Temporal Variation of *Ljungan Virus* Antibody Levels in Relation to Islet Autoantibodies and Possible Correlation to Childhood Type 1 Diabetes

A.-L. Nilsson[*,1], E. Lagerquist[2], K.F. Lynch[3], Å. Lernmark[2,3] and O. Rolandsson[4]

[1]*Department of Pediatrics, Östersund Hospital, Östersund, Sweden*

[2]*Department of Medicine, University of Washington, Seattle, WA, USA*

[3]*Department of Clinical Sciences, Lund University/CRC, University Hospital MAS, Malmö, Sweden*

[4]*Department of Public Health and Clinical Medicine, University of Umeå, Umeå, Sweden*

Abstract: Viral infection may trigger islet autoimmunity, type 1diabetes (T1D), or both. Fluctuating population density of bank voles as a putative reservoir of *Ljungan virus* has been claimed to be associated with variations in T1D incidence rate (IR). We tested the hypothesis that *Ljungan virus* antibodies reflecting prior exposure(s) to the virus may be associated with islet autoimmunity, childhood diabetes or both. Incident, 0-18y, T1D patients (n = 63) were studied along with age and sample time matched controls (n = 126). The younger children (< 9 years) tended to have a higher incidence rate during winter (IR = 67.6, 95%CI 41.9-103.5) compared to summer (IR = 33.6, 95%CI 15.3-63.9) months. The proportion of children with high level antibodies against *Ljungan virus* (LVAb) were both younger compared to the rest of the children ($p < 0.002$) and correlated with half yearly T1D IR ($r = 0.78$, $p = 0.005$). High level LVAb fluctuating with season and correlating with T1D IR indicates that past exposure to *Ljungan virus* may be associated with T1D.

1. INTRODUCTION

The etiology and pathogenesis of T1D is multifaceted and involve both genetic risk and environmental factors [1]. Sweden and neighbouring Finland have the highest incidence rate (IR) in the world of T1D among children and adolescents [2, 3]. Explanations to the high IR might be the high frequency of predisposing HLA [4], climate [5] or dietary factors [6]. The possible influence of viral infections as a trigger of islet autoimmunity leading to the appearance or clinical onset of T1D has been reported in several studies [7-9] including reports that Enterovirus infection of mothers during pregnancy is a risk factor for T1D in the offspring [10, 11].

Virus may affect T1D risk in different ways. One way may be as a trigger of islet autoimmunity following virus-induced beta-cell lysis, islet antigen presentation and the subsequent formation of autoantibodies against GAD65, IA-2 or insulin [4, 12]. Another possible way to induce an islet autoimmune process would be by "molecular mimicry", which may be induced because of structural similarities between a viral protein and a beta-cell specific autoantigen. This hypothesis does not require beta-cell destruction per se but rather structural similarities between a virus antigen and a target autoantigen [6]. Other possible trigger mechanisms include an activation of a chronic bystander islet inflammatory process by cells secreting damaging cytokines or other inflammatory mediators [6, 7, 12]. Finally, a virus-infection may affect T1D risk by increasing insulin resistance and insulin requirements that may precipitate the clinical onset of T1D in children already positive for one or several islet autoantibodies [13].

Studies in different countries have suggested that enterovirus may be associated with the appearance of either islet autoimmunity, T1D, or both [8, 9]. The *Ljungan virus* (LV), a *Parechovirus*, possibly pathogenic to humans [14, 15] has been suggested to contribute to T1D [16, 17]. The *Ljungan virus* was defined as a separate species in the family *Picornavirus* as the nucleotide sequence was found to be related to the *Parechovirus* genus [15, 18]. The LV was first isolated in a bank vole, *Myodes glareolus* (previously referred to as *Clethrionomys glareolus*), from the valley of the river Ljungan in northern Sweden [14]. It was also reported that bank voles captured in the wild develop diabetes [17] and had not only LVAb but also increased levels of autoantibodies against GAD65, IA-2 and insulin [19]. Levels of LVAb were increased in young age at onset T1D children but a possible relationship to T1D IR could not be determined [19]. The seasonal variations in the IR of T1D suggest possible influence by one or more environmental factor. Multiannual population cycles with high amplitudes has been characteristic for northern voles [20]. The very high IR of T1D among young people noted in 2000 in the County of Jämtland, Sweden, initiated this study to test whether presence of LVAb may be associated with type 1 diabetes incidence in young children. The hypothesis was that *Ljungan virus* antibody levels would reflect prior exposure(s) to the virus and might be associated with islet autoimmunity, childhood diabetes, or both.

2. MATERIALS AND METHODOLOGY

2.1. Subjects

All newly diagnosed patients (n = 63) aged 11 months-18 years of age and living in the county of Jämtland, Sweden, were studied at the time of T1D clinical onset, which

*Address correspondence to this author at the Department of Pediatrics, Östersund Hospital, S-831 83 Östersund, Sweden; Tel: +46 63 15 39 77; Fax: +46 63 15 45 05; E-mail: anna-lena.nilsson@jll.se

1874-3099/09

occurred between October 1st 2000 and February 15th 2006 (Table **1**).

Blood samples were obtained at the clinical onset from all incident T1D patients after obtaining informed consent by the parents (for the youngest patients) or both patient and parents. Samples were taken at or within one day from the diagnosis of T1D except from three patients. Of these three one was diagnosed in October 2000 and the sample was obtained in February 2001 and the other two were diagnosed in November and December 2000, respectively, but the samples were obtained in March 2001. Serum was prepared from the blood samples and kept frozen at – 20º C until the analyses of LVAb, GAD65Ab, IA-2Ab and IAA.

Table 1. Incident (2000-2006) Type 1 Diabetes Patients and Controls, Jämtland County. Age, Gender, Seasonal Distribution and Islet Autoantibodies

		New Onset T1D Patients	Controls
N		63	126
F/M ratio		24/39	62/64
Age (years)	mean	8.9	8.5
	Range	1-17	1-18
Blood Sampling	Winter[a]	39	78
	Summer[a]	24	48
Islet Autoantibodies[b] (any)		60/63 (95%)	12/126 (10%)
GAD65Ab		46/63 (73%)	6/126 (5%)
IA-2Ab		54/63 (86%)	5/126 (4%)
IAA		28/63 (44%)	6/126 (5%)

[a]Summer months = April-September, Winter months = October to March.
[b]Positive and negative for autoantibody is defined as above or below the 95th percentile of controls.

The control children represented 126 non-diabetic individuals (Table **1**). These children were from the same geographical area and a blood sample was obtained in the same month as the sample from the patient. The samples were primarily analyzed for gliadin antibodies at Umeå University Hospital Virus laboratory. All samples for gliadin antibody analysis from Jämtland County were sent to this laboratory. The gliadin test was also performed in many children with mild symptomatology. After approval from the regional ethic review board, two controls per patient in each study month were selected at random from these samples. None of the controls were diagnosed with celiac disease or had any diagnosis of other autoimmune disease. All children in the county diagnosed with an autoimmune disease are registered and none of the controls had been recorded. The controls were matched for ages 0-18 years but not necessarily by age for every patient to control.

T1D incidence data for the Jämtland County in 1978-2005 was obtained from the Swedish Childhood Diabetes Register [13].

The Regional Ethical Review Board in Umeå, Sweden, approved the study.

2.2. Antibody Analyses

Ljungan virus antibody (LVAb) analyses were performed using a radioligand binding assay as previously described [19]. In the LVAb assay, mouse (antiserum 87-012 #10) and guinea pig (antiserum 174F#4) antisera (both antisera kindly donated by Bo Niklasson, Stockholm, Sweden) were used as positive in-house controls along with normal mouse serum and normal guinea pig serum as negative reference samples. A standard curve generated with various dilutions of the mouse 87-012#10 LV-antiserum was used in each assay. LV-antibody levels were either reported in U/ml or as index values to correct for inter-assay variation. The antibody index was obtained from the formula: index = (counts per minute (cpm) of the unknown sample - average cpm of three negative standards)/(cpm of the positive standard - average cpm of three negative standards).

2.3. Autoantibodies

Autoantibodies against GAD65 [21-23] and IA-2 [22] were determined in standardized radiobinding assays using coupled *in vitro* transcription translation [21] of autoantigen cDNA to label the recombinant proteins with 35S-methionine. GAD65Ab and IA-2A levels were reported in WHO units (U/ml) [24] or as an index as for LVAb.

The intra-assay % coefficient of variation (CV) in the GAD65Ab assay was 8% and the inter-assay CV was 6%. The corresponding CV in the IA-2A assay was 11% and 10%, respectively.

IAA were determined as previously described [19, 25] using an in-house IAA standard. The data are expressed in either arbitrary units or as an index. The intra-assay % CV in the IAA assay was 9% and the inter-assay CV was 9%.

Positivity for GAD65Ab, IA-2Ab and IAA was defined as above the 95th percentile among the controls.

2.4. Statistical Analyses

The number of patients with newly diagnosed T1D were presented as Incidence Rate (IR) per 100 000 person years. The number of children residing in Jämtland by age and year were obtained from the Statistics Sweden website (www.scb.se). Exact 95% confidence intervals (95%CI) for incidence rates were calculated assuming a poisson distribution [26].

As higher than normal LVAb levels may also be present among non-diabetic children, the LVAb distribution among controls were first evaluated using Quintile-Quintile normality plots. A departure from a linear correlation between LVAb levels and quintiles of a standard normal distribution would indicate the distribution is not normally distributed. The LVAb index among controls and cases were also divided into quartiles and tested for statistical difference. Differences in proportions across groups were performed using exact chi-square tests with Monte Carlo approximation. Correlation between T1D IR and proportion of subjects determined to have high LVAb levels were tested by Pearson correlation tests. Mann-Whitney tested for difference in continuous variables between groups. Graphs were drawn in GraphPad 4.0 and statistical analyses were done using SPSS

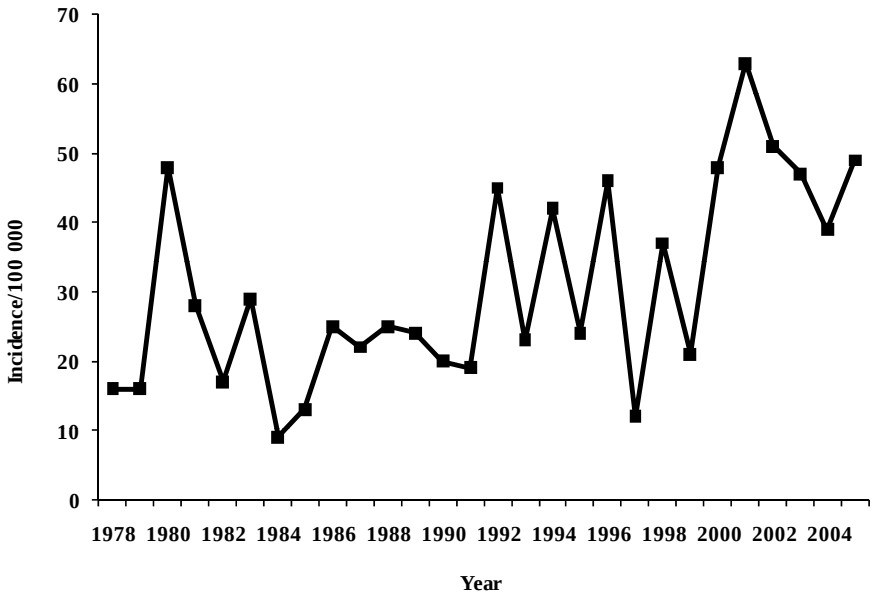

Fig. (1). Incidence rates in 1978-2005 of childhood type 1 diabetes in the county of Jämtland, Sweden. Only children 0-14 years of age are shown as the number of incident patients for the age group 15-18 years was not available for 1978-1999.

(www.spss.com). A p-value < 0.05 was considered statistically significant.

3. RESULTS

The T1D IR in the Jämtland County in the present and past 20 year period revealed annual fluctuations (Fig. **1**). Between October 2000 and February 2006, the IR of T1D among the 0-18 years olds was 44.5 (95% CI 34.2-57.0) per 100 000 person years. The IR tended to be higher in younger patients during winter (October to March) compared to the summer (April to September) months (Table **2**).

The frequency of GAD65Ab, IA-2Ab and IAA levels were increased in the newly diagnosed T1D patients compared to controls (Table **1**). As previously shown, IAA but not GAD65Ab and IA-2A was observed more often in young T1D children (< 9 years of age) (Table **3**).

Table 2. Type 1 Diabetes Incidence Rates in the County of Jämtland by Season and Patient Age at Diagnosis

Age at Diagnosis Years	Season[a]	Number of Patients	Person Years[b]	Incidence Rate (IR)/100 000 Person Years (95%CI)
0-8	Winter	21	31030	67.6 (41.9-103.5)
	Summer	9	26750	33.6 (15.3-63.9)
9-17	Winter	18	44950	40.0 (23.7-63.3)
	Summer	15	38750	38.7 (21.7-63.8)
0-17	Winter	39	75980	51.3 (36.5-70.2)
	Summer	24	65500	36.6 (23.5-54.5)

a = summers = April-September for years 2001-2005, winter = October to March for years 2000/1-2005/6, b = estimated from population statistics (www.scb.se).

There was no difference in GAD65Ab, IA-2Ab and IAA distribution between summer and winter months in the T1D children (data not shown).

As expected, LVAb levels among controls were not normally distributed (Fig. **2**, panel **a**). The control population was therefore analyzed in quartiles (Fig. **2**). 1st quartile represents children with the lowest levels of LVAb while the children in the 4th quartile are those with the highest level LVAb. These children will be referred to as "high level LVAb" children. As the LVAb levels were also not normally distributed in the patients, their results were similarly expressed (Fig. **2**, panel **b**). There was no difference in levels between patients and controls (p = NS).

Children with high level LVAb were next compared to the rest of the children. Both among patients (p = 0.008) and controls (p = 0.037), children with high level LVAb were found to be younger compared to the rest of the children (Table **3**). There was no difference in gender. Presence of GAD65Ab, IA-2Ab or IAA with high level (4th quartile) of LVAb did not differ from other patients (data not shown).

Table 3. Age of Children with High Levels of Lvab, Upper 4th Quartile (> 75 Percentile Among Controls), Compared to Low Levels of Lvab, Lower Three Quartiles, Both Among Patients and Controls

Subjects	4th Quartile LVAb	Number of Subjects n %	Age of Children (Years)	
			Median (Inter-Quartiles Range)	p-Value
All	Yes	44 (23%)	7.0 (2.3-9.0)	
	No	145 (77%)	10.0 (6.0-13.5)	0.002
Patients	Yes	13 (21%)	6.0 (2.5-8.0)	
	No	50 (79%)	10.0 (6.8-14.0)	0.008
Controls	Yes	31 (25%)	7.0 (2.0-10.0)	
	No	95 (75%)	10.0 (5.0-13.0)	0.037

Table 4. Frequency of Patients with Islet Autoantibodies within Different Study Years

Study Year	Year of Diagnosis	Number	GAD65Ab % pos	IA-2Ab % pos	IAA % pos	% of Patients with > 1 Antibody
1	Oct 2000 – Sept 2001	14	78.6	92.9	35.7	92.9
2	Oct 2001 – Sept 2002	13	84.6	92.3	46.2	76.9
3	Oct 2002 – Sept 2003	9	88.9	100.0	66.7	100.0
4	Oct 2003 – Sept 2004	10	90.0	80.0	80.0	100.0
5	Oct 2004 – Sept 2005	10	50.0	70.0	0.0	30.0
6	Oct 2005 – Febr2006	7	28.6	71.4	42.9	42.9
			$P^a = 0.02$	$P^a = 0.31$	$P^a = 0.007$	$p^a < 0.001$

a = p-value testing for difference in proportion across years of diagnosis

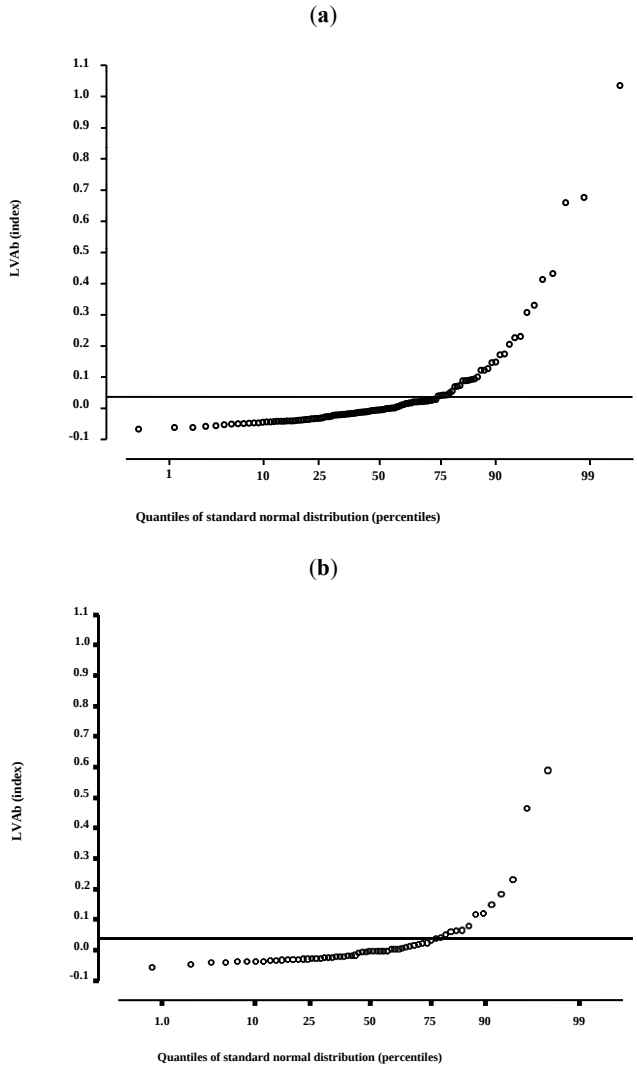

Fig. (2). *Ljungan virus* antibody levels (LVAb (index)) in 126 non-diabetic controls (panel **a**) and in 63 newly diagnosed type 1 diabetes patients (panel **b**) shown as QQ normality plots. The horizontal line represents the upper (4^{th}) quartile of controls (75^{th} percentile = 0.0395 LVAb index).

In order to increase statistical power, patients and controls were combined to test whether LVAb levels varied both with season and year (Fig. **3**). This analysis showed that the proportion of subjects with high level LVAb correlated with the half yearly T1D incidence rate (r = 0.78, p = 0.005).

Yearly percentage of patients or controls with high level LVAb did not differ (20-33%) except for year three where only 1/26 children (3.7%) had LVAb levels in the 4^{th} quartile (Fig. **3**). In contrast, among patients GAD65Ab and IAA as well as the number of islet autoantibodies differed by study year (Table **4**).

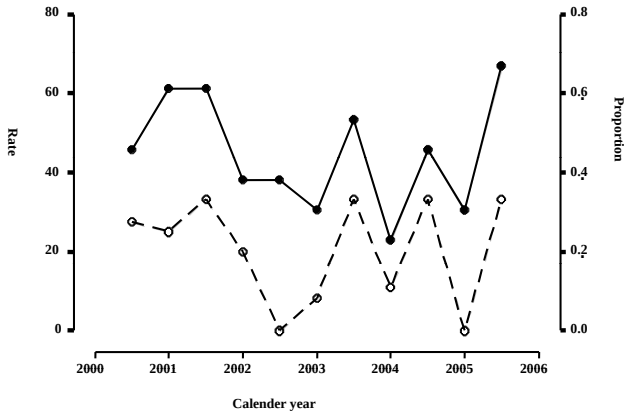

Fig. (3). Half yearly (summer compared to winter months) seasonal trend in type 1 diabetes incidence rate (Rate; solid line) compared to the proportion of patients and matched controls with LVAb in the 4^{th} quartile (Proportion; dash line). The proportion of subjects with the 4^{th} quartile = high level LVAb.

4. DISCUSSION

This study supports the hypothesis that antibodies against *Ljungan virus* may be associated with islet autoantibodies and temporal variations of T1D by three major findings.

First, the T1D IR in the Jämtland County tended to be increased in younger (< 9 years) patients during the winter months of 2000-2006.

Second, patients with high level LVAb (4^{th} quartile) were younger by more than three years. A similar association between LVAb levels and age was not only reported previously [19] but also observed among the control children supporting the view that exposure to LV is common particularly among the young.

Third, the proportion of subjects with high LVAb levels correlated with the half yearly (winter compared to summer months) IR of T1D.

Although direct evidence is lacking, these results may suggest that the increased IR of T1D in younger patients during winter months may correlate to a possible prior exposure to LV. As LV may be widespread and common, exposure to LV would not be unique to children developing T1D but would be rather common in the overall childhood population. It is speculated that LV rather than being a trigger of islet autoimmunity may affect the age of clinical onset of T1D in islet autoantibody-positive children. The higher LVAb levels in younger children whether patients or not suggest that early exposure to LV may be important perhaps also to explain the increased T1D IR in younger children [13].

Taken together, it cannot be excluded that LV infection may contribute to the seasonal as well as annual variation in T1D IR. With respect to seasonal variation, our study of the Jämtland County showed similar fluctuation in T1D IR as previously reported for Sweden [13]. T1D IR also fluctuates annually but it is difficult to distinguish such short term increases and decreases from random variation [13].

It is well known and carefully recorded that the population of bank voles in northern Sweden varies from year to year [20]. This makes it interesting that changes in T1D incidence rate was positively correlated with changes in bank vole density [16], where about two years delay between high number of bank voles and peak T1D IR was reported. Significant temporal correlation to other diseases such as myocarditis was reported as well supporting the view that etiological agent(s) may be using the bank vole as reservoir and vector [16].

In contrast to the T1D IR, we did not observe a seasonal but rather an annual fluctuation of GAD65Ab and IAA as well as in the number of islet autoantibodies observed at the time of clinical onset. It remains to be determined if annual fluctuations of islet autoantibodies reflect either T1D IR or merely the incidence rate of islet autoimmunity. On the other hand, the seasonal but not annual variation in LVAb levels would be consistent not only with T1D IR but also with the population density of bank voles as vectors of *Ljungan virus* [16, 20].

The strength of this study is the research design with a complete coverage of a geographically defined area with a relatively stable population. The incidence rates were based on the Swedish Childhood Diabetes Register [13]; [Annual numbers recevied as a personal communication from Gisela Dahlquist]. All new patients in the Jämtland County have been reported to this register since 1978. Another important factor in the study design was that two Jämtland County controls were selected for each patient. These controls were matched not only by age but also for the time of blood sampling. Furthermore, the islet autoantibody assays used are standardized and it is common to express GAD65Ab, IA-2Ab and IAA as index or arbitrary units in relation to international standards [24, 25]. Using a cut off at the 95th percentile for the islet autoantibodies the frequency of GAD65Ab, IA-2A and IAA was comparable to similar investigations of newly diagnosed Swedish T1D patients [21, 27, 28]. The LVAb analysis was also consistent with previous analysis of these virus antibodies in a larger number of children diagnosed with T1D in Stockholm, Sweden [19]. However, both in this and the previous study neither the levels nor the frequency of LVAb were increased among the T1D patients compared to controls. A potential type 2 error was made previously as seasonal and annual fluctuations were not analyzed. In future studies, it will therefore be important to ascertain blood samples prospectively not only for LVAb-in this study representing immunoglobulin G as the antibodies are detected with Protein A [21] but also for LV-IgM as for the virus itself.

Which one of the several possible mechanisms of LV exposure may explain the data? Using LVAb as a proxy for prior exposure we speculate that the virus is widespread and may affect a large proportion of children. The next question is whether LV would be able to induce islet autoimmunity. Injecting mice with LV induced high titer LVAb but not islet autoantibodies [19]. In contrast, injecting bank voles with LV induced both LVAb and islet autoantibodies, however, it is not clear what these results mean as the bank voles were neither inbred nor virus free [19]. Until samples from longitudinal studies such as the TEDDY study [29] become available, it remains an open question whether exposure to LV would induce islet autoimmunity (e.g. by beta cell lysis or molecular mimicry) or accelerate the disease process in children positive for islet autoimmunity.

5. CONCLUSION

From the current study, limited to one county of Sweden, using a carefully selected control group, it can be concluded that signs of LV infection fluctuate between summer and winter months. Patients in particular but also controls below the age of 9 years show high levels of LVAb, which also correlated with the T1D IR. A possible triggering may be the fluctuating population of bank voles, which are common in the Jämtland County and known to be the reservoir and carrier of LV. Further analyses of newly diagnosed patients and matched controls also from other counties of Sweden for the presence of LV RNA, and different subsets of immunoglobulins should provide a better insight into the possible relationship between LV exposure and T1D.

ACKNOWLEDGEMENTS

This study was supported by grants from the Syskonen Persson Foundation and FoU-unit, Mittuniversitetet, Östersund, Jämtland, Sweden (AL N) as well as from the National Institutes of Health (grants DK2910, DK53004 to ÅL), the Swedish Diabetes Association and the Roberth H Williams Endowed Chair. We thank Bo Niklasson for the gift of mouse and guinea-pig antisera against *Ljungan virus*. The nurses and laboratory personnel in the Departments of Pediatrics and Chemical Laboratory at the Östersund Hospital are acknowledged for blood sampling and processing of the samples.

ABBREVIATIONS

T1D = Type 1 diabetes

GAD65Ab = GAD65 autoantibodies

IAA = Insulin autoantibodies

IA-2A = IA-2 autoantibodies

IR = Incidence rate

LV = *Ljungan virus*

LVAb = *Ljungan virus* antibody/ies

REFERENCES

[1] Notkins AL. Immunologic and genetic factors in type 1 diabetes. J Biol Chem 2002; 277(46): 43545-8.

[2] EURODIAB. Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. Lancet 2000; 355(9207): 873-6.

[3] Soltesz G, Patterson CC, Dahlquist G. Worldwide childhood type 1 diabetes incidence-what can we learn from epidemiology? Pediatr Diabetes 2007; 8(Suppl 6): 6-14.

[4] Lernmark A. Type 1 diabetes. Clin Chem 1999; 45(8 Pt 2): 1331-8.

[5] Dahlquist G. The aetiology of type 1 diabetes: an epidemiological perspective. Acta Paediatr Suppl 1998; 425: 5-10.

[6] Dahlquist GG. Viruses and other perinatal exposures as initiating events for beta-cell destruction. Ann Med 1997; 29(5): 413-7.

[7] Lonnrot M, Salminen K, Knip M, *et al*. Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: A prospective study. Childhood Diabetes in Finland (DiMe) Study Group. J Med Virol 2000; 61(2): 214-20.

[8] Salminen K, Sadeharju K, Lonnrot M, *et al*. Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. J Med Virol 2003; 69(1): 91-8.

[9] Cabrera-Rode E, Sarmiento L, Tiberti C, *et al*. Type 1 diabetes islet associated antibodies in subjects infected by echovirus 16. Diabetologia 2003; 46(10): 1348-53.

[10] Dahlquist G, Frisk G, Ivarsson SA, *et al*. Indications that maternal coxsackie B virus infection during pregnancy is a risk factor for childhood-onset IDDM. Diabetologia 1995; 38(11): 1371-3.

[11] Hyoty H, Hiltunen M, Knip M, *et al*. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. Diabetes 1995; 44(6): 652-7.

[12] Yoon JW, Jun HS. Autoimmune destruction of pancreatic beta cells. Am J Ther 2005; 12(6): 580-91.

[13] Dahlquist G, Mustonen L. Analysis of 20 years of prospective registration of childhood onset diabetes time trends and birth cohort effects. Swedish Childhood Diabetes Study Group. Acta Paediatr 2000; 89(10): 1231-7.

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

[15] Lindberg AM, Johansson S. Phylogenetic analysis of *Ljungan virus* and A-2 plaque virus, new members of the Picornaviridae. Virus Res 2002; 85(1): 61-70.

[16] Niklasson B, Hornfeldt B, Lundman B. Could myocarditis, insulin-dependent diabetes mellitus, and Guillain-Barre syndrome be caused by one or more infectious agents carried by rodents? Emerg Infect Dis 1998; 4(2): 187-93.

[17] Niklasson B, Hornfeldt B, Nyholm E, *et al*. Type 1 diabetes in Swedish bank voles (Clethrionomys glareolus): Signs of disease in both colonized and wild cyclic populations at peak density. Ann N Y Acad Sci 2003; 1005: 170-5.

[18] Johansson S, Niklasson B, Maizel J, *et al*. Molecular analysis of three *Ljungan virus* isolates reveals a new, close-to-root lineage of the Picornaviridae with a cluster of two unrelated 2A proteins. J Virol 2002; 76(17): 8920-30.

[19] Niklasson B, Heller KE, Schonecker B, *et al*. Development of type 1 diabetes in wild bank voles associated with islet autoantibodies and the novel *Ljungan virus*. Int J Exp Diabesity Res 2003; 4(1): 35-44.

[20] Hornfeldt B, Hipkiss T, Eklund U. Fading out of vole and predator cycles? Proc Biol Sci 2005; 272(1576): 2045-9.

[21] Grubin CE, Daniels T, Toivola B, *et al*. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 1994; 37(4): 344-50.

[22] Vandewalle CL, Falorni A, Lernmark A, *et al*. Associations of GAD65- and IA-2- autoantibodies with genetic risk markers in new-onset IDDM patients and their siblings. The Belgian Diabetes Registry. Diabetes Care 1997; 20(10): 1547-52.

[23] Falorni A, Ortqvist E, Persson B, Lernmark A. Radioimmuno-assays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. J Immunol Methods 1995; 186(1): 89-99.

[24] Mire-Sluis AR, Gaines Das R, Lernmark A. The World Health Organization International Collaborative Study for islet cell antibodies. Diabetologia 2000; 43(10): 1282-92.

[25] Bingley PJ, Bonifacio E, Mueller PW. Diabetes Antibody Standardization Program: First assay proficiency evaluation. Diabetes 2003; 52(5): 1128-36.

[26] Ulm K. A simple method to calculate the confidence interval of a standardized mortality ratio (SMR). Am J Epidemiol 1990; 131(2): 373-5.

[27] Hagopian WA, Karlsen AE, Gottsater A, *et al*. Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. J Clin Invest 1993; 91(1): 368-74.

[28] Graham J, Hagopian WA, Kockum I, *et al*. Genetic effects on age-dependent onset and islet cell autoantibody markers in type 1 diabetes. Diabetes 2002; 51(5): 1346-55.

[29] Hagopian WA, Lernmark A, Rewers MJ, *et al*. TEDDY-the environmental determinants of diabetes in the young: an observational clinical trial. Ann NY Acad Sci 2006; 1079: 320-6.

Received: August 18, 2009 Revised: September 4, 2009 Accepted: October 25, 2009