The association of different enteroviruses with atopy and allergic diseases in early childhood

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Abstract

Background: Enterovirus (EV) infections, being among the most prevalent viruses worldwide, have been associated with reduced risk of allergic diseases. We sought to determine the association of EVs with allergic sensitization and disease in early childhood. Methods: The study was carried out in a nested case-control setting within a prospective birth cohort in Finland. We included 138 case children who had specific IgE (s-IgE) sensitization at the age of 5 years and 138 control children without s-IgE sensitization. Allergic disease was recorded at study visits and asked with ISAAC questionary. We screened for the presence of serotype specific antibodies against 41 EVs at 1 to 5 years of age and assessed their association with allergic sensitization and disease. Results: The overall number of EV infections did not differ between s-IgE-sensitized children and non-sensitized control children. However, there was a tendency of case children with an allergic disease having less EV infections than their controls. This observation was statistically significant for species A EVs in case children with atopic dermatitis vs. control children: OR 0.6 (95 % CI 0.36-0.99), P = 0.048. Conclusion: This study supports the evidence that EV exposure and development of allergic disease are inversely associated. Interestingly, the inverse association was not observed for bare atopic IgE sensitization, but for IgE sensitization coupled with clinical atopic disease. This suggests that environmental factors influencing IgE sensitization may differ from those influencing progression to clinical allergic disease.

Introduction

Atopy is defined as a tendency to produce IgE antibodies in response to ordinary harmless exposures to allergens. The prevalence of atopic IgE sensitization in developed countries is extremely high, rising from around 12% at the age of 1 year to around 30% at the age of 6 years[1] and staying at around 20-50% throughout adulthood [2]. The rapid increase in the prevalence during the last half a century [3] suggests involvement of environmental factors.

According to the hygiene/biodiversity hypothesis [4,5]the increase in atopy and allergic diseases is caused by reduction in microbial exposures in childhood and thus, skewing of the immune responses towards allergy prone Th2-based reactions. Viruses guide the immune system towards Th1-direction [6]. One could thus

hypothesize that virus infections reduce the risk of atopy and allergy. Accordingly, low-grade inflammation, induced for example by microbial exposure during infancy, has been shown to decrease allergen specific IgE (s-IgE) sensitization[7]. Multiple viruses have been linked to reduction of allergic diseases and/or IgE sensitization[8–13] but there are also reports contradicting the role of viruses [3,9,14,15].

Enterovirus infections (EVs) are among the most common human virus infections worldwide. The classic human EVs can be classified into four species (EV-A, -B, -C and -D) comprising more than 100 serotypes. The pathophysiology of different serotypes differs depending on their tropisms to different tissues and cells. Enterovirus infections are mostly asymptomatic or mild such as common cold or hand, foot and mouth disease, but also serious diseases such as meningitis, myocarditis, and paralysis may occur [16]. In *in vitro* models EVs have been shown to bias the immune reactions towards the mature Th1-like pathway [17,18]. They have also been linked with reduced risk of allergic diseases/atopy [11,12,19], but the results are contradictory [20,21].

We contribute new insight into the association between EVs and atopy with respect to the individual EV serotypes. This study is the most comprehensive of its kind, containing 41 different human EV serotypes analyzed in a large case-control setting.

Methods

Study population

The study population was derived from the prospective population-based Type 1 Diabetes Prediction and Prevention (DIPP) birth-cohort study described in detail previously [22]. Briefly, children born in the Tampere, Turku, and Oulu University Hospitals in Finland are screened at birth for HLA genotypes conferring to increased risk of type 1 diabetes. Written informed consent was obtained from caretakers both for HLA screening and follow-up. The study protocol was approved by the ethics committees of the participating hospitals. The present study included children born between years 1995 and 2004 in Tampere and Oulu where children carrying the HLA-DQB1 genotypes *02/*0302 and *0302/x (x [?]*02), *0301 or *0602 were invited to follow-up. Blood samples were drawn at 3, 6, 12, 18, and 24 months of age and once a year thereafter. Atopic case and non-atopic control subjects were identified among children with an available serum sample for both EV antibody assay (serum sample between 1 and 5 years of age) and s-IgE antibody assay (serum sample at 5 years of age). Altogether 138 case children with a positive s-IgE test and 138 matched control children with negative s-IgE test were identified. Of the 138 case children 65% were sensitized to the food allergen mixture and 71% to the inhalant allergen mixture. Of those positive for foods 57% tested positive for cow's milk, 32% for egg, 22% for wheat, and 6% for fish. Of those positive for inhalants 57% were positive for birch, 37% for timothy, 3 % for cat, and 10% for house dust mite. The pairs were matched by gender and age at the EV antibody measurement (+/- 3 months). The proportion of boys was 65%.

Allergic diseases were identified at 5 years of age by ISAAC questionnaire questions: "Has your child ever had asthma/hay fever or some other allergic rhinitis/atopic eczema diagnosed by a doctor?". ISAAC-data was missing in 39 children. Their allergic disease diagnoses were retrieved from the DIPP database: atopic dermatitis (ICD-code L20.x), asthma (J45.x) and allergic rhinitis/hay fever (J30.x) reported and confirmed by a physician by 5 years of age. Sixty-four (46%) out of the 138 IgE-sensitized case children reported an allergic disease (47 atopic dermatitis, 27 allergic rhinitis and 15 asthma) and formed a case subgroup for analysis of enteroviruses' association with clinical disease. These 64 case children were compared to their IgE-negative matched control children. Some of the 64 IgE-negative control children also reported an allergic disease (n = 17), but were eligible as controls, as IgE sensitization was the feature defining case vs. control status. Thirty-one (22.5%) out of the total of 138 IgE-negative control children reported an atopic disease (25 atopic dermatitis, 2 allergic rhinitis and 8 asthma reports).

Background data of the participants retrieved from the DIPP database included information of the age at the entry of daycare, duration of breastfeeding, level of maternal and paternal education, HLA-DQB1-genotype, the presence of type 1 diabetes associated islet autoantibodies, pets at home, older siblings and maternal smoking during pregnancy. Parental atopy background information was available in the ISAAC-data for all

but 39 children. Maternal smoking was excluded from the analyses as it was reported by only five mothers.

Testing for allergen specific IgE

The s-IgE antibodies were tested at the age of 5 years against a mixture of airborne and food allergens (a multi-allergen test Phadiatop®, Thermo Fisher Scientific, Uppsala, Sweden) using the ImmunoCAP® enzyme immunoassay (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's protocol. The test was regarded positive if the food or inhalant allergen mixture showed a result of 0.35 kU/L or more. Individual s-IgE results were available for cow's milk, hen's egg, fish, wheat, house dust mite, cat, birch, and timothy grass.

Enterovirus seroneutralization assays

Neutralizing antibodies were measured in serum or plasma against 41 EV serotypes listed in Table 2 as previously described[23]. Viruses were isolated and cultivated, and seroneutralization assays were performed using the A549, Vero, RD (all bought from American Type Culture Collection) and GMK (from the National Institute for Health and Welfare, Finland) cell lines. Most of the viruses were analyzed using a standard plaque neutralization assay[24], whereas viruses that did not form clear plaques were analyzed using a microneutralization assay (CAV5, CAV6, CAV10, CAV16, EV71, EV78 and EV94). The neutralization assay was considered positive when the serum dilution 1:4 reduced the number of plaques by more than 75% (plaque assay) or inhibited the ability of the virus to kill cells (microneutralization assay). The serum/plasma samples analyzed were taken between 1 and 5 years of age. The sample volume was adequate for the analysis of neutralizing antibodies against all 41 serotypes in 183 children. In 83 children the volume was adequate for the analysis of 39-40 serotypes and in nine children for 4-21 serotypes.

Statistical Methods

The statistical analyses were performed using R version 4.0.2 (2020-06-22, The R Foundation for Statistical Computing, https://www.R-project.org). Data are presented as medians, interquartile ranges (IQRs), and ranges, as the continuous variables were not normally distributed. Wilcoxon test was used to assess differences in continuous variables. For determining the risk effect of different exposures conditional logistic regression models were applied. The results are presented as odds ratios (OR) and 95% confidence intervals (CI). P value was considered significant when < 0.05.

Results

Prevalence of neutralizing antibodies

The median age for the EV antibody analysis was 2.0 years and the median age difference between case and control children was 7 days. All children were seropositive for at least one EV serotype. The median number of positive serotypes was eight (range 1-21). Most common serotypes were Echo33 (antibody prevalence 77%), Echo30 (74%), EV74 (58%), CAV10 (56%), and CVB1 (52%).

Background factors

None of the background factors differed statistically significantly between the case and control children (**Table 1**).

Exposure to individual EV serotypes and risk of atopy

Seropositivity for individual EV serotypes is shown in **Table 2** . Antibodies against Echo3 and Echo14 were less prevalent among case children as compared to control children (OR 0.11, 95% CI 0.01-0.88, $P = 0.037$ and OR 0.17, 95% CI 0.04-0.75, $P = 0.019$, respectively). EV74 antibodies were more prevalent among case children (OR 1.68, 95% CI 1.02-2.76, $P = 0.04$). In a subgroup analysis of cases with higher likelihood of clinical symptoms (s-IgE-levels over 0.7 kU/L) CAV6 was a strong protective element for atopic sensitization (OR 0.22, 95% CI 0.08-0.66, $P = 0.007$) while EV74 continued to show a risk association (OR 1.82, 95% CI 1.01-3.29, $P = 0.047$).

When case children with an allergic disease were compared to their matched controls CAV6, CAV16, and Echo14 seropositivity were significantly less frequent in cases (OR 0.23, 95% CI 0.07-0.81,$P = 0.022$; OR 0.33, 95% CI 0.12-0.92, $P = 0.033$ and OR 0.11, 95% CI 0.01,0.88, $P = 0.037$, respectively).

The prevalence of EV seropositivity was analysed separately in case boys vs. control boys and case girls vs. control girls. Two EV serotypes showed significant inverse assocation with atopic sensitization among boys: CAV6 (OR 0.27, 95% CI 0.09-0.80, $P = 0.019$) and Echo14 (OR 0.10, 95% CI 0.01-0.78, $P = 0.028$). In addition, CAV6, CAV16, and Echo5 showed significant inverse assocation with allergic disease among case boys compared to their controls (OR 0.18, 95% CI 0.04-0.82,$P = 0.027$; OR 0.21, 95% CI 0.06-0.75, $P = 0.015$ and OR 0.31, 95% CI 0.1-0.94, $P = 0.039$, respectively). None of the serotypes showed assocation with atopic sensitization or allergic disease among girls.

Cumulative enterovirus exposure

The cumulative exposure to different EV serotypes illustrates the total number of different EVs that the child has encountered. We hypothesized that cumulative EV exposure would protect from allergic sensitization but found no such association (**Figure 1**). However, there was a trend of an inverse association between cumulative number of EV infections and allergic disease (**Figure 1**). This association was statistically significant between the cumulative exposure to group A EVs and cases with atopic dermatitis (OR 0.6, 95% CI 0.36-0.99,$P = 0.048$).

Discussion

For the first time, antibodies against a vast panel of different EVs were analyzed to shed light on the possible role of EVs as risk-modifying factors in the development of allergic sensitization and/or disease. The cumulative number of infections by different EV serotypes was not associated with IgE sensitization. However, the cumulative number of species A EV infections showed a trend for protective association with the development of allergic disease. This inverse association was significant for IgE-sensitized cases with atopic dermatitis, whereas for hay fever and asthma only a statistically insignificant inverse tendency was observed. Especially asthma is a heterogeneous disease and many patients lack atopic background[25], which could explain the lack of association. Another explanation could be limited statistical power, since the number of cases with asthma or hay fever were lower than those with atopic dermatitis.

Certain individual EV serotypes showed an association with IgE sensitization and/or allergic disease, including Echo3 and 14 which both showed an inverse association and EV74 which showed a risk association. Interestingly, Echo3 has been reported to associate with a reduced risk of allergic diseases also in a previous study [11]. Additionally, we wish to highlight CAV6, CAV16, Echo5, and Echo14 as intriguing protective players in the development of allergic diseases. Especially CAV6 exposure, a common virus causing hand, foot and mouth disease, was significantly less frequent in cases with allergic disease than in their matched controls and CAV6 was also less frequent in cases with stronger (> 0.7kU/L) IgE sensitization. The inverse CAV6 association was observed for boys with IgE sensitization and boys with atopic disease, but not for girls. This sex difference is parallel with our previous report of rhinoviruses' association with reduced risk of atopy within boys but not in girls[21], and indicates that viruses' influence on atopy risk may be more pronounced in males. The fact that only few of the 41 EV serotypes showed association with atopy may relate to immunofenotype differences between virus serotypes and strains, as suggested by our previous report showing very different immune system activation by individual CBV1 strains[27]. It is also known that EVs differ in their ability to infect various white blood cell populations[26]. Thus, it is possible that certain EV types, such as CAV6, have a unique "anti-allergic" immunophenotype due to their specific interaction with the immune system. This hypothesis requires further studies.

Previous studies have not identified any specific microbes driving the inverse association between microbial exposures and the risk of allergic disease. Instead, it seems that a vast variety of microbes, perhaps even the total microbiome, is involved[4]. Our finding of an inverse association between the cumulative exposure to different EV types and allergic disease fits with the hypothesis of more than a single culprit microbe involved. Accordingly, even though we found some significant associations when analyzing the individual serotypes,

we want to encourage opting for as large virus panel as possible in future studies on EVs and atopy.

Strengths of this study are the comprehensive panel of the most common circulating EV serotypes and the utilization of a highly serotype specific neutralization assay, which can be considered the gold standard for determining serotype specific EV antibodies. The endpoints of our study were also solid, as the IgE sensitization was determined with a wide allergen panel including both inhalant and food allergens, and information on allergic diseases was based on either a validated atopy questionnaire (ISAAC) or a diagnosis made by a physician.

The limitations of the study include the lack of correction of *P*values for the great number of comparisons performed when analyzing the individual EV serotypes. In our study using a Bonferroni coefficient of 41 (number of EV serotypes studied) leads to a statistical significance cutoff value $P < 0.0012$ and wipes out the significant associations for individual serotypes. Bonferroni correction has been criticized for being overly conservative, and therefore we held to the generally accepted P value <0.05. One must keep in mind that without correction for multiple comparisons the results for individual EV serotypes must be kept preliminary and they need confirmation in further studies.

A further limitation is that the study subjects did not represent the general population but carried specific type 1 diabetes-associated HLA haplotypes. However, no consistent relationship has been shown between allergic diseases and type 1 diabetes [27,28], and neither islet autoimmunity nor HLA was associated with atopic sensitization in the current cohort. Lastly, we were unable to ascertain the chronological order of the EV infections and IgE sensitization, as the latter was measured only at the age of 5 years.

In conclusion, we show that there is an inverse association between cumulative EV infections and allergic disease but not with allergic sensitization alone. Whether this relationship is causal and how EVs could obstruct the disease progression from sensitization to allergic disease needs to be further studied. We also want to highlight serotypes Echo3, Echo5, Echo14, CAV6 and CAV16 as possible protective serotypes and EV74 as a possible risk factor in allergic sensitization and disease.

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Impact statement: Hygiene hypothesis has long been proposed to explain the increasing incidence of allergic sensitization and disease. Enteroviruses, the most common viruses infecting human beings, have been associated with the risk of allergy and asthma, but it is not known whether this association links to certain enterovirus types. The present study utilized the most comprehensive serotype panel thus far applied and was able to strengthen previous indications of a possible role of enteroviruses in the regulation of the risk of allergic diseases.

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Table1. Background data of case children with allergic sensitization and controls

	Cases n = 138	Controls n = 138	OR (95 % CI), *P* value
Older siblings (yes)	77 (56 %)	78 (57 %)	OR 0.97 (0.59-1.6), *P* =0.898
Indoor pets (yes)	38 (29 %)	52 (39 %)	OR 0.7 (0.43-1.13), *P* =0.148
Entry in daycare (months, median (range))	24 (9-87)	24 (9-97)	OR 1.0 (0.99-1.12), *P* =0.424
Paternal education			
Elementary/lower secondary	65 (48 %)	53 (39 %)	ref
Matriculation examination/higher secondary	71 (52 %)	84 (61 %)	OR 0.71 (0.45-1.14), *P* =0.159
Maternal education			
Elementary/lower secondary	42 (31 %)	31 (22 %)	ref
Matriculation examination/higher secondary	95 (69 %)	107 (78 %)	OR 0.66 (0.39-1.11), *P* =0.118
Parental atopy			
Neither	32 (27 %)	31 (26 %)	ref
Either or both	86 (73 %)	86 (74 %)	OR 1 (0.53-1.86), *P* =1
Breastfeeding			
Total (months, median (range))	7 (0-25)	8 (0-24)	OR 0.99 (0.94-1.05), *P* =0.758
Exclusive breastfeeding (months, median (range))	1.2 (0-5.5)	1.3 (0-6)	OR 1.01 (0.86-1.19), *P* =0.889
HLA risk for type 1 diabetes			
Moderate risk	94 (68 %)	101 (73 %)	ref
High risk	44 (32 %)	37 (27 %)	OR 1.37 (0.76-2.47), *P* =0.299
Islet autoantibody positivity	28 (20 %)	27 (20 %)	OR 1.05 (0.58-1.9), *P* =0.879

Table 2. Prevalence of neutralizing antibodies against enterovirus serotypes in cases and controls and the risk for allergic sensitization associated with enterovirus serotypes.

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FIGURE LEGENDS

Figure 1. Association between cumulative enterovirus exposure for any enterovirus (EV) serotype / species A-serotype (A group) / species B-serotype (B group) and allergic sensitization or allergic disease in cases vs. controls (left) and for cases with a specific allergic disease vs. their matched controls (right).