Activation of Human Eosinophils *In Vitro* by Respiratory Syncytial Virus¹

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ABSTRACT. To determine the nature of the interaction between viruses and eosinophils, normodense eosinophils were separated from the blood of healthy volunteers and incubated *in vitro* with respiratory syncytial virus (RSV). After incubation for 2 h with the virus, $29.5 \pm 15.8\%$ of the eosinophils demonstrated specific binding of the virus to the cell membrane, as detected by fluorescent staining with an anti-RSV Mab. Superoxide production and leukotriene C₄ release were measured as determinants of cell activation. Using a cytochrome *c* reduction assay, superoxide could be detected in the supernatant 30 min after exposure to RSV. Maximal release was reached at 3 h postexposure (5.88 ± 2.19 nmol cytochrome *c* reduction/ 5×10^5 cells). The virus-induced superoxide generation varied in magnitude among different subjects and ranged from 0.6 to 11.5 nmol cytochrome *c* reduction/ 5×10^5 cells. RSV also appeared to prime eosinophils to the effects of other known cell activators, as demonstrated by an increase in superoxide production upon subsequent stimulation of RSV-primed cells with phorbol-12-myristate-13-acetate (21.4 ± 5.8 versus 9.4 ± 2.7 nmol cytochrome *c* reduction/ 5×10^5 cells for primed and unprimed cells, respectively) ($p < 0.04$). RSV did not directly induce leukotriene C₄ release from the eosinophils but primed the cells to exhibit a more vigorous response on subsequent challenge with the calcium ionophore A23187 (9.16 ± 1.04 versus 4.2 ± 1.3 ng leukotriene C₄/ 1×10^6 cells) ($p < 0.005$). These findings indicate that RSV can activate or prime eosinophils to release various inflammatory mediators. Such virus-induced effects on inflammatory cells may play a role in the pathogenesis of RSV bronchiolitis and may also be critical for the development of persistent airway hyperreactivity after viral infections. (*Pediatr Res* 32: 160–164, 1992)

Abbreviations

RSV, respiratory syncytial virus
PMA, phorbol-12-myristate-13-acetate
PAF, platelet activating factor
LTC₄, leukotriene C₄
HBSS, Hanks' balanced salt solution
SOD, superoxide dismutase
m.o.i., multiplicity of infection

RSV is the leading cause of pneumonia and bronchiolitis during infancy (1). It has been speculated that RSV infection in infancy plays a role in the development of hyperreactive airway disease in later life (2). The pathogenesis of the inflammation of the airways during acute RSV infection and the mechanisms underlying the possible development of subsequent airway hyperreactivity are not completely understood.

Recent evidence suggests an important role for eosinophils in a variety of inflammatory states (3). Although the role of eosinophils in natural RSV infection is unclear, reports of field studies with a formalin-inactivated RSV vaccine carried out in the late 1960s suggest a role for eosinophils in disease due to RSV. Lymphocytes and eosinophils predominated in the airways on autopsy of two patients who died as a consequence of severe RSV bronchiolitis after vaccination (4), and children who had more severe bronchiolitis when subsequently infected with the natural virus demonstrated an increased blood eosinophilia compared to the controls (5). In addition, the release of LTC₄ in the respiratory tract during RSV infection has been described (6, 7), and we recently showed higher levels of eosinophil cationic protein in nasopharyngeal secretions of children with natural RSV bronchiolitis in comparison with children affected only by RSV upper respiratory tract infection or non-RSV respiratory illness (8). The present study was therefore undertaken to examine *in vitro* the nature of interaction between RSV and eosinophils.

MATERIALS AND METHODS

Human volunteers. Peripheral blood (120 mL) was obtained in heparinized syringes from healthy adult volunteers who had a differential eosinophil count of 1–4%. The subjects had no respiratory disease for at least 3 wk and had not taken any medication for at least 3 d before sampling. Signed statements of informed consent were obtained. All subjects had serum antibodies to RSV as measured by ELISA (9).

Preparation of eosinophil suspensions. Percoll dilutions were prepared as described previously, with modifications (10). Briefly, a stock solution of Percoll was prepared by adding 1 part HBSS 10× to 9 parts Percoll. The osmolality of this solution was measured with a freezing-point osmometer (The Advanced Micro-Osmometer model 3MO; Advanced Instruments, Needham Heights, MA) and adjusted to 340 ± 1 mosmol/L. The density was measured with a glass 2-mL pycnometer (Ace Glass Incorporated, Vineland, NJ). From this stock solution (density 1.123 g/mL), 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 1×. All densities were verified by measurement with a pycnometer. Manipulations of the solutions were carried out under sterile conditions and the Percoll solutions were checked regularly for bacterial or fungal contamination by microscopic examination and culture.

Discontinuous Percoll gradients were prepared in 15-mL conical plastic tubes (Falcon Plastics, Los Angeles, CA), using a

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peristaltic pump (Peristaltic Pump P1; Pharmacia Fine Chemicals, Uppsala, Sweden). Two and one half mL of 1.080 g/mL Percoll were 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.

Five parts blood were mixed with one part 4.5% dextran and left at room temperature for 45 min to sediment the red cells. Twenty-mL aliquots of the leukocyte rich supernatant were underlayered with 10 mL Ficoll-Hypaque (1.076 g/mL) and centrifuged ($450 \times g$, 20 min at room temperature). The cell pellet was collected and washed twice with HBSS 1 $\times$ without calcium or magnesium and finally resuspended in HBSS 1 $\times$ with 5% neonatal calf serum. A cell count was performed with an automated counter (Coulter counter S+IV; Coulter Corporation, Hialeah, FL), and the suspension adjusted to 20×10^6 cells/mL. Two mL of this cell suspension were layered on top of each Percoll gradient and centrifuged ($600 \times g$, 20 min at room temperature).

Cells from the three lower interfaces (1.100–1.095, 1.095–1.090, and 1.090–1.085) were collected and washed twice in HBSS 1 $\times$ with 0.1% gelatin. Residual red blood cells were lysed by hypotonic shock. The number of cells in each band was determined and a differential cell count was performed on cyto-centrifuge slides (Cytospin 2; Shandon, Sewickley, PA) after staining with a modified Wright's stain (Stat Stain, VWR Scientific, Piscataway, NJ).

Normodense eosinophils obtained from the 1.095–1.100 interface were used in subsequent experiments. The cell population was $87.8 \pm 2.0\%$ pure ($n = 17$). Viability of the cells after the separation was always more than 98% as determined by trypan blue exclusion. Contaminating cells were exclusively neutrophils.

Virus preparation. RSV (Long strain) was grown in HEP-2 culture monolayers as described (11). 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 virus was 5×10^5 to 5×10^6 plaque-forming units/mL, as determined by plaque-forming assay.

Immunofluorescence. Eosinophils were incubated with RSV at an m.o.i. of 1 in serum-free HBSS for 2 or 24 h at 37°C with 5% CO_2 . After incubation, the cells were washed, fixed on glass slides, and stained with a FITC-conjugated monoclonal anti-RSV antibody (Bartels Immunodiagnostic Supplies, Sacramento, CA). The slides were then examined under a fluorescence microscope (Olympus Optical Co. Ltd., Shinjuku-Tokyo, Japan) by two independent investigators.

Superoxide generation. Generation of superoxide was determined as SOD-inhibitable reduction of ferricytochrome *c* as previously described (12). The tests were carried out in flat-bottom, 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA), and the color change was measured in an automated ELISA reader at 550 nm (Titertek Multiskan Plus MKII; Flow Laboratories, McLean, VA). After adding the stimuli to the wells, the plate was read six times in the first 30 min and at 15-min intervals thereafter until the moment a plateau phase in superoxide production was reached. Between readings, the plate was incubated at 37°C with 5% CO_2 . The mean of triplicate wells was calculated and SOD control and spontaneous release were subtracted from the test wells. Superoxide generation was expressed as nmol cytochrome *c* reduction/ 5×10^5 cells per min as described (12).

Leukotriene production. One million eosinophils were incubated overnight in 1 mL of RSV-conditioned medium (RPMI 1640 supplemented with 5% FCS) at an m.o.i. of 2 or in an equal amount of control medium, at 37°C and 5% CO_2 . After incubation, the cells were washed twice with HBSS and counted, and the viability was determined by trypan blue exclusion. The cells were subsequently incubated in a total volume of 1 mL with calcium ionophore A23187 at a final concentration of $2.5 \mu\text{M}$ for 10 min in a shaking water bath. The reaction was stopped by

addition of 1.5 mL of ice-cold methanol. Twenty ng of prostaglandin B_2 were added as an internal standard for HPLC analysis. The resultant mixture was centrifuged at $1000 \times g$ for 15 min at 4°C . The supernatant was applied to a C18 reversed phase extraction cartridge (Sep-Pack; Millipore, Waters Associates, Milford, MA) that had been washed previously with 10 mL of distilled HPLC-grade water and activated with 10 mL HPLC-grade methanol. After the sample was placed on the cartridge, it was washed with 10 mL of distilled water and the leukotrienes were eluted with 10 mL of 70% methanol. The methanol fraction was evaporated under vacuum in a rotary evaporator (Rotavapor, Buchi, Switzerland) and the residue was redissolved in 500 μL of 30% methanol and stored at -70°C until testing. The samples were injected into a C18 reverse-phase column (Beckman, San Ramon, CA) and eluted isocratically at a flow rate of 1 mL/min in a HPLC system (Pharmacia, Piscataway, NJ) with a solvent system of methanol/water/acetic acid (70/28/2 vol/vol/vol) adjusted to pH 5.5 with ammonium hydroxide. The eluate was monitored at 280 nm by a wavelength spectrometric detector (Pharmacia). For identification and quantification of LTC_4 in the samples, different mixtures of purified LTC_4 and prostaglandin B_2 were prepared in HBSS and processed as described above. Each sample was tested in parallel with these standards.

Reagents. HBSS with and without calcium and magnesium, RPMI 1640, and neonatal calf serum were purchased from Gibco Laboratories (Grand Island, NY). Gelatin was obtained from Bio-Rad Laboratories (Richmond, CA). Dextran, Percoll, PMA, calcium ionophore A23187, formyl-methionyl-leucyl-phenylalanine, SOD, horse-heart ferricytochrome *c* (type VI), prostaglandin B_2 , and platelet activation factor $\text{L-}\alpha$ -phosphatidylcholine, β -acetyl- γ -O-(octadec-9-*cis*-enyl) (PAF) were purchased from Sigma Chemical Co. (St. Louis, MO). The chemicals were aliquoted and stored at -20°C or -70°C as recommended by the manufacturer. HPLC solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ficoll-Hypaque was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Purified LTC_4 was a generous gift of Dr. J. Rokach, Merck Frosst, Montreal, Quebec, Canada.

Statistical methods. The *t* test for paired samples was used to compare group means. All values are expressed as mean $\pm$ SEM.

RESULTS

Association of RSV with eosinophils. Eosinophils were incubated with live RSV and then stained with FITC-conjugated MAb to RSV. After 2 h of incubation, $29.5 \pm 15.8\%$ of the eosinophils demonstrated significant staining for viral antigen ($n = 4$). The staining appeared as bright spots rather diffusely distributed over the eosinophil cell membrane, sometimes confluent to a larger area of immunofluorescence on the cell (Fig. 1). However, cells incubated with RSV for 24 h revealed no RSV-specific staining in any of the eosinophil preparations studied ($n = 3$). To exclude the possibility that contaminating neutrophils might show the positive staining, only cell preparations consisting of more than 95% eosinophils were used for the immunofluorescence studies. Cells incubated with virus-free HEP-2 cell supernatant or control culture medium did not show any fluorescence.

Superoxide production. Eosinophil preparations were stimulated with PMA in a final concentration of 1 or 10 ng/mL or PAF at a final concentration of 10^{-6} M to establish a reference curve for eosinophil superoxide production kinetics (12). As shown in Figure 2, peak superoxide production was observed after 180 min of incubation with PMA 1 ng/mL or PAF 10^{-6} M. On the other hand, superoxide production by the eosinophils stimulated with PMA 10 ng/mL reached a peak level at 30 min. The maximum superoxide production was 38.2 ± 6 nmol cytochrome *c* reduction/ 5×10^5 cells for the high concentration of PMA versus 8.9 ± 2.7 nmol cytochrome *c* reduction/ 5×10^5

Fig. 1. Immunofluorescence of eosinophils after exposure to RSV for 2 h.

Fig. 2. Kinetics of eosinophil superoxide production after stimulation with PMA 10 ng/mL ($n = 5$) and 1 ng/mL ($n = 8$) and PAF 10^{-6} M ($n = 3$).

cells for the low PMA concentration and 11.5 ± 7.5 nmol cytochrome *c* reduction/ 5×10^5 cells for PAF.

Eosinophils were incubated with live RSV at an m.o.i. of 1, and the kinetics of superoxide release was determined over the following 180 min (Fig. 3). Eosinophils of three volunteers did not demonstrate any reactivity toward RSV, although the superoxide production by control cells stimulated with PMA was appropriate (data not shown). Eosinophils from two volunteers demonstrated a very low but consistent superoxide release after incubation with the virus (maximal release 1.05 ± 0.15 nmol cytochrome *c* reduction/ 5×10^5 cells). Conversely, cells of three other donors demonstrated a high superoxide production after stimulation with RSV (9.3 ± 1.17 nmol cytochrome *c* reduction/ 5×10^5 cells). Cells of one of these volunteers demonstrated a rather late onset of maximal superoxide production. For the three volunteers whose cells exhibited a high response to RSV, the degree of oxygen radical release was comparable to the amount released by PMA 1 ng/mL or PAF 10^{-6} M. To study the possible confounding effect of superoxide production by contaminating neutrophils in the cell suspensions, purified neutrophils of the same volunteers were incubated with RSV. No

Fig. 3. Kinetics of superoxide production after stimulation with RSV ($n = 5$). Eosinophils from three other donors did not generate superoxide when stimulated with RSV.

RSV-induced superoxide production by the neutrophils could be detected, although the cells were activated appropriately by PMA and formyl-methionyl-leucyl-phenylalanine (data not shown). In addition, different percentages of neutrophils (5–15%) in the cell suspensions did not affect the activation of eosinophils (data not shown). The stock RSV preparation did not contain measurable amounts of superoxide.

To evaluate if RSV had a priming effect on eosinophils, resulting in a more pronounced production of superoxide after subsequent challenge, eosinophils were incubated with the virus at an m.o.i. of 1 for 30 min at 37°C and 5% CO_2 . After two washing steps with HBSS, the cells were plated and challenged with PMA at a concentration of 1 ng/mL. The data on eosinophil preparations of four different volunteers are presented in Figure 4. The maximal PMA-induced superoxide release by cells preincubated with RSV was 32.6, 16.5, 7.3, and 29 nmol cytochrome *c* reduction/ 5×10^5 cells in each of four experiments. These values are 90 to 250% higher than those in corresponding control experiments on cells incubated in virus-free medium (9, 9.2, 3.1, and 16.4 nmol cytochrome *c* reduction/ 5×10^5 cells, respectively). The mean peak of superoxide production after PMA challenge was 21.4 ± 5.8 nmol cytochrome *c* reduction/ 5×10^5 eosinophils incubated in the presence of virus versus 9.4 ± 2.7 nmol cytochrome *c* reduced/ 5×10^5 cells incubated in control medium ($p < 0.04$) ($n = 4$).

Eosinophils incubated with HEP-2 cell supernatant did not produce detectable superoxide, nor was a priming effect noted.

Leukotriene release. Incubation of 1×10^6 purified eosinophils from different donors for up to 12 h in the presence of live RSV or HEP-2 cell supernatant did not induce release of any sulfido-peptide leukotrienes. Shorter incubations (30, 60, or 120 min) did not result in LTC_4 release either (data not shown). However, incubation of the eosinophils for 12 h in control medium followed by a challenge with a suboptimal concentration of calcium ionophore A23187 resulted in the release of detectable amounts of LTC_4 (4.2 ± 1.3 ng LTC_4 / 1×10^6 cells). When the eosinophils were preincubated with RSV for 12 h and then challenged with calcium ionophore, significantly higher amounts of LTC_4 were released (9.16 ± 1.04 ng LTC_4 / 1×10^6 cells) ($n = 6$) ($p < 0.005$) (Fig. 5). HEP-2 cell-conditioned medium, however, did not show any priming effect on the eosinophils.

Fig. 4. Maximal superoxide production by PMA 1 ng/mL for cells preincubated with RSV (filled bars) or control medium (open bars) by eosinophils of four different donors.

Fig. 5. LTC₄ release by eosinophils after challenge with calcium ionophore A23187 at a final concentration of 2.5 μ M. Cells were preincubated with RSV or control medium ($n = 6$).

DISCUSSION

The findings of this study suggest that RSV is capable of stimulatory interaction with eosinophils. One third of the cells demonstrated specific fluorescent membrane staining after 2 h of exposure to the virus. This finding contrasts with a previous report in which positive staining was not obtained after incubation of polymorphonuclear granulocytes with RSV and subsequent staining of the cells with anti-RSV MAb (13). However, nonpurified granulocyte suspensions were used in that study, and the cells were only examined after 24 h. In the present study, RSV-eosinophil interactions reversed spontaneously by 24 h of incubation.

Activation of eosinophils of healthy subjects by respiratory syncytial virus described in the present study was evident from the release of superoxide and LTC₄. The magnitude of superoxide production due to RSV stimulation varied among the subjects but was clearly detectable in five of eight individuals (63%) tested. Furthermore, the virus primed the cells to release signifi-

cantly more superoxide on subsequent challenge with PMA and also sensitized eosinophils to release higher amounts of LTC₄ in response to a subsequent challenge with calcium ionophore.

The mechanism and initiating steps of the oxidative metabolism of neutrophils is well understood. A similar understanding of the respiratory burst of eosinophils has been inhibited because of technical difficulties in separating cells from peripheral blood and because superoxide assays have required large numbers of cells. In the present study, a recently described microassay for measurement of superoxide production by small numbers of phagocytic cells was used (12, 14, 15). PMA stimulates eosinophils to release toxic oxygen metabolites through a mechanism bypassing the initial receptor-mediated activation and starting the chain of superoxide generation directly at the level of phosphokinase C. PAF stimulates the cells through interaction with specific membrane receptors (16). The mechanism by which RSV activates the eosinophils to release oxygen radicals is not clear. Nonspecific phagocytosis of the virus by the cells is possible, but the interaction of RSV with a thus far unidentified putative receptor on the eosinophils cannot be ruled out. The possibility that eosinophils are indirectly activated by factors released by HEP-2 cells infected with RSV cannot be ruled out completely by this study, although to our knowledge there exists no evidence suggesting HEP-2 cells as a source of cytokines or other factors inducing or mediating stimulation of eosinophils.

The interindividual variability in superoxide generation cannot be explained at the present time. The donors had no symptoms of atopic disease and did not report other recent illness, nor were they taking any medication that could blunt or accentuate the response. Serum-specific anti-RSV antibody titers as determined by ELISA were similar for all the subjects (data not shown), probably excluding variability in immune complex formation as a mechanism.

RSV did not induce directly a release of LTC₄ from the eosinophils. However, cells incubated in the presence of the virus exhibited a significantly stronger response when challenged subsequently with calcium ionophore. The priming of eosinophils by monokines and eosinophil chemotactic factors has been previously described (17, 18). Ingestion of the viral particles by the cell or interaction of RSV with the eosinophil membrane may render the cells more active upon stimulation with a chemical stimulus such as calcium ionophore.

To date, the only reports describing the activation of granulocytes by RSV *in vitro* involve the interaction of RSV and neutrophils. Neutrophils produce superoxide and thromboxane B₂ when challenged with RSV (19), demonstrate a significant chemiluminescence when incubated with RSV-antibody immune complexes (20), attach to RSV-infected HEP-2 cells (11), and are cytotoxic for RSV-infected targets (21). The present study provides information on RSV-specific activation of purified eosinophils.

The demonstration that RSV can both induce the production of superoxide and prime the eosinophils to release higher amounts of mediators after subsequent challenge leads to the speculation that some of the inflammatory signs seen in the airways of children with RSV bronchiolitis might be eosinophil-induced. Indeed, it has been shown that eosinophil mediators such as major basic protein and products of the respiratory burst can induce damage of respiratory epithelium and airway smooth muscle *in vitro* (22–26). Viral damage to respiratory epithelium could work in concert with toxic damage by eosinophil products and edema with bronchoconstriction induced by LTC₄ to bring about the airway obstruction seen in RSV bronchiolitis.

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