Original Article

# Alteration in CD8$^+$ T cell subsets in enterovirus-infected patients: An alarming factor for type 1 diabetes mellitus

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**Abstract** Type 1 diabetes is a multi-factorial disease that can develop due to the combination of genetic and environmental factors. Viruses, particularly enteroviruses, are major environmental candidates in the pathogenesis of type 1 diabetes, even though the mechanisms of pathogenicity of these viruses and their effects on the immune system have not been understood very well yet. Previous studies show that any imbalance in the population of different lymphocyte subsets could develop autoimmune diseases. Our theory is that enteroviral infection causes an impairment in the distribution of lymphocyte subtypes and consequently results in the diabetes onset in some individuals. Therefore, in this project, we evaluated the distribution of T CD8+ lymphocytes and their subsets in type 1 diabetes patients. This study was conducted to investigate the relationship between enteroviral infection and type 1 diabetes mellitus in an Iranian population, and suggestion a predicting approach for susceptible subjects.

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## Introduction

Autoimmunity refers to the system of immune responses in which immune cells and involved proteins in this system attack their cells and tissues [1]. Causes of autoimmunity are complex and sometimes unknown but generally, the genetic susceptibility and presence of environmental risk factors can play a role in the development of autoimmunity [1]. Growing incidence of these diseases has attracted much attention. Type 1 diabetes is a cell-mediated auto-immune disease [2] in which pancreatic islet beta cells are destroyed [2—4]. This autoimmune disease is due to the combination of various genetic and environmental factors [5]. In the recent years, having determined genetic factors involved in the development of type 1 diabetes [6,7], efforts have sought to determine the environmental factors of this disease.

Viruses are considered the main environmental causes of autoimmune diseases [8]. Among the viral families, enteroviruses are considered to be involved in the path-ogenesis of type 1 diabetes [9,10], although the mecha-nism(s) of pathogenicity of these viruses has (have) not yet been sufficiently understood. Iran has a low rate of type 1 diabetes incidence (3.7 per 100,000 people) [11], but the frequency of enteroviral infection in type 1 dia-betes patients, and their potential effects on the immune system have not been adequately studied in this country. In this study, we investigated enteroviruses genome in peripheral blood mononuclear cells (PBMCs) using a sen-sitive assay, RT-PCR, and the primers for 5′-untranslated region which are shared by all enteroviruses, to determine the prevalence of infection in Iranian patients with type 1 diabetes.

Previous studies suggested that imbalance in the pop-ulation of naive, effector and memory lymphocyte subsets could cause autoimmune diseases [12]. It has been shown that enteroviral infections mainly have an immunomodu-latory effect and, in rare cases, directly lyse beta cells [5,13]; in other words, these viruses serve as immune modifiers, not just simple triggers. Therefore, we evalu-ated the potential effects of enterovirus on the distribu-tion of T CD8$^+$ lymphocyte, as main cells involved in the destruction of the beta islet cells, and its subsets in type 1 diabetes patients. First, we looked for the presence of enteroviral infection in patients with diabetes. In the next step, the patient group was divided into two groups con-sisting of the enterovirus-infected group and non-enterovirus-infected group. Then, we investigated alter-ation in the distribution of T CD8$^+$ lymphocytes population and their subsets in these groups compared with a control group.

## Materials and methods

### Patients and clinical specimens

In this case—control study, samples were taken from 35 patients with type 1 diabetes aged 2—27 [mean: 13.7 ± 6.6] years. The patients were followed up by the Department of Internal Medicine of the Shahrekord University of Medical Sciences. After research purposes were explained, the people who volunteered to participate in the study pro-vided informed consent for their voluntary participation. In case of collecting samples from under 18 years-old patients and volunteers, we gained informed consents from their parents or legal guardian after explanations the aims of the research. It should be noted that patients were selected based on their diagnostic criteria by a specialist for this study. We did not sample from diabetic patients with any autoimmune background in their family since we wanted to study on enteroviruses, as an external factor involved in type 1 diabetes diseases, and their probable function on alteration of CTLs subsets.

Then, suitable samples were taken from the patients. Moreover, appropriate samples of 35 healthy people (1—27 (mean: 13.6 ± 7.3) years), who were age and sex-matched with patients, were taken in the same year with controls. The serum samples were kept at −80 °C until assays with anti-enterovirus VP1 protein and autoantibody ELISA kits. RNA was extracted immediately from a portion of freshly PBMCs for RT-PCR enterovirus genome detection. Another part of the collected PBMCs was used to identify pheno-types of T CD8$^+$ subsets using flow cytometry.

### RNA extraction and RT-PCR

PBMCs of the patients and controls were isolated from whole blood by Ficoll density gradient centrifugation. We extracted total RNA from PMBCs using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufac-turer's instructions. Total RNA was transferred immediately to and kept in a freezer at −80 °C till cDNA synthesis. cDNA was synthesized with reverse transcriptase kit (Takara, Japan).

Two μl PrimeScript Buffer 5X (containing MgCl$_2$ and dNTP), 0.5 μl PrimeScript RT Enzyme Mix I (containing RNase Inhibitor), 50 pmol Random Hexamer, 1 pmol Gene-specific primer F1: 5′-CACCGGATGGCCAATCCA-3′, and 1.5 μl nuclease free water were added to 5 μl extracted total RNA. This mixture was incubated for 15 min at 42 °C in one cycle for reverse transcription. Then, it was incubated for 5 s at 85 °C for inactivation of reverse transcriptase with heat treatment.

Produced cDNA was used for amplification in nested RT-PCR. We used two-step RT-PCR to detect enteroviral RNA. At the first step, 22.5 μl master mix solution containing 0.5 μl F1 primer, 0.5 μl R1 primer: 5′-CCTCCGGCC CCTGAATG-3′ [9], ready-to-use PCR Buffer (containing Tris—HCl, NH$_4$SO$_4$, EDTA and 2-mercaptoethanol in pH = 8.8), 0.5 μl MgCl$_2$, 0.5 μl dNTP, 0.1 μl Taq DNA po-lymerase 1.2 U and 17.9 μl nuclease-free water was added to 2.5 μl cDNA. The first step of PCR was performed on a total volume of 25 μl of the mixture in the following ther-mal cycle program: At the first step, we performed initial denaturation at 94 °C for 5 min. Then, five cycles were conducted at 90 °C for 30 s, 59 °C for 25 s and 73 °C for 40 s. These five cycles were followed by 30 cycles in the following thermal cycle program: 94 °C for 45 s, 57.7 °C for 25 s and 73 °C for 45 s. At the end of the first step of PCR, a final extension was conducted at 73 °C for 10 min.

In the second step of the nested-PCR test, 1.6 μl of the first PCR product was diluted at a ratio of 1:20 by nuclease free water. Two and a half μl diluted DNA was mixed in the

total of 22.5 μl prepared PCR master mix but with the change in primers to F2: 5′-ATTGTCACCATAAGCAGCCA-3′ and M13-R2: 5′-TATGTAAAACGACGGCCAGT-GCCCCTGAAT GCGGCTAAT-3′ (Table 1). Nested-PCR was performed in a similar manner to the first step, but 30 cycles in nested-PCR were followed in new annealing temperature (at 60 °C). M13-primer was used to sequence PCR products (the data of sequencing are not shown). PCR products were electrophoresed on 1.5% agarose gel (Sigma, Germany) and ethidium bromide used for staining bands. In the first step, 197-bp products were synthesized, and in the nested-PCR, the final product was 165-bp.

## Anti-VP1 antibody and autoantibody to GAD65

Anti-VP1 antibodies of three different classes (IgA, IgG, and IgM) were measured in the patients' and controls' serum samples by an ELISA kit (Serion/Virion, Germany). In addition, antibodies against GAD65 were measured by GAD65-Ab ELISA kit (BIOMERICA, USA).

## Analysis of immunological markers of cytotoxic lymphocytes

PBMCs were isolated by Ficoll density gradient centrifugation from freshly heparinized blood and washed twice in PBS solution. Approximately $10^5$ PBMCs were collected from each sample. The cells were collected in separated tubes. The antibody which was used to identify cytotoxic lymphocyte cells was anti-CD8 conjugated with PE-Cy5 (Becton Dickinson, UK). To further distinguish cytotoxic lymphocytes subsets, anti-CD45RO conjugated with phycoerythrin and anti-CD45RA conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson, UK) were added to each tube. The PBMCs in the tubes alongside sufficient amounts of these monoclonal antibodies were incubated for 20 min at 4 °C, and then 20 min at room temperature.

After staining tubes, to remove extra and unbounded conjugated antibodies, cells in the tubes were washed twice with PBS. The phenotypes of the cells were analyzed by three-color Partec CyFlow Space flow cytometer (Partec GmbH, Germany). At least 10.000 cells were counted and analyzed per each sample. Analysis of naive, effector and memory CD markers of the cytotoxic lymphocytes was performed by Flowjo software. We also gated population of lymphocytes to calculate more accurate percentages of lymphocytes and their subsets based on respective antibodies. By this gating, we did not count the monocytes,

remaining red blood cells, granulocytes, dead cells and other debris. The percentage of positive cells was determined by setting the lower limit line over the non-specific fluorescence of unstained samples and samples with suitable isotype control.

## Statistical analysis

The flow cytometric findings are expressed as means ± SD. The percentages of cytotoxic T lymphocytes and their subsets in the two groups of the patients and the controls were compared by two-tailed student's t-test for unpaired samples with the normal distribution. The results of ELISA on detection of antibodies and autoantibodies were compared by Fisher's exact test, and those of nested-PCR were compared among the groups by chi-square test. Statistical analysis was conducted by SPSS 23.0. P value less than 0.05 was considered the level of significance.

## Results

### ELISA

Antibodies against GAD65 were detected in 10 (28.5%) of the 35 patients with type 1 diabetes, and 1 (2.8%) of the 35 controls ($P = 0.003$). Furthermore, anti-GAD65 antibody titer was significantly higher in enterovirus-infected patients with type 1 diabetes than in the non-enterovirus-infected patients and controls ($P = 0.001$ and $P < 0.001$, respectively).

Anti-EV VP1-IgA antibodies were detected in the serum of 10 (28.5%) patients and anti-EV VP1 IgG antibodies in 13 (37.1%) of 35 patients. Moreover, we found no anti-EV VP1-IgM antibodies in the patients' serum. Anti-EV VP1-IgM antibodies were not measured in the serum samples of the controls.

### Nested-PCR

As we can see in Figure 1, the Nested-PCR was positive in 12 (34.3%) of 35 patients with type 1 diabetes, and only 1 (2.9%) of 35 the controls with a significant difference between the two groups of the patients and the controls ($P = 0.001$).

Generally, anti GAD65, as an autoantibody, can observe in the control subjects. It is also true in terms of positive PCR result [9]. So we did not omit the data of autoantibody and PCR positive healthy subjects in statistical analysis.

**Table 1** Some characteristics of nested-PCR primers used in the study.

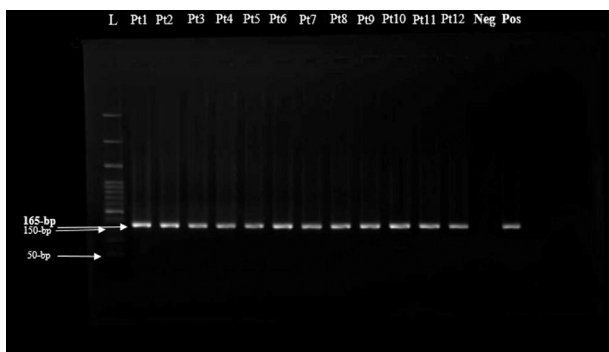| Primers | Sequence | Temperature (°C) | Location | Product size |
|---------|----------|-----------------|----------|--------------|
| R1 | 5′-CACCGGATGGCCAATCCA-3′ | 59.7 | 645−628 | 197-bp |
| F1 | 5′-CCTCCGGCCCCTGAATG-3′ | 59.4 | 448−464 | |
| M13-R2 | 5′-TATGTAAAACGACGGCCAGT-GCCCCTGAATGCGGCTAAT-3′ | 60.5[a] | 450−468 | 165-bp |
| F2 | 5′-ATTGTCACCATAAGCAGCCA-3′ | 57.4 | 596−577 | |

[a] Regardless of M13-primer.

**Figure 1.** The results of nested-PCR in patients with type 1 diabetes; Pt: Patient; **Neg**: Negative control; **Pos**: Positive control; and **L**: DNA Ladder (Fermentas 50bp-Ladder, USA).

## Flow cytometry technique

Analysis of flow cytometry plots (Figure 2) showed that the percentage of CD8$^+$ T cells was significantly higher in patients with diabetes than in the controls ($24.7 \pm 4.2\%$ vs.

$21.4 \pm 5.1\%$, $P = 0.007$) (Table 2). However, CD8 frequency was not significantly different between enterovirus-infected and non-enterovirus-infected patients with type 1 diabetes ($P > 0.05$). The results obtained from experiments on CD8$^+$ cytotoxic lymphocyte subsets are as follows: The percentage of CD8$^+$ CD45RA$^+$ lymphocytes increased in both infected and non-infected patients compared to the controls ($24.6 \pm 3.9\%$ and $21.4 \pm 3.2\%$ vs. $18.2 \pm 5.5\%$, $P < 0.001$ and $P = 0.012$, respectively) with no significant difference between the two groups of the patients.

We did not observe alterations of the CD8$^+$ memory subset in the studied groups. The percentage of CD8$^+$ CD45RO$^+$ lymphocytes was not significantly different among enterovirus-infected patients, non-enterovirus-infected patients, and the controls ($7.6 \pm 1.8\%$ and $8.0 \pm 3.3\%$ vs. $7.2 \pm 2.0\%$, respectively, $P > 0.05$). The percentage of cytotoxic effector lymphocytes or CD8$^+$ CD45RA$^+$CD45RO$^+$ lymphocytes, which co-express naive and memory markers and are the most important cells involved in type 1 diabetes pathogenesis and islet beta cells destruction, decreased very significantly in type 1 diabetic
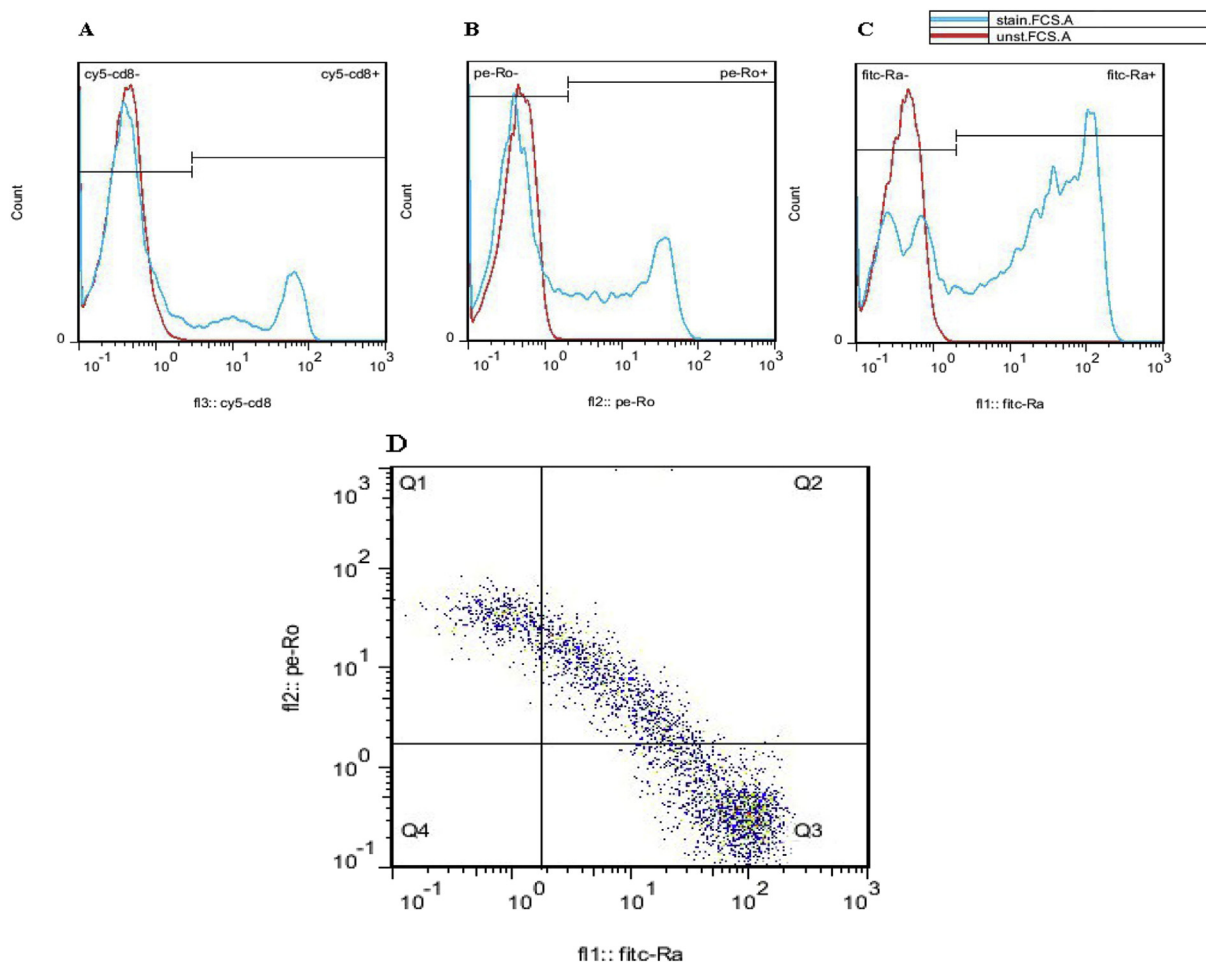


**Figure 2.** Three-color flow cytometry, histogram analysis of human cytotoxic lymphocytes and their subsets; **A**. The third filter, color PE-Cy$^5$; the CD8$^+$ cells; **B**. The second filter, color PE; the CD8$^+$ CD45RO$^+$ or memory cytotoxic cells; **C**. The first filter, color fluorescein isothiocyanate, the CD8$^+$ CD45RA$^+$ or naive cytotoxic cells; and **D**. dot plot, determination of the distribution of each cytotoxic cell subsets on isolated PBMCs after compensation process and CD8 gating on FL1 and FL2 filters for measuring the naive, memory and effector (CD8$^+$ CD45RA$^+$CD45RO$^+$) cytotoxic subsets. The percentages of the cytotoxic cells and their naive, memory and effector subsets were measured in the enterovirus-infected and non-enterovirus-infected patients with type 1 diabetes and healthy controls.

**Table 2**  Distribution of cytotoxic T lymphocytes (CTLs) and their subsets in enterovirus-infected and non-enterovirus-infected patients with type 1 diabetes and healthy controls.

| Percentage | Diabetic patients | | Healthy controls |
|---|---|---|---|
| CD8$^+$ (CTLs) | 24.7 ($\pm$4.2)$^\ddagger$ | | 21.4 ($\pm$5.1) |
| | Enterovirus-infected patients with type 1 diabetes | Non-enterovirus-infected patients with type 1 diabetes | Healthy controls |
| CD8$^+$ CD45RA$^+$ (Naive) | 24.6 ($\pm$3.9)$^{\dagger\ddagger}$ | 21.4 ($\pm$3.2)$^\dagger$ | 18.2 ($\pm$5.5) |
| CD8$^+$ CD45RO$^+$ (Memory) | 7.6 ($\pm$1.8) | 8.0 ($\pm$3.3) | 7.2 ($\pm$2.0) |
| CD8$^+$ CD45RA$^+$ RO$^+$ (Effector) | 21.0 ($\pm$3.9)$^{\ddagger*}$ | 24.6 ($\pm$4.3) | 25.6 ($\pm$4.4) |

The percentage is reported as mean ($\pm$SD); subsets are given as a percentage of the CTLs in peripheral blood. $\dagger P < 0.05$ vs. healthy controls. $\ddagger P < 0.01$ vs. healthy controls. $\dagger\ddagger P < 0.001$ vs. healthy controls. $*P < 0.05$ vs. non-enterovirus-infected patients with type 1 diabetes.

patients with enterovirus infection compared to the controls ($21.0 \pm 3.9\%$ vs. $25.6 \pm 4.4\%$, $P = 0.002$). Non-infected type 1 diabetic patients also had a decrease in the percentage of cytotoxic effector lymphocytes compared to controls, but this decrease was not significant ($24.6 \pm 4.3\%$ vs. $25.6 \pm 4.4\%$, $P = 0.299$).

All in all, our findings in the present study showed that the prevalence of enteroviral infection in people who developed type 1 diabetes after 10 years of age was significantly higher than those developing diabetes before this age (Table 3). Also our results showed that enteroviral infection was not associated with sex (P = 0.689). Enteroviral infection in people who developed type 1 diabetes after 10 years of age was significantly higher than those developing type 1 diabetes at younger ages (P = 0.030).

## Discussion

Autoimmune diseases, such as type 1 diabetes, result from the combination of environmental factors and genetic susceptibility. Environmental agents along with genetic factors predispose a person to type 1 diabetes. Therefore, any changes in environmental factors in genetically susceptible people may be considered a strategy to prevent the development of type 1 diabetes [4]. Investigation of these factors can expand our understanding of this disease and lead to new methods to predict the risk of developing type 1 diabetes, preventing it, or slowing down its progression.

Moreover, to investigate the possible relationship between enteroviral infection, as an important environmental factor, and development of type 1 diabetes among Iranian patients, we investigated immunological phenotypic alteration in CD8$^+$ T cells with emphasis on the possible role of enteroviruses. Such studies about CTLs subsets alteration in patients with type 1 diabetes, enterovirus-infected patients, in particular, have not conducted yet. Currently, the risk of type 1 diabetes development is assessed by detection of antibodies against pancreatic autoantigens in pre-diabetes people [4,9]. GAD65 is considered as the main autoantigen in type 1 diabetes so that the presence of antibodies against this autoantigen precedes the disease [9], although the absence of anti-GAD65 antibody does not necessarily represent no risk of type 1 diabetes.

Therefore, alongside detection of antibodies against GAD65 autoantigen, other factors may be measured to predict better the risk of developing type 1diabetes. CD8$^+$ T cells are, on the one hand, the main cells causing cell-mediated autoimmune responses and, one the other hand, are involved in fighting the viruses. These cells are functionally divided into three subsets: naive, effector and memory cells [2,12]. According to Bossowski et al.'s study, any changes in the distribution of these cells result in autoimmunity [14]. Viruses and autoantigens may cause such alterations in the balance of their cells and therefore investigation of this issue was one of the aims of this study. According to the flow cytometric results, we observed a decrease in CD8$^+$ CD45RO$^+$CD45RA$^+$ population in peripheral blood of enterovirus-infected patients. This decrease

**Table 3**  The demographic characteristics of patients with type 1 diabetes and healthy controls.

| | Number | Age (mean $\pm$ SD) | Range of age (years) | Sex Male/Female | anti-VP1 IgA | anti-VP1 IgG | anti-VP1 IgM | Positive Nested-PCR Male/Female |
|---|---|---|---|---|---|---|---|---|
| Patients group | 35 | 13.6$\pm$(7.3) | 2—27 | 13/22 | 10 | 13 | 0 | 5/7 |
| Control group | 35 | 13.75$\pm$(6.0) | 1—27 | 17/18 | 2 | 2 | — | 1/0 |

| Enteroviruses | | | |
|---|---|---|---|
| Age at diabetes onset | Positive Nested-PCR | Negative Nested-PCR | Total |
| Under 10 years old | 5$^a$ | 18 | 23 |
| Over 10 years old | 7$^b$ | 5 | 12 |

$^a$ Three male patients and two female patients.
$^b$ Two male patients and five female patients.

in cytotoxic effector cells can be interpreted concerning Graham et al.'s and Faustman's findings [15,16].

This active subset for performing cytotoxic functions and triggering other actions of the cells, such as macrophages, re-migrates to the islets [15]. Thus, the population of this subset naturally decreases in peripheral blood. Significant increase in naïve CD8$^+$ subset in both (enterovirus-infected and non-enterovirus-infected) groups of patients with type 1 diabetes compared to the controls, and also lack of any change in percentage of memory cytotoxic subset in all groups, showed that differentiation of naive cytotoxic T subset into memory one was blocked in the patients, especially enterovirus-infected group. Besides that, we observed an increase in cytotoxic T cell population in the patients. Taken together, it can be hypothesized that blocking of differentiation pathway to memory subsets causes excess accumulation in the population of effector cytotoxic T subsets in the patients. Because of migration of effector CTLs to the pancreas via the circulation, it is obvious that increase in the number of these subsets is not detectable in peripheral blood. This possible mechanism can exacerbate beta cells destruction and cause the development of type 1 diabetes.

Enteroviruses formed dsRNA to develop a persistent infection [13,17]. This causes lasting immune response and probably phenotypic changes in enterovirus-infected patients with diabetes. Enteroviruses are likely to block differentiation of naive cytotoxic subset to the memory cells through certain molecular mechanisms and to trigger or enhance effector subsets immigration to pancreas where they lyse beta cells [13]. As a result, the distribution of cytotoxic subsets is disrupted and therefore the disease is developed. Based on the findings in this study, the titer of anti-GAD65 is significantly higher in enterovirus-infected patients with type 1 diabetes than in non-enterovirus-infected patients. Previous studies showed that enterovirus-infected people could produce antiviral responses which cross-reacted with beta cell self-antigens by a mechanism called molecular mimicry [9,13,18]. The results of this study can be explained in the light of homology between the 2C protein in VP1 antigen of enteroviruses and pancreatic GAD protein [9,13]. Because of this similarity between the antigens and molecular mimicry mechanisms, enterovirus-infected people are likely to have higher GAD65 antibodies titers than non-enterovirus-infected and healthy people. Regarding that IgG to enterovirus VP1 protein in the serum of people with type 1 diabetes remains for many years [19], this antibody may be considered a better indicator of the incidence of enteroviral infection. Atkinson and Eisenbarth's study showed that the highest incidence of type 1 diabetes usually occurs before 10 years of age [6]. Similarly, based on our results in Table 3, if someone was already infected with enteroviruses in the first years of life, the risk of developing diabetes reduced. It can be argued that most probably, genetic factors play a role in type 1 diabetes in the early years of life (before 10 years of age) and then, the role of environmental factors, especially enteroviruses, becomes more prominent.

Therefore, age can play an important part in the detection of susceptible people. The results of this study indicated that the prevalence of enteroviral infection in people with type 1 diabetes (34.2%) was significantly higher than in the controls, which is consistent with Kawashima et al.'s study conducted in Japan [9]. The incidence rate of enteroviral infection in patients with type 1 diabetes in this study is higher than those reported by the studies conducted in some European countries [20—22] and Cuba [19].

The issue becomes more complicated when genetic, immunological and viral factors are simultaneously considered. Certainly, genetic and environmental factors are intertwined in the onset of type 1 diabetes. The most important genes involved in the development of type 1 diabetes are located on the short arm of chromosome 6 [1,5]. This gene region contains HLA class I and class II loci. Investigating the polymorphisms of HLA class I genes, as molecules, that present self and viral peptides to CTLs, has demonstrated that HLA-A*0201 is responsible for presenting GAD peptides and enteroviral 2C proteins [2]. The molecular mimicry mechanism in people who have this allele of HLA, especially if it is associated with HLA-DR4, is likely to cause infiltration of the effector CTL subset and development of the disease in pancreatic phase. The cytotoxic effector cells have been found to produce IFN-$\gamma$ [23]. Local IFN-$\gamma$ produced by these cells and CXCL10 increase [24] exacerbate progression of the disease by increased HLA class I molecules and molecules involved in responding to viruses such as MDA-5 [13] as well as furthered production of CD8$^+$ CD45RA$^+$ CD45RO$^+$ subset. Such mechanism may disrupt the balance of cytotoxic subsets in enterovirus-infected people with type 1 diabetes more severely than in non-enterovirus-infected patients.

Genetic investigation of this disease is very complex as the countries with high incidence of type 1 diabetes have been reported to have the lower prevalence of enteroviral infection than those with the low incidence of diabetes type 1. In countries with low incidence of diabetes, this finding may be due to the high prevalence of the genes that have a role in cross reaction and molecular mimicry or cause an excessive antiviral response. Undoubtedly, further studies about the polymorphism of HLA class I alleles and MDA-5 as well as the presence of HLA-A*0201 and HLA-DR4 can contribute greatly to detection of susceptible people.

Since autoantibodies, such as anti-GAD65, have been demonstrated to be present even prior to the onset of the disease, detection of susceptible people is conducted only by detection of autoantibodies in serum. But, it is noteworthy that in cases where viral causes are predisposing to type 1 diabetes, measurement of autoantibodies alone does not suffice to identify susceptible people. If the virus is present in the body and takes the full replicative form to reproduce, destruction of pancreatic beta cells occurs without the presence of any autoantibodies or certain immunological responses. In such cases, detection of susceptible people indeed may not be possible only through measuring autoantibodies.

## Conclusion

According to the results of this study, it can be inferred that in addition to the measurement of autoantibodies, especially the presence of anti-GAD65 in susceptible people's

serum, other factors such as enteroviral infections, counting the percentages of cytotoxic T cells and particularly their effector (CD8$^+$ CD45RA$^+$CD45RO$^+$) subset and, for advanced diagnosis, looking for HLA-A*0201 and HLA-DR4 or detection increased CXCL10 mRNA can be used to detect susceptible people. Our results suggested that enteroviral infections had a direct relationship with type 1 diabetes and enteroviruses caused pathogenesis in patients with type 1diabetes possibly through inducing alterations in the cellular distribution of various CTLs subsets. However, the potential molecular mechanism by which enteroviruses can disrupt the distribution balance of cytotoxic lymphocyte subsets has not yet been known.

Further studies are needed to figure out the mechanisms by which the enteroviruses develop diabetes, because if the enterovirus can directly cause the destruction of beta cells, enterovirus vaccine can be considered. In contrast, if immunological cross-reactivity or exposure to autoantigens by molecular mimicry mechanism causes inflammation, autoimmune responses in diseases are exacerbated by vaccination [13,25]. We reckoned that enteroviruses make the alteration in CD8 T subsets in type 1 diabetic patients, accordingly, we considered this alteration as one of a risk factor in DM development. We think assessment CD8$^+$ T subsets in type 1 diabetic patients can be noted as a low price-high sensitive screening test to recognition susceptible objects. Thus, the full understanding of the functional mechanism of the virus in the pathogenesis of type 1 diabetes may lead to more effective diagnosis and treatment strategies. To achieve this purpose, recognition of susceptible people is the first step to take.

## Acknowledgement

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