Alteration of airway neuropeptide expression and development of airway hyperresponsiveness following respiratory syncytial virus infection

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RESPIRATORY SYNCYTIAL VIRUS (RSV) is the most common cause of acute bronchiolitis that affects children under the age of 2 years (17). Lower respiratory tract infection by RSV is considered a major risk factor for the development of persistent wheezing and asthma during childhood (35–37). However, not all children will go on to develop long-term sequelae following RSV infection, suggesting that environmental, genetic, and host response factors may be important determinants in the development of altered airway function following RSV infection (26).

In animal models of RSV infection, airway inflammation contributes to the development of airway hyperresponsiveness (AHR), defined as an exaggerated bronchoconstrictive response to methacholine (5, 34). There is also some evidence that RSV infection can alter the neurogenic control of airway function (8, 9), but the mechanisms responsible for such alterations have not been fully identified. In rats, RSV infection upregulates the mRNA expression of neurokinin (NK)-1, the high-affinity receptor of substance P in the airways and this receptor is thought to play a significant role in the development of exaggerated neurogenic airway inflammation, as indicated by increased inflammation and vascular leakage in the airways of infected animals (2, 22). Recent studies in mice have described increased levels of the tachykinin substance P in the bronchoalveolar lavage (BAL) fluids recovered from RSV-infected animals and attributed a role for this neuropeptide in the development of the lymphocytic response to RSV infection (39, 40). This notion was supported by the findings that lymphocytes recovered from the airways of these animals expressed the NK-1 receptor and proliferated in response to stimulation with substance P in vitro.

Because airway function is controlled by neural mechanisms (21) and it is known that airway inflammation can alter this control (41), we hypothesized that RSV infection may alter the expression of sensory neuropeptides in the airways thereby contributing to the development of altered airway function. In the present study, we focused on substance P and calcitonin gene-related peptide (CGRP) as potential neuromodulators of airway function as both neuropeptides can coexist in many nerve fibers within the airways (27). The study was specifically designed to examine the effects of RSV infection on the expression of both sensory neuropeptides in the airways of mice and to relate the changes to the development of altered airway function. The expression and tissue localization of substance P and CGRP were analyzed by quantitative immunohistochemistry, and their involvement in modulating airway function was defined by antagonizing their function in vivo. Airway function was analyzed at the postjunctional level, by measuring the changes in airway resistance to inhaled methacholine in vivo, and at the prejunctional level, by measuring the contractile responses of isolated tracheal smooth muscle (TSM) segments to electrical field stimulation (EFS) in vitro.

METHODS

Reagents. CGRP, CGRP(8–37), and the highly selective neurokinin (NK)-1 receptor antagonist Sendide (53) were obtained from Bachem California (Torrance, CA) and were administered at the prejunctional level, by measuring the changes in airway resistance to inhaled methacholine in vivo, and at the prejunctional level, by measuring the contractile responses of isolated tracheal smooth muscle (TSM) segments to electrical field stimulation (EFS) in vitro.

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**Animals.** BALB/c mice, 8 to 10 wk of age, were obtained from Jackson Laboratories (Bar Harbor, ME) and were used under a protocol approved by the Institutional Animal Care and Use Committee.

**Virus.** The strain A2 of human RSV (cat. no. VR-1302) was obtained from American Type Culture Collection (ATCC, Manassas, VA). Stocks of purified RSV were prepared under endotoxin-free conditions and were assessed to be free of endotoxin contamination using a Limulus Amoebocyte Lysate (LAL)-based Pyrogen Plus Gel Clot assay with a sensitivity of 0.06 endotoxin U/ml (cat. no. N283–06, BioWhittaker, Walkersville, MD). The virus was propagated in monolayers of HEP-2 cells (ATCC, cat. no. CCL-23) grown in Eagle’s minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% FBS. At maximum cytopathic effect, the cells were harvested and disrupted by sonication in the same culture medium. The suspension was clarified by centrifugation at 10,000 g for 15 min at 10°C and the resulting supernatant was layered on top of a sucrose gradient (30% in 50 mM Tris-buffered normal saline solution containing 1 mM EDTA, pH 7.5) and further centrifuged at 100,000 g for 2 h at 10°C. The pellet containing purified virus was resuspended in 10 mM PBS (pH 7.4) containing 15% sucrose and stored in aliquots at −70°C. Viral titers were determined by standard plaque assay combined with immunostaining for RSV using a biotinylated goat anti-human RSV antibody (Accurate Chemical & Scientific, Westbury, NY) and an Avidin-Biotin Peroxidase detection system (DAKO, Carpinteria, CA). Uninfected HEP-2 cell cultures were similarly processed to obtain a control, virus-free sham preparation.

**Experimental design.** Mice were inoculated under light anesthesia (Avertin 2.5%, 0.010 ml/g body wt) by intranasal instillation of 25 μl of endotoxin-free PBS containing 10° plaque-forming units of purified RSV. Control mice received sham inoculum, consisting of virus-free preparation. Groups of mice were treated by intraperitoneal injections of CGRP, CGRP(8–37), Sendide, or vehicle (saline), administered either as a prophylactic treatment, i.e., 1 day before and on days 1, 3, and 5 after inoculation with RSV, or as a therapeutic treatment, i.e., 2 h before assessment of airway function on days 1, 3, and 5. Airway function, lung inflammation, and neuropeptide expression were analyzed on day 6 postinoculation.

** Messenger RNA expression.** CGRP and substance P mRNA expression were analyzed in lung tissue by the RT-PCR method using specific oligonucleotide primers (Table 1). Lung infection was confirmed by RT-PCR detection of RSV nucleoprotein gene (12). Briefly, total RNA was extracted from each individual mouse lung using TRIZol Reagent (Invitrogen) and cleaned up with RNeasy mini columns (Qiagen, Valencia, CA). Ten micrograms of extracted RNA were reverse-transcribed into cDNA in an 80-μl reaction volume using M-MLV reverse transcriptase and oligo (dT)12-18 primers (Invitrogen). Two microliters of transcribed cDNA (equivalent to 250 ng transcribed total RNA) were amplified by 30 cycles of PCR in an 80-μl reaction volume with specific oligonucleotide primers and Taq polymerase (Invitrogen). This cycle number was selected from cycle-dependent PCR amplification data to allow for determination of relative target mRNA abundance within the linear range of amplification. PCR cycle conditions were as follows: 1 cycle of 95°C for 4 min; 30 cycles of 94°C for 30 s, 55°C for 20 s, 72°C for 40 s; and 1 cycle of 72°C for 5 min. The PCR products were resolved by electrophoresis on ethidium bromide-stained agarose gels and visualized by ultraviolet light illumination. Images of the gels were captured with a digital camera and transferred to a G4 Macintosh computer for densitometric measurements of the signal intensity for each band. The relative mRNA abundance for each target was determined by measuring the ratio of target to β-actin mRNA signals using National Institutes of Health (NIH) Scion Image software (version 1.62, developed at the U.S. NIH and available on the Internet at http://rsb.info.nih.gov/). A detailed description of mRNA expression determination is presented in the Results section.

**Extraction and quantification of lung neuropeptides.** Neuropeptides were extracted from whole lung tissue and corresponding BAL fluid. The lungs were weighed and homogenized in 2 M acetic acid (100 μg tissue/ml). The homogenates were boiled for 30 min followed by centrifugation at 20,000 g for 15 min. The supernatants were saved and the pellets were resuspended in the same initial volume of 2 M acetic acid and boiled again for a second round of extraction. After centrifugation, as before, the second supernatants were recovered and pooled with the first supernatants. To remove contaminants, which can interfere with enzyme immunoassays (EIA), neuropeptides were purificated from supernatants of lung tissue extracts and acidified BAL fluids (diluted 1:4 with 4% acetic acid) by passage of these samples through activated C-18 solid-phase extraction columns (Waters, Milford, MA). After the columns were washed with 4% acetic acid, the retained neuropeptides were eluted from the columns with a mixture of acetonitrile and 1% trifluoroacetic acid (60:40 vol/vol). The eluates were dried by centrifugation under vacuum to remove the solvent phase, and the resulting residues were reconstituted to their original sample volume with EIA buffer. The levels of substance P and CGRP were determined using commercially available EIA kits following the manufacturer’s recommendations (Cayman Chemical, Ann Arbor, MI). The detection limits of the assays were as follows: 8.2 pg/ml substance P and 5 pg/ml CGRP.

**Immunohistochemistry.** CGRP and substance P were detected by immunoperoxidase staining of formalin-fixed, paraffin-embedded lung tissue sections. After blocking endogenous peroxidase by incubation with 0.3% H2O2 in 0.1% NaNO2 for 10 min, the sections were rinsed in 50 mM TBS (pH 7.6) and incubated for 30 min with 5% normal goat serum diluted in TBS containing 0.05% Tween 20 and 1% bovine serum albumin (TTBS/BSA) to prevent nonspecific binding of secondary antibody. The sections were then incubated for 2 h with polyclonal rabbit antibodies to CGRP, substance P, and the pan-neuronal marker PGP9.5 (Biogenesis, Sandown, NH), optimally diluted in TTBS/BSA. After three washes for 5 min each in TBS, the sections were incubated for 2 h with biotinylated goat anti-rabbit immunoglobulins (DAKO), diluted 1:300 in TTBS/BSA, followed by washes in TBS and incubation for 30 min with avidin-biotin peroxidase complex (ABC; DAKO). After a final wash in TBS, the sections were incubated with 3,3′-diaminobenzidine (DAB)-containing peroxidase substrate (Sigma FAST DAB substrate; Sigma, St. Louis, MO), rinsed in distilled water, and counterstained with Harris’s hematoxylin (Fisher Scientific, Pittsburgh, PA).

The results of immunohistochemistry were quantified as previously described (13), by digital image analysis using Scion Image software. Briefly, images of stained tissue sections were captured under the microscope with a Kodak MDS 120 digital camera (Eastman Kodak, Rochester, NY) and transferred to a G4 Macintosh computer for the analysis. Digital images of a micrometer scale (10-μm

<table>
<thead>
<tr>
<th>Table 1. Oligonucleotide primers used for RT-PCR</th>
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<tr>
<td><strong>Target</strong></td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
<tr>
<td>Substance P</td>
</tr>
<tr>
<td>CGRP</td>
</tr>
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</table>

FP, forward primer; RP, reverse primer; CGRP, calcitonin gene-related peptide.
subdivisions) were also captured, under the same magnifications as for tissue sections, and were used for linear calibration of the measurements. Immunoreactive areas were outlined automatically using the slice density function of the program, to highlight only the pixels that belong to the staining, and measured for each of the staining on consecutive tissue sections. When applicable, the number of immunoreactive cells was also determined. All measurements (the groups were blinded to the observer) were performed on at least three serial tissue sections cut from the paraffin blocks every 50 μm and were normalized to the perimeter of the basement membrane of the adjacent airway epithelium. The measured values were averaged for each animal and the mean values were determined for each group.

In vivo assessment of airway function. Airway function was assessed in vivo in anesthetized, mechanically ventilated mice by measuring changes in lung resistance (RL) in response to increasing doses of inhaled methacholine, as previously described (38). Baseline values were recorded from data obtained after challenge with aerosolized saline. The data are presented as a percent of change from baseline RL.

In vitro assessment of airway function. Airway smooth muscle responsiveness was assessed in vitro at the prejunctional level, as previously described (24), by measuring the contractile response of isolated TSM segments in response to increasing frequencies (0.5 to 20 Hz) of EFS. The data are presented as frequency-response curves with corresponding electrical stimulation (ES)50 values, representing the frequency of EFS causing 50% of maximal response.

Statistical analysis. Data are presented as means ± SE of n = 6 to 8 animals in each group. Statistical significance at a P value of <0.05 was determined by ANOVA using Statview 4.5 statistical analysis software package (Abacus Concepts, Berkeley, CA). Differences between the groups were determined by using Fisher’s protected least-significant difference test for multiple comparisons of the means.

RESULTS

RSV infection alters the expression of sensory neuropeptides in the airways. Infection of mice with RSV resulted in the development of a predominantly mononuclear cell infiltration of peribronchial and perivascular airway tissue, peaking on day 6 postinfection (Fig. 1). Associated with this response, a mild mucus production was induced in central intrapulmonary airways of RSV-infected animals. In the BAL, the inflammatory cellular response was characterized by significant increases in peribronchial and perivascular airway tissue infiltration by mononuclear cells (arrowheads) and significant mucus production (arrows). A and C: hematoxylin and eosin stain. B and D: periodic acid-Schiff (PAS) stain. Scale bar = 100 μm.

Table 2. Kinetics of RSV-induced airway inflammation and goblet cell mucus production

<table>
<thead>
<tr>
<th>Group</th>
<th>Day Postinfection</th>
<th>Control</th>
<th>RSV 3</th>
<th>RSV 6</th>
<th>RSV 14</th>
<th>RSV 21</th>
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<tr>
<td></td>
<td>Total cells (×10^3)</td>
<td>79.3 (3.4)</td>
<td>89.5 (4.9)</td>
<td>137.0* (6.9)</td>
<td>91.5 (5.4)</td>
<td>82.0 (3.9)</td>
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<td></td>
<td>Macrophages (×10^3)</td>
<td>77.4 (3.0)</td>
<td>68.0 (7.2)</td>
<td>99.0* (7.9)</td>
<td>80.6 (5.5)</td>
<td>80.1 (3.4)</td>
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<td></td>
<td>Lymphocytes (×10^3)</td>
<td>0.4 (0.2)</td>
<td>6.8 (1.2)</td>
<td>30.0* (2.4)</td>
<td>8.9 (1.4)</td>
<td>0.4 (0.3)</td>
</tr>
<tr>
<td></td>
<td>Neutrophils (×10^3)</td>
<td>1.4 (0.4)</td>
<td>12.4* (2.2)</td>
<td>6.6* (1.0)</td>
<td>1.3 (0.6)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td></td>
<td>Eosinophils (×10^3)</td>
<td>0.2 (0.2)</td>
<td>0.4 (0.3)</td>
<td>1.3 (0.6)</td>
<td>0.7 (0.5)</td>
<td>0.2 (0.2)</td>
</tr>
<tr>
<td></td>
<td>Mucus production</td>
<td>4.5 (1.0)</td>
<td>6.5 (1.9)</td>
<td>23.5* (3.9)</td>
<td>8.5 (2.6)</td>
<td>4.3 (1.7)</td>
</tr>
</tbody>
</table>

Values in parentheses are SE. *Significant difference when compared with control group, P < 0.05. RSV, respiratory syncytial virus; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff.
numbers of mononuclear cells (macrophages and lymphocytes) and neutrophils (Table 2). Temporally, these inflammatory changes were maximal on day 6 but waned on day 14 and resolved by day 21 following RSV infection. Analyses of airway responsiveness to inhaled methacholine revealed that these animals also developed a significant AHR that peaked on day 6 and completely resolved by day 21 after infection (Fig. 2).

To define the effects of RSV infection on substance P and CGRP levels in the lung, we analyzed the expression of these neuropeptides at both mRNA and protein levels in a time-related manner, during the induction and resolution of RSV-induced airway inflammation and AHR. The results of mRNA expression analysis show that RSV infection induced marked increases in substance P expression but significant decreases in CGRP expression that reached maximum levels on day 6 postinfection (Fig. 3, A and B). Analysis of lung neuropeptide content by EIAs revealed parallel changes in substance P and CGRP levels occurring at the protein levels on day 6 following RSV infection (Fig. 3C). CGRP was detected only in extracts from lung tissue but not from BAL fluid, and the recovered tissue amounts were significantly decreased on day 6 postinfection. By contrast, increased amounts of substance P were recovered at the same time point in extracts from both BAL fluids and lung tissue of RSV-infected animals. In tissue, CGRP and substance P were localized by immunohistochemistry and their expression was analyzed by quantitative mor-

Fig. 2. Kinetics of development and resolution of RSV-induced airway hyperresponsiveness (AHR) in mice. AHR was monitored in anesthetized, mechanically ventilated animals on days 3, 6, 14, and 21 following RSV infection by measuring changes in lung resistance to increasing doses of inhaled methacholine (MCH). Mice infected with RSV developed significant AHR that was maximal on day 6 but completely resolved by day 21 postinfection. Data are means ± SE (n = 6 mice/group). *Significant difference compared with control group, P < 0.05.

Fig. 3. Kinetics of RSV-induced changes in substance P (SP) and calcitonin gene-related peptide (CGRP) mRNA and protein levels in the lung. A: agarose gels showing RT-PCR products for β-actin, substance P, and CGRP mRNA. Data are representative of 2 experiments with n = 3 mice/group in each experiment. B: relative mRNA abundance of substance P and CGRP. C: substance P and CGRP protein levels. RSV induced significant increases in substance P mRNA and protein levels but decreases in CGRP mRNA and protein levels that were maximal on day 6 postinfection. The expression of both neuropeptides returned to baseline levels on day 21 postinfection. ND, not detected; BAL, bronchoalveolar lavage. *Significant difference compared with control group, P < 0.05, n = 6 mice/group.
phometry. In uninfected control mice, CGRP was constitutively expressed in the airways and localized to submucosal airway nerve fibers and neuroepithelial bodies (NEBs), consisting of innervated clusters of neuroepithelial cells localized within the epithelium at the branching points of the airways (Fig. 4A). After RSV infection, the expression of CGRP was decreased in both airway tissue locations (Fig. 4, B and C). By contrast, the expression of substance P was dramatically increased in airway tissue following RSV infection (Fig. 5). More particularly, in RSV-infected animals, substance P localized not only to submucosal airway nerve fibers but also to subsets of epithelial cells mostly in central pulmonary airways. In uninfected control animals, substance P was expressed at low levels in airway tissue, localizing exclusively to submucosal nerve fibers with no expression in the airway epithelium. Further analyses of airway tissue immunoreactivity for the pan-neuronal marker PGP9.5 revealed that the overall airway nerve density was not altered following RSV infection (Fig. 6).

Substance P contributes to the development of altered airway function following RSV infection. To define the role of substance P in the development of altered airway function following RSV infection, we examined the effects of prophylactic or therapeutic treatments of mice with Sendide, a highly selective antagonist of NK-1 receptor administered as described in METHODS. Compared with control, sham-inoculated mice, RSV-infected mice developed significant AHR, measured as increases in Rrs in response to inhaled methacholine (Fig. 7A). Prophylactic treatment with Sendide significantly inhibited the development of AHR in RSV-infected mice. The increases in Rrs induced after RSV infection were attenuated by ~50% at the maximal dose of inhaled methacholine in Sendide-treated animals compared with saline-treated animals (P < 0.05). Administration of Sendide as a therapeutic treatment, 2 h before the assessment of airway function on day 6 postinfection, failed to inhibit the development of AHR to methacholine RSV-infected mice (Fig. 7C).

In vitro assessment of airway function further revealed that RSV infection also altered the airway smooth muscle (ASM) responsiveness to EFS. RSV infection did not alter EFS-induced maximal tension, but it resulted in an increased ASM responsiveness to EFS characterized by a marked leftward shift to lower frequency-dependent EFS-induced ASM contraction (Fig. 8). As a consequence, the frequency of EFS50 was significantly decreased in RSV-infected animals compared with control, uninfected animals (P < 0.05). Prophylactic treatment with Sendide inhibited the development of ASM hyperresponsiveness to EFS in RSV-infected animals (Fig. 8). However, Sendide failed to inhibit the increases in ASM responsiveness to EFS when administered in vivo, as a therapeutic treatment to RSV-infected mice, or when added in vitro to isolated tracheas from RSV-infected animals (data not shown).

Analysis of inflammatory cells recovered by BAL showed that prophylactic treatment with Sendide significantly reduced the numbers of lymphocytes accumulating in the airspace of RSV-infected animals (Table 3). Therapeutic treatment with Sendide, however, failed to do so (data not shown).

CGRP normalizes airway function following RSV infection. To investigate the role of CGRP in the development of altered airway function following RSV infection, we treated mice with CGRP(8–37), a highly selective antagonist of CGRP type 1 receptor. Alternatively, we examined the effects of treatment of mice with exogenous CGRP. As shown in Fig. 7A, prophylactic treatment with CGRP(8–37) did not inhibit AHR in RSV-infected animals but resulted in slight increases in Rrs in response to inhaled methacholine compared with treatment with saline, clearly indicating that endogenous CGRP is not a
mediator of AHR in these animals. In contrast, prophylactic administration of exogenous CGRP significantly inhibited the development of AHR in RSV-infected mice. Assessment of ASM function in vitro demonstrated that prophylactic treatment of animals during the course of RSV infection with exogenous CGRP, but not CGRP(8–37), prevented the development of altered airway responsiveness to EFS (Fig. 8). Analysis of inflammatory cells recovered in BAL fluids from these animals indicated that the numbers of lymphocytes were further increased after treatment with CGRP(8–37) but decreased following treatment with CGRP (Table 3).

Interestingly, when administered as a therapeutic treatment on day 6 postinfection only, 2 h before in vivo assessment of airway responsiveness to methacholine, CGRP suppressed the development of AHR in RSV-infected animals (Fig. 7B). This suppressive effect was mediated by CGRP receptor as the effects were abolished by pretreatment of mice with CGRP(8–37). However, neither therapeutic administration of CGRP nor its addition to isolated tracheas resulted in an inhibition of EFS-induced ASM hyperresponsiveness in vitro (data not shown). In addition, the profile of inflammatory cells recovered in the BAL fluids was not modified by treatment on day 6 following RSV infection in these animals (data not shown).

Effects of substance P and CGRP on lung viral titers. As substance P and CGRP were found to modulate airway inflammation and AHR, we examined whether these effects were associated with altered replication or clearance of RSV in the lungs of treated animals. As shown in Table 4, none of the prophylactic treatments with either Sendide or CGRP or its receptor antagonist CGRP(8–37) resulted in significant changes in lung viral titers at the study points.

Effects of substance P and CGRP on mucus production. To determine if substance P and CGRP are involved in RSV-mediated mucus production by airway epithelial cells, lung tissue sections were stained with the periodic acid-Schiff (PAS) method and examined for mucus expression in airway tissue (Fig. 9). Quantitative analysis of mucus (PAS positive)-producing airway epithelial cells indicated that treatment of mice with Sendide or exogenous CGRP, but not CGRP(8–37), significantly reduced mucus production by airway epithelial cells in RSV-infected animals (Table 3).

DISCUSSION

The results of this study demonstrate that RSV infection alters the expression of both substance P and CGRP in the airways of mice. At both the messenger RNA level and protein level, substance P expression was increased, whereas CGRP expression was decreased following RSV infection (Fig. 3). These changes occurred at the peak response, on day 6 following RSV infection, when airway inflammation, mucus production, and AHR were maximal. We therefore investigated the consequences of antagonizing endogenous activities of substance P and CGRP and also examined the effects of administering exogenous CGRP given the induced deficit in CGRP expression. When administered as prophylactic treatments during the course of RSV infection, both Sendide and CGRP, but not CGRP(8–37), inhibited the development of AHR as measured in vivo by increased R₉ₒ to inhaled methacholine (Fig. 7A). Similar effects were also observed in vitro when isolated

Fig. 6. Effect of RSV infection on PGP9.5 immunoreactivity in airway tissue. The pan-neuronal marker PGP9.5 (arrows) was detected by immunohistochemistry in the airways of uninfected control mice (A) and RSV-infected mice (B) and quantified by morphometry (C). Data were obtained from n = 8 mice from each group. RSV infection did not alter the overall density of airway nerve fibers in mice. Scale bar = 50 μm.

Fig. 7. Role of CGRP and substance P in the development of AHR following RSV infection. A: effect of prophylactic treatments with CGRP, CGRP(8–37), and Sendide. Groups of mice (n = 8/group) were administered saline [RSV/Saline], CGRP [RSV/CGRP], its antagonist [RSV/CGRP(8–37)], or Sendide [RSV/Sendide] 1 day before and on days 1, 3, and 5 after RSV infection. Airway responsiveness to inhaled methacholine was assessed in vivo on day 6 postinfection. Both Sendide and CGRP, but not CGRP(8–37), inhibited AHR in RSV-infected mice. *Significant difference when compared with uninfected control mice. §Significant difference when compared with RSV-infected mice. P < 0.05. B: effects of therapeutic treatment with CGRP. Groups of mice (n = 8/group) were administered saline [RSV/Saline], CGRP [RSV/CGRP], or CGRP(8–37) followed by CGRP [RSV/CGRP(8–37)+CGRP] just 2 h before the assessment of airway function on day 6 following RSV infection. CGRP inhibited AHR in RSV-infected mice with previously established airway inflammation. *Significant difference between animal groups, P < 0.05. C: effects of therapeutic treatment with Sendide. Sendide was administered to RSV-infected mice (n = 8) 2 h before assessment of airway function on day 6 following infection. Sendide did not inhibit AHR in RSV-infected mice with previously established airway inflammation.
TSM segments were tested for their contractile response to EFS (Fig. 8). When administered as a therapeutic treatment, just 2 h before assessment of lung function, Sendide failed to inhibit AHR (Fig. 7C), whereas CGRP normalized airway responsiveness to inhaled methacholine despite increased levels of substance P and established airway inflammation in RSV-infected animals (Fig. 7B). Analysis of lung viral titers indicated that neither substance P, as shown by other investigators (18), nor CGRP appeared to alter viral replication and clearance in the lungs of treated animals.

The tachykinin substance P is primarily produced from a neuronal source in vivo but inflammatory cells including lymphocytes (14, 23), monocytes/macrophages (19), and eosinophils (29) may also contribute as nonneuronal sources of substance P in vivo. In the present study, we identified epithelial cells as a nonneuronal source for substance P production in RSV-infected mice (Fig. 4). However, when normal human bronchial and small airway epithelial cells, including the human type 2 alveolar epithelial cell line A549, or the mouse lung epithelial cell line LA4 were infected in vitro with RSV, they did not produce substance P (A. Dakhama, A. Balhorn, E. W. Gelfand, unpublished observations), implying that the observed in vivo induction of substance P expression in mouse airway epithelial cells might result from an indirect effect of RSV infection, possibly through elicited inflammatory stimuli.

In mammals, substance P localizes predominantly to sensory C-fibers in the airways (25) and its known effects relevant to asthma include plasma extravasation, mucus hyperproduction, and potentiation of airway cholinergic function (4). In asthmatic airways, these events of “neurogenic inflammation” are mediated essentially by the high-affinity receptor of substance P, NK-1 (3). In addition, substance P mediates proinflammatory effects, namely stimulation of hematopoiesis, recruitment and activation of inflammatory cells, stimulation of cytokine production, and proliferation of endothelial cells, fibroblasts, and smooth muscle cells in vitro (21).

Previous studies have documented increased levels of substance P in the lungs of RSV-infected animals and attributed a role for this neuropeptide in the development of the lymphocytic response to RSV infection (40). This notion was supported by the findings that, in these animals, lymphocytes expressed the NK-1 receptor (2, 39) and proliferated in vitro in response to stimulation with substance P (40). Furthermore, neutralization of substance P or NK-1 blockade resulted in a significantly reduced influx of lymphocytes into the lungs of RSV-infected animals (2, 18). Our data confirm these findings and further suggest a possible role for substance P in mucus production during RSV infection. However, this effect on mucus is distinct from the inhibitory effect obtained with

### Table 3. Role of substance P and CGRP in RSV-induced airway inflammation and goblet cell mucus production

<table>
<thead>
<tr>
<th>Group Treatment</th>
<th>Control None</th>
<th>RSV Saline</th>
<th>RSV CGRP</th>
<th>RSV CGRP(8–37)</th>
<th>RSV Sendide</th>
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<tr>
<td>BAL cellularity</td>
<td></td>
<td></td>
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<tr>
<td>Total cells (×10³)</td>
<td>80 (2)</td>
<td>155* (4)</td>
<td>117*† (9)</td>
<td>173* (8)</td>
<td>127*† (4)</td>
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<tr>
<td>Macrophages (×10³)</td>
<td>78 (2)</td>
<td>110* (3)</td>
<td>102* (5)</td>
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<td>Lymphocytes (×10³)</td>
<td>0 (0)</td>
<td>40* (4)</td>
<td>28* (3)</td>
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<td>Neutrophils (×10³)</td>
<td>1 (1)</td>
<td>5* (1)</td>
<td>2 (1)</td>
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<td>Eosinophils (×10³)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
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<td>0 (0)</td>
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<td>Mucus production</td>
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<tr>
<td>PAS+ cells/mm BM</td>
<td>4.8 (0.8)</td>
<td>22.7* (2.9)</td>
<td>10.4* (0.8)</td>
<td>21.3* (2.4)</td>
<td>9.9*† (0.9)</td>
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</table>

Values in parentheses are SE. *Significant difference when compared with control group, P < 0.05. †Significant difference when compared with RSV/saline group, P < 0.05. BM, basement membrane.

Fig. 8. Role of CGRP and substance P in the development of altered tracheal smooth muscle (TSM) responsiveness to electrical field stimulation (EFS) following RSV infection. Groups of mice (n = 6/group) were administered saline [RSV/Saline], CGRP [RSV/CGRP], its antagonist [RSV/CGRP(8–37)], or Sendide [RSV/Sendide] 1 day before and on days 1, 3, and 5 after RSV infection. On day 6 postinfection, tracheas were isolated and tested for contractile responsiveness to EFS in vitro. RSV infection increased the sensitivity of TSM to EFS-induced contraction, as indicated by a significant leftward shift to lower frequency-dependent EFS-induced contraction (A) resulting in decreased ES₅₀ values (B). This effect was inhibited by treatments of mice with Sendide or CGRP but not CGRP(8–37). Maximal tension values were not significantly different between the groups and treatments [control: 122.20 ± 29.05; RSV/Saline: 115.20 ± 18.70; RSV/CGRP: 126.50 ± 13.38; RSV/CGRP(8–37): 113.80 ± 16.39; RSV/Sendide: 120.75 ± 19.64]. ES₅₀, frequency of electrical stimulation causing 50% of maximum contractile response. *Significant difference when compared with control animal group, P < 0.05.
exogenous CGRP, which could be mediated through a possible anti-inflammatory action, as antagonizing endogenous CGRP by treatment with CGRP(8–37) did not alter mucus production in RSV-infected animals. In addition, the present data establish a role for substance P as a mediator in the development of AHR following RSV infection. Thus prophylactic treatment with Sendide, which resulted in reduced accumulation of lymphocytes in the airways of RSV-infected animals, also inhibited the development of AHR in the same animals. Furthermore, the data indicate that the effects of substance P on airway function are likely transduced at the prejunctional level as blockade of the NK-1 receptor inhibited EFS-induced contraction of TSM. However, this effect was not observed when Sendide was added in vitro to isolated tracheas, suggesting that the NK-1-mediated effects on airway function occurred in vivo in these animals. Together, our data suggest that substance P may contribute to the development of AHR in at least two ways, by proinflammatory and by neurogenic mechanisms. The latter may involve the known potentiating effects of substance P on cholinergic responsiveness by facilitating acetylcholine release in the airways (10, 44).

CGRP is produced by alternative splicing of the calcitonin gene in the central and peripheral nervous systems (1). CGRP is constitutively expressed in normal airways, localizing to NEBs and submucosal nerve fibers in several animal species, including human (33, 42). Unlike the tachykinin substance P, CGRP does not induce mucus secretion or plasma protein extravasation in the airways (27, 45). In a mouse model of allergic airway inflammation and hyperresponsiveness, we showed that allergen exposure results in a profound depletion of CGRP in the lungs of sensitized animals, which developed a robust airway inflammatory response (13). Treatment of these animals with exogenous CGRP, during or after allergen exposure, restored normal airway responsiveness to inhaled methacholine without a marked effect on airway inflammation. However, when administered during allergic sensitization (at the time of T cell priming), CGRP prevented the development of a TH2-dominated primary immune response by inhibiting antigen-specific T cell activation as detected by diminished IL-2 production (11).

Previous studies showed that CGRP can inhibit IL-2 production by directly interacting with T cells (43), whereas others suggested that it may also inhibit T cell responses by decreasing the expression of CD86 and major histocompatibility class II molecules on antigen-presenting cells (6). Accordingly, the results of this study suggest that CGRP may modulate the lymphocytic response to RSV infection in a similar fashion, as prophylactic treatment of mice with the CGRP antagonist resulted in significantly increased numbers of lymphocytes, whereas prophylactic treatment with exogenous CGRP reduced the numbers of lymphocytes that accumulated in the airways of RSV-infected mice. In addition to these potential anti-inflam-

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**Table 4. Lung viral titers**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>RSV</th>
<th>RSV</th>
<th>RSV</th>
<th>RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Saline</td>
<td>CGRP</td>
<td>CGRP(8–37)</td>
<td>Sendide</td>
</tr>
<tr>
<td>Viral titers, PFU/g lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>ND</td>
<td>2,120 (229)</td>
<td>1,960 (221)</td>
<td>2,200 (269)</td>
<td>2,060 (213)</td>
</tr>
<tr>
<td>Day 6</td>
<td>ND</td>
<td>294 (82)</td>
<td>300 (49)</td>
<td>378 (69)</td>
<td>306 (52)</td>
</tr>
</tbody>
</table>

PFU, plaque-forming units; ND, not detected.

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Fig. 9. Role of CGRP and substance P in RSV-mediated mucus production by airway epithelial cells. Groups of mice (n = 8/group) were administered saline (A), Sendide (B), CGRP (C), or its antagonist CGRP(8–37) (D) 1 day before and on days 1, 3, and 5 after RSV infection. Mucus production was assessed on day 6 postinfection by staining lung tissue sections, using the PAS staining method for detection of mucus-producing epithelial cells (arrows). Both Sendide and CGRP, but not CGRP(8–37), inhibited mucus production in RSV-infected animals. Scale bar = 100 µm.
matory effects, the results demonstrate that CGRP can normalize airway function in animals with previously established airway inflammation as a result of either RSV infection, as shown in the present study (Fig. 7B), or allergic sensitization and airway challenge as shown previously (13).

The mechanisms by which CGRP modulates airway function are unknown. However, CGRP is known to activate the adenyl cyclase and mediate increases in cAMP levels, a response usually associated with bronchodilation (16). At the neuromuscular junction of the skeletal muscle, neuronal CGRP can be coreleased with ACh from motor nerves, but from distinct vesicles (28). At this level, CGRP appears to play a trophic role by facilitating desensitization of nicotinic acetylcholine receptors by phosphorylation of certain receptor subunits (30, 31). In cultured chick myotubes, CGRP has been shown to increase the synthesis of acetylcholinesterase by a cAMP-dependent mechanism (7). In the autonomic nervous system and in the brain, these effects of neuronal CGRP are thought to be important processes in the regulation of nicotinic synaptic neurotransmission (15). Whether similar effects occur at the smooth muscle level needs to be further investigated.

In summary, the results of this study demonstrated that RSV infection induces significant alterations in the expression of sensory neuropeptides in the airways. These alterations are associated with the development of altered airway function. Substance P mediates, whereas CGRP protects against, the development of RSV-induced airway dysfunction. These sensory neuropeptides may serve as potential targets for therapeutic intervention to normalize airway function during or after RSV infection.

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