Identification of tyrosine phosphatase 2 (256-760) construct as a new, sensitive marker for the detection of islet autoimmunity in type 2 diabetic patients (NIRAD Study 2)

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Running Title: IA-2 immunoreactivity in LADA patients

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ABSTRACT

Objective: The presence of autoantibodies to islet antigens GAD and/or IA-2 in type 2 diabetic patients identifies those subjects (LADA) at high risk to develop insulin dependency. Aim of this study was to dissect humoral anti-IA-2 immune response in Caucasian LADA patients, identifying the most sensitive construct to evaluate IA-2 immunoreactivity and comparing LADA IA-2 epitope specificities to those found in type 1 diabetes.

Research Design and Methods: Patients: 177 LADA and 978 type 2 diabetic patients with different disease duration, collected in a nationwide italian survey (NIRAD Study) aimed at assessing prevalence and characteristics of autoimmune diabetes in type 2 diabetic patients; 106 newly-diagnosed type 1 diabetic patients (53 children, 53 adults). Method: analysis by radioimmunoassay of humoral immunoreactivity to 7 IA-2 constructs [IA-2PTP(687-979), IA-2(761-964), IA-2(256-760), IA-2JM(601-630), IA-2IC(605-979), IA-2BDC(256-556:630-979), IA-2FL(1-979)].

Results: IA-2(256-760) fragment was identified as the marker with the highest sensitivity for detection of humoral IA-2 immunoreactivity in LADA, identifying IA-2 autoantibodies in almost 30% of GADA positive LADA and in 3.4% of GADA negative type 2 diabetic patients. LADA IA-2(256-760)A positivity was associated to an increased frequency of autoimmune diabetes HLA susceptible genotypes and to a higher risk for developing thyroid autoimmunity compared to autoantibody negative type 2 diabetic patients. At disease diagnosis, adult-onset type 1 diabetic and LADA patients showed a lower IA-2 C-terminal immunoreactivity compared to childhood-onset type 1 diabetic patients.

Conclusions: IA-2 immunoreactivity in LADA patients was so far underestimated and IA-2(256-760) autoantibody detection may represent a novel diagnostic tool for the identification of islet autoimmunity in these patients.

KEYWORDS. IA-2 autoantibodies, epitopes, LADA, type 1 diabetes, type 2 diabetes
Autoimmune diabetes is characterized by the presence of circulating autoantibodies directed against several islet proteins, including insulin, glutamic acid decarboxylase (GADA) and tyrosine phosphatase 2 (IA-2A) (1). Among these diabetes-related autoantibodies, only GADA are not age-dependent, thus representing a sensitive marker for the study of childhood as well as of adult autoimmune diabetes (2). In addition, GADA identify that subset of patients with type 2 diabetes mellitus, who initially do not require insulin treatment, but that may develop insulin dependency within a few years after diagnosis (3-5). This form of diabetes has been variably indicated (6-9). Throughout this manuscript, we will refer to it as Latent Autoimmune Diabetes in Adults (LADA) (7). The study of GAD65 antigenic target domains in Caucasian subjects with type 2 diabetes, demonstrated that the presence of autoantibodies directed against the GAD COOH-terminal epitopes is strongly associated with a type 1 diabetes phenotype (10,11). However, in Japanese LADA subjects (12), a relationship between N-terminal binding of GADA and time to progression to insulin requirement was reported, suggesting that genetic background may influence GAD epitope specific immunoreactivities in LADA patients. Despite GADA were shown to be the most sensitive marker to identify autoimmune diabetes in adult type 2 diabetes (3-5), they are not the sole islet-related autoantibody detected in these patients. IA-2A were found in 2.2% of type 2 diabetic patients and their presence, in addition to GADA, increases the relative risk of these patients to require insulin therapy (13). To date, IA-2A are detected using sensitive and specific radioimmunoassays, differing in terms of which of the radiolabeled IA-2 constructs is utilized, usually chosen among the full-length IA-2FL (a.a.1-979), the truncated NH2 terminally spliced IA-2 variant lacking exon 13 IA-2BDC (a.a.256-556:630-979) and the intracytoplasmic IA-2IC (a.a.605-979) construct (14,15). A recent study (16) showed that cytoplasmic IA-2IC (605-979) is the construct detecting IA-2A with the highest sensitivity both in newly diagnosed type 1 diabetic and prediabetic patients, thus suggesting that such construct should be used in type 1 diabetes-related autoantibody screening studies. In the same study (16), it was also demonstrated that IA-2IC (605-979) immunoreactivity did not account for the whole anti-IA-2 humoral immune response in type 1 diabetes, as other IA-2 constructs investigated showed additional immunoreactivities otherwise undetected by the IA-2IC (605-979) construct. No data are currently available of which, among IA-2FL (a.a.1-979), IA-2BDC (a.a.256-556:630-979) and IA-2IC (605-979), has the highest sensitivity and specificicity for detecting IA-2A in LADA patients and whether other IA-2 constructs may identify additional immunoreactivities in the sera of these patients. It was demonstrated an association between IA-2 epitope specificities and age of onset in recent onset Japanese type 1 diabetic and long-standing type 2 diabetic patients (17). To our knowledge, no information is at present available on IA-2 epitope specificity in Caucasian LADA patients. On the basis of these and previous considerations, the aim of this study was to dissect the humoral autoimmune response to IA-2 in LADA patients of Caucasian origin. More specifically, we aimed at identifying the IA-2 construct able to detect IA-2 immunoreactivity with the highest sensitivity in Caucasian LADA patients and at establishing the frequency of autoimmune response against such construct in a large cohort of patients with type 2 diabetes. Finally, the epitope pattern of IA-2 immunoreactivity of LADA patients at
disease diagnosis was compared to that of type 1 diabetic patients.

**RESEARCH DESIGN AND METHODS**

During NIRAD Study, a nationwide survey supported by the Società Italiana di Diabetologia aimed at assessing prevalence and characteristics of adult autoimmune diabetes in Italy in patients attending diabetes clinics with a clinical diagnosis of type 2 diabetes, 4250 type 2 diabetes patients with a disease duration <5 years, with no insulin requirement and no evidence of ketosis for at least 6 months from diagnosis, were enrolled and screened for GAD and IA-2\textsubscript{IC(605-979)} autoantibodies. One hundred ninety-one (4.5%) and 39 (0.9%) type 2 diabetic patients were found GADA and IA-2\textsubscript{IC(605-979)}A positive, respectively (18). Of the 4250 NIRAD patients, 1020 were collected at the “Sapienza” University of Rome (572M, 406F; age range 21.1-85.8 years; median 57.6 years; mean disease duration 21.9±18.0 months). Forty-two (4.1%) and 13 (1.3%) of these 1020 patients were GADA and IA-2\textsubscript{IC(605-979)}A positive, respectively, whereas 978 patients were GADA and IA-2\textsubscript{IC(605-979)}A negative.

In the first step of the present study, we aimed at evaluating which, among seven IA-2 constructs had the highest sensitivity to detect humoral IA-2 immunoreactivity in Italian LADA patients. To this end, we analyzed all GADA positive LADA patient sera available from the NIRAD Study (n=177, 92M, 85F; age range 23.7-86.6 years; median 54.3 years; mean disease duration 24.0±18.8 months).

In the second step of the study, the immunoreactivity of the IA-2\textsubscript{(256-760)} construct, identified as the marker with the highest sensitivity for the detection of IA-2 autoantibodies in the group of GADA positive LADA patients, was analyzed in all above mentioned 1020 samples (978 GADA and IA-2\textsubscript{IC}A negative + 42 GADA positive type 2 diabetic patients) screened at the “Sapienza” University of Rome during the NIRAD Study.

In addition, among the above mentioned 1020 patients, those found GADA or IA-2\textsubscript{(256-760)}A positive were tested for Thyroid Peroxidase autoantibodies (TPO-A) and, when possible, typed for HLA DRB1-DQB1 polymorphisms; 114 (for TPO-A) and 64 (for HLA polymorphisms) GADA negative/IA-2\textsubscript{(256-760)}A negative type 2 diabetic patients of comparable age and sex were analyzed as well.

Finally, in another set of experiments, we compared the IA-2 epitope target domains recognized at disease diagnosis by LADA, adult-onset and childhood-onset type 1 diabetic sera. To this end, by utilizing the 7 constructs represented in Figure 1, the following groups of patients at disease diagnosis were studied:

- 33 GADA positive LADA (22M, 11F, median age 44.8 years, age range 25-73 years);
- 53 GADA positive type 1 diabetic children (28M, 25F, median age 7.6 years, age range 2-12 years);
- 53 GADA positive type 1 diabetic adults (29M, 24F, median age 29.0 years, age range 18-48 years).

The 106 type 1 diabetic patients are part of 537 newly-diagnosed patients recruited between 1990 and 2004 at “Sapienza” University of Rome. The 53 type 1 diabetic children represent all GADA-positive patients aged <12 years consecutively recruited between 2001 and 2004, whereas the 53 type 1 diabetic adults represent all GADA-positive patients aged >18 years available in this cohort of patients. All type 1 and type 2 diabetic patients analyzed in the present study were diagnosed according to American Diabetes Association criteria (19).

**IA-2 constructs utilized in the study.** cDNAs encoding IA-2\textsubscript{(761-964)} were amplified by PCR from full-length IA-2 using 5’ ACCATGAGCGATTACATCAACGCACCCGCA-3’ and 5’ TCAGCAGCTACAGTCAGAATT-3’
primers. Ligation and transformation of fresh PCR products were performed as previously described for IA-2(256-760) construct (16). After purification, insert were sequenced in both directions using ABI 377 sequencer (Applied Biosystems Inc, Foster City, CA, USA). No deletions or truncations were found in this IA-2 construct. IA-2BDC was prepared as reported (20) and kindly provided, as the IA-2 (256-760) construct, by Dr G.S. Eisenbarth (University of Colorado, Denver, USA). IA-2FL(1-979), IA-2IC(605-979), IA-2JM(601-630) and IA-2PTP(687-979) cDNAs were kindly provided by Dr.E. Bonifacio (San Raffaele Scientific Institute, Milan, Italy).

AUTOANTIBODY MEASUREMENTS

**IA-2A detection.** Each IA-2 fragment was in vitro transcribed and translated in the presence of [\(^{35}\)S]-methionine (NEN™) using the TNT-coupled rabbit reticulocyte system (Promega, Madison, USA) with Sp6 RNA polymerase. Autoantibodies against each single IA-2 construct were detected by a slightly modified quantitative radioimmunoprecipitation assay (13) using 50% protein A-Sepharose to separate free [\(^{35}\)S]-methionine from antibody-bound labeled products. Results were expressed as an index defined as follows: (sample cpm – negative standard control cpm)/(positive standard control cpm – negative standard control cpm). Positive autoantibody indexes, defined as values above 99th percentile of 211 healthy control sera (102F, 109M, median 27 years; range 3-77 years) were 0.094, 0.010, 0.073, 0.064, 0.138, 0.049 and 0.094 for IA-2FL, IA-2IC, IA-2BDC, IA-2(256-760), IA-2JM, IA-2PTP and IA-2(761-964), respectively. Intra- and inter-assay coefficients of variation were 5.7% and 10.3% for IA-2(256-760), 6.2% and 10.9% for IA-2JM, 5.8% and 10.0% for IA-2(761-964), 5.1% and 9.2% for IA-2PTP, 5.6% and 7.6% for IA-2BDC, 4.8% and 9.9% for IA-2IC, 5.0% and 8.5% for IA-2FL. In this study, IA-2(761-964) and IA-2PTP(687-979) constructs were utilized to evaluate IA-2 C-terminal immunoreactivities, whereas IA-2JM(601-630) and IA-2(256-760) constructs were used to detect IA-2 middle-domain immunoreactivities. The remaining 3 IA-2 fragments (IA-2FL, IA-2IC, IA-2BDC) are those most commonly utilized to evaluate IA-2 immunoreactivity in diabetic-related screenings. IA-2A in the NIRAD Study were detected using IA-2IC (19). IA-2ICA assay obtained 72% sensitivity and 99% specificity at 2007 4th assay proficiency evaluation (lab155) of the Diabetes Antibody Standardization Program (DASP).

**IA-2A competition experiments.** To evaluate the specificity of antibody binding to [\(^{35}\)S]-IA-2(256-760) in comparison to [\(^{35}\)S]-IA-2IC construct, the mutual inhibition activity of different concentrations of unlabeled IA-2IC and/or IA-2(256-760) fragments were tested. The unlabeled fragments were prepared by in vitro transcription and translation as described above, but replacing [\(^{35}\)S]-methionine with unlabeled methionine in the aminoacid mixture. Unlabeled recombinant IA-2(256-760) and/or IA-2IC (0.5-1-2 and 4 folds the amount of [\(^{35}\)S]-labeled protein) were added to each tube and incubated overnight at 4°C with patient sera. The following day, after incubation with radiolabelled [\(^{35}\)S]-IA-2(256-760) or [\(^{35}\)S]-IA-2IC proteins, samples were processed with the usual radioimmunoprecipitation assay. In competition experiments, 10 type 2 diabetic patients sera found IA-2(256-760)A positive/IA-2ICA negative (n=6), IA-2(256-760)A negative/IA-2ICA positive (n=2) or IA-2(256-760)A positive/IA-2ICA positive (n=2), respectively, were analyzed.

**GADA detection.** GADA in type 1 diabetic and LADA sera were detected by a slightly modified fluid-phase radioimmunoprecipitation assay (21) utilizing a human recombinant full-length GAD65 construct kindly furnished by Dr. Å. Lernmark (Dept of Medicine, University of Washington, Seattle, WA, USA). GADA assay obtained
IA-2 immunoreactivity in LADA patients

80% sensitivity and 98% specificity at 2007 4th DASP (lab 155).

*Thyroid Peroxidase autoantibodies (TPO-A).* TPO-A were measured by a commercial radioimmunoassay kit (cod. 14752, Adaltis, Italy).

**HLA class II genotyping**

Genomic DNA was extracted using the salting-out method. High-resolution typing for DRB1*04 and DQB1 loci was performed using allele group-specific amplifications. A reverse line blot method, kindly provided by H.A. Erlich and T. Bugawan (Roche Molecular System, Alameda, CA), was used as the detection system (22). HLA genotypes were classified in three risk categories (high, moderate and low), based on the absolute risk values for type 1 diabetes previously estimated in the Italian population (23).

**Statistical analysis.** Statistical analyses were performed using SPSS software, version 13 (SPSS, Illinois, USA). Frequency differences were calculated by χ² test with Yates’ correction, whenever appropriate, or by Fisher’s exact test. A p value < 0.05 was considered significant.

**RESULTS**

**IA-2 epitope immunoreactivities in GADA-positive LADA patients.** Of 177 LADA patients, 59 (33.3%) were positive for at least one of the 7 IA-2 constructs analyzed. IA-2_{256-760} was the fragment showing the highest sensitivity, identifying IA-2 immunoreactivity in 29.4% (52/177) LADA sera (Table 1), significantly more frequently than IA-2_{PTP} (11.3%, 20/177, p < 0.0001), IA-2_{(761-964)} (9.6%, 17/177, p < 0.0001), IA-2_{JM} (9.6%, 17/177, p < 0.0001), IA-2_{FL} (18.1%, 32/177, p = 0.017), IA-2_{BDC} (13.0%, 23/177, p = 0.0002) and IA-2_{IC} (19.8%, 35/177, p = 0.048). Of 59 IA-2A positive sera, 20 (33.9%) reacted with only one of the 7 constructs (17 with IA-2_{256-760}, 2 with IA-2_{IC} and 1 with IA-2_{BDC}). All IA-2_{PTPA} (n = 20) and IA-2_{(761-964)A} (n = 17) positive sera reacted with IA-2_{IC}. Thirteen sera reacted with IA-2_{PTP} as well IA-2_{(761-964)}. All IA-2_{JMA} positive sera reacted with IA-2_{256-760} construct. No significant difference in terms of sex, age, disease duration and BMI was detected between the 59 GADA/IA-2A positive and the 118 GADA positive/IA-2A negative LADA patients.

**IA-2_{256-760} immunoreactivity in 978 GADA and IA-2_{IC}A negative T2DM patients.** Autoantibodies to IA-2_{256-760} were detected in 33/978 (3.4%) of GADA/IA-2_{IC}A negative type 2 diabetic patients. Table 2 reports GAD, IA-2_{IC} and IA-2_{256-760} immunoreactivities of the whole group of type 2 diabetic patients (n = 1020) analyzed. In this group of patients, 4.9% (50/1020) sera were found IA-2_{256-760}A positive, in a higher percentage not only vs IA-2_{IC}A (1.3%, 13/1020, p < 0.0001), but also vs GADA (4.1%, 42/1020). IA-2_{IC} construct detected as positive 3 patients negative for IA-2_{256-760}A, which on the other hand detected as positive 7 GADA positive LADA and 33 GADA negative type 2 diabetic individuals resulted negative for IA-2_{IC} antibodies.

**Clinical, immunological and genetic features of type 2 and LADA diabetic patients.** Table 3 reports sex, age at disease diagnosis, BMI and fasting glucose of the 1020 type 2 diabetic patients classified according to their different GAD or IA-2_{(256-760)} immunoreactivities. The GADA positive, but not the IA-2_{(256-760)A} positive LADA patients, showed significantly lower BMI, mean age at diagnosis and higher fasting glucose values than antibody negative type 2 diabetic patients. Table 4 reports the TPO antibody frequencies of GAD or IA-2_{(256-760)A} positive LADA in comparison to 114 GADA/IA-2_{(256-760)A} negative type 2 diabetic patients of comparable age and sex. TPO antibody frequency were significantly higher in both groups of GADA positive or IA-2_{(256-760)A} positive patients vs type 2 diabetic GADA/IA-2_{(256-760)A} negative patients (p = 0.001 and p = 0.032, respectively). LADA patients positive for GADA or IA-2_{(256-760)A}
IA-2 immunoreactivity in LADA patients

showed a significant higher frequency of high and moderate HLA risk genotypes compared with type 2 diabetic patients negative for GADA and IA-2\((256-760)\)A (p<0.05) (Table 5).

**IA-2Ab competition experiments.** Figure 2 shows the results of representative competition experiments performed with sera single positive for IA-2\((256-760)\) or IA-2\(_{IC}\) and double positive for IA-2\((256-760)\)/IA-2\(_{IC}\) autoantibodies. An IA-2 fragment specific, dose dependent reduction of antibody binding was observed in each of the 3 sera analyzed according to their relative IA-2\((256-760)\)/IA-2\(_{IC}\) autoantibody pattern. Specific results are detailed in Figure 2 legend. Similar data were found for other 7 sera investigated, according to the corresponding autoantibody pattern (data not shown).

**Comparison of GADA-positive LADA and type 1 diabetes IA-2 epitope immunoreactivities at disease diagnosis.** C-terminal immunoreactivities: Altogether, at disease diagnosis, immunoreactivity against C-terminal containing IA-2\(_{(761-964)}\) and/or IA-2\(_{PTP(687-979)}\) constructs was found in 58.5% (31/53) of childhood-onset type 1 diabetic patients, in a significantly higher percentage vs adult-onset type 1 diabetic (35.8%, 19/53, p=0.032) and LADA patients (24.2%, 8/33, p=0.003). In particular, in childhood-onset type 1 diabetic patients, IA-2\(_{(761-964)}\)Ab frequency (47.2%) was significantly higher (p<0.01) vs LADA patients (18.2%), whereas IA-2\(_{PTP(687-979)}\)Ab frequency (52.5%) was significantly higher (p<0.01) vs both adult-onset type 1 diabetic (20.8%) and LADA (21.2%) patients (Figure 3A).

Middle-domain immunoreactivities: Altogether, at disease diagnosis, immunoreactivity against IA-2\((256-760)\) and/or IA-2\(_{JM(601-630)}\) constructs was found in 35.8% (19/53) childhood-onset type 1 diabetic patients, a lower, but not significantly different percentage vs adult-onset type 1 diabetic (45.3%, 24/53) and LADA (42.4%, 14/33) patients. However, in childhood-onset type 1 diabetic patients, single IA-2\(_{JM(601-630)}\)A frequency (5.7%) was significantly lower (p<0.01) vs adult-onset type 1 diabetic (26.4%) and LADA (24.2%) patients. IA-2\((256-760)\) immunoreactivities in LADA (39.4%), adult-onset (39.6%) and childhood-onset type 1 diabetic patients (30.2%) were not significantly different (Figure 3B).

IA-2 constructs utilized in international screenings: At disease diagnosis, IA-2\(_{IC}\), IA-2\(_{FL}\), and IA-2\(_{BDC}\) autoantibody frequencies (69.8%, 62.3% and 62.3%, respectively) in childhood-onset type 1 diabetic patients (Figure 3C) were significantly higher in comparison to adult-onset type 1 diabetic (45.3% p<0.02, 39.6% p=0.03 and 30.2% p<0.002, respectively) and LADA patients (27.3% p<0.001, 27.3% p=0.002 and 21.2% p<0.001, respectively).

**DISCUSSION**

IA-2 is one of the major autoantigens in type 1 diabetes, target of both humoral (24-29) and T-cell reactivity (30,31). IA-2A have also been detected in small percentages of type 2 diabetic patients, but only few cases in addition to GADA (18,13). IA-2A presence, in addition to GADA, increases the risk of LADA patients to require future insulin therapy (13). The first aim of the present study was to identify the IA-2 construct able to detect IA-2 immunoreactivity with the highest sensitivity in Italian LADA GADA positive patients. We found that IA-2\(_{(IC(605-979)}\), at present considered as the most sensitive construct for IA-2A detection in autoimmune diabetes (16), reacted with 19.8% of LADA patients investigated, a significantly lower percentage in comparison to the 29.4% of IA-2\((256-760)\), a construct utilized for the first time in LADA to investigate IA-2 middle-domain immunoreactivity. By the use of competition experiments, we showed that these two fragments represent distinct IA-2 immunoreactive epitopes. The different immunoreactivities of IA-2\(_{(IC(605-979)}\) and IA-
2(256-760) fragments seem to be related to the age at diagnosis; as a matter of fact, C-terminal domain of the IA-2 protein was found to be the major IA-2 autoantigenic region in childhood-onset type 1 diabetes, whereas in adult-onset type 1 diabetic and LADA GADA positive patients there is a lower IA-2 C-terminal immunoreactivity that results in a significant decrease of diagnostic sensitivity of all IA-2 constructs containing a C-terminal residue. The finding that IA-2(256-760) construct reacts with more LADA patients than IA-2FL(1-979), even if the former is a portion of the latter, might be due to a different conformation of the 256-760 aminoacidic residues of the two constructs or to steric hindrances, ultimately leading to variable autoantibody binding affinities. Several studies demonstrated that IA-2A in type 1 diabetic patients are directed against multiple epitopes of the intracellular cytoplasmic portion of the protein (a.a.601-979), located in the juxtamembrane region (JM, a.a.605-682), as well as in the protein (PTP)-like C-terminal domain (a.a.630-979) (32-39). To date, an immune response against the extracellular domain of the IA-2 protein has not been demonstrated in type 1 diabetes. In our cohort of LADA patients, immunoreactivity against the IA-2JM(601-630) fragment was almost 3 times less frequent than IA-2(256-760) construct, thus suggesting that the main epitopes target of IA-2A in LADA may be located either between aminoacids 631 and 760 of the protein, and/or, more interestingly, between aminoacids 256-600, a domain comprising the extracellular portion of the protein.

The second aim of our study was to evaluate the frequency of IA-2(256-760) immunoreactivity in a large cohort of type 2 diabetic patients negative for GADA and IA-2IC(605-979)A. Surprisingly, 33 of these patients were IA-2(256-760)A positive. To date, IA-2A presence, in absence of GADA, was considered a rare phenomenon in type 2 diabetes (13,40). However, our results clearly demonstrate that most IA-2 autoantibody screenings performed so far in type 2 diabetic patients, underestimated the frequency of immunoreactivity against the IA-2 autoantigen, and, as a consequence, of the number of patients with autoimmunity. The double positivity for GAD and IA-2 autoantibodies in type 2 diabetes (11,40) suggests a pathogenetic link more consistent with type 1 rather than with type 2 diabetes, identifying patients who progress towards insulin dependency within a relatively brief period of time. In the present study, we found that the LADA patients positive for GADA have significantly lower mean age, BMI and higher fasting glucose levels compared to type 2 diabetic patients negative for GADA and IA-2(256-760)A. IA-2(256-760)A positivity does not seem to determine a similar metabolic phenotype, however genetic analysis demonstrates that LADA patients positive for GADA or IA-2(256-760)A have a significant higher frequency of autoimmune diabetes HLA susceptible genotypes compared to type 2 diabetic patients negative for both GADA and IA-2(256-760)A.

GADA positivity in type 2 diabetic patients identifies also those at high risk for developing thyroid autoimmunity (41). Interestingly, in our cohort of Caucasian patients, a similar result was found not only for GADA (18), but also for IA-2(256-760)A positive type 2 diabetic patients. TPO-A occurred significantly more frequently in IA-2(256-760)A positive (regardless of GADA positivity) than in islet-related autoantibody negative type 2 diabetic patients, thus supporting the hypothesis that some of these patients may represent a phenotypic expression of a complex autoimmune polyendocrine syndrome.

Recently, it was shown that the islet proteins recognized by T-cells and autoantibodies in type 1 diabetic and LADA patients may be in part different (42) and that
measures of multiple islet protein T-cell responses in type 2 diabetic patients may improve the identification of patients with autoimmune diabetes compared with autoantibody assessment alone (43). It was hypothesized that the higher sensitivity of T-cell responses could be due to the capacity of T-cells to react with unknown islet antigens (43). In that study, however, IA-2 immunoreactivity was evaluated with IA-2IC(605-979) and not with IA-2(256-760) construct. It is possible that IA-2(256-760) A detection might contribute to reduce the bias of sensitivity between T-cell response and autoantibody analysis for identification of LADA patients. It is also of potential interest that IA-2(256-760) construct contains in its sequence a number of IA-2 T-cell epitopes recognized by human CD4 T-cells (44).

Finally, our data, related to Caucasian patients, confirm and extend those reported in Japanese diabetic populations (17), where an association between IA-2 autoantibody epitope specificities and age at onset was found. These results suggest that the mechanisms responsible for the generation of IA-2A in Caucasian and Japanese LADA patients are similar, and that different genetic backgrounds probably do not influence IA-2A epitope specificity, in contrast with what has been reported for GAD epitope immunoreactivity (12).

In summary, by analyzing IA-2 epitope immunoreactivity in Caucasian LADA and type 1 diabetic patients, we found that:

a) IA-2(256-760), an IA-2 construct lacking the C-terminal portion of the protein, may represent a new sensitive marker for the study of the humoral IA-2 immunoreactivity in LADA patients, being able to identify IA-2 immunoreactivity also among GADA-negative type 2 diabetic patients;

b) IA-2(256-760)A presence is associated at increased risk for developing thyroid autoimmunity and higher frequency of autoimmune diabetes HLA susceptible genotypes;

c) the specificity of IA-2 humoral immune response in autoimmune diabetic patients is related to the age at diagnosis, with an increased IA-2 C-terminal immunoreactivity in childhood-onset type 1 diabetes.

In conclusion, the results of the present study suggest that IA-2 immunoreactivity in type 2 diabetic, as well as LADA patients, is more frequent than previously demonstrated and that the analysis of IA-2(256-760) immunoreactivity in type 2 diabetic patients may represent an additional, important diagnostic tool for a more appropriate classification of diabetes.

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TABLE 1

Immunoreactivities of 7 IA-2 constructs in 177 GADA positive LADA patients

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</tbody>
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<sup>a</sup> p < 0.0001 vs IA-2NC and IA-2FL and IA-2FL; p = 0.048 vs IA-2FL; p = 0.017 vs IA-2FL; p = 0.0002 vs IA-2NC

<sup>b</sup> p = 0.03 vs IA-2FL and IA-2FL

<sup>c</sup> p = 0.01 vs IA-2FL and IA-2FL, p = 0.033 vs IA-2FL
TABLE 2

**GAD, IA-2**<sub>250-760</sub> **and IA-2<sub>IC</sub> Immunoreactivities in LADA and T2DM patients**

<table>
<thead>
<tr>
<th></th>
<th>T2DM analyzed in Rome n = 1020</th>
<th>LADA GADA positive n = 42 (4.1%)</th>
<th>T2DM GADA negative n = 978 (95.8%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA-2&lt;sub&gt;IC&lt;/sub&gt;A+</td>
<td>n = 13 (1.3%)</td>
<td>13 (30.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;250-760&lt;/sub&gt;A+</td>
<td>n = 50 (4.9%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 (40.5%)</td>
<td>33 (3.4%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;IC&lt;/sub&gt;A+ : IA-2&lt;sub&gt;250-760&lt;/sub&gt;A+</td>
<td>n = 10 (1.0%)</td>
<td>10 (23.8%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;IC&lt;/sub&gt;A+ : IA-2&lt;sub&gt;250-760&lt;/sub&gt;A neg</td>
<td>n = 3 (0.3%)</td>
<td>3 (7.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;IC&lt;/sub&gt;A neg : IA-2&lt;sub&gt;250-760&lt;/sub&gt;A+</td>
<td>n = 40 (3.9%)</td>
<td>7 (16.7%)</td>
<td>33 (3.4%)</td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;IC&lt;/sub&gt;A neg : IA-2&lt;sub&gt;250-760&lt;/sub&gt;A neg</td>
<td>n = 967 (94.8%)</td>
<td>22 (52.4%)</td>
<td>945 (96.8%)</td>
</tr>
</tbody>
</table>

<sup>a and b</sup> p < 0.0001 vs IA-2<sub>IC</sub>A+
## TABLE 3

Clinical characteristics of type 2 and LADA diabetic patients

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>M:F</th>
<th>Age at diagnosis (years)</th>
<th>Disease duration (months)</th>
<th>BMI (Kg m²)</th>
<th>Fasting glucose (mg dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYPE 2 DIABETIC patients</strong></td>
<td>945</td>
<td>538/407</td>
<td>55.3 ± 11.1</td>
<td>21.9 ± 18.0</td>
<td>30.3 ± 6.5</td>
<td>148.6 ± 44.0</td>
</tr>
<tr>
<td>GADA and IA-2 288-299Ab neg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUTOIMMUNE patients</strong></td>
<td>42</td>
<td>26/16</td>
<td>44.3 ± 12.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.2 ± 18.7</td>
<td>24.8 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>177.0 ± 67.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GADA +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUTOIMMUNE patients</strong></td>
<td>50</td>
<td>30/20</td>
<td>52.2 ± 11.9</td>
<td>22.6 ± 19.6</td>
<td>29.0 ± 6.8</td>
<td>153.7 ± 63.6</td>
</tr>
<tr>
<td>IA-2 288-299Ab +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>p<0.001 vs T2DM patients,  
<sup>b</sup>p<0.001 vs T2DM and IA-2 288-299Ab<sup>+</sup> patients,  
<sup>c</sup>p<0.001 vs T2DM patients
**TABLE 4**

Frequency of TPO-Abs in LADA patients according to their pattern of autoantibody positivity

<table>
<thead>
<tr>
<th></th>
<th>II</th>
<th>TPO % Ab +</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYPE 2 DIABETIC patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADA and IA-2&lt;sub&gt;255-799&lt;/sub&gt;Ab neg</td>
<td>114</td>
<td>10.5&lt;sup&gt;a&lt;/sup&gt; (4m / 8 f)</td>
</tr>
<tr>
<td><strong>AUTOIMMUNE patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADA +</td>
<td>42</td>
<td>33.3 (9m / 5 f)</td>
</tr>
<tr>
<td><strong>AUTOIMMUNE patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;261-799&lt;/sub&gt;Ab +</td>
<td>50</td>
<td>24.0 (6m / 6 f)</td>
</tr>
</tbody>
</table>

<sup>a</sup>: p=0.001 vs GADA+ and p=0.032 vs IA-2<sub>255-799</sub>Ab+ patients
TABLE 5
Frequency of HLA class II in LADA patients according to their pattern of autoantibody positivity

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>High risk % (n)</th>
<th>Moderate risk % (n)</th>
<th>Low risk % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYPE 2 DIABETIC patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADA and IA-2&lt;sub&gt;266-706&lt;/sub&gt;Ab neg</td>
<td>64</td>
<td>1.6 (1)</td>
<td>9.2 (6)</td>
<td>89.2 (57)</td>
</tr>
<tr>
<td><strong>AUTOIMMUNE patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADA +</td>
<td>42</td>
<td>9.5 (4)</td>
<td>21.5 (9)</td>
<td>60.0 (28)</td>
</tr>
<tr>
<td>IA-2&lt;sub&gt;266-706&lt;/sub&gt;Ab +</td>
<td>36</td>
<td>2.8 (1)</td>
<td>22.2 (8)</td>
<td>75.0 (27)</td>
</tr>
</tbody>
</table>

nHigh: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 genotype (DRB1*04 different from 0403, 06, 11);

Moderate: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302x and DRB1*03x (X different from DRB1*03, DRB1*04-DQB1*0302; DRB1*04 not 0403, 06, 11, or DQB1*0602/03) genotypes

Low: other genotypes

*χ²: 2 x 2; (high and moderate vs low risk HLA genotypes) 1 vs 2 and 3 p<0.05
**FIGURE LEGENDS**

**Figure 1. Schematic representation of IA-2 constructs utilized in the various phases of the study**
Numbers refer to amino acid position on the deposited sequence of IA-2 whole protein. The upper portion of the figure represents IA-2$_{PTP(687-979)}$ and IA-2$_{(761-964)}$, the two constructs utilized to detect IA-2 C-terminal immunoreactivity. The central portion of the figure represents IA-2$_{(256-760)}$ and IA-2$_{(601-630)}$, the two constructs utilized to detect IA-2 middle-terminal immunoreactivity. The lower portion of the figure represents IA-2$_{FL(1-979)}$, IA-2$_{BDC(256-556; 630-979)}$ and IA-2$_{IC(605-979)}$ are represented, the three IA-2 fragments commonly used for the determination of IA-2Abs in type 1 diabetes autoantibody screenings. Dashed grey area in IA-2$_{BDC}$ fragment represents aminoacids missing in the construct, a spliced IA-2 variant lacking exon 13. JM= Juxtamembrane. TM = Transmembrane.

**Figure 2. IA-2Ab competition experiments**
Competition binding to radiolabeled IA-2$_{(256-760)}$ or IA-2$_{IC(605-979)}$ in 3 LADA patient sera positive only for IA-2$_{IC(605-979)}$A (a), only for IA-2$_{IC(605-979)}$A (b), or for both IA-2$_{(256-760)}$ and IA-2$_{IC(605-979)}$A (c), respectively. Y axis represents the amount of precipitated $^{35}$S-radiolabeled IA-2$_{(256-760)}$ or IA-2$_{IC(605-979)}$ expressed in counts per minute (cpm). X axis represents the amount of unlabeled IA-2$_{(256-760)}$ or IA-2$_{IC(605-979)}$ added, which was 0.5, 1, 2 and 4 fold that of the amount of $^{35}$S-antigen utilized in the assay. An IA-2 fragment-specific, dose dependent reduction of antibody binding was observed in each of the 3 sera analyzed according to their relative IA-2 autoantibody pattern. Specifically, panel (a) shows that the IA-2$_{(256-760)}$A positive serum, could be inhibited by unlabeled IA-2$_{(256-760)}$, but not by unlabeled IA-2$_{IC}$ protein. Conversely, panel (b) shows that IA-2$_{IC}$A positive serum, could be inhibited by unlabeled IA-2$_{IC}$, but not by unlabeled IA-2$_{(256-760)}$ protein. Panel (c) shows that serum autoantibody binding of a double positive serum could be partially or entirely inhibited by the use of a single or both unlabeled IA-2$_{(256-760)}$ and IA-2$_{IC}$ constructs, respectively.

**Figure 3. Comparison of IA-2 immunoreactivities of 7 IA-2 constructs in 33 Caucasian LADA and 106 type 1 diabetic sera at disease diagnosis**
Type 1 diabetic patients were subdivided into two groups according to age at diagnosis: n=53 type 1 diabetic children aged <12 years and n=53 type 1 diabetic adult patients aged >18 years. Y axis indicates, for each construct analyzed, the autoantibody frequency in the 3 groups of patients investigated. Immunoreactivity against IA-2 C-terminal domains (A and C constructs) was significantly higher in type 1 diabetic children compared to type 1 diabetic adult and LADA patient sera. Conversely, IA-2 middle-domain immunoreactivity was directed against a significantly higher percentage of type 1 diabetic adult and LADA patient sera only in the case of the B construct IA-2$_{(601-630)}$. 

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IA-2 immunoreactivity in LADA patients

![Diagram showing IA-2 immunoreactivities and constructs used to detect IA-2 Abs in international screenings]

**Figure 1**
IA-2 immunoreactivity in LADA patients
IA-2 immunoreactivity in LADA patients

A  IA-2 C-terminal reactivities

B  IA-2 middle-domain reactivities

C  Reactivities of the constructs utilized in IA-2RI screenings

Figure 3