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Proximal airway mucous cells of ovalbumin-sensitized and -challenged Brown Norway rats accumulate the neuropeptide calcitonin gene-related peptide

Shawnessy D. Larson,¹ Charles G. Plopper,¹ Greg Baker,¹ Brian K. Tarkington,² Kendra C. Decile,¹ Kent Pinkerton,¹ James K. Mansoor,³ Dallas M. Hyde,¹ and Edward S. Schelegle¹

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Larson, Shawnessy D., Charles G. Plopper, Greg Baker, Brian K. Tarkington, Kendra C. Decile, Kent Pinkerton, James K. Mansoor, Dallas M. Hyde, and Edward S. Schelegle. Proximal airway mucous cells of ovalbumin-sensitized and -challenged Brown Norway rats accumulate the neuropeptide calcitonin gene-related peptide. *Am J Physiol Lung Cell Mol Physiol* 287: L286–L295, 2004. First published April 2, 2004; 10.1152/ajplung.00369.2003.—Mucous cell hypersecretion and increased neuropeptide production play a role in the exacerbation of symptoms associated with asthma. The source of these neuropeptides have been confined to the contributions of small afferent nerves or possibly neuroendocrine cells. We tested the hypothesis that repeated exposure to allergen would alter the sources and abundance of neuropeptides in airways. Right middle lobes from rats (8 wk old) exposed to 2.5% ovalbumin (OVA) for five episodes (30 min each) or filtered air were inflated fixed with paraformaldehyde. The lobes were dissected to expose the airway tree, permeabilized with DMSO, and incubated in antibody to rat calcitonin gene-related peptide (CGRP), followed with a fluorochrome-labeled second antibody. CGRP-positive structures were imaged via confocal microscopy. Airways were later embedded in plastic and sectioned for cell identification. In animals challenged with OVA, CGRP-positive cells, not neuroendocrine or neuronal in origin (confirmed by a lack of protein gene product 9.5 signal), were recorded along the axial path. In section, this fluorescent signal was localized to granules within epithelial cells. Alcian blue/periodic acid-Schiff staining of these same sections positively identify these cells as mucous cells. Mucous cells of animals not challenged with OVA were not positive for CGRP. We conclude that episodic allergen exposure results in the accumulation of CGRP within mucous cells, creating a new source for the release of this neuropeptide within the airway.

asthma; protein gene product

CALCITONIN GENE-RELATED PEPTIDE (CGRP), a pleiotropic neuropeptide with potent vasodilatory activity, is located within various tissues of neural and nonneural origin in mammalian species. The primary sources of CGRP within the respiratory tract are generally thought to be nonmyelinated and thinly myelinated sensory nerve fibers and neuroendocrine cells. In addition, Baluk et al. (6) detected weak immunostaining for CGRP within the granules of serous-type cells of the trachea and, to a lesser extent, the mainstem bronchi of F344 rats. Beyond its potent vasodilatory effect (11), CGRP has numerous other effects that would be expected to influence the maintenance and function of the lower respiratory tract in health and disease. CGRP has been shown to modulate sub-

stance P-induced inflammation (32) and increase ciliary beat frequency (41) and has the potential to play a role in airway repair by inducing epithelial proliferation and migration (26, 38, 47). In addition, CGRP has been shown to have several immunomodulatory effects. CGRP is a chemoattractant for CD4+ and CD8+ T lymphocytes (17), whereas at least one fragment of CGRP, produced by the degradation of CGRP by neutral endopeptidase, is a chemoattractant for eosinophils (16). In contrast, CGRP also has anti-immune properties that include inhibiting the proliferative response of T lymphocytes to mitogen (45) as well as inhibiting macrophage secretion and the capacity of macrophages to activate T lymphocytes (31, 44).

Allergen sensitization followed by allergen inhalation induces significant changes in the airway environment, including vasodilation, lymphocyte and eosinophil infiltration, airway smooth muscle hyperresponsiveness, and increased airway mucous that is the result of an increase in the production and the number of airway mucous cells (2, 18, 27, 34, 39). Given the stimulatory effect of inflammatory mediators released as a consequence of allergen inhalation on neuropeptide-containing nerves (7) and the observation that substance P and neurokinin receptor-1 expression (14) and plasma CGRP (13) are increased in human asthmatics, it is likely that the distribution and source of airway neuropeptides, including CGRP, is altered in allergic airway disease. In addition, the observations of Bousbaa and Fleury-Feith (10) indicate that sensitization and challenge of guinea pigs with ovalbumin (OVA) increase the number of airway neuroendocrine cells, suggesting at least one source of increased CGRP within the allergic airway.

The purpose of the present study was to examine the possible changes in the distribution and source of airway CGRP and its possible role in regional epithelial cell proliferation in response to allergen inhalation in a model of the allergic airway disease, asthma, in Brown Norway rats. In pathogen-free Brown Norway rats, we defined the distribution of CGRP using immunofluorescence techniques. Using whole mount preparations from microdissected pulmonary airways, we compared the differences in the distribution of CGRP along the entire pulmonary airway. Neural sources (nerve fibers and neuroendocrine cells) of CGRP were confirmed using the nonspecific neural marker protein gene product (PGP) 9.5. To further confirm the cellular source of an abundant epithelial CGRP signal, the whole mount preparations were embedded, sec-

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tioned, and imaged by epifluorescence microscopy. These same sections were then stained with alcian blue/periodic acid-Schiff (AB/PAS) to identify mucous-containing cells and 1% toluidine blue for morphological analysis. Airway epithelial cell proliferation over the entire allergen inhalation regimen was determined using the incorporation of 5'-chloro-2'-deoxyuridine (CldU), a thymidine analog, and was correlated with changes in CGRP distribution and airway morphology among treatment groups.

MATERIALS AND METHODS

Animals and Experimental Protocols

Male Brown Norway rats (BN/SsNHsd) obtained from a specific pathogen-free colony, barrier 218B (Harlan Sprague Dawley, Prattville, AL), were used in this study. The experimental protocol was approved by the University of California, Davis Animal Use and Care Administrative Advisory Committee following both Animal Welfare Act and Health Research Extension Act guidelines. Animals were housed in inhalation chambers ventilated with chemical-bacteriological-radiological-filtered air. Food and deionized filtered water were provided *ad libitum*, and rats were allowed at least 1 wk to acclimate before procedures were started. Of 50 rats, 8 wk of age, 32 were sensitized to OVA and 16 were sham sensitized. Two sentinel rats were not sensitized in any way. Sensitization was produced with a subcutaneous injection of 1 mg of chicken OVA and 200 mg of aluminum hydroxide (Sigma, St. Louis, MO) in 1 ml of sterile saline and 1 ml of *Bordetella pertussis* vaccine (Bioport, Lansing, MI) containing 6×10^9 heat-killed organisms delivered intraperitoneally. Sham sensitization was achieved by two saline injections, subcutaneous and intraperitoneal. Two weeks later, under deep anesthesia [50 mg/ml of ketamine (Schering-Plough Animal Health, Union, NJ) and 10 mg/ml of xylazine (Miles Animal Health Products, Shawnee Mission, KS), intraperitoneally], rats received subcutaneous osmotic pumps (14 day, 5 μ l/h; ALZA, Palo Alto, CA) filled with 2 ml of CldU (42.5 mg/ml; Sigma). Once animals had awakened from anesthesia, the first challenge was administered. Sixteen animals from the sensitized group and one sentinel were exposed to five 30-min cycles of OVA aerosol at an exposure interval of one exposure every third day. The remaining rats, 16 from the sensitized group and 16 from the sham-sensitized group along with one sentinel, received filtered air only.

Validation of the model of allergic airway disease in Brown Norway rats. To validate that the OVA sensitization and challenge protocol used in this study produced Brown Norway rats with an airway phenotype consistent with allergic asthma, 24 rats (8 per group) were studied for their airway responsiveness to methacholine and conducting airway eosinophil and mucous cell content. Rats were studied 3 days after the last OVA airway challenge. Animals were anesthetized with a mixture of α -chloralose and urethane (0.1 g α -chloralose/kg body wt, 10 g urethane/kg body wt ip) and orally intubated with a 14-gauge catheter. The airway responsiveness to double doses of methacholine was measured, and the lungs were then removed and fixed at an inflation pressure of 30 cmH₂O using zinc-formalin (Z-fix; Anatech, Battle Creek, MI). The main axial airway path of the left caudal lobe was examined for airway eosinophil and mucous cell content.

Distribution of CGRP. Two days after the final OVA aerosol inhalation, 24 rats (8 per group) were anesthetized with pentobarbital sodium (7.8 mg/ml, 70 mg/kg) and killed by exsanguination. Lungs were fixed in the chest cavity via tracheal cannula for 2 h with 1% paraformaldehyde. The right middle and cranial lobes were microdissected to expose the airway tree (37). Airways from the right cranial lobe were used for measuring CldU uptake, and airways from the right middle lobe were used for defining the distribution of nervous elements. Sentinel rats housed with experimental animals for the duration

of the study were sent to the Comparative Pathology Laboratory at University of California, Davis, for health screening to confirm the absence of respiratory pathogens, virus antibodies, and parasites. Comprehensive health screening, which included microbiology, histopathology, and serology, indicated that the rats were free of known pathogenic agents. Screening of two rats immediately after delivery was also performed; the results were consistent with the health monitoring report furnished by the supplier.

Generation of OVA Aerosol

OVA, 2.5% by weight diluted in PBS, was nebulized with a high-flow rate nebulizer (HEART; Westmed, Tucson, AZ). The resulting droplets were diluted with dry air and conveyed through a krypton-85 discharging column to reduce electrostatic charge. The aerosol was mixed with the inlet air stream of a 4.2-m³-volume exposure chamber to produce an aerosol of solid particles composed of allergen with a salt residue (39). The aerosol was characterized by samples drawn from the animal breathing zone of the chamber. Collected samples were used to determine total mass concentration and protein content of the aerosol (33). Aerodynamic size distributions were determined using a seven-stage Mercer-type cascade impactor (ARIES, Davis, CA) (30). The content of chloride anion derived from saline residue in the particles was measured on each impactor stage and the after-filter by ion chromatography (DX-120; Dionex, Sunnyvale, CA). A log-normal distribution was fitted to each sample set of data. The total mass concentration of the aerosol was 12.63 ± 0.84 mg/m³ ($n = 10$), and the protein content was 6.9 ± 1.19 mg/m³ ($n = 6$). The mass median aerodynamic diameter was 1.93 ± 0.15 μ m ($n = 3$) with a geometric standard deviation (σ_g) of 1.99 ± 0.08 . All aerosol data are means $\pm$ SD.

Measurement of Airway Responsiveness

Changes in airway responsiveness were examined by delivering aerosolized methacholine for 1 min and measuring the changes in airway resistance for the next 3 min. Rats were placed in a whole body plethysmograph, and their tracheal cannulas were attached to a constant volume positive pressure ventilator (model 683, Harvard) set at a tidal volume of 0.7 ml/100 g body wt and a frequency of 90 breaths/min. The rats were then paralyzed with succinyl chloride (0.2 ml at 20 mg/ml ip), and the plethysmograph was closed. Airflow was measured using a pneumotachograph (model 8300, Hans Rudolph) mounted into the side of the plethysmograph. Airway pressure was measured using a differential pressure transducer (Validyne DP15; Northridge, CA) with one side attached near the tracheal cannula port of the ventilator and the other side attached to a fluid-filled cannula, with side ports near the tip, and whose tip was placed in the esophagus in the midthoracic region. Pulmonary flow resistance (R_L) was calculated according to the method of Amdur and Mead (3) with airflow and airway pressure being measured at an isovolume of 70% of tidal volume using a digital data acquisition system (PO-NE-MAH; Gould Instrument Systems, Valley View, OH). A starting dose of 0.125 mg/ml of methacholine was used and continued in double doses until airway resistance had doubled or a concentration of 64 mg/ml of methacholine had been reached. The concentration of methacholine required to double lung resistance (EC_{200} R_L) was obtained by linear interpolation between the two concentrations bounding the point at which lung resistance reached 200% of control on a log-log plot.

Assessment of Lung Histology

Immediately after airway responsiveness evaluation, the lungs were removed and fixed at a volume of 30 cmH₂O using zinc-formalin (Z-fix). After fixation of the lungs, tissue sections were prepared by cutting transverse lung slices immediately cranial and caudal to the hilum of the left lobe. Each tissue slice was embedded in paraffin and sectioned using a Microm HM 355 rotary microtome (Zeiss, Thorn-



wood, NY). The main axial airway paths of the left caudal lobe of each animal were examined. All sections were cut 5- μ m thick. Serial tissue sections were stained with hematoxylin and eosin to observe general pulmonary structures, AB/PAS for epithelial distribution of mucin, and combined eosinophil/mast cell stain for visualization of eosinophils and mast cells. The airway was examined in cross section at the level of the third to sixth generation to confirm that the same general airway site would be described for all animals studied. Images were collected using a $\times 10$ objective with an Olympus BH2 microscope interfaced to a computer with a video camera (Dage MTI, Michigan City, IN). The abundance of AB/PAS-positive mucosubstances and eosinophils within the epithelium was determined by area analysis using the NIH Image program (Bethesda, MD) with the density gradient feature to highlight positively stained areas of the epithelium. All measurements were normalized to the basal lamina length of each airway.

Whole Mount Immunohistochemistry

Microdissected airways from the right middle lobe were permeabilized with DMSO (3 times, 10 min each), rinsed in PBS, and incubated in primary antibody (1:100 mouse monoclonal anti-PGP 9.5, Biogenesis and 1:200 rabbit anti-rat-CGRP, Sigma) overnight at 4°C. Control experiments to test for nonspecific staining were done using nonimmune rabbit serum (1:200) or PBS in place of primary antibodies. After a 4-h rinse in PBS, airways were incubated in a secondary antibody conjugated to goat anti-mouse Alexa 488 or goat anti-rabbit Alexa 568 (1:100) (Molecular Probes, Eugene, OR) for 14 h at room temperature and then mounted on glass slides and stored in PBS until viewing.

Imaging

Images of immunopositive structures in airway whole mounts were obtained using water-immersion objectives ($\times 10$ to $\times 40$ magnification) on a confocal laser scanning microscope with LaserSharp software (MRC 1024; Bio-Rad Laboratories, Hercules, CA). Previously imaged airways were later embedded in glycol methacrylate (Polysciences, Warrington, PA) and sectioned to a 2- μ m thickness for high-resolution imaging. The conserved fluorescent signal was captured on a microscope (BH-2; Olympus, Tokyo, Japan) using a $\times 60$ lens and Cool Snap imaging software. The identity of cells containing granules immunoreactive for CGRP were confirmed by AB/PAS stain. Fluorescent images and AB/PAS images were taken from the same section. Thin sections were also stained with 1% toluidine blue for morphological analysis and captured on an Olympus microscope using a $\times 40$ lens and Cool Snap imaging software. Images were later imported into the Stereology Toolbox software program (Morphometrics, Davis, CA) for analysis.

CldU Detection

Microdissected right cranial lobes were embedded in paraffin. Hydrated thick sections (30 μ m) were boiled in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA), treated with 2.5 N HCl for 30 min to denature DNA, and rinsed with borate buffer. Sections were then treated with 3% peroxide for 30 min to block endogenous peroxides and 10% bovine serum albumin. Sections were incubated with monoclonal mouse anti-bromodeoxyuridine (BrdU; 1:50; Dako, Carpinteria, CA) overnight at 4°C. After rinsed sections were incubated in biotinylated rabbit anti-mouse immunoglobulin (1:200; Dako), immunoreactive CldU was visualized with the Vectastain Elite ABC kit (Vector Laboratories) using 3',3'-diaminobenzidine tetrachloride (Sigma) as a chromagen. The number of CldU-positive nuclei per surface area of basement membrane was counted from sites selected by the computerized CAST grid system (Olympus).

Morphometry

The number of CGRP-positive cells per surface area of epithelium (N_s) was estimated using an unbiased optical "disector" in an optical series of confocal images captured using a $\times 40$ objective. Those cells that intersected the top section and two sides of the counting frame were excluded. Stacks of serial sections (spaced 8- μ m apart and with a depth of focus of 8 μ m) varied from 8 to 41 images, depending on the orientation of the airway surface in the dissected whole mounts. Luminal surface area of the airway epithelium was estimated by applying a quadratic lattice to each section and counting the number of intersections with the epithelial surface in both x and y directions (20). Because the points of the lattice are rays in the z direction sweeping through space, intersections in the z direction were recorded by transitions within and without the epithelial surface between confocal images. We determined N_s by dividing the numerical density by surface density (Sv) using the formula

$$N_s = Nv/Sv = (N \times L_p \times P_{epi}) / (P_{epi} \times A_p \times H \times 2I) \\ = (N \times L_p) / (A_p \times H \times 2I) \quad (1)$$

L_p is length of lattice per point, H is the distance between and height of the sections, A_p is the area of lattice per point, N is the number of CGRP-positive cells, Nv is number per volume, and I is the total number of luminal epithelial intersections in x , y , and z directions. Note that P_{epi} , the points that hit epithelium, divide to 1 in the equation.

The number of CldU-positive nuclei per surface area of luminal epithelium was estimated in local vertical thick sections (30 μ m) by 1) scanning through the section using a $\times 100$ objective and counting the number of CldU-positive nuclei within the epithelium and 2) recording the number of basement membrane intercepts with the cycloid arc test system (46). A microcator gauge mounted on the microscope and the computerized CAST grid system (Olympus) allowed definition of the frame boundaries of our optical disector. In one representative frame per section, we imposed a cycloid arc counting frame to record the number of intersections with the basement membrane. The number of CldU-positive nuclei per surface area of basement membrane was estimated using the formula listed above where L_p = (no. of cycloid/no. of points) $\times$ length of the cycloid and A_p = area of frame per point. The number of basement membrane intersections with the cycloid arc grid is represented by I . N is the number of CldU-positive nuclei, and H is the height of the bounded frame.

The mass and abundance of ciliated and nonciliated cells within the pulmonary airways were estimated using procedures described by Hyde et al. (21). All measurements were made using a $\times 40$ objective and 2.0- μ m sections. The proportion of airway epithelial cells either ciliated or nonciliated was estimated using point counting. The mass, as measured by volume (μ m³) of airway epithelium per unit area (μ m²) of basement membrane (V_s), was estimated from point and intercept counts with a cycloid arc grid and local vertical sections. We used the formula

$$V_s = (P_o \times L_p) / (2 \times I) \quad (2)$$

where P_o is the number of points falling on the cell of interest, L_p is the length of cycloid curve per point, and I is the number of basement membrane intersections with cycloid arcs.

Statistical Analysis

We analyzed the number of CGRP-immunoreactive epithelial cells per surface area of luminal epithelium (N_s) and the number of CldU-immunoreactive epithelial cells per surface area of basement membrane (N_s) using ANOVA, where sensitization (sensitized with OVA, sham sensitized) and exposure (filtered air, challenged with OVA) were the grouping factors. Differences among the four groups (sham sensitized with filtered air, sensitized with filtered air, sham



sensitized with OVA exposure, and sensitized with OVA exposure) were analyzed using ANOVA and Fisher's least significant differences test (Statview, version 5.01; SAS Institute, Cary, NC). We analyzed the volume density of epithelial cell types and the volume of ciliated or nonciliated cells per surface area of basement membrane in polar groups (sham sensitized with filtered air, sensitized with OVA exposure) using Student's *t*-test (Statview, version 5.01). All data are presented as means $\pm$ 1 SE. Statistical significance was considered $P < 0.05$.

RESULTS

Airway Responsiveness to Methacholine

The effective concentration of methacholine at which airway resistance doubled ($EC_{200} R_L$) was significantly reduced in the sensitized/challenged group compared with the control and sensitized groups (Fig. 1A). There was no significant difference in $EC_{200} R_L$ between the control and sensitized groups.

Central Airway Eosinophil Content

Central airway eosinophil number relative to basal lamina length was significantly increased ($P < 0.05$) in the sensitized/challenged group compared with the control and sensitized groups in the conducting airways (Fig. 1B).

Central Airway Mucin Levels

Central airway mucin area relative to basal lamina length in the sensitized/challenged group mucin area was significantly greater than the control or sensitized groups ($P < 0.05$; Fig. 1C).

Whole Mount Confocal Microscopy

In control rats, one-half of which were sensitized to OVA, small, unmyelinated afferent nerves and a few neuroendocrine cells labeled with anti-CGRP antibodies conjugated to Alexa 568 fluorochrome are easily visualized within the epithelium (Fig. 2A, top). Animals who received five episodes of exposure to OVA (whether or not they were sensitized to OVA) displayed a large increase in the number of CGRP-positive epithelial cells (Fig. 2A, bottom). Nerves, although still present, are more difficult to visualize against the high epithelial signal. When stacks of confocal images captured at third generation airways were analyzed, the mean number of N_s in animals sensitized and challenged with OVA was significantly greater than both filtered air control groups (sensitized, sham sensitized). The mean increase in CGRP N_s between the sensitized challenged rats and the sham-sensitized filtered air control group or the sham-sensitized challenged rats was 6.6-fold or 7.9-fold, respectively (Fig. 2B). Neuroendocrine cells that were immunoreactive for PGP 9.5 as well as CGRP were identified in the airways of all three treatment groups. However, the incidence of neuroendocrine cells was too low to accurately represent their density using the same unbiased optical disector that we used to estimate the number of CGRP-positive mucous cells per surface area of luminal epithelium.

Double-Labeled Airway Tissue

Airways double labeled with anti-CGRP antibodies and the nonspecific neural indicator PGP 9.5, conjugated to rhodamine

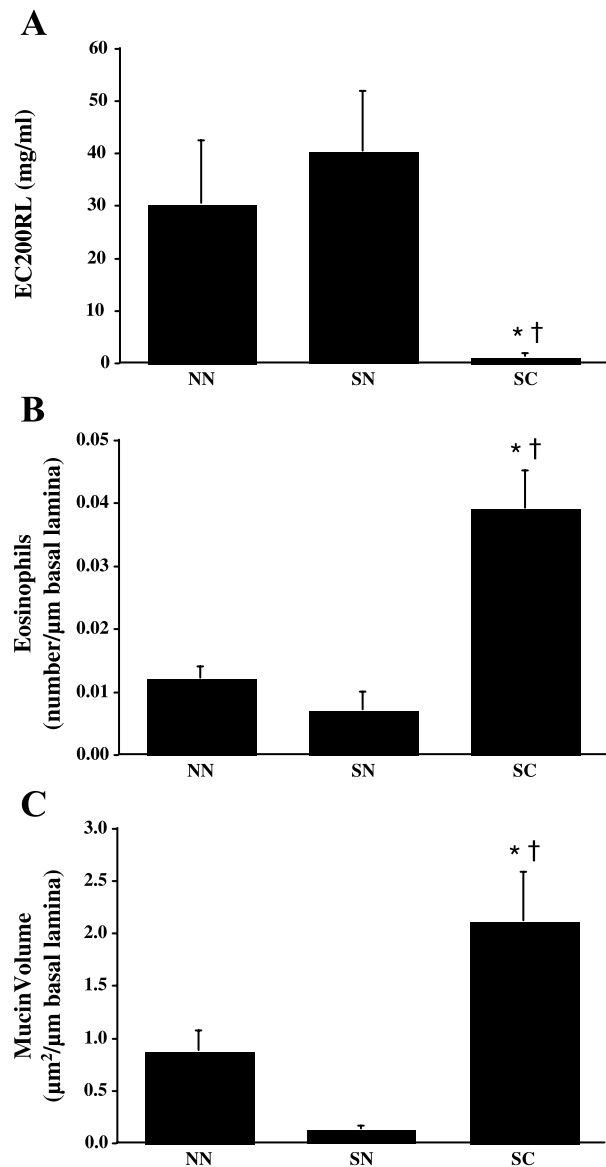


Fig. 1. Validation of the ovalbumin sensitization and challenge protocol used to produce hallmarks of allergic airway disease or asthma in 12-wk-old Brown Norway rats whose treatments were NN (sham sensitization, filtered air only), SN (sensitized to ovalbumin, filtered air only), and SC (sensitized to ovalbumin, challenged with ovalbumin). A: effective concentration of methacholine at which airway resistance doubled ($EC_{200} R_L$). B: central airway eosinophil number relative to basal lamina length. C: central airway mucin area relative to basal lamina length. Values are means $\pm$ SE. *Significant difference from control group. †Significant difference from sensitized group. Significance was set at $P < 0.05$.

red X (red) and FITC (green), respectively, were embedded, sectioned, and imaged at high magnification to better define the cellular location of CGRP and the relationship between CGRP-positive epithelial cells and local neural components. In the left column of Fig. 3, the CGRP-positive cells present postexposure are defined by red fluorescent granules within the body of the cell. These CGRP-positive epithelial cells are not positive for PGP 9.5; an approaching PGP 9.5-positive nerve fiber does indicate neural association if not direct contact. In the right

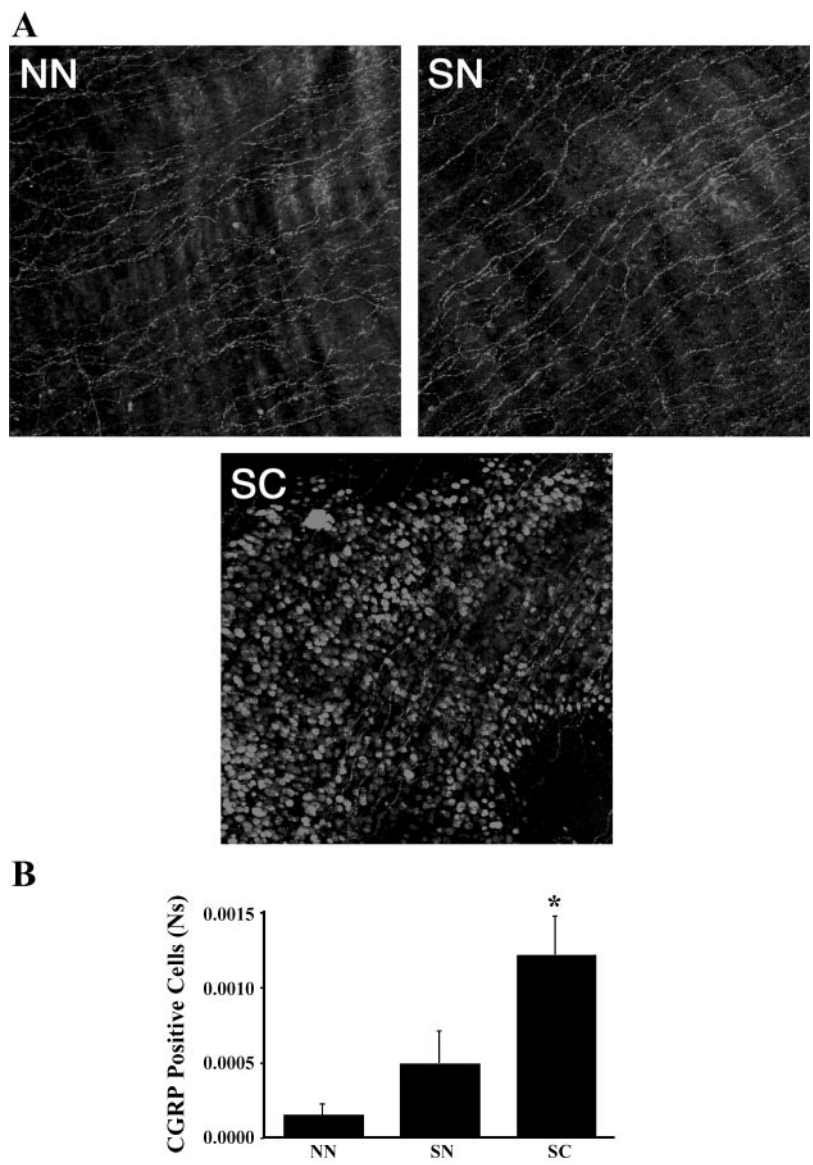


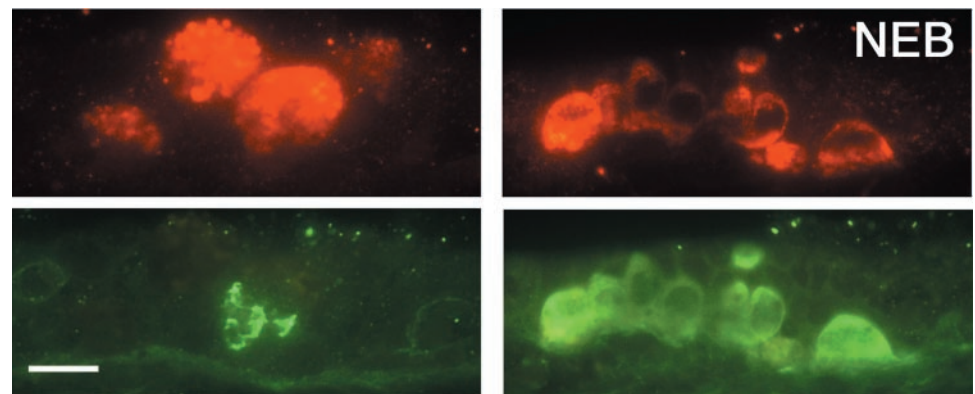
Fig. 2. *A*: confocal images of proximal pulmonary airway structures immunoreactive for the neuropeptide calcitonin gene-related peptide (CGRP) in 12-wk-old Brown Norway rats whose treatments were as follows: NN, SN, and SC. *B*: analysis of confocal images from 7 lungs per group at third-generation airways. The number of CGRP-positive epithelial cells per surface area of epithelium (N_s ; μm^2) + SE of data is shown. *SC significantly differs from SN and NN. $P < 0.05$, Fisher's protected least significant differences (PLSD) test.

column of Fig. 3, a rare neuroepithelial body is identified. The neuroendocrine cells that make up the neuroepithelial bodies are positive for both CGRP and PGP 9.5 and can be easily distinguished from the aforementioned epithelial cells on the basis of their shape and density (Fig. 3).

Defining Mucous Cells and CGRP-Positive Cells in Section

When airway whole mounts that had previously been labeled and imaged were embedded and sectioned, we were able to capture epifluorescent images displaying a conserved CGRP

Fig. 3. Epifluorescent sections from a Brown Norway rat sensitized and challenged with ovalbumin. The airway was labeled for protein gene product (PGP) 9.5 (green), a nonspecific neural indicator, and CGRP (red). Neuroepithelial cells shown (*right*) label for both PGP 9.5 and CGRP. Because CGRP-positive cells (*left*) are larger, more granulated, and not positive for PGP 9.5, we conclude they are not neuroendocrine cells. Neural innervation of these CGRP-positive epithelial cells by a PGP 9.5-positive nerve fiber is indicated (*bottom left*). NEB, neuroendocrine body. Mag bar = 10 μm .





signal and later identify mucous cells in the same sections via an AB/PAS stain. Figure 4 shows paired CGRP epifluorescent and AB/PAS images in the epithelium lining fourth-generation airways in all groups. Whereas the mucous cells from rats not exposed to OVA were not immunoreactive for CGRP, the mucous cells from rats sensitized and challenged with OVA labeled for CGRP. A few exceptions were noted in section; one such instance of a mucous cell labeling for CGRP in an unchallenged animal is shown in Fig. 4 [SN(A)]. The overall incidence of unchallenged animals displaying CGRP-positive cells within the epithelium in third-generation airways is depicted in Fig. 2B.

Defining Airway Distribution of CGRP-Positive Epithelial Cells

In airways labeled for CGRP, we also evaluated the distribution of CGRP-positive epithelial cells along the entire length

of the pulmonary airway. We captured these differences using confocal microscopy on airway whole mounts and epifluorescence in thin sections. Both techniques produce similar results; the density of CGRP-positive cells in rats sensitized and challenged with OVA decreases as airway generation number increases. In other words, the more distal the airway location, the fewer epithelial CGRP-positive cells present (Fig. 5). In sensitized and challenged animals, it was unusual to see any CGRP-positive mucous cells beyond the seventh airway generation. No CGRP-positive mucous cells were observed within terminal bronchioles, the most distal conducting airway generation (Fig. 5).

Evidence Against Mucous Cell Proliferation

Using CldU incorporation in replicating cells as an index of proliferation, we compared the degree of epithelial proliferation among all three groups. Our CldU marker was delivered via a subcutaneous osmotic pump enabling us to label proliferating cells throughout the challenge protocol (5 cycles). Figure 6 shows the mean number of proliferating cells per square micrometer of basement membrane (N_s). We were not able to detect a statistical difference in CldU incorporation within the epithelium of proximal airways among any of the three groups. In the most distal airways, we were also unable to detect a statistical difference in airway epithelial cell CldU incorporation (Fig. 6).

Morphological Cell Type Analysis in Proximal Pulmonary Airways

We found no significant change in the density of ciliated or nonciliated cells with sensitization and exposure to aerosolized allergen. Forty-eight hours after the final challenge (filtered air or OVA), the mean volume density of ciliated cells or nonciliated cells in animals sham sensitized and challenged with filtered air or sensitized and challenged with OVA was not significantly different ($P < 0.05$, unpaired Student's *t*-test). Moreover, the mean volume (μm^3) of ciliated cells or nonciliated cell epithelium per surface area (μm^2) of basement membrane was not significantly different between challenged and filtered air animals (Fig. 7).

DISCUSSION

This study indicates a new source for the release of the neuropeptide CGRP within pulmonary airways of rats with experimentally induced allergic airway disease. Sensitization and repeated exposure to allergen induced CGRP accumulation in mucous cells of proximal pulmonary airways of the Brown Norway rats studied. We suspect this new population of CGRP-positive mucous cells to have originated from the serous cell population present in proximal pulmonary airways before animal sensitization and exposure. In animals cyclically exposed to allergen, we found 1) an increase in the abundance of CGRP-positive cells in proximal airways; 2) the localization of CGRP granules to pulmonary airway mucous cells; 3) the absence of an increase in epithelial cell division; and 4) no significant change in the density of ciliated or nonciliated cells in sensitized animals exposed to allergen.

Phenotypes in airway epithelium in which CGRP has been observed previously include neuroendocrine cells, serous-type secretory cells, and small afferent nerve fibers (6, 12, 19, 23,

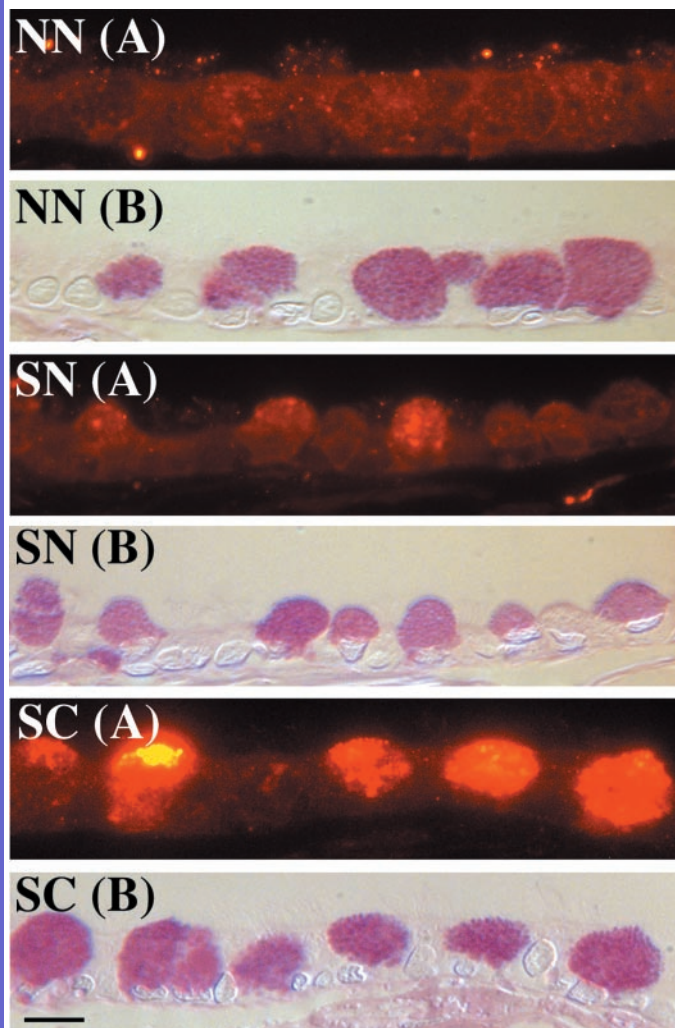


Fig. 4. Images captured from fourth-generation pulmonary airways from 12-wk-old Brown Norway rats whose treatments were as follows: NN, SN, and SC. After images of CGRP-immunoreactive cells were captured by epifluorescence (A), mucous cells were identified in these 2- μm sections by way of an alcian blue/periodic acid-Schiff stain (B). Although mucous cells were present at the fourth generation in each group, only the mucous cells of challenged animals contained abundant amounts of the neuropeptide CGRP. Mag bar = 10 μm .

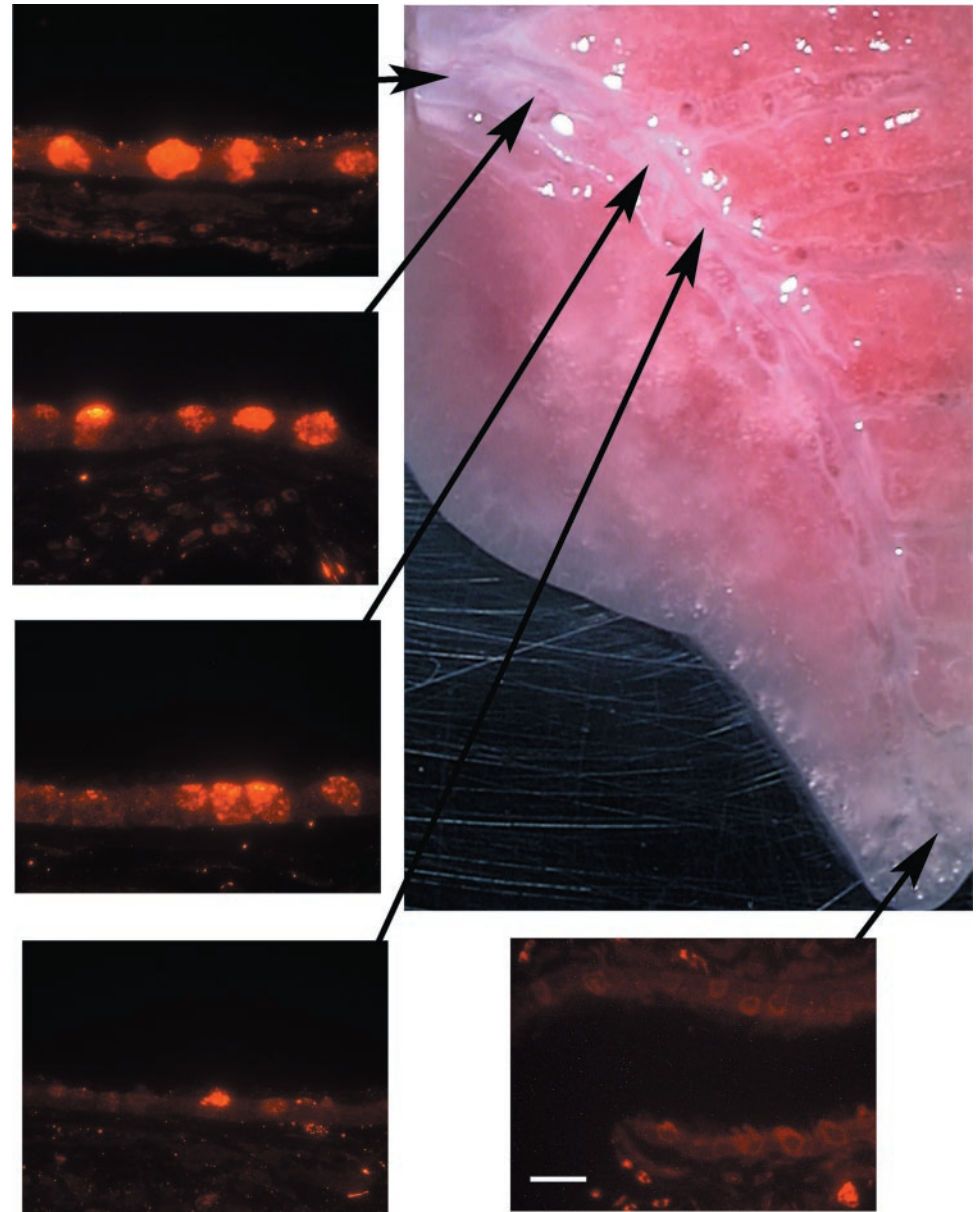


Fig. 5. Images obtained from the left lung lobe of a 12-wk-old Brown Norway rat that was sensitized and challenged with ovalbumin. The image at top right is of the dissected main axial airway from which the confocal images of CGRP-positive mucous cells were captured. Arrows indicate the sampling site for each of the confocal images shown. The density of CGRP-positive mucous cells decreased as generation number increased. Mag bar = 20 μ m.

25, 29, 40, 43). The CGRP-positive cells we detected do not fall into previously described CGRP-positive cell types because 1) the identified cells were not positive for PGP 9.5, a nonspecific neural indicator that labels both nerves and neuroendocrine cells, and 2) the positive cells contained large amounts of AB/PAS-positive material, indicating the presence on both acidic and basic mucins, thereby discounting the identification of these cells as serous cells. Moreover, the shape and distribution of these CGRP-positive cells postchallenge correspond to the distribution of mucous cells in Brown Norway rats that have been sensitized and challenged with OVA (34).

We are confident in our identification of the CGRP-immunoreactive mucous cell in challenged animals for a number of reasons. First, we obtained animals from a barrier maintained free of specific pathogens. We confirmed this with sentinel animals upon delivery. Second, sentinel rats killed at the end of the experiment were found to be free of known pathogens,

including rat coronavirus, Sendai virus, pneumonia virus of mice, parvovirus (includes H-1, KRV, RPV), *Mycoplasma pulmonis*, reovirus 3, lymphocytic choriomeningitis, Theiler's murine encephalitis virus, mouse adenovirus 1, cilia-associated respiratory bacillus, and Hanta virus. Third, the mucous cells from rats challenged with filtered air displayed little CGRP immunoreactivity; if an unknown virus was responsible for our CGRP immunoreactivity, one would expect indiscriminant labeling among groups. Last, secondary antibodies did not bind to cells of interest in the absence of anti-CGRP primary antibody.

An increase in the number of airway mucous cells in asthmatics is a well-documented phenomenon (1). In animal models of asthma, similar increases in the number of airway goblet cells have been induced through a variety of allergen protocols (8, 15, 34, 35, 39, 42). A study in Brown Norway rats attempted to better define the mechanism of increasing airway mucous cells by using BrdU incorporation as an index of

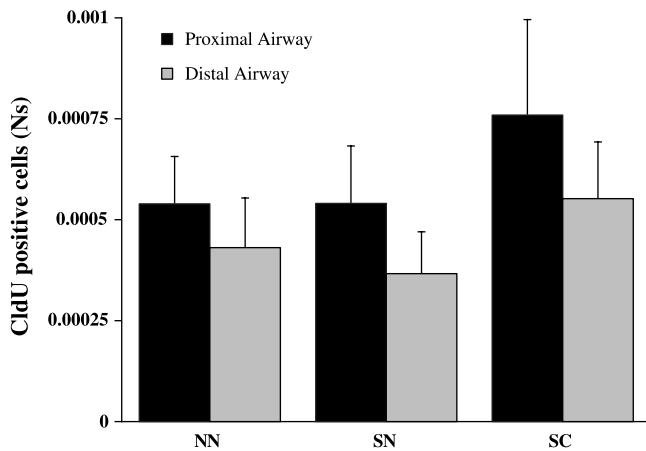


Fig. 6. Analysis of pulmonary airway sites from 12-wk-old Brown Norway rats whose treatments were as follows: NN, SN, and SC. Using 5'-chloro-2'-deoxyuridine (CldU) incorporation as an index of proliferating epithelial cells, we compared proximal and distal airway epithelium from all 4 groups. Twenty sites per animal were selected by the computerized CAST grid system. Results are mean CldU number per surface area of basement membrane (μm^2) $\pm$ SE of data obtained from 7–8 lungs per treatment group. No significant differences between treatment groups were found ($P < 0.05$, Fisher's PLSD).

proliferation. Using a BrdU pulse-labeling technique, Panetier et al. (35) found that there was a significant increase in epithelial cell proliferation associated with the third of three OVA challenges. Because CGRP had been shown to induce epithelial proliferation in vitro (47), we considered a correlation between CGRP mucous cell accumulation and an increase in mitosis. In our study, we identified a significant increase in the number of CGRP-positive cells (N_s) in the proximal pulmonary airways of animals challenged with OVA. Throughout the course of all five cyclic OVA challenges, we continuously labeled cells transitioning through S-phase with CldU using subcutaneous osmotic pumps and were not able to identify a significant increase in the number of epithelial cells (N_s) undergoing proliferation in the proximal pulmonary airway generations, the same generations with significant differences in the number of CGRP-positive cells.

Unable to verify a significant increase in the number of mitotic cell divisions within the epithelium of the pulmonary airways exposed to OVA, we believe this increase in the number pulmonary airway mucous cells to be the result of trans-differentiating nonciliated secretory cells. This conclusion is based on morphological data obtained from tissue sections. We found no significant difference in the density or proportion of nonciliated or ciliated cells when comparing sensitized challenged animals and control animals. The size, shape, and distribution of serous cells in the proximal pulmonary airway of the rat make them a likely progenitor cell. In fact, serous cells have previously been shown to differentiate into mucous cells (4, 22, 24). A serous-to-mucous cell conversion would certainly explain the increase in proximal airway mucous cells in Brown Norway rats periodically exposed to allergen, particularly in the absence of any significant alteration in the rate of baseline epithelial proliferation. Previous studies reporting CGRP immunoreactivity in serous-like cells may have actually visualized cells in the process of trans-differentiation (6). Small amounts of environmental allergens could easily induce allergic asthma in Brown Norway rats, a susceptible population.

Our observation of mucous cell metaplasia and increases in CGRP immunoreactivity of mucous cells with OVA sensitization and challenge may or may not be related events. Mucous cell metaplasia is thought to be driven by the immune response with the cytokine IL-13 playing a critical role (48). In contrast, the increase in mucous cell CGRP content could be the result of the immune response that drives metaplasia and/or nonspecific airway irritation. Peake et al. (36) found an increase in airway epithelial CGRP in response to acute airway injury. The increased CGRP expression was attributed to neuroendocrine expression, but since no additional markers to confirm cell types were used, the true identity of these CGRP-positive cells remains in question. One possible nonspecific mechanism would be the release of neuropeptides from small sensory nerve fibers and the subsequent autocrine upregulation of CGRP from epithelial cells much like the mechanism previously demonstrated with substance P and human keratinocytes (5). Alternatively, the elevated content of CGRP within mucous cells of OVA-sensitized and -challenged Brown Norway rats may be the result of the uptake of CGRP released from sensory nerves and/or neuroendocrine cells. If this were the case, then it would indicate a marked increase in the release of CGRP from sensory nerves and/or neuroendocrine cells with sensitization and challenge since the mucous cells that were present in the control group were not positive for CGRP.

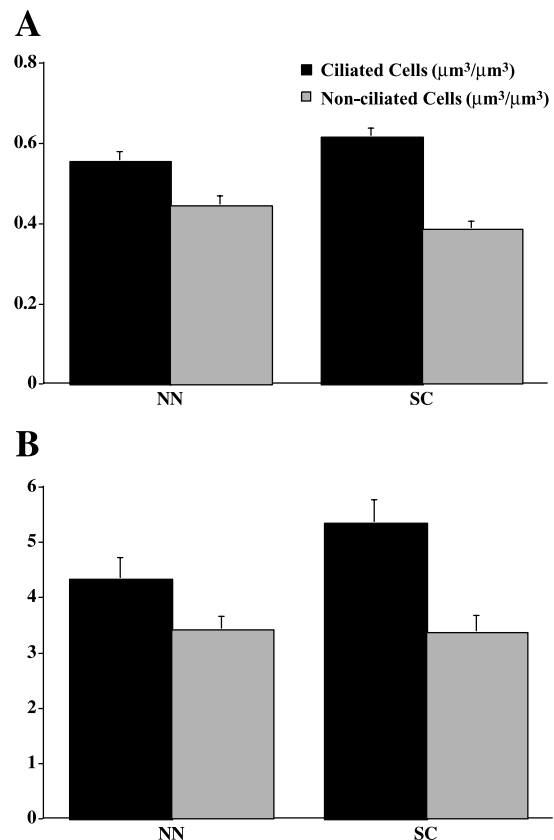


Fig. 7. Morphological analysis from third-generation pulmonary airways from 12-wk-old Brown Norway rats sensitized and challenged with ovalbumin (SC) or given sham treatments (NN). A: mean ciliated and nonciliated cell density. B: ciliated and nonciliated cell volume (μm^3) per surface area (μm^2) of basement membrane. Results are means $\pm$ SE of data obtained from 8 lungs per treatment group. No significant difference between any of the compared groups was found ($P < 0.05$, unpaired *t*-test).



The identification of CGRP receptors in rodent airway epithelium would be consistent with either of the latter two mechanisms (28).

Regardless of the mechanism that leads to the accumulation of CGRP within the mucous cells of sensitized and challenged rats, our results demonstrate a marked increase of CGRP within the airway. The concentration of CGRP in the airway may be important in the modulation of the asthmatic response. CGRP released from sensory nerves and neuroendocrine cells and/or mucous cells might help to modulate the immune response by acting as a chemoattractant for both T lymphocytes and eosinophils (16, 17). But while acknowledging CGRP's part in potentiating immune responses, we also believe CGRP may play a role in limiting inflammation as evidenced by its inhibition of T lymphocyte proliferation and macrophage activity (31, 45). The specific role CGRP plays in mucous cell metaplasia, a hallmark of asthma precipitated by Th2 lymphocytes, remains to be determined. The abundance of airway epithelial CGRP evident in asthmatic Brown Norway rats indicates a profound role for CGRP in the modulation of inflammatory and immune processes of the airway.

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