Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma

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Background: Recent evidence has shown that nitric oxide (NO) levels are increased in asthmatic airways. Although the role of NO in asthma is unknown, reactive metabolites of NO may lead to nitrotyrosine formation and promote airway dysfunction.
Objective: The aim of this study was to determine whether nitrotyrosine, as a marker of nitrating species, could be found in the airways and lung parenchyma of subjects with asthma who died of status asthmaticus or other nonrespiratory causes.
Methods: Lung tissue specimens were obtained from 5 patients who died of status asthmaticus, 2 asthmatic patients who died of nonrespiratory causes, and 6 nonasthmatic control subjects who died of nonrespiratory causes. Lung sections were stained for immunofluorescence with use of an antinitrotyrosine antibody, followed by a indiocarbocyanine (Cy5, Jackson Immunochemicals, Westgrove, Pa)–conjugated secondary antibody.
Results: Nonasthmatic lungs showed little or no nitrotyrosine staining, whereas asthmatic lungs demonstrated significantly more staining of nitrotyrosine residues distributed in both the airways and lung parenchyma.
Conclusion: This study demonstrates the presence of nitrotyrosine, and hence evidence of formation of nitrating species, in the airways and lung parenchyma of patients with asthma who died of status asthmaticus or other nonrespiratory causes. This finding supports the concept that widespread airway and parenchymal inflammation occurs in asthma, and, more specifically, that NO and its reactive metabolites may play a pathophysiologic role in asthma. (J Allergy Clin Immunol 1999;104:747-54.)

Abbreviations used
H&E: Hematoxylin and eosin
NO: Nitric oxide

Key words: *Asthma, nitric oxide, nitrotyrosine, peroxynitrite*

Mounting evidence has shown that the concentration of nitric oxide (NO) in exhaled gas is elevated in asthmatic patients.[1-4] This elevation in NO is thought to be the result of the airway inflammation that is intrinsic to asthma because NO increases in response to controlled allergen challenge,[3] correlates with the degree of inflammation as assessed by sputum eosinophils,[4] and is suppressed after treatment with corticosteroids.[2] Although the relationship between inflammation and airway hyperresponsiveness is not clearly defined, elevated levels of NO also correlate with measures of airway hyperresponsiveness.[4] Thus exhaled NO has been considered a "surrogate marker of inflammation in asthma."[4]

However, it is possible that NO may be causally involved in the pathogenesis of asthma (Fig 1). Direct roles of NO in asthma may include the protective effect of bronchodilation[5] but also the deleterious effects of airway edema[6] and epithelial injury.[7] In addition, NO may participate in biochemical reactions that result in the formation of other reactive nitrogen species. For example, NO combines with the free radical superoxide anion to form peroxynitrite, a potent oxidizing agent that can initiate lipid peroxidation and cause the oxidation of sulfhydryl groups[8] and the nitration of tyrosine residues.[9] NO may also be metabolized to nitrite, which may promote nitrotyrosine formation by reaction with hypochlorous acid or myeloperoxidase.[10]

These oxidative and nitrating effects of reactive nitrogen species may have significant deleterious effects on protein function.[11] For example, peroxynitrite-induced inhibition of surfactant function is thought to be mediated by oxidative damage to both the protein and lipid components of surfactant.[12] An important functional consequence of peroxynitrite formation in relation to asthma is the finding that peroxynitrite induces airway hyperresponsiveness in guinea pigs,[13] suggesting a direct

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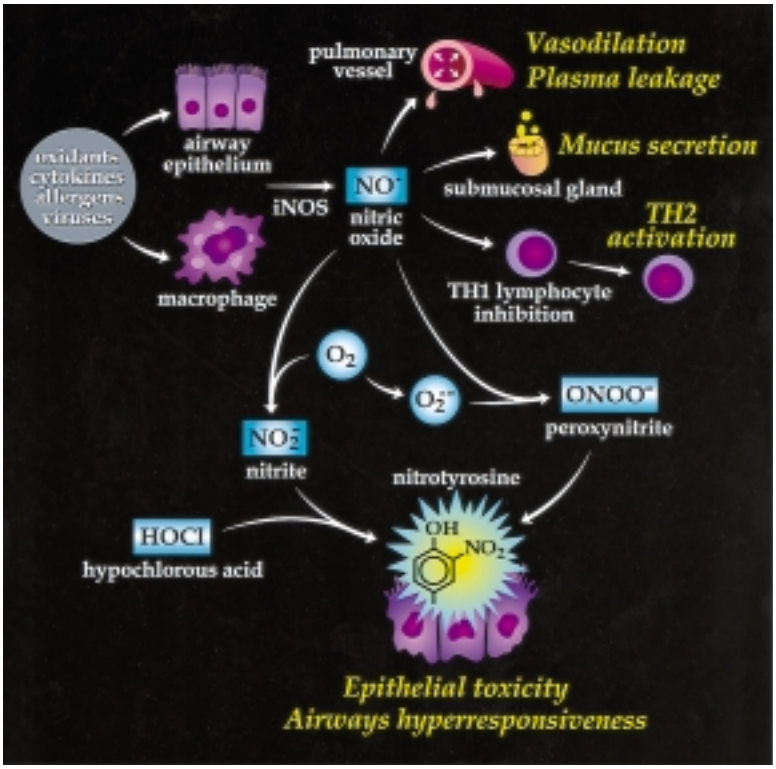

FIG 1. Pathways by which NO and its metabolites contribute to inflammation and nitrotyrosine formation in asthma. Exposure of airway epithelium or alveolar macrophages to various stimulants, such as oxidants, cytokines, allergens, or viruses, may lead to enhanced expression of the enzyme inducible nitric oxide synthase *(iNOS),* resulting in increased production of NO in lung. NO contributes to inflammation in asthma by causing pulmonary blood vessel dilation and plasma leakage, enhanced mucus secretion, and indirect activation of Th2 lymphocytes by inhibition of Th1 lymphocytes. In addition, NO reacts with superoxide *(O_2^-)* to form the potent oxidant peroxynitrite *($ONOO^-$).* Exposure of airway epithelium to peroxynitrite results in epithelial toxicity and the functional consequence of increased airway hyperresponsiveness. A marker of such exposure to peroxynitrite is formation of nitrotyrosine residues on airway epithelial proteins. Because nitrotyrosine may also be found after exposure of proteins to the NO metabolite nitrite *(NO_2^-)* in association with hypochlorous acid *(HOCl)* or myeloperoxidase, the finding of nitrotyrosine residues is a general marker of presence of the reactive nitrogen species.

pathophysiologic role for NO-derived metabolites in asthma.

Evidence for oxidative damage from peroxynitrite exposure in human lung disease has been described in patients with acute lung injury[9,14] and idiopathic pulmonary fibrosis.[15] Most recently, evidence for peroxynitrite formation has been found in the airways and inflammatory cells of asthmatic patients.[16] This latter study found that peroxynitrite formation not only correlated inversely with measures of airway function and hyperresponsiveness but also was inhibited by treatment with inhaled corticosteroids. These findings further support the association of NO and asthmatic airway inflammation and are suggestive of a pathophysiologic role for peroxynitrite or other reactive nitrogen species in asthma.

Our group has been especially interested in the involvement of the lung parenchyma in the pathophysiologic mechanisms of asthma.[17] Accordingly, the aim of this study was to determine whether evidence of the formation of reactive nitrogen species could be found not only in the airways but also in the lung parenchyma of patients with asthma. To obtain lung tissue that would contain both airways and parenchyma, we examined lung tissue obtained at autopsy of patients who died of severe asthma. We also analyzed lung tissue from patients with mild asthma who died from nonrespiratory causes. The formation of nitrotyrosine residues is considered a "footprint" of exposure to reactive nitrogen species[11] and can be detected by the use of a specific antinitrotyrosine antibody. Therefore we hypothesized that patients who died of severe asthma or who had asthma but died of nonrespiratory causes would have more nitrotyrosine staining in their airways and lung parenchyma than would nonasthmatic subjects who died of nonrespiratory causes.

METHODS

Tissue specimens and routine histologic examination

Lung tissue specimens were obtained at autopsies performed at Fletcher Allen Health Care, the teaching hospital of the University of Vermont College of Medicine, and from the Sir Charles Gairdner

TABLE I. Clinical characteristics and tissue section immunofluorescence of study subjects

Subject	Age (y)	Sex	Time to autopsy (h)	Duration of asthma (y)	Cortico-steroids	Allergies	Smoking	Other diag-noses	Immuno-fluore-scence	Figure location
Fatal asthma										
1	16	F	20	10	Yes	Yes	No	None	3	Figs 1; 2, *C;* 3, *C, D*
2	55	M	31	15	Yes	Yes	No	None	3.5	Fig 3, *A, B*
3	31	M	27	20	Yes	Yes	No	None	2	Fig 2
4	33	F	28	24	Yes	Yes	No	None	4	Fig 2, *D*
5	18	F	63	3	Yes	NA	No	None	1	Fig 2
									Mean 2.7	
Asthma: nonrespiratory death										
6	29	M	28	25	No	Yes	Yes	CO poisoning	4	Fig 2, *A*
7	27	M	18	15	No	Yes	Yes	CO poisoning	2	Fig 2, *B*
									Mean 3.0	
Nonasthmatic controls										
8	44	M	17	NA	No	No	Yes	CO poisoning; pest controller	1	Fig 1
9	36	M	12	NA	No	No	No	Asphyxiation; nurse	1.25	
10	22	M	28	NA	No	No	No	CO poisoning; welder, boilermaker	0.25	
11	47	F	18	NA	No	No	NA	Hanging; occupational therapist	0	
12	47	M	21	NA	No	No	NA	CO poisoning; farmer	1.5	
13	30	M	11	NA	No	No	Yes	Aortic dissection	0	Fig 1
									Mean 0.7	

F, Female; *M,* male; *NA,* not available; *CO,* carbon monoxide.

Hospital of Nedlands, Australia. Clinical data on the subjects whose lung tissue was analyzed were collected from review of coroner and hospital records. The protocol was approved by the Committees on Human Research in the Medical Sciences at the University of Vermont and the Sir Charles Gairdner Hospital Research Institutional Ethics Committee. Tissue samples from asthmatic patients were analyzed from 7 patients with asthma: 5 who died from status asthmaticus and 2 with a history of asthma who died from nonrespiratory causes. Comparisons were made with lung tissue samples from 6 patients without asthma who died from nonrespiratory causes (Table I). Routine microscopic sections were prepared by fixation in 10% neutral buffered formalin, and staining of paraffin-mounted sections was performed with Mayer's hematoxylin and acidic eosin (H&E).

Immunofluorescence

Lung sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions with use of standard procedures. Subsequently, sections were permeabilized in PBS containing 1% Triton X-100 for 30 minutes, and nonspecific staining was blocked with 1% BSA in PBS (2×30 minutes). Sections were incubated with a rabbit polyclonal antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) at 2 μg/mL for 1 hour. After three 20-minute washes in PBS-BSA, sections were incubated with an indodicarbocyanine (Cy5, Jackson Immunochemicals, Westgrove, Pa)–conjugated secondary antibody (Jackson Immunoresearch, Westgrove, Pa) at 20 μg/mL for 1 hour. This far red fluorophor was selected because it allowed us to detect fluorescence at wavelengths that would not be interfered with by the autofluorescence commonly seen with formalin fixation and paraffin embedding. After the incubation with secondary antibody, slides were rinsed 3 times in PBS and once in deionized water. Propidium iodide (50 μg/mL) was used as a counterstain to visualize the lung architecture by nuclear staining and was present in the mounting medium (Vector, Burlingame, Calif).

To verify nitrotyrosine reactivity, a tissue sample from a nonasthmatic control patient was reacted with 1 mmol/L peroxynitrite (provided by Dr Sadis Matalon, University of Alabama) for 10 minutes in PBS before incubation with the antinitrotyrosine antibody ("positive-staining" control). Furthermore, to ascertain the specificity of nitrotyrosine immunofluorescence in tissue sections from asthmatic subjects, the nitrotyrosine antibody was incubated with a 10-fold weight excess of 3-nitrotyrosine (Sigma, St Louis, Mo) overnight at 4°C. This neutralized antibody preparation was subsequently reacted with a tissue sample from a patient with asthma, and the lack of immunofluorescence was confirmed ("negative-staining" control). Similarly, antibody specificity was tested by omitting the primary antinitrotyrosine antibody from the staining protocol.

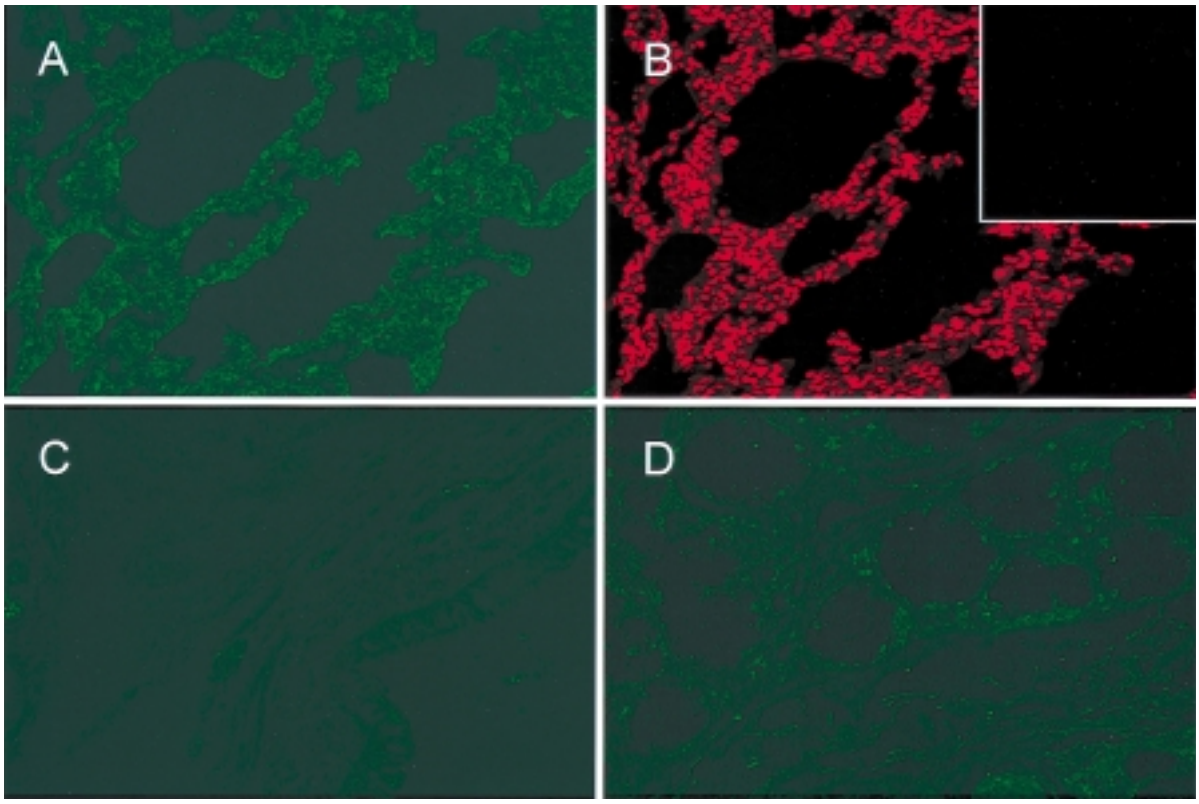

FIG 2. Nitrotyrosine immunofluorescence in tissue samples serving as "negative-staining" control **(A, B)** and from tissue samples of 2 of the nonasthmatic control subjects **(C, D)**. **A,** Lung tissue from asthmatic subject staining positively (green) for nitrotyrosine. To control for antibody specificity, a serial section of this sample was then incubated with antinitrotyrosine antibody that had been previously neutralized with 10-fold weight excess of 3-nitrotyrosine **(B)**. Because this neutralized control specimen lacked any immunofluorescence for nitrotyrosine (insert), the tissue was visualized with propidium iodide (red) to confirm the integrity of the tissue specimen. Specimens shown in **C** and **D** are representative of nonasthmatic control samples and demonstrate some of the variability in staining intensity seen in tissue samples from nonasthmatic control subjects. (Confocal microscopy of Cy5 and propidium iodide immunofluorescence, original magnification ×200.)

Confocal microscopy

Fluorescence detection of Cy5 (green) or propidium iodide (red) signals was accomplished by confocal microscopy with use of an Olympus BX50 upright microscope attached to a Biorad (Hercules, Calif) MRC 1000 confocal laser scanning microscope equipped with a 15 mW mixed-gas krypton argon laser. Instrument settings were used at a laser power of 30%, confocal iris aperture of 2.5 mm, and an optical gain setting of 1400 V. Sections were scanned at a magnification of ×200 and optimized digital images were collected and captured with use of the COMOS software package (Biorad, Hercules, Calif).

Semiquantitation of staining intensity

To provide a more objective assessment of staining intensity, 2 observers blinded to the identity of each tissue sample and trained in techniques of immunofluorescent microscopy were asked to grade each sample on a scale of 0 to 4, with 0 representing little or no staining and 4 representing intense, bright staining. This approach is similar to that used by other investigators.[16] Because concordance between the 2 observers was excellent (Pearson r^2 = 0.98), the mean of the 2 scores was chosen as the final score for each section. Scores between asthmatic and nonasthmatic subjects were compared with the unpaired Student *t* test, and statistical significance was assigned to a *P* value of <.05.

RESULTS

The clinical characteristics of the subjects involved in the study are shown in Table I. There were 7 asthmatic and 6 nonasthmatic subjects. Of the 7 asthmatic patients, 5 had died of status asthmaticus and 2 had died from other nonrespiratory causes. These latter 2 subjects were both smokers and atopic and were taking inhaled β-agonists regularly but were not receiving corticosteroids nor were ever hospitalized for asthma. The 5 subjects with fatal asthma were all nonsmokers and atopic and were receiving corticosteroid therapy. The 6 nonasthmatic control subjects all died from nonrespiratory causes, although 3 may have had respiratory occupational exposures (subjects 8, 10, and 12). The smoking history among the nonasthmatic controls was variable. Included in Table I are the immunofluorescence scores and the locations of the sections within the figures.

On routine histologic examination with H&E staining (examples shown in Fig 4), all specimens from asthmatic lungs showed microscopic changes consistent with asthma, including mucus plugging of airways, epithelial sloughing, subepithelial thickening, smooth muscle

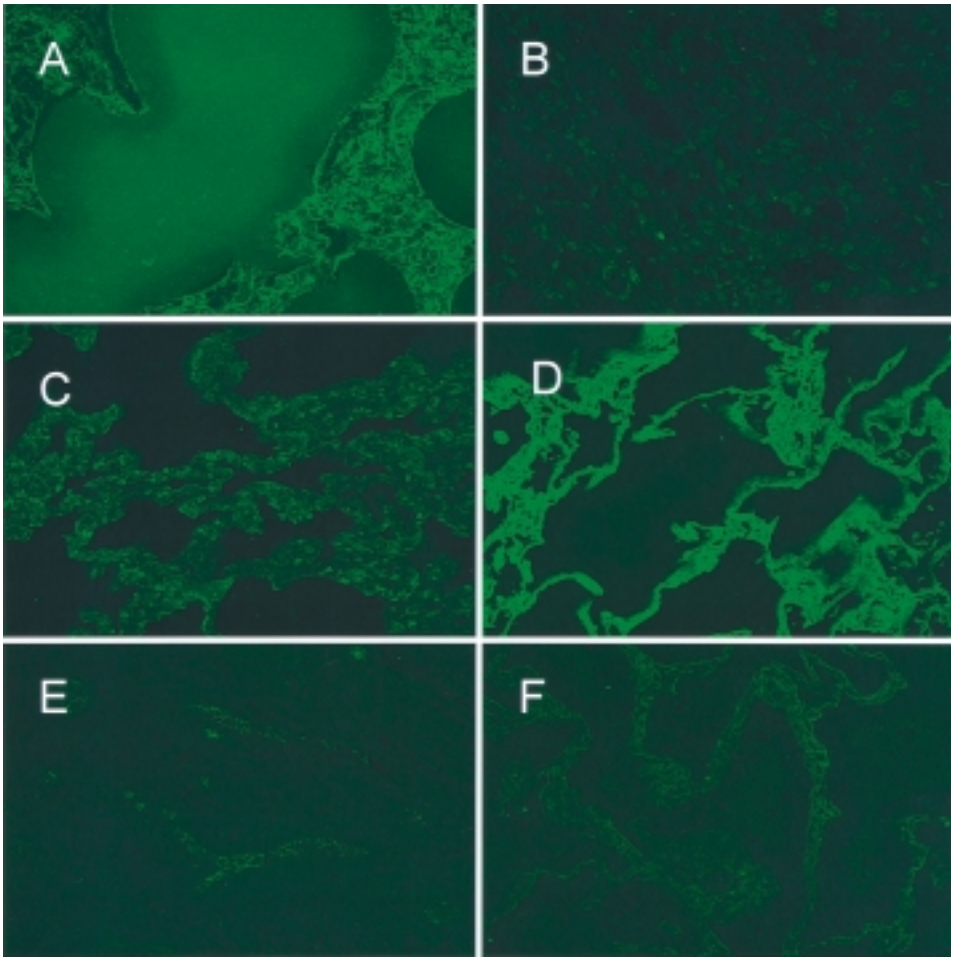

FIG 3. Nitrotyrosine immunofluorescence from the 2 asthmatic subjects who died from nonrespiratory causes **(A, B)** and from 4 of the asthmatic subjects who died from status asthmaticus (**C** to **F**). Nitrotyrosine staining appears cytoplasmic throughout predominantly parenchymal structures seen in these sections. (Confocal microscopy of Cy5 immunofluorescence, original magnification ×200.)

hyperplasia, and peribronchial inflammation. Control lung specimens demonstrated relatively normal-appearing microscopic lung architecture (not shown).

The specificity of the antinitrotyrosine antibody is demonstrated in Fig 2. Fig 2, *A,* shows a tissue section from an asthmatic subject incubated with the antinitrotyrosine antibody followed by the Cy5-conjugated secondary antibody. The diffuse green staining represents a positive reaction indicative of the presence of nitrotyrosine in the tissue; the color of the staining is similar to that seen with the "positive-staining" control section (not shown). Fig 2, *B,* demonstrates the "negative-staining" control and shows a serial tissue section from the same asthmatic subject incubated with antinitrotyrosine antibody that had been previously neutralized with a 10-fold excess (by weight) of 3-nitrotyrosine. Because nitrotyrosine staining was completely absent (dark field in upper right corner), this image was visualized with propidium iodide to confirm the integrity of the tissue specimen. A similar lack of immunofluorescence was observed when the primary antinitrotyrosine antibody was omitted from the staining protocol.

Also in Fig 2 are representative images from 2 of the nonasthmatic control subjects. The average staining intensity was graded as 0.7 for all control subjects. Note that faint nitrotyrosine staining can be seen in *D,* which was tissue from a smoker who worked in the pest control industry and therefore may have had some exposure to toxic organic chemicals. Minimal nitrotyrosine staining was evident in *C,* which was tissue from a control subject who was also a smoker.

Tissue sections from the 2 subjects who had asthma but died of nonrespiratory causes are shown in Fig 3, *A* and *B.* The average staining intensity was graded as 3.0 in these subjects.

Finally, tissue sections from 4 of the subjects who died of severe asthma are shown in Fig 3, *C, D, E,* and *F.* These subjects showed variable degrees of staining, but the average staining intensity was graded as 2.7. The average staining intensity between asthmatic and

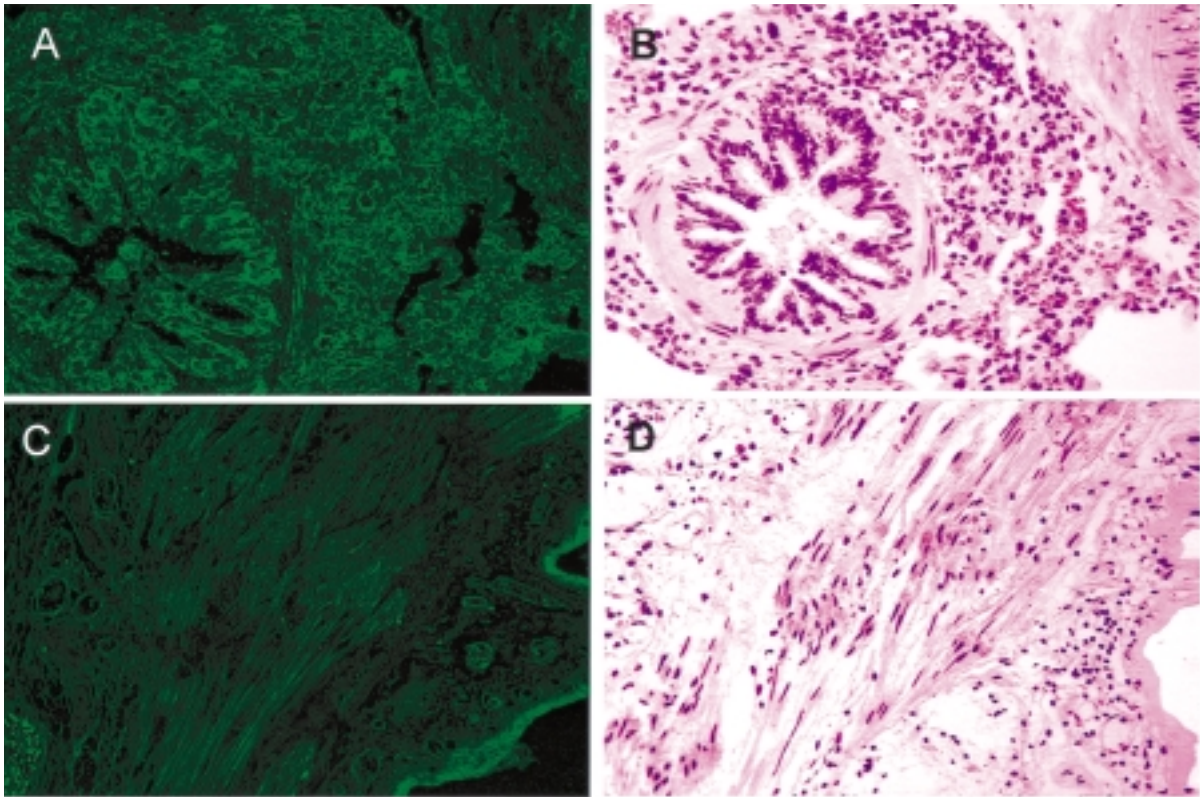

FIG 4. Nitrotyrosine immunofluorescence from 2 asthmatic subjects who died of status asthmaticus **(A, C)** together with their corresponding H&E-stained images (**B** and **D,** respectively). H&E-stained images were obtained to better visualize underlying lung histologic features and thereby better localize nitrotyrosine staining. Specimen in **A,** from the one asthmatic who died of status asthmaticus not shown in Fig 3, illustrates diffuse nature of staining, including both small airway and lung parenchyma. Specimen in **C** (from same subject as *C* in Fig 3) is shown to especially highlight staining of airway smooth muscle. (**A** and **C**: confocal microscopy of Cy5 immunofluorescence, original magnification ×200. **B** and **D**: H&E staining, original magnification ×200.)

nonasthmatic sections was significantly different (2.8 ± 0.4 vs 0.7 ± 0.3, $P = .002$, mean ± SEM), but there was no difference in staining intensity between the asthmatics who died of asthma compared with those who did not.

Nitrotyrosine staining appeared to be primarily cytoplasmic, with involvement of small airways as well as lung parenchyma. Airway staining included the epithelium, subepithelial region, and smooth muscle. Within the parenchyma nitrotyrosine was present in the alveolar epithelium as well as the interstitium. Inflammatory cells within the interstitium as well as the alveolar space also stained for nitrotyrosine. These findings are confirmed and highlighted in Fig 4, which depicts nitrotyrosine staining in 2 of the subjects with fatal asthma and their corresponding lung histologic features as seen by H&E staining.

DISCUSSION

This study has demonstrated the presence of nitrotyrosine and hence evidence of formation of reactive nitrogen species in the airways and lung parenchyma of patients with asthma who died of status asthmaticus or other nonrespiratory causes.

Before the implications of these findings are considered, a few technical considerations are important. First, we assumed that the presence of immunoreactivity against nitrotyrosine was evidence for the formation of reactive nitrogen species. Nitrotyrosine formation is well documented after exposure to peroxynitrite[11] or to nitrite in association with hypochlorous acid or myeloperoxidase.[10] Although species such as nitrogen dioxide or acidified nitrate can also produce nitrotyrosine, they exist in quantities insufficient in vivo for significant in vitro nitration.[11] In addition, direct experimental evidence has failed to show significant nitrotyrosine formation after exposure to hydrogen peroxide, superoxide anions, hydroxyl radicals, or NO alone.[9] Thus the presence of nitrotyrosine specifically implicates the formation of reactive nitrogen species.

Second, tissue-preserving techniques or degeneration of tissue on cell death may have contributed to the formation of NO and other reactive nitrogen species and therefore of subsequent nitrotyrosine residues. The time to autopsy

was variable in the subjects involved, up to 60 hours in one case. This may be one reason why even nonasthmatic subjects demonstrated some faint immunoreactivity to nitrotyrosine. However, there were clearly still differences between the autopsy tissues from the asthmatic and nonasthmatic subjects.

Third, the severe asthmatic subjects used corticosteroids to a variable and unknown extent before the development of status asthmaticus. The use of corticosteroids would likely modify the resulting degree of nitrotyrosine immunoreactivity observed.[16] Clearly, the tissue specimens from the 2 asthmatics who died of nonrespiratory causes stained strongly for nitrotyrosine, and these subjects were not receiving corticosteroid therapy. However, because the use of corticosteroids would be expected to decrease the inflammatory process, the intense nitrotyrosine reactivity seen in the tissue specimens of the asthmatic subjects who died of status asthmaticus despite corticosteroid therapy may underestimate the actual degree of inflammation and nitrotyrosine formation involved. It is interesting to speculate that perhaps status asthmaticus is characterized by a failure of corticosteroids to control reactive nitrogen species formation.

Fourth, atopy and smoking status may be confounding factors in this study. With regard to atopy, all the subjects who had asthma had a history of allergies, whereas none of the control subjects did. Some studies have shown differences in the biochemical responses to allergen challenge in atopic versus nonatopic asthmatics,[18] but this study does not allow us to sort out the independent effects of atopy. Similarly, the effects of smoking may have complicated the underlying degree of NO metabolism, with smoking possibly promoting[19] or attenuating[20] reactive nitrogen species formation. However, given that none of the asthmatic subjects who died of asthma were current smokers, we believe we can at least state that nitrotyrosine formation is not dependent on smoking. In addition, the 2 asthmatics (subjects 6 and 7) who died of nonrespiratory causes were smokers. Because these 2 asthmatic subjects showed intense staining similar to that of nonsmoking asthmatics who died of asthma and because both smoking (subjects 8 and 13) and nonsmoking controls (subjects 9 and 10) had significantly less staining than asthmatic subjects, smoking status does not appear to be independently associated with either enhanced or attenuated nitrotyrosine formation. Further work with additional numbers of patients with differing histories of atopy and smoking will need to be performed to verify the specificity of our results with regard to these factors.

Last, the formation of reactive nitrogen species relies on the availability of molecular oxygen, and this availability may have been a confounding factor in explaining the degree of nitrotyrsosine formation. Most of the asthmatic subjects no doubt received supplemental oxygen before their deaths, perhaps enhancing the formation of reactive nitrogen species. However, 1 asthmatic subject (subject 1) clearly died outside the hospital without the addition of supplemental oxygen and yet had significant nitrotyrosine immunoreactivity. Likewise, at least 1 nonasthmatic control subject (subject 13) died after being in the hospital at least 12 hours undergoing emergency surgery and yet, despite the addition of supplemental oxygen, had minimal nitrotyrosine formation. Therefore, because of the inconsistent degree to which asthmatic and nonasthmatic subjects were exposed to supplemental oxygen, we do not feel that this was a confounding factor.

A similar concern is that some of our nonasthmatic control subjects died from asphyxia or carbon monoxide poisoning and thus may not have had sufficient exposure to oxygen to allow the formation of reactive nitrogen species, thereby explaining their minimal staining. Although this may be true of the 2 subjects who died of asphyxia, we do not believe this was an important confounding factor because we again recall the asthmatic subject (subject 1) who died outside the hospital of status asthmaticus and therefore was essentially asphyxiated and yet had significant nitrotyrosine formation. In the subjects who died of carbon monoxide poisoning, they too would have continued to have had exposure to ambient oxygen in their alveoli and dissolved oxygen in their blood because carbon monoxide is a cellular asphyxiant that interferes with hemoglobin binding of oxygen and oxygen utilization at the level of the electron transport chain.

The finding of evidence for reactive nitrogen species is consistent with previous reports showing increased levels of exhaled NO in patients with asthma. However, the effects of reactive nitrogen species do not appear to be specific for asthma alone. Indeed, other studies have also found nitrotyrosine immunoreactivity in human acute lung injury[9,14] and idiopathic pulmonary fibrosis.[15] NO and its metabolites likely are involved in generalized mechanisms of inflammation shared by a variety of pulmonary and nonpulmonary diseases.

The presence of nitrotyrosine in fatal asthma suggests that nitration of lung cell proteins may contribute, at least in part, to the airway and parenchymal inflammation and epithelial cytotoxicity observed in severe asthma. In addition, the observation that peroxynitrite causes airway hyperresponsiveness in guinea pigs[13] suggests a possible important functional role for NO and its metabolites in mediating the airway hyperresponsiveness of human asthma. Indeed, a recent publication demonstrating nitrotyrosine staining of airway and inflammatory cells in asthmatic subjects, which is related to airway function and diminished by treatment with corticosteroids, is strong evidence that reactive nitrogen species are present during the disease in life as well and may influence the degree of lung dysfunction.[16]

The diffuse location of lung nitrotyrosine immunoreactivity in this study is consistent with recent evidence supporting the view that asthma is a disease involving the lung parenchyma as well as the airways.[17,21] Inflammation in asthma has been associated with the production of reactive oxygen species by cells obtained from bron-

choalveolar lavage.[22] Such cells are thought to originate in the alveolar spaces, and their characteristics may represent events occurring at the level of the lung parenchyma. The reactive oxygen species superoxide anion is necessary for the generation of peroxynitrite from NO; thus the necessary ingredients for nitration of protein residues in the lung periphery are present during the inflammatory process in asthma.

Lung parenchymal involvement by inflammation would likely worsen the overall clinical severity of asthma because gas exchange would be expected to be adversely affected. In addition, tissue inflammation, by virtue of peribronchial edema and airway wall remodeling, would be expected to enhance the uncoupling of parenchyma and airway and thereby allow more severe airway narrowing to occur.[23] The fact that airway and parenchymal nitrotyrosine staining is observed even in the subjects with milder asthma who died of nonrespiratory causes is further evidence that inflammation, in this case involving reactive nitrogen species extending out to the lung parenchyma, starts early in the disease process, even when lung function may still be preserved.[24]

In summary, this study has demonstrated the presence of nitrotyrosine residues as markers of reactive nitrogen species formation in airway and lung parenchymal tissue from patients who died of severe asthma, as well as in the airways and lung parenchyma of patients with asthma who died of nonrespiratory causes. These residues were not detected to the same degree in lung tissue of nonasthmatic control subjects. Although nitrotyrosine formation may be a marker of severe inflammation, the known damaging effects of peroxynitrite suggest a pathophysiologic role for reactive nitrogen species in asthmatic inflammation. Additional studies are necessary to define further the role of reactive nitrogen species in asthma.

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