

Substance P and its receptor neurokinin 1 expression in asthmatic airways

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Background: Neural mechanisms have been suggested to contribute to the pathogenesis of chronic asthma. The expression of neuropeptides such as substance P may be regulated by infectious pathogens, including *Mycoplasma* species. In contrast to substance P, the substance P receptor neurokinin 1 has not been examined at the protein level in asthmatic airways.

Objective: This study evaluated substance P and neurokinin 1 protein expression and mucus content in endobronchial biopsy specimens from normal control subjects and asthmatic subjects. Detection of *Mycoplasma pneumoniae* was performed in both biopsy and bronchoalveolar lavage specimens.

Methods: Biopsy specimens were collected from 10 normal control subjects and 18 asthmatic subjects before and after a 6-week treatment with a macrolide antibiotic (n = 11) or placebo (n = 7) and were stained for substance P, neurokinin 1, and mucus. *M pneumoniae* was evaluated by PCR.

Results: At baseline, compared with normal control subjects, asthmatic subjects demonstrated increased expression of substance P and neurokinin 1 and mucus content in the airway epithelium. Epithelial mucus content correlated with epithelial substance P expression ($r_s = 0.45$, $P = .04$) and FEV₁ percent predicted ($r_s = -0.51$, $P = .019$). After antibiotic treatment, both epithelial substance P and neurokinin 1 expression were significantly reduced in asthmatic subjects. *M pneumoniae* was found in 8 of 18 asthmatic subjects. Asthmatic subjects with *M pneumoniae*, compared with those without *M pneumoniae*, showed higher baseline epithelial neurokinin 1 expression, which was significantly reduced after antibiotic treatment ($P = .02$).

Conclusion: Our data suggest that abnormalities in neural mechanisms may exist in the epithelium of asthmatic airways, and *M pneumoniae* is possibly involved in this process. Antibiotic intervention may be effective in the treatment of asthma partly through the downregulation of the neurogenic process. (J Allergy Clin Immunol 2000;106:713-22.)

Key words: Asthma, neuropeptides, substance P, neurokinin 1, mucus, *Mycoplasma pneumoniae*

Recently, sensory nerves have been suggested to alter airway function.¹⁻³ Different sensory nerve-derived neuropeptides and their receptors have been examined in asthmatic airways. An early study demonstrated an

Abbreviation used

TBS: TRIS-buffered saline

increase in substance P-immunoreactive nerves in the submucosa of asthmatic endobronchial biopsy specimens.⁴ Elevated levels of substance P were also found in the bronchoalveolar lavage fluid and sputum of asthmatic subjects.^{5,6} However, recent studies have not demonstrated increased substance P in asthmatic lungs.^{7,8} Messenger RNA for the substance P receptors neurokinin 1 and neurokinin 2 have also been evaluated in two studies.^{9,10} Bai et al⁹ demonstrated an increase of neurokinin 2 but not neurokinin 1 in asthmatic subjects compared with that found in normal control subjects. In contrast, Adcock et al¹⁰ showed more neurokinin 1 messenger RNA but not neurokinin 2 messenger RNA expression in asthmatic airways. Thus far, no studies have looked at the neurokinin 1 protein expression in asthmatic airways.

Interestingly, recent studies have suggested that viral or mycoplasmic infections may contribute to the neurogenic inflammation.¹¹⁻¹⁴ Neurogenic inflammation was induced in a murine model of respiratory infection with *Mycoplasma pulmonis* and sensory nerve stimulation with capsaicin. The infected rat airway demonstrated an increased neurokinin 1 expression on blood vessels and enhanced mucus production in the trachea epithelial layer.¹²⁻¹⁴ Treatment with an antibiotic oxytetracycline in this animal model significantly reduced the airway neurogenic inflammation and the number of infecting organisms.¹⁴ We have reported that chronic asthma is associated with *Mycoplasma pneumoniae* in the airway.¹⁵ Additionally, improvement in lung function was followed with a macrolide antibiotic treatment.¹⁶

In the current study we hypothesized that a neural mechanism may be involved in the pathogenesis of asthma with *M pneumoniae* infection/loading contributing to this neurogenic process. Therefore macrolide antibiotics, such as clarithromycin, may be effective in attenuating this process. We compared the expression of substance P and its receptor neurokinin 1 by immunostaining in endobronchial biopsy specimens of asthmatic subjects and normal control subjects, as well as the effect of an antibiotic on the expression, function, or both of these neurogenic markers in asthmatic airways. To evaluate the possible functions of substance P and neurokinin 1, mucus staining of the biopsy tissue was additionally analyzed.

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METHODS

Subjects

Twenty-eight subjects were recruited by means of newspaper and radio advertisements from the general community of Denver, Colorado. Eleven of these subjects came from our previous report of *M pneumoniae* detection by means of PCR,¹⁵ and 17 subjects were new to this study. The asthmatic subjects fulfilled criteria for asthma exhibiting a PC₂₀ to methacholine of less than 8 mg/mL and reversibility of lung function by at least 15% with a bronchodilator. Control subjects exhibited no evidence of atopy or bronchial hyper-responsiveness (PC₂₀ >18 mg/mL). Exclusion criteria included inpatient status; upper or lower respiratory tract infection within 3 months of the study; use of macrolides, tetracyclines, or quinolones within 3 months of study; smoking history greater than 5 pack-years or any cigarettes within the previous 2 years; significant nonasthmatic pulmonary disease; or other medical problems. No subject was a hospital worker to insure that contact with potential patients infected with *M pneumoniae* was minimized.

Bronchoscopy

Both asthmatic subjects (n = 18) and control subjects (n = 10) underwent bronchoscopy, as previously described.¹⁷ Endobronchial biopsy tissues were obtained for staining protocol and *M pneumoniae* PCR analysis.¹⁵ The location of the biopsy specimens was randomized to the right or left lower lobe. Two to 4 tissue specimens were obtained under direct visualization from the fourth or fifth generation airways. Asthmatic subjects were then randomly divided into two subgroups with different treatments. One subgroup of asthmatic subjects (n = 11) received oral clarithromycin at 500 mg twice daily for 6 weeks. The remainder of the asthmatic subjects (n = 7) received placebo for 6 weeks. Bronchoscopy and biopsy were then repeated in both asthma groups. All subjects gave written informed consent, and the protocol was approved by the institutional review board.

Tissue processing and staining

Endobronchial biopsy specimens were processed as previously reported.¹⁸ Briefly, specimens were fixed in acetone at -20°C overnight and then embedded in glycol methacrylate resin. Tissue blocks were stored at -20°C. Serial 2-μm sections were cut from the tissue blocks with a Reichert Ultracut E ultramicrotome (Leica Inc, Deerfield, Ill) and placed on poly-L-lysine-coated slides.

All the biopsy tissue sections were eligible for immunostaining with rabbit polyclonal substance P (R5, 1:1200 dilution) and neurokinin 1 (1:500 dilution) antibodies. The characteristics of these antibodies have been previously described.^{19,20} They have specificity to human and rat tissue. To reduce the variation of immunostaining between different batches, all the substance P or neurokinin 1 staining was performed in the same batch. Sections were treated with 0.3% hydrogen peroxide in 0.05 mol/L TRIS-buffered saline (TBS, pH 7.6) for 30 minutes to inhibit endogenous peroxidase, followed by incubation with 1% normal goat serum for 30 minutes to block potential nonspecific binding sites. The slides were then incubated with the primary antibodies mentioned above for 2 hours at room temperature, followed by incubation with biotinylated goat anti-rabbit IgG for 1 hour at room temperature. Thereafter, avidin-biotin-peroxidase complex (Vector Labs, Burlingame, Calif) was added to the slides for 45 minutes at room temperature. After rinsing the slides in TBS, 0.03% aminoethylcarbazole in 0.03% hydrogen peroxide was used as a substrate to develop a peroxide-dependent red color reaction. Slides were counterstained with Mayer's hematoxylin and covered with Crystallmount.

Appropriate control slides were included. First, substance P and neurokinin 1 antibodies were preabsorbed for 4 hours with excessive amount of substance P and neurokinin 1 peptides, which were

used to generate the antibodies. The preabsorbed antibodies were then used to replace the substance P and neurokinin 1 antibodies in the above staining procedure. Second, slides were similarly treated but with the primary antibodies replaced by rabbit serum or TBS.

Mucus in the tissue was demonstrated by using the Alcian blue/periodic acid-Schiff staining method.²¹ The slides were covered with permount. For mucus analyses, only those biopsy tissues of a reasonable size (area ≥0.5 mm²) and intact ciliated epithelial cells were used. Therefore 9 normal control subjects and 11 asthmatic subjects were included at baseline. Ten asthmatic subjects qualified for mucus analyses after antimicrobial therapy or placebo treatment.

Analyses of substance P, neurokinin 1, and mucus staining

Because substance P and neurokinin 1 staining was seen in many tissue components, a semiquantitative method was used to evaluate the expression of substance P and neurokinin 1 in airway epithelium and submucosa separately. The submucosa in the biopsy tissue is defined as the area underneath the epithelial layer, which includes connective tissue, inflammatory cells, blood vessels, smooth muscle, glands, and cartilage. A scale of 0 to 3 was used to judge the staining with 0 for no staining and 1, 2, and 3 for mild, moderate, and strong staining, respectively.^{22,23}

A National Institute of Health Scion image analysis program was used to evaluate the percentage of mucus staining in the intact epithelium of the biopsy specimens. Black and white pictures were taken and transferred to the computer by means of a video camera. The stained area for mucus was black and high in intensity compared with the nonstained area (white). The "density slice function" of the scion image program automatically and selectively identified the mucus staining at a predetermined density. The area of the mucus staining was then automatically measured. The total area of the epithelium was manually circumscribed and measured. The result was expressed as the percentage of the total area of the epithelium occupied by mucus staining.²¹

The same observer read all slides. The reader was completely blinded to every aspect of the subjects. The same observer only had one scale difference (scale range, 0-3) in less than 10% of total substance P- and neurokinin 1-stained slides and a less than 10% coefficient of variation for mucus measurement on repeated measurement. We performed a pilot study to test the interobserver variability for some randomly selected slides. The agreement between the observers was a 0 to 1 difference for semiquantitative analysis and a less than 10% coefficient of variation for mucus measurement.

Statistical analyses

Within-group comparisons were performed by using the paired *t* test or the Wilcoxon rank-sum test, depending on the distribution of the data. Between-group comparisons were performed by using the unpaired *t* test or a nonparametric Kruskal-Wallis ANOVA, depending on data distribution. Correlations were performed by using the Spearman rank correlation coefficient. All tests were two sided, and a *P* value of .05 or less was regarded as significant.

RESULTS

Subjects

The characteristics of asthmatic and normal control subjects are shown in Table I. The sex distribution and the age of subjects were similar between asthmatic and normal control subjects. The baseline FEV₁ percent predicted and PC₂₀ were significantly lower in asthmatic than normal control subjects. Five of 18 asthmatic subjects were receiving inhaled steroids. None of the asthmatic

subjects received oral steroids. At baseline, no significant differences were present between asthmatic subjects treated with the macrolide or placebo in regard to age, sex, FEV₁ percent predicted, duration of asthma, PC₂₀, and inhaled steroid use. After treatment, improvement of FEV₁ was seen in 9 (81.8%) of 11 antibiotic-treated asthmatic subjects and 3 (42.3%) of 7 placebo-treated asthmatic subjects ($P = .08$). PC₂₀ was increased in 8 (72.7%) of 11 antibiotic-treated asthmatic subjects and 2 (28.6%) of 7 placebo-treated asthmatic subjects ($P = .07$).

Substance P and neurokinin 1 expression in the airways of normal control and asthmatic subjects

One to two tissue pieces of good quality from a bronchoscopy were used for the analyses of substance P and neurokinin 1. The location, but not the extent, of the staining for substance P and neurokinin 1 in the airway tissue of normal control and asthmatic subjects is similar. Substance P immunoreactivity was observed in both the epithelium and submucosa of the two groups. In the epithelium patchy substance P staining was localized mainly at the lower half of the epithelial layer. In the submucosa the staining was observed around blood vessels and smooth muscles and on some inflammatory cells. Similar to substance P, neurokinin 1 immunoreactivity was also found in both the epithelium and submucosa. In the epithelium neurokinin 1 expression was mainly seen on cell surfaces of the upper half of the epithelial layer. Goblet cells appeared to be the cells with the strongest staining. In the submucosa neurokinin 1 was primarily localized on the endothelial cells of the blood vessels, the surfaces of inflammatory cells, and some smooth muscle cells.

Fig 1 demonstrates substance P and neurokinin 1 immunostaining in normal control (Fig 1, A and B) and asthmatic subjects (Fig 1, C and D). The specificity of the immunostaining was confirmed by negative staining on tissue slides incubated with either rabbit serum or substance P and neurokinin 1 antibodies preabsorbed with substance P and neurokinin 1 peptides (Fig 1, E and F).

In the epithelium asthmatic subjects had significantly higher expression of substance P (score, 1.4 ± 0.2 vs 0.5 ± 0.2 ; $P = .048$) and neurokinin 1 (score 1.6 ± 0.2 vs 0.7 ± 0.2 , $P = .014$) than normal control subjects (Fig 2). However, substance P and neurokinin 1 expression were similar in the submucosa between asthmatic and normal control subjects (Fig 2).

Effects of antibiotic and placebo on airway substance P and neurokinin 1 expression in asthmatic subjects

At baseline, asthmatic subjects treated with an antimicrobial or placebo showed similar levels of substance P (1.5 ± 0.3 vs 1.6 ± 0.3 , $P = .96$) and neurokinin 1 (1.9 ± 0.4 vs 1.9 ± 0.3 , $P = .81$) expression in the epithelium. Similar levels of substance P (1.3 ± 0.3 vs 1.7 ± 0.4 , $P = .50$) and neurokinin 1 (1.4 ± 0.2 vs 1.6 ± 0.3 , $P = .83$)

TABLE I. Subject characteristics

	Asthmatic subjects (n = 18)	Control subjects (n = 10)
Sex (M/F)	10/8	6/4
Age (y)	34.0 ± 2.2	30.7 ± 2.4
Duration of asthma (y)	22.9 ± 2.8	—
FEV ₁ (L)	2.4 ± 0.2	$4.2 \pm 0.3^*$
FEV ₁ (% predicted)	65.6 ± 4.8	$103.8 \pm 5.2^*$
PC ₂₀ (mg/mL)	0.27 ± 0.06	$>18^*$
Medications	ICS (n = 5), β-agonist (n = 18)	None

ICS, Inhaled corticosteroids.

* $P < .01$ compared with asthmatic subjects.

expression were also found in the submucosa between the two asthmatic groups.

A significant reduction of substance P expression was seen in the epithelium after 6 weeks of antibiotic treatment (Fig 3, A). However, neither substance P expression in the submucosa nor neurokinin 1 expression in both the epithelium and submucosa were significantly changed (Fig 3, A and B). Because some asthmatic subjects showed low levels of baseline substance P and neurokinin 1 expression, further reduction of substance P and neurokinin 1 expression may be difficult to obtain. Therefore we reanalyzed the data in those asthmatic subjects with a sum score of 3 or greater from the epithelium and submucosa for substance P or neurokinin 1 staining (Fig 3, A and B). Asthmatic subjects (n = 10) with a sum score of 3 or greater for substance P still demonstrated a significant decrease of substance P expression in the epithelium ($P = .02$) but not in the submucosa. Interestingly, asthmatic subjects (n = 5) with a sum score of 3 or greater for neurokinin 1 staining now showed a significant reduction of neurokinin 1 expression in both the epithelium ($P = .003$) and submucosa ($P = .03$).

In contrast to antimicrobial treatment, placebo did not significantly alter the substance P and neurokinin 1 expression in either the epithelium or submucosa of asthmatic airways, regardless of whether all asthmatic subjects (n = 7) or those (n = 3) with a sum score of 3 or greater from the epithelium and submucosa for substance P or neurokinin 1 staining were included for the analyses.

Mucus content in the epithelium

Compared with normal control subjects, asthmatic subjects showed a significant increase in mucus staining area in the epithelium ($5.8\% \pm 1.2\%$ vs $1.9\% \pm 0.5\%$, $P = .03$; Fig 1, G and H, and Fig 4). For asthmatic subjects, complete paired data were available in 6 subjects treated with the antimicrobial and 4 subjects treated with placebo. Reduced mucus area was seen in 4 (67%) of 6 asthmatic subjects receiving active treatment. Although statistically significant differences were not reached in these 6 asthmatic subjects ($P = .20$) after the active treatment, a significant decrease of mucus area was indeed seen in those asthmatic subjects (n = 3, $3.9\% \pm 1.3\%$ vs $0.8\% \pm 0.1\%$, $P = .049$) with a sum neurokinin 1 score of 3 or

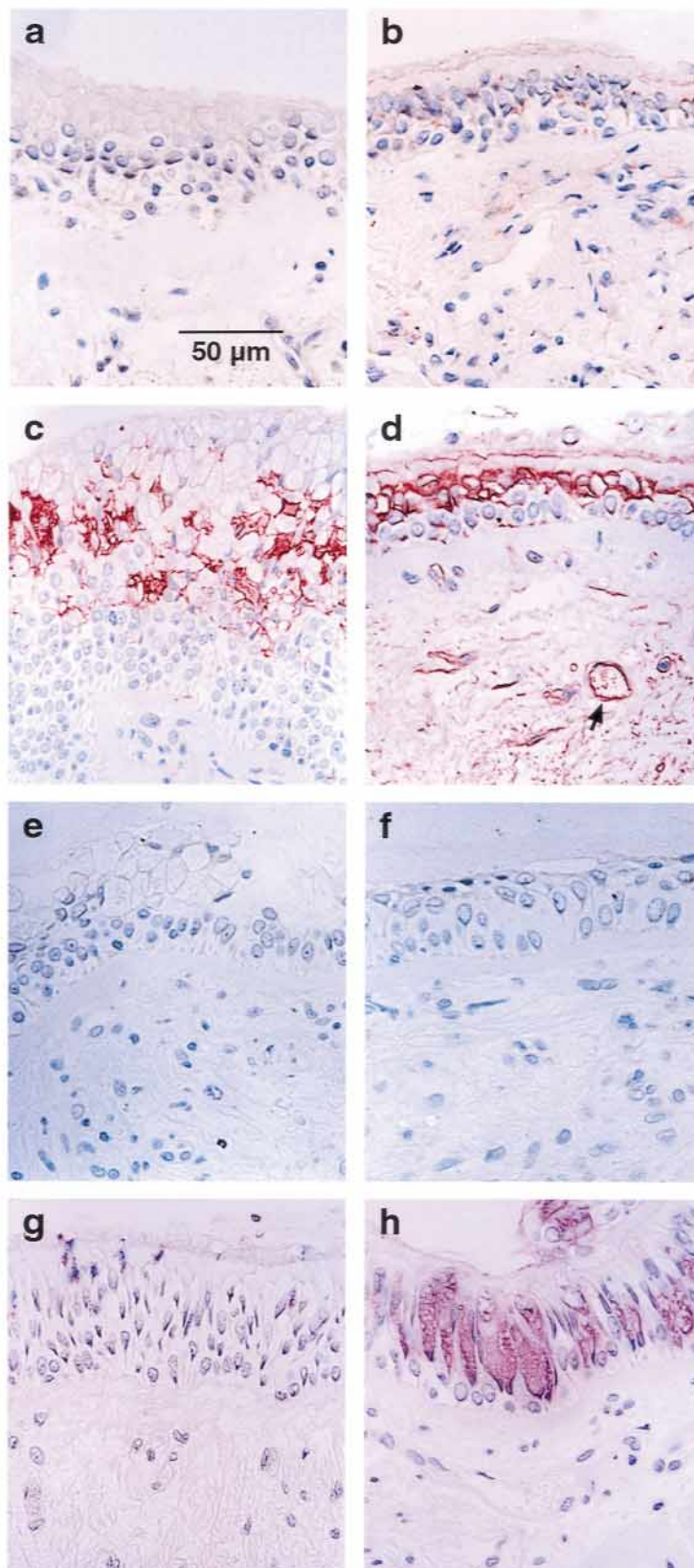


FIG 1. Representative photomicrographs of immunostaining on endobronchial biopsy samples. Substance P (**a**) and neurokinin 1 (**b**) staining in a normal control subject. Substance P (**c**) and neurokinin 1 (**d**) staining in an asthmatic subject. The arrow in **d** indicates the neurokinin 1 staining on endothelial cells of a blood vessel in the submucosa. Asthmatic biopsy sections incubated with either substance P peptide preabsorbed substance P antibody (**e**) or neurokinin 1 peptide preabsorbed neurokinin 1 antibody (**f**). Alcian-blue/periodic acid-Schiff staining for mucus in a normal control subject (**g**) and an asthmatic subject (**h**). (Original magnification, 400 $\times$.)

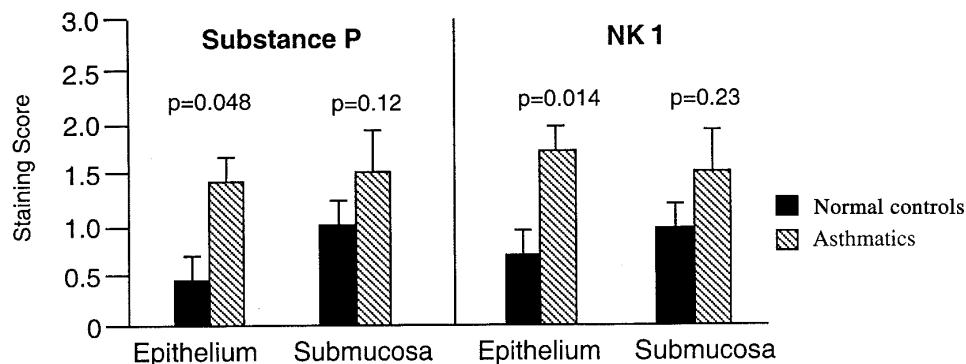


FIG 2. Comparisons of substance P and neurokinin 1 (*NK1*) expression in endobronchial biopsy samples between normal control subjects and asthmatic subjects.

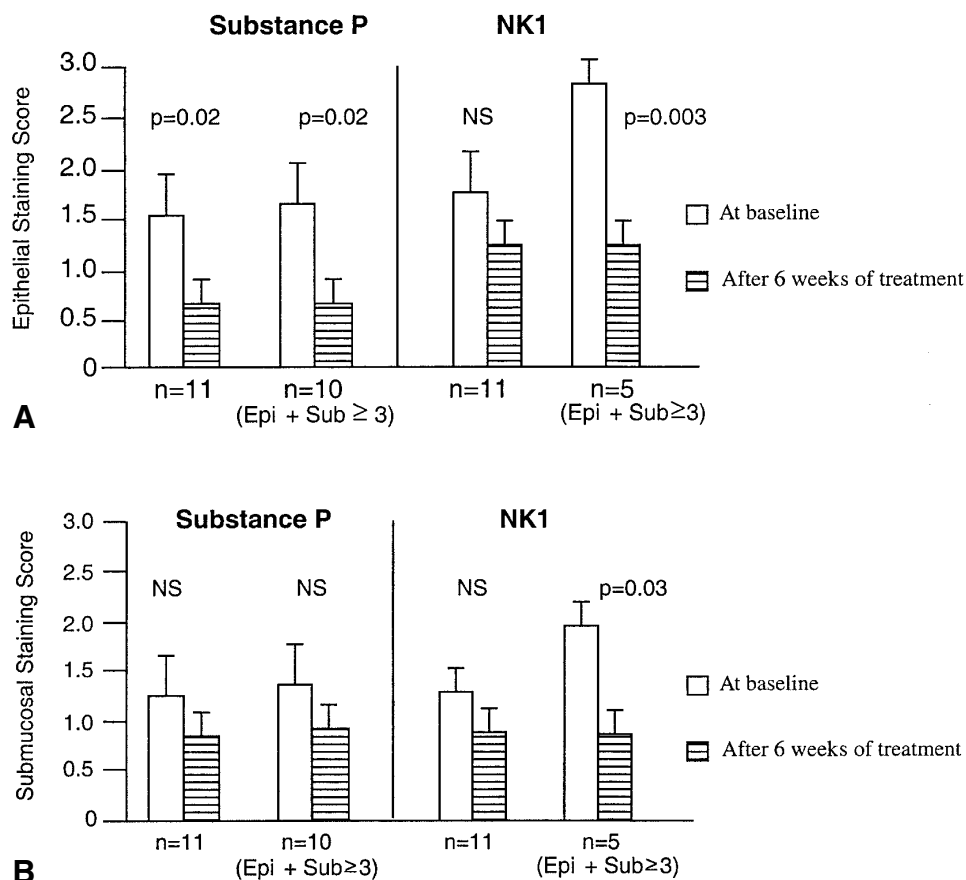


FIG 3. A, Epithelial substance P and neurokinin 1 (*NK1*) expression in asthmatic subjects at baseline and after 6 weeks of clarithromycin treatment. **B,** Submucosal substance P and neurokinin 1 (*NK1*) expression in asthmatic subjects at baseline and after 6 weeks of clarithromycin treatment.

greater from the epithelium and submucosa. Those asthmatic subjects ($n = 5$) with a sum substance P score of 3 or greater from the epithelium and submucosa tended to have a reduced mucus area ($5.4\% \pm 1.3\%$ vs $2.3\% \pm 1.0\%$, $P = .07$) after treatment. After placebo, asthmatic subjects did not show significant changes in mucus area ($5.3\% \pm 3.3\%$ vs $1.7\% \pm 0.5\%$, $P = .80$).

Correlations among substance P and neurokinin 1 expression, epithelium mucus content, FEV₁, and PC₂₀

Because substance P and neurokinin 1 have been suggested to regulate mucus production, and mucus production has been proposed to affect the pathophysiology of

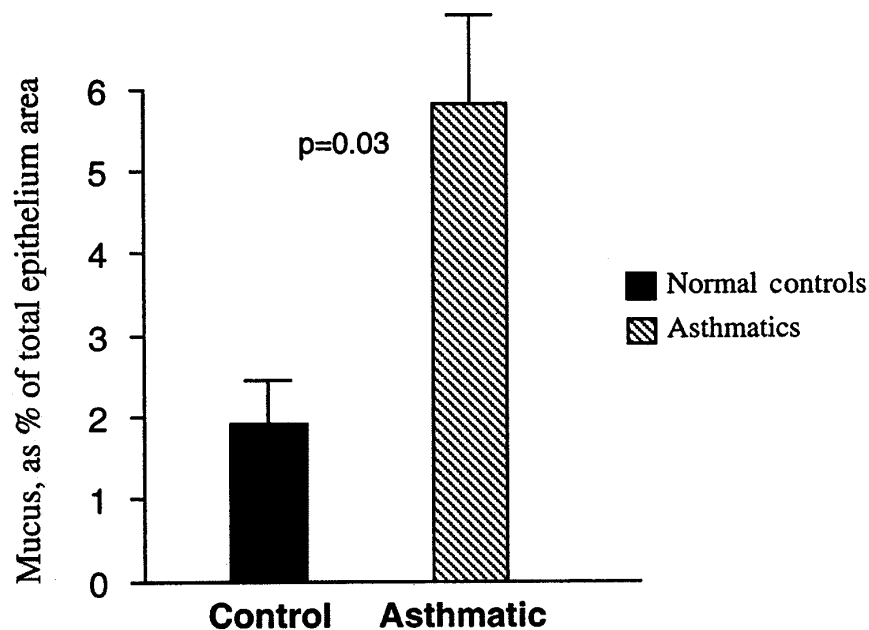


FIG 4. Comparisons of epithelial mucus production in endobronchial biopsy samples of normal control subjects and asthmatic subjects at baseline.

asthma, correlations among substance P and neurokinin 1 expression, epithelium mucus content, FEV₁, and PC₂₀ were performed. For both asthmatic and normal control subjects at baseline, substance P expression in the epithelium but not in the submucosa significantly correlated with the epithelial mucus content ($r_s = 0.45$, $P = .04$; Fig 5, A). However, significant correlations did not exist between neurokinin 1 expression in either the epithelium or submucosa and the epithelial mucus content ($r_s = 0.30$, $P > .05$ and $r_s = -0.16$, $P > .05$, respectively). Epithelial mucus content was negatively correlated with FEV₁ percent predicted ($r_s = -0.51$, $P = .019$; Fig 5, B). PC₂₀ did not correlate significantly with substance P, neurokinin 1, or epithelial mucus content.

***M pneumoniae* detection and its association with substance P and neurokinin 1 expression and epithelium mucus content**

PCR analysis for *M pneumoniae* in bronchoalveolar lavage fluid, endobronchial biopsy tissue, or both demonstrated *M pneumoniae* positivity in 8 (44.4%) of 18 asthmatic subjects. For those asthmatic subjects with *M pneumoniae* positivity, 6 were in the antibiotic-treated group, and 2 were in the placebo-treated group. *M pneumoniae* PCR data were only available for 5 normal control subjects. One of the control subjects had positive test results for *M pneumoniae*. Because of the small sample size of normal control subjects ($n = 5$), comparisons of *M pneumoniae* positivity were not performed between asthmatic and normal control subjects. However, our previous data did show

that *M pneumoniae* positivity was significantly higher ($P = .02$) in asthmatic than normal control subjects.¹⁵

As shown in Table II, at baseline, asthmatic subjects ($n = 8$) with *M pneumoniae* had significantly higher neurokinin 1 expression in the epithelium (2.4 ± 0.4 vs 1.4 ± 0.3 , $P = .02$) and marginally higher submucosal substance P (2.0 ± 0.3 vs 1.1 ± 0.3 , $P = .06$) and neurokinin 1 (1.9 ± 0.3 vs 1.2 ± 0.2 , $P = .07$) expression than those ($n = 10$) without *M pneumoniae*. Substance P expression and mucus area in the epithelium were similar between asthmatic subjects with and without *M pneumoniae*. Compared with normal control subjects, asthmatic subjects with *M pneumoniae* ($n = 8$) showed significantly higher substance P and neurokinin 1 expression in both the epithelium (substance P: 1.5 ± 0.3 vs 0.5 ± 0.2 , $P = .04$; neurokinin 1: 2.4 ± 0.4 vs 0.7 ± 0.2 , $P = .01$) and submucosa (substance P: 2.0 ± 0.3 vs 1.1 ± 0.2 , $P = .03$; neurokinin 1: 1.9 ± 0.3 vs 1.0 ± 0.2 , $P = .04$), whereas asthmatic subjects without *M pneumoniae* ($n = 10$) did not. After antibiotic treatment, asthmatic subjects with *M pneumoniae* ($n = 6$) showed a significant reduction of neurokinin 1 expression in the epithelium ($\Delta = -1.5 \pm 0.3$ vs $\Delta = 0.1 \pm 0.3$, $P = .02$) and a marginal decrease of substance P expression in the submucosa ($\Delta = -0.8 \pm 0.5$ vs $\Delta = 0.3 \pm 0.3$, $P = .10$) compared with those without *M pneumoniae* ($n = 5$). The changes in submucosal neurokinin 1 ($\Delta = -0.5 \pm 0.5$ vs $\Delta = -0.6 \pm 0.2$, $P = .88$) and epithelial substance P expression ($\Delta = -0.8 \pm 0.3$ vs $\Delta = -1.2 \pm 0.6$, $P = .34$) were not significantly different between asthmatic subjects with *M pneumoniae* and asthmatic subjects without *M pneumoniae*.

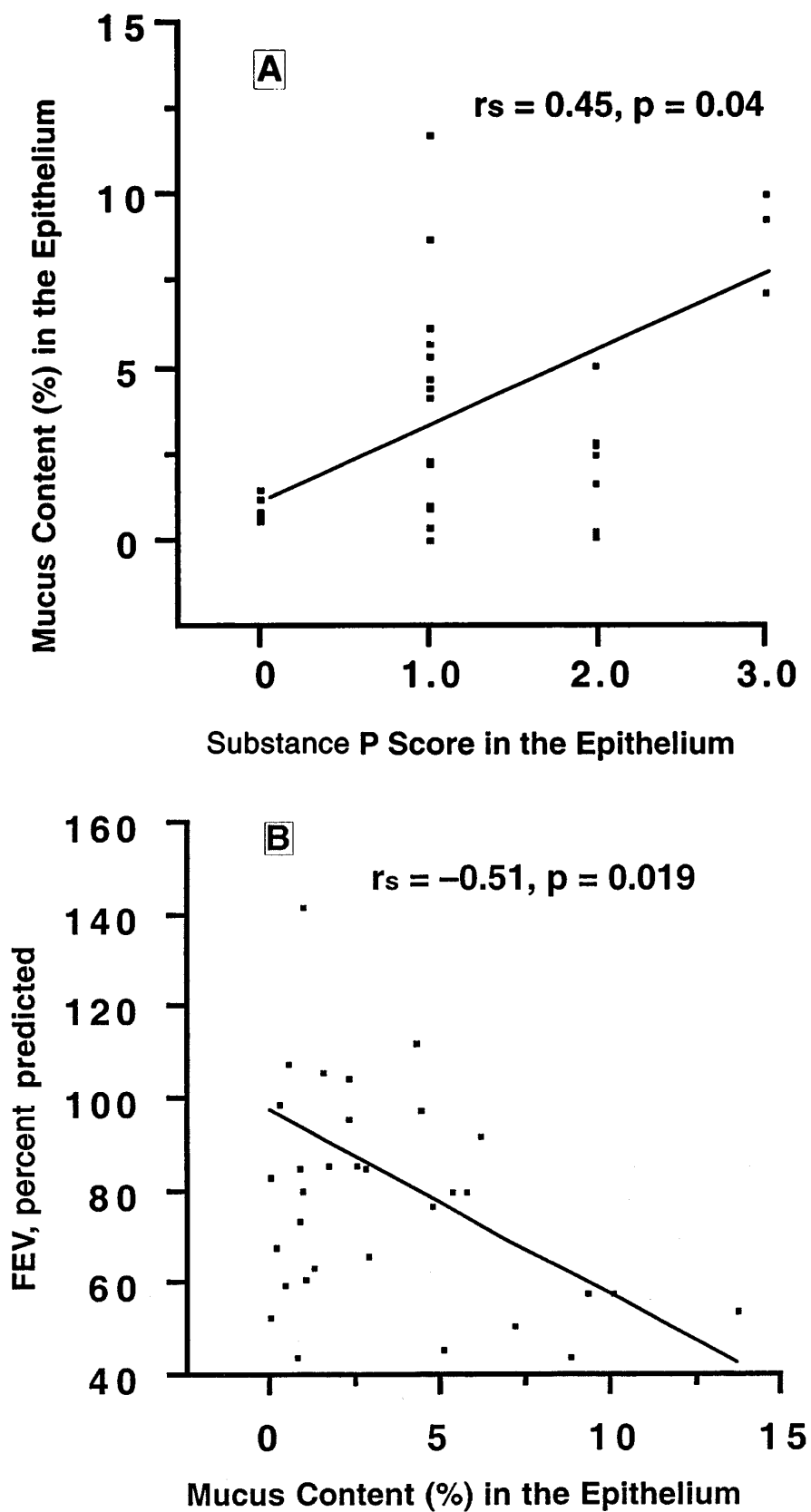


FIG 5. A, Correlation between epithelial substance P expression and epithelial mucus content. B, Correlation between epithelial mucus content and FEV₁ percent predicted.

TABLE II. Comparisons between asthmatic subjects with and without *M pneumoniae*

	<i>M pneumoniae</i> -positive asthmatic subjects	<i>M pneumoniae</i> -negative asthmatic subjects	P value
Substance P: epithelium score	1.5 ± 0.3	1.6 ± 0.3	.85
Substance P: submucosa score	2.0 ± 0.3	1.1 ± 0.3	.06
Neurokinin 1: epithelium score	2.4 ± 0.4	1.4 ± 0.3	.02
Neurokinin 1: submucosa score	1.9 ± 0.3	1.2 ± 0.2	.07
Mucus in epithelium (%)	4.3 ± 1.5	7.4 ± 1.9	.34

All values are given as mean ± SEM.

DISCUSSION

This study demonstrates that compared with normal control subjects, asthmatic subjects have increased expression of substance P and its receptor neurokinin 1 in the airway epithelium but not in the submucosa. Consistent with this finding, increased mucus production was also observed in asthmatic subjects, and it was inversely correlated with FEV₁. The increased epithelial substance P and neurokinin 1 expression in asthmatic subjects appears to be associated with the detection of *M pneumoniae* in the lower airways. Antibiotic therapy reduced the epithelial substance P and neurokinin 1 expression in asthmatic subjects. Our data suggest that abnormalities in neurogenic mechanisms may exist in the epithelium of asthmatic subjects. *M pneumoniae* infection/loading may be involved in this neurogenic process, which could be attenuated by antibiotic therapy.

Substance P is one of the 5 tachykinin neuropeptides described thus far in the sensory nerves. The others include neurokinin A, neuropeptide K, neuropeptide γ, and neurokinin B. Neurokinin A and especially substance P are the two best studied.²⁴ Although substance P has been suggested to be mainly localized to sensory nerves in the bronchial epithelium, and in and around blood vessels and smooth muscle, recent studies suggest that airway epithelial cells and some inflammatory cells, such as eosinophils, macrophages, and dendritic cells, can also express substance P.²⁵⁻²⁸ Our current study suggests that substance P immunoreactivity is primarily localized on the bronchial epithelium and inflammatory cells in the endobronchial biopsy specimens. The exact cellular sources of substance P immunostaining in the tissue were not determined in this study. Because of the small size of the biopsy sample and the thin tissue sections, the current study also cannot tell whether some substance P immunoreactivity is indeed in the nerves. Three distinct tachykinin receptors exist.²⁴ They are neurokinin 1, neurokinin 2, and neurokinin 3. Each tachykinin neuropeptide preferentially binds a receptor. Substance P has the highest binding affinity with neurokinin 1 and less with neurokinin 2, and neurokinin A has the highest binding affinity with neurokinin 2 and less with neurokinin 1. Neurokinin 1 receptor activation may result in mucus secretion, microvascular leakage, vasodilation, migration, and activation of inflammatory cells, including lymphocytes, mast cells, macrophages, neutrophils, and eosinophils. The neurokinin 1 receptor has been found in

bronchial epithelial cells, mucous glands, blood vessels, and some inflammatory cells. The immunolocalization of the neurokinin 1 receptor in our current study is consistent with previous results.^{9,12} The airway neurokinin 2 receptor is mainly localized in smooth muscle. Neurokinin 2 activation is therefore involved directly with bronchoconstriction.²⁴

The immunohistochemical study of substance P in asthma is still controversial, which may be explained by differences in the type of study subjects, tissue processing, and staining procedures. Because substance P can be rapidly digested by neutral endopeptidase, it is very important to fix and process the tissue samples promptly.²⁹ We carefully processed biopsy samples and performed appropriate controls for the immunostaining in the current study. Our finding of no differences in substance P immunoreactivity in the submucosa between asthmatic and normal control subjects does not support data from the study by Ollerenshaw et al,⁴ which showed increased substance P immunostaining in asthmatic airway submucosa. However, our data do support recent studies that indicate that substance P is not increased in the airway submucosa of asthmatic subjects.^{7,8,30} Interestingly, our current study demonstrates increased expression of substance P in the airway epithelium of asthmatic subjects. Similar to substance P, increased neurokinin 1 expression was also found in the epithelium but not in the submucosa of asthmatic airways. To our knowledge, this is the first report that asthmatic subjects have increased neurokinin 1 protein expression in the epithelium. The coexistence of increased substance P and its receptor neurokinin 1 in the epithelium indicates that neurogenic mechanisms may play a role in the activation of bronchial epithelial cells. The fact that increased epithelial mucus production is also present may further support a neurogenic mechanism in asthma. As in other studies,^{31,32} our current study further confirms the overproduction of mucus in asthmatic airways. Hypersecretion of mucus has been suggested to play an important role in the pathophysiology of asthma.

One of the important questions in this study is what causes or potentiates the expression of neuropeptides, such as substance P, and their receptors, including neurokinin 1, in the asthmatic airways. Some of the well-known factors capable of inducing neurogenic inflammation include capsaicin, bradykinin, histamine, SO₂, and hyperosmolar solutions.² Recent studies suggest that air-

way viral infection may also be involved in the neurogenic inflammatory process.¹¹ *Mycoplasma pulmonis* infection in rats has been suggested to contribute to the airway neurogenic inflammation characterized by increased substance P and neurokinin 1 expression, hypersecretion of mucus, angiogenesis, and musosal inflammatory cell infiltrate.¹²⁻¹⁴ However, the association of airway *M pneumoniae* infection/loading with neurogenic inflammation was not addressed in asthma or animal models. We have shown that asthmatic subjects have an increased frequency of *M pneumoniae* infection/loading in the lower airway compared with that of normal control subjects.¹⁵ Asthmatic subjects with positive test results for *M pneumoniae* have increased expression of neurokinin 1 expression, substance P expression, or both in the airway tissue, especially in the bronchial epithelium, compared with those asthmatic and normal control subjects with negative test results for *M pneumoniae*. These data suggest that the airway neurogenic process, as represented by increased substance P and neurokinin 1 expression in the epithelium, may be associated with *M pneumoniae* infection/loading. Previous studies have demonstrated that *M pneumoniae* is mainly localized to the cilia of bronchial epithelial cells of animals and human subjects.^{33,34} The colocalization of substance P-neurokinin 1 and *M pneumoniae* would support the possible involvement of *M pneumoniae* in the airway neurogenic process.

If *M pneumoniae* is involved in asthma pathogenesis, a macrolide antibiotic with anti-*M pneumoniae* activity should alter the neurogenic characteristics. Asthmatic subjects treated with clarithromycin showed reduction of substance P, neurokinin 1, and mucus in the epithelium. Reduction of epithelial neurokinin 1 expression was more prominent in asthmatic subjects with *M pneumoniae* than in those without *M pneumoniae*. These data suggest that antibiotic therapy is capable of inhibiting the neurogenic mechanisms on the surface of airways. Because clarithromycin has been suggested to have some direct anti-inflammatory effects,^{35,36} we cannot rule out the possibility that there may be a combination effect of antibiotic therapy (ie, antimicrobial and direct anti-inflammatory effect).

Because asthma pathogenesis is very complex, it is conceivable that *M pneumoniae* infection/loading in the airway may be only part of the disease process in asthma. In fact, recent studies suggest that airway infection with other pathogens, such as *Chlamydia pneumoniae*, and viruses may also contribute to the pathogenesis of asthma.^{11,37} Because we have not yet evaluated the potential effect of mycoplasma on other aspects of airway inflammatory or remodeling processes, it is difficult to determine the role of antimicrobial therapy in asthma.

In conclusion, our current study suggests that neurogenic mechanisms in the bronchial epithelium may be involved in the pathogenesis of asthma. *M pneumoniae* is associated with increased expression of the neuropeptide substance P and its receptor neurokinin 1. Antimicrobial therapy may reduce the expression of these neurogenic markers. Further

study is needed to examine the functional changes of bronchial epithelial cells after *M pneumoniae* infection and the role of antimicrobial therapy in chronic asthma.

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