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ASSOCIATIONS BETWEEN HLA CLASS II HAPLOTYPES, ENVIRONMENTAL FACTORS AND TYPE 1 DIABETES MELLITUS IN LITHUANIAN CHILDREN WITH TYPE 1 DIABETES AND CONTROLS

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ABSTRACT

Introduction. The onset of type 1 diabetes (T1D) is determined by genetic predisposition and environmental factors.

Aim. The aim of our work was to identify associations between human leukocytes antigen (HLA) class II alleles, environmental factors and T1D in Lithuania.

Materials and methods. Our case-control study included 124 diabetic children (mean age 9.19±3.94 years) and 78 controls (mean age 10.77±3.36 years). The age ranged from 0 to 15 years. HLA–DRB1, DQA1 and DQB1 alleles were genotyped using polymerase chain reaction. Information concerning the environmental factors was collected via questionnaires.

Results. Logistic regression model indicated that three haplotypes: (DR3)––DQA1*0501–DQB1*0201, (DR4)–DQA1*0301–DQB1*0302 and (DR1)–DQA1*010––04–DQB1*0501, increased the T1D risk statistically significantly 18.1, 12.3 and 3.4 times, respectively, while (DR11/12/13)–DQA1*05–DQB1*0301 haplotype decreased the risk of T1D 9.1 times.

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Several different regression models included environmental factors and different sets of risk and protective haplotypes. The results suggest that living in a remote area with lower population density during pregnancy increased the risk of T1D, as well as short breastfeeding, introduction of eggs before 5th month of age and infections during the last 6 months before diagnosis. Smoking during pregnancy as well as rubella and varicella virus infections seemed to decrease the risk of T1D. These associations were revealed while evaluating only environmental factors and when different HLA haplotypes together with environmental factors were included in the regression model.

Discussion. The HLA typing shows that the differences in the incidence of T1D between Lithuania and neighboring countries cannot be explained only by genetics, but lifestyle and/or environmental factors should be considered. A number of studies presented here, have shown conflicting results regarding environmental factors and their associations with T1D.

Conclusions. Both genetic and environmental factors play a major role in diabetes development and protection. However, even quite rapidly ongoing changes of environmental factors and lifestyle in Lithuania have not helped us to reveal any clear picture.

Key words: type 1 diabetes (T1D), children, human leukocytes antigens (HLA), environmental risk factors, case-control study.

INTRODUCTION

Type 1 diabetes mellitus (T1D) is a slowly progressive autoimmune disease caused by a selective destruction of the insulin-producing pancreatic beta cells. Genetic predisposition is important, but not sufficient, for the disease to develop. Human leukocytes antigen (HLA) genes contribute the most to genetic susceptibility for T1D, although other genes are also likely to be involved but with much less importance [12]. Certain HLA genes can also provide protection from diabetes. Among Caucasians, T1D is positively associated with DR3–DQ2 and DR4–DQ8 haplotypes and negatively associated with DR2–DQ6 [9, 24]. Together with genetic predisposition, several facts, such as the rapidly increasing incidence, prove that environmental factors play a crucial role for the development of T1D [10]. Prenatal events, growth during the first years of life, nutrition early in life and rapid weight gain are those factors which can cause beta cell stress [2, 14]. Several studies have observed that non-breast-fed infants gain weight more rapidly than breast-fed children. This fact may explain the protective effect of breast-feeding against T1D [14]. Viral infections, social factors, psychological stress, etc., can also modify the risk for the disease [4, 16].

Thus, T1D develops in genetically susceptible individuals as a response to the interaction with lifestyle and/or environmental agents. However, convincing evidence

for some major environmental factors to be the initiators of the disease process has so far not been presented. Being aware of many extensive studies in this field, we still thought that studies on this topic in Lithuania might yield additional new information. In Lithuania, the incidence of T1D is rather low – 14.2 per 100 000 children in a year [28] – as compared to neighboring countries: 64.2 per 100 000 in a year in Finland [7], 37.8 per 100 000 in a year in Sweden [27] and 22.7 per 100 000 in a year in Norway [1]. The incidence is increasing year by year on average from 1.3% in Norway to 3.3% per year in Sweden [21], where in 2008–2009, the incidence amounted to more than 40 cases per 100 000 in a year (Samuelsson U., Sweden; unpublished data, 2009).

AIM

The aim of our work was to identify associations between HLA class II alleles, environmental factors and T1D in Lithuania against the background of rapid changes in lifestyle and environmental factors in Lithuania.

MATERIALS AND METHODS

Our study is part of a larger study (Diabetes and Environment at the Baltic Sea, DEBS) which was designed as a case-control study. The group consisting of 286 children with newly diagnosed T1D during the period of 1 August, 1996, and 1 August, 2000, in Lithuania and 813 age and sex matched double randomly selected healthy controls participated in that study, which has been presented earlier [23].

All parents, together with their children, filled in the questionnaires about nutrition in early life, duration of exclusive and total breast-feeding, time of introduction of cow's milk based formula, cereal, eggs and other solid foods. There were questions regarding exposure during pregnancy, neonatal period and first year of life, social factors such as living conditions and residence, mother's education, occupation, employment, child attendance to kindergarten, infections and vaccinations. The questionnaire has been described previously [23].

HLA testing was performed in 124 diabetic children (55 male and 69 female, mean age 9.19±3.94 years) and compared with 78 controls (43 males and 35 females, mean age 10.77±3.36 years). The ages ranged from 0 to 15 years. Blood samples were obtained from children with diabetes as well as control children and stored at -20°C. DNA was extracted from blood leukocytes by the standard phenol-chloroform method and then was dissolved in sterile double-distillate water. HLA–DRB1, DQA1 and DQB1 alleles for diabetic children were genotyped using polymerase chain reaction (PCR) with amplification of the second exon of the genes as described earlier [25]. Amplified product was manually dot blotted onto nylon membranes. Synthetic sequence-specific oligonucleotide (SSO) probes were 3'-end labeled with (αP^{32})dCTP and used for hybridization followed by stringency washes and autoradiography. Laboratory analysis was carried out in the Department of Molecular Immunogenetics, Karolinska Institute, Sweden.

HLA–DRB1, DQA1 and DQB1 alleles for control children were genotyped using PCR with sequence-specific primers (SSP–PCR) supplied by Protrans and following manufacturer's recommendations (Protrans, Germany). The amplified products were determined by means of agarose gel electrophoresis. Laboratory analysis was carried out in the Laboratory of Immunology and Genetics, Kaunas Medical University Hospital, Lithuania.

The study was approved by the Research Ethics Committee of Kaunas University of Medicine, Lithuania.

Statistical analysis

Comparisons of means between the groups of cases and controls were performed by the Student's *t*-test or Mann-Whitney *U*-test (non-parametric values). Proportions were compared using Pearson's χ^2 or Fisher's exact test. Odds ratios (OR) with 95% confidence intervals (CI) were calculated. Risk factors' models were analyzed using logistic regression analysis. Differences were considered significant at $P < 0.05$.

RESULTS

A binary logistic regression model was performed to assess the importance of different haplotypes and environmental factors on T1D development. We tried several logistic regression models that included the previously mentioned environmental factors and HLA haplotypes.

The first logistic regression model (Tab.1) indicated that three haplotypes: (DR3)–DQA1*0501–DQB1*0201, (DR4)–DQA1*0301–DQB1*0302 and (DR1)––DQA1*0101–04–DQB1*0501, increased the risk of diabetes statistically significantly 18.1, 12.3 and 3.4 times, respectively, while (DR11/12/13)–DQA1*05–DQB1*0301 haplotype decreased the risk of T1D 9.1 times.

Tab. 1. Logistic regression model for haplotypes predicting the likelihood of T1D mellitus

Haplotype	OR	95% CI	*P*
(DR1)–DQA1*0101–04–DQB1*0501	3.36	(1.27–8.86)	0.014
[DR2(DR15)]–DQA1*0102–DQB1*0602	0.000	–	0.998
(DR3)–DQA1*0501–DQB1*0201	18.06	(5.07–64.36)	< 0.001
(DR4)–DQA1*0301–DQB1*0302	12.31	(4.25–35.61)	< 0.001
(DR11/12/13)–DQA1*05–DQB1*0301	0.11	(0.03–0.38)	0.001

$\chi^2 = 134.53$, $df = 5$, n = 202; $P < 0.001$

In other logistic regression models, we included all environmental factors mentioned above that had significant associations with diabetes. The final logistic re-

gression model including environmental factors is shown in Tab. 2. The mother's residence during pregnancy in a village or remote house, eggs introduction in infant nutrition before 5th month of age and child infections during the last 6 months before diabetes onset significantly increased diabetes risk. Viral infectious such as rubella and varicella seemed to decrease the risk of T1D.

Tab. 2. Logistic regression model for environmental factors predicting or protecting the likelihood of T1D mellitus

Environmental factor	OR	95% CI	*P*
Mother's residence during pregnancy in village or remote house	6.46	1.74–23.94	0.005
Egg introduction before 5th month of age	3.70	1.77–7.75	0.001
Tetanus, diphtheria and pertussis vaccine	4.02	0.99–16.32	0.052
Varicella infection	0.47	0.22–0.97	0.042
Rubella infection	0.33	0.14–0.77	0.011
Infection during the last 6 months before diagnosis of T1D	3.85	1.79–8.27	0.001
Stressful event previous 6 months before diagnosis	2.38	0.85–6.62	0.097

$\chi^2 = 57.09$, $df = 7$, n = 187; $P < 0.001$

Finally, to several different regression models, we included environmental factors and different sets of risk and protective haplotypes (Tab. 3–5). These results are based on a quite small sample and show heterogeneous pictures of associations. Although sometimes statistically significant, they are difficult to interpret. Logistic regression analysis showed that living in a remote area with lower population density during pregnancy seemed to increase the risk, as well as short breast-feeding. Smoking during pregnancy as well as rubella and varicella infections rather seemed to decrease the risk of T1D. These associations were observed while evaluating only environmental factors and when different HLA haplotypes together with environmental factors were included in the regression model.

Tab. 3. Logistic regression model for protective haplotypes and environmental risk factors influencing the likelihood of T1D mellitus

Environmental factor	OR	95% CI	*P*
Mother's residence during pregnancy in a village or remote house	12.68	0.82–197.18	0.07
Total breast-feeding less than 3 months	3.41	1.09–10.62	0.035
At least one of the protective HLA haplotypes	0.003	0.001–0.03	<0.001

$\chi^2 = 104.59$, $df = 3$, $n = 145$; $P < 0.001$

Tab. 4. Logistic regression model for risk haplotypes and environmental protective factors influencing the likelihood of T1D mellitus

Environmental factor	Odds ratio	95% CI	*P*
Mother's smoking during pregnancy	0.07	0.01–0.93	0.04
Varicella infection	0.35	0.15–0.80	0.013
Rubella infection	0.33	0.13–0.82	0.017
At least one of the risk HLA haplotypes	23.30	9.37–57.93	< 0.001

χ^2=90.05, *df*=7, n=193; *P*<0.001

Tab. 5. Logistic regression model for grouped haplotypes and environmental factors predicting or protecting the likelihood of T1D mellitus

Environmental factor	Odds ratio	95% CI	*P*
Mother's residence during pregnancy in a village or remote house	17.74	1.41–222.99	0.026
Total breast-feeding less than 3 months	3.46	1.14–10.50	0.028
Rubella infection	0.19	0.06–0.64	0.007
Infection during the last 6 months before diagnosis of T1D	2.49	0.86–7.22	0.09
At least one of the risk HLA haplotypes	12.48	4.31–36.16	< 0.001
At least one of the protective HLA haplotypes	0.03	0.01–0.11	< 0.001

χ^2 = 151.39, *df* = 6, n = 191; *P* < 0.001

DISCUSSION

This study is important because of the specific situation in Lithuania regarding the low incidence of T1D as compared with other European, especially neighboring, countries. Even considering some limitations of this study, such as small sample size and the retrospective data collection with the risk of bias, the present study provides useful and essential information about T1D etiology.

The HLA typing shows that the differences in the incidence of T1D between Lithuania and neighboring European countries such as Sweden, Finland, Norway or Estonia [1, 7, 19, 28] cannot be explained only by genetics, but lifestyle and/or environmental factors should be considered. A number of studies have shown conflicting results regarding environmental factors and their associations with T1D. In some studies, long-term breast-feeding has been shown to have a protective effect [22], while other studies have not found such association [18]. Our study confirms that total breast-feeding for three months or less increased the risk of T1D only when other environmental factors and HLA haplotypes were included in logistic regression analyses. Some recent studies have confirmed associations between the early

introduction of cow's milk and development of diabetes [23, 30]. Other studies do not support such associations [18]. Early introduction of cereal, eggs and other solid foods may increase the risk of T1D too [20]. Our study showed no impact of early introduction of cow's milk or any solid food except for early introduction of eggs on the development of T1D. We found that living in an area with low population density was the factor increasing the risk of T1D mostly, which might fit into the "hygiene hypothesis" suggesting that less exposure to certain infection/antigens early in life might counteract the maturation of the immune system and increase the risk of autoimmune disease like T1D [5]. Anyhow, early contact with microbial antigens may prevent autoimmune diabetes [15]. However, data from other studies show conflicting results. Some studies proposed that residence in urban areas increased the risk of diabetes [8], while other studies found the lowest incidence of T1D in urban areas [11]. Possibly, our finding that smoking during pregnancy has a protective effect is related to the "hygiene hypothesis", or smoking has a direct effect on the maturation of the immune system [13]. Our results are in concordance with those of Svensson et al., who found that maternal smoking during pregnancy was associated with a decreased risk of T1D in the offspring [26].

Exposure to common infections during the first half year of life has been reported to be associated with reduced diabetes risk [15]. However, some viruses such as rubella and enteroviruses could directly destroy beta cells in susceptible individuals. Similarly, another study showed that only one of the childhood infections (morbilli, pertussis, rubella, etc.) was not related to diabetes risk [3]. In our study, we found that varicella and rubella infections in association with at least one of the risk HLA haplotypes in early childhood decreased diabetes risk. However, infections during the last six months before the onset of diabetes may increase diabetes risk [29]. Other studies have shown that only the most common infections may influence diabetes development [3].

Vaccinations have been proposed to protect against diabetes [4, 15] or increase the risk of diabetes [17]. No association between the risk of T1D and any of routinely recommended childhood vaccines was found.

Stressful events in early life or stress during the last half year have also been associated with an increased risk of T1D [6, 29]. Such events have been hypothesized to accelerate a pre-existing autoimmune process [6]. However, no associations between the development of diabetes and stressful events were observed in our study.

CONCLUSIONS

Environmental factors and lifestyle together with genetic predisposition certainly play an important role in the development of T1D, so etiology is complex. Our study showed that nutrition in early life as well as factors related to increased hygiene might contribute to T1D development. However, even quite rapidly ongoing changes of environmental factors and lifestyle in Lithuania have not helped us to reveal any clear picture.

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