Steroids Fail to Down-Regulate Respiratory Syncytial Virus-Induced IL-8 Secretion in Infants

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ABSTRACT

In the first year of life, respiratory syncytial virus (RSV) is the major cause of bronchiolitis and is characterized by extensive inflammatory cell influx to airways. We investigated whether this might reflect a failure to down-regulate secretion of the chemokine IL-8, which has been identified as a key chemoattractant during host defense to RSV. Two milliliters of blood were obtained from infants, children aged 1–12 y, and adults. Peripheral blood mononuclear cells (PBMC) were isolated and infected with RSV, and IL-8 secretion was measured by ELISA. The effect of preincubation of PBMC with either 0.1–10 μM dexamethasone or 1–100 ng/mL of one of the down-regulatory T helper 2 cytokines IL-4, IL-10, or IL-13 before RSV infection was examined. RSV stimulated IL-8 secretion in a dose-dependent manner similarly in all age groups. IL-8 secretion occurred mainly within 24 h of infection, with maximal concentrations of 30,000–46,000 pg/10^6 cells. IL-4 caused modest inhibition and IL-10 and IL-13 caused no inhibition of IL-8 secretion in all groups. Dexamethasone inhibited IL-8 secretion by 34 ± 8% in children and by 41 ± 3% in adults but had no effect on infant PBMC. In summary, RSV-induced IL-8 secretion from infant PBMC is equal to that in children and adults and relatively unaffected by down-regulatory cytokines. However, the inhibitory effects of steroids on IL-8 secretion are absent in infants, which may partly explain why they develop more severe bronchiolitis, and why steroid therapy is unsuccessful in clinical practice. (***Pediatr Res 52: 368–372, 2002***)

Abbreviations
RSV, respiratory syncytial virus
PBMC, peripheral blood mononuclear cells
MOI, multiplicity of infection
LPS, lipopolysaccharide

RSV is the major pathogen causing bronchiolitis in infants, resulting in at least 90,000 hospital admissions in the United States annually (1), imposing a major economic burden (2). RSV infection is a risk factor for development of wheezing in childhood (3). Serious RSV infection is uncommon in older children and immunocompetent adults. Host immune responses are important in pathophysiology of infection, as demonstrated by the sometimes fatal exacerbations of bronchiolitis in vaccinated infants (4). Bronchiolitis is partly due to marked inflammatory cell influx to infected airways (5, 6), although whether this reflects excessive up-regulation of proinflammatory mediators or failure to down-regulate normal antiviral immune responses is unknown.

Cellular recruitment is dependent on secretion of chemokines, including IL-8. High IL-8 concentrations were present in nasal fluid from RSV-infected children (7) and in plasma from infants with severe RSV (8), where concentrations may reflect disease severity (9). RSV-infected macrophages and mononuclear cells secrete high IL-8 concentrations (10, 11), although they are not the only cellular sources of IL-8 (12–15).

The relative immaturity of immune responses in infants may contribute to severity of RSV infection. Neonatal lymphocytes, mononuclear cells, and macrophages secrete fewer cytokines in response to a number of stimuli than their adult counterparts (16–18). Reduced IL-4 and IFN-γ secretion were reported as markers of severe RSV-bronchiolitis (9), although other data indicate a predominant T helper 1 cytokine profile in RSV infection (19). There are no data on age-dependent chemokine secretion in RSV infection.

METHODS

Subject selection. Three groups of subjects were studied: infants (<1 y), children (1–12 y), and adults. The infants and children were undergoing minor, elective surgical procedures and had no evidence of systemic or respiratory illness on questionnaire screening and clinical examination. Two milliliters of blood was venesected before the surgery at the time of routine cannulation. Similar volumes of blood were taken from

a group of healthy adult volunteers. A detailed history, including enquiry about previous hospital admissions for respiratory illness, family and individual atopic history, breast-feeding, and family smoking history, was obtained for each subject. No subjects were receiving any form of immunosuppressive medication. The study was approved by the St. George's Hospital Research Ethics Committee. Informed consent from those who participated in this study was obtained from parents of infants and children or directly from adult volunteers.

Virus culture and titration. RSV (strain A2) was propagated in HEp-2 cells according to methods described (20) and partially purified by centrifugation at $10,000 \times g$ for 10 min at 4°C, to remove cell supernatant. Cells were inoculated with 0.1 MOI RSV and harvested once >80% cell detachment was observed. The cell suspension was spun at $13,000 \times g$, and the virus-containing pellet rapidly resuspended in fresh media, aliquoted, snap frozen, and stored at −80°C. Virus titer was quantitated using the microplaque immunoperoxidase method (21). Control aliquots generated using uninfected HEp-2 cells had no effect on IL-8 secretion.

Preparation of PBMC. Immediately after collection, whole blood was diluted 1:1 with sterile 0.9% normal saline solution and layered on Ficoll-Paque Plus (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.) in 1.5-mL microtubes. PBMC were separated by density gradient centrifugation at $500 \times g$, washed three times in sterile Hanks' balanced salt solution, and then resuspended in serum-free Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, U.S.A.), supplemented with 2 mM glutamine and 10 $\mu g/mL$ ampicillin. The PBMC were plated at 1.5×10^5 cells per well in 0.5 mL media in 24-well plates and maintained in 5% CO_2 at 37°C. The total number of PBMC obtained from the 2 mL blood from infants was approximately $4-8 \times 10^6$ cells.

PBMC stimulation. PBMC were infected with RSV at a MOI of 0.1, 0.3, or 1 and cultured for either 24 or 48 h. Cells cultured in serum-free medium alone were the negative control. Time points were selected on the basis of preliminary studies in PBMC and similar work from this and other laboratories investigating respiratory epithelial cells (22, 23). In specific experiments investigating the effects of steroids, PBMC were preincubated for 1 h with and then cultured in the presence of serial dilutions of dexamethasone (0.1, 1, or 10 μM) and then infected with RSV at a MOI of 0.3. To investigate the effects of IL-4, IL-10, or IL-13 (Peprotech. London, U.K.), PBMC were pretreated for 1 h with either 1, 10, or 100 ng/mL of each cytokine before RSV infection. Each T helper 2 cytokine remained in the tissue culture medium for the duration of the experiment. After the culture period, supernatants were harvested and stored at −80°C for IL-8 analysis. These inhibitors did not cause significant cell death over the 24-h study period (data not shown).

IL-8 ELISA and analysis of data. IL-8 concentrations in supernatants were measured by specific ELISA, using matched-paired antibodies and recombinant standards from R & D Systems Europe (Oxford, U.K.). The lower limit of sensitivity of the assay was 16–31 pg/mL. IL-8 concentrations within samples are expressed as picograms per 10^6 cells, and stimulated levels of IL-8 were corrected for unchallenged

release levels. The mean ± SEM was calculated for each data point, and data within groups were compared using Wilcoxon matched pair tests. Comparisons between age groups were performed using Mann-Whitney U tests.

RESULTS

Subject characteristics. We studied 12 infants (mean age 19 ± 4 wk), 16 children (60 ± 10 mo), and 12 adults (35 ± 3 y), none of whom had a current respiratory illness (Table 1). Two infants and five children had previously been admitted to hospital with respiratory infections or asthma, none of which none were due to RSV. In the United Kingdom, it would be expected that by 24 mo of age, over 90% individuals will have a detectable immunologic response to RSV. Therefore, it is likely that virtually all children and adults had been exposed to RSV, although this was not tested. No adult had been admitted for a chest infection within 20 y. There was no unexpectedly high incidence of drug allergies or atopy (including asthma, eczema, and hay fever) in subjects or their families. Parental smoking is a risk factor for respiratory illness (24) and about 50% infants and children came from households with one or more smokers. Breast-feeding is protective against respiratory tract illnesses in early life (25), and both infants' and children's groups contained approximately equal proportions of breast-fed subjects.

Table 1. *Clinical characteristics of study groups*

	Infants	1–12 y	Adults
No. subjects	12	16	12
Age range	19 ± 4 wk	60 ± 10 mo	35 ± 3 y
M:F ratio	10:2	15:1	8:4
No. that were previous inpatients for respiratory illness	2	5	0*
No. breast fed	6	10	ND
No. atopic patients	0	7	8
No. with family history of atopy	6	12†	5†
No. with smokers in household	5	8	4

* Information unavailable for early childhood.

† In each case, one value is missing, as information unavailable.
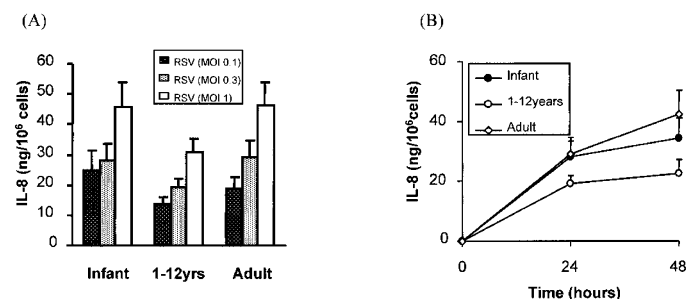
ND, no data.

Figure 1. IL-8 secretion from RSV-infected PBMC from infants ($n = 12$), children ($n = 16$), and adults ($n = 12$). (*A*) The effect of increasing infectious dose. PBMC were infected with RSV at MOI of 0.1, 0.3, or 1. Supernatants were then harvested after 24 h and IL-8 concentrations measured by ELISA. (*B*) Kinetics of IL-8 secretion. PBMC were infected with RSV (MOI = 0.3) and cultured for up to 48 h. IL-8 concentrations were then measured in culture supernatants. All data are given as mean ± SEM, in picograms per 10^6 cells, for each age group. p = NS for all comparisons between age groups by unpaired Mann-Whitney tests.

RSV-induced IL-8 secretion. Infection with RSV induced IL-8 secretion from PBMC at 24 h of culture, dependent on the MOI (Fig. 1*A*). IL-8 concentrations secreted by infant RSV-infected PBMC were 24,720 $\pm$ 6520, 28,280 $\pm$ 5180, and 45,710 $\pm$ 8010 pg/10^6 cells in response to 0.1, 0.3, and 1 MOI of RSV, respectively. At all ages, the increase in IL-8 concentrations after stimulation by RSV at an MOI of 1.0 compared with an MOI of either 0.1 or 0.3 were significant ($p < 0.001$). Comparing IL-8 secretion after exposure to RSV at an MOI of 0.1 with RSV at an MOI of 0.3, the increase in IL-8 secretion was significant in both children and adults ($p < 0.01$) but not in the infant group, possibly due to the number of patients studied. There were no significant differences in RSV-induced IL-8 secretion between the three age groups. Most IL-8 secretion occurred within 24 h and did not increase greatly between 24 and 48 h (Fig. 1*B*). IL-8 secretion did not relate to clinical characteristics and was unaffected by household smokers or breast-feeding.

Effect of dexamethasone on IL-8 secretion. Preincubation of PBMC with dexamethasone caused dose-dependent, partial inhibition of RSV-induced IL-8 secretion in adults and older children. Maximal reduction of IL-8 secretion was 34 $\pm$ 8% in children and 41 $\pm$ 13% in adults ($p < 0.05$ compared with untreated cells by paired Wilcoxon test). In contrast, dexamethasone did not alter IL-8 secretion from infant PBMC (Fig. 2), which were 28,280 $\pm$ 5180 pg/10^6 from untreated cells, and 27,270 $\pm$ 9500 pg/10^6 from cells pretreated with 10 μM dexamethasone (p = NS). In contrast to the differences between the three groups, within each group there was no discernible trend in terms of IL-8 secretion in relation to age. For example, in the infant group, only two patients showed suppression of IL-8 in response to dexamethasone, of whom one was aged 8 wk and one 18 wk (mean age group 19 $\pm$ 4 wk).
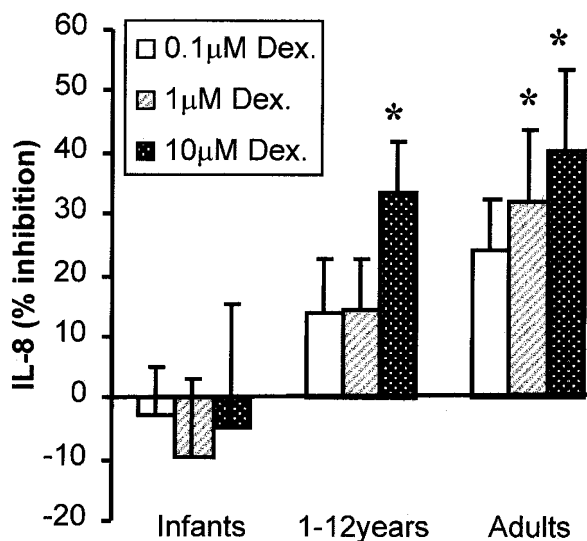

Figure 2. Effect of increasing doses of dexamethasone on RSV-induced IL-8 secretion. PBMC from infants, 1–12 y olds, or adults were preincubated with increasing concentrations (0.1, 1, 10 μM) of dexamethasone for 1 h, before infection with RSV (MOI = 0.3). Supernatants were harvested after 24 h culture, and IL-8 measured by ELISA. Data are expressed as percentage inhibition, and given as mean $\pm$ SEM for each age group. *$p < 0.05$ compared with untreated cells infected with RSV (paired Wilcoxon test).

In contrast, dexamethasone did not at any age decrease RANTES secretion, which we found was induced by RSV in a similar dose- and time-dependent manner to IL-8, although at a lesser magnitude.

Effect of IL-4, IL-10, and IL-13 on RSV-induced IL-8 secretion. Finally, the effects of IL-4, IL-10, and IL-13 on RSV-induced IL-8 secretion were examined. Table 2 shows that only 100 ng/mL IL-4 exerted any inhibitory action, decreasing IL-8 secretion by 31 $\pm$ 6% in infants, 36 $\pm$ 7% in children, and 34 $\pm$ 14% in adults ($p < 0.05$ compared with untreated). IL-10 and IL-13 had no significant effects on RSV-induced IL-8 secretion from PBMC at any concentration used in this study. There were also no differences observed between the responses of RSV-infected PBMC to IL-4, IL-10, or IL-13 in the different age groups.

DISCUSSION

This study demonstrates that the magnitude and kinetics of IL-8 secretion from RSV-infected PBMC are similar in infants, adults, and children, even with increasing infectious load (MOI). Although the blood volumes available were insufficient to perform parallel studies using LPS, these data are consistent with earlier work showing that LPS-stimulated cord blood mononuclear cells secrete as much IL-8 as maternal monocytes (26). In contrast, neonatal monocyte-derived macrophages and lymphocytes secrete less IFN-γ than adult cells (16) and PBMC secrete less IL-3, IL-15, and granulocyte-macrophage colony stimulating factor (27). The data concerning age-dependent responses to RSV are inconsistent with both increased and reduced cell-mediated immunity reported (28, 29). Such differences may be stimulus and mediator specific. RSV-infected neonatal macrophages secrete the proinflammatory cytokines tumor necrosis factor-α and IL-6 to a normal concentration (30). Together with our findings, the data indicate that although neonatal cells display immaturity in some immune responses, they have a mature proinflammatory response, including IL-8 secretion.

IL-8 secretion occurred within 24 h of RSV infection, suggesting that viral replication is not required; RSV is known to infect but not multiply within mononuclear cells (31). We did not determine the source of IL-8, but it is likely to be lymphocyte and monocyte derived. *Ex vivo* infection of PBMC by RSV is a useful, relevant model for investigating immune responses inasmuch as these cells are recruited to airways early in infection. Furthermore, circulating mononuclear cells from infants with RSV express viral antigen (31), and measuring RSV RNA in whole blood by reverse transcriptase PCR during clinical bronchiolitis has been used to confirm systemic spread of virus (32). Infant monocytic cells may even be more prone than adult cells to RSV infection (33).

The most striking observation was that in infants but not in children or adults, dexamethasone did not to suppress PBMC IL-8 secretion. However, within each group, there were no clear age-dependent trends in IL-8 secretion. Potentially, this may result in prolonged, proinflammatory cell influx to sites of infection. The observation is consistent with the lack of benefit, in terms of disease resolution or severity, of administration of

Table 2. *IL-8 concentrations (ng/10^6 cells) after RSV infection of PBMC: effect of pretreatment with IL-4, IL-10, or IL-13*

Pretreatment concentration (ng/mL)	None	IL-4				IL-10				IL-13			
	0	1	10	100	p*	1	10	100	p†	1	10	100	p‡
Infant	38 ± 9	40 ± 10	31 ± 9	28 ± 8	<0.05	43 ± 12	42 ± 11	38 ± 9	NS	41 ± 9	35 ± 10	33 ± 9	NS
Child	25 ± 4	25 ± 5	19 ± 3	17 ± 3	<0.05	28 ± 5	21 ± 4	22 ± 4	NS	18 ± 3	22 ± 4	27 ± 5	NS
Adult	40 ± 8	36 ± 8	29 ± 5	26 ± 4	<0.05	38 ± 7	37 ± 7	36 ± 7	NS	32 ± 5	34 ± 6	40 ± 7	NS

* Comparison between IL-8 secretion for RSV alone and maximal concentration (100 ng/mL) of IL-4 (paired Wilcoxon test).

† Comparison by Wilcoxon tests of IL-8 secretion from untreated PBMC *vs* PBMC pretreated with IL-10 (all concentrations).

‡ Comparison by Wilcoxon tests of IL-8 secretion from untreated PBMC *vs* PBMC pretreated with IL-13 (all concentrations).

dexamethasone in RSV infection (34, 35). The mechanism underlying this observation is the subject of ongoing research. However, this effect appears specific and, in preliminary studies, dexamethasone reduced LPS-induced IL-8 secretion at all ages, which is consistent with data from Taniguchi *et al.* (26). In addition, the effect seems chemokine-specific in that this steroid had no effect on RANTES secretion.

IL-4, IL-10, and IL-13, T helper 2 cytokines, which often down-regulate monocyte chemokine secretion (36, 37), did not effectively block IL-8 secretion from RSV-infected PBMC, although high IL-4 concentrations partially inhibited secretion in all age groups. The specific response to IL-4 may reflect differences in their mechanisms of action; for example, IL-10 blocks LPS-induced nuclear factor-κB activation in monocytes, whereas IL-4 decreases stability of transcribed mRNA (37). In addition, T cells possess functional IL-4 but not IL-13 receptors. The fact that IL-4 had the most effect (even though this was a modest one) is consistent both with experimental observations in murine models in which a critical role for IL-4 in development of airway hyperresponsiveness was found, with overexpression of IL-4 delaying clearance of RSV (38, 39). The lack of effect of IL-10 was somewhat unexpected inasmuch as endogenous IL-10 increases during clinical RSV infection (40) and has been associated with subsequent development of recurrent wheezing (41). However, our data are consistent with the fact that *ex vivo* IL-10 secretion does not relate to severity of RSV as reflected by required duration of mechanical ventilation (42). Although recent murine studies have once again emphasized a role for IL-13 in airways hyperreactivity (43), our data indicate that this is not mediated by effects on acute IL-8 (or RANTES) secretion in humans.

In summary, infant PBMC display a normal IL-8 activation in response to RSV, compared with children and adults, that is not easily inhibited by down-regulatory cytokines. However, infant PBMC do not respond to down-regulation by dexamethasone. The efficient secretion of "adult" concentrations of IL-8 in response to RSV, coupled with a relative resistance to down-regulatory stimuli, coupled with the immature immune system, may contribute to exaggerated and prolonged inflammatory response in RSV-infected infants. These data may also explain part of the mechanism underlying the lack of benefit from steroids in treatment of RSV bronchiolitis. Further studies in RSV-infected infants are required to dissect out the mechanisms and further define the importance of the current observations.

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