Research Article

Assessment of plasma CD3, CD4 and CD8 levels in β-thalassemic patients with different treatment modalities

Mohamed GB*, Hakeem GLA*, Higazi AM and Hassan Z***
* Department of Pediatrics, Faculty of Medicine, Minia University.
**Department of Clinical Pathology, Faculty of Medicine, Minia University.

Abstract
Background*:* β Thalassaemia major is one of the chronic hemolytic anemias resulting from defect in β globin chain. It requires frequent blood transfusion plus other treatment modalities. These treatment modalities may be associated with certain immunologic modulations. **Objective**: To asses various immunological parameters in some β thalassemic children under different treatment modalities. **Subjects and Methods**: Forty-eight children were enrolled and classified into four groups. Twelve β thalassemic patients treated only with blood transfusion (group I). Twelve patients treated with transfusion and iron chelation (group II). Twelve patients treated with transfusion, iron chelation and subjected to splenectomy (group III). Group IV involved twelve healthy age and sex matched children. CBC plus serum levels of ferritin, were measured along with detection of CD3, CD4 and CD8 percentages. **Results**: Significant statistical differences regarding CD3, CD4 and CD8 T lymphocytes percentages within thalassemic groups when compared to each other's and to healthy controls except for CD3 T lymphocytes in group II versus III. Splenectomized patients had higher significant levels regarding serum ferritin (p=0.02), CD4, CD8 and CD3 (p=0.001) compared to non splenectomized ones. **Conclusion**: Immune modulation occurs in thalassemic patients with regional specific variations. This may also be related to the difference in treatment modalities**.**
Keywords: Thalassaemia; Immunity; Treatment modalities; El Minia region.
Abbreviations: CBC-Complete Blood Count.

Introduction
Thalassemia is defined as a group of inherited disorders that arise as a result of certain mutations in hemoglobin (Hb) genes. β-thalassemia major (βTM) has high prevalence in the Mediterranean region including Egypt, Middle East, Indian subcontinent, and South East Asia[1]. Though, it is a growing global health problem due to extensive population migrations. About 1.5% of the world populations are carriers of the β -thalassemia gene[2].

Volumetric and multiple transfusions lead to alloimmunization, accumulation of iron in different tissues and are associated with increased risk for transmitted infections. As well, the risk of sepsis in splenectomized patients is as high as 7% over a 10-year period and almost 25% of splenectomized patients are at risk of severe infections[3]. Accordingly, βTM patients present with many problems rather than severe anemia. One of these problems is the increased susceptibility to bacterial infections. Infectious complications constitute the second most common cause of mortality and a major cause of morbidity in β-thalassemia after heart failure[4].

Several studies on immune competence in β-thalassemia had revealed numerous quantitative and functional defects, involving T and B lymphocytes, immunoglobulin in addition to impairment in components of the complement system[5,6]. Additionally, immunologic disorders in patients with βTM comprising decreased absorption and phagocytic ability of segmented neutrophils, changes in cytokines production as well as dysfunction of macrophages, properdin and lysozyme[7]. However, it has been reported that distribution of mutations on the β-globin genes differs among the ethnic groups in addition to its regional and individual's variation. So far, around 230 different mutations have been reported on β-globin gene worldwide. These mutations affect the structure of Hb in red blood cells which leads to certain pathophysiological disorders[8].

Therefore, patients showed different phenotypes with regard to genotypes. Furthermore, there is an increasing body of evidence suggesting that clinical features or complications of βTM plus their immunologic characters and even their response to various therapies are population dependence comprising patient's age, sex and even blood group[3].

Our aim was to compare the immune competence between those thalassemic children who receiving multiple blood transfusions either alone or with iron chelating agents plus or minus splenectomy with respect to CD3, CD4 and CD8.

Subjects and Methods

Subjects:

This case-control study was carried out from ***18-5-2015*** to ***18-9-2015***. Forty eight children were included in this study which involved a group of 36 thalassemic patients and 12 healthy age and sex matched control group.

The thalassemic patients group was further subdivided into three subgroups according to the difference in their treatment regimens. These subgroups comprised patient group I (No. 12) receiving only blood transfusion, patient group II (No 12) receiving blood transfusion and chelation therapy as well as patient group III (No 12) receiving blood transfusion, chelation therapy and splenectomized. Blood was taken from thalassemic patients just prior to a scheduled transfusion.

All patients were selected from pediatric hematology outpatient clinic in Minia University Hospital while controls were selected from apparently healthy children from pediatric growth clinic.

All patients and controls included were subjected to complete history taking (name, age, sex, residence, family history, consanguinity, age of 1st blood transfusion, amount of transfusion per year, duration of splenectomy in splenectomized patients in addition to history of exposure to infections). Moreover, the involved children were examined carefully (general examination, anthropometric measures which were plotted on percentile growth charts, vital data as well as examination of chest, heart and abdomen).

The exclusion criteria were patients who had bone marrow transplant. Finally, the laboratory investigations were performed to all subjects. These investigations were comprised Complete Blood Count (CBC), blood smears, Hemoglobin (Hb) electrophoresis for controls, serum ferritin plus CD3, 4 and 8.

Blood Sampling

Venus blood samples were collected from both patients and controls under complete aseptic conditions. About 2 ml of blood sample were withdrawn in K3-EDTA anticoagulant tubes for complete blood count, peripheral blood smears as well as flow cytometric analysis (immunephenotyping) then analyzed within 24 hrs. About 3 ml of blood in a plain tube without any anticoagulant left to clot and centrifuged at 3000 revolutions per minute (rpm) for 5 minutes. The serum was then separated, liquated and stored at - 70°C till used for serum ferritin.

Blood samples were withdrawn from thalassemic patients just prior to a scheduled transfusion. The blood samples from control group were taken while coming for follow up of their growth. All controls had normal hemoglobin levels for their age and sex with normal red blood cell indices. At the time of sampling all patients and controls were free from infection.

Laboratory methodology

Complete blood count was performed using automated blood counter (Sysmex KX-21N). Additionally, peripheral blood smears were stained by Leishman stain. The absolute lymphocytic and neutrophils counts were calculated for patients. Serum ferritin was measured using human in vitro Enzyme-Linked Immunosorbent Assay (ELISA) kit from Abcam Inc, Cambridge, USA, catalog No ab108837. The assay was performed according to the manufacturer's instructions. Final ferritin values were expressed in (ng/ml) nanogram/ milliliter[9,10].

Flow cytometric immunophenotypic analysis

T- Lymphocyte subsets in whole blood samples were enumerated using fluoroisothiocyanate (FITC) conjugated CD4 (Becton Dickinson, Bioscience, USA), phycoerythrin (PE) conjugated CD8 (Becton Dickinson, Bioscience,

USA) and peridinium-chlorophyll-protein (Per-CP) conjugated CD3 (Becton Dickinson, Bioscience, USA). Flow cytometric analysis was done by FACS Calibur flow cytometry with Cell Quest software (Becton Dickinson Biosciences, USA). An isotypem-atched negative control was used with each sample. Forward and side scatter histogram was used to define the lymphocyte population (R1). Percentage (%) of CD3+ cells refer to the total T Lymphocytes, CD4+ cells are the helper cells and CD8+ are the cytotoxic cells.

Statistical analysis

The clinical and laboratory data were recorded on performed sheets. These data were analyzed using statistical program for social science SPSS software version 17 (SPSS Inc., Chicago, IL, USA). Quantitative variables were presented as mean ± standard deviation (SD). On the other hand, description of qualitative variables was presented as number (No.) and percentage (%). Results were expressed as tables and figures. Graphics were done by Excel Microsoft Office 2010. Student test was used to compare results between groups as regards quantitative variable. Correlation was performed by using Pearson correlation coefficient (r). For all tests a probability (p) was considered non-significant if >0.05, significant if ≤ 0.05 and highly significant if ≤ 0.001. One Way ANOVA test was used for comparison of quantitative data between more than two groups. Chi-square test was used to compare qualitative variables between groups. The Mann-Whitney test compares the medians from two groups.

Results

Some demographic and clinical data of the subjects in different studied groups

Comparing thalassemic patients (group I, II, III) with controls (group IV) was showing no statistically significant difference in ages of the included subjects. The patients and controls were sex matched as well. Significant differences were found when comparing group, I, III to controls ($p=0.02$, 0.001 respectively) regarding weight centile and between group III and controls regarding BMI ($p=0.001$) (Table 1). The demographic characteristics of all included subjects plus some of their clinical findings, history of repeated infections and HCV positivity were reported in Table 1.

Table 1: Comparison between studied groups regarding some demographic and clinical data

parameters			Patient			Control
			Group I	Group II	Group III	
Age		Range	2-8	5-12	10-16	3-14
		Mean±SD	4.3±2.1	7.6±2.2	13.3-2.7	6.5±3.7
		Median	4	7.5	14	5.8
Comparison with control			p=0.3	p=0.9	p=0.06	-
Sex	Male	NO.	6	4	7	6
		%	50%	33.3%	58.3%	50%
	Female	NO.	6	8	5	6
		%	50%	66.7%	41%	50%
Comparison with control			p=0.91	p=0.09	p=0.99	-
Wt. centile		Range	3-90	5-90	3-25	5-75
		Mean±SD	20.7±25.6	38.3±29.1	7.3±6.3	50.8±28.6
		Median	10	25	5	62.5
Comparison with control			p=0.02*	p=0.9	p=0.001**	-
Ht. centile		Range	3-75	3-90	3-5	3-50
		Mean±SD	10.1±20.5	18.8±26.6	3-3±0.8	23.8±20.6
		Median	3	3	3	17.5
Comparison with control			p=0.64	p=0.89	p=0.11	-
BMI. centile		Range	10-95	25-90	3-50	50-95
		Mean±SD	60±29.1	52.5±20	25.1±17.3	75.8±14.3
		Median	62.5	50	25	75
Comparison with control			p=0.42	p=0.05*	p=0.001**	-
History of recurrent infection	+ve	No	3	3	6	--------
		%	25%	25%	50%	
	-ve	No.	9	9	6	---------
		%	75%	75%	50%	
HCV infection	+ve	No	0	0	3	--------
		%	0%	0%	25%	
	-ve	No.	12	12	9	---------
		%	100%	100%	75%	

No: Number; Wt: Weight; Ht: Height; BMI: Body Mass Index; HCV: Hepatitis C Virus *= Significant ($p \leq 0.05$); **= Significant ($p \leq 0.001$).

Comparison of some laboratory results and immunological parameters between β thalassemia major children under different treatment modalities and controls

Significant difference was found on comparison between thalassemia patients (group I, II, III) and control group regarding Hb levels (p=0.015), ANC (p=0.024), CD3 (p=0.044), CD4 (p=0.03), CD8 (p=0.003) (Table 2). Also, differences within the 3 thalassemic groups regarding CD3, CD4 and CD8 were summarized in Table 3.

Table 2: Comparison between studied groups regarding some laboratory data;

Parameters		Patient			Control	p-values (patients vs controls)
		Group I	Group II	Group III		
Hb level (g/dl)	**Mean±SD**	6.7 ± 1.6	6.8 ± 1.5	6.9 ± 1.1	12.3 ± 0.93	0.015*
WBCs count (×10³ cells/µl)	**Mean±SD**	14.9 ± 1.6	9.24 ± 2.0	10.1 ± 2.9	8.8 ± 3	0.08
Platelet count (×10³ cells/µl)	**Mean±SD**	510 ± 25	449.8 ± 16	570.9 ± 21	350 ± 14	0.18
ANC (×10³ cells/µl)	**Mean±SD**	5.90 ± 0.17	5.98 ± 0.2	6.07 ± 0.3	4.55 ± 0.2	0.024*
ALC (×10³ cells/µl)	**Mean±SD**	3.49 ± 1.5	3.28 ± 0.8	3.66 ± 1.64	4.01 ± 1.2	0.38
Serum ferritin (ng/ml)	**Mean±SD**	2136 ± 17.6	3031.7 ± 14.2	3974.2± 13.7	70.03±18.8	0.013*
AOBT/Y (ml/kg/year)	**Mean±SD**	1995 ± 11.6	4585.42± 13.6	2907.8±74.3	-------	-
CD4 (%)	**Mean±SD**	51.1 ± 5.2	30.8 ± 6.7	46.7 ± 4.9	32.4 ± 6.1	0.03*
CD8 (%)	**Mean±SD**	46.9 ± 5.5	19.8 ± 6.6	41.4 ± 7.1	27.2 ± 6.1	0.003*
CD3 (%)	**Mean±SD**	21.3 ± 7.1	41.9 ± 4.8	40.2 ± 11.7	59.9 ± 12.1	0.044*

vs: versus; Hb: Hemoglobin; WBCs: White Blood Cells; ANC: Absolute Neutrophils Count; ALC: Absolute Lymphocyte Count; AOBT/Y: Amount of Blood Transfusion per Year; *= Significant ($p \leq 0.05$); **= Significant ($p \leq 0.001$).

The comparison between splenectomized patients (group III) and non splenectomized ones (group I plus II) concerning the studied immunological parameters was illustrated in Figure 1. CD3, 4 and 8 percentages were statistically significant higher in splenectomized group in comparison to non splenectomized patients ($p \leq 0.001$).

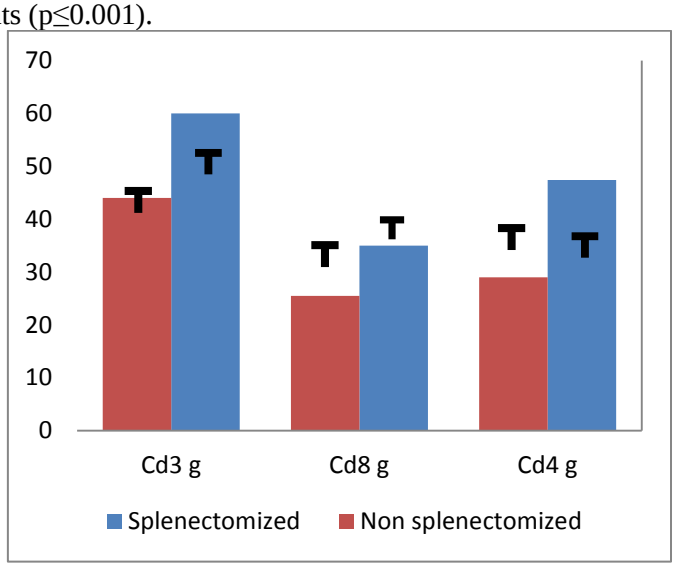

Figure 1: Splenectomized versus non splenectomized patients concerning CD3, CD8 and CD4 ($p \leq 0.001$).

Table 3: Comparison between studied patients regarding CD3, 4 and 8 vs.

parameter	Group I vs II	Group I vs III	Group II vs III
	P-value		
CD3	<0.001**	<0.001**	0.64
CD4	<0.001**	0.04*	<0.001**
CD8	<0.001**	0.05*	<0.001**

Non sig. >0.05, Sig. ≤ 0.05*, High sig. ≤ 0.001**.

Serum ferritin levels within β-thalassemic subgroups in correlation to immunological characteristics

The mean ± SD of serum ferritin levels within thalassemic groups as well as controls were shown in Table 2. There was significant increase in serum ferritin levels when patients were compared with controls (p=0.013). Regarding splenic status, serum ferritin levels were elevated in splenectomized patients (group III) compared with non splenectomized ones (group I, II) (p=0.02) (Figure 2). Significant moderate negative correlation was found between CD3 and serum ferritin (p=0.04, r=-0.6) in group I patients (Table 4). There were no other significant correlations between ferritin and the rest of immunological parameters (Table 4).

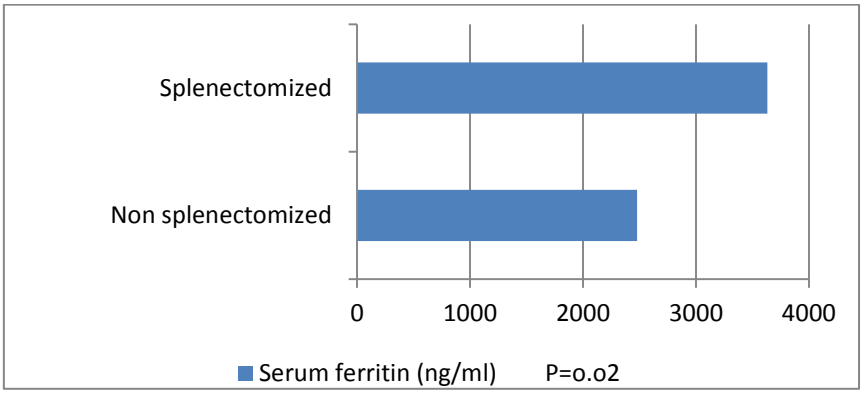

Figure 2: Splenectomized versus non splenectomized patients as regards serum ferritin; *= Significant (p ≤ 0.05).

Table 4: Correlation between CD3, CD4,CD8 and serum ferritin levels within patient's groups;

Group/ Variable			CD3	CD4	CD8
Group I	**Serum ferritin**	**P**	0.04*	0.6	0.4
		r	-0.6	-0.2	-0.3
Group II		**P**	0.9	1	0.5
		r	-0.02	-0.02	0.2
Group III		**P**	0.8	0.8	0.2
		r	-0.09	0.07	-0.4

r=0.75-1(strong correlation); r=0.5- .74(moderate correlation); r=0.25-0.49(fair correlation);r=0.1-0.24 (weak correlation); Non sig. >0.05, Sig. ≤ 0.05* .

Discussion

β-thalassemia major is defined as one of the most prevalent disorders in Mediterranean regions with approximately 1.5% of the populations worldwide who are carriers of the β – thalassemia[1,2]. Patients with β-thalassemia major are presented with many problems in addition to their severe anemia, including increased susceptibility to infections which is the second commonest reason for death within thalassemic patients[4,11]. It had been shown that the immunecapability of thalassemic patients has been altered[6,12]. β-thalassemia itself leads directly to a constant immune stimulation. Besides, the presence of abnormal erythrocytes leads to a continuous activation of monocytes that is responsible for immune clearance[13].

Furthermore, the variations and long periods of treatment modalities have great relevance to the incidence of these immune alterations. The therapeutic approaches for βTM patients are variable. Repeated blood transfusion alone or in combination with iron chelators are the widely spread schedules. Splenectomy is added to them when indicated. Recently, gene therapy and

bone marrow transplant or stem cell therapy are promising hopes for those patients[14] but they are out of our scope here. It has been reported that βTM-related immunologic changes differ according to therapeutic procedures used and in a population dependence manner[3]. The aim of this work is to evaluate the immune status of some children suffering from β-thalassemia major and receiving different treatment modalities within El Minya region.

Repeated transfusion is followed by risk of transmission of lots of bacterial infections plus many viruses -with immunosuppressive properties- such as cytomegalovirus (CMV), Ebstein-Barr virus (EBV) and hepatitis C virus (HCV)[15]. Additionally, Yersinia enterocolitica is usually associated with the use of deferoxamine (DFO) treatment as an iron chelating agent[16]. Therefore, depending on this fact that postulates thalassemic patients as more vulnerable individuals to repeated infections which in turn stimulate the immune system[17], we classified patients into two groups (patients with positive history of recurrent infection and patients with negative or queerly history of recurrent infection). In the current study, highly significant increase in lymphocytes count was reported in patients with positive history of recurrent infection than in patients with negative history of recurrent infection (data not shown). Also, children in group III (splenectomized) had history of more recurrent infections than those in group I and II. Likewise, all thalassemic patients had history of more recurrent infections than controls. This was in harmony with Rahav, et al., who reported that splenectomy accounts for the highest risk of infection in thalassemic patients[16]. Most patients in our study with positive history of recurrent infections are of higher ages compared with those without history of recurrent infection (data not shown).

Previous study performed by Rahav, et al., stated that main predictor for infection in thalassemia major was duration of thalassemia since the patient diagnosed or duration of splenectomy.[16] This was in agreement with Ahluwalia et al. and Lee et al., who found that splenectomy and aged more than 10 years are risk factors for severe infection in thalassemic patients[17,18].

The underlying causes of increased susceptibility to infections in thalassemic patients may be attributed to many factors including anemia per-ci, reticuloendothelial system dysfunction and transfusion related infections[17]. Also, ferritin levels affect immune response, which resulted in higher susceptibility to infections.. In addition, iron overload generates oxygen free radicals and causes peroxidative tissue injury leading to accelerated ageing of immune system with subsequent gradual decline in responsiveness to antigens and abnormal T cell function[20,21]. Likewise, splenectomy has been correlated with quantitative lymphocyte changes and aggravation of the immunological effects of multiple transfusions due to the reduced clearance of immune cells[22]. Lymphocytes are crucial in cell mediated immunity. Lymphocyte subsets include Helper T cells (CD4 T-cells), Cytotoxic T cells (CTLs or CD8 T-cells), Memory T cells and Regulatory T cells (Treg cells). CD4 T cells become activated upon foreign antigens exposure and secrete cytokines while the CD8 T-cells destroy virally infected cells. CD4+ cells along with CD8+ cells represent the majority of T lymphocytes[22]. We measured the levels of CD4+ and CD8+ T lymphocytes beside CD3+ T lymphocytes as representative of the total T Lymphocytes. In the present study, there were significant statistical differences regarding CD4, CD8 and CD3 T lymphocytes percentages within thalassemic groups when compared to each other's and to healthy controls. Patients of group I had significantly elevated CD4+ and CD8+ percentages, when compared to controls, group II and group III. Group II patients had significantly lower CD4+ and CD8+ percent-tages when compared to controls and other thalassemic groups but group III had significantly elevated CD4+ and CD8+ percentages in comparison to controls. These findings were in agreement with Vento et al., and Gharagozloo et al.,[4,19] In contrast, Noulsri, et al. had reported an insignificant difference in T-cell subsets CD3, CD4 and CD8 between patients and controls[20]. Though, all thalass-emic patients had lower CD3 T lymphocyte compared to healthy controls which is in agreement with Vento, et al., and Del Vecchio, et al.,[4,24].

Blood transfusion cause activation of chronic infections and constant stimulation of immune system which might induce the T regulatory cells and thus suppress the T cells effector functions. β-thalassemia itself leads directly to the same effect with continuous activation of monocytes that are responsible for immune clearance[13]. Additionally, majority of the patients in this study were found to have not only increased CD8+ cells but also higher CD4+ cell percentages except those in group II who had lower CD4+ and CD8+ percentages, when compared to healthy controls and other thalassemic groups. This may be related to deferrioxamine (DFX) induced reduction in the iron overload or could possibly be due to a direct effect of DFX on the immune system[23]. When classifying the studied patients into splenectomized and non splenectomized groups; splenectomized patients had higher significant levels regarding serum ferritin, CD4+, CD8+ and CD3+ compared to non splenectomized ones ($p \leq 0.05$) which was in agreement with Lee et al., and Gharagozloo et al., [18,19]. Increased T lymphocyte count on post-splenectomy might be associated with antigen that could not effectively filtered by spleen. This suggests that spleen could play some roles in the regulation of lymphocyte counts and act as a reservoir for lymphocytes produced in the body[20]. Iron overload indicated by ferritin was higher on post-splenectomy group than non-splenectomy group. This condition was due to iron absorption in response to ineffective erythropoiesis[25]. Therefore, iron overload, both from increased iron absorption or chronic blood transfusions, may affect the regulation and redistribution of lymphocyte subsets from the spleen and lymph nodes to the circulating pool and vice versa. Also, it has been reported that iron overload increased CD8+ populations[19].

Conclusion

Our results support that thalassemic patients are immunologically different from normal children and that treatment approaches are an essential player in this immune modulations. Many factors including therapeutic modalities are involved in this immune alteration.

Ethical Considerations

The study was approved by the research ethics committee of Minia University. The rationale, nature and possible risks of the experiments were fully explained to the parents. All parents gave written, informed consents at the beginning of the study and all data were kept confidential and used for research purposes only.

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