Activated Peripheral Blood Lymphocyte Subpopulation in Newly Diagnosed Type 1 Diabetes Mellitus Children

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SUMMARY:

BACKGROUND:

In Type 1 Diabetes Mellitus (T1DM), numerous changes in the cellular as well humoral immune response have been identified. However, it is not known whether both the CD_4^+ and CD_8^+ subpopulation or only one of these or CD_{19}^+contains increased numbers of activated cells.

OBJECTIVE:

The aim was to study the activated lymphocyte subpopulation by use of monoclonal antibodies to T-cell and B-cell antigens which is known to be expressed on activated cells.

METHODS:

A total of 60 T1DM patients who had newly onset of the disease (diagnosed was from one week up to five months) were included in the present study, all the patients were treated with daily replacement doses of insulin. Fifty apparently healthy control subjects underwent the PBL phenotyping. Phenotyping of surface antigens was done by direct Immunoflurocent (IFT) technique using mouse antihuman CD_3, CD_4, CD_8, $CD_{45}RA$, CD_{19}, and activated markers $CD_{45}RO$, DR-antigen and CD_{38}.

RESULTS:

T1DM patients showed a remarkable lowering in CD_3^+, CD_8^+, and $CD_{45}RA^+$ cells ($p<0.0001$), but the decrease in CD_4^+ cells percentage was not significant. In contrast, a significant elevation of activation markers includes ($CD_{45}RO^+$, HLA-DR^+ and CD_{38}^+ cells) were observed in patients in addition to a significant increase of CD_{19}^+ cell percentage and CD_4^+: CD_8^+ ratio in the patients.

CONCLUSION:

This study provides evidence that abnormalities of T-cells regulation are detectable in patients with T1DM.

KEY WORDS: T1DM, CD markers, immunophenotyping

INTRODUCTION:

Type 1 Diabetes Mellitus (T1DM) is a chronic autoimmune disease resulting from selective destruction of β cells in the islet of langerhans. At diagnosis patients usually show both cellular and humoral immune changes in their peripheral blood, including the production of autoantibodies to islet cells and insulin, and activation of T- cells [1, 2]. No consensus has been reached to date regarding T- cell activation and the lymphocyte subsets involved due to the heterogeneity of applied methodology and of the patients evaluated. Reported alterations include, besides the absence of any deviation from normal [3], elevated proportion of circulating T-cells carrying HLA-DR [4, 5], increased levels of activation markers have been described on both CD_4^+ and CD_8^+ T- cells [4]. Moreover, abnormal elevated levels of HLA-DR, CD_8^+ T-cells demonstrated in the peripheral blood of monozygotic twins patients in prospective studies, whereas twins that remained normoglycemic had lower levels of activated CD_8^+ T-cells [6]. An Iraqi study reported low percentage of peripheral blood CD_3^+, CD_4^+, $CD_8^+$and HLA-DR molecules with a decreased CD_4^+ / CD_8^+ ratio in early onset and long term T1DM patients [7]. T-lymphocytes has been seen to be activated mostly in the prediabetic state and to fade with increasing destruction of beta cells [3], suggesting that a combination of cellular and humoral immune changes with their tendency to persist may be highly predictive of progression to clinical T1DM [8].

By analysis of $CD_{45}RA^+$ isoforms, the reciprocal subpopulation of $CD_{45}RA^+$ (naive, unprimed) and $CD_{45}RO^+$(memory, primed) may be differentiated among CD4 and CD8 T-cells [9]. In T1DM of recent onset, the proportion of $CD_{45}RA^+$ cells in the CD_4^+ T-cells subsets have been described to be normal (3) or increased (5).

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CD_{38} is a single chain type II transmembrane glycoprotein expressed on the surface of monocytes, platelets, NK-cells, T and B lymphocytes, myeloid cells, vascular endothelium and many tissues, used as a phenotypic markers of differentiation and activation of hematopoietic cells [10]. It was detected that anti-CD_{38} autoantibodies were found in 9.7% of type II diabetic patients and in 13.1% of T1DM patients vs. 1.3% in control group [11], and prolonged exposure of human pancreatic islets to sera containing CD_{38} antibodies impairs their function and viability [12].

Type 1 Diabetes Mellitus involve the interaction of different subsets of lymphocytes and antigen presenting cells (APCs). These responses involves both, CD_4 and CD_8 - cells responding to antigen present by B-cells, macrophages, and dendritic cells [13]. Although B-cells do not present antigens as efficiently as dendritic cell, they bind antigen specifically via cell surface immunoglobulin and thus can present soluble proteins much more effectively than cells that do not bear specific receptors and the specificity of the immunoglobulin directs processing of the protein [14].

In the present study, we have investigated the peripheral blood lymphocyte subsets in children at the onset of T1DM by monoclonal antibodies directed at the cell surface antigens of lymphocyte subpopulation.

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 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 T1DM, the criteria as listed in the expert committee of diagnosis and classification of diabetes mellitus was used [15]. 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 apparently 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 displaced into glass universal tubes containing Heparin (10 IU /ml) as anticoagulant. The mononuclear Lymphocytes were isolated and assayed the same day. Lymphocytes were separated from the whole blood using Ficoll- Isopaque density centrifugation (Flow-Laboratories,U.K.), according to Schendel *et al.* [16]. Finally the collected lymphocytes were resuspended in 2 ml warm RPMI-1640 (Euroclone, UK) 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 $2\text{-}3x10^6$ cells/ml.

Phenotyping of surface antigens of PBL of both patients and controls was performed by direct Immunoflurocent (IFT) technique. In the present study, eight monoclonal antibodies were used including: mouse antihuman CD_3, CD_4, CD_8, CD_{19}, $CD_{45}RA$ (Naive cells), and activated markers includes $CD_{45}RO$ (Memory cells), DR-antigen and CD_{38} (Serotec, UK). All the mcAbs were purified IgG conjugated to fluorscein isothiocynate isomer-1 (FITC). The method of IF-labeling of fixed cells was done as described by Wigzell and Anderson [17]. Slides were ready for examination with IF-microscope 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, the results were expressed as means ± standard error (SE), and also Pearson Correlation (R).

RESULTS:

PBL phenotyping can give an idea of the immunological status in patients with T1DM and it can be considered as a mirror image of the immunity.

Total T-Cells (CD_3^+), T-helper Cells (CD_4^+) and T-cytotoxic Cells (CD_8^+):

As shown in Table (1) T1DM patients ≤10 years old have shown CD_3^+ cells percentage (66.03%) which was significantly lower than the control group (73.76%) (P_1=0.0001). On other hand, the same result was obtained among patients group >10 years

old in which CD_3^+ cells percentage decreased significantly (64.75%) in comparison with control group (75.31%) (P_1=0.0001). Decreased percentage means of CD_4^+ 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 (P_1= 0.12; 0.098 respectively). There was a highly significant decrease in mean percentage of CD_8^+ cells in patients compared to controls (23.5 vs. 28.43% respectively, P_1= 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). No statistically differences was shown in the mean percentage of CD_3^+ (P_2=0.44); CD_4^+ (P_2= 0.2) and CD_8^+ (P_2= 0.71) between patients in both age groups, table (1)

CD_4^+/CD_8^+ Ratio:

The CD_4^+/CD_8^+ ratio was significantly higher among patients in ≤10 years old group than controls (1.78 vs. 1.52, P_1= 0.015). In other hand, a significant difference was also found between patients and controls in >10 years old group concerning the CD_4^+/CD_8^+ ratio (1.64 vs. 1.42) respectively (P_1= 0.034) (Table- 2). No significant differences were shown in CD_4^+/CD_8^+ ratio between the patients in both age groups (P_2= 0.30).

Table- 2: The difference in mean peripheral CD_4^+ / CD_8^+ lymphocyte ratio between control and diabetic patients.

Age	Groups	No.	CD4/CD8 ratio				P_1	P_2
			Mean	SE	Mean	SE		
≤10 yrs	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 yrs	Controls	29	1.42	0.07	1.05	2.55	0.034 (S)	
	T1DM	24	1.64	0.10	1.11	2.76		

P_1: Patients vs. controls P_2: Patients ≤10 years vs. patients >10 years. NS: Not significant

It is important to know which one of these determinants is the master key for the determination of CD_4^+/CD_8^+ ratio in T1DM patients. By applying the pearson correlation and linear regression equation it was found that both CD_4^+ cell population and CD_8^+ cells population were correlated with CD_4^+/CD_8^+ ratio. CD_4^+ cell subsets showed a significant direct positive correlation with CD_4^+/CD_8^+ ratio (r= 0.83, P=0.001), on the other hand CD_8^+ cells showed a highly significant negative correlation with CD_4^+/CD_8^+ ratio (r= -0.79, P=0.0001).

$CD_{45}RA^+$ and $CD_{45}RO^+$ Cells:

Table (3) showed that there was highly significant decrease in mean percentage of $CD_{45}RA^+$ (naive, unprimed) lymphocytes in patient group ≤10 years old (64.33 % compared to control group (72.67%) (P_1= 0.001). This highly statistical decrease was shown also in patients >10 years old than controls (53.08 vs. 61.14% respectively, P_1= 0.001). The $CD_{45}RO^+$ (memory, primed) cells were statistically high among diabetic patients in comparison with healthy individuals (34.75 vs. 25.05% respectively, P_1= 0.0001) in age group ≤10 years old. This statistical increase was also demonstrated among patients in age group >10 years than controls (46.75 vs. 38.14% respectively, P_1= 0.0001) (table -3). The results indicated highly significant increase of the mean percentage of activation $CD_{45}RO^+$ cell subset among patients >10 years old (46.75%) than patients ≤10 years old (34.75%) (P_2= 0.0001) and this significant level reflected on the mean percentage of $CD_{45}RA^+$ cells in patients ≤10 years old 64.33% vs 53.08% in >10 years old patient (P_2=0.0001). There was negative correlation between the percentage of CD_3^+ and $CD_{45}RA^+$ cells subsets in patients (r= -0.57, P=0.0001) whereas significant direct positive correlation was demonstrated between the percentage of CD_3^+ and $CD_{45}RO^+$ cells subsets (r= 0.57, P=0.0001).

Table- 1: The differences in the mean percentage of peripheral CD_3^+, CD_4^+ and CD_8^+ lymphocytes between control and T1DM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
CD_3^+	Controls	21	73.76	0.95	60	79	0.000 (HS)	Controls	29	75.31	1.17	63	89	0.000 (HS)	0.44 (NS)
	T1DM	36	66.03	1.13	49	80		T1DM	24	64.75	1.44	49	78		
CD_4^+	Controls	21	42.67	0.78	33	48	0.12 (NS)	Controls	29	41.17	1.24	29	56	0.098 (NS)	0.2 (NS)
	T1DM	36	40.39	1.19	29	51		T1DM	24	37.88	1.51	27	49		
CD_8^+	Controls	21	28.43	0.79	21	35	0.000 (HS)	Controls	29	29.62	0.87	20	37	0.000 (HS)	0.71 (NS)
	T1DM	36	23.50	0.67	17	31		T1DM	24	23.92	0.87	17	35		

P_1: Patients vs. controls P_2 : Patients ≤10 years vs. patients >10 years. NS: Not significant HS: High significant

Table -3: The differences in mean peripheral $CD_{45}RA^+$ and $CD_{45}RO^+$ lymphocyte % between control and T1DM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
$CD_{45}RA^+$	Controls	21	72.67	0.95	65.00	80.0	0.001 (HS)	Controls	29	61.14	1.11	51.0	71.0	0.001 (HS)	0.0001 (HS)
	T1DM	36	64.33	1.38	52.0	77.0		T1DM	24	53.08	1.35	42.0	64.0		
$CD_{45}RO^+$	Controls	21	25.05	1.32	17.0	35.0	0.0001 (HS)	Controls	29	38.14	1.04	30.0	48.0	0.0001 (HS)	0.0001 (HS)
	T1DM	36	34.75	1.39	22.0	47.0		T1DM	24	46.75	1.29	37.0	57.0		

HLA-DR$^+$ Lymphocytes:

T1DM patients ≤10 years old and >10 years showed increased percentage means of HLA-DR$^+$ cells (32.09% and 31.38% respectively) as compared to the control groups (28.47% and 28.08% respectively). Both differences were significant (P_1 value = 0.005 and 0.038 respectively). But the differences were not significant (P_2 = 0.75) between the patients in both age groups (Table-4). There was direct positive correlation between the mean percentage of CD38 cells and HLA-DR+ cells population (r=0.581).

Table-4: The differences in mean peripheral HLA-DR$^+$ lymphocyte percentage between control and T1DM patient groups.

Age	Groups	No.	HLA-DR$^+$				P_1	P_2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	28.47	0.86	20.00	37.00	0.005 (S)	0.79 (NS)
	T1DM	36	32.09	0.89	25.00	39.00		
>10 years	Controls	29	28.08	1.17	20.00	38.00	0.038(S)	
	T1DM	24	31.38	1.01	23.00	40.00		

CD_{38}^+ Lymphocytes:

Increased percentage of activation marker CD_{38}^+ cells were observed in T1DM patients (24.72%, 23.83%) as compared with the control group (16.86%, 15.97%) in the age group ≤10 years and >10 years old respectively. These differences were highly significant (P_1=0.0001) between the patients and healthy individuals, but failed to reach a significant level (P_2= 0.44) between the patients in both age groups (Table-5).There was strong direct positive correlation between the mean percentage of CD_{38}^+ cells and CD_4^+ cells (r= 0.808) and CD_{19}^+ cells (r= 0.602).

Table -5: The differences in mean peripheral CD_{38}^+ lymphocyte % between control and T1DM patients groups.

Age	Groups	No.	CD_{38}^+ lymphocyte %				P1	P_2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	16.86	0.76	13.00	23.00	0.0001 (HS)	0.44 (NS)
	T1DM	36	24.72	0.81	15.00	38.00		
>10 years	Controls	29	15.97	0.63	12.00	23.00	0.0001 (HS)	
	T1DM	24	23.83	0.82	15.00	31.00		

B-Lymphocytes (CD_{19}^+):

B-lymphocytes were tested and counted as in T-lymphocytes. As demonstrated in table (6), increased percentage means of CD_{19}^+ 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 (P_1 value =0.003 and 0.0001 respectively), but the difference failed to reach a significant level (P_2 = 0.681) between patients in both age groups. The statistical analysis revealed that there was a strong direct positive correlation between CD_{19}^+ cells and HLA-DR$^+$ activation marker cells subsets (r = 0.92, P = 0.0001). CD_{19}^+cell population were found to be correlated negatively and significantly with the

$CD_{45}RA^+$ cell subset in T1DM patients (r = -0.62, P= 0.0001). Moreover the present finding also revealed significant direct positive correlation between CD_{19}^+ cells and activated $CD_{45}RO^+$ cells subset in T1DM patient (r = 0.63, P = 0.0001), and with activated CD_{38}^+ cell subsets (r = 0.602).

Table -6: The differences in mean peripheral CD_{19}^+ lymphocyte % between control and T1DM patients group.

Age	Groups	No.	CD_{19}^+				P_1	P_2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	14.95	1.03	6.00	25.00	0.003 (S)	0.68 (N)
	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		

DISCUSSION:

CD_3^+, CD_4^+, CD_8^+ Cell Subsets, CD_4^+/ CD_8^+ 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 percentage of pan T-cells (CD_3^+), cytotoxic T-cells (CD_8^+) accompanied by non significant decrease of helper/inducer T-cells (CD_4^+) subsets. CD_4^+/ CD_8^+ ratio is considered as an index of immune activation or suppression. In T1DM patients CD_4^+/ CD_8^+ ratio was higher than normal controls, their high CD_4^+/ CD_8^+ ratio was due to a lower extent of CD_8^+ cells and also to low CD_4^+ cell population. These results come in agreement with the study on T1DM Iraqi patients (7) and also with other reported findings [18, 19]. The reduction in the amount of CD_8^+ 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 [20]. This hypothesis is confirmed by Buschard and his team, 1990 who found a low percentage of CD_8^+ 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 (4). The reduction of the cytotoxic phenotype CD_8^+ cells at the onset of the disease agrees with the classical theory of pathogenesis of autoimmune disease as the depressed immunological suppressive functions trigger the autoaggressive processes [21].

$CD_{45}RA^+$ and $CD_{45}RO^+$ Cell subsets:

In the present study, a significant decrease in $CD_{45}RA^+$ cells percentage (naive/resting) and increase the percentage of $CD_{45}RO^+$ cell subset (memory/activated) were found among T1DM patients. CD_{45} family represents a family of surface protein tyrosin phosphatase, which is present in all human leukocytes. $CD_{45}RA$ antigen present in approximately 50% of CD_4^+ cells, 78% of CD_8^+ cells and essentially on all B-lymphocytes and NK cells [22]. A selective loss of $CD_{45}RA^+$ has been seen in autoimmune disease and viral disease [23]. $CD_{45}RO^+$ activated marker is present on about 40% of PBLs including CD_4^+ and CD_8^+ T-cells population. It is present early in T-cell maturation cycle, but upon activation by mitogen or alloantigens, naive T-cells loss the $CD_{45}RA^+$ and reciprocally acquire the $CD_{45}RO^+$ antigen (10). The reduction in the $CD_{45}RA^+$ cells and increase the proportion of $CD_{45}RO^+$ cell subsets in T1DM patients may be due to vigorously responds of these cells to recall antigens. The results in other reports confirmed these finding [24,19].

HLA-DR^+ Cells:

High significant elevation of mean percentage of

HLA-DR$^+$ activation marker was demonstrated in our patients. It has been suggested that one of the important immunoregulatory abnormalities in T1DM is related to HLA-DR$^+$ cells and activation of lymphocytes by different stimuli increases their expression of surface markers (10). This fact is confirmed in our finding that there were strong positive linear correlation between HLA-DR$^+$ and CD_{19}^+ cells (r = 0.90), CD_4^+ cells (r = 0.78) and inverse negative correlation with CD_8^+ cells (r =- 0.39). Many studies confirm these facts (25). Buschard *et al.*, 1990 also found higher percentage of HLA-DR$^+$ cells in T1DM patients at diagnosis and after one month, but their percentage decline after 7 months (4). In contrast, a decline of HLA-DR$^+$ cells percentage was demonstrated among type I and type II Iraqi diabetic patients diagnosed within two years of onset (7).

CD_{38}^+ Cell Subsets:

The results detected a very high significant elevated percentage of activation CD_{38}^+ antigen in PBL of T1DM patients. CD_{38} is (ADP/ ribosyl cyclase/ ADP ribose hydrolase) an integral membrane glycoprotein. Human CD_{38} is highly expressed on early T-cell precursors migrating to the thymus and on CD_4^+ CD_8^+ double positive thymocytes. During the process of negative selection, CD_{38}^+ expression is decreased and mature single positive T-cells express low levels of CD_{38} [26]. It is present on approximately all pre-B-lymphocytes, in 18% of Th and some Tc cells [23], and in tissues such as human pancreatic islets [27]. In pancreatic beta-cells, this enzyme appears to play a role in glucose induce insulin release via a mechanism involves its cyclase activity which leading to increase cytoplasmic Ca^{+2} concentration and insulin release (11). Mature T-cells isolated from peripheral blood can acquire CD_{38}^+ cell surface expression during antigen activation [26].

A strong positive linear relationship is found between CD_{38}^+ cells and CD_4^+ cells (r = 0.808), with CD_{19}^+ cells (r = 0.602) and with HLA-DR$^+$ cell population (r = 0.581). CD_{38}^+ act as positive and negative regulator of cell activation and proliferation depending on cellular environment. Thus, mature B-cells proliferate wheras the opposite occurs in immature B-cells in the bone marrow. The CD_{38} signaling pathway in this environment blocks B-lymophopoiesis, mostly by inducing apoptosis [28]. CD_{38} involved in adhesion between human lymphocytes and endothelial cells. Presence of autoantibodies with anti- CD_{38} specifity in patients with type I and type II diabetes has been reported to down regulate CD_{38} expression in lymphoid cells (11).

CD_{19}^+ Cell subsets:

The present finding reported a significant elevation of CD_{19}^+ 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. It was found 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 (14). Many potential human studies focus on treatment or prevent early diabetes via depletion of B-cells with anti-CD_{20} treatment [29, 30]. 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 a significant elevation of activation markers $CD_{45}RO^+$, CD_{38}^+ and HLA-DR$^+$ cells percentage with the CD_{19}^+ cell subset in the patients. No significant differences in the percentage of CD_4^+ cell subsets was observed in the patients in comparison to controls, while a significant decrease of CD_3^+, CD_8^+, $CD_{45}RA^+$ and CD_{56}^+ cells percentage with significant elevation of CD_4^+: CD_8^+ ratio were observed.

Recommendations:

Further studies of the induction of Fas (CD_{95}) receptor on β-cells and ligation with ($CD_{95}L$) on the surface of CD_4^+ or CD_8^+ cells as possible role of autoimmune β-cell death.

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