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پروپوزال نویسی
Evaluation of Immunological Parameters in Diabetic Patients: Are These Patients Immunodeficient?

Zoleikha Moazzezi¹, Azam Hosseinian¹, Ensiyeh Ahmad Moazam¹, Mohamed-Bagher Eslami², Ezatollah Mosavi³, Hahel Akhavan-Niaki³, Ali Bijani⁵, Nanette Schloot⁶, and Amrollah Mostafazadeh⁴

¹ Department of Endocrinology, Ayatollah Rohani Hospital, Babol University of Medical Sciences, Babol, Iran
² Department of Pathobiology, Faculty of Health, Tehran University of Medical Sciences, Tehran, Iran
³ Department of Microbiology and Immunology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
⁴ Cellular and Molecular Biology Research Center, Babol University of Medical Sciences, Babol, Iran
⁵ Non-Communicable Pediatrics Diseases Research Center, Babol University of Medical Sciences, Babol, Iran
⁶ Department of Immunobiology, German Diabetes Research Center, Dusseldorf, Germany

ABSTRACT

It has been widely thought that diabetic patients are prone to infections due to hyperglycemia induced immunodeficiency; the present study was designed to examine this opinion.

In diabetic patients and normal control groups T-cell reactivity to hsp-60 molecule, tetanus toxoid recall antigen (TT) and phytohemagglutinin-A (PHA) mitogen were evaluated. The number of circulating IFN-γ, IL-10 and IL-13 cytokine producing cells stimulated with above antigens or mitogen as well as the serum levels of Th1/Th2 type cytokines were determined. Total serum immunoglobulins (IgG, IgA, IgM), C3, C4 and CH50 were also measured.

Diabetic patients showed a positive circulating T-cell reactivity to human recombinant hsp60. However, this reactivity was significantly lower in comparison to control group ($p < 0.001$). All other examined factors were not significantly different between diabetic and normal subjects except for the number of IFN-γ and IL-13 producing cells in response to PHA stimulation, which was higher in control group ($p = 0.006$, $0.018$, respectively). The mean serum concentration of IgA in diabetic patients was 245.86±115.05 mg/dl versus 192.96±105.33 mg/dl in healthy control group ($p<0.018$).

We were not able to demonstrate any substantial mitigation in cellular arms of immune reaction to some prominent T-cell antigens and mitogens, as well as, in main parameters of humoral immunity of diabetic patients, thus, the common notion of believing that patients with diabetes suffering from immunodeficiency should be revised. It is much more appropriate that “altered immunity” is applied instead of “immunodeficiency” to explain the immunity condition in this group of patients.

Keywords: Adaptive Immunity; Complement System Proteins, Cytokines, Diabetes Mellitus, Infection

INTRODUCTION

Diabetes is one of the most common metabolic...
disorders in which hyperglycemia is considered to be a laboratory hallmark.\textsuperscript{1,2} Due to sedentary and corpulent lifestyle, the number of people with this disorder is increasing worldwide.\textsuperscript{3,4} Among health care experts, there is almost a consensus on diabetic patients’ susceptibility to some infections due to the presumed existence of hyperglycemia induced immunodeficiency in these subjects.\textsuperscript{5,11} On the contrary, there are some evidences implying that the immune system does not undergo a significant deficiency in individuals with diabetes, even in some circumstances hyperglycemia causes the immune-hyperactivity and may act as a predisposing factor of the complications of diabetes such as acute diabetic foot syndrome and probably for autoimmune/auto-inflammatory disease in general.\textsuperscript{12-16} Indeed in some literatures, type 2-diabetes is considered as the auto-inflammatory diseases.\textsuperscript{17,18} Our previous works showed that the levels of total anti-heat shock protein-60 antibody as well as IgA anti hsp60, IgG and its two subclasses IgG1 and IgG4 specific for this protein are the same in type 1 diabetic patients and healthy controls.\textsuperscript{19} In another study on subjects with type 2 diabetes, we showed the increased levels of IgA anti-hsp60 antibody at borderline significant in comparison to normal controls.\textsuperscript{20} We were not able to judge whether this increase originated from intrinsic tendency of diabetic patients to IgA production or was directed against hsp60 only.

Heat shock protein-60 (Hsp-60), a ubiquitous and phylogenically highly conserved protein, which its expression alters in different stress conditions including hyperglycemia has a prominent role in diabetes.\textsuperscript{21} As part of its danger signal activity, this protein can also induce the expression of inflammatory cytokines such as tumor necrosis factor-\textalpha\, (TNF-\textalpha) and Interleukin-6 (IL-6) which in turn these cytokines can trigger inflammation, the other diabetes hallmark.\textsuperscript{22,23} 

Along with antibody, the complement system which consists of more than 25 proteins constitutes the other major arm of humoral immune system. In addition to defense against pathogens, its immunoregulatory function is also paramount. The third component of this system (C3) has a special role in this regard. Thus, regarding its important role in human health, in routine clinical practice, the efficacy of this system was evaluated by measuring its two components with highest serum concentrations i.e., the third and fourth components (C3 and C4), as well as by assessing the whole complement hemolytic activity (CH50).\textsuperscript{24}

In the present study, in order to grasp an overall view about the effect(s) of chronic hyperglycemia on immune system, humoral and cell immunity were evaluated in diabetic patients and healthy controls. To this end, in vitro T-cell reactivity to hsp-60 molecule, tetanus toxoid (TT) recall antigen as well as phytohemagglutinin-A (PHA) as T-cell polyclonal activator, were used to evaluate cell-mediated immunity and total serum immunoglobulins G, A, M (IgG, IgA, IgM), serum levels of C3, C4 and CH50 were also determined to evaluate humoral immunity.

**PATIENTS AND METHODS**

**Patients and Normal Controls for Evaluation of Cellular Immunity**

Twenty-one subjects (12 females, 9 males, average age 30.3 y, range 15-43 y) with newly onset type 1 diabetes (T1D) and twenty age- and sex-matched controls (11 females, 9 males, average age 28.5 y) who had no clinical manifestations of diabetes were recruited in the present study. Patients referred to Diabetic Clinic at the Diabetes Research Institute in Dusseldorf, Germany for diagnosis or treatment. All patients had a positive result for glutamic acid decarboxylase (GAD-65) and (Islet Antigen) IA-2 antibody and fasting blood sugar (FBS) equal or more than 126 mg/dl when tested by clinical laboratory methods.

Peripheral blood mononuclear cells (PBMCs) were prepared from 10 ml of venous blood sample of each subject. All subjects gave their written consent to participate in this study.

**Patients and Controls for Evaluation of Humoral Immunity**

Fifty patients with diabetes mellitus (DM) type 2 (40 females, 10 males) who were consecutively referred to the clinic of Shahid Beheshti Teaching Hospital in Babol City, Iran were enrolled in this study. The mean±SD age of patients was (48±17.31 y). All patients had glycosylated hemoglobin A1c (HbA1c) more than 7%. Patients with acute diseases, chronic complications (retinopathy, nephropathy, neuropathy), hyperthyroidism, hypothyroidism, autoimmune diseases, chronic digestive diseases, hepatitis, as well as subjects under immunosuppressive drug therapy were excluded from the study. Also as normal controls, fifty subjects (41 females, 9 males, mean age;
normal controls had clinical diabetic manifestations, confirmed by our research group’s endocrinologist. All subjects gave their written consent to participate in this study.

Five ml venous blood was taken from each subject for serum preparation. Isolated sera were stored at -80°C until the time of conducting the relevant test.

**Lymphocyte Transformation Test (LTT)**

As representatives of cellular immunity, the in vitro T-cell reactivity to a recall antigen such as TT and to a ubiquitous and phylogenically highly conserved self antigen i.e heat shock protein (hsp-60) as well as PHA as a T-cell mitogen were investigated by proliferation test in patients with T1D and their age- and sex-matched controls. LTT was performed according to the method previously described. Briefly, PBMCs were isolated from the patients and normal controls from Na-heparinated blood by ficoll-hypaque gradient centrifugation. In 96 wells- cell culture plate, 1.5×10⁶ of isolated PBMCs were incubated separately at 37°C and 5% CO2 with either 10µg/ml of recombinant human hsp-60 (Peptor Germany), 2% TT (Peptor, Germany) or 0.3ug/ml PHA. As background control under the stated condition, the same number of PBMCs were cultured without any stimulation. After 5 days of incubation, luci of H-thymidine was added to each well and incubation was extended for another 18 hr. Then, cellular DNA was transferred onto the filtered plate and 50µL of scintillation liquid was added to each well and the beta radiation was counted after 30 min. Lymphocyte reactivity to antigen or stimulator (Stimulation Index (SI)) was determined by dividing the count value obtained from each related antigen stimulated well to the count value of the well without stimulation (background control).

**Determination of the Number of Interferon (IFN)-γ, IL-10 and IL-13 Producing Cells by Enzyme Linked Immunospot (ELISPOT)**

ELISPOT is a technique that enables single cell level counting of special cytokine producing cells. One and a half million PBMCs from each patient and normal control individuals were suspended in 0.5ml of RPMI-1640 medium supplemented by 10% human serum of AB+ blood group (Biowhittakar, Netherlands), 200µg/ml L-glutamine (Gibco company) and 500µg/ml penicillin and Erythromycin were incubated in 48 wells cell culture plate (Nunc, Denmark) at 37°C and 5% CO2 separately with 10µg/ml of Hsp60 and 0.3µg/ml PHA, 10ng/ml PMA/1µg lonomycine and 2% TT as recall antigen. After 24hr, 0.5ml RPMI-1640 supplemented with 5% AB+ human serum was added to each well and incubation was continued up to 48hr. After which, the non-adherent cells were transferred into sterile test tubes and after 3 times of washing with sterile phosphated-buffered Saline (PBS) (Gibson company) at 270g, for 3min, then 300000 stimulated lymphocytes in duplicated wells were suspended in RPMI-1640, 1% human serum AB+ was transferred into the Elispot plate which was already coated with capture antibody specific for either IL10, IL-13 or IFN-γ (U-cytech, Netherlands) and blocked with 1% Bovine serum albumin (U-cytech, Netherlands) on its antibody uncoated surface. According to the manufacturer’s instruction, after incubation for 5hr at 37°C and 5% CO2, the supernatants were removed without shaking and then 200µl cold (2-8°C) deionized water was added to each well, on ice, for disrupting the cell membrane. After 10min., the cold water was discarded and the plates were thoroughly washed 10 times with 300µl of PBS-tween, 0.05%. After adding 100µl Biotin conjugate detector antibody reactive to IFN-γ and IL-13, IL-10 and incubating at 37°C for 1hr, the wells were thoroughly washed and 50µl of Gold Labeled Anti Biotin Antibiot (GABA) was added /supplemented to each well and then the spots were developed by adding the gold activator solution (U-Cytech, Netherlands ready for use). The spot developing reaction was stopped after 20-30min by deionized water washing. The number of spots in each plate was detected by Elispot plates reader (Biorereader, Germany).

**Determination of Serum Levels of IFN-γ, IL-10, IL-13 by Enzyme Linked Immunosorbert Assay (ELISA)**

For investigating serum cytokine levels in diabetic patients, some selected Th1 (IFN-γ) and Th2 type cytokines (IL-10, IL-13) were determined by Elisa and compared with healthy control subjects. To this end, we used the selected cytokines Elisa kits supplemented by Pharmingen USA. Briefly, maxisorb microtitration plates (Nunc, Denmark) were coated with mouse monoclonal antibody specific to each cytokine
Evaluation of Immunological Parameters in Diabetic Patients

(Pharmingen, USA) and the uncoated sites of plates were blocked with PBS solution containing 0.5% skim milk (Sigma). Then, 50uL of diluted sera in PBS-milk 0.5% solution was dispensed into the duplicated wells devoted to each cytokine. After 1hr incubation at room temperature (RT) and washing it thoroughly, 50uL of biotin-conjugated second antibodies (Pharmingen, USA) were added to each well. The plates were incubated for another 1hr at RT and then thorough washing, 50uL streptavidine conjugated with horse radish peroxidase (HRP) enzyme (Pharmingen USA) was dispensed to each well. Following 30min. incubation at RT, 100uL of tetra methyl benzidin (TMB) was added and the enzymatic reaction was stopped by 0.1N of sulfuric acid. The optical density (OD) was read by Elisa reader (Titerteck Multiscan, UK) and cytokine concentrations (expressed in pg/ml) were calculated for each cytokine according to its related standard curve.

Determination of the Serum Levels of Total IgG, IgA, IgM and Complement Components (C3, C4)

The serum levels of IgG, IgA, IgM, as well as the third and fourth components of complement system (C3,C4) were assessed by the ready- to-use kits (Baharafshan, Iran), based on the single radial immunodiffusion technique. Briefly, the plate was adjusted to RT and then 3-5ul of patients and controls sera and 3-5ul of each 3 standard sera were placed into each related well. Standard sera had a definite concentrations (in mg/dl) at low, intermediate and high levels of IgG, A, M, C3, and C4. After 24-48hr of incubation at RT, the diameter of developed precipitation ring in each well was measured by a ruler. The optical density (OD) was read by Elisa reader (Titerteck Multiscan, UK) and cytokine concentrations (expressed in pg/ml) were calculated for each cytokine according to its related standard curve.

Whole Complement Hemolytic Activity (CH50%) Assessment

The CH 50% was determined in serum samples by using the Miller et al method.\(^2\) Briefly; serum samples were diluted in veronal buffered saline (VBS) at 1:60 and then 1, 1.2, 1.5, 2, 2.5, 2.0 ml of diluted sera were dispensed to six related tubes, numbered 1 to 6, respectively. Two other tubes number 7, 8 were used as controls. Tube number 7 contained 6.5ml of VBS without any serum and the tube number 8 contained only 6.5mL of distilled water (without serum and VBS). This tube contained no serum and no VBS. Sheep red blood cell (SRBC) sensitization was performed with incubation at room temperature for 10 min of the cells with anti-SRBC (Biogen Iran). One ml of sensitized –SRBC was added to each test tube except tube number 6. After 30min of incubation at 37\(^{\circ}\)C in water bath on the shaker, the content volumes of all tubes were adjusted to 7.5mL with VBS. The tubes span at 300rpm for 5min and then the OD of supernatants was obtained by spectrophotometer (Clement, England) which already the absorption at 410 nm wavelength was adjusted to zero by supernatant of tube number 6. The OD value of tube number 7 was subtracted from other tubes’ OD and the “y” value was obtained.

Table 1. The levels of serum Immunoglobulins, complement factors, complement activity and mean values of stimulation index in response to different stimulator in diabetic and normal subjects.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>Diabetic patients Mean+/-SD</th>
<th>Normal controls Mean+/-SD</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG(mg/dl)</td>
<td></td>
<td>2540.82±1528.62</td>
<td>3035.76±1588.01</td>
<td>0.116</td>
</tr>
<tr>
<td>IgA(mg/dl)</td>
<td></td>
<td>245.86±115.05</td>
<td>192.96±105.33</td>
<td>0.018</td>
</tr>
<tr>
<td>IgM(mg/dl)</td>
<td></td>
<td>305.54±121.66</td>
<td>323.58±110.78</td>
<td>0.44</td>
</tr>
<tr>
<td>C3(mg/dl)</td>
<td></td>
<td>101.68±4.31</td>
<td>93.12±3.67</td>
<td>0.133</td>
</tr>
<tr>
<td>C4(mg/dl)</td>
<td></td>
<td>43.12±3.51</td>
<td>38.46±2.51</td>
<td>0.250</td>
</tr>
<tr>
<td>CH50(U/ml)</td>
<td></td>
<td>29.66±0.64</td>
<td>30.18±0.77</td>
<td>0.605</td>
</tr>
<tr>
<td>SI(Hsp60)</td>
<td></td>
<td>3.65±4.06</td>
<td>6.91±8.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SI(TT)***</td>
<td></td>
<td>39.84±36.70</td>
<td>23.27±22.51</td>
<td>0.148</td>
</tr>
<tr>
<td>SI(PHA)****</td>
<td></td>
<td>17.53±32.58</td>
<td>83.95±121.47</td>
<td>0.303</td>
</tr>
</tbody>
</table>

** Human recombinant hsp60 was used for stimulation, *** tetanus toxoid was use for stimulation, **** Phytohemagglutinin-A was as the stimulator
(lysis%), for each tube were obtained by dividing of the OD value of the desired tube to OD value of tube number 8, in which 100% of SRBC were lysed by hypotonic distilled water. Then the “x” value for each tube was calculated by y/1-y formula.

The curve was plotted against “x” on X axis and the “volume of serum dispended to each tube” on Y axis for all serum samples on the logarithmic paper. The point of x=1 was found on the curve for each subject perpendicular to Y axis to determine the serum volume required for SRBC 50% lysis. The CH50 values which were expressed in U/ml were calculated by dividing 60 (reciprocal of serum dilution) to serum volume needed for SRBC 50% lysis (Table 1).

### Statistical Analysis

The normality of data distribution has been examined by Kolmogorov-Smirnov test. For the comparison of means of the data with Gaussian distribution, Independent sample t-test and for the data without Gaussian distribution, Mann-Whitney U tests were carried out. Pearson test was used for the correlation between age and IgA levels. In all tests, \( p < 0.05 \) was considered statistically significant.

### RESULTS

#### Proliferation of PBMCs of Diabetic Patients in Response to TT, hsp60 and PHA

In response to stimulation with TT, hsp60 antigen and PHA, the diabetic patients and normal subjects showed positive results for all stimulators (the Stimulation Index (SI) \( \geq 3 \) is considered as positive response in LTT) (Table 1). Even in response to TT, the diabetic patients showed a trend of having a higher reactivity in comparison to normal subjects (SI mean; 39.84±36.70 in diabetic group versus 23.27±22.51 in normal control group). However, this difference was not statistically significant.

In response to hsp60 antigen, the mean of SI in diabetic group was 3.65±4.06 and 6.91±8.47 in normal group. Statistically, this difference was significant (\( p=0.006, 0.018 \), respectively). However, between the two groups, there was no significant difference in number of circulating IFN-\( \gamma \), IL-10 and IL-13 producing cells stimulated with hsp60 and TT (Table 2).

#### No Significant Difference in Serum Levels of Th1 type (IFN-\( \gamma \)) and Th2 (IL-10, IL-13) between Diabetic Patients and Healthy Controls

Table 3 presents the mean values for serum levels of Th1/Th2 type cytokines in two study groups. The diabetic patients' serum levels of IFN-\( \gamma \), IL-10, IL-13 were lower than healthy control group. However, the differences were not statistically significant.

#### Positive Correlation between Age of Subjects and Serum IgA Levels

Direct correlation between age and serum IgA was found when we examined the correlation between these two variables in whole subjects of our study by Pearson test (\( r=0.33, p<0.001 \)). This direct correlation was also observed while diabetic group was analyzed separately, (\( r=0.31, p=0.028 \)) (Figure 2).

Table 1 presents the serum concentrations of C3, C4 and CH50 in diabetic patients and healthy controls. The diabetic patients showed similar levels of serum C3 and C4 when compared with healthy control subjects. Also, between the two groups, there was no significant difference in CH50 activity in which all complement
Table 2. The number of circulating cytokine producing cells stimulated with heat shock protein-60 (hsp-60), phytohemagglutinin A (PHA) and tetanus toxoid (TT) in type 1 diabetic patients and normal controls.

<table>
<thead>
<tr>
<th>Cytokines &amp; Stimulant</th>
<th>Groups</th>
<th>Diabetic patients’ Cytokine producing cell number Mean± SD(n)</th>
<th>Healthy subjects’ Cytokine producing cell number Mean+/−SD(n)</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ (hsp60)</td>
<td>4.84±7.63(19)</td>
<td>6.35±8.55(20)</td>
<td>0.102</td>
<td></td>
</tr>
<tr>
<td>IFN-γ (PHA)</td>
<td>4.92±1.68(14)</td>
<td>25.21±47.46(19)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>IFN-γ (TT)</td>
<td>8.8±12.80(15)</td>
<td>23.85±52.79(20)</td>
<td>0.150</td>
<td></td>
</tr>
<tr>
<td>IL-10 (hsp60)</td>
<td>7.74±9.71(19)</td>
<td>7.15±7.90(20)</td>
<td>0.671</td>
<td></td>
</tr>
<tr>
<td>IL-10 (PHA)</td>
<td>41.37±17.6(16)</td>
<td>60.79±62.06(19)</td>
<td>0.544</td>
<td></td>
</tr>
<tr>
<td>IL-10 (TT)</td>
<td>17.2±33.97(15)</td>
<td>28.95±64.89(20)</td>
<td>0.738</td>
<td></td>
</tr>
<tr>
<td>IL-13 (hsp60)</td>
<td>3.067±2.6(15)</td>
<td>3.25±3.34(16)</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>IL-13 (PHA)</td>
<td>3.15±0.55(13)</td>
<td>15.93±23.31(17)</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>IL-13 (TT)</td>
<td>16.33±21.67(12)</td>
<td>15.06±18.58(17)</td>
<td>0.756</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. IgA concentration (mg/dl) in serum of patients with diabetes and normal control subjects.

Table 3. Serum levels of IFN-γ, IL-10 and IL-13 in type 1 diabetic patients and healthy subjects.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Study groups</th>
<th>Diabetic patients (n=4) Mean±SD</th>
<th>Healthy subjects (n=13) Mean±SD</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>2.43±2.15</td>
<td>3±13.87</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>IL-10(pg/ml)</td>
<td>0.48±0.48</td>
<td>0.56±0.59</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>IL-13(pg/ml)</td>
<td>0.15±0.67</td>
<td>2.36±7.68</td>
<td>0.47</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The most important finding in our study was that the patients with diabetes showed higher levels of serum IgA in comparison to normal control subjects. This finding is consistent with the data reported by Gonzales-Quinетеla A et al. Interestingly, as in A. Gonzales-Quintile’s study, we also found a positive correlation between serum IgA levels and the age of patients and normal subjects. In comparison to the other classes of immunoglobulins, IgA has the highest concentrations in body’s fluids such as saliva and also in mucus covering the surface of intestine and respiratory tracts.

In the interpretation of why diabetic patients synthesized higher levels of IgA compared to normal subject group, we supposed that the permeability of mucosal surfaces in these patients has increased thereby increasing higher antigen uptake by intestinal mucosa membrane and in reaction to these antigens, IgA antibody is synthesized. It seems logical because according to the study reported by Outi, the diabetic patients have leaky intestine with increased levels of permeability. There was no significant difference in serum levels of IgM and IgG between the two groups. Thus, the data generated by this study shows that the diabetic patients produce adequate serum IgG, A, M required not to succumb to pathogenic microorganisms. By referring to the presented values in the paper published by Furst in related to the minimal levels of serum immunoglobulins, requiring to an appropriate defense against infectious germs and consequently not succumbing to infections, the diabetic patients of our study showed higher levels of serum IgG, A, M therefore they are not immune deficient.

There was no significant difference in serum levels of C3, C4 and CH50 between patients and control groups. Complement system especially C3 component has important role in induction of the specific immunity memory and antibody production. Thus, the normal levels of serum immunoglobulins were consistent with the normal levels of serum C3 concentrations in diabetic patients evaluated in our study. Also complement activation can lead to the direct killing of some infectious agents including Nisseria. The normal results of C3, C4 and CH50 tests in patients with diabetes imply that these patients may exhibit a normal reaction to some pathogens (such as Nisseria) in which for their eradication, complement plays a pivotal role.
As some representatives of the cell mediated immunity, we evaluated the PBMC response to PHA as a T-cell polyclonal activator, TT as a recall antigen and hsp60 as a ubiquitously expressed and phylogenically conserved protein. We also found that isolated PBMCs from most of the study subjects proliferated when stimulated with human recombinant hsp60. Human hsp60 has a high amino acid sequences homology with GroEL molecule which is synthesized by E.Coli, one of the best known normal commensal intestinal microbiota in human. We supposed that the existence of natural T-cells, reactive to self hsp60 /GroEL in blood of almost all individuals resulted in this finding. This supposition is supported by other studies. Handely HH et al. have shown that all sera from normal subjects and patients with some autoinflammatory diseases had autoantibodies directed to human hsp60 induced by GroEL molecule. Varbiro S et al. also reported that human anti-hsp60 autoantibodies have the basic features of natural autoantibodies. The work of Konen-Waisman S et al. proved that in normal subjects, self and bacterially originated hsp60 T-cell epitope peptides serve as immunogenic carriers for a T-cell-independent sugar antigen.

Although the diabetic patients showed a positive T-cell response to hsp60, this response is significantly lower than the response of normal control subjects but it seems to be adequate to support other immune cells including B-Lymphocytes. This judgment is supported by our previous findings demonstrating that diabetic patients showed the same levels of serum IgG, G1, G4 and IgA anti hsp60 when compared to normal controls. Although, the PBMCs prepared from diabetic patients and normal control subjects showed a higher stimulation index when stimulated with TT and PHA, due to the existence of high variation in related data, we were not able to see any statistically significant difference between the two groups. Despite PBMCs from normal control showed a higher proliferation rate in response to hsp60, the Elispot data generated by this study was not able to provide any evidence implying existence of higher number of hsp60 or TT stimulated IFN-γ or other cytokine producing cells in control subjects. Indeed, in agreement with Elispot data, both normal and diabetic groups showed the same levels of IFN-γ, IL-10 and IL-13 in serum. Elispot data demonstrated that diabetic patients revealed a significantly lower number of circulating IFN-γ and IL-13 but not IL-10 producing cells upon stimulation by PHA mitogen, however, the two groups had no significant difference in proliferation response to this mitogen. It has been previously reported that some cytokines including IL-2 and IFN-γ play pivotal roles in cell growth of PBMCs induced by antigen or PHA, in this context the role of IL-2 is more significant than IFN-γ. Neither the number of IL-2 producing cells in PBMC nor the IL-2 levels in serum was evaluated in this study. Moreover, we did not assess directly the IFN-γ and IL-2 levels in lymphocyte proliferation test cell culture supernatant. For these reasons, our judgment which was based on the number of cytokine producing cells, was insufficient.

Taken together, the data generated by this study implicated that there was no significant decrement in the main components of the immune system and even the production of IgA was increased. Some basic and clinical evidence make this conclusion reasonable. Upon T-cell activation, via CD28-mediated PI3k/Akt-dependent pathway which leads to the induction and trafficking of glucose transporter -1 (GIUt-1) onto the cell membrane, the adequate levels of glucose are imported into the T-cells. Thus, T cells do not need insulin-induced glucose transporter Glut-4 to transport glucose into the cytoplasm. Moreover, lymphocytes and macrophages can use glutamine as an energy supplier instead of glucose. Also, CD28 activation pathway through mTOR (mammalian target of rapamycine) can facilitate the transport of amino acids into the activated T-cells where there are a high demands for these in order to synthesize a dozen of cytokines and growth factors. This is another role of insulin hormone that in conditions like its absence or its receptor dysfunction which may present in diabetic patients, will be compensated by engaging the CD28 activation pathway in one of the most vital immune cells i.e. T-helper cells. In turn, as helper, these cells would influence a variety of functions of immune system including antibody production and cell mediated immunity. Indeed by blocking this pathway, normal T-cells undergo anergic condition despite the presence of signal 1 and signal 2 which are required for full activation of T-lymphocytes. It has also been shown that anergic T-cells are metabolically anergic. Jeffrey C. Rathmell reported that T-cells do not typically respond directly to insulin. From these evidences, it could be concluded that T-cells may metabolically function as insulin independent cells and...
consequently, the immune system would not have serious complications. In other words, the immune system has adequate requirements and sufficient competency to combat against invading microorganisms or to elicit a normal immune response to vaccines. For example, Pozzilli P reported that the humoral and cell-mediated immune responses to the 3 virus strains constituent of WHO recommended anti influenza vaccine were similar in people with diabetes and healthy individuals. Also Solomonova indicated that the antibody in response to tetanus reimmunization in diabetic patients is unimpaired.

Collectively, the above mentioned evidences and also the data generated by this study demonstrated that chronic hyperglycemia does not cause a substantial mitigation in both humoral and cellular arms of immune system and such a common belief that patients with diabetes are prone to infections should be revised. Along with this conclusion, Grumach and et al. also reported that there was no strong correlation between diabetic immune deficiency and susceptibility to infections, the paper published by Joshi also confirms this conclusion.

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