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Divergence of Mechanisms Regulating Respiratory Burst in Blood and Sputum Eosinophils and Neutrophils from Atopic Subjects

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Eosinophil respiratory burst is an important event in asthma and related inflammatory disorders. However, little is known concerning activation of the respiratory burst NADPH oxidase in human eosinophils. Conversely, neutrophils are known to assemble NADPH oxidase in intracellular and plasma membranes. We hypothesized that eosinophils and neutrophils translocate NADPH oxidase to distinct intracellular locations, consistent with their respective functions in $O_2^-$-mediated cytotoxicity. PMA-induced $O_2^-$ release assayed by cytochrome $c$ was 3.4-fold higher in atopic human eosinophils than in neutrophils, although membrane-permeable dihydrorhodamine-123 showed similar amounts of release. Eosinophil $O_2^-$ release was dependent on Rac, in that it was 54% inhibited by Clostridium difficile toxin B (400–800 ng/ml). In eosinophils stimulated with PMA, a pronounced shift of cytosolic Rac to p22phox-positive plasma membrane was observed by confocal microscopy, whereas neutrophils directed Rac2 mainly to intracellular sites coexpressing p22phox. Similarly, ex vivo sputum eosinophils from asthmatic subjects exhibited predominantly plasma membrane-associated immunoreactivity for Rac, whereas sputum neutrophils exhibited cytoplasmic Rac2 staining. Thus, activated sputum eosinophils, rather than neutrophils, may contribute significantly to the pathogenesis of asthma by extracellular release of tissue-damaging $O_2^-$. Our findings suggest that the differential modes of NADPH oxidase assembly in these cells may have important implications for oxidant-mediated tissue injury. The Journal of Immunology, 2003, 170: 2670–2679.

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espiratory burst is an important event in many inflammatory conditions, characterized by production of superoxide anion ($O_2^-$) and related reactive oxygen species (ROS), including $H_2O_2$ and $OH^-$ (1). Eosinophils from atopic subjects generate elevated amounts of ROS (2, 3), which may directly injure tissues in the airways (4) and can react with eosinophil peroxidase to produce further tissue-damaging microbicidal products (1). Neutrophils are also well characterized for their ability to release $O_2^-$ (5). Interestingly, neutrophils from asthmatic subjects have been shown to generate more $O_2^-$ than those from normal individuals (6, 7). In all cell types exhibiting respiratory burst, regulated generation of $O_2^-$ is dependent on assembly and activation of the normally latent NADPH oxidase complex in cell membranes (8, 9).

The molecular mechanisms associated with NADPH oxidase assembly and activation have been studied in more detail in neutrophils (5, 8, 10–13) and cell-free assays (10, 14) than in eosinophils (15–19). This complex is composed of five essential subunits, the membrane-bound cytochrome $b_{558}$ (a complex of two subunits, p29phox and gp91phox) which associates with cytosolic subunits Rac, p47phox, and p67phox during stimulation (8). Rac, a small monomeric GTP-binding protein of ~21 kDa, belongs to the Rho family of GTPases (20) and is bound to cytosolic guanine nucleotide exchange factor (RhoGDI) under basal conditions. Rac1 shares 92% amino acid homology with Rac2, and these are functionally interchangeable in their ability to activate NADPH oxidase (14, 21–23), whereas other GTPases, including K-Ras, Rap1A, Rap1B, RhoA, and Cdc42Hs, are unable to activate this complex in cell-free assays (24). Rac2 expression is limited to hematopoietic cells (25). During respiratory burst, p47phox and p67phox (in a complex with p40phox) become phosphorylated and translocate to cell membranes to bind cytochrome $b_{558}$. Concurrent phosphorylation of a putative guanine nucleotide exchange factor induces dissociation of Rac from RhoGDI and binding of activated Rac-GTP to gp91phox and p67phox in the membrane (24).

Studies on eosinophil NADPH oxidase activation have shown strong similarities between eosinophils and neutrophils in assembly and activation of this complex. However, eosinophils generate up to 10-fold more extracellular $O_2^-$ than neutrophils, which may be caused by elevated expression of NADPH oxidase in eosinophils (15–17, 26, 27). Several reports have alluded to the possibility that stimulated neutrophils mainly generate $O_2^-$ intracellularly to assist in phagocytic killing (28, 29). However, there is a lack of direct evidence of expression and location of NADPH oxidase in human eosinophil vs neutrophil respiratory burst.

In this study, we hypothesized that eosinophils preferentially assemble NADPH oxidase in the plasma membrane to generate extracellular $O_2^-$. In addition, we investigated whether human eosinophils express Rho-related GTPases, which are required for $O_2^-$
release through NADPH oxidase activation. Using a combination of subcellular fractionation and confocal laser scanning microscopic techniques, we describe a clear divergence in the spatial distribution of the critical oxidase regulator Rac between eosinophils and neutrophils both in vitro and ex vivo. Understanding the molecular details regulating this process and distinct patterns of regulated O$_2^-$ release in these two cell types may contribute to development of novel and cell-specific therapeutic targets to modulate mediator secretion in asthmatic inflammation.

Materials and Methods

Materials
Nycodenz was purchased from Life Technologies (Burlington, Ontario, Canada). Baculovirus-generated recombinant Rac2 and rabbit polyclonal antiserum to recombinant human Rac2 were generously provided by Dr. G. M. Bokoch (The Scripps Research Institute, La Jolla, CA) (30, 31). Rabbit polyclonal Ab to RhodGDI was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Rac Abs were purchased from Upstate Biotechnology (Lake Placid, NY) and BD Transduction Laboratories (Mississauga, Canada). Dihydrorhodamine-123 (DHR-123) was obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal anti-sera anti-recombinant human p47$^\text{phox}$ and p67$^\text{phox}$ were kindly supplied by Dr. W. Nauseef (University of Iowa, Iowa City, IA). Mouse monoclonal anti-human p22$^\text{phox}$ and gp91$^\text{phox}$ proteins were generously provided by Dr. M. T. Quinn (Montana State University, Bozeman, MT), Clostridium difficile toxin B was purchased from List Biological Laboratories (Campbell, CA).

Preparation of eosinophils and neutrophils
To isolate eosinophils, peripheral blood (100 ml) was obtained from mild atopic asthmatic and atopic nonasthmatic subjects displaying eosinophilia (>2%, and who were not receiving oral corticosteroids (32, 33). Briefly, whole blood was subjected to erythrocyte sedimentation in 6% dextran, and upper phase cells were centrifuged on a single-step Ficoll (Pharmaea, Peapack, NJ) gradient before highly purified CD16$^+$ eosinophils (>99%) were isolated by negative immunomagnetic selection. For neutrophils, 50–100 ml of peripheral blood was obtained from normal subjects (except for experiments using infants, as described in text). Eosinophils and neutrophils were obtained from the same donor), which was subjected to erythrocyte sedimentation in 6% dextran, followed by density centrifugation on Ficoll. Using this isolation technique, neutrophil purity usually averaged >98%.

Measurement of O$_2^-$ release from eosinophils
Generation of extracellular O$_2^-$ from cells in suspension was measured as previously described (19). Briefly, cells (1–2 x 10$^6$) were suspended in 1-ml microcuvets containing supplemented PBS (PBS), pH 7.4 (with 3 mM MgCl$_2$, 5 mM KCl, 0.5 mM CaCl$_2$, 5 mM glucose, and 0.1% BSA) and 50 $\mu$M ferricytochrome c at 25°C. The mixture was blanked at 550 nm in a Beckman DU 640 spectrophotometer (Beckman Instruments, Mississauga, Canada) before adding PMA at doses ranging from 1 to 1000 ng/ml. Superoxide dismutase-inhibitable OD was calculated using $\epsilon = 2.11 \times 10^4$ M$^{-1}$ cm$^{-1}$ for reduced cytochrome c. To inhibit O$_2^-$ production, C. difficile toxin B (40–800 ng/ml) was added to 1 x 10$^7$ cells/ml in RPMI 1640 (Life Technologies) containing 15% FCS and incubated at 37°C for 2–20 h before treatment with PMA.

Dihydrorhodamine-123 (DHR-123, 1 $\mu$M), a membrane-permeable probe sensitive to oxidation by ROS to produce fluorescein, was separated at 37°C for 10 min with cells (1–2 x 10$^6$) before adding 1 $\mu$g/ml PMA for 10 min using a modification of previously published protocols (34, 35). Cells were then subjected to flow cytometric analysis (FACScan; BD Biosciences, San Jose, CA). MFI values were obtained from gated regions on dot plots for each sample.

RT-PCR
Highly purified batches of eosinophils and neutrophils (≥99%) were subjected to total RNA extraction using a Qiagen RNeasy MiniKit (Qiagen, Mississauga, Canada) using 2 x 10$^6$ cells per extraction (producing 0.2–1 $\mu$g RNA) (19). Primer sequences used for detecting Rac1/2 mRNA were generated in our laboratory for Rac1 (product size, 575 bp), with forward and reverse sequences as follows: 2–24 bp, 5'-TGGAGGCCCATTAG GTGGTGTTG-3' and (554–576 bp) 5'-CACAGGAGCCATTTTCT TCC-3'. Rac2-specific primers (product size, 576 bp) were (3–25 bp) 5'- CGAGGGCATTACAGGTGGTTG-3' and (556–578 bp) 5'-TAGAG GAGGGTGAGCGGCGT-3', respectively. Both sets of primers are intron spanning (36, 37). Reactions were carried out in a PTC 100 Thermal Controller (M-J Research, Watertown, MA) using an annealing temperature of 57°C (Rac1-specific primers) or 56°C (Rac2-specific primers). Nonreverse-transcribed samples were included as controls.

Western blot analysis
Samples were subjected to acrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane blots (19). Primary mouse mAbs to Rac (1/1000), p22$^\text{phox}$ (1/1000), gp91$^\text{phox}$ (1/1000), and p67$^\text{phox}$ (1/1000) were used before introduction of secondary Abs (1/5000 sheep anti-mouse IgG or 1/5000 donkey anti-rabbit IgG conjugated to HRP, Amersham Canada, Oakville, Canada). Chemiluminescence was developed by addition of SuperSignal substrate solution (Pierce, Rockford, IL).

Subcellular fractionation of eosinophils and marker enzyme assays
Eosinophils were subjected to homogenization through a ball-bearing cell homogenizer (HGM Precision Engineering, Heidelberg, Germany), followed by production of postnuclear supernatant from which organelles were separated by linear density gradient centrifugation.

PMA-stimulated eosinophils (>5 x 10$^7$ cells) were prewarmed to 37°C in 5 ml of PBS with 250 U/ml catalase and 50 U/ml superoxide dismutase for 5 min (19). PMA was added to a final concentration of 500 ng/ml for 8 min at 37°C. The reaction was terminated by addition of 10 ml ice-cold PBS, and cells were homogenized as described above.

Profiles of marker enzyme activities were obtained using previously reported techniques (19, 33, 38). Fractions enriched in cytosol (lactate dehydrogenase), plasma membrane/small secretory vesicles (CD9), cristalloid granules (eosinophil peroxidase), and crystallloid/small granules (β-hexosaminidase) were determined in supernatants and pellets using modifications of microtitre plate assays (33). Plasma membrane activity was determined by dot blot analysis with mAb to CD9 (32).

Double labeling and confocal laser scanning microscopy
Granulocyte cytospins (50 μl of 0.8 x 10$^7$ cells/ml in RPMI 1640 supplemented with 20% FCS) were prepared by Cytospin 2 centrifugation (Shandon, Astmoor, Runcorn, U.K.) as previously optimized (39). Primary labeling was conducted with 20 μg/ml mouse monoclonal anti-human Rac, specific for both Rac1 and Rac2 (mouse IgG2b; Upstate Biotechnology). Immunoreactivity to Rac was detected using 1.4 μg/ml Rhodamine Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) (33, 40). Slides were double labeled with 20 μg/ml anti-human p22$^\text{phox}$ antibody. Bound anti-Rac was detected by incubating 20 μg/ml BODIPY FL-labeled goat anti-mouse IgG (Molecular Probes). Mouse IgG1 and IgG2b (20 μg/ml) were used as isotype controls (R&D Systems, Minneapolis, MN; Sigma-Aldrich, St. Louis, MO). Cells were counterstained with 4',6-diamidino-2-phenylindole nuclear stain. Slides were mounted with 30 μl of antibleaching agent (0.4% n-propyl gallate; Sigma) in glycerol-TBS (3:1) before coverslip attachment and then examined using a ×40 objective on a Zeiss confocal laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were collected and processed as described (33).

Sputum collection and analysis
Sputum was induced from normal and well-characterized asthmatic subjects by inhalation of nebulized saline at increasing concentrations (up to 5%) (41). Atopic asthmatic subjects (5), as determined by the skin prick test, exhibited forced expiratory volume at 1 s scores of <80% of predicted values at the time of diagnosis. All asthmatics were receiving medication (inhaled corticosteroids and β-agonists). After inhalation of nebulized saline, subjects rinsed their mouths and then coughed sputum into sterile collection containers. Mucus plugs were manually removed and fixed immediately in 4% paraformaldehyde in PBS for 2 h. Samples were paraffin embedded via butanol in a tissue array fashion (42). For immunohistochemical analysis, samples were processed as described above for cytospins for Rac and major and basic protein (MBP) immunoreactivity. To detect MBP, 1% mouse mAb to human MBP (BMK-13, generated in house) (43) was applied to sections. Bound BMK-13 was detected by 5 μg/ml BODIPY FL-conjugated goat anti-mouse IgG. Slides were analyzed by confocal laser scanning microscopy on a Zeiss system, described above, and by deconvolution restoration microscopy using a DeltaVision microscopy system (Applied Precision, Issaquah, WA).
Data presentation

Enzyme activities corresponding to granule, membrane and cytosol constituents after fractionation were expressed as frequency distributions (32). Data were analyzed by ANOVA or Kruskal-Wallis statistical comparison with individual samples compared by Tukey’s or Dunn’s multiple comparison test.

Results

Extracellular vs intracellular O$_2^-$ levels generated by eosinophils and neutrophils

Eosinophils and neutrophils generated extracellular O$_2^-$ in response to PMA stimulation, although there was a significant discrepancy in amounts of O$_2^-$ produced from each cell type. Continuous spectrophotometric measurement of cytochrome c reduction in response to increasing doses of PMA (Fig. 1, A and B) revealed that the peak rate of O$_2^-$ release was 3.4-fold higher from eosinophils than from neutrophils, similar to a value of 3.2-fold obtained previously (44) (Fig. 1C). At the maximally stimulating dose of 50 ng/ml PMA, the average peak rate of O$_2^-$ release from eosinophils was 15.9 ± 0.6 nmol O$_2^-$/10$^6$ cells/min compared with 6.2 ± 0.7 nmol O$_2^-$/10$^6$ cells/min for neutrophils.

These values are close to those obtained for PMA-induced O$_2^-$ production in eosinophils and neutrophils in a previous study, which reported 13.2 ± 1.1 nmol O$_2^-$/10$^6$ cells/min in eosinophils and 7.2 ± 1.3 nmol O$_2^-$/10$^6$ cells/min in neutrophils in response to 100 ng/ml PMA (27). In addition, the peak rate of O$_2^-$ generation in eosinophils always exceeded that of neutrophils at all doses of PMA >1 ng/ml (Fig. 1C). These findings support those of earlier reports (17, 27, 45). Although the response reached a plateau within 5–10 min of stimulation, the saturation appears not to be due to consumption of total cytochrome c in the reaction because lowering the concentration of cells did not result in loss of the plateau (data not shown).

A fluorescent probe assay coupled with flow cytometric analysis was used to determine ROS production in eosinophils and neutrophils. The membrane-permeable fluorochrome DHR-123 is unable to discriminate between intracellular and extracellular production of ROS, because it primarily reacts with H$_2$O$_2$, which is also membrane permeable and has the capacity to re-enter cells during PMA incubation before FACS analysis (46). Thus, total ROS production (both intra- and extracellular) may be measured using this fluorescent probe. Cells were incubated in the presence of DHR-123 at 37°C for 10 min before addition of 1 μg/ml PMA for 10 min at the same temperature and then subjected to flow cytometric analysis. Using this assay, we observed an average increase of 3014 MFI U in PMA-stimulated eosinophils compared with 3857 MFI in similarly stimulated neutrophils (Table I). The increase in MFI in both cell types was significant compared with unstimulated cells (p < 0.01 for eosinophils; p < 0.05 for neutrophils). However, there was no significant difference in the PMA-stimulated MFI values between cell types. These data suggest that neutrophils may generate total amounts of intra- and extracellular ROS similar to those for eosinophils during respiratory burst, in contrast to previously published data (17, 27, 44, 45).

Is Rho-related GTPase activation essential for O$_2^-$ release in eosinophils?

We determined whether Rho-related GTPases are required for NADPH oxidase activation in eosinophils by incubating cells with C. difficile toxin B. As shown in Fig. 2, increasing doses of toxin B reduced O$_2^-$ generation in response to a suboptimal dose of PMA (10 ng/ml). Inhibition was significant at 400 ng/ml toxin B (p < 0.05) and was further suppressed at 800 ng/ml (54% inhibition; p < 0.01). No significant inhibition was detected after 2 and 16 h of incubation with toxin B at these doses (data not shown). These findings indicate that Rho-related GTP-binding proteins may be involved in regulation of PMA-induced O$_2^-$ release in eosinophils.

Table I. Measurement of MFI in eosinophils and neutrophils loaded with DHR-123 (1 μM) and stimulated for 10 min with PMA (1 μg/ml) at 37°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>253 ± 56</td>
<td>1112 ± 148</td>
</tr>
<tr>
<td>PMA stimulated (1 μg/ml)</td>
<td>3267 ± 905**</td>
<td>4969 ± 903*</td>
</tr>
<tr>
<td>PMA stimulated (10 μg/ml)</td>
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* Values represent average MFI obtained in five separate experiments.

**p < 0.05
***p < 0.01.
alter organelle densities. Before stimulation, Rac, RhoGDI, and p47phox were unchanged (data not shown), indicating that PMA did not mediate by marker enzyme assays before and after PMA stimulation. Marker enzyme activities and corresponding immunoreactivities were determined in eosinophils, although neutrophils are well known for their expression of oxidase components in specific granules (48, 49). We sought to determine whether eosinophils translocate Rac to cell membranes in correlation with extracellular O2 generation.

Double labeling of Rac and p22phox demonstrates divergence in eosinophil and neutrophil NADPH oxidase assembly

Intracellular sites of NADPH oxidase assembly have not yet been determined in eosinophils, although neutrophils are well known for their expression of oxidase components in specific granules (48, 49). We sought to determine whether eosinophils translocate Rac to cell membranes in correlation with extracellular O2 generation.

Rac immunofluorescence was cytosolically distributed in unstimulated eosinophils (Fig. 5A), whereas p22phox was membrane associated (Fig. 5B) with some intracellular staining. Overlaid images showed little colocalization between Rac and p22phox under b55k immunoreactivity, although no discernable Rac translocation occurred. A small shift in immunoreactivities for p22phox and gp91phox toward lower density fractions (fractions 5 and 6) was observed in PMA-stimulated cells, suggesting that cytochrome b55k may be present in a population of small secretory vesicles that fuse with the plasma membrane on activation.

Translocation of NADPH oxidase components to cell membrane fractions occurs in parallel with O2 generation

Membrane translocation of cytosolic NADPH oxidase components (Rac1/2, p47phox, and p67phox) has been previously shown to correlate with O2 production in neutrophils (30, 31) and guinea pig eosinophils (18, 19). To determine whether similar translocation occurred in human eosinophils, 5 \times 10^7 eosinophils were subjected to subcellular fractionation and immunoblot analysis before and after stimulation with PMA. Fig. 4 shows the profiles of marker enzyme activities and corresponding immunoreactivities for these components. Positions of intracellular organelles determined by marker enzyme assays before and after PMA stimulation were unchanged (data not shown), indicating that PMA did not alter organelle densities. Before stimulation, Rac, RhoGDI, p47phox, and p67phox were predominantly expressed in cytosolic fractions, whereas the cytochrome b55k subunits, p22phox and gp91phox, were localized to plasma membrane-rich fractions. After 8 min of stimulation with PMA (500 ng/ml), p47phox and p67phox, but not RhoGDI, translocated to fractions containing cytochrome b55k immunoreactivity, although no discernable Rac translocation occurred. A small shift in immunoreactivities for p22phox and gp91phox toward lower density fractions (fractions 5 and 6) was observed in PMA-stimulated cells, suggesting that cytochrome b55k may be present in a population of small secretory vesicles that fuse with the plasma membrane on activation.

Eosinophil expression of Rac1, Rac2, and Cdc42 message and protein

To determine expression of Rho-related proteins in eosinophils, we generated primers detecting human Rac1 (using GenBank accession number NM_006908.2) and Rac2 (using GenBank accession number M29871). Primers specific for Rac1 and Rac2 generated products migrating to sizes similar to those from human neutrophils (575 bp for Rac1; 576 bp for Rac2) (Fig. 3A). We also generated primers for human Cdc42. However, these were not intron spanning as the gene sequence for human Cdc42 (GenBank accession number M35543) does not contain any introns (47). Consequently, we obtained PCR products for Cdc42 in non-reverse-transcribed control RNA samples, indicating contamination by genomic DNA (data not shown).

Western blot analysis of whole cell homogenates using Abs raised against human Rac1, Rac2, and Cdc42 demonstrated that Rac and Cdc42 proteins were expressed in eosinophils (Fig. 3B). We were unable to determine whether eosinophils express Rac2 in preference to Rac1, since currently available Abs do not specifically recognize Rac1.

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Rac immunofluorescence was cytosolically distributed in unstimulated eosinophils (Fig. 5A), whereas p22phox was membrane associated (Fig. 5B) with some intracellular staining. Overlaid images showed little colocalization between Rac and p22phox under
basal conditions (Fig. 5C). Stimulation of $O_2^-$ release in eosinophils resulted in a pronounced translocation of Rac from the cytoplasm to the cell periphery (Fig. 5D), to colocalize with membrane p22phox immunofluorescence (Fig. 5F).

Resting neutrophils exhibited a pattern of Rac and p22phox immunofluorescence similar to that of eosinophils (Fig. 5, G–I). However, stimulated neutrophils showed a distinct translocation of Rac from that of eosinophils, in which increased intracellular Rac staining was detected which colocalized with the p22phox label (Fig. 5, J and L). These findings suggest that, unlike eosinophils, activated NADPH oxidase predominantly assembled at intracellular sites in neutrophils.

**Eosinophils and neutrophils in asthmatic sputum samples exhibit distinct patterns of Rac distribution**

We examined eosinophils in sputum from atopic asthmatics to examine patterns of Rac staining ex vivo. We were surprised to find that granulocytes in sputum exhibited Rac staining similar to those stimulated in vitro. As shown in Fig. 6, B–F, morphologically intact MBP$^+$ eosinophils in asthmatic sputum samples exhibited peripheral immunostaining for Rac along cell membranes, analogous to that found in PMA-stimulated eosinophils (Fig. 5D). In contrast, MBP$^+$ eosinophils in normal sputum samples, which were rare, expressed low levels of cytosolic Rac (Fig. 6G), comparable with unstimulated
peripheral blood eosinophils (Fig. 5A). Moreover, MBP– polymorphonuclear neutrophils in asthmatic sputum, identified based on their multilobular nuclear morphology using 4′,6′-diamidino-2-phenylindole nuclear counterstaining, displayed substantial intracellular Rac staining (Fig. 6H), similar to those stimulated in vitro with PMA (Fig. 5J).

**Discussion**

Translocation and assembly of NADPH oxidase are essential for regulated $\text{O}_2^-$ generation in phagocytes. Activation of this complex is critically dependent on receptor stimulation of intracellular regulatory Rho-related GTPases, principally Rac1 or its homolog Rac2. In this study, PMA-induced $\text{O}_2^-$ generation in eosinophils was shown to require Rac stimulation of NADPH oxidase, as demonstrated by its sensitivity to toxin B inhibition. Although toxin B inhibits Rho, Rac, and Cdc42 by monoglucosylation at Thr37 or Thr35 (50), it is likely that the inhibitory effect of toxin B on $\text{O}_2^-$ production in eosinophils was mediated through blockade of Rac1 or Rac2. This is based on findings from cell-free assays that demonstrated that only Rac1 and Rac2, and not Rho or Cdc42, were able to activate NADPH oxidase in reconstituted lipid bilayers (24).

In addition, human eosinophils were shown to express both Rac1 and Rac2 mRNA, although only Rac2 protein could be positively identified by Western blot analysis. This is similar to guinea

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**FIGURE 5.** Confocal analysis of respiratory burst in eosinophils and neutrophils. Cells were double labeled for Rac and p22phox, followed by DAPI nuclear counterstain (blue). Rac immunofluorescence (A, D, G, and J), shown in red, was overlaid with green p22phox immunofluorescence (B, E, H, and K) to determine colocalization as indicated by yellow-orange color (C, F, I, and L). Anti-Rac from Upstate Biotechnology was used in these experiments. A–C, Resting eosinophils; D–F, PMA-stimulated (500 ng/ml) eosinophils. Neutrophils, shown in G–I, were compared with PMA-stimulated cells (J–L). Original magnification, ×40.
pig eosinophils, which may produce both isoforms (18, 19). Human neutrophils, in contrast, have been shown to predominantly express Rac2 protein (21, 31). Rac2 binds with a 6-fold higher affinity with p67phox than Rac1 in two-hybrid assays, suggesting that it may be a more effective inducer of oxidase activity than Rac1 (51). Previous studies have demonstrated expression of p40phox, p47phox, p67phox, and cytochrome b558 in human eosinophils (15, 17, 52–55) but did not show expression of Rho-related GTPases. Eosinophils may preferentially express Rac2 rather than Rac1 based on a shared hemopoietic lineage with neutrophils, although we were unable to determine Rac1 expression based on our Western blot data.

Translocation of oxidase components during respiratory burst has not previously been demonstrated in human eosinophils. In our

**FIGURE 6.** Confocal and deconvolution restoration microscopic analysis of eosinophils and neutrophils in sputum samples. Confocal images are shown for sputum eosinophils from normal (A) and asthmatic (B) subjects after labeling with anti-Rac, shown in red fluorescence, and MBP, as indicated by green. Deconvolution restoration microscopy was also conducted on eosinophils (C–F) and neutrophils (H) from asthmatic sputum. All panels were produced from combined images of double labeling. A sputum eosinophil from a normal donor (G) is shown for comparison. Original magnification, ×63. Sections of asthmatic sputum shown here are representative of samples obtained from five atopic asthmatics.
study, translocation of cytosolic p47^phox and p67^phox to plasma membrane at 8 min of PMA stimulation in eosinophils was similar to earlier observations on guinea pig eosinophils (18, 19) and correlated with observations in neutrophils (30). This study demonstrated that p47^phox and p67^phox translocated to the membrane during respiratory burst on an equimolar basis with Rac. The majority of cellular cytochrome b_{558} is localized to specific granule membranes in neutrophils, which is transferred to plasma or phagosomal membranes on activation (48, 49, 56). Cytosolic p47^phox and p67^phox associate with cytochrome b_{558} after phosphorylation of specific serine/threonine sites (8). These bind through Src homology 3 and pleckstrin homology domains to allow association of Rac to plasma membrane and p67^phox. Collectively, these subunits work to initiate electron transfer from NADPH to flavin adenine di-nucleotide through cytochrome b_{558}, ultimately resulting in formation of O_2^- from O_2 on the external or luminal surface of the membrane (8).

The mobilization and assembly of NADPH oxidase to cell membranes in human eosinophils correlated with maximal O_2^- generation as determined by cytochrome c reduction assays. This procedure measures only extracellular production of O_2^-, since O_2^- and cytochrome c are membrane impermeable. Previous studies using immunoblot analysis have demonstrated that eosinophils produce more NADPH oxide than neutrophils, which was thought to explain why eosinophils generate more O_2^- than neutrophils (15, 17, 18). However, the discrepancy in O_2^- production between eosinophils and neutrophils may also be attributable to neutrophils preferentially generating O_2^- inside cells. We tested this notion by stimulating cells with PMA in the presence of DHR-123, which can detect intracellular ROS production by reacting with H_2O_2 and forming a fluorescent product inside the cells (46). Total ROS production was found to be equivalent in PMA-stimulated neutrophils and eosinophils using DHR-123. These findings suggest that neutrophils preferentially generate O_2^- intracellularly, which may partially account for the discrepancy in cytochrome c measurements of O_2^- release from eosinophils and neutrophils.

The confocal data in this study supported the possibility that eosinophils generate most of their O_2^- extracellularly in correlation with the translocation of Rac to the cell membrane. We observed by confocal microscopy that, in striking contrast to neutrophils, eosinophils preferentially translocated Rac to the plasma membrane rather than intracellular sites following PMA stimulation. Neutrophils did not appear to translocate Rac2 to plasma membrane, and instead directed Rac2 to intracellular sites in association with p22^phox of PMA stimulation. Interestingly, eosinophils are able to phagocytose extracellular Escherichia coli and Staphylococcus aureus but are unable to kill these as efficiently as neutrophils (57), which was believed to correlate with an inability to modify amino acids through the peroxidase-H_2O_2-Cl^- system. Our findings indicate that eosinophils may be less efficient at killing due to the lack of significant intracellular O_2^- production.

Oxidase assembly may occur in two distinct pools in the neutrophil which are regulated by different pathways. The mechanism of action of PMA is not well understood, although it is frequently assumed to activate NADPH oxidase through protein kinase C. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), has been shown to block only intracellular production of O_2^- and not extracellular release induced by PMA (58). This indicates that a PI3K-dependent pathway may activate only intracellular O_2^- production in neutrophils, whereas a PI3K-independent pathway regulates extracellular release of O_2^- in contrast, the effect of wortmannin on eosinophil respiratory burst is not well understood. Thus, whereas eotaxin-induced O_2^- release was inhibited by wortmannin in human eosinophils, it was without effect on leukotriene B_4^-induced respiratory burst in guinea pig eosinophils (1). It remains to be determined whether eosinophils utilize a PI3K-independent pathway in PMA-induced oxidase activation to release O_2^- extracellularly.

We did not detect significant expression of oxidase components in eosinophil crystalloid granule-enriched subcellular fractions, but rather in plasma membrane/light membrane fractions (including small secretory vesicles; Fig. 4), which supports the findings of Calafat et al. (53). NADPH oxidase activation is therefore unlikely to involve crystalloid granules in eosinophils. This is in contrast to neutrophils, which express cytochrome b_{558} in specific granules (48, 49, 53, 59). The majority of NADPH oxidase activity in neutrophils undergoing arachidonate- or PMA-induced respiratory burst was found to localize to specific granules (58, 60). The function of cytochrome b_{558} expression in neutrophil-specific granules is thought to be associated with NADPH oxidase activation after fusion of the specific granules with newly phagocytosed particles, with the purpose of carrying out oxygen-dependent intracellular killing of phagocytosable microorganisms (24). The lack of expression of NADPH oxidase components on crystalloid granules lends further support to the suggestion that eosinophils do not generate significant intracellular O_2^- during respiratory burst and instead direct O_2^- towards extracellular regions. Although translocation of Rac to the cell membrane was not evident in Western blot analysis, it was detectable in confocal microscopy analysis, suggesting that the latter technique may be substantially more sensitive to translocation events than Western blot. When Rac2 translocation was shown in PMA-stimulated guinea pig eosinophils in our previous report (19), the quantity translocated was barely detectable by immunoblot analysis.

The distinct pattern of Rac immunoreactivity in activated eosinophils in vitro was observed in ex vivo sputum samples from asthmatic patients. Rac immunofluorescence in normal sputum eosinophils was less than that of asthmatic cells and did not exhibit a peripheral membrane pattern. In contrast, sputum eosinophils from asthmatic patients exhibited intense Rac immunofluorescence around the cell membrane, suggesting that these cells were stimulated and were actively releasing O_2^- into the tissues and airways. These observations indicate that sputum eosinophils in unstable asthma may be activated by in vivo stimuli to produce extracellular O_2^- and contribute to oxidant-mediated tissue injury. These novel observations indicate that eosinophil respiratory burst may be important in the pathogenesis of asthma.

In conclusion, eosinophils appear to assemble NADPH oxidase similarly to neutrophils at the level of molecular complex formation, whereas intracellular distribution of NADPH oxidase may differ significantly between these two cell types. This divergence, reflected in a predominantly plasma membrane association of Rac with p22^phox in eosinophils vs a mainly intracellular location in neutrophils, may parallel distinct functional roles that these two cell types have in innate immunity. Thus, our findings suggest that sputum eosinophils from individuals with unstable asthma may be activated to produce more extracellular O_2^- than neutrophils, with the potential to induce tissue damage and contribute to the pathogenesis of this disease. Neutrophils may produce comparatively less extracellular O_2^- in sputum from these individuals. The differential manner of NADPH oxidase assembly in these cells may have important implications for determining the activation status of airway eosinophils and, ultimately, treatment of oxidant-mediated tissue injury in asthma.

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