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IMMUNOLOGIC AND GENETIC MARKERS IN INSULIN-DEPENDENT DIABETES MELLITUS (TYPE 1) IN AN ARGENTINE POPULATION

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Abstract The objective was to evaluate the prevalence and association of several markers (islet cell antibodies: ICA, ainsulin autoantibodies: IAA, glutamic acid decarboxylase antibodies: GADA and ICA512 antibodies: ICA512A) along with HLA DQB1 genotype in type 1 diabetes mellitus of recent onset, including siblings and individuals without any history of this disease, in an Argentine population. A total of 79 children with type 1 diabetes mellitus of recent onset were studied, as well as 79 control children, and 68 healthy siblings of type 1 diabetic cases. IAA, ICA, GADA, ICA512A and HLA DQB1 alleles were determined. Sensitivity was 67.1% for ICA, 36.7% for IAA, 74.6% for GADA and 63.4% for ICA512A. None of the control subjects was positive for the immunological markers. Combined sensitivity of ICA-IAA-GADA was 89.8%, similar to the ICA512A- GADA (87.3%) or ICA512A-GADA-IAA combination (91.1%). GADA correlated positively with ICA, but no such correlation was found between IAA, ICA512A and ICA. IAA correlated negatively and GADA positively with age. IAA was associated to DQB1*0201, whereas ICA and ICA512A associated to DQB1*0302. Among siblings, 3/68 (4.4%) were positive for IAA and a single case (1.5%) was positive for GADA and one for ICA512A. Our findings show that the combination of multiple tests increases the sensitivity for prediction, with the ICA512A-GADA combination proving highly sensitive and equivalent to other proposed combinations, such as ICA-IAA-GADA.

Key words: type 1 diabetes mellitus, autoantibodies, ICA, insulin autoantibodies, glutamic acid decarboxylase antibodies, ICA512 antibodies, HLA.

Resumen ***Estudio argentino de marcadores inmunológicos y genéticos en diabetes mellitus insulino dependiente (tipo 1).*** El objetivo fue evaluar la prevalencia y asociación de los marcadores inmunológicos (anticuerpo anti-islote pancreático: ICA, autoanticuerpo anti-insulina: IAA, anticuerpo anti-decarboxilasa del ácido glutámico: GADA y anticuerpo anti ICA512: ICA512A) con el genotipo HLA DQB1en diabetes tipo 1 de reciente debut, hermanos de diabéticos y personas sin historia de enfermedad autoinmune en población argentina. Se estudiaron 79 niños con diabetes tipo 1 de reciente debut, 79 niños controles y 68 hermanos sanos de niños con diabetes tipo 1. En todos ellos se determinaron IAA, GADA, ICA, ICA512A y alelos HLA DQB1. La sensibilidad para ICA fue de 67.1%, para IAA de 36.7%, para GADA de 74.6% y para ICA512A de 63.4%. Ninguno de los niños control presentó marcadores inmunológicos positivos. La sensibilidad combinada de ICA- IAA- GADA fue de 89.8%, similar a la de ICA512A - GADA (87.3%) a la combinación de ICA512- GADA-IAA (91.1%). El valor de GADA presentó correlación positiva con el de ICA, no encontrándose correlación alguna entre los valores de IAA, ICA512 A e ICA. El valor de IAA presentó correlación negativa y el de GADA positiva con la edad de los pacientes. La presencia de IAA se asoció con DQB1 *0201, mientras que la de ICA e ICA512A con DQB1 *0302. Entre los hermanos, 3/68 (4.4%) fueron positivos para IAA, uno (1.5%) lo fue para GADA y otro para ICA512A. Nuestros resultados muestran que la combinación de múltiples marcadores incrementa la sensibilidad de predicción, siendo la asociación ICA512A GADA altamente sensible y equivalente a otras combinaciones propuestas como ICA-IAA-GADA.

Palabras clave: diabetes mellitus tipo 1, autoanticuerpos, ICA, anti-insulina, anti-ácido glutámico decarboxilasa, anti-ICA512, HLA

Type 1 diabetes mellitus is one of the greatest challenges in public health and one of the most frequent chronic diseases in the pediatric age. In our country, data from the Province of Buenos Aires for 1985-1990 show a mean annual incidence of 6.66 per 100,000 children under 15 years of age[1].

The capability to predict the disease has allowed therapeutic intervention with diverse strategies in an attempt to prevent its development[2, 3]. The most recent work recommends using combinations of multiple

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markers to improve sensitivity when employed for screening purposes. These include islet-cell antibodies (ICA), autoantibodies to ICA512/IA-2 (ICA512A), glutamic acid decarboxylase antibodies (GADA) and insulin autoantibodies (IAA).

The general goal of the present work was to establish guidelines for our prediction programs in first-degree relatives of patients with type 1 diabetes in Argentina, as a stage prior to the potential application of prevention programs.

Specific objectives were to determine the sensitivity and specificity of the group of early markers of autoimmune beta cell aggression and their products, ICA, IAA, GADA and ICA512A, as well as to identify associated HLA DQB1 genotypes in our population; to establish the combined sensitivity of the 4 immunological markers and their association with genetic data in the diabetic populations and their siblings.

Material and Methods

Population: studied diabetic patients comprised 79 children and adolescents, 40 males and 39 females, consulting at the Service of Nutrition, J. P. Garrahan National Pediatrics Hospital, admitted from June 1994 to July 1996 with recent onset of their disease. Serum and blood samples were collected from all patients within 72 hr after initiation of insulin treatment. Mean age (± SD) was 10.23 ± 3.6 years, ranging from 0.6 to 19 years. Type 1 diabetes was diagnosed according to WHO criteria[4] that defines this form of diabetes with permanent insulinopenia prone to ketoacidosis, result from a cellular-mediated autoimmune destruction of the beta cells of the pancreas. As control subjects, a total of 79 children and adolescents were studied, matched by sex and age with the diabetic patients, none of whom had either a personal or family history of diabetes or other autoimmune pathologies. Sixty eight healthy siblings of type 1 diabetic cases, 44 females and 24 males, mean age 10.7 ± 5.3 years, ranging from 1.7 to 21 years, were also studied. Samples of serum and blood were taken for immunological and HLA allele typing tests, respectively. The ethnicity of our population was mainly Caucasian.

The present study was approved by the Teaching and Research Committee and the Ethics Committee of the hospital. Each family provided informed consent before samples were collected.

Immunological studies

ICA was determined by indirect immunofluorescence (IIF). Determinations were carried out by using international standards of known Juvenile Diabetic Foundation Units (JDFU).

GADA, IAA and ICA512A were determined by reference radiobinding assays (RBA). The GADA results were expressed as GAD index regarding an international reference serum[5], the IAA results were expressed as percentages of ^{125}I-labeled insulin binding and the ICA512A results were expressed as binding percentages regarding total counts (B%).

The ICA assay was controlled externally by an international proficiency test, in which our laboratory achieved the following scores: 75% sensitivity, validity, specificity and consistency (Immunology Diabetes Workshop, University of Florida, USA, 1993).

RBA procedures for single-antigen IAA, GADA and ICA512A were controlled externally by international proficiency tests, in which our laboratory achieved the following scores: 82% sensitivity, 82% validity, and 100% specificity and consistency for IAA (Immunology Diabetes Workshop, University of Florida, USA, 1996); 100% sensitivity, validity, specificity and consistency for GADA in the third (1998) and fourth (1999) GADA Proficiency Tests (Research Institute for Children, Harahan LA) and 100% sensitivity, validity, specificity and consistency for ICA512A in the third IA-2 Proficiency Test (Research Institute for Children, Harahan LA, 1999).

The DNA locus of HLA DQB1 genotype was typed using the Polymerase Chain Reaction (PCR) and Sequence-Specific Oligonucleotide (SSO) probes[6].

Statistical methods

Mean values, ranges and standard deviations were obtained for normally distributed values. Categorical variables were compared by means of the Chi square test. For values lower than 5 the Fisher test was performed. The Spearman rank test with a significance level lower than 0.05 was used for correlations.

Results

Immunological markers and HLA DQB1 typing prevalence from patients, siblings and controls are presented in Table 1. Since no control sample proved positive for any marker, specificity was 100% throughout.

Sensitivity of combined markers

In the diabetic population, 71 / 79 patients were positive to at least one marker when the ICA-IAA-GADA

TABLE 1.- Immunological markers and HLA DQB1 allele prevalence

	Diabetic Patients % n=79	Siblings % n=68	Controls % n=79
ICA	67.0	0	0
IAA	36.7	4.4	0
GADA	74.6	1.5	0
ICA512A	63.4	1.5	0
Aspartic 57neg/neg	76.9	67.5	-
Aspartic 57neg/pos	23.1	30.0	-
Aspartic 57 pos/pos	0	12.5	-
Alleles *0302/other	81	53.7	-
Alleles *0201/other	55	41	-
Alleles *0302/*0201	44.0	9.8	-
Alleles *0602/other	0	7.3	-

Note: ICA: islet cell antibodies, IAA: insulin autoantibodies, GADA: glutamic acid decarboxilase antibodies, ICA512A: ICA512 antibodies

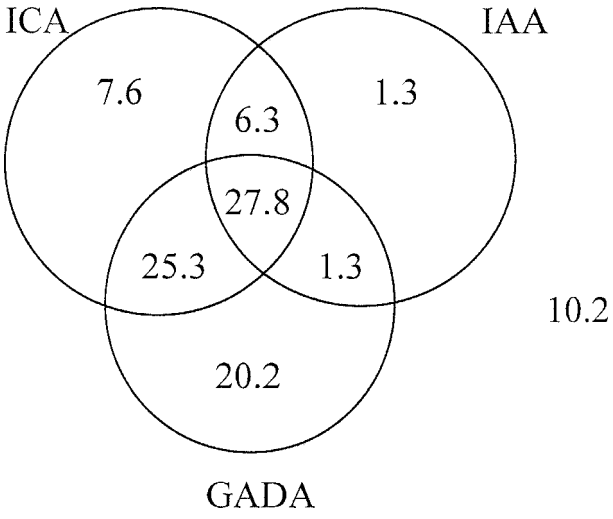

Fig. 1.— Percentage antibody frequency in an Argentine diabetic population (ICA, IAA, GADA and their combinations)

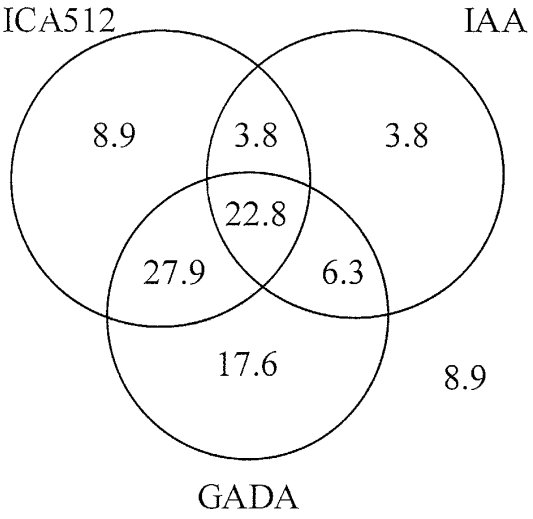

Fig. 2.— Percentage antibody frequency in an Argentine diabetic population (ICA512A, IAA, GADA and their combinations)

combination was used, so that these three autoantibodies jointly presented 89.8% sensitivity (Figure 1).

The combination of ICA512A and GADA proved positive for 69 (87.3%) patients, with a sensitivity equivalent to that of ICA-IAA-GADA (Figure 2). When the combination ICA512A-IAA-GADA was used, 72 of the patients presented at least one marker, with 91.1% sensitivity (Figure 2). The presence of multiple immunological markers was quite frequent in the diabetic population: 48 (60.7%) patients presented 2 or more markers, versus 24 (30.3%) patients who only presented a single marker. According to the autoantibodies employed, 5 (6.3%) diabetic patients were negative for all 4 markers.

As indicated in Table 1, 3/68 (7.4%) of the siblings of type 1 diabetic patients exhibited a positive marker, but there were no cases with muliple positive markers.

With regard to age, there were no differences in ICA512A prevalence. No correlation was found between values and subject ages. IAA tended to be more prevalent among the population under 5 years of age (50%) vs children aged 5 to 10 (34%) and those over 10 years (35%), although differences lacked statistical significance. IAA values presented a significant negative correlation with age ($p < 0.05$). GADA was more frequent among those over 10 years of age (84%), although the difference was not statistically significant vs those under 5 years (60%), nor vs those between 5 and 10 years (65.6%). However, the GAD index presented a significant positive correlation with patient age. The ICA marker also tended to be more frequent in children over 5 and 10 years of age (70 and 67%, respectively) vs those under 5 years (55%), though such differences lacked statistical significance. However, ICA values greater than 20 JDFU correlated positively with ages ($p < 0.05$).

When the correlation among markers was studied, the values of GAD index showed a significant linear correlation with those of ICA in JDFU ($p < 0.03$; $r = 0.24$). Such positive correlation was maintained for all ICA values, including those exceeding 20 JDFU ($p < 0.002$; $r = 0.44$). No significant correlation was found between ICA512A or IAA and ICA titers.

Associations between HLA and markers

Among diabetic patients, IAA was associated more frequently with DQB1*0201 than with DQB1*0302 (67% vs 59% for both alleles), while ICA and ICA512A presented closer association with DQB1*0302 (80% and 81%, respectively) than with DQB1*0201 (55% and 78%, respectively). The presence of at least two immunological markers was more frequently associated with DQB1*0302 (82%) than with DQB1*0201 (56%). Heterozygous patients presented greater positive frequency than homozygous ones for ICA (99% vs 69%), GADA (91% vs 50%), IAA (65% vs 25%) and ICA512A (86% vs 50%). In addition, the DQB1*0302/*0201 genotype presented greater frequency than other genotypes in patients positive for GADA (92% vs 80%), IAA (67% vs 53%) and ICA512A (82% vs 80%) markers. Heterozygosis was associated with the presence of multiple markers (87% vs 75%), whereas among homozygous subjects the presence of a single immunological marker was more frequent (25% vs 5%).

Two out of the 68 siblings with positive markers presented the DQB1*0201/0302 genotype: one was

homozygous for DQB1*0201 and the other for DQB1*0302.

Discussion

The main goal was to establish the methodological basis to predict the population at risk within our mainly Caucasian ethnic group and to determine the minimal set of tests potentially applicable to routine screening or in larger predictive programs. As a preliminary test, genetic risk and the presence of antibodies were studied in the population at risk comprising, in this phase of the study, 68 healthy siblings of the type 1 diabetic patients. The sensitivity for ICA was 67%, a value that may be ascribed to the appreciable number of borderline serum samples considered to be negative. We think that our pancreatic sections influence assay results, causing differences in detection limits. In this case, like in the proficiency control, our group privileged the specificity of the tests even in detriment of the sensitivity. IAA presented a sensitivity of 36.7%, exhibiting values somewhat lower than those reported by other laboratories[7]. For GADA and ICA512A the sensitivity recorded in our population was 74.6 and 63.4%, respectively, in agreement with findings described in the literature[8-15].

The frequency of HLA DQB1 Asp 57 (-/-) alleles in our group with type 1 diabetes (76.9%), as well as in siblings (67.5%), was remarkably high, since previous studies in healthy Argentine populations have found 19% prevalence of such alleles[16]. Allele DQB1*0302 presented greater prevalence both in diabetic patients (81%) and in siblings (53.7%) than DQB1*0201 (55% in diabetic patients and 41% in siblings), in agreement with the high prevalence of the former allele in Swedish[9] and Belgian populations[17]. This suggests that despite the wide differences in the incidence of type 1 diabetes mellitus throughout the world, the genetic risk conferred by HLA seems quite uniform. Forty-four of our diabetic patients presented the high risk heterozygous genotype DQB1*0302/0201, whose prevalence proved much greater than that described for other diabetic populations, in which it ranges from 29% to 39%[9, 17]. Allele DQB1*0602 was not present in our diabetic patients, but was detected in heterozygosis in 7.3% of siblings. Since such allele is strongly though negatively associated with type 1 diabetes[18, 9], it is considered as a protector for the disease. Based on these data, Trucco et al. proposed limiting HLA identification to DQB1*0302 or *0201 (though not *0602), associated to DQA1*0501 or *0301 as a first step in prediction[19], while the ongoing Diabetes Prediction Trial (DPT-1) uses for screening the protective allele as a single genetic marker.

Our results analyzing multiple immunological markers confirm that it is the combination of autoantibodies rather than the presence of any one in particular that increases the sensitivity for diagnostic support of diabetes mellitus mediated by autoimmunity. The analysis of antibody combinations demonstrated that 60.7% of our diabetic patients presented more than one immunological marker, while 30.3% were only positive for a single marker, which agrees with the widely-known fact[20, 21] that the antibodies present in diabetes are in response to multiple antigens released during beta cell destruction.

In our experience, the IAA-GADA-ICA512A combination exhibited greater sensitivity (91.1%) than that of ICA, IAA and GADA (89.8%). This coincides with the findings of Verge et al. in 1996[22], who documented greater risk of type 1 diabetes mellitus with a growing number of markers, regardless of ICA. More recently, when studying the combined sensitivity of the ICA512A-IAA-GADA combination, Feeney et al.[15] concluded that the inclusion of a positive ICA result failed to increase the positivity significantly. In our study, ICA512A-GADA combination sensitivity (87.3%) proved practically equivalent to that of the ICA-GADA combination (88.5%). On the basis of similar results, Seissler[23] proposed that the ICA512A-GADA combination could replace ICA. Likewise, Yokota et al.[20] detected 93% sensitivity for the ICA512A-GADA combination in the Japanese population. The high sensitivity obtained with the ICA512A-GADA-IAA combination allows ICA to be excluded, particularly considering that the IIF method suffers from numerous limitations. Accordingly, on the basis of obtained results we agree with other authors in that the combination of 2 or 3 markers (GADA and ICA512A with or without IAA) affords an advantageous alternative to replace ICA[9, 10, 13, 22].

Concerning age, the higher frequency of GADA and/or ICA in postpuberal versus prepuberal patients has been amply documented[8, 14, 15, 24, 25]. In our population we confirmed this finding in the subgroup over 10 years with a positive correlation between GAD index and/or ICA exceeding 20 JDFU versus age. In agreement with numerous reports[8, 15, 24], IAA proved more frequent among patients under 5 years and values tended to correlate negatively with increasing age, though no statistical significance was found. Our confirmation of the results, suggests that for predictive purposes the age group under study should be kept in mind when selecting markers.

As in numerous previous studies[8, 11, 23, 24], we found positive correlation between GADA and ICA values. This finding suggests that GADA and other markers could represent distinct ICA subgroups or a simultaneous immune response against several antigens of the pancreatic islet, detected jointly as ICA[25]. In our study, we failed to confirm a statistically significant correlation between ICA512A and ICA values, as described by Seissler[23], Gorus[12] and others. When the association between immunological and genetic markers was

analyzed, Hagopian[9] reported an association between IAA and DQB1*0302. However, in our population, such association could not be discerned, but instead a close correlation of IAA with DQB1*0201 was documented. On the other hand, ICA and ICA512A associated with DQB1*0302, in agreement with findings in other populations by Gorus[12] and Vandewalle[17].

Lastly, the DQB1*0302/0201 genotype presented positive association with GADA, IAA and ICA512A antibodies. Asp 57 (+/-) associated closely with the presence of each one of the antibodies separately and with multiple antibody positivity, while Asp 57 (-/-) subjects presented greater prevalence of a single positive marker, which coincides with findings in a Finnish population by Akerblom[11].

To sum up, the goal of the present work was to provide sensitivity and specificity data of immunological and genetic markers in an Argentine population of type 1 diabetic patients selected by means of a panel of 4 markers and HLA DQB1 typing. Our findings demonstrate that a combination of 2 (GADA and ICA512A) or 3 (GADA, ICA512A and IAA) markers affords the basis for systematic prediction studies as the first indispensable step for potential prevention protocols applicable to our community.

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