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Rotavirus infections and development of diabetes-associated autoantibodies during the first 2 years of life

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SUMMARY

Rotavirus, the most common cause of childhood gastroenteritis, has been implicated as one of the viral triggers of diabetes-associated autoimmunity. To study the possible association between rotavirus infections and the development of diabetes-associated autoantibodies, we measured the prevalence of rotavirus antibodies in serum samples collected at 3–6-month intervals up to the age of 2 years from 177 children selected from consecutive newborns because they carried HLA-DQB1 alleles associated with increased risk for type 1 diabetes. Twenty-nine of the children developed at least two of four diabetes-associated autoantibodies (ICA, IAA, GADA or IA-2A) during the first 2 years of life (the cases), whereas 148 children remained autoantibody-negative matched with the cases for date of birth, gender, living region and HLA-DQB1 alleles. The temporal association between the development of the first-appearing diabetes-associated autoantibody and rotavirus infections was studied by analysing whether the cases had a diagnostic increase in rotavirus antibody titre more often during the 6-month period that preceded seroconversion to autoantibody positivity than the controls. By the age of 12 months one of the 13 case children (7%), who had a serum sample drawn at that age and who had developed at least one type of diabetes-associated autoantibodies, had experienced a rotavirus infection, while 12 of the 61 (20%) autoantibody-negative control children had had a rotavirus infection. By 18 months, four of the 22 autoantibody-positive cases (18%) and 18 of the 89 controls (20%) had rotavirus antibodies, and by the age of 24 months the respective numbers were five of the 27 cases (19%) and 32 of the 113 (28%) controls. A rotavirus infection occurred during the 6 months preceding the sample which was positive for an autoantibody in four of the 25 periods (16%) for which both necessary samples were available, while the controls had a rotavirus infection during 55 of the 370-such periods (15%). Accordingly, our data suggest that rotavirus infections are unlikely triggers of beta-cell autoimmunity in young children with genetic susceptibility to type 1 diabetes.

Keywords aetiology autoantibody prospective rotavirus Type 1 diabetes mellitus

INTRODUCTION

Worldwide, rotaviruses are one of the most common causes of gastroenteritis in young children and a major cause of gastroenteritis-associated hospitalizations. Severe infections seldom occur before the age of 6 months because of protective maternal antibodies, and a large proportion of the infections are mild or subclinical in older children and adults. Neutralizing antibodies, probably together with rotavirus-primed cytotoxic T lymphocytes, effectively protect man and experimental animals from reinfections [1].

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Honeyman and coworkers [2] reported recently that diabetes-associated autoantibodies appeared in their study children concomitantly with a rise in rotavirus IgG antibody titre. Because rotaviruses share homologous amino acid sequences with two prominent diabetes-associated autoantigens, the 65 kDa isoform of glutamic acid decarboxylase (GAD65) and the protein tyrosine phosphatase related IA-2 molecule, the data suggested that the autoimmune reaction may have been induced by molecular mimicry [3]. Rotavirus also changes the permeability and cytokine balance in the intestinal mucosa, and may thereby enhance autoimmunity. Cytokines released during a rotavirus gastroenteritis are clearly a major cause of the severe epithelial dysfunction that commonly associates with the disease. Interestingly, T cells are apparently also able to modify intestinal ion secretion in rotavirus gastroenteritis, at least in a murine model of the disease

[4]. While the cytokine IFN-gamma affects the permeability at tight junctions in the mucosa [5], TNF-alpha and IFN-gamma, produced excessively during a rotavirus infection, are directly toxic to, e.g. human colonic epithelial cells [6].

We now have evaluated the association between the appearance of diabetes-associated autoantibodies and serological signs of rotavirus infection in serum samples collected in the Type 1 Diabetes Prediction and Prevention project (DIPP) at the JDRF Center for Prevention of Type 1 Diabetes in Finland [7,8]. The samples, obtained at birth and then at 3–6-month intervals from children selected from consecutive newborns because of increased HLA-DQ gene-mediated diabetes risk, allowed us to determine when rotavirus infections occurred, and when the children began to produce diabetes-associated autoantibodies.

RESEARCH DESIGN AND METHODS

Subjects

Twenty-nine children (13 boys) were selected from those DIPP study children (see below) who had developed diabetes-associated autoantibodies during their first 2 years of life. All study children had either the HLA-DQB1*02/*0302 or the HLA-DQB1*0302/x (*x *02, *0301 or *0602) genotype, known to be highly associated with susceptibility to type 1 diabetes. From four to seven control children matched for the HLA-DQB1 risk genotype, date of birth (median 0·5 days after, from 54 days before to 50 days after; the closest ones in the study), gender and living region (regions in or around the cities of Turku, Oulu or Tampere in Finland) were selected for each case child. The control children (N = 148; 67 boys) have all remained autoantibody negative at least up to the age of 3 years. Special care was taken that all samples used in the study for comparisons were always collected from the case and his/her controls within a short time frame (median 1 day before, from 60 days before to 78 days after). The study was approved by the hospitals' ethics committees. Informed consent was obtained from each study subject before participation.

According to the DIPP protocol [7,8] ICA alone were first measured in all samples, and if the child had ICA, antibodies against the biochemically characterized autoantigens (insulin, GAD65 and IA-2) were also analysed in all previous and all future samples of that child. All children regarded in this study as autoantibody-positive had developed at least two types of autoantibodies by the age of 2 years. In all analyses, the case children were regarded to have seroconverted to autoantibody positivity when the first autoantibody that remained positive at least to the age of 2 years was detected. In fact, all autoantibodies found in these study children persisted at least up to that age.

Production of virus antigen

Cultures of LLC-MK2 cells were infected with Nebraska calf diarrhoea (NCD) virus in the presence of trypsin [9]. After adsorption, basal medium containing bovine serum albumin and trypsin was added. The cells were harvested when the virus had caused an advanced cytopathic effect. The material was pooled, frozen and diluted after thawing to be used as virus antigen in antibody determinations.

Virus antibodies

IgG antibodies against rotavirus were measured with an indirect EIA method using NCD virus as an antigen. Microtitre plates (Nunc Immunoplate, Nunc, Roskilde, Denmark) were coated with the virus antigen at 5 μg/ml concentration in carbonate buffer (1·6% Na_2CO_3, 2·9% $NaHCO_3$, 0·2% NaN_3) and incubated overnight at room temperature. Sera were incubated for 2 h at 1/100 dilution in PBS + 1% BSA + 0·05% Tween 20 in duplicate wells. After washes peroxidase conjugated antihuman IgG antibody (Dako, Copenhagen, Denmark) was added at 1/1000 dilution. After incubation for 1 h, o-phenylenediamine and H_2O_2 in citrate-Na_2HPO_4 buffer were added, the reaction was stopped by adding 1 M HCl and the colour intensity was measured with a Multiscan Plus spectrophotometer (Labsystems, Helsinki, Finland). A threefold stronger absorbance than that caused by a negative control serum was considered positive. A twofold increase in absorbance between two consecutive samples was considered to indicate that an infection had occurred between the time-points.

Autoantibody assays

ICA were detected by indirect immunofluorescence using blood group O human pancreas [10] and fluorescein-conjugated rabbit antihuman IgG. Dilutions were quantified using end-point titration. The titres were expressed in juvenile diabetes foundation units (JDFU) [10].The threshold of detection was 2·5 JDFU. The sensitivity of this assay was 100% and the specificity 98% according to the fourth round of the international workshops for ICA standardization [11].

GAD antibodies (GADA) were analysed by a radioligand method using an *in vitro* transcribed and translated GAD65 antigen [12]. A standard curve was prepared by mixing a sample from a pooled positive sample with GADA-negative serum. The antibody concentrations were expressed in relative units (RU) based on the curve. The cut-off limit for antibody positivity was set at the 99th percentile in 373 healthy Finnish children and adolescents, i.e. at 5·35 RU. The disease sensitivity of this assay was 69% and the specificity 100% based on 140 samples from the 1995 multiple autoantibody workshop [13].

IA-2 antibodies (IA-2A) were measured using a similar assay principle as for GADA [14]. The IA-2A titres were expressed in relative units based on a standard curve. The cut-off limit for antibody positivity was 0·43 RU, representing the 99th percentile in 374 non-diabetic subjects. This assay had a disease sensitivity of 62% and a specificity of 97% based on 140 samples derived from the 1995 multiple autoantibody workshop [13].

IAA were quantified with a method modified from that described by Williams *et al*. [15]. Serum samples were incubated with mono-[^{125}I]-TyrA14-human insulin (Amersham, Little Chalfont, Bucks, UK) for 72 h in the absence or presence of an excess of unlabelled insulin. Immunocomplexes were precipitated by adding protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) and the quantity of complexes was counted with a scintillation counter. The results were expressed in relative units (RU). The limit for antibody positivity was 1·56 RU, which represents the 99th percentile in 374 healthy Finnish children and adolescents. The disease sensitivity and specificity of this assay was 35% and 100%, respectively, based on the 140 samples from the 1995 multiple autoantibody workshop [13].

Statistical analysis

Fisher's exact test was used to compare the frequencies of rotavirus infection between the case and the control children. P-values <0·05 were regarded as significant.

RESULTS

Most children in this study had maternal IgG antibodies against rotavirus in the cord blood sample, but these antibodies showed a continuous decline in titre after birth and became undetectable by the age of 6 months. None of the study children had maternal diabetes-associated autoantibodies in cord blood. Fifteen of the 29 case children, who all developed at least two types of diabetes-associated autoantibodies before reaching the age of 2 years, lost the maternal rotavirus antibodies during the first few months of life, after which the rotavirus antibody titres remained continuously low for the rest of the study period indicating that no rotavirus infections had occurred (Fig. 1a).

The remaining 14 case children also had declining rotavirus antibody titres after birth, but the titres then increased markedly, implying that the children had had a rotavirus infection (Fig. 1b). Using these criteria, the 29 case children and the 148 control children had altogether 93 detectable rotavirus infections during the first 2 years of life.

Only one child showed evidence of a rotavirus infection before the age of 6 months. He was one of the three case children (33%) who had already developed diabetes-associated autoantibodies by that age, while none of the 12 control children had had a rotavirus infection (Table 1).

At the age of 12 months, only this same child of the 13 autoantibody-positive case children (7%) had rotavirus antibodies whereas 12 of the 61 matched control children (20%) had antibodies against rotavirus. The frequency of diabetes-associated autoantibodies in the case children and seropositivity for rotavirus antibodies increased then in parallel in the two groups of children, so that at the age of 2 years, five of the 27 autoantibody positive case children (19%) had antibodies against rotavirus while the respective number among the 113 autoantibody-negative control children was 32 (28%). Accordingly, children with autoantibodies showed no evidence of having experienced more rotavirus infections than the control children at any time-point analysed (Fig. 2).

Also, when rotavirus infection frequencies were calculated in case children who had developed different types of diabetes-associated autoantibodies or different combinations of autoantibodies, the findings were closely similar to those in their control children at all ages studied.

The possible inter-relationship between rotavirus infections and the appearance of individual autoantibodies or their combination was studied further by dividing the follow-up series into 6-month periods. At least one new diabetes-associated autoantibody was detected during 25 such 6-month periods in the 29 case children, whereas no new autoantibodies appeared during a total of 370 periods in the case and control children (Table 2).

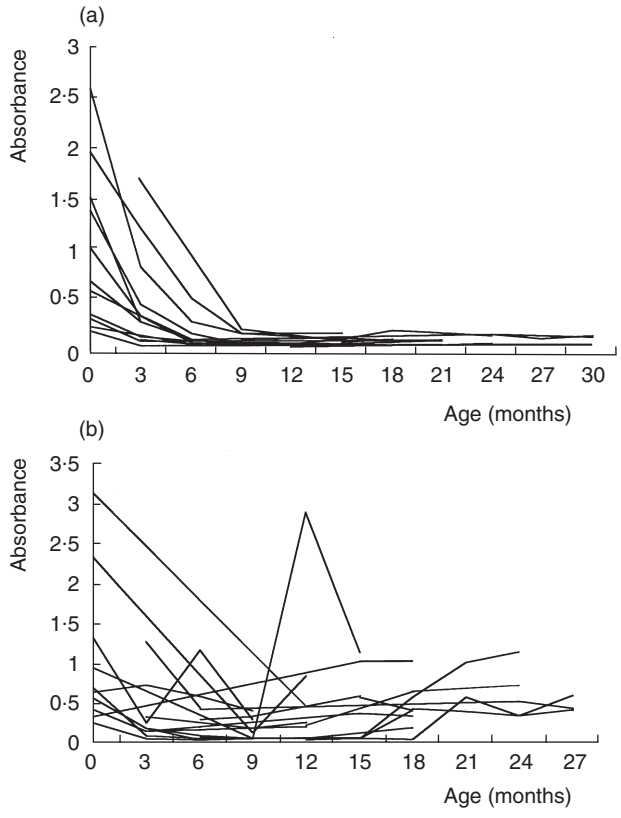

Fig. 1. Rotavirus antibody in the case children who developed diabetes-associated autoantibodies during the first 2 years of life. (a) Children who did not have increases in IgG levels during the first 2 years of life (n = 15). (b) Rotavirus antibody levels in those children who based on these antibody titres were deduced to have experienced a rotavirus infection (n = 14).

Table 1. Frequency of rotavirus infections experienced by study subjects before the seroconversion of diabetes associated autoantibodies

	Seropositive for rotavirus antibodies				
	Cases		Matched controls		
Time of autoantibody appearance	*n*	%	*n*	%	*p*
Any autoantibody					
6 months	1/3	33	0/12	0	0·20
12 months	1/13	7	12/61	20	0·44
18 months	4/22	18	18/89	20	>0·99
24 months	5/27	19	32/113	28	0·34
ICA					
6 months	0	0	0	0	
12 months	2/8	25	16/35	46	0·43
18 months	6/18	33	24/66	36	>0·99
24 months	8/25	32	44/96	46	0·36
IAA					
6 months	1/3	33	0/12	0	0·20
12 months	1/13	8	12/63	19	0·45
18 months	4/21	19	19/89	21	>0·99
24 months	4/24	17	26/101	26	0·43
GADA					
6 months	0	0	0	0	
12 months	2/8	25	10/27	37	0·69
18 months	6/19	32	23/65	36	>0·99
24 months	7/21	33	31/76	41	0·62
IA-2					
6 months	0	0	0	0	
12 months	1/2	50	2/6	33	>0·99
18 months	3/9	33	8/17	47	0·68
24 months	3/11	27	10/27	37	0·71

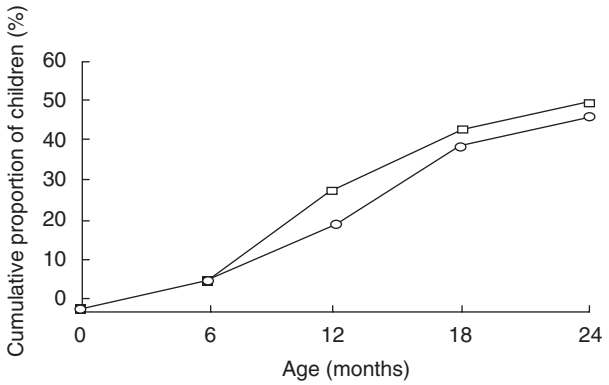

Fig. 2. Cumulative proportion of case children (○) and control children (□) who had experienced at least one rotavirus infection by the age shown on the *x*-axis.

Table 2. Periods with the appearances of autoantibodies and occurrence of rotavirus infections based on rotavirus antibody rises

	Infection	No infection	Total	*P*
Any autoantibody	4 (16%)	21 (84%)	25	0·78
ICA	2 (14%)	12 (86%)	14	>0·99
IAA	2 (13%)	14 (88%)	16	>0·99
GADA	1 (9%)	10 (91%)	11	>0·99
IA-2	0	5 (100%)	5	>0·99
No autoantibody	55 (15%)	315 (85%)	370	
Total	59 (15%)	336 (85%)	395	

Four of the 25 periods during which a new autoantibody emerged in the case children (16%) were periods with a rotavirus infection, while the respective number in the control periods was 55 (15%). If the first-appearing autoantibody only in each child was taken into account in this analysis, four of 20 such periods had a rotavirus infection (20%).

We also analysed how often the case children seroconverted to autoantibody positivity during the 6-month period following a rotavirus infection. Only one of these 25 6-month periods (4%) that in the case children comprised a seroconversion to autoantibody positivity was preceded by a period of rotavirus infection, while periods without autoantibody appearance were preceded in 58 cases by a period with a rotavirus infection. When the different autoantibodies were analysed separately, the findings were closely similar and failed to support the hypothesis that rotavirus infections may associate with the appearance of diabetes-associated autoantibodies in young children.

DISCUSSION

In recent years, several viruses have been implicated as playing a role in the pathogenesis of type 1 diabetes. Children with a congenital rubella infection commonly acquire type 1 diabetes [16] and children who develop diabetes have experienced more enterovirus infections than control subjects before the appearance of autoantibodies and in fetal life [17–19]. The appearance of diabetes-associated autoantibodies also coincides with enterovirus infections more often than predicted by chance [19], and an enterovirus has been isolated from the pancreas of a child who died at the diagnosis of diabetes [20]. Single reports have also connected mumps [21] and cytomegalovirus infections [22] with type 1 diabetes.

The recent data of Honeyman and coworkers [2] suggested that rotavirus infections might be related to the primary appearance of diabetes-associated autoantibodies. They analysed rotavirus IgA and IgG antibodies and Coxsackie B IgM antibodies in serum samples taken during follow-up of 24 infants with at least two autoantibodies or one islet antibody detected on two occasions and 17 unrelated control children from the Australian Babydiab study. A rotavirus infection was associated with the first appearance of insulin, GAD and IA-2 autoantibodies in 83%, 50% and 86% of the children with a first-degree relative with type 1 diabetes. In striking contrast to these observations, we found no evidence for an association between rotavirus infections and the appearance of the first or any of the later diabetes-associated autoantibodies in our large cohort of children followed prospectively with increased genetic risk for type 1 diabetes.

The discrepant results of the two studies may reflect a true difference between the two study populations or methodological differences. In the Australian series consecutive increases in rotavirus antibody titres were interpreted as separate infections, whereas we in our study population found no clear signs suggesting reinfections with rotavirus. We feel that reinfections are probably rare, since our cumulative figures show that approximately half of our study children seroconverted to rotavirus antibody positivity by age of 2 years. Furthermore, epidemiological data on rotavirus infections indicates that there is usually one major annual epidemic. Prolonged rotavirus shedding may occur after an acute rotavirus infection and it could cause prolonged rotavirus antibody production. Indeed, a previous study shows that 11 of 37 children with rotavirus diarrhoea excreted rotavirus for 20–57 days after the onset of diarrhoea, and the virus genotype changed from the P to the G genotype in only one of the children [23]. Extended secretion of rotavirus was accompanied by a boost in rotavirus IgA antibody titres during the follow-up.

Rotavirus infections are common in young children as, e.g. in Washington DC more than 90% of all children acquire rotavirus antibodies before the age of 4 years [24]. A large proportion of adults also have rotavirus antibodies, their levels enhanced probably by occasional subclinical reinfections. Ruuska and Vesikari [25], who observed a cohort of 336 Finnish children from birth to the age of 24–32 months, found that rotavirus infections were uncommon during the first 6 months of life but that 22% of the boys and 16% of the girls had a symptomatic rotavirus diarrhoea during the later follow-up. Interestingly, they observed that each child experienced rotavirus diarrhoea only once and that the annual rotavirus epidemic usually lasted from December to May. These figures agree well with our data, which show that about half of the study children had had a rotavirus infection by the age of 2 years.

A clear increase in rotavirus IgG antibody titre reliably detects rotavirus infections [26,27]. Bishop and coworkers [28] followed 68 mother–infant pairs for 12–17 months to study the dynamics of their rotavirus antibodies. Sera were obtained from the children at 6-month intervals and faecal specimens weekly. The rotavirus antigen was found in the stools of 15 infants during

the follow-up, while rotavirus IgG antibodies were detected in 12 of the children. When serum samples were collected at appropriate intervals, rotavirus IgG antibodies were detected in all children who excreted the rotavirus antigen.

Antigenic relationship between human and animal rotaviruses has been well established [29,30] and forms the basis for the use of animal viruses in human vaccines. The NCDV antigen-based antibody detection using radioimmunoassay has excellent sensitivity and specificity [9]. When the NCDV antigen detection test was compared with human rotavirus EIA and human rotavirus RIA in 98 rotavirus negative and 118 rotavirus positive stools samples of children, the NCDV antigen detection test was practically as sensitive and specific for rotavirus infection as the human rotavirus EIA [31].

In conclusion, our findings fail to support the hypothesis that rotaviruses are important triggers of beta-cell autoimmunity in young children at increased genetic risk for type 1 diabetes.

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