doi:10.1006/jaut.2001.0577, available online at http://www.idealibrary.com on IDEAL® Journal of Autoimmunity (2002) **18,** 191–196

ICA512(IA-2) Epitope Specific Assays Distinguish Transient from Diabetes Associated Autoantibodies

Dongmei Miao, Liping Yu, Claudio Tiberti[1], David D. Cuthbertson[2], Marian Rewers, Umberto Di Mario[1], George S. Eisenbarth and Francesco Dotta[1]

Barbara Davis Center for Childhood Diabetes, University of Colorado, Denver
[1]Department of Medical Sciences, Endocrinology, University 'La Sapienza', Rome
[2]H. Lee Moffitt Cancer Center and Research Institute, University of South Florida

Received 9 August 2001
Accepted 27 November 2001

Key words: ICA512(IA-2), autoantibodies, epitopes, radioassay, diabetes

ICA512/IA-2, a tyrosine phosphatase-like protein, is one of the major autoantigens in type 1 diabetes. Following phage display characterization of ICA512 autoantigenic epitopes, we developed fluid phase autoantibody radioimmunoassays for a series of ICA512 fragments (F1 [amino acids (aa): 761–964], F2A [aa 256–760], F2B [aa 761–928], and F2C [aa 929–979]). With the hypothesis that 'non-diabetes associated' ICA512 autoantibodies would differ from diabetes associated ICA512 autoantibodies in terms of epitopes recognized, we analyzed ten such serum samples (two from normal control individuals, one from a general population subject with transient ICA512 autoantibodies and seven from relatives of patients with type 1 diabetes who had single transient ICA512 positivity). All but one of the 'non-diabetes associated' ICA512 positive samples (9/10) did not react with Fragment 1 which contains the major antigenic epitopes of the molecule that were recognized by almost all (51/52) ICA512 positive new onset patient samples and pre-diabetic relatives ($P<10^{-6}$). The great majority of samples (44/52) from the new onset patients and pre-diabetic relatives reacted with at least two fragments and 60% (31/52) with three or more fragments. In contrast, only one sample of the ICA512 'non-diabetes associated' sera reacted with multiple fragments ($P<10^{-4}$). Our findings suggest that diabetes associated anti-ICA512 autoantibodies react with multiple ICA512 epitopes while non-diabetes associated ICA512 autoantibodies may usually represent reactivity of antibodies with determinants of ICA512 unrelated to type 1 diabetes. The ability to distinguish diabetes associated from non-diabetes associated anti-ICA512 autoantibodies should provide prognostic information and more importantly suggests that even with highly specific radioassays positivity may occur unrelated to type 1 diabetes.

Introduction

Multiple autoantigens are targets of the autoimmune response in type 1 diabetes. One of these is the islet antigen ICA512 (IA-2) [1, 2], a transmembrane glycoprotein localized in dense core secretory granules of peptide-secreting endocrine cells and neurons [3] that belongs to the tyrosine phosphatase-like protein family. ICA512/IA2 autoantibodies (ICA512AA) are present in patient sera months to years before the diagnosis of type 1 diabetes and about 60-70% of newly diagnosed patients and prediabetic relatives are positive [4, 5]. ICA512AA are predictive of type 1 diabetes in first-degree relatives and in the general population [4–11]. With binding and competition analysis using multiple chimeric ICA512/ IA-2β constructs (another member of the protein tyrosine phosphatase family), we and others found that major epitopes of ICA512 are located in the carboxy-terminus of the molecule, protein tyrosine phosphatase (PTP) domain, and an epitope located within the juxtamembrane domain [12, 13]. The epitopes in the PTP domain of ICA512 were recently further characterized with human monoclonal autoantibodies isolated from the peripheral blood of the newly diagnosed patients with type 1 diabetes [14]. The peripheral T-cell responses to the ICA512 molecule from the patients and the normal controls with HLA-DR*0401 and/or DQ*0302 have been studied by the several laboratories and indicated that the PTP domain contains several major T-cell epitopes [15–17].

Two different constructs (naturally occurring, alternative splice variants) are often used for the determination of ICA512 (IA-2) autoantibodies in international workshops (ICA512bdc, amino acids

Correspondence to: George S. Eisenbarth, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 4200 East 9th Avenue, B-140, Denver, CO 80262. Tel: (303)315-4891; Fax: (303)315-4892; E-mail: george.eisenbarth@uchsc.edu

0896–8411/02/020191+06 $35.00/0

256–556:630–979, and ICA512ic, amino acids 605–979). Using both constructs in a DPT-1 ancillary study [18], no difference in overall positivity was found in analysis of 2,151 relatives though both constructs detected a small number samples not detected by the other construct (7/2,151 ICA512bdc+/ICA512ic−; 7/2,151 ICA512ic+/ICA512bdc−). In the current study the ICA512bdc construct was utilized for initial screening for ICA512 autoantibodies.

Recent studies indicated that some relatives of patients with type 1 diabetes and individuals from the general population can express transient anti-islet autoantibodies without known genetic risk factors for type 1 diabetes [19]. The sera with transient autoantibodies usually had low levels of antibodies reacting with a single islet antigen. Individuals with transient autoantibodies rarely expressed autoantibodies more than once. In present study, we applied ICA512 fragment assays to sera from individuals with transient ICA512 autoantibodies. The pattern of epitope recognition differs markedly from newly diagnosed type 1 diabetes patients and pre-diabetic relatives.

Research Design and Methods

Subjects

The 10 sera with transient ICA512 autoantibodies were collected from 10 subjects, seven relatives through DPT–1 (Diabetes Prevention Trials–Type 1) and DAISY (Diabetes Autoimmunity Study in the Young), one individual from the general population through DAISY, and two normal control subjects. All the subjects were confirmed to be positive for ICA512 autoantibodies by repeating the tests at least three times on different days and all converted to negative in later follow up except one control subject who was not re-tested. ICA512 autoantibodies occurred only on a single serum sample for all these individuals and they never expressed any other anti-islet autoantibody (GAD65 and insulin autoantibodies).

Forty-two sera of new onset patients with type 1 diabetes within 7 days of diagnosis and 10 sera from pre-diabetic relatives (mean time to diabetes=1.5 years, range from 0.3 to 3 years) were from the Barbara Davis Center and confirmed as ICA512 autoantibody positive. We also specifically included 13 new onset patients with low index values of ICA512 autoantibodies. Subjects gave informed consent to participate in the study.

Construction of ICA512 fragment cDNA (Figure 1)

Using the technology of screening bacteriophage cDNA expression libraries of the ICA512bdc clone (256–556:630–979) with positive patient sera, we identified [20] two major ICA512 epitope fragments: the Fragment 1 (aa 761–964) and the Fragment 2C (aa 929–979). These fragments were *in vitro* transcribed and translated and utilized in fluid phase radioassays. Fragment 1 (761–964) was recognized by virtually all ICA512bdc autoantibody-positive sera. Two new fragment constructs, Fragment 2A (aa 256–760) and the Fragment 2B (aa 761–928), were produced resulting in three non-overlapping fragments spanning the ICA512 molecule from aa 256 to the end of the molecule (aa 979). The fragments were created with PCR with full length ICA512 as a template followed by cloning and introduction of a start-codon (ATG), at the beginning of each fragment for *in vitro* translation except for Fragment 2C (aa 929–979) which has a natural methionine in position 929. Dr. Ezio Bonifacio kindly provided the cDNA construct of the intracellular domain of ICA512 (ICA512ic, aa 605–979). All fragment constructs of ICA512 used in the present study are summarized in Figure 1.

Autoantibody radioimmunoassays

Serum samples were stored at −20°C before testing. ICA512 and all its fragment peptides were produced by *in vitro* transcription/translation technique (Promega) and the products were labeled with ^{35}S-methionine during *in vitro* translation. Autoantibody reactivity was measured in duplicate using an assay format as described previously [21]. The inter-assay coefficient of variation is 10% and the intra-assay coefficient of variation 5% for the ICA512 autoantibody assay. The intra-assay coefficient of variation for Fragment 1, 2A, 2B, and 2C are 4%, 9%, 5%, and 3%, respectively. The cut-off for positivity was defined as the 99th percentile of 198 healthy controls for ICA512bdc and ICA512ic assays and the 99th percentile of 100 healthy controls for ICA512 fragment assays. The index at the 99th percentile for each assay is listed in the Table 1. Index is calculated as (cpm of sample−cpm of negative control)/(cpm of positive control−cpm of negative control). In the Immunology of Diabetes Society's Combined Autoantibody Workshop of 1995, sensitivity (for diabetes at less than age 30 years) for ICA512AA assay was 73% and specificity 100%.

Statistical analysis

Categorical variables were analyzed using Fisher's exact tests. All tests were two-sided, using a significance level of 0.05. Statistical analyses were performed utilizing True Epistat (Round Rock, Richardson, TX).

Results

As previously published autoantibody assays using either ICA512bdc or ICA512ic gave discordant results in less than 10% of new onset patients and prediabetic relatives. For the present 52 new onset patients and pre-diabetic relatives selected to be ICA512bdc positive, five were negative for ICA512ic. All samples

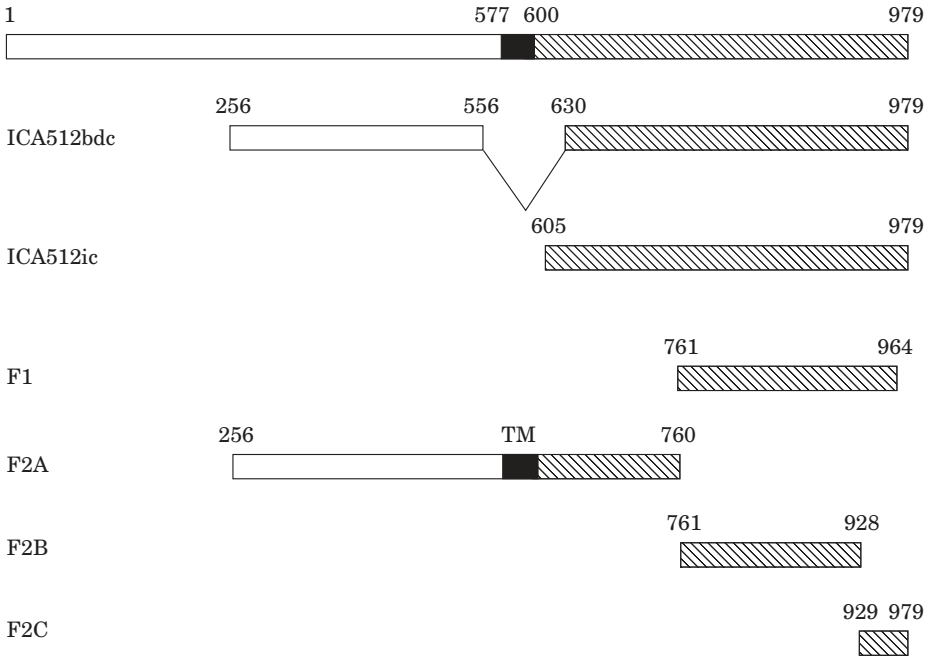

Figure 1. The maps of all fragment constructs of ICA512 used in the present study. TM=transmembrane domain.

except one from new onset patients (41/42) or pre-diabetic relatives (10/10) were positive using Fragment 1 (aa 761–964). Fragment 1 contains 70% of the PTP domain (aa 687–979). The mini Fragment 2C (929–979) contains only 51 amino acids of the PTP domain C-terminus. Seventy-six percent (32/42) of serum samples from new onset patients and 80% (8/10) of the pre-diabetic relatives that were positive with ICA512bdc construct reacted with Fragment 2C. Fragment 2A (256–760) which contains the juxtamembrane region had the lowest sensitivity (16/42 positive for new onset patients and 3/10 positive for pre-diabetic relatives). Interestingly, nine samples positive for Fragment 1 were negative for both Fragments 2B and 2C, although the latter two fragments overall cover the whole length of Fragment 1.

Levels of autoantibodies (index) of ICA512 positive sera from individuals with transient ICA512 autoantibodies and the patient samples are listed in Table 1. Amongst the 10 transient and 'control' ICA512 positive samples, 9/10 did not react with any PTP domain fragments including Fragments 1, 2B, and 2C. Three ICA512 transient positive samples reacted with the Fragment 2A, which contains the juxtamembrane region and did not react with other fragments. Only one transient positive sample reacted with the PTP fragments and it was positive with multiple fragments. In contrast, 44/52 ICA512 positive samples from new onset patients and pre-diabetic relatives reacted with 2 fragments ($P<10^{-4}$) and only one sample (1/52) reacted with Fragment 2A alone, not reacting with the other fragments (PTP domain fragments). A summary of auto-reactivities to all the fragments between the ICA512 transient autoantibodies and the patient samples is summarized in the Table 2. Of note, Tables 1 and 2 include data on diabetic subjects specifically selected to have an ICA512bdc index below the highest transient subject. Similar to the whole group of 'diabetes associated' autoantibodies, 12/13 of these sera recognized Fragment 1 and 7/13 recognized multiple fragments.

Discussion

ICA512 (IA-2) autoantibodies are probably the most specific marker of type 1 diabetes currently available. We were able to find relatively few individuals with transient ICA512 autoantibodies with the screening more than 71,000 individuals of DPT for ICA512 autoantibodies. As expected from our prior studies, a subset of samples were ICA512bdc positive and ICA512ic negative. We believe it is likely that if we had screened with the ICA512ic variant, we would find ICA512ic positive and ICA512bdc negative samples as we have reported previously for analysis of approximately 2,100 DPT samples [18].

Major epitopes of ICA512 recognized by sera from type 1 diabetic patients and prediabetic relatives are located in the intracellular PTP domain of the molecule. Not surprisingly, almost all the samples of new onset patients and pre-diabetic relatives in the present study were positive for PTP domain fragments. From the current data, four ICA512 fragments were analyzed with three in the PTP domain represented by Fragment 1 and three non-overlapping fragments, Fragment 2A, 2B, and 2C. The largest PTP fragment, Fragment 1, remarkably gave near 100% positivity in diabetic and pre-diabetic patients (51/52) compared to only 1/10 of transiently positive subjects. In our additional preliminary studies, Fragment 1 detected as positive (>99th percentile) the majority of those samples giving non-concordant results for ICA512bdc

Table 1. *The results (index) of all fragment analysis for all samples. The numbers in bold are positive results*

Samples	Group	ICA512bdc	ICA512ic	F1	F2A	F2B	F2C
Cut-off		0.049	0.01	0.051	0.201	0.047	0.045
Control-1	Transient	**0.079**	**0.113**	0.019	**0.225**	−0.015	0.001
Control-2	Transient	**0.061**	**0.044**	0.013	**0.359**	−0.048	−0.005
Daisy-1	Transient	**0.156**	−0.005	−0.023	−0.010	−0.040	−0.006
Daisy-2	Transient	**0.071**	−0.003	−0.002	0.134	−0.030	−0.004
DPT-1	Transient	**0.136**	**0.036**	**0.058**	0.058	**0.133**	**0.242**
DPT-2	Transient	**0.110**	0.003	0.019	**0.384**	0.023	0.021
DPT-3	Transient	**0.164**	−0.001	−0.012	−0.055	0.006	0.001
DPT-4	Transient	**0.116**	0.012	0.037	0.079	0.026	0.043
DPT-5	Transient	**0.127**	−0.001	0.036	0.138	−0.005	−0.016
DPT-6	Transient	**0.267**	−0.001	0.009	0.129	0.027	−0.016
530636	new DM	**1.397**	**0.539**	**1.233**	**0.703**	**1.441**	**0.920**
530635	new DM	**1.532**	**0.845**	**0.933**	**0.390**	**0.623**	**0.841**
532189	new DM	**1.046**	**0.481**	**0.846**	**0.363**	**0.381**	**0.521**
532958	new DM	**0.908**	**0.842**	**0.757**	**0.540**	**0.747**	**0.583**
533691	new DM	**0.967**	**0.757**	**0.546**	**0.370**	**0.496**	**0.740**
533543	new DM	**0.928**	**0.781**	**0.812**	**0.854**	**0.682**	**0.832**
533437	new DM	**1.003**	**0.787**	**0.196**	**0.240**	**0.210**	**0.696**
532422	new DM	**1.215**	**0.780**	**0.809**	**0.431**	**0.066**	**0.088**
531914	new DM	**1.100**	**0.893**	**0.665**	**0.235**	**0.710**	**0.647**
530650	new DM	**1.134**	**0.993**	**0.923**	**0.738**	**0.730**	**0.829**
530637	new DM	**0.765**	**0.548**	**0.402**	0.099	**0.082**	**0.355**
531589	new DM	**1.660**	**0.889**	**1.116**	0.171	**0.232**	**0.396**
532949	new DM	**0.991**	**0.867**	**0.804**	0.116	**0.265**	**0.328**
532659	new DM	**1.340**	**0.984**	**0.557**	0.088	**0.667**	**0.794**
539866	new DM*	**0.074**	**0.042**	**0.275**	−0.083	**0.060**	**0.055**
542605	new DM*	**0.151**	**0.089**	**0.144**	−0.077	**0.256**	**0.048**
533997	new DM	**0.413**	**0.822**	**0.638**	0.004	**0.261**	**0.432**
531365	new DM	**1.573**	**0.767**	**1.031**	0.047	**0.197**	**0.495**
532711	new DM	**0.722**	**0.661**	**0.517**	−0.019	**0.145**	**0.386**
533892	new DM	**0.347**	**0.553**	**0.580**	−0.030	**0.070**	**0.337**
531764	new DM	**1.596**	**0.592**	**1.058**	**0.345**	0.012	**0.085**
530146	new DM*	**0.084**	0.004	**0.178**	**0.255**	−0.006	**0.253**
533935	new DM	**0.777**	**0.830**	**0.366**	**0.519**	−0.012	**0.202**
530387	new DM	**1.478**	**0.630**	**0.827**	0.032	0.025	**0.161**
530478	new DM	**1.326**	**0.558**	**0.662**	0.134	0.008	**0.517**
531847	new DM	**1.123**	**0.099**	**0.931**	−0.047	−0.025	**0.147**
539722	new DM*	**0.214**	**0.223**	**0.581**	0.063	0.015	**0.066**
538709	new DM*	**0.175**	**0.390**	**0.505**	−0.097	−0.014	**0.093**
531920	new DM	**0.464**	**0.386**	**0.369**	0.061	0.033	**0.240**
532465	new DM	**0.107**	**0.132**	**0.108**	−0.027	0.015	**0.074**
532071	new DM	**0.307**	−0.004	**0.243**	−0.028	−0.032	**0.053**
533591	new DM	**0.375**	−0.002	**0.330**	−0.051	0.004	**0.063**
532995	new DM*	**0.252**	**0.192**	**0.808**	**0.246**	−0.026	0.004
532151	new DM	**0.666**	**0.241**	**1.607**	**0.793**	−0.035	−0.002
540381	new DM*	**0.140**	**0.053**	**0.304**	−0.141	**0.066**	0.006
539129	new DM*	**0.123**	**0.385**	**0.423**	−0.090	0.002	0.001
542932	new DM*	**0.169**	**0.056**	**0.223**	−0.078	−0.003	0.012
543213	new DM*	**0.137**	**0.309**	**0.393**	−0.062	−0.021	−0.005
543621	new DM*	**0.171**	**0.112**	**0.256**	−0.122	0.002	−0.005
543450	new DM*	**0.196**	**0.061**	**0.341**	0.016	−0.015	0.032
532158	new DM	**0.174**	0.001	**0.264**	−0.036	0.004	0.038
538261	new DM*	**0.089**	0.005	0.001	**0.292**	0.017	0.003
528287	pre DM	**1.148**	**1.459**	**1.118**	**0.809**	**1.545**	**1.515**
532099	pre DM	**0.865**	**1.068**	**1.055**	0.192	**0.379**	**0.671**
534052	pre DM	**0.634**	**0.376**	**0.828**	0.008	**0.773**	**0.904**
529275	pre DM	**0.832**	**0.553**	**1.069**	0.006	**0.676**	**0.908**
529055	pre DM	**0.462**	**0.338**	**0.886**	−0.041	**0.244**	**0.476**
527450	pre DM	**0.282**	**0.175**	**0.575**	0.002	**0.239**	**0.225**
527112	pre DM	**0.393**	**0.260**	**0.722**	−0.003	**0.141**	**0.320**
531751	pre DM	**0.466**	**1.048**	**0.957**	**1.757**	0.021	0.282
531166	pre DM	**0.541**	**0.541**	**0.946**	**0.367**	0.019	0.019
527407	pre DM	**0.547**	**0.438**	**0.768**	−0.048	−0.049	−0.034

*Serum samples from new onsets of type 1 diabetes specifically selected to have an ICA512bdc index below the highest of the transient subjects.

Table 2. *Autoantibody recognition of ICA512 epitope fragments*

Amino acid:	ICA512bdc 256–556 630–979	ICA512ic 601-979	Frag. 1 761–964	Frag. 2A 256–760	Frag. 2B 761–928	Frag. 2C 929–979	Fragments+ 1	>=2
Transient positive sera	10/10	3/10	1/10	3/10	1/10	1/10	4/10	1/10
New onset IDDM Sera	42/42	37/42	41/42	16/42	21/42	32/42	42/42	35/42
Pre-DM sera	10/10	10/10	10/10	3/10	7/10	8/10	10/10	9/10
P value	n.s.	0.0001	$<10^{-6}$	n.s.	0.01	0.0001	$<10^{-5}$	$<10^{-4}$

and ICA512ic constructs (Fragment 1 detected as positive 7/8 sera ICA512bdc+/ICA512ic− and 6/7 sera ICA512ic+/ICA512bdc−) and this fragment will be one of the most sensitive constructs for assessing ICA512 autoantibodies. Fragment 2C, which only contains the 51 C-terminal amino acids, retained nearly 80% of the sensitivity (40/52) of ICA512bdc and was similarly specific, present in only 1/10 of the transiently positive subjects. Most of the samples of new onset patients and pre-diabetic relatives reacted with two or more PTP domain epitopes. Fragment 2A which contains the juxtamembrane region had a lower sensitivity compared with the PTP domain fragments and when sera were positive with this fragment their indexes were relatively low. The true frequency of positivity for the juxtamembrane region epitope is likely to be slightly increased to that found since others have identified patients with antibodies only against juxtamembrane epitope and these may have been missed by selection of ICA512 positivity using the ICA512bdc clone.

Surprisingly, 9/10 ICA512 transient positive samples did not react with any of the three PTP domain fragments which contain major antigenic epitopes of the molecule and were recognized by almost all ICA512 positive sera (51/52) from new onset patient and prediabetic relative samples ($P<10^{-6}$). Three samples from ICA512 transient positive subjects reacted only with a single construct, Fragment 2A containing the juxtamembrane region sequences. The great majority of samples (44/52) from the new onset patients and prediabetic relatives reacted with at least two fragments and 60% (31/52) with three or more fragments. In contrast, only one ICA512 transient positive sample reacted with multiple fragments ($P<10^{-4}$) and most of the sera (7/10) were negative for ICA512ic molecule (aa 605–979) ($P=0.0001$). Reactivity with multiple fragments was not simply determined by the level of ICA512 autoantibodies. The level of ICA512 autoantibodies from the transient positive sample that reacted with multiple fragments was not the highest. Thirteen samples of new onset patients were specifically selected to have low levels of ICA512 autoantibodies. They were similar to those randomly selected ICA512 positive samples in that almost all reacted with PTP domain epitopes and most reacted with multiple fragments and thus again very different from the 'non-diabetes associated' samples. The majority of ICA512 transient autoantibodies, either from relatives or from the general population, bind to a single epitope and the epitope is not identical to major diabetes related epitopes. This suggests that transient ICA512 autoantibodies represent 'false positive' (non-diabetes associated) autoantibodies that bind to a single epitope of this islet autoantigen. Studies of the epitopes recognized by transient and persistent GAD65 and insulin autoantibodies should allow further testing of the hypothesis that transient 'autoantibodies' usually do not react with epitopes characteristic of diabetes related sera. With epitope specific radioassays, diabetes associated ICA512 autoantibodies may be distinguished.

Acknowledgements

Samples kindly provided from Diabetes Prevention Trial-1 and Diabetes Autoimmunity Study in the Young serum bank. This work was supported by NIH grants DK32083 and 5-M01-RR-00069 (Clinical Research Centers Program) from the NIH; JDF grant 1-1999-679; DK-57516 (DERC) autoimmunity center excellence and autoimmunity prevention center; and Telethon grant E660. Bayer Corporation provided ancillary funding for DPT 'biochemical' autoantibody analysis. The DPT-1 was supported through cooperative agreements by the Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK, NIH, and by NIAID, NICHD, and National Center for Research Resources, NIH; the American Diabetes Association; the Juvenile Diabetes Foundation International; and various corporate sponsors.

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