

HLA-Class II Risk Alleles Control T-Lymphocyte Proliferation in Response to Enterovirus and Adenovirus Antigens and IgG Antibody Prevalence in Newly Diagnosed T1DM Children

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Abstract

Background: Viral infections are implicated in the pathogenesis of type 1 diabetes mellitus (T1DM) in a number of studies, and playing a role in the initiation of beta-cell damaging process.

Objective: To evaluate the T-cell proliferation in response to enterovirus antigens including coxsackievirus B and poliovirus in addition to adenovirus in an HLA-matched population of children with T1DM and children who were healthy, in addition to screening for specific anti-viral IgG antibodies.

Subjects and methods: A total of 60 Iraqi T1DM children were included in the present study. They were newly diagnosed diabetics. For the purpose of comparisons, 50 apparently healthy children were selected. HLA typing was measured by microlymphocytotoxicity, while MTT assay was used for lymphocyte proliferation by culturing peripheral blood lymphocytes (PBLs) with Con-A, Coxsackievirus B₅ (CVB5), Adenovirus 3, 4, and 7 serotypes, and Poliovaccine. Serum IgG against these viruses were detected quantitatively with an indirect ELISA.

Results & conclusion: No significant differences were shown in the PBL proliferative

percentage in response to Con-A mitogen and tested viruses (CVB₅ and adenovirus) between T1DM and healthy controls, but PBL proliferative percentage of patients showed a significant decline in response to poliovaccine. Strong T-cell proliferation in response to the tested viral antigens were observed and was related to HLA-DR4 and HLA-DQ3 antigens, whereas the HLA-DR3 and HLA-DQ2 alleles were associated with weak responsiveness to the same antigens. High significant mean proliferative percentage for all tested viruses were detected in those patients who were sero-positive IgG as compared to the sero-negative IgG diabetic children.

Conclusion: In children with new-onset diabetes, responses were generally decreased, but higher in children who carried risk HLA-class II alleles and who were sero-positive to anti-viral IgG antibodies.

Key Words: T1DM, HLA class II alleles, Lymphocyte proliferation, Anti-CVB5 IgG, Anti-polio IgG, Anti-Adeno IgG.

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Introduction:

Epidemiological studies indicate that autoimmune diseases such as type 1 diabetes mellitus (T1DM) have a strong environmental component to their

pathogenesis (1,2). There is a considerable body of evidence suggesting that involvement of several groups of viruses, but particularly those of the enterovirus genus, in the development and / or acceleration of T1DM (3). Coxsackie virus B4 (CVB4) - specific IgM responses are more common in newly diagnosed subjects with T1DM than in healthy control subjects (4). The finding of viral RNA in circulation at the onset of the disease have further support the role of enteroviruses (5).

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Viral infection like other environmental risk factors can probably induce β -cell damaging processes only in individuals with genetic T1DM susceptibility. The most important risk genes are located within the HLA gene complex, where HLA-DQ alleles associated with increased susceptibility to or protection against T1DM can be defined (6). Enterovirus (EV) infections possibly occur predominantly in individuals with the DQA1*0501. DQB1*02 haplotype, who usually are also positive for HLA-B8 and HLA-DR3 alleles (7, 8). HLA may also influence immune responses to EV antigens in comparison occurs between patients and control individuals (9).

Few studies have focused on T- cell / virus interaction. In the present study T-cell proliferation in response to enterovirus antigens including coxsackie virus B and poliovirus in addition to adenovirus was analyzed in an HLA-matched population of children with T1DM and children who were healthy, and whether HLA alleles modified the cellular immune responses to the viral antigens.

Subjects, Materials and Methods

Subjects:

Sixty Iraqi T1DM children were subjected to this study. The patients were attending the National Diabetes Center at Al-Mustansiriya University during the period May 2004 - October 2005. Their ages ranged from 3 -17 years, and they were new onset of the disease (diagnosis

was from one week up to five months). For the diagnosis of Diabetes Mellitus, the criteria as listed in the Expert Committee of Diagnosis and Classification of Diabetes Mellitus, 2003 was used. All the patients were treated with daily replacement doses of insulin at the time of blood sampling. Fifty healthy children were selected, who have no history or clinical evidence of type 1 diabetes or any chronic diseases and obvious abnormalities as a control group. These children were compared with T1DM children and matched for sex, age (4-17), and HLA-DR and DQ risk alleles as represented in table -1 and 2. The patient and control subjects were divided into two groups according to their ages, equal or less than 10 years and more than 10 years old.

Collection of Blood Samples:

Ten milliliters of venous blood were collected from each subject. Eight milliliters of blood were put in heparinised test tube (10 U/ml) and used for lymphocyte separation for the detection of HLA polymorphism and lymphocyte proliferation. Heparinised blood was processed as soon as possible. The remaining blood was drawn into plain test tube and the serum was separated by centrifugation at 2500 rpm for 10 min., divided into aliquot and kept at -20°C until used.

HLA Typing: It was carried out by microlymphocytotoxicity assay as described by (11).

Table- 1: distribution of HLA-DR antigens in T1DM children and control groups.

Group	DR3/DR4	DR3	DR4	Others
T1DM (60)	25	7	5	23
Controls (50)	12	6	2	30

Table- 2: distribution of HLA-DQ antigens in T1DM children and control groups.

Groups	DQ2/DQ3	DQ3	DQ2	Others
T1DM (60)	9	15	11	25
Controls (50)	-	12	9	29

Lymphocyte proliferation using MTT assay:

Peripheral blood lymphocytes (PBLs) were isolated using Ficoll-isopaque gradient centrifugation (Flow-Laboratories, UK). The washed PBLs were resuspended in complete RPMI-1640 medium (Euroclone, UK) supplemented with 10% heat inactivated AB serum (National blood transfusion center); HEPES; crystalline penicillin (1,000,000 IU) and streptomycin (1gm)(Pharma-intersprl, Belgica), and the final lymphocyte concentration was adjusted to 1-2x10⁶ cells / ml.

Triplicate incubations of 100 µl of cell suspension with antigen(s) in 96 flat-bottom microculture plates for 3 days at 37°C in a humidified 5% CO₂ incubator. Then 20 µl of 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Germany) working solution was added to each culture well and the culture were incubated for further 4 hrs. The converted dye was solubilized by adding acidic isopropanol. The absorbancy was read using microculture plate reader using a wave length of 570 nm (12).

The antigens were:

CVB5 antigen solution (1:5 dilution) (KBR-CF antigen Vero, France), poliovirus Trivalent Vaccine (1:5 dilution) (Polioral Trivalent; Chiron), and adenovirus type 3,4,7 solution (1:10 dilution) (KBR-CF antigen type 3, 4, 7, Vero). The final concentration or dilution for the three viral antigens was achieved according to the result of MTT serial dilution run of these antigens.

Con-A mitogen (100 µg/ml) was used as a mitogen positive control.

The percent of proliferative response of lymphocytes was calculated by the following formula:

$$\% \text{ Proliferation} = \left[\frac{\text{Absorbancy of experimental wells} - 1}{\text{Absorbancy of control wells}} \right] \times 100$$

Virus antibodies:

IgG class antibody was measured against purified CVB5; adenovirus antigen (serotype 4, 5, 7) and poliovaccin using indirect ELISA method as described by (13, 14). Sample value lie below the cutoff value (mean negative + 2 SD) were considered negative. Those who were equal or greater than cutoff value were considered positive (15).

Statistical analysis

Regarding of HLA and disease association the frequency distribution for selected variables was done. Student t-test was used to measure the differences between two means; the results were expressed as means ± standard error (SE). The single Factor ANOVA (F-test) was used in this study to find out whether the difference between more than two groups of samples is significant or not. Pearson Correlation (R), which measures to what degree the two variable observations are correlated to each other was employed in addition to Chi Square test.

Results

Lymphocyte Proliferation: This test was performed to study whether the different viral antigens have any association with the proposed cell mediated immune (CMI) activation or not after incubation

with peripheral blood lymphocytes (PBLs) of T1DM patients and healthy controls. The results of mean proliferative percentage in response to Con-A were represented in table (3). A similar mean lymphocyte proliferation percentage in response to Con-A mitogen was observed among patients and control groups, but newly diagnosed T1DM patients tended to have a lower non significant proliferative percentage than control subjects ≤ 10 years old (83.33 vs. 85.93%, $P_1=0.82$) and in >10 years old group (86.04 vs. 92.7% , $P_1= 0.62$).

Role of Viral Antigens in Functional Activation of PBL:

Considering the response to different viral antigen, a lower mean proliferative percentage was seen among patients ≤ 10 years old in response to CVB₅ compared to controls (36.67 vs 49.16%) and among patients >10 years old than controls (38.87 vs 51.20%). Those differences failed to reach significant levels in both age groups ($P_1=0.061$, and 0.14 respectively), (Table - 4).

Significant decline of proliferative response against poliovaccine was seen in T1DM patients (34.44%) compared to controls (47.38%) ($P_1= 0.045$) in ≤ 10 years old group and >10 years old group (28.30 vs. 40.86%, $P_1= 0.004$) (Table -4).

A non significant ($P_1= 0.82$) proliferative percentage decline in response to adenovirus was observed in ≤ 10 years old patients (19.97%) compared to controls (20.67%) and also in patients >10 years old (23.02%) in comparison with controls (28.61%) ($P_1=0.23$).

No statistical differences appeared in the mean lymphocyte proliferative percentage between patients in both age groups against CVB₅ ($P_2=0.57$), poliovaccine ($P_2=0.14$) and Adenovirus ($P_2=0.57$).

Relation of HLA Class II Alleles with the PBL Proliferation Percentage in T1DM Patients:

At HLA-class II region, highly significant increased frequencies of DR3 (53.33 vs. 26.25%) and of DR4 (50.0 vs. 12.5%) were observed in the patients compared to controls ($P=9.7 \times 10^{-3}$ and 1×10^{-5} respectively) (data was not shown). At HLA-DQ loci, two antigens DQ2 and DQ3 were significantly increased in the patients compared with controls (DQ2: 33.33 vs. 15.0%, $P=0.009$; DQ3: 40.0 vs. 20.0%, $P=0.008$).

To find out any relation between the HLA-class II risky alleles (genetic factors) and proliferative percentage of MTT (CMI level), ANOVA test was applied to compare the proliferative percentage in patients with HLA-DR risky alleles (DR3; DR4 and DR3/DR4) with those patients who had other alleles. The results represented in table -5 showed that the mean PBL proliferative percentage in response to different tested viral antigens was significantly higher in the patients with DR4, DR3 and DR3/DR4 serotypes compared with the children carrying other alleles. The significant levels scored $P= 0.021$ in response to CVB₅, $P=0.031$ in response to poliovaccine, and $P= 0.041$ in response to adenovirus. Moreover, the mean proliferative percentage was significantly higher in patients carrying DR4 allele than those patients with DR3 alleles in response to CVB₅ (62.67 vs. 43.32%, $P=0.038$), to poliovaccine (59.86 vs 38.40%, $P=0.031$) and to adenovirus (46.02 vs. 22.48%, $P= 0.046$).

Concerning the HLA-DQ risky alleles (DQ2, DQ3, DQ2/DQ3), our results presented in table -6 showed a significant increase of proliferative percentage in patients carrying different HLA-DQ risky alleles compared with the patients who lack these alleles. The

results scored as significant levels of $P=0.032$ in response to CVB₅, $P=0.038$ in response to poliovaccine, and ($P=0.042$) in response to adenovirus ($P=0.042$).

As detected in table -6, the proliferative percentages were significantly higher in patients with DQ3 alleles than in patients with DQ2 alleles in response to all tested viral antigens.

Anti-Viral IgG in T1DM Patients

Seropositivity against the 3 viral antigens was significantly higher in diabetics than controls. Only 12 patients out of 60 were sero-positive (20%) compared to 4 healthy individuals out of 50 (8%) who were sero-positive for anti-CVB₅ IgG. These differences were statistically significant ($P=0.048$). Nineteen patients (31.67%) were sero-positive for anti-polio-IgG compared to 13 (26%) healthy controls, and no difference appeared between both groups ($P=0.649$), whereas only 4 patients were sero positive for anti-Adeno IgG (6.67%) compared with the control group who were all sero-negative. This difference was not significant between the two groups.

Relation between Mean Lymphocyte Proliferation Percentage and Anti-Viral IgG in T1DM Patients

To detect any relation that can clarify if the PBLs were primed previously by the same viral antigen. The results represented in table -7 showed a significant increase of mean proliferative

percentage in response to CVB₅ in the patients who were sero-positive for anti-CVB₅-IgG compared with the sero-negative patients (50.58 vs 22.99%) ($P=0.048$).

The mean proliferative percentage for sero-positive and sero negative anti-polio-IgG patients is illustrated in table -8. It was found that the patients who were sero-positive for anti-polio IgG had higher proliferative percentage reading in response to polio-vaccine (31.48%) than those patients who were sero-negative (20.61%) and these differences were significant ($P=0.039$).

The study also demonstrated increased mean proliferative percentage of PBLs in response to adenovirus in sero-positive anti-Adeno IgG patients in comparison to sero-negative anti-Adeno IgG patients (30.10 vs. 14.16%) and again these differences reach the significant level ($P=0.042$) (Table -9).

Moreover, the present findings also revealed a significant positive correlation between the PBL proliferative percentage in response to CVB₅ and anti-CVB₅-IgG ($r=0.412$). Strong negative correlation was also detected between proliferative percentage in response to adenovirus and anti-Adeno-IgG ($r=-0.635$) while the correlation found with the anti-polio-IgG was weakly positive ($r=0.101$).

Table- 3: t-test between controls and T1DM patient groups regarding comparison of MTT proliferation percentage in response to Con-A.

Mitogen	≤10 years					>10 years					P ₂
	Groups	No.	Mean	SE	P ₁	Groups	No.	Mean	SE	P ₁	
Con-A	Controls	21	85.93	10.60	0.82 (NS)	Controls	29	92.70	10.2	0.62 (NS)	0.57 (NS)
	T1DM	36	83.33	5.60		T1DM	24	86.04	8.27		

Table- 4: Comparison of mean proliferation percentage of PBL between controls and T1DM patients in response to CVB₅, poliovaccine and adenovirus.

Viral antigens	≤10 years					>10 years					P ₂
	Groups	No.	Mean	SE	P ₁	Groups	No.	Mean	SE	P ₁	
CVB ₅	Controls	21	49.16	5.88	0.061 (NS)	Controls	29	51.20	5.97	0.14 (NS)	0.57 (NS)
	T1DM	36	36.67	3.08		T1DM	24	38.87	5.08		
Polio Vaccine	Controls	21	47.38	5.83	0.045 (S)	Controls	29	40.86	3.28	0.004 (S)	0.14 (NS)
	T1DM	36	34.44	2.79		T1DM	24	28.30	3.28		
Adeno-virus	Controls	21	20.67	2.24	0.82 (NS)	Controls	29	28.61	3.73	0.23 (NS)	0.35 (NS)
	T1DM	36	19.97	1.61		T1DM	24	23.02	3.27		

P₁: T1DM patients vs. control

P₂: T1DM patients ≤10 years vs. patients >10 years old.

Table- 5: Relation of mean lymphocyte proliferation percentage in response to different viral antigens with the HLA-DR risky alleles in T1DM patients.

Viruses	DR3/DR4 (n=25)	DR3 (n=7)	DR4 (n=5)	Others (n=23)	ANOVA F-test	P
CVB ₅	40.37	43.32	62.27	29.73	8.585	0.021 (S)
Polio vaccine	34.42	38.4	59.86	25.27	7.689	0.031 (S)
Adenovirus	29.44	22.48	46.02	26.14	5.704	0.041 (S)

Table- 6: Relation of mean lymphocyte proliferation percentage in response to different viral antigens with the HLA-DQ risky alleles in T1DM patients.

Viruses	DQ2/DQ3 (n=9)	DQ3 (n=15)	DQ2 (n=11)	Others (n=25)	ANOVA F-test	P
CVB ₅	42.84	60.90	26.41	33.63	7.975	0.032 (S)
Polio vaccine	39.31	48.09	23.21	27.29	6.695	0.038 (S)
Adenovirus	22.26	37.41	31.37	26.74	5.684	0.042 (S)

Table-7: Relation of mean PBL proliferative percentage in response to CVB₅ with the anti- CVB₅ IgG.

CVB ₅		No.	Proliferation percentage	SE	P
Anti-CVB ₅ IgG	+ve	12	50.58	10.09	0.048 (S)
	-ve	48	22.99	3.27	

t = 2.62

Table-8: Relation of mean PBL proliferative percentage in response to poliovaccine with the anti-polio IgG.

Poliovaccine		No.	Proliferation percentage	SE	P
Anti-Polio IgG	+ve	19	31.48	5.83	0.039 (S)
	-ve	41	20.61	2.92	

t = 3.85

Table-9: Relation of mean PBL proliferative percentage in response to adenovirus with the anti-adeno IgG.

Adenovirus		No.	Proliferation percentage	SE	P
Anti-Adeno IgG	+ve	4	30.10	6.45	0.042 (S)
	-ve	56	14.16	2.22	

t = 2.66

Discussion:

Functional Activity of PBL:

The use of lymphocyte proliferation technique is based on the capability of the lymphocytes for responding to an antigen (specific response), which has induced memory lymphocyte, either by vaccination or by natural infection. These lymphocytes, when they are repeatedly contacted with antigens, have a blastogenic transformation (16).

The proliferative percentage of PBLs has been found lower in T1DM patients than in healthy controls in response to Con-A. Considering the responses to

viral antigens, proliferative responses against CVB₅ and adenovirus were tended to have a lower percentage in T1DM patients than controls, but these values were not statistically different, while the proliferative responses against poliovaccine were significantly lower in patients especially in >10 years old group than controls. The low proliferative responses against CVB₅ antigen at disease onset is in agreement with other studies showing reduced T-cell proliferation against CVB₄ (17), while the same investigators found in previous

study, no differences in T-cell proliferation against CVB₄-infected lysate between diabetic patients and healthy-non diabetic individuals (18). Another report conducted by Juhela *et al.*, 2000 found that PBLs of the children at onset of T1DM had significant weaker responses to purified CVB₄ and non-significant decrease in response to poliovirus type 1 and 3 than healthy children, while the responses to adenovirus did not differ between patients and controls. Temporary decline in T-cell responsiveness at diabetes onset has also described in GAD peptide that contains the homology region to the CVB₄ 2C protein (20).

The results of these studies and the present study are subjected to several interpretations. One explanation is that, decreased responses of PBLs are due to redistribution of virus-specific T-cells, with virus-responder cells presumed to have homed to the pancreas and therefore unavailable for detection in peripheral blood (21), and so T-cell responses to various viral antigens may be suppressed at the onset of the disease. On the other hand, Varela-Calvino *et al.*, 2002 indicated abundance of circulating primed CVB₄ specific responder T-cells that secrete IFN- γ in T1DM patients with relative lack of proliferation. These findings have been related to two broadly defined phenotypes of memory T-cells characterized by (22). Primed (memory) T-cells with the capacity to proliferate termed as "central memory" TCM cells, and lack immediate effector function and predominantly produce IL-2, the major T-cell growth factor to support proliferation and express CCR₇, a chemokine receptor, that direct homing to lymph nodes. In contrast the primed memory cell subsets that produce the proinflammatory

cytokines IFN- γ during an immune response termed "effector memory" subset TEM, those cells do not express CCR₇, present in the circulation at sites of infection or tissue inflammation and release cytokines.

Relation of Lymphocyte Proliferation with HLA:

The present results indicated that stronger T-cell proliferation in response to CVB₅, poliovaccine and adenovirus were related to HLA-DR4 allele and HLA-DQ3 allele; whereas the HLA-DR3 and HLA-DQ2 were associated with weak responsiveness to the same antigens. These results are in agreement with a report of Bruserud *et al.*, 1985 who found that DR4, which is in linkage disequilibrium with the HLA-DQB1*0302 allele, is associated with strong T-cell responses; whereas HLA-DR3 associated with HLA-DQB1*02 allele associates with weak T-cell responses to enterovirus antigens. Another study conducted by Juhela *et al.*, 2000 demonstrated the same observation in T-cell responses to enterovirus antigens in T1DM patients.

Anti-Viral IgG

The present results described finding of IgG antibodies against CVB₅ to be more frequent (20%) in T1DM patients than in controls (8%). A low prevalence of specific CVB-IgG may be due to the use of only one CVB serotype (CVB₅) and there may be another CVB serotype in the sera of T1DM patients, which is not detected. The frequency of IgG antibodies against poliovirus (Oral sabin) was more (31.67%) in diabetic patients than in controls (26%). Also IgG antibodies against adenovirus were detected in only four diabetic children (6.67%).

The presence of CVB₅, poliovirus and adenovirus specific IgG antibodies

are evidence of previous infection in T1DM children. This fact was confirmed by measuring the PBLs proliferative percentage in sero-positive IgG diabetic children *in vitro* in response to CVB5, poliovirus and adenovirus, and the results indicated a high significant mean proliferative percentage for all tested viruses in those patients as compared to the sero-negative IgG diabetic children. This means that PBLs of sero-positive IgG patients were boosted earlier either by natural infection or vaccination.

The low prevalence of anti-polio-IgG determined in healthy children may indicate a failure of poliovaccine to enhance the immune system, although these children presumably had taken many boosted doses of oral poliovaccine.

Several studies have found CVB-specific IgM antibodies to be more common in newly diagnosed children compared to healthy individuals (19,23). Others detected an increase of anti-enterovirus antibody levels (both IgM and IgG) preceding the appearance of signs of autoimmunity reflected either by synthesis of several autoantibodies or the development of clinical disease (24). In contrast Tuvemo *et al.*, 1989 and Emekdas *et al.*, 1992 found no evidence of increased antibody frequencies against CVB1-6 serotypes at the onset of childhood diabetes. A lower antibody titer against CVB3-5 serotypes and adenovirus-7 were also demonstrated in newly diagnosed T1DM children than in healthy controls (27).

Enteroviruses could be involved in the pathogenesis of T1DM. During infection, viruses may reach the pancreatic islet and destroy insulin-producing β -cells by virus-induced cytolysis (28). Alternatively, β -cell damage might result from virus-induced

inflammatory reactions through producing inflammatory cytokines (IL- 1β , IFN- α ... etc.) (29), in addition β -cell destruction might be based on molecular mimicry, because immunological cross-reactions between enteroviruses and β -cell autoantigens (GAD-65, Tyrosin phosphatase IAR/IA3 can take place at least *in vitro* (14).

In conclusion, the present results show that T- cell proliferation in new onset Type 1 Diabetic children were decreased, but higher in children who carried risk HLA- class II alleles and who were sero positive to anti- viral IgG antibodies.

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