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Rotavirus-specific T cell responses and cytokine mRNA expression in children with diabetes-associated autoantibodies and type 1 diabetes

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Summary

Rotavirus infections have been implicated as a possible trigger of type 1 diabetes. We elucidated this connection by comparing peripheral blood T cell responses to rotavirus between children with newly diagnosed type 1 diabetes ($n = 43$), healthy children with multiple diabetes-associated autoantibodies ($n = 36$) and control children carrying human leukocyte antigen (HLA)-conferred susceptibility to type 1 diabetes but without autoantibodies ($n = 104$). Lymphocyte proliferation assays based on stimulation with an antigen were performed using freshly isolated peripheral blood mononuclear cells (PBMC) and IgG and IgA class rotavirus antibodies were measured using plasma samples collected from the children. The expression of interferon (IFN)-γ, interleukin (IL)-4, IL-10 and transforming growth factor (TGF)-β in PBMC was studied with real-time polymerase chain reaction (PCR) in a subgroup of 38 children. No differences were observed in the strength or frequency of positive T cell responses to rotavirus between children with overt diabetes, children with multiple autoantibodies and control children. Children with diabetes-associated autoantibodies had, instead, stronger T cell responses to purified coxsackie B4 virus than control children. Rotavirus-stimulated lymphocytes from autoantibody-positive children produced more IL-4 and phytohaemagglutinin (PHA)-stimulated lymphocytes more IL-4 and IFN-γ than lymphocytes from control children. PHA-stimulated lymphocytes from children with diabetes also produced more IL-4 and purified protein derivative (PPD)-stimulated lymphocytes less TGF-β than lymphocytes from autoantibody-negative control children. In conclusion, our lymphocyte proliferation studies did not provide evidence supporting an association between rotavirus infections and the development of type 1 diabetes or diabetes-associated autoantibodies in young children.

Keywords: cytokine, rotavirus, T cell proliferation, type 1 diabetes

Introduction

Type 1 diabetes (T1D) is caused by immune-mediated destruction of the pancreatic insulin-producing beta cells. Genetic background, largely polymorphism within the class II region of the human leukocyte antigen (HLA) complex, defines the susceptibility to the disease [1]. Environmental factors contribute to the penetrance of the disease, as demonstrated by the conspicuously increased incidence rate of the disease during the last decades [2,3]. Infectious factors including viruses have been implicated as possible triggers of the destructive autoimmune process, and in particular the role of enteroviruses has recently been discussed extensively [1,4].

Honeyman and coworkers [5] have reported that rotavirus infections preceded the appearance of diabetes-associated autoantibodies in an Australian prospective study. They also observed that rotavirus was able to grow in monkey pancreatic islets *in vitro* [6]. However, we did not find any association between the development of rotavirus antibodies and appearance of diabetes-associated autoantibodies in young children in our prospective study [7].

Rotavirus infections are documented to induce rotavirus-specific T cell and cytokine responses in children [8–11],

although in our previous follow-up study T cell responses in young children declined shortly after infection. Persistent responsiveness to rotavirus measured as lymphocyte proliferation was observed in adults [11]. We have now extended our T cell proliferation studies to evaluate further the possible connection between rotavirus and T1D and analysed T cell responsiveness to rotavirus in children with T1D or T1D-associated autoantibodies. Moreover, as a part of cell-mediated immune response to rotavirus, we have evaluated the capability of rotavirus-specific T cells to produce cytokines. As T1D is a T cell-mediated disease [12], we speculated that if a link between rotavirus infections and the disease exists, cellular responsiveness to viral antigens might be altered in children with T1D-associated autoantibodies and/or T1D.

Subjects and methods

Subjects

Altogether 183 children (122 boys) taking part in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study [13] at Turku University Hospital, Finland, were included in the study. Forty-three of them had newly diagnosed T1D [median age 8·4 years, interquartile range (IQR) 5·3–11·3 years] and an additional 36 children had developed two or more T1D-associated autoantibodies (median age 6·3 years, IQR 4·5–8·2 years). Altogether, 104 children who had not developed autoantibodies served as control subjects (median age 5·0 years, IQR 3·5–6·9 years). Eleven children with T1D, seven additional children with autoantibodies and 20 autoantibody negative control children were also studied for the expression of interferon (IFN)-γ, interleukin (IL)-4, IL-10 and transforming growth factor (TGF)-β. All children in the study carried the HLA–DR3–DQ2 (DQA1*05–DQB1*02) and/or DR4–DQ8 (DQB1*0302) haplotype, predisposing to T1D, and did not have protective DQ alleles (DQA1*0201–DQB1*02, DQB1*0301 or DQB1*0602). The children were vaccinated according to the standard Finnish vaccination protocol in which, for example, bacillus Calmette–Guérin (BCG) immunization is given to infants within the first few days after birth and diphtheria, pertussis and tetanus (DPT) vaccination at the ages of 3, 4, 5 and 20–24 months. The study was approved by the Ethics Committee of South-West Finland Health Care District and blood was drawn with the consent of all subjects and/or their guardians.

Antigens

The Wa strain (G serotype 1, P serotype 1A) of human rotavirus was propagated in fetal green monkey kidney (MA104) cells in the presence of trypsin. The Nebraska calf diarrhoea (NCD) strain (G serotype 6, P serotype 6) of bovine rotavirus and coxsackie B4 virus were propagated in rhesus monkey kidney epithelial (LLC–MK2) cells. The cells were harvested, as the virus had caused an advanced cytopathic effect. Negative control antigens were prepared identically from uninfected MA104 and LLC cells. Human rotavirus [11] and coxsackie B4 virus [14] were purified with sucrose gradient centrifugation. Purified human rotavirus (PRV) and purified coxsackie B4 virus (PCB), human rotavirus lysate (RV) and uninfected MA104 cell lysate were used at 1 μg/ml concentration and NCDV, coxsackie B4 virus lysate (CBV) and uninfected LLC cell lysate at 10 μg/ml concentration. Tetanus toxoid (TT; 1 μg/ml; National Public Health Institute, Helsinki, Finland) and purified protein derivative (PPD) (2·5 μg/ml; Staatens Seruminstitut, Copenhagen, Denmark) were used as positive control antigens. Phytohaemagglutinin (PHA; 100 μg/ml in T cell proliferation assays and 6·25 μg/ml in cytokine assays; Difco, Detroit, MI, USA) was used as a mitogen control. Cell culture medium served as negative background and control for purified antigens.

T cell proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples with Ficoll-Paque gradients (Pharmacia, Uppsala, Sweden). The freshly isolated cells were washed twice with RPMI-1640 medium supplemented with 0·01% gentamycin and resuspended in RPMI-1640 medium containing 7·5% human AB serum (Finnish Red Cross, Helsinki, Finland), 2% HEPES, 0·2% glutamine and 0·01% gentamycin. The cells (5×10^4 PBMC in a final volume of 200 μl of complete medium) were cultured with antigens in quadruplicate in 96-well round-bottomed microtitre plates (Corning Incorporated, Corning, NY, USA). The cells were incubated at 37°C in 5% CO_2 for 6 days to be labelled with tritiated thymidine (2 μCi/ml, Amersham, Buckinghamshire, UK) for 18 h. The cells were harvested on glass fibre filters with Tomtec 93 Mach Manual Harvester (Tomtec, Orange, CT, USA) and the incorporated radioactivity was measured with a Micro Beta scintillation counter (Wallac, Turku, Finland). Stimulation indices (SI) were calculated by dividing median experimental counts per minute (cpm) by the median control cpm. Sis ≥ 3 were regarded as positive.

Total RNA extraction and reverse transcription

The cells (1×10^5 PBMC in a final volume of 200 μl of complete medium) were cultured with either PRV, PCB, PPD, PHA or cell culture medium only in duplicate in 96-well round-bottomed microtitre plates (Corning). After 48 h at 37°C in 5% CO_2, the cells were collected into 1·5 ml Eppendorf tubes (Sarstedt, Nümbrecht, Germany) and centrifuged for 5 min at 800 ***g***. The supernatant was extracted and the cell pellet was resuspended in 250 μl lysis solution (Applied Biosystems, Foster City, CA, USA) and frozen at −70°C. Total RNA was isolated with the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems). Isolation was performed

according to the manufacturer's instructions, with slight modifications. The loading time in the first step of isolation was extended from 120 s to 180 s and the vacuum was set at 30%. The elution time was also extended from 120 s to 180 s. We used 500 μl, 500 μl and 250 μl wash solution 2 in the three wash steps, respectively. Extracted RNA was suspended in 150 μl elution solution (Applied Biosystems) and stored at −70°C.

The reverse transcription (RT) reaction was performed with 0·04 U pd(T)$^{12-18}$ primer (Amersham, Piscataway, NJ, USA). The samples were denatured for 5 min at 70°C and cooled for 3 min on ice. RT-enzyme mixture containing 1 × Moloney murine leukemia virus (M-MLV) reverse transcriptase buffer [50 mM Tris-HCl, pH 8·3, 3 mM $MgCl_2$, 75 mM KCl and 10 mM dithiothreitol (DTT)] (Promega, Madison, WI, USA), 0·5 mM deoxyribonucleoside triphosphate (dNTP) (Amersham), 4 U recombinant Rnasin ribonuclease inhibitor (Promega) and 40 U M-MLV reverse transcriptase Rnase H Minus (Promega) was added to the samples. Reaction was performed on a standard polymerase chain reaction (PCR) machine (PerkinElmer Cetus DNA Thermal Cycler, Norwalk, CT, USA) for 60 min at 42°C and the samples were denatured for 5 min at 90°C. The cDNA samples were stored at −70°C.

Primers and probes

The primers and probes are described in Table 1. The probe and reverse primer sequences for the detection of IFN-γ and the primers used for the production of β-actin standard have been published by Stordeur and coworkers [15], the primer sequences for the detection of β-actin and the production of IFN-γ and IL-4 standards were described in our earlier study [16] and the other sequences used were designed for this study with Primer3 software [17] according to the guidelines of Stordeur *et al.* [15]. The primers and probes were synthesized at Eurogentec (Seraing, Belgium) except for the primers used in the production of TGF-β standards, which were obtained from TIB Molbiol (Berlin, Germany).

Real-time PCR

Either LightCycler (Roche Diagnostics, Mannheim, Germany) or Rotor-Gene 3000 (Corbett Research, Sydney, Australia) was used for analysing the samples. The PCR reaction was carried out in a total volume of 20 μl in glass capillaries (LightCycler) or in 0·1 ml plastic tubes (Rotor-Gene) containing: (1) up to 20 μl H_2O; (2) 1 × QuantiTect Probe PCR Master Mix (HotStar*Taq* DNA polymerase, QuantiTect Probe PCR buffer, dNTP mix and ROX dye) (Qiagen, Hilden, Germany); (3) 6 μM forward and reverse primers, final concentration 300 nM (Table 1); (4) 4 μM probe (final concentration 200 nM, see Table 1); and (5) 2 μl cDNA or standard dilution. The enzyme mixture was pipetted in a separate PCR-clean room in which PCR products are not handled. The PCR protocol consisted of an initial denaturation step at 95°C for 15 min and a two-step temperature cycling consisting of 95°C for 0 s/60°C for 60 s (LightCycler) or 94°C for 5 s/60°C for 60 s (Rotor-Gene). Fluorescence was read at the end of the second step and a total of 50 cycles were performed. A standard curve was constructed from serial dilutions of purified DNA and mRNA amounts were expressed in copy numbers normalized against β-actin mRNA. In later runs with Rotor-Gene, stored parameters were used to calculate the standard curve adjusted with a single standard concentration in duplicate reactions for each run. PBMC were stimulated with PHA and total RNA was isolated for TGF-β, IL-10 and β-actin cDNA standard production. PCR reaction was carried out in a total volume of 20 μl with (1) 1 × QuantiTect SYBR Green PCR master mix (HotStar*Taq* DNA polymerase, QuantiTect SYBR green PCR buffer, dNTP, SYBR green I and ROX dye) (Qiagen); (2) 6 μM forward and reverse primers, final concentration 300 nM (Table 1); and (3) 5 μl cDNA template. Amplification was performed with an initial denaturation step of 95°C for 15 min followed by 45 cycles of 95°C for 15 s/58°C for 30 s/72°C for 30 s. The PCR products were run on 2% agarose gel with ethidium bromide staining and bands of appropriate size were excised with a scalper. The PCR product was then purified with the GFX DNA and gel band purification kit (Amersham) according to the manufacturer's recommendations. The calculation of copy numbers was based on spectrophotometry measurements of PCR product concentrations. Standard DNA for IFN-γ and IL-4 were prepared similarly except for the PCR amplification step, which was performed as described in our earlier study [16]. mRNA copy number in each sample was calculated from the standard curve with instrument software and Cycle threshold value, defined as the number of cycles needed for the fluorescence signal to reach the automatically set background threshold. The Ct value was correlated inversely with the number of template nucleic acid present in the reaction.

Rotavirus antibodies

Microtitre plates (Nunc, Roskilde, Denmark) were coated by incubating them with NCD virus (1·0 μg/well) in a carbonate buffer (1·6% Na_2CO_3, 2·9% $NaHCO_3$, 0·2% NaN_3) at room temperature overnight. For the detection of rotavirus IgG-antibodies, plasma samples were diluted 1 : 200 in phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) + 0·05% Tween 20 and incubated for 2 h at + 37°C. The plates were washed and peroxidase-conjugated anti-human IgG antibody (Dako, Copenhagen, Denmark) was added in a 1 : 1000 dilution. After a 1-h incubation, O-phenylenediamine and H_2O_2 in citrate-Na_2HPO_4 buffer were added. For the detection of rotavirus-specific IgA antibodies, the virus-coated plates were first residual-coated with PBS + 1% BSA. Plasma samples were diluted 1 : 10 in PBS + 0·2% BSA + 0·05% Tween 20 and incubated on the

Table 1. Primers and probes.

Target	Type[1,2]	Code[3]	T_m[4]	Length	Sequence	Exon no.	Product size
IFN-γ	St. for	IFN-g1	57·7	29	ATGAAATATACAAGTTATATCTTGGCTTT	1	494
	St. rev	IFN-g2	69·6	26	GATGCTCTTCGACCTCGAAACAGCAT	4	494
	For	F327	59·4	23	CTGACTAATTATTCGGTAACTGACTTG	3/4	107
	Rev*	R433	60·4	19	GCGACAGTTCAGCCATCAC	4	107
	Probe	P383	65·2	24	TCCAACGCAAAGCAATACATGAAC	4	
IL-4	St. for	IL-4/1	63·6	21	ATGGGTCTCACCTCCCAACTG	1	614
	St. rev	IL-4/2	56·7	20	TCAGCTCGAACACTTTGAATAT	4	614
	For	F166	60·0	20	ATCTTTGCTGCCTCCAAGAA	2/3	102
	Rev	R267	60·3	19	GCAGCGAGTGTCCTTCTCA	3	102
	Probe	P216	70·0	20	TGCGACTGTGCTCCGGCAGT	3	
TGF-β	St. for	ex6F	65·8	20	GGCTGGAAGTGGATCCACGA	6	243
	St. rev	ex7R	68·0	21	GCAGGAGCGCACGATCATGTT	7	243
	For	F949	60·7	20	GGCTACCATGCCAACTTCTG	6	100
	Rev	R1048	60·3	20	CCGGGTTATGCTGGTTGTAC	7	100
	Probe	P987	68·8	21	CATTTGGAGCCTGGACACGCA	6	
IL-10	St. for	F109	59·2	20	CTGCCTAACATGCTTCGAGA	1	494
	St. rev	R602	59·5	20	CCAGATCCGATTTTGGAGAC	5	494
	For	F373	60·7	20	CGCTGTCATCGATTTCTTCC	3/4	88
	Rev	R460	59·6	23	TGCCTTTCTCTTGGAGCTTATTA	4/5	88
	Probe	P405	69·9	20	GAGCAAGGCCGTGGAGCAGG	4	
β-actin	St. for	F745	58·1	19	CCCTGGAGAAGAGCTACGA	4	509
	St. rev	R1253	58·2	20	TAAAGCCATGCCAATCTCAT	6	509
	For	F926	63·9	19	TTGCCGACAGGATGCAGAA	5	76
	Rev	R1001	62·3	23	TCAGGAGGAGCAATGATCATTTGAT	5/6	76
	Probe*	P954	72·7	20	TGCCCTGGCACCCAGCACAA	5	

[1]St., standard; For, forward; Rev, reverse. [2]A slight modification of the reference sequence is indicated by (*). [3]Code for primers designed for this study indicates nucleotide position from the start codon (atg). [4]T_m, primer melting temperature, calculated with Primer 3 software using default parameters. IFN: interferon; IL: interleukin; TGF: transforming growth factor.

plates for 2 h at + 37°C. The plates were washed and biotinylated anti-human IgA antibody (Vector Laboratories, Burlingame, CA, USA) was added in a 1 : 100 dilution. After a 1·5 h incubation and washes, alkaline phosphatase–streptavidin (Zymed, San Francisco, CA, USA) was used as a secondary conjugate in a 1 : 1000 dilution. The plates were incubated for 1 h, washed and P-nitrophenyl phosphate substrate tablets (Sigma, St Louis, MO, USA) were added in a carbonate buffer (0·05 M $NaHCO_3$, 0·05 M Na_2CO_3, 0·02% $MgCl_2$, 0·02% NaN_3). The colour intensities were measured with spectrophotometer. Each specimen was tested in duplicate. Positive and negative standards were included on each plate in four different concentrations. A ≥threefold absorbance compared to negative control specimen exceeding the cut-off level of seropositivity, 0·150 optical density units, was considered positive.

Autoantibodies

Islet cell antibodies (ICA), GAD antibodies (GADA) and IA-2 antibodies (IA-2 A) were measured as described previously [18,19]. Insulin autoantibodies (IAA) were analysed with a slightly modified method of Williams *et al.* [20]. The threshold for detection of ICA was 2·5 Juvenile Diabetes Foundation units (JDFU). The sensitivity for our ICA assay was 100% and the specificity 98% in the fourth round of the International Workshop on the Standardization of the ICA assay. The detection limits for positivity for IAA, GADA and IA-2 A were set at the 99th percentile [1·56 relative units (RU) for IAA, 5·36 RU for GADA and 0·43 RU for IA-2 A] in more than 370 non-diabetic Finnish children. The disease sensitivity for our IAA assay was 44%, GADA assay 82% and IA-2 A assay 62% and the disease specificity was 100%, 98% and 100%, respectively, in the 2002 Centers for Disease Control and Prevention-sponsored Diabetes Autoantibody Standardization Program Workshop.

Statistics

Linear regression method was selected to compare T cell proliferation between groups. Age and HLA genotypes (HLA–DR4–DQ8/x or HLA–DR4–DQ8/DR3–DQ2) were included in the analysis as potential confounding factors. Due to skewed distributions, a logarithmic transformation was conducted for stimulation indices (SIs) to TT, PCB and CBV and 1/x transformation for SIs to rotavirus antigens. The Mann–Whitney *U*-test was used to compare cytokine production between groups. Frequencies of positive T cell responses between two groups were assessed with Fisher's exact test. Correlations between different parameters were calculated

with Spearman's rank correlation. In correlation analysis, the SIs less than 1 were regarded as 1. Statistical analysis was conducted with the SAS system for Windows, release 9·1 (SAS Institute, Cary, NC, USA). The level of significance was set at 0·05.

Results

Lymphocyte proliferation and rotavirus antibodies

As immunization with BCG and tetanus toxoid (TT) are both included in the routine Finnish vaccination schedule, the lack of an apparent (SI ≥ 3) response to either PPD or TT was considered a probable indicator of a poor condition of the cells and those few samples were excluded from the analysis. The proliferation induced by uninfected MA-104 and LLC cell lysates was similar to background proliferation levels in all groups of children (median SI 1·4 and 1·3 in children with T1D, 1·5 and 1·2 in children with autoantibodies and 1·3 and 1·0 in control children, for MA-104 and LLC, respectively). A significant correlation was observed between age and T cell responses to human RV ($r = 0{\cdot}32$ $P < 0{\cdot}0001$), bovine NCDV, $r = 0{\cdot}20$, $P = 0{\cdot}001$), PCB, $r = 0{\cdot}32$, $P < 0{\cdot}0001$), PPD ($r = 0{\cdot}19$, $P = 0{\cdot}01$) and a borderline correlation in response to TT ($r = 0{\cdot}15$, $P = 0{\cdot}04$) (Fig. 1).

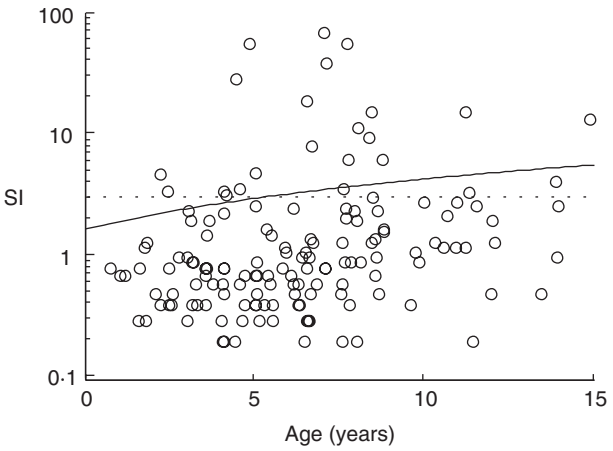

Fig. 1. T cell responses to human rotavirus lysate expressed as stimulation indices (SIs) showed a moderate correlation with age ($r = 0{\cdot}32$, $P < 0{\cdot}0001$). The dotted line was drawn at SI ≥ 3 as the cut-off limit of a positive response.

Altogether 130 of 180 (72%) children from whom information on rotavirus antibodies was available had IgA and/or IgG antibodies to rotavirus. Eighty-four (65%) of them had both IgA and IgG antibodies, nine (7%) children IgA antibodies only and 37 (28%) children IgG antibodies only. Information on T cell responsiveness to at least two rotavirus antigens was available from 156 children, 54 of whom (35%) had a positive (SI ≥ 3) T cell response to at least one of the rotavirus antigens. In 153 children from whom information on both serological and cellular responsiveness to rotavirus was available, children with rotavirus antibodies had more frequently positive T cell responses to rotavirus (50 of 112, 45%) than children without antibodies to rotavirus (four of 41, 10%) ($P < 0{\cdot}0001$). Further, cell responses to all rotavirus antigens were stronger in children with rotavirus antibodies than in children without these antibodies ($P = 0{\cdot}010$, $P = 0{\cdot}0031$ and $P < 0{\cdot}0001$ for PRV, RV and NCDV, respectively) (Table 2). The proportion of children having antibodies to rotavirus was similar in children with T1D (33 of 43, 77%) or T1D-associated autoantibodies (35 of 44, 80%) and control children negative for autoantibodies (68 of 101, 67%) ($P = 0{\cdot}32$ and $P = 0{\cdot}17$, for children with T1D and T1D-associated autoantibodies and control children, respectively). The frequencies of positive T cell responses to any of the rotavirus or coxsackie B4 virus antigens did not differ between children who had or had not developed T1D-associated autoantibodies (Table 3). Children with clinical T1D had positive responses to coxsackie B4 virus more frequently and they also tended to have responses to TT and rotavirus antigens more frequently than control children. This may be due, however, to age-related changes in cellular responsiveness to these antigens as the children with T1D (median age 8·4 years) were older than the control children (median age 5·0 years).

All children carrying either HLA–DR4–DQ8/x (21 of 30 children with T1D, 26 of 44 children with T1D-associated autoantibodies and 43 of 77 control children) or HLA–DR4–DQ8/DR3–DQ2 genotype (nine of 30 children with T1D, 18

Table 2. T cell responsiveness to purified protein derivative (PPD), tetanus toxoid (TT), purified human rotavirus (PRV), human rotavirus lysate (RV) and Nebraska calf diarrhoea virus (NCDV) in children who had or had not IgG and/or IgA antibodies to rotavirus.

	Rotavirus antibody positive children			Rotavirus antibody negative children			
	Median	*n*	Interquartile range	Median	*n*	Interquartile range	*P*
PPD	96·9	130	35·5–195·7	82·7	50	23·3–181·0	0·77
TT	9·1	130	2·1–31·0	9·0	50	1·7–35·5	0·84
PRV	0·9	129	0·5–2·0	0·7	50	0·3–1·0	0·010
RV	1·0	112	0·5–2·6	0·7	41	0·4–0·9	0·0031
NCDV	1·1	112	0·6–4·4	0·6	41	0·4–0·9	< 0·0001

Table 3. The proportions of children having a positive T cell response (stimulation index ≥ 3) to at least one of the rotavirus antigens (purified human rotavirus, human rotavirus lysate, bovine Nebraska calf diarrhoea virus), coxsackie B4 virus antigens (purified and lysate coxsackie B4 virus) and tetanus toxoid in children with newly diagnosed type 1 diabetes, children with diabetes-associated autoantibodies and control children.

	Children with diabetes	Control children	*P*	Children with autoantibodies	Control children	*P*
Rotavirus	18/38 (47%)	26/84 (31%)	0·10	15/42 (36%)	26/84 (31%)	0·69
Coxsackie B4 virus	27/38 (71%)	41/84 (49%)	0·030	26/42 (62%)	41/84 (49%)	0·18
Tetanus toxoid	35/43 (81%)	67/104 (64%)	0·050	34/44 (77%)	67/104 (64%)	0·19

of 44 children with T1D-associated autoantibodies and 34 of 77 control children) were included in linear regression analysis. No difference was observed in T cell responses to rotavirus antigens between children who had developed T1D or T1D-associated autoantibodies and autoantibody negative control children (Fig. 2), nor did responsiveness to TT differ between these groups. Responsiveness to PPD was slightly stronger in children with T1D-associated autoantibodies than in control children ($P = 0{\cdot}026$), but this was due to differences in the ages of children in these groups ($P = 0{\cdot}015$). Children with autoantibodies also had stronger T cell responses to PCB than autoantibody negative control children ($P = 0{\cdot}0003$). Autoantibody status ($P = 0{\cdot}033$) and age ($P = 0{\cdot}001$) were observed to affect cellular responsiveness to PCB. In children with T1D, a similar tendency was observed ($P = 0{\cdot}001$; $P = 0{\cdot}058$ and $P = 0{\cdot}007$ for T1D and age, respectively). Further, the children with T1D had higher responsiveness to CBV lysate antigen ($P = 0{\cdot}013$), but this was due to differences in ages and HLA genotypes between the groups ($P = 0{\cdot}028$ and $P = 0{\cdot}039$, for age and HLA genotype, respectively). Cell-mediated immunity to rotavirus was also analysed separately in those children who, based on the presence of specific antibodies in their sera, had experienced rotavirus infections. This analysis did not either reveal any differences in cellular responsiveness to rotavirus between children with T1D-associated autoantibodies or T1D compared to autoantibody negative control children (data not shown).

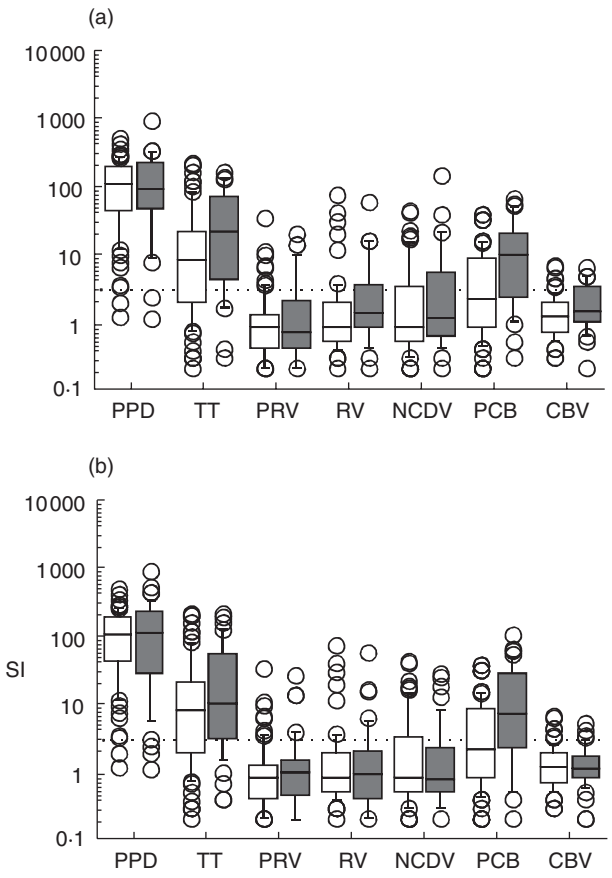

Fig. 2. T cell responses shown as stimulation indices (SIs) to a panel of antigens [purified protein derivative (PPD), tetanus toxoid (TT); purified human rotavirus (PRV); human rotavirus lysate (RV); Nebraska calf diarrhoea virus (NCDV); purified coxsackie B4 virus (PCB); lysate coxsackie B4 virus (CBV)] in children with diabetes-associated autoantibodies (a) and in children with type 1 diabetes (b). T cell responses to PCB were stronger in children with clinical diabetes and autoantibodies (grey boxes, $P = 0{\cdot}0012$ and $P = 0{\cdot}0003$, respectively) than in control children (white boxes) as well as responses to CBV in children with T1D ($P = 0{\cdot}013$) and responses to PPD in children with diabetes-associated autoantibodies ($P = 0{\cdot}026$). Median value in each box is shown with a horizontal line in the box. The box plots delineate values between the 25th and 75th percentiles and the whiskers values between the 10th and the 90th percentiles. The values outside this range are indicated with circles.

Cytokine production

We also wanted to assess cytokine responsiveness in association of T cell responses to rotavirus. The production of IL-4, IFN-γ, TGF-β and IL-10 was studied in non-stimulated and PHA-, PPD-, PRV- and PCB-stimulated PBMC from 11 children with T1D, seven additional children with T1D-associated autoantibodies and in 20 control children. In PRV-stimulated cells, a correlation was found between the age of the child and the production of IL-4 and IFN-γ (data not shown).

The production of IFN-γ and IL-4 correlated with the lymphocyte proliferation response to the same antigen in PBMC stimulated with PPD ($r = 0{\cdot}69$, $P < 0{\cdot}0001$ and $r = 0{\cdot}54$, $P = 0{\cdot}0005$ for IFN-γ and IL-4, respectively), PRV ($r = 0{\cdot}48$, $P = 0{\cdot}003$ and $r = 0{\cdot}46$, $P = 0{\cdot}004$, respectively)

Table 4. Median cytokine production of peripheral blood mononuclear cells from control children and children with diabetes-associated autoantibodies.

	Children with autoantibodies			Control children			
	Median	*n*	Interquartile range	Median	*n*	Interquartile range	*P*
IFN-γ							
Medium	6·4	10	0·0–18·6	2·9	20	0·0–9·0	0·44
PHA	2307·3	10	588·2–7822·1	709·2	20	177·1–1715·2	0·028
PPD	592·9	10	452·2–1168·9	1027·3	20	120·2–1743·4	0·71
PRV	126·8	10	35·0–608·0	22·8	20	7·0–134·0	0·18
PCB	1885·6	10	64·3–4859·6	672·9	20	48·1–2491·0	0·55
IL-4							
Medium	1·2	10	0·0–2·2	0·0	20	0·0–1·7	0·21
PHA	38·6	10	11·5–58·3	14·6	20	3·4–28·6	0·039
PPD	2·2	10	0·0–6·6	6·8	20	0·0–13·1	0·17
PRV	3·2	10	0·0–11·0	0·0	20	0·0–1·2	0·037
PCB	1·4	10	0·0–8·4	0·0	19	0·0–8·6	0·82
IL-10							
Medium	60·6	10	15·2–167·5	15·9	20	5·9–53·3	0·19
PHA	11·5	10	4·3–40·0	8·5	20	2·5–23·5	0·29
PPD	14·3	10	6·0–17·1	14·0	20	7·3–24·6	0·79
PRV	143·0	10	112·2–201·8	101·5	20	62·8–164·0	0·31
PCB	121·1	10	29·3–221·3	43·1	20	22·4–177·7	0·36
TGF-β							
Medium	29·2	10	18·8–58·0	21·1	20	16·1–40·6	0·43
PHA	17·3	10	5·2–23·6	11·6	20	5·2–19·9	0·72
PPD	21·4	10	13·3–24·7	31·2	20	20·3–44·3	0·095
PRV	36·4	10	22·0–67·8	35·0	20	30·0–49·3	0·57
PCB	30·4	10	21·8–47·0	21·9	20	13·5–42·8	0·25

IFN: interferon; IL: interleukin; TGF: transforming growth factor.

and PCB ($r = 0{\cdot}61$, $P < 0{\cdot}0001$ and $r = 0{\cdot}38$, $P = 0{\cdot}019$, respectively). The production of TGF-β did not correlate with proliferation responses to any of the studied antigens, but the production of IL-10 in PRV- and PCB-stimulated lymphocytes correlated with T cell responses to these antigens ($r = 0{\cdot}36$, $P = 0{\cdot}026$ and $r = 0{\cdot}39$, $P = 0{\cdot}015$, respectively). A trend towards a higher production of IFN-γ was observed in PRV-stimulated PBMC from seropositive children compared to children without rotavirus antibodies ($P = 0{\cdot}084$).

In children with T1D-associated autoantibodies, IL-4 expression in PRV-stimulated and IFN-γ and IL-4 expression in PHA-stimulated PBMC were higher than in control children (Table 4). When cytokine production in PRV-stimulated cells was studied in rotavirus seropositive children only, no difference was observed in between children with clinical T1D or T1D-associated autoantibodies and control children, due possibly to the small amount of children in each group. In children with T1D, PHA-stimulated PBMC also expressed more IL-4 and PPD-stimulated cells less TGF-β than in autoantibody negative control subjects (Table 5). No difference was seen in the relative amount of cytokine gene expression in stimulated *versus* non-stimulated lymphocytes between healthy children with and without autoantibodies. The relative amount of TGF-β in PPD-stimulated *versus* non-stimulated cultures was lower in children with T1D than in autoantibody negative control children ($P = 0{\cdot}011$).

Discussion

We did not find any association between rotavirus-specific T cell responses and the presence of T1D-associated autoantibodies or T1D, nor did cytokine responses in rotavirus-stimulated PBMC considerably differ between children with overt T1D, children with autoantibodies or control children. Our results are concordant with those of Jones and Crosby [21], who studied T cell responses to the 69M strain of serotype G8 rotavirus in newly diagnosed T1D patients and control subjects and found strong rotavirus-specific T cell responses in six of 26 diabetics and four of 24 control subjects. The present observations are also consistent with our previous study, in which we did not find any association between serologically diagnosed rotavirus infections and the appearance of T1D-associated autoantibodies in a cohort of 177 children at increased genetic risk for T1D [7]. Twenty-nine children developed multiple T1D-associated autoantibodies during the follow-up, but the appearance of autoantibodies did not occur simultaneously with rotavirus infections and the frequency of infections was similar to that seen in matched control children. Controversially, in a prospective study with 41 Australian children at increased

Table 5. Median cytokine production of peripheral blood mononuclear cells from control children and children with type 1 diabetes.

	Children with diabetes			Control children			
	Median	*n*	Interquartile range	Median	*n*	Interquartile range	*P*
IFN-γ							
Medium	3·4	11	0·0–24·9	2·9	20	0·0–9·0	0·76
PHA	588·2	11	342·2–5165·9	709·2	20	177·1–1715·2	0·32
PPD	717·4	10	530·0–3495·1	1027·3	20	120·2–1743·4	0·84
PRV	67·2	11	9·4–123·4	22·8	20	7·0–134·0	0·56
PCB	2081·2	11	232·7–11285·1	672·9	20	48·1–2491·0	0·19
IL-4							
Medium	0·9	11	0·0–4·8	0·0	20	0·0–1·7	0·24
PHA	58·3	11	19·1–97·8	14·6	20	3·4–28·6	0·0064
PPD	5·9	10	2·7–16·7	6·8	20	0·0–13·1	0·96
PRV	0·0	11	0·0–11·0	0·0	20	0·0–1·2	0·15
PCB	0·0	11	0·0–19·8	0·0	19	0·0–8·6	0·76
IL-10							
Medium	48·1	11	20·2–80·2	15·9	20	5·9–53·3	0·099
PHA	8·7	11	4·9–22·8	8·5	20	2·5–23·5	0·54
PPD	9·2	10	6·3–17·1	14·0	20	7·3–24·6	0·63
PRV	180·9	11	80·4–260·3	101·5	20	62·8–164·0	0·12
PCB	144·6	11	50·6–278·4	43·1	20	22·4–177·7	0·19
TGF-β							
Medium	24·1	11	18·8–89·3	21·1	20	16·1–40·6	0·17
PHA	29·5	11	12·9–63·3	11·6	20	5·2–19·9	0·060
PPD	20·3	10	14·5–26·0	31·2	20	20·3–44·3	0·025
PRV	28·8	11	24·2–49·4	35·0	20	30·0–49·3	0·48
PCB	21·8	11	17·9–30·4	21·9	20	13·5–42·8	0·59

IFN: interferon; IL: interleukin; TGF: transforming growth factor.

genetic risk to develop T1D, 86% of the increases in IA-2 A, 62% of the increases in IAA and 50% of the increases in GADA were temporally associated with rotavirus antibody seroconversions [5]. The differences between these studies may be due to real differences between populations or predominating virus strains. Also different virus strains used in these studies or methodological differences in interpreting antibody data may affect these results.

Shared epitopes between autoantigens and viruses or a deficient response related to a persistent infection has been suggested to be possible mechanisms for the association between some viral infections and T1D [22]. Honeyman *et al.* have observed homology between VP7 protein of the P strain of serotype G3 rotavirus and epitope peptides in the T1D-associated autoantigens GAD65 and IA-2 [23]. They reported 75% identity and 92% similarity between GAD65 and VP7 peptide containing amino acids (aa) 17–28 (ILLNYVLKSLTR) and 56% identity and 100% similarity between IA-2 and VP7 peptide containing aa 40–50 (IIVILSPLLNA). In the cases of molecular mimicry between rotavirus and β-cell antigens or an increased number of rotavirus infections, one might expect an increased level of cellular responsiveness to rotavirus. Alternatively, a chronic infection might also be established due to the lack of specific immune responsiveness. Accordingly, we also analysed T cell responses to rotavirus separately in children seropositive for rotavirus IgG and/or IgA antibodies to detect whether they exhibited abnormal responsiveness to rotavirus. However, we did not find any differences in proliferation responses between children with T1D or T1D-associated autoantibodies and control children in these comparisons. In the present study, we have used G1 and G6 serotypes of rotavirus because G1 is the most prevalent serotype in Finland [24] as well as in the whole of Europe, and here serotype G3 represents only a few per cent of rotavirus infections [25]. Of the two peptides in VP7 protein that Honeyman *et al.* observed to resemble T1D-associated autoantigens, the aa 17–28 sequence of G1 serotype rotavirus contains two different amino acids than G3 serotype. More variation is present in the aa 40–50 sequence, where five amino acids are substituted by different amino acids. The significance of these differences is uncertain, as it has been shown that direct homology between amino acid stretches is not needed in molecular mimicry [26]. Consequently, a few amino acids binding to critical sites in the HLA molecule may be enough to induce a cross-reactive immune response [27].

The ages of children in this study varied from 3·5 to 11·3 years. Naturally, the frequency of positive T cell or antibody responses to viral antigens increases with age. Linear regression models appeared to be the most powerful way to

analyse this type of data as age could be included in these analyses as explanatory variables. However, the use of linear regression analysis was not possible in all analysis settings, e.g. when frequencies of positive responses were compared between groups of children. Age differences between the children may thus affect some results, but the effect of age was excluded in the main analyses comparing T cell responsiveness between children with and without T1D and/or T1D-associated autoantibodies.

Coxsackie B4 virus antigens served as a reference in our study as this virus has often been connected to T1D and β cell autoimmunity. In our previous studies, we found increased T cell responsiveness to CBV in children positive for ICA and/or GADA and in T1D patients within 4–72 months from diagnosis, but not in patients with newly diagnosed T1D [28,29]. Jones and Crosby [21], in contrast, found strong T cell responses to a lysate coxsackie B4 virus antigen in newly diagnosed T1D patients. In the present study, we observed stronger cellular responsiveness to PCB but not to CBV in children with T1D-associated autoantibodies compared to autoantibody negative control children. In this study, coxsackie B4 virus lysate antigen was a remarkably poor inducer of any T cell responses. This may reflect technical problems with this particular antigen as cell lysate-type antigens often show inhibitory effects at higher concentrations.

In cytokine expression studies with PRV-stimulated PBMC from children with autoantibodies, the expression of IL-4 was higher than in control children. In PHA-stimulated PBMC, we found higher IL-4 expression in children with clinical T1D and T1D-associated autoantibodies and higher IFN-γ expression in children with autoantibodies than in control children. We also observed lower TGF-β expression in PPD-stimulated PBMC from children with T1D than in control children. We used several stimulators to measure four different cytokines and, as *P*-values lose their significance if multiplied by the number of comparisons, the differences found may represent chance effects. However, in our earlier study we consistently observed increased mRNA expression of both IL-4 and IFN-γ in PHA-stimulated PBMC in 22 children with newly diagnosed T1D compared to 20 healthy control children [30]. Kallman and coworkers found higher titres of IFN-γ but not IL-4 in PHA-stimulated PBMC from 53 newly diagnosed T1D patients than in 56 control subjects [31]. Kretowski and coworkers reported lower titres of TGF-β1 in PHA-stimulated PBMC from 22 newly diagnosed T1D patients and their 24 first-degree relatives than in 18 control subjects [32]. Controversial observations have been reported by Berman *et al.* [33] and Lohmann *et al.* [34] ,who observed decreased titres of IL-4 and no difference or decreased production of IFN-γ in children with newly diagnosed T1D compared to age-matched control children. These controversial observations may also be related to different methodologies used, as mRNA expression may not be directly comparable with cytokine concentrations measured with enzyme-linked immunosorbent assay (ELISA). Also the length of *in vitro* stimulation with PHA [35] and the duration of T1D might affect cytokine production pattern.

In conclusion, our cellular immunity studies did not provide any evidence supporting an association between rotavirus infections and T1D or the presence of T1D-associated autoantibodies in young children.

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