Cytokine production of RSV/PHA-stimulated tonsillar mononuclear cells: influences of age and atopy

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Cytokine production of RSV/PHA-stimulated tonsillar mononuclear cells: influences of age and atopy. S. Ichinohe, I.R. Hussain, S.L. Johnston. ©ERS Journals Ltd 2003.

ABSTRACT: Links between immune responses to respiratory syncytial virus (RSV), age and atopic sensitisation are poorly understood.

This study investigated the induction of target organ type-1, type-2 and pro-inflammatory cytokine responses to RSV and/or phytohaemagglutinin (PHA) in tonsillar mononuclear cells from children, in relation to age and atopic status.

In comparison with the control medium, RSV induced production of the type-1 cytokines interferon (IFN)- γ and interleukin (IL)-18, the pro-inflammatory cytokines IL-6, -8 and RANTES (regulated on activation, normal T-cell expressed and secreted), but not any of the type-2 cytokines IL-4, -5, -10 and -13. Induction of IL-6, -8 and RANTES, but not IFN- γ or IL-18, were shown to be dependent on virus replication. PHA induced all except IL-12, -13, and -15. Induction of IFN- γ , IL-6, -8, and RANTES was significantly increased in atopic children. Induction of both IFN- γ and IL-4 increased in parallel in relation to age, with no change in the IFN- γ :IL-4 ratio.

These data are compatible with the hypothesis that immature type-1 immunity during early childhood plays a role in both respiratory syncytial virus bronchiolitis and in its relationship with atopy.

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Asthma now affects up to 30% of the childhood population in westernised societies [1]. Respiratory syncytial virus (RSV) bronchiolitis is an important risk factor for asthma and atopic sensitisation at 7 yrs of age [2, 3]. However, the pathogenesis of RSV-induced bronchiolitis in early childhood and its link with wheezing and atopic sensitisation later in life is poorly understood.

Both CD4⁺ and CD8⁺ T-cell memory immune responses can be divided into type-1 and type-2 responses depending on the profile of cytokines they produce [4]. Interferon (IFN)- γ has antiviral activity and is the major effector type-1 cytokine. Interleukin (IL)-12, -15 and -18 are all produced by antigen presenting cells and induce type-1 activity in T-cells. IL-4, -5, -10 and -13 are produced by type-2 T-cells and their excess production is implicated in the pathogenesis of allergic sensitisation and asthma [5, 6].

The relationship between virus-associated wheeze and asthma disease in childhood is complex [7, 8], but a possible explanation for the link could be deficient production of type-1 cytokines in at risk infants. However, the data regarding type- and type-2 cytokine production in RSV bronchiolitis are conflicting. VAN SCHAİK *et al.* [9] demonstrated that IFN- γ concentrations in nasopharyngeal aspirates of infants with viral bronchiolitis were higher than in infants with upper respiratory infection, suggesting that a type-1 cytokine response is predominant in the respiratory tract in bronchiolitis. In contrast, several studies investigating peripheral

blood mononuclear cells (MNCs) have observed reduced IL-12 or IFN- γ and/or increased type-2 cytokine production in association with RSV bronchiolitis [10–13]. Some of these conflicting results could stem from all but one [9] of these investigations being carried out in samples not derived from the target organ. Therefore, the authors elected to study cells derived from tonsils, which are the major draining upper respiratory lymphoid organ in RSV infection, since it is extremely difficult to obtain lower airway samples from children.

The authors wished to address the hypothesis that allergic/atopic disease is associated with impaired type-1 cytokine production and might be associated with increased bystander production of proinflammatory cytokines. To investigate these hypotheses the authors studied type 1 (IL-12, -15, -18 and IFN- γ) and type 2 (IL-4, -5, -10 and -13), as well as proinflammatory cytokines (IL-6, -8 and RANTES (regulated on activation, normal T-cell expressed and secreted)) from purified tonsillar MNCs from children with and without a history of allergic disease, in response to RSV, the nonspecific mitogen phytohaemagglutinin (PHA) and the combination of RSV and PHA.

The association of wheezing with RSV decreases significantly with increasing age [3]. In addition, immune responses are known to be immature in the early years of life. Pregnancy is known to be a type-2 cytokine-rich environment, and postnatal microbial stimulation is known to be important

to the development of mature type-1 cytokine responses [7]. However, the balance between type-1 and type-2 cytokine responses to RSV in relation to age have not yet been investigated. The authors hypothesised that in infants a reduced capacity to produce IFN- γ along with increased type-2 cytokine production could impair virus clearance, allow RSV to spread to the lower respiratory tract and result in bronchiolitis. Therefore, the authors investigated the balance between type-1 and type-2 cytokine responses in tonsillar MNCs in relation to age.

Materials and methods

Virus preparation

RSV A2 strain (Medical Research Council Common Cold Unit, Salisbury, UK) was propagated to high titre in HEp2 cells (European Collection of Animal Cell Cultures, Salisbury, UK) at 37°C, in a humidified, 5% carbon dioxide (CO₂) incubator. Virus was harvested, aliquoted and stored at -70°C. Titration of stored aliquots was carried out by inoculating serial dilutions of virus stock onto HEp2 cells according to standard methods. For each experiment, a new vial was rapidly thawed and used immediately. The HEp2 cells, media and viral stocks were tested for contamination with mycoplasma species using semiquantitative detection of digoxigenin-labelled products by a hybridisation-based microtitre plate assay (Boehringer Mannheim, Mannheim, Germany) and proved negative.

Patients

Informed consent was obtained from parents in all cases and the study was approved by the Southampton Joint Hospitals Ethics Subcommittee. Tonsils were obtained from 18 patients aged 3–21 yrs who had routine clinical indications for tonsillectomy and no history of recent infections. Children were categorised as atopic if they or their parents recorded positive responses to a questionnaire inquiring whether they had a past or present history of allergic diseases including asthma, hay fever, eczema or urticaria/hives. There were 11 atopic children (six male and five female, median age 8.5 yrs, range 3–21 yrs) and seven nonatopic children (three male and four female, median age 9 yrs, range 4–20 yrs). There were no significant differences in age or sex between the two groups. The authors were unable to define atopic status objectively by the use of skin-prick testing or total or specific immunoglobulin E determination, as the requested permission for these studies was declined by the Ethical Committee. Informed consent was obtained in all cases and the study was approved by the Southampton Joint Hospitals Ethics Subcommittee.

Purification and culture of tonsillar mononuclear cells

Tonsils were cut into small pieces immediately after tonsillectomy. The pieces were ground on a sieve, suspended and washed once with phosphate-buffered saline (PBS). MNCs were separated by Ficoll centrifugation (Histopaque1077; Sigma-Diagnostics, St Louis, MO, USA), washed twice with PBS and resuspended with medium (Rockwell Park Memorial Institute 1640 with Glutamax (Gibco BRL; Paisley, UK) containing 10% foetal calf serum and 1% penicillin/streptomycin). Cell viability by trypan blue dye exclusion was >95% for all cell preparations.

Stimulation of tonsillar mononuclear cells

The MNCs were cultured at a concentration of 1×10^6 mL⁻¹ in 1 mL of medium in 24-well plates at 37°C in 5% CO₂ with RSV (multiplicity of infection (MOI) of 10), PHA ($10 \mu\text{g} \cdot \text{mL}^{-1}$), PHA and RSV combined, ultraviolet (UV)-inactivated RSV (UV-RSV; $1200 \mu\text{J} \cdot \text{cm}^{-2}$, 15 min), or medium alone as control. Supernatants were harvested at 24 h and stored at -70°C for analysis for cytokine concentrations. The dose and time point for stimulation were determined based on preliminary dose/response and time/course experiments and the data on RSV replication (see below). Cells were harvested at 0 and 24 h after stimulation with RSV for analysis of surface expression of RSV antigens and 0, 4, 24 and 48 h for determination of RSV F-protein messenger ribonucleic acid (mRNA) expression.

Cytokine assays

Cytokine concentrations in MNC culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). The capture antibodies, detecting antibodies and recombinant human cytokines for IFN- γ , IL-6, -8, -13, -15, -18 and RANTES (R&D Systems, Abingdon, UK), IL-4, -10 and -12 (Biosource, Nivelles, Belgium), paired antibodies for IL-5 (PharMingen, Oxford, UK) and recombinant human IL-5 (Pepro Tech EC LTD, London, UK) had varying sensitivities of the assays of $2.0 \text{ pg} \cdot \text{mL}^{-1}$ for IFN- γ , IL-5, -6, -8, -10, -13, -15, -18 and RANTES, $0.05 \text{ pg} \cdot \text{mL}^{-1}$ for IL-4, and $0.4 \text{ pg} \cdot \text{mL}^{-1}$ for IL-12.

Mononuclear cell surface marker expression

To determine the cellular composition of the tonsillar MNC preparations, the surface phenotype of cells within the MNC preparations were analysed by flow cytometry in eight samples. MNCs cultured for 24 h were stained with fluorescein isothiocyanate or phycoerythrin conjugated monoclonal antibodies to CD4, CD8, CD20, and CD14 (PharMingen, Oxford, UK) and 5,000–10,000 cells from each sample were analysed on a FACScan flow cytometer (Becton, Dickinson and Company, San Diego, CA, USA).

Respiratory syncytial virus surface antigen and gene expression in respiratory syncytial virus-stimulated mononuclear cells

RSV antigens expressed on cell surfaces at 0 and 24 h after infection were analysed preliminarily in four samples by flow cytometry using a monoclonal antibody against RSV (DAKO, Ely, UK). Expression was determined within B-cells (CD20+), helper (CD4+) and suppressor (CD8+) T-cells.

Copy numbers of RSV F-protein mRNA in MNCs cultured with RSV, UV-RSV, or control medium at 0, 4, 24 and 48 h were measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the TaqMan fluorogenic detection system (Perkin Elmer Corp./Applied Biosystems, Foster City, CA, USA) in three samples. Total RNA was extracted from 10^6 MNCs using Trizol (Gibco BRL, Paisley, UK) according to the manufacturer's instruction. RT was then performed using random hexamers (Promega, Southampton, UK) according to standard protocols. PCR was performed in a total volume of 25 μL PCR mixture containing 5 μL of complementary deoxyribonucleic acid, 2.5 μL of TAQMAN buffer A, 1 μM of MgCl₂, 500 nM

of Taqman probe, 200 nM of deoxyadenosine triphosphate/deoxycytidine triphosphate/deoxyguanosine triphosphate, 400 nM of deoxyuridine triphosphate, 0.01 u·mL⁻¹ of AmpErase UNG, 0.05 u·mL⁻¹ of AmpliTaq Gold, and 300 nM of RSV F-protein primers (sense; TGC AGT CAC ATT TTG TTT TGC TT, anti-sense; TTC TCA GAG CAC TAA GAT AGC CTT TG) or reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA primers (sense; GGG AAG GTG AAG GTC GGA GT, anti-sense; TGG AAG ATG GTG ATG GGA TTT C) for 50 cycles. Dilutions of plasmids constructed from the F-protein of RSV A2 strain and GAPDH were used to generate standard curves. The lower limits of detection were five gene copies for each PCR. To control for possible variation in cell numbers over time or between conditions, RSV F-protein gene expression was expressed as the ratio between F and GAPDH copy numbers.

Statistical analyses

Except where indicated otherwise, all data are described as median and range and analysed by the Wilcoxon rank test for paired data or the Mann-Whitney U-test for nonpaired data. Correlations were performed with Spearmans rank correlation. A $p \leq 0.05$ was accepted as statistically significant for all analyses.

Results

Surface phenotype of purified tonsillar mononuclear cells

The subpopulations of MNCs purified from tonsils were B cells (CD20 expression $57.4 \pm 7.5\%$ (mean $\pm$ SD)), T-helper (CD4, $26.6 \pm 1.8\%$) and suppressor (CD8, $8.1 \pm 1.3\%$) cells and monocytes (CD14, $1.4 \pm 0.8\%$).

Limited respiratory syncytial virus infection in tonsillar mononuclear cells

The ability of RSV to replicate in tonsillar MNCs was investigated by determining surface antigen and mRNA expression in MNCs at various time points after RSV inoculation. RSV surface antigen positive cells were always $<1.0\%$ positive cells within in CD4+, CD8+ and CD20+ cell populations at both 0 and 24 h. There were no significant differences in expression between samples with and without RSV.

The ratios of RSV F-protein:GAPDH mRNA copy numbers in MNCs cultured with RSV were greater than those cultured with UV-RSV or control medium at all time points studied ($p < 0.05$, fig. 1). Peak RSV-F gene expression was observed at 24 h. However, there was no statistically significant difference in the copy number ratios between 4, 24, and 48 h after infection of RSV compared with those of 0 h ($p = \text{not significant (NS)}$, fig. 1).

These results suggest RSV was able to attach to and enter tonsillar MNCs ($>98\%$ B and T-cells), but that active replication was relatively limited.

Cytokine production by tonsillar mononuclear cells in response to phytohaemagglutinin

Production of the type-1 cytokine IFN- γ , the type-2 cytokines IL-4, -5, -10 and -13, the pro-inflammatory cytokine IL-6, the pro-inflammatory CXC chemokine IL-8, the pro-inflammatory C-C chemokine RANTES and the monocyte/macrophage derived type-1 cytokines IL-12, -15 and -18 was determined in supernatants of MNCs stimulated for 24 h with PHA or medium alone. This time point was chosen to match

Fig. 1.—The ratio of respiratory syncytial virus (RSV) F-protein:glyceraldehyde-phosphate dehydrogenase (GAPDH) gene copy numbers in mononuclear cells cultured with RSV (■), ultraviolet-RSV (○), or control medium (▲) with time postinoculation. Data are presented as mean $\pm$ SD. *: $p < 0.05$.

the time point used for analysis of RSV-stimulated cultures, as virus load had peaked at 24 h (see below). The results are shown in table 1.

Significant increases in cytokine production in MNCs stimulated with PHA were observed for IFN- γ , IL-4, -5, -6, -8, -10, and RANTES ($p < 0.01$), IL-18 ($p < 0.05$) but not for IL-12, -13 and -15 compared with those without stimulation.

Cytokine production by tonsillar mononuclear cells in response to respiratory syncytial virus

The results are shown in table 1. Induction of the type-1 cytokines IFN- γ ($p < 0.05$) and IL-18 ($p < 0.01$) was observed following 24 h stimulation with RSV. Similarly, significant production of the pro-inflammatory cytokines IL-6, -8, and RANTES ($p < 0.01$) was observed with RSV. However, no induction of any type-2 cytokine, or of the monocyte-derived type-1 cytokines IL-12 and -15 was observed compared with production without stimulation ($p = \text{NS}$).

Virus specificity of cytokine production by tonsillar mononuclear cells in response to respiratory syncytial virus

The RSV stock used to inoculate the MNC cultures was confirmed by ELISA not to contain measurable IFN- γ , IL-4, -5, -10, -12, -13 and -15. However, it contained IL-6, -8, -18 and RANTES at detectable levels (data not shown), presumably since RSV infected epithelial cells have previously been shown to produce most of these cytokines [14, 15]. For this reason the authors analysed levels of all cytokines following stimulation with UV-RSV to confirm virus replication-specificity of induction of these cytokines, rather than detection of cytokines added with the virus inoculum.

Levels of cytokines in the UV-inactivated RSV-stimulated cultures were significantly increased compared with control cultures for IFN- γ and IL-5 ($p < 0.05$) and IL-6, -8, -18 and RANTES ($p < 0.01$, table 1).

In comparison with levels observed with live RSV, significantly lower levels of IL-6, -8 and RANTES were observed following stimulation with UV-RSV ($p < 0.01$) and there was a strong similar trend for a reduction in IL-18. However, the difference just failed to reach statistical significance ($p = 0.055$) (table 1). These data suggest that the

Table 1. – Type-1, type-2 and pro-inflammatory cytokine production from tonsillar mononuclear cells stimulated for 24 h with phytohaemagglutinin (PHA), respiratory syncytial virus (RSV) and ultraviolet (UV)-inactivated RSV

Cytokine pg·mL ⁻¹	Control	PHA	<i>P</i> versus control	RSV	<i>P</i> versus control	UV-RSV	<i>P</i> versus RSV
IFN- γ	16.5 <2–88	209 42–2414	<0.01	27 <2–145	<0.05	39* <2–274	NS
IL-12	12.0 <0.4–91	10.1 <0.4–66	NS	12.1 <0.4–66	NS	13.8 <0.4–62	NS
IL-15	<2 <2–12.8	2.5 <2–16	NS	2.4 <2–31	NS	<2 <2–17.6	NS
IL-18	77 <2–111	90 <2–1035	<0.05	3752 595–6181	<0.01	2610** 1396–5414	0.055
IL-4	0.05 <0.05–2.7	10.5 <0.05–56	<0.01	0.05 <0.05–3.7	NS	0.05 <0.05–4.0	NS
IL-5	4.5 <2–24	13.5 5–58	<0.01	6.5 <2–38	NS	10.5* 7–48	<0.05
IL-10	16.0 <2–45	95 4–613	<0.01	19 <2–60	NS	11.0 <2–74	NS
IL-13	7.0 <2–105	10.0 <2–176	NS	3.0 <2–123	NS	2.0 <2–75	NS
IL-6	29 <2–180	758 59–2863	<0.01	5683 3437–6315	<0.01	3410** 193–4407	<0.01
IL-8	611 73–2282	1342 224–2940	<0.01	6722 3282–7438	<0.01	6094** 4218–6376	<0.01
RANTES	<2 <2–78	57 11–861	<0.01	2806 880–3560	<0.01	732** 322–1174	<0.01

Data are presented as median and range unless otherwise stated. IFN- γ : interferon-gamma; NS: not significant; IL: interleukin; RANTES: regulated on activation, normal T-cell expressed and secreted. *: $p < 0.05$; **: $p < 0.01$ versus control.

induction of the pro-inflammatory cytokines IL-6, -8 and RANTES was at least in part dependent on virus replication. In contrast, production of IFN- γ was not seen to be dependent on virus replication.

Cytokine production by tonsillar mononuclear cells in response to combined stimulation with phytohemagglutinin and respiratory syncytial virus

The authors observed no evidence of synergistic interactions between RSV and PHA, as the levels of cytokine induction observed with combined stimulation were not statistically different from the summation of the individual stimuli ($p = \text{NS}$, data not shown).

Influence of atopy on cytokine production

To examine the influence of atopy on cytokine production, differences in cytokine production were tested in unstimulated cultures and in cultures stimulated with PHA or PHA+RSV for 24 h, between atopic and nonatopic groups.

Production of IFN- γ , IL-6, IL-8 and RANTES following stimulation with PHA were significantly higher in the atopic group than in the nonatopic group (fig. 2).

Similar findings were observed following stimulation with RSV+PHA (data not shown). Additionally, production of IL-6 and IL-8 with medium alone were significantly higher in the atopic group than in the nonatopic group ($p < 0.05$ for both cytokines, data not shown).

Influence of age on type-1 and type-2 cytokine production

To determine the influence of age on type-1 and type-2 cytokine production, correlations between age and cytokine production stimulated with PHA+RSV after 24 h were tested.

A significant correlation between IFN- γ production stimulated

with PHA+RSV and age was observed ($r = 0.54$, $p = 0.022$, fig. 3). A similar correlation was observed between age and IL-4 production stimulated with PHA+RSV ($r = 0.72$, $p = 0.0001$, fig. 3). There were no statistically significant correlations between other cytokine concentrations and age and no correlation between the IFN- γ :IL-4 ratio and age.

Discussion

Type-1 and type-2 cytokine production

The authors were unable to demonstrate induction of any type-2 cytokine following stimulation with RSV alone, although several previous investigations have demonstrated the possibility [16, 17]. RSV stimulation significantly increased IFN- γ production in tonsillar MNCs compared with those without RSV stimulation. However, the magnitude of this induction was small compared with that induced by PHA (table 1). Previous studies demonstrated that IFN- γ protein production did not increase in RSV infected peripheral blood MNCs. However, induction of IFN- γ mRNA could be detected [18–20]. Therefore, the current study is generally consistent with these reports.

The authors were also unable to detect induction of the monocyte-derived type-1 inducing cytokines IL-12 and IL-15 in response to either RSV or PHA stimulation. One of the possible reasons for this failure to observe induction of these cytokines is that the authors were unable to detect clear-cut evidence of RSV replication within the tonsillar MNCs, and only 1.4% of the tonsillar preparation was CD14 positive monocytes. This suggests that if replication of RSV does occur within mononuclear cells, it is likely to occur within monocytes.

Influence of atopy on cytokine production

The authors were able to investigate whether production of some type-1 and type-2 cytokines in response to PHA was different in children with atopy compared with those without.

Fig. 2.—Comparison of tonsillar mononuclear cells (MNC) cytokine production between atopic and nonatopic children. a) ○: Interferon- γ +phytohaemagglutinin (PHA); ●: interleukin (IL)-6+PHA; □: IL-8+PHA. b) Regulated on activation, normal T-cell expressed and secreted+PHA. *: $p<0.05$; **: $p<0.01$. Horizontal bars show median values.

Fig. 3.—Correlations between cytokine production and age with a) interferon- γ (IFN- γ) and b) interleukin-4 (IL-4). Data were analysed by calculating the Spearman rank correlation coefficient. a) $n=18$, $r=0.54$, $p=0.022$ and b) $n=17$, $r=0.72$, $p=0.0001$.

The results showed that production of IFN- γ , IL-6, IL-8 and RANTES in tonsillar MNCs of children who have a history atopic disease were significantly higher than those of children who have no history (fig. 2). Production of the proinflammatory cytokines IL-6 and IL-8 was also significantly increased in atopic children without *in vitro* stimulation. The increased cytokine production observed may be a result of increased cellular activation among children with atopic disease.

Influence of age on cytokine production

This study investigated responses to RSV+PHA, so that cytokines induced by either of the stimuli could be included in the analysis. The results clearly demonstrated that both IFN- γ and IL-4 producing capacities increase in proportion to age. Cytokine producing capacities in children are different from adults. Previous studies observed less IFN- γ and less IL-4 production in neonatal cord blood MNCs compared with adult peripheral blood MNCs [21, 22] and in peripheral blood MNCs from children compared to those from adults [23, 24]. Observations from this study are in agreement with these studies. However, there are no studies reporting the development of the IFN- γ :IL-4 ratio with age in childhood. No correlation was observed between IFN- γ :IL-4 ratio and age, suggesting that the two arms of the T-cell immune response mature in parallel.

No correlations were observed between age and levels of other investigated cytokines that were induced upon stimulation. These observations suggest that the capacities to produce these cytokines have already reached adult levels by the age of 3 yrs (the youngest age included in the current study).

In conclusion, these studies indicate that respiratory syncytial virus induces a wide range of type-1 and pro-inflammatory cytokines from the target lymphoid organ in children. Phytohaemagglutinin stimulation also induced type-2 cytokines in addition to the above. Pro-inflammatory cytokine release in response to phytohaemagglutinin was increased in atopic compared with nonatopic children. Finally, production of the antiviral cytokine interferon- γ with phytohaemagglutinin and respiratory syncytial virus increased with increasing age. These data are compatible with the hypothesis that immature type-1 immunity during early childhood plays a role in the pathogenesis of respiratory syncytial virus bronchiolitis and in its relationship with subsequent atopic diseases.

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