THE POSSIBLE ROLE OF AUTOIMMUNITY IN THE PATHOGENESIS OF DIABETES IN \(\beta\)-THALASSEMIA MAJOR

L. MONGE, S. PINACH, L. CARAMELLINO (1), M.T. BERTERO (2), A. DALL’OMO (3), Q. CARTA

SUMMARY - Objective: To evaluate the possible role of autoimmunity in the pathogenesis of diabetes associated with \(\beta\)-thalassemia, we studied a cohort of 53 \(\beta\)-thalassemic individuals, under long term blood transfusion, that included twelve patients with diabetes (22.6%).

Material and Methods: To evaluate the activation of an autoimmune response, individuals were tested for islet cell antibodies (ICA), glutamic acid decarboxylase (GAD) autoantibodies, insulin autoantibodies (IAA) and serum anti-nuclear antibodies (ANA).

Results: Nine of the total \(\beta\)-thalassemic population (16.98%) were ICA-positive. The frequency of ICA-positive subjects among thalassemic individuals was higher than in the general population. Five (41.6%) of the ICA-positive individuals were diabetic. Of these, three were serum C-peptide-negative (<0.21 nmol/l). HLA class II typing of our thalassemic population did not reveal significantly different allelic frequencies with respect to the control population.

Conclusions: Our study demonstrates evidence of immune system activation against pancreatic \(\beta\)-cells in \(\beta\)-thalassemia and we propose that iron deposition may, through oxidative damage, act as an environmental factor that triggers the autoimmune response. Therefore, we speculate that pancreatic autoimmunity may contribute to selective \(\beta\)-cells damage in the pathogenesis of diabetes associated with \(\beta\)-thalassemia.

Key-words: thalassemia, diabetes, ICA, GAD, iron.

RÉSUMÉ - Le rôle possible de l’auto-immunité dans la pathogénie du diabète dans la \(\beta\)-thalassémie majeure.

Objectifs : Pour évaluer le rôle possible de l’auto-immunité dans la pathogénie du diabète associé à la \(\beta\)-thalassémie, nous avons étudié 53 sujets \(\beta\)-thalassémiques polytransfusés de longue date dont 12 diabétiques (22,6 %).

Matériel et Méthodes : Pour évaluer l’activation d’une réponse auto-immunatoire, les anticorps anti-cellules d’îlots pancréatiques (ICA), anti-glutamate décarboxylase (GAD), anti-insuline (IAA), et antinucléaires (ANA) ont été mesurés.

Résultats : Neuf patients sur l’effectif total \(\beta\)-thalassémique (16,98 %) étaient ICA positifs, soit une fréquence plus forte que dans la population générale. Cinq (41,6 %) parmi les sujets ICA positifs étaient diabétiques, dont trois C peptide sérique négatifs (<0,21 nmol/l). Le typage HLA classe II dans notre population thalassémique ne révèle pas de différence de distribution allélique par rapport à la population témoin.

Conclusions : Notre étude indique qu’il existe une activation du système immunitaire contre la cellule \(\beta\)-pancréatique dans la \(\beta\)-thalassémie et nous proposons que l’excès de fer tissulaire puisse, par un stress oxydatif, agir comme un facteur environnemental inducteur de la réponse auto-immunatoire. C’est pourquoi nous suggérons que l’auto-immunité pancréatique puisse contribuer à l’atteinte de la cellule \(\beta\)-pancréatique dans la pathogénie des diabètes associés à la \(\beta\)-thalassémie.

Mots-clés : thalassémie, diabète, ICA, GAD, fer.
Diabetes is one of the principal metabolic complications of β-thalassemia. Hypertransfusion therapy has improved both the quality and expectancy of life in patients with β-thalassemia. However, despite chelation therapy, the frequent transfusions cause iron deposition in tissues with consequent organ damage [1]. Many authors have suggested that iron deposition in the pancreas can lead to progressive glucose intolerance [2]. The high incidence of diabetes in idiopathic hemochromatosis [3] and in posttransfusional iron overload [4] supports this hypothesis. Recent reports suggest that early chelation therapy offers protection against the development of diabetes [5, 6].

Insulin resistance and hyperinsulinemia can be detected before the onset of diabetes [7, 8]; in the presence of a normal β-cell response to glucose, hyperinsulinemia may result from reduced hepatic clearance which in turn is probably related to iron overload in the liver [8]. There are many common characteristics in the pathogenesis of diabetes between thalassemia and haemochromatosis [9]. The onset of diabetes seems to be the consequence of insulin resistance associated with a concomitant β-cell defect. Although the mechanisms responsible for the progressive loss of β-cell function have not been elucidated, oxygen free radicals are known to play a role in causing tissue damage and iron is important for free radical production [10].

In β-thalassemia, cases of diabetes are not always characterized by a gradual progression from reduced tolerance to overt diabetes: rather, sometimes, the onset can be acute with rapid ketosis and severe insulin deficiency. Furthermore, analysis of β-cell secretion by means of the glucagon test and the intravenous glucose tolerance test demonstrates that insulin secretion is severely compromised in some diabetic thalassemic patients [11]. In fact, an immunohistochemical and ultrastructural study on the haemochromatotic pancreas of insulin-dependent diabetic patients shows a selective damage of β-cells [12]. On the other hand, in thalassemic patients, glucagon secretion appears normal or even elevated both in basal condition and after stimulation [13].

In the light of these observations, the aim of this work was to find evidence of immune system activation and to evaluate the possible role of autoimmunity in the pathogenesis of diabetes mellitus associated with β-thalassemia major.

■ MATERIALS AND METHODS

Subjects – A cross-sectional study was carried out on a group of thalassemic individuals attending the hematology clinic of the Hospital of S. Giovanni Battista in Turin, Italy. This cohort consisted of 53 individuals (24 males, 29 females, average age 33 ± 8 years, average BMI 21.6 ± 2.7); 12 were diabetic, 11 had impaired fasting glucose (IFG) and 30 were not diabetic. All the patients presented a long-term blood transfusion therapy (27.9 ± 6.7 years) with a chelation therapy of 20 ± 5.3 years. All procedures were carried out in accordance with the Helsinki Declaration.

Metabolic parameters and ferritin – Glucose tolerance was evaluated according to the criteria of the American Diabetes Association [14]. Endogenous insulin secretion was evaluated by measuring the basal plasma C-peptide by RIA: normal values were 0.36-1.17 nmol/l (Biochem Immunosystems, Italy). Insulin dependence, as proposed by Veterans Affairs Cooperative Study, was defined as a basal plasma C-peptide value < 0.21 nmol/l [15].

Serum ferritin was assayed by ELISA (Eurogenetics Italia): normal values were 20-200 ng/ml for men and 15-150 ng/ml for women.

Antibody and hepatitis C virus (HCV) assays – Anti-islet cell antibodies (ICA) were assayed by indirect immunofluorescence on frozen sections of human blood group 0 pancreas with fluorescein isothio-cyanate-conjugated rabbit antibodies [16]. ICA positivity was expressed in Juvenile Diabetes Foundation units (JDF-u), by a standard curve based on the international JDF-u reference sera sample. An ICA ≥ 5 JDF-u was considered as positive. Insulin autoantibodies (IAA) were evaluated in a competitive liquid-phase radiobinding assay [17], in the presence of an excess of unlabelled insulin, using monoclonal insulin as antigen and polyethylene glycol as precipitating agent. The normal range in our laboratory is < 70 nU/ml, with values above the mean ± 3 SD of the control means considered as positive. Glutamic acid decarboxylase (GAD) autoantibodies were also measured by a radioligand assay using human recombinant GAD 65 as antigen (RSR limited - Cardiff, UK); immunocomplexes were precipitated with protein A, according to the method of Schmidli [18]. Our laboratory’s normal range was < 1U/ml. We used an age and sex-matched group of 104 healthy blood donors (40 males, 60 females, average age 39 ± 10 years) as controls for ICA, IAA and GAD assays. We participate regularly in the international proficiency program for ICA, IAA and GAD antibodies of the Research Institute for Children, New Orleans, Louisiana, USA, repeatedly achieving 100% sensitivity and 100% specificity for the three islet autoantibodies.

To evaluate non-organ-specific autoantibodies, antinuclear antibodies (ANA) were evaluated by indirect immunofluorescence using the human epithelioid cell line HEp-2 derived from a laryngeal carcinoma (Anafluor – Incstar - Stillwater, Minnesota, USA). Hepatitis C virus antibodies were detected by ELISA (Ortho Diagnostic System, Johnson & Johnson Company, USA).
**RESULTS**

The fifty-three thalassemic subjects enrolled in this study were divided into three groups according to their glucose tolerance: twelve were diabetic (22.6%), eleven had IFG (20.7%) and thirty were normal. Characteristics of these three groups were presented in Table I. The wide standard deviation of ferritin in our group of patients could not allow to obtain any informations from this parameter.

In the control group of healthy blood donors we did not observe any positivity to pancreatic autoimmunity. ICA positivity was high in thalassemic group (16.98%), significantly higher in comparison to control group (p < 0.000), while no differences were found for IAA and GAD antibodies. In the thalassemic group the presence of autoantibodies in each of the glucose tolerance groups is shown in Table II. When the nine ICA-positive samples were divided according to antibody titer, in seven cases the titer ranged from 5 to 10 JDF-u, 1 case had a titer between 10 and 40 JDF-u and another had a titer > 40 JDF-u. ICA analysis revealed four cases (one IFG and three non-diabetic) in which fluorescence staining was not limited to the endocrine islets but was also distributed among exocrine cells. Morphologically, the fluorescence was distributed regularly throughout the cytoplasm and homogeneously in the interlobular ducts; there were no differences in staining between exocrine acini and endocrine islets. These cases, of uncertain interpretation, were considered ICA-negative. The search for GAD antibodies was negative in all individuals. IAAAs were weakly positive in two individuals: one IFG, one normal. ANAs were positive in four cases: three were also ICA-positive (two with diabetes, one with IFG) and one showed diffuse fluorescence (normal).

The positivity for HCV in the cohort was 73.07%: respectively 90.90% in diabetic, 75.00% in IFG and 64.52% in normal patients. No patient received alpha-interferon treatment.

Some relevant data for the diabetic group are shown in Table III.

We divided the diabetic patients in two group using the criteria of insulin dependence (basal plasma C-peptide value < 0.21 nmol/l) but no significant differences were found for age, sex, BMI, fasting plasma glucose, fructosamine, ferritin between the two groups.

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**Table I. Characteristics of the thalassemic patients divided according to glucose tolerance.**

<table>
<thead>
<tr>
<th></th>
<th>Normal (n. 30)</th>
<th>IFG (n. 11)</th>
<th>Diabetic (n. 12)</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male %</td>
<td>46.7</td>
<td>36.4</td>
<td>50.0</td>
<td>NS</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>33 ± 8</td>
<td>34 ± 8</td>
<td>33 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>22.2 ± 3</td>
<td>20.9 ± 2</td>
<td>21.8 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>FBG (mg/dl)</td>
<td>84 ± 9</td>
<td>115 ± 5*</td>
<td>195 ± 55*</td>
<td>0.000</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>249 ± 27 §</td>
<td>254 ± 20 §</td>
<td>363 ± 87</td>
<td>0.000</td>
</tr>
<tr>
<td>Basal C-peptide (mmol/l)</td>
<td>0.75 ± 0.61 ^</td>
<td>1.11 ± 0.46 ^</td>
<td>0.38 ± 0.38</td>
<td>0.008</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>1473 ± 1357</td>
<td>2418 ± 1594</td>
<td>1576 ± 1853</td>
<td>NS</td>
</tr>
</tbody>
</table>

* p < 0.000 vs Normal; ^ p < 0.002 vs IFG.
§ p < 0.000 vs Diabetic.
Regarding HLA DRβ*01-16 typing, no significant differences in phenotypic frequencies were found between patient and control groups. The phenotypic frequencies of DQβ1*01-04 alleles also showed no significant differences nor did intermediate resolution DQβ*01 typing. In eleven of the 34 patients with alterations of glucose metabolism, the DRβ1*03, DRβ1*04 and DQβ1*0302 (DQ8) alleles were not significantly increased. HLA typing of six of the nine ICA-positive individuals revealed no increase in the frequency of particular alleles.

**DISCUSSION**

In our study group the frequency of diabetes (22.6%) was found to be higher than that reported in the literature, where it varies from 6.5 to 18% [21, 22]. Our finding is also higher than the recently reported value for the Italian population of 4.9% [5]. These discrepancies can be explained by differences in diagnostic criteria and by the fact that the average age of our thalassemic patients (33 ± 8.2 years) is higher than that of the Italian prevalence study (18.1 ± 4.8 years). It follows that our cohort, on long term blood transfusion, has been exposed for a longer period of time to diabetogenic factors.

ICA are present in 70-90% of Type 1 diabetics before or at the onset of disease whereas their presence is rare in the healthy population (0.2-4.1%) with considerable variation depending on the titer and the age group being considered. The study by Betterle reports 0.5% positivity in the Italian population [23].

**TABLE II. Autoantibody positivity among thalasemic patients divided according to glucose tolerance.**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>IFG</th>
<th>Diabetic</th>
<th>P =</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Pn</td>
<td>(P %)</td>
<td>N</td>
</tr>
<tr>
<td>ICA</td>
<td>30</td>
<td>2</td>
<td>6.7</td>
<td>11</td>
</tr>
<tr>
<td>GADAb</td>
<td>21</td>
<td>0</td>
<td>0.0</td>
<td>7</td>
</tr>
<tr>
<td>IAA</td>
<td>21</td>
<td>1</td>
<td>4.76</td>
<td>8</td>
</tr>
<tr>
<td>ANA</td>
<td>28</td>
<td>1</td>
<td>3.57</td>
<td>11</td>
</tr>
</tbody>
</table>

Values represent number of cases (N); number (Pn) and percentage (%) of antibody-positive individuals.

**TABLE III. Main clinical characteristics of diabetic patients subgroup.**

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Sex</th>
<th>BMI</th>
<th>Duration of DM (yrs)</th>
<th>Basal Serum C-peptide (nmol/l)</th>
<th>ICA (JDF-u)</th>
<th>HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>M</td>
<td>27.8</td>
<td>8</td>
<td>1.04</td>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>22.4</td>
<td>10</td>
<td>0.91</td>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>27</td>
<td>M</td>
<td>20.7</td>
<td>5</td>
<td>0.74</td>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>18.6</td>
<td>4</td>
<td>0.73</td>
<td>10</td>
<td>POS</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>23.0</td>
<td>3</td>
<td>0.47</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>31</td>
<td>M</td>
<td>19.5</td>
<td>16</td>
<td>0.16</td>
<td>5</td>
<td>POS</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>21.8</td>
<td>12</td>
<td>0.16</td>
<td>80</td>
<td>POS</td>
</tr>
<tr>
<td>32</td>
<td>M</td>
<td>21.3</td>
<td>15</td>
<td>0.13</td>
<td>5</td>
<td>POS</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>21.9</td>
<td>16</td>
<td>0.05</td>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>19.8</td>
<td>19</td>
<td>0.05</td>
<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>31</td>
<td>M</td>
<td>26.5</td>
<td>12</td>
<td>0.05</td>
<td>5</td>
<td>POS</td>
</tr>
<tr>
<td>38</td>
<td>F</td>
<td>18.8</td>
<td>19</td>
<td>0.05</td>
<td>NEG</td>
<td>POS</td>
</tr>
</tbody>
</table>
In our group ICA positivity was high in the entire cohort (16.98%). In two small groups of children with thalassemia, ICA positivity was not detected [7,24], while Viallet et al. [25] detected ICA in an analogous percentage with respect to the control group [25]. In a group of thalassemic subjects without diabetes Malizia found a low percentage (8.6%) of IAA positivity [26]. In our thalassemic diabetic group, we found a very high frequency of ICA positivity (41.67%). Although ICA bind to molecules found in all islet cells, they are certainly heterogeneous, having multiple and variable target antigens. In our cohort, ICA positivity is associated with complete negativity for GAD and IAA. If the latter negativity may be due to the relatively high average age of the sample [27], this observation suggests that, in our study group, other subtypes of islet antigens contribute to ICA reactivity.

The basal serum C-peptide values give us a more complete clinical picture, the division into three groups according to glucose tolerance highlights the lower values of serum C-peptide in diabetics with respect to individuals with normal glucose tolerance and IFG. Among the 12 diabetic individuals, 6 were found to have C-peptide values under 0.21 nmol/l. In 3 of these cases, there was an association with ICA positivity. In ICA-positive patients with a residual insulin secretion over 0.21 nmol/l, 2 were not yet diabetic, 2 presented with IFG and there was one case of recent abrupt onset. Among diabetic patients, 3 who were ICA-negative still had valid insulin secretion.

The presence of ANA positivity in 2 ICA-positive diabetic individuals highlights a greater frequency of non-organ-specific autoimmune manifestations. The immunofluorescence positivity of pancreatic exocrine tissue is likely due to antibodies directed non exclusively against pancreatic antigens.

HLA Class II typing of 34 thalassemic patients did not reveal significant differences in allelic frequency with respect to the control population. Among the 11 patients with altered glucose metabolism (IFG and diabetes), the DRβ1*03, DRβ1*04 and DQβ1*0302 (DQ8) allele frequency was not significantly increased. In particular, the susceptibility haplotype DQβ1*04/DQβ1*0302 was found in only one of the 34 patients. So far, this patient presents neither metabolic alterations nor pancreatic autoimmunity.

With lacking evidence of a genetic predisposition, we can speculate on a relationship between the activation of autoimmune phenomena in the pancreas and damage induced by iron deposition in that tissue. Iron is considered to be an environmental factor that can be directly connected with the onset of diabetes, as chelation therapy in thalassemia prevents diabetes [6]. Iron deposition through oxidative damage may contribute to β-cell damage with aspecific chronic inflammation and consequent fibrosis [12]. We suggest that membrane damage may lead to the uncovering of antigens and the triggering of autoimmune phenomena and subsequent insulitis. Where specific β-cell autoimmune-mediated injury is present, it may contribute with the chronic inflammatory damage to bring about a situation of total insulin deficiency.

Hypertransfusions are responsible for the high incidence of HCV positivity in the thalassemic population [28]. Allison et al. have proposed that HCV may play a pathogenetic role in the development of diabetes [29], mediated above all by autoimmune mechanisms, although Ando et al. do not confirm this association in diabetic patients with chronic hepatitis C [30]. Therefore, we cannot exclude that the presence of HCV positivity in almost all of the diabetic subjects (91.66%) in our sample may also contribute to the autoimmune manifestations.

In summary, our data show that diabetes in β-thalassemia major, on long-term blood transfusion, has heterogeneous clinical features. In fact, diabetic individuals with prevalent insulin-resistance (i.e., good insulin secretion and mostly negativity of immunological markers) are found alongside diabetics that are prevalently insulinoenopics (i.e., insulin dependence often associated with the presence of immunological markers).

Our data suggest that the chronic islet cell damage occurring during thalassemia may trigger islet autoimmunity in the absence of high risk Type 1 diabetes haplotypes. We would further speculate that iron may represent the environmental triggering factor. Therefore, we suggest that pancreatic autoimmunity, contributing to selective β-cell damage, may be added to several diabetogenic factors responsible for diabetes in β-thalassemia.

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REFERENCES


