Pediatr Allergy Immunol 2003: 14: 363–370
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PEDIATRIC ALLERGY AND IMMUNOLOGY

Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants

van Benten I, Koopman L, Niesters B, Hop W, van Middelkoop B, de Waal L, van Drunen K, Osterhaus A, Neijens H, Fokkens W. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants.
Pediatr Allergy Immunol 2003: 14: 363–370. © 2003 Blackwell Munksgaard

Respiratory infections in infancy may protect against developing Th2-mediated allergic disease (hygiene hypothesis). To estimate the relative contribution of particular viruses to the development of the immune system and allergic disease, we investigated longitudinally the prevalence of respiratory viral infections in infants. One hundred and twenty-six healthy infants were included in this prospective birth cohort study in their first year of life. Physical examination was performed and nasal brush samples were taken during routine visits every 6 months and during an upper respiratory tract infection (URTI) (sick visits). The prevalence of respiratory viral infections in infants with URTI, infants with rhinitis without general malaise and infants without nasal symptoms was studied. Rhinovirus was the most prevalent pathogen during URTI and rhinitis in 0- to 2-year-old infants (~40%). During URTI, also respiratory syncytial virus (~20%) and coronavirus (~10%) infections were found, which were rarely detected in infants with rhinitis. Surprisingly, in 20% of infants who did not present with nasal symptoms, rhinovirus infections were also detected. During routine visits at 12 months, a higher prevalence of rhinovirus infections was found in infants who attended day-care compared with those who did not. We did not observe a relation between breast-feeding or smoking by one or both parents and the prevalence of rhinovirus infections. The parental history of atopy was not related to the prevalence of rhinovirus infection, indicating that the genetic risk of allergic disease does not seem to increase the chance of rhinovirus infections. In conclusion, rhinovirus infection is the most prevalent respiratory viral infection in infants. It may therefore affect the maturation of the immune system and the development of allergic disease considerably.

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Key words: allergy; infant; day-care; prevalence; respiratory infection; rhinovirus; RSV

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Accepted 16 February 2003

The human immune system can respond in various ways to environmental factors. Two of the main responses are the production of T helper 1 (Th1) cytokines during viral and bacterial infections and Th2 cytokine production upon parasite infection (1–3). Particularly in the Western world, there has been an increase in the number of subjects who respond to common allergens such as grass pollen and house dust mite, a response which is characterized by Th2 cytokine production (4). A change in exposure to pathogens may underlie this increased prevalence of allergic disease.

The infant immune system differs from that of adults as it is skewed towards a Th2 response after birth, developing into a normal adult Th1 cytokine profile in subsequent years (5). As postulated in the hygiene hypothesis (6), frequent infections during childhood may affect this maturation process. Th1 cytokine production during infection may skew the infant immune response from Th2 to Th1. As a consequence, infants suffering from many infections may have a diminished risk of developing Th2-mediated allergic disease.

Several types of infection may account for the reduced risk of allergic disease. Lactobacilli

colonizing the gut immediately after birth and bacterial infection or exposure to bacterial products during childhood may reduce the risk of allergic disease (7, 8). Because viruses induce the majority of respiratory tract infections, these pathogens have been most extensively studied in relation to allergic disease (9). Epidemiological studies have demonstrated a reduced risk of allergic disease in children with older siblings and children attending day-care, who are known to be all heavily exposed to viral infections (10, 11). Recently, the first direct evidence for this protective effect was provided by a study of Illi and colleagues, who found a twofold reduction of asthma in children of school age with repeated viral respiratory tract infections in the first 3 years of life (12).

Some data show that viral respiratory tract infections can trigger the development of allergic disease. The high risk of wheezing symptoms after bronchiolitis in infants induced by respiratory syncytial virus (RSV) has suggested the possibility of the subsequent development of asthma. Indeed, Sigurs and colleagues were able to show that children with bronchiolitis had an increased risk of allergic disease at age 7 (13). But others were not able confirm these observations. Although Stein and colleagues found an increased risk of recurrent wheezing in 7-year-old children after bronchiolitis, no increased risk of allergic disease was found at age 14 (14).

The VIGALL (Dutch abbreviation for 'Virus mediated allergy') birth cohort study aims at investigating the effect of upper respiratory tract infections (URTI) on the development of allergic disease and the maturation of the infant immune system. Both the prevalence of a particular virus and the host cellular antiviral immune response probably determine the general contribution of the virus infection on the maturation of the immune system. Initially, then, we need to investigate the prevalence of viruses during URTI in infants. In adults and children of school age, rhinovirus is the most prevalent inducer of respiratory pathology, which is found in up to 80% of patients with a common cold (15, 16) and during the majority of asthma exacerbations (17). In infants, viruses have mainly been studied during bronchiolitis, but they have hardly been studied at all during URTI. RSV, and occasionally parainfluenzavirus (PIV) and influenzavirus infections, have been mainly found in bronchiolitis (18–20). Only recently, rhinovirus infections have been reported in common colds in infants (21). In this paper, we analyzed the prevalence of respiratory viruses in 0- to 2 year-old infants in relation to severity of symptoms, day-care attendance and the age of the child.

Methods

Participants

The aim of the VIGALL study was to investigate the relation between viral URTI and the development of allergy during childhood. One hundred and twenty-six healthy infants, living in the Rotterdam area (the Netherlands), were included in this prospective birth cohort study and were followed until 2 years of age. Recruitment of children took place at the Department of Obstetrics (Sophia's Children Hospital), at health centres, and among participants of the PIAMA birth cohort study (22). Eighty-six infants (68%) were selected who had a family history of atopy, defined as allergic disease in one or both parents. Parents with self-reported asthma, hay-fever, house dust mite allergy, or pet allergy were considered to be allergic. This was established with a validated screening questionnaire (23). Forty infants were selected with a negative family history of atopy; infants of whom both parents reported not to have allergic disease. The Erasmus University Medical Ethics Committee in Rotterdam approved the study design and parents of all infants gave informed consent.

Data collection

Information was collected on birth characteristics (duration of pregnancy, gender, birth weight, numbers of siblings, season of birth), indoor environmental and lifestyle factors (smoke exposure, breast-feeding, day-care attendance) using questionnaires completed by the parents when the child reached 3, 12 and 24 months. General symptoms of illness such as rhinorrhoea, cough, fever, and symptoms of allergic disease such as skin rash and wheezing were scored by the parents on weekly symptom cards.

Parents were asked to contact the study doctor when their child had signs of an URTI, defined as rhinorrhoea and fever, general malaise, sleeping difficulties or loss of appetite. During the subsequent sick visits, infants were considered to have a common cold and were assigned to the 'URTI group'. Physical examination was performed and nasal brush samples were taken. A medical history was taken on general illness symptoms (fever, general malaise, loss of appetite), upper respiratory tract disease (rhinorrhoea, sore throat), lower respiratory tract disease (wheeze, cough, dyspnoea), skin rash and

numbers of upper respiratory tract infections in the preceding follow-up period. During sick visits, infants were grouped around 6 (2–9), 12 (10–15), 18 (16–21) and 24 (22–29) months of age.

Children visited the Sophia's Children Hospital for routine visits at the age of 6, 12, 18 and 24 months. A physical examination was performed, and a medical history and nasal brush sample were taken. During routine visits, infants were assigned to two groups. Infants with rhinorrhoea, but without signs of fever, general malaise, sleeping difficulties and loss of appetite were assigned to the 'rhinitis group'. Children without rhinorrhoea were considered not to have upper respiratory tract disease and were assigned into the 'nasal-symptom-free group'.

Parentally reported episodes of rhinorrhoea

The numbers of rhinorrhoea episodes that infants at 12 and 24 months of age had suffered from in the preceding year of life were calculated from the weekly symptom cards. Missing data were obtained from medical histories taken at 6, 12, 18 and 24 months of age. Infants were classified into three groups: (1) infants with 0–3 episodes a year, (2) infants with 3–8 episodes a year and (3) infants with continuous rhinorrhoea, defined as rhinorrhoea for more than 16 weeks a year.

Day-care facilities

Infants were considered to attend day-care when they were cared for regularly by relatives or foster parents and have contact with small numbers of children (<5 children) other than their siblings, and when infants attended large day-care facilities (usually >10 children).

Viral diagnostics in nasal brushes

Cells were harvested from the nasal cavity with a cytobrush (Medscand Medical, Malmö, Sweden) and processed as previously described (24). Cells were collected in 7 ml of RPMI-1640 medium (Life Technologies, Breda, the Netherlands). After centrifugation, 4 ml of supernatant was used for the isolation of influenzavirus, parainfluenzavirus, RSV, adenovirus, cytomegalovirus (CMV), enterovirus and echovirus and 3 ml was frozen and stored at −80°C. Cells were stained with fluorescent labeled antiviral antibodies to detect RSV, influenzavirus, parainfluenzavirus and adenovirus. Rhinovirus and coronavirus were detected by the isolation of viral RNA from 0.5 ml of frozen nasal brush supernatant using the MagnaPure LC Instrument (Roche Applied Science, Penzberg, Germany) and amplification by RT-PCR followed by hybridization with either rhinovirus- or coronavirus-specific radiolabeled probes (25).

Statistical analysis

For the purposes of a simultaneous evaluation of the prevalence of individual viruses, logistic regression was used (GEE estimation using the GENMOD module from SAS; SAS Institute Inc., Cary, NC, USA), allowing for differences between, as well as within, individuals. The age of the child, symptom severity, season of sampling (winter: October to March, summer: April to September), a family history of atopy and day-care attendance were the factors examined. Fisher's exact test was used to evaluate the relation between rhinovirus prevalence and several genetic and environmental factors at 12 and 24 months separately. The McNemar test was used to compare prevalences of different viruses within symptom and age groups. Kaplan–Meier survival analysis was used to examine differences between children who dropped out at 12, 18 or 24 months compared with those who completed the study. Differences were considered statistically significant if p was ≤ 0.05.

Results

Patient characteristics

One hundred and twenty-six infants were followed for the first 2 years of life and examined during routine and sick visits. Table 1 shows the number of visits as well as the clinical symptoms of the child during the visit. None of the infants were admitted to hospital due to severe lower respiratory tract infection. Forty-two children (33%) dropped out for various reasons. Fifteen children dropped out at 12 months of age, 22 children at 18 months and 5 children at 24 months of age.

Table 1. Clinical symptoms in infants (%) during routine and sick visits

	Sick visits	Routine visits	
	URTI	Rhinitis	Nasal-symptom-free
n	80	133	221
Rhinorrhoea	80 (100%)	133 (100%)	0 (0%)
Loss of appetite	47 (59%)	0 (0%)	4 (2%)
General malaise	66 (83%)	0 (0%)	4 (2%)
Fever	40 (50%)	0 (0%)	0 (0%)
Wheeze	14 (18%)	13 (10%)	5 (2%)
Cough	68 (85%)	76 (57%)	41 (19%)

URTI: Upper respiratory tract infection.

No differences were found between the children who dropped out and those who did not in terms of family history of atopy, day-care attendance, numbers of episodes of rhinorrhoea, and development of atopic dermatitis in the preceding follow-up period (data not shown).

Prevalence of respiratory viruses in infants

The prevalence of viral respiratory pathogens was examined in infants with URTI, with rhinitis or without nasal symptoms separately around 6, 12, 18 and 24 months of age (Table 2). During URTI, a viral infection was diagnosed in 58% (6 months) to 80% (24 months) of the infants. At 6 months of age, rhinoviruses (27%), RSV (18%) and coronaviruses (21%) were mainly found during URTI. In children of 12 months and older, the most prevalent pathogen during URTI was rhinovirus (43% at 12 months to 60% at 24 months). In infants with rhinitis, a positive virus diagnosis was found in 34% (18 months) to 58% (12 months) and in infants without nasal symptoms in 16% (24 months) to 33% (6 months). These were mainly rhinoviruses (85% and 75% of virus infections, respectively). At 6 months of age in the nasal-symptom-free group, in addition to rhinovirus, significantly more CMV was detected compared with PIV, influenzavirus, adenovirus, and enterovirus. Multiple-virus infections were diagnosed in 15% of infants with URTI, 8% of infants with rhinitis and 3% of infants without nasal symptoms. Rhinovirus and coronavirus infections were mostly found in combination with any other virus.

Viral prevalence in relation to symptomatology and age of the child

Multiple logistic regression was conducted to investigate whether the age of the child and the severity of symptoms affected viral prevalences in infants. Age- and severity-related differences in viral prevalences did not depend on day-care attendance, a family history of atopy and season of sampling.

Rhinovirus. Rhinovirus was the most prevalent inducer of respiratory infection in all symptom groups (Fig. 1a, Table 2). The prevalence of rhinovirus in the URTI group increased with age from 27% at 6 months to 60% at 24 months and remained unchanged in the rhinitis group (31% at 18 months – 52% at 12 months) and in infants without nasal symptoms (14% at 24 months – 28% at 12 months). No statistically significant differences were found between age categories in any symptom groups. We did observe significantly more rhinovirus in the URTI group and rhinitis group than in infants without nasal symptoms ($p < 0.001$ and $p = 0.001$, respectively),

Table 2. Prevalences of respiratory viruses in infants with URTI, with rhinitis or infants without nasal symptoms at 6, 12, 18 and 24 months of age

	n	Any virus detected (%)	Rhino-virus (%)	RSV (%)	Corona-virus (%)	PIV (%)*	Influenza-virus (%)†	CMV (%)	Other viruses‡ (%)
URTI									
6 months	33	19 (58)	9 (27)	6 (18)	7 (21)	2 (6)	2 (6)	1 (3)¶	2 (6)**
12 months	14	11 (79)	6 (43)	3 (21)	0 (0)*	2 (14)	1 (7)	0 (0)**	0 (0)**
18 months	28	20 (71)	15 (54)	3 (11)¶	1 (4)¶	0 (0)¶	1 (4)¶	0 (0)¶	2 (7)¶
24 months	5	4 (80)	3 (60)	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rhinitis									
6 months	46	25 (54)	19 (41)	1 (2)¶	3 (7)¶	3 (7)¶	0 (0)¶	3 (7)¶	1 (2)¶
12 months	33	19 (58)	17 (52)	1 (3)¶	1 (3)¶	0 (0)¶	0 (0)¶	0 (0)¶	1 (3)¶
18 months	29	10 (34)	9 (31)	1 (3)**	0 (0)¶	0 (0)¶	0 (0)¶	2 (7)**	1 (3)¶
24 months	25	13 (52)	12 (48)	1 (4)¶	1 (4)¶	0 (0)¶	0 (0)¶	0 (0)¶	0 (0)¶
Nasal-symptom-free									
6 months	70	23 (33)	12 (17)	2 (3)**	3 (4)**	1 (1)¶	1 (1)¶	9 (13)§	2 (3)¶
12 months	64	20 (31)	18 (28)	0 (0)¶	0 (0)¶	0 (0)¶	0 (0)¶	3 (5)¶	1 (2)¶
18 months	38	12 (32)	10 (26)	0 (0)¶	0 (0)¶	0 (0)¶	0 (0)¶	2 (5)**	0 (0)¶
24 months	49	8 (16)	7 (14)	0 (0)**	1 (2)	0 (0)**	0 (0)**	0 (0)**	0 (0)**

Prevalences were calculated as a percentage of total numbers of infants (n) per age and symptom group.
RSV: respiratory syncytial virus; PIV: parainfluenzavirus; CMV: cytomegalovirus.
*One PIV1, 2 PIV2 and 5 PIV3 infections.
†Four influenza virus A and 1 influenza B infection.
‡Adenovirus and enterovirus.
§$p < 0.01$ CMV vs. PIV and vs. influenza virus, $p < 0.05$ vs. 'other viruses'.
¶$p < 0.01$ vs. rhinovirus.
**$p < 0.05$ vs. rhinovirus.

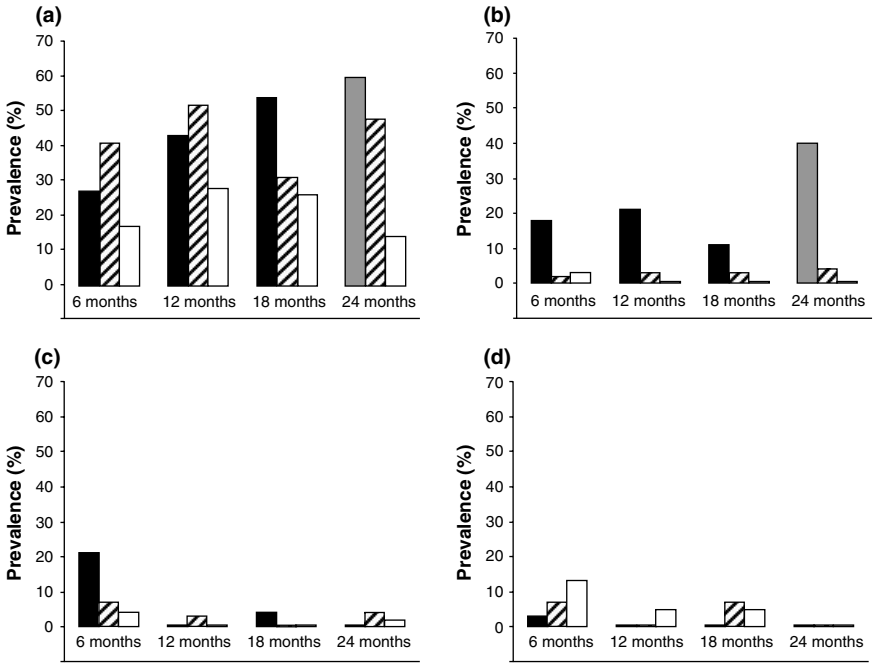

Fig. 1. Prevalences of rhinovirus (a), RSV (b), coronavirus (c) and CMV (d) in infants with URTI (black bars), rhinitis (cross-hatched bars) or without nasal symptoms (white bars) related to the age of the child. Data represented by the gray bar are based on only a few infants (n = 5).

whereas the prevalence of rhinovirus did not differ between the URTI and rhinitis groups.

RSV. A significantly higher prevalence of RSV was found in infants with URTI than in infants with rhinitis or in infants without nasal symptoms ($p = 0.001$ and $p < 0.001$, respectively; Fig. 1b, Table 2). No differences in prevalence of RSV were found between age groups.

Coronavirus. Coronavirus was predominantly found during URTI episodes in 6-month-old infants (21%), decreasing to 0% at 24 months of age ($p = 0.02$; Fig. 1c, Table 2). Significantly more coronavirus infections were found in the URTI group than in the rhinitis and nasal-symptom-free groups ($p = 0.05$ and $p = 0.004$, respectively).

CMV. Some CMV infections were detected in children without nasal symptoms. Prevalences decreased gradually with age from 13% at 6 months to 0% at 24 months ($p = 0.001$; Fig. 1d, Table 2). CMV infections were significantly more prevalent in the nasal-symptom-free group compared with the URTI group ($p = 0.01$) but did not differ from infants in the rhinitis group.

Genetic and environmental factors in rhinovirus prevalence

During routine visits (rhinitis and symptom-free groups), the majority of the viruses found were rhinoviruses. We therefore examined the relationship between several genetic and environmental factors and the prevalence of rhinovirus during routine visits at 12 and 24 months of age separately.

At the age of 12 months, a higher prevalence of rhinovirus was found in children in day-care (51%) compared with other children (19%; $p = 0.01$; Table 3). No relation was found

Table 3. Prevalence of rhinovirus at 12 months of age in relation to genetic and environmental factors

		Rhinovirus positive/n (%)*	p-value
Day-care attendance	Yes	31/61 (51%)	
	No	4/21 (19%)	0.01
Siblings	Yes	19/42 (45%)	
	No	17/47 (36%)	0.40
0–3 episodes of rhinorrhoea		7/34 (21%)	
3–8 episodes of rhinorrhoea		17/40 (43%)	
Continuous rhinorrhoea		12/20 (60%)	0.01†
Breast-feeding	Yes	12/22 (55%)	
	No	21/61 (34%)	0.13
Smoking parents	Yes	7/22 (32%)	
	No	29/58 (50%)	0.21
Girls		19/46 (41%)	
Boys		18/51 (35%)	0.68
Born in winter‡		12/27 (44%)	
Born in summer		25/70 (36%)	0.49
Family history of atopy	Yes	26/70 (37%)	
	No	11/26 (42%)	0.81

*n not always 97 due to occasional missing data.
†Test for trend.
‡Winter: October–March, Summer: April–September.

between the presence or absence of siblings and rhinovirus prevalence. We were able to confirm the reliability of parental symptom scores since the prevalence of rhinovirus increased from 21% in children with 0–3 episodes of rhinorrhoea to 60% in children with continuous rhinorrhoea during the preceding year of life ($p_{trend} = 0.01$). Simultaneous evaluation using logistic regression of the significant factors (day-care attendance and rhinorrhoea episodes) showed that both these factors are significant predictors of increased rhinovirus prevalence. A trend for similar relations was observed between day-care attendance and episodes of rhinorrhoea and rhinovirus prevalence at the age of 24 months, but this did not attain statistical significance (data not shown). Breast-feeding, smoking parents, season of birth, gender and a family history of atopy did not affect the prevalence of rhinovirus during routine visits at 12 and 24 months. Comparable results were obtained when genetic and environmental factors were related to a positive diagnosis for any virus in infants at 12 and 24 months of age (data not shown).

Discussion

In the VIGALL birth cohort study, we found that rhinovirus was the most prevalent viral pathogen in infants from 0 to 2 years. Rhinovirus was found in ~40% of children with URTI and also in ~40% of children with rhinitis without general malaise. Rhinovirus was even detected in ~20% of infants without nasal symptoms.

RSV and parainfluenzavirus have usually been considered the most important viral pathogens in infants because these viruses have been found to induce lower respiratory tract infection (bronchiolitis) (18–20). However, the importance of rhinovirus in infants has been underestimated. It is only in recent years that PCR techniques have become available. These techniques can detect rhinoviruses more accurately than the viral culture methods used previously (26). In adults, rhinovirus has already been shown to be the most prevalent viral pathogen during URTI (15, 16). We have now shown that rhinovirus is the most frequently detected viral pathogen during URTI and rhinitis in infants as well. This is in accordance with recent data from the Finnish Otitis Media Cohort Study that observed similar high rates of rhinovirus infection in infants during episodes of URTI and acute otitis media (21, 27). It can therefore be concluded that rhinovirus frequently infects both infants and adults.

Increased prevalences of rhinovirus were found in children attending day-care. Epidemiological studies indicated that frequent respiratory tract infection in infants, associated with day-care attendance or the numbers of siblings, may reduce the risk of developing allergic disease (10–12). On the basis of extensive virus diagnostics, this study found prevalences of viral infections in infants in day-care, in particular rhinovirus, which were ~2.7 times higher than those for other children. We therefore concluded that the indirect indications of protection by recurrent respiratory infections in epidemiological studies, such as by day-care attendance, are probably rhinovirus infections to a large extend. A well-known protective factor for lower respiratory infections during childhood is breast-feeding whereas a parental history of allergic disease and exposure to tobacco smoke have been described as risk factors (22, 28, 29). Remarkably, in contrast to lower respiratory tract infections, the same studies showed that breast-feeding, a parental history of allergic disease and exposure to tobacco smoke did not relate to upper respiratory tract infections. In line with these reports, we also did not observe a relation between a family history of atopy, breast-feeding or smoking by one or both parents and the prevalence of rhinovirus infections during routine visits at 12 months.

At the age of 6 months, RSV and coronaviruses were also found in addition to rhinovirus in infants with URTI. Only a few coronavirus infections were detected in children of 12 months and older. Below the age of 24 months, the prevalence of RSV remained ~20%. This is likely to decrease during later childhood because prevalences up to 13% have been reported in adults (30). At the age of 18 months, most children (75%) will have been infected with RSV at least once (31). The prevalence of rhinovirus increases slowly to more than 50%. In adults, a prevalence of 80% has been reported for rhinovirus during the common cold (16). This indicates that rhinovirus prevalence found in infants in the present study may increase further. Why does the prevalence of rhinovirus remain unaltered or even increase gradually, whereas the prevalence of RSV decreases with age? For RSV, which has few different serotypes, humoral and cellular responses may possibly protect the child from frequent re-infections. By contrast, over 100 different serotypes of rhinovirus are present (32). Re-infection with rhinovirus is therefore likely to occur with a new serotype for which the child does not yet have induced humoral and cellular responses.

Frequent viral infections are believed to be important modulators of the natural maturation of the infant immune system from Th2- to Th1-skewed and may affect the development of allergic disease (5, 12). This maturation is likely to result from repeated stimulation by pathogens, rather than from a single viral infection. The general effect of a particular virus is dependent on both the prevalence of the virus and on the type of host immune response upon infection. RSV is capable of inducing a general inflammatory response in infants which largely induces an immune response towards Th1 (33). Since now rhinovirus has been found to be more prevalent than RSV in infants, the question arises to what extend rhinovirus infections are able to influence the infant immune maturation and modulation. We are currently investigating what type of immune response a rhinovirus infection can induce in infants and whether this type of immune response differs between infants with URTI and rhinitis (van Benten et al., unpublished data).

Interestingly, rhinovirus was also detected in infants without nasal symptoms (~20%). It is unlikely that the high prevalence of rhinovirus results from RT-PCR detection that is too sensitive, because this assay is not designed to detect a single viral particle. Rhinovirus detection could indicate imminent or past infection because ~70% of the infants had symptoms of URTI or rhinitis during the week before or after sampling.

In the other children without nasal symptoms, rhinovirus could have colonized the nasal mucosa without inducing symptoms. This is in accordance with a recent Finish study that detected picornavirus in 20% of nasopharyngeal samples from children without any past or recent respiratory infection (34). As the nasal mucosa is colonized by bacteria, it is likely that it can also harbor respiratory viruses (35). These rhinovirus infections may perhaps only induce clinical symptoms when the host immune system is temporarily compromised. It is unclear whether these subclinical rhinovirus infections may also affect immune maturation in infants.

In conclusion, rhinovirus infection has been found to be the most prevalent respiratory viral infection, in ~40% of infants with URTI and rhinitis, and it may well stimulate the maturation of immune system and prevent the development of allergic disease. More information is subsequently needed on the immune modulating capacity of rhinovirus infections in this context.

Acknowledgments

We would like to thank all parents and children for their participation in the VIGALL study. This study was supported by the Dutch Asthma Foundation (Nr 93.96.1) and the Netherlands Organization for Health Science (Nr 940-35-025). We received an additional research grant from the Foundation 'Vereniging Trustfonds Erasmus Universiteit Rotterdam' in the Netherlands (Nr 97030).

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