COMBINATION OF AUTOANTIBODY MARKERS AND RISK FOR DEVELOPMENT OF TYPE 1 DIABETES: RESULTS FROM A LARGE FRENCH COHORT OF FAMILY MEMBERS

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on behalf of the GRADI study

SUMMARY - Background: The aims of the study were to characterize the relatives at high risk of progression to diabetes and to determine whether rate of progression to diabetes varied according to age, specific combination of antibodies and genetic markers of susceptibility.

Methods: Family members of type 1 diabetic patients were examined through a large medical network for the presence of specific antibodies to beta cell constituents and high risk DQB1 alleles. Antibodies to insulin, GAD and IA-2 as well as ICA were examined in 4,044 family members recruited in a large prospective family study in the Rhone-Alpes region (the GRADI study). Among them, 3,951 non diabetic first degree relatives have been tested on a median of 2.2 occasions and were followed for up to 16 years.

Results: Presence of antibodies to GAD (3.6%), IA-2 (4.9%), insulin (2.2%) and ICA at titers equal or above 20JDF units (1.1%) were noticed at the first determination and prevalence increased among ICA positive relatives versus ICA negative relatives. All combinations of markers resulted in specificities above 90%. The positive predictive value of antibodies was dependent on the number of positive antibodies. Combination of antibodies to GAD and IA-2 or GAD and IAA had higher predictive values and sensitivities than ICA titers above 20JDF units. In addition, positivity of IAA increased the predictive value but reduced the sensitivity of the screening procedure. Using a combi GAD/IA-2 assay increased the sensitivity of the screening up to 87.8% but reduced the predictive value to 13.8%.

Conclusions: These data confirm in a large French cohort of first degree relatives of type 1 diabetic patients that combinations of antibodies to beta cell constituents can replace ICA in the first screening procedure. We report that combi GAD/IA-2 assay is well suited for screening purposes. However, time to diabetes in antibody positive relatives appears to be more heterogeneous to what has been described. The importance of genetic markers needs further evaluation.

Key-words: diabetes, immunology, prediction.

RÉSUMÉ - Combinaison d’auto-anticorps et risque de développer un diabète de type 1 : résultats d’une large cohorte française d’apparentés du 1er degré.

Contexte : Cette étude a pour buts de tester différents moyens de dépister les apparentés à risque de diabète de type 1 et d’évaluer l’influence de l’âge, des combinaisons de marqueurs sérologiques et génétiques dans la susceptibilité vis-à-vis de la maladie.

Méthodes : Les apparentés du 1er degré de diabétiques de type 1 ont été recrutés à travers un large réseau médical avec la recherche d’auto anticorps vis-à-vis des constituants de la cellule bêta et des allèles DQB1 à haut risque. Les anticorps dirigés contre l’insuline, la GAD et l’IA-2 de même que les ICA ont été recherchés chez 4 044 apparentés dans le cadre d’une étude familiale de la région Rhône-Alpes (étude GRADI). Parmi ceux-ci, 3 951 apparentés non diabétiques ont été suivis jusqu’à un maximum de 16 années et testés à une fréquence moyenne de 2,2 occasions.

Résultats : La présence des anticorps anti-GAD (3,6 %), anti-IA-2 (4,9 %), anti-insuline (2,2 %) et ICA à un titre supérieur ou égal à 20 unités JDF (1,1 %) a été détectée lors du 1er test. La prévalence est plus importante chez les sujets ICA positifs par rapport aux sujets ICA négatifs. Toutes les combinaisons d’auto anticorps ont une spécificité supérieure à 90 %. La valeur prédictive des tests était dépendante du nombre de spécificités d’anticorps. Les combinaisons des anticorps anti-GAD et IA-2 ou anti-GAD et anti-insuline avaient des valeurs prédictives positives et des sensibilités supérieures à un titre d’ICA supérieur ou égal à 20 unités JDF. Une positivité des anti-insuline en plus de gAD et IA-2 augmentait la valeur prédictive positive mais diminuait la sensibilité du dépistage. L’utilisation d’un test combiné GAD/IA-2 a augmenté la sensibilité à 87,8 % mais diminué la valeur prédictive à 13,8 %.

Conclusions : Ces résultats ont confirmé dans une large cohorte d’apparentés de diabétiques de type 1, que la combinaison des auto-anticorps dirigés contre des constituants de la cellule bêta peut remplacer le test ICA dans le cadre du test initial. Toutefois, le délai d’apparition du diabète chez les apparentés anticorps positifs était plus variable que dans la littérature. La place des tests génétiques est en cours d’analyse.

Mots-clés : diabète, immunologie, prédicton.
he burden of the disease, the inadequacy of treatment to prevent chronic complications and the risk of severe hypoglycemia justify the research of preventive strategies for type 1 diabetes. Although studies like DCCT [1] clearly demonstrated the relationships between tight blood glucose control and reduced risk of chronic complications, it is clear that intensive insulin therapy is not applicable to all diabetic patients and that physiological control of blood glucose excursions is difficult to reproduce. A large body of data indicates that type 1 diabetes is of autoimmune etiology [2, 3]. Lymphocytic infiltration of islets containing insulin secreting cells is the hallmark of the autoimmune process that lead to massive and selective beta cell destruction [4]. Surrogates of cell-mediated cytotoxicity have been difficult to establish, probably due to the low amount of pathogenic T cells in the peripheral blood [5]. Although autoantibodies to beta cell constituents are at present the best indicators of ongoing beta cell destruction in humans [6-8], they probably do not contribute to pathogenesis based on extensive studies in animal models and in development of the disease despite B lymphocyte deficiency [9]. Recent pathological studies in diabetic patients of recent onset revealed a good correlation between insulinis and presence of multiple autoantibodies [10]. The quest for prevention of diabetes has been made feasible by the unraveling of the immunogenetics of the disease and the identification of at risk subjects by immunological markers. The characterization and cloning of several candidate autoantigens have facilitated the identification of high risk individuals before onset of clinical diabetes. However, epidemiological studies conducted in several countries have revealed that type 1 diabetes is an heterogeneous disease thus emphasizing the need to conduct studies in large and representative cohorts. With increasing age at clinical onset, signs of diabetes become less pronounced and prevalence of predictive markers appears to vary [11]. These variations lead to differences in the rate of beta cell loss or impairment [12]. In the present study, we investigated the sensitivity, specificity and predictive value of different combinations of antibody markers in the initial screening and the performance of a combi GAD/IA-2 assay.

RESEARCH DESIGN AND METHODS

Subjects

The Lyons’ [13] and GRADI (Groupe Rhône-Alpes pour le dépistage du diabète insulinodépendant) [14] studies recruited parents, siblings and offsprings of patients with type 1 diabetes who were diagnosed before 25 years of age in the Rhone-Alpes region in France. Between January 1982 and December 2000, a total of 1,517 type 1 diabetic patients and their family members were enrolled on a voluntary basis by physicians participating in the registry. Recruitment for the Lyons’ family study took place between 1982 and 1992 and recruitment for the GRADI study took place from 1992 onward. By December 2000, 4044 first-degree relatives were reported. Among them, 3973 non diabetic first-degree relatives were tested on a median of 2.2 occasions (range 1 to 16) with 7657 serum samples. Type 1 diabetes was diagnosed according to the criteria of the national Diabetes data group on glucose concentrations and the need for insulin at onset [15]. Follow-up of nondiabetic subjects was performed by each participating center that referred to the coordinating center each new case of diabetes. The clinical data were compared to the antibody data file concerning newly diagnosed type 1 patients which was centralized in Lyon.

Assays

Antibodies

Antibody immunoassays to in vitro translated [35S] glutamate decarboxylase (GAD65) and [35S] protein tyrosine phosphatase like antigen (IA-2ic) were measured as previously described [16]. Plasmid cDNA encoding for full length human GAD65 cloned into the pcDNAII-vector (Dr A Lernmark, Seattle Washington), cDNA encoding for the cytoplasmic part of IA-2 (aa603-979) cloned into the pSP64poly(A) vector (Dr MC Christie London UK) were amplified in Escherichia Coli XL1 blue. Anti-GAD65 and anti-IA-2 Abs were measured in duplicate using in vitro transcribed and translated recombinant human protein and precipitation with protein A- sepharose as previously reported [17]. After washing, bound counts were expressed as arbitrary units using a pool of positive sera with an arbitrary value of 1 for GAD and 100 for IA-2. Combi GAD-IA-2 test consisted in a single step immunoprecipitation of both radiolabeled proteins in 96 well plates using 5µl of serum instead of 2µl in the usual GAD and IA-2 assays. The cutoff index levels for combi-test, anti-GAD65 and anti-IA-2 Ab assays were determined using 200 healthy control subjects. Sera with indexes above index plus 3 times SD were regarded as positive. Anti-GAD65 and anti-IA-2 Ab assays were assessed during international Diabetes proficiency workshops and obtained the following results at the last combined islet autoantibody workshop: GAD assay, sensitivity 78%, specificity 95% and IA-2 assay, sensitivity 70%, specificity 98%. Insulin autoantibodies were detected by conventional radioligand using mono-iodinated human insulin. Islet cell cytoplasmic antibodies (ICA) titers were converted to Juvenile Diabetes Foundation units using a JDF standard reference serum on frozen human pancreatic sections from a blood O group donor.
Genotyping

During the Lyon’s study, DR and DQ alleles were determined by serology in 1347 (34%) relatives. More recently, DQ alleles were determined in the GRADI study by molecular biology in 598 (15%) relatives. DNA was extracted from EDTA blood. The DQB1 genes were amplified by polymerase chain reaction (PCR) and hybridized to a panel of allele specific probes. Altogether, determination of the high susceptibility alleles DQ2-DQB1*0201 and DQ8-DQB1*0302 was obtained in 1945 relatives. Previous haplotype studies performed in 537 type 1 diabetic patients and 2047 normal individuals revealed in our population a relative risk of 2.62 and 5.07 for DQB1*0201 and DQB1*0302 alleles respectively.

Statistical analysis

Differences in prevalences were tested by X² test with Yates’ correction or Fischer’s exact test, when appropriate. Comparisons of the survival rates according to presence of antibodies were performed using the log-rank test. Differences were considered statistically significant at p < 0.05.

RESULTS

Prevalence of serological markers

None of the 200 control subjects had a positive result for more than one of antibodies, indicating that presence of two or more antibody specificities was highly specific. Of 3951 initial screening samples, 79 (2%) were ICA positive including 44 with titers equal or above 20 JDF units and 3872 were ICA negative (98%). Among the 79 ICA positive relatives, 51 (64%, 1.3% of total relatives screened) were GAD positive, 34 (43%, 0.8% of total relatives) were IAA positive and 29 (36.7%, 0.7% of total relatives) were IA-2 positive. Among the individuals positive for GAD and/or IA-2, 45% (88 of 195) were IAA positive and 29% (37 of 128) of IAA positive relatives were also ICA positive. The levels of GAD antibodies of ICA positive relatives (n = 51, median 0.58 units, range 0-1.2) were significantly higher than the levels of ICA negative relatives (n = 72, median 0.36 units, range 0-2, median test: p < 0.001). The levels of IA-2 antibodies of ICA positive relatives (n = 29, median 73.9 units, range 0.01-150) were significantly higher than the levels of ICA negative relatives (n = 43, median 11.8 units, range 0-87, median test: p < 0.0001). The titers of IAA in ICA positive were not statistically different from those of ICA negative relatives (3.06 vs 2.09 units, p = 0.18). The median titers of ICA were 53.6 and 9.9 JDF units for GAD and/or IA-2 positive relatives versus GAD and IA-2 negative subjects (p < 0.0001). Within antibody positive relatives, siblings had significantly higher prevalence of IAA than parents (6.62% vs 2.65%, p < 0.05). Prevalence of ICA was higher in siblings than in parents (3.14% vs 1.32%, p < 0.05) with no significant difference in ICA titers. Frequencies of antibodies according to age at entry in the study are presented in Table 1. Prevalence of IAA were more frequent in relatives of younger age but no significant difference could be noticed for the other antibody specificities. The combi GAD-IA-2 test was performed in the initial serum sample of 1550 relatives. Among them, 145 (9.4%) were positive with median test value of 18.2 units range 2-105.

Predictive value of antibody combinations

Among the 33 relatives (0.83%) who developed diabetes during follow-up, 13 (39.4%) had positive results at first determination for IAA, 22 (66%) for GAD65, 12 (36.3%) for IA-2 and 23 (69.6%) for one or more of these antibodies. Among them, 16 (48.5%) had positive results for ICA. Among the 10 relatives who developed diabetes despite the absence of antibody positivity at the first determination, 8 (80%) relatives converted to antibody positivity during follow-up after a mean period of 49 months. Mean (range) age of these 8 subjects was 11.5 (4-33). The first antibody to occur was GAD in 3 subjects, IA-2 in one subject, and more than two antibodies simultaneously in 4 subjects. Only two relatives developed diabetes despite the absence of specific autoantibodies. These were 2 sisters of respectively 19 and 9 years old at onset sharing the DR3-DQ2 haplotype with their diabetic proband. These two relatives were tested at three occasions within 5 years before diabetes onset and were negative for ICA, IAA, IA-2 and GAD65 antibodies. When the combi GAD/IA-2 test was used, 29 of the 33 relatives who subsequently developed diabetes were positive. In addition, 6 of the 10 patients with negative GAD or IA-2 tests were positive at the first determination. Using survival analysis to allow for the different lengths of follow-up, presence of combination of antibodies in the first serum sample was associated with an increased risk of diabetes as shown in Figure 1. Relatives with both GAD and IA-2 or more positive antibodies had a significantly higher

Table I. Autoantibodies in first degree relatives according to age.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Age at entry (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10</td>
</tr>
<tr>
<td>GAD65</td>
<td>10 (2.8 %)</td>
</tr>
<tr>
<td>IA2</td>
<td>5 (1.4 %)</td>
</tr>
<tr>
<td>IAA</td>
<td>18 (5.2 %)</td>
</tr>
</tbody>
</table>
risk compared with those with only GAD positivity (p<0.01). As shown in Table II, the combination of GAD and IA2 antibodies had higher predictive value and sensitivity for future development of diabetes to positive ICA at titer equal or above 20 JDF units since 19/42 (45.2%) vs 17/44 (38.6%) relatives became diabetic during follow-up. Results from the combi GAD/IA-2 test was available for 1858 relatives and in 1550 first serum samples. When dividing the combi positive relatives into subjects with low, moderate and high titers, we observed a relationship between combi titers and prevalence of diabetes as shown in Figure 2. Estimates of the sensitivity, specificity and accuracy of the different combinations of risk markers as listed in Table II revealed that this assay had the best sensitivity (87.8%) and that this performance was optimized in siblings up to 91.6%. However, the positive predictive value of the combi test was poor indicating that additional controls are needed in combi positive individuals.

DQ alleles and risk of progression to diabetes

Combination of genetic markers and antibodies further increased the sensitivity of the screening giving a 70% risk of diabetes within 8 years.

The distribution of high risk DQ alleles is shown in Table III. As expected the distribution of high risk haplotypes was increased in the relatives due to the number of shared haplotypes with their diabetic probands. More importantly, there was no preferential association between the presence of ICA, antibodies to GAD, IA-2, or IAA and DQ8/DQB1*0302 or DQ2/DQB1*0201. When the analysis was conducted in siblings, the predictive value of the combination of GAD and IA-2 antibodies dramatically increased, since 100% of the antibody positive siblings carrying the DQ8/DQB1*0302 (n = 9) or the heterozygous for DQ8/DQB1*0302 and DQ2-DQB1*0201 haplotypes (n = 4) developed diabetes within 14 years. This indicates that combination of immunologic and genetic markers may be useful.

**DISCUSSION**

Clinical onset of type 1 diabetes is associated with genetic and autoimmune markers for which age dependent patterns and ethnic background have been described [2]. The identification of potential future cases of type 1 diabetes for prevention trials is laborious. At the moment, the best surrogate markers of prediabetes are the presence of autoantibodies against beta cell antigens. In family members of patients with type 1 diabetes, multiple diabetes related antibodies are associated with a high risk of diabetes within a few years [18, 19]. In the present study, we observed that combination of antibodies to GAD and IA-2 gave the best predictive value. The additional benefit of IAA in the screening was not observed. Several explanations may explain these differences. Age at entry in the study may differ from other family studies. Another difference might result from the differences in the genetic background.
Results of our combi GAD/IA-2 test are very promising and indicate that this test is adapted for an initial screening of large number of individuals due to a very good sensibility since 29/30 of the relatives who subsequently developed diabetes were detected with the first serum sample. However, due to the poor predictive value of the combi test, further analysis of antibody specificities are clearly needed to improve the predictive value of the test procedure. In line with what has been shown in a previous study [20], we observed a correlation between the combi titers and the predictive value of the test. We interpret the improvement over the separate antibody determination as the consequences of differences in assay sensitivity.

### Table II. Sensitivity, specificity, positive predictive value and accuracy of antibody markers for predicting type 1 diabetes in first degree relatives.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Sensitivity</th>
<th>PPV</th>
<th>Specificity</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICA&gt;20u JDF</td>
<td>51.5</td>
<td>38.6</td>
<td>99.3</td>
<td>49.9</td>
</tr>
<tr>
<td>Siblings (31)</td>
<td>57.7</td>
<td>50</td>
<td>99.0</td>
<td>48.4</td>
</tr>
<tr>
<td>GAD and IA2</td>
<td>60</td>
<td>47.6</td>
<td>99.4</td>
<td>50</td>
</tr>
<tr>
<td>Siblings (28)</td>
<td>62</td>
<td>53.6</td>
<td>99.2</td>
<td>50.1</td>
</tr>
<tr>
<td>GAD and IAA</td>
<td>57.5</td>
<td>47.5</td>
<td>99.4</td>
<td>50</td>
</tr>
<tr>
<td>Siblings (31)</td>
<td>66.6</td>
<td>50.1</td>
<td>99.1</td>
<td>51.6</td>
</tr>
<tr>
<td>GAD-IA2-IAA</td>
<td>42.4</td>
<td>66</td>
<td>99.8</td>
<td>50</td>
</tr>
<tr>
<td>Siblings (15)</td>
<td>50</td>
<td>80</td>
<td>99.8</td>
<td>50</td>
</tr>
<tr>
<td>Combi test</td>
<td>87.8</td>
<td>13.8</td>
<td>90.1</td>
<td>48.2</td>
</tr>
<tr>
<td>Siblings (101)</td>
<td>91.6</td>
<td>21.8</td>
<td>89.9</td>
<td>48.7</td>
</tr>
</tbody>
</table>
due to a two-fold increase in the amount of serum used for the assay in the combi test. We confirm previous observations indicating that antibodies to GAD and IA-2 were the best markers to use as a first line screening strategy while IAA and ICA could be used as a second step [21]. However, it is still difficult to extrapolate our results to other populations especially to relatives of younger age due to the higher prevalence of IAA in these subjects.

Several prospective family studies addressed the question of the time course of the immunological events that lead to beta cell loss. Prospective studies in children of diabetic mothers such as the BABY-DIAB study [22] or DAISY [23] clearly indicated that autoantibodies to insulin may occur as soon as 2 years of age. A systematic follow-up of newborns with high risk DBQB1 alleles in Finland, also revealed early antibody positivity [24]. Nevertheless, serum converters in our study were not as frequent as previously described when using the combi-test, indicating that autoimmunity to beta cells were already active at entry into the study. However, interactions with environmental factors are certainly complex and antibody negativity during childhood does not exclude the possibility of future diabetes in genetically predisposed individuals. On the other hand, a small group of relatives with antibodies to GAD, IA-2 and IAA over more than 10 years did not develop diabetes. It is not known whether these relatives are definitively protected from diabetes or whether they will develop late onset type 1 diabetes. Beside evaluating the degree and type of islet cell infiltration by pancreatic biopsies [10] or T cell imaging, additional peripheral markers are lacking and longer periods of follow-up are needed to solve this important issue. The recent observation of a woman developing type 1 diabetes despite complete B lymphocyte deficiency but specific anti-GAD T lymphocytes emphasized on the importance of cellular immunity in human diabetes [25]. The HLA system and the DQ region in particular significantly contribute to the genetic predisposition of type 1 diabetes [26]. Several associations have been described in newly diagnosed patients between antibodies to GAD, IA-2, IAA and specific DR or DQ alleles [27, 28]. Here, we observed a comparable association with high risk alleles. However, DQ risk allele determination greatly improved the predictive value of antibody markers. This may be interpreted by the role of DR and DQ molecules in T cell activation. However, it is interesting to note that the determination of DBQB1 risk alleles did not influence the susceptibility to type 1 diabetes of antibody positive schoolchildren [29]. In this regard, epidemiological studies in the general population after newborn screening of HLA markers will be determinant to determine whether the results obtained in first degree relatives can be extrapolated to the general population [30, 31].

Altogether, our study confirms to a certain extend the usefulness of a combinatorial approach of antibody screening of genetically at risk subjects. At present, applicability of this approach to the general population needs confirmation. The availability of simple and inexpensive genetic tests may facilitate the initial screening and increase the predictive value of the immunological markers. Improving the predictive value of the initial test without impairment of metabolic beta cell function is a critical issue to increase the chances of success of early prevention trials.

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REFERENCES


| TABLE III. Genetic background of autoantibody positive relatives as determined by serological or molecular biology typing. |
|-----------------|--------|--------|--------|--------|
| DQ allele       | Total  | ICA    | GAD    | IA-2   | IAA    |
| Serological Typing (1347) |       |        |        |        |        |
| DQ8             | 514    | 18     | 27     | 20     | 45     |
|                 | 38.1%  |        |        |        |        |
| DQ2             | 808    | 22     | 37     | 18     | 49     |
|                 | 59.9%  |        |        |        |        |
| DQ8/2           | 167    | 7      | 14     | 10     | 21     |
|                 | 12.4%  |        |        |        |        |
| Molecular Biology (598) |       |        |        |        |        |
| DQB1*302        | 255    | 10     | 14     | 10     | 13     |
|                 | 42.6%  |        |        |        |        |
| DQB1*201        | 305    | 6      | 9      | 7      | 16     |
|                 | 51%    |        |        |        |        |
| DQB1*201/302    | 80     | 5      | 7      | 5      | 8      |
|                 | 13.4%  |        |        |        |        |
COMBINATION OF AUTOANTIBODIES AND PREDICTION OF TYPE 1 DIABETES


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