An Association of T-cell proliferation and anti-Coxsackie Virus B5, anti-Polio, and anti-Adeno IgG antibodies in T1DM Children

Eman M. Saleh *

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.

To evaluate the T-cell proliferation in response to Enterovirus antigens including Coxsackievirus B and Poliovirus in addition to Adenovirus in children with T1DM, and screening for specific anti-viral IgG antibodies.

In 60 Iraqi children with recent onset of T1DM, Lymphocyte proliferation was analyzed using MTT assay by culturing Peripheral Blood Lymphocytes (PBLs) with Coxsackie Virus B5 (CVB5), Adenovirus, and Polio vaccine. Serum IgG against these viruses were detected quantitatively with an indirect ELISA.

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 it showed a significant decline in patients in response to Polio vaccine. High significant mean proliferative percentage for all tested viruses were detected in those patients who were sero-positive for anti-viral IgG as compared to the sero-negative IgG diabetic children.

In children with new-onset diabetes, mean proliferative percentage of PBL was generally decreased, but higher in children who were sero positive to anti-viral IgG antibodies.

Keywords: Type 1 Diabetes Mellitus, Lymphocyte Proliferation, Anti-CVB5 IgG, Anti-Polio IgG, Anti-Adeno IgG.

1. INTRODUCTION

Type 1 Diabetes Mellitus (T1DM) is an autoimmune disease thought to be caused by the activation of autoreactive CD4 and CD8 effector cells that recognize islet-cell antigens resulting in the destruction of most pancreatic insulin-producing β-cells (Chapel et al., 1999). Environmental factors, and/or infectious agents such as viruses have been implicated as possible cause of diabetes (Lönnort et al., 2000a).

The evidence that viral infection might cause T1DM is derived from studies where virus particles known to cause cytopathic or autoimmune damage to β-cells have been isolated from the Pancreas (Yoon et al., 1979). Several viruses have been implicated including Enteroviruses (EVs) that have been indicated to be associated with the onset of T1DM in both epidemiological, serological as well as by the studies of the viral antigen (Dahlquist, 1997). A definite islet-cell tropism of EVs was demonstrated in the human Pancreas (Yilpaasto et al., 2004). Several prospective studies showing that the children who later developed T1DM had more EV infections than control children years before the diagnosis of the disease (Hyote et al., 1995). Others were demonstrated high levels of specific IgM antibodies to Coxsackie virus B (CVB) in most newly diagnosed T1DM children (Yin et al., 2002), and high levels of specific IgM antibodies to Poliovirus derived VP1 peptide at onset of T1DM (Harkonen et al., 2003). The finding of viral RNA in circulation (Lönnort, et al., 2000b), and EV mRNA in serum samples taken from children at the time of diagnosis (Crig et al., 2003) have further support the role of EVs. Enteroviruses are transient inhabitants of the human alimentary tract and

* Department of Microbiology, Al-Kindy College of Medicine, University of Baghdad, Baghdad, Iraq. Received on 15/1/2008 and Accepted for Publication on 11/11/2008.
may be isolated from the throat or lower intestine (Nester et al., 2004).

Adenoviruses are commonly infecting human causing acute illness, mainly of the respiratory (the common cause of colds with fever) and intestinal tract (Nester et al., 2004).

The goal of the present study was to analyzed the T-cell proliferation in the presence of anti- CVB5, anti-Polio and anti- Adeno IgG antibodies in a population of children with T1DM and children who were healthy.

2. SUBJECTS, MATERIALS AND METHODS

Sixty Iraqi T1DM children (28 males and 32 females) 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 (mean= 9.45±3.9), and they were new onset of the disease (diagnosis was from one week up to five months). Diagnosis of Diabetes Mellitus and selection of patients was accomplished with the assistance of the consultant medical staff in the National Diabetes Center. All the patients were treated with daily replacement doses of insulin at the time of blood sampling. The patients were divided into two groups according to their ages in order to assess the aggressive of immune responses: 36 children equal or less than 10 years and 24 children up to 10 years. For the purpose of comparisons, 50 healthy control subjects matched for age (4-17 years old, mean= 11.26±3.73) and sex (25 males and 25 females) were selected who have no history or clinical evidence of type 1 diabetes or any chronic diseases and obvious abnormalities as a control group. The controls were divided into two groups 21 children equal or less than 10 years, and 29 children up to 10 years.

Collection of Blood Samples:

Eight milliliters of venous blood were collected from each subject. Five milliliters of blood were put in heparinised test tube (10 U/ml) and used for the lymphocyte proliferation. 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 for detection of anti- viral IgG.

Lymphocyte Proliferation Using Methylthiazol tetrazolium (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 human AB serum; Hepes; crystalline penicillin (1,000,000 IU) and streptomycin (1gm)(Pharma-intersprl, Belgica), and the final lymphocyte concentration was adjusted to 1-2x10^6 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 was incubated for further 4 hrs. Adding acidic isopropanol solubilized the converted dye. The absorbency was read using microculture plate reader using a wavelength of 570 nm (Mosmann, 1983).

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

Virus Antibodies

Serum IgG class antibody was measured against purified CVB5; Adenovirus antigen (serotype 3, 4, 7) and Polio vaccine using indirect ELISA method as described by (Davidkin et al., 1998; Harkoneen et al., 2003). 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 (Voller et al., 1980).

Statistical Analysis

Student t-test was used to measure the differences between two means; the results were expressed as means ± standard error (SE). The 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.

3. RESULTS

Lymphocyte Proliferation

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 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% respectively, \( P_1=0.82 \)) and in >10 years old group (86.04 vs. 92.7% respectively, \( P_1=0.62 \)).

**Table 1. Comparison of mean proliferation percentage of PBL in response to Con-A, CVB5, Polio vaccine and Adenovirus between controls and T1DM patients**

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>≤10 years</th>
<th>&gt;10 years</th>
<th>( P_1 )</th>
<th>( P_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Groups</td>
<td>No.</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Con-A</td>
<td>Controls</td>
<td>21</td>
<td>85.93</td>
<td>10.60</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>36</td>
<td>83.33</td>
<td>5.60</td>
</tr>
<tr>
<td>CVB5</td>
<td>Controls</td>
<td>21</td>
<td>49.16</td>
<td>5.88</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>36</td>
<td>36.67</td>
<td>3.08</td>
</tr>
<tr>
<td>Polio vaccine</td>
<td>Controls</td>
<td>21</td>
<td>47.38</td>
<td>5.83</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>36</td>
<td>34.44</td>
<td>2.79</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Controls</td>
<td>21</td>
<td>20.67</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>36</td>
<td>19.97</td>
<td>1.61</td>
</tr>
</tbody>
</table>

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

**Table 2. Prevalence of sero positive / negative IgG against CVB5, Poliovirus, and Adenovirus in control and T1DM patient groups**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Groups</th>
<th>No.</th>
<th>Sero positive</th>
<th>Sero negative</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td></td>
</tr>
<tr>
<td>CVB5</td>
<td>Controls</td>
<td>50</td>
<td>4 8.0</td>
<td>46 92.0</td>
<td>0.048 (S)</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>60</td>
<td>12 20.0</td>
<td>48 80.0</td>
<td>0.649 (NS)</td>
</tr>
<tr>
<td>Polio</td>
<td>Controls</td>
<td>50</td>
<td>13 26.0</td>
<td>37 74.00</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>60</td>
<td>19 31.67</td>
<td>41 68.33</td>
<td>0.57</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Controls</td>
<td>50</td>
<td>0 0</td>
<td>50 100.00</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>T1DM</td>
<td>60</td>
<td>4 6.67</td>
<td>56 93.33</td>
<td>0.57</td>
</tr>
</tbody>
</table>

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 \( (P_1=0.061,\text{and } 0.14 \text{ respectively}), \) (Table - 1).

Significant decline of proliferative response against Polio vaccine 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 1).

A non significant \( (P_1=0.82) \) mean 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 \( (P_2=0.57) \), Polio vaccine \( (P_2=0.14) \) and Adenovirus \( (P_2=0.57) \).

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 (Table 2). 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) (Table 2), whereas only 4 patients were sero positive for anti-Adeno IgG (6.67%) compared with the control group who were all sero-negative (Table 2). 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 same viral antigen primed the PBLs previously. The results represented in table 3 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 3. It was found that the patients who were sero-positive for anti-Polio IgG had higher mean 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 3).

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).

4. DISCUSSION

Functional Activity of PBLs

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 (Chapel et al., 1999).

The mean 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. No differences in immune responses were found between patients in the two age groups. The low proliferative responses against CVB5 antigen at disease onset is in agreement with other studies showing reduced T-cell proliferation against CVB5 (Varela-Calvino et al., 2002). Another report conducted by Juhela et al., (2000) found that PBLs of the children at onset of T1DM had significant weaker responses to purified CVB5 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.

The results of 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 (Varela-Calvino and Peakman, 2003), 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 CVB5 specific responder T-cells that secretes IFN-γ in T1DM patients with relative lack of proliferation.

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%).
Table 3. Relation of mean PBL proliferative percentage with the anti-CVB5, anti-Polio, and anti-Adeno IgG in T1DM patients

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Proliferation percentage</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CVB5 IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>12</td>
<td>50.58</td>
<td></td>
<td>0.048 (S)</td>
</tr>
<tr>
<td>-ve</td>
<td>48</td>
<td>22.99</td>
<td></td>
<td>0.039 (S)</td>
</tr>
<tr>
<td>Anti-Polio IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>19</td>
<td>31.48</td>
<td></td>
<td>0.042 (S)</td>
</tr>
<tr>
<td>-ve</td>
<td>41</td>
<td>20.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Adeno IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>4</td>
<td>30.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ve</td>
<td>56</td>
<td>14.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 Polio vaccine to enhance the immune system, although these children presumably had taken many boosted doses of oral Polio vaccine. An increase of anti-Enterovirus antibody levels (both IgM and IgG) was found preceding the appearance of signs of autoimmunity reflected either by synthesis of several autoantibodies or the development of clinical disease (Lonnrot et al., 1998). In contrast, a lower antibody titer against CVB3-5 serotypes and adenovirus-7 were also demonstrated in newly diagnosed T1DM children than in healthy controls (Buschard and Madsbad, 1984).

5. CONCLUSIONS

The present results show that T-cell proliferation in new onset Type 1 Diabetic children were decreased, but higher in children who were sero positive to anti-CVB5, Polio, and Adenovirus IgG antibodies.

REFERENCES

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IgG YAL results showed that the children's
calculation of P < 0.05 with respect to IgG levels,
Adenovirus B5-YAL were positive for Adenovirus
calculated 100% at the level of 5.0 IgG, YAL for
the child, when his first increase in the level of
Figure C were significant (P < 0.05).

The data are consistent with the data of the
IgG ELISA test for the small child. It was found
that the child's level of 5.0 IgG was higher than
5.0 in the control level. Con-A viral lesion and
influenza virus were found by ELISA test and
PCR test, respectively. The children were treated
with antireoviral agents. In the first day of
antireoviral treatment, the children's level of
IgG was lower than 5.0 in the control level. The
antireoviral treatment was started with the
use of antireoviral agents.

IgG YAL results showed that the children's
level of 5.0 IgG was higher than 5.0 in the control
level. The children were treated with antireoviral
agents. In the first day of antireoviral treatment,
the children's level of 5.0 IgG was lower than
5.0 in the control level. The antireoviral treatment
was started with the use of antireoviral agents.

*P < 0.05