Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus


ABSTRACT: The mechanisms by which respiratory syncytial virus (RSV) infection induces bronchiolitis and airway disease are unclear. The presence of large numbers of polymorphonuclear leukocytes (PMN) in the airways of infants with RSV infection suggests a potential role of PMN in airway injury associated with RSV infection.

To investigate the potential role of neutrophils in RSV bronchiolitis, human alveolar type II cells (A549 cells) were infected with different doses of RSV for 6–48 h. A 51Cr-releasing assay was used to measure PMN-induced damage and image analysis was used to determine PMN adhesion and detachment of epithelial cells.

The results showed that RSV infection of epithelial cells enhanced PMN adherence in a dose- and time-dependent pattern, RSV infection alone could damage and detach epithelial cells to a limited extent and PMN significantly augmented RSV infection-induced damage and detachment of epithelial cells.

These data suggest that respiratory syncytial virus infection of respiratory epithelial cells enhances neutrophil adhesion to the epithelium and that activated neutrophils augment the damage and detachment of epithelium infected with the virus. Polymorphonuclear leukocytes may contribute to the pathogenesis of respiratory syncytial virus airway disease by inducing epithelial damage and cell loss.

37°C in a humidified incubator in an atmosphere of 5% CO₂/air, the cells in the chambers or wells formed confluent monolayers, numbering about 1.5×10⁵ in each chamber or well.

**Study design**

For the PMN adhesion study, the confluent epithelial cells were infected with different doses of RSV (multiplicity of infection (MOI) of 0.01, 0.1 and 1.0) for 6, 24 and 48 h. PMN were then added to the chamber slides with RSV-infected epithelial cells and co-cultured for 30 min. The number of PMN adhered to epithelial cells was determined with image analysis. On the basis of the adhesion study, confluent epithelial cells were infected with different doses of RSV for 24 h for both the cytotoxicity and detachment studies. Thereafter, PMN were added to the wells and co-cultured up to different time points (from 2 to 48 h). A ⁵¹Cr-releasing assay and image analysis were used to determine PMN-induced leakage and detachment of epithelial cells, respectively. The following three controls were used in the experiments: culture medium, sterile A549 cell debris and inactivated RSV (RSVi).

**Virus preparation**

The characterized long strain of RSV was originally obtained from P. Young (Sir Albert Sakzewski Virus Centre, Royal Children’s Hospital, Brisbane, Australia). The virus was propagated in A549 cells. The virus-infected cells were harvested, sonicated and stored in aliquots in liquid nitrogen. The titre was determined by median tissue culture infective dose (TCID₅₀). TCID₅₀ end-point was determined by the method of Reed and Muench [11]. The titres of RSV were 10⁷ TCID₅₀ units·mL⁻¹. Control sonicates from uninfected A549 cells were processed in the same manner as the RSV samples.

**Viral infection of epithelial cells**

Different doses of RSV (MOI of 0.01, 0.1 and 1.0) were used in the infection of epithelial cells. RSV diluted in DMEM plus 2% FCS were added separately to the confluent A549 monolayers in each chamber of eight-chamber plates (for adhesion or detachment experiments) or one well of 48-well plates (for leakage experiments) for 2 h in the incubator at 37°C in humidified 5% CO₂/air (to allow virus adsorption). The supernatant was removed and all chambers or wells were washed once with DMEM. Then, 0.25 mL DMEM plus 2% FCS was added to each chamber or well and cultured to specific time points in the incubator.

**Controls for RSV infection**

In an attempt to clarify the true contribution of RSV infection, three controls were used: 1) Culture medium, which was maintained free of viruses. 2) Sterile A549 cell debris, sonicated A549 cell debris was diluted into a series of doses or concentrations as equivalent MOI 0.01, 0.01 and 1.0, which were determined according to the number of cells used for culturing the same dose of live RSV. The culture process and conditions were the same as for live RSV. 3) RSVi, the virus samples were rendered noninfec-
tious by ultraviolet (UV) light [12]. In brief, an RSV suspension in a six-well cluster tray, which had been titrated before inactivation and dilutions had been determined from this titre, was exposed to an intense UV light source for 5 min (UV Box, 4×15 W germicidal lamps set at a distance of 8 cm to give a flux density of 6.7×10^4 erg·cm⁻²·s⁻¹; Flinders Medical Centre, SA, Australia). The lack of infectivity of these preparations was confirmed by cultivating them in A549 monolayers and observing the absence of viral cytopathic effect (CPE) for 5 days. The dilutions for RSVi were made that corresponded to those used with live virus. The culture process and conditions were the same as for live RSV.

**Preparation of neutrophils**

Immediately before each experiment, peripheral blood neutrophils from normal, healthy volunteers were isolated by Lymphoprep (Hycomed Pharma AS, Oslo, Norway) and 3% dextran (T500; Pharmacia Biotech, Uppsala, Sweden) sedimentation techniques [13]. Residual erythrocytes in the granulocyte-rich fraction were eliminated by hypotonic lysis in 0.2% sodium chloride twice for 20 s each time. This resulted in a cell fraction containing >97% neutrophils with >97% viability as determined by trypan blue exclusion. Neutrophils were suspended at appropriate concentrations in suitable media and were put on a rocking platform at room temperature until use.

**Neutrophil adherence assay**

At 6, 24 and 48 h after RSV infection of the epithelial cells, the supernatants in the chambers of RSV infected cells plus the three controls (negative, A549 cell debris and RSVi) were removed and the chambers were washed twice with 0.25 mL DMEM. Then, 6×10⁵ PMN suspended in DMEM were added to each chamber and incubated with the epithelial cells for 30 min (derived from time course studies) at 37°C in the incubator. As phorbol myristate acetate (PMA; Sigma Chemical Co., St Louis, MO, USA) is a potent PMN stimulator, uninfected epithelial cell monolayers plus PMN plus PMA (final concentration 50 ng·mL⁻¹, derived from dose course studies) served as a positive control [14, 15].

The nonadherent neutrophils were removed by repeated immersion of the plates (after the chamber walls have been removed) in phosphate-buffered saline (PBS) (20 times per culture) kept at 37°C [16]. The plates were air dried for at least 10 min in the dark (myeloperoxidase is inactivated by light), fixed for 30 s in commercial 37% formaldehyde, diluted 1:9 with 95% ethanol, washed for 2 min in deionized water, then air dried again for at least 10 min in the dark [17]. The plates were incubated in the dark for 6 min at 24°C in the incubation medium (0.05% 3′,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma), 0.06% hydrogen peroxide in 38 mM Tris-HCl buffer, pH 7.4) [18]. After being washed in water, the plates were air dried again. The number of neutrophils within six fields of each chamber were counted utilizing image analysis (Videopro; Leading Edge Pty, Adelaide, SA, Australia) by microscopy. The average number of neutrophils per square millimetre was determined.

**Cytotoxicity (leakage) assay**

Neutrophil-induced cytotoxicity was measured by a modified ⁵¹Cr-release assay [14]. After A549 cells had...
reached confluence, RSV (MOI 0.01, 0.1 and 1.0) in 0.25 mL DMEM plus 2% FCS was added to each well of 48-well plates. After 16 h of incubation, 1 μCi Na2CrO4 (51Cr; ICN Pharmaceuticals, Irvine, CA, USA; counted as total load of 51Cr, (T)) was added per well and the cells were incubated for an additional 8 h (total time for infection=24 h). Supernatants from some wells were collected and each well was washed twice with 0.25 mL DMEM. The supernatants and washing medium were counted together (as unloading 51Cr, (U)). Then, 6×10⁴ of neutrophils in 0.25 mL DMEM plus 0.5% bovine serum albumin (BSA; fraction V powder; Sigma) were added to each well and cocultured to different time points (12, 16 and 20 h). After the plates (with neutrophils) had been incubated at 37°C in 5% CO2 for 15 min, during which time the neutrophils sedimented onto the epithelial cell monolayers [14], neutrophil activator PMA (at a final concentration of 50 ng·mL⁻¹, diluted in DMEM) was gently added to each PMN well (epithelial cells, PMN and PMA) in a volume of 0.05 mL. At the same time, 0.05 mL DMEM was added to every other well.

As PMA had been shown to have the strongest stimulating effect on PMN-induced cytotoxicity [14], the PMN wells (uninfected epithelial cells plus PMN plus PMA) were taken as positive controls. Uninfected epithelial cells without PMN were used as negative controls and baseline. The effects of uninfected epithelial cells plus PMN were also investigated. After 12, 16 or 20 h of co-culture with PMN, supernatants were collected and each well was washed once with 0.25 mL DMEM (counted as leaked 51Cr, (L)).

The amount of 51Cr radioactivity of each sample was measured. After 8 h of loading, the loaded or bound 51Cr (B) to cells in each well is: B= T-U. Using this information, the percentage of specific 51Cr release from RSV-infected cells was calculated by: percentage of specific 51Cr release=[L-C]/([B-C]×100, where L is the counts per minute (cpm) leaked into the medium of the test sample, C is the baseline cpm released from the negative control and B is the total cpm initially bound or loaded to the cells at the beginning of the experiment [14].

**Detachment assay**

After the A549 cells had reached confluence, RSV (MOI 0.01, 0.1 and 1.0) in 0.25 mL DMEM plus 2% FCS was added to the confluent A549 monolayers in each chamber of eight-chamber plates and cultured for 2 h at 37°C (to allow virus adsorption). The supernatant was removed and all chambers were washed once with DMEM. Then, 0.25 mL DMEM plus 2% FCS was added to each chamber and cultured up to 24 h at 37°C in a humidified incubator in an atmosphere of 5% CO2/air. After 24 h of RSV infection, supernatants were removed and each chamber was washed twice with 0.25 mL DMEM. Then, 6×10⁴ of neutrophils in 0.25 mL DMEM plus 0.5% BSA were added to each chamber and incubated for periods of up to 20 h at 37°C. Uninfected epithelial cells cultured in DMEM plus 0.5% BSA were used as negative controls. Uninfected epithelial cells plus neutrophils plus PMA in a final concentration of 50 ng·mL⁻¹ were used as positive controls.

The slides bearing the monolayers were removed from the chamber, washed gently in PBS at 37°C, dried in air, then fixed and stained with a quick Giemsa stain. Detachment was assessed with an image-analysis system by microscopy. The whole area and the undetached area of a field were counted, then the detached area in one field was estimated. Six fields from each chamber were analysed. The detached area was determined as the mean detached percentage (%).

**Statistical analysis**

Values are reported as mean±standard error (±). Data were analysed using a one-way analysis of variance (ANOVA). A Bonferroni t-test (making allowance for multiple comparisons) was used to identify differences between individual groups. A p-value <0.05 was considered significant.

**Results**

**PMN adhesion to RSV-infected respiratory epithelial cells**

In preliminary studies, the PMN adherence with different numbers of PMN (0.75×10⁵, 1.5×10⁵, 3×10⁵, 6×10⁵ and 12×10⁵ PMN-chamber⁻¹), which were co-cultured with the respiratory epithelial cells for 30 min, with both negative and positive controls (PMA), was investigated. The PMN adherence increased almost linearly with increasing number of PMN in each chamber from 0.75×10⁵ to 6×10⁵. This linearity reached a plateau between 6×10⁵ and 12×10⁵ in the PMA control wells (data not shown). Therefore, 6×10⁵ PMN were added into each chamber in the adhesion experiments.

RSV infection increased the number of adhered PMN in epithelial cell cultures. Significantly more PMN were adherent to RSV-infected A549 cells than to uninfected A549 cells, including the negative control and A549 cell debris control at most time and dose points (figs. 1 and 2). At 48 h of infection with RSV at MOI of 0.1, the PMN adher-
ence to RSV infected epithelial cells was also significantly greater than that to epithelial cells pretreated with inactivated RSV (RSVi) (fig. 1). The PMN adhesion to RSV-infected A549 cells increased in a dose- and time-dependent pattern. However, at 48 h, the PMN adhesion to infected cells (with 1.0 MOI of RSV) became lower than that at the 24 h time point. In these chambers, the RSV infection-induced CPE was very marked and some monolayers had detached. Hence, the lower adhesion rates at 48 h (1.0 MOI of RSV) are likely to be artefactual, owing to epithelial monolayer detachment.

RSVi increased PMN adherence, but to a lesser extent than active RSV. The PMN adherence to A549 cells infected with RSV (MOI=1.0) became lower than that at the 24 h time point. In these chambers, the RSV infection-induced CPE was very marked and some monolayers had detached. Hence, the lower adhesion rates at 48 h (1.0 MOI of RSV) are likely to be artefactual, owing to epithelial monolayer detachment.

Neutrophil-induced cytotoxicity (leakage) to RSV-infected epithelial cells

In preliminary studies, PMN-induced damage using the chromium release assay was investigated at 2, 4, 6, 8 and 10 h, and 12, 16, 20, 24, 36 and 48 h of co-culture. The effect of different PMN numbers (0.75×10⁵, 1.5×10⁵, 3×10⁵ and 6×10⁵ PMN-chamber⁻¹) was also studied with different RSV doses (MOI 0.01, 0.1 and 1.0). From RSV co-culture time 2 to 10 h, there was almost no leakage change in either the negative control (cell monolayers only and cell monolayers+PMN) or RSV infection (+PMN) and there was only a slight change in the positive control (PMN+PMA). The significant changes occurred from 16 to 20 h of PMN co-culture, after 24 h of RSV infection and with 6×10⁵ PMN-chamber⁻¹ (data not shown). Therefore, the effects of 6×10⁵ PMN-chamber⁻¹ were investigated at 12, 16 and 20 h of PMN co-culture after 24 h of RSV infection with different MOI.

Neither PMN or PMA alone could increase the specific leakage of ⁵¹Cr at each co-culture time point (12, 16 and 20 h). RSV infection alone induced low levels of ⁵¹Cr release from the epithelial cells, particularly with 0.1 and 1.0 MOI of RSV at 16 and 20 h time points, but these effects...
Confluent A549 monolayers grown in 8-chamber plates were infected with RSV at a multiplicity of infection (MOI) of 0.01 (B1, B2), 0.1 (C1, C2) and 1.0 (D1, D2). A1 is just the monolayer as control. A2 is monolayer plus PMN. B2, C2 and D2 are monolayers infected with increasing doses of RSV plus PMN. With increasing MOI of RSV without added PMN (column 1) there are slight increases in epithelial cell loss. With added PMN (column 2) there is marked augmentation of epithelial cell loss. (Internal scale bars = 25 µm.)

Fig. 5. – Polymorphonuclear leukocyte (PMN)-induced detachment of epithelial cell monolayers infected with respiratory syncytial virus (RSV). Confluent A549 monolayers grown in 8-chamber plates were infected with RSV at a multiplicity of infection (MOI) of 0.01 (B1, B2), 0.1 (C1, C2) and 1.0 (D1, D2). A1 is just the monolayer as control. A2 is monolayer plus PMN. B2, C2 and D2 are monolayers infected with increasing doses of RSV plus PMN. With increasing MOI of RSV without added PMN (column 1) there are slight increases in epithelial cell loss. With added PMN (column 2) there is marked augmentation of epithelial cell loss. (Internal scale bars = 25 µm.)
were not statistically significant. Neutrophils, however, induced significant $^{51}$Cr release in the RSV-infected epithelial cells in a RSV dose- and time-dependent pattern. The effect of PMN at the 20 h co-culture time point with RSV infection at MOI of 1.0 was most significant (fig. 3).

**Effect of neutrophils on the detachment of RSV-infected epithelial cell monolayers**

In preliminary studies, the effect of PMN (6×10$^5$ PMN·chamber$^{-1}$) (as suggested in the cytotoxicity assay) was also investigated with different RSV doses (MOI 0.01, 0.1 and 1.0) at 8, 12, 16, 20 and 24 h of co-culture. It was found that 20 h of co-culture with PMN was the best time point at which to investigate detachment, as at 24 h there was too much detachment at MOI 1.0 of RSV plus PMN after the slides had been washed and stained.

At 20 h of co-culture, PMN or PMA alone could not detach epithelial cells significantly. RSV infection alone detached epithelial cell monolayers significantly in a dose-dependent pattern. Neutrophils markedly augmented RSV infection-induced detachment, even at 0.01 MOI of RSV. The highest percentage of detachment (81.9±2.3%) was seen with 1.0 MOI of RSV, co-cultured with PMN for 20 h (figs. 4 and 5).

**Discussion**

These results show that RSV infection of respiratory epithelial cells increased PMN adherence to the epithelial cells in a dose- and time-dependent pattern. Neutrophils were found to augment RSV infection-induced damage and detachment of epithelial cells.

The data show that RSV infection of A549 cells increased PMN adherence to the cells in a dose-dependent manner. These results suggest that RSV infection could increase PMN adherence to epithelial cells even at a very low dose of RSV. The inoculation of RSV used in these studies approximates to that found clinically and is less than that used in another recent study [9]. There are no published data on the viral load encountered in vivo in infants with RSV infection. A recent study by the present authors (unpublished data) indicated that at the time of presentation to the hospital, viral loads in the upper respiratory tract are falling and are broadly comparable to the MOI used in this study. In a previous study, RSV infection of A549 cells, with 3–5 plaque-forming units (PFU)·cell$^{-1}$ and after 2–3 days of incubation, was found to increase PMN adherence to the epithelial cells [9], but whether the increased PMN adherence was dose-dependent or time-dependent was not examined.

RSV infection of A549 cells increased PMN adherence in a time-dependent manner, as the duration of infection increased from 6 to 24 to 48 h. These results indicate that enhanced PMN adherence to infected epithelial cells could occur in a very early stage of infection, but PMN adherence is enhanced with longer duration of infection. The kinetics of RSV growth varies considerably with RSV strain, cell type, MOI and other factors [19]. In A549 cells, the kinetics of RSV growth and the expression of adhesion molecules on the cell surface after RSV infection are unclear. However, in Hep-2 cells infected with 2–5 MOI of RSV strain A2, the synthesis of viral proteins and ribonucleic acid (RNA) can be detected by 2–6 h after infection, and progeny virus by 10–12 h [19].

Inactivated RSV also increases neutrophil binding. This increase was less than that observed with active RSV. These results indicate that inactivated or dead RSV could also affect the interaction between PMN and epithelial cells by some means at an early stage. Up to now, ICAM-1 is the only known ligand on respiratory epithelial cells for neutrophil binding. However, anti-ICAM-1 monoclonal antibody (mAb) inhibited PMN adhesion to the epithelial cells by only 30% [9]. Therefore, it was postulated that a non-ICAM-1 ligand for PMN was present on the epithelial cell cultures [9, 20]. Because the enhancement of ICAM-1 expression required an infectious virus [12], some RSV proteins or antigens from inactivated RSV may stimulate epithelial cells and induce other adhesion molecule expression. However, because RSV infection of epithelial cells induces interleukin (IL)-1 release that stimulates ICAM-1 expression [12] and this cytokine is not destroyed by UV irradiation, the increased PMN adhesion by UV-inactivated RSV (containing RSV-infected-A549 cell debris) may be cytokine dependent.

Neutrophil-mediated cytotoxicity is most efficient under conditions of cell-to-cell adhesion [21]. Therefore, neutrophil adhesion to respiratory epithelium is not only a crucial early event in the initiation of inflammatory reactions, but also important in retaining neutrophils at the sites of inflammation and in contributing to their effector functions [22–24]. However, it has been unclear previously whether the airway neutrophilia observed in RSV infection is primarily protective or damaging.

Our results showed not only that RSV infection could increase neutrophil adherence to RSV-infected epithelial cells, but also that neutrophils could augment RSV infection-induced damage to epithelial cells in a dose and time-dependent pattern. Such results imply that neutrophils play a role in the airway epithelial damage that occurs in RSV infection. The $^{51}$Cr-release assay was used to determine the cytotoxicity induced by RSV infection and PMN. The $^{51}$Cr-release assay has been widely used to assess damage to respiratory epithelial cell cultures [14, 25, 26].

Neutrophils may contribute to damage of airway epithelial cells in many inflammatory conditions [14]. Previous studies have found that human neutrophils, stimulated by PMA, kill monolayers of rat alveolar type II cells, by a process that does not require neutrophil-generated reactive oxygen metabolites; however, pretreatment of neutrophils with an antibody (anti-Mo1) that reduced neutrophil adherence to epithelial cells limits killing [14]. These observations support the view that neutrophil adhesion to epithelial cells is an important prerequisite for neutrophils to induce damage to epithelial cells.

RSV infection has previously been shown to induce airway epithelial cells to produce cytokines such as IL-8, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) that have effects on neutrophils as chemokines and/or activators [27, 28]. Therefore, as RSV interacts with the airway epithelium, it may not only cause direct CPE, but also may enhance local injury through stimulation of adhesion molecules and cytokines to promote neutrophil-induced airway epithelial damage [9].

In addition to damage, the present results also showed that RSV infection alone could detach epithelial cell monolayers in a dose-dependent pattern and neutrophils augmented RSV infection-induced detachment very significantly. These results are consistent with clinical findings.
(data not shown) which show that there are significant numbers of detached epithelial cells in the nasal washing from infants infected with RSV. This contrasts with control infants, who have very few detached epithelial cells from nasal lavages.

Two previous studies have investigated PMA-activated neutrophil-induced epithelial damage. Neutrophils activated by PMA induced significant detachment (29% detachment) and damage (18% release of 51Cr) of epithelial cells [29]. A recent study [30] investigating the role of tetradecanoyl phorbol acetate (TPA)-induced neutrophil activation showed that detachment was increased when the neutrophils were activated in situ with TPA and after longer incubation periods. Some cytokines, such as IL-8, produced and released from RSV-infected epithelial cells [27], may activate neutrophils and lead to damage and detachment of respiratory epithelial cells. Clearly, further studies need to be performed to characterize the mechanisms of PMN-induced epithelial damage and detachment in RSV infection.

In summary, it is hypothesized that neutrophils play a significant role in the pathology of respiratory syncytial virus airway disease. The virus activates neutrophils, enhancing neutrophil adhesion to the epithelium. The activated neutrophils also damage the epithelium, inducing cell loss.

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References

3. Holberg CJ, Wright AL, Martinez FD. Risk factors for respiratory syncytial virus airway disease. The virus activates neutrophils, enhancing neutrophil adhesion to the epithelium. The activated neutrophils also damage the epithelium, inducing cell loss.

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