

Serum Levels of cytokines in poly-transfused patients with Beta-Thalassemia major: Relationship to splenectomy

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Abstract : Beta thalassemia is the most common chronic haemolytic anemia in Egypt. A major cause of morbidity and mortality in -thalassemic patients is infections, assumed to be the result of immunological changes. Cytokines production by immune cells is superior representative of phenotypes and functions of lymphocytes, but results of previous researches are not satisfactory and in some cases are controversial, due to differences in their experimental designs. So the aim of this study was to determine the possible defect, we investigated the cytokine IL-2 and IL-8 productions by blood cells of -thalassemic patients. The study was conducted on fifty one patients with homozygous beta-thalassemia major (23 of them were splenectomized group 1), who attending the Haematology Clinic, New Paediatric Hospital, Faculty of Medicine, Cairo University. Beside 17 healthy subjects served as control, with the same age matched group. All subjects were subjected to: full clinical examination, complete blood counting, liver function tests, and renal function tests. Determination of IL-2 was done by an immunoenzymometric assay for the quantitative measurement (Biosource IL-2 EASIA kit), and Determination of IL-8 by AviBion Human Interleukin-8 ELISA kits. The result showed that, there were significant increase ($P < 0.05$) in the serum level of IL-8 among group 1 (mean level was 526.4 ± 65.7 U/ml) as compared to control group (mean level was 208.67 ± 35.53 pg/ml) as well as group 2 (mean level was 438.21 ± 58.063 pg/ml). Also group 2 had significant increase ($P < 0.05$) in the serum level of IL-8 as compared to control group. While, the levels of serum IL-2 showed no significant changes ($P > 0.05$) between the thalassaemic groups as well as the control group. In conclusion, the study revealed that beta-thalassemia major patients had increased level of IL-8 which was more prominent in splenectomized patients. The potential role of IL-8 and the interactions between different cytokines in thalassaemic patients require further investigation. Multi-transfusions could be responsible for a change in circulating cytokines that could contribute to a state of partial immune deficiency in beta-thalassaemic patients, which is more prominence among the splenectomized patient.

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1.Introduction:

Thalassemia is an inherited autosomal recessive [blood disease](#). In thalassemia, the genetic defect results in reduced rate of synthesis of one of the globin chains that make up hemoglobin. Reduced synthesis of one of the globin chains can cause the formation of abnormal hemoglobin molecules. Beta-thalassemias are a group of hereditary blood disorders characterized by anomalies in the synthesis of the beta chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. The total annual incidence of symptomatic individuals is estimated at 1 in 100,000 throughout the world (Galanello & Origa, 2010). Beta-thalassemia is characterized by reduced synthesis of the hemoglobin subunit beta (hemoglobin beta chain) that results in microcytic hypochromic anemia, an abnormal peripheral blood smear with nucleated red blood cells, and reduced

amounts of haemoglobin A (HbA) on haemoglobin analysis. Individuals with thalassemia major have severe anemia and hepatosplenomegaly; they usually come to medical attention within the first two years of life. Without treatment, affected children have severe failure to thrive and shortened life expectancy (Geetha *et al.*, 2004).

The haemoglobin disorders are the most common clinically serious single gene disorders in the world. Beta thalassemia is the most common chronic haemolytic anemia in Egypt. So, it was estimated that 1,000/1.5 million per year live births will suffer from thalassemia disease in Egypt (total live births 1,936,205 in 2006) El-Beshlawy & Youssry 2009. A major cause of morbidity and mortality in -thalassemic patients is infections, assumed to be the result of immunological changes (Ghollam *et al.*, 2006).

Patients with thalassemia major commonly develop hypersplenism after 5-10 years of regular blood transfusions. As a result of splenic enlargement transfusion requirement steadily increase, accelerating the influx of toxic iron. Splenectomy is one form of the management of thalassemia disease Nasim et al., 2000. Splenomegaly tends to develop later and splenectomy can be postponed until the second decade of life or later. The commonly adopted criteria for splenectomy is a blood consumption greater than 50% above the mean requirement of the splenectomized population, i.e. more than 200-250 ml/kg/year of pure red cells, to maintain a pre-transfusion Hb around 9 g/d L (LISA *et al.*, 2008) Infectious complications constitute the second most common cause of mortality and a main cause of morbidity among major -thalassemia patients. These could be the results of functional alteration in the immune system due to multiple blood transfusions. Recent studies on immune competence in -thalassemia have revealed numerous quantitative and functional defects involving T and B lymphocytes, immunoglobulin production, neutrophils and macrophages, as well as the complement system (Farmakis *et al.*, 2003) suggested to be the results of iron overload and. There are also reports showing the phenotype of lymphocytes changes and increase in the circulating level of C-reactive protein occurs among the post-splenectomized patients, while there is no significant changes among non-splenectomized. Cytokines production by immune cells is superior representative of phenotypes and functions of lymphocytes, but results of previous researches are not satisfactory and in some cases are controversial, due to differences in their experimental designs. For example; Lombardi and colleagues in 1994 showed that serum level of IL-2 and IL-6 are undetectable or within the normal range in all their -thalassemic patients, while results obtained by Aggeli and co-workers in 2005 indicate an increase in the circulating level of IL-6 among these patients. It is known that interleukin-6 (IL-6) and interleukin-8 (IL-8) are important components of the pro-inflammatory response. The plasma levels of these cytokines may be relevant in the pathophysiology of beta-thalassemia (Oztürk *et al.*, 2001). For this purpose, the aim of this study plasma level of IL-2 and IL-8 cytokine were measured among both non-splenectomized and splenectomized thalassaemic patients.

1. Patients and Methods:

The study was conducted on fifty one patients with homozygous beta-thalassemia major (they were diagnosed by clinical examination and hemoglobin electrophoresis), who were attending the Haematology Clinic, New Paediatric Hospital, Cairo University, for

receiving blood transfusion and treatment. Beside 17 age and sex matched healthy person served as control group. The patients were divided into two groups:

1- Group 1(+ ve splenectomy)

Comprised (23) patients 12 Male (52.2 %) and 11 female (47.8 %), they were blood transfusion dependent and had splenectomy since 10 month. Their ages ranged from 4 to 19 years.

2- Group 2 (-ve splenectomy)

Comprised (28) patients 18 Male (64.3 %) and 10 female (35.7%), they were blood transfusion dependent and without splenectomy. Their ages ranged from 3 to 18 years.

1.1. Blood sampling

Ethically, patients were informed and written statements of their agreements were collected before sample collection. Peripheral fasting venous blood samples were collected from each patient and healthy person. The blood was left to clot at room temperature to separate sera after centrifuging for 10 minutes at 3000 r. p. m. Sera were divided into several aliquots and stored at -70°C until assay.

1.2. All patients were subjected

- Full clinical history and clinical examination
- Complete blood count: included haemoglobin concentration, hematocrite, and rectics, using Coulter counter and examination of Lishman or Wright-stained peripheral blood smears.
- Determination of serum alanine transaminase (ALT) and serum aspartate transaminase (AST) levels by using the method recommended by Committee on enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1976). The test was performed using already commercially available kit from Boehringer-Mannheim Company, Germany.
- Determination of serum bilirubin level by colorimetric method using available kit from Bio-Merieux Company, France (Perry *et al.*, 1983).
- Determination of serum urea level was done by colorimetric method according to Tietz (1995) the Kit from Croma Test Company Spain.
- Determination of serum creatinine by enzymatic method for creatinine utilizes a multi-step approach ending with a photometric end-point reaction. The enzyme creatinine amidohydrolase is used to convert creatinine to creatine. Creatine is broken down to sarcosine and urea by creatine amidinohydrolase. Further enzyme linked steps with sarcosine oxidase and peroxidase yield a colored chromogen read at 545nm according to (Young *et al.*, 1990)

- Determination of IL-2 by an immunoenzymometric assay for the quantitative measurement (BIOSOURCE IL-2 EASIA kit). It is a solid phase Enzyme Amplified Sensitivity Immunoassay. Cat. No. KAC1241-KAC1242, BioSource Europe S.A., Rue de L'Industrie, 8 B-1400 Nivelles Belgium (ROBB, 1984).
- Determination of IL-8 by AviBion Human Interleukin-8 ELISA kits. It is an enzyme-linked immunosorbent assay for quantitative detection of human IL-8. The assay recognizes both natural and recombinant Hu IL-8. Ref. IL08001, Ani Biotech Oy, Orgenium Laboratories Business Unit, Tiilitie 3, Fin-07120 Vantaa FINLAND (Baggiolini *et al.*, 1989).

1.3. Statistical analysis

The data were coded and entered in a personal computer using the Statistic packages SPSS (11.5 version) computer program. Results are expressed as mean \pm standard Error. Different groups were compared using student t test and the *p* values of less than 0.05 were considered statistically significant.

2. Results

The study was conducted on fifty one patients with beta-thalassemia major, their age ranged from 3-19 years; they were divided into two groups one with splenectomy and the other without splenectomy. Besides seventeen normal healthy control subjects matched for the age and sex were enrolled in this study.

The clinical data of the patients showed, pallor in 56.5% in group 1 while 57.1 % in group 2, and typical mongoloid facies (large head, prominence of the check bone tend to obscure the base of the nose and exposed the upper teeth, frontal bossing, and protuberance of the abdomen) appeared almost in all patients as evident by clinical examination and X rays. Hepatomegaly was present in 39.1% of group 1 while it present in 39.3% of group 2 as evident by the patients abdominal ultra

sonography. Splenomegaly was present in 14.3% among group 2 as shown in Table (1).

Table 2 showed the laboratory finding of the patient groups in relations to control group, the mean level of red blood count in group 1 was $3.76 \pm 0.67 \times 10^6$ cells/ml while in group 2 it was $3.04 \pm 0.8 \times 10^6$ cells/ml which were significant reduced ($P < 0.05$) as compared to the control group.

The mean level of haemoglobin concentrations in both groups were reduced, in group 1 it was 16.1 ± 0.36 g/dl while in group 2 it was 6.53 ± 0.182 g/dl which they were significant ($P < 0.05$) as compared to the control group.

As regards the total leucocytic count there were no statistical significant ($P > 0.05$) between the studied groups apart a slight increase in group 1, which was not significant.

Liver function tests (ALT and AST) and renal function tests (urea and creatinine) showed no significant variation ($P > 0.05$) between the studied groups, while the serum level of bilirubin were increased in the two groups of patients, the mean level of bilirubin was 1.92 ± 0.43 in group 1 and it was 1.53 ± 0.11 in group 2. They were a significant increase ($P < 0.05$) of serum bilirubin in both groups as compared to controls, as shown in Table (2).

Table 3 showed that, there were significant increase ($P < 0.05$) in the serum level of IL-8 among group 1 (mean level was 526.4 ± 65.7 U/ml) as compared to control group (mean level was 208.67 ± 35.53 pg/ml) as well as group 2 (mean level was 438.21 ± 58.063 pg/ml). Also group 2 had significant increase ($P < 0.05$) in the serum level of IL-8 as compared to control group as shown in table (3) & figure (1).

Serum IL-2 levels showed no significant changes ($P > 0.05$) between the thalassaemic groups as well as the control group as shown in table (3) & figure (2).

Table (1): Demographic data and clinical finding present in the studied groups.

	Control	Group 1 (+ve)	Group 2 (-ve)
Number	17	23	28
Age(years)	Range:	4-19	3-18
	mean \pm SE	9.184 ± 0.81	8.7 ± 0.83
Male/Female	10/7	12/ 11	18/10
Pallor	-	13 (56.5%)	16 (57.1%)
Jaundice	-	9 (39.1%)	15 (53.7%)
Mongoloid facies	-	21 (91.3%)	27 (96.4%)
Hepatomegaly	-	9 (39.1%)	11 (39.3%)
Splenomegaly	-	-	4 (14.3 %)

- Group1(+ve) : Patient with splenectomy.
- Group1(-ve) : Patient without splenectomy.

Table (2): Laboratory finding of the patient and control groups. (Data expressed as Range & mean \pm SE) and Statistical Variation.

	Control	Group 1 (+ve)	Group 2 (-ve)
Number	17	23	28
Red blood cells (x 10 ⁶ cells/mL)	4.3 – 5.1 4.67 \pm 0.33	2.5 - 4.2 3.76 \pm 0.76	2.1 – 3.8 3.04 \pm 0.8
Haemoglobin (g/dL)	9.9–14.1 13.0 \pm 1.1	3.9 -9.5 6.1 \pm 0.36*	5.1 – 8.7 6.53 \pm 0.182*
Leukocytic count (x10 ⁹ /L)	3.9 – 10.5 8.75 \pm 0.12	4.9 – 11.9 9.8 \pm 0.67	5.1 – 10.7 9.6 \pm 0.23
Liver function tests:			
<u>ALT (U/L)</u>	12-42 28.27 \pm 1.01	14-47 31.72 \pm 1.45	13-46 30.16 \pm 1.72
<u>AST (U/L)</u>	17- 41 29.1 \pm 1.71	19 – 48 31.92 \pm 1.67	23 – 54 34.93 \pm 1.4
<u>Bilirubin (mg/dL)</u>	0.2-1.01 0.7 \pm 0.02	0.8-3.1 1.92 \pm 0.43*	0.4 -2.8 1.533 \pm 0.11*

ALT= Alanine transaminase, AST= Aspartate transaminase

* Statistical significant compared to control group P<0.05

Table (3): Range and Mean \pm SE for Serum Interleukin 8 and Interleukin 2 Levels in the Different Studied Groups and Statistical Variation.

	Control No. 17	Group 1 No. 23	Group 2 No. 28
IL-8 (pg/ml)			
Range	47.76 -528.78	116.35 - 1445	77.05 – 1099
Mean \pm SE	208.678 \pm 35.53	526.40 \pm 65.72	438.213 \pm 58.063
P value : Group 1vs. Cont.		0.000219*	0.018364*
Group 2vs. Cont.		-	0.049783
Group 1 vs. group 2		-	-
IL-2 (U/ml)			
Range	0.35 - 2.33	0.29 - 3.14	0.45 -2.79
Mean \pm SE	1.686 \pm 0.179	1.87 \pm 0.19	1.905 \pm 0.167
P value : Group 1 vs. Cont.		0.191298	0.095193
Group 2 vs. Cont.			0.282238
Group 1 vs. group 2			

*P value :- P<0.05 considered significant.

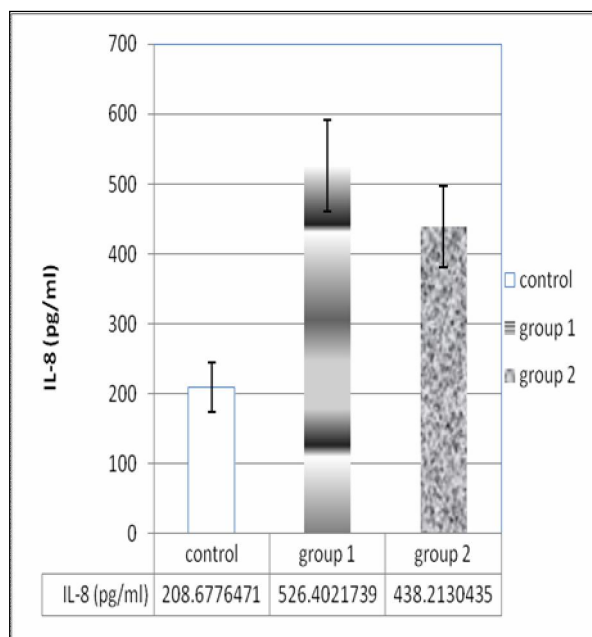


Figure 1: Mean ± S.E. for Serum Levels of Interleukin-8 (IL-8) (pg/ml.) in the Different Studied Groups

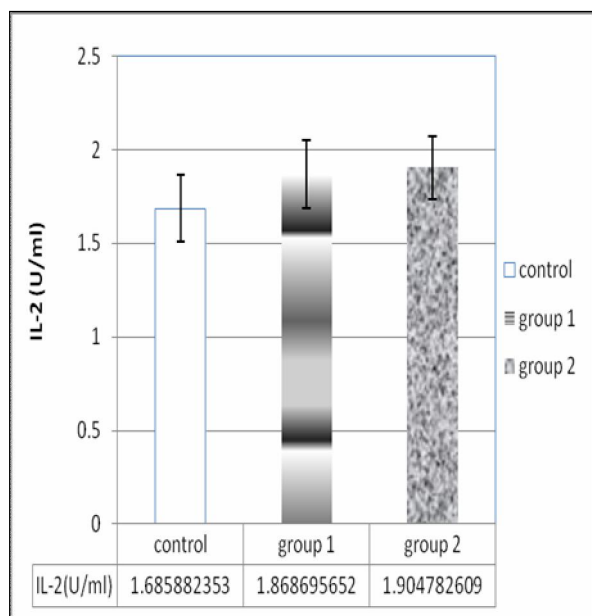


Figure 2: Mean ± S.E. for Serum Levels of Interleukins-2 (IL-2) (U/ml) in the Different Studied Groups

3. Discussion

Several, sometime contradictory immunological defects have been reported in patients with beta-thalassemia. They include: impaired activity of monocytes and neutrophils, defective activity of the complement alternative pathway, increased serum immunoglobulin levels, numerical or functional alternation of different peripheral lymphocytes and blood anomalies of serum level of cytokines. The mechanism of these abnormalities is not clarified. Factors such as splenectomy, iron overload, repeated exposure to foreign antigens at the time of blood transfusions and the use of chelating agent deferoxamine, known to have profound effects on the immune system. The role of immunologic alternations on the clinical course of beta thalassemia is not established, although they have been considered relevant to infectious episodes that these patients suffer (Consolini *et al.*, 2001).

The results of this study showed that there was significant increase in the serum IL-8 level in the thalassaemic patients with a profound increased in splenectomized patients (group 1). These increment in the serum level of IL-8 could be due to several immunological defects can be found in patients with beta-thalassaemia, among which the impairment of neurophil and macrophage phagocytic and killing functions and the production of some cytokines are the most important. It is known that interleukin-6 (IL-6) and interleukin-8 (IL-8) are important components of the pro-inflammatory response. The plasma levels of these cytokines may be relevant in the pathophysiology of beta-thalassaemia (Oztürk *et al.*, 2001).

These results were in accordance with Oztürk et al study who found that plasma IL-8 levels in the patients who had blood transfusions over 100 times were significantly higher than those of under 100 times ($p < 0.05$), whereas there was no statistical difference for IL-6. Markedly increased plasma IL-6 and IL-8 levels were documented in patients with beta-thalassemia. Increased production of IL-6 and IL-8 might have contributed to abnormalities in iron metabolism and it is probably due to overstimulation of macrophages (Oztürk *et al.*, 2001).

Also, Meliconi found that high IL-8 serum concentrations in the majority of beta-thalassaemic patients. A likely cause of this IL-8 production could

be ascribed to the transfusion-related continuous antigenic stimulation and iron overload with consequent macrophage activation. They concluded that macrophages (and fibroblasts) can be responsible for IL-8 production either directly or indirectly via TNF synthesis (Meliconi *et al.*, 1993).

The cloned interleukin-8 (IL-8), also known as neutrophil-activating peptide-1 (NAP-1), acts primarily and almost exclusively on neutrophils, stimulating chemotaxis and degranulation. This peptide can be produced by a variety of cell types, including large granular lymphocytes, macrophages, endothelial cells, fibroblasts, and synovial cells. Activated monocytes, endothelial cells, and fibroblasts may produce IL-8 in response to exogenous and endogenous stimuli, such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), and IL-1 (Strieter *et al.*, 1989).

Increased serum levels of IL-8 and TNF were reported in homozygous polytransfused beta-thalassemia major (Ugucioni *et al.*, 1993). In this study the authors suggested that the main causes for the rise in these cytokines were macrophage activation due to iron overload and the antigenic stimulation related to chronic transfusion therapy. Since it has also been reported that during erythrophagocytosis activated monocytes may produce different cytokines to enhance their phagocytic function (Simms *et al.*, 1991) and that IL-8 may increase in response to endogenous stimuli such as tumor necrosis factor and IL-1 (Strieter *et al.*, 1989). Also, in a study done by Fausto *et al.*, in 1995, they thought that the elevated serum IL-8 levels found in untransfused thalassemia syndromes could be related to the phagocytosis of red blood cells by hyperactive monocytes.

The result of this study showed that, there was slight non-significant increase in the serum level of IL-2 in the both groups of thalassemia as compared to control group. The reason for the low or normal serum levels of IL-2, a potent inducer of B-lymphocyte differentiation and of their capacity to synthesize immunoglobulin, despite strong stimulation of the B-immune system such as that described in our patients with b-thalassemia is not clear (Lombardi *et al.*, 1994).

On the other hand Gharagozloo *et al.* (2008) studied the immunologic abnormalities of Iranian beta-thalassemia major patients. Their results showed that Patients with thalassemia showed significantly increased absolute lymphocyte counts compared with the control group. An increased number of activated T cells and higher levels of serum neopterin were also observed in thalassemia patients, which suggest chronic stimulation of immune system. On the contrary, T-cell proliferation and interleukin 2 (IL-2),

interferon gamma (IFN-gamma), and IL-4 production were suppressed in patients compared to controls.

Also, Moshtaghi-Kashanian *et al.*, (2006), showed that IL-2 production of thalassaemic patients' groups were significantly ($p < 0.01$) lower than corresponding value obtained for the control group. They concluded that, multi-transfusions could be responsible for a change in the subset of circulating lymphocytes that could contribute to a state of partial immune deficiency in beta-thalassaemic patients, which is more prominence among the splenectomized patient.

4. Conclusion

The study revealed that beta-thalassemia major patients had increased level of IL-8 which was more prominent in splenectomized patients. The potential role of IL-8 and the interactions between different cytokines in thalassaemic patients require further investigation. Multi-transfusions could be responsible for a change in circulating cytokines that could contribute to a state of partial immune deficiency in beta-thalassaemic patients, which is more prominence among the splenectomized patient. So evaluation of the serum levels of selected cytokines may be a useful tool in improving our knowledge about these functional immunological defects in b-thalassemia.

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