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

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Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus. S-Z. Wang, H. Xu, A. Wraith, J.J. Bowden, J.H. Alpers, K.D. Forsyth. ©ERS Journals Ltd 1998.

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 6–48h. A ^{51}Cr-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.

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Respiratory syncytial virus (RSV) causes significant respiratory disease, infecting almost all children during the first 2 yrs of life [1, 2]. RSV is the most frequent cause of bronchiolitis and pneumonia in infants requiring hospitalization [3]. Moreover, at least 50% of infants who have acute viral bronchiolitis due to RSV have subsequent episodes of wheezing consistent with asthma [4].

The mechanism of airflow obstruction in RSV bronchiolitis is poorly understood, although current evidence suggests that the cellular immune response contributes to airway injury [5]. Lymphocytes are accorded the dominant role in the pathogenesis of RSV infection based on animal studies [5]. The role that neutrophils or polymorphonuclear leukocytes (PMN) may play in RSV disease has not been well studied. In calf models of RSV infection, PMN were closely associated with RSV-infected epithelial cells and evidence of PMN fusion with infected epithelial cells was seen [6]. In infants with RSV infection, PMN accounted for 93% of inflammatory cells in the upper airway recovered by nasopharyngeal aspirates and 76% of inflammatory cells in the lower airway recovered by bronchial lavage [7]. Most recently, it was reported that RSV-inoculated guinea-pigs had significantly increased bronchiolar PMN infiltrates [8]. These observations suggest that RSV infection may provide the necessary stimulus for PMN migration to inflammatory sites in airways and that PMN may play an important role in the pathogenesis of RSV infection.

It was speculated that PMN may augment injury to airway epithelium induced by RSV. The aim of this study was to investigate the interrelationship between PMN and RSV-infected epithelial cells. The specific goals were to determine whether RSV infection of respiratory epithelial cells could increase PMN adherence in a dose- and time-dependent pattern and to investigate whether PMN contribute to the pathogenesis of RSV disease by inducing epithelial damage and cell loss.

Materials and methods

Epithelial cell culture

A549, an immortalized human alveolar type II epithelial cell line (American Type Culture Collection, Rockville, MD, USA), was selected as a source of respiratory epithelial cell for these studies [9, 10] because of its standardized use as a respiratory epithelial cell line and its low baseline expression of intercellular adhesion molecule-1 (ICAM-1; a ligand for neutrophil binding to epithelial cells). A549 cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco BRL, Life Technologies, New York, USA), with high glucose, L-glutamine and 5% foetal calf serum (FCS). One chamber (1 cm^2) of eight-chamber plates (Nunc, Naperville, IL, USA) or one well (1 cm^2) of 48-well plates (Costar Co., Cambridge, MA, USA) was seeded with 0.75×10^5 cells. After 24–36 h of culture at

37°C in a humidified incubator in an atmosphere of 5% CO_2/air, the cells in the chambers or wells formed confluent monolayers, numbering about 1.5×10^5 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 ^{51}Cr-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 ($TCID_{50}$). $TCID_{50}$ end-point was determined by the method of Reed and Muench [11]. The titres of RSV were 10^7 $TCID_{50}$ 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 % CO_2/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 noninfectious 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 culturing 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, 6×10^5 PMN suspended in DMEM were added into 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 ^{51}Cr-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 Na_2CrO_4 (^{51}Cr; ICN Pharmaceuticals, Irvine, CAm, USA; counted as total load of ^{51}Cr, (*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 unloaded ^{51}Cr, (*U*)). Then, 6×10^5 of neutrophils in 0.25 mL DMEM plus 0.5% bovine serum albumin (BSA; fraction V powder; Sigma) were added to each well and co-cultured to different time points (12, 16 and 20 h). After the plates (with neutrophils) had been incubated at 37°C in 5% CO_2 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 PMA 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 PMA 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 ^{51}Cr, (*L*)).

The amount of ^{51}Cr radioactivity of each sample was measured. After 8 h of loading, the loaded or bound ^{51}Cr (*B*) to cells in each well is: $B= T-U$. Using this information, the percentage of specific ^{51}Cr release from RSV-infected cells was calculated by: percentage of specific ^{51}Cr release=$(L-C)/(B-C)\times100$, 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% CO_2/air. After 24 h of RSV infection, supernatants were removed and each chamber was washed twice with 0.25 mL DMEM. Then, 6×10^5 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 (SE). 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 (0.75×10^5, 1.5×10^5, 3×10^5, 6×10^5 and 12×10^5 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 0.75×10^5 to 6×10^5. This linearity reached a plateau between 6×10^5 and 12×10^5 in the PMA control wells (data not shown). Therefore, 6×10^5 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-

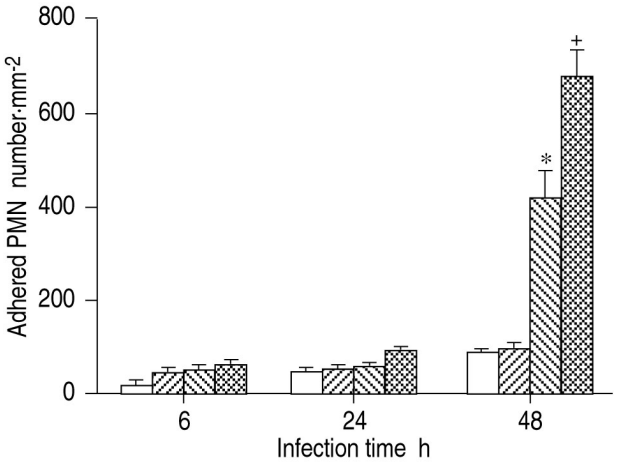

Fig. 1. – Polymorphonuclear leukocyte (PMN) adherence to epithelial cells infected with respiratory syncytial virus (RSV) at a multiplicity of infection (MOI) of 0.1. Comparison of PMN adherence to A549 monolayers infected with RSV (▩ ; MOI=0.1) or pretreated with inactivated RSV (RSVi; ▨) and A549 cell debris (▨) (with equivalent MOI= 0.1) at different time points (6, 24 and 48 h). □ : control. The data were compiled from four experiments and values are expressed as mean±SE. *: $p<0.05$, compared with both control group and A549 group; +: $p<0.05$, compared with control group, A549 group and RSVi group.

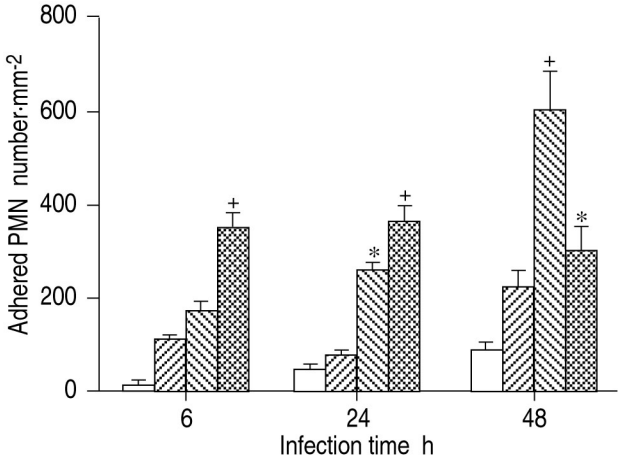

Fig. 2. – Polymorphonuclear leukocyte (PMN) adherence to epithelial cells infected with respiratory syncytial virus (RSV) at a multiplicity of infection (MOI) of 1.0. Comparison of PMN adherence to A549 monolayers infected with RSV (▩ ; MOI=1.0) or pretreated with inactivated RSV (RSVi; ▨) and A549 cell debris (▨) (with equivalent MOI= 1.0) at different time points (6, 24 and 48 h). □ : control. The data were compiled from four experiments and values are expressed as mean±SE. *: p<0.05, compared with control group; +: p<0.05, compared with both control group and A549 group. The lower number of adherent neutrophils at 48 h in the active RSV group is likely to be due to epithelial detachment.

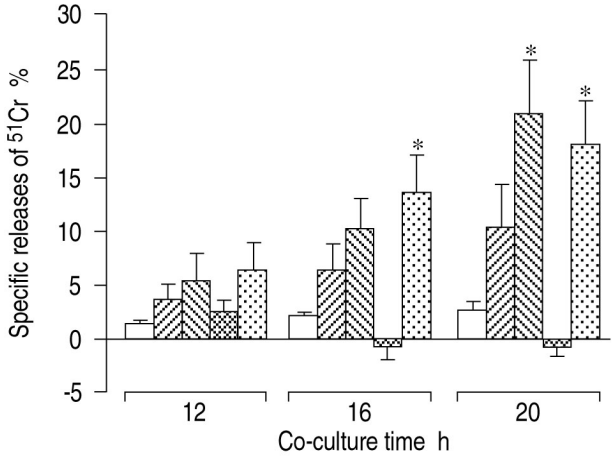

Fig. 3. – Polymorphonuclear leukocyte (PMN)-induced specific leakage of epithelial cells in respiratory syncytial virus (RSV) infection. PMN-induced specific leakage of radiolabelled chromium (^{51}Cr) in A549 cells infected with RSV (multiplicity of infection=1.0) at various co-culture time points (12, 16 and 20h). □ : PMN; ▨ : RSV 1.0; ▨ : RSV 1.0+PMN; ▩ : phorbol myristate acetate (PMA); ⬚ : PMA+ PMN. The data shown are the specific leakage (the background from the negative control has been subtracted). The data were compiled from seven experiments and values are expressed as mean±SE. *: p<0.05, treatment groups *versus* PMN groups.

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-in-fected 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 pretreated with RSVi was also significantly increased compared with the negative controls and A549 cell debris control at same time and dose points. However, it was still lower than that to RSV-infected epithelial cells at most time and dose points (figs. 1 and 2).

Sterile A549 cell debris did not increase PMN adherence significantly at all time and dose points, compared with the negative controls.

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 (0.75×10^5, 1.5×10^5, 3×10^5 and 6×10^5 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 6×10^5 PMN·chamber^{-1} (data not shown). Therefore, the effects of 6×10^5 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 ^{51}Cr at each co-culture time point (12, 16 and 20 h). RSV infection alone induced low levels of ^{51}Cr 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

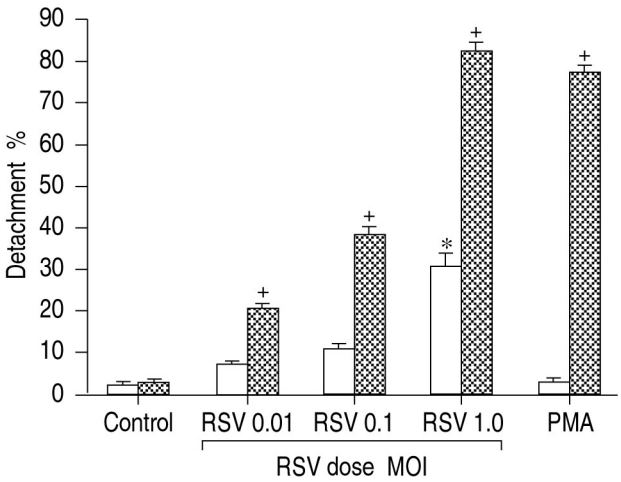

Fig. 4. – Polymorphonuclear leukocyte (PMN)-induced detachment of epithelial cells infected with respiratory syncytial virus (RSV). PMN-induced detachment of A549 monolayers infected with various doses of RSV (multiplicity of infection (MOI) 0.01, 0.1 and 1.0) at 20 h of co-culture. □ : negative; ▩ : PMN. The data were compiled from four experiments and values are expressed as mean±SE. *: p<0.05, RSV groups *versus* control; +: p<0.05, RSV or phorbol myristate acetate (PMA) plus PMN groups *versus* both control and RSV or PMA groups

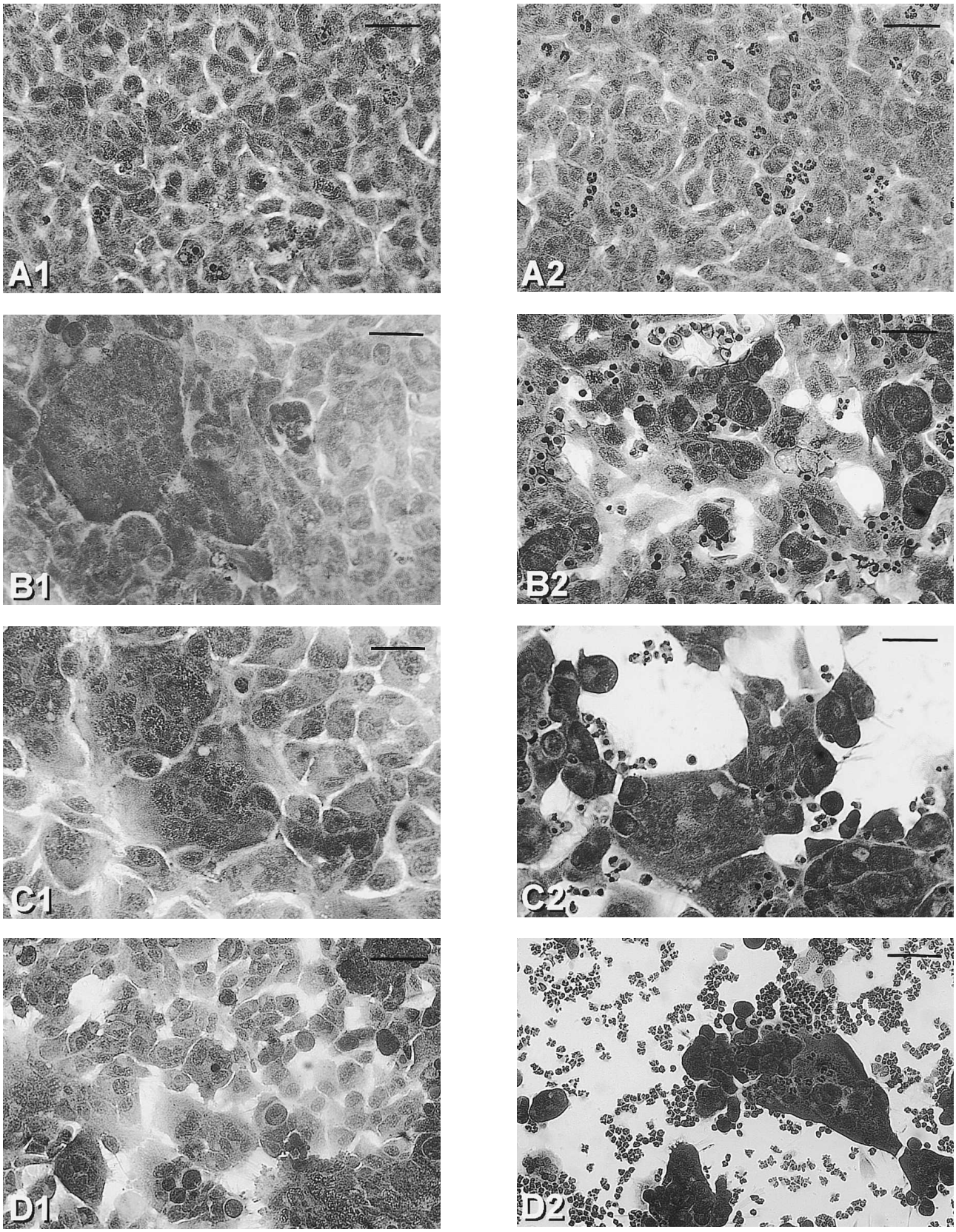

Fig. 5. – Polymorphonuclear leukocyte (PMN)-induced detachment of epithelial cell monolayers infected with respiratory syncytial virus (RSV). Confluent A549 monolayers grown in 8-chamber plates were infected with RSV at a multiplicity of infection (MOI) of 0.01 (B1, B2), 0.1 (C1, C2) and 1.0 (D1, D2). 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 ^{51}Cr) 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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