

An ultrastructural study of the interaction of human eosinophils with respiratory syncytial virus

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It was shown previously that eosinophils are activated *in vivo* and *in vitro* by respiratory syncytial virus (RSV) (Garofalo et al., J Pediatr 1992; 120: 28–32; Kimpen et al., Pediatr Res 1992; 32: 160–4). For study of the interaction of eosinophils and RSV on the ultrastructural level, normodense eosinophils were purified from peripheral blood of healthy human volunteers. After incubation with RSV in the presence or absence of autologous serum, the eosinophils were examined with immunofluorescence microscopy employing an RSV-specific monoclonal antibody, and with transmission electron microscopy. After 2-h incubation in the presence of live RSV, $25.6 \pm 12.9\%$ of the eosinophils demonstrated positive fluorescence. This increased to $62.8 \pm 8.9\%$ when fresh autologous serum was added during incubation ($P = 0.015$). The effect was abolished when the serum was heat-treated, indicating Fc-receptor-independent enhancement of viral uptake by the cells. In transmission electron microscopy, virions were seen in phagocytic vacuoles at the periphery of the cells. Eosinophil activation characterized by piecemeal degranulation was evident. In conclusion, activation of eosinophils during RSV bronchiolitis probably occurs in part by direct interaction of the cells with the virus.

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Respiratory syncytial virus (RSV) is the major cause of acute lower respiratory tract disease of infants and young children (1). Patients with congenital heart disease or bronchopulmonary dysplasia are most severely affected (2, 3). RSV bronchiolitis has been implicated as a possible cause of hyperreactive airway disease later in childhood (4). Both specific and nonspecific immune mechanisms contribute to the intense inflammation of the terminal bronchioles during RSV infection, culminating in the clinical and pathologic picture of bronchiolitis (5–7). Most research to date has focused on the role of RSV-specific antibodies and cytotoxic T cells in the clearance of the virus from the lungs and the pathogenesis of RSV-induced illness. Indeed, pathologic examination of lungs of children dying of severe bronchiolitis shows an intense mononuclear cell infiltrate around the terminal bronchioles and in the interstitium (8, 9).

The possible role of eosinophils in severe RSV disease was already suggested during the trials with a formalin-inactivated candidate RSV vaccine (10, 11). More recently, eosinophil cationic protein

(ECP) has been demonstrated in the respiratory secretions of patients with RSV bronchiolitis (12). In addition, it has been shown that eosinophils can be activated *in vitro* by RSV to produce superoxide and leukotriene C₄ (13). These findings suggest that release of eosinophil granular proteins and other inflammatory mediators which are toxic to respiratory epithelium (14, 15) may contribute to the pathogenesis of bronchiolitis.

The present studies were undertaken to gather more information about the nature of the interaction of RSV with eosinophils. We studied whether eosinophils phagocytose RSV either alone or in immune complexes, and whether the interaction of RSV with eosinophils induces ultrastructural changes of the cells compatible with activation.

Material and methods

Human volunteers

Peripheral blood (120 ml) was obtained in heparinized syringes from healthy adult volunteers whose total circulating leukocyte population consisted of

2–4% eosinophils. The subjects had had no respiratory disease for at least 3 weeks and had not taken any medication for at least 3 days before sampling. Signed statements of informed consent were obtained. All subjects had serum antibodies to RSV as measured by ELISA (16).

Preparation of eosinophil suspensions

Discontinuous Percoll gradients were prepared as described previously (13). Briefly, from stock solution of Percoll (1.123 g/ml, 340 ± 1 mosm/l) (Sigma Chemical Co, St Louis, MO, USA), five Percoll solutions with gradually increasing densities (1.080, 1.085, 1.090, 1.095, and 1.100 g/ml) were prepared by adding calculated amounts of HBSS 1X (Gibco Laboratories, Grand Island, NY, USA). Manipulations of the solutions were carried out under sterile conditions. The solutions were checked regularly for bacterial or fungal contamination by microscopic examination and culture.

The gradients were prepared in 15-ml conical plastic tubes (Falcon Plastics, Los Angeles, CA, USA) with a peristaltic pump (Peristaltic Pump P1, Pharmacia Fine Chemicals, Uppsala, Sweden). A volume of 2.5 ml of 1.080 g/ml Percoll was underlayered subsequently with 3 ml of 1.085 g/ml, 3 ml of 1.090 g/ml, 2.5 ml of 1.095 g/ml, and 1.5 ml of 1.100 g/ml Percoll. Red blood cells were sedimented with 4.5% dextran (Sigma), and mononuclear cells were separated by centrifugation of the white blood cell suspension over Ficoll-Hypaque (1.076 g/ml) (Pharmacia). The cell pellet, containing predominantly granulocytes, was collected, washed with HBSS 1X without calcium and magnesium, and resuspended in HBSS 1X with 5% neonatal calf serum (Gibco) at a final concentration of 20×10^6 cells/ml. A 2-ml volume of this cell suspension was layered on top of each Percoll gradient and centrifuged (600 g, 20 min at room temperature).

Normodense eosinophils obtained from the 1.095–1.100 interface were used in subsequent experiments. The cell population was always more than 85% pure as determined by a modified Wright stain of cytocentrifuge slides. Contaminating cells were exclusively neutrophils. Viability of the cells after separation was more than 98%, as determined by Trypan blue exclusion.

Virus preparation

RSV (Long strain) was grown in HEp-2 culture monolayers, as previously described (17). When more than 90% cytopathic effect was observed (48–72 h), the virus was harvested. The virus was stored at -70°C . HEp-2 cell supernatant was processed in the same way to serve as control. The titer of the vi-

rus was $5 \times 10^5 - 5 \times 10^6$ pfu/ml, as determined by plaque-forming assay.

Immunofluorescence

Eosinophils were incubated with RSV at a multiplicity of infection (m.o.i.) of 1:1 in HBSS for 2 h at 37°C with 5% CO_2 with or without 10% autologous serum (13). After incubation, the cells were washed, fixed on glass slides, and stained with FITC-conjugated monoclonal anti-RSV antibody (Bartels Immunodiagnostic Supplies, Sacramento, CA, USA). The slides were examined with a fluorescence microscope (Olympus Optical Co, Shinjuku, Tokyo, Japan) by two independent, blinded investigators. Maximally, 100 cells were counted per cytospin preparation.

Electron microscopy

Eosinophils ($1-2 \times 10^6$ cells) were incubated with RSV at an m.o.i. of 1:1 in the conditions described before. After incubation, the cells were pelleted in conical plastic tubes and processed for routine transmission electron microscopy, as described previously (18). Five electron microscopy experiments were performed with cells of three different donors.

Statistics

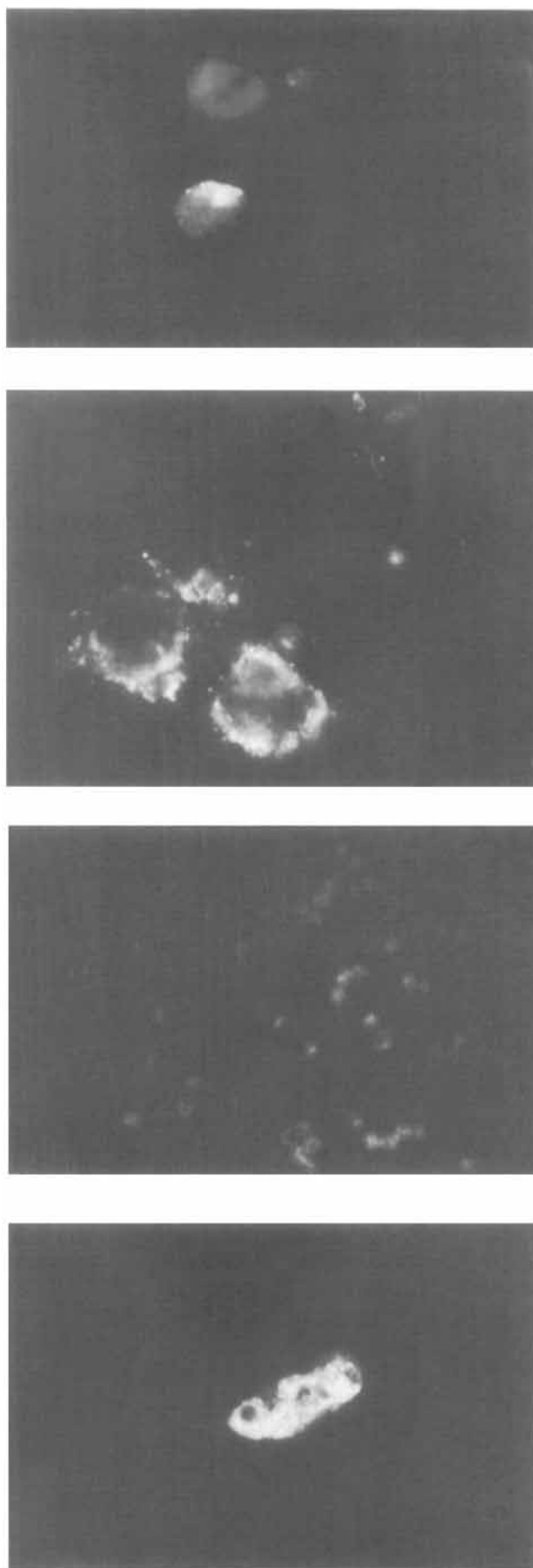
Two-tailed Student's *t*-tests were used to compare group means. Results are expressed as mean $\pm$ SEM.

Results

Immunofluorescence

For immunofluorescence studies, eosinophil preparations of more than 95% purity were used to eliminate the possibility that contaminating neutrophils were confounding the results. When eosinophils were incubated with RSV for 2 h, and stained afterward with an FITC-conjugated anti-RSV monoclonal antibody, $25.6 \pm 12.9\%$ of the cells stained positive. The fluorescence appeared either at one side of the cell, resembling a capping phenomenon (Fig. 1a), or as an evenly distributed punctate staining over the entire cell membrane (Fig. 1b). Control cells incubated with uninfected HEp-2 cell supernatant did not demonstrate any fluorescence (Fig. 1c).

When 10% fresh autologous serum was added to the eosinophil-RSV suspension during incubation, more cells demonstrated positive fluorescence ($62.8 \pm 8.9\%$) ($P = 0.015$) (Fig. 2), and the staining was qualitatively more intense (Fig. 1d). Eosinophils incubated with RSV in the presence of heat-inactivated serum did not show increased fluorescence in



comparison with eosinophils incubated with RSV alone. Cells incubated with uninfected HEP-2 cell supernatant in the presence or absence of serum did not demonstrate positive immunofluorescence (data not shown).

Electron microscopy

In the next set of experiments, eosinophils were processed for electron microscopy after incubation with RSV (m.o.i. 1:1) and autologous serum.

Single, round viral particles measuring 150–250 nm in diameter were seen inside phagocytic vacuoles at the periphery of the eosinophils (Fig. 3a–b). The virion consists of an electron-dense viral envelope surrounding a core in which electron-dense dots are seen, corresponding to the nucleoprotein of the virus, as shown at a higher magnification (19) (Fig. 3c). Fine irregular projections protrude from the envelope surface.

Eosinophils incubated with RSV and autologous serum showed piecemeal degranulation, i.e., emptying of granules in the cytoplasm. This piecemeal degranulation is characteristic of activation of eosinophils (18). Especially the matrix area of the secondary granules showed an aspect of empty lumen, while the electron-dense core remained remarkably stable (Fig. 4). Eosinophils incubated with uninfected HEP-2 cell supernatant in the absence or presence of autologous serum did not show piecemeal degranulation.

Discussion

The present studies describe ultrastructural aspects of the interaction of RSV with eosinophils. Incubation of eosinophils with live RSV alone resulted in phagocytic uptake of the virus. The addition of 10% fresh autologous serum increased the amount of virus taken up by the eosinophils, as evidenced by increased immunofluorescence staining. The use of heat-inactivated serum or secretory IgA did not enhance any of these activities. Therefore, an Fc-receptor-independent mechanism of enhanced uptake of virus by eosinophils is most likely, possibly via complement-fixing RSV immune complexes binding to complement receptors on the cell surface (20). Presumably, interaction with Fc-receptors for IgG on the cell membrane occurs, but the failure of RSV plus heat-inactivated serum to stimulate eosinophils suggests that this interaction is relatively minor. Future research should focus on blocking ex-

Fig. 1. (From top: 1a, 1b, 1c, and 1d). Immunofluorescence of eosinophils after staining cells with RSV-specific monoclonal antibody; a and b) incubation with RSV for 2 h without autologous serum; c) incubation with control HEP-2 cell supernatant; d) incubation with RSV in presence of 10% autologous serum.

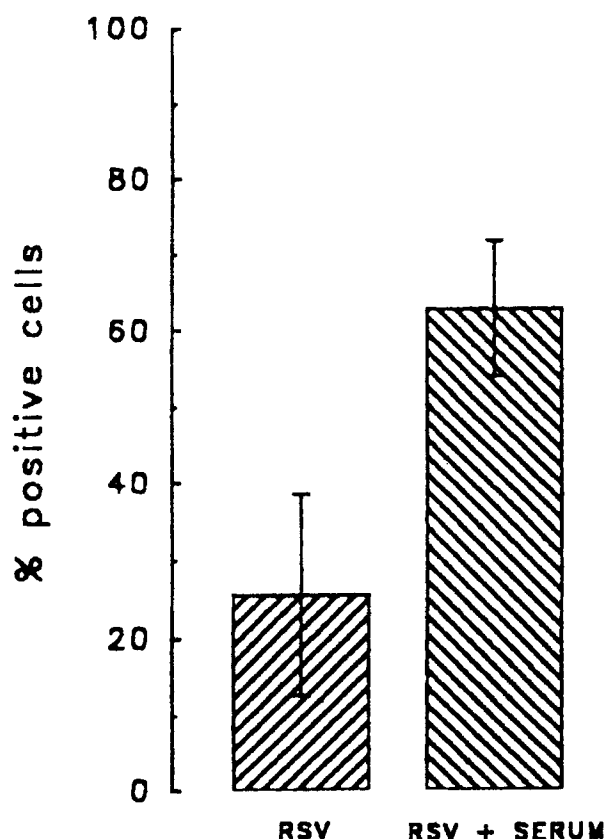


Fig. 2. Immunofluorescence of eosinophils after staining with RSV-specific monoclonal antibody in presence or absence of autologous serum ($n = 5$).

periments with monoclonal antibodies to the complement receptors or Fc-receptor on the eosinophil cell membrane.

There is a discrepancy between the amount of viral antigen detected by immunofluorescence and the small number of viral particles seen in electron microscopy. A likely explanation of this observation is the fact that at an m.o.i. of 1:1, 100–1000 times higher loads of noninfectious viral antigen are present, reacting in the immunofluorescence, but not visible on electron micrographs.

To our knowledge, there are no published studies describing the interaction of RSV with eosinophils. Midulla et al. studied the interaction of RSV with polymorphonuclear leukocytes (21). Using immunofluorescence techniques like those described herein, they found no interaction of virus with these cells. However, these investigators (12) apparently assessed the cells only at 24 h of incubation and not earlier. In the present study, positive fluorescence was seen at 2 h, but not at 24 h when RSV was incubated with eosinophils in the absence of serum. The addition of autologous serum to the cells during incubation induced positive staining at 24 h (data not shown). Other investigators have described activa-

tion of neutrophils after interaction with RSV (17). Minimal activation occurred as a result of direct RSV–neutrophil interactions, and this was enhanced by addition of fresh serum, as was demonstrated in the present report. RSV is also apparently capable of

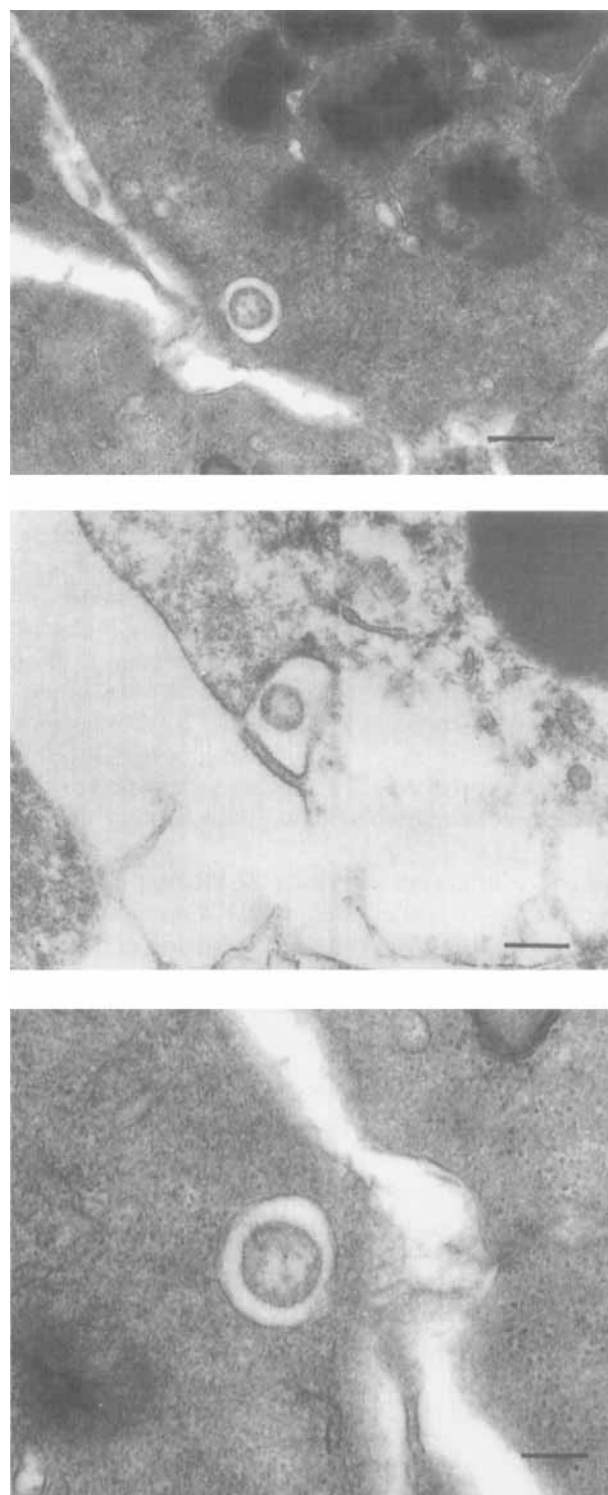


Fig. 3. Electron microscopy of eosinophils after incubation with RSV in presence of autologous serum (m.o.i. of 1:1) Scale a and b = 400 nm ($\times 25\,000$); c = 200 nm ($\times 50\,000$).

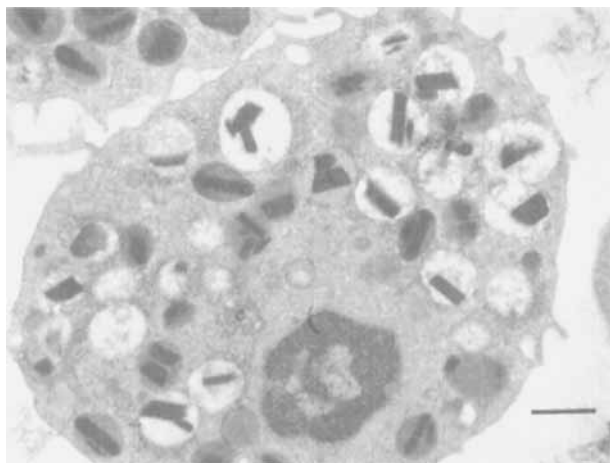


Fig. 4. Electron microscopy of eosinophils after incubation with RSV at m.o.i. of 1:1, showing piecemeal degranulation (scale = 1000 nm).

infecting monocytes and alveolar macrophages (21, 22), resulting in the release of mediators which influence the nature of the immune response of the host to RSV (23, 24). Mononuclear cells were separated from eosinophil preparations in the present study, and there was only minimal contamination by neutrophils. Therefore, the effects of interactions of these different cells with eosinophils could not be evaluated in the present study. Nevertheless, it is conceivable that cytokines released *in vivo* by mononuclear cells, epithelial cells, neutrophils, or eosinophils themselves could either prime eosinophils for activation by RSV or directly cause eosinophil degranulation or release of other inflammatory mediators (25–27).

Finally, in the present study, no attempt was made to determine whether eosinophils were actually infected with RSV. Such studies, involving cultures of supernatants of eosinophils, might require stimulation of cells with cytokines which are known to prolong eosinophil survival (28, 29).

In conclusion, eosinophils have been shown to interact *in vitro* with RSV. This interaction may lead to activation of the eosinophils with release of mediators that are toxic to the respiratory epithelium. These findings support a possible role for eosinophils in airway damage during RSV infection.

Acknowledgments

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