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Prospective Trial Evaluating Immunocytochemical-based Sputum Techniques for Early Lung Cancer Detection: Assays for Promotion Factors in the Bronchial Lavage

Frank Scott, PhD[1], Frank Cuttitta, PhD[1], Anthony M. Treston, PhD[1], Ingalill Avis, BA, RN[1], Prabodh Gupta, MD[2], John Ruckdeschel, MD[3], Karen Kelly, MD[4], Steven Piantadosi, MD, PhD[5], Melvyn Tockman, MD, PhD[5], and James Mulshine, MD[1]

[1] Biomarkers & Prevention Research Branch, National Cancer Institute, Rockville, MD
[2] University of Pennsylvania, Philadelphia, PA
[3] H. Lee Moffitt Cancer Center, University of South Florida, Tampa, FL
[4] University of Colorado, Denver, CO
[5] Johns Hopkins School of Medicine, Baltimore, MD

Abstract To confirm the results of a previous report on the use of monoclonal antibodies in immunocytochemical assays of sputums for the early detection of lung cancer, we designed a new prospective trial in an independent clinical trial population. Since well-characterized Stage I resected non-small cell lung cancer patients have a low rate of tumor relapse and a high (1–3%/year) chance of developing a second primary lung cancer, they comprise a very favorable group for conducting an early lung cancer detection trial. The rate of new lung cancer is about 10-fold in excess of a standard "high" risk population of smokers.

To optimize the chance for a favorable outcome, all of the technical components for the trial have been systematically evaluated to ensure that optimal procedures are employed. For example, automated immunostaining of the sputum specimens will be performed.

Bronchial lavages will be analyzed in a subset of the trial participants to define additional targets for early lung cancer detection. Two markers will be quantitated, including gastrin releasing peptide and peptidyl glycine α-amidating monooxygenase activity. These two markers assess the epithelium's capacity to produce growth factors which may be central to the biology of tumor promotion. Since these assays have not been performed in this context before, we attempted to optimize the specimen handling to permit the receipt of the material from a range of collaborating clinical sites in a condition that permits accurate quantitation of these two biomarkers.

Efforts to standardize the assay endpoint stimulated the development of computer-assisted methods of immunocytochemical analysis. An algorithm for image analysis was developed as a result of systematic analysis of a range of potentially quantifiable assay endpoints with a panel of teaching cases. When a sampling of the original immunostained material from the first monoclonal antibody-based early lung cancer detection report was reanalyzed using the image analysis algorithm, a 90% concurrence with the original immunostaining interpretation was observed. These results suggest that there was an objective basis to the first report and that image analysis can greatly refine the process of early lung cancer detection research.

Address correspondence to Dr. James Mulshine, Biomarkers & Prevention Research Branch, DCPC, National Cancer Institute, 9610 Medical Center Drive Room 300, Rockville, MD 20850.

An update of the current trial status will be summarized emphasizing the unique challenges presented in early cancer detection research.

Key words: lung cancer, early detection, immunocytochemistry, bronchial lavage

Lung cancer is a profoundly important cause of premature mortality in the industrialized world. Despite intensive efforts to improve this situation, the diagnosis of lung cancer still results in a 5 year mortality rate of 87% [1]. The absence of effective systemic agents for metastatic lung cancer is, in large measure, responsible for this high level of lethality [2]. In response to this, a reconsideration of early detection approaches is necessary.

We have reported an approach to early lung cancer detection using an immunocytochemical technique that in a prospective, non-concurrent analysis of archival sputum specimens, demonstrated a predictive accuracy of approximately 90% two years prior to the detection of lung cancer using conventional techniques [3,4]. To confirm the efficacy of this approach, we have begun a new prospective trial in collaboration with institutions and investigators previously associated with the defunct Lung Cancer Study Group (LCSG). The LCSG was a large number of carefully evaluated Stage I non-small cell lung cancer patients who had an extremely high rate of developing new primary lung cancers (1–3% cumulatively per year)[5]. A number of Stage I resected lung cancer patients, including many from LCSG surgical protocols, have now enrolled in a new prospective early lung cancer detection study. The current participating sites are collectively called the Lung Cancer Early Detection Working Group (LCEDWG).

After standardizing the immunostaining procedure as previously reported, an annual sputum sample is acquired and analyzed using the same two antibodies employed in the first report. The antibodies, 624H12 and 703D4, were generated using a small cell and a non-small cell lung cancer cell line, respectively [3]. With accrual of about 1,000 resected Stage I patients after four years, sufficient new lung cancers will occur to permit the analysis of immunostaining and clinical outcome to independently determine the precision of this approach. Anticipating population-based applications of sputum immunocytochemistry for early lung cancer detection, we previously reported the validation of a computer algorithm to permit objective scoring of immunostaining using image analysis [6].

Using a Zeiss Axiomat microscope to identify informative characteristics of the immunostained bronchial epithelial cells, a series of objective parameters including transmission spectra, optical texture and morphometry were determined [6]. This information was analyzed for a series of specimens as part of a validation study. Multivariate analysis of the clinical correlations from the specimens in the training set established which combination of measurements most closely reflected the classification of cell status as originally determined by the reference pathologist from the first successful report of sputum immunostaining [3]. This type of research leads to identification of a reproducible, objective endpoint, a factor crucial to the successful broad clinical application of sputum immunostaining if it can be confirmed as an effective early lung cancer detection method.

Since the initial positive early lung cancer detection report [3], we have been challenged to find the scientific basis for that success. We postulate that sputum is the right specimen to analyze for lung cancer early detection because it permits analysis of the composition of shed bronchial epithelial cells. Since lung cancer arises as an epithelial process, the epithelium should be the first site probed for the development of an invasive cancer [7]. We also speculate that at least one of the monoclonal antibodies used in the study by Tockman *et al.* [3] detected oncofetal antigens which reflect a more primitive cellular phenotype. This is consistent with epithelial cell changes in bronchial tissues which have been exposed to critical levels of carcinogens. This phenomenon has been termed a "cancer field" by Slaughter [8]. Successful early detection efforts may depend on understanding cancer field dynamics and use of relevant biomarkers to detect and monitor such

critical aspects of cancer field biology as early cancer promotion.

Based on our long-standing studies of growth factors in lung cancer, we speculate that autocrine growth factor biology may be central to certain types of lung cancer during the early stages of tumor promotion [9–13]. The paradigm for this work has been the autocrine growth factor, gastrin releasing peptide (GRP), a member of the bombesin-like family of peptides. In addition to GRP's mitogenic effect on normal and malignant lung epithelium [9,14], this peptide also plays a central role in fetal lung development [15]. A clinical trial to determine the effect of serotherapy with a monoclonal antibody that neutralizes the proliferative effect of GRP in advanced small cell lung cancer patients is ongoing [12,16]. Other growth factors such as IGF-I and a transferrin-like molecule also appear to mediate proliferative effects in lung cancer [10,11,13]. This suggests that generic strategies may be required to control lung cancer promotion. In many organs, cells possessing neuroendocrine histological features are associated with the potential for synthesizing an array of peptide growth factors. An understanding of the neuroendocrine properties of lung cancer cells in relation to overall lung cancer biology will be essential to develop these new strategies.

Different lung cancer types express neuroendocrine features to varying degrees, suggesting that lung cancer originates from a common cell type which may be neuroendocrine in nature. Neuroendocrine properties studied in lung cancer cell lines include the expression of dopa-decarboxylase activity, dense core granules, synaptophysin, neural cell adhesion molecule (NCAM) antigen, chromogranin A, and the production of various neurohormonal peptides [17,18]. Dopa-decarboxylase, an enzyme which catalyzes an essential step in the production of the neurotransmitter dopamine, has been shown to be the key indicator of the neuroendocrine nature of a cell line. Dense core granules are structural features which are believed to be related to the storage of neurohormonal peptides in many cell lines. Synaptophysin, NCAM, and chromogranin A are neuroendocrine marker antigens usually studied by immunocytochemical means [17,18]. The neuroendocrine features of a lung cancer cell line may reflect the biology of the corresponding tumor. Classic small cell lung cancer usually exhibits neuroendocrine features, and is particularly aggressive.

The production of peptides is often attributed to neuroendocrine cells. About 50% of gastrointestinal and neuroendocrine peptides are α-carboxyamidated, a modification usually required for full biological activity [19,20]. Therefore, peptide α-amidating activity may be a useful marker for neuroendocrine differentiation. Human small cell lung cancer tumors and cell lines, as well as other neuroendocrine tumors and cell lines, produce a variety of peptide growth factors, at least one of which—GRP—is an amidated peptide known to act as an autocrine growth factor [9,21,22].

All known carboxy-amidated peptides/hormones are derived from their corresponding carboxyl-terminal glycine-extended intermediate proforms. During the final steps of post-translational processing, glycine is enzymatically oxidized to form the carboxy-amide of the neighboring amino acid (penultimate to glycine) and glyoxylate [23,24] (Fig. 1). Until recently, this modification reaction was believed to be catalyzed by a single copper and ascorbate-dependent enzymatic activity, designated peptidylglycine α-amidating monooxygenase (PAM) [19,24,25]. The DNA sequence of PAM from several species, including humans, is known, but the enzymes responsible for human peptide α-amidating activity have not been characterized [18,20,26–28]. Rat and bovine studies give evidence that the amidation process is catalyzed by two separate enzymatic activities [29–31] (Fig. 1). The first enzymatic activity, designated peptidylglycine α-hydroxylating monooxygenase (PHM), catalyzes the formation of an α-hydroxylated intermediate from a glycine extended peptide. The second activity, designated peptidyl-α-hydroxyglycine α-amidating lyase (PAL), catalyzes the cleavage of the α-hydroxylated intermediate to form an amidated peptide product and glyoxylate [20].

The degree to which enzymes involved in peptide α-amidation are present on the bronchial epithelium may indicate the capability of epithelial cells to process bioactive peptide factors during cancer promotion [22]. To determine whether peptide amidating enzymes could potentially indicate early neoplastic changes in the respiratory epithelium, it is necessary to try

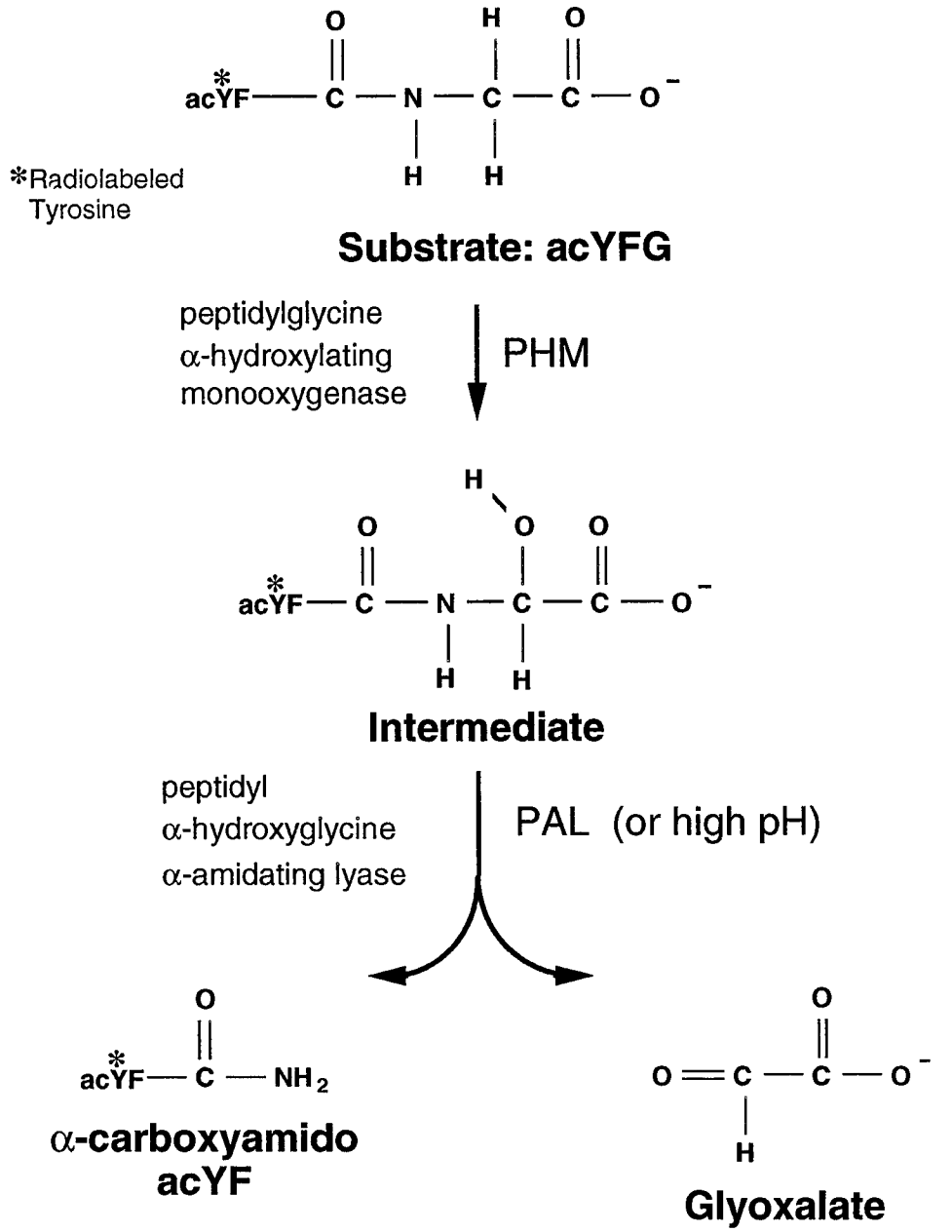

Fig. 1. The reaction mechanism of α-amidation of glycine-extended peptides illustrated using a tripeptide substrate, acYFG, used in assaying the peptide amidating enzymes peptidylglycine α-hydroxylating monooxygenase and peptidyl-α-hydroxyglycine α-amidating lyase [19,20].

measuring the levels of these enzymes in bronchial secretions. In confirming the utility of sputum immunocytochemistry in a cohort of Stage I resected non-small cell lung cancer patients, we can identify additional early detection tools that enhance our diagnostic capabilities. We propose performing fiberoptic bronchoscopy in a subset of patients to obtain bronchioalveolar lavage (BAL) fluids to quantitate the expression of specific growth factors such as GRP. This also allows quantitation of the processing enzymes required for α-amidation and evaluation of these enzymes as tools for early lung cancer detection. When the clinical outcome of the trial participants is known at trial maturity, we can correlate the levels of growth factors or amidating activity and clinical status.

Chronic injury leading to invasive lung cancer impacts the overall function of the respiratory epithelium, frequently resulting in coexistent conditions such as chronic bronchial infection, which may post a serious challenge to accurate biomarker determination. The goal in developing appropriate assay methodology consists of recovering evanescent biological species such as GRP or quantitating activity levels of amidating enzymes in this "polluted" *in vivo* setting. In this manuscript, we will describe in detail several of these issues and our working proposal for quantitation of biomarkers in bronchial lavage fluid.

METHODS

Bronchoscopic Procedures

Selected subjects from the LCEDWG trial underwent fiberoptic bronchoscopy to determine levels of GRP and amidating enzymes in their bronchial airways. After routine sedation, a flexible fiberoptic scope is used for the bronchoscopy. The bronchoscope is wedged into one of the subsegments of the anterior segment of the right upper lobe for lavage. In the event of right upper lobectomy, the anterior segment of the left upper lobe is lavaged. Examination of the airway for suspicious lesions and lesion evaluation (if any, with brush or biopsy) is conducted after the BAL. An aliquot of 60 ml of sterile normal saline (preservative-free) at room temperature is instilled through the bronchoscope. The fluid is immediately, but gently, hand-aspirated using the instilling syringe. The recovered injectate is placed in a sterile specimen cup (to which a cocktail of protease inhibitors has already been added per instructions). Saline is instilled an additional three times for a total injected volume of 240 ml.

Participating sites of the LCEDWG are sent a concentrated solution of protease inhibitors to be added to the BAL sample at the time of collection of the fluid. The protease inhibitors in the mixture, their final concentration in the BAL fluid and the targeted class of proteases are listed in Table I. All of these inhibitors, with the exception of EDTA, are purchased from Sigma Chemical Company, St. Louis, MO. EDTA was obtained from Fisher Scientific, Chicago, IL. Protease inhibitors are chosen to inhibit a number of proteases which may be present in the lavage fluid (Table I). Some of the inhibitors are chosen based on their ability to stabilize GRP from proteolysis in cell extracts. Bacitracin, which may inhibit proteolysis indirectly by preventing bacterial contamination, is also included in the mixture at 110 μM. The BAL fluid with protease inhibitors added, is shipped back on ice by overnight express for analysis.

The BAL fluid is divided into two portions for analysis (Fig. 2). One portion is brought to a concentration of 2 N acetic acid by adding glacial acetic acid and boiling for 10 minutes. The acidified portion is intended for preservation of peptide growth factors. After centrifugation at 2000 $\times$ g for 10 minutes, the acid supernatant is divided into aliquots and frozen at –30°C. The acid pellet is homogenized with a Polytron homogenizer, divided into aliquots, and frozen at –30°C. The non-acidified half of the lavage sample is divided into three portions and centrifuged at 200 $\times$ g for 10 min. The supernatant is frozen in aliquots at –30°C. One of the pellets is homogenized and frozen at –30°C. The second is frozen intact and the third is frozen in Saccomanno's fixative.

GRP assay. The assay for quantitation of GRP in BAL fluid, a competitive binding assay which uses a radiolabeled monoclonal antibody (MoAb 2A11) highly specific for the amidated amino terminal portion of GRP, was performed according to the method of Kasprzyk *et al.* [32]. The MoAb 2A11, a murine $IgG_{1\kappa}$ antibody was described by Cuttitta *et al.* [9]. The assay is

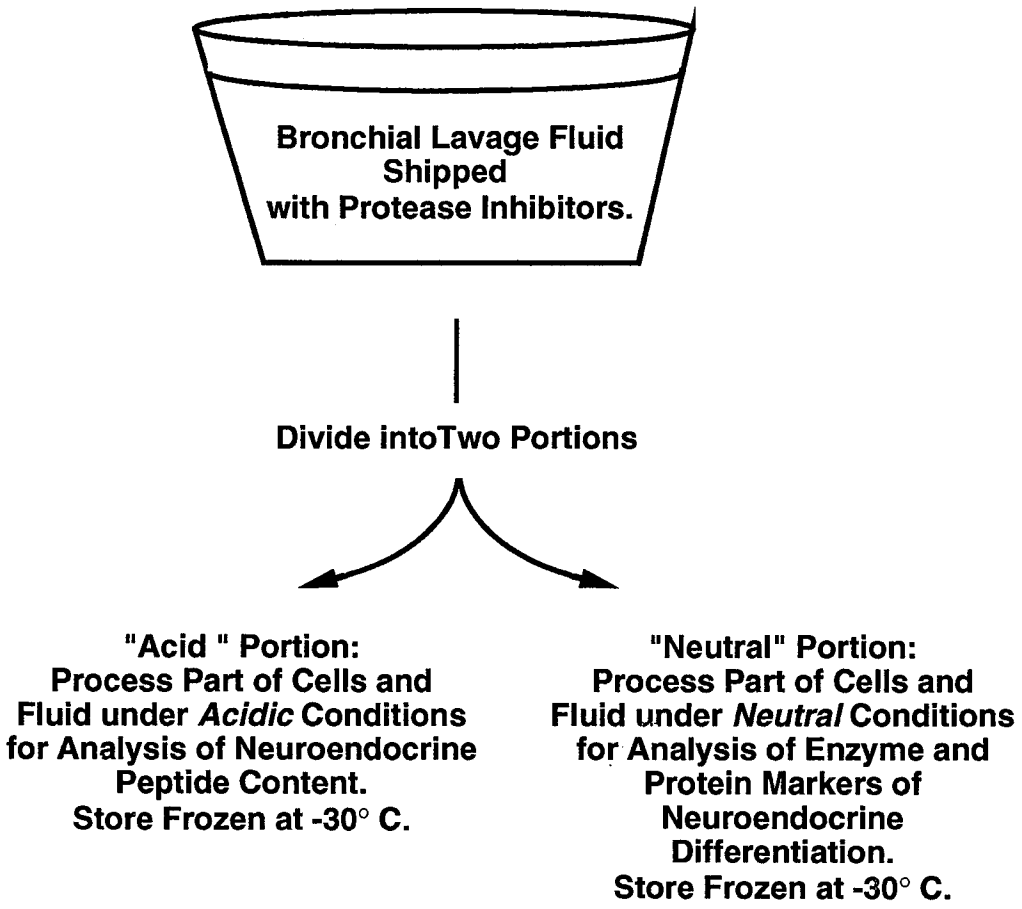

Fig. 2. Schematic representation of processing of bronchial lavage samples for biomarker analysis.

TABLE I. Protease Inhibitors* Used in Collection of Bronchioalveolar Lavage Fluid

Protease Inhibitor	Final Concentration	Protease Target
Aprotinin	7.8 μM	Serine proteases
Ethylenediamine tetra-acetic acid (EDTA)	100 μM	Metalloproteases
Leupeptin	45 μM	Serine and cysteine proteases
Pepstatin	1.0 μM	Acid proteases
Phenylmethyl sulfonyl-fluoride (PMSF)	0.5 mM	Serine and thiol proteases

* [34,35]

performed by competition of unlabeled GRP in the BAL for ^{125}I-2A11 to solid phase GRP. Aliquots of the acidified portion of BAL samples were dried under vacuum to remove acetic acid and redissolved for assay. The peptide amount in the sample is quantitated by comparison to a standard curve of known GRP concentrations and expressed in terms of total protein determined by the Pierce bicinchoninic acid reagent.

Radiolabeling of 2A11 antibody. 10 μg of MoAb 2A11 is radiolabeled with ^{125}I by the chloramine-T method as described by Kasprzyk *et al.* [32] to a specific activity of approximately 40 μCi/μg and stored at 4°C in a 0.2 M borate buffer pH 7.65, containing 1% BSA until use.

Solid phasing of human GRP_{1-27}. Polyvinyl chloride 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) are treated with 60 μl of 5% glutaraldehyde in 0.02 M sodium phosphate pH 7.4, 0.15 M sodium chloride, 0.02% sodium azide (PBS) per well for 1 hour at room temperature (RT), followed by washing with PBS. Human GRP_{1-27} (100 ng/well) in a 50 μl volume is added for overnight incubation at 37°C, 95% relative humidity. All wells are washed with PBS; 150 μl of PBS containing 1% bovine serum albumin (PBS/BSA) per well is added prior to storage at 4°C.

Peptidyl α-amidation assay. Assay methods are variations of Mizuno *et al.* [26] and Perkins [28,30,31] (Fig. 1). For the PHM and PAL assays, 2 ml of thawed, non-acidified BAL fluid are concentrated about tenfold using an Amicon Centricon-10 microconcentrator spun at 2000 × g.

PHM assay. *N*-acetyl-Tyr-Phe-Gly (acYFG) is converted to the C-terminal amidated analog *N*-acetyl-Tyr-Phe-amide (acYFamide) by sequential PHM and PAL activity. The assay is carried out in polypropylene microfuge tubes with 35 μl of assay mix and 5 μl of enzyme preparation. A set of assay mixes for PHM activity are prepared which contain approximately 150 mM MES buffer pH 6.25, and 20,000 cpm [^{125}I-Tyr]-acYFG, 0.2 μM unlabeled acYFG, 5 μl bovine catalase, 0.5 mM ascorbate, and a range of Cu^{2+} (as $CuSO_4$) concentrations from 100 to 500 μM.

In order to measure the level of PHM activity in a BAL sample, which potentially contains both PHM and PAL activity, any α-hydroxyglycine intermediate (*N*-acetyl-Tyr-Phe-α-hydroxyglycine, acYF(αHO)G) remaining in the PHM (pH 6.25) reaction mix is converted to acYF-amide by addition of 50 μl of 0.2 M sodium hydroxide for five minutes at 37°C. This base-catalyzed conversion step ensures that the assay is independent of PAL, and is therefore a measure of PHM activity only.

PAL assay. The assay is a modification of the method used by Perkins [28,30,31]. An aliquot of purified bovine or human PHM with no PAL activity is used with a PHM reaction mixture at reduced pH (150 mM MES, pH 5.5) to produce acYF(αHO)G. After five hours at 37°C, the PHM reaction is terminated by the addition of 100 μM of the covalent PHM inhibitor 4-phenyl-3-butenoate for 30 minutes [33]. The PAL reaction is carried out with 35 μl of this mixture and 5 μl of BAL sample for two hours at 37°C. Generally about 2–3% of total radioactivity is extracted into the organic phase in the absence of added PAL, whereas addition of NaOH before ethyl acetate extraction results in transfer of about 85% of the radioactivity into the organic phase. This amount represents almost complete conversion of acYFG to the PAL substrate acYF(αHO)G by purified PHM.

For all assays, the reaction is stopped by the addition of 200–250 μl 1.0 M HEPES (pH 7.5) buffer saturated with ethyl acetate, and 650 μl ethyl acetate saturated with the same HEPES buffer. The tubes were capped and mixed to extract the α-amide product into the ethyl acetate, which is counted in a gamma counter. Both acYFG and acYF(αOH)G are acidic and do not extract from aqueous pH 7.5 solution into organic solvent. The extraction procedure is validated with iodinated authentic [^{125}I-Tyr]-acYFG and [^{125}I-Tyr]-acYFamide; approximately 2% of radiolabeled acYFG compared to 90% of radiolabeled authentic acYFamide is extracted into the organic phase. Specific activities are calculated from respective enzyme activities and protein concentrations determined using Pierce bicinchoninic acid reagent in a microtiter plate format.

RESULTS AND DISCUSSION

A major concern in organizing the collection of BAL specimens from a number of different geographic locations is stabilizing the collected fluid from proteolytic degradation during shipping and processing. Proteolytic enzymes which

may be present on the bronchial epithelium come primarily from neutrophil cells [34]. Alveolar macrophages, T-lymphocytes, and mast cells are also capable of releasing proteases into the lung lining [34,35]. Examples of proteolytic enzymes in BAL include elastases and collagenases, which are metalloproteases, and cathepsins, which may be serine or cysteine proteases [34,35]. Plasminogen activator and chymotrypsin-like protease activities, which are serine proteases, have both been reported to be produced by alveolar macrophages [34,36]. In order to stabilize peptide growth factors which may be present in BAL fluids against proteolysis by these and other enzymes, a cocktail of protease inhibitors aimed at a wide range of proteases has been included in the collection of BAL specimens.

Eighteen BAL samples have been received from patients on the LCEDWG protocol. Fifteen samples have been assayed for the peptide amidating enzymes PHM and PAL and/or for bombesin-like immunoreactivity. PHM and PAL activities ranged from 0 to 87 pmol/hr/mg and 0 to 183 pmol/hr/mg, respectively. Bombesin-like immunoreactivity ranged from 32 to 206 fmol/mg.

Efforts are being made to measure dopa decarboxylase activity and epidermal growth factor levels in the BAL specimens but further refinement will be required before a critical appraisal of the utility of those assays as potential biomarkers is feasible.

Further experience with diverse patient populations is required to establish the normal range of expression of potential early detection markers such as PHM, PAL and GRP. Long-term clinical follow-up to determine which subjects on the LCEDWG protocol subsequently develop a new primary lung cancer may permit a preliminary assessment of the utility of these assays as early lung cancer detection tools. This process (neutralizing growth factors which drive the early phases of lung cancer progression) is proposed as an early step in the systematic development of more effective early detection approaches for this devastating cancer [37]. Rigorous assay validation to ensure the precision of biomarker determination under realistic study conditions is critical to the identification of potentially useful early lung cancer detection tools.

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