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RESEARCH ARTICLE

Interaction of enterovirus infection and cow's milk-based formula nutrition in type 1 diabetes-associated autoimmunity

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Abstract

Background Enteral virus infections and early introduction of cow's milk (CM)-based formula are among the suggested triggers of type 1 diabetes (T1D)-associated autoimmunity, although studies on their role have remained contradictory. Here, we aimed to analyse whether interactions between these factors might clarify the controversies.

Materials The study population comprised 107 subjects developing positivity for at least two T1D-associated autoantibodies and 446 control subjects from the Finnish diabetes prediction and prevention cohort. Enterovirus, rotavirus, adenovirus, respiratory syncytial virus and bovine insulin-binding antibodies were analysed from prospective serum samples at 3–24 months of age. Data on infant cow's milk exposure were available for 472 subjects: 251 subjects were exposed to cow's milk before 3 months of age and 221 subjects later in infancy.

Results Signs of an enterovirus infection by 12 months of age were associated with the appearance of autoimmunity among children who were exposed to cow's milk before 3 months of age. Cox regression analysis revealed a combined effect of enterovirus infection and early cow's milk exposure for the development of ICA and any of the biochemically defined autoantibodies ($p = 0.001$), of IAA ($p = 0.002$), GADA ($p = 0.001$) and IA-2A ($p = 0.013$).

Conclusions The effect of enterovirus infection on the appearance of T1D-associated autoimmunity seems to be modified by exposure to cow's milk in early infancy suggesting an interaction between these factors. Moreover, these results provide an explanation for the controversial findings obtained when analysing the effect of any single one of these factors on the appearance of T1D-associated autoimmunity. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords autoantibody appearance; cow's milk formula exposure; coxsackievirus B4 infection; enterovirus infection; interaction; type 1 diabetes

Introduction

Type 1 diabetes (T1D) is an autoimmune disease resulting from the destruction of the pancreatic β cells by an autoimmune process. Genetic predisposition to the loss of self tolerance is well-known. Among the T1D susceptibility genes, the human leukocyte antigen (HLA) region is the major determinant, being responsible for about 50% of the inherited disease risk. Outside the HLA region, the *INS* gene polymorphism and *PTPN22* Arg620Trp polymorphism seems to be important contributors to the autoimmune process leading to T1D [1].

Despite the evident role of the inherited component of T1D risk, only a small proportion of the individuals with predisposing genotypes present with clinical

disease. Thus, in addition to the genetic background, environmental factors are crucial for the initiation and progression of the disease process. Both virus infections, especially enterovirus and rotavirus infections, and early exposure to a cow's milk (CM)-containing diet during infancy have been implicated as triggering factors of the autoimmune process [2]. However, the outcomes of several recent studies have remained controversial, and accordingly, the role of these environmental factors in the disease process is still unknown [2].

In a recent study from our group, an association between enteral virus infections during early infancy and increased bovine insulin-binding antibody levels was observed, indicating a role for early acquired enteral virus infections (enterovirus, rotavirus or adenovirus) in the induction of insulin immunity [3]. The effect of enteral virus infections on the humoral insulin immunity was more common among subjects exposed to CM-based formula feeding during early infancy, suggesting an interaction between these two environmental factors in the formation of insulin immunity. Therefore, we now set out to confirm this finding in a larger cohort and to further investigate the combined effect of early acquired enteral virus infections and CM-based formula exposure on the emergence of T1D-associated humoral autoimmunity and clinical T1D. For comparison with other virus infections, respiratory syncytial virus (RSV) infection during infancy was also analysed.

Subjects and methods

Subjects

The study subjects were participants in the Finnish diabetes prediction and prevention (DIPP) study and carried HLA-*DQB1* genotypes associated with an increased risk for T1D. According to the study protocol, the subjects were prospectively followed at 3-month intervals until the age of 12 months, 3–6-month intervals up to 24 months and 6–12-month intervals thereafter depending on the study centre for the appearance of serological signs of T1D-associated autoantibodies [islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA) and antibodies to the protein tyrosine phosphatase-related IA-2 molecule (IA-2A)] [3,4]. The study subject was considered to be positive for a specific autoantibody if the autoantibody test was positive in at least two consecutive serum samples. Timing of primary exposure to CM-based formula in infancy was asked about during the regular follow-up visits at 3 and 6 months of age.

The current study population consisted of participants from two research centres, Turku and Oulu, comprising 99 subjects who turned positive for at least two of the T1D-associated autoantibodies during the follow-up, and 477 control subjects matched for HLA-*DQB1* genotype, gender and place and date of birth (3–5 controls per case, median 5 controls, follow-up 4.5–12.0 years) who were not included in the pilot study group [3]. During the study implementation, 23 of the control subjects developed positivity for a single autoantibody and eight controls presented with multiple autoantibodies. Controls developing single autoantibody positivity were excluded from further analysis, and controls emerging with multiple autoantibodies were analysed with the case group. Descriptive characteristics of the case-control cohort used in the analysis are provided in Table 1. Forty-seven of the case subjects developed clinical T1D during the follow-up. Information on the exposure to CM-based formula feeding was available from 472 study subjects: 251 subjects were exposed to formula feeding before 3 months of age and 221 subjects at the age of 3 months or later. The information on CM-based formula exposure was supported by the higher levels of bovine insulin-binding antibodies at the age of 3 months among subjects exposed to CM-based formula before 3 months of age compared with those exposed later during infancy [$p = 0.007$, Mann–Whitney U test (MWU test)]. All subjects with clinical T1D were diagnosed according to the WHO criteria.

The study protocol was approved by the local ethical committees, and informed consent was obtained from the parents of the study participants.

Analyses of enterovirus, adenovirus and rotavirus IgG and IgA antibodies

IgG and IgA antibodies against purified coxsackievirus B4 (CBV4) antigen and adenovirus hexon protein were measured by enzyme immunoassay (EIA) as described earlier [3,5]. A twofold or higher increase in absorbance between consecutive samples, exceeding the cut-off level (0.20 optical density units) was considered evidence of an infection. The detection of rotavirus-specific serum IgG and IgA antibodies was performed as described previously [3]. For the rotavirus IgG and IgA assays, a positive result was defined as a twofold or higher increase in

Table 1. Descriptive characteristics of the final autoantibody positive case and autoantibody negative control subjects

Final cohort of AAB positive case and AAB negative control subjects			
		n (%) of AAB positive subjects	*n* (%) of AAB negative subjects
Gender	Female	43 (40.2)	184 (41.3)
	Male	64 (59.8)	262 (58.7)
HLA DQB1	*02/*0302	44 (41.1)	162 (36.3)
	*0302/x[a]	61 (57.0)	275 (61.7)
	*02/y[a]	2 (1.9)	9 (2.0)
Centre	Turku	53 (49.5)	209 (46.9)
	Oulu	54 (50.5)	237 (53.1)
T1D	T1D	47 (43.9)	0 (0)
	Healthy	60 (56.1)	446 (100.0)
Follow-up	Range (years)	0.9–12.1	4.5–12.1
	Mean	6.9	8.2
	Median	7.5	8.0

AAB, autoantibody; T1D, type 1 diabetes
[a]For x and y, see the Methods.

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the absorbance between consecutive samples, or a threefold or higher absorbance compared with a negative control specimen, exceeding the cut-off level (0.15 optical density units).

Analysis of RSV IgG antibodies

Respiratory syncytial virus-specific IgG antibodies were analysed using EIA. Briefly, lysate antigen of RSV A (Randall strain)-infected VERO cells was used. Microstrip 96-well plates (Immunoplate, Nunc, Roskilde, Denmark) were coated with RSV antigen diluted in phosphate-buffered saline (PBS) and incubated over night at room temperature (RT). Plates were washed with wash buffer (1% Tween in PBS) and serum samples diluted in 1:100 assay buffer (5% pork serum, 0.5% Tween in PBS) were added to the plate and incubated at +37 °C for 2 h. Each sample was tested in duplicate, and all samples from one subject were tested on the same plate. After washes, peroxidase conjugated antihuman IgG antibody (Dako, Copenhagen, Denmark) was added in 1:2500 dilution, and the plates were incubated at +37 °C for 1 h. *O*-phenylenediamine tablets (Kem-En-Tec Diagnostics, Copenhagen, Denmark) were used as substrate. The reaction was stopped by 1 M HCl, and the absorbance at 490 nm (A_{490}) was measured using a spectrophotometer. A positive result was defined as a twofold or higher increase in the absorbance between two consecutive samples or by an absorbance three times higher or more than the negative control exceeding the cut-off level (0.15 optical density units).

Analysis of bovine insulin-binding IgG antibodies

Bovine insulin-binding IgG antibodies were detected using an EIA method [6]. Briefly, microtitre plates (Combiplate Enhanced Binding, Labsystems, Helsinki, Finland) were coated with bovine insulin (Sigma, St. Louis, MO, USA) (1 μg/well in PBS) and incubated at +4 °C overnight. The plates were washed with buffer containing 0.05% Tween 20 in PBS and residual coated with 1% human serum albumin. Samples were diluted 1:10 in PBS + 0.2% human serum albumin + 0.05% Tween 20 and incubated at RT for 2 h. After washes, alkaline phosphatase-conjugated rabbit antihuman IgG antibody (Vector Laboratories, Burlingame, CA, USA) was added in a 1:100 dilution, and the plates were incubated at RT for 90 min. P-nitrophenyl phosphatase tablets (Sigma) were used as substrate, and the absorbance was read on a spectrophotometer.

The autoantibody analysis

The antibody assays have been described previously [7]. The detection limit for ICA was 2.5 Juvenile Diabetes Foundation Units. The cut-off limits for IAA, GADA and IA-2A positivity were 1.56 RU, 5.36 RU and 0.43 RU, respectively, representing the 99th percentiles in a series comprising more than 370 nondiabetic Finnish children and adolescents. The disease sensitivity of the IAA, GADA and IA-2A assays were 58%, 82% and 72%, respectively, while the specificity values were 98%, 96% and 100%, respectively, based on the 2005 Diabetes Autoantibody Standardization Programme Workshop.

The genetic analysis

The methods for the HLA-*DQB1* and HLA-*DQA1* genotyping typing were performed as described previously [8].

Statistical analysis

All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, Il, USA). The Log Rank test was used in the Kaplan–Meier analysis to compare the appearance of autoantibodies or T1D between the groups. Cox regression was employed to analyse the combined effect of viral infections and CM milk formula exposure on the appearance of autoimmunity. ANOVA for repeated measurements was used to compare the antibody levels between the groups over time and the MWU-test to analyse the differences in the antibody levels at specific time points.

Results

The effect of enterovirus, adenovirus, rotavirus and RSV infections in infancy on bovine insulin-binding antibody levels

The effect of enterovirus, adenovirus, rotavirus and RSV infection by 6 and 12 months of age on the development of bovine insulin-binding antibodies at the age of 6, 12, 18 and 24 months was analysed. Among the study group, 15 subjects with enterovirus, 13 subjects with adenovirus, 28 subjects with rotavirus and 42 subjects with RSV infections were detected by 6 months of age. By the age of 12 months, 83, 68, 97 and 152 subjects had signs of these infections, respectively. Occurrence of enterovirus, adenovirus or rotavirus infection before 6 months of age was associated with elevated bovine insulin-binding antibody over time ($p = 0.01$, ANOVA for repeated measurements). When time points were analysed separately, bovine insulin-binding antibody levels were higher at 6 months of age among subjects with any of the enteral virus infections by that age, but no differences were observed at the age of 12, 18 or 24 months (for 6 months of age $p < 0.001$, MWU-test). Positivity for antibodies against any of these enteral virus infections by 12 months of age did not affect the bovine insulin-binding antibody levels in the subsequent samples analysed.

When each virus infection was analysed separately, rotavirus infection before 6 months of age was associated

with elevated bovine insulin-binding antibody levels over the follow-up period ($p = 0.03$, ANOVA for repeated measurements) and at the age of 6 months ($p = 0.02$, MWU-test). When analysed separately, adenovirus, enterovirus and RSV infections before 6 and 12 months of age did not affect the bovine insulin-binding antibody levels in infancy.

The effect of infections and CM formula-based feeding on the appearance of T1D-associated autoimmunity

When the effect of the virus infections on the appearance of T1D-associated autoantibodies (ICA, IAA, GADA or IA-2A) or clinical T1D was analysed, no association between enterovirus, adenovirus or RSV infection by 6 or 12 months of age on appearance of autoantibodies or clinical T1D could be observed. No effect of CM-based formula nutrition was observed on the appearance of T1D-associated humoral immunity or clinical T1D when comparing subjects exposed to CM formula nutrition before the age of 3 months or later in infancy. However, enhanced emergence of GADA was observed among subjects with rotavirus infection before 6 months of age (among subjects with rotavirus infection, seven of 28 (25.0%) subjects developed GADA compared with 65 of 465 (14.0%) subjects in the group without rotavirus infection by that age, $p = 0.04$, respectively, Kaplan–Meier analysis, Log rank test). No effect of rotavirus infection later in infancy on the appearance of autoimmunity could be observed.

The combined effect of enteral virus infection and exposure to CM-based formula in infancy on the appearance of T1D-associated autoimmunity and clinical disease

When the effect of various virus infections on the appearance of humoral autoimmunity was analysed separately among subjects exposed to CM-based formula before 3 months of age and among subjects exposed to CM later in infancy, the predisposing effect of the rotavirus infection before 6 months of age was observed only among subjects exposed to CM-based formula in early infancy (Table 2). However, Cox Regression analysis showed no significant association when analysing the interaction between early rotavirus infection and CM formula exposure before 3 months of age. No effect on autoimmunity appearance was seen for other infections before 6 months of age when the two dietary groups were analysed separately.

When the same comparison was made for infections experienced before 12 months, the appearance of ICA, IAA, GADA and IA-2A was enhanced among subjects having an enterovirus infection during the first year of life

Table 2. The cumulative appearance of islet cell antibodies with any of the biochemically defined autoantibodies or specifically of insulin autoantibodies, glutamic acid decarboxylase, antibodies to the protein tyrosine phosphatase-related IA-2 molecule and type 1 diabetes [autoantibody positive or type 1 diabetes (*n*) /total (*n*), (%)] among subjects with or without rotavirus antibodies indicating an infection before the age of 6 months in the group exposed to cow's milk-based formula before 3 months of age or in the group exposed to cow's milk formula later in infancy

	Rotavirus	ICA	*p*-value*	IAA	*p*-value*	GADA	*p*-value*	IA-2A	*p*-value*	T1D	*p*-value*
CM <3 months	Positive	6/19 (31.6)		4/19 (21.1)		5/19 (26.3)		4/19 (21.1)		3/19 (15.8)	
	Negative	32/206 (15.5)	0.014	24/206 (11.7)	0.14	26/206 (12.6)	0.031	24/206 (11.7)	0.11	14/206 (6.8)	0.078
CM ≥3 months	Positive	1/7 (14.3)		1/7 (14.3)		1/7 (14.3)		0/7 (0)		1/7 (14.3)	
	Negative	38/195 (19.5)	0.88	33/195 (16.9)	0.98	30/195 (15.4)	0.92	26/195 (13.3)	0.37	14/195 (7.2)	0.34

ICA, islet cell antibodies; IAA, insulin autoantibodies; GADA, glutamic acid decarboxylase; IA-2A, to the protein tyrosine phosphatase-related IA-2 molecule; T1D, type 1 diabetes; CM, cow's milk.
*Kaplan–Meier analysis, Log Rank test

in the early CM exposure group (Table 3, Figure 1). No clear interaction between adenovirus or RSV infection and early CM-based formula exposure could be observed. Similarly, no interaction between rotavirus infection by 12 months of age and early CM exposure could be seen (Table 3).

In Cox Regression analysis for the combined effect of enterovirus infection in infancy and CM formula exposure, we observed an association between enterovirus infection before 12 months of age and the appearance of ICA together with any of the biochemically defined autoantibodies, the appearance of IAA, GADA and IA-2A among subjects exposed to CM-based formula before 3 months [for ICA $p = 0.001$, hazard ratio (HR) for interaction term 7.4, 95% CI 2.2–25.4, for IAA $p = 0.002$, HR 9.5, 95% CI 2.3–38.4, for GADA $p = 0.001$, HR 9.7, 95% CI 2.5–38.2, for IA-2A $p = 0.013$, HR 5.2, 95% CI 1.4–18.7], but the effect on progression to clinical T1D remained nonsignificant ($p = 0.15$, HR 3.8, 95% CI 0.60–24.1).

Discussion

Both the limited role of genetic predisposition and the rapid increase in the disease rate indicate that the role of environmental factors in the pathogenesis of T1D is crucial. In Finland, the incidence of T1D has doubled over the last 25 years, and the disease incidence continuous to increase [9]. Moreover, in Europe, the highest proportional increase in the disease rate is currently observed in countries in Eastern Europe that have so far been considered to have low T1D incidence, suggesting a rapid change in the impact of environmental factors [10].

An extensive series of studies on the role of environmental agents in the T1D disease process has been published during the recent decades. Currently, the environmental triggers most strongly implicated include viral infections, especially enterovirus infections, and early CM exposure. Supporting the hypothesis of the role of enterovirus infection in the induction of T1D-associated autoimmunity, a persistent or recurrent enterovirus infection in gut mucosa has recently been described among T1D patients, suggesting a reduced clearance of enterovirus among affected patients [11]. In addition, enteroviruses have been shown to infect pancreatic islets [12], and enteroviruses have been detected in pancreatic islets in patients with T1D [13,14].

Several case-control series have reported not only elevated enterovirus antibody levels among T1D patients [15–17] but also contradictory results [18,19]. Moreover, in Finnish prospective series, a temporal association has been observed between the emergence of T1D-associated autoantibodies and enterovirus infections [5,20–23]. Accordingly, a temporal association between the appearance of humoral signs of autoimmunity and the presence of enterovirus RNA in blood has been observed repeatedly in Finnish studies [22,24,25], although no such associations were reported among other populations [26–28].

In addition to enterovirus infections, rotavirus infection has been associated with temporary increases in the levels of T1D-associated autoantibodies in an Australian study [28], but a Finnish prospective cohort failed to find any association between rotavirus infection and the appearance of T1D-associated autoantibodies and overt T1D [29]. However, a recent study from our group showed an association between enteral virus infection, especially rotavirus infection, in early infancy and elevated bovine insulin-binding antibody levels during infancy, suggesting a role for these infections in the formation of insulin immunity [3].

The role of CM-based formula nutrition as a triggering factor of T1D autoimmunity is unclear, and contradictory results have been published. Several studies have shown an association between early CM exposure or short exclusive breastfeeding and the emergence of β-cell autoimmunity or clinical T1D [30–33], but numerous other surveys have failed to show any relationship between these two factors [34–37]. However, the role of dietary bovine insulin in the formation of insulin immunity and tolerance is conceivable [6,38,39]. Moreover, the only prospective intervention study so far comparing the effect of hydrolyzed formula feeding and conventional CM-based formula nutrition on the development of T1D-associated autoimmunity suggested a decreased appearance of humoral signs of β-cell autoimmunity among subjects receiving the hydrolyzed formula [30,40].

In our series, no direct association between the enhanced emergence of T1D-associated autoimmunity and enterovirus infection during infancy or early exposure to CM-based formula could be observed among subjects carrying the HLA-*DQB1* genotypes associated with T1D risk. However, when the combined effect of these two factors was analysed, enterovirus infection before 12 months of age was found to enhance the appearance of T1D-associated autoimmunity among subjects exposed to CM before 3 months of age. This result proposes a more complex pathway in the initiation of β-cell autoimmunity. In line with this, we observed in an earlier study that the presence of cellular responses to enterovirus antigens during early infancy and early exposure to CM formula were associated with an increased humoral response towards bovine insulin [41]. This result suggests that among at-risk subjects, enterovirus infection during infancy may modify the developing tolerance to dietary bovine insulin. Thus, this finding supports our hypothesis of the necessity of multiple separate factors contributing to the process resulting in β-cell destruction.

In the present study cohort, rotavirus infection during infancy was associated with a subsequent increase in bovine insulin-binding antibody levels. In addition, signs of association between early rotavirus infection and appearance of humoral β-cell autoimmunity could be observed. Rotavirus infection in gut mucosa increases the permeability of the gut, which may lead to increased absorption of and enhanced immunity to food proteins, and may thus facilitate the generation of autoimmunity to autologous insulin [42]. In contrast, the mechanisms

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Table 3. Survival analysis of the effect of enterovirus, rotavirus, adenovirus and respiratory syncytial virus infection before 12 months of age on the appearance of islet cell antibodies with any of the biochemically defined autoantibodies or specifically of insulin autoantibodies, glutamic acid decarboxylase, antibodies to the protein tyrosine phosphatase-related IA-2 molecule and clinical type 1 diabetes in the group exposed to cow's milk-based formula before 3 months of age or in the group exposed to cow's milk formula later in infancy [autoantibody positive or type 1 diabetes (*n*) /total (*n*), (%)]

	Virus infection analysed	ICA	*p*-value*	IAA	*p*-value*	GADA	*p*-value*	IA-2A	*p*-value*	T1D	*p*-value*
	Enterovirus										
CM <3 months	Positive	12/28 (42.9)		10/28 (35.7)		12/28 (42.9)		10/28 (35.7)		4/28 (14.3)	
	Negative	31/202 (15.2)	0.001	22/202 (10.9)	<0.001	25/202 (12.4)	<0.001	23/202 (11.4)	0.001	14/202 (6.9)	0.17
CM ≥3 months	Positive	4/37 (10.8)		3/37 (8.1)		3/37 (8.1)		4/37 (10.8)		2/37 (5.4)	
	Negative	39/174 (22.4)	0.08	34/174 (19.5)	0.08	32/174 (18.4)	0.10	26/174 (14.9)	0.42	16/174 (9.2)	0.40
	Rotavirus										
CM <3 months	Positive	15/57 (26.3)		11/57 (19.3)		11/57 (19.3)		11/57 (19.3)		6/57 (10.5)	
	Negative	28/177 (15.8)	0.04	20/177 (11.3)	0.10	25/177 (14.1)	0.24	21/177 (11.9)	0.11	12/177 (6.8)	0.26
CM ≥3 months	Positive	6/28 (21.4)		4/28 (14.3)		6/28 (21.4)		4/28 (14.3)		4/28 (14.3)	
	Negative	34/177 (19.2)	0.72	30/177 (16.9)	0.74	26/177 (14.7)	0.33	23/177 (13.0)	0.84	12/177 (6.8)	0.16
	Adenovirus										
CM <3 months	Positive	9/30 (30.0)		7/30 (23.3)		8/30 (26.7)		8/30 (26.7)		4/30 (13.3)	
	Negative	34/200 (17.0)	0.11	25/200 (12.5)	0.09	29/200 (14.5)	0.07	25/200 (12.5)	0.03	14/200 (7.0)	0.20
CM ≥3 months	Positive	5/31 (16.1)		5/31 (16.1)		3/31 (9.7)		4/31 (12.9)		2/31 (6.5)	
	Negative	38/180 (21.1)	0.47	32/180 (17.8)	0.75	32/180 (17.8)	0.24	26/180 (14.4)	0.77	16/180 (8.9)	0.63
	RSV										
CM <3 months	Positive	17/71 (23.9)		12/71 (16.9)		16/71 (22.5)		12/71 (16.9)		6/71 (8.4)	
	Negative	26/163 (15.9)	0.12	19/163 (11.7)	0.26	20/163 (12.3)	0.04	20/163 (12.3)	0.31	12/163 (7.3)	0.75
CM ≥3 months	Positive	11/64 (17.2)		10/64 (15.6)		8/64 (12.5)		8/64 (12.5)		4/64 (6.3)	
	Negative	29/141 (20.6)	0.61	24/141 (17.0)	0.79	24/141 (17.0)	0.45	19/141 (13.5)	0.85	12/141 (8.5)	0.62

ICA, islet cell antibodies; IAA, insulin autoantibodies; GADA, glutamic acid decarboxylase; IA-2A, to the protein tyrosine phosphatase-related IA-2 molecule; T1D, type 1 diabetes; CM, cow's milk.
*Kaplan–Meier analysis, Log Rank test

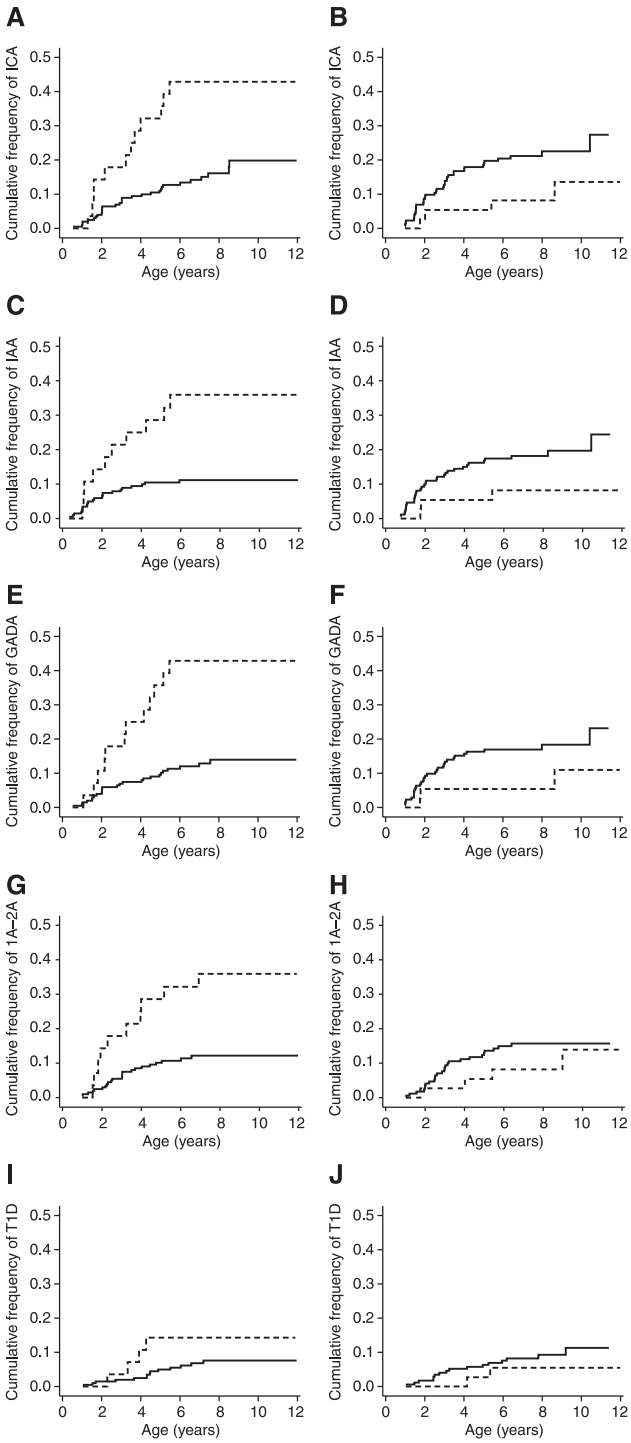

Figure 1. In Kaplan–Meier survival analysis, the appearance of islet cell antibodies with any of the biochemically defined autoantibodies (a), of insulin autoantibodies (c), glutamic acid decarboxylase (e) and antibodies to the protein tyrosine phosphatase-related IA-2 molecule (g) was enhanced among subjects with enterovirus infection by 12 months of age (dotted line) compared with subjects without enterovirus infection by that age (continuous line) among subjects exposed to cow's milk by 3 months of age ($p = 0.001$, <0.001, <0.001, 0.001, respectively, Log Rank test) but no significant difference in the development of clinical type 1 diabetes could be observed [$p = 0.17$ (i)]. No such effect of enterovirus infection on the emergence of type 1 diabetes-associated autoimmunity was observed in the group exposed to cow's milk later in infancy [for islet cell antibodies $p = 0.08$ (b), for insulin autoantibodies $p = 0.08$ (d), for glutamic acid decarboxylase $p = 0.10$ (f), for antibodies to the protein tyrosine phosphatase-related IA-2 molecule $p = 0.42$ (h), for type 1 diabetes $p = 0.40$ (j)]

of the facilitation of the autoimmunity by enterovirus infection might be different as no enhancement of bovine insulin specific antibody response was observed after enterovirus infection in infancy. In addition, the timing of the diabetes-predisposing infection seems to differ between infection caused by rotavirus or enterovirus also suggesting a different role of these infections in the pathogenesis of β-cell autoimmunity. In this respect, it is interesting that CBV4 infection has been shown to induce a bystander activation effect in the non-obese diabetic (NOD) mouse model but

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only if a critical threshold level of β-cell-specific autoreactive T cells have accumulated indicating that the timing of the coxsackie virus infection rather than its presence or absence may be important for the role of the infection in the development of T1D-associated autoimmunity [43].

In the current study, we analysed a cohort derived from the DIPP study, which is a prospective cohort tightly following subjects with HLA conferred risk for developing T1D. The number of subjects in the current study and HLA selection criteria limit the power of the study when generalizing the finding to background population. Moreover, we did not see any significant effect of early exposure to CM-based formula and enterovirus infection during infancy on the development of clinical disease. However, this is most likely explained by the lower number of case subjects, and the longer observation period needed to see the development of clinical disease. The trend observed in clinical disease was in the same direction as found in the autoantibody markers, and the value of the autoantibody combinations used in the study as surrogate markers of T1D is well-established [2].

In conclusion, our results suggest an interplay between CM-based formula exposure in early infancy and enterovirus infections during the first months of life in the appearance of β-cell-specific humoral autoimmunity and clinical T1D among subjects with HLA-conferred susceptibility to T1D. As suggested by NOD mouse model earlier, current results could be explained by enterovirus infection induced activation of the existing β-cell-specific autoreactive T cells, together with the hypothesis that early CM-based formula exposure acts as a primary trigger of diabetes-associated insulin autoimmunity. The suggested interaction between these two environmental factors may also provide an explanation for the contradictory findings on the role of any one of these factors in the appearance of autoimmunity leading to subsequent T1D. More studies are needed to clarify the role of the suggested interaction of these two environmental factors in the pathogenesis of T1D.

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Conflict of interest

None declared.

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