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Interferon-gamma levels in nasopharyngeal secretions of infants with respiratory syncytial virus and other respiratory viral infections

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SUMMARY

Respiratory syncytial virus (RSV) infection, one of the most common causes of hospitalization of children in developed countries, has been implicated as a cause of asthma. We aimed to characterize the cytokine profile in nasopharyngeal aspirates (NPAs) taken from infants during upper respiratory tract infection to investigate whether RSV induced a unique immune response as compared with other viruses. Additionally, we sought to determine whether this profile was influenced by the infants' atopic status. A prospective birth cohort of babies at high risk of atopy was recruited. Ratios of a T-helper 1 (Th1) cytokine, interferon gamma (IFN-γ) and a T-helper 2 (Th2)-like cytokine, interleukin-10 (IL-10), in NPAs were determined during episodes of respiratory tract infections in the first year. The viral aetiology of the respiratory tract infections was determined using polymerase chain reaction (PCR), culture and immunofluorescence. Atopic status was ascertained at 1 year of age using skin prick tests. Participants were recruited antenatally and subsequently followed in the community. Sixty babies with one or both parents atopic were enrolled into the study. IFN-γ: IL-10 ratios in NPAs during upper respiratory tract infections and their correlation with viral aetiology and atopic status were the main outcome measures. The mean IFN-γ: IL-10 ratio was significantly lower (due to lower IFN-γ) during RSV infections than during infections with other viruses ($P = 0{\cdot}035$). The cytokine ratio, however, did not differ between infants with or without wheeze during URTIs ($P = 0{\cdot}44$), or between infants who were atopic or non-atopic ($P = 0{\cdot}49$). This study suggests that RSV is associated with lower IFN-γ production in young babies, regardless of their atopic status, compared to upper respiratory tract infections where either another virus is detected or where no viral identification is made.

Keywords cytokines interferon–gamma interleukin-10 respiratory syncytial virus

INTRODUCTION

There is evidence that frequent infections early in life protect against the development of atopy [1–4]. It is not clear, however, which infections are protective and whether some infections may in fact promote the development of atopy. The Th1/Th2 paradigm is central to hypotheses about the relationship between infections and the development of atopy. Th1 ($CD4^+$) cells that primarily produce IFN-γ and interleukin-2 (IL-2) are involved in cell mediated immunity and inhibit B cell isotype switching to IgE production. Th2 cells produce interleukin-4 (IL-4), which promotes IgE production, interleukin-5 (IL-5), which is an important eosinophil growth factor, and IL-10, which is at least functionally a Th2 type cytokine because it inhibits IFN-γ production [5,6]. In theory, multiple infections early in life may enhance the development of Th1 type cells, thus protecting against further Th2 differentiation and possibly atopy.

The role of respiratory viral infections in the development of atopy and asthma presents an enigma, in that many studies have shown an increased incidence of wheeze and other respiratory symptoms after severe RSV infections in childhood when compared with controls who were not hospitalized for RSV [7–9]. The question then arises: does RSV cause or uncover asthma (at least in childhood) and if so, what is the mechanism? One possibility is that RSV promotes a Th2-type immune response in babies.

We aimed, first, to measure IFN-γ and IL-10 levels in the NPAs of babies during respiratory tract infections and to compare these levels in atopic and non-atopic babies. Secondly, we sought to ascertain whether RSV infection promotes a different cytokine

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profile to that induced by other viral infections. Our third aim was to see if there was a difference in the nasopharyngeal cytokine profiles during respiratory tract infections in children who wheezed and those who did not wheeze during the acute illness.

METHODS

Subjects

Babies due to be born to parents of whom one or both were atopic were recruited antenatally from clinics at KGV Hospital, Camperdown, NSW and the Westmead Hospital in Westmead, NSW. After obtaining informed consent, skin prick tests (SPTs) against common allergens were performed on each parent. If one or both parents was positive to at least one of the allergens, their baby was included in the study.

Specimen collection

The method for NPA collection has been reported in detail previously [10]. Briefly, NPAs were collected by suctioning the nasopharynx with a portable suction unit. In each collection, the child was placed in the supine position. A sterile feeding tube with an attached mucus trap (Maerisk Indoplas, Sydney, Australia) was passed into the nasopharynx. Gentle suction was then applied. No saline was instilled prior to suction. At least 0·5 ml of aspirate was collected from each child. Specimens were placed on ice and transported immediately to the laboratory. All specimens were received in the laboratory within 1 h of their collection.

Specimen processing

Each specimen was diluted to a volume of 2 ml using phosphate buffered saline (PBS). The specimen was centrifuged at 375 ***g*** for 5 min and visible cellular debris removed.

Cytokine measurements

We measured both IL-10 and IFN-γ using enzyme-linked immunosorbent assays (ELISA). We also measured IL-4 and IL-5 but could not detect these cytokines in NPAs, so discontinued these measurements. The IL-10 assays were performed using a technique already standardized in our laboratory [10]. Purified IL-10 (Pharmingen, San Diego, CA, USA) was used as a standard. The minimum detection level for IL-10 was 4 pg/ml. IFN-γ levels were measured using Pelikine Compact™ Human IFN-γ ELISA Kits (Pelikine, the Netherlands) which have a lower limit of detection of 4 pg/ml for IFN-γ. For data analysis, cytokine levels higher than background readings but lower than the lower limit of detection were recorded as half the lower limit of detection. The ratio of IFN-γ: IL-10 was calculated in order to control for any dilutional factors in NPAs.

Viral identification

Immunofluorescence for RSV using fluorescein-labelled RSV antiserum (bioMérieux, France) and viral culture on monkey kidney cells and diploid fibroblast cells were performed by the Departments of Virology at the Royal Alexandra Hospital for Children and Westmead Hospital. Briefly, the nasal aspirate was gently suspended in cold MEM and after centrifugation, the supernatant was removed and reserved for immediate inoculation into tissue culture tubes. The cell pellets were used for viral immunofluorescence. The viral cultures were incubated at 34°C for 21 days and checked for cytopathic effect every second day.

Polymerase chain reaction (PCR)

Reverse transcriptase (RT)-PCR for rhinovirus and coronaviruses was performed by Dr David Smith at Sir Charles Gardiner Hospital in Perth, Australia. The method used for the rhinovirus PCR has been described in full by Ireland *et al*. [11]. Briefly, a seminested RT-PCR directed at conserved sequences in the 5′ untranslated region was used. The first run detected picornaviruses, and a second run with a rhinovirus primer was used for more specific identification [11].

The coronavirus PCR was a multiplex nested RT-PCR directed at the nucleocapsid genes of coronaviruses 229E and OC43, described in full by Myint *et al*. [12]

Patient follow-up (birth to 1 year)

Parents were asked to report any respiratory symptoms that they noted during their child's first year of life. In addition, they were contacted once weekly and asked a standard series of questions pertaining to the presence of respiratory symptoms including rhinorrhoea, cough and wheeze. If parents reported any respiratory symptoms, a home visit was performed. At the home visit, the child was examined, and if rhinorrhoea was present an NPA was collected as per the above protocol. In order to identify new episodes of illness, an NPA was not collected if it was less than 10 days since the last collection. To increase the yield for viral identification, specimen collections were performed within 4 days from the first reported symptoms.

Assessments at 1 year of age

The study nurse, who was blinded to the results of any laboratory tests conducted previously on that child, administered a detailed questionnaire with regard to demographic details and the baby's health over the past year. The questionnaire was based on the International Study of Asthma and Allergies in Childhood (ISAAC) protocol, which provides a standardized set of questions to assess the presence of symptoms and signs of these diseases [13]. A paediatrician also examined the child if there was a positive answer to any of the eczema questions. All children had SPTs performed and venous blood collected. For the purposes of this study, atopy was defined as a positive reaction to one or more skin tests. The allergens tested were standardized protein extracts (Greer) of milk, egg, peanut, Der p1 from *Dermatophagoides pteronyssinus*, Der f1 from *D. farinae*, alternaria, Fel d1 cat allergen and grass mix.

Ethics

The study was approved by the ethics committees of the Royal Alexandra Hospital for Children, the Royal Prince Alfred Hospital (for KGV) and Westmead Hospital.

Statistics

The IFN-γ and IL-10 values were not normally distributed and were therefore presented as medians and ranges. Differences in cytokine concentrations between RSV^+ and RSV^- samples were assessed using the Mann–Whitney rank sum test. Measurement of cytokine levels alone did not correct for dilutional factors therefore geometric mean cytokine ratios were compared using independent sample *t*-tests. All data were analysed using SPSS for Windows™ software.

Table 1. Viral identification in NPAs and the rate of cough and wheeze

	No. of NPAs	Age mean (days)	Sex % female	Cough (% total)	Wheeze (% total)
Virus	57	200	50	45 (78)	16 (28)
No virus	44	199	49	41 (92)	14 (33)

RESULTS

Sixty babies were enrolled in the study and 101 samples were collected and analysed. The mean number of infections per child was 1·68 per year with a range of 1–7. There were two hospital admissions of 24 h or longer. RSV was identified in the two hospitalized infants. The remainder of the babies were treated in the community. There were no statistically significant differences in the incidence of cough or wheeze in babies with and without a positive viral identification (Table 1).

RSV was identified in 16 of the samples (15·8%), rhinovirus in 35 (34·6%), parainfluenza virus type 1 in four samples and adenovirus, influenza A and coronavirus in one sample each (the sample with coronavirus was also positive for rhinovirus). A virus was identified in 57 samples (56·4%). Of the rhinovirus samples, 12 were identified using culture and the remainder using PCR. There was no virus identified in 44 of the samples.

The demographic data are summarized in Table 2. There was no significant difference in the age, sex or skin prick status of the babies who were RSV^+ or RSV^-.

Ratios of IFN-γ: IL-10 were used as the primary method of comparing samples because individual cytokine concentrations would be confounded by differences in the volume of secretions obtained. This method of comparison has been described previously [14]. The geometric mean of the IFN-γ: IL-10 ratio in the NPA samples from individuals with RSV was 0·74 (95% CI 0·3–1·7), which was significantly lower than the geometric mean in NPAs of children without RSV (1·9, 95% CI 1·36–2·7, $P = 0{\cdot}035$) (Fig. 1). The level of IFN-γ in non-RSV-infected samples was significantly higher than in RSV-infected samples ($P < 0{\cdot}05$) while there was no difference in IL-10 levels between the two groups (Table 3).

The geometric mean IFN-γ: IL-10 ratio in NPAs where rhinovirus was identified was 2·1 (95% CI 1·4–3·3) and this was not significantly different to the geometric mean ratio in samples where rhinovirus was not identified (1·4, 95% CI 0·9–2·2; $P = 0{\cdot}22$). The geometric mean ratio of IFN-γ: IL-10 in RSV^+ samples (0·74, 95% CI 0·3–1·7) was significantly lower than the same ratio in $rhinovirus^+$ samples (2·1, 95% CI 1·4–3·3, $P = 0{\cdot}021$). The geometric mean IFN-γ: IL-10 ratio in the 57 samples where any virus was identified was 1·56 (95% CI 1·06–2·3) and this was not significantly different to the ratio in the 44 samples where there was no virus identified (1·74, 95% CI 1·01–2·98, $P = 0{\cdot}75$). The geometric mean IFN-γ: IL-10 ratio in RSV^+ samples (0·74, 95% CI 0·3–1·7) was lower than in the specimens where no virus (1·74, 95% CI 1·01–2·98), or in the six samples where a virus other than rhinovirus virus (1·6, 95% CI 0·58–4·35), was identified but this difference did not reach significant levels ($P = 0{\cdot}09$ and 0·3, respectively).

Thirty-three of the samples were collected from SPT^+ babies and 67 from SPT^- babies. The geometric mean ratio in samples from subjects with positive SPTs was 1·42 (95% CI 0·85–2·4) and this was not significantly different to those with negative SPTs (1·81,95% CI 1·2–2·7,$P = 0{\cdot}49$).

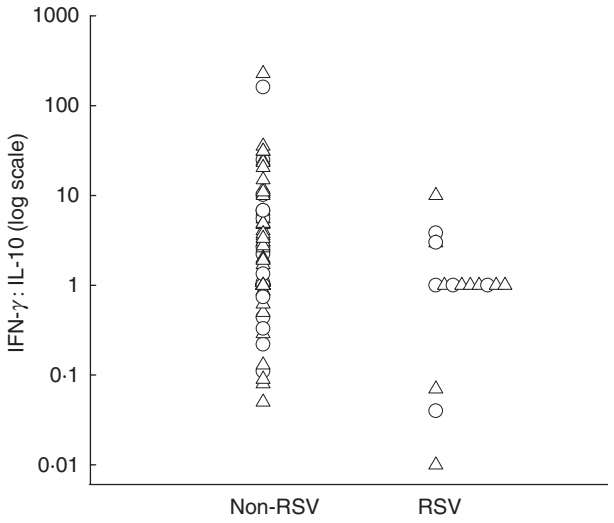

Fig. 1. Comparison of IFN-γ: IL-10 ratios in NPAs from RSV^+ and RSV^- infants. RSV = RSV identified in NPA. Non-RSV = either no virus or a virus other than RSV identified in the NPA. ○, SPT^+ ρ, SPT^-.

Wheeze was heard on 34 occasions when NPAs were collected. Subjects who wheezed during respiratory tract infections had a median age of 241 days and were significantly older than subjects who did not wheeze (median age 176 days, $P = 0{\cdot}044$) (Table 2). The geometric mean of the IFN-γ: IL-10 ratio in children who wheezed during respiratory tract infections was 1·35 (95% CI 0·76–2·4) and in children who did not wheeze was 1·8 (95% CI 1·23–2·65, $P = 0{\cdot}44$). Although children who wheezed tended to have lower ratios, this difference was not significant (Table 2).

DISCUSSION

We found that the ratio of IFN-γ: IL-10 is lower in the RSV^+ NPAs of infants when compared with RSV^- NPAs. The level of IFN-γ was lower in samples where RSV was identified than non-RSV-infected samples while there was no difference in IL-10 levels between the two groups.

This difference was not found between $rhinovirus^+$ and $rhinovirus^-$ samples, nor between NPAs in which a virus was identified compared with no viral isolation. We did not detect a difference in IFN-γ: IL-10 ratios between atopic and non-atopic babies nor between babies with and without wheeze during respiratory tract infections.

We studied a cohort of babies at high risk of atopy in order to control for the considerable influence of family history in the development of atopy and for the fact that babies with a positive family history of atopy also have lower IFN-γ levels at birth [15,16].

Our earlier work has shown that IL-2 levels in babies are comparable in secretions from the upper and lower airways [10]. Van Schaik *et al*., using similar collection techniques to ours, also demonstrated a strong correlation ($r = 0{\cdot}97$) between IFN-γ: IL-4

Table 2. Relationship between demographic data, wheeze and virus isolation in 60 children at high risk of atopy during 101 respiratory infections in the first year of life

	RSV	Non-RSV	Rhinovirus	Non-rhinovirus	Wheeze with URTI	No wheeze
No.	16	85	35	66	34	67
% females	62	53	51·5	56	59	52
Age (median days)	231	201	224	201	231**	176**
% SPT^+	36·8	33·3	34·2	33·8	26·3	37·3
Geometric mean IFN-γ: IL-10 ratio (95% CI)	0·74* (0·3–1·7)	1·9* (1·4–2·7)	2·1 (1·4–3·3)	1·4 (0·9–2·2)	1·35 (0·76–2·4)	1·8 (1·2–2·6)

*$P = 0{\cdot}035$ using independent sample *t*-test. **$P = 0{\cdot}044$ using independent sample *t*-test.

Table 3. Median cytokine concentrations in samples from RSV^+ and RSV^- samples

	RSV^- (pg/ml)	RSV^+ (pg/ml)	*P*
IFN-γ	3·4 (2–457)	2 (2–27)	0·04
IL-10	2 (2–115)	2 (2–222)	0·8

ratios in NPAs and endotracheal aspirates of intubated babies [14].

The rate of positive viral identification (56·4%) in this study is consistent with the findings of another study of age-comparable infants that reported a positive rate of 49·9% [17]. In our community-based study, rhinovirus was the most frequently identified viral pathogen and this concurs with previously reported data where rhinovirus caused 59 of 250 (24%) identified community-based infections [18].

Early studies have demonstrated a lower production of interferon in nasal washes during RSV infections when compared to other viral infections [19,20]. It is likely, however, that IFN-α was the interferon measured most easily, as reported in a subsequent study [21]. The lower airways of asthmatics have increased Th2 cytokines when compared with controls as measured in bronchoalveolar fluid [22,23]. Our study shows that the IFN-γ: IL-10 ratio is significantly lower in the respiratory tract secretions of babies who have RSV infection and that absolute IFN-γ concentrations are also lower when compared with babies where another virus or no virus is identified as a cause of respiratory tract symptoms. This suggests a suppression of Th1 cytokine responses at the respiratory tract level during RSV infections. It would also suggest that other viral infections, in particular rhinovirus, up-regulate IFN-γ production during acute infections. Moreover, our results did not demonstrate any differences in cytokine responses between atopic and non-atopic babies, which suggests that the cytokine response to RSV was not due to the babies' pre-existing atopic status.

Sheeran *et al*. [24] found that concentrations of IL-10 in respiratory secretions obtained from children with RSV infection were significantly greater than in samples obtained from control children, also suggesting a Th2 type response during RSV infections. Increased Th2 cytokine expression has been reported in the peripheral blood mononuclear cells (PBMCs) of healthy adults infected with RSV [25], as has a Th2 type response from the PBMCs of babies hospitalized with acute bronchiolitis [26]. Renzi *et al*. [27] found that, of children hospitalized with acute RSV infection, those who developed asthma had significantly lower IFN-γ levels produced by their PBMCs at the time of the acute RSV infection than those with no asthma. This suggests that the children who will go on to develop asthma after bronchiolitis either have an existing deficiency in IFN-γ production or that they respond to the virus in a different way to those children who do not develop asthma.

An animal study has shown that mice sensitized to ovalbumin during RSV infection had a Th2 profile on stimulation of PBMCs with ovalbumin compared with mice who were sensitized during a sham infection and that mice sensitized to ovalbumin after RSV infection developed increased airway responsiveness to methacholine. The authors concluded that RSV infection results in airway hyperresponsiveness in the acute phase and leads to changes in immune function that can enhance the effects of airway sensitization to antigen after infection [28]. In light of the animal model described, one can speculate that if a susceptible individual has RSV in early infancy and is exposed to an appropriate allergen at this critical time, their immune system may be more 'primed' to an atopic response.

This study shows that acute RSV infection, unlike rhinovirus and other respiratory viral infections, is associated with a lower IFN-γ level in NPAs, regardless of their atopic status. Unlike other childhood respiratory infections, RSV may serve as an exception to the hygiene hypothesis during acute infections. This raises the possibility that the pathogenesis of wheeze and perhaps asthma following RSV in susceptible individuals is related to the development of this cytokine profile in the respiratory tract. Asthma may not become apparent or manifest until these infants are older and we therefore plan to follow this cohort with regards to atopy and asthma. It will be valuable to relate these outcomes to the current cytokine measurements.

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