ORIGINAL ARTICLE

Immunological Evaluation of β-Thalassemia Major Patients Receiving Oral Iron Chelator Deferasirox

Aamer Aleem[1], Zahid Shakoor[2], Khalid Alsaleh[1], Farjah Algahtani[1], Zafar Iqbal[3] and Abdulkareem Al-Momen[1]

ABSTRACT

Objective: To determine the immune abnormalities and occurrence of infections in transfusion-dependent β-thalassemia major patients receiving oral iron chelator deferasirox (DFX).

Study Design: An observational study.

Place and Duration of Study: Hematology Clinics, King Khalid University Hospital, Riyadh, Saudi Arabia, from July to December 2010.

Methodology: Seventeen patients with β-thalassemia major (12 females, median age 26 years) receiving deferasirox (DFX) for a median duration of 27 months were observed for any infections and had their immune status determined. Immune parameters studied included serum immunoglobulins and IgG subclasses, serum complement (C3 and C4) and anti-nuclear antibody (ANA) level, total B and T-lymphocytes, CD4+ and CD8+ counts, CD4+/CD8+ ratio, and natural killer (NK) cells. Immunological parameters of the patients were compared with age, gender, serum ferritin level and splenectomy status. Lymphocyte subsets were also compared with age and gender matched normal controls.

Results: A considerable reduction in serum ferritin was achieved by DFX from a median level of 2528 to 1875 μmol/l. Serum IgG levels were increased in 7 patients. Low C4 levels were found in 9 patients. Total B and T-lymphocytes were increased in 14 patients each, while CD4+, CD8+ and NK cells were increased in 13, 12 and 11 patients respectively. Absolute counts for all lymphocyte subsets were significantly higher compared to the normal controls ($p \leq 0.05$ for all parameters). Raised levels of IgG were associated with older age, female gender, splenectomized status and higher serum ferritin levels but this did not reach statistical significance except for the higher ferritin levels ($p=0.044$). Increased tendency to infections was not observed.

Conclusion: Patients with β-thalassemia major receiving DFX exhibited significant immune abnormalities. Changes observed have been described previously, but could be related to DFX. The immune abnormalities were not associated with increased tendency to infections.

Key Words: *β-thalassemia major. Immune system. Deferasirox. Infections.*

INTRODUCTION

Beta-thalassemia is a hereditary anemia due to defects in the production of β-globin chain. Patients with β-thalassemia major are prone to several complications including tendency to develop infections.[1] The morbidity and mortality from infections vary in different parts of the world depending on the level of care, preventive strategies adopted, epidemiology of various infections and the socio-economic level of each country. Infections have been found to be the second common cause of death after heart failure in patients with β-thalassemia major.[2-5]

The susceptibility to infections in thalassemia is multifactorial and appears to be related to the disease itself, altered immune system secondary to blood transfusions, iron overload and splenectomy.[1,6] A wide range of abnormalities of the humoral and cell mediated immunity, along with other aspects of immune system have been reported in patients with β-thalassemia major.[7-10] The abnormalities observed are both quantitative and functional, and involve several components of the immune system.[6,7] Patients may react differently to immune stimulation/modulation by various factors like blood transfusions, iron overload, splenectomy and the presence of infections like HBV or HCV. A variable influence of these factors may explain different findings in patients from different geographical areas. Iron overload has been implicated as an important factor in the development of many of these abnormalities.[7] The direct evidence that iron overload is responsible for the immune abnormalities has been well documented in a study describing restoration of defective neutrophil phagocytic function by iron chelation in patients with thalassemia.[8] It is, therefore, possible that the administration of chelating agents in thalassemia not only reduces the iron overload but may also have a beneficial effect on the associated immune abnormalities.

Deferoxamine (DFO) has been an effective iron chelator in thalassemia patients but is known for its predis-

[1] *Department of Medicine, Division of Hematology/Oncology,*
[2] *Department of Pathology, College of Medicine and King Khalid University Hospital, King Saud University, Riyadh, Saudi Arabia.*
[3] *Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud Bin Abdulaziz University of Health Sciences, Riyadh, Saudi Arabia.*

Correspondence: Dr. Aamer Aleem, Department of Medicine (38), King Khalid University Hospital, Riyadh-11472, P.O. Box 7805, Saudi Arabia.
E-mail: ameralem@ksu.edu.sa

Received: February 08, 2012; Accepted: April 23, 2014.

position to infections with yersinia family of bacteria.[9] There are conflicting reports of immune system abnormalities in patients with β-thalassemia major associated with the use of deferiprone, another iron chelating agent.[10,11] Deferasirox (ICL670, Exjade®, Novartis) was introduced few years ago as an oral iron chelator in the clinical practice and has proven to be useful in reducing iron overload in β-thalassemia major patients.[12] Whether DFX has any effect on the immune system in β-thalassemia major patients is not known and has not been investigated. We performed immunologic evaluation in 17 β-thalassemia major patients receiving iron chelation therapy with DFX and present our findings here.

METHODOLOGY

Immune parameters were determined in 17 consecutive β-thalassemia major patients being followed at King Khalid University Hospital, Riyadh, during a 6 months period from July to December 2010, while the development of any infections was observed from the start of deferasirox (DFX) therapy. Patients were 15 years or older and were receiving regular blood transfusions from an early age. All patients had homozygous β-thalassemia major and were transfused to maintain a hemoglobin level of above 9.0 g/dl. Majority of the patients received blood transfusions at a mean interval of 28 ± 4 days. All of these patients previously received deferoxamine for iron chelation and they were shifted to DFX when it became available in the institution in July 2007. The initial dose of DFX in most of the patients was 20 mg/kg and it was increased to 30 mg/kg in patients who did not have a satisfactory response.

Blood samples were collected for assays before the monthly blood transfusions. Clinical and laboratory information was retrieved from the patients' notes and computer records. Patients' data were collected for the following clinical and laboratory parameters: age, gender, serum ferritin before starting DFX and during the study period, splenectomy status, dose and duration of DFX treatment, evidence of infection with hepatitis B or C and history of any infections during the past 2 years from the start of DFX. Immunological parameters studied included: serum immunoglobulins and IgG subclasses, serum complement (C3 and C4) and anti-nuclear antibody (ANA) levels, total B and T-lymphocyte counts, CD4+ and CD8+ counts, CD4+/CD8+ ratio, and natural killer (NK) cells.

For immunoglobulin and complement assessment, five milliliters of venous blood was collected in clean tubes without any anticoagulant from all patients. Serum was separated after centrifugation and IgG along with the subclasses, IgM, IgA, and complement levels were assessed by BN ProSpec Nephelometer (Siemens Healthcare Diagnostics, Marburg, Germany).

For lymphocyte assays a 5-ml sample of peripheral blood was collected from each individual using ethylene-diamine-tetra-acetic acid (EDTA) as anticoagulant. Immunophenotyping for lymphocyte subsets was performed by flow cytometry according to the protocol of the Centre for Infectious Diseases, USA. Briefly, 100 µl aliquots of peripheral blood collected in EDTA were added to 20 µl of relevant monoclonal antibodies (mAbs). The labeled mAbs used in the study against cell surface markers included anti-CD3, CD4, CD8, CD19, CD56+CD16 and HLA-DR. Isotypic controls were IgG1 labeled with fluorescein isothiocyanate (FITC) and IgG2 labeled with phychoerythrin (PE) mouse antibodies. Following incubation with the relevant mAbs, erythrocytes were lysed using 2 ml of fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, Biosciences Pharmigen, San Diego, CA). After lyzing the erythrocytes, cells were washed twice with 0.5 ml of phosphate-buffered saline containing 0.01% sodium azide. Cells were fixed in 200 ml of FACS fix solution (10 g/l paraformaldehyde, 1% sodium cacodylate, 6.65 g/l sodium chloride, 0.01% sodium azide). Flow cytometric data acquisition was performed with a Becton Dickinson FACS caliber instrument. CELLQUEST TM software (BD Bioscience, San Jose, CA, USA) provided by the manufacturer was used for data acquisition and analysis.

Statistical analysis of the data was performed using SPSS software (version 20). Lymphocyte subsets of the patients were compared with 20 age and gender matched normal controls. All continuous data were compared using two-tailed independent-samples t-test. A p-value of ≤ 0.05 was considered significant. Multivariate analysis was performed to look for any significant association between possible candidate variables and the outcome. The independent variables were: age, gender, initial ferritin level and splenectomy status; dummy variable was created with zero being non-splenectomized or female and 1 for splenectomized cases or male. The dependent variable was IgG level. The variables were introduced into a single model using the “ENTER” procedure in SPSS.

RESULTS

Patients' characteristics are shown in Table I. There were 12 female and 5 male patients with a median age of 26 years (range 15 - 32). Fourteen patients were splenectomised. Serum ferritin decreased in 12 patients, increased in 3 and remained stable in 2 patients. Two patients were infected with both hepatitis B and C and one of these patients received anti-viral treatment in 2007. All the patients were negative for human immunodeficiency virus (HIV). A 30 years old female patient with insulin-dependent diabetes mellitus and poor sugar control had 3 episodes of febrile illnesses during the study within a 12 months period. All the work

Table I: Characteristics of the patients with β-thalassemia major.

Variable	Patients' values
Age (years)	
Median	26
Range	15-32
Gender	
Female	12
Male	5
Median treatment period with DFX in months (range)	27 (24-30)
Splenectomy	14
Serum ferritin (μmol/l)	
At start of DFX	
Median	2528
Range	974-13166
At last follow-up	
Median	1875
Range	563-6643
HCV and HBSAg positivity (no. of patients)	2

DFX; deferasirox, HCV-hepatitis-C virus, HBSAg-hepatitis-B surface antigen.

Table II: Changes in the immunological parameters of 17 β-thalassemia major patients in relation to the normal reference ranges.

Immunological parameter	Increased	Normal	Decreased
IgG	7	10	-
IgM	2	14	1
IgA	4	12	1
IgG1	5	11	-
IgG2	1	16	-
IgG3	2	13	2
IgG4	2	15	-
C3	-	15	2
C4	-	8	9
Total B-lymphocytes	14	3	-
Total T-lymphocytes	14	2	1
Total CD4+ cells	13	3	1
Total CD8+ cells	12	3	2
Total NK-cells	11	6	-
CD4+/CD8+ ratio	8	7	2

up for a possible underlying infection was negative. No clear cause of these episodes could be established and she received multiple courses of antibiotics. She remained well subsequently for almost one year till the end of study. Rest of the patients did not suffer from any notable infectious episodes during the period of DFX administration.

Among the various immunological parameters investigated, multiple abnormalities were noted in the lymphocyte subsets of the patients with β-thalassemia major. In comparison to the normal reference ranges, absolute counts for total B and T-lymphocytes were high in 14 (82%) patients each, 13 (76.5%) patients had high counts for CD4+ cells, 12 (70.5%) had high CD8+ counts and 11 (65%) patients had high absolute number of natural killer (NK) cells. Whereas CD+/CD8+ ratio was mildly increased in 8 (47%) patients, it was normal in 7 patients and reversed in 2 patients. Although majority of the patients had normal immunoglobulin levels, 7 (41%) patients had high IgG levels, 5 (29.5%) patients had high

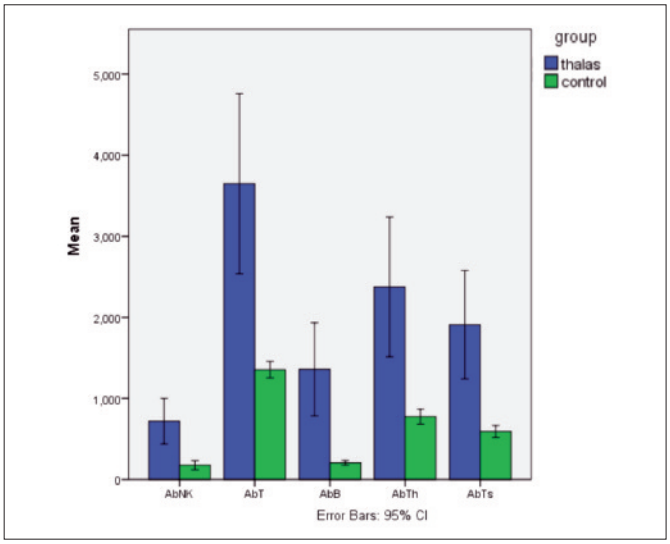

Figure 1: Comparison of the absolute counts of lymphocyte subsets between β-thalassemia major patients and normal controls. Blue bars represent the patients with β-thalassemia major (n = 17) and the green bars represent normal controls. P-value for all comparisons is ≤ 0.05. AbNK = absolute NK cells, AbT=absolute T-cells, AbB=absolute B-cells, AbTh = absolute T-helper cells, AbTs=absolute T-cytotoxic cells.

IgG1 and 4 (23.5%) patients had high IgA levels. Low C4 levels were found in 9 (53%) patients and low C3 in 2 patients. Anti-nuclear antibodies were detected in 2 patients whereas no patient tested positive for anti-double-stranded DNA antibodies. Number of patients with changes in each immunological parameter in relation to the normal reference ranges is given in Table II.

Figure 1 shows the comparison of lymphocyte subsets between patients and the normal controls. Absolute number of all the cell types including total T-cells, T-helper cells (CD4+), T-cytotoxic cells (CD8+), total B-cells and NK cells ($p \leq 0.05$ for all parameters) were significantly higher in the patients when compared with the normal controls. Using a multivariate regression, raised levels of IgG were associated with older age, female gender, splenectomized status and higher serum ferritin levels, but all these factors did not reach statistical significance except for the higher ferritin levels ($p=0.044$).

DISCUSSION

This study showed that treatment with DFX achieved considerable reduction in the serum ferritin levels in patients with β-thalassemia major. Patients were found to have significantly higher absolute counts for all the peripheral blood lymphocyte subsets. In addition, over 50% of patients had low C4 complement levels, and high immunoglobulin levels were detected in 23.5 - 41% of patients. Increased tendency to infections was not observed in this cohort.

Many abnormalities of the immune system have been reported in β-thalassemia major patients receiving multiple blood transfusions, although the relationship of

these abnormalities with the increased incidence of infections remains doubtful. Whether all or some of these abnormalities have clinical importance, is not known. Lombardi *et al*. studied the abnormalities of lymphocyte subsets and cytokines in polytransfused patients with β-thalassemia major but did not find any clinical relevance of these findings.[13]

Iron and its binding proteins have immune modulating properties and excess of iron may produce deleterious effects on the immune system.[7,14] Effects of the iron overload include alterations in T-lymphocyte subsets and modification of lymphocyte distribution in different compartments of the immune system.[14-18] Iron overload has been associated with the increase of CD8+ cells whereas a decrease in the CD4+ cell counts has also been reported.[7,15-18] Iron overload has been implicated in the increase of CD8+ cells irrespective of the splenectomy status of the patients.[15-17] Based on these observations, a reduction of CD4+/CD8+ ratio would be expected in patients with chronic iron overload, and a low CD4+/CD8+ ratio has been reported in thalassemia patients.[19] Majority of the patients in this study were found to have not only increased CD8+ cells but also higher CD4+ cell counts resulting in mildly increased or normal CD4+/CD8+ ratios. These findings might be related to DFX induced reduction in the iron overload or could possibly be due to a direct effect of DFX on the immune system.

Consistent with the findings of the present study, other investigators have reported higher counts of circulating B-cells in patients with β-thalassemia major.[14-16] Following treatment with DFX, higher levels of circulating immunoglobulins were found in some of the patients in this study, indicating increased functional activity of B-cells. Increase in the number of peripheral blood B-cell counts and raised serum immunoglobulin levels in thalassemia patients have been attributed to splenectomy, which is often performed as a therapeutic measure.[20] Majority of the patients in the present study had their spleens removed and splenectomy was associated with higher immunoglobulin levels, so it is possible that this was the contributing factor influencing peripheral blood B-cell counts and serum immunoglobulin levels.

Al-Awadhi *et al*. reported lymphocyte subsets in multitransfused β-thalassemia major patients from neighbouring Kuwait. T-cell marker levels were comparable between the patients and the controls, but the B-cell marker (CD19) was significantly higher and a lower percentage levels of NK (CD56) cells was found in the patients.[21] This patients group is likely to be ethnically similar to our patient population and the differences in the findings of lymphocyte subsets between the two groups raise the possibility that the changes observed in our patients could be related to DFX.

This study also found low complement C4 levels in over half of the patients. Low levels of complement C4 were shown to be associated with high serum ferritin levels in a recent study in which β-thalassemia major patients with serum ferritin levels higher than 3000 ng/ml were consistently found to have low serum complement C4 levels.[22] This association, however, appears to be an isolated finding and C4 levels do not appear to be influenced by the changes in serum ferritin levels, as reported by other investigators.[23] This was also evident in the present study where despite a significant reduction in serum ferritin levels after treatment with DFX, over 50% of the patients had low complement C4 levels. Low levels of complement C3 and factor-B have been reported in β-thalassemia major patients suffering from hepatitis-B virus infection.[23] Decreased complement C4 levels found in this study do not appear to be related to infection with hepatitis-B virus or autoimmunity. Furthermore, decreased serum complement C4 levels in the present study were not associated with concurrent reduction in complement C3 levels indicating that the low levels of C4 were not due to classical activation of the complement pathway but possibly due to circulating immune complexes related to blood transfusions.

Data regarding immune alterations in patients with β-thalassemia major receiving DFX are lacking. Although this study identified certain immune abnormalities in β-thalassemia major patients receiving DFX, it was, however, not possible to draw firm conclusion because of the relatively small number of patients. Further studies are recommended to gain a better understanding of DFX related immune alterations in β-thalassemia major patients with chronic iron overload.

CONCLUSION

Significant abnormalities of the immune parameters were found in β-thalassemia major patients treated with DFX. The changes could be related to DFX. The immune abnormalities were not associated with increased tendency to infections.

Acknowledgement: This study was supported by the College of Medicine Research Center, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

REFERENCES

1. Ricerca BM, Di Girolamo A, Rund D. Infections in thalassemia and hemoglobinopathies: focus on therapy-related complications. *Mediterr J Hematol Infect Dis* 2009; **1**: e2009028.
2. Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, *et al*. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. *Haematologica* 2004; **89**: 1187-93.

3. Ladis V, Chouliaras G, Bedousi H, Kanavakis E, Kattamis C. Longitudinal study of survival and causes of death in patients with thalassemia major in Greece. *Ann N Y Acad Sci* 2005; **1054**:445-50.
4. Chern JP, Su S, Lin KH, Chang SH, Lu MY, Jou ST, *et al*. Survival, mortality, and complications in patients with beta-thalassemia major in northern Taiwan. *Pediatr Blood Cancer* 2007; **48**:550-4.
5. Wanachiwanawin W. Infections in E-beta thalassemia. *Pediatr Hematol Oncol* 2000; **22**:581-7.
6. Farmakis D, Giakoumis A, Polymeropoulos E, Aessopos A. Pathogenetic aspects of immune deficiency associated with beta-thalassemia. *Med Sci Monit* 2003; **9**:RA19-22.
7. Walker EM, Walker SM. Effects of iron overload on the immune system. *Ann Clin Lab Sci* 2000; **30**:354-65.
8. Cantinieaux B, Hariga C, Ferster A, Toppet M, Fondu P. Desferrioxamine improves neutrophil phagocytosis in thalassemia major. *Am J Hematol* 1990; **35**:13-7.
9. Quintiliani L, Mastromonaco A, Giuliani E, Buzzonetti A, Sisti P, Guglielmetti M, *et al*. Immune profile alterations in thalassaemic patients. *Boll Ist Sieroter Milan* 1983; **62**:524-30.
10. Mehta J, Singhal S, Mehta BC. Deaths in patients receiving oral iron chelator L1. *Br J Haematol* 1993; **85**:430-1.
11. Loebstein R, Dalal I, Nisbet-Brown E, Berkovitch M, Meydan N, Andrews D, *et al*. Immune function in patients with beta-thalassaemia receiving the orally active iron-chelating agent deferiprone. *Br J Haematol* 1997; **98**:597-600.
12. Cappellini MD, Cohen A, Piga A, Bejaoui M, Perrotta S, Agaoglu L, *et al*. A phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with beta-thalassemia. *Blood* 2006; **107**:3455-62.
13. Lombardi G, Matera R, Minervini MM, Cascavilla N, D'Arcangelo P, Carotenuto M, *et al*. Serum levels of cytokines and soluble antigens in polytransfused patients with beta-thalassemia major: relationship to immune status. *Haematologica* 1994; **79**:406-12.
14. Cunningham-Rundles S, Giardina PJ, Grady RW, Califano C, McKenzie P, De Sousa M. Effect of transfusional iron overload on immune response. *J Infect Dis* 2000; **182**:S115-21.
15. Dua D, Choudhury M, Prakash K. Altered T and B lymphocytes in multitransfused patients of thalassemia major. *Indian Pediatr* 1993; **30**:893-6.
16. Pattanapanyasat K, Thepthai C, Lamchiagdhase P, Lerdwana S, Tachavanich K, Thanomsuk P, *et al*. Lymphocyte subsets and specific T-cell immune response in thalassemia. *Cytometry* 2000; **42**:11-7.
17. Grady RW, Akbar AN, Giardina PJ, Hilgartner MW, de Sousa M. Disproportionate lymphoid cell subsets in thalassaemia major: the relative contributions of transfusion and splenectomy. *Br J Haematol* 1985; **59**:713-24.
18. Dwyer J, Wood C, McNamara J, Williams A, Andiman W, Rink L, *et al*. Abnormalities in the immune system of children with beta-thalassemia major. *Clin Exp Immunol* 1987; **68**:621-9.
19. Del Vecchio GC, Schettini F, Piacente L, De Santis A, Giordano P, De Mattia D. Effects of deferiprone on immune status and cytokine pattern in thalassemia major. *Acta Haematol* 2002; **108**:144-9.
20. Akbar AN, Giardina PJ, Hilgartner MW, Grady RW. Immunological abnormalities in thalassemia major. A transfusion-related increase in circulating cytoplasmic immunoglobulin-positive cells. *Clin Exp Immunol* 1985; **62**: 397-404.
21. Al-Awadhi AM, Alfadhli SM, Al-Khaldi D, Borhama M, Borusly M. Investigation of the distribution of lymphocyte subsets and zinc levels in multi-transfused beta-thalassemia major patients. *Int J Lab Hematol* 2010; **32**:191-6.
22. Ghaffari J, Vahidshahi K, Kosaryan M, Soltantooyeh Z, Mohamadi M. Humeral immune system state in ß-thalassemia major. *Med Glas Ljek komore Zenicko-doboj kantona* 2011; **8**:192-6.
23. Sinniah D, Yadav M. Elevated IgG and decreased complement component C3 and factor B in β-thalassemia major. *Acta Paediatr Scand* 1981; **70**:547-50.