Original article

Age- and infection-related maturation of the nasal immune response in 0–2-year-old children

Background: The hygiene hypothesis suggests that exposure to micro-organisms influences development of the immune system in children.

Methods: In this study, we examined nasal immune responses in the first 2 years of life in relation to age of children and the number of viral infections they have experienced. Nasal brushes were taken during rhinovirus- (n = 20) or respiratory syncytial virus (RSV)-induced (n = 7) upper respiratory tract infections (URTI), and of controls (n = 40).

Results: The number of macrophages were higher during URTI and increased with age. The number of T lymphocytes increased with age in controls and were higher during URTI at all ages. We found an age-related decrease in the number of interleukin (IL)-4- and IL-10-positive cells in controls, while the number of IL-12-positive cells remained unchanged. Changes in T lymphocyte and IL-4 cell number were stronger related to the age of the child than to the number of respiratory infections, while the opposite was true for macrophages.

Conclusions: In infants, we found an infection- and age-related increase respectively for nasal macrophages and T lymphocytes during URTI. Furthermore, the number of IL-4- and IL-10-positive cells decreased with age. Whether this maturation reflects a natural age-related maturation, the degree of exposure to respiratory infections, or possibly both, could not be resolved and needs further study.

The immune system in infants is not fully developed. This is reflected by the high susceptibility of children to respiratory infections during the first few years of life (1). In general, host immune responses to viral pathogens are mediated by T-helper (Th1)-type cytokines (2). As the production of Th1 cytokines can be inhibited by cytokines secreted by Th2 lymphocytes (3), an adequate balance of Th2 and Th1 cytokines is essential for the efficient eradication of pathogens.

The high risk of infections in infants may be a consequence of an immature cellular host immune response. In vitro experiments with peripheral and cord blood cells have shown that cells from infants respond less well to infection-related stimuli, than those from adults, with a lower production of both Th1-like cytokines [interleukin (IL)-2, IL-12, interferon (IFN)γ] and Th2-like cytokines (IL-5) (4–6). Furthermore, the production of Th2 cytokines is favoured over Th1 cytokines, in infants (7). This may result from a preferential Th2 milieu that prevails during pregnancy (8).
response in infants may therefore persevere and predispose a child to the later development of a Th2-mediated allergic disease.

No data are available about whether postulated changes in the developing immune system of young children are also reflected in the nasal mucosa. In our study, we therefore examined whether the magnitude of nasal macrophage and T-lymphocyte responses, as well as Th1-driving (IL-12), Th2-driving (IL-4) and regulatory cytokine responses (IL-10), during upper respiratory tract infections (URTI) in 0-2-year-old children, were affected by age and/or the history of respiratory tract infections.

Materials and methods

Participants and data collection

In all, 24 infants (age 2-22 months, eight boys) participating in the VIGALL birth cohort study (13) were examined during 27 episodes of URTI in the first 2 years of life either caused by rhinovirus (n = 20) or by respiratory syncytial virus (RSV) (n = 7). URTI was defined as a runny nose and at least one of the symptoms such as fever, malaise, sleeping difficulties, or loss of appetite. This case-control study was confined to children in whom a single type of virus was detected during URTI. Sixteen children had a positive family history of atopy (FHA; one or both parents had allergic disease) and 11 children had a negative FHA (no allergic disease in the 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 (14).

A control group comprised 36 other children (age 6-26 months, 18 boys) from the same birth cohort study (matched for FHA, age, and gender) who were completely free of any symptoms (no runny nose, fever, malaise, cough, wheeze, sleeping difficulties, or loss of appetite) in the 2 weeks spanning the visit. The children had no virus infection during sampling, and had not suffered from atopic dermatitis before the age of 2 years (determined using the UK Working Party’s Diagnostic Criteria for Atopic Dermatitis) (15).

Viral diagnostics

Cells were harvested from the nose with a cytobrush (Medscand Medical, Sweden) and collected in 7 ml of RPMI 1640 medium (Life Technologies, Breda, the Netherlands). After centrifugation, the supernatant was used to detect influenza virus, parainfluenzavirus, RSV, adenovirus, cytomegalovirus (CMV), enterovirus and echovirus on cultured HEp-2 cells with immunofluorescence. Additionally, nasal brush cells from the pellet were stained directly with fluorescent-labelled antiviral antibodies to detect RSV, influenza virus, parainfluenzavirus and adenovirus (16). Finally, rhinovirus and coronavirus were detected by the isolation of viral RNA from nasal brush supernatant using reverse transcriptase-polymerase chain reaction (RT-PCR), followed by virus-specific hybridization (17).

Immunohistochemical detection of macrophages and T lymphocytes

The immunohistochemical staining procedure was as described elsewhere (18) to detect macrophages (αCD68, 4.6 μg/ml, clone EBM11; DAKO, Glostrup, Denmark) and T lymphocytes (αCD3, 4.2 μg/ml, clone T3-4B5; DAKO) in nasal brush samples with an isotypic antibody as negative control. The number of positive cells with red cytoplasmic and/or cell membrane staining were counted per 1000 nasal brush cells and stated as percentages of positive cells.

Statistical analysis

The relationship between the number of effector cells and cytokine-positive cells on the one hand and age and FHA on the other hand were investigated with regression analysis for repeated measurements (using the ‘proc mixed’ module from SAS 6.12 for windows; SAS, Cary, NC, USA). In order to obtain approximate linear relations in these analyses, the outcomes were transformed logarithmically. The same method was used to evaluate the effects of number of respiratory infection episodes. Differences were considered statistically significant when the P-value was ≤ 0.05.

Results

Age-related macrophage and T-lymphocyte responses during URTI

In control children, the number of macrophages was low (range 0-2%) and this did not depend on the age of the child. In the group of children with URTI, we observed a marked increase of macrophages in the nose at every age (P < 0.001) (Fig. 1A). Moreover, the number of macrophages attracted to the nose during URTI...
increased 2.7-fold per 12 months of age (P = 0.02; Table 1). A URTI episode in 6-month-old children resulted in an increase in the median percentage of macrophages from 1% at baseline to 3% during infection, whereas this percentage increased to 10% in children aged 22 months. We did not find any difference in number of macrophages between RSV and rhinovirus infections at any time point, nor were the number of macrophages dependent on the FHA of the child.

The number of T lymphocytes in nasal brushes of these children also revealed age-related changes (Fig. 1B). In contrast to the macrophage response, a comparable increase in the number of T lymphocytes with the advancing age of the child was observed in controls (P = 0.01) and during URTI (P = 0.01). The median percentage of T lymphocytes of controls increased from 0.5% at 6 months of age to 4% at 22 months. During URTI, an influx of T lymphocytes into the nose was observed at all ages (P = 0.03), which was comparable between rhinovirus and RSV infections. At the age of 6 months, the median number of T lymphocytes increased from 0.5% at baseline to 1% in children with URTI, while in 22-month-old children this increased from 4 to 8%. This represents a 3.8-fold increase per 12 months of age during URTI (Table 1). Moreover, here the changes in T lymphocytes did not depend on the FHA of the child.

Age-related cytokine responses

Figure 2A shows a significant age-dependent decrease in the number of Th2-driving cytokine IL-4-positive cells (P = 0.01), which decreased from a median number of 20% at 6 months to 10% at 24 months [factor 0.6 (40%) per 12 months of age] (Table 1). For the regulatory cytokine IL-10, a trend was observed towards an age-related decrease in the number of cells (P = 0.09; Table 1). The median number of IL-10-positive cells were reduced from 41% at 6 months to 29% at 24 months of age (Fig. 2B). The median number of the Th1-driving IL-12 cytokine-positive cells remained unchanged (median 6%) with age (Fig. 2C). No significant differences were found in nasal cytokine responses between children with a positive or negative FHA.

Table 1. Relation between immunological parameters and the age of the child or the number of respiratory infections experienced

|                | Age    | P-value | Adjusted | Respiratory Infections | P-value | Adjusted |
|----------------|--------|---------|----------|------------------------|---------|----------|
| Macrophages    | 2.7    | 0.02    | NS       | 1.7 (1.1–2.5)          | 0.02    | 0.05     |
| T lymphocytes  | 3.8    | 0.01    | 0.05     | 1.5 (1.1–2.2)          | 0.04    | NS       |
| IL-4           | 0.6    | 0.01    | 0.09     | 0.8 (0.7–1.0)          | 0.08    | NS       |
| IL-10          | 0.8    | 0.09    | NS       | 0.9 (0.8–1.1)          | NS      | NS       |
| IL-12          | 1.1    | NS      | 1.1 (0.8–1.5) | NS | NS      |

Data given are the factors (95% confidence interval) at which the levels increase per 12 months of age or per doubling of the number of respiratory infections. P-values showing a statistically significant relation (P ≤ 0.05) and P-values showing a trend (P ≤ 0.10) are represented (NS, not significant).

* P-value for the relation with age adjusted for number of respiratory infections.
† P-value for relation with number of respiratory infections adjusted for age.
Age-related or infection-related immune maturation in children?

The cumulative incidence of respiratory infections increased from approximately two episodes at 6 months to approximately three at 12 months, approximately five at 18 months and approximately eight episodes at 24 months of age ($P < 0.001$) and did not depend on the FHA. As the cumulative incidence of respiratory infections was positively related to age, we would expect the nasal immune responses to be also related to the number of respiratory infections. A positive relation was indeed found between number of respiratory infections and the number of macrophages or T lymphocytes ($P = 0.02$ and 0.04, respectively; $n = 20$). The number of macrophages increased 1.7-fold and T lymphocytes 1.5-fold per doubling of the number of respiratory infections (Table 1). Additionally there was a trend towards a decrease in IL-4-positive cells ($P = 0.08$, $n = 32$) (Table 1), while no relation was found between IL-12 ($n = 30$) or IL-10 ($n = 26$) and the number of respiratory infections.

To discriminate between age-related or infection-related maturation, we performed a regression analysis for repeated measurements that included both variables. However, because of the high correlation between the age of the child and the number of respiratory infections, the effect of both variables on the IL-10 response proved difficult to separate. The strongest predictor for the T lymphocyte response during URTI and the IL-4 response in children during baseline turned out to be the age of the child. The age of the child remained related to both responses, even after adjusting for the number of respiratory infections ($P = 0.05$ for T lymphocytes, $P = 0.09$ for IL-4; Table 1). This is supported by the observation that, when the relation between the number of respiratory infections and the IL-4 and T-lymphocyte responses was adjusted for age, this relation was not statistically significant ($P = 0.77$ and 0.85, respectively). However, the strongest predictor for the macrophage response during URTI turned out to be the number of respiratory infections, as the relation remained statistically significant, even after adjusting for the age of the child ($P = 0.05$; Table 1).

**Discussion**

The hygiene hypothesis suggests that exposure to microorganisms contributes to the development of the immune system in children. This study is the first to show an age-related nasal immune maturation in 0–2-year-old children, with number of Th2-driving (IL-4) and regulatory (IL-10) cytokine-positive cells decreasing with age. Interestingly, the percentages of nasal macrophages and T lymphocytes attracted to the nose during URTI were low until the age of 6 months, but increased rapidly thereafter with the age of the child. In 2-year-old children, the
number of macrophages and T lymphocytes from control children and from the group of children with URTI were comparable with those in adult patients with a common cold, as reported in our previous study (20). This indicates that recruitment of inflammatory cells to the nose is in full potential at the age of 2 years. An assessment of whether the age-related increase in the number of T lymphocytes in nasal brush samples is due to an increase of a particular type of T lymphocytes was not possible, but memory T lymphocytes are likely candidates. While children show high percentages of naïve T lymphocytes (approximately 82%) and only few memory T lymphocytes (approximately 16%) in peripheral blood, adult T lymphocytes show the opposite phenotype (approximately 48% naïve, 49% memory) (21).

A change in the natural balance between Th1 and Th2 cytokine production with the age of the child from Th2-skewed at birth to Th1-skewed in adulthood has been suggested (7). This could explain the age-related increase in effector cell responses observed during URTI. Indeed, in our study, an age-related decrease in Th2-driving (IL-4) cytokine-positive cells was observed in the noses of children. It is difficult to relate our in vivo data to known in vitro data obtained from cord blood and peripheral blood samples. Prescott and colleagues found an age-related decrease in the expression of Th2 cytokine IL-4 mRNA in allergen-stimulated cord blood and peripheral-blood mononuclear cells in 0–18-month-old children (22). However, others found an age-related increase in Th2 cytokine responses during the first year of life when peripheral blood cells were activated polyclonally or with allergen (9, 23).

In the present study also, the number of nasal IL-10-positive cells decreased with age. These high IL-10 responses in the nose of infants are in line with studies showing a high expression of IL-10 by peripheral blood mononuclear cells of infants compared with adults (24). The role of IL-10 in immune maturation is complex. IL-10 can inhibit the production of Th1-driving cytokine IL-12 by dendritic cells (25), and therefore a decrease in the number of nasal IL-10-positive cells with age would allow an age-related increase in nasal IL-12 responses. This in turn would stimulate Th1 cytokine production, as IL-12 is a key factor for Th1 differentiation (26). Recently it has become clear that IL-10 can inhibit many more cytokines (TNFα, IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-18) and therefore IL-10 is nowadays regarded as an important regulatory cytokine (27). In this quality high expression of IL-10 in infancy may induce tolerance in early life and may regulate a balanced Th1 and Th2 maturation. This regulatory role of IL-10 in immune maturation has already been suggested by van den Biggelaar et al. (28), who found that high production of IL-10 during helminth infection was inversely related to the development of Th2-mediated allergic disease in children.

The reduction in the number of IL-10- and IL-4-positive cells is not paralleled by an increase in number of Th1 driving IL-12-positive cells. Although this does not preclude an increase in protein production. Lower production of IL-12 protein by LPS-stimulated neonatal DCs has been observed by comparison with adult DCs (29). Recently, Upham et al. (30) showed that protein production of IL-12p70 increased gradually from infancy to adulthood after stimulation of peripheral and cord-blood cells. Unfortunately, our approach did not allow us to determine whether levels of IL-12 protein expression rather than cell number are upregulated.

Children with a positive or negative FHA did not differ in terms of nasal cytokine responses (IL-4, IL-10, IL-12). This concurs with findings from in vitro studies where polyclonally stimulated cord blood T lymphocytes from both groups of children did not differ in the production of Th1 and Th2 cytokines (31). However, the absence of a difference between children with positive or negative FHA could also stem from the relative small number of children included in the study. This small sample size also did not allow us the study the same children for 2 years both during and outside an URTI episode. Differences in cytokine production have been observed between children that do and do not develop allergic disease. Van der Velden et al. (11) showed that protein levels of Th2 cytokines (IL-4) produced by polyclonally stimulated blood lymphocytes increased from birth to 12 months of age in children with a high genetic risk for allergy who developed allergic disease at 2 years of age, whereas cytokine levels did not change in children who remained healthy. However, as none of our children had developed atopic dermatitis at the age of 2 years and a proper diagnosis of asthma and allergic rhinitis cannot be made in these young children, we were not able to verify these observations in our study. A follow-up study on the development of allergic disease at the age of 6 years will help us address this question.

The hygiene hypothesis suggests that repeated respiratory infections influence immune maturation in children towards stronger Th1 responses and limit Th2 responses, resulting in a diminished chance of developing Th2-mediated allergic disease (12). This is partly supported by the present study as macrophage responses during URTI were more strongly related to the increasing number of respiratory tract infections than to the age of the child. However, our data on the development of nasal IL-4 and T-lymphocyte responses show that these responses were more strongly related to the age of the child. At present, we are not able to distinguish between a strict age-related or infection-related immune maturation and it may well be that both factors are important for a correct maturation. We have necessarily restricted ourselves to viral infections in our study and not evaluated possible effects of bacterial infections or colon colonisation. Given our results, a larger study seems called for that encompasses these factors.

No differences were observed in children with a high or a low genetic risk of developing allergic disease. Neither
the incidence of respiratory infections nor the nasal macrophage and T lymphocyte response upon URTI differed, indicating that both groups of children are equally susceptible to respiratory infections. This is in agreement with studies showing that a parental history of allergic disease is a risk factor for lower respiratory tract infections, but not for URTI (32). The design of our study also allowed an analysis of potential differences on the effect of upper vs lower respiratory tract infections on immune maturation. This would be interesting, as we have previously shown a strong nasal IL-18 response in RSV-induced lower respiratory tract infection in children, where such a response is absent during a RSV-induced URTI (33).

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