No Association Between Ljungan Virus Seropositivity and the Beta-Cell Damaging Process in the Finnish Type 1 Diabetes Prediction and Prevention Study Cohort

Anne J. Jääskeläinen[1], PhD, Noora Nurminen,[2] MSc, Pekka Kolehmainen,[1,3] PhD, Teemu Smura[1], PhD, Sisko Tauriainen[3], PhD, Jorma Toppari[4,5], MD, PhD, Jorma Ilonen[6], MD, PhD, Riitta Veijola[7], MD, PhD, Mikael Knip[8,9,10,11], MD, PhD, Heikki Hyöty[2,12], MD, PhD, Olli Vapalahti[1,13], MD, PhD, and Hannimari Kallio-Kokko,[1] PhD

Corresponding author: Anne J. Jääskeläinen, Associate professor, PhD, Hospital microbiologist, Helsinki University and Helsinki University Hospital, Department of Virology, P.O.B. 21 (Haartmaninkatu 3), FIN-00014 Univ. of Helsinki, Finland. Email: anne.jaaskelainen@helsinki.fi, Mobile phone: +358 50 5420743, Office phone: +358 9 19126671, Fax: +358 9 19126491

Abbreviated title: No Association Between Ljungan Virus and Type 1 Diabetes

Running head: Ljungan Virus and Type 1 Diabetes

[1] Helsinki University and Helsinki University Hospital (HUSLAB), Department of Virology, Finland

[2]Department of Virology, School of Medicine, University of Tampere, Tampere, Finland

[3]Department of Virology, University of Turku, Turku, Finland

[4]Department of Physiology, Institute of Biomedicine, University of Turku, Turku, Finland

[5]Department of Pediatrics, Turku University Hospital, Turku, Finland

[6]Immunogenetics Laboratory, University of Turku and Turku University Hospital, Turku, Finland

1

[7]Department of Pediatrics, PEDEGO Research Unit, Medical Research Center, Oulu

University Hospital and University of Oulu, Oulu, Finland

[8]Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki,

Finland

[9]Research Programs Unit, Diabetes and Obesity, University of Helsinki, Helsinki, Finland

[10]Folkhälsan Research Center, Helsinki, Finland

[11]Tampere Center for Child Health Research, Tampere University Hospital, Tampere,

Finland

[12]Fimlab Laboratories, Pirkanmaa Hospital District, Tampere, Finland

[13]Faculty of Veterinary Medicine, Department of Veterinary Biosciences, University of Helsinki,

Finland.

2

Abstract

Background

Ljungan virus (LV) has not confirmed to associate with any human disease, but a possible connection with type 1 diabetes (T1D) has been suggested. Ljungan virus (LV) is a rodent-borne picornavirus that induces a diabetes-like condition in rodents. Approximately 30% of adults and 60% of children are seropositive in Finland. The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study enabled the use of very well characterized sample panels from children seroconverted to positivity for multiple islet autoantibodies during their prospective observation from birth, in addition samples from age, sex, HLA and residence area matched control children.

Methods

We analyzed LV IgG seroprevalence in 102 case children (65 had also developed T1D), in addition to non-diabetic control children. LV and human parechovirus (HPeV) immunofluorescence assays were used to analyze LV and HPeV specific IgG from 102 plasma samples taken at the time of islet autoantibody appearance, and from 204 samples from the matched control children.

Results

Altogether 46.1% of the case and 50.7% of the control children were positive for LV IgG (odds ratio 0.8, 95% CI 0.47-1.36, p=0.416), and 67.6% vs. 79.8% were positive for HPeV IgG, respectively (odds ratio 0.49, 0.27-0.9, p=0.023).

Conclusions:

Thus, no risk associations between LV or HPeV -specific IgG, and islet autoimmunity were observed. However, a trend for significantly higher prevalence of HPeV antibodies in control

3

children (p=0.023) suggests a possible protective association of this virus with islet autoimmunity.

Keywords: Ljungan virus, LV, human parechovirus, HPeV, DIPP, diabetes, type 1 diabetes

Introduction

Parechoviruses (*Picornaviridae*: *Picornavirus*) are small non-enveloped single-stranded RNA viruses. The genus *Parechovirus* comprises four species, and species of *Parechovirus A* [formerly *Human parechovirus* (HPeV)] and *Parechovirus B* [formerly *Ljungan virus* (LV)] consist of 16 HPeV and 4 LV types, (www.picornaviridae.com website, November 2017). LV is a rodent-borne virus (1) while HPeVs infect humans with symptoms including mild gastrointestinal and respiratory infections in children, and neonatal sepsis-like disease and encephalitis in infants (2).

The external triggers of human type 1 diabetes (T1D) have been the subject of extensive research, and certain virus infections, particularly enteroviruses have been implicated as the main candidates (3). Possible association of LV and diabetes has been studied using both nucleic acid detection methods and serological methods. In a MIDIA cohort study (4), LV RNA was not detected in stool samples collected from children in Norway. In Sweden, LV IgG antibodies were found to be associated with both positivity for HLA-DQB1*0302 (DQ8) allele and T1D-associated islet autoantibodies (5, 6). In addition to LV IgG, the prevalence of HPeV IgG was also increased in T1D patients with insulin autoantibodies and HLA-DQ8 when analyzed using multiplex immunoassay. However, the authors stated that cross-reactions could not be ruled out with this assay (6).

Our aim was to screen both LV- and HPeV-specific IgG antibodies from children who have seroconverted for multiple T1D-associated autoantibodies as a marker of an ongoing beta-cell damaging process and from their matched healthy control children in the DIPP study. This study design enabled the identification of infections which preceded the initiation of the beta-cell damaging process and could thus contribute to the initiation of this process.

5

Methods

Study Subjects

All study subjects were participants in the DIPP study. The present study cohort included children recruited at the University Hospital in Tampere. Blood samples were drawn at the ages of 3, 6, 12, 18, and 24 months and once a year thereafter, and all follow-up sera were screened for T1D-associated autoantibodies [islet cell autoantibodies (ICA) as well as autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA) and the protein tyrosine phosphatase-related IA-2 antigen (IA-2A)]. The DIPP study has been approved by the ethical committees of the participating university hospitals. A written informed consent was obtained from all participating families.

The following criteria were used to select children for the nested case-control study: The case children (N=102, 64% males) had turned positive for two or more of the T1D-associated autoantibodies mentioned above and remained autoantibody positive in all later follow-up samples. Of these, 65 have also progressed to clinical T1D. Two autoantibody negative and non-diabetic control subjects (N=203, only one control child was available for one of the case children) were selected for each case child and matched for time of birth (± 2 months), age at sample collection (± 3 months), sex and T1D-associated HLA-DQ alleles. The first autoantibody positive sample of the case children and the corresponding sample from their matched control children were selected for virus antibody analyses (median age was 48 months and range 17-133 months at sample collection). The children were born in 1995-2009.

Diabetes-Associated Autoantibodies and HLA Genotyping

ICA was analyzed using indirect immunofluorescence assay (IFA) and IAA, GADA and IA-2A using radiolabel-binding assays (7). Genotypes associated with susceptibility to, or protection

6

against, type 1 diabetes were identified by analyzing HLA-DQB1 alleles (DQB1*02, *03:01, *03:02, and *06:02/3) from cord blood of the children. The genotyping was based on lanthanide labeled oligonucleotide probe hybridization and time-resolved fluorometric detection (8). Children carrying the high-risk HLA-DQB1*02/DQB1*0302 genotype or the moderate-risk DQB1*0302/x genotype (x ≠ DQB1*03:02, *06:02, or *06:03) were invited for follow-up. The diagnosis of type 1 diabetes was based on the World Health Organization criteria.

Virus antibodies

LV- and HPeV-specific IgG was detected using LV (LV strain 145SLG; described in Jääskeläinen et al. 2013) and HPeV IFAs. The HPeV IFA was set up using HPeV1 strain Harris and the method described in Jääskeläinen et al. (9), and was further evaluated by comparing the results with those obtained from 9 LV-antibody negative plasma samples using a HPeV microneutralization assay (MNT; 10). Two commercial hyperimmune antisera against HPeV1 and HPeV2 [VR-1063AS/HO™, VR-1064AS/HO™; produced in horse, LGC Standards (ATCC), Teddington, United Kingdom] were used as controls.

Statistical Analyses

The prevalence of virus antibodies in both case and control groups was analyzed using conditional logistic regression and STATA 12.1 statistical software (StataCorp, College Station, TX, USA). A p value of less than 0.05 was set as indicator of statistical significance.

Results

In the comparison of HPeV IFA test with microneutralization assay, concordant results were obtained and HPeV IFA detected all HPeV1-6 types (8 positive samples). The titers for HPeV1-6 varied from 64 until 16384, and LV IFA was negative in all cases. One MNT negative sample was negative in both HPeV and LV IFA. In total, 49% (150/305) of all DIPP children were

seropositive for LV IgG and 76% (231/305) HPeV IgG in IFA. Only one child was HPeV negative but LV positive. However, 84 children (27.5%; 84/505) were HPeV positive but LV negative (Table 1). The prevalence of LV antibodies did not differ between the case and control children (Table 1). This was also true when only those case children who progressed to clinical T1D and their controls were included. However, case children tended to be less frequently positive for HPeV antibodies than control children (Table 1). This trend was observed in both younger and older children and it was particularly clear among boys and in the subgroup of cases that had progressed to clinical T1D. Similar trend was further seen in children who developed IAA as the first appearing autoantibody and in those who seroconverted first to positivity for GADA (data not shown). The parallel presence of antibodies against LV and HPeV showed a significant correlation ($\chi2 = 69.04$, df = 1, p < 0.00001) in whole study cohort.

Discussion

LV infections have been linked to human T1D in some studies (16, 17) but prospective studies are lacking. In this study, both LV (49%) and HPeV (76%) antibodies were frequently detected. LV IFA was previously evaluated and no cross-reactivity was detected for HPeV1-2 and 4-6 (9). In the present study we tested the HPeV IFA using plasma samples positive for neutralizing antibodies (MNT) against HPeV1-6. All MNT positive samples were positive by HPeV IFA but negative by LV IFA.

LV seropositivity was not associated with the beta-cell damaging process as 46% of the case children, compared to 51% of their age, sex and HLA matched control children, were positive for LV antibodies (p=0.416). This was also the case for HPeV IFA where 68% vs. 80% of the children analyzed were positive (p=0.023), respectively. Thus, the prevalence of HPeV antibodies was significantly lower in case children than in control children. The reason to this

8

difference is not known. Overall, the control children were more HPeV seropositive than cases (p=0.023), especially when the group of clinical TD1, boys and children over three years were examined (p=0.007, 0.009, 0.029, respectively; Table 2). The case children had no diabetes-associated metabolic changes, since the virus antibodies were analyzed in the early prediabetic phase when the children were still normoglycaemic. However, we cannot exclude the possibility that the function of the immune system is affected by the autoimmune process, leading to lower levels in HPeV antibodies. Alternatively, it is possible that HPeV infections could somehow protect against islet autoimmunity, possibly by stimulating the protective elements of the gut immune system. Accordingly, for enteroviruses, a link between infections with low-virulence virus strains and protection against T1D has been suggested (11). It should also be noted that maternal virus antibodies could not have biased these findings since all children were older than one year and maternal antibodies should have disappeared by that age.

In Sweden, HLA-DQB*0302 and islet autoantibodies, particularly insulin autoantibodies, were suggested to be associated with LV and HPeV IgG antibodies (5, 6). In Norway and Finland, the children participating in the MIDIA and DIPP studies, respectively, were studied for parechovirus nucleic acids and/or antibodies by Tapia et al. (4), and Zhao et al. (12). No LV RNA positive feces in the MIDIA cohort (4) nor DIPP cohort (12). All children in the analyzed DIPP cohort samples have been DQB1*0302 positive based on earlier eligibility criteria and it has thus not been possible to explore the HLA association here.

This is a prospective study covering the time before the initiation of islet autoimmunity. This is the strength of this study, as well as, that the control children were carefully matched with to cases, and the LV and HPeV IgG IFAs were analyzed in parallel using validated methods.

9

However, this study was carried out only in Finland, and this can be considered as a limitation to make any final conclusions.

The data also indicated that control children were statistically more positive for HPeV than case group, even when clinically T1D cases were analyzed. This is interesting finding, and raised the question do the HPeV antibodies somehow protect from T1D?

References

1. Niklasson B, Kinnunen L, Hornfeldt B, et al. A new picornavirus isolated from bank voles (Clethrionomys glareolus). *Virology* 1999; 255:86-93

2. Verboon-Maciolek MA, Krediet TG, Gerards LJ, et al. Severe neonatal parechovirus infection and similarity with enterovirus infection. *Pediatr Infect Dis J.* 2008; 27:241-5.

3. Nurminen N, Oikarinen S, Hyöty H. Virus infections as potential targets of preventive treatments for type 1 diabetes. *Rev Diabet Stud Winter* 2012; 9:260-271

4. Tapia G, Cinek O, Rasmussen T, et al. No Ljungan virus RNA in stool samples from the Norwegian environmental triggers of type 1 diabetes (MIDIA) cohort study. *Diabetes Care* 2010; 33:1069-1071

5. Nilsson AL, Vaziri-Sani F, Andersson C, et al. Relationship between Ljungan virus antibodies, HLA-DQ8, and insulin autoantibodies in newly diagnosed type 1 diabetes children. *Viral Immunol* 2013; 26:207-215

6. Nilsson AL, Vaziri-Sani F, Broberg P, et al. Serological evaluation of possible exposure to Ljungan virus and related parechovirus in autoimmune (type 1) diabetes in children. *J Med Virol.* 2015; 87:1130-1140.

7. Knip M, Virtanen SM, Seppä K, et al.; Finnish TRIGR Study Group. Dietary intervention in infancy and later signs of beta-cell autoimmunity. *N Engl J Med* 2010; 363:1900–1908

8. Sjöroos M, Iitiä A, Ilonen J, et al. Triple-label hybridization assay for type-1 diabetes-related HLA alleles. *Biotechniques.* 1995; 18:870-7.

9. Jääskeläinen AJ, Kolehmainen P, Voutilainen L, et al. Evidence of Ljungan virus specific antibodies in humans and rodents, Finland. *J Med Virol* 2013; 85:2001-2008

10. Kolehmainen P, Koskiniemi M, Oikarinen S, et al. Human parechovirus and the risk of type 1 diabetes. *J Med Virol.* 2013; 85:1619-1623

11. Christen U, von Herrath MG. Do viral infections protect from or enhance type 1 diabetes and how can we tell the difference? *Cell Mol Immunol* 2011; 8:193-198

12. Zhao G, Vatanen T, Droit L, et al. Intestinal virome changes precede autoimmunity in type I diabetes-susceptible children. *Proc Natl Acad Sci U S A.* 2017; 114:E6166-E6175

Table 1. Prevalence of LV and HPeV antibodies in children with β-cell autoimmunity (case children) and autoantibody-negative control children.

Group (*N*= in total)		Seroprevalence of LV and HPeV antibodies	Cases[1] N (%)	Controls[2] N (%)	OR	95% CI	p-value[3]
Summary of all (Cases *N*=102, Controls *N*=203; All *N*=305)		LV	47 (46.1)	103 (50.7)	0.80	0.47, 1.36	0.416
		HPeV	69 (67.6)	162 (79.8)	0.49	0.27, 0.90	**0.023**
		LV and HPeV	45 (44.1)	102 (50.2)	0.77	0.45, 1.33	0.354
		LV pos, HPeV neg	0 (0)	1 (0.5)	NA	NA	NA
		HpeV pos, LV neg	24 (23.5)	60 (30.5)	0.67	0.36-1.23	0.196
Divided by sex (Cases *N*=102, Controls *N*=203; All *N*=305)	Boys (*N*=192)	LV	27 (42.2)	68 (53.1)	0.56	0.28, 1.12	0.102
		HPeV	41 (65.1)	104 (81.2)	0.35	0.16, 0.77	**0.009**
	Girls (N=113)	LV	20 (52.6)	35 (46.7)	1.35	0.59, 3.12	0.481
		HPeV	29 (78.4)	59 (79.7)	0.91	0.31, 2.62	0.855
Divided by age of 3 years (Cases *N*=102, Controls *N*=203; All *N*=305)	Age ≤3 years	LV	16 (47.1)	31 (45.6)	1.09	0.41, 2.89	0.868
		HPeV	22 (64.7)	49 (73.1)	0.63	0.22, 1.77	0.380
	Age >3 years	LV	31 (45.6)	72 (53.3)	0.71	0.38, 1.33	0.283
		HPeV	48 (72.7)	114 (84.4)	0.43	0.20, 0.92	**0.029**

13

Cases progressed to T1D (*N*=65 cases, their child matched controls *N* = 129)	LV	33 (50.8)	66 (51.2)	1.00	0.51, 1.95	1.000
	HPeV	43 (66.2)	107 (82.9)	0.35	0.17, 0.75	**0.007**

LV, Ljungan virus; HPeV, human parechovirus; T1D, type 1 diabetes; NA, not applicable;.

[1] in total 102 children, gender: 64 males/38 females

[2] in total 203 children, gender: 128 males/75 females

[3] p-value <0.05 considered significant

** In total, 65 individual cases progressed to clinical T1D. None of the matched control children presented with T1D (n=129; one TD1 case had only one control child).

14