INFECT DIS TROP MED 2015; 1 (2): E88

Antibodies against Coxsackievirus B4 in Sardinian schoolchildren with positivity to islet cell antibodies

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ABSTRACT:

— ***Background:*** Infections caused by enteroviruses, particularly Coxsackievirus B4 (CVB4), have been suggested to be diabetogenic in genetically predisposed subjects. The onset of autoimmune processes leading to Type 1 Diabetes Mellitus (T1DM) may precede clinical diagnosis by years, and it is unknown at what stage viral infection might be significant. The Sardinian Island has the second highest incidence of T1DM in the world, close to Finland. To date, no studies have been performed to evaluate a possible role of CBV4 infection on T1DM development in Sardinian population. In our study, we evaluated the possible relationship between CBV4 infection and pre-diabetic (Islet Cell Antibodies, ICA-positivity) status in Sardinian schoolchildren. The relationships between CBV4 infection and the appearance of other diabetes-related autoantibodies, namely glutamic acid decarboxylase antibodies (GADA), and protein tyrosine phosphatase-2 antibodies (IA2-A), whose positivity dramatically increases the PPV for T1DM development, were also studied.

— ***Patients and Methods:*** 69 ICA-positive schoolchildren and 69 ICA-negative controls were studied. ICA were measured by conventional indirect immunofluorescence, and antibodies against CVB4 (CBV4-Ab) were measured by microneutralization test. GADA and IA2-A were measured by radioimmunoprecipitation on the sera of 121 out of the 138 subjects of the study.

— ***Results****:* The number of schoolchildren which showed positivity to any titer of CBV4-Ab was 44 in the ICA-positive group (63.8%), and 51 (73.9%) in the ICA-negative group (p = NS). The CBV4-Ab frequency in GADA-positive and GADA-negative subjects, and in IA2-A-positive and IA2-A-negative subjects was not different.

— ***Conclusions****:* Our study, in Sardinian schoolchildren, does not support a role of CBV4 infection in the appearance of pancreatic islet autoimmunity.

— ***Key words:*** Children, Coxsackievirus, Diabetes, Enterovirus, Islet cell antibodies, Type 1 diabetes mellitus.

INTRODUCTION

Type 1 Diabetes Mellitus (T1DM) is a multifactorial disease, resulting from environmental factors acting on genetically susceptible individuals[1]. Microbial infections and their immunological consequences are suspected to take part in the pathogenesis of T1DM[2,3]. Infections caused by enteroviruses, particularly Coxsackievirus B4 (CVB4), have been suggested to be diabetogenic in genetically predisposed subjects[4].

The onset of autoimmune processes leading to T1DM may precede clinical diagnosis by years, and it

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is not known at what stage viral infection might be significant[5].

Previous studies have suggested that enterovirus infection in childhood is likely to be associated with the development of Islet Cell Antibodies (ICA), which indicate an autoimmune reaction to islet cells, and are known to precede the onset of T1DM by months or even years[6,7].

ICA alone have limited Positive predictive value (PPV) for T1DM development[8]. Thus, understanding the relationships between CBV4 infection and the appearance of other diabetes-related autoantibodies, namely glutamic acid decarboxylase antibodies (GADA), and protein tyrosine phosphatase-2 antibodies (IA2-A), whose positivity dramatically increases the PPV for T1DM development[9,10], appears to be essential.

The incidence of T1DM differs greatly among populations, with oriental populations showing the lowest rates (0.1/100,000 in China), and Finland and Sardinia (Italy) showing the highest incidence rates (34-36/100000 children up to 14 years)[11,12]. The reasons for this different prevalence rate are still largely unknown.

To date, no studies have been performed to evaluate a possible role of CBV4 infection on T1DM development in Sardinian population. Assuming that this virus may play a role in the initiation of autoimmune insulitis, the association of recent CBV4 infections with the appearance of ICA in the pre-diabetic period should be noted. The aim of this study was therefore to search for possible relationships between CBV4 infection and pre-diabetic (ICA-positivity) status in Sardinian schoolchildren.

PATIENTS AND METHODS

Study population

We studied the sera of 69 ICA-positive schoolchildren (30 girl, mean age 11.47 years, range 7.2-16.2 years; 39 boys, mean age 12.03 years, range 7.1-16.6 years) coming from a biological repository obtained during a large study carried out in non-selected schoolchildren in the province of Sassari, Sardinia. For each ICA-positive individual, a serum sample from an age and sex-matched classmate was selected, the sample being collected on the same day (girls control group: mean age 11.23 years, range 7.0-15.9 years; boys control group: mean age 12.21 years, range 7.3-16.1 years). The study was approved by the Local Ethic Committee.

ICA, GADA and IA2-Aassays

ICA was measured by conventional indirect immunofluorescence on blood group 0 human pancreas sections as previously described[13]; the cut-off value for positivity was ≥ 5 Juvenile Diabetes Foundation Units.

GADA and IA2-A were measured by radioimmunoprecipitation[14,15]; the cut-off points for both assays were established as the 99th percentile of autoantibody levels, and corresponded to 10 Arbitrary Units (AU) and 5 AU for GADA and IA-2A, respectively. The assay was performed on the sera of 121 out the 138 subjects of the study (87.7%).

Antibodies against CBV4 (CBV4-Ab) assay

Virus source, inoculation, and titer determination

CVB strains were originally obtained from the American Type Culture Collection (Rockville, Md). Virus stocks were grown on KB cells, a human cell line derived from an epidermoid carcinoma. KB monolayers were infected at a low multiplicity of infection (MOI = 0.1) and incubated in serum-free RPMI 1640 for 24 h at 37°C. KB cultures displaying > 90% cytopathic effect (CPE) were further disrupted by two freeze-thaw cycles. Cell debris was removed by centrifugation; cell-free supernatants were subjected to titer determination and stored at -70°C. The endotoxin levels in the virus preparations were 0.01 U/ml (*Limulus* assay). Virus titers were obtained by a micromethod involving KB cells, and expressed as 50% tissue culture infective dose (TCID50) per ml[16].

CBV4- neutralizing antibodies (microneutralization test).

Sera were inactivated (56°C, 30 min), and 75 µl of four-fold dilutions (1:8 to 1:572) in growth medium were prepared in duplicate in 96-well tissue culture microtiter plates (Falcon, Becton Dickinson, Le Pont de Claix, France) by using an automated microdilutor (Finnpipette; Labsystems). A 75 µl volume of growth medium containing 30 to 300 50% tissue culture infective doses of virus was added to each well. A single row of serum dilutions containing no virus was prepared for each serum. A virus back titration (100 to 0.1 50% tissue culture infective doses per 50 µl) was done in quadruplicate. Four wells containing only growth medium were included with each plate. The plates were gently agitated and then incubated at 37°C for 1 h in a 5% CO_2 atmosphere. A suspension of KB cells was prepared in growth medium, and 50 µl were added to each well. The plates were gently agitated reincubated for 5 days, and examined microscopically for a viral cytopathic effect[17].

Statistical analysis

Fisher's exact test was used to compare the antibody frequencies of ICA-positive and ICA-negative control subjects.

RESULTS

Results are summarized in Table 1 and Table 2.
The number of schoolchildren showing positivity to any titer of CBV4-Ab was 44 (63.8%) in the ICA-positive group, and 51 (73.9%) in the ICA-negative group (p = NS). In particular, CBV4-Ab-positivity was detected in 19 ICA-positive and 20 ICA-negative girls (p = NS), and in 25 ICA-positive and 31 ICA-negative boys (p = NS).

Table 1. Total subjects: 60 (30 ICA-positive and 30 ICA-negative girls).

ICA-positive girls					ICA-negative girls				
Subject n°	ICA	GADA	IA2-A	CBV4-Ab	Subject n°	ICA	GADA	IA2-A	CBV4-Ab
1	13	4	0	0	31	<5	1	0	0
2	24	1	1	0	32	<5	1	0	256
3	85	505	0	0	33	<5	2	0	64
4	9	2	0	0	34	<5	n/d	n/d	0
5	9	0	0	0	35	<5	2	0	16
6	80	1	1	512	36	<5	2	0	0
7	6	0	0	32	37	<5	0	1	64
8	6	0	1	64	38	<5	1	0	16
9	56	1	1	0	39	<5	0	0	256
10	6	1	0	0	40	<5	1	0	64
11	9	4	0	64	41	<5	n/d	n/d	0
12	6	1	0	64	42	<5	n/d	n/d	0
13	45	n/d	n/d	64	43	<5	0	0	0
14	14	2	1	256	44	<5	0	0	64
15	6	1	0	64	45	<5	0	0	0
16	6	1	0	256	46	<5	4	0	0
17	85	149	1	256	47	<5	1	0	128
18	6	3	1	256	48	<5	2	1	16
19	6	1	0	512	49	<5	3	0	128
20	12	n/d	n/d	256	50	<5	2	0	16
21	24	2	1	512	51	<5	2	1	64
22	85	0	0	512	52	<5	1	0	64
23	39	2	0	0	53	<5	2	0	64
24	14	71	1	256	54	<5	n/d	n/d	64
25	6	0	0	0	55	<5	0	0	0
26	26	2	0	16	56	<5	1	0	32
27	12	0	0	64	57	<5	0	0	0
28	6	3	0	256	58	<5	0	0	256
29	5	1	0	0	59	<5	3	0	64
30	5	1	0	0	60	<5	0	0	64

ICA (Islet Cell Antibodies); cut-off point: ≥ 5 Juvenile Diabetes Foundation Units
GADA (Glutamic Acid Decarboxylase Antibodies); cut-off point: ≥ 10 Arbitrary Units
IA2-A (Protein Tyrosine Phosphatase-2 Antibodies); cut-off point: ≥ 5 Arbitrary Units
n/d: not determined

GADA positivity was observed in six subject: three girls (all ICA-positive: one CBV4-Ab-negative, and two CBV4-Ab-positive: subjects 3, 17, and 24), and three boys (two ICA-positive, and one ICA-negative, all CBV4-Ab-negative: subjects 62, 99, and 121). IA-2A positivity was observed in two ICA-positive boys (one CBV4-Ab-positive, and one CBV4-Ab-negative: subjects 80 and 99).

A post-hoc analysis performed by using ≥ 256 NU as a cut-off for high CBV4-Ab titer (HT) revealed that the number of girls (but not boys) with HT was significantly higher in the ICA-positive group (11 *vs*. 3; $p = 0.03$) (Figure 1).

DISCUSSION

The Sardinian Island has the second highest incidence of T1DM in the world, (34.4/100,000/year), close to Finland (36.4)[11], and far from other Italian regions, such as Lazio (7.9)[18], that lies opposite to Sardinia across the Tyrrhenian Sea.

The attempts to detect in our Island the factor(s) responsible for this increased incidence of T1DM have been so far inconclusive. For example, no associations have been found between T1DM and nitrate concentrations in drinking water[19], duration of breastfeeding[20], rate of Helicobacter Pylori infection[21], seasonal patterns[22], and other factors (for a comprehensive review, see[23]). One possible explanation for these inconsistent results may be represented by the long incubation period before T1DM becomes clinically apparent, so that, when testing for a putative etiological factor a T1DM population, factors compromising beta-cell function in earlier stages of life might be overlooked.

ICA, indicating an autoimmune reaction to pancreatic islet cells[24], are known to precede the onset of T1DM by months or even years[6,7]. Consequently, studies conducted in ICA-positive (prediabetic) subjects may unravel exposure to agent(s) acting very early in the T1DM pathogenesis.

The incidence of CBVs infection is known to vary with age, sex, season, and geographical area[25], and most studies might not adequately match control to cases for these factors. The requirement for appropriate control subjects is ideally met in our study, where age- and sex-matched classmates of the probands were selected.

Table 2. Total subjects: 78 (39 ICA-positive and 39 ICA-negative boys)

ICA-positive boys					ICA-negative boys				
Subject n°	**ICA**	**GADA**	**IA2-A**	**CBV4-Ab**	**Subject n°**	**ICA**	**GADA**	**IA2-A**	**CBV4-Ab**
61	6	*n/d*	*n/d*	0	100	<5	0	0	8
62	85	907	0	0	101	<5	3	0	128
63	6	0	0	32	102	<5	0	0	1024
64	6	*n/d*	*n/d*	256	103	<5	1	0	64
65	6	*n/d*	*n/d*	64	104	<5	0	0	128
66	45	*n/d*	*n/d*	32	105	<5	0	0	64
67	28	*n/d*	*n/d*	0	105	<5	1	0	256
68	6	*n/d*	*n/d*	128	107	<5	1	0	0
69	8	2	1	128	108	<5	1	0	64
70	6	2	0	0	109	<5	2	2	64
71	6	1	0	0	110	<5	0	0	64
72	24	2	0	0	111	<5	0	0	0
73	6	0	0	64	112	<5	0	0	16
74	6	0	0	128	113	<5	*n/d*	*n/d*	128
75	6	3	0	64	114	<5	*n/d*	*n/d*	16
76	56	0	0	32	115	<5	*n/d*	*n/d*	128
77	6	0	0	16	116	<5	2	0	128
78	6	0	0	256	117	<5	2	0	256
79	85	1	0	64	118	<5	*n/d*	*n/d*	256
80	45	8	315	8	119	<5	1	0	8
81	6	0	0	0	120	<5	2	0	128
82	9	4	0	128	121	<5	13	0	0
83	13	1	0	0	122	<5	2	0	512
84	13	0	0	256	123	<5	1	0	0
85	13	1	0	128	124	<5	0	0	32
86	5	2	2	0	125	<5	1	1	256
87	5	0	0	0	126	<5	1	1	128
88	5	0	0	1024	127	<5	4	0	512
89	69	2	0	512	128	<5	1	1	0
90	6	3	36	256	129	<5	2	1	8
91	9	4	0	1024	130	<5	0	0	64
92	12	2	1	0	131	<5	*n/d*	*n/d*	64
93	85	0	0	256	132	<5	1	2	32
94	26	0	0	4096	133	<5	2	1	0
95	12	3	1	4096	134	<5	1	0	0
96	14	1	1	0	135	<5	3	1	0
97	5	2	1	0	136	<5	2	1	64
98	5	1	0	512	137	<5	0	0	1024
99	85	883	412	0	138	<5	2	1	512

ICA (Islet Cell Antibodies); cut-off point: ≥ 5 Juvenile Diabetes Foundation Units
GADA (Glutamic Acid Decarboxylase Antibodies); cut-off point: ≥ 10 Arbitrary Units
IA2-A (Protein Tyrosine Phosphatase-2 Antibodies); cut-off point: ≥ 5 Arbitrary Units
n/d: not determined

The mechanism through which CBV4 may participate in the pathogenesis of T1DM is presently unknown. Interestingly, CBV4 infection-associated tissue damage can result in the release of islet antigens from beta cells[26]. Following this release, these antigens could then be presented to autoreactive T cells and initiate the disease. The primary role of CBV4 in the T1DM pathogenesis could then be the induction of tissue damage, with subsequent release and presentation of sequestered islet antigen. Interestingly, a recent study showed that the rate of progression from islet autoimmunity to T1DM was significantly increased in children after the detection of enterovirus RNA in serum[27]. However, despite these intriguing observations, in our population we found no association between CBV4 infection and the appearance of diabetes-related autoantibodies.

A post-hoc analysis performed by using ≥ 256 NU as a cut-off to define high CBV4-Ab titer (HT) revealed that the number of girls (but not boys) with HT was significantly higher in the ICA-positive group (11 *vs*. 3; $p = 0.03$). However, the cut-off we used to define CBV4-Ab HT is arbitrary, and was not established *a priori*. Thus, even though this observation might generate speculations based on the well known sexual dimorphism of autoimmune diseases[28], we cannot use these findings to formulate data-supported conclusions.

In conclusion, our study carried out in Sardinian schoolchildren does not support a role for CBV4 infection in the appearance of pancreatic islet autoimmunity. The attempts to detect in our Island the environmental factor(s) responsible for the increased incidence of T1DM remain still inconclusive.

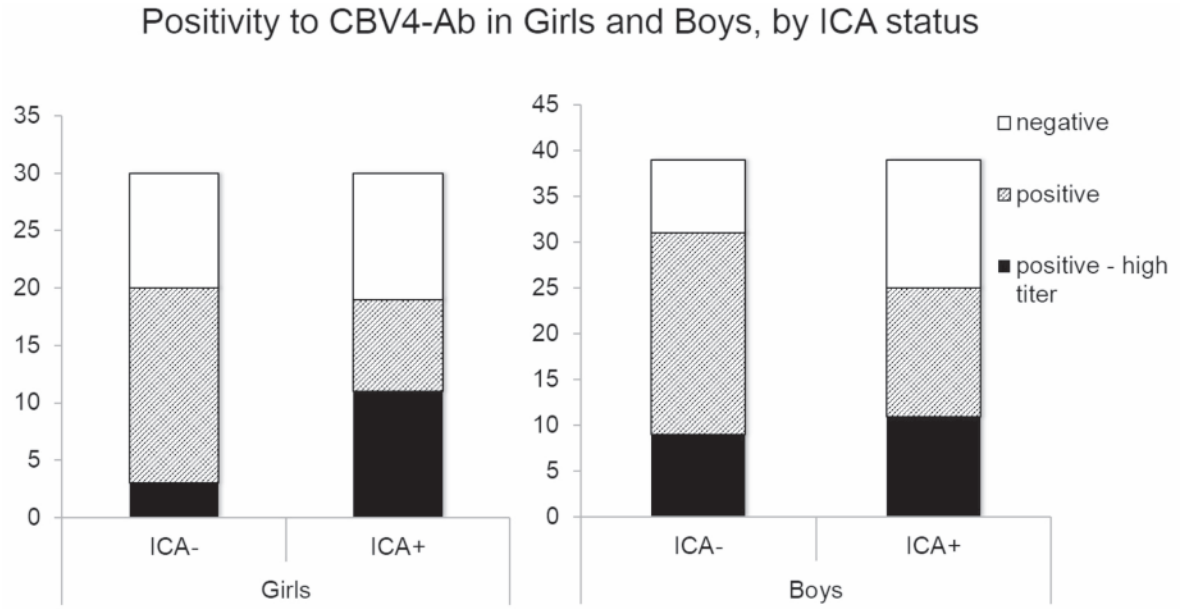

Figure 1. Positivity to CBV4-Ab in girls and boys by ICA status. In the girl group, the number of subjects with high titer of CBV4-Ab (≥ 256 NU) was significantly higher in the ICA-positive subgroup (11 *vs*. 3; $p = 0.030$). No significant differences were found in the boy group.

Conflict of interest.

The Authors declare that they have no conflict of interests.

Acknowledgments

We are deeply indebted to Prof. Saverio Stranges (Warwick University) for his careful reading of the manuscript and his constructive remarks.

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