A Study of Autoimmunity in Children with Thalassemia Major

KHALED H. TAMAN, M.D. 1; KHALED M. SALAMA, M.D. 2; ABDEL FATTAH M. AHMAD, Ph.D. 3; EITHAR K. EL ADHAM, Ph.D. 4 and MOHAMAD M. BAYOUMI, Ph.D. 5

The Departments of Pediatrics, Institute of Postgraduate Childhood Studies, Ain Shams University 1, Pediatrics, Faculty of Medicine, Cairo University 2, Biochemistry 3, Pediatrics 4 and Physiology 5, Atomic Energy Authority.

Abstract

Aim: This study was subjected to estimate the most common autoimmune complications in thalassemics and the prevalence of different auto antibodies so as to evaluate its correlation with personal data including age, sex, growth parameters, hepatitis C antibody status, ferritin level and type of iron chelation therapy.

Methodology: Different Abs including anticardiolipin, antihistone, ANA, anterythropoietin by ELISA technique, hepatitis C virus by ELISA and. Ferritin by IRMA (Immune radiometric assay), result of the study were analyzed by appropriate statistical methods.

Results: ANA were positive in 13.3%, anterythropoietin antibodies were positive in 15% of cases while antierythropoietin antibodies were positive in 93.3% of cases and they were not significantly related to HCV antibody status or type of chelation. The mean age of hepatitis C virus negative cases was significantly lower than hepatitis C virus positive cases, ferritin levels were significantly higher in patients on deferipone than those on desferrioxamine.

Conclusion: The presence of auto antibodies especially anterythropoietin antibodies is prevalent among thalassemia major patients.

Key Words: Autoimmunity – Thalassemia.

Introduction

Repeated transfusions for the treatment of thalassemia major cause an insult to the patients immune system. It provokes post transfusion purpura and hemolytic reactions that can be severe and life threatening [2].

There are numerous autoantibodies found in the sera of thalassemic patients receiving iron chelators. The presence of antihistone suggests an alteration in the humoral immune response. The condition is associated with a lupus like picture concerning clinical complications as autoimmune hemolytic reactions, arthritis and impaired renal functions [3].

HCV is the most prevalent transfusion transmitted infection in Egyptian thalassemics. HCV infection has been associated with autoimmune diseases (i.e. SLE) and an increased risk of thromboembolic events. Anticardiolipin Ab, lupus anticoagulants and antiphospholipids are detected in thalassemics with HCV infection and proved to be involved in inflammatory process [4].

Resistance to recombinant human erythropoietin therapy was observed in some thalassemic patients receiving regular transfusion therapy. Anterythropoietin Ab should be evaluated in such patients and it may play a role in rhEpo resistance [5].

Aim of the study:

This study was subjected to estimate the most common autoimmune complications in thalassemics and the prevalence of different auto antibodies so as to evaluate its correlation with personal data including age, sex, growth parameters, hepatitis C antibody status, ferritin level and type of iron chelation therapy.

Abbreviations:

TMB : Tetramethy Benzedrine.
HRP : Horseradish Peroxides.
OD : Optical Density.

Correspondence to: Dr. Khaled H. Taman, The Department of Pediatrics, Institute of Postgraduate Childhood Studies, Ain Shams University.
Patients and Methods

This crosssectional clinical laboratory study was conducted on 60 thalassemic patients attending the Pediatric Hematology Clinic of Cairo University Children’s Hospital (CUCH).

Inclusion criteria:

All patients in the study fulfilled the following criteria:

- Thalassemia major patients following-up at the hematology outpatient clinic.
- Age ranges between 5-15 years old.

Exclusion criteria:

- Patients with thalassemia intermedia.

Patients were classified into groups according to:

- Type of chelation therapy whether they are using Desferral (n=28) or Deferipone (n=32).
- HCV+ve (n=45) or HCV-ve (n=15) cases.

All patients were subjected to:

Full history taking including:

- Onset, course and duration of the illness.
- Affected siblings and consanguinity.
- Frequency of blood transfusion.
- History of chelation therapy either in the form of S.C. infusion pumps or oral chelation therapy.
- History of hemolytic crises.
- History of splenectomy.

Clinical examination:

- Physical examination including height, weight and BMI.
- Cardiac examination.
- Joint examination.

Laboratory Investigations:

- HB%.
- Serum Ferritin by IRMA.

Autoimmune profile:

- Indirect Coombs test.
- Anticardiolipin Ab by ELISA.
- ANA, RF.
- Antihistone Ab by ELISA.
- Antierthropoietin by ELISA.
- Antiplatelet Ab.

Antihistone antibodies:

Principle of the test:

Quantitative measurement of antihistone antibodies by QUANTALITE TM histone which is an enzyme-linked immunosorbent assay (ELISA) for the semiquantitative measurement of histone antibodies in human serum.

Procedure of the test:

Purified histone antigens are bound to the wells of polystyrene microwell plate under conditions that will preserve the antigen in its native stage. Prediluted controls and dilute patient sera are added to separate wells allowing any histone antibodies present to bind to the immobilized antigen. Unbound sample is washed away and an enzyme labeled anti human IgG conjugate is added to each well. A second incubation allows the enzyme labeled antihuman IgG conjugate to bind to any patient antibodies, which have become attached to the microwells. After washing away any unbound enzyme labeled antihuman IgG, the remaining enzyme activity is measured by adding achromogenic substrate and measuring the intensity of the color that develops. The assay can be evaluated spectrophotometrically by measuring and comparing the color intensity that develops in the patient wells with the color in the control wells.

Reagents:

1- Polystyrene microwell ELISA plate coated with a purified histone antigen (12-1 x 8 wells), with holder in foil package containing desiccants.
2- ELISA Negative control.
3- Histone ELISA Law positive, 1 vial of buffer containing preventative and human serum antibodies to histone, prediluted 1.2ml.
4- Histone ELISA high positive, 1 vial of buffer containing preservative and human serum antibodies to histone prediluted, 1.2ml.
5- HRP sample diluents, 1 vial-colored pink containing Tris-buffered saline.
6- Concentrated HRP wash buffer.
7- HRP IgG conjugate, antihuman IgG, 1 vial-coloured blue containing buffer, protein stabilizers and preservative 10 ml.
8- TMB chromogen, 1 vial containing stabilizers 10ml.
9- HRP substance solution will stop the reaction.

Quality control:

The histone ELISA low positive, the histone ELISA high positive and ELISA negative control
should be run with every batch of samples to ensure that all reagents and procedures perform properly.

**Calculation of the results:**

The average OD for each set of duplicates is first determined. The reactivity for each sample that can be calculated by dividing the average OD of the sample by the average OD of the histone ELISA low positive. The result is multiplied by the number of units assigned to the histone ELISA low positive bound on the label:

\[
\text{Sample value} = \frac{\text{Sample OD}}{\text{Histone ELISA low positive OD}} \times \text{Histone ELISA low positive OD}
\]

The sample can be classified as negative, weak positive, moderately positive or strong positive according to the Table:

<table>
<thead>
<tr>
<th>Units</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Weak positive</td>
<td>1-1.5</td>
</tr>
<tr>
<td>Moderate positive</td>
<td>1.6 – 2.5</td>
</tr>
<tr>
<td>Strong positive</td>
<td>&gt;2.5</td>
</tr>
</tbody>
</table>

**Anterythropoietin antibodies:**

Quantitative measurement of erythropoietin antibodies by enzyme immune assay by REE DE 560 11.

**Procedure of the test:**

Test procedure is done on 2 successive days.

**On the 1st day:**

1. Pipette 150ul of the diluent into each well of the Microtiter plate.
2. Pipette 50ul of each standard control and patient sample into respective well.
3. Cover plate with adhesive foil, incubated for 4 hours at room temperature.
4. Remove adhesive foil.
5. At the end of incubation time 100ul of freshly prepared erythropoietin antiserum was added into each well.
6. Cover plate with adhesive foil. Incubate for 16-20 hrs at 2-8C.

**Second day:**

- Remove adhesive foil.
- Pipette 100ul of freshly prepared enzyme conjugate into each well.
- Approximately 10min before end of incubation prepare PNPP substrate solution.
- Wash plates with buffer. Remove excess solution by topping the inverted plate on paper towel.
- Pipette 200M of freshly prepared PNPP substrate solution into each well.
- Cover plates and incubate 120min at room temp on an orbital shaker.
- Measure the optical density with photometer at 45nm.

**Quality control:**

All standards and kit controls must be found within the acceptable ranges as stated on the QC certificate.

The samples are classified as negative, weak positive, moderate positive or strong positive.

**Anticardiolipin Ab:**

Anticardiolipin IgM antibodies are detected using REAADs test which is an enzyme linked immunosorbent assay (ELISA) for the semi quantitative determination of IgM anticardiolipin Abs.

**Procedure of the test:**

The test is performed as an indirect ELISA. Diluted serum samples, calibrator sera, and controls are incubated in cardiolipin coated microwell, allowing a CL antibodies present in the samples to react with the immobilized antigen. After the removal of unbound serum proteins by washing antibodies specific for human IgM labeled with horseradish peroxides (HRP) is added forming complexes with the cardiolipin bound antibodies.

The concentration of IgM acl is determined through another washing step, the bound enzyme-antibody conjugated is assayed by the addition of tetramethy Benzedrine (TMB) and hydrogen peroxide \((H_2O_2)\) the chromogenic substrate color develops in the wells at an intensity proportional to the serum concentration of acl antibodies.

Results are obtained by reading the optical density (OD) of each well with a spectrophotometer calibrator sera is provided with IgM acl concentrations expressed in MPL units. The OD values of all samples are multiplied by the conversion factor to obtain IgM acl antibody concentration in standard units.

One MPL unit is equivalent to 1ug/ml of an affinity purified standard IgM sample.

**Quality control:**

The OD value of calibrator should be at least 0.400 to assure that the kit is functioning properly.
Calculation of the results:

Anticardiolipin concentration of sample conversion factor absorbance of the sample (OD) IgM concentration more than 15 MPL is considered positive.

Serum Ferritin by IRMA:

Principle of the procedure:

Coat A count Ferritin IRMA is a solid phase immunoradiometric assay based on monoclonal and polyclonal anti-ferritin polyclonal antibody in liquid phase and monoclonal anti-ferritin antibody immobilized to the wall of a polystyrene tube.

In the procedure:

Ferritin is captured between monoclonal anti-ferritin antibodies immobilized on the inside surface of the polystyrene tube and the radiolabeled polyclonal antiferritin tracer.

Unbound I125 labeled anti-ferritin antibody is removed by decanting the reaction mixture and washing the tube, this reduces non specific binding to a very low level, and ensures excellent low end precision.

Ferritin concentration is directly proportional to the radioactivity present in the tube after the wash step. The radioactivity is measured by gamma counter after which a concentration of ferritin in the patient sample is obtained by comparing the patient counts per minute and those obtained for the use of calibrators.

Indirect Coomb's test: (Coombs RRA, Mourant AE, Race RP, A test for the detection of weak and "incomplete" Rh agglutinins).

First stage:

Washed test red blood cells (RBCs) are incubated with a test serum. If the serum contains antibodies to antigens on the RBC surface, the antibodies will bind onto the surface of the RBCs.

Second stage:

The RBCs are washed three or four times with isotonic saline and than incubated with antihuman globulin. If antibodies have bound to RBC surface antigens in the first stage, RBCs will agglutinate when incubated with the antihuman globulin (also known Coombs reagent) in this stage and the indirect Coombs test will be positive.


Reagents prepared from the serum of patients with immune thrombocytopenic purpura incubated with patient’s serum and observed for agglutination.

All cases were negative concerning the antiplatelet Antibodies.

Statistical analysis of data collected:

Computer software package SPSS 9 was used for qualitative variables. Frequency and percent were estimated. While mean (as a measure for central tendency), standard deviation minimum and maximum (as a measure for dispersion) were estimated for qualitative variable, test (student test) was done to estimate the difference in quantitative variables comparing 2 groups.

Results

The study has been conducted on sixty patients with thalassemia major attending the New Children Hospital of Cairo University for follow-up. Cases were 38 males and 22 females with male: female ratio 1.7:1.

Patients were classified into 2 groups: First they were classified according to the chelation, there were 32 patients on Deferiprone and 28 patients on Desferioxamine.

The second classification was according to HCV infection, we have 45 seropositive patients and 15 patients are seronegative.

Concerning the clinical data for the selected cases, the age ranged between 10-15 years with a mean value of 12.2 ± 1.88, the weight ranged between 20-40 with a mean value of 29.55 ± 5.05 and the height ranged between 75-142 with a mean of 120.92 ± 14.66 BMI ranged between 11.7-26.2 with a mean of 20.88 ± 6.6.

Table (1) shows a comparison of age between thalassemic cases who are HCV+ve (45) and those who are HCV-ve (15).

The age of HCV-ve cases was significantly lower than HCV+ve cases with a \( p \)-value of 0.039.

Table (2) and Fig. (1) compared the serum ferritin levels among cases according to their chelation therapy. Cases on oral chelation, deferipone showed a significantly higher serum ferritin levels than those on desferioxamine therapy \( (p=0.044) \).
Table (2): Ferritin levels according to the type of chelation therapy.

<table>
<thead>
<tr>
<th></th>
<th>Deferiprone</th>
<th>Desferioxamine</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>32</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>131-4450</td>
<td>790-4400</td>
<td>0.044 (S)</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2640.03±1004.6</td>
<td>2197.18±963.45</td>
<td></td>
</tr>
</tbody>
</table>

S: Statistically significant difference.

Fig. (1): Ferritin levels according to the type of chelation therapy.

ANA were positive in 13.3%, antierythropoietin antibodies were positive in 15% of cases while antierythropoietin antibodies were positive in 93.3% of cases (Table 3).

Table (3): Distribution of positive antibodies among the 60 cases.

<table>
<thead>
<tr>
<th></th>
<th>+ve n (%)</th>
<th>–ve n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>8 (13.33)</td>
<td>52 (86.67)</td>
</tr>
<tr>
<td>Anti histone</td>
<td>2 (3.33)</td>
<td>58 (96.67)</td>
</tr>
<tr>
<td>Anti CL</td>
<td>9 (15.00)</td>
<td>51 (85.00)</td>
</tr>
<tr>
<td>Anti EP</td>
<td>56 (93.33)</td>
<td>4 (6.67)</td>
</tr>
</tbody>
</table>

* Data presented in no. of cases (%).

Table (4) and Fig. (2) showed the auto antibodies positivity according to HCV seropositivity. No significant difference was noted between the positive and negative cases.

Table (4): Positive antibodies according to HCV infection.

<table>
<thead>
<tr>
<th></th>
<th>HCV(–)ve (n = 15)</th>
<th>HCV(+)ve (n = 45)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA *</td>
<td>1 (6.67)</td>
<td>7 (15.56)</td>
<td>0.661 (NS)</td>
</tr>
<tr>
<td>Anti histone*</td>
<td>0 (0.00)</td>
<td>2 (4.44)</td>
<td>1.000 (NS)</td>
</tr>
<tr>
<td>Anti CL*</td>
<td>1 (6.67)</td>
<td>8 (17.78)</td>
<td>0.531 (NS)</td>
</tr>
<tr>
<td>Anti EP*</td>
<td>15 (100.00)</td>
<td>41 (91.11)</td>
<td>0.550 (NS)</td>
</tr>
</tbody>
</table>

* Data presented in no. of cases (%).
NS: Statistically non-significant difference.

Table (5) and Fig. (3) showed no significant differences concerning the existence of auto antibodies in thalassemic patients on different chelation modalities.

Table (5): Positive antibodies according to the chelation therapy.

<table>
<thead>
<tr>
<th></th>
<th>Deferiprone (n = 32)</th>
<th>Desferioxamine (n = 28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA *</td>
<td>6 (18.75)</td>
<td>2 (7.14)</td>
<td>0.384 (NS)</td>
</tr>
<tr>
<td>Anti histone*</td>
<td>2 (6.25)</td>
<td>0 (0.00)</td>
<td>0.532 (NS)</td>
</tr>
<tr>
<td>Anti CL*</td>
<td>4 (12.50)</td>
<td>5 (17.86)</td>
<td>0.828 (NS)</td>
</tr>
<tr>
<td>Anti EP*</td>
<td>28 (87.50)</td>
<td>28 (100.00)</td>
<td>0.156 (NS)</td>
</tr>
</tbody>
</table>

* Data presented in no. of cases (%).
NS: Statistically non-significant difference.

Table (5) and Fig. (3) showed no significant differences concerning the existence of auto antibodies in thalassemic patients on different chelation modalities.

Table (5): Positive antibodies according to the chelation therapy.

<table>
<thead>
<tr>
<th></th>
<th>Deferiprone (n = 32)</th>
<th>Desferioxamine (n = 28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA *</td>
<td>6 (18.75)</td>
<td>2 (7.14)</td>
<td>0.384 (NS)</td>
</tr>
<tr>
<td>Anti histone*</td>
<td>2 (6.25)</td>
<td>0 (0.00)</td>
<td>0.532 (NS)</td>
</tr>
<tr>
<td>Anti CL*</td>
<td>4 (12.50)</td>
<td>5 (17.86)</td>
<td>0.828 (NS)</td>
</tr>
<tr>
<td>Anti EP*</td>
<td>28 (87.50)</td>
<td>28 (100.00)</td>
<td>0.156 (NS)</td>
</tr>
</tbody>
</table>

* Data presented in no. of cases (%).
NS: Statistically non-significant difference.

Fig. (2): Distribution of positive antibodies according to HCV infection.

Antiplatelate antibodies were negative in all cases of the study.

Discussion

Homzygous B-thalassemia usually results in the condition of thalassemia major, a clinically severe anemia in which affected patients require
treatment by regular blood transfusion and iron chelation therapy from early infancy for survival [6].

A wide spectrum of immune abnormalities has been described by numerous studies involving B-thalassemia patients undergoing multiple transfusions [7].

The study was conducted on 60 patients with thalassemia major with mean age of 12.2 ± 1.88 and male: female ratio of 1.7 : 1.

Among our 60 thalassemic patients, ANA was positive in 8 (13.33%) of them. This result is near to those obtained by many authors. Economidou, et al. reported a 13% (10/75) incidence of ANA in poly transfused thalassemic patients, and Agrawal et al. found ANA to be positive in 12 out of their 83 thalassemia major patients (14.5%). The study done by Mehta, et al. reported ANA positivity in 9 out of their 90 patients (10%). A higher result was reported by Voulgari, et al. who reported an incidence of 25.9% (15/58) of positive ANA in thalassemic patients but, this could be explained by the higher mean age of this study population (32.4 ± 9.6 years).

Concerning the type of chelation, six ANA positive cases were on Deferiprone (6/32, 18.75%) while 2 were on Desferioxamine (2/28, 7.14%) Mehta et al. found ANA positive in 25.9% of patients on Deferiprone versus 3.2% in those not receiving oral iron chelation. On the other hand, Choudhry et al. and Ayub et al. reported that def eriprone treatment was not associated with any increase in ANA seropositivity.

Only one of the ANA positive cases was HCV seronegative (1/15, 6.67%) while the others were HCV seropositive (7/45, 15.56%), this result is less than that obtained by Siagris et al. who reported that ANA positivity was found in 11.42 (26.2%) of thalassemic patients with chronic hepatitis C virus infection, this can be attributed to the younger age of our study group with less allo-antigenic stimulation by frequent blood transfusions. Two of our cases (3.33%) had positive antihistone antibodies both of them were on oral iron chelater deferiprone and were ANA positive. One of them had also anticardiolipin antibodies.

Mehta et al. stated that their investigation showed that there is a small amount of background ANA-positivity in thalassemia major patients but the antihistone antibodies were always negative. But when investigating patients receiving deferiprone the frequency of ANA positivity was higher and some of whom also had positive ANA which is consistent of the drug induced lupus.

Different results were reported by Predhan et al. [3] as he found that antihistone antibodies and other auto antibodies were found to be more prevalent among thalassemia patients receiving iron chelation therapy. He reported a markedly higher percentage than the present study and were higher in those receiving desferioxamine (48%) than patients on deferiprone therapy (30%).

The presence of antihistone antibodies together with ANA is specific for drug induced lupus [8].

Anticardiolipin antibodies (IgM) was positive in 9 patients (15%) and no history of thrombosis was given by the patients. No significant relation was present between it and anti HCV antibodies positivity.

In a study done by Sharma et al. [4] IgM anticardiolipin antibodies were detected in 6% of thalassemia major cases with no statistically significant association between anti HCV antibodies and IgM anti cardiolipin antibodies. Also, none of their patients had thrombotic manifestations. In a study done in Iran anticardiolipin antibodies were detected in 42.7% of cases but they measured the IgG type (which is more prevalent than the IgM type) and it was not statistically related to HCV infection (Kashef et al., 2008). Also in an older study by Grordano et al., in 1998 no significant relation was found between HCV infection and anticardiolipin antibody positivity and none of the positive patients for anticardiolipin antibodies mentioned any thrombotic manifestations.

On the other hand, a study done by Eldor, et al. reported that none of their thalassemic patients had anticardiolipin antibody.

Antierthropoietin antibodies have been described in patients with SLE and pure red cell aplasia [9] and it was later postulated that antibodies to erythropoietin may contribute to the pathogenesis of SLE associated with pure red cell aplasia [10].

Antierthropoietin was the most frequently encountered antibody among our patients. It was positive in 56/60 (93.33%) cases.

These cases included all who were positive to other performed antibody testing fifty percent of them were on desferioxamine therapy and the other half was an oral iron chelator deferiprone. HCV
status had no effect on the presence of anti erythropoietin antibody. None of our patients was on treatment by recombinant human erythropoietin.

Voulgar, et al. detected for the first time the presence of anti erythropoietin antibodies in thalassemic patients and he postulated that this may contribute to recombinant human erythropoietin resistance encountered in some cases. All his positive cases were on recombinant human erythropoietin therapy while non of our cases was receiving this mode of therapy [11].

The clinical data as age, weight, height, BMI show no variation between the different groups regarding the type of chelation, but regarding HCV infection the age was significantly higher in HCV positive cases than HCV negative cases with a p-value of 0.039 as frequency of transfusion, iron overload and the systemic complication of the disease increase by age [12].

In this study ferritin level was significantly higher in patients using Deferipone than those on Desferral, so, Desferal proved to be a better chelator than Deferipone in the cases of this study, however recent studies suggest that the use of both chelators on the long run is better to reduce iron overload and cardiac complications [13].

Conclusions:
The presence of auto antibodies especially antieythropoietin antibodies is prevalent among thalassemia major patients. Autoimmunity should be assessed in thalassemics as it may be a cofactor in the development of life threatening complications.

References