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Neutrophil survival is prolonged in the airways of healthy infants and infants with RSV bronchiolitis

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ABSTRACT: Large numbers of neutrophils in the airway of infants infected by respiratory syncytial virus (RSV) are recruited by chemokines, such as interleukin-8, and specific inflammatory molecules can delay apoptosis increasing their longevity. The aim of this study was to investigate whether airway secretions in RSV bronchiolitis contain factors that influence neutrophil apoptosis.

Nasal lavage fluid (NLF) was obtained from 24 infants with RSV bronchiolitis (31 infant controls and 12 adults). Neutrophils isolated from healthy adult volunteers were incubated with the NLF in Dulbecco modified Eagle medium (DMEM) for 24 h, and apoptosis and necrosis were quantified using Hoechst 33342 and propidium iodide viability dyes. The presence of putative factors that delay neutrophil apoptosis was investigated using inhibitors to leukotriene-B_4, lipopolysaccharide and the IL-8 receptor CXCR2, and blocking antibodies to granulocyte-monocyte colony-stimulating factor. Characterisation of NLF involved tests of thermal instability, proteolysis, deoxyribonuclease digestion and molecular filtration.

NLF from infants with RSV bronchiolitis and controls significantly delayed neutrophil apoptosis, whereas NLF from healthy adults did not. None of these inhibitor molecules blocked this delay in apoptosis but activity was heat liable and >3 kDa.

The study showed that nasal lavage fluid from infants significantly delays neutrophil apoptosis. The speculation is that the prolonged survival of neutrophils in the infant airway contributes to the characteristic accumulation of neutrophils in the airways of infants with respiratory infections.
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Respiratory syncytial virus (RSV) is responsible for annual epidemics of respiratory disease affecting the whole population [1–3] and particularly infants of whom the majority are infected during their first winter [1, 4, 5]. Of these, 20–30% develop lower respiratory tract symptoms and 0.5–2% are hospitalised with RSV bronchiolitis [1, 2, 4, 5]. It is increasingly recognised that RSV is a major cause of respiratory morbidity in the elderly [6, 7]. Several prospective studies have also indicated that lower respiratory tract RSV infections in hospitalised [8] and nonhospitalised [9] infants, result in an excess of recurrent lower respiratory tract symptoms in subsequent years. These symptoms decline progressively over the first decade of life but the cause for this excess morbidity remains unclear [10].

There has been much speculation regarding the nature of the host/virus interaction [3, 11] and more than 30 yrs ago it was proposed that RSV bronchiolitis is a consequence of a specific immunopathology. Despite much research to demonstrate this, no distinct immunopathological process has been identified. The current authors have shown that neutrophils are the predominant inflammatory cell in the upper and lower airway in RSV infections in infants, accounting for ~80% of cells recovered from the tracts [12]. These finding have been subsequently replicated by others [13]. Increased protease activity in airway secretions from these infants, with neutrophil elastase (NE) accounting for most of this protease activity [14], and the presence of degraded fibronectin in the lavage samples have also been demonstrated [15]. NE may play a role in the genesis of recurrent symptoms by directly damaging the immature airway, inducing airways hyperreactivity and inducing glandular and goblet cell hyperplasia [16]. The role of neutrophils in virus clearance, if any, is unclear.

Neutrophil accumulation within the airway can be influenced by their rate of migration and longevity. Interleukin (IL)-8 is a major chemokine for neutrophils in this condition and RSV infection stimulates IL-8 release from various cells *in vitro*, including epithelial cells, macrophages, and neutrophils [17, 18]. The current authors have reported elevated IL-8 in nasal lavage fluid (NLF) from babies with acute RSV bronchiolitis [19]. There may also be a correlation between disease severity and IL-8 concentration [20], although it is unclear whether the IL-8 levels reflect or

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contribute to disease severity. Neutrophil lifespan is regulated by apoptosis [21]. Apoptotic neutrophils can be phagocytosed by resident macrophages *via* the vitronectin or phosphatidylserine receptor [22], therefore limiting the release of harmful neutrophil products into the airways, which would occur if the neutrophils died by necrosis. A number of factors likely to be present in airway secretion of infants with RSV bronchiolitis, such as IL-8, leukotriene-B_4 (LTB_4) and granulocyte-monocyte colony-stimulating factor (GM-CSF), inhibit neutrophil apoptosis [21, 23], thereby prolonging cell survival. A previous group studying neutrophils recovered from the airways of infants with RSV bronchiolitis have suggested that neutrophil apoptosis may be accelerated in this condition [24]. However, this study assessed the rate of apoptosis in neutrophils already resident in the airways for an unknown duration and compared this to the survival of circulating neutrophils. Without knowing how long these neutrophils have been resident in the airways it is difficult to determine whether their survival has been shortened or prolonged.

The aim of this study was to determine whether factors released into the airway secretions of infants with RSV bronchiolitis influence the rate of apoptosis of neutrophils. Additional studies were performed in an attempt to identify factor(s) that may be responsible for these effects.

Materials and methods

Subjects

NLF was obtained from infants hospitalised with RSV bronchiolitis (RSV-NLF), who were confirmed positive for RSV by indirect immunofluorescence on nasopharyngeal aspirates before they were recruited. NLF was also obtained from healthy infants and adults. The healthy infants were recruited at routine "well-baby clinics" having no past history of respiratory or other clinical illness at the time of sampling. Parents were contacted 2–3 days later to ensure that these infants were not incubating a respiratory illness at the time of sampling. The adults were healthy volunteers with no history of respiratory problems.

Ethical approval (South Sheffield Ethics Committee, Sheffield, UK) and informed consent was obtained from the parents of the infants and from the adult volunteers.

Collection and processing of nasal lavage fluid

NLF was collected as described by NOAH *et al.* [25], by introducing 5 mL sterile isotonic saline into one nostril while holding the infant's head tilted to the opposite side. The saline drained into the opposite nostril and was collected in a container. The process was then repeated for the opposite nostril. The 10 mL of NLF were pooled, centrifuged at 875×*g* for 5 min at 4°C, and stored in aliquots at -70°C. The protein concentration of each NLF sample from both the infants and adults was determined using a sensitive Bicinchoninic acid protein assay (BCA assay; Pierce, IL, USA).

Isolation of neutrophils

Neutrophils were isolated from the peripheral whole blood of healthy adult volunteers as described by SMITH *et al.* [26], layered on Histopaque-1077 (Sigma Chemical Company Ltd, Poole, UK) and centrifuged. Pelleted cells were collected and washed in endotoxin-free phosphate buffered saline (BioWhittaker, MD, USA). The erythrocytes were then lysed in ammonium chloride solution, and the neutrophils were centrifuged at 160×*g* for 8 min to pellet.

The viability of the neutrophil preparation was routinely >95% and purity, as assessed by automated haemocytometer analysis, was >95%, with the total yield varying between $0.75–1.5\times10^6\cdot mL^{-1}$ of blood for these donors.

Incubation of neutrophils with nasal lavage fluid

The purified neutrophils were resuspended at $1\times10^6\cdot mL^{-1}$ in Dulbecco modified Eagle medium (DMEM; Sigma Chemical Company Ltd) with 2 mM glutamine and 100 $\mu g\cdot mL^{-1}$ penicillin and 50 $\mu g\cdot mL^{-1}$ streptomycin (termed DMEM-AP). Some of these neutrophils were resuspended in 20% autologous plasma (removed at the time of centrifugation). The antibiotics were also added to the NLF samples.

The authors seeded 1×10^5 neutrophils in 100 μL aliquots into wells of a 96-well tissue culture dish (Corning Costar Ltd, High-Wycombe, UK) in triplicate for each sample/treatment, added a further 100 μL of neat, diluted, or treated NLF and gently mixed the contents. The viability of the neutrophils was measured in 200 μL of DMEM-AP in each experiment. The cells were incubated at 37°C in a humidified 95% air, 5% carbon dioxide in an incubator (Sanyo, Osaka, Japan) for 24 h.

Assessment of neutrophil viability

After 24-h incubation at 37°C, neutrophils were stained with 8 μM Hoechst 33342 (Sigma Chemical Company Ltd) and 5 μM propidium iodide (Molecular Probes, Cambridge Biochemicals, Cambridge, UK) for 15 min at 37°C. They were then examined without further manipulation using a fluorescent inverted Leica DMIRB microscope (Leica, Wetzlar, Germany). The morphology and characteristics of the viable and apoptotic cells (Hoechst stained) and necrotic cells (propidium iodide stained) are shown in figures 1a and b. Both fluorescent dyes were visible under the ultraviolet filter and could be counted simultaneously. The incidence of viable, apoptotic and necrotic cells was obtained by counting using a Whipple graticule in five separate random grid areas in each of three wells for each treatment.

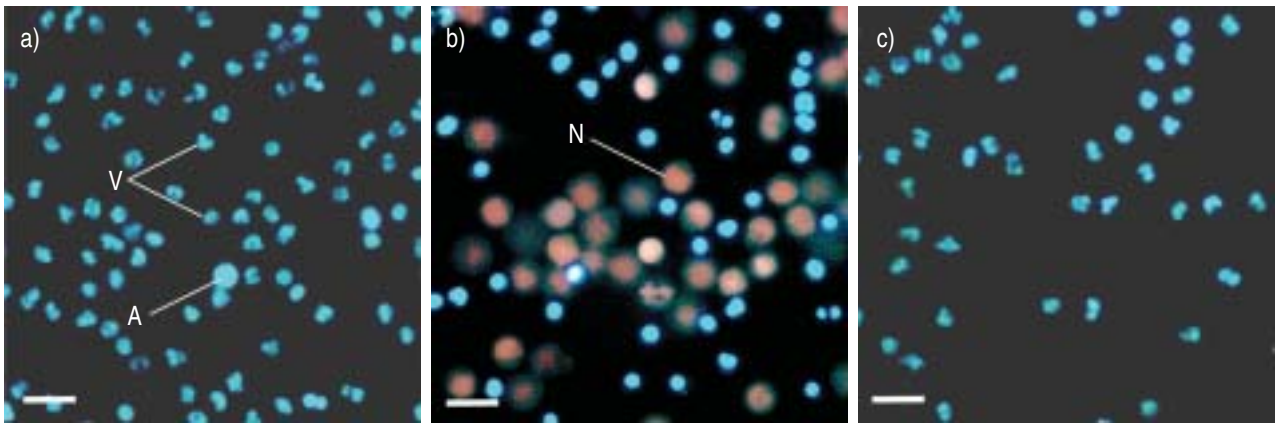

Fig. 1. – Viable (V), apoptotic (A), and necrotic (N) donor neutrophils stained with fluorescent deoxyribonucleic acid binding dyes Hoechst 33342 (Sigma Chemical Company Ltd) and propidium iodide (Molecular Probes, Cambridge Biochemical). Freshly isolated neutrophils a) after 3 h in culture without nasal lavage fluid (NLF), b) after 24 h in medium culture without NLF and c) after 24 h in medium with 50:50 dilution of NLF obtained from infants hospitalised with respiratory syncytial virus bronchiolitis. Scale bars=25 μM.

Biochemical characterisation of the apoptosis inhibiting activity in nasal lavage fluid

In some experiments, NLF was treated before adding it to the neutrophils. NLF samples with similar high activity from the RSV group were also pooled to help with the biochemical characterisation.

To determine whether the activity affecting apoptosis was labile, RSV-NLF (n=5) was frozen in liquid nitrogen and thawed in five repeated cycles. RSV-NLF (n=5) was also heated to 90°C for 15 min, cooled, and then used in the viability assay.

Bacterial deoxyribonucleic acid (DNA) has potential pro-inflammatory properties but degraded host DNA is reported to have immunosuppressive actions [27]. To determine whether DNA released from damaged respiratory tract cells affected neutrophil apoptosis, pooled RSV-NLF (n=5) was digested for 2 h at 37°C with 500 $\mu g \cdot mL^{-1}$ deoxyribonuclease (DNAse) type I (Sigma Chemicals Ltd). The degradative activity of the DNAse was confirmed using calf sperm DNA under similar conditions and demonstrating smearing following separation on 1% agarose gels stained with ethidium bromide.

RSV-NLF (n=5) was subjected to limited digestion in 0.5% bovine trypsin (Sigma Chemical Company Ltd) for 2 h at 37°C to investigate the role of proteins in the NLF. The trypsin activity was stopped with 100 $\mu g \cdot mL^{-1}$ of Soybean trypsin inhibitor (Sigma Chemical Company Ltd). Limited proteolysis was confirmed by examining the digested NLF on a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with silver. Controls included the addition of the trypsin and soya bean trypsin inhibitor complex but with no NLF.

To determine whether NLF contained low molecular weight activity, 0.5 mL samples of RSV-NLF were subjected to centrifugal filtration using 3 K molecular weight Centricon cut-off filters (Amicon, Millipore Inc., Bedford, MA, USA). The volumes of the filtrate and retentate were accurately measured and both made up to 500 μL using sterile saline solution. Five-hundred μL of unfiltered NLF was also diluted by an equivalent amount using sterile saline. The filtrate, retentate and unfiltered NLF were then incubated with the neutrophils.

Characterisation of LTB_4, IL-8, GM-CSF, and LPS activity in the NLF samples

These factors have been reported to delay neutrophil apoptosis in experimental models [21, 23] and were examined for their contribution to the activity of NLF.

The leukotriene LTB_4 inhibitor VML 295 (LY29311; gift from G. Parker, Vanguard Medica, Surrey, UK) was dissolved in dimethyl sulfoxide (DMSO). VML 295 (10 μM and 100 μM) was dissolved in pooled and individual NLF samples and then added to the neutrophils.

SB-237844 (gift from S. Sarau, SmithKline Beecham, King of Prussia, USA), a competitive antagonist of the CXCR2 IL-8 receptor, was dissolved in DMSO and added to the neutrophils from 10–0.05 μM for 5 min before the addition of pooled and individual sample NLF.

The lipopolysaccharide (LPS) inhibitor Polymyxin-B was added at 1–100 $\mu g \cdot mL^{-1}$ to the neutrophils for 5 min before the addition of pooled or individual NLF samples. The effect of Polymyxin-B was confirmed by its inhibition of 10 $\mu g \cdot mL^{-1}$ *Escherichia coli* LPS (serotype 0.26:B6; Sigma Chemical Company Ltd), which was shown to significantly delay the rate of neutrophil apoptosis.

To block the cytokine GM-CSF, 10 $\mu g \cdot mL^{-1}$, 50 $\mu g \cdot mL^{-1}$ and 100 $\mu g \cdot mL^{-1}$ of a blocking goat immunoglobulin (Ig) G antihuman GM-CSF (R&D Systems, Cambridge Biochemical, Cambridge, UK) was incubated with pooled or individual NLF for 30 min at 37°C. The NLF antibody mixture was then added to the neutrophils. Controls for these inhibition experiments included equivalent dilutions of solvents used for some of the compounds and nonimmune goat IgG at similar dilutions as for the GM-CSF blocking experiments.

Statistical analysis of data

Differences between and within groups were analysed by nonparametric Mann-Whitney U-test and by Kruskal-Wallis nonparametric tests, respectively. The relationship between the level of apoptosis and protein content of the nasal lavage samples was examined by the Spearman correlation coefficient. Significant differences were taken at $p<0.05$. The data is shown in the text as the mean±95% confidence limits.

Results

Subjects

Twenty-four infants admitted to hospital with RSV bronchiolitis were recruited with a median age of 120 days (range 33–242) and samples were obtained at a median of 3 days after onset of symptoms (range 1–4). Control samples were obtained from 31 infants (median age 149 days (range 19–433)) and 12 adults (median age 38 yrs (range 21–48)) with no symptomatic evidence of airway infection. Thirty-three per cent of the infants with bronchiolitis and 27% of infant controls were passively exposed to smoke. None of the adults were smokers.

Nasal lavage fluid protein content

The median protein concentration of the NLF samples from the infants with RSV bronchiolitis was 55.9 $\mu g \cdot mL^{-1}$ (range 7.6–386.5). The value for infant controls was 62.3 $\mu g \cdot mL^{-1}$ (19.9–212.0 range) and the value for adult controls was 19.5 $\mu g \cdot mL^{-1}$ (3.1–53.1 range), and they were significantly lower ($p<0.001$) than the two infant groups.

Effect of nasal lavage fluid on neutrophil survival

Neutrophils were incubated in medium alone or with RSV-NLF, control infants' NLF and healthy adults' NLF, and after 24-h changes to cell viability were determined. The mean amount of viable cells in the medium alone was 27.5% (±3.3), with 49.3% of the cells being apoptotic (±3.1) and 23.4% being necrotic (±3.5), as shown in figure 2a. Following the addition of RSV-NLF, there was a significant increase to 77.5% (±3.3) viable cells, and a decrease to 19.5% apoptotic (±2.9) and 3.0% necrotic (±0.9), compared to medium

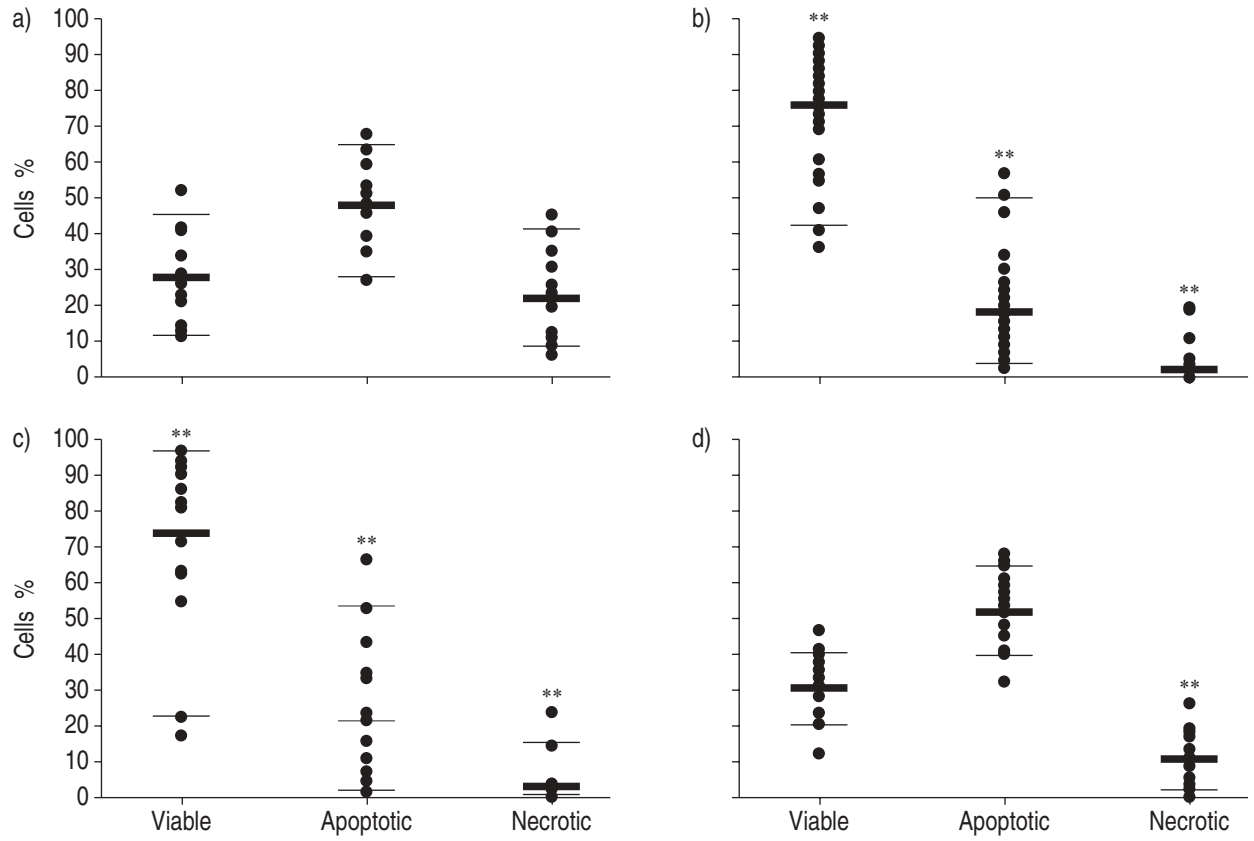

Fig. 2.–Measurement of the percentage of viable, apoptotic and necrotic neutrophils in Dulbecco modified Eagle medium after 24 h of incubation at 37°C. a) Medium only (n=13). b) Nasal lavage fluid (NLF) obtained from infants hospitalised with respiratory syncytial virus bronchiolitis (RSV-NLF) (n=25 samples). c) Infant control NLF (n=19 samples). d) Adult control NLF (n=18 samples). Each sample data point is shown with the median (thick bars) and 95% confidence limits (thin bars) shown for each data set. Each cell count (viable, apoptotic and necrotic) for each sample group (RSV-NLF, infant NLF and adult NLF) was compared to cells grown in medium alone. **: $p<0.01$, obtained by Mann-Whitney tests.

alone (fig. 2b). These changes were also seen in the neutrophils incubated in the NLF from the infant controls, where 76.3% (±5.3) of cells were viable, 20.4% (±3.3) apoptotic and 3.3% (±1.4) necrotic, compared to the medium alone (fig. 2c). However, when incubated in NLF from adults, the patterns were comparable to cells in medium alone, with 33.2% (±2.1) of cells viable, 54.5% (±2.2) apoptotic, and a small but significant decrease to 12.1% (±1.6) necrotic cells (fig. 2d).

Characterisation of the apoptosis-inhibiting activity

Thermal stability. A significant loss in the anti-apoptotic activity was observed when RSV-NLF samples were heated at 90°C for 5 min. In this experiment (n=5), the amount of viable cells in the presence of the RSV-NLF was 85.5% (±10%) and significantly ($p<0.05$) changed to 37.0% (±27) following heating. Apoptotic cells increased significantly ($p<0.05$) from 24.2% (±16) to 53.5% (±20), but the increase in necrotic cells (from 0.8% (±0.8) to 9.8% (±9.0)) was not significant. These levels of viability following heat treatment were comparable to those seen in medium alone.

Repeated freeze thawing of the samples did not change the capacity of RSV-NLF to delay neutrophil apoptosis.

DNAse digestion. Treatment of pooled (n=5) RSV-NLF samples with DNAse did not reduce the anti-apoptotic effects compared to nontreated RSV-NLF (91.2% (±7.0) *versus* 89.2% (±8.7) viable cells).

Diafiltration. Pooled RSV-NLF (n=5) was subjected to diafiltration using an ~3 kDa cut-off pore membrane to establish whether there were low molecular weight constituents of NLF, such as ions, that influenced the rate of apoptosis. The filtrate from the RSV-NLF did not increase the viability of the neutrophils (6.3% (±6.0)), suggesting that the apoptosis inhibiting activity was >3 kDa. The RSV-NLF above the filter supported an increase in neutrophil viability (38.8% (±24.1)) compared to medium alone (17% (±3.6)), but was less active than the unfiltered NLF (viability of 91% (±8.0)).

Effects of blocking LTB_4, GM-CSF, LPS and IL-8

In all of these experiments, a concentration range consistent with effective activity but minimal toxicity was first determined by adding these agents to neutrophils incubated in medium alone.

LTB_4 inhibitor. No decrease in the viability of neutrophils was seen when the LTB_4 inhibitor was added to pooled (n=8) RSV-NLF at 10 μM (79.2% (±8.3)) or 100 μM (85.4% (±13.5)), compared to the effect of the sample alone (74.3% (±16.5)).

GM-CSF inhibitor. A nonsignificant increase in the viability (81.0% (±14.2)) of neutrophils was seen when 100 $\mu g \cdot mL^{-1}$ of the blocking antibody to GM-CSF was added to RSV-NLF (n=8), compared to RSV-NLF alone (72% (±22.1)). No significant differences were observed using the antibody at other concentrations. Nonimmune goat IgG at equivalent concentration also had no affect upon the levels of viability in RSV-NLF.

Polymixin B. LPS (10 $\mu g \cdot mL^{-1}$) alone significantly ($p<0.01$) increased the viability (82.6 (±6.6)) of the neutrophils compared to cells maintained only in medium (33.6 (±14.2)). Polymixin (10 $\mu g \cdot mL^{-1}$) decreased ($p<0.01$) neutrophil viability (66.7% (±9.4)) in the presence of pooled RSV-NLF (n=5), compared to RSV-NLF alone (80.0±(8.6)), but this change was not significant. At this concentration, there were no toxic effect of Polymyxin when added to neutrophils cultured in medium alone (viability 53% (±19.3)), compared to cells in medium alone (42.5 (±16.8)).

CXCR2 (IL-8) receptor inhibitor. IL-8 added to neutrophils from 5–100 $ng \cdot mL^{-1}$ supported a dose-dependent increase in viability and reduced cell death with increasing concentration of the cytokine (fig. 3). In the presence of a low (5 $ng \cdot mL^{-1}$) or a high concentration (100 $ng \cdot mL^{-1}$) of IL-8, the CXCR2 inhibitor (from 0.05–10 μM) showed no reversal of the effects of IL-8. The CXCR2 inhibitor also had no effect on the activity of RSV-NLF, *e.g.* with 100 μM of the inhibitor the viability was 82% (±8.4) *versus* 72.1% (±24.8) for RSV-NLF alone.

Discussion

These results demonstrate that airway secretions from infants significantly delay neutrophil apoptosis, compared to unexposed neutrophils and neutrophils exposed to NLF from healthy adults. The observed effects of the RSV-NLF were not unexpected, since a

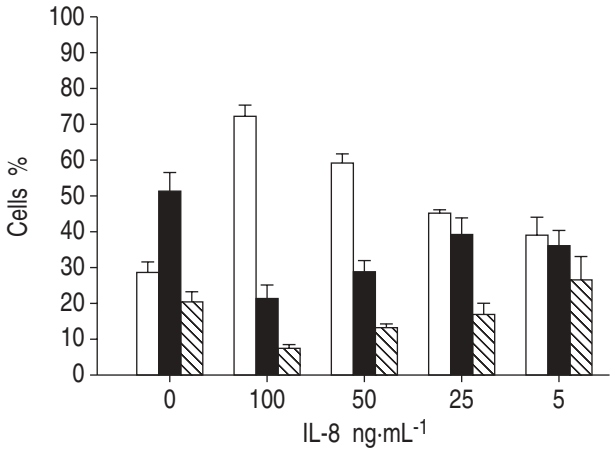

Fig. 3.–Measurement (n=8) of the percentage of viable (□), apoptotic (■) and necrotic (▨) neutrophils in Dulbecco modified Eagle medium after 24 h incubation at 37°C in the presence of increasing concentrations of interleukin (IL)-8. Addition of increasing IL-8 dose resulted in significant differences ($p<0.01$) for each of these viability indices as demonstrated by the Kruskal-Wallis procedure.

number of factors that inhibit neutrophil apoptosis may well be present in airway secretions. However, the equivalent effects of NLF from apparently healthy asymptomatic infants were a surprise. The effects of infant NLF were negated by thermal denaturation, were not reduced by limited proteolysis, and were not caused by small molecular weight constituents, DNA, or LTB_4 and GM-CSF. In the presence of purified IL-8 (>25 ng·mL^{-1}), a dose-dependent decrease in apoptosis was observed, which was consistent with other reports [28]. Such concentrations exceed those measured in RSV-NLF [19] but could reflect localised concentrations at the cell surface due to sequestration of IL-8 by glycosaminoglycans. However, a CXCR2 inhibitor did not block the suppression of apoptosis by IL-8 and had no affect in NLF, although it was tested within a concentration range (50 nM–10 μm) that displaces IL-8 from CXCR2 [28]. This suggests that an IL-8-mediated block on apoptosis is not mediated by signalling through CXCR2, and if this chemokine contributes to the apoptotic suppressing activity of infant NLF, it must mediate its effects through CXCR1.

Whilst other studies have shown anti-apoptotic effects of LTB_4, IL-8, GM-CSF and lipopolysacaride [21, 23], these agents have been studied in isolation *in vitro*. The inability of these inhibitors to significantly affect the prolonged survival of neutrophils incubated with NLF from infants, suggests that *in vivo* this effect may be mediated either by a combination of pro-inflammatory factors or by another as yet unidentified factor. One nonspecific factor that may influence the survival of these neutrophils is the protein concentration of the NLF. Although the median protein content of the adult NLF samples was less than those of the two infant groups, there was considerable overlap at the individual level and no significant correlation between protein concentration and levels of apoptosis were observed for any of the study groups. Furthermore, the current authors have recently developed a defined serum-free medium to support optimal survival of neutrophils. In these experiments, it was found that protein (albumin) concentrations of >2 mg·mL^{-1} were required to enhance survival by ~10%. The median protein concentrations seen in the infant NLF samples were 40-times lower than this (~60 μg·mL^{-1}), while the infant NLF samples increased the neutrophil survival from ~27% to ~77%, indicating that the prolonged survival was not attributable to nonspecific effects of increased protein in the infant samples.

The results indicate prolonged neutrophil survival in infants with RSV bronchiolitis and would therefore appear to contradict the results of WANG *et al.* [24]. However, these two observations may not be contradictory if a prolonged life span results in an eventual increase in the proportion of neutrophils undergoing apoptosis within the airway. WANG *et al.* [24] suggested that accelerated apoptosis could protect the airways by limiting the release of inflammatory products. However, the present study could suggest the opposite, *i.e.* neutrophils accumulate in the airways explaining the increase in neutrophil elastase activity measured in infants particularly those with RSV bronchiolitis [14, 15, 19]. Prolonged neutrophil survival and function could contribute to the symptoms characteristic of RSV infection. Interestingly, studies have indicated that neutrophil chemotaxis in the immediate postnatal period is relatively impaired [29], possibly explaining why acute bronchiolitis is uncommon in the first month of life.

The observation that NLF from apparently healthy infants inhibited apoptosis to a similar degree to RSV-NLF, suggests there are age-related developmental changes promoting inflammatory cell survival in the infant airway. Prolonged survival of these cells could act as a nonspecific defence mechanism aimed at countering the frequent respiratory infections experienced in early life. Further work is required to explore this possibility in more detail and would involve obtaining samples from infants and young children. One, albeit very unlikely, alternative is that all of the healthy control infants were experiencing subclinical infections. The follow-up telephone contacts confirmed that none of these infants were at a preclinical stage of an overt respiratory illness when sampled.

Neutrophilic inflammation is a recognised characteristic response to a number of respiratory viruses [30], and 15–25% of bronchiolitis is due to viruses other than RSV. Therefore, it is likely that no specific immunopathology exists in the airway of infants with RSV bronchiolitis, and the high prevalence of infant bronchiolitis every winter suggests that there is no effective herd immunity to this virus. As a consequence, RSV infects most infants each winter, a minority of whom develop severe lower respiratory tract symptoms requiring hospitalisation.

In summary, nasal lavage fluid obtained from infants, including those with respiratory syncytial virus bronchiolitis, significantly delayed apoptosis in neutrophils. This could account for the characteristic accumulation of these cells in the airways during respiratory syncytial virus bronchiolitis and may also promote a prolonged lifespan for these cells to protect the airway against infection by pathogens entering the healthy infant airway.

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References

1. Kim HW, Arrobio JO, Brandt CD, *et al.* Epidemiology of respiratory syncytial virus infection in Washington DC. 1. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. *Am J Epidemiol* 1973; 98: 216–225.
2. Everard ML. Respiratory syncytial virus bronchiolitis and pneumonia. *In*: Taussig L, Landau L, eds. Textbook of Paediatric Respiratory Medicine. St. Louis, Mosby, 1998.
3. Gilchrist S, Torok TJ, Gary HE Jr, Alexander JP,

Anderson LJ. National surveillance for respiratory syncytial virus, United States, 1985–1990. *J Inf Dis* 1994; 170: 986–990.
4. Clarke SKR, Corner BD, Haines C. Respiratory syncytial virus infection: admissions to hospital in industrial, urban and rural areas. Report to the MRC Subcommittee on Respiratory Syncytial Virus Vaccines. *BMJ* 1978; 2: 796–798.
5. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and re-infection with respiratory syncytial virus. *Am J Dis Child* 1986; 140: 543–546.
6. Fleming DM, Cross KW. Respiratory syncytial virus or influenza. *Lancet* 1993; 342: 1507–1510.
7. Falsey AR, Walsh EE. Respiratory syncytial virus infection in adults. *Clin Microbiol Rev* 2000; 13: 371–384.
8. Pullan CR, Hey EW. Wheezing, asthma and pulmonary dysfunction 10 year after infection with respiratory syncytial virus in infancy. *BMJ* l982; 284: 1665–1669.
9. Stein RT, Sherrill D, Morgan WJ, *et al.* Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13. *Lancet* 1999; 354: 541–545.
10. Everard ML. What link between early respiratory viral infections and atopic asthma. *Lancet* 1999; 354: 527–528.
11. Ruuskanen O, Ogra PL. Respiratory syncytial virus. *Curr Probl Pediat* 1993; 23: 50–79.
12. Everard ML, Swarbrick A, Wrightham M, *et al.* Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. *Arch Dis Child* 1994; 71: 428–432.
13. Smith P, Wang SZ, Dowling K, Forsyth K. Leukocyte populations in respiratory syncytial virus-induced bronchiolitis. *J Paediatr Child Health* 2001; 37: 146–151.
14. Evans GS, Pettitt E, Elphick HE, Jones A, Everard ML. Identification of neutrophil elastase as the major uninhibited protease in the airways of children with RSV positive bronchiolitis. *Am J Respir Crit Care Med* 1999; 159: A189.
15. Evans GS, Pettitt E, Jones A, Elphick HE, Everard ML. Fibronectin degradation is associated with raised neutrophil elastase activity in the upper airways of infants with viral induced wheeze and RSV bronchiolitis. *Eur Respir J* 1999; 14: Suppl. 30, s21.
16. Blyth DI, Pedrick MS, Savage TJ, Bright H, Beesley JE, Sanjar S. Induction, duration, and resolution of airway goblet cell hyperplasia in a murine model of atopic asthma: effect of concurrent infection with respiratory syncytial virus and response to dexamethasone. *Am J Respir Cell Mol Biol* 1998; 19: 38–54.
17. Becker S, Quay J, Soukup J. Cytokine (tumor necrosis factor, IL-6, IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J Immunol1991*; 147: 4307–4312.
18. Arnold R, Humbert B, Werchau H, Gallati H, Konig W. Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type 1 release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. *Immunology* 1994; 82: 126–133.
19. Abu-Harb M, Bell F, Rao WH, *et al.* IL-8 and neutrophil inflammatory mediators in the upper respiratory tract of infants with RSV bronchiolitis. *Eur Respir J* 1999; 14: 150–154.
20. Schultz C, Richter N, Moller JC, Bucsky P. IFN-gamma response and IL-8 plasma levels in neonates with respiratory syncytial virus bronchiolitis. *Eur Respir J* 2000; 17: 321–324.
21. Lee A, Whyte MK, Haslett C. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukocyte Biol* 1993; 54: 283–288.
22. Fadok VA, Savill JS, Haslett C, *et al.* Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognise and remove apoptotic cells. *J Immunol* 1992; 149: 4029–4035.
23. Sampson AP. The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* 2000; 30: Suppl. 1, 22–27.
24. Wang SZ, Smith PK, Lovejoy M, Bowden JJ, Alpers JH, Forsyth KD. The apoptosis of neutrophils is accelerated in respiratory syncytial virus (RSV)-induced bronchiolitis. *Clin Exp Immunol* 1998; 114: 49–54.
25. Noah TL, Henderson FW, Henry MM, Peden DB, Develin RB. Nasal lavage cytokines in normal, allergic and asthmatic school-age children. *Am J Respir Crit Care Med* 1995; 152: 1290–1296.
26. Smith JD, Cortes NJ, Evans GS, Read RC, Finn A. Induction of β2 integrin-dependent neutrophil adhesion to human alveolar epithelial cells by type 1 Streptococcus pneumoniae and derived soluble factors. *J Inf Dis* 1998; 177: 977–985.
27. Pisetsky DS. Antibody responses to DNA in normal immunity and aberrant immunity. *Clin Diagn Lab Immunol* 1998; 5: 1–6.
28. White JR, Lee JM, Young PR, *et al.* Identification of a potent, selective nonpeptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration. *J Biol Chem* 1998; 273: 10095–10098.
29. Carr R, Pumford D, Davies JM. Neutrophil chemotaxis and adhesion in preterm babies. *Arch Dis Child* 1992; 67: 813–817.
30. Turner RB. The role of neutrophils in the pathogenesis of rhinovirus infections. *Ped Inf Dis J* 1990; 9: 832–835.