T-Cell Responses to Enterovirus Antigens in Children With Type 1 Diabetes

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Enterovirus infections, implicated in the pathogenesis of type 1 diabetes in a number of studies, may precipitate the symptoms of clinical diabetes and play a role in the initiation of the β-cell damaging process. The aim of this study was to evaluate whether cellular immune responses to enterovirus antigens are abnormal in children with type 1 diabetes. Lymphocyte proliferation responses to enterovirus antigens were analyzed in 41 children with new-onset type 1 diabetes, 23 children with type 1 diabetes for 4–72 months, and healthy control children in subgroups matched for HLA-DQB1 risk alleles, sex, and age. Children with diabetes for 4–72 months more often had T-cell responses to the Coxsackievirus B4–infected cell lysate antigen than children with new-onset diabetes ($P < 0.01$) or control children ($P < 0.01$). Responses to recombinant nonstructural protein 2C of Coxsackievirus B4 were also more frequent in children with type 1 diabetes for 4–72 months when compared with control subjects ($P = 0.03$), whereas the responses to purified Coxsackievirus B4 and recombinant VP0 protein, which did not contain nonstructural proteins, did not differ. These data suggest that T-cell responses to Coxsackievirus B4 proteins and particularly to the antigens containing the nonstructural proteins of the virus are increased in children with type 1 diabetes after the onset of the disease. However, in children with new-onset diabetes, responses were normal or even decreased. This phenomenon was specific for enteroviruses and could be caused by trapping of enterovirus-specific T-cells in the pancreas. *Diabetes* 49:1308–1313, 2000

For 3 decades, enterovirus infections, especially Coxsackievirus B (CBV) infections, have been suggested to be associated with the development of type 1 diabetes. CBV4-specific IgM responses are more common in newly diagnosed subjects with type 1 diabetes than in healthy control subjects (1,2), and CBV has been isolated from the pancreas of a child who succumbed at the onset of type 1 diabetes (3). The findings of viral RNA in circulation at the onset of type 1 diabetes have further supported the role of enteroviruses (4,5). Furthermore, recent prospective studies indicate that enterovirus infections may play a role in triggering the autoimmune processes leading to diabetes because children who later developed type 1 diabetes had more enterovirus infections than control children years before the diagnosis of type 1 diabetes (6). The infections also occurred predominantly at times when exceptional numbers of children first developed diabetes-related autoantibodies or showed increases in autoantibody titers (7). Fetal enterovirus infections may also increase the risk for future type 1 diabetes (6,8).

Enterovirus infections, like other environmental risk factors, can probably induce β-cell–damaging processes only in individuals with genetic type 1 diabetes susceptibility. The most important risk genes locate within the HLA gene complex, where HLA-DQ alleles associated with increased susceptibility to or protection against type 1 diabetes can be defined (9). The association of genetic risk with 2 major haplotypes characterized by DQ molecules encoded by the HLA-DQA1*0301-DQB1*0302 and DQA1*0501-DQB1*02 alleles may indicate heterogeneity in the etiology of the disease. Enterovirus infections possibly occur predominantly in subjects with the latter haplotype, who usually are also positive for the HLA-B8 and HLA-DR3 alleles (10–12). HLA may also influence immune responses to enterovirus antigens (13) and bias comparisons between patients and control subjects.

In this investigation, T-cell proliferation to enterovirus antigens was analyzed for the first time in an HLA-matched population of children with type 1 diabetes and children who were healthy. We also studied whether children's HLA genotype modified cellular immune responses to the enterovirus antigens.

RESEARCH DESIGN AND METHODS

Subjects. In total, 41 children with new-onset type 1 diabetes (blood sample taken within 1 week after diagnosis), 23 children with type 1 diabetes for 4–72 months, and 57 healthy control subjects participating in prediction studies of type 1 diabetes (14) were analyzed. These children were compared with each other in subgroups, which were matched for HLA-DQB1 risk alleles, sex, and age. The descriptions of these subgroups are presented in Table 1. Serum samples for virus antibody analysis were not available from every child, and in the serological analysis, group 1 had 13 pairs, group 2 had 12 pairs, and group 3 had 24 pairs. The effect of HLA on immune responses was additionally analyzed by comparing T-cell responses in children with different HLA-DQB1 alleles. In these comparisons, the children were matched for age and sex (Table 2).

HLA typing. The HLA-DQB1 alleles that associate with type 1 diabetes risk or protect from type 1 diabetes were measured as described (15).

Lymphocyte proliferation assay. Heparinized venous blood was collected, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden). The washed

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Received for publication 17 August 1999 and accepted in revised form 20 April 2000.

CAV16, Coxsackievirus A16; CBV, Coxsackievirus B; EIU, enzyme immunoassay unit; GST, glutathione S–transferase; PBMC, peripheral blood mononuclear cell; OD, optical density; PPD, purified protein derivative of tuberculin; PV, poliovirus; SI, stimulation index; TT, tetanus toxoid.

TABLE 1
Ages of patients and control subjects in various comparison groups

Comparison group	Subjects	Number of pairs	Age at time of study (years)
1	Children with type 1 diabetes for 4–72 months	15	10.1 ± 3.3
	Children with new-onset type 1 diabetes		9.7 ± 3.7
2	Children with type 1 diabetes for 4–72 months	13	7.9 ± 3.3
	Healthy children		7.9 ± 2.8
3	Children with new-onset type 1 diabetes	29	7.4 ± 3.4
	Healthy children		7.2 ± 3.5

Data are *n* or means ± SD.

PBMCs were resuspended in RPMI 1640 medium supplemented with 10% human male AB serum (Finnish Red Cross, Helsinki, Finland), glutamine, HEPES, and gentamycin (10 µg/ml). Quadruplicate incubations of 50,000 PBMCs with antigen(s) in a final volume of 200 µl continued to incubate in 96-well round-bottomed microtiter plates for 6 days. Tritiated thymidine (2 µCi/ml; Amersham, U.K.) was added 18 h before the cells were harvested on glass fiber filters using a Tomtec 93 Mach III Manual Harvester (Tomtec, Orange, CT). Incorporated radioactivity was measured with a Micro-Beta scintillation counter (Wallac, Turku, Finland).

Antigens. CBV4 (ATCC), Coxsackievirus A16 (CAV16) (strain G-10), and poliovirus (PV)-3 (Sabin) were grown in LLC-MK_2 cells (ATCC). Infected cell cultures were harvested when the cells detached freely from the surface. The infected cells and supernatant fluid were frozen and thawed 3 times, and the viruses were inactivated using 0.02% final concentration of β-propiolactone at 4°C for 18 h. Uninfected LLC-MK_2 cells were harvested at the same time for preparation of the control antigen. The protein concentrations of enterovirus-infected cell lysate and purified antigen preparations were measured using the Pierce BCA protein assay reagent (Pierce, Rockford, IL). Enterovirus cell lysates and uninfected cell lysates were used at concentrations of 10, 1, and 0.1 µg/ml. Maximal responses were observed with 10 µg/ml, and this concentration was used in the results.

Purified CBV4 and PV1 were prepared using sucrose gradient centrifugation and used at 2 concentrations (1 and 0.1 µg/ml) (16). The maximal responses were observed with 1 µg/ml, and this concentration was used in the results. Adenovirus hexon protein (10, 1, and 0.1 µg/ml) (17), tetanus toxoid (TT) (1 µg/ml) (National Public Health Institute, Helsinki, Finland), and purified protein derivative of tuberculin (PPD) (10 µg/ml; Statens Serumintitut, Copenhagen, Denmark) were used as control antigens, and pokeweed mitogen (1 µg/ml) was used as a mitogen control. Maximal responses to adenovirus hexon protein were observed with 10 µg/ml, and this concentration was used in the results. Preparation of CBV4 VP0 and 2C proteins has been described elsewhere (18). Full-size CBV4 cDNA was used as a template to amplify the genes by polymerase chain reaction. The genes were cloned into expression vector pGEX-2T and expressed as a fusion protein with glutathione S-transferase (GST) (GST-VP0 and GST-2C) in *Escherichia coli*. GST-VP0 and GST-2C were purified on GST-sepharose columns (GST Gene Fusion System, Pharmacia Biotech). Two concentrations (1 and 0.1 µg/ml) of VP0 and 2C protein were used, and the maximal response was observed with 1 µg/ml; this concentration was used in the results. Stimulation index (SI) values for purified enterovirus antigens, PPD, TT, and adenovirus hexon protein were calculated by dividing the median count of antigen-stimulated quadruplicate wells by the median count of identical number of control wells. SI values for enterovirus-infected cell lysate antigens were calculated by dividing the median value of lysate antigen-stimulated quadruplicate wells by the median value of similar number of wells containing cells with noninfected cell lysate. SI values for VP0 and 2C proteins were calculated accordingly by dividing the median value of the antigen-stimulated quadruplicate wells by the median of a similar number of GST-stimulated wells. An SI value exceeding 3 was regarded as a positive proliferation response.

Virus antibodies. IgA and IgG class antibodies were measured against a synthetic enterovirus peptide (KEVPALTAVETGAT) and purified adenovirus hexon antigen (serotype 5) using an indirect enzyme immunoassay method (6,19,20). Virus antibody levels were expressed in enzyme immunoassay units (EIUs), which were calculated according to the following formula.

$$100 \times [(\text{optical density [OD]}_{\text{sample}} - \text{OD}_{\text{negative reference serum}})/ (\text{OD}_{\text{positive reference serum}} - \text{OD}_{\text{negative reference serum}})]$$

An EIU value exceeding 10 was considered positive.

Statistical analysis. A 2-tailed Mann-Whitney *U* test was used for comparisons of the SI levels between 2 groups. The Kruskal-Wallis test was used when appropriate. A χ^2 test was used to compare frequencies of positive responses, and Fisher's exact test was used when appropriate. Wilcoxon's signed-rank test was used when comparing longitudinal data of children with type 1 diabetes.

Ethics. This study has been approved by the Joint Ethics Committee of the Turku University and Turku University Hospital. Informed consent was obtained from all parents/guardians.

RESULTS

Comparison group 1: immune responses in children with new-onset type 1 diabetes and in children with type 1 diabetes for 4–72 months. The children with type 1 diabetes for 4–72 months had markedly stronger T-cell responses to the CBV4-infected cell lysate antigen than the newly diagnosed patients matched for HLA, sex, and age (Fig. 1*A*). The proportions of children with positive responses (SI >3) to the CBV4 lysate antigen was 53% (8/15) vs. 7% (1/15), respectively (P = 0.007, Fisher's exact test). The proportion of children with positive responses to recombinant nonstructural protein 2C of CBV4 was also higher in children with type 1 diabetes for 4–72 months than in children with new-onset disease (12/15 vs. 6/15; P = 0.03, χ^2 test). However, the responses to purified CBV4 or to recombinant VP0, which included only the structural proteins VP2 and VP4 of CBV4, did not differ between the groups. The responses to CAV16- and PV3-infected cell lysates and to purified PV1 were also similar in both groups.

TABLE 2
Children in each group separated by HLA-DQB1 risk alleles

Comparison group	Subjects	Children positive for HLA-DQB1* alleles			
		02/X†	0302/Y‡	02/0302	Z/Z§
4	Healthy children	9	9	9	9
5	Children with new-onset type 1 diabetes	10	10	10	5
6	Children with type 1 diabetes for 4–72 months	6	6	6	2

Data are *n*. †X, DQB1 allele other than *0302; ‡Y, DQB1 allele other than *02; §Z, neither *02, *n*, or *0302.

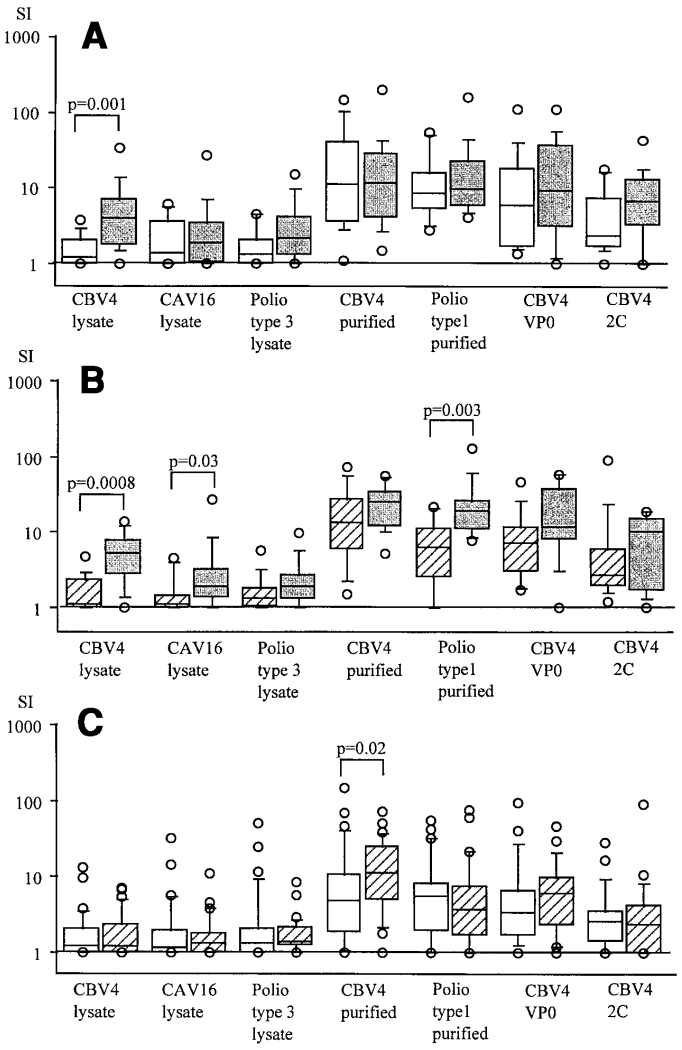

FIG. 1. T-cell responses to all studied enterovirus antigens of comparison group 1 (*A*), including children with new-onset type 1 diabetes (□) and children with type 1 diabetes for 4–72 months (■); comparison group 2 (*B*), including control children (▨) and children with type 1 diabetes for 4–72 months (■); and of comparison group 3 (*C*), including children with new-onset type 1 diabetes (□) and healthy control children (▨). The boxes comprise values between the 25th and 75th percentiles. The bars outside the boxes represent the 10th and 90th percentiles of the values. The open circles are values outside this range. For statistical analysis, the Mann-Whitney *U* test was used. Note the logarithmic scale on the *y*-axis.

The spontaneous proliferation in unstimulated wells was very low and did not differ between the groups (median counts per minute in unstimulated wells was 229 in children with type 1 diabetes for 4–72 months and 231 in children with new-onset disease, NS). The responses to uninfected cell lysate were similar to those of unstimulated cells (median SI 1.0 [interquartile range 0.6] in children with type 1 diabetes for 4–72 months and 1.0 [0.2] in children with acute disease). The proliferation responses to the adenovirus hexon protein and to TT also did not differ between the groups.

Levels of IgG and IgA class antibodies to synthetic enterovirus peptide did not differ between children with new-onset type 1 diabetes and children with type 1 diabetes for 4–72 months (Mann-Whitney *U* test), but the proportion of children with IgG class as well as IgA class antibodies to enterovirus peptide was higher in children with type 1 diabetes for 4–72 months than in children with new-onset disease ($P = 0.05$, Fisher's exact test; $P = 0.04$, χ^2 test, respectively) (Table 3). The proportion of children who tested positive for adenovirus antibodies did not differ between the groups.

Blood samples were available from 7 children, both at time of onset of disease and at a later point. The mean duration of the disease was 10 months (6–14 months) at the time when the second sample was taken. At disease onset, median SI (interquartile range) for CBV-infected cell lysate was 1.0 (0.5) and at a later time, 2.6 (4.9) ($P = 0.03$, Wilcoxon's signed-rank test). T-cell responses to purified CBV4 were also lower at the onset of the disease. Median SI (interquartile range) for purified CBV4 was 5.9 (3.8) at the onset of type 1 diabetes and 11.5 (17.7) after the onset of disease ($P = 0.04$, Wilcoxon's signed-rank test). The proliferation responses to recombinant VP0 and 2C were only available from 4 individuals at both time points. Even though the responses to 2C protein had a tendency to be stronger after the onset of disease, the difference did not reach significance. The proliferation responses to other studied antigens were similar at both time points.

Comparison group 2: immune responses in children with type 1 diabetes for 4–72 months and in nondiabetic control subjects. Children studied 4–72 months after diagnosis of type 1 diabetes had stronger T-cell responses to CBV4- and CAV16-infected cell lysates and purified PV1 than the HLA-, sex-, and age-matched healthy control subjects (Fig. 1*B*). The proportion of children with positive responses (SI >3) to CBV4 lysate antigen was 69% (9/13) in diabetic children versus 8% (1/13) in control children ($P = 0.001$, Fisher's exact test). Responses to other studied enterovirus antigens as well as the responses to TT, PPD, and adenovirus hexon protein did not differ. The level of antibodies to the enterovirus peptide did not differ between the groups and neither did the proportion of children positive for the enterovirus peptide (Table 3). The antibody responses to adenovirus hexon protein were also comparable in both groups.

Comparison group 3: immune responses in children with new-onset type 1 diabetes and in control subjects. Children with new-onset type 1 diabetes had weaker responses to purified CBV4 than control children ($P = 0.02$, Mann-Whitney *U* test; Fig. 1*C*). The proportion of positive responses (SI >3) to purified CBV4 was also lower in children with new-onset diabetes when compared with control children (17/29 and 25/29, respectively; $P = 0.05$, Fisher's exact test). This difference was not observed in responses to purified PV1 or to enterovirus-infected cell lysates and or in responses to recombinant VP0 or 2C of CBV4 proteins. The proliferation responses to uninfected cell lysate did not differ between the groups, and the responses to this control antigen were similar to the responses of unstimulated cells (median counts per minute in unstimulated wells was 198 in patients and 206 in control children; median SI [interquartile range] for uninfected cell lysate was 1.1 [0.2] in children with new-onset diabetes and 1.0 [0.2] in control children). The responses to adenovirus hexon protein and to TT did not differ between children with new-onset type 1 diabetes and control subjects. Levels of IgG and IgA class antibodies to synthetic enterovirus peptide and to adenovirus hexon protein were similar in children with new-onset diabetes and in control subjects.

TABLE 3
Median titer and interquartile range of enterovirus and adenovirus IgG and IgA class antibodies and the proportion of positive (>10 EIU) responses

	Peptide IgG		Peptide IgA		Adenovirus IgG		Adenovirus IgA	
Subjects	Titer	Children at titer >10 EIU	Titer	Children at titer >10 EIU	Titer	Children at titer >10 EIU	Titer	Children at titer >10 EIU
Children with type 1 diabetes for 4–72 months	17 (37)	7/13*	20 (28)	11/13†	7.0 (36)	6/13	7.4 (27)	5/13
Children with new-onset type 1 diabetes	0 (4.2)	2/13*	6.9 (26)	6/13†	20 (48)	9/13	3.1 (3.8)	1/13
Children with type 1 diabetes for 4–72 months	2.5 (13)	3/12	10 (9.2)	8/12	27 (38)	7/12	14 (26)	7/12
Control subjects	3.0 (4.2)	1/12	5.3 (12)	4/12	13 (45)	6/12	2.8 (17)	5/12
Children with new-onset type 1 diabetes	1.4 (12)	6/24	8.6 (16)	11/24	21 (41)	17/24	4.0 (22)	6/24
Control subjects	5.4 (12)	7/24	5.1 (13)	8/24	21 (31)	13/24	8.2 (16)	11/24

Data are medians (interquartile range) or proportions. *P = 0.05, Fisher's exact test; †P = 0.04, χ^2 test.

Comparison groups 4–6: effect of HLA on immune responses. There was a significant heterogeneity in the lymphocyte proliferation response to PV3 lysate antigen in control subjects grouped according to HLA-DQ genotypes (P = 0.03, Kruskal-Wallis test; Fig. 2). The proportion of children with positive T-cell responses to CAV16-, CBV4-, and PV3-infected cell lysate was higher in children with the HLA-DQB1*0302/X genotype compared with children with HLA DQB1*02/Y or HLA-DQB1*02/*0302 genotypes (P < 0.05, Fisher's exact test). There were no differences in responsiveness to PPD, TT, or pokeweed mitogen as well as to adenovirus hexon protein between children carrying different HLA-DQB1 genotypes. HLA genotypes did not have the same effect on T-cell proliferation to enterovirus antigens in diabetic patients as in healthy children (data not shown).

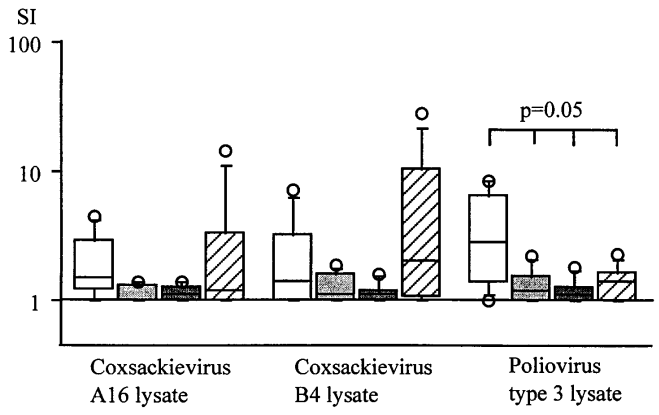

FIG. 2. T-cell responses to enterovirus-infected cell lysates in healthy children divided into groups according to the defined HLA-DQB1 alleles. □, Children with HLA-DQB1*0302/X (X equals DQB1 allele other than *0302); □, children with HLA-DQB1*02/Y (Y equals DQB1 allele other than *02); ■, children with HLA-DQB1*02/0302; ▨, children with HLA-DQB1*Z/Z (Z equals neither *02 nor *0302). For details, see legend to Fig. 1. Statistical significance for the difference between SI values was calculated by the Kruskal-Wallis test.

DISCUSSION

This study shows that T-cell responses to CBV4-infected cell lysate are higher in children with type 1 diabetes after the onset of disease compared with children with new-onset type 1 diabetes or healthy children. Furthermore, the responses to purified CBV4 and VP0 protein do not differ between the groups, suggesting that the enhanced reactivity is targeted to the nonstructural CBV4 proteins. Previously, we have studied enterovirus-specific T-cell responses in children with CBV4 infections verified by a plaque neutralization assay (21). Children with CBV infection had stronger T-cell responses to CBV4 antigen when compared to either children with other enterovirus infections or children without any serological evidence of enterovirus infection. These data demonstrated the specificity of our assay to CBVs despite the large cellular cross-reactivity between enteroviruses, which also contributes to the responsiveness (22,23).

The strong responses of children with type 1 diabetes to coxsackie-infected cell lysates are concordant with the findings of Jones and Crosby (24), who showed that type 1 diabetic patients have stronger T-cell responses to CBV-infected cell lysates. In addition to CBV4, a stronger response was also observed to purified PV1; all our children had a history of several doses of inactivated Salk polio vaccination. The strong cross-reactivity of T-cell responses to different enterovirus serotypes (22,23) suggests that the stronger response to purified PV antigen may be associated with an increased number of various enterovirus infections in diabetic children.

Children with new-onset type 1 diabetes showed no enhanced reactivity to CBV4-infected cell lysates or 2C protein, and the responses to purified CBV4 were even lower in these patients than in matched control subjects. Temporary decline in T-cell responsiveness at diabetes onset has also been described in GAD peptides that contain the homology region to the CBV4 2C protein (25,26). This apparently controversial finding may be related to the redistribution of the specific lymphocyte population from peripheral blood, which might be caused by recent or ongoing enterovirus infection or cross-reactive autoimmune process. Repeated cycles of enterovirus infections with largely cross-reactive immunity may resemble

the situation in chronic infections; acute *Malaria falciparum* infection is also associated with the low T-cell reactivity to *M. falciparum* proteins (27,28). Experimental models have demonstrated the trapping of insulin-reactive T-cells in the pancreas and lack of measurable T-cell reactivity to insulin in the peripheral blood at the time of the most severe insulitis (29,30). Poor proliferation response to CBV3 has been reported during CBV3–induced experimental myocarditis in sensitized mice at the time of histologically severe myocarditis (31), whereas strong responses are elicited during the preceding time and recovery.

The development of stronger responsiveness to enterovirus-infected cell lysates cannot be evaluated in the present study because the preclinical samples were not available from children with type 1 diabetes. However, we have previously shown that healthy children with diabetes-associated autoantibodies who represent early phases of prediabetes have stronger enterovirus-specific T-cell responses compared with healthy children without these autoantibodies (32). This reactivity was targeted to the CBV4-infected cell lysate antigen but not to purified enteroviruses, just like in the reactivity in this study. If enterovirus infections initiate an autoimmune process by either a molecular mimicry–based cross-reaction or by affecting immune regulation, repeated infections might very well also accelerate and finally precipitate the infection (33).

In this study, the healthy children with the HLA-DQB1*0302 allele have stronger T-cell responses to enterovirus-infected cell lysates, whereas the HLA-DQB1*02 allele is associated with weak responsiveness to the same antigens. Previously, Bruserud and colleagues (13,34) found that DR4, which is in linkage disequilibrium with the HLA-DQB1*0302 allele, associates with strong T-cell responses, whereas HLA-DR3, associated with the HLA-DQB1*02 allele, associates with weak T-cell responses to enterovirus antigens. For unknown reasons, the HLA alleles influenced T-cell responses only in control subjects but not in patients with type 1 diabetes. Particularly, the HLA-DQB1*02 allele was not associated with low responses to enteroviruses in diabetic patients. The similar level of responsiveness to enterovirus antigens in diabetic children with HLA-DQB1*02 and HLA-DQB1*0302 alleles could reflect an increased number of enterovirus infections, particularly in HLA-DQB1*02–carrying children developing type 1 diabetes. This result has also been suggested by the finding that the seropositivity for CBV4 IgM class antibodies in type 1 diabetic patients is associated with HLA-DR3 (10).

Previous data on enterovirus antibodies in patients with new-onset type 1 diabetes are controversial (35). We measured IgG and IgA class antibodies to a synthetic enterovirus peptide (KEVPALTAVETGAT), which is reported to be a common antigenic determinant of several enteroviruses and is able to detect antibodies to a variety of enterovirus serotypes, including all CBV serotypes (20). We could not find any difference in antibody levels to this enterovirus peptide between type 1 diabetic patients and healthy control children. However, the children with new-onset type 1 diabetes had a decreased proportion of enterovirus antibody seropositivity compared with diabetic children with a longer duration of type 1 diabetes, suggesting that infections are associated with disease onset.

In conclusion, our results show that type 1 diabetes associates with increased T-cell responses to nonstructural CBV4 proteins, which may be suppressed at the clinical manifestation of this disease. The enhanced responsiveness to the nonstructural CBV4 proteins may reflect increased exposure to enterovirus infections because nonstructural proteins are highly conserved or because nonstructural viral proteins have a specific role in the β-cell damaging process.

ACKNOWLEDGMENTS

Grant support was obtained from the Sigrid Jusélius Foundation, Academy of Finland, Foundation for Diabetes Research in Finland, Juvenile Diabetes Foundation International (grants 395019, 197058, and 4-1998-274), Päivikki and Sakari Sohlberg Foundation, and Research and Science Foundation of Farmos.

The expert technical assistance of Maria Kivivirta and Eeva Jokela is gratefully acknowledged.

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