COMBINED ANALYSIS OF LONG-TERM ANTI-β-CELL HUMORAL REACTIVITY IN TYPE 1 DIABETES WITH AND WITHOUT THYROID DISEASE

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SUMMARY - The prevalence and levels of islet-cell antibodies (ICA) decrease in the years following diabetes onset but may persist, particularly in patients with concomitant autoimmune disease. The aim of this cross-sectional study was to investigate the frequencies, associations and levels of the major anti-β-cell antibodies in long-standing diabetic patients (median duration: 14 years; range 5-47 years) with and without autoimmune thyroid disease (ATD) in order to consider the specific anti-pancreatic immunologic features associated with endocrine autoimmunity. Both ICA and glutamic acid decarboxylase (GAD) antibody (GAD-A) frequencies were increased in diabetic patients with ATD (38 vs 23 %, \( p = 0.03 \) and 70 vs 21 %, \( p < 10^{-4} \) respectively). Although IA2-A frequency tended to be higher in diabetic patients with ATD, no significant difference was seen (37 vs 26 %, \( p = 0.14 \)). GAD median level was significantly higher in the diabetic group with ATD (15 vs 5 units, \( p < 10^{-4} \)). IA2-A and ICA median levels were similar in both groups. Regardless of the combined analysis performed (ICA/GAD-A, ICA/IA2-A or GAD-A/IA2-A), the prevalence of combined antibody positivity was higher in diabetic patients with than without ATD. In both diabetic populations, ICA and GA-DA were significantly associated (\( p < 10^{-4} \)), and their levels were correlated (\( r = 0.42, p < 10^{-4} \) and \( r = 0.584, p < 10^{-4} \) respectively). No significant correlation was seen between IA2-A levels and either ICA or GAD-A titres. It is concluded that Type 1 diabetes mellitus with ATD is characterised by increased persistent humoral islet-related reactivity, particularly directed towards GAD. Diabetes & Metabolism 1999, 25, 28-33

Key-words: Type 1 diabetes mellitus, long-term duration, GAD antibodies, IA2 antibodies, islet cell antibodies, thyroid autoimmune disease.

RÉSUMÉ - Analyse combinée de la réactivité humorale anti-cellules β persistante au cours du diabète de type 1 avec ou sans mala- die thyroïdienne associée. La fréquence et le taux des ICA diminue avec le temps après la découverte de la maladie mais persiste plus particuliè- rement chez les patients diabétiques porteurs d’une autre maladie au- toimmune. Le but de cette étude transversale était d’étudier l’évolution des principaux anticorps anti-îlots chez les patients diabétiques de type 1 de longue durée (durée médiane : 14 ans, 5-47 ans) porteurs ou non d’une maladie autoimmune thyroïdienne (MAT) afin de mieux connaître les par- ticularités immunologiques humorales anti-pancréatiques associées à l’autoimmunité anti-thyroïdienne au cours du diabète de type 1. A la fois la fréquence des ICA et celle des anti-GAD étaient significativement aug- mentées chez les diabétiques avec MAT comparé à ceux sans (38 versus 23 %, \( p = 0.03 \) et 70 versus 21 %, \( p < 10^{-4} \) respectivement). Bien que la fréquence des anticorps anti-IA2 était augmentée dans le groupe avec MAT, aucune différence significative n’était notée entre les 2 groupes (37 versus 26 %, NS). Les niveaux médians des anticorps anti-GAD étaient significativement plus élevés dans le groupe des diabétiques avec MAT (15 versus 5 units, \( p < 10^{-4} \)). Ceux des anti-IA2 et des ICA étaient semblables dans les 2 groupes. Quelle que soit la combinaison analysée (ICA/GAD, ICA/IA2, GAD-A/IA2-A), la fréquence de la positivité si- multanée à 2 anticorps était plus haute chez les diabétiques avec MAT. Dans les 2 groupes de diabétiques, ICA et anticorps anti-GAD étaient associés (\( p < 10^{-4} \)) et leurs titres étaient corrélés (\( r = 0.42, p < 10^{-4} \) and \( r = 0.584, p < 10^{-4} \) respectivement). Il n’existait par contre pas de corré- lation entre les titres des IA2 et ceux des ICA soit des anti-GAD. En conclusion, le diabète de type 1 associé à une MAT est caractérisé par une persistance plus importante d’une réactivité humorale anti-cellules β, particulièremment vis-à-vis de la GAD. Diabetes & Metabolism 1999, 25, 28-33

Mots-clés : Diabète de type 1, durée> 5 ans, anticorps anti-GAD, anti- corps anti-IA2, ICA, maladie autoimmune thyroïdienne.
Type 1 diabetes mellitus results from the autoimmune destruction of pancreatic β-cells [1]. The autoimmune phenomena associated with Type 1 diabetes include lymphocytic infiltration of pancreatic islets and circulating antibodies to various islet-specific antigens such as glutamic acid decarboxylase (GAD), IA2 and insulin [review in 2]. The prevalence and levels of islet cell antibodies (ICA) have been found to decrease in the years following the onset of diabetes [3, 4]. Type 1 diabetes mellitus can be associated with autoimmune thyroid disease (ATD) [5,6], and it has been reported that ICA and GAD/64 kD protein antibodies persist particularly in some Type 1 diabetic patients with concurrent autoimmune disease [3, 7-11]. Although IA2 is a major antigen of autoimmune β-cell reactivity and contributes to ICA reactivity [12, 13], the long-term frequency of IA2 antibodies [4, 12, 14-16] has not been investigated according to the presence or absence of extrapancreatic autoimmune disease.

The aim of this cross-sectional study was to investigate the frequencies, associations and levels of the major anti-β-cell antibodies (ICA, GAD-A and IA2-A) in long-standing diabetic patients with and without thyroid disease in order to consider the specific anti-pancreatic immunologic features associated with endocrine autoimmunity.

### MATERIALS AND METHODS

**Patients** – Sera of long-term (> 5 years) Type 1 diabetic patients with and without ATD, which had been sent to the serology laboratory of our clinic, were retrospectively tested for anti-pancreatic antibodies independent of the original autoantibody request. The sera were consecutively collected after informed consent of patients, aliquoted and stored at ~30°C. Median diabetes duration was similar in the two groups: 14 years (range 5-41 in the group with diabetes alone and 5-47 in the group with diabetes and ATD). Sera of Type 1 diabetic patients with ATD came from 60 subjects (13 male and 47 female). Median age was 26 years (range: 3-56) at diabetes diagnosis, and 42 years (range: 19-69) at sampling. Diabetes was associated in 39 cases with Graves’ disease and in 31 cases with hypothyroidism secondary to chronic autoimmune thyroiditis. The diagnosis of Graves’ disease was based on the presence of clinical and biological symptoms of hyperthyroidism, homogeneous goiter, diffuse thyroid neotectum uptake at scintigraphy, ophthalmopathy, and detection of thyroid-stimulating antibodies (TSAb). Diagnosis of autoimmune thyroiditis was based on the presence of clinical and biological symptoms of hypothyroidism, thyroglobulin and/or thyroperoxidase antibodies detected by the radioimmunoassay (RIA) method, and multifocal or diffuse areas of thyroid hypochogenicity at ultrasonography, with goiter for Hashimoto’s thyroiditis or small thyroid gland for atrophic thyroiditis.

Sera of patients with Type 1 diabetes alone were obtained from 97 consecutively recruited subjects (51 males, 46 females). Median age was 17 years (range: 1-45) at diabetes diagnosis and 34 years (range: 16-62) at sampling. These patients were carefully selected for the absence of extrapancreatic autoantibodies including thyroperoxidase (TPO) antibodies by the RIA dynotest (Berlin, Germany), anti-gastric parietal cells and/or adrenal antibodies by indirect immunofluorescence on human tissue sections and also for the absence of a family history of other autoimmune disease.

All diabetic patients were diagnosed according to National Data Group criteria [17]. They displayed ketosis and an absolute requirement for insulin at diagnosis, without clinical, biological or morphological evidence of secondary diabetes.

**ICA determination on human pancreas** – ICA were detected by indirect immunofluorescence on sections of human frozen pancreas, as previously described [18]. Sera were also tested after prolonged (18 h) incubation in the presence of aprotinin [19]. Antibody titres were determined by serial dilutions to end-point, using a fluoresceinated anti-human IgG serum (Wellcome, Dartford, Kent, UK). Results are expressed in Juvenile Diabetes Foundation (JDF) units. One JCA-positive internal standard and reference sera from international workshops were included [20]. In the 11th International ICA Workshop, our laboratory had 100% sensitivity and 100% specificity in blinded analysis of test serum samples, with a detection limit of 2.5 JDF units.

**GAD and IA-2 antibodies** – GAD-65 and IA-2 autoantibodies were determined by radioligand assay [13]. Briefly, in vitro transcription and translation reaction of 2 µg of pGEM-3 DNAs, corresponding to GAD-65 and IA-2 specificities, were performed. These cDNAs were kindly provided by E. Bonifacio, San Rafaele, Milan, Italy. After labelling with 1 mCi/ml 35S-methionine (Amersham, Aylesbury, UK) and separation from non-incorporated 35S-methionine by chromatography, 18,000 cpm aliquots of 35S-methionine GAD 65 and 20000 cpm aliquots of 35S-methionine IA-2 were incubated at 4°C with 2 µl of serum. The amount of labelled protein precipitated by patient sera was quantified after IgG isolation with 1 mg protein A Sepharose. In each experiment, the same positive and negative standard sera were included in duplicates. Results are expressed in arbitrary units (U) calculated as follows: U = (test serum cpm – negative standard serum cpm) × 100/positive standard serum cpm – negative standard serum cpm. The intraassay coefficient of variation was less than 5%, and the interassay coefficient of variation less than 10%. The cut-off for antibody positivity was set at mean + 3 SD of antibody levels in 130 normal control sera (8 GAD-U and 5 IA2-U respectively). Control sera were collected from healthy blood donor volunteers without a personal or family history of diabetes and/or other endocrine diseases.

The sensitivity of the GAD assay was 84% and the specificity 100%, based on blind analysis using the samples included in the second GAD Antibody Proficiency Workshop [21].

In a series of 100 consecutively recruited adult Type 1 diabetic patients (> 15 years), GAD-A were found in 85% and IA2-A in 50% at diabetes diagnosis.

**Statistical analysis** – The significance of differences among antibody values between the two groups was determined by the non-parametric Mann-Whitney test. The significance of differences among frequencies was determined by chi-square analysis with Yates’ correction or by Fisher’s exact test, if appropriate. Differences were considered significant for p less than 0.05.

### RESULTS

Frequencies of anti-islet β-cell antibodies in long-standing diabetes (Table I, II and III) – Both ICA and GAD-A frequencies were significantly increased in Type 1 diabetic patients with ATD as compared to those with diabetes alone (38 vs 23%, p = 0.03 and 70 vs 21%, p < 10^-4 respectively). Although IA2-A frequency tended to be higher in diabetic patients with ATD, no significant difference was seen (37 vs 26%, 70 vs 21%, p = 0.03).
In the diabetic group with ATD, the frequencies of ICA, GAD-A and IA2-2 did not differ between patients with Graves’ disease and those with thyroiditis. GAD-A frequency was significantly increased compared to that of ICA and IA2-A (p = 5.10^{-4} for both). In the group with diabetes alone, the frequencies of the three islet β-cell markers were not significantly different.

In both diabetic populations with and without ATD, the frequencies of each antibody did not differ according to sex or age at diagnosis (< 15 years and > 15 years) (Table II).

The frequencies of antipancreatic markers in Type 1 diabetes with and without ATD, as a function of disease duration, are shown in Table III. When patients were assigned to three groups according to disease duration (5-10 years, 11-20 years and > 20 years), a significant increase in GAD-A frequency was found in each duration subgroup of the diabetic population with ATD (Table III). In the Type 1 diabetic group with ATD, the percentage of ICA-positive patients dropped from 67% for the 5- to 10-year interval to 25% for the 10- to 20-year interval; a significant increase, as compared to patients with diabetes alone, was found only for the 5- to 10-year interval (p = 0.03, Table III). IA2-A positivity decreased with time. Although IA2-A frequency tended to be higher in patients with ATD after 10 years of diabetes duration, no significant difference was seen between the diabetic populations.

**Levels of islet-related antibodies in long-standing diabetes** – Compared to patients with diabetes alone, GAD-A median level was significantly higher for the entire diabetic group with ATD (15 units (range: 2-86) vs 5 units (range: 3-92); p < 10^{-4}) and in each subgroup according to disease duration (Fig. 1). The median level of IA2-A was similar in the two groups (3 units (range: 1-74) in diabetes with ATD vs 3 units (range: 1-63) in diabetes without ATD; NS). Similar results were found when only patients positive for GAD-A or IA2-A were analysed. Although median ICA levels tended to be higher in ICA-positive diabetic patients with than without ATD, the difference was not significant (20 units vs 10 units, p = 0.09).

**Combined analysis of the three anti-β-cell markers** (Table IV). Most patients with diabetes alone displayed no markers, as compared to diabetic patients with ATD (57% vs 17%, p < 10^{-4}). Only 14 subjects displayed all 3 markers (6 diabetic patients without and 8 with ATD), 10 of whom had a 5- to 10-year diabetes duration.

Regardless of the combination of two markers analysed (ICA/GAD-A, ICA/IA2-A or GAD-A/IA2-A), the prevalence of combined antibody positivity was higher when diabetes was associated with ATD (Table IV).

In both diabetic populations, ICA and GAD-A were significantly associated (p < 10^{-4} in Type 1 diabetes...
without ATD and p < 0.005 in diabetes with ATD). An association between ICA and IA2-A was found in patients with diabetes alone (p = 0.03), but not in the other populations. No association between GAD-A and IA2-A was noted.

When median GAD-A and IA2-A levels were analysed according to the presence or absence of ICA, median GAD-A levels were significantly increased in ICA-positive patients of both diabetic groups (p < 5.10^{-4} and p < 10^{-4} respectively). Median IA2-A levels were similar in ICA-positive and ICA-negative patients of both diabetic groups. Finally, ICA and GAD-A titres were correlated in both diabetic groups (r = 0.42, p < 10^{-4} and r = 0.584, p < 10^{-4} respectively). In both populations, no significant correlation was noted between IA2-A levels and either ICA or GAD-A levels.

**DISCUSSION**

This cross-sectional study found that persistent diabetes-related reactivity was higher in patients with than without ATD. We previously showed in recent-onset diabetes that GAD-A, 37/40 kD antibodies and the simultaneous positivity of both markers were more frequent in patients with than without ATD [22]. In long-term diabetes, the frequencies of ICA, GAD-A and IA2-A decreased with time in both types of diabetes, and only a few patients displayed both GAD-A and IA2-A, whereas IA2-A were slightly higher and GAD-A frequency was dramatically increased in long-standing diabetes with ATD.

The increased GAD-A frequency in long-term Type 1 diabetes with ATD, and the trend for higher frequencies of other antibodies, may reflect a slower decline of β cells in this type of diabetes [23]. Although we did not investigate patients with delayed insulin dependency, it is noteworthy that patients with ATD

**Table III** Frequencies of antipancreatic markers in type 1 diabetes mellitus with autoimmune thyroid disease (DM with ATD) or without (DM alone) according to the duration of the disease.

<table>
<thead>
<tr>
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<th>5-10 years</th>
<th>10-20 years</th>
<th>&gt; 20 years</th>
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<tbody>
<tr>
<td><strong>ICA</strong></td>
<td></td>
<td></td>
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<tr>
<td>DM with ATD</td>
<td>14/21 (67 %)*</td>
<td>6/24 (25 %)</td>
<td>3/15 (20 %)</td>
</tr>
<tr>
<td>DM alone</td>
<td>10/28 (36 %)</td>
<td>9/46 (20 %)</td>
<td>3/23 (13 %)</td>
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<tr>
<td><strong>GAD-A</strong></td>
<td></td>
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<td></td>
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<tr>
<td>DM with ATD</td>
<td>18/21 (86 %)**</td>
<td>16/24 (67 %)**</td>
<td>8/15 (53 %)****</td>
</tr>
<tr>
<td>DM alone</td>
<td>7/28 (25 %)</td>
<td>11/46 (24 %)</td>
<td>2/23 (8 %)</td>
</tr>
<tr>
<td><strong>IA2-A</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DM with ATD</td>
<td>9/21 (43 %)</td>
<td>9/24 (37 %)</td>
<td>4/15 (27 %)</td>
</tr>
<tr>
<td>DM alone</td>
<td>14/28 (50 %)</td>
<td>8/46 (17 %)</td>
<td>3/23 (13 %)</td>
</tr>
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* p = 0.03, ** p = 10^{-4}, ***p = 5.10^{-4}, ****p = 0.002, versus DM alone.

**Figure 1.** GAD-A levels in type 1 long-term diabetes alone (a) and with autoimmune thyroid disease (b) according to disease duration. Data are presented in box plots (from 25th to 75th percentiles) showing median, 10th and 90th percentiles. Significant differences were shown between diabetes with and without autoimmune thyroid disease in each duration period: whole duration and 5-10 years (p< 0.0001); 11-20 years (p< 0.001); > 20 years (p< 0.01).

**Table IV.** Frequencies of combined antibody positivity in long-term duration type 1 diabetic patients with and without autoimmune thyroid disease (ATD).

<table>
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<th>Type 1 diabetes mellitus</th>
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<tr>
<td></td>
<td>with ATD (n = 60)</td>
<td>without (n = 97)</td>
<td>significance (p)</td>
</tr>
<tr>
<td><strong>No marker</strong></td>
<td>10 (17 %)</td>
<td>55 (57 %)</td>
<td>10^{-4}</td>
</tr>
<tr>
<td><strong>All markers</strong></td>
<td>8 (13 %)</td>
<td>6 (6 %)</td>
<td>0.12</td>
</tr>
<tr>
<td>ICA and GAD-A</td>
<td>21 (35 %)</td>
<td>12 (12 %)</td>
<td>7.10^{-4}</td>
</tr>
<tr>
<td>ICA and IA2-A</td>
<td>20 (33 %)</td>
<td>11 (11 %)</td>
<td>8.10^{-4}</td>
</tr>
<tr>
<td>GAD-A and IA2-A</td>
<td>15 (25 %)</td>
<td>8 (8 %)</td>
<td>0.004</td>
</tr>
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</table>

were older at diabetes onset than those with diabetes alone. Although GAD-A was associated with better β-cell secretion after moderate diabetes duration [24], other studies have suggested that GAD-A persistence is not exclusively explained by a preserved beta-cell function [11, 25]. Thus, 20 years after onset, half of Type 1 diabetic patients with ATD displayed GAD-A.

Several studies have previously investigated GAD-A frequency in long-standing diabetic patients [10, 11, 15, 16, 25-29]. It has been suggested that the clinical characteristics of diabetic populations with and without ATD could influence the positivity of GAD-A [15, 27, 30]. Hermitte et al. [15] have recently shown an increased frequency of GAD-A in diabetic patients with post-pubertal onset. Our patients with ATD, who were older than those without, were more frequently positive for GAD-A. To test this, we evaluated GAD-A frequency with respect to the age of diabetes onset and found no difference. GAD-A frequency has sometimes been reported to be higher in females than in males [15, 27, 30]. Interestingly, female predominance has also been associated with diabetes with ATD. However, we found no significant difference between GAD-A frequencies of male and female patients in our populations with and without ATD.

The maintenance of immune responses to GAD antigen, despite a decline in both endogenous β-cell function and immune responses to other islet cell antigens, could also be interpreted in several other ways. Firstly, a potential link between persistent GAD-A and diabetic autonomic neuropathy, owing to the fact that GAD is abundant in autonomic nervous structures [26], was suggested in a first report [31], but not confirmed in others [25, 26, 28, 32, 33]. Secondly, some authors have suggested that polyclonal activation of the immune system secondary to impairment of non-specific suppressor cell activity [34] is a mechanism leading to excessive production of autoantibodies in organ-specific autoimmune disease [9, 10, 35]. However, this hypothesis could not account for the long-term differential response to various β-cell antigens and was not confirmed in other works [36, 37]. Thirdly, GAD humoral immune response could potentially be stimulated by GAD antigen or cross-reactive epitopes to GAD antigen expressed in tissues other than pancreatic β cells [38]. Thus, the observation that GAD is found in the thyroid gland may explain, at least in part, why GAD reactivity is more frequent at higher levels than IA2 reactivity in diabetes with ATD.

GAD and IA2 antigens have been identified as the major ICA specificities at diabetes onset [12, 13, 41], and GAD accounts predominantly for persistent ICA reactivity [12, 41]. We did not investigate the relationship between ICA and either GAD-A or IA2-A directly by blockade experiments, but our results provide indirect confirmation of previous findings indicative of GAD involvement in the persistent reactivity of ICA. A strong association between ICA and GAD-A was noted in both diabetic groups, regardless of disease duration, and median GAD-A levels were higher in ICA-positive than ICA-negative sera. Finally, a positive correlation was found between ICA and GAD-A levels.

Although GAD contributed to ICA reactivity, most GAD-positive sera in patients with ATD were negative for ICA, confirming previous reports [11, 26, 32, 41]. This discrepancy may reflect the lower sensitivity of immunofluorescence as compared to RIA. As shown in other publications [11, 26] and discussed above, median GAD-A levels were higher in ICA-positive than ICA-negative sera. Furthermore, our ICA-negative/GAD-positive sera were found predominantly in diabetes with the longest duration, when GAD-A level was lowest. Thus, ICA immunofluorescence attributable to GAD was only detectable when GAD-A levels were high. An alternative and/or complementary hypothesis is that GAD epitopes recognized in radiobinding assays are not exposed in substrate tissue during immunofluorescence and therefore not detectable [26]. It has been shown that reacting GAD epitopes were not the same in different GAD-A-positive populations studied [42-45]. Thus, the epitopes recognized could differ according to whether diabetes progresses or not and the slowness of the process. In this context, we showed that the discrepancy between ICA and GAD-A frequencies was only apparent in patients with ATD who later progressed to Type 1 diabetes. It may be concluded that Type 1 diabetes mellitus with ATD is characterised by increased persistent humoral islet-related reactivity, predominantly directed towards GAD.

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