Identification of Tyrosine Phosphatase $2_{(256–760)}$ Construct as a New, Sensitive Marker for the Detection of Islet Autoimmunity in Type 2 Diabetic Patients

The Non–Insulin Requiring Autoimmune Diabetes (NIRAD) Study 2*

Claudio Tiberti,[1] Carla Giordano,[2] Mattia Locatelli,[3] Emanuele Bosi,[4] Gian Franco Bottazzo,[3] Raffaella Buzzetti,[1] Domenico Cucinotta,[5] Aldo Galluzzo,[2] Alberto Falorni,[6] and Francesco Dotta[7]

OBJECTIVE—The presence of autoantibodies to islet antigens GAD and/or tyrosine phosphatase 2 (IA-2) in type 2 diabetic patients (latent autoimmune diabetes in adults [LADA]) identifies subjects at high risk to develop insulin dependency. The 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—We analyzed 177 LADA and 978 type 2 diabetic patients with different disease duration, collected in a nationwide Italian survey, the Non–Insulin Requiring Autoimmune Diabetes (NIRAD) study aimed at assessing prevalence and characteristics of autoimmune diabetes in type 2 diabetic patients and 106 newly diagnosed type 1 diabetic patients (53 children, 53 adults). By radioimmunoassay, we analyzed humoral immunoreactivity to seven IA-2 constructs: $IA\text{-}2_{PTP\ (687–979)}$, $IA\text{-}2_{(761–964)}$, $IA\text{-}2_{(256–760)}$, $IA\text{-}2_{JM\ (601–630)}$, $IA\text{-}2_{IC\ (605–979)}$, $IA\text{-}2_{BDC\ (256–556:630–979)}$, and $IA\text{-}2_{FL\ (1–979)}$.

RESULTS—$IA\text{-}2_{(256–760)}$ fragment was identified as the marker with the highest sensitivity for detection of humoral IA-2 immunoreactivity in LADA patients, identifying IA-2 autoantibodies in ~30% of GAD antibody (GADA)-positive LADA patients and in 3.4% of GADA-negative type 2 diabetic patients. LADA $IA\text{-}2_{(256–760)}$A positivity was associated with an increased frequency of autoimmune diabetes HLA-susceptible genotypes and with a higher risk for developing thyroid autoimmunity compared with autoantibody-negative type 2 diabetic patients. At disease diagnosis, adult-onset type 1 diabetic and LADA patients showed a lower IA-2 COOH-terminal immunoreactivity compared with childhood-onset type 1 diabetic patients.

CONCLUSIONS—IA-2 immunoreactivity in LADA patients has thus far been underestimated, and $IA\text{-}2_{(256–760)}$ autoantibody detection may represent a novel diagnostic tool for the identification of islet autoimmunity in these patients. ***Diabetes*** **57: 1276–1283, 2008**

Autoimmune diabetes is characterized by the presence of circulating autoantibodies directed against several islet proteins, including insulin, GAD, and tyrosine phosphatase 2 (IA-2) (1). Among these diabetes-related autoantibodies, only GAD autoantibodies (GADAs) are not age dependent, thus representing a sensitive marker for the study of childhood and of adult autoimmune diabetes (2). In addition, GADAs identify the subset of patients with type 2 diabetes who initially do not require insulin treatment but who may develop insulin dependency within a few years after diagnosis (3–5). This form of diabetes has been variably indicated (6–9). Throughout this article, we 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 NH_2-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. Although GADAs 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 autoantibodies detected in these patients. IA-2 autoantibodies (IA-2As) were found in 2.2% of type 2 diabetic patients, and their presence, in addition to GADAs, increases the relative risk of these patients to require insulin therapy (13). To date, IA-2As are detected using sensitive and specific radioimmunoassays, differing in terms of which of the radiolabeled IA-2 constructs is used, usually chosen among the full-length $IA\text{-}2_{FL\ (1–979)}$, the truncated NH_2-terminally spliced IA-2 variant lacking exon

From the [1]Department of Clinical Sciences, University of Rome "La Sapienza," Rome, Italy; the [2]Department of Endocrinology, University of Palermo, Palermo, Italy; the [3]Scientific Institute, Bambino Gesù Hospital, Rome, Italy; [4]General Medicine, Diabetes, and Endocrinology, San Raffaele Scientific Institute and Vita Salute University, Milan, Italy; the [5]Department of Internal Medicine, University of Messina, Messina, Italy; the [6]Department of Internal Medicine, University of Perugia, Perugia, Italy; and the [7]Department of Internal Medicine, Endocrine and Metabolic Sciences, and Biochemistry, University of Siena, Siena, Italy.

Corresponding author: Claudio Tiberti, Department of Clinical Sciences, Policlinico Umberto I, Sapienza University of Rome, Viale del Policlinico 155, 00161, Rome, Italy. E-mail: claudio.tiberti@uniroma1.it.

Received for publication 28 June 2007 and accepted in revised form 23 January 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 10 March 2008. DOI: 10.2337/db07-0874.

*A complete list of the NIRAD study investigators and committees can be found in an online appendix at http://dx.doi.org/10.2337/db07-0874.

DASP, Diabetes Antibody Standardization Program; GADA, GAD autoantibody; IA-2, tyrosine phosphatase 2; IA-2A, IA-2 autoantibody; LADA, latent autoimmune diabetes in adults; NIRAD, Non–Insulin Requiring Autoimmune Diabetes; TPO-A, thyroid peroxidase autoantibody.

13 IA-$2_{BDC\ (256–556:630–979)}$ and the intracytoplasmic IA-$2_{IC\ (605–979)}$ construct (14,15). A recent study (16) showed that cytoplasmic IA-$2_{IC\ (605–979)}$ is the construct detecting IA-2As with the highest sensitivity in both newly diagnosed type 1 diabetic and pre-diabetic 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-$2_{IC\ (605–979)}$ immunoreactivity did not account for the whole anti–IA-2 humoral immune response in type 1 diabetes because other IA-2 constructs investigated showed additional immunoreactivities otherwise undetected by the IA-$2_{IC\ (605–979)}$ construct. No data are currently available regarding which construct among IA-$2_{FL\ (1–979)}$, IA-$2_{BDC\ (256–556:630–979)}$, and IA-$2_{IC\ (605–979)}$ has the highest sensitivity and specificity for detecting IA-2As in LADA patients and whether other IA-2 constructs may identify additional immunoreactivities in the sera of these patients. 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 was demonstrated (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 to identify the IA-2 construct able to detect IA-2 immunoreactivity with the highest sensitivity in Caucasian LADA patients and to establish 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 with that of type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

During the Non–Insulin Requiring Autoimmune Diabetes (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, 4,250 type 2 diabetic 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-$2_{IC\ (605–979)}$ autoantibodies. One hundred ninety-one (4.5%) and 39 (0.9%) type 2 diabetic patients were found to be GADA and IA-$2_{IC\ (605–979)}$A positive, respectively (18). Of the 4,250 NIRAD patients, 1,020 were collected at the "Sapienza" University of Rome (572 men and 406 women; 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 1,020 patients were GADA and IA-$2_{IC\ (605–979)}$A positive, respectively, whereas 978 patients were GADA and IA-$2_{IC\ (605–979)}$A negative.

In the first step of the present study, we aimed at evaluating which IA-2 constructs among the seven studied here had the highest sensitivity to detect humoral IA-2 immunoreactivity in Italian LADA patients. To this end, we analyzed all $GADA^+$ LADA patient sera available from the NIRAD study (n = 177: 92 men and 85 women; 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-$2_{(256–760)}$ construct, identified as the marker with the highest sensitivity for the detection of IA-2 autoantibodies in the group of $GADA^+$ LADA patients, was analyzed in all abovementioned 1,020 samples (978 $GADA^-$ and IA-$2_{IC}A^-$ plus 42 $GADA^+$ type 2 diabetic patients) screened at the "Sapienza" University of Rome during the NIRAD study.

In addition, among the abovementioned 1,020 patients, those found GADA or IA-$2_{(256–760)}$A positive were tested for thyroid peroxidase autoantibodies (TPO-As) and, when possible, typed for HLA DRB1-DQB1 polymorphisms; 114 (for TPO-A) and 64 (for HLA polymorphisms) $GADA^-$/IA-$2_{(256–760)}A^-$ 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 using the seven constructs represented in Fig. 1, the following groups of patients were studied at disease diagnosis: 33 $GADA^+$ LADA patients (22 men and 11 women; median age 44.8 years; age range 25–73 years); 53 $GADA^+$ type 1 diabetic children (28 boys and 25 girls; median age 7.6 years; age range 2–12 years); and 53 $GADA^+$ type 1 diabetic adults (29 men and 24 women; 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 $GADA^+$ patients aged <12 years consecutively recruited between 2001 and 2004, whereas the 53 type 1 diabetic adults represent all $GADA^+$ 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 used in the study

cDNAs encoding IA-$2_{(761–964)}$ were amplified by PCR from full-length IA-2 using 5′-ACCATGAGCGATTACATCAACGCCA-3′ and 5′-TCAGCAGCTACAGT CAGAATT-3′ primers. Ligation and transformation of fresh PCR products were performed as previously described for IA-$2_{(256–760)}$ construct (16). After purification, inserts were sequenced in both directions using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). No deletions or truncations were found in this IA-2 construct. IA-2_{BDC} was prepared as reported previously (20) and was provided, as the IA-$2_{(256–760)}$ construct, by Dr. G.S. Eisenbarth (University of Colorado, Denver, CO). IA-$2_{FL\ (1–979)}$, IA-$2_{IC\ (605–979)}$, IA-$2_{JM\ (601–630)}$, and IA-$2_{PTP\ (687–979)}$ cDNAs were 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, WI) 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 >99th percentile of 211 healthy control sera (102 women and 109 men; median age 27 years; age range 3–77 years) were 0.094, 0.010, 0.073, 0.064, 0.138, 0.049, and 0.094 for IA-2_{FL}, IA-2_{IC}, IA-2_{BDC}, IA-$2_{(256–760)}$, IA-2_{JM}, IA-2_{PTP}, 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-2_{JM}, 5.8 and 10.0% for IA-$2_{(761–964)}$, 5.1 and 9.2% for IA-2_{PTP}, 5.6 and 7.6% for IA-2_{BDC}, 4.8 and 9.9% for IA-2_{IC}, and 5.0 and 8.5% for IA-2_{FL}. In this study, IA-$2_{(761–964)}$ and IA-$2_{PTP\ (687–979)}$ constructs were used to evaluate IA-2 COOH-terminal immunoreactivities, whereas IA-$2_{JM\ (601–630)}$ and IA-$2_{(256–760)}$ constructs were used to detect IA-2 middle-domain immunoreactivities. The remaining three IA-2 fragments (IA-2_{FL}, IA-2_{IC}, and IA-2_{BDC}) are those most commonly used to evaluate IA-2 immunoreactivity in diabetes-related screenings. IA-2As in the NIRAD study were detected using IA-2_{IC} (19). IA-2_{IC}A assay obtained 72% sensitivity and 99% specificity at the 2007 4th assay proficiency evaluation (lab 155) of the Diabetes Antibody Standardization Program (DASP).

IA-2A competition experiments. To evaluate the specificity of antibody binding to ^{35}S-labeled IA-$2_{(256–760)}$ in comparison with ^{35}S-labeled IA-2_{IC} construct, the mutual inhibition activity of different concentrations of unlabeled IA-2_{IC} 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 amino acid mixture. Unlabeled recombinant IA-$2_{(256–760)}$ and/or IA-2_{IC} (0.5-, 1-, 2-, and 4-fold 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 radiolabeled ^{35}S–IA-$2_{(256–760)}$ or ^{35}S–IA-2_{IC} proteins, samples were processed with the usual radioimmunoprecipitation assay. In competition experiments, IA-$2_{(256–760)}A^+$/IA-$2_{IC}A^-$ (n = 6), IA-$2_{(256–760)}A^-$/IA-$2_{IC}A^+$ (n = 2), or IA-$2_{(256–760)}A^+$/IA-$2_{IC}A^+$ (n = 2) sera, respectively, were analyzed from 10 type 2 diabetic patients.

GADA detection. GADA in type 1 diabetic and LADA sera were detected by a slightly modified fluid-phase radioimmunoprecipitation assay (21) using a human recombinant full-length GAD65 construct furnished by Dr. Å. Lernmark (Department of Medicine, University of Washington, Seattle, WA). GADA assay obtained 80% sensitivity and 98% specificity at the 2007 4th DASP assay proficiency evaluation (lab 155).

TPO-A. TPO-As were measured by a commercial radioimmunoassay kit (cod.14752; Adaltis, Roma, 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, 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).

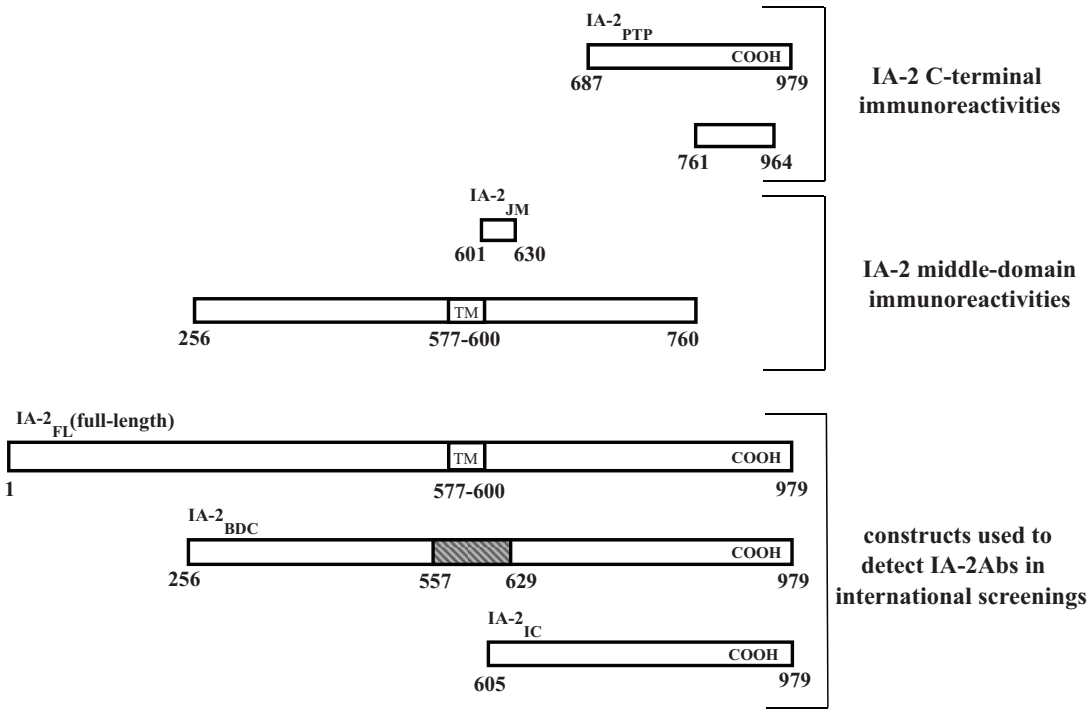

FIG. 1. Schematic representation of IA-2 constructs used in the various phases of the study. Numbers refer to amino acid position on the deposited sequence of IA-2 whole protein. The *top* portion of the figure represents IA-$2_{PTP\ (687–979)}$ and IA-$2_{(761–964)}$, the two constructs used to detect IA-2 COOH-terminal immunoreactivity. The *middle* portion of the figure represents IA-$2_{(256–760)}$ and IA-$2_{(601–630)}$, the two constructs used to detect IA-2 middle-terminal immunoreactivity. The *bottom* portion of the figure represents IA-$2_{FL\ (1–979)}$, IA-$2_{BDC\ (256–556:630–979)}$, and IA-$2_{IC\ (605–979)}$, the three IA-2 fragments commonly used for the determination of IA-2Abs in type 1 diabetes autoantibody screenings. Dashed gray area in IA-2_{BDC} fragment represents amino acids missing in the construct, a spliced IA-2 variant lacking exon 13. JM, juxtamembrane. TM, transmembrane.

Statistical analysis

Statistical analyses were performed using SPSS software, version 13 (SPSS, Chicago, IL). Frequency differences were calculated by χ^2 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$^+$ LADA patients

Of 177 LADA patients, 59 (33.3%) were positive for at least one of the seven IA-2 constructs analyzed. IA-$2_{(256–760)}$ was the fragment showing the highest sensitivity, identifying IA-2 immunoreactivity in 29.4% (52 of 177) of LADA sera (Table 1), significantly more frequently than IA-2_{PTP} (11.3%, 20 of 177, $P < 0.0001$), IA-$2_{(761–964)}$ (9.6%, 17 of 177, $P < 0.0001$), IA-2_{JM} (9.6%, 17 of 177, $P < 0.0001$), IA-2_{FL} (18.1%, 32 of 177, $P = 0.017$), IA-2_{BDC} (13.0%, 23 of 177, $P = 0.0002$), and IA-2_{IC} (19.8%, 35 of 177, $P = 0.048$). Of 59 IA-2A$^+$ sera, 20 (33.9%) reacted with only one of the seven constructs (17 with IA-$2_{(256–760)}$, 2 with IA-2_{IC}, and 1 with IA-2_{BDC}). All IA-2_{PTP}A$^+$ ($n = 20$) and IA-$2_{(761–964)}$A$^+$ ($n = 17$) sera reacted with IA-2_{IC}. Thirteen sera reacted with IA-2_{PTP} and IA-$2_{(761–964)}$. All IA-2_{JM}A$^+$ 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$^+$ and the 118 GADA$^+$/IA-2A$^-$ LADA patients.

IA-$2_{(256–760)}$ immunoreactivity in 978 GADA$^-$ and IA-2_{IC}A$^-$ type 2 diabetic patients

Autoantibodies to IA-$2_{(256–760)}$ were detected in 33 of 978 (3.4%) GADA/IA-2_{IC}A$^-$ 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 ($N = 1,020$) analyzed. In this group of patients, 4.9% (50 of 1,020) of sera were found to be IA-$2_{(256–760)}$A positive, a higher percentage not only versus IA-2_{IC}A (1.3%, 13 of 1,020, $P < 0.0001$) but also versus GADA (4.1%, 42 of 1,020). The IA-2_{IC} construct detected as positive three IA-$2_{(256–760)}$A$^-$ patients and on the other hand detected as positive seven GADA$^+$ LADA and 33 GADA$^-$ type 2 diabetic individuals negative for IA-2_{IC} antibodies.

TABLE 1
Immunoreactivities of seven IA-2 constructs in 177 GADA$^+$ LADA patients

	IA-2 COOH-terminal domains		IA-2 middle domains		IA-2 constructs utilized in international screenings		
Fragment	IA-$2_{(761–964)}$	IA-$2_{PTP\ (687–979)}$	IA-$2_{(256–760)}$	IA-$2_{JM\ (601–630)}$	IA-$2_{IC\ (605–979)}$	IA-$2_{FL\ (1–979)}$	IA-$2_{BDC\ (256–556:630–979)}$
% A$^+$	9.6	11.3	29.4*	9.6	19.8†	18.1‡	13.0
n A$^+$ ($N = 177$)	17	20	52	17	35	32	23

*$P < 0.0001$ vs. IA-$2_{(761–964)}$, IA-2_{PTP}, and IA-2_{JM}; $P = 0.048$ vs. IA-2_{IC}; $P = 0.017$ vs. IA-2_{FL}; $P < 0.002$ vs. IA-2_{BDC}. †$P = 0.01$ vs. IA-$2_{(761–964)}$ and IA-2_{JM}; $P = 0.039$ vs. IA-2_{PTP}. ‡$P = 0.03$ vs. IA-$2_{(761–964)}$ and IA-2_{JM}.

TABLE 2
GAD, IA-$2_{(256–760)}$, and IA-2_{IC} immunoreactivities in LADA and type 2 diabetic patients

	Type 2 diabetes: analyzed in Rome, n = 1,020	LADA: GADA$^+$, n = 42 (4.1%)	Type 2 diabetes: GADA$^-$, n = 978 (95.9%)
IA-$2_{IC}A^+$	13 (1.3)	13 (30.9)	0 (0)
IA-$2_{(256–760)}A^+$	50 (4.9)*	17 (40.5)	33 (3.4)*
IA-$2_{IC}A^+$/IA-$2_{(256–760)}A^+$	10 (1.0)	10 (23.8)	0 (0)
IA-$2_{IC}A^+$/IA-$2_{(256–760)}A^-$	3 (0.3)	3 (7.1)	0 (0)
IA-$2_{IC}A^-$/IA-$2_{(256–760)}A^+$	40 (3.9)	7 (16.7)	33 (3.4)
IA-$2_{IC}A^-$/IA-$2_{(256–760)}A^-$	967 (94.8)	22 (52.4)	945 (96.6)

Data are n (%). *$P < 0.0001$ vs. IA-$2_{IC}A^+$.

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 1,020 type 2 diabetic patients classified according to their different GAD or IA-$2_{(256–760)}$ immunoreactivities. The GADA$^+$ but not the IA-$2_{(256–760)}A^+$ LADA patients showed significantly lower BMI and 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-$2_{(256–760)}A^+$ LADA in comparison with 114 GADA/IA-$2_{(256–760)}A^-$ type 2 diabetic patients of comparable age and sex. TPO antibody frequencies were significantly higher in both groups of GADA$^+$ or IA-$2_{(256–760)}A^+$ patients versus type 2 diabetic GADA/IA-$2_{(256–760)}A^-$ patients (P = 0.001 and 0.032, respectively). LADA patients positive for GADA or IA-$2_{(256–760)}$A showed a significantly 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-2A competition experiments

Fig. 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 three sera analyzed according to their relative IA-$2_{(256–760)}$/IA-2_{IC} autoantibody pattern. Specific results are detailed in the Fig. 2 legend. Similar data were found for the other seven sera investigated, according to the corresponding autoantibody pattern (data not shown).

Comparison of GADA$^+$ LADA and type 1 diabetic IA-2 epitope immunoreactivities at disease diagnosis

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

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 of 53) of childhood-onset type 1 diabetic patients, a lower but not significantly different percentage versus adult-onset type 1 diabetic (45.3%, 24 of 53) and LADA (42.4%, 14 of 33) patients. However, in childhood-onset type 1 diabetic patients, single IA-$2_{JM\ (601–630)}$A frequency (5.7%) was significantly lower ($P < 0.01$) versus 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 (Fig. 3*B*).

IA-2 constructs used 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 (Fig. 3*C*) were significantly higher in comparison with 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, a target of both humoral (24–28) and T-cell reactivity (29,30). IA-2As have also been detected in small percentages of type 2 diabetic patients but only in a few cases in

TABLE 3
Clinical characteristics of type 2 diabetic and LADA patients

	Type 2 diabetic patients: GADA$^-$ and IA-$2_{(256–760)}A^-$	Autoimmune patients: GADA$^+$	Autoimmune patients: IA-$2_{(256–760)}A^+$
n	945	42	50
Sex (male/female)	538/407	26/16	30/20
Age at diagnosis (years)	55.3 ± 11.1	44.3 ± 12.9*	52.2 ± 11.9
Disease duration (months)	21.9 ± 18.0	21.2 ± 18.7	22.5 ± 19.5
BMI (kg/m^2)	30.3 ± 5.5	24.8 ± 3.5†	29.0 ± 6.8
Fasting glucose (mg/dl)	148.6 ± 44.0	177.0 ± 67.2‡	153.7 ± 53.6

Data are means ± SE. *$P < 0.001$ vs. type 2 diabetic patients, †$P < 0.001$ vs. type 2 diabetic and IA-$2_{(256–760)}{}^+$ patients, ‡$P < 0.001$ vs. type 2 diabetic patients.

TABLE 4
Frequency of TPO-A in LADA patients according to pattern of autoantibody positivity

	n (male/female)	TPO % A^+
Type 2 diabetic patients: $GADA^-$ and IA-$2_{(256–760)}A^-$	114 (71/43)	10.5 (4/8)*
Autoimmune patients: $GADA^+$	42 (26/16)	33.3 (9/5)
Autoimmune patients: IA-$2_{(256–760)}A^+$	50 (30/20)	24.0 (6/6)

*$P = 0.001$ vs. $GADA^+$ and $P = 0.032$ vs. IA-$2_{(256–760)}{}^+$ patients.

addition to GADAs (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 $GADA^+$ 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 with the 29.4% of IA-$2_{(256–760)}$, a construct used 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; COOH-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 $GADA^+$ patients, there is a lower IA-2 COOH-terminal immunoreactivity that results in a significant decrease of diagnostic sensitivity of all IA-2 constructs containing a COOH-terminal residue. The finding that IA-$2_{(256–760)}$ construct reacts with more LADA patients than IA-$2_{FL\ (1–979)}$, even if the former is a portion of the latter, might be due to a different conformation of the 256–760 amino acidic residues of the two constructs or to steric hindrances, ultimately leading to variable autoantibody binding affinities. Several studies demonstrated that IA-2As in type 1 diabetic patients are directed against multiple epitopes of the intracellular cytoplasmic portion of the protein (amino acids 601–979), located in the juxtamembrane region (JM, amino acids 605–682), and in the protein (PTP)-like COOH-terminal domain (amino acids 630–979) (31–36). 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-$2_{JM\ (601–630)}$ fragment was almost three 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 amino acids 631 and 760 of the protein and/or, more interestingly, between amino acids 256 and 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-$2_{IC\ (605–979)}$A. Surprisingly, 33 of these patients were IA-$2_{(256–760)}$A positive. To date, IA-2A presence in the absence of GADA has been considered a rare phenomenon in type 2 diabetes (13,37). 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, the number of patients with autoimmunity. The double positivity for GAD and IA-2 autoantibodies in type 2 diabetes (11,37) suggests a pathogenetic link more consistent with type 1 rather than with type 2 diabetes, identifying patients who progress toward 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 and BMI and higher fasting glucose levels compared with 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 significantly higher frequency of autoimmune diabetes HLA susceptible genotypes compared with type 2 diabetic patients negative for both GADA and IA-$2_{(256–760)}$A.

GADA positivity in type 2 diabetic patients also identifies those at high risk for developing thyroid autoimmunity (38). Interestingly, in our cohort of Caucasian patients, a similar result was found not only for $GADA^+$ (18) but also for IA-$2_{(256–760)}A^+$ type 2 diabetic patients. TPO-A occurred significantly more frequently in IA-$2_{(256–760)}A^+$ (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 (39) 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 assess-

TABLE 5
Frequency of HLA class II in LADA patients according to pattern of autoantibody positivity

	n	High risk	Moderate risk	Low risk
Type 2 diabetic patients: $GADA^-$ and IA-$2_{(256–760)}A^-$	64	1.6 (1)	9.2 (6)	89.2 (57)
Autoimmune patients: $GADA^+$	42	9.5 (4)	21.5 (9)	69.0 (29)
Autoimmune patients: IA-$2_{(256–760)}A^+$	36	2.8 (1)	22.2 (8)	75.0 (27)

Data are *n* (%). High risk: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 genotype (DRB1*04 different from 0403, 06, 11). Moderate risk: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302/X and DRB1*03/X (X different from DRB1*03, DRB1*04-DQB1*0302; DRB1*04 not 0403, 06, 11, or DQB1*0602/03) genotypes. Low risk: other genotypes. $P < 0.05$ by χ^2 2 × 2 (high and moderate vs. low-risk HLA genotypes) for type 2 diabetic $GADA^-$ and IA-$2_{(256–760)}A^-$ patients vs. autoimmune $GADA^+$ and IA-$2_{(256–760)}A^+$ patients.

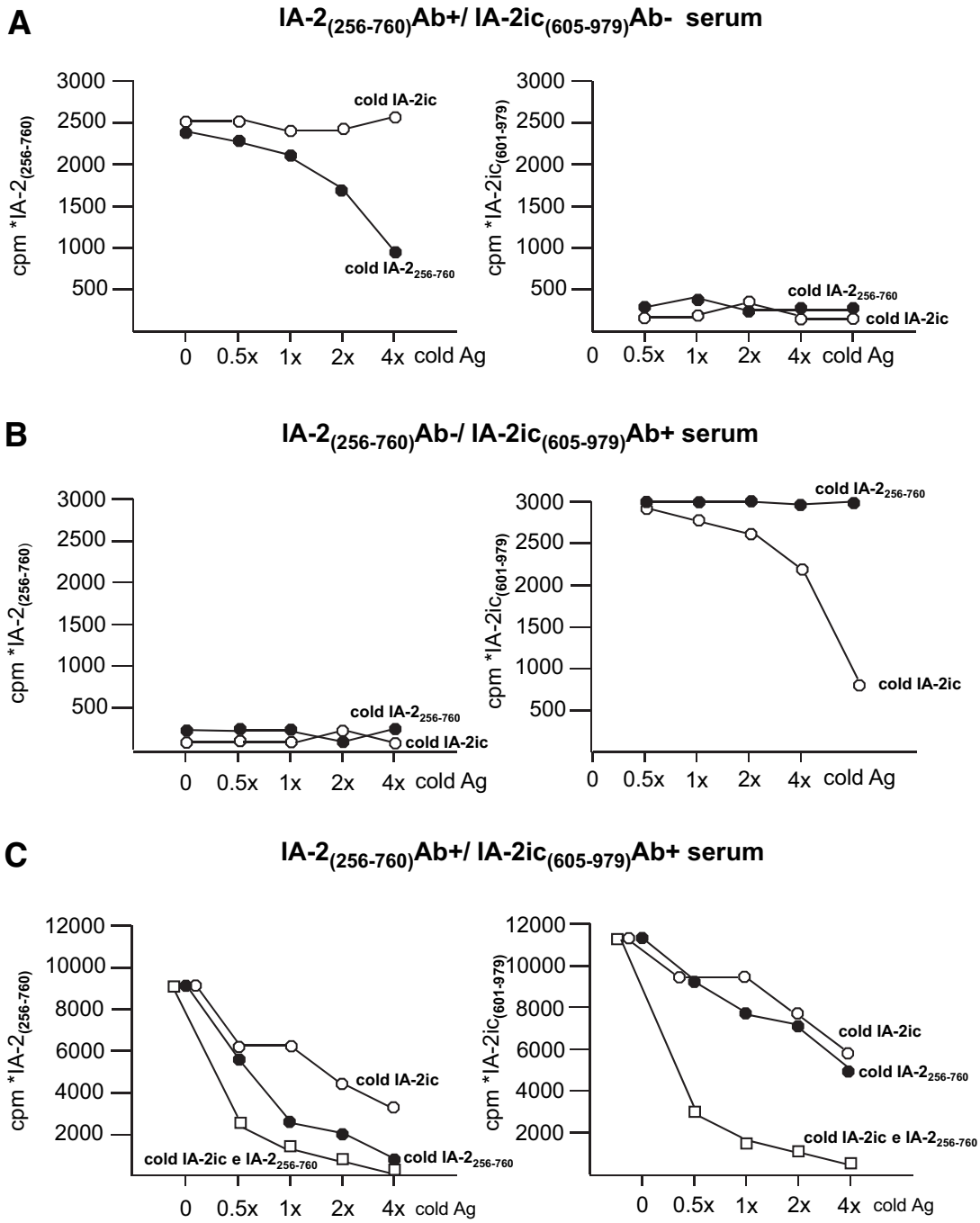

FIG. 2. IA-2A competition experiments. Competition binding to radiolabeled IA-$2_{(256–760)}$ or IA-$2_{IC\ (605–979)}$ in three LADA patient sera positive only for IA-$2_{(256–760)}$A (*A*), only for IA-$2_{IC\ (605–979)}$A (*B*), or for both IA-$2_{(256–760)}$ and IA-$2_{IC\ (605–979)}$A (*C*), respectively. *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-labeled antigen used in the assay. An IA-2 fragment-specific, dose-dependent reduction of antibody binding was observed in each of the three sera analyzed according to their relative IA-2 autoantibody pattern. Specifically, *A* shows that the IA-$2_{(256–760)}A^+$ serum could be inhibited by unlabeled IA-$2_{(256–760)}$ but not by unlabeled IA-2_{IC} protein. Conversely, *B* shows that IA-$2_{IC}A^+$ serum could be inhibited by unlabeled IA-2_{IC} but not by unlabeled IA-$2_{(256–760)}$ protein. *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.

ment alone (40). 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 (40). In that study, however, IA-2 immunoreactivity was evaluated with IA-$2_{IC\ (605–979)}$ 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 (41).

Finally, our data, related to Caucasian patients, confirm and extend those reported in Japanese diabetic populations (17), in which an association between IA-2 autoantibody epitope specificities and age at onset was found. These results suggest that the mechanisms responsible for

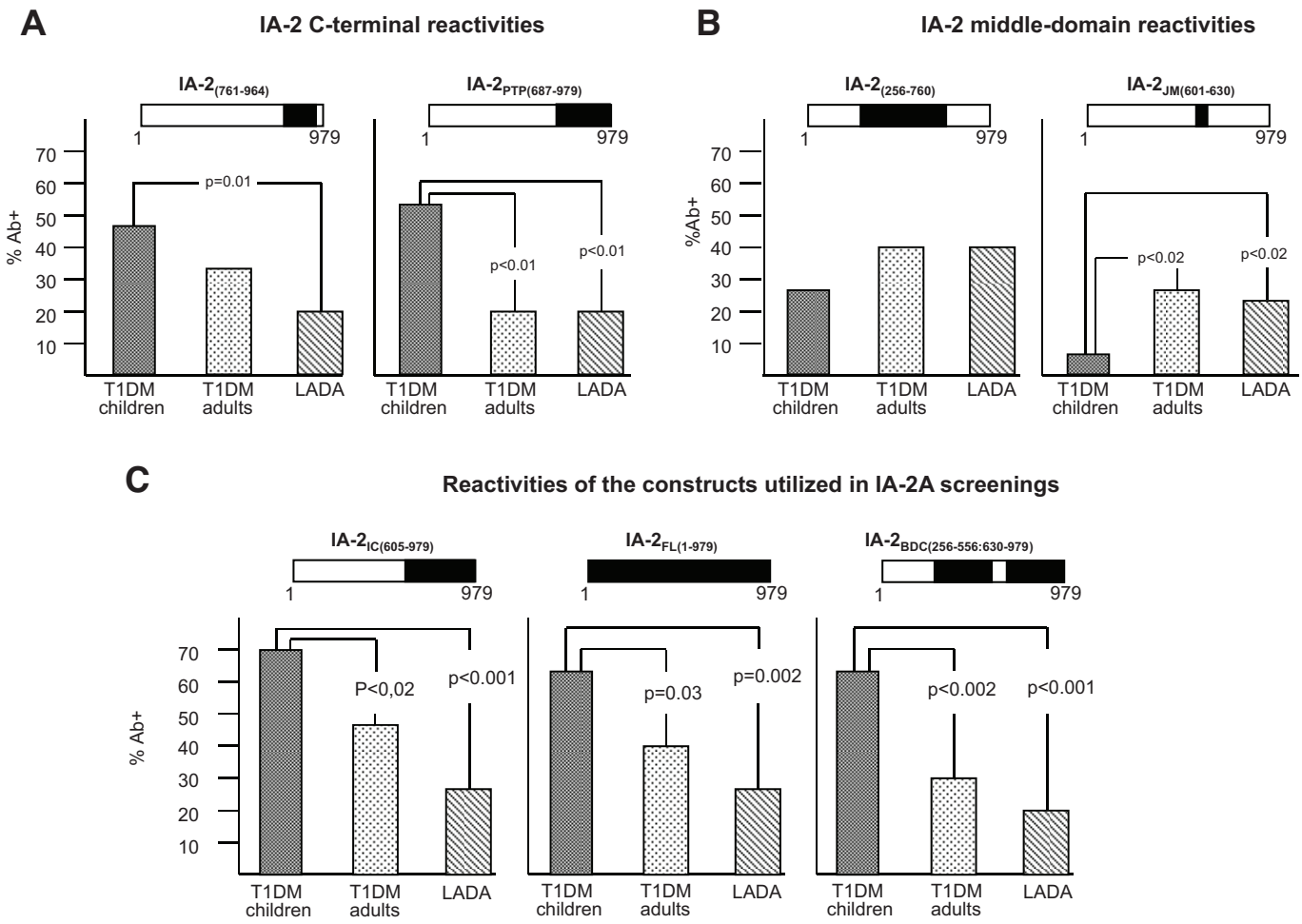

FIG. 3. Comparison of IA-2 immunoreactivities of seven IA-2 constructs in 33 Caucasian LADA and 106 type 1 diabetic sera at disease diagnosis. Type 1 diabetic patients were subdivided into two groups according to age at diagnosis: $n = 53$ type 1 diabetic children aged <12 years and $n = 53$ type 1 diabetic adult patients aged >18 years. *y*-Axis indicates, for each construct analyzed, the autoantibody frequency in the three groups of patients investigated. Immunoreactivity against IA-2 COOH-terminal domains (*A* and *C* constructs) was significantly higher in type 1 diabetic children compared with 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-$2_{(601–630)}$.

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 *1*) IA-$2_{(256–760)}$, an IA-2 construct lacking the COOH-terminal portion of the protein, may represent a new sensitive marker for the study of the humoral IA-2 immunoreactivity in LADA patients, by being able to identify IA-2 immunoreactivity also among $GADA^-$ type 2 diabetic patients; *2*) IA-$2_{(256–760)}$A presence is associated with increased risk for developing thyroid autoimmunity and higher frequency of autoimmune diabetes HLA susceptible genotypes; and *3*) the specificity of IA-2 humoral immune response in autoimmune diabetic patients is related to the age at diagnosis, with an increased IA-2 COOH-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 and 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.

ACKNOWLEDGMENTS

The NIRAD study is supported by Foundation for the Research of the Società Italiana di Diabetologia (FoRiSID) based on an unconditioned research grant from Novo Nordisk Italy.

We are indebted to the past President of FoRiSID, Prof. Riccardo Giorgino, for his effort in promoting and supporting this study and to Prof. Riccardo Vigneri, present advisor of the study, for his invaluable continuous support.

This article is dedicated to the memory of Prof. Umberto Di Mario, who greatly contributed to the design and implementation of the study.

REFERENCES

1. Atkinson MA, Eisenbarth GS: Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221–229, 2001
2. Vandewalle CL, Falorni A, Svanholm S, Lernmark Å, Pipeleers DG, Gorus FK: High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. *J Clin Endocrinol Metab* 80:846–851, 1995
3. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR:

Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with non–insulin-dependent onset of diabetes. *Diabetes*: 42:359–362, 1993
4. Turner R, Stratton I, Horton V, Manley S, Zimmet P, Mackay IR, Shattock M, Bottazzo GF, Holman R, UK Prospective Diabetes Study Group: UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. *Lancet* 350:1288–1293, 1997
5. Fourlanos S, Dotta F, Greenbaum CJ, Palmer JP, Rolandsson O, Colman PG, Harrison C: Latent autoimmune diabetes in adults (LADA) should be less latent. *Diabetologia* 48:1960–1967, 2005
6. Kobayashi T, Tamemoto Nakanishi K, Kato N, Okubo M, Kajio H, Sugimoto T, Murase T, Kosaka K: Immunogenetic and clinical characterization of slowly progressive IDDM. *Diabetes Care* 16:780–788, 1993
7. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA: Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to Glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet Med* 11:299–303, 1994
8. Juneja R, Palmer JP: Type 1 ½ diabetes: myth or reality? *Autoimmunity* 29:65–83, 1999
9. Pozzilli P, Di Mario U: Autoimmune diabetes not requiring insulin at diagnosis (latent autoimmune diabetes of the adult): definition, characterization, and potential prevention. *Diabetes Care* 24:1460–1467, 2001
10. Falorni A, Gambelunghe G:, Forini F, Kassi G, Cosentino A, Candeloro P, Bolli GB, Brunetti P, Calcinaro F: Autoantibody recognition of COOH-terminal epitopes of GAD65 marks the risk for insulin requirement in adult-onset diabetes mellitus. *J Clin Endocrinol Metab* 85:309–316, 2000
11. Maioli M, Alejandro E, Tonolo G, Gilliam LK, Bekris L, Hampe CS, Obinu DA, Manconi A, Puddu L, Lynch K, Lernmark Å: Epitope-restricted 65-kilodalton glutamic acid autoantibodies among new-onset Sardinian type 2 diabetes patients define phenotypes of autoimmune diabetes. *J Clin Endocrinol Metab* 89: 5675–5682
12. Kobayashi T, Tanaka S, Okubo M, Nakanishi K, Murase T, Lernmark Å: Unique epitopes of glutamic acid decarboxylase autoantibodies in slowly progressive type 1 diabetes. *J Clin Endocrinol Metab* 88:4768–4775, 2003
13. Bottazzo GF, Bosi E, Cull CA, Bonifacio E, Locatelli M, Zimmet P, Mackay IR, Holman RR: IA-2 antibody prevalence and risk assessment of early insulin requirement in subjects presenting with type 2 diabetes (UKPDS 71). *Diabetologia* 48:703–708, 2005
14. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley BJ, Eisenbarth GS: Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: combinatorial islet autoantibody workshop. *Diabetes* 47:1857–1866, 1998
15. Bingley PJ, Bonifacio E, Mueller P, participating laboratories: Diabetes antibody standardization program: first assay proficiency evaluation. *Diabetes* 52:1128–1136, 2003
16. Tiberti C, Verrienti A, Fiore B, Yu L, Eisenbarth GS, Dotta F, Di Mario U: IA-2 combined epitope assay: a new, highly sensitive approach to evaluate IA-2 humoral autoimmunity in type 1 diabetes. *Clin Immunol* 115:260–267, 2005
17. Kawasaki E, Sera Y, Fujita N, Yamauchi M, Ozaki M, Abe T, Yamakawa K, Uotani S, Takino H, Yamasaki H, Yamaguchi Y, Uchigata Y, Matsuura N, Eguchi K: Association between IA-2 autoantibody epitope specificities and age of onset in Japanese patients with autoimmune diabetes. *J Autoimmun* 17:323–331, 2001
18. Buzzetti R, Di Pietro S, Giaccari A, Petrone A, Locatelli M, Suraci C, Capizzi M, Arpi ML, Dotta F, Bosi E, for the NIRAD Study Group: High titre of autoantibodies to GAD identifies a specific phenotype of adult-onset autoimmune diabetes. *Diabetes Care* 30:932–938, 2007
19. American Diabetes Association: Diagnosis and classification of diabetes mellitus. *Diabetes Care* 29:S43–S48, 2006
20. Park YS, Kawasaki E, Kelemen K, Yu L, Schiller MR, Rewers M, Minuta M, Eisenbarth GS, Hutton JC: Humoral autoreactivity to an alternatively spliced variant of ICA512/IA-2 in type 1 diabetes. *Diabetologia* 43:1293–1301, 2000
21. Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark Å: A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 37: 344–350
22. Erlich H, Bugawan T, Begovich AB, Scharf S, Griffith R, Saiki R, Higuchi R, Walsh PS: HLA-DR, DQ and DP typing using PCR amplification and immobilized probes. *Eur J Immunogenet* 18:33–55, 1991
23. Buzzetti R, Galgani A, Petrone A, Del Buono ML, Erlich HA, Bugawan TL, Lorini R, Meschi F, Multari G, Pozzilli P, Locatelli M, Bottazzo G, Di Mario U: Genetic prediction of type 1 diabetes in a population with low frequency of HLA risk genotypes and low incidence of the disease (the DIABFIN Study). *Metab Res Rev* 20:137–143, 2004
24. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E: Identification of protein tyrosine phosphatase-like (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 155:5419–5426, 1995
25. Lan MS, Wasserfall C, Maclaren NK, Notkins AL: IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc Natl Acad S USA* 93:6367–6370, 1996
26. Bingley BJ, Bonifacio E, Williams AJK, Genovese S, Bottazzo GF, Gale EAM: Prediction of IDDM in the general population: strategies based on combination of autoantibody markers. *Diabetes* 46:1701–1710, 1997
27. Hawa M, Rowe R, Lan MS, Notkins AL, Pozzilli P, Christie MR, Leslie RDG: Value of antibodies to islet protein tyrosine phosphatase-like molecule in predicting type 1 diabetes. *Diabetes* 46:1270–1275, 1997
28. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, Chase HP, Eisenbarth GS: Prediction of type 1 diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 45:926–933, 1996
29. Ellis TM, Schatz DA, Ottendorfer EW, Lan MS, Wasserfall C, Salisbury PJ, She JX, Notkins AL, Maclaren NK, Atkinson MA: The relationship between humoral and cellular immunity to IA-2 in IDDM. *Diabetes* 47:566–569, 1998
30. Dotta F, Dionisi S, Viglietta V, Tiberti C, Matteoli MC, Cervoni M, Bizzarri C, Marietti G, Testi M, Multari G, Lucentini L, Di Mario U: T-cell mediated autoimmunity to the IA-2 islet tyrosine phosphatase in type 1 diabetes mellitus. *Eur J Endocrinol* 141:272–278, 1999
31. Lampasona V, Bearzatto M, Genovese S, Bosi E, Ferrari M, Bonifacio E: Autoantibodies in insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosine phosphatase-like IA-2 autoantigen. *J Immunol* 157:2707–2711, 1995
32. Zhang B, Lan MS, Notkins AL: Autoantibodies to IA-2 in IDDM. *Diabetes* 46:40–43, 1997
33. Kawasaki E, Yu L, Rewers MJ, Hutton JC, Eisenbarth GS: Definition of multiple ICA/512 phogrin autoantibody epitopes and detection of intermolecular epitope spreading in relatives of patients with type 1 diabetes. *Diabetes* 47:733–742, 1998
34. Naserke HN, Ziegler AG, Lampasona V, Bonifacio E: Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes. *J Immunol* 161:6963–6969, 1998
35. Seissler M, Schlott N, Morgenthaler G, Sherbaum WA: Mapping of novel autoreactive epitopes of the diabetes-associated autoantigen IA-2. *Clin Exp Immunol* 122:157–163, 2000
36. Kolm-Litty V, Berlo S, Bonifacio E, Bearzatto M, Engel AM, Christie M, Ziegler AG, Wilde T, Endl J: Human monoclonal antibodies isolated from type 1 diabetes patients define multiple epitopes in the protein tyrosine phosphatase-like IA-2 antigen. *J Immunol* 165:4676–4684, 2000
37. Falorni A, Brozzetti A: 2005 Diabetes-related antibodies in adult diabetic patients. *Best Pract Res Clin Endocrinol Metab* 19:119–133, 2005
38. Gambelunghe G, Forini F, Laureti S, Murdolo G, Toraldo G, Santeusanio F, Brunetti P, Sanjeevi CB, Falorni A: Increased risk for endocrine autoimmunity in Italian type 2 diabetic patients with GAD65 autoantibodies. *Clin Endocrinol* 52:565–573, 2000
39. Palmer JP, Hampe CS, Chiu H, Goel A, Brooks-Worrell BM: Is latent autoimmune diabetes in adults distinct from type 1 diabetes or just type 1 diabetes at an older age? *Diabetes* 54:S62–S67, 2005
40. Goel A, Chiu H, Felton J, Palmer JP, Brooks-Worrell B: T-cell responses to islet antigens improves detection of autoimmune diabetes and identifies patients with more severe β-cell lesions in phenotypic type 2 diabetes. *Diabetes* 56:2110–2115, 2007
41. Di Lorenzo TP, Peakman M, Roep BO: Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes. *Clin Exp Immunol* 148:1–16, 2007