14.03       | 56.79 ± 39.74           | -3.01 | 0.001 |
| DQB1*030201      | 26.68 ± 24.27       | 53.22 ± 49.19           | -1.32 | 0.331 |
| DQB1*030101/DQB1*0201 | 114.72 ± 42.99   | 24.09 ± 25.39           | 2.77 | <0.001 |
| DQB1*040101/DQB1*0302 | 111.29 ± 49.97   | 52.12 ± 38.92           | 0.786 | 0.007 |
| DQB1*040101/DQB1*0302 | 19.34 ± 15.36   | 60.24 ± 56.90           | -3.24 | 0.001 |
| DQB1*110101/DQB1*030101 | 17.32 ± 15.56   | 98.30 ± 50.10           | -3.25 | 0.001 |

**Note:** Positive, patients carrying the specific allele/haplotype; negative, other patients; Z, Z score. The values are means ± SD antibody titers (AU/ml); differences in antibody titers between the patient groups were determined using a two-tailed Student t test.

| Allele/haplotype | Antibodies | Z | P |
|------------------|------------|---|---|
| DRB1*030101      | Anti-GAD   | 3.87 | <0.001 |
| DRB1*040101      | Anti-GAD   | 1.26 | 0.209 |
| DRB1*070101      | Anti-GAD   | -3.30 | 0.001 |
| DRB1*110101      | Anti-GAD   | -3.51 | <0.001 |
| DQB1*030101      | Anti-GAD   | -3.01 | 0.001 |
| DQB1*030201      | Anti-GAD   | -1.32 | 0.331 |
| DQB1*030101/DQB1*0201 | Anti-GAD | 2.77 | <0.001 |
| DQB1*040101/DQB1*0302 | Anti-GAD | 0.786 | 0.007 |
| DQB1*040101/DQB1*0302 | Anti-GAD | -3.24 | 0.001 |
| DQB1*110101/DQB1*030101 | Anti-GAD | -3.25 | 0.001 |

## Table 4. Matched odds ratios associated with antibody titers in class II alleles and haplotypes

| Allele/haplotype | Anti-GAD | Anti-IA2 |
|------------------|----------|----------|
| DRB1*030101      | aOR (95% CI) | aOR (95% CI) |
| DRB1*040101      | 5.23 (2.21–12.40) | 1.37 (0.60–3.09) |
| DRB1*070101      | 1.52 (0.61–3.78) | 2.00 (0.91–2.81) |
| DQB1*030101      | 0.93 (0.31–2.70) | 0.424 (0.21–1.70) |
| DQB1*030201      | 0.75 (0.66–0.86) | 0.269 (0.13–0.58) |
| DQB1*030101      | 0.21 (0.07–0.67) | 0.424 (0.21–1.70) |
| DQB1*040101      | 0.14 (0.04–0.46) | 0.144 (0.03–0.47) |
| DQB1*040101      | 15.76 (5.83–42.57) | 1.51 (0.87–2.64) |
| DQB1*030101      | 0.21 (0.07–0.67) | 0.168 (0.08–0.34) |
| DQB1*030201      | 0.75 (0.66–0.86) | 0.380 (0.20–0.77) |

**Note:** Only alleles and haplotypes significantly associated with altered risk of T1DM in Tunisians (7).

**Boldface indicates statistical significance.**
CD4+ T cells present on antigen-presenting cells have critical roles for the activation of helper cell reactivity. HLA class II molecules expressed on antigen-presenting cells contribute to susceptibility to T1D, and it is likely that anti-GAD positivity and IA-2 positivity may progress toward tissue destruction or protection (17, 30). The genetic modifiers of susceptibility, which are also attributed to HLA molecules, the dominant genetic association with T1D is that primary mechanistic influence by biasing or adding to the specific subtype of DR4 present on the same haplotype (30). It is tempting to speculate that this is based on the selection of specific dominating epitopes favored during disease progression (12, 21, 30). Other genetic modifiers of susceptibility, which are also attributed to HLA molecules, the dominant protection conferred by HLA-DQ alleles, may also have a primary mechanistic influence by biasing or adding to the specific epitope recognition cascade (12, 17, 30).

In this way, T1D can be viewed as a disease of epitope selection and immunological focusing, whereby autoimmunity may progress toward tissue destruction or protection (17, 30). While specific HLA alleles and haplotypes clearly impact susceptibility to T1D, it is likely that anti-GAD positivity and T1D-susceptible locus HLA types translate into increased T cell reactivity. HLA class II molecules expressed on antigen-presenting cells have critical roles for the activation of helper T cells (mainly CD4+ T cells) and may influence antibody status in the periphery. This was highlighted by the findings of Itoh et al., in which HLA DR9/X-positive T1D patients had significantly higher numbers of GAD-reactive IFN-γ-producing T cells than those with other loci (9).

HLA polymorphism affects autoimmune responses according to the binding capacities of antigenic peptides, and the repertoire of T cell receptors of reactive T cells (25) and different HLA-DR/DQ molecules might have different binding affinities to disease-associated peptides (18). These facts may explain our results showing unique and different HLA associations with autoantibodies from the Caucasian populations. In conclusion, our study of TIDM among Tunisians reveals that both GAD and IA-2 antibodies and the HLA-DR and -DQ alleles are critical in determining the risk for the disease.

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