Application of Monoclonal Antibody 44-3A6 in the Cytodiagnosis and Classification of Pulmonary Carcinomas

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Thirty-five pulmonary carcinomas were studied retrospectively with monoclonal antibody (MCA) 44-3A6 raised against a human adenocarcinoma cell line. The antibody was applied to cytologic smears of bronchial brushings originally stained with the Papanicolau method, and to conventional tissue sections. Ten of 12 adenocarcinomas (ADC) immunostained strongly in sections and smears, as did five of seven large-cell "undifferentiated" carcinomas (LCUC). Eight neuroendocrine carcinomas (NEC) and eight squamous-cell carcinomas (SCC) were negative, except for rare weakly positive foci. We conclude that MCA 44-3A6 can be effectively applied on cytologic smears, and that it could be valuable in the precise classification of pulmonary carcinomas. The immunoreactivity of the ADC and SCC was predictable. Positive immunostaining in some LCUC confirms that these constitute a heterogeneus tumor class that includes cases that are phenotypically ADC despite the absence of obvious glands. Occasional immunostaining in NEC suggests focal exocrine differentiation as previously noted by electron microscopy. Diagn Cytopathol 1985;1:300–7.

Key Words: Pulmonary carcinomas; Classification; Cytodiagnosis; Immunocytochemistry; Monoclonal antibody

Current therapeutic approaches to pulmonary carcinomas vary considerably according to the histologic classification into the four major types: squamous-cell (SCC); adenocarcinoma (ADC); large-cell undifferentiated (LCUC); and neuroendocrine carcinoma (NEC).[1,2] Cytologic preparations, including bronchial brushings or fine needle aspirates, may be the earliest, or only, diagnostic material available at the time therapeutic decisions are made. The overall diagnostic accuracy for detecting malignancy in bronchial brushings is 89.6% for central and 70.9% for peripheral lesions,[3] but the accuracy in assigning histologic types is lower, especially for large-cell undifferentiated carcinoma (42%–91%).[3,4] Thus, alternative and/or adjunct modalities are needed to increase the accuracy of phenotypic classification required by current therapeutic protocols.

Cytoplasmic markers demonstrable by immunohistochemistry have proven to be useful in the study and diagnosis of pulmonary neoplasms in histologic sections. These include neuroendocrine markers,[2,5] carcinoembryonic antigen (CEA), beta-human gonadotropin (bHCG), pregnancy-specific β_1 glycoprotein (SP1),[6] and intermediate filaments.[5–7] We[8,9] and others[10] have used immunohistochemical techniques to demonstrate neuron-specific enolase (NSE) and several neuropeptides in cytologic preparations from cases of small-cell NEC, but surprisingly few investigations on the application of immunohistochemical markers in the cytodiagnosis of lung neoplasms have been published to date.

Recently, a newly developed monoclonal antibody (MCA), 44-3A6, raised against a human pulmonary adenocarcinoma cell line has been shown to distinguish adenocarcinomas and some large-cell carcinomas from other types of pulmonary carcinoma in histologic sections.[11,12] We undertook the present study to determine if this antibody would be similarly applicable on cytologic preparations previously fixed in 95% alcohol and stained by the Papanicalou technique. We also wished to determine if it would be as discriminating in the cytologic material as in tissue sections.

Materials and Methods

Thirty-five cases of pulmonary carcinoma that had both positive bronchial brushings and tissue specimens avail-

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able for review were selected from the files of Rush-Presbyterian-St. Luke's Medical Center. We required that a definite diagnosis and classification of the tumor could be made on the tissue specimen, and that ample cells be present in both types of specimens for the application and unambiguous interpretation of the immunohistochemical findings. Tumors were classified as ADC, LCUC, NEC, and SCC. Histologic classification was based on the WHO system as modified by Gould et al to include neuroendocrine carcinomas[1,2]; cytologic classification was made according to Kato et al.[3] Patient charts were reviewed to determine the extent of work-up, to ascertain that the tumors under study were indeed primary pulmonary carcinomas, and to establish whether they had been subjected either to radiotherapy or to chemotherapy prior to sampling. In two cases, radiation therapy was administered prior to resection, and in one case prior to the brushings as well.

Case Material

Twelve cases of adenocarcinoma included eight men and four women, age range 52–88. Although minimal clinical work-up had been performed to exclude other primaries, in all cases none was apparent. Three cases had metastases in a distribution compatible with lung primary at the time of presentation; only one patient who presented with widespread metastases, including the chest wall lesion studied here, represented a questionable pulmonary primary, although no other primary was demonstrable clinically. Seven cases of large-cell undifferentiated carcinoma included three men and four women, age range 33–73 yr; all were considered to be lung primaries. Eight cases of neuroendocrine carcinoma included six men and two women, age range 54–71; all cases were established as pulmonary primaries. Eight cases of squamous-cell carcinoma included seven men and one woman, age range 62–81; all cases were convincingly established as pulmonary primaries.

Immunohistochemical Studies

MCA 44-3A6 was produced using the well-described hybridoma technology; the antibody was raised against the human pulmonary adenocarcinoma cell line culture NCI-A549. It is a murine antibody of IgG1 isotype that recognizes a protein antigen of about 40,000 kd.[11]

Tissue specimens were formalin-fixed, paraffin-embedded, and stained with H&E for light microscopy. Immunohistochemistry was performed on deparaffinized sections cut from the same blocks. Cytologic specimens consisted of direct smears of brushings made at the time of bronchoscopy; these were fixed immediately in 95% alcohol and stained by the Papanicalou technique. Two slides with numerous well-preserved tumor cells distributed both singly and in clusters were selected from every case. Immunohistochemistry was performed directly on one slide; the other was destained first by passing it through graded alcohols. Immunostaining was performed with Avidin-Biotin complex (ABC) method (Vector Laboratories, Burlingame, CA) as previously described.[11,12] MCA 44-3A6 was used at a concentration of approximately 1 μg/ml. Slides were slightly counterstained with hematoxylin for 1.5 min, hydrated, and mounted conventionally. Negative controls were performed by omitting primary antibody and substituting a nonreactive MCA of the same isotype.

Results

Immunostaining was obtained in all slides as judged by staining in the nonneoplastic respiratory epithelial cells, which were the built-in controls (Table I). There was no significant difference between slides that had been immunostained directly and those that had been destained first. Reactive alveolar lining cells and macrophages were occasionally positive, but they could easily be distinguished from the tumor cells and did not pose a problem in interpretation. In the two cases with prior radiation therapy, immunostaining was weaker and less consistent in tissue than in the brushings.

Adenocarcinoma

Tissue specimens consisted of six biopsies, five resections, and one biopsy of a chest wall metastasis. One patient had received radiation therapy prior to both brushings and resection. Microscopically, the tumors, as defined, exhibited gland formation in over 50% of the sample. Cells were of intermediate or large size, with vesicular nuclei and prominent eosinophilic nucleoli (Fig. C-1*A*). The radiated case showed extensive necrosis and marked stromal fibrosis. Immunohistochemical staining was

Table I. Immunocytochemical Staining in Cytologic Smears and Tissue Sections of 35 Cases of Pulmonary Carcinoma

		Cytology			*Tissue*		
Tumor type	*Number of cases*	*Strong positive*	*Weak positive*	*Negative*	*Strong positive*	*Weak positive*	*Negative*
Adenocarcinoma	12	10	2	0	10	2	0
Large cell carcinoma	7	5	1	1	2	2	3
Neuroendocrine carcinoma	8	0	1	7	0	1	7
Squamous carcinoma	8	0	2	6	0	3	5

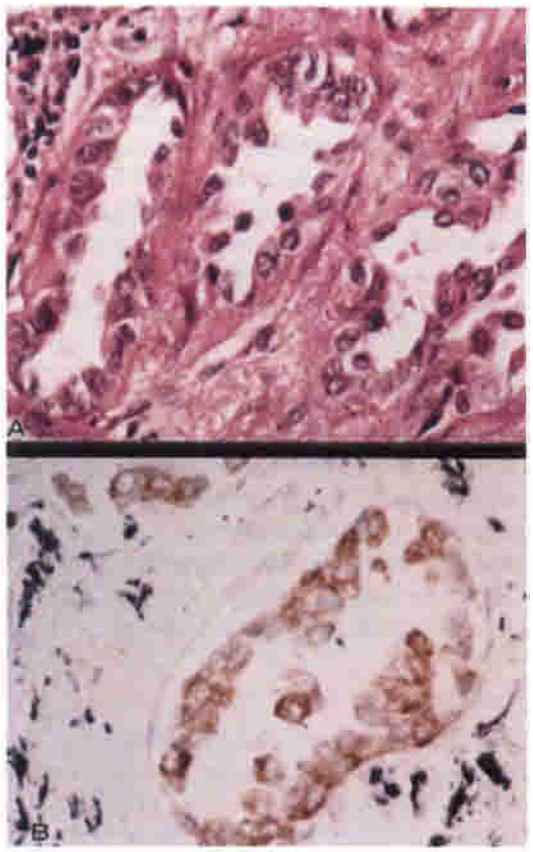

Fig. C-1.

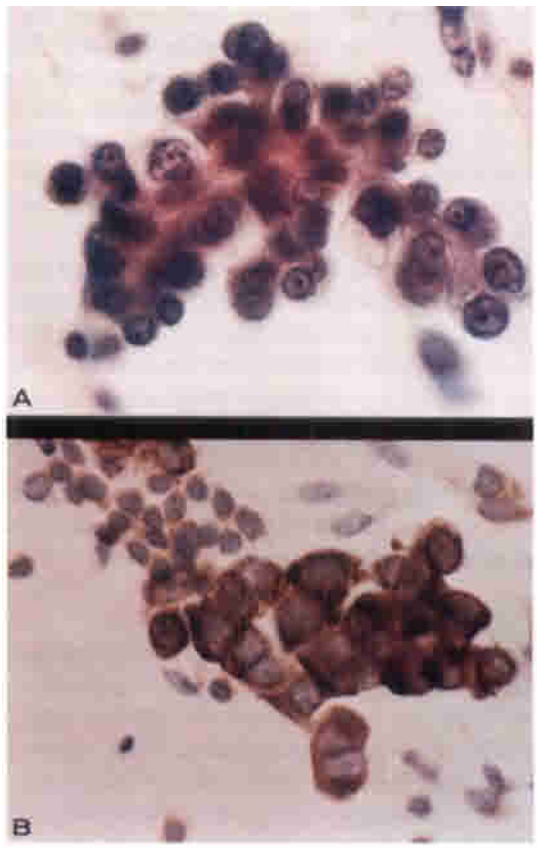

Fig. C-2.

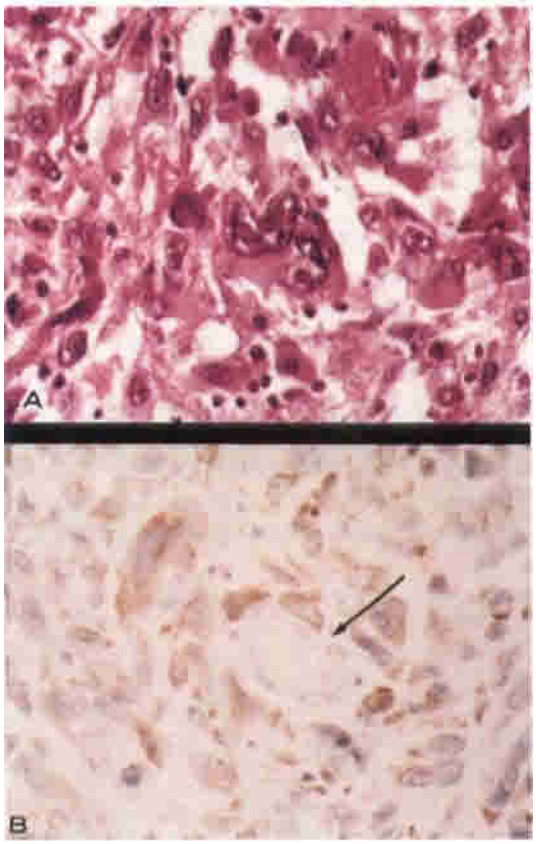

Fig. C-3.

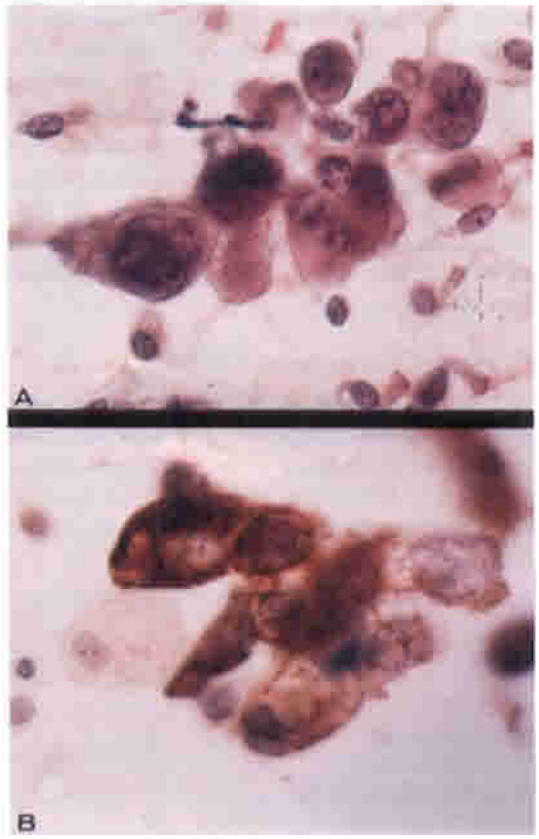

Fig. C-4.

strongly and diffusely positive in ten cases (Fig. C-1*B*). In two cases, tumor cells showed variably intense positivity, with some cells exhibiting only faint staining. One of these was the radiated case, and the other was a bronchioloalveolar carcinoma. In one additional case, a few foci of squamous differentiation were notably negative.

Cytologically, the brushings from seven cases had been diagnosed as adenocarcinoma; four were called poorly differentiated carcinoma with features of adenocarcinoma, and one case was diagnosed as large-cell undifferentiated carcinoma with features of adenocarcinoma. Tumor cells in the brushings were distributed singly and in clusters. Nuclei ranged from 8 to 14 μm in greatest dimension and were round or oval with only mild pleomorphism. Nuclear membranes were thick. Eosinophilic nucleoli were prominent; perinucleolar parachromatin clearing was frequently seen. Cytoplasm was abundant and delicately structured; vacuoles were frequent. Occasional mitoses were present in cell clusters (Fig. C-2*A*).

In the immunohistochemical preparations of the brushings, tumor cell clusters were easier to identify than single cells and more reliable to evaluate for positivity due to better preservation of the cytoplasm within the clusters. In 10 of the 12 cases, including the radiated one, all tumor cells were strongly positive (Fig. C-2*B*). The remaining two cases showed mostly positive and occasional negative clusters.

Large-Cell Undifferentiated Carcinoma

Tissue specimens included five biopsies and two lobectomies; one of the latter was performed after radiation therapy. Microscopically, the tumors consisted of nests and sheets of large cells with marked nuclear pleomorphism, prominent eosinophilic nucleoli, and abundant eosinophilic cytoplasm (Fig. C-3*A*). Mitoses were frequent. The previously radiated tumor displayed extensive necrosis. Four cases exhibited focal formation of gland lumens while two were felt to have some squamous features.

Immunostaining was strong and diffuse in two cases (Fig. C-3*B*) and focal in two cases; three cases did not immunostain. The negative cases included one with features of adenocarcinoma.

Cytologically, five cases had been diagnosed as large-cell undifferentiated carcinoma, and two as adenocarcinoma. All contained tumor cells distributed both singly and in clusters. Nuclei were 9–21 μm in greatest dimension and contained prominent, often large or irregular eosinophilic nucleoli and coarse chromatin. Cytoplasm was abundant; it ranged from delicate and vacuolated to optically dense (Fig. C-4*A*).

Immunohistochemical staining was strongly and diffusely positive in five cases (Fig. C-4*B*), variable in one case, and negative in one case. The negative case also had negative histology but showed questionable features of adenocarcinoma both cytologically and histologically.

There were three cases with discrepancies in staining between cytology and tissue specimens. In two cases cytology was positive and tissue was negative; in the third, the radiated patient, cytology was strongly positive and tissue was weakly positive.

Neuroendocrine Carcinoma

Tissue specimens included seven biopsies and one lobectomy. Six cases were diagnosed as neuroendocrine carcinoma, small-cell type, and two as intermediate cell type. The latter pair included the case with lobectomy, which was performed for the initial—incorrect—diagnosis of adenocarcinoma. The diagnosis was subsequently revised after examination of more abundant tissue from the lobectomy; electron microscopic study of this case showed prominent membrane-bound granules. The small-cell carcinomas were present as sheets of cells underneath the bronchial mucosa (Fig. C-5*A*). Necrosis and crushing artifact were prominent. Preserved nuclei were round, oval, or fusiform. Nuclear molding, sparse cytoplasm, and inconspicuous nucleoli were distinctive features. The intermediate cell NE carcinomas consisted of larger cells whose pleomorphic nuclei had more stippled chromatin and a more distinct cytoplasmic rim.

Immunohistochemical staining was negative in all but the resected case of intermediate cell neuroendocrine carcinoma, where a few cells were positive (Fig. C-5*B*).

Cytologically, all of these cases had been originally diagnosed as small-cell carcinoma except the resected case, which had been diagnosed as adenocarcinoma. Tumor cells were dispersed singly and in loosely cohesive groups in mucous strands. Small-cell nuclei ranged from 5 to 10 μm. All small-cell cases showed round or fusiform nuclei, stippled chromatin, absent nucleoli, and prominent nuclear molding. Necrosis was prominent (Fig. C-6*A*). The intermediate cell cases differed by having larger nuclei, 8–13 μm in diameter, a looser chromatin pattern, identifiable though not prominent nucleoli, and considerably more cytoplasm. Cytologically, intermediate cell NE carcinomas resembled adenocarcinomas; indeed, the one case had been originally diagnosed as such.

None of these cases immunostained with MCA 44-3A6

Fig. C-1. Pulmonary adenocarcinoma. **(A)** Tissue section (H&E stain, × 300). **(B)** Positive MCA 44-3A6 immunostaining (× 300). **Fig. C-2.** Pulmonary adenocarcinoma. **(A)** Bronchial brushings (Papanicolaou stain, × 480). **(B)** Positive MCA 44-3A6 immunostaining in cluster of tumor cells (× 480). **Fig. C-3.** Large-cell undifferentiated carcinoma. **(A)** Tissue section (H&E stain, × 300). **(B)** Positive MCA 44-3A6 immunostaining in some tumor cells. Note negative vessel (arrow) (× 300). **Fig. C-4.** Large-cell undifferentiated carcinoma. **(A)** Bronchial brushings (Papanicolaou stain, × 480). **(B)** Positive MCA 44-3A6 immunostaining in cluster of tumor cells (× 480).

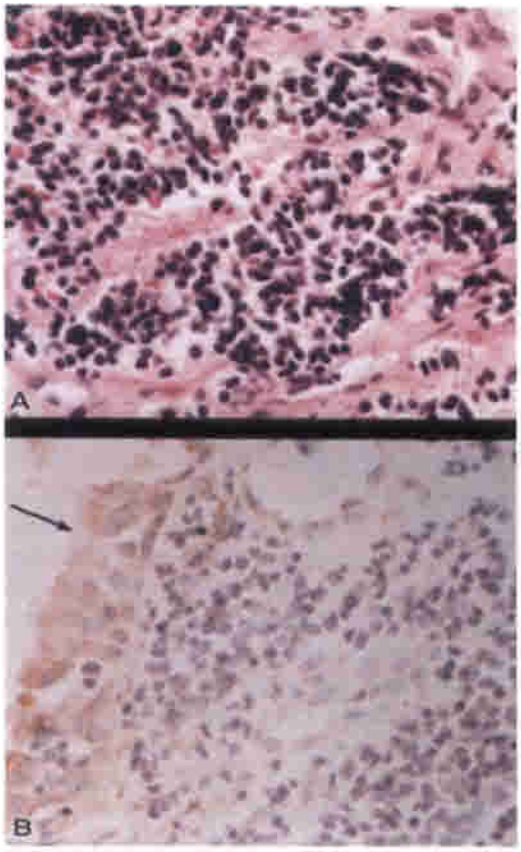

Fig. C-5.

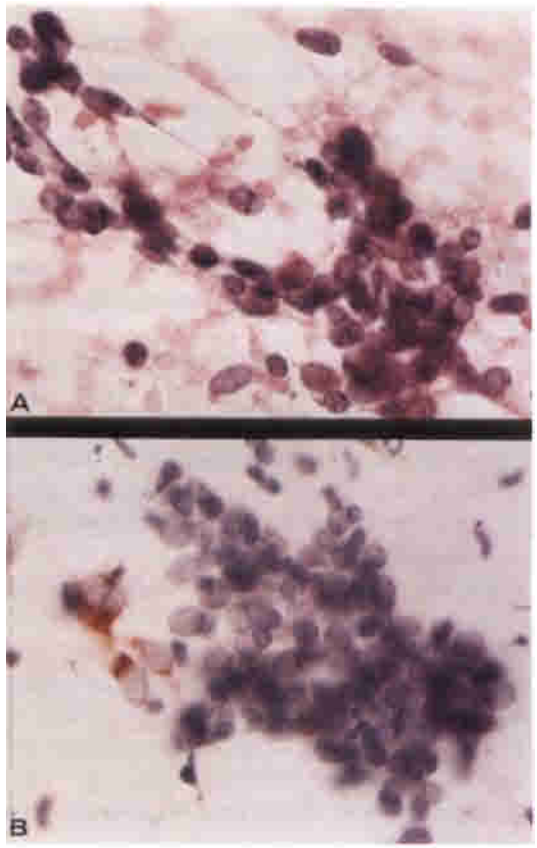

Fig. C-6.

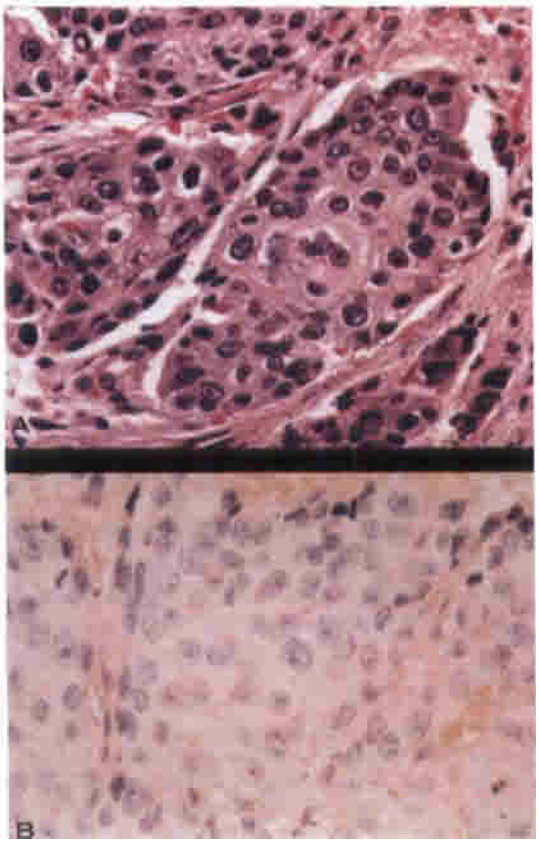

Fig. C-7.

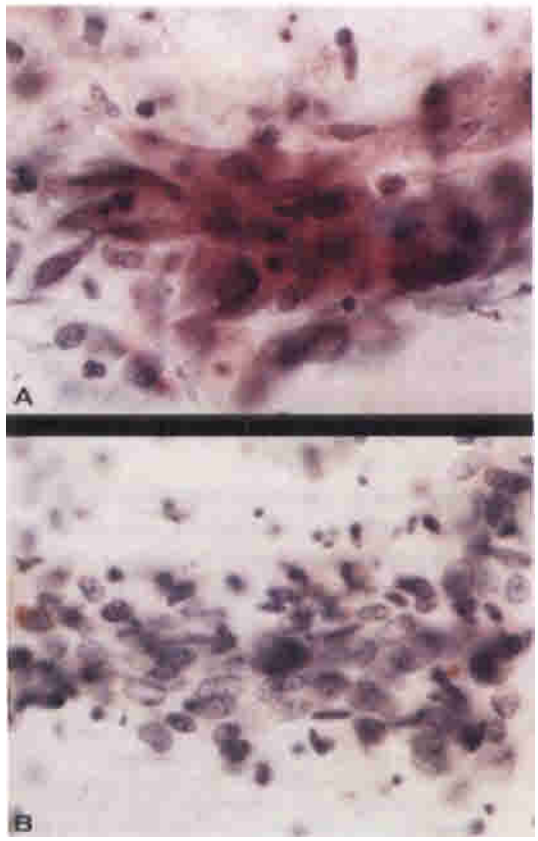

Fig. C-8.

(Fig. C-6*B*) except for the case originally called adenocarcinoma, in which there were occasional positive cells, as was the case in the corresponding surgical specimen.

Squamous Carcinoma

Tissue specimens consisted of six biopsies and two resections. The tumor was well differentiated in one case, moderately differentiated in six cases (Fig. C-7*A*), and poorly differentiated in one case.

Immunohistochemical staining was negative in five cases; in three cases there was faint positivity around the edges of clusters, often where cells were markedly fragmented. None of these cases was convincingly positive (Fig. C-7*B*).

Cytologically, neoplastic cells were present singly or in irregular groups (Fig. C-8*A*). There was cellular and nuclear pleomorphism. Nuclei were hyperchromatic or vesicular, with coarse chromatin and eosinophilic nucleoli. They ranged in diameter from 9 to 19 μm. Cytoplasm was plentiful, optically dense, and either eosinophilic or cyanophilic. There was scattered necrotic debris. These tumors had been diagnosed as well (two), moderately (three), or poorly (two) differentiated based on the number of keratinized cells and the degree of cellular and nuclear pleomorphism. One case had been called large-cell undifferentiated carcinoma.

Immunocytochemistry was negative in six cases (Fig C-8*B*). Faint positivity was found in occasional cells in two cases, one of which had been faintly positive in the tissue sample.

Discussion

Current therapeutic protocols require high diagnostic accuracy in the detection and classification of tumors. Frequently, a cytology specimen is the earliest or only material available for this decision-making process. The overall accuracy for the cytologic detection of pulmonary neoplasms ranges from 80% to 98%.[13] In the series of Kato et al., accuracy ranged from 65% for sputums, to 81% for bronchial brushings, to 89% for percutaneous needle aspirates.[3] When studied according to tumor location, bronchial brushings detected accurately 89.6% of central lesions and 70.9% of peripheral lesions.[3] These excellent overall detection rates for pulmonary neoplasms are offset, however, by distinctly lower accuracy and considerable observer variability regarding precise cytomorphologic types, particularly LCUC. Accuracy in correlating cytologic and histologic typing of lung tumors ranges from 75% to 94% for SCC, 68% to 86% for ADC, 42% to 91% for LCUC, and 83% to 96% for small-cell NEC.[3,4,14] In one study, interobserver variability in typing histologic sections of lung tumors ranged from 2%–5% for well-differentiated adeno- and squamous carcinomas, to 23%–25% for undifferentiated large-cell and small-cell carcinoma, to 40%–42% for poorly differentiated adeno- and squamous carcinomas.[15]

In our study, cytology-histology correlation was 100% for ADC, with the caveats that in four cases cytology samples were diagnosed as poorly differentiated carcinoma with features of adenocarcinoma, and one case was diagnosed as large-cell undifferentiated carcinoma with features of adenocarcinoma. Correlation for LCUC was 75% accurate; two cases were called adenocarcinoma on brushings. Correlation for NEC was 88% accurate; one case of intermediate cell NEC was called adenocarcinoma on brushings while the other was called small-cell NEC. In the former case, MCA 44-3A6 staining was strongly positive and would have allowed proper classification as an ADC. Accuracy in correlation for SCC was 88%; one case had been diagnosed as large-cell undifferentiated carcinoma on brushings.

Thus, our observations as well as those of others indicate that the two major problems in the cytologic classification of lung tumors are: (1) to distinguish among poorly differentiated variants of adeno- and squamous carcinoma and intermediate cell NEC, and (2) to properly subclassify LCUC into phenotypically adeno-, squamous, or truly undifferentiated categories.

The nebulous status of LCUC in the overall classification of pulmonary neoplasms doubtless contributes to diagnostic inaccuracy. Indeed, although LCUC is defined as a tumor comprised of large cells without obvious glandular or squamous differentiation in the WHO classification,[1] other investigators regard LCUC as a group actually comprising large-cell variants of both adeno- and squamous carcinomas.[3,16] In the past these tumors have been classified with both squamous and adenocarcinoma.[13,17] In our series, the LCUC group displayed the most variability of immunostaining with MCA 44-3A6 and the most discrepancies between cytology and tissue interpretations. Thus, our findings support the notion that LCUCs constitute a phenotypically heterogeneous group of neoplasms. Interestingly, criteria for nuclear size are not given in many descriptions of LCUC,[3,4,16] and a subjective estimate of what constitutes "large" seems surprisingly acceptable to most diagnosticians. We were

Fig. C-5. Pulmonary neuroendocrine carcinoma, small-cell type. **(A)** Tissue section (H&E stain, × 300). **(B)** Negative MCA 44-3A6 immunostaining in tumor and vessels. Note weak positivity in surface epithelium (arrow) (× 300). **Fig. C-6.** Pulmonary neuroendocrine carcinoma, small-cell types. **(A)** Bronchial brushings (Papanicolaou stain, × 480). **(B)** Negative MCA 44-3A6 immunostaining in cluster of tumor cells. Four respiratory epithelial cells are positive (× 480). **Fig. C-7.** Pulmonary squamous-cell carcinoma. (A) Tissue section (H&H, × 300). **(B)** Negative MCA 44-3A6 immunostaining (× 300). **Fig. C-8.** Pulmonary squamous-cell carcinoma. **(A)** Bronchial brushings (Papanicolaou stain, × 480). **(B)** Negative MCA 44-3A6 immunostaining in cluster of tumor cells (× 480).

similarly surprised to note that actual determination of nuclear dimensions carried out retrospectively in the brushings were not significantly different among our cases of large-cell carcinoma (9–21 μm; average, 14 μm), adenocarcinoma (8–14 μm; average, 11 μm), and squamous carcinoma (9–19 μm; average, 12 μm). Thus, given the reported levels of accuracy, the acknowledged interobserver variability, and other pitfalls in the diagnosis of LCUCs, it is not surprising that the group, as currently defined, includes tumors of variable phenotypic characteristics. Adjunct diagnostic modalities including the immunohistochemical demonstration of phenotype-specific antigens may become a useful means to refine our concepts and to improve the diagnostic identification of these tumors.

Immunohistochemical techniques have been widely and consistently applied in the diagnosis of pulmonary tumors in histologic sections; the NEC group has been most extensively studied.[2,5,18] Cytoplasmic markers most frequently and reliably demonstrable in nonneoplastic and neoplastic neuroendocrine cells of the lung include NSE, serotonin, and a broad spectrum of neuropeptides.[2,18] Recent studies of both the cytoskeletal proteins and neuropeptide hormones using immunocytochemical, electron microscopic, and two-dimensional gel electrophoresis techniques have demonstrated coexpression of cytokeratins and neuroendocrine markers such as NSE, bombesin, etc., in all variants of pulmonary neuroendocrine tumors, supporting the notion that NE substances can be synthesized in cells that, as defined by their cytoskeletal features, are typically epithelial.[5] These observations are not limited to neuroendocrine neoplasms of the lung since similar findings and conclusions have been reported with regard to neuroendocrine neoplasms of the skin.[19] Numerous additional studies have been performed on pulmonary carcinomas utilizing antibodies against intermediate filaments[6,7,20–23]; unfortunately, in some of those studies the antibodies were poorly characterized, and the antigens were very probably altered or masked by conventional fixation methods, thus resulting in apparently contradictory and, at times, demonstrably invalid conclusions.[22,24] Other types of antigens have been investigated in attempts to differentiate more accurately among different types of pulmonary carcinomas; they include CEA and bHCG,[6,23] SP1,[6] IgA and secretory piece, alpha-fetoprotein, and surfactant high-molecular-weight glycoprotein.[23]

To date, immunohistochemical techniques have not been widely applied in the cytologic diagnosis of lung tumors. Springall et al.[10] demonstrated NSE immunoreactive cells in pleural and ascitic fluid specimens and imprints of bronchial biopsies from patients with small-cell NEC of the lung using the peroxidase-antiperoxidase method. With the same method, we demonstrated NSE and leu-enkephalin immunoreactive tumor cells in both cytologic preparations of positive pleural fluids and histologic sections of tumor tissue from cases of pulmonary neuroendocrine carcinomas.[9] Yam and Winkler[25] were able to distinguish between small-cell NEC and lymphoma in pleural fluid using an oat-cell-carcinoma–specific monoclonal antibody with an immunoalkalin-phosphatase technique. The groups of adeno- and squamous carcinoma, particularly the poorly differentiated variants, and the large-cell undifferentiated carcinomas still defy accurate classification for lack of reliable markers; CEA and keratins, while readily demonstrable, do not discriminate between these groups.

Monoclonal antibody 44-3A6 was raised against a human adenocarcinoma cell line NCI-A549 in tissue culture. The antigen appears to be related to cell membrane components but is distinct from CEA.[11] The role of this antigen is not clear, but it may be related to cell differentiation toward glandular or secretory activity.[12] Immunoreactivity has been shown in reactive type II pneumocytes and some but not all normal bronchial epithelial cells. Immunoreactivity in pulmonary tumors was observed in all adenocarcinomas and some of the better differentiated NE tumors, particularly carcinoids and well-differentiated NE carcinomas.[12] The latter observation is not surprising given the known presence of phenotypically "exocrine" cells in some NE neoplasms.[2,18] Small-cell NE carcinomas, squamous-cell carcinomas, and bronchioloalveolar carcinomas were negative.[12] Our findings in the tissue sections appear to differ from those of Lee et al. only in that our two bronchioloalveolar carcinomas did immunostain with MCA 44-3A6 whereas in their study tumors of this type did not.[12] These apparently conflicting results may be readily explained if we consider that bronchioloalveolar carcinomas constitute a highly heterogeneous group that may include Clara cells, mucin-secreting bronchial cells, type II alveolar cells, and even endocrine phenotypes.[23,26–28] We may thus have a highly discriminating antibody for exocrine or adenocarcinoma phenotype; however, it should be stated that, as of now, we have no evidence that this marker can distinguish between primary and metastatic pulmonary adenocarcinomas. Our meager data from two cases suggest that radiation may diminish but not eradicate immunostaining in ADC and LCUC.

In conclusion, a monoclonal antibody derived from a human lung adenocarcinoma cell line in culture and directed against an antigen apparently related to some features of exocrine differentiation has been shown to immunoreact not only with obvious—pulmonary—adenocarcinomas but also with some presumably "undifferentiated" large-cell carcinomas, thus suggesting that some LCUC are phenotypically adenocarcinomas despite the absence of recognizable glands by light microscopy.

The antigen is retained and can be readily demonstrated immunohistochemically in conventionally fixed, processed, and paraffin-embedded tissue sections and in alcohol-fixed, Papanicalou-stained cytologic preparations. We tentatively suggest that MCA 44-3A6, together with antibodies to various molecular weight cytokeratin polypeptides, NSE, and some selected neuropeptides such as bombesin, would constitute an excellent immunomarker panel that could notably enhance our capability to accurately classify pulmonary carcinomas on conventional cytologic specimens.

Acknowledgments

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