

Severe respiratory syncytial virus infection in early life is associated with increased type 2 cytokine production in Gambian children

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Summary

Background Severe respiratory syncytial virus (RSV) infection in early childhood has been associated with subsequent wheezing and atopy. The aim of this study was to test if severe RSV infection in early life was associated with an increase in type 2 cytokine production and atopy in Gambian children 5 years later.

Methods A cohort of children with severe RSV infection during the first year of life ('cases', $n = 66$) and without ('controls', $n = 122$) was followed-up at 5 years of age. Immediate hypersensitivity to common allergens, airway reactivity, serum IgE concentration and the production of IFN- γ , IL-5 and IL-13 by lymphocytes activated *in vitro* with RSV F-G or control antigens was determined.

Results After adjustment for confounders, cases produced significantly higher concentrations of IL-13 in response to RSV F-G and of IL-5 and IL-13 in response to tuberculin. Cases were more likely to have presented with a wheezy lower respiratory tract infection in the first 3 years of life (adjusted odds ratio = 9.9; 95% CI 1.6–61.0), but not thereafter. Cases and controls had similar skin response to allergens, airway reactivity and serum IgE concentrations.

Conclusion Severe RSV infection in early life is associated with a higher production of type 2 cytokines in Gambian children at 5 years of age. However this does not appear to result in increased risk of atopy or clinical allergy at that age.

Keywords RSV infection, cohort study, type 2 cytokines, atopy, wheeze, The Gambia

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Introduction

Respiratory syncytial virus (RSV) is recognized as the most common viral cause of severe lower airway disease in infants world-wide [reviewed in 1 and 2]. An association of severe RSV disease in early life with subsequent wheezing has been observed in children from both developed and developing countries [3–6]. A recent community-based study showed a significant increase in risk of subsequent wheeze during the first 10 years of life, but this excess risk disappeared by the age of 13 [7]. Another study reported an association between RSV infection and subsequent allergic sensitization at age 7 years [5].

Studies indicate that the association between severe RSV infection in early life and subsequent wheezing and atopy could be related to an allergic reaction induced by an excessive production of type 2 cytokines [8]. In mice, the RSV attachment protein G induces lung pathology by activating T helper type 2 (Th2) lymphocytes producing IL-4 and IL-5 [8–10]. In contrast, mice sensitized with the fusion F protein induces a Th1 response and no eosinophilia. The Th2-type response induced by RSV

can also promote allergic reactions to non-RSV antigens. Indeed, in mice recurrent RSV infections lead to airway hyper-responsiveness to allergens [11].

Some studies indicate that infants with severe RSV infection have predominant Th2-type responses with decreased Th1-type responses, but this issue remains controversial [12–14]. The role of type 2 cytokines in the pathogenesis of wheezing subsequent to RSV infection in humans has not been established. In most clinical studies wheezing was not associated with increased allergic reactions [7]. It has been suggested that two groups of children with wheezing following RSV infection should be differentiated [3, 15]. The first group includes children who have increased serum concentrations of IgE, skin reactions to allergens and persistent wheezing [5]. Recent data suggest that these children have increased Th2-type response to RSV antigens [16]. The second group includes the majority of children who have normal concentrations of IgE and skin reactions to allergens. In these children, wheezing is transient and disappears within several years after RSV infection. The pathogenesis of transient wheezing may be independent of type 2 cytokines and be predominantly associated with small airways, as determined by lung function measurements [3, 15].

We previously reported a cohort study indicating a transient increase in the incidence of wheezing in Gambian children who had severe RSV disease during the first year of life [6]. At 1 year

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of age, these children were eight times more likely to present with episodes of wheezing than controls but the incidence decreased to control values by the age of 3 years. The aim of the current study was to investigate the mechanisms underlying wheezing associated with severe RSV infection in The Gambia. We followed up the same cohort of RSV cases and controls when children were 5 years old, evaluated their atopic status and studied the *in vitro* production of type 2 cytokines in response to RSV and control antigens.

Materials and methods

Study population

Children who participated in this study were recruited to a cohort study of severe RSV disease in infancy, as previously described [6]. Briefly, between October 1993 and December 1994, children below the age of 1 year admitted either of the two major hospitals in the periurban region near Banjul, the capital of The Gambia, living up to a distance of no more than approximately 40 km away, with RSV infection confirmed by immunofluorescence, were recruited as cases. At the same time, two groups of children not admitted with severe RSV infection were recruited as controls. One group consisted of age-matched children who did not present with RSV disease, but were likely to have been exposed (age-matched controls). The other controls were children born after the annual outbreaks (August–December) and therefore very unlikely to have been exposed to RSV during the first 6 months of life (non-age-matched controls). Mothers were asked to bring their children for evaluation if they developed any clinical problem. Data on clinically diagnosed wheezing, asthma, allergic rhinitis and eczema over 5 years of regular clinical follow-up were recorded prospectively on study forms by a physician blinded to the RSV history. Between April and June 1999 all children were invited to participate in the current study, following parental consent. A questionnaire was administered to record details of risk factors for atopy, including indicators of socio-economic status, smoke exposure and history of anti-helminthic treatment. Details of immunizations were recorded from child health cards. A venous blood sample was collected for full blood count, serum IgE measurement and *in vitro* cytokine production. Anaemia was defined as a haemoglobin concentration below 11.0 mg/dL. A stool sample was obtained and immediately analysed under light microscopy for the presence of parasites. Lung function tests were carried out using a portable spirometer (Microlab 3300, Microlab, Gillingham, Kent, UK) and by brief airflow interruption using the RINT method (Microlab 4000^R, Micro Medical, Gillingham, Kent, UK) [17] at rest and at 2 and 10 min after running. Examiners were not aware of the RSV history of the child.

Immediate and delayed hypersensitivity

Skin prick reactivity to common allergens (*Dermatophagoides pteronyssinus*, cockroach and mould mix) was measured after 15 min on the volar aspect of the left arm using histamine and glycerol as positive and negative controls, respectively. A positive result was defined as a reaction wheel greater than 3 mm in diameter. Skin reactivity to tuberculin (2 units purified protein derivative (PPD), RT23, Statens Serum Institute, Copenhagen,

Denmark) was evaluated after 48 h on the volar aspect of the right arm.

Serum IgE concentration

Total serum IgE concentration was determined from cases and age-matched controls using a microparticle enzyme immunoassay (IMX^R, Abbot, Maidenhead, Berkshire, UK). Two controls were run with each carousel and the interassay coefficient of variation was 5.0–5.5%. All the controls were within the specified ranges in all the runs.

In vitro cytokine production

Five millilitres of heparinized venous blood were collected from cases and age-matched controls. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway) and resuspended in RPMI 1640 medium (BioWittaker, Verviers, Belgium) supplemented with nonessential amino acids (Gibco Life Technologies, Paisley, Scotland), 1 mM sodium pyruvate (Gibco Life Technologies), 2 mM L-glutamine (Gibco Life Technologies), 0005 mM 2-ME (Gibco Life Technologies), 100 UI/mL penicillin, 100 µg/mL streptomycin and 10% heat-inactivated pooled human AB serum (Sigma Chemicals, St Louis, MO, USA). PBMC were incubated in 96-well plates (2×10^5 cells/well) for various periods of time at 37 °C in 5% CO₂ in the presence of RSV recombinant chimaseric fusion (F) and attachment (G) protein (2 µg/mL, kindly provided by SmithKline Beecham Biologicals, Rixensart, Belgium), tuberculin (5 µg/mL, PPD RT49, Statens Serum Institute), tetanus toxoid (2 µg/mL, TT, Behring), phytohaemagglutinin (5 µg/mL, PHA, Sigma, Poole, UK) or medium alone. Supernatants were collected on day 3 from PHA stimulated wells and on day 6 from antigen stimulated and control wells for cytokine measurements. Commercially available ELISA kits were used to measure the concentrations of IFN-γ, IL-5 (BioSource Europe, Fleurus, Belgium) and IL-13 (Diacalone, Besancon, France).

Statistical analysis

Statistical analysis was performed using Stata 6 (Stata Corporation, College Station, TX, USA). Differences between means were assessed using Student *t*-tests, and differences in proportions by using Chi-square test. Differences in cytokine production between the RSV group and controls were assessed using the Mann–Whitney *U*-test. Cytokine production was also dichotomised and the odds ratio for RSV was estimated using logistic regression with cytokine production as the outcome variable. Multivariate models were used to assess the effect of RSV on outcome adjusting for potential confounding variables. Only those variables which confounded the relationship between case/control status and a specific outcome were retained in each final model.

Statistical significance was assigned by a two-sided alpha level of 0.05.

The study was approved by the Gambia Government/Medical Research Council Ethics committee.

Results

Three-hundred and six children were originally recruited into the cohort. During the 5 years of follow-up, six children (2.0%)

had died, 11 (3.6%) refused further participation, and 78 (25.5%) had moved out of the study area. The mean attrition rate of the cohort was therefore 7.5% per year. Of the remaining 211 children, 188 (89.1%; 66 cases and 122 controls) participated in this follow-up study and answered the questionnaire and 166 of those (88.3%) came to the clinic for further investigations. Table 1 shows demographic and health characteristics of cases and both groups of controls. The differences observed between cases and controls reflect those that were present at the initial recruitment of the cohort [18]. No difference was observed between the two groups of controls [Table 1]. Data on clinical allergy, skin reactivity to allergens and airway reactivity from both groups of controls were combined in the analysis.

Clinical allergy, wheezing, and atopy in RSV cases and controls

During the 1994–99 period of follow-up, 56 cases (84.8%) and 94 controls (77.0%) attended the clinic at least once with a medical complaint ($P = 0.2$). During these 5 years, cases were at higher risk for a diagnosis of a wheezy lower respiratory tract

infection (LRI) than controls (16.7% vs. 5.7%, OR = 3.3, 95% CI 1.2–9.0). However, Table 2 shows that the difference between cases and controls for wheezing was only significant during the first 3 years of life and not thereafter, confirming our previous observation [6]. The association between cases and early wheezing was confounded by the number of people in the compound, birth order, measles vaccination, helminth infection, and anaemia [Table 2]. Very few diagnoses of other clinical manifestations of allergy were made during the follow-up. Three children (two cases and one control) were diagnosed with asthma, three children (one case and two controls) with eczema and none with allergic rhinitis.

Airway reactivity testing confirmed that cases were no longer at increased risk of wheezing five years after RSV disease. Among the 26 cases and 50 controls who completed spirometry testing, two cases and three controls had a forced expiratory volume decline after one second (FEV₁) of 20% or more after 2 ($P = 0.3$) or 10 min of strenuous exercise ($P = 0.5$). Fifty-six cases and 103 controls performed adequately on the RINT method. Among them, cases and controls had similar airway resistance at baseline (0.83 vs. 0.78; $P = 0.6$) as well as similar

Table 1. Demographic, socio-economic and health characteristics of the RSV cohort on follow-up, means (SD) and percentage given

	Cases	Age-matched controls	After epidemic controls
N	66	66	56
Age (years)	5.4 (0.5)	5.3 (0.4)	4.8 (0.5)
% boys	56.1	38.5	46.4
Birth order	4.5 (2.5)	3.9 (2.4)	3.9 (2.0)
Follow-up period (years)	4.6 (0.4)	4.5 (0.4)	4.4 (0.6)
People in compound	9.8 (5.0)	8.6 (4.8)	9.1 (4.9)
Children 0–2 years in compound	1.6 (0.9)	2.0 (1.0)	1.9 (1.3)
% flush toilet in compound vs. pitlatrine or no toilet	7.6	9.1	5.4
% tap water in compound vs. other water sources	16.7	15.2	8.9
% with a smoking father/mother	37.9	34.9	35.7
% with a deceased sibling	50.0	38.5	44.6
% immunized against measles	57.6	75.8	71.4
Age at measles vaccination in days	324 (85)	356 (190)	313 (52)
% helminthiasis in stool	12.8	6.4	19.4
Haemoglobin (g/dL)	11.9 (1.5)	11.5 (1.6)	11.3 (1.8)
% WHZ score ≤ 2	20.7	18.6	12.2

WHZ: weight for height Z.

Table 2. Univariate and multivariate analysis of the association of severe RSV with wheezing and atopy

Outcome	Unadjusted OR (95% CI)	P	Adjusted ² OR (95% CI)	P	n
Diagnosed with wheezing ARI < 3 year of age	8.10 (1.6–40.0)	0.01	9.89 (1.6–61.0)	0.01	128
Diagnosed with wheezing ARI ≥ 3 year of age	0.57 (0.1–5.6)	0.6	0.48 (0.03–8.2)	0.6	128
Positive skin response to allergens	1.16 (0.6–2.4)	0.7	1.27 (0.6–2.8)	0.6	124
PPD skin test response ¹	0.93 (0.4–1.9)	0.9	0.78 (0.4–1.7)	0.5	124
Increased serum IgE ¹	0.60 (0.2–1.4)	0.3	0.55 (0.2–1.6)	0.3	78

¹The PPD skin test responses and serum IgE concentrations are dichotomised based on the median level in the non-RSV group. ²Adjusted for timing of measles vaccination (≥ 300 days, 0–300 days, no vaccination), anaemia, birth order (first born vs. other), number of people in the compound (> 5 vs. ≤ 5) and helminth infection. Unadjusted and adjusted odds ratios were calculated on children without missing values. Unadjusted odds ratios based on the total available sample size (and not just restricted to the sample size available for the multivariate model) showed comparable effects on the association of severe RSV with wheezing and atopy.

mean changes after 2 (0.86 vs. 0.79; $P = 0.7$) and 10 min (0.84 vs. 0.78; $P = 0.8$) of exercise.

Skin reactivity to common allergens was tested in 57 cases (86.4%) and 97 controls (79.5%). There was a tendency among cases to show more hyper-reactivity to one or more allergens (49.4%) than controls (41.4%), also after adjustment for confounders, but this difference was not significant [Table 2]. A comparable OR was obtained after adjustment for potential confounders. Similar results were obtained when 4 or 5 mm was used as cut-off for a positive test (cases vs. controls; 4 mm: 29.8 vs. 18.0%, $P = 0.09$; 5 mm: 14.0 vs. 8.0%, $P = 0.2$). Skin reactivity to PPD was assessed in 55 cases (83.3%) and 105 controls (86.1%). As shown in Table 2, cases and controls had similar reactivity to PPD. Serum IgE concentration was measured in 45 cases (68.2%) and 50 age-matched controls (75.8%). Cases and controls had similar serum IgE concentrations (median (interquartile range): 426 (195–1395) vs. 545 [312–936]) also after adjusting for confounders with serum IgE concentration dichotomised [Table 2]. Mean relative and absolute eosinophil counts were also similar among cases and controls before or after adjusting for current helminth infection (data not shown).

Type 2 cytokine production in response to RSV and other antigens in RSV cases and controls

The production of type 1 cytokine, IFN- γ , and type 2 cytokines, IL-5 and IL-13, in response to RSV-FG and control antigens was studied in 50 cases and 52 age-matched controls. The crude data [Fig. 1] show that cases and controls apparently produced similar concentrations of IFN- γ , IL-5 and IL-13 in response to RSV-FG, PPD and TT. A lower median concentration of IFN- γ was induced by PHA (Mann–Whitney test, $P < 0.05$) in cases as compared to controls but the concentrations of IL-5 and IL-13 were similar in both groups. Cytokine concentrations were then dichotomised based on the median values in the control group in order to enable adjustment for confounders. Adjustment for confounders (time of measles vaccination, number of people in the compound, anaemia and birth order) increased the odds for increased cytokine production in RSV cases as compared to controls in response to all antigens (data not shown). However, in most instances, the OR was not significantly different from 1 and in particular the production of IFN- γ induced by PHA did not differ in cases and controls, in contrast to results obtained before adjustment. As shown in Table 3, after adjustment for confounders, the production of IL-13 induced by RSV-FG and that of IL-5 induced by PPD

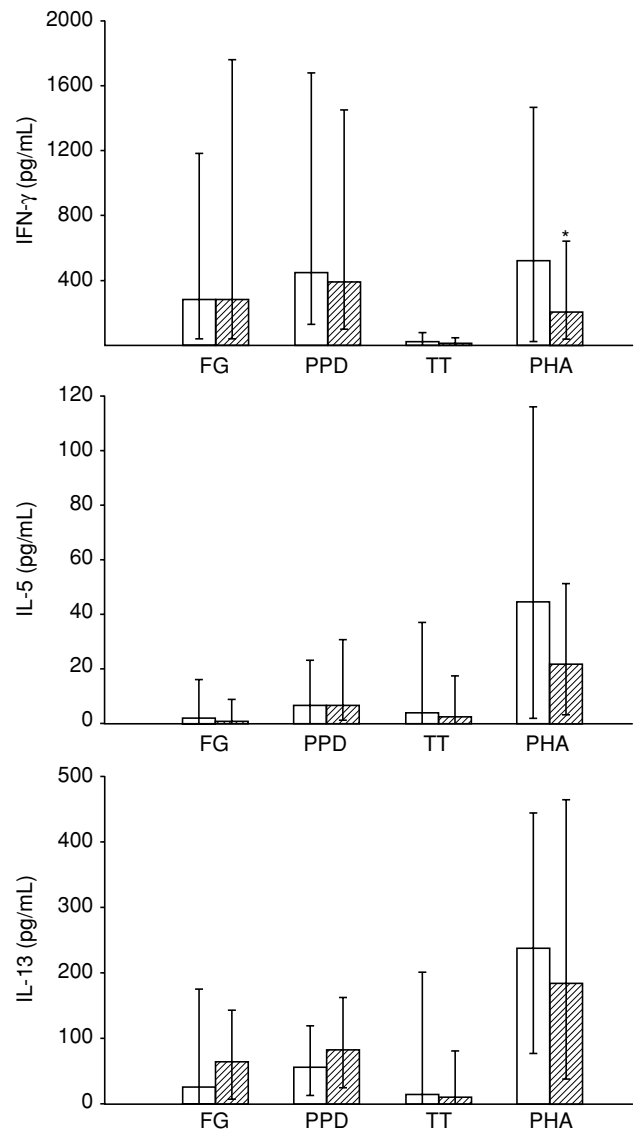


Fig. 1. Cytokine production induced by RSV and other antigens. PBMC obtained from cases ($n = 50$) and age-matched controls ($n = 52$) were incubated in the presence of RSV-FG (2 $\mu\text{g/mL}$), PPD (5 $\mu\text{g/mL}$), TT (2 $\mu\text{g/mL}$) or PHA (5 $\mu\text{g/mL}$). IFN- γ , IL-5 and IL-13 concentrations were measured in supernatants by immunoassays. Reported values for PHA were obtained after 3 days of incubation; for the others after 6 days of incubation, after verifying that an early response did not occur on day 3. Data are presented as median and upper and lower quartiles. Open bars: cases; shaded bars: controls. * $P < 0.05$ as compared to controls; Mann–Whitney U -testing.

Table 3. Univariate and multivariate analysis of the association of severe RSV with cytokine production, after stimulation by various antigens

Outcome		Unadjusted OR (95% CI)	<i>P</i>	Adjusted OR (95% CI)	<i>P</i>	<i>n</i>
RSV-FG	IL-13	1.30 (0.53–3.17)	0.56	2.98 ¹ (1.08–8.25)	0.035	77
PPD	IL-5	1.48 (0.65–3.36)	0.35	3.65 ² (1.19–11.19)	0.023	93
	IL-13	1.49 (0.60–3.69)	0.39	2.78 ³ (0.93–8.34)	0.068	77

The cytokine response is dichotomised based on the median level in the control group.¹adjusting for timing of measles vaccination (≥ 300 days, 0–300 days, no vaccination), anaemia, birth order (first born vs. other) and helminth infection.²adjusting for timing of measles vaccination (≥ 300 days, 0–300 days, no vaccination), anaemia, birth order (first born vs. other), number of people in compound (> 5 vs. ≤ 5) and ethnic group.³adjusting for timing of measles vaccination (≥ 300 days, 0–300 days, no vaccination), number of people in compound (> 5 vs. ≤ 5), anaemia, birth order (first born vs. other), sex and helminth infection. Unadjusted and adjusted odds ratios were calculated on children without missing values only. Unadjusted odds ratios based on the total available sample size (and not just restricted to the sample size available for the multivariate model) showed comparable effects on the association of severe RSV with wheezing and atopy.

were significantly higher in cases as compared to controls. The production of IL-13 induced by PPD was also higher in cases and reached borderline statistical significance [Table 3].

Discussion

RSV infection in early life in The Gambia is associated with increased wheezing during the first 3 years of life. This study shows that after 5 years of follow-up, children who had wheezing following severe RSV in early life produced higher concentrations of type 2 cytokines in response to RSV and unrelated antigens during childhood, while they did not appear to be at increased risk of atopy or clinical allergy. These associations were confounded by several environmental factors including timing of measles vaccination, birth order and helminth infection, which are thought to influence immune responses in childhood [19, 20]. Our results indicate that a viral infection may influence cytokine responses to other infections. Thus RSV infection in early life was associated with an increased production of IL-5 and IL-13 in response to tuberculin after 5 years. We do not think our results are substantially affected by bias due to a selective loss to follow-up, which was similar across the groups, and who had similar baseline characteristics. However, it is possible that children who develop severe RSV disease in infancy are already genetically predisposed towards a type 2 response. A prospective birth cohort is needed to explore this association further.

Other studies have suggested that type 2 cytokines play a pathogenic role in severe RSV disease and in wheezing subsequent to RSV infection. Data reported by Renzi et al. indicate that early wheezing following bronchiolitis is associated with increased type 2 response to allergens [21]. Experiments performed in mice have demonstrated the pathogenic role type 2 cytokines can play in the severity of the disease, but data obtained in humans have been controversial [9, 11, 12]. The increased production of IL-13 in response to RSV antigens in our study population is compatible with the hypothesis that a type 2 response may be implicated in disease severity in humans. However, the number of children who wheezed on follow-up in our study was probably not large enough to have detected an association with type 2 cytokine production. Further studies are needed to characterize the immune response to RSV during the acute phase of the infection and when subsequent wheezing occurs.

Assessment of bronchial hyper-reactivity using spirometry in this young age group was difficult. Nearly all children were able to perform adequately on the airway interrupter technique, but due to poor performance on the spirometry, we could not compare the two techniques [22]. More work is needed with the interrupter technique in developing countries to establish reference values to assist in the assessment of airway hyper-reactivity and lung function.

Recently, studies in The Gambia of adults (≥ 15 years) and children (aged 8–12 years) showed high prevalences of atopy as assessed by immediate skin prick reactivity and high IgE levels, but a relatively low prevalence of asthma and bronchial hyper-reactivity upon challenge [23, 24]. Other studies in Nigeria [25] and Ethiopia [26] found a similar dissociation between clinical allergy and skinprick reactivity. However, the prevalence of atopic disease is rapidly increasing in many countries in the

world, and places a considerable burden on the health services. It has been hypothesized that this increase is associated with decreased exposure to infectious agents in early life linked to improved hygiene. Such decreased exposure would affect the maturation of the immune system and could result in a type 2 dominance. Recently it has been proposed to broaden this 'hygiene hypothesis' into a broader 'counter-regulatory hypothesis'. This model hypothesizes that chronic or repeated infections in early life lead to an up-regulation of counter-regulatory mechanisms, including regulatory T cells. Such an up-regulation would secondarily suppress an underlying predisposition to the development of atopy and atopic disease (type 2 dominance) or autoimmune disease (type 1 dominance) [27]. This hypothesis would suggest a different effect of severe RSV on subsequent atopy in industrialized countries where decreased incidence of other infections in early life could lead to a relative deprivation of this counter-regulatory immunological feedback, compared to the effect in countries like The Gambia, where longer term effects of severe RSV might be modulated by the effect of other antigenic pressures. Prospective, community-based studies are urgently needed to further define risk factors for atopy and atopic disease.

Our findings indicate that severe RSV infection in early life was not associated with clinical atopy beyond 3 years of age and may not be an important risk factor for the development of clinical allergy and atopy in Gambian children. However, severe RSV infection in early life was associated with a persistent increase in the production of type 2 cytokines, which could play a role in the pathogenesis of RSV infection and could influence the immune response to other pathogens.

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