Increased Levels of Bombesin-like Peptides in the Lower Respiratory Tract of Asymptomatic Cigarette Smokers

Samuel M. Aguayo,* Madeleine A. Kane,‡ Talmadge E. King, Jr.,§ Marvin I. Schwarz,‖ Lana Grauer,¶ and York E. Miller**
**Pulmonary Sciences Division, Department of Medicine, Veteran's Administration Medical Center, University of Colorado Health Sciences Center, Denver, Colorado 80220; ‡Medical Oncology Division, Department of Medicine, Veterans Administration Medical Center, University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, Colorado 80220; §Pulmonary Sciences Division, Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80206; ‖Pulmonary Sciences Division, Department of Medicine, Veterans Administration Medical Center, University of Colorado Health Sciences Center, Denver, Colorado 80262; ¶Hybritech, Inc., San Diego, California 92121-1104; and **Pulmonary Sciences and Medical Oncology Divisions, Department of Medicine, Veterans Administration Medical Center, Eleanor Roosevelt Institute for Cancer Research, University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, Colorado 80220*

Abstract

Bombesin-related peptides are growth factors for a variety of cells, including normal human bronchial epithelial cells. An ELISA for bombesin-like peptides (BLP) has been devised using the MAb BBC353, which is specific for the biologically active carboxy-terminal fragment shared by all known BLP. Using this ELISA, we measured bronchoalveolar lavage (BAL) fluid levels of BLP in normal cigarette smokers (n = 15) and normal nonsmokers (n = 18). Smokers' BAL fluid contained increased levels of BLP, whether expressed in terms of BAL fluid volume (P = 0.0001) or protein content ($P < 0.05$). BLP levels did not correlate with any cellular constituent in the BAL fluid but immunostaining of lung tissue with BBC353 revealed an intense specific staining of neuroendocrine cells, implying these as a potential source. Two peaks of bombesin-like immunoreactivity were purified using sequential reverse phase and gel filtration HPLC. Both BLP have apparent molecular weights similar to gastrin-releasing peptide on gel filtration HPLC analysis. However, the amino acid composition of these BLP is different from that of gastrin-releasing peptide or neuromedin B, the only known mammalian forms of BLP, suggesting either incomplete purification or novel peptides. Sequence analysis could not be performed due to blocking groups at the amino terminus of these peptides. Our data demonstrate that cigarette smoking is associated with increased levels of pulmonary BLP and imply a potential role for these neuropeptides in the lung's response to tobacco smoke.

Introduction

Cigarette smoking is associated with an increased risk for developing chronic bronchitis, emphysema, and lung cancer (1). A common feature of these diverse lung diseases is the abnormal proliferation of bronchial epithelium (2). Although the etiology of this abnormal cell proliferation is unknown, we hypothesized that alterations in growth factor production may play a role. Pulmonary neuroendocrine (NE)[1] cells, which are prominent in normal lung development and are found in close association with the bronchial epithelium (3), may be a potential source of such growth factors. Pulmonary NE cells contain and secrete an array of neuropeptides including gastrin-releasing peptide (GRP) (4). GRP, a mammalian homologue of the amphibian neuropeptide bombesin, and other bombesin-related peptides have been shown to be potent mitogens for normal human bronchial epithelial cells (5), small cell lung cancer (SCLC) cell lines (6), and Swiss 3T3 cells (7). Furthermore, NE cells are increased in lungs from patients with various pulmonary diseases, including emphysema and SCLC (8). Increased serum levels of immunoreactive calcitonin and chromogranin A, two peptides produced by the NE cells, have been reported in patients with tobacco-associated lung diseases (9, 10). This suggests that, in smokers, pulmonary NE cells are not only increased in number but also may secrete increased quantities of neuropeptides. Previous reports of increased NE cells or increased NE cell products have been in patients with clinical abnormalities. Here, we report that asymptomatic cigarette smokers without clinically detectable lung disease exhibit increased levels of low molecular weight peptides with bombesin-like immunoreactivity in bronchoalveolar lavage (BAL) fluid when compared with normal nonsmokers. These findings suggest that an increased production of pulmonary neuropeptides may be an early response to cigarette smoke inhalation, implying a potential direct link to abnormal bronchial epithelial cell proliferation.

Methods

Subjects. We performed BAL in 33 healthy volunteers, 15 smokers (13 current smokers, 2 recent quitters) and 18 people who had never smoked. Informed consent was obtained from each subject, and the protocol was approved by the Institutional Human Subjects Review Committee. These subjects denied respiratory symptoms, and had normal physical examinations, chest radiographs, spirometric test results, lung volumes, and diffusing capacity for carbon monoxide (Table I). A detailed smoking history was obtained from every subject. None of the volunteers had a history of smoking nontobacco products.

This work was presented in part at the 1987 National Meeting of the American Federation for Clinical Research and as an abstract (*Clin. Res.* 1987. 35:530*a*. [Abstr.]).

Address reprint requests to Dr. Samuel M. Aguayo, Department of Medicine, Veterans Administration Medical Center, Respiratory Care 111A, 1055 Clermont Street, Denver, CO 80220.

Received for publication 31 January 1989 and in revised form 5 May 1989.

The Journal of Clinical Investigation, Inc.
Volume 84, October 1989, 1105–1113

1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; BLP, bombesin-like peptides; GRP, gastrin-releasing peptides; NE, neuroendocrine; SCLC, small cell lung cancer; TFA, trifluoracetic acid.

Table I. Clinical Characteristics, Pulmonary Function and BAL Fluid Information

Demographic data	Nonsmokers	Smokers	*P* Value (*t* test)
n	18	15	
Age (*yr*)	45.1±12.4	49.6±10.9	NS
Sex (M/F)	10/8	10/5	
Smoking History (pack/yr)	0	25.7±13.2	
Pulmonary function			
FEV_1 (*% pred*)	104.2±15.4	103.3±17.0	NS
FVC (*% pred*)	97.4±9.5	94.5±15.9	NS
FEV_1/FVC (*% pred*)	80.8±6.3	81.7±6.8	NS
TGV (*% pred*)	90.7±16.3	97.9±28.9	NS
DLCO (*% pred*)	111.8±15.1	110.6±18.2	NS
BAL			
Fluid recovery (%)	67.9±14.6	65.1±8.6	NS
Total cells (*$\times 10^4$/ml*)	27.4±16.5	58.8±61.7	NS
Macrophages (%)	83.4±8.4	83.6±11.4	NS
Lymphocytes (%)	14.3±8.3	11.3±8.0	NS
Neutrophils (%)	1.8±1.6	4.4±4.4	0.0266
Eosinophils (%)	0.4±0.7	0.7±1.1	NS
Total protein (*mg/ml*)	0.065±0.023	0.119±0.115	NS
BLP (*pmol/ml*)	26.6±24.8	98.7±44.9	0.0001
BLP (*nmol/mg protein*)	0.7±0.6	1.4±1.1	0.0446

Abbreviations for pulmonary function tests: DLCO, diffusing capacity for carbon monoxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; TGV, thoracic gas volume at normal end-expiration.

A carboxyhemoglobin level of < 1.5% was documented in every nonsmoker, thus minimizing the possibility of significant passive smoking exposure.

BAL fluid. BAL fluid was collected as previously reported (11). Briefly, BAL was performed with a 5.9-mm flexible fiberoptic bronchoscope (model BFB3; Olympus Corp. of America, New Hyde Park, NY), after intramuscular premedication with atropine (0.4 mg) and codeine (30 mg). Topical anesthesia was achieved with nebulized 4% lidocaine to the mouth and nose and 1% lidocaine to the trachea and bronchi. Supplemental oxygen (2 liters/m) was administered during and for 30 min after the procedure. After inspection of the segmental orifices, the tip of the bronchoscope was wedged into a subsegment of the right middle lobe (or lingula if the right middle lobe was technically inaccessible). Sterile normal saline at room temperature was instilled through the bronchoscope in 60-ml aliquots to a total of 240 ml, with harvest of the fluid by immediate gentle hand suction applied to each instilling syringe. The BAL fluid was immediately placed in ice water. After centrifugation at 800 *g* for 10 min, 5 ml of supernatant BAL fluid was saved for protein determination (12). Acetic acid was added to the remaining cell-free BAL fluid supernatant to a final concentration of 2 N. This solution was boiled for 10 min and stored at −70°C. 24 h before assay, aliquots of BAL fluid were vacuum dried in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY), and reconstituted to isovolume with 0.05 M Hepes buffer, pH 7.0, in HPLC-grade water.

BAL cell counts. The cell pellet was resuspended in HBSS (without calcium or magnesium), with no added serum. Cytocentrifuge slides of resuspended cells were prepared using a cytospin-2 (Shandon Southern Instruments, Sewickley, PA). Differential counts of 400 cells were performed on Wright-Giemsa-stained cytocentrifuge preparations.

Production of antibombesin MAb BBC353. The antibombesin MAb BBC353 was obtained as previously reported (13). Briefly, lys^3-bombesin (Peninsula Laboratories, Belmont, CA) was conjugated to bovine thyroglobulin (Calbiochem-Behring Corp., La Jolla, CA) using a ratio of 110 mol of peptide per mol of carrier in 7 mM glutaraldehyde, 100 mM sodium phosphate pH 7.0. The reaction was allowed to proceed for 18 h with constant stirring at room temperature. The conjugate was dialyzed against 50 mM sodium phosphate, pH 7.0. Mice were injected intraperitoneally with 100 μg of conjugate on days 0 and 21. 4 d before fusion, the mice received a final boost of 100 μg of conjugate. Spleen cells were removed from the mice and fused with the myeloma line P3.653 according to the procedure of Kohler and Milstein (14). The MAb BBC353 was selected based on its ability to bind synthetic bombesin by ELISA and ^{125}I (Tyr^4)-bombesin in a liquid-phase RIA.

Immunohistochemistry. Lung tissue samples were obtained from patients enrolled in the National Heart, Lung, and Blood Institute Interstitial Lung Diseases Specialized Centers of Research program and from the University of Colorado Health Sciences Center Department of Pathology. The tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4-μm sections, and mounted on glass slides. Before immunohistochemical staining, slides were deparaffinized by three 5-min xylene washes followed by three 10-min ethanol washes. Endogenous peroxidase was quenched by a 30-min incubation in methanol with 0.2% H_2O_2. Slides were then incubated for 1 h in 0.3% Triton in PBS, rinsed in tap water, and then incubated for 30 min with 150 μl normal horse serum. An equal volume (150 μl) of the primary MAb BBC353, at a concentration of 350 ng/ml, was added to the slides before overnight incubation in a humidified chamber. Subsequently, slides were rinsed and incubated with 150 μl of biotinylated horse antimouse immunoglobulins (Vector Laboratories, Burlingame, CA) at a concentration of 20 μg/ml. After washing three times, slides were incubated for 1 h with 150 μl of 25 μg/ml avidin-peroxidase complex (Vector Laboratories). Slides were washed and the stain was developed using 40 μg/ml diaminobenzidine and 0.01% H_2O_2, rinsed, incubated for 5 min in 3% $CuSO_4$, and then briefly counterstained with hematoxylin. To examine the specificity of staining with the primary antibody, working dilutions of BBC353 were preincubated with bombesin, ranatensin, litorin, neuromedin B, or neurotensin before overlaying on slides.

BLP immunoassay. Immunoreactive bombesin-like peptides (IR-BLP) content was determined by a competition ELISA. Nunc-Immuno I testing plates (InterMed; Roskilde, Denmark) were incubated overnight at 4°C with lys^3-bombesin (50 ng/well) then blocked with 0.05% Tween 20 in PBS for 2 h at 4°C. After washing twice with PBS, either standard concentrations of bombesin, other peptides, or unknown BAL fluid samples (50 μl/well) were added to the wells and incubated for 1 h at room temperature with the antibombesin MAb BBC353 (50 μl of a 50-ng/ml solution). Unbound BBC353 was removed by washing twice with PBS, and horseradish peroxidase-conjugated goat anti-mouse serum (Kirkegaard-Perry, Gaithersburg, MD) was added to the plates (diluted 1:5,000 in PBS containing 1% bovine serum and 0.05% Tween-20) for 1 h. Unbound second antibody was removed by washing twice with PBS. The chromogenic substrate 2,2′-azino-bis-3′-ethylbenz-thiazoline sulfonic acid, 0.8 mM, was added in 0.1 M sodium citrate, pH 5.0, with 0.001% H_2O_2 and absorbance (414 nm) determined after 30 min. BAL fluid samples were analyzed in quadruplicate versus a standard bombesin control curve in each plate. The results were reported in picomoles of IR-BLP per milliliter of BAL fluid or nanomoles of BLP per milligram of protein.

HPLC analysis. For biochemical characterization of IR-BLP, 82 ml of BAL fluid from a smoker with an initial BLP level of 31 pmol/ml (~ 2.5 nmol total BLP) were subjected to sequential reverse-phase and gel filtration HPLC. Reverse phase HPLC analysis of BAL fluid was performed on a Nucleosil C-18 column (150 × 4.6 mm, All-Tech; Applied Sciences Laboratories, Waltham, MA), eluted with a linear gradient of acetonitrile (0–100%) in 0.1% trifluoroacetic acid (TFA) over 30 min at a flow rate of 1 ml/min. For all of these analyses, the

column eluates were monitored for UV absorbance at 254 nm because of excessive noise/signal ratio at 214 nm. Control blanks and standards were always analyzed with each series of samples. 1-ml fractions were collected and, after being vacuum dried and reconstituted to isovolume with 0.05 M Hepes buffer (pH 7.0) in HPLC-grade water, analyzed for their IR-BLP content. The immunoreactive fractions were pooled and further analyzed using an isocratic 20% acetonitrile/0.1% TFA elution run over 5 min followed with a 20–60% acetonitrile/0.1% TFA gradient over 30 min at 1 ml/min using the same column. Again, 1 ml fractions were collected, vacuum dried, reconstituted in 0.05 M Hepes buffer (pH 7.0) HPLC-grade water, and analyzed for IR-BLP content. The immunoreactive fractions were pooled, vacuum dried, and reconstituted in 2 N acetic acid/HPLC-grade water prior to gel filtration analysis. Preparative gel filtration HPLC of the immunoreactive fractions was performed on a TSK 20 column (300 × 7.5 mm, Bio-Gel; Bio-Rad Laboratories, Richmond, CA) eluting the samples with 2 N acetic acid in HPLC-grade water over 30 min at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected, vacuum dried, reconstituted in 0.05 M Hepes buffer (pH 7.0) HPLC-grade water, and analyzed for IR-BLP content.

Amino acid composition analysis. Purified BLP were prepared for analysis by vacuum drying and reconstitution in 2 N acetic acid HPLC-grade water. Amino acid analysis was performed using the method of Jones (15), after hydrolysis of the BLP in 6 M ethiolglycolic acid at 155°C for 35 min, using an amino acid analyzer (model 655A-11; Hitachi Ltd., Tokyo). Sequencing of the purified peptides (~ 50 pmol) was attempted on a protein sequencer equipped with an Autoanalyzer (models 470A and 120A, respectively; Applied Biosystems, Foster City, CA) using the 03RPTH program. The column for the separation of the phenylthiohydantoin-amino acid derivatives was the phenylthiohydantoin C-18 (220 × 2.1 mm, 5 μm) with a gradient of: 0 min, 8% B, 120 μl/min; 0.1 min, flow to 200 μl/min; 11 min, 12% B; 30 min, 30% B, flow to 230 μl/min; 37 min, 36% B; 37.1 min, 80% B; 40 min, flow to 300 μl/min; 48 min, 80% B. The buffers were: A, 5% tetrahydrofuran in HPLC-grade water with 28.8 ml/liter of 3 M sodium acetate, pH 3.8, and 6.7 ml/liter of 3 M sodium acetate, pH 4.6; and B, acetonitrile.

Results

Immunohistochemistry. Immunostaining of lung tissue from three subjects with NE cell hyperplasia (neonate with bronchopulmonary dysplasia, normal smoker, and smoker with pulmonary eosinophilic granuloma) using the MAb BBC353 revealed an intense, specific staining of NE cells throughout the airways (Fig. 1). Both the solitary NE cells and the NE cell clusters termed neuroepithelial bodies were immunostained with BBC353. In addition, similar NE cells also stained with anticalcitonin and antichromogranin A antisera (data not shown), thus confirming their identity. No other cells were demonstrated to stain with BBC353. The staining was totally abolished when the primary antibody BBC353 was preincubated with 5 μg/ml of either bombesin, ranatensin, litorin, or neuromedin B, but not with the unrelated neuropeptide neurotensin at concentrations of 5 or 25 μg/ml, suggesting that the antigenic domain recognized by the MAb is the highly conserved carboxy-terminal region shared by all of the BLP.

Immunoassay. The ELISA detected bombesin with a sensitivity of 0.2 pmol/ml and was linear from 1 to 10 pmol/ml (Fig. 2 *A*). It also detected lys^3-bombesin, tyr^4-bombesin, and the bombesin-related peptides GRP 1–27, GRP 20–27, GRP-10 (also commonly referred to as GRP 18–27 or neuromedin C), and GRP 14–27 (Fig. 1 *A*). The minimal fragment recognized by BBC353 is the biologically active carboxy-terminal octapeptide shared by all of these BLP. GRP 1–16 was not detected by the ELISA. Oxidized bombesin and GRP 1–27, prepared by incubation with 30% H_2O_2, were detected with the same sensitivity as the nonoxidized peptides. In the ELISA, as in the immunohistochemistry, the MAb BBC353 also detected ranatensin, litorin, and neuromedin B, other bombesin-related peptides that differ at their carboxy terminus by conservative substitutions of one and two amino acids, respectively (Table II). However, different sensitivities were displayed for ranatensin, litorin, and neuromedin B when compared to the other bombesin-related peptides (Fig. 2 *A*). In addition to GRP 1–16, the unrelated peptides bradykinin, neurotensin, eledoisin, and substance P did not crossreact with the ELISA (Fig. 2 *B*). These results, in concert with the immunohistochemistry, further confirm the specificity of the MAb BBC353 for the carboxy-terminal domain shared by all known BLP.

BLP levels. Detectable levels of BLP were present in the BAL fluid of all smokers and most nonsmokers (Fig. 3). BAL fluid BLP produced ELISA curves comparable to the synthetic bombesin and other BLP control curves (data not shown). In smokers, the BLP levels ranged from 30.9 to 172.8 pmol/ml. In the nonsmokers, the BLP levels ranged from undetectable to 98.8 pmol/ml. The four subjects with undetectable BLP were assigned the minimum detectable value of the ELISA (0.2 pmol/ml) for statistical purposes, resulting in a mean value for the nonsmokers of 26.6±24.8 pmol/ml, compared with a mean of 98.7±44.9 pmol/ml for the smokers ($P = 0.0001$, t test). No correlation was found between the BLP levels and the BAL fluid protein content ($r = 0.0330$) suggesting that the difference between smokers and nonsmokers is not due to nonspecific protein leakage into the airways. Furthermore, if BLP levels were expressed as nanomoles/milligram protein, the significant difference between smokers and nonsmokers persisted ($P = 0.0446$, t test). In addition, there was no correlation between the BLP levels and any of the cellular constituents in the BAL fluid, including neutrophils, although these were increased in the smokers group as a whole (Table I). This suggests that the increased levels of pulmonary BLP observed in smokers were not related to parameters of airways inflammation. A significant correlation was observed between the BAL fluid BLP levels and the subjects' age ($r = 0.35$), and there was also a tendency for BLP levels to be lower when BAL fluid recoveries were greater ($r = 0.28$). However, there was no difference in age or BAL fluid recovery between smokers and nonsmokers (Table I), suggesting that these factors do not account for the difference in BAL fluid BLP levels. No correlation was found between BAL fluid BLP levels and any of the pulmonary function tests performed. Finally, there was no correlation between BAL fluid BLP levels and the extent of smoking history expressed as packs per years.

HPLC analysis. To determine the chemical nature of this BLP we performed sequential reverse-phase and gel filtration HPLC analysis. In the reverse-phase TFA/acetonitrile solvent system, the BAL fluid samples were resolved into a series of peaks, two of which contained all the BLP detected by our ELISA. Other peaks present in the BAL fluid did not contain BLP (Figs. 4 and 5). These immunoreactive peaks had different retention times compared with bombesin, GRP 1–27 and various GRP fragments, including GRP-10. However, the failure of these BLP to coelute in reverse-phase HPLC with synthetic BLP does not necessarily imply that the BAL fluid BLP are different because the retention time of peptides in crude mixtures can be different from the retention time of the same

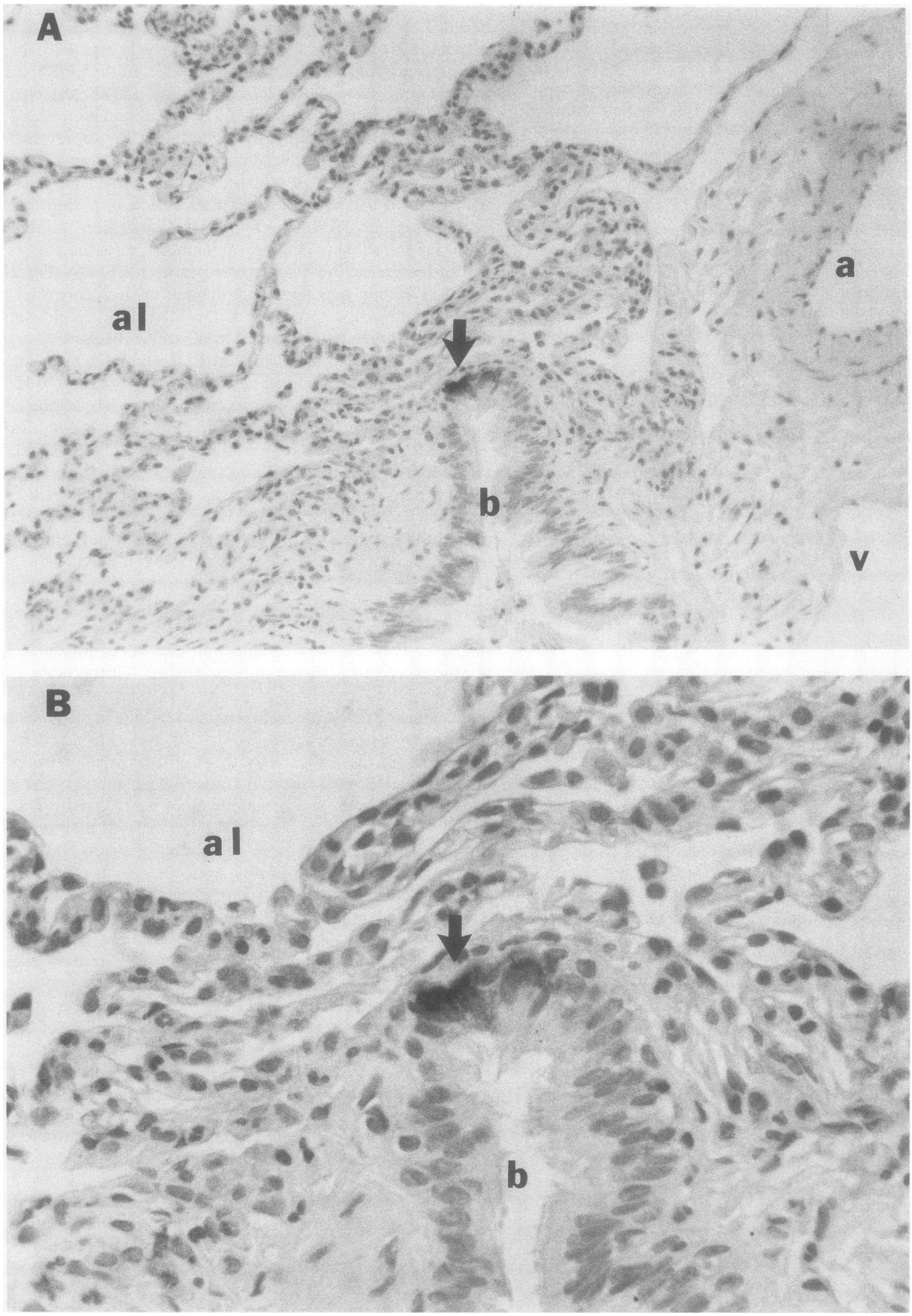

Figure 1. BLP immunohistochemistry in normal smoker lung. Immunoperoxidase with BBC353 (350 ng/ml) demonstrates BLP-positive neuroendocrine cells in a bronchiole. Section is counterstained with hematoxylin. *Arrows* point to positively stained cells. (*a,* arteriole; *al,* alveolus; *b,* bronchiole; *v,* venule). *A,* ×160; *B,* ×400.

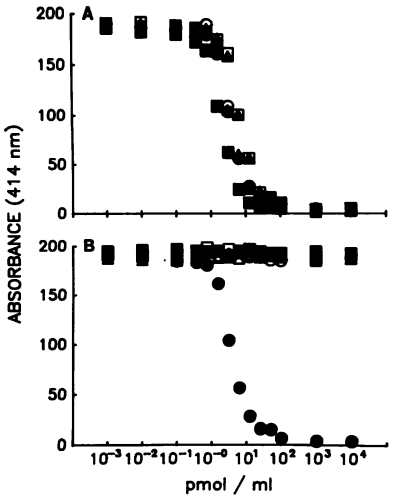

Figure 2. Immunoassay for bombesin-like peptides. (*A*) (●) Bombesin; (○) GRP 1-27; (△) neuromedin C; (▲) ranatensin; (□) litorin; and (■) Neuromedin B. (*B*) (●) Bombesin; (○) GRP 1-16; (△) neurotensin; (▲) bradykinin; (□) substance P; and (■) eledoisin.

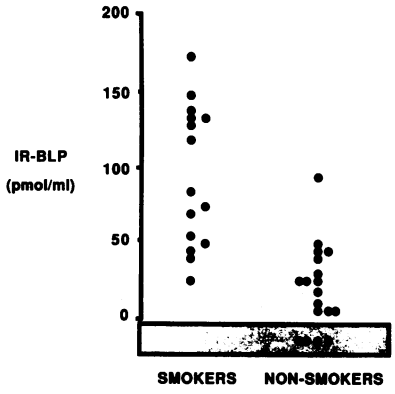

Figure 3. Immunoreactive BLP (IR-BLP) levels in BAL fluid samples from 15 smokers and 18 nonsmokers. The shadowed area represents value below the sensitivity of the ELISA (0.2 pmol/ml).

pure peptides (16). Preparative gel filtration HPLC was performed with material from each of the two immunoreactive peaks obtained by reverse phase analysis and both peaks were demonstrated to be of a similar molecular size, despite the significant difference in reverse phase mobility (Fig. 6). Human GRP eluted in the same gel filtration fraction, suggesting a molecular weight of ~ 2,500–3,000. The recovery efficiency for synthetic BLP using this protocol was 5%. For BAL fluid BLP the recovery efficiency was estimated to be 4%.

Amino acid composition and sequence analysis. The amino acid compositions of these two BLP were very similar to one another (Table III) and both had all the amino acids required for the BLP carboxy-terminal fragment detected by the MAb BBC353. The presence of phenylalanine in fraction B, but not in fraction A, may account for the different retention times in reverse phase HPLC. These BLP, however, are different from human GRP in that both fraction A and fraction B contain serine, isoleucine, and glutamate residues (not present in human GRP), whereas lysine and threonine (both present in human GRP) are not present in either fraction. Another difference from GRP is that both fractions A and B appear to be blocked at the amino terminus. Initial attempts at removing the blocking group have been unsuccessful.

Discussion

We have demonstrated increased levels of two peptides, which react with an antibombesin MAb, in the lower respiratory tract of clinically normal smokers. The potential biologic relevance of this observation is enhanced by the specificity of the immunoassay for the biologically active carboxy-terminal domain shared by all known BLP.

There is no consensus in the literature regarding nomenclature for this family of neuropeptides, but Spindel and coworkers (17) have referred to members of the bombesin family (bombesin, GRP, neuromedin C), ranatensin family (ranatensin, litorin, neuromedin B), and phyllolitorin family as the bombesin-like peptides, based on highly conserved structures at their carboxy termini (Table II). We have used this nomenclature when referring to the immunoreactive peptides detected in our BAL fluid samples because the MAb BBC353 does not distinguish between members of this neuropeptide family. Although this cross-reactivity may suggest some nonspecificity of the antibody, it can be easily explained based on the conservative phenylalanine-leucine substitution (nonpolar hydrophobic aminoacids with similar size side-chain), unlikely to be distinguished by an MAb. In addition, no cross-reactivity could be demonstrated with peptides that have no homology to the carboxy-terminal region present in the BLP, either in the ELISA or by immunohistochemistry. Finally, the specificity of the BBC353 immunostaining of human lung NE cells supports its utility for these studies and suggests that we are measuring NE cell BLP.

Table II. Amino Acid Sequences of Bombesin and Bombesin-like Peptides

Bombesin and bombesin-like peptides	Amino acid sequence
Bombesin	pGlu Gln Arg Leu Gly Asn Gln Trp Ala Val Gly His Leu Met NH_2
Human GRP	Val Pro Leu Pro Ala Gly Gly Gly Thr Val Leu Thr Lys Met Tyr Pro Arg Gly Asn His Trp Ala Val Gly His Leu Met NH_2
Neuromedin c	Gly Asn His Trp Ala Val Gly His Leu Met NH_2
Ranatensin	pGlu Val Pro Gln Trp Ala Val Gly His Phe Met NH_2
Litorin	pGlu Gln Trp Ala Val Gly His Phe Met NH_2
Neuromedin b	Gly Asn Leu Trp Ala Thr Gly His Phe Met NH_2
Leu-8 phyllolitorin	pGlu Leu Trp Ala Val Gly Ser Leu Met NH_2
Phe-8 phyllolitorin	pGlu Leu Trp Ala Val Gly Ser Phe Met NH_2

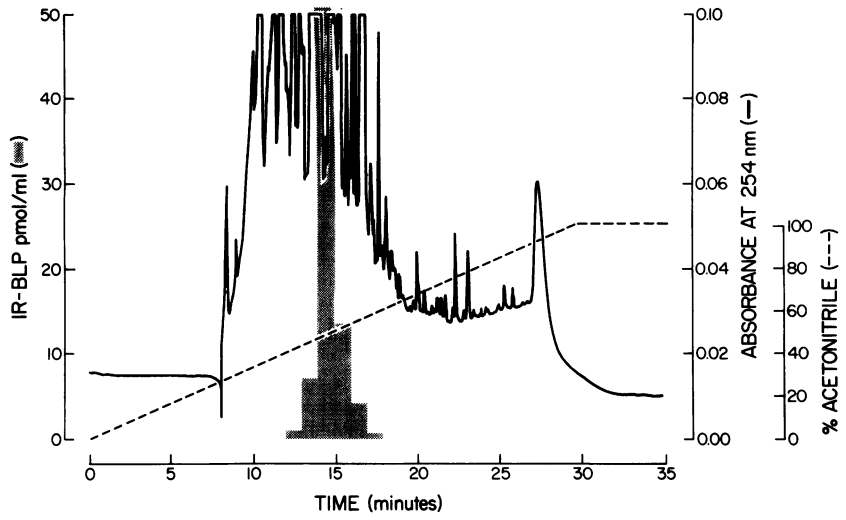

Figure 4. First reverse-phase HPLC of BAL fluid. Sample, 0.5 nmol of BAL fluid BLP. Flow rate, 1 ml/min. Fraction size, 1 ml/tube. Column, All-Tech Nucleosil C-18 (150 × 4.6 mm). Solvent system, Linear gradient elution from *A* to *B*. *A*, 0.1% TFA/HPLC-grade water-acetonitrile, 100:0. *B*, 0.1% TFA/HPLC-grade water-acetonitrile, 0:100. Control retention times, GRP 10, 12.4 min; bombesin, 21.1 min; human GRP, 26.7 min.

Although the exact amino acid sequences of these BLP have not been determined, due to the presence of blocked amino termini in both BLP, amino acid composition analysis demonstrates that all the amino acids necessary for the BLP carboxy-terminal fragment detected by the MAb BBC353 are present in both BLP species purified from BAL fluid. The apparent molecular weights of these BLP are similar to GRP. However, several findings suggest that these BLP may be different from human GRP, the only pulmonary BLP fully characterized on a molecular level to date. Most important is the absence of lysine and threonine from BAL fluid BLP. A possible explanation for this could be that BAL fluid BLP represents a GRP fragment, such as GRP-10 (neuromedin C), but that these BLP have different retention times on reverse-phase HPLC and are both blocked at the amino terminus suggest that they are distinct from previously described GRP fragments. In addition, gel filtration HPLC analysis suggests a molecular weight closer to that of whole GRP. The presence of phenylalanine in one of the BAL fluid BLP (fraction B) may indicate that this peptide is related to the ranatensin-litorin family (18). However, the only known mammalian homologue of these peptides, neuromedin B, is not blocked at the amino terminus and contains threonine in its carboxy terminus (17, 19). Finally, the presence of serine in both BAL fluid BLP raises the possibility of these being related to the phyllolitorin family (17). Whereas mammalian homologues for bombesin (GRP) and ranatensin (neuromedin B) have been described, a mammalian phyllolitorin homologue has yet to be demonstrated. These results further stress the necessity for additional biochemical characterization of BLP, as most reports of BLP in lung have relied only on immunological techniques that may not differentiate between multiple BLP species.

The levels of BAL fluid BLP reported here are many fold higher than those reported by other investigators in human plasma (20) and in conditioned media using SCLC cell lines (21). However, comparable levels have been reported in milk (22), and more recently, in urine (23). A possible explanation for these findings is that a slower degradation rate for BLP in BAL fluid than plasma may contribute to the accumulation of these neuropeptides (24). Another possibility is that we could be measuring novel BLP with a higher affinity for the antibody and thus overestimating the true amounts of peptide.

It is still unclear what the source of these BAL fluid BLP

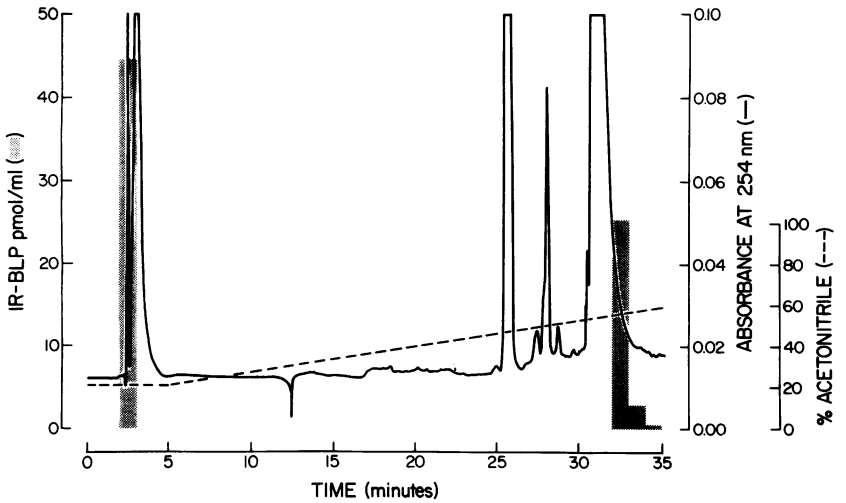

Figure 5. Second reverse-phase HPLC of BAL fluid. Sample, fraction 14–15 in Fig. 3 (one-half portion). Flow rate, 1 ml/min. Fraction size, 1 ml/tube. Column, All-Tech Nucleosil C-18 (150 × 4.6 mm). Solvent system, Isocratic 20% acetonitrile/0.1% TFA/HPLC-grade water for 5 min followed by linear gradient elution from *A* to *B*. *A*, 0.1% TFA/HPLC-grade water-acetonitrile, 80:20. *B*, 0.1% TFA/HPLC-grade water-acetonitrile, 40:60. Control retention times: GRP 10, 7.3 min; bombesin, 24.0; human GRP, 26.7 min.

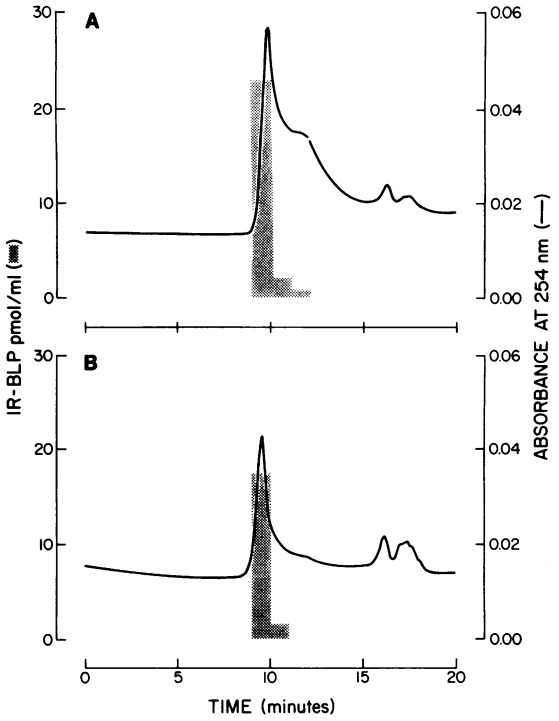

Figure 6. Gel filtration HPLC of IR-BLP fractions. Sample: (*A*) Fraction 3 in Fig. 4 (one quarter-portion). (*B*) Fraction 33 in Fig. 4 (one-quarter portion). Flow rate, 0.5 ml/min. Fraction size, 0.5 ml/tube. Column, Bio-Gel TSK 20 (300 × 7.5 mm). Solvent system, isocratic elution with 2 N acetic acid in HPLC-grade water. Control elution times, GRP 10, 11.2 min; bombesin, 10.8 min; human GRP, 9.4 min.

might be. However, pulmonary NE cells are a likely candidate. Increased neuroepithelial bodies and solitary NE cells have been observed in pathological specimens of smokers with chronic obstructive pulmonary disease (8). Alternatively, alveolar macrophages may be the source of, or contribute to, the BAL fluid levels of BLP. Human alveolar macrophages have been reported to contain BLP (25), but is unclear whether they synthesize BLP or accumulate it from their microenvironment. In our study, we found no correlation between BAL fluid BLP levels and any of the cellular constituents in the BAL fluid, including alveolar macrophages. In addition, immunostaining of lung tissue with the antibody BBC353 demonstrated intense specific staining in NE cells but not in macrophages, neutrophils or any other cells. Steady-state mRNA and in situ hybridization studies, using a human GRP cDNA probe, have indicated that pulmonary NE cells and related tumor cell lines, such as carcinoid and SCLC, express mRNA homologous to GRP mRNA (26). We are aware of no studies demonstrating GRP message in alveolar macrophages, but this does not exclude the possibility that they may produce other forms of BLP. Although both pulmonary NE cells and alveolar macrophages are potential sources of the elevated BLP in smokers' BAL fluid, the current evidence favors NE cell synthesis.

Table III. Amino Acid Composition of BAL Fluid BLP and Human GRP

Amino acid	Bombesin-like peptides (measured)		Human GRP (calculated)
	Fraction A	Fraction B	
	molar ratio		
Asp	3.0	2.9	1
Glu	6.0	4.1	0
Ser	3.3	7.6	0
His	2.2	1.1	2
Gly	14.7	14.6	5
Thr	0	0	2
Arg	2.6	1.9	1
Ala	4.4	4.2	2
Tyr	1	1.1	1
Met	2.4	1.1	2
Val	2.4	2.0	1
Phe	0	1.0	0
Ile	1.0	1.6	0
Leu	4.4	4.3	3
Lys	0	0	1
Trp	(Not detected by this method of analysis)		1

Several possible mechanisms exist for increased levels of BLP in the lower respiratory tract of clinically normal smokers. Stimuli such as nicotine and nitrosamines have been shown to increase the number of pulmonary NE cells and neuroepithelial bodies in various animal models (27, 28). It thus is possible that multiple substances contained in cigarette smoke may cause NE cell hyperplasia. A distinct hyperplasia of neuroepithelial bodies has also been found in lungs from individuals living at high altitude, presumably as an adaptive response to chronic hypoxia (8). Therefore, it is also possible that BAL fluid BLP levels in our subjects were affected by altitude. However, the altitude in Denver (~ 1,600 m) is significantly less than in the cited study (> 3,400 m). Even if physiologically relevant, altitude would not explain the difference between smokers and nonsmokers. Hypoxia and nicotine cause in vivo exocytosis of NE cell dense core vesicles and presumably neuropeptide secretion (29). Therefore, the increased BLP in the lower respiratory tract of smokers may reflect not only an increased number of NE cells but also an increased production and secretion by individual cells. Interestingly, two of the subjects in the smoker group had stopped smoking 24 and 20 mo before BAL, yet demonstrated BLP levels of 129.6 and 74 pmol/ml, respectively. The persistent elevation in BLP levels in these subjects suggests that the increased pulmonary BLP represent primarily NE cell hyperplasia and not nicotine-induced BLP hypersecretion.

The expression of increased levels of GRP mRNA and antigen during normal lung development suggests that the production of GRP from pulmonary NE cells may play a role in pulmonary ontogeny (3). Perhaps increased amounts of these putative pulmonary growth factors contribute to tissue repair processes elicited by tobacco-induced lung injury. Because bombesin-related peptides stimulate proliferation of normal human bronchial epithelial cells in vitro at concentrations comparable to those found in the BAL fluid samples (5), it is possible that the BLP detected by the ELISA stimulates proliferation of the bronchial epithelium in vivo. Furthermore, because GRP activates protein kinase C (30), as do phorbol ester tumor promoters, persistently elevated levels of pulmonary BLP may mimic the role of tumor promoters.

Although increased serum concentrations of neuropeptides

(calcitonin and chromogranin A) have been previously observed in smokers with lung disease (9, 10), this is the first report of increased neuropeptide levels in the respiratory secretions of smokers without clinically detectable lung disease. Neuropeptide levels in the lung-lining fluid may be a better reflection of the bronchial epithelial cell environment than serum measurements. In addition, this observation suggests an early role for the NE system in tobacco-associated lung disorders and may support the hypothesis that NE cell hyperplasia precedes the induction of lung tumors (31). The wide range of BLP levels in smokers may be a reflection of individual differences in response to cigarette smoke. If so, extreme elevations of pulmonary BLP levels could be a risk marker for specific diseases associated with cigarette smoking, such as lung cancer or chronic obstructive pulmonary disease. If increased levels of BLP prove to be an important link between smoking and lung disease, perhaps manipulation of the NE system may become a rational therapeutic approach.

Addendum

Tabassian and colleagues have recently reported that subchronic exposure of hamsters to cigarette smoke results in increased levels of pulmonary calcitonin and bombesin-like peptide, as well as neuroendocrine cell hyperplasia. (Tabassian, A. R., E. S. Nylen, R. I. Linnoila, R. H. Snider, M. M. Cassidy, and K. L. Becker. 1989. Stimulation of hamster pulmonary neuroendocrine cells and associated peptides by repeated exposure to cigarette smoke. *Am. Rev. Respir. Dis.* In press.)

Acknowledgments

We thank R. Ellison, J. Stewart, R. Lewis, and P. A. Bunn for helpful discussions; B. Kao and K. Sherritt for expert technical assistance; and T. Peterson for manuscript preparation.

Samuel M. Aguayo is a Robert Wood Johnson Foundation fellowship awardee (Minority Medical Faculty Development Program). This work was also supported by American Cancer Society Institutional Research grant IN5Z (S. M. Aguayo), National Institutes of Health grant R01 HL-29891, Minority Investigator Supplemental Research Award (S. M. Aguayo), Veterans Administration Career Development and Merit Review Grants (Y. E. Miller and M. A. Kane), National Cancer Institute First Award IR29-CA44763-01 (M. A. Kane), and by Specialized Centers of Research grant National Heart, Lung, and Blood Institute HL-27353 (T. E. King, Jr. and M. I. Schwartz). This is contribution #906 from the Eleanor Roosevelt Institute for Cancer Research.

References

1. U.S. Department of Health, Education, and Welfare: The Health Consequences of Smoking. Chronic Obstructive Lung Disease. 1984. U.S. Department of Health and Human Services, Public Health Service, Office of Smoking, Rockville, MD. DHHS (PHS) 84-SD205.

2. Auerbach, O., A. P. Stout, E. C. Hammond, and L. Garfinkel. 1961. Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. *N. Engl. J. Med.* 265:253–258.

3. Spindel, E. R., M. E. Sunday, H. Hofler, H. J. Wolfe, J. F. Habener, and W. W. Chin. 1987. Transient elevation of mRNAs encoding GRP, a putative pulmonary growth factor, in human fetal lung. *J. Clin. Invest.* 80:1172–1179.

4. Cutz, E., W. Chan, and N. S. Track. 1981. Bombesin, calcitonin and leuenkephalin immunoreactivity in endocrine cells of the human lung. *Experientia (Basel).* 37:765–767.

5. Willey, J. C., J. F. Lechner, and C. C. Harris. 1984. Bombesin and the C-terminal tetradecapeptide of gastrin-releasing peptide are growth factors for normal human bronchial epithelial cells. *Exp. Cell Res.* 153:245–248.

6. Cuttitta, F., D. N. Carney, J. Mulshine, T. W. Moody, J. Fedorko, A. Fishler, and J. D. Minna. 1985. Bombesin-like peptides can function as autocrine growth factors in human small cell lung cancer. *Nature (Lond.).* 316:823–826.

7. Rozengurt, E., and J. Sinnett-Smith. 1983. Bombesin stimulation of DNA synthesis and cell division in cultures of Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 80:2936–2940.

8. Gould, V. E., R. I. Linnoila, V. A. Memoli, and W. H. Warren. 1983. Neuroendocrine components of the bronchopulmonary tract: hyperplasias, dysplasias, and neoplasms. *Lab. Invest.* 49:519–537.

9. Becker, K. L., D. Nash, O. L. Silva, R. H. Snider, and C. F. Moore. 1981. Increased serum and urinary calcitonin levels in patients with pulmonary disease. *Chest.* 79:211–216.

10. Sobol, R. E., D. T. O'Connor, J. Addison, K. Suchocki, I. Royston, and L. J. Deftos. 1986. Elevated serum chromogranin A concentrations in small-cell lung carcinoma. *Ann. Intern. Med.* 105:698–700.

11. Watters, L. C., M. I. Schwarz, R. M. Cherniack, J. A. Waldron, T. L. Dunn, R. E. Stanford, and T. E. King. 1987. Idiopathic pulmonary fibrosis. Pretreatment bronchoalveolar lavage cellular constituents and their relationships with lung histopathology and clinical response to therapy. *Am. Rev. Respir. Dis.* 135:696–704.

12. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87:206–210.

13. Rittman-Grauer, L., and J. P. Leung. 1985. Characterization of monoclonal antibodies to bombesin. *Proc. Am. Assoc. Cancer Res.* 26:228. (Abstr.)

14. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* 256:495–497.

15. Jones, B. N., S. Paabo, and S. Stein. 1981. Amino acid analysis and enzymatic sequence determination of peptides by an improved *O*-phthaldialdehyde precolumn procedure. *J. Liq. Chromatogr.* 4:565–586.

16. Hancock, W. S., editor. 1984. CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins. Vol. II. CRC Press, Inc., Boca Raton, FL. 522 pp.

17. Krane, I. M., S. L. Naylor, D. Helin-Davis, W. W. Chin, and E. R. Spindel. 1988. Molecular cloning of cDNAs encoding the human bombesin-like peptide neuromedin B. Chromosomal localization and comparison to cDNAs encoding its amphibian homolog ranatensin. *J. Biol. Chem.* 263:13317–13323.

18. Chronwall, B. M., J. J. Pisano, J. F. Bishop, T. W. Moody, and T. L. O'Donahue. 1985. Biochemical and histochemical characterization of ranatensin immunoreactive peptides in rat brain: lack of coexistence with bombesin/GRP. *Brain Res.* 338:97–113.

19. Minamino, N., K. Kangawa, and H. Matsuo. 1983. Neuromedin B: a novel bombesin-like peptide identified in porcine spinal cord. *Biochem. Biophys. Res. Commun.* 114:541–548.

20. Pert, C. B., and U. K. Shumacher. 1982. Plasma bombesin concentrations in patients with extensive small cell carcinoma of the lung. *Lancet.* i:509.

21. Moody, T. W., E. K. Russell, T. L. O'Donahue, C. D. Linden, and A. F. Gazdar. 1983. Bombesin-like peptides in small cell lung cancer: biochemical characterization and secretion from a cell line. *Life Sci.* 32:487–493.

22. Jahnke, G. D., and L. H. Lazarus. A bombesin immunoreactive peptide in milk. *Proc. Natl. Acad. Sci. USA.* 81:578–582.

23. Aguayo, S. M., M. A. Kane, W. Silvers, L. M. Nett, and Y. E. Miller. 1988. Urinary levels of bombesin-like peptide in asymptomatic smokers. *Chest.* 94:20S. (Abstr.)

24. Aguayo, S. M., M. A. Kane, T. E. King, and Y. E. Miller. 1988. In vitro degradation of bombesin by human plasma, urine and bronchoalveolar lavage fluid. *Clin. Res.* 36:502*a*. (Abstr.)

25. Wiedermann, C. J., M. E. Goldman, J. J. Plutchok, K. Sertl, M. A. Kaliner, A. Johnston-Early, M. H. Cohen, M. R. Ruff, and C. B. Pert. 1986. Bombesin in human and guinea pig alveolar macrophages. *J. Immunol.* 137:3928–3932.

26. Sunday, M. E., L. M. Kaplan, E. Motoyama, W. W. Chin, and E. R. Spindel. 1988. Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab. Invest.* 59:5–24.

27. Wang, N. S., M. F. Chen, D. E. Schraufnagel, and Y. T. Yao. 1984. The cumulative scanning electron microscopic changes in baby mouse lungs following prenatal and postnatal exposures to nicotine. *J. Pathol.* 144:89–100.

28. Resnik-Schuller, H. 1976. Proliferation of endocrine (APUD-type) cells during early N-diethylnitrosamine-induced carcinogenesis in hamsters. *Cancer Lett.* 1:255–258.

29. Lauweryns, J. M., M. Cokelaere, M. Deleersnyder, and M. Liebens. 1977. Intrapulmonary neuro-epithelial bodies in newborn rabbits. Influence of hypoxia, hyperoxia, hypercapnia, nicotine, reserpine, L-DOPA and 5-HTP. *Cell Tissue Res.* 182:425–440.

30. Zachary, I., P. J. Woll, and E. Rozengurt. 1987. A role for neuropeptides in the control of cell proliferation. *Dev. Biol.* 124:295–308.

31. Minna, J. D., F. Cuttitta, J. F. Battey, J. L. Mulshine, I. Linnoila, A. F. Gazdar, J. Trepel, and E. A. Sausville. 1988. Gastrin-releasing peptide and other autocrine growth factors in lung cancer: pathogenetic and treatment implications. *In* Important Advances in Oncology. 55–64.