

Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways

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Background: Asthma is a heterogeneous inflammatory airway disorder that involves eosinophilic and noneosinophilic phenotypes. Unlike in healthy lungs, eosinophils are often present in atopic asthmatic airways, although a subpopulation of asthmatic subjects predominantly experience neutrophilic inflammation. Recently, it has been demonstrated that eosinophils and neutrophils generate bactericidal extracellular traps consisting of DNA and cytotoxic granule proteins. **Objective:** We sought to explore whether living eosinophils and neutrophils infiltrating human atopic asthmatic airways actively form extracellular DNA traps *in vivo*. **Methods:** Quantitative analysis of eosinophils releasing DNA was performed in endobronchial biopsy specimens from 20 human subjects with mild atopic asthma at baseline and after local allergen challenge and 10 healthy subjects. DNA was stained with propidium iodine and major basic protein with specific antibody. Differential cell counts and cytokines/chemokines were assessed in bronchoalveolar lavage fluid. **Results:** Asthmatic airways were infiltrated with a significantly higher number of eosinophils than healthy airways (39.3 ± 4.6 vs 0.4 ± 0.9 , $P < .0001$). All asthmatic subjects but only 1 control subject expressed eosinophils releasing DNA that colocalized with major basic protein (33.65 ± 20.33 vs 0.3 ± 0.9 per high-power field, $P < .0001$). Four asthmatic subjects mostly expressed neutrophilic inflammation and neutrophil DNA traps. Allergen challenge had no significant quantitative effect on eosinophil or neutrophil DNA traps. Airway eosinophils or DNA traps did not correlate with either bronchoalveolar lavage levels of IL-5, IFN- γ , or eotaxin or the provoking doses of methacholine or allergen in asthmatic subjects. **Conclusions:** Extracellular DNA traps are generated by eosinophils and neutrophils in human atopic asthmatic airways *in vivo*. The mechanism and role of this new finding will

necessitate further investigation. (J Allergy Clin Immunol 2011;127:1260-6.)

Key words: Atopic asthma, bronchoalveolar lavage fluid, biomarker, eosinophils, extracellular DNA traps, human, neutrophils

Asthma is a heterogeneous inflammatory airway disorder that involves eosinophilic and noneosinophilic phenotypes. The latter are frequently subdivided into neutrophilic and paucigranulocytic subtypes.¹ Eosinophils are unique bone marrow–derived granulocytic leukocytes with potent cytotoxic activities and functions linked to both innate and adaptive immunity.² Although eosinophils are mainly tissue leukocytes, they are rarely present in healthy lungs.³ In contrast, eosinophils are commonly found in asthmatic airways, and their numbers increase during allergen-induced asthma exacerbation in most atopic asthmatic subjects.⁴ Nonetheless, some atopic asthmatic subjects predominantly have neutrophilic airway inflammation⁵ and react to allergen with marked airway influx of neutrophils.⁶ Patients with neutrophilic asthma usually have more severe disease and respond poorly to corticosteroids.⁷ Currently, the cause and pathophysiologic consequences of these phenotypic discrepancies among allergic asthmatic subjects are not well understood.

Recently, it has been reported that eosinophils primed with IL-5 or IFN- γ and stimulated with bacterial LPS, eotaxin, or complement 5a (C5a) rapidly release mitochondrial DNA and granule proteins, such as major basic protein (MBP) or eosinophil cationic protein (ECP), to form bactericidal traps in the extracellular space,⁸ which in this article are called eosinophil extracellular traps (EETs). It has been shown that viable neutrophils primed with GM-CSF and stimulated with Toll-like receptor 4 receptor agonist or C5a release mitochondrial DNA and form neutrophil extracellular traps (NETs).⁹ Active expulsion of mitochondrial DNA by viable eosinophils and neutrophils differs from the previously described microbe-killing extracellular traps generated by dead neutrophils¹⁰ and mast cells¹¹ passively releasing nuclear DNA, histones, and proteases after reactive oxygen species–mediated cell damage.¹² Although active release of mitochondrial DNA by viable eosinophils and neutrophils is also reactive oxygen species dependent, the process does not lead to cell death.^{8,9} Formation of extracellular traps by eosinophils, neutrophils, and mast cells could be viewed as a double-edged sword providing an important mechanism of host innate immunity against infections and simultaneously causing cell and tissue injury.⁸

In the present study we tested the hypothesis that eosinophils and neutrophils infiltrating airways in human atopic asthmatic subjects would form extracellular DNA traps *in vivo*.

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Abbreviations used

Atp6: ATP synthase subunit 6 gene
BAL: Bronchoalveolar lavage
C5a: Complement 5a
ECP: Eosinophil cationic protein
EET: Eosinophil extracellular trap
Gapdh: Glyceraldehyde-3-phosphate dehydrogenase gene
hpf: High-power field
MBP: Major basic protein
NET: Neutrophil extracellular trap

METHODS

Subjects

Twenty subjects with mild allergic asthma based on National Asthma Education and Prevention Program guidelines¹³ and 10 healthy volunteers were recruited from the middle Tennessee community (Table I). All asthmatic volunteers had positive skin test results with common aeroallergens, methacholine challenges, and inhaled allergen challenges during screening before enrollment into the study. Oral antihistamines and inhaled corticosteroids (1 patient) were discontinued 48 hours before procedures. Pregnant women were excluded by means of urine human chorionic gonadotropin testing. No volunteer had a history of smoking, other concomitant lung disease, or a respiratory tract infection in the 6 weeks preceding the study. Volunteers consented to our protocol verbally and in writing. The protocol was approved by the Vanderbilt University Committee for the Protection of Human Subjects.

Study protocol

Twenty asthmatic volunteers underwent bronchoscopy with baseline (before allergen challenge) bronchoalveolar lavage (BAL) and airway biopsy in the lingua of the left upper lobe followed by segmental allergen challenge in a subsegment of the right middle lobe. The allergen-challenged subsegment underwent lavage, and biopsy specimens were obtained 24 hours later. At least 3 weeks after the bronchoscopic studies, asthmatic subjects underwent methacholine challenge followed by an inhaled allergen provocation the next day. Ten healthy volunteers underwent baseline bronchoscopy with collection of BAL fluid and airway biopsy specimens.

Allergy skin tests

Skin prick testing was performed with diluent and histamine controls in parallel with a battery of standardized aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat hair, Bermuda grass, Kentucky bluegrass, fescue meadow grass, orchard grass, redtop grass, ryegrass, sweet vernal grass, timothy grass, and short ragweed; Greer Laboratories, Lenoir, NC).

Bronchoscopic studies

Volunteers fasted overnight. Midazolam (1–2 mg) and fentanyl (50–100 µg) were administered intravenously if patients wished conscious sedation. Topical lidocaine (≤ 7 mg/kg) was used for airway anesthesia. BAL was performed with two 50-mL aliquots of normal saline and immediately suctioned back. Four endobronchial biopsy specimens were taken with disposable alligator forceps (Olympus FB-211D; Olympus, Ltd, Japan) at bifurcations of the subsegmental airways, fixed in 4% paraformaldehyde, and embedded in paraffin. Segmental allergen challenge was done with 2 incremental doses of the allergen to which the volunteers had a positive skin test result, with each dose in a 5-mL aliquot of normal saline. The first dose of the allergen was 10-fold higher than the concentration that produced a 2+ skin reaction on intradermal testing (5–8 mm wheal and 20–30 mm erythema). The subsegment was watched for 3 minutes. None of the volunteers had a visible local inflammatory reaction after the first dose of allergen. Thus in each subject the second dose of the same allergen was instilled at a concentration 100-fold higher than that eliciting the 2+ skin reaction, and the bronchoscope was then removed.¹⁴ Allergens used for challenge included (number of subjects in

TABLE I. Patients' demographics, baseline characteristics, and history of atopy, asthma, and asthma-medication use

Characteristic	Mean/range
Sex, F/M	10/10
Average age (y), F/M	31.7 (23–44)/27.6 (22–29)
Race	
White	18
African American	2
Family history of atopy	20
History of allergic rhinitis	12
Frequency of daily asthma symptoms	
≤ 2 d/wk	15
>2 d/wk but not daily	5
Nighttime awakenings	
≤ 2 d/wk	20
>2 d/wk but not daily	0
Baseline spirometry (% predicted)	
FEV ₁ (% predicted)	90 (75–109)
FEV ₁ /FVC ratio	0.69 (0.56–0.76)
Methacholine PC ₂₀ (mg/mL)	5.5 (0.15–10)
Medications	
H1-antihistamine	8
Albuterol ≤ 2 d/wk	20
Fluticasone/salmeterol	1
Leukotriene-modifying agents	0

brackets) *Dermatophagoides pteronyssinus* (n = 4), *Dermatophagoides farinae* (n = 2), cat hair (n = 6), Bermuda grass (n = 1), Kentucky bluegrass (n = 2), timothy grass (n = 1), ray grass (n = 1), and short ragweed (n = 3).

Methacholine challenge

Methacholine (Provacholine; Methapharm, Inc, Brantford, Ontario, Canada) challenge was undertaken according to American Thoracic Society guidelines¹⁵ by using a Salter dosimeter (Salter Labs, Arvin, Calif) and Flow Screen computerized spirometer (VIASYS Healthcare GmbH, Hoechberg, Germany).

Inhaled allergen challenge

Allergen inhalation challenge was carried out as previously described.¹⁶ Allergen aerosols were generated by using a Wright nebulizer connected to a 2-way Hans Rudolph valve mouthpiece (Roxon, Montreal, Canada) attached to a wall oxygen source at 50 psi. Spirometry was performed with a Flow Screen computerized spirometer.

Determination of eosinophil and neutrophil DNA traps

EETs and NETs were identified as previously described.^{8,9} Briefly, endobronchial biopsy specimens were paraformaldehyde fixed and paraffin embedded. Staining was performed on 4-µm-thick sections. DNA was stained with propidium iodide. MBP and elastase were detected by means of indirect immunofluorescence. Polyclonal anti-MBP antibody was a kind gift of Dr Gerald Gleich (Salt Lake City, Utah), and monoclonal anti-elastase antibody was obtained from DakoCytomation (Glostrup, Denmark). After incubation with primary antibody, tissues were incubated with the appropriate fluorescein isothiocyanate-conjugated secondary antibody (Amersham Pharmacia Biotech, Duebendorf, Switzerland) and analyzed by means of confocal laser-scanning microscopy (LSM 510; Carl Zeiss Microimaging, Jena, Germany). To show the extracellular DNA in its full extension, we performed image and data processing, as described previously.⁸ It should be noted that this technology allowed identification of the cellular source of the released DNA.⁸ There are currently no fluorescent dyes available that would allow distinguishing between nuclear and extracellular DNA in tissues.

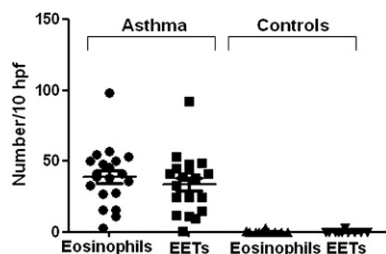


FIG 1. Eosinophil and EET counts per 10 hpf in airway biopsy specimens from asthmatic and control subjects. Values are presented as single numbers, as well as mean levels $\pm$ SEMs.

Cell death assay

Cell death was assessed on 4- μ m-thick, paraformaldehyde-fixed, paraffin-embedded lung tissue sections by using a TdT-mediated dUTP-biotin nick end-labeling assay (Roche Diagnostics Corp, Rotkreuz, Switzerland). Esophageal tissues from patients with eosinophilic esophagitis were used as a positive control.¹⁷ In these assays eosinophils were labeled with a monoclonal anti-ECP antibody (EG1; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Slides were analyzed by means of confocal microscopy.

Fluorescent *in situ* DNA analysis

DNA was assessed on 4- μ m-thick, paraformaldehyde-fixed, paraffin-embedded lung tissue sections by using fluorescence-labeled probes (Cy3) for the ATP synthase subunit 6 gene (*Atp6*) and glyceraldehyde-3-phosphate dehydrogenase gene (*Gapdh*) genes by using the Label IT labeling kit, according to the manufacturer's instructions (Mirus Bio LLC, Madison, Wis).⁸ Hybridization was performed overnight at 42°C by using the Label IT fluorescent *in situ* hybridization kit (Mirus Bio LLC).⁸ Slides were mounted by using antifade 4'-6-diamidino-2-phenylindole dihydrochloride counterstain and analyzed by means of confocal microscopy.

Measurement of cytokines in BAL fluid

IL-5 and IFN- γ levels in BAL fluid were measured with a 10-plex MSD Multi-Spot assay (Meso Scale Discovery, Gaithersburg, Md; IL-5 and IFN- γ), and GM-CSF and eotaxin levels were measured with a Cytometric Bead Array (CBA; BD Biosciences, San Jose, Calif).

Statistical analysis

A 2-tailed Mann-Whitney test was used for comparison of asthmatic and healthy control subjects, the Wilcoxon signed-rank test was used for comparisons within the asthmatic group, and the Spearman rank correlation coefficient was used for correlation analyses with the software package SPSS (SPSS, Inc, Chicago, Ill). Data were expressed as means $\pm$ SEMs. Significance was accepted at a *P* value of less than .05.

RESULTS

Extracellular DNA traps generated by eosinophils are a common phenomenon in subjects with atopic asthma

Atopic asthmatic airways were infiltrated with a significantly higher number of eosinophils than healthy airways (39.3 ± 4.6 vs 0.4 ± 0.9 , $P < .0001$). Eosinophils were detected in all asthmatic biopsy specimens at baseline, although their numbers varied among subjects (range, 3-98 eosinophils per 10 high-power fields [hpf]; mean, 39.3). Small numbers of eosinophils were found in 2 normal biopsy specimens (range, 1-3 per 10 hpf; mean, 0.4). All atopic asthmatic subjects but only 1 healthy subject expressed EETs (33.65 ± 20.33 vs 0.3 ± 0.9 per 10 hpf, $P < .0001$,

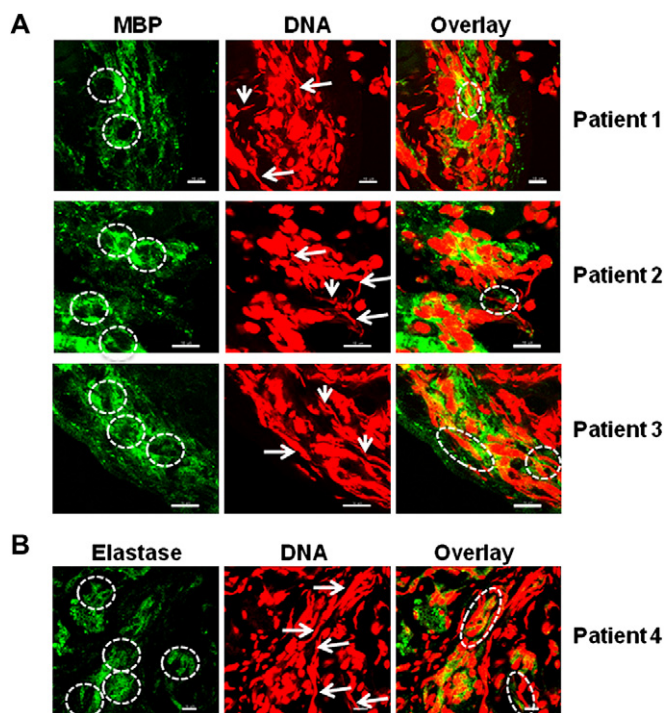


FIG 2. Representative endobronchial biopsy specimens from atopic asthmatic subjects showing EETs and NETs. **A**, EETs generated by eosinophils contain DNA and MBP. Biopsy specimens were from 3 different patients. In the *left panels* infiltrating eosinophils expressing MBP are circled by *dashed lines*. In the *middle panels* examples of extracellular DNA are indicated by *arrows*. In the *right panels* colocalization of extracellular DNA and MBP is circled by *dashed lines*. **B**, NETs generated by neutrophils contain DNA and elastase. Representative results from 1 patient are shown. In the *left panel* infiltrating neutrophils expressing elastase are circled by *dashed lines*. In the *middle panel* examples of extracellular DNA are indicated by *arrows*. In the *right panel* colocalization of extracellular DNA and elastase is circled by *dashed lines*. Bars = 10 μ mol/L. Control antibodies showed no staining (data not shown).

Fig 1). The percentage of EETs varied among asthmatic subjects (range, 1-92; mean, 33 per 10 hpf) but correlated with the total number of eosinophils on biopsy specimens (Spearman coefficient, 0.82; $P < .0001$). The extracellular DNA colocalized with MBP (Fig 2, A) in a fashion similar to the structures previously described in patients with Crohn disease and intestinal infections.⁸ It should be noted that colocalization cannot always be demonstrated in a 2-dimensional picture. However, when using a z-stack, colocalization at the same extracellular DNA can often be seen, but then on other places of the image, it can no longer be demonstrated. In addition, because it is unlikely that the EETs are preformed intracellularly, not all DNA and granule proteins necessarily colocalize in the extracellular space. In fact, extracellular MBP depositions were also seen not adjacent to DNA-releasing eosinophils. In analogy to the NETs, which also contained DNA and granule proteins,¹⁰ we decided to call the structures released by eosinophils EETs.

Neutrophil extracellular DNA traps are seen in patients with neutrophilic asthma

Four subjects with atopic asthma expressed a high number of neutrophils and neutrophils generating NETs that exceeded the

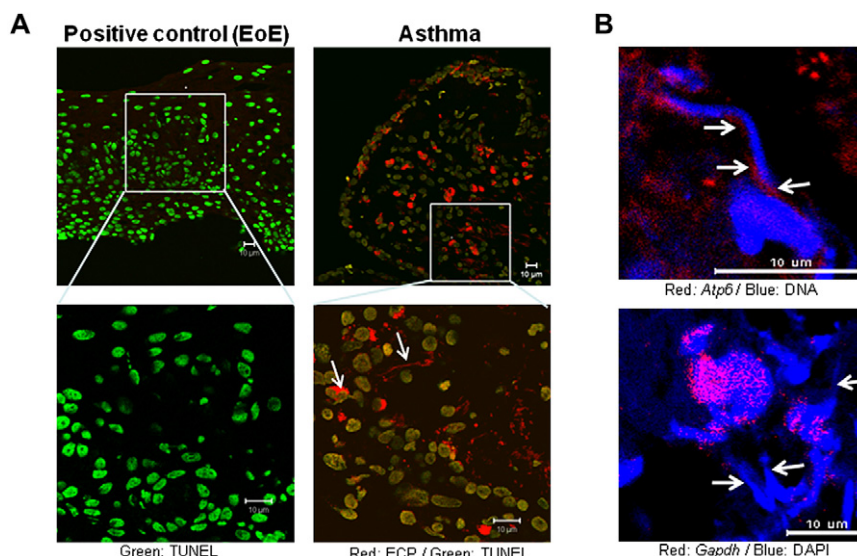


FIG 3. Extracellular DNA is released from viable eosinophils. **A**, Positive TdT-mediated dUTP-biotin nick end-labeling (*TUNEL*) analysis of an esophageal biopsy specimen obtained from a patient with eosinophilic esophagitis (*EoE*; left panel) and an asthmatic airway biopsy specimen showing a negative *TUNEL* assay (right panel). Eosinophils and EETs were identified by using anti-ECP antibody (arrows). Lower panels represent magnifications of the indicated fields within the upper panels. **B**, Fluorescent *in situ* analysis of asthmatic biopsy specimens. Positive signals within the extracellular DNA (arrows) were seen with an *Atp6* but not with a *Gapdh* probe. The data in each panel are representative of at least 3 independent experiments. Bars = 10 μ m. *DAPI*, 4'-6-Diamidino-2-phenylindole dihydrochloride.

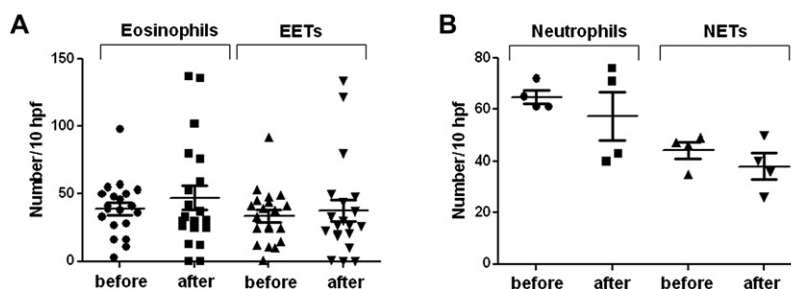


FIG 4. No effect of segmental allergen challenge on numbers of EETs and NETs in airway biopsy specimens of atopic asthmatic subjects. **A**, Eosinophils and EETs before and 24 hours after allergen challenge. **B**, Neutrophils and NETs before and 24 hours after allergen challenge in asthmatic subjects with neutrophil infiltration. Values are presented as single numbers, as well as mean levels $\pm$ SEMs per 10 hpfs. Changes are not significant.

number of eosinophils and eosinophils generating EETs (61/35 vs 3/1, 65/47 vs 16/12, and 61/46 vs 38/38 total neutrophils/NETs vs total eosinophils/EETs per 10 hpfs, respectively). The DNA released by neutrophils colocalized with the granule protein elastase (Fig 2, B).

Extracellular DNA traps appear to be generated by viable eosinophils

Cell death was assessed on selected asthmatic biopsy specimens by using the TdT-mediated dUTP-biotin nick end-labeling technique. Eosinophils were stained with anti-ECP antibody, which also detected extracellular ECP. The morphology of the extracellular ECP structures suggested that ECP was bound to DNA and a component of EETs. In these experiments we did not obtain any evidence that eosinophils would undergo cell death,

suggesting active generation of EETs by living eosinophils (Fig 3, A). This assumption is supported by the observation that the mitochondrial *Atp6* gene signal was readily detectable in extracellular DNA released by tissue-infiltrating eosinophils, as assessed by means of fluorescent *in situ* analysis (Fig 3, B). In contrast, the *Gapdh* gene signal was selectively seen in nuclei (Fig 3, B). Together these data suggest that the DNA is actively released by eosinophils in asthmatic lungs and of mitochondrial but not nuclear origin.

Allergen challenge does not increase eosinophil or neutrophil extracellular DNA traps

Neither the number of total eosinophils nor eosinophils generating EETs in the asthmatic airways significantly increased after allergen challenge (Fig 4, A). Likewise, neither the number

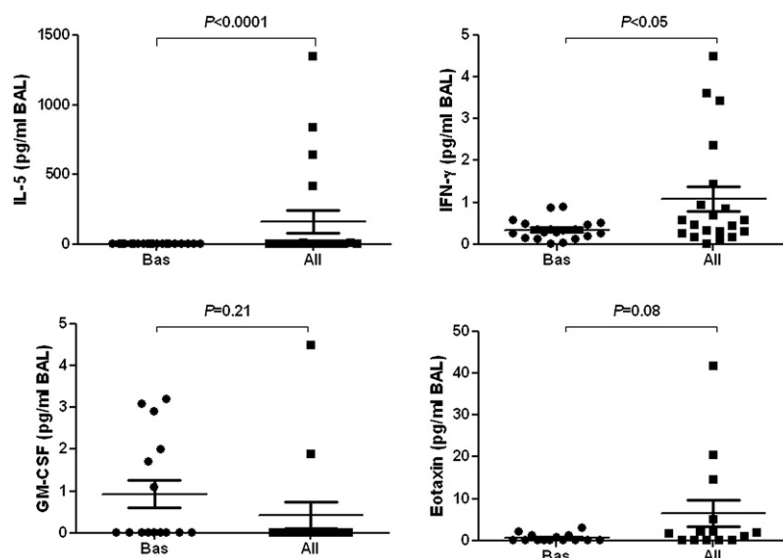


FIG 5. Effect of segmental allergen challenge on cytokine/chemokine concentrations in BAL fluid of atopic asthmatic subjects. The indicated cytokines/chemokines were measured before and 24 hours after allergen challenge. Values are presented as single numbers, as well as mean levels $\pm$ SEMs. *P* values are indicated.

TABLE II. Relationship between percentages of EETs and NETs in asthmatic airways, provocative thresholds of methacholine and allergen, and levels of cytokines in BAL fluid (correlation coefficient and significance [*r* and *P* values, respectively])

	Methacholine PC ₂₀	Allergen PC ₂₀	IL-5	IFN- γ	Eotaxin	GM-CSF
EETs						
<i>r</i>	-0.43	-0.22	-0.1	-0.43	-0.44	—
<i>P</i> value	.054	.34	.107	.06	.11	—
NETs						
<i>r</i>	0.56	0.4	—	—	—	-0.86
<i>P</i> value	.44	.75	—	—	—	.33

of total neutrophils nor neutrophils generating NETs was significantly higher after allergen provocation (Fig 4, B). In contrast, allergen produced a significant increase in the BAL fluid eosinophils and neutrophils in asthmatic subjects ($2.1\% \pm 0.67\%$ vs $18.25\% \pm 5.9\%$ BAL cells [$P < .0002$] and $2.5\% \pm 0.56\%$ vs $21.1\% \pm 4.7\%$ BAL cells [$P < .0003$], respectively). Interestingly, asthmatic subjects with more pronounced neutrophilic airway wall inflammation had predominantly BAL neutrophilia at 24 hours after allergen challenge.

The levels of IL-5 and IFN- γ in BAL fluid significantly increased after allergen challenge, although the degree of the response markedly differed among subjects. The increase in the allergen-stimulated increase in eotaxin was nearly statistically significant. On the other hand, allergen did not significantly affect generation of GM-CSF in BAL fluid (Fig 5). No correlation was found between the total number of eosinophils or eosinophils generating EETs in biopsy specimens and concentrations of IL-5, IFN- γ , or eotaxin in BAL fluid either before or after allergen challenge. Likewise, the number of tissue eosinophils or eosinophils generating EETs did not correlate with methacholine PC₂₀ and allergen PC₂₀ in the atopic asthmatic subjects (Table II).

DISCUSSION

Our study demonstrates that EETs composed of DNA and MBP are almost universally formed in atopic asthmatic airways. Airway biopsy specimens from some asthmatic subjects showed both EETs and NETs. One patient expressed predominantly extracellular NETs. The DNA-releasing eosinophils and neutrophils had no features of damage or apoptosis and appeared to release mitochondrial DNA, suggesting an active mechanism of extracellular DNA trap formation. In contrast to asthmatic subjects, a small number of EETs were found in 1 healthy volunteer only, and none of the normal biopsy specimens expressed NETs. This is a striking finding because all asthmatic subjects participating in our study had only mild persistent or mild intermittent asthma and normal baseline airway function. The presence of EETs and NETs has not previously been described in human atopic asthmatic airways *in vivo*. Presently, however, the specificity of the newly described feature of airway inflammation in atopic asthma is unknown because subjects with other inflammatory airway disorders were not involved in our study.

The mechanism of EET and NET formation in atopic asthmatic airways will necessitate additional studies. It has been demonstrated that development of EETs requires priming of eosinophils with IL-5 or IFN- γ and stimulation with LPS, eotaxin, or C5a *in vitro*.⁸ Increased generation of IL-5 is a characteristic feature of airway inflammation in a high proportion of atopic asthmatic subjects.¹⁸ Likewise, increased frequency of IFN- γ -producing cells¹⁹ and increased expression of eotaxin²⁰ has previously been described in asthmatic subjects compared with control subjects. In this study we obtained evidence for increased IL-5, IFN- γ , and eotaxin levels in asthmatic BAL fluid, at least after allergen challenge. Moreover, endotoxin is a natural environmental exposure factor,²¹ and C5a levels are increased in blood and epithelial lining fluid in asthmatic subjects.²² Similarly, GM-CSF, which primes viable neutrophils to generate NETs in response to Toll-like receptor 4 or C5a receptor

stimulation *in vitro*,⁹ is upregulated in asthmatic airways.²³ Hence the airway environment generated by immune and inflammatory responses in atopic asthmatic subjects might favor EET and NET formation by viable eosinophils and neutrophils *in vivo*. We found no significant correlation between the numbers of eosinophils, neutrophils, EETs, or NETs and the levels of IL-5, IFN- γ , eotaxin, or GM-CSF in BAL fluid either at baseline or after allergen challenge in our patients. However, these results need to be interpreted with caution because BAL findings do not necessarily exactly reflect the tissue environment in the asthmatic airways.²⁴ Moreover, it is likely that the extracellular DNA is rapidly destroyed by tissue nucleases. Different kinetics in the degradation of BAL fluid cytokines and extracellular bronchial DNA could, at least partially, explain the lack of correlation between these parameters.

The physiologic role, pathophysiologic role, or both of EET and NET formation in asthmatic airways is unclear and also will require additional investigations. Asthma is associated with a loss of the structural integrity of airway epithelium and dysfunction of the physical barrier, which protects airways from external harmful factors, including infections.²⁵⁻²⁸ Thus EETs and NETs, which have a potent bactericidal activity,^{8,9} could serve as an important innate immunity mechanism protecting the airway wall from invasion by infectious factors. On the other hand, cytotoxic cationic proteins released by activated eosinophils might significantly contribute to the pathomechanism of airway injury² and remodeling²⁹ and adaptive immune responses involved in the pathomechanisms of atopic asthma.³⁰

We found no correlation between the number of eosinophils or eosinophils generating EETs in airway biopsy specimens and eosinophils in BAL fluid either before or after allergen challenge. Likewise, our study did not reveal a correlation between the number of eosinophils or eosinophils generating EETs and nonspecific airway hyperresponsiveness to methacholine or airway sensitivity to specific allergen. Previous studies with humanized mAbs against IL-5, such as mepolizumab, demonstrated marked discrepancy between the BAL fluid and bronchial mucosal eosinophilia in human asthmatic subjects.²³ Despite a significant reduction in BAL fluid eosinophilia, sputum eosinophilia, or both, mepolizumab had no significant effect on airway tissue eosinophilia and airway function in asthmatic subjects.³¹ Although a recent study demonstrated reduction of severe asthma exacerbations and improvement in asthma scores in patients with refractory eosinophilic asthma treated with mepolizumab, the role of mucosal eosinophilia in the pathophysiology of specific and nonspecific airway hyperresponsiveness in human asthmatic subjects remains currently unclear.^{32,33}

In summary, in this study we describe formation of eosinophil and neutrophil extracellular DNA traps representing a novel feature of viable eosinophils and neutrophils infiltrating the airway wall in human atopic asthmatic subjects *in vivo*. The mechanism of EET and NET formation, the physiologic role, pathophysiologic role, or both of these structures in allergic asthma, and the influence of pharmacologic drug treatments will necessitate further investigation.

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Clinical implications: EETs (1) are frequently seen in subjects with atopic asthma, (2) consist of DNA and granule proteins, and (3) might be a new useful biomarker reflecting eosinophil activation.

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