Journal of Genetic and Environmental Resources Conservation, 2014, 2(1): 1-9.
www.jgerc.com

Profile of HLA-class I alleles, autoantibodies and C-peptide in type 1 diabetes mellitus patients and their sibs

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Abstract

The association between HLA-class I alleles (A, B and C) and type 1 diabetes mellitus (T1DM) was investigated in 25 patients, 25 sibs and 25 controls. Serum level of anti-GAD (glutamic acid decarboxylase) and anti-IA-2 (tyrosine phosphatase) autoantibodies and C-peptide was also assessed in addition to anti-cytomegalovirus (CMV) and anti-rubella antibodies. For HLA, only A*1 allele showed a corrected significant (Pc = 2.3 $\times 10^{-3}$) increased frequency in patients as compared with controls (56.0 *vs*. 8.0%). With respect to serum level of autoantibodies and C-peptide; anti-GAD was significantly increased in patients (51.66 IU/ml) as compared with patient's sibs (5.44 IU/ml) or controls (5.49 IU/ml). Anti-IA-2 antibody behaved in a similar manner (35.31 *vs*. 13.86 and 10.94 IU/ml, respectively), while C-peptide was significantly decreased in patients and their sibs (9.65 and 6.55 ng/ml, respectively) as compared with controls (16.66 ng/ml). Assessment of anti-viral antibodies revealed that 28% of patient's sera were positive for anti-CMV antibody, while it was less frequent in patient's sibs or controls (12 and 8%, respectively). Anti-rubella antibody positive cases shared the frequency of anti-CMV antibody in patients (28%), but the frequency was higher in patient's sibs (36%), while in controls, it was 16%.

Keywords: HLA, T1 Diabetes mellitus, Anti-GAD, Anti-IA2, C-peptide, Cytomegalovirus, Rubella.

Introduction

Type 1 diabetes mellitus (T1DM) is the most common metabolic disease found in children and adolescents and affects around 4 million people in USA and Europe, and it seems that the disease is increasing; and this will certainly impact public health (Forouhi and Wareham, 2006). It is a chronic disease caused by the absence of insulin synthesis, by its secretion or action defects, and it is associated with a damage and long term dysfunction of several organs and tissues (Craig *et al*., 2009).

T1DM is an autoimmune disease characterized by a variable silent period before the overt disease occurs, resulting in a destruction of β cells. Primary damage is due to a cell-mediated immune response where T helper 1 (TH1; CD4+) cells activate *in situ* specific T cytotoxic (Tc; CD8+) cells directed against β cells. These cells show an increased expression of MHC class I and class II molecules that have been confirmed in biopsies of T1DM patients (Stadinski *et al*., 2010). However, autoantibodies against several fractions are produced secondarily; for instance, anti-glutamic acid decarboxylase (GAD), anti-islet cell (ICA), anti-cytoplasmic (ICCA), anti-insulin (IAA) and anti-tyrosine phosphatase (IA-2) autoantibodies, but, since they are found in pre-diabetic first degree family members of T1DM, they are important in the predictive evaluation of the disease (La Torre, 2012). Therefore, autoantibodies comprise a very useful tool for an early diagnosis. However, even genetically susceptible individuals who develop islet cell antibodies do not necessarily develop T1DM, implying that individuals with existing autoimmunity to the pancreas need a trigger to develop the destructive autoimmunity that destroys β cells (Nokoff and Rewers, 2013).

Environmental factors such as diet and viral infections have been associated with an increased risk of T1DM in genetically susceptible individuals. Some viruses have been claimed to unchain the disease; inducing autoimmunity or facilitating this process by molecular mimicry or non-specific activation mediated by cytokines. Homology between a highly conserved sequence of Coxackie B4 (P2C) and GAD-65 has been found (Yoon and Jun, 2004). The response is probably not T1DM specific, since antibodies have been found in healthy controls as well (van der Werf *et al*., 2007). Early exposure to enterovirus may induce autoimmunity, because 10–20% of children with congenital rubella infection and

specific human leukocyte antigen (HLA) susceptibility markers have a greater chance to develop T1DM (Strom, 2009).

The HLA region encodes several molecules that play key roles in the functions of immune system, and a strong association between HLA alleles and autoimmune diseases (AID) has been established for over fifty years, especially those components of HLA class II encoded HLA-DRB1-DQA1-DQB1 haplotype (Klein and Sato, 2000). Molecules encoded by this region play a key role in exogenous antigen presentation to CD4+ T helper cells, indicating the importance of this pathway in AID initiation and progression, and although other components of HLA region (i.e. class I and III subregions) have also been investigated for association with AID, apart from the association of HLA-B*27 with ankylosing spondylitis, it has been difficult to determine additional susceptibility loci independent of the strong linkage disequilibrium (LD) with HLA class II genes (Petrovsky and Brusic, 2004). However, recent advances in statistical analysis of LD and the recruitment of large AID datasets have allowed investigation of the HLA class I region to be re-visited, and association of HLA class I region, independent of known HLA class II effects, has now been detected for several AIDs, including strong association of HLA-B locus with T1DM (Nejentsev *et al*., 2007) and HLA-C locus with multiple sclerosis and Graves' disease (Gough and Simmonds, 2007). These results provide further evidence of a possible role for bacterial or viral infection and CD8+ T cells in AID onset. Worldwide studies and many other individual reports have clearly shown that HLA-class II loci have the most intense susceptibility determinants for T1DM (Gorodezky *et al*., 2006), and although, the strongest contribution is widely recognized to come from the class II DRB1 and DQB1 genes, various reports have indicated that DPB1, class I and class III loci can modify susceptibility to this disease. Accordingly, other genes within the MHC have been suggested, because it has been found that HLA-class I alleles may modify the risk of expression, and therefore influencing the evolution, age at onset and the degree of β cell destruction (Nejentsev *et al*., 2007).

Accordingly, the present study was planned with the aim to shed light on the association between HLA-class I alleles (A, B and Cw) and T1DM, in the ground of their individual frequencies or two-locus association in patients and their sibs who were younger than the patients. Equally important, the serum level of anti-GAD and anti-IA-2 autoantibodies and C-peptide was assessed in terms of their means in patients or their sibs; furthermore, the impact of HLA alleles on these levels was also determined. Finally, the viral aetiology of T1DM was targeted through assessment of subject's sera for two anti-viral antibodies (anti-cytomegalovirus and anti-rubella). Such collective evaluations may help for a further understanding of the immunogenetic background of T1DM in a sample of Iraqi patients.

Materials and Methods

A total of 25 T1DM patients (12 males and 13 females) were enrolled in the study. They were at the early onset of disease (less than four months); with an age range of 8-19 years at the time of investigation. The patients were referred to the out-patient clinic of National Diabetes Centre, Al-Mustansiriyah University (Baghdad; January-March 2009) and Ibn Balady Hospital (Baghdad; March-June 2009) for diagnosis, treatment or follow-up evaluation, which was carried out by the consultant medical staff at these clinics. Patients were free of acute illness or infection at the time of study. The diagnosis of T1DM was made on the basis of recommended criteria by WHO (1999). In addition to patients, further two groups were also investigated. The first included 25 sibs (15 males and 10 females) of patients who were one year younger and were healthy and not diabetic. The second group included 25 apparently healthy subjects, but they had no evidence of diabetes or a history of diabetes in their nuclear families (grandparents, parents and sibs). They were age- (6-19 years), gender- (13 males 12 and females) and ethnicity- (Iraqi Arabs) matched with patients.

Venous blood (8 ml) was collected from each subject and transferred immediately to two different test tubes (heparin tube; 5 ml and plain tube; 3 ml). Heparinized blood was subjected to a density gradient centrifugation using lymphoprep (Evans, England) as a separating medium to collect lymphocytes. The collected cells were phenotyped for HLA-class I alleles by the microlymphocytotoxicity test (Ad'hiah, 1990), using a panel of monoclonal antibodies (Biotest, Germany) that were able to recognize 8 HLA-A (A*1, A*2, A*3, A*9, A*10, A*11, A*19 and A*28), 16 HLA-B (B*5, B*7, B*8, B*12, B*13, B*14, B*15, B*16, B*17, B*18, B*21, B*22, B*27, B*35, B*37 and B*40) and 7 HLA-C (Cw*1, Cw*2, Cw*3, Cw*4, Cw*5, Cw*6 and Cw*7) alleles on the surface of tested lymphocytes. Serum collected from clotted blood was used for assessments of anti-GAD and -IA-2 autoantibodies (Euroimmun, UK) and C-peptide (DRG Instruments, Germany), as well as, anti-CMV and -rubella antibodies (Biokit, USA) by means of ELISA method using commercial kits.

Statistical Analysis: The association between HLA alleles and T1DM was expressed in terms of relative risk (RR), etiological fraction (EF) and preventive fraction (PF). The RR value can range from less than one (negative association) to more than one (positive

association). If the association was positive, the EF was calculated, while if it was negative, the PF was calculated. The significance of such association (positive or negative) was assessed by Fisher's exact probability (P), which was corrected for the number of alleles tested at each locus (Pc). Calculations of such parameters were carried out using the computer Programmes for Epidemiologists (PEPI) version 4.0. The HLA system was further characterized in terms of gene frequencies of its alleles, in which square-root formula was applied, and from gene frequencies, expected percentage frequency of two-locus association between two alleles was estimated (Ad'hiah, 1990). Anti-GAD, anti-IA-2 and C-peptide parameters were analyzed using the statistical package SPSS version 13.0. Their data were given as mean ± standard error (S.E.), and differences between means were assessed by ANOVA followed by LSD or Duncan's tests. The difference was considered significant when P value was ≤ 0.05.

Results and Discussion

HLA Allele Frequencies

At HLA-A locus, A*1 (56.0 *vs*. 8.0%) and A*10 (36.0 *vs*. 12.0%) alleles showed a significant increased percentage in T1DM patients as compared with controls, while A*11 was presented with a significant decreased percentage in patients (4.0 *vs*. 24.0%). However, a corrected significant level (Pc = 2.3 $\times 10^{-3}$) was maintained for A*1 only, in which the RR was 14.64, and the EF was 0.52. Such allele also maintained a corrected significant (Pc = 4.1$\times 10^{-4}$) increased percentage (56.0 *vs*. 4.0%) in patients as compared to their sibs, but the RR value was almost doubled (RR = 30.55) and the EF was also increased to 0.54. Comparing diabetic sibs with controls revealed variation in only one HLA-A allele, which was A*10 (40.0 *vs*. 12%), but the difference was significant before correction (P = 0.025). At HLA-B locus, only allele B*35 showed an increased frequency in T1DM patients as compared with controls (24.0 *vs*. 4%), and such difference was associated with RR value of 7.58 and EF value of 0.21, but again the P value was significant before correction (P = 0.049). Antigen B*8, which is reported in the literature to be increased in T1DM patients, showed an increased frequency in the patients as compared to controls (24.0 *vs*. 12.0%) but the difference failed to attend any significant level (P > 0.05). For HLA-C locus, T1DM patients showed no deviation in allele frequencies as compared to controls, but patient's sibs contradicted such observation and two alleles demonstrated differences in comparison with controls. Cw*1 (28.0 *vs*. 4.0%) and Cw*2 (32.0 *vs*. 4%) alleles showed increased frequencies in patient's sibs, but the differences were significant before correction (P = 0.024 and 0.012, respectively); however, such differences accounted for RR value of 9.33 and 11.29, respectively and EF value of 0.25 and 0.29, respectively. Comparing patients with their sibs also revealed no significant variation in HLA-C alleles (Table 1).

Table (1): HLA alleles showing significant variations between type 1 diabetes mellitus patients, their sibs and controls.

HLA Alleles	No.	%	No.	%	RR	EF	PF	P	Pc
	Type 1 Diabetes Mellitus Patients *versus* Controls								
A*1	14	56.0	2	8.0	14.64	0.52	-	2.9×10^{-4}	2.3×10^{-3}
A*10	9	36.0	3	12.0	4.13	0.27	-	0.048	N.S.
A*11	1	4.0	6	24.0	0.13	-	0.19	0.049	N.S.
B*35	6	24.0	1	4.0	7.58	0.21	-	0.049	N.S.
	Type 1 Diabetes Mellitus Patient's Sibs *versus* Controls								
A*10	10	40.0	3	12.0	4.89	0.32	-	0.025	N.S.
Cw*1	7	28.0	1	4.0	9.33	0.25	-	0.024	N.S.
Cw*2	8	32.0	1	4.0	11.29	0.29	-	0.012	N.S.
	Type 1 Diabetes Mellitus Patients *versus* their Sibs								
A*1	14	56.0	1	4.0	30.55	0.54	-	5.1×10^{-5}	4.1×10^{-4}

RR: Relative risk; EF: Etiological fraction; PF: Preventive fraction; P: Probability; Pc: Corrected P; N.S.: Not significant.

HLA Gene Frequencies and Two-Locus Association

The gene frequencies of HLA alleles were estimated for two purposes. For the first, it was aimed to shed light on the most frequent allele that may characterize each investigated group, while for the second, which was the more important, included estimation of two-locus association between alleles that showed variations (A*1, A*10, B*8, B*35, Cw*1 and Cw*2). With respect to the first aim, T1DM patients were mainly characterized by the allele A*1, which scored the highest frequency (gene frequency = 0.337) and accounted for more than 30% of the total pool of HLA-A alleles, while at HLA-B and -C loci, the corresponding alleles were B*12 and Cw*6 (0.152 and 0.226, respectively). The corresponding alleles in patient's sibs were A*10 (0.226), B*5, B*12 and B*17 (each with a gene frequency of 0.106) and Cw*4 (0.226), while they were A*19 (0.252), B*5 (0.226) and Cw*4 (0.106) in controls (Data not shown).

Two HLA-locus associations between two alleles belong to two HLA loci were estimated from gene frequencies (expected percentage frequencies) of the respective alleles, and at the same time, the co-occurrence of the two alleles was inspected in each subject and was presented as an observed percentage frequency in each group. No statistical analysis was carried out between the observed and expected numbers because of low sample size (Table 2). As shown in the table, most of the observed frequencies were higher than the expected frequencies in the three investigated groups. However, certain HLA allelic combinations occurred more frequently (observed and expected frequencies) in the patients (A1-B8, A1-Cw1, A1-Cw2, A10-B8, A10-B35 and B8-Cw1), while increased observed and expected frequencies of A10-Cw1 and A10-Cw2 were observed in patient's sibs in comparison with T1DM patients or controls.

Table (2): Observed and expected percentage frequencies of two-HLA-locus combinations in type 1 diabetes mellitus patients, their sibs and controls.

HLA Combination	Percentage Frequency					
	Diabetic Patients (No.= 25)		Controls (No.= 25)		Patient's Sibs (No.= 25)	
	Observed	Expected	Observed	Expected	Observed	Expected
A1-B8	**16.0**	**4.347**	4.0	0.254	4.0	0.130
A1-B35	4.0	4.347	0.0	0.086	0.0	0.086
A1-Cw1	**8.0**	**2.830**	0.0	0.086	8.0	0.319
A1-Cw2	**12.0**	**3.572**	0.0	0.086	4.0	0.369
A10-B8	**8.0**	**2.580**	4.0	0.384	0.0	1.401
A10-B35	**8.0**	**2.580**	0.0	0.130	0.0	0.936
A10-Cw1	**8.0**	**1.680**	0.0	0.130	**12.0**	**3.435**
A10-Cw2	**12.0**	**2.120**	4.0	0.130	**28.0**	**3.977**
A11-B8	4.0	0.270	0.0	0.799	4.0	0.657
A11-B35	0.0	0.270	4.0	0.434	0.0	0.270
A11-Cw1	0.0	0.176	0.0	0.270	4.0	1.611
A11-Cw2	0.0	0.222	0.0	0.270	4.0	1.865
B8-Cw1	**8.0**	**1.083**	0.0	0.130	0.0	0.942
B8-Cw2	4.0	1.367	0.0	0.130	0.0	1.091
B35-Cw1	4.0	1.083	0.0	0.044	4.0	0.623
B35-Cw2	4.0	1.367	0.0	0.044	0.0	0.721

Anti-GAD and -IA-2 Autoantibodies and C-peptide

Serum level mean of two autoantibodies (anti-GAD and -IA-2) and C-peptide was assessed in T1DM patients, their sibs and controls. With respect to anti-GAD antibody, it was significantly increased in patients (51.66 IU/ml) as compared to patient's sibs (5.44 IU/ml) or controls (5.49 IU/ml). Anti-IA-2 antibody behaved in a similar manner (35.31 *vs*. 13.86 and 10.94 IU/ml, respectively), while C-peptide showed a different variation; it was significantly decreased in patients and their sibs (9.65 and 6.55 ng/ml, respectively) as compared with controls (16.66 ng/ml) (Table 3).

HLA Impact on Autoantibodies and C-peptide

To understand the relationship between HLA alleles and the investigated parameters, serum level of each autoantibody and C-peptide was assessed in subjects (patients, patient's sibs and controls) positive or negative for specific HLA alleles. HLA alleles that showed variations in this regard were A*10 and Cw*2. With respect to anti-GAD antibody, patients positive for A*10 were presented with a significant decreased level as compared with A*10 negative

patients (19.79 *vs*. 62.91 IU/ml). Anti-IA-2 antibody acted in the patient's sibs, in which A*10 positive sibs had a significant increased level as compared with A*10 negative sibs (17.31 *vs*. 11.15 IU/ml). A much more clear effect was observed in C-peptide, which was significantly decreased in A*10 positive T1DM patients, their sibs and controls (3.32, 4.74 and 8.86 ng/ml, respectively) as compared with the corresponding A*10 negative subjects (12.63, 7.97 and 18.14 ng/ml, respectively) (Table 4). For Cw*2 allele, each antibody level showed its own variation in the three investigated groups. Patients positive for Cw*2 showed a decreased level of anti-GAD antibody as compared with negative patients (22.59 *vs*. 59.74 IU/ml). Anti-IA-2 antibody demonstrated a significant decreased level in positive patients (11.52 *vs*. 41.26 IU/ml), while it was significantly increased in positive patient's sibs (21.18 *vs*. 11.02 IU/ml) as compared with the corresponding Cw*2 negative subjects. The C-peptide showed a significant decreased level in Cw*2 positive sibs as compared with Cw*2 negative sibs (3.37 *vs*. 7.79 ng/ml) (Table 5).

Table (3): Serum level of anti-GAD and -IA-2 autoantibodies and C-peptide in type 1 diabetes mellitus patients, their sibs and controls.

Investigated Parameters	Mean ± S.E.*		
	Diabetic Patients (Number = 23)	Controls (No. = 25)	Patient's Sibs (No. = 25)
Anti-GAD (IU/ml)	51.66 ± 13.52^{A}	5.49 ± 0.03^{B}	5.44 ± 0.03^{B}
Anti-IA-2 (IU/ml)	35.31 ±12.07^{A}	10.94 ± 0.14^{B}	13.86 ± 2.88^{B}
C-peptide (ng/ml)	9.65 ± 2.58^{AB}	16.66 ± 4.15^{A}	6.55 ±1.06^{B}

***Different letters: Significant difference (P ≤ 0.05) between means of the same row.**

Table (4): Serum level of anti-GAD and -IA-2 autoantibodies and C-peptide in type 1 diabetes mellitus patients, their sibs and controls distributed by HLA-A*10 allele.

Investigated Parameters	HLA	Mean Serum Level ± S.E.		
		Diabetic Patients	Controls	Patient's Sibs
Anti-GAD (IU/ml)	A*10+ve	19.79 ± 14.22♦	5.59 ± 0.09	5.46 ± 0.04
	A*10-ve	62.91 ± 16.96	5.47 ± 0.03	5.42 ± 0.03
Anti-IA-2 (IU/ml)	A*10+ve	34.09 ± 15.63	10.84 ± 0.09	17.31 ± 6.56♦
	A*10-ve	35.89 ± 16.45	10.96 ± 0.17	11.15 ± 0.19
C-peptide (ng/ml)	A*10+ve	3.32 ± 0.57♦	8.86 ± 3.34♦	4.74 ± 1.30♦
	A*10-ve	12.63 ± 3.59	18.14 ± 4.86	7.97 ± 1.54

♦Significant difference between A*10+ve and A*10-ve subjects for each parameter.

Table (5): Serum level of anti-GAD and -IA-2 autoantibodies and C-peptide in type 1 diabetes mellitus patients, their sibs and controls distributed by HLA-Cw2 phenotype.

Investigated Parameters	HLA	Mean Serum Level ± S.E. (pg/ml)*		
		Diabetic Patients	Controls	Patient's Sibs
Anti-GAD (IU/ml)	Cw*2+ve	22.59 ± 7.08♦	5.35●	5.49 ± 0.06
	Cw*2-ve	59.74 ± 16.30	5.49 ± 0.03	5.42 ± 0.03
Anti-IA-2 (IU/ml)	Cw*2+ve	11.52 ± 0.31♦	10.61●	21.18 ± 5.29♦
	Cw*2-ve	41.26 ± 14.85	10.96 ± 0.15	11.02 ± 0.15
C-peptide (ng/ml)	Cw*2+ve	6.52 ± 2.52	18.74●	3.37 ± 0.37♦
	Cw*2-ve	10.43 ± 3.17	16.57 ± 4.33	7.79 ± 1.37

♦Significant difference between Cw2+ and Cw2- subjects for each parameter.
●Only one subject was reported in this group.

Table (6): Observed numbers and percentage frequencies of anti-cytomegalovirus and anti-rubella antibody positive sera of type 1 diabetic mellitus patients, their sibs and controls.

Anti-Viral Autoantibody	Antibody Positive Sera					
	Diabetic Patients (No. = 25)		Controls (No. = 25)		Patient's Sibs (No. = 25)	
	No.	%	No.	%	No.	%
Anti-Cytomegalovirus antibody	7	28.0	2	8.0	3	12.0
Anti-Rubella antibody	7	28.0	4	16.0	9	36.0

Anti-cytomegalovirus and Anti-rubella Antibodies

To shed light on the possible association between T1DM and viral infection (cytomegalovirus or rubella), the sera of patients, their sibs and controls were screened for anti-cytomegalovirus (CMV) and anti-rubella antibodies. However, no statistical analysis was made because the sample size could not give fruitful analysis, and instead they were presented as percentage frequencies. For anti-CMV antibody, 28% of patient's sera were positive for such antibody, while it was less frequent in patient's sibs or controls (12 and 8%, respectively). Anti-rubella antibody positive cases shared the frequency of anti-CMV antibody in patients (28%), but the frequency was higher in patient's sibs (36%), while in controls, it was 16% (Table 6).

The present study demonstrated that HLA-class I region is still of importance in aetiology of T1DM, and the region harbours genes that can predispose or protect the individuals from triggering the episodes of the disease. It was also augmented that these genes can be more effective when they are presented in terms of their different two-locus combinations, and the results highlighted the importance of LD in such effects. Furthermore, the impact of HLA-class I alleles on serum level of autoantibodies (anti-GAD and anti-IA-2) and C-peptide was also observed in the patients as compared with their sibs or controls. In agreement with such findings, the evidence from many studies has established the importance of genetic factors in aetiology of T1DM, and accordingly, many genetic models have been proposed and most of them have been based on the assumption that an "T1DM susceptibility" gene (or genes) is closely linked to the HLA complex. The studies of 1970s made a concern of HLA-class I alleles in aetiology of T1DM, while in 1980s and 1990s, the concern was shifted to HLA-class II region (Gorodezky *et al*., 2006). However, later studies of the 2000s made a recall for HLA-class I region; especially their role in modifying the risk of a disease expression and influencing evolution, age at onset and degree of β cell destruction in T1DM patients (Nejentsev *et al*., 2007). Furthermore, Qu and Polychronakos (2009) supported the impact of HLA-class I antigens in the production of autoantibodies in T1DM; a finding that was also observed in the present sample of patients.

The present study findings together with the demonstrations of other studies came to highlight and confirm the role of HLA gene products in aetiopathogenesis of T1DM, which were supported by more than one line of evidence. The first line was based on a population approach that has consistently found very strong positive associations between T1DM and HLA alleles (Gorodezky *et al*., 2006 and results of present study). Linkage analyses in families having T1DM represented the second line of evidence that a genetic predisposition for the disease is associated with genes in the HLA regions (class I and II), and in multiple sib case families (i.e. those families with 2 or more T1DM children), diabetic siblings shared HLA haplotypes much more often than the expected frequencies (Alizadeh and Koeleman, 2008). Both approaches were targeted in the present study, and HLA analyses in terms of their individual frequencies or two-locus combinations revealed that HLA class I (A, B and C) alleles contribute to T1DM aetiology, especially if the difference between the observed and expected frequencies of two-locus combinations was considered, because it strongly highlights the concept of LD between alleles of HLA-class I subregion and the importance of such phenomenon in aetiopathogenesis of T1DM and the mechanism that initiates the autoimmune cascade, which leads to a destruction of β cells in the patients. Such conclusion is supported by animal model studies. In non-obese diabetic mouse model of TIDM, MHC class I molecules and class-I-restricted CD8+T cells were found to be central to the development of autoimmune diabetes. Such findings are correlated with the observations that in TIDM patients, cells infiltrating pancreatic islets are predominantly CD8+ and islet cells hyper-express MHC class I molecules (Reviewed by Nejentsev *et al*., 2007). Taken together with our results, it can be concluded that class-I-mediated anti-islet β-cell responses are critical in T1DM and may accelerate disease onset. Furthermore, HLA-class I alleles have been functionally and directly linked to T-cell autoreactivity to insulin (Sia and Weinem, 2005).

The results of individual HLA-class I alleles showed significant variations in allele frequencies between T1DM patients, their sibs and controls. For instance, A*1 showed a significant variation in patients versus controls and patients versus their sibs, and the EF values of such variations were

14.64 and 30.55, respectively. Such finding agrees with Tuomilehto-Wolf and Tuomilehto (1991) who referred to A*1 as one of the significant alleles that was associated with T1DM. A*10 allele also showed a significant increase in TIDM patients versus controls and sibs versus controls, while Huh *et al*. (1986) and Tuomilehto-Wolf and Tuomilehto (1991) recorded a non-significant association between A*10 allele and T1DM. Such discrepancy can be explained in the ground of racial differences, especially if we consider that HLA alleles show different frequencies in different populations including Iraqis (Ad'hiah, 2009). In contrast, A*11 showed a significant decrease in patients versus control, and such finding may highlight its protective effect against the development of T1DM, especially if we consider a PF value of 0.19. However, Murphy *et al*. (1983) recorded a non-significant variation of A*11 allele in T1DM patients versus controls. B*35 was also significantly increased in the patients; a finding that has also been shared by Tait *et al*. (2003) and Sia and Weinem (2005).

The present study also made some concern of the autoantibody status in T1DM patients, their sibs and controls, in addition to the impact of HLA-class I alleles on such status. The autoantibodies of interest were anti-GAD and anti-IA-2 antibodies, and both antibodies showed a significant increased serum level in the patients as compared to their sibs or controls; an observation that marks their importance in the aetiopathogenesis of T1DM. Several studies have indicated the presence of two or three anti β-cell antibodies (anti-IC, -GAD and IA-2 autoantibodies) at diagnosis to be predictive of severe deterioration of β cell function within a few years, whereas that of either anti-GAD or anti-IA-2 alone is associated with slower progression of the disease. However, other studies demonstrated a high prevalence of anti-GAD antibody at the time of T1DM diagnosis (Graham *et al*., 2002; Kawaski and Eguchi, 2004; Urakami *et al*., 2009), as well as, prevalence of anti-IA-2 antibody in newly diagnosed T1DM patients (Pihoker *et al*., 2005).

The results of HLA impact on the level of these autoantibodies revealed that certain HLA-class I alleles were operating and they had some role to effect the serum level of autoantibodies. For anti-GAD antibody, B*35 allele was able to occupy 33% of the total serum level of such antibody in T1DM patients only, while no such effect was observed in siblings of patients or controls, and such observation may highlight the causal relationship between anti-GAD antibody and the allele B*35, but by which mechanism; it is not known. However, for HLA-class II alleles, anti-GAD antibody was reported to be associated with HLADQA1*0501/B1*0201 (DQ2) allele, and also with DR*3 allele and DR3-DQB1*02 haplotype. It was also reported an increased anti-GAD antibody frequency among siblings of affected probands with DR4-DQB1*0302 haplotype, and in DR3/4 heterozygous subjects (Kulmala *et al*. 2000). For anti-IA-2 antibody, a different allele was operating in sibs, but not in controls, which was Cw*2. Such observation is important in the ground of T1DM prediction, because anti-IA-2 antibody has been observed to be the most predictive autoantibodies among first-degree relatives (Mrena *et al*., 2006). Prospective studies have also established that the appearance of these autoantibodies in the circulation presages by months to years the onset of clinical T1DM, especially in patients or first-degree relatives of specific HLA alleles or phenotypes (Dejkhamron *et al*., 2007).

The serum level of C-peptide was also evaluated, and the results revealed that its level was decreased in T1DM patients, but a much more decreased level was observed in sera of sibs. Furthermore, such level was monitored by different HLA alleles in each of the three investigated groups. It was Cw*1 in patients, A*1 in sibs and Cw*2 in controls. Such decline of C-peptide came to confirm earlier studies that stated that a decline in C-peptide is very gradual up until six months prior to diagnosis, and during the last six months prior to diagnosis, stimulated C-peptide declines more rapidly (Chailurkit *et al*., 2007). Such findings confirm the long-held clinical opinion that the decline in β-cell function is more rapid in the pre-diagnosis time period of T1DM. In this regards, several factors have been proposed to affect the rate of C-peptide decline and the loss of β cell function in patients with T1DM. These factors include age at diagnosis, degree of metabolic control, immune status based upon markers such as IL-1Ra and antibody levels and genetics (i.e. HLA and insulin gene) (Palmer, 2009). With respect to HLA alleles, their impact on C-peptide level has also been suggested, and Spoletini *et al*. (2007) stated that fasting C-peptide in T1DM subjects with low HLA genetic risk was significantly higher when compared with subjects with moderate or

high HLA genetic risk from time of diagnosis and up to 12 months.

The final part of this study was concerned with environmental agents (CMV and rubella virus) that are suspected to be triggers of T1DM in genetically susceptible individuals. A high percentage of positive cases for CMV was observed in patients, while a high percentage of positive cases for rubella virus was observed in patient's sibs. However, the results were not conclusive as some control cases were also positive for both viruses, but the association between viruses and T1DM is still under investigation in humans. The T1DM outcome strongly suggests that viral infections should be considered in terms of "viral profiles" (i.e. cell tropism, cross-reactivity, nature, extent and localization of inflammation), rather than in terms of viral types (Fillipi and herrath, 2005). It has also been shown that certain T cells may express two TCRs of different specificities, due to productive gene rearrangement of both alleles, which makes it a potential risk factor for development of autoimmunity. T cells with specificities for both viral and self-antigen could also respond to self antigen when activated during viral infection (Alba *et al*., 2005). Infectious agents are the most studied environmental factors potentially involved in autoimmunity induction, because they represent a potent stimulus for the immune system and may contribute to the selection and triggering of auto-reactive lymphocytes in susceptible individuals. Data from experimental animals, as well as, *in vitro* studies indicate that various viruses are clearly able to modulate the development of T1DM via different mechanisms, including direct β cell lysis, bystander activation of autoreactive T cells, loss of regulatory T cells and molecular mimicry (van der Werf *et al*., 2007).

Conclusion

The study suggests the role of HLA-class I alleles, autoantibodies and viral infection in aetiopathogenesis of T1DM, but further studies are certainly required.

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