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A M E R I C A N C O L L E G E O F
 C H E S T
P H Y S I C I A N S

Airway Inflammation and Cellular Stress in Noneosinophilic Atopic Asthma*

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Study objectives: It has been suggested that patients with noneosinophilic asthma (NEA) show increased numbers of sputum neutrophils and a lack of response to therapy with corticosteroids, which are features that are commonly related to COPD. The aim of our study was to test the hypothesis that airway inflammation in NEA patients is different from that seen in patients with eosinophilic asthma (EA) and is similar to COPD.

Design: Sputum cellular stress markers and neutrophilic and eosinophilic fluid-phase mediators were analyzed in asthma and COPD patients. NEA patients were identified based on a sputum eosinophil count of $\leq 2.2\%$ of the total nonsquamous cell count, and were compared to EA and COPD patients.

Setting: University Hospital of Heraklion, Department of Thoracic Medicine.

Patients: A total of 37 atopic asthmatic patients and 25 patients with COPD.

Measurements: Sputum cell counts, cellular expression of heme oxygenase-1, inducible nitric oxide synthase, and nitrotyrosine, and sputum levels of eosinophilic cationic protein (ECP), myeloperoxidase (MPO), interleukin-8, and granulocyte macrophage colony-stimulating factor.

Results: A total of 17 asthmatic patients (46%) belonged to the NEA group and 20 patients (54%) to the EA group. Patients with NEA showed no difference in neutrophil counts, fluid-phase mediators, or cellular stress markers compared to patients with EA. Compared to COPD patients, NEA patients showed the following significant differences: lower total cell counts ($p < 0.03$); lower neutrophil counts ($p < 0.01$); lower nitrotyrosine positive cell counts ($p < 0.003$); lower ECP levels ($p < 0.005$); lower MPO levels ($p < 0.000$); higher lymphocyte counts ($p < 0.01$); and higher macrophage counts ($p < 0.03$).

Conclusions: Despite low eosinophil counts, airway inflammation in NEA patients may share common features with that in EA patients but is distinct from COPD. Larger studies are needed to investigate further the clinical and inflammatory characteristics of NEA before we are able to categorize asthma patients into those with or without eosinophilic inflammation.

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Key words: asthma; atopy; COPD; eosinophils; inflammation; neutrophils; sputum

Abbreviations: DTT = dithiothreitol; EA = eosinophilic asthma; ECP = eosinophilic cationic protein; GM-CSF = granulocyte macrophage colony-stimulating factor; HO = heme-oxygenase; IL = interleukin; iNOS = inducible nitric oxide synthase; MPO = myeloperoxidase; NEA = noneosinophilic asthma

Airway inflammation is considered to be the hallmark in asthma pathogenesis. However, the known hypothesis that T-cells produce T-helper type 2 cytokines, which trigger an eosinophilic inflammatory response in atopic asthmatic patients, has been

questioned. There is now significant evidence that noneosinophilic asthma (NEA) exists.¹

Although NEA has been associated with increased numbers of pulmonary neutrophils,² other studies^{3,4} have failed to verify this finding. Furthermore, it is unclear whether asthma patients with noneosinophilic airway inflammation show a clinical phenotype

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that is clearly different from that seen in "classic" eosinophilic asthma (EA). NEA might be more frequent in patients with severe persistent asthma, and it might predict a poor response to steroid treatment.^{3,5-8} However, a substantial number of patients with mild and moderate asthma also have demonstrated noneosinophilic airway inflammation.⁶ Interestingly, both airway neutrophilia and steroid resistance are major features of COPD.⁹⁻¹¹ Although this suggests that there may be similarities in airway inflammation between NEA and COPD patients, there is no evidence so far to support such an hypothesis.

Sputum induction is a safe and reproducible technique, and sputum analysis offers insight into many biological processes of the airways.¹² Characteristic inflammatory changes in patients with asthma have been identified in sputum and include, besides airway eosinophilia, increased numbers of cytokines and chemokines, and increased expression of stress-related proteins, like heme-oxygenase (HO) and inducible nitric oxide synthase (iNOS).¹³⁻¹⁵ Only a few studies have investigated changes in those parameters in patients with NEA.²

In the present study, indexes of neutrophilic and eosinophilic airway inflammation (*ie*, sputum cell counts, sputum supernatant eosinophilic cationic protein [ECP], myeloperoxidase [MPO], interleukin [IL]-8, and granulocyte macrophage colony-stimulating factor [GM-CSF] levels) and cellular stress (*ie*, HO-1, iNOS, and nitrotyrosine expression) were evaluated in sputum samples from patients with NEA, and were compared to those from EA and COPD patients. The primary aim was to test the hypothesis that NEA shows a distinct pattern of airway inflammation, primarily neutrophilic, compared to EA. Considering that NEA has been related to increased asthma severity, a relative increase in stress-related protein levels would also be expected. The secondary aim of the study was to test the hypothesis that airway inflammation in NEA patients resembles that seen in COPD patients. Our results did not show any difference between NEA and EA patients. Moreover, the inflammatory profile of NEA patients was different from that seen in COPD patients.

MATERIALS AND METHODS

Subjects

Induced sputum samples were obtained from 37 asthmatic patients and 25 patients with COPD who had been recruited from the Pulmonary Outpatient Clinic of the University Hospital of Heraklion in Crete, Greece. A diagnosis of asthma was made according to the National Heart, Lung, and Blood Institute

criteria.¹⁶ A diagnosis of COPD was made according to the current guidelines.^{9,10} All asthmatic patients were life-long atopic nonsmokers. All COPD patients were ex-smokers with irreversible airflow limitation (*ie*, increases in FEV₁ of <12% of the predicted baseline FEV₁ after the inhalation of 400 µg of salbutamol and/or increases in FEV₁ of <200 mL). None of the COPD patients reported a history of allergic diseases or showed atopy. Atopy was defined by at least one positive skin-prick reaction test result or a positive radioallergosorbent test result.

All COPD and asthmatic patients were receiving therapy with inhaled steroids (at least 400 mg/d fluticasone) and inhaled bronchodilators (short-acting β₂-agonists and/or long-acting β₂-agonists). No subject was receiving other medication or had another pulmonary or systemic disease.

At the time of the study, all patients were clinically stable. No subject had experienced a respiratory infection or an exacerbation for at least 6 weeks prior to entering the study. The Hospital Ethics Committee approved the protocol, and all subjects gave their written consent.

Study Design

Spirometry and reversibility tests, skin-prick reaction tests, blood sampling for total serum IgE count, and radioallergosorbent tests for a battery of common allergens were performed on the first visit. Spirometry was performed with a computerized system (MasterLab, 2.12; Jaeger; Wuerzburg, Germany) according to standardized guidelines.¹⁷ Subjects refrained from using long-acting and short-acting bronchodilators for 12 h and 6 h, respectively, prior to the measurements being made and did not smoke or drink tea or coffee during the morning of the investigation. Reversibility was assessed by the inhalation of 400 µg of salbutamol via a metered-dose inhaler.

Sputum induction was performed within 2 weeks after the first visit. According to sputum cytology findings, patients with NEA were identified and compared to EA and COPD patients. Using the data on the normal ranges of sputum cellular counts in the study by Belda et al,¹⁸ NEA was defined by the presence of eosinophils comprising ≤2.2% of the total nonsquamous cell count.

Sputum Induction and Analysis

Sputum was induced and processed as previously described.¹⁹ Based on previous reports²⁰ that induction time significantly affects the cellular and fluid-phase measurements in induced sputum, we chose 20 min as the standard duration of sputum induction in all subjects. Total and differential cell counts were performed using standard methods.

Immunocytochemical Analysis

Immunostaining procedures for HO-1, iNOS, and nitrotyrosine were performed as previously described.²¹ On immunostained slides, polymorphonuclear cells and macrophages were identified by morphologic analysis, and positively stained cells within 300 cells of each leukocyte population were counted using a light microscope. The results were expressed as the percentage of positive cells within each leukocyte population. All analyses were performed in a blind fashion by two investigators, and the results were averaged. Three replicate measurements were performed by each observer in 10 randomly selected slides. Both the intraobserver and interobserver coefficient of variation were <10%.

Measurement of Soluble Mediators

Commercial immunoassays were used to measure sputum supernatant IL-8, GM-CSF, MPO, and ECP levels, as previously

described.²¹ All measurements were performed twice for the same sample, and the results were averaged. The mean intrasample coefficient of variation was < 4%.

All assays were validated according to European Respiratory Society guidelines.¹³ Spiking recoveries of ECP, MPO, IL-8, and GM-CSF levels were assessed by adding a known quantity of purified mediator to different aliquots of sputum samples. Considering that recovery might differ between individuals due to differences in factors in the sputum that interfere with detection, sputum from a range of subjects with asthma and COPD was used (NEA, 5 patients; EA, 5 patients; COPD, 5 patients). The expected concentration of the spiked mediator, assuming 100% recovery, was in the mid-part of the standard curve. The spiking recovery of each mediator was calculated as the percentage of the expected value, which was the sum of the spiked purified mediator and the mediator concentration in the original sputum sample. The median recovery values were 88.3% (range, 71.2 to 98.9%) for ECP, 82.1% (range, 47.8 to 94.6%) for MPO, 84.5% (range, 73.4 to 95.3%) for IL-8, and 82.8% (range, 67.4 to 92.7) for GM-CSF.

Statistical Analysis

Before between two group comparisons were made, differences among three groups were tested using the Kruskal-Wallis test for nonnormally distributed variables and the analysis of variance test for normally distributed variables. Differences between two groups were tested using the Mann-Whitney test for nonnormally distributed variables and the *t* test for normally distributed variables. Normality was tested by the Shapiro-Wilk test. A statistical software package (StatsDirect; Camcode; Cambridge, UK) was used. A *p* value of < 0.05 was considered to be significant.

RESULTS

Among the 37 asthmatic patients included in the study, 17 patients (46%) belonged to the NEA group and 20 patients (54%) belonged to the EA group. Table 1 shows the characteristics of the two groups of asthmatic patients and of COPD patients. No difference was found between the two asthmatic groups. However, there was a tendency toward lower

bronchodilator response in NEA patients (*p* = 0.1). COPD patients were significantly older and were more often men with more severe airflow limitation that did not respond to therapy with inhaled bronchodilators.

Table 2 shows cellular counts in the three groups. The mean ($\pm$ SD) viability of the cells (as the percentage of the total number of nonsquamous cells) was $81 \pm 13\%$ in NEA patients, $85 \pm 15\%$ in EA patients, and $83 \pm 16\%$ in COPD patients. The mean percentage of squamous epithelial cells (as the percentage of the total of sputum cells) was $9 \pm 5\%$ in NEA patients, $8 \pm 4\%$ in EA patients, and $6 \pm 5\%$ in COPD patients. No other difference except in eosinophil counts was demonstrated between the NEA and EA groups. Compared to COPD patients, NEA patients showed lower total cell counts and neutrophil percentages, and higher macrophage and lymphocyte percentages.

Figure 1 shows the immunopositivity for HO-1 (Fig 1, *top left, a1*, and *top right, a2*), iNOS (Fig 1, *middle left, b1*, and *middle right, b2*), and nitrotyrosine (Fig 1, *bottom left, c1*, and *bottom right, c2*), expressed as percentages of positive inflammatory cells (*ie*, polymorphonuclear cells and macrophages) and as absolute counts of positive cells per gram of sputum. Comparisons across the three groups revealed no differences in immunopositivity for HO-1 and iNOS, but a significant difference (*p* = 0.003) for nitrotyrosine in at least one sample population vs one other sample population. Further analyses of the comparison between two groups showed that NEA patients did not differ from EA patients but demonstrated lower nitrotyrosine-positive percentages (*p* = 0.07) and absolute counts (*p* < 0.003) in comparison with COPD patients.

Figure 2 shows the levels of inflammatory mediators. ECP, MPO, and IL-8 concentrations were

Table 1—Demographic Characteristics of NEA Patients, EA Patients, and COPD Patients*

Variables	Asthma Patients		COPD Patients (n = 25)
	NEA Patients (n = 17)	EA Patients (n = 20)	
Age, yr	45 $\pm$ 18	49 $\pm$ 16	69 $\pm$ 8†
Sex			
Male	6	7	23‡
Female	11	13	2‡
FEV ₁ , % predicted	77 $\pm$ 29	74 $\pm$ 25	43 $\pm$ 13‡
FEV ₁ /FVC ratio	71 $\pm$ 14	74 $\pm$ 16	49 $\pm$ 17‡
Δ FEV ₁ , % predicted from baseline	8 $\pm$ 7	14 $\pm$ 10	3 $\pm$ 3‡
ICS,§ μ g/d	500 (400–1,000)	450 (400–1,000)	640 (400–1,000)

*Values are given as the mean $\pm$ SD or median (range). Δ FEV₁ = change in FEV₁ after the inhalation of 400 μ g of salbutamol; ICS = inhaled corticosteroid.

†*p* < 0.01 (NEA vs COPD patients).

‡*p* < 0.001 (NEA vs COPD patients).

§Dose was calculated as fluticasone equivalents, where 1 μ g of fluticasone = 2 μ g of beclomethasone = 2 μ g of budesonide.

Table 2—Sputum Differential Cell Counts Within the Total Non-Squamous Cell Count and in Absolute Numbers of Cells in NEA Patients, EA Patients, and COPD Patients*

Variables	Asthma Patients		COPD Patients
	NEA Patients	EA Patients	
TCC, $\times 10^6$ cells/g sputum	6 (0.3, 78)	8 (4, 34)	20 (0.7, 77)†
MAC, %	44 $\pm$ 18	42 $\pm$ 14	18 (1, 41)‡
AC, $\times 10^6$ cells/g sputum	4 (0.01, 19)	3 (0.3, 20)	4 (0, 19)
NEU, %	51 $\pm$ 21	48 $\pm$ 16	79 (50, 99)‡
AC, $\times 10^6$ cells/g sputum	3 (0.2, 62)	4 (1, 14)	12 (0.5, 69)‡
LYM, %	2 (0, 8)	3 (0, 5)	1 (0, 6)†
AC, $\times 10^6$ cells/g sputum	0.1 (0, 0.8)	0.2 (0, 1)	0.1 (0, 1)
EOS, %	0 (0, 2)	8 (3, 28)§	0.5 (0, 8)
AC, $\times 10^6$ cells/g sputum	0 (0, 0.2)	0.6 (0.1, 3)§	0.02 (0, 3)

*Values are given as the mean $\pm$ SD and median (minimum, maximum). TCC = total cell count; MAC = macrophages; NEU = neutrophils; LYM = lymphocytes; EO = eosinophils; AC = absolute count.

†p < 0.01 (NEA vs COPD patients).

‡p < 0.001 (NEA vs COPD patients).

§p < 0.001 (NEA vs EA patients).

detected in 100% of sputum supernatant samples, and GM-CSF concentrations were detected in 79% of sputum supernatant samples. Comparisons across the three groups revealed no difference in IL-8 and GM-CSF concentrations, but a significant difference in ECP concentration ($p = 0.01$) and in MPO concentration ($p < 0.0001$) in at least one sample population vs one other sample population. Further analyses of the comparisons between two groups showed that NEA patients did not differ from EA patients, but that they had significantly lower ECP and MPO levels compared to COPD patients.

DISCUSSION

Sputum cytology, inflammatory mediators, and stress-related proteins were measured in NEA, EA, and COPD patients. Despite previous evidence of neutrophilia and increased asthma severity in NEA patients, no difference could be detected between NEA and EA patients. Compared to COPD patients, NEA patients showed lower neutrophil counts and lower airway inflammatory and oxidative load. These findings suggest that there might be similarities in airway inflammation between NEA and EA patients, but that NEA patients were clearly different compared to COPD patients.

There are several limitations regarding our study. First, due to the relatively small number of subjects included in the two asthma groups and to the large scatter of values for sputum mediators, the study in these particular comparisons was underpowered. It is obvious that the data from this study are not sufficient to exclude that there are differences in fluid-phase mediators between NEA and EA patients, and that further studies are needed in order to

duplicate these findings. Second, all of our subjects were clinically stable and receiving treatment with inhaled steroids. Therefore, it is possible that a significant proportion of patients with EA did not have eosinophilia at the time of our study due to a steroid effect or because they were in a clinically stable condition. We decided to study only steroid-treated subjects for ethical reasons and in order to keep the results between groups comparable. We could have recruited only steroid-naïve subjects, but this would have limited us to a smaller population of asthmatic patients with mild disease. Similarly, previous studies^{2,3,6} on NEA have evaluated steroid-treated asthmatic patients. Even if our patients were clinically stable and underinhaled the steroid treatment at the time of the assessment, we were still able to demonstrate two distinct groups according to sputum eosinophilia. Moreover, the existence of NEA has been confirmed in steroid-naïve subjects and in subjects with current symptoms.^{6,22–24} Third, were we have used previously published data to set the normal sputum eosinophil count at < 2.2% of the total cell count.¹⁸ Although it is a commonly accepted approach to defining sputum eosinophilia based on eosinophil percentages, as can be seen from Table 2, there are NEA patients with higher absolute eosinophil counts than those for EA patients. This finding raises considerations as to whether absolute sputum eosinophil counts rather than percentages should be used to define sputum eosinophilia. Finally, dithiothreitol (DTT), a reducing agent that we used to homogenize sputum, might have affected the detection of inflammatory mediators in our sputum sol phase.²⁵ However, spiking experiments showed good recovery for all mediators, and other investigators^{26–28} have shown no effect of

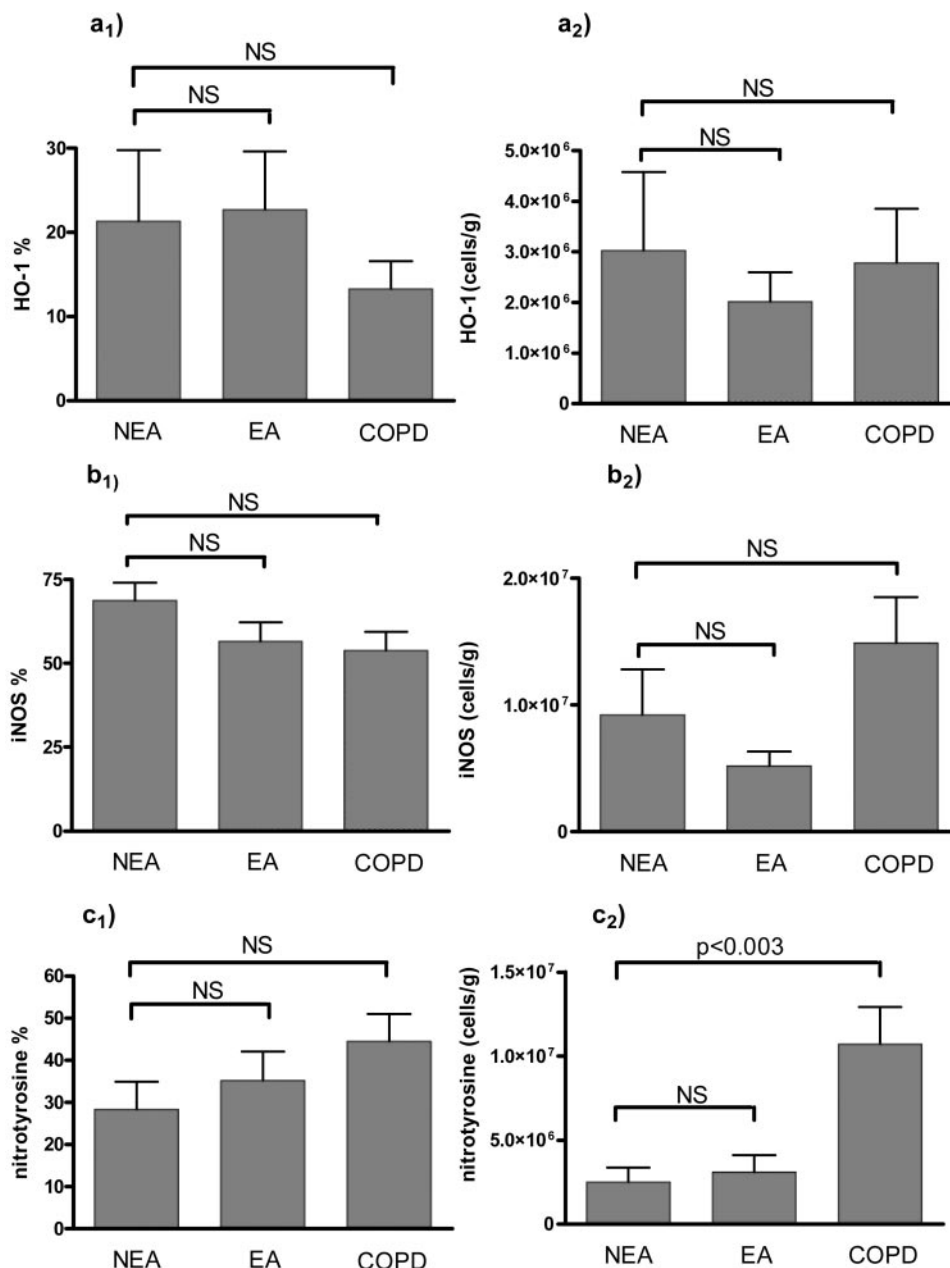


FIGURE 1. *Top left, a1*: percentages of HO-1-positive inflammatory cells (*ie*, polymorphonuclear cells and macrophages) in the total number of inflammatory cells in NEA patients, EA patients, and COPD patients. The results are expressed as the mean (SEM). *Top right, a2*: the absolute number of HO-1-positive inflammatory cells (*ie*, polymorphonuclear cells and macrophages) per gram of sputum in NEA, EA, and COPD patients. The results are expressed as the mean (SEM). *Middle left, b1*: percentages of iNOS-positive inflammatory cells, as expressed in *top left, a1*. *Middle right, b2*: absolute number of iNOS-positive inflammatory cells, as expressed in *top right, a2*. *Bottom left, c1*: percentages of nitrotyrosine-positive inflammatory cells, as expressed in *top left, a1*. *Bottom right, c2*: absolute number of nitrotyrosine-positive inflammatory cells, as expressed in *top right, a2*. NS = not significant.

DTT on their standard curves. Even if DTT has a small effect on any of these measurements, comparability between samples should be preserved.

Airway inflammation in asthma patients is currently the subject of a number of studies investigating both the nature of inflammatory cells and of the

cytokines present. The eosinophil is thought to be the most important cell involved in asthma pathogenesis, and for many years all asthma cases were assumed to be related to increased eosinophilic inflammation.²⁹ However, the surprising finding that a subgroup of severe asthmatic patients shows nor-

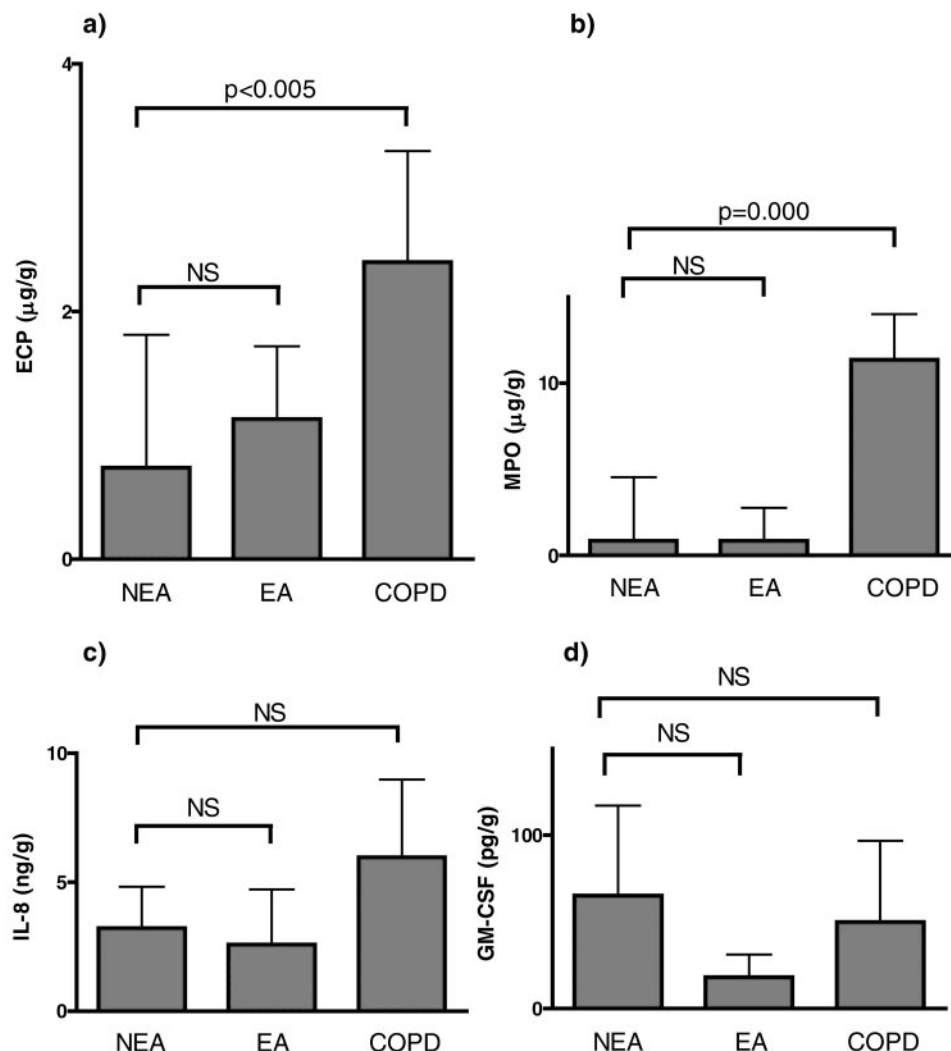


FIGURE 2. Sputum ECP levels (top left, a), MPO levels (top right, b), IL-8 levels (bottom left, c), and GM-CSF levels (bottom right, d) in NEA patients, EA patients, and COPD patients. The results are expressed as the mean (SEM). See Figure 1 legend for expansion of abbreviation.

mal airway eosinophil counts and high neutrophil counts led to the hypothesis that other inflammatory mechanisms, primarily neutrophilic, might be involved in the development of airway obstruction in these patients.^{2,30} There is now increasing evidence that NEA is much more frequent than previously thought, even among patients with mild asthma, and neutrophilia is presumed to be triggered by the activation of innate immunity.^{1,6}

We have evaluated a cohort of atopic asthmatic subjects with a wide range of airflow limitation, from mild to severe, and found that only 54% of asthmatic patients belong to the eosinophilic phenotype. Similar percentages have been reported by other investigators.¹ Patients with NEA showed no significant difference in the severity of airflow limitation or the response to short-acting β -agonists compared to

patients with EA. However, the bronchodilator response tended to be smaller in NEA patients ($p = 0.01$). Accordingly, Green et al⁶ studied a large cohort of asthmatic patients and found no correlation between sputum eosinophil count and the FEV₁ percent predicted. However, a weakly negative correlation was observed between the provocative concentration of methacholine causing a 20% fall in FEV₁ and sputum eosinophil counts among atopic patients. Moreover, NEA patients with sputum neutrophilia showed irreversible airflow limitation after inhaled steroid treatment. Taken together, all of these findings and ours suggest that low sputum eosinophil counts might not predict increased asthma severity, but may indicate decreased bronchial hyperresponsiveness and poor response to treatment.

Several investigators^{2,30,31} have addressed the important role of neutrophilic inflammation in NEA patients. Green et al⁶ identified in their cohort of asthmatic patients a subgroup with low eosinophil counts and sputum neutrophilia (23% of 259 asthmatic patients); however, not all NEA patients showed high neutrophil numbers. In another sputum study by Simpson et al,³¹ 28% of 93 asthma patients were noneosinophilic and neutrophilic. In our group of 37 asthmatic patients, only 2 patients (5%) showed high neutrophil counts (if the normal range for neutrophils is set to 65.3%, as in the study by Green et al⁶). As expected, we did not find any difference in sputum neutrophil counts between subjects with sputum eosinophilia and those without. Similarly, Wenzel et al³ failed to find any connection between eosinophil and neutrophil numbers in endobronchial biopsy specimens from patients severe asthma. However, both our study and the study by Wenzel et al³ included a limited number of subjects, which perhaps did not allow the identification of an adequate number of neutrophilic asthmatic patients for the further differentiation of NEA into neutrophilic and nonneutrophilic. Moreover, we have evaluated only atopic asthmatic patients, while Green et al⁶ included equal numbers of atopic and nonatopic patients in their study. In fact, they found a connection between the subgroup of patients with neutrophilia and the absence of atopy. According to these results, which are in agreement with ours, neutrophilia might be a characteristic of nonatopic NEA. Toward this result, Douwes et al¹ have supported the hypothesis that nonatopic asthma derives from the activation of innate immunity, which triggers the neutrophilic response. Still, Simpson et al³¹ recruited mainly atopic asthmatic patients and were still able to identify a significant percentage of neutrophilic subjects. Therefore, the relationship between nonatopic asthma and neutrophilia is not yet clear.

It is important to notice that by defining sputum neutrophilia at neutrophil percentages of > 65.3% or > 61%, respectively, Green et al⁶ and Simpson et al³¹ included a large number of asthmatic patients in the neutrophilic subgroup. However, Belda et al¹⁸ analyzed sputum samples from a large cohort of healthy subjects and set the upper normal limit for neutrophil percentages at 77.7%, according to which neutrophilic NEA is less frequent than what these authors showed.³¹

Having found no difference in neutrophil numbers between patients with EA and those with NEA, no difference was also found in the levels of neutrophilic markers, such as MPO and IL-8. Gibson et al² also reported similar MPO levels in NEA and EA patients, but increased IL-8 levels in NEA patients.

However, in that study NEA patients showed increased numbers of neutrophils.

Despite low eosinophil numbers in NEA patients, no difference was found in ECP or GM-CSF levels compared to EA patients. GM-CSF has been designated as the main cytokine enhancing the survival of eosinophils in the asthmatic airways, but it affects multiple leukocyte lineages and has much broader functional activities.^{32,33} ECP is an eosinophil degranulation product; however, there have been reports²¹ from patients with noneosinophilic airway disease, such as COPD, of increased ECP levels. Previous studies^{21,34} have shown a close correlation between total cell count and ECP levels, as well as between the severity of airflow limitation and ECP levels. It is possible that the few eosinophils that are present in the airways of NEA patients are highly degranulated due to an increase in the presence of degranulating agents. There is also evidence that other types of cells except eosinophils, like neutrophils, contain ECP.³⁵ Therefore, our finding of similar ECP levels in NEA and EA patients might be associated with the similar neutrophil counts in the two groups of asthma patients.

We have gone further in evaluating NEA and measured sputum immunopositivity for stress-related proteins such as HO-1, iNOS, and nitrotyrosine. Inflammatory and oxidant stimuli induce the cellular expression of iNOS and HO-1.^{36,37} Nitric oxide under aerobic conditions reacts with oxygen and superoxide anion radicals to yield peroxynitrite. Peroxynitrite leads to tyrosine nitration, making nitrotyrosine an indicator of the involvement of nitric oxide in irreversible oxidative reactions. We have used these molecules to assess the inflammatory and oxidative load imposed on the cells present in the airways of EA and NEA patients, and have found no difference between the two groups. Previous investigators^{14,15,38} have demonstrated increased numbers of HO-1-positive, iNOS-positive, and nitrotyrosine-positive cells in sputum and bronchial or lung biopsy specimens from asthmatic patients compared to those from healthy subjects. To the best of our knowledge, until today no study had evaluated the differences in these parameters between NEA and EA patients. Our results showing no difference in stress-induced enzyme and nitrotyrosine levels indicate a similar intensity of inflammatory and oxidant stimuli in the airways of NEA and EA patients. This finding is in accordance with the similar severity of airflow limitation that we found in NEA and EA patients, and does not favor the hypothesis that NEA is predictive of increased asthma severity.

In the second part of our study, we compared NEA to COPD patients. Considering previous reports^{2,6,30} of neutrophilia and steroid resistance in

NEA patients, we looked for similarities in airway inflammation and cellular stress between these two groups. Our study showed decreased neutrophil counts, lower MPO levels, and a tendency toward decreased IL-8 levels in NEA patients compared to those in COPD patients ($p = 0.17$). These results suggest that neutrophilic inflammation is less prominent in the airways of NEA patients compared to those of COPD patients. The lack of a statistically significant difference in IL-8 levels despite the strong difference in neutrophil numbers could be due to the fact that other mediators, such as leukotriene B₄, might be more powerful neutrophil chemoattractants than IL-8.³⁹ Total cell counts were also decreased in NEA patients, which probably reflects a less intense inflammatory process in the airways of these patients. An interesting finding, but one that was not surprising, was that of decreased ECP levels in NEA patients compared to those in COPD patients. This is very much associated with the decreased neutrophil numbers found in NEA patients and with the reported release of ECP from these cells.³⁵

Previous investigators have shown that an important factor that might influence the number of sputum neutrophils is age and that neutrophil numbers increase with increasing age (specifically, numbers in persons > 50 years of age).⁴⁰ Therefore, one might attribute the increased number of neutrophils that we found in COPD patients compared to those in NEA patients to the significant age difference between the two groups. We did not succeed in recruiting patients in similar age groups because, in general, the first diagnosis of COPD is established in subjects who are > 40 to 50 years of age.⁴¹ However, we think that the difference found between NEA and COPD patients is disease specific rather than age specific because the neutrophil numbers in our COPD group were higher than the number that should be expected from healthy subjects in similar age group (mean, 68.5%; SD, 20.6%), based on data from the study by Thomas et al.⁴⁰

Previous studies^{14,15,38} have shown the increased expression of HO-1 and iNOS, and the increased formation of nitrotyrosine in asthmatic patients and COPD patients. Ichinose et al³⁸ have demonstrated a relative increase in nitrotyrosine levels in COPD patients compared to those in asthma patients, but no difference in iNOS levels, suggesting that NO is mainly consumed via oxidative reactions in COPD patients compared to asthma patients. Our results confirm and extend these findings to patients with NEA, and indicate that NEA, like EA, is associated with lower degrees of airway oxidative stress compared to COPD.

Although COPD patients showed increased oxida-

tive stress, a similar increase in the expression of HO-1, which is induced by oxidant stimuli, was not detected. Maestrelli et al⁴² have reported deficient HO-1 expression in patients with severe COPD compared to control smokers. Moreover, it has been shown⁴³ that microsatellite polymorphism in the HO-1 gene promoter is associated with the susceptibility to emphysema. These findings and ours suggest that an impairment of the HO-1 pathway in COPD patients may be involved in the pathogenesis of the disease.

In conclusion, despite previous evidence for airway neutrophilia and steroid resistance in NEA patients, as in COPD patients, this study reported significantly decreased neutrophilic inflammation and oxidative stress in NEA patients compared to COPD patients. This evidence clearly suggests that NEA is distinct from COPD. Contrary to previous observations, in which similar numbers of neutrophils, and neutrophilic and eosinophilic markers were seen, a similar expression of stress-related proteins was found to exist in atopic patients with NEA and EA, which suggests that there might be similarities in airway inflammation between NEA and EA patients. However, larger studies are needed to investigate further the clinical and inflammatory characteristics of NEA before we are able to categorize asthma patients into those with or without eosinophilic inflammation. Moreover, to be able to interpret whether NEA is a distinct phenotype it is necessary to show whether the phenotype is reproducible.

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