ASSOCIATION BETWEEN HLA-CLASS II ALLELES AND T-CELL PROLIFERATION IN RESPONSE TO ENTEROVIRUSES AND ADENOVIRUS ANTIGENS IN NEWLY DIAGNOSED CHILDREN WITH TYPE 1 DIABETES MELLITUS

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ABSTRACT

Viruses may be involved in the pathogenesis of Type 1 Diabetes Mellitus (T1DM), either through direct β-cell infection or as triggers of autoimmunity. T- cell proliferation was evaluated in response to Enteroviruses antigens including Coxsackievirus B and Poliovirus in addition to Adenovirus in an HLA- matched population of children with T1DM and in children who were apparently healthy. A total of 60 Iraqi T1DM children were included in the presents study. They were with new onset of the disease. For the purpose of comparisons, 50 apparently healthy control subjects were also selected. HLA typing was measured by microlymphocytotoxicity, while methylthiazoltetrazolium (MTT) assay was used for lymphocyte proliferation by culturing peripheral blood lymphocytes (PBL) with Coxsackievirus B_5, Adenovirus 3, 4 and 7 and Poliovaccine. No significant differences were shown in the PBL proliferative percentage in response to Con-A mitogen and tested viruses (CVB_5 and Adenovirus) between T1DM and healthy controls, but PBL proliferative percentage of patients showed a significant decline in response to Poliovaccine. HLA class II (-DR3, DR4, DQ2 and DQ3) antigens were significantly increased in T1DM patients and they played an important role in the etiology of the disease. Strong T-cell proliferation in response to the tested viral antigens were observed to be related to HLA-DR4 and HLA-DQ3 antigens, whereas the HLA-DR3 and HLA-DQ2 alleles were associated with week responsiveness to the same antigens. However, in children with new- onset diabetes, responses were decreased and this could be caused by trapping of virus- specific T- cells in the pancreas.

Key words: T1DM, HLA, Lymphocyte proliferation, Enteroviruses, Adenovirus.

العلاقة بين مستضدات التطابق النسيجي- الصنف الثاني والفعالية الوظيفية للخلايا اللمفية بعد تحفيزها بمستضدات الحمات المعوية والحمات الغدية في الاطفال المصابين حديثاً بالسكري من النوع الأول

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الخلاصة

أثبتت العديد من الدراسات أن للفايروسات دوراً تؤديه في احداث امراضية مرض السكر من النوع الاول بالاص ابة المباشرة لخلايا بيتا في البنكرياس أو بتحفيز المناع ه الذاتية . أجريت الدراسة لغرض تقييم الفعالية الوظيفية للخلايا اللمفية بعد تحفيزها بمستضدات الحمات المعوية والتي تشمل فايروس الكوكساكي نوع- ب وفايروس شلل الاطفال فضلا عن الحمة الغدية في مجموعة من الاطفال المصابين بمرض السكري من النوع الاول ومجموعة من الاطفا ل الاصحاء المطابقين لمستضدات التطابق النسيجي من الصنف الثاني. شملت الدراسة ستون مريضاً حديثي الاصابة بمرض السكري النوع الاول فضلا عن مجموعة السيطرة المكونة من 50 طفلا يبدون أصحاء ظاهريا لغرض التحري عن وجود أليلات الخطورة لمستضدات التطابق النسيجي- الصنف الثاني. تم قياس الفعالية الوظيفية للخلايا اللمفية بعد حضنها مع الفايروسات انفة الذكر . أظهرت النتائج انخفاضا غير معنويا في الفعالية الوظيفية للخلايا اللمفية كأستجابة للمشطر وكذلك بعد حضنها مع فايروس الكوكساكي النوع المصلي ب-5 وفايروس الحمة الغدية للانواع المصلية 3، 4, و 7 في الاطفال المرضى مقارنة بالاصحاء . ولكن هذا الانخفاض كان معنويا فقط عند استخدام فايروس شلل الاطفا ل .هناك زيادة معنوية في نسبة الاطفال الحاملين لاليلات الخطورة من الصنف الثاني مقارنة بالاصحاء HLA class II (-DR3, DR4, DQ2 and DQ3) antigens وهناك علاقة مباشرة وقوية بين الفعالية الوظيفية للخلايا اللمفية بعد حضنها مع المستضدات الفايروسية ومستضدات التطابق النسيجي , بينما كانت العلاقة ضعيفة مع المستضدات الاخرى HLA- DR4, HLA- DQ3 .DQ2- ;HLA-DR عموما كانت الاستجابة قليلة في الاطفال المرضى ولكن هناك زيادة محسوسة عند الاطفال الحاملين لاليلات التطابق النسيجي من النوع الثاني.

INTRODUCTION

There are a considerable body of evidences suggesting that involvement of several groups of viruses including: Congenital rubella, Rotaviruses, Retroviruses, Herpes viruses, Cytomegalovirus, Measles, Hepatitis C, and particularly those of the Enterovirus genus, in the development and / or acceleration of Type 1 Diabetes Mellitus (T1DM) (1). Several epidemiological and prospective studies showed that some cases of T1DM are strongly associated with Enterovirus (EV) infections (2), and the children who later developed T1DM had more EV infections than control children years before the diagnosis of the disease (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). Several mechanisms have been proposed to explain this putative link with T1DM pathogenesis, including molecular mimicry (6), bystander activation through release of autoimmune mediators like proinflammatory Cytokines IL-1β; TNF-α; and IFN-γ (7), and super antigen effect (8).

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 locate within the HLA gene complex, where HLA-DQ alleles associated with increased susceptibility to or protection against T1DM can be defined (9). Enterovirus 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 (10,11). HLA may also influence immune responses to EV antigens in comparison occurs between patients and control individuals (12).

Few studies had 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.

MATERIALS AND METHODS

Sixty Iraqi T1DM children were subjected to this study. The patients were attending to National Diabetes Center at Al-Mustansiriya University during the period May 2004 to 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 (13), was used. All the patients were treated with daily replacement doses of Insulin at the time of blood sampling. For the purpose of comparisons, 50 apparently healthy control subjects matched for age (4-17) years, sex and ethnic back ground (Iraqi Arabs) were selected who had no history or clinical evidence of type 1 diabetes or any chronic diseases and obvious abnormalities as a control group. The patients 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

Eight milliters of blood was placed in heparinised test tube (10 U/ml) used for lymphocyte separation for the detection of HLA polymorphism and lymphocyte proliferation. Heparinised blood was processed as soon as possible.

HLA typing: were measured by microlymphocytotoxicity assay as described by Stoker and Bernoco (14).

Lymphocyte proliferation using methylthiazoltetrazolium (MTT) assay

Heparinized venous blood was collected, and 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% AB serum (National blood transfusion center); Hepes; Crystalline penicillin (1,000,000 IU) and Streptomycin (1gm)(Pharma-intersprl, Belgica), the final lymphocyte concentration was adjusted to 1-2x10^6 cells / ml.

Triplicate incubations of 1-2x10^6 PBL/ ml with antigen(s) in a final volume of 100µl continued to incubate 96 flat-bottom microculture plates for 3 days at 37°C in a humidified 5% CO_2 incubator. Then 20µl of (MTT) 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (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 (15).

Antigens

- Coxsackievirus B5 (CVB5) antigen solution (1:5 dilution), (KBR-CF antigen Vero, France).
- Poliovirus Trivalent Vaccine (1:5 dilution), (Polioral Trivalent; Chiron).
- Adenovirus type 3,4,7 solution (1:10 dilution), (KBR-CF antigen type 3, 4, 7, Vero).
- Concavalin-A (Con-A) mitogen (100 µg/ml), was used as a mitogen positive control.
- **:** The final concentration or dilution for the three viral antigens was achieved according to the result of MTT serial dilution run of these antigens.

The **Note** percent of proliferative response of lymphocytes was calculated by the following formula (15):

$$\%\ \text{Proliferation} = \left[\frac{\text{Absorbancy of experimental wells}}{\text{Absorbancy of control wells}} - 1 \right] x100$$

Statistical analysis

Regarding 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 among more than two groups of samples is significant or not, and Pearson Correlation (R), which measures to what degree the two variable observations are correlated to each other, and the type of this correlation.

RESULTS

Lymphocyte Proliferation

This test was performed to study whether the different viral antigens have any association with 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 (1). A similar mean lymphocyte proliferation percentage in response to Con-A mitogen was seen 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% respectively, P_1=0.82) and in >10 years old group (86.04 vs. 92.7% respectively, P_1= 0.62).

Table(1) : t-test between controls and T1DM patient groups regarding comparison of MTT proliferation percentage in response to Con-A.

Mitogen	Age ≤10 years					Age >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		

P_1: T1DM patients vs. control
P_2: T1DM patients ≤10 years vs. patients >10 years old.

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 CVB_5 compared to controls (36.67 vs. 49.16% respectively) and among patients >10 years old than controls (38.87 vs. 51.20% respectively). This differences failed to reach significant levels in both age groups (P_1=0.061, P_1= 0.14 respectively) ,Table (2).

Significant decline of proliferative response against Poliovaccine was seen in T1DM patients (34.44%) than controls (47.38%) (P_1= 0.045) in ≤10 years old group and (28.30 vs. 40.86% respectively, P_1= 0.004 in >10 years old group ,Table (2).

Table(2) : Comparison of mean proliferation percentage of PBL between controls and T1DM patients in response to CVB_5, poliovaccine and adenovirus.

Viral antigens	Age ≤10 years					Age >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**		

P_1: T1DM patients vs. control
P_2: T1DM patients ≤10 years vs. patients >10 years old.

A non significant proliferative percentage decline in response to adenovirus was observed in patients (19.97%) and controls (20.67%) (P_1= 0.82) in ≤10 years old group 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 CVB_5 (P_2=0.57), Poliovaccine (P_2=0.14) and Adenovirus (P_2=0.35).

Mitogenic Properties of Tested Viral Antigens *In Vitro*

To confirm the immunostimulatory effect of CVB_5, Polio and Adenovirus, which compared with Con-A mitogen as a control positive for PBL mitogenesis and with control negative (Table-3). Statistical analysis, had shown that CVB_5 had a mitogenic potential *in vitro*. By comparing the mean of MTT OD value of CVB_5 (0.369) with control negative (C-ve) (0.270) in patient group ≤10 years old, it was found that control negative is significantly lower than CVB_5 mean of MTT reading (P_1= 0.045). The same statistical difference was seen among patients >10 years old between OD value of CVB_5 and control negative (0.368 vs. 0.265, P_2=0.037). This indicates that CVB5 may have a role in inducing the disease in those patients. The mean MTT reading of CVB_5 was significantly lower than Con-A mean MTT reading in patients ≤10 years old (0.369 vs. 0.495 respectively, P_1=0.045) and (0.368 vs. 0.493 respectively, P_1= 0.045) in patients >10 years old. This means that CVB_5 has a good mitogenic potential, but does not reach the high level of Con-A.

Table (3): Paired t-test among CVB_5, polio vaccine, adenovirus, Con-A and control negative MTT (OD) reading means for the comparison among T1DM patients.

Age≤ 10 years (n= 36)		P_1	Age>10 years (n= 24)		P_1
CVB_5	C-ve	0.045 (S)	CVB_5	C-ve	0.037 (S)
0.369	0.270		0.368	0.265	
CVB_5	Con-A	0.045 (S)	CVB_5	Con-A	0.045 (S)
0.369	0.495		0.368	0.493	
Polio	C-ve	0.054 (S)	Polio	C-ve	0.074 (NS)
0.363	0.270		0.340	0.265	
Polio	Con-A	0.037 (S)	Polio	Con-A	0.017(S)
0.363	0.495		0.340	0.493	
Adeno	C-ve	0.21 (NS)	Adeno	C-ve	0.17 (NS)
0.324	0.270		0.326	0.265	
Adeno	Con-A	0.006 (S)	Adeno	Con-A	0.007(S)
0.324	0.495		0.326	0.493	

Concerning the Poliovaccine and Adenovirus, it was found that control negative mean of MTT OD value (0.270) was lower than Poliovaccine (0.369) and Adenovirus (0.324) mean of MTT readings in patients group ≤10 years old. These means were weakly significant among Poliovaccine (P_1= 0.054) and not statistically different among Adenovirus. On the other hand, the same results were demonstrated among patients >10 years old, (0.265 vs. 0.340, P_2= 0.074) for Poliovaccine and (0.265 vs. 0.326 P_2= 0.17) for Adenovirus. This indicates that compared with that of CVB_5, although Poliovaccine had a weak mitogenic potential in patients group ≤10 years old and might have weakly immunostimulatory activity *in vivo* ,Table(3).

Lymphocyte proliferation percent against both Poliovaccine and Adenovirus showed significant positive correlation with lymphocyte proliferative percent in response to CVB_5 (r =0.38, r = 0.25 respectively, P<0.05), nevertheless a positive correlation between Poliovaccine and Adenovirus (r = 0.45, P<0.05).

Relation of HLA class II alleles with the PBL proliferation percentage in T1DM patients:

At HLA-class II region (DR-loci), highly significant increased frequencies of DR3 (53.33 vs. 26.25%) and of DR4 (50.0 vs. 12.5%) were observed in the patients ($P=9.7x10^{-3}$ and $1x10^{-5}$ respectively) (data was not shown). At HLA-DQ loci, two antigens DQ2 and DQ3 were significantly increased in the patients compared with controls (33.33 vs. 15.0%, P=0.009) for DQ2 while (40.0 vs. 20.0%, P=0.008) for DQ3. The distribution of HLA-DR and -DQ antigens in T1DM children and controls were represented in Tables (4 , 5).

Table (4) : 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 (5) : 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

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. Results represented in Table (6) showed that the mean PBL proliferative percentage in response to different tested viral antigens were 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 CVB_5, (P=0.031) in response to Poliovaccine, and (P= 0.041) in response to Adenovirus. Moreover, the mean proliferative percentage were significantly higher in patients carrying DR4 allele than those in patients with DR3 alleles in response to CVB_5 (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).

Table(6) : 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)

Concerning the HLA-DQ risky alleles (DQ2, DQ3,and DQ2/DQ3), our results represented in table (7) 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 CVB_5, (P= 0.038) in response to Poliovaccine, and (P= 0.042) in response to Adenovirus (p= 0.042).

Table (7) : 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)

As detected in table (7), the proliferative percentages were significantly higher in patients with DQ3 alleles than in patients with DQ2 alleles in response to all tested viral antigens.

DISCUSSION

Functional Activity of PBL

The use of lymphocyte proliferation is one of the more frequently used "*in vitro*" techniques for the study of the specific and non-specific stimulation capability of lymphocytes(15). The 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).

MTT has been used in the measurement of proliferative percentage of PBL which 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 CVB_5 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 was significantly lower in patients especially in >10 years old group than controls. The low proliferative responses against CVB_5 antigen at disease onset is in agreement with other studies showing reduced T-cell proliferation against CVB_4 (17), while the same investigators found in previous study, no differences in T-cell proliferation against CVB_4-infected lysate between diabetic patients and healthy-non diabetic individuals (6). Another report conducted by Juhela,*et al.*(18) found that PBL of the children at onset of T1DM had significant weaker responses to purified CVB_4 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 glutamic acid decarboxylase (GAD) peptide that contains the homology region to the CVB_4 2C protein (19).

The present study results are open to several interpretations. One explanation is that, decreased responses of PBL 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 (8), and so T-cell responses to various viral antigens may be suppressed at the onset of the disease. On the other hand, Varela-Calvino and his team(17) in their study indicated abundance of circulating primed CVB_4 specific responder T-cells that secretes IFN-γ in T1DM patients with relative lack of proliferation. These finding have been related to two broadly defined phenotypes of memory T-cells characterized by Sallusto and Lanzavecchia(20). The Primed (memory) T-cells with the capacity to proliferate termed as "central memory" TCM cells. These cells lack immediate effector function and predominantly produce IL-2, the major T-cell growth factor to support proliferation and express CCR_7, a chemokine receptor, that direct homing to lymph nodes. In contrast the primed memory cell subsets that produces the proinflammatory Cytokines IFN-γ during an immune response termed "effector memory" subset TEM, those cells do not express CCR_7, present in the circulation at sites of infection or tissue inflammation and release Cytokines.

On the other hand most studies showed that CVB5 infection caused meningitis, respiratory, gastrointestinal and cardiac diseases, while T1DM most commonly caused by CVB4 and CVB3 (21). Our result showed that CVB5 may be associated with the disease.

elation 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 the report of Bruserud *et al.*,(12) who found that DR4, which is in linkage disequilibrium with the HLA-DQB1*0302 allele, associates with strong T-cell responses; whereas HLA-DR3 associated with HLA-DQB_1*02 allele associates with weak T-cell responses to Enterovirus antigens. Juhela and her team(18), also reported the same observation in T-cell responses to Enterovirus antigens in T1DM patients.

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