Original Article

Abnormal Lymphocyte Subsets in Children With Type 1 Diabetes Mellitus

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Abstract:

This study was designed to investigate the cellular changes of peripheral blood lymphocytes (PBL) in children with type 1 diabetes mellitus (T1DM) by determining the surface antigens (CD markers). Sixty T1DM patients who were newly diagnosed as type1diabetetics (duration of their illness is less than five months) were selected from the National Diabetes Center at Al-Mustansiriya University during the period May 2004 to October 2005. All the patients were treated with daily replacement doses of insulin. Their age ranged from (3-17) years. Fifty apparently healthy control subjects, matched with age (4-17) years and sex underwent the PBL phenotyping. Phenotyping of lymphocyte surface antigens was done by direct Immunoflurocent (IF) technique using mouse antihuman CD3, CD4, CD8, CD19 and CD56.

T1DM patients showed a remarkable lowering in CD3+, CD8+, and CD56+ cells but the decrease in CD4+ cells percentage was not significant in patients in comparison to healthy controls. In contrast, a significant elevation of CD19+ cell percentage and CD4+: CD8+ ratio were observed in the patients.

Key words: T1DM, CD markers, Immunophenotyping

Introduction:

Type 1 Diabetes Mellitus (T1DM) is a slow autoimmune disease associated with the selective destruction of β cells in the islet of langerhans. The autoimmune- mediated destruction of pancreatic β cells is characterized by two features, autoantibodies and insulitus [1]. Autoantibodies present in T1DM are directed against a variety of β cell antigens, including insulin, glutamic acid decarboxylase (GAD65 and 67) and membrane proteins that are homologous to tyrosin- phosphatase (ICA512 and IA-2) [2]. Insulitus is characterized by inflammatory infiltrates in the islet consisting primarily of CD8+ cells, but also of CD4+ cells, B cells, Macrophages, and Natural killer (NK) cells [1].

During the past years, there have been conflicting reports concerning possible abnormalities of T-lymphocytes in T1DM patients. Most studies point to altered ratios of CD4+ cells (helper) and CD8+ cells (cytotoxic / suppressor) T- cells in the blood of T1DM patients at diagnosis [3]. In addition increased number of activated T- cells have been found in the blood of pre-diabetic people at diagnosis and to a certain degree after diagnosis [4]. Significant abnormalities of lymphocyte subpopulations was observed in T1DM patients including: reduced percentage of CD4+ cells (helper / inducer) [5], or normal percentage [4] or predominant activation of CD8+ cells (cytotoxic/suppressor) [6]. An Iraqi study reported low percentage of peripheral blood CD3, CD4+, CD8+ and HLA-DR molecules with a decreased CD4+ / CD8+ ratio in early onset and long term TIDM patients [7].

Natural Killer (NK) Cells make up to 10-15% of the recirculating lymphocyte population. Most surface antigens detectable on NK-cells by mAbs are shared with T-cells (CD56) or macrophages (CD16) [8]. A decreased number of NK-cells were reported in T1DM children at onset [5], while other report showed that both before and after clinical onset of T1DM, the level of NK-cells in peripheral blood reduced [9]. On other hand some investigators demonstrated that the level of NK-cells in peripheral blood of T1DM patients remain normal [4, 10].

Type 1 Diabetes Mellitus involve the interaction of different subsets of lymphocytes and antigen presenting cells (APCs). These responses involves both, CD4+ and CD8+ T- cells responding to antigen

presented by B- cells, machrophages, and dendritic cells [11].

In the present study, we evaluated the phenotypic characteristics of peripheral blood lymphocyte subsets in children at the onset of T1DM.

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Subjects, Materials and Methods:

Sixty Iraqi T1DM children (28 males and 32 females) were subjected to this study. The patients were attending to National Diabetes Center at Al-Mustansiriya University/ College of Medicine during the period May 2004 to October 2005. Their ages range 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 report of the expert committee on the diagnosis and classification of diabetes mellitus (2003) [12], was used. 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: 36 child equal or less than 10 years and 24 child more than 10 years.

For the purpose of comparisons, 50 healthy control subjects matched for age (4-17) years old and sex were selected who have no history or clinical evidence of type 1 diabetes or any chronic diseases and obvious abnormalities as a control group. Five ml of venous blood were drawn from each subject (patients and controls). The collected blood was placed into glass universal tubes containing heparin (10 IU /ml) as anticoagulant. The mononuclear Lymphocytes were isolated and assayed on the same day. Lymphocytes were separated from the whole blood using Ficoll-Isopaque density centrifugation (Flow-Laboratories ,UK). This technique originally described by Boyum, (1968) and reported by Schendel et al., (1997). The collected cells were suspended in washing medium (RPMI-1640 free serum cultured media) (Euroclone, UK) and centrifuged three times, then the lymphocytes were resuspended in 2 ml warm RPMI-1640 supplemented with 10% heat inactivated human type AB serum and determined their viability. The viability accepted should be 95% and above. The final lymphocyte concentration was adjusted to 2-3x106 cells/ml.

Phenotyping of surface antigens of PBL of both patients and controls was done by direct Immunoflurocent (IF) technique. In the present study, five monoclonal antibodies were used including: mouse antihuman CD3 (pan T-lymphocytes), CD4 (Th), CD8 (Tc), CD19 (B-lymphocytes), CD56 (NK-cells) (Serotec, UK). All the mcAbs were purified IgG conjugated to fluorescein isothiocynate isomer-1 (FITC).

The method of IF-labeling of fixed cells was done as described by Wigzell and Anderson, (1971):

The isolated lymphocytes were adjusted to a cell count of 2-3x106 cell / ml in RPMI-1640 medium, cells should be >95% viable as determined by trypan blue dye exclusion. Then 10 µl per well on IF-slides was added. Samples were allowed to dry completely at room temperature.

Slides were dipped into Buffered Formal Aceton (BFA) fixative for 1-3 min, left to dry completely at room temperature.

Slides then were checked with a microscope for even spread of cells, then covered with aluminum foil and stored at -20°C till assayed.

Labeling for Direct IF Test

The IF-slides pre-coated with lymphocytes were removed from freezer, allowed to reach room temperature, unwrapped and washed by dipping into phosphate buffered saline (PBS)-filled jar with stirring for 10 min.

Slides were laid flat section side up in humidity chamber, then 10 µl of a specific anti-CD marker antibody at 1:5 dilution with common antibody diluents were added to each well. The chamber was covered , and the slides were left undisturbed in incubator at 37°C for 2 hrs. Slides then were transferred to staining jar filled with PBS at room temperature. One to two drops of mounting media (glycerol 7: phosphate buffer 3) was added to each well. Then cover slips were lowered into place slowly to avoid bubbles. Slides were ready for examination with IF-microscope at 490 nm immediately or up to 3 days as a maximal duration.

The number of the only stained cells was counted. This maneuver was repeated till 200 cells had been counted. Positive cells give green-apple color.

The tests which have been used for statistical analysis were Student t-test was used to measure the differences between two means. The results were expressed as means ± standard error (SE) and Pearson Correlation (R), which measures to what degree the two variable observations are correlated to each other, and the type of this correlation whether direct, inverse, or no correlation at all.

Results:

1. Total T-Cells (CD3+), T-helper Cells (CD4+) and T-cytotoxic/ suppressor Cells (CD8+)

As shown in Figure (1) T1DM patients ≤10 years old have shown CD3+ cells percentage (66.03%) which was significantly lower than the control group (73.76%) (P1=0.0001). On other hand, the same result was obtained among patients group >10 years old in which CD3+ cells percentage decreased significantly (64.75%) in comparison with control group (75.31%) (P1=0.0001).

Decreased percentage means of CD4+ cells were observed in patients (40.39%) as compared to controls (42.67%) in the age group ≤10 years old and the same decreased percentage means were observed also in patients (37.88%) than controls (41.17%) in age group >10 years. These differences were not significant (P1= 0.12; 0.098 respectively).

There was a highly significant decrease in mean percentage of CD8+ cells in patients compared to controls (23.5 vs 28.43% respectively, P1= 0.000) in age group ≤10 years old, and the same significant decrease was shown among patients >10 years old 23.92% than controls 29.62%, P= 0.0001 (Figure-1).

No statistically differences was shown in the mean percentage of CD3+ (P2=0.44); CD4+ (P2= 0.2) and CD8+ (P2= 0.71) between patients in both age groups.

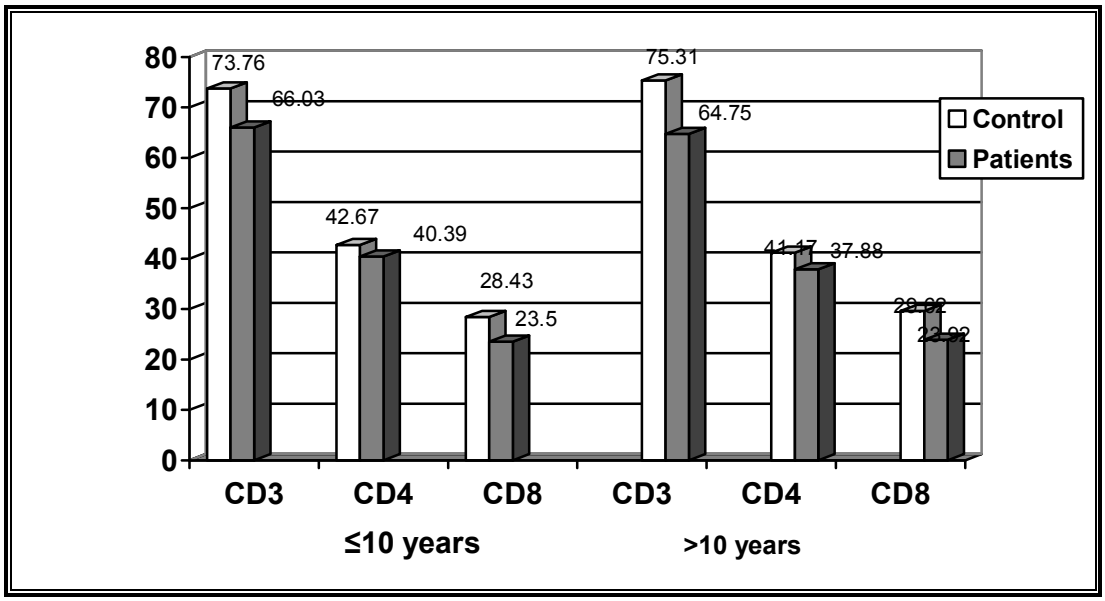

Figure- 1: Bar chart of the mean percentage of CD3+, CD4+ and CD8+ cell populations for the healthy controls and TIDM patients

2. CD4+/CD8+ Ratio

CD4+/CD8+ ratio is important because it represents an index that refers to immunological balance between T-helper cells and T-cytotoxic cells in the immune system in that the higher CD4+/CD8+ ratio, the nearer balance point would be to T-helper cells, which means lower cytotoxic activity and higher other forms of CMI and humoral immunity. The CD4+/CD8+ ratio was significantly higher among patients in ≤10 years old group than controls (1.78 vs 1.52, P1= 0.015). In other hand, a significant difference was also found between patients and controls in >10 years old group concerning the CD4+/CD8+ ratio (1.64 vs 1.42) respectively (P1= 0.034) (Table- 1). No significant differences were shown in CD4+/CD8+ ratio between the patients in both age groups (P2= 0.30).

Table- 1: The difference in mean peripheral CD4+ / CD8+ lymphocyte ratio between control & diabetic patients.

Age	Groups	No.	CD4/CD8 ratio				P1	P2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	1.52	0.05	1.08	2.14	0.015 (S)	0.30 (NS)
	T1DM	36	1.78	0.9	1.1	2.83		
>10 years	Controls	29	1.42	0.07	1.05	2.55	0.034 (S)	
	T1DM	24	1.64	0.10	1.11	2.76		

P1: TIDM patients vs. control
S: Significant

P2: TIDM patients ≤10 years vs. patients >10 years old.
NS: Not significant

CD4+/CD8+ ratio is governed by two cell population namely CD4+ cells and CD8+ cells. So it is important to know which one of these determinants is the master key for the determination of CD4+/CD8+ ratio in T1DM patients. By applying the pearson correlation and linear regression equation it was found that both CD4+ cell population and CD8+ cells population were correlated with CD4+/CD8+ ratio and dynamically do control the ratio. In T1DM patients CD4+ cell subsets showed a significant direct positive correlation with CD4+/CD8+ ratio (r= 0.83, P=0.001), on the other hand CD8+ cells showed a highly significant negative correlation with CD4+/ CD8+ ratio (r= -0.79, P=0.0001).

3. CD56+ Lymphocytes

Significant decreased percentage means of CD56+ cells were observed in patients ≤10 years old as compared with controls (8.17 vs 10.67% respectively, P1 = 0.001), whereas a highly significant decreased percentage means of CD56+ cells were observed among patients >10 years old than controls (9.21 vs 13.07%, respectively, P1 = 0.0001). This deviation was not significant between patients in both age groups (P2=0.13), (Table- 2).

Table- 2: The differences in mean peripheral CD56+ cells (NK) % between control and T1MD patient group.

Age	Groups	No.	CD4/CD8 ratio				P1	P2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	10.67	0.55	7.00	17.00	0.001 (S)	0.13 (NS)
	T1DM	36	8.17	0.47	4.00	13.00		
>10 years	Controls	29	13.07	0.52	7.00	19.00	0.000(S)	
	T1DM	24	9.21	0.48	5.00	14.00		

P1: TIDM patients vs. control
P2: TIDM patients ≤10 years vs. patients >10 years old.
S: Significant
NS: Not significant

4. Peripheral Blood B-Lymphocytes (CD19+)

B-lymphocytes were tested and counted as in T-lymphocytes. As demonstrated in table (3), increased percentage means of CD19+ cells were observed in patients ≤10 years old (20.28%) and in patients >10 years old (20.88%) as compared to controls (14.95% and 14.72% respectively). Both differences were significant (P1 value =0.003 and 0.0001 respectively), but the difference failed to reach a significant level (P2 = 0.681) between patients in both age groups.

Table -3: The differences in mean peripheral CD19+ lymphocyte % between control and T1DM patients group.

Age	Groups	No.	CD4/CD8 ratio				P1	P2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	14.95	1.03	6.00	25.00	0.003 (S)	0.68 (NS)
	T1DM	36	20.28	0.90	11.00	29.00		
>10 years	Controls	29	14.72	0.61	8.00	20.00	0.0001 (HS)	
	T1DM	24	20.88	1.14	12.0	29.0		

P1: TIDM patients vs. control
P2: TIDM patients ≤10 years vs. patients >10 years old.
S: Significant
NS: Not significant

Discussion:

1. CD3+, CD4+, CD8+ Cell Subsets, CD4+/ CD8+ Ratio:

This study provides evidence that abnormalities of T-cells regulation are detectable in patients with T1DM. Thus, in newly diagnosed patients, the main alteration found was decrease in the pan T-cells (CD3+), cytotoxic/suppressor T-cells (CD8+) accompanied by non significant decrease of helper/inducer T-cells (CD4+) subsets. CD4+/ CD8+ ratio is considered as an index of immune activation or suppression. In T1DM patients CD4+/ CD8+ ratio was higher than normal controls, although the patients had a lower CD4+ cell population than controls. This did not mean that T1DM patients had a good immune activity, but the reverse, their high CD4+/ CD8+ ratio was due to a lower extent of CD8+ cells and also to low CD4+ cell population. Thus, in general CD4+/CD8+ was mainly determined by both CD4+ T-cells and CD8+ T-cells which was

statistically confirmed in this study by revealing a clear direct liner relationship with CD4+ cells (r = 0.83) and inverse linear relationship with CD8+ cells (r = -0.79). These results come in agreement with the study conducted by Al-Samarrai,(2001) on T1DM Iraqi patients and also with other reported findings by Buscard et al., (1983) and Michalkova et al.,(2000). The reduction in the ratio of suppressor / cytotoxic cells and inducer / helper could theoretically be due to the metabolic dearrangement of the patients at the diagnosis of T1DM and the examination was done under insulin treatment which affect circulating PBL leading to normalizing the T-cell defect [15]. This hypothesis is confirmed by Buscard et al., (1990) who found a low percentage of CD8+ cells at the diagnosis of T1DM, followed by normalization in the remission period, and may reflect decreased pathogenetic activity as indicated by constant level of C-peptide.

The most pronounced outcome of the present study is the reduction of the cytotoxic / suppressor phenotype CD8+ cells at the onset of the disease. This agrees with the classical theory of pathogenesis of autoimmune disease as the depressed immunological suppressive functions trigger the autoaggressive processes [8]. This result is in disagreement with other studies which reported increase in CD8+ cells percentage and lowering CD4+ cell subsets [17], or lowering CD8+ cells and elevating CD4+ cell subsets in T1DM patients [4].

2. CD56+ Cell Subsets

Other lineage of cell subsets is NK cells. In the present study the percentage of these cells bearing CD56+ antigen significantly decreased in T1DM patients. Although NK cells have powerful antitumor effects, mediating their cytotoxicity by an NK-like effector mechanism that is IL-12 dependent [18]. They also serve as regulators for the speed of immune responses by secreting IL-4, IL-13 and pro-Th2 factor that inhibit Th1 mediated cytotoxic T-lymphocyte (CTL) responses [19]. However, their reduced percentage could lead to the genesis of autoimmunity through a deficiency in Th2 cell function [11]. This finding was in agreement with other studies [5,9,10].

3. CD19+ Cell subsets

The present finding reported a significant elevation of CD19+ cell subsets in the patients. T1DM involves the interaction of different subsets of lymphocytes and APCs. The question of whether antigen presentation or production of autoantibodies by B-cells is important in diabetes development. One of the important aspects of the function of B-cells as APCs is the ability to concentrate the soluble antigen many folds by the virtue of the antigen specificity of the B-cell receptor (BCR) [20]. This will allow antigen-specific T-cells to expand, if appropriately stimulated by relevant B-cell populations. This finding was strengthened by other study who confirmed that the expression of membrane Ig transgene increased insulitis in NOD mice, and the ability of B-cells to produce antibodies is not necessary for B-cells to have some effect on the development of diabetes [21]. Many potential human studies focus on treatment or prevent early diabetes via depletion of B-cells with anti-CD20 treatment [22, 23]. We presume that such therapy has a much greater effect on B-cells as antigen presentation rather than it does on antibody levels.

Conclusion:

Defining of PBL phenotypes by means of CD markers showed no significant differences in the percentage of CD4+ cell subsets in the patients in comparison to controls, while a significant decrease of CD3+, CD8+, and CD56+ cells percentage with significant elevation of CD4+: CD8+ ratio were observed.

A significant elevation of the CD19+ cell subset were detected in the patients.

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