

THE SECOND K. PRATHAP MEMORIAL LECTURE

CANCER OF THE LUNG: AN IMMUNOCYTOCHEMICAL, HISTOLOGICAL AND ULTRASTRUCTURAL STUDY.

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Summary

A light and electron microscopical study of lung cancer combined with immunocytochemistry using monoclonal antibodies has led to the conclusion that this disease should be considered a single entity. The various classifications used are largely meaningless from the point of view of histogenesis as nearly all lung cancers exhibit cellular heterogeneity with any individual tumour showing characteristic features of squamous, glandular and small cell differentiation. It is suggested that the practice of grading tumours for prognostic purposes might be replaced by a system which identifies the percentage of cells labelling with the monoclonal antibody Ki67 which specifically labels an antigen in nuclei of proliferating cells. Attention is drawn to the frequent co-expression of intermediate filament proteins in lung cancer.

Key words: Lung neoplasms, immunohistochemistry, monoclonal antibodies, intermediate filament proteins.

INTRODUCTION

Cancer of the lung is the leading cause of mortality from malignant disease in the Western world. A distinctly unusual disease, or at least a rarely diagnosed one, until the 1920's it has now reached the proportions of an epidemic. The possibility that this increase in incidence was apparent, due to more accurate methods of diagnosis, was shown to be invalid by the epidemiological investigations conducted by Doll¹ and others. Environmental and occupational factors, among which the most notable is cigarette smoking, are implicated in this striking increase. In the 1950's men were affected more frequently than women; in 1959 the ratio of men to women dying of lung cancer was 6.7 but 20 years later in 1979 it had dropped to 3.6. There is a long lag period between onset of smoking and development of lung cancer and this change is thought to be due to the fact that women did not start smoking cigarettes until 25 years after the habit had become common in men. Aside from cigarette smoking other factors are of relatively minor significance but there are certain well established hazards notably in industries

involving mining of radioactive ores, asbestos and production of coal gas. Although incidence of lung cancer correlates well on a world wide basis with tobacco use it has been claimed that there are variations in geographic pattern. Thus Jindal *et al*² found that in Northern India patients were younger and there was a higher proportion of non-smokers than in Western countries.

There has been some debate as to the type of lung cancer involved and it was popularly believed that adenocarcinoma bore little relation to smoking habits. Kreyberg and Saxen³ working in Norway and Finland reported that smoking-related cancers were epidermoid or oat cell. In contrast Yesner, Gelfman and Feinstein⁴ found that among smokers the quantity of cigarettes smoked was directly related to prevalence rates for small cell cancer but not epidermoid and other cell types. In Wales Harley⁵ analysed 132 women with lung cancer and found the following percentage distribution – epidermoid 28.5, oat cell 35.9, adenocarcinoma 27.2, large cell 5.8, other types 2.5. It has usually been maintained

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that adenocarcinoma of the lung has only a weak relationship to smoking but in the United States one review of 1,682 cases⁶ over a period of 13 years found that adenocarcinoma was becoming progressively more prevalent compared with other forms of lung cancer. This was considered to be due to the increasing number of women who smoked. Yet it has been suggested⁷ that lung carcinoma of all types may be related to cigarette smoking and indeed **some** studies noted a clear relationship between smoking and adenocarcinoma in both **men**⁸ and **women**.⁹ Much more stringent epidemiological evidence has recently been forthcoming in a study of white women in Los Angeles¹⁰ where in over half of a series of 149 cases with adenocarcinoma smoking appeared to be the main aetiological factor.

CLASSIFICATION OF LUNG CANCER

These apparent discrepancies with regard to cell type are reflected in the complexities found in various classifications of lung cancer. First among these is that produced by WHO¹¹ in 1967 and revised in 1982.¹² This is both elaborate and detailed including all tumours whether benign, malignant, mesothelial, epithelial or connective tissue. Here we are only concerned with epithelial neoplasms (Table 1). Scrutiny of this table at once indicates that the nomenclature is somewhat confusing and practising histopathologists attempting to place a tumour into one of the categories listed encounter some difficulty. This is because careful perusal of any given tumour will often reveal it to contain more than one cell type although it is usual for one variety to be dominant.

These classifications have all been based on conventional light microscopy and attempts have been made to cope with difficulties by using such terms as adenosquamous or even ignoring the situation altogether and stating, for instance, that predominantly squamous carcinomas with evidence of small areas of mucus secretion should be placed firmly in the squamous category. A further point of importance is that in many instances the majority of diagnoses are based on bronchial biopsy material which is of necessity small in quantity and represents the most superficial part of the tumour.

It has been one of the tenets of tumour pathology that each cancer has a parent cell type from which it arises. Thus squamous carcinoma is derived from squamous epithelium, metaplastic in nature in the case of the

bronchus, adenocarcinoma from glandular tissue and so forth. This seems an oversimplification and for this reason an investigation was undertaken^{13,14} into the pathology of a series of lung cancers using only resection specimens so that the entire tumour was available for examination, employing not only conventional light microscopy and histochemistry but also immunocytochemistry using monoclonal antibodies, and electron microscopy. The question we wished to answer was 'Do different histological categories of lung cancer originate from separate cell types?'

MATERIALS AND METHODS

All lung specimens were received fresh from the operating theatre. Representative samples were taken from the tumour, snap frozen and stored in liquid nitrogen. Adjacent small samples were selected for ultrastructural examination and fixed immediately in 4 per cent glutaraldehyde at 4°C. The remainder of the material was fixed in formol saline and samples processed for light microscopy.

For immunocytochemical examination cryostat sections were cut from snap frozen material and stained by a three-stage immunoperoxidase method or the alkaline phosphatase antialkaline phosphatase technique^{15,16} using a panel of monoclonal antibodies as summarised in Table 2.

Initially 66 tumours were available and these were independently classified on light microscopy by two observers into one of the following four groups – squamous cell carcinoma, small (oat) cell carcinoma, adenocarcinoma, large cell carcinoma or adenosquamous carcinoma. The criteria used to allocate a tumour to one or other of these categories has been given elsewhere.⁴

The ultrastructural features which were considered to indicate differentiation towards a given class of tumour were as follows:

Squamous carcinomas at their most differentiated were characterised by tonofilaments converging on well developed desmosomes or by isolated tonofilaments in the cytoplasm. Keratohyaline granules and wide intercellular spaces bridged at desmosomal sites were also useful indicators.

Adenocarcinoma showed intracellular secretory vacuoles, well developed endoplasmic reticulum, prominent Golgi apparatus and plentiful mitochondria.

Small cell carcinoma typically contained dense core cytoplasmic neurosecretory granules in their cytoplasm. These were by

TABLE 1
REVISED WHO CLASSIFICATION OF LUNG
CARCINOMA

-
1. Squamous cell carcinoma
Variant:
 Spindle cell carcinoma
 2. Small cell carcinoma
 - (a) Oat cell carcinoma
 - (b) Intermediate cell type
 - (c) Combined oat cell carcinoma
 3. Adenocarcinoma
 - (a) Acinar adenocarcinoma
 - (b) Papillary adenocarcinoma
 - (c) Broncho-alveolar cell carcinoma
 - (d) Solid carcinoma with mucus formation
 4. Large cell carcinoma
Variants:
 - (a) Giant cell carcinoma
 - (b) Clear cell carcinoma
 5. Adenosquamous carcinoma
-

TABLE 2
MONOCLONAL ANTIBODIES USED FOR
ANALYSIS OF HUMAN LUNG TUMOURS

Anti EMA	HMFG-1, HMFG-2, E29
Anti Cytokeratin	LE61, LP 64 Hep 29, Hep 54, Hep 111
Anti prekeratin	K20, K92
Anti CEA	11, 285, 14
Anti neural antigen	UJ 13 A

no means plentiful and had to be searched for with great care.

Large cell carcinoma is a term reserved for malignant epithelial tumours which on light microscopy cannot be placed into one of the above categories. Yet electron microscopic examination of such tumours has nearly always revealed features of one of the above categories.^{7,18}

RESULTS

Immunocytochemistry

Immunocytochemical investigation of tumours classified by light microscopy into one of the WHO categories revealed a considerable overlap in antigenic profile (Table 3). Epithelial membrane antigen, also known as human milk fat globule membrane antigen, is found in normal lung tissue on luminal surfaces of bronchial epithelium and bronchial mucous glands, so it is not surprising that virtually all lung carcinomas examined are positive for this antigen. There is, however, some difference in emphasis in distribution. Squamous carcinoma shows focal positive staining often localised to less well differentiated areas whereas adenocarcinomas, and to a lesser extent oat cell carcinomas, show uniform labelling throughout the tumour (Figs. 1 and 2).

The cytokeratin antibodies LE61 and LE34 which detect intermediate filaments between 44 and 67 kd label all the tumours but are more strongly positive in poorly differentiated areas. There is striking reciprocal staining with the antibodies K20 and K92, specific for prekeratin, which label keratinising squamous areas (Fig. 3). Adenocarcinomas seldom stain with K20 and K92 antibodies but focally positive areas are often found in oat cell carcinoma (Fig. 4).

The antibody to carcinoembryonic antigen has been employed more frequently and for a longer period than any other in tumour pathology. No normal tissues were stained with this in our series, carcinoid tumours were negative, oat cell and adenocarcinomas were diffusely labelled and there was focal labelling of squamous tumours.

The antibody UJ13A appeared to be the most effective for recognising an antigen present on a wide variety of neural and neurally related tissues and does not label normal bronchial or alveolar tissue. It is diffusely present in oat cell carcinomas (Fig. 5) and in carcinoid tumours but of greater interest is the focally positive staining which can be seen in squamous (Fig. 6) and adenocarcinomas often in areas which on review show differentiation towards an oat cell type tumour.

Immunohistology related to histological category

Although tumours showing clear differentiation towards one of the well recognised classes in the WHO classification tended to demonstrate a characteristic immunocytochemical profile there was often crossreactivity. Thus all tumours expressed positive staining with one or more of the anticytokeratin anti-

TABLE 3
ANTIGENIC PROFILE OF VARIOUS WHO CATEGORIES OF LUNG CANCER*

WHO Classification	Epithelial Membrane Antigen	Cytokeratins	Prekeratins	CEA	Neural Antigens
Squamous cell carcinoma	+	+	- (+)	+	(+)
Small (oat) cell carcinoma	+	+	- (+)	+	+
Carcinoid	+	+	-	-	+
Adenocarcinoma	+	+	- (+)	+	

* Modified from Gatter et al ¹³

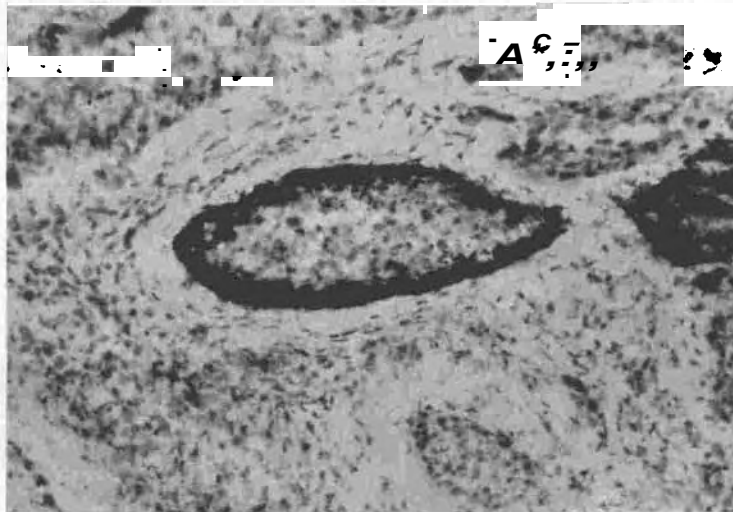


FIG. 1: Squamous carcinoma treated with antibody against epithelial membrane antigen using the immunoperoxidase method. There is positive staining in the less well differentiated areas. Frozen section x 120.

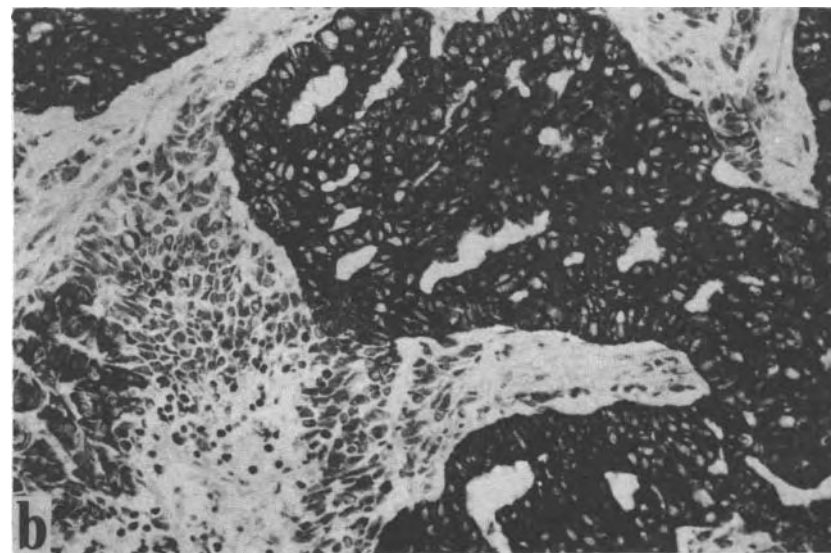
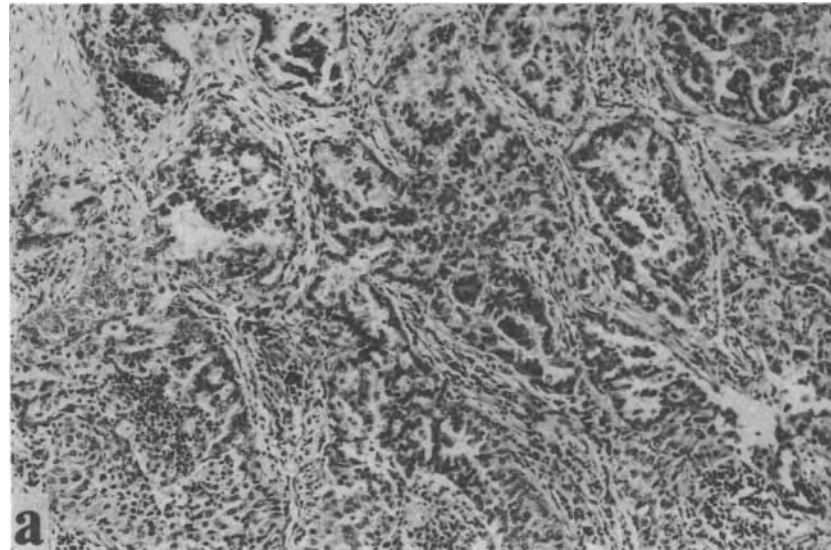


FIG. 2: Adenocarcinoma of lung (a) haematoxylin and eosin section of paraffin embedded material x 120. (b) stained with antibody against epithelial membrane antigen. There is diffuse staining of tumour cells. Frozen section. Immunoperoxidase x 180.

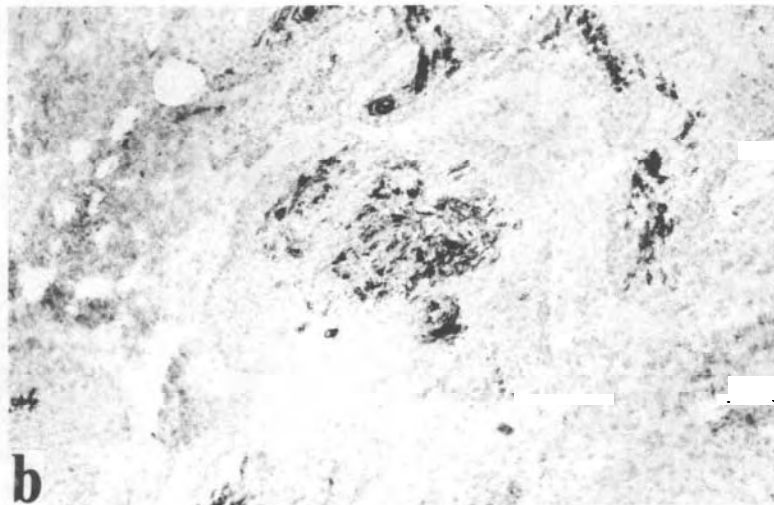
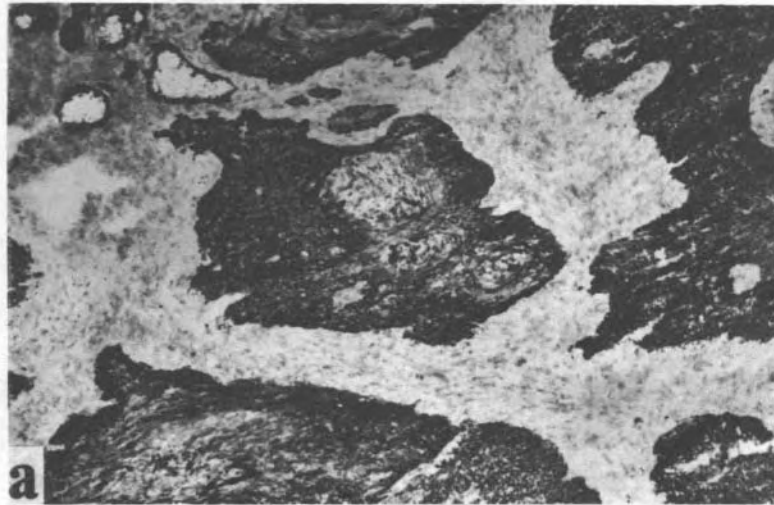


FIG. 3: Squamous carcinoma (a) stained with antibodies to cytokeratin showing a positive reaction in less well differentiated areas. (b) stained with antibody to prekeratin showing positive staining in keratinising zones. Frozen sections x 120.

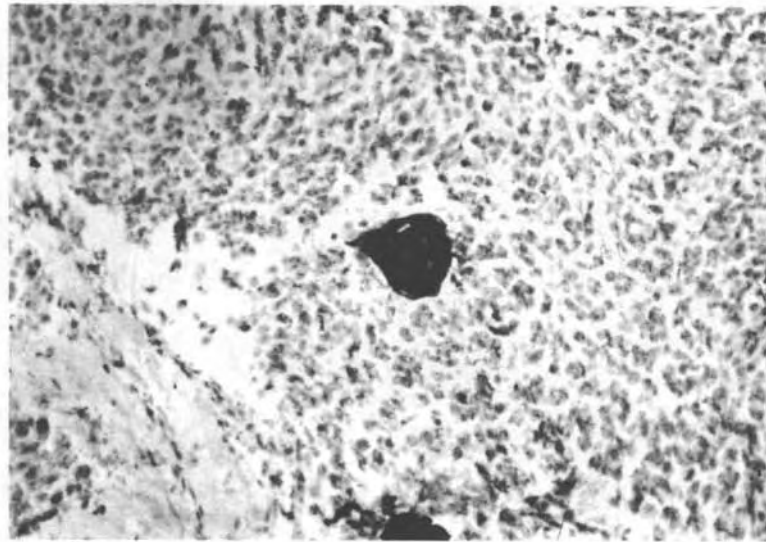


FIG. 4: **Small** (oat) cell carcinoma exhibiting focal staining with antibody to prekeratin. Frozen section **x 180**.

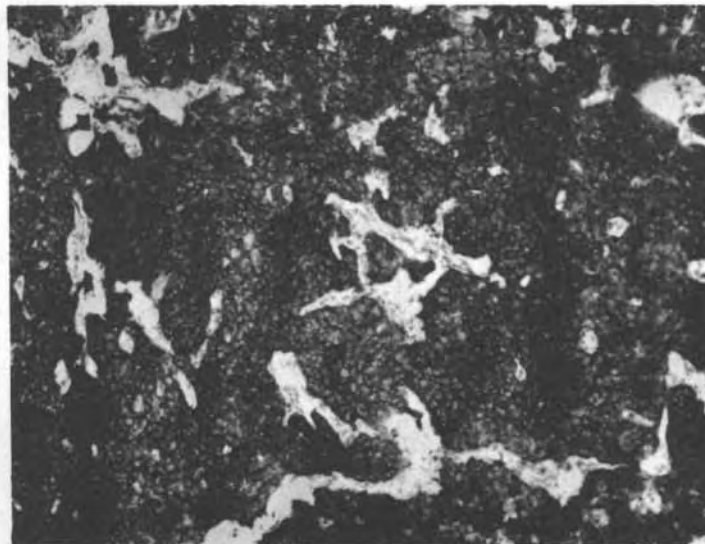


FIG. 5: Small (oat) cell carcinoma showing diffuse staining with antineural antibody UJ13A. Frozen section **x 120**.

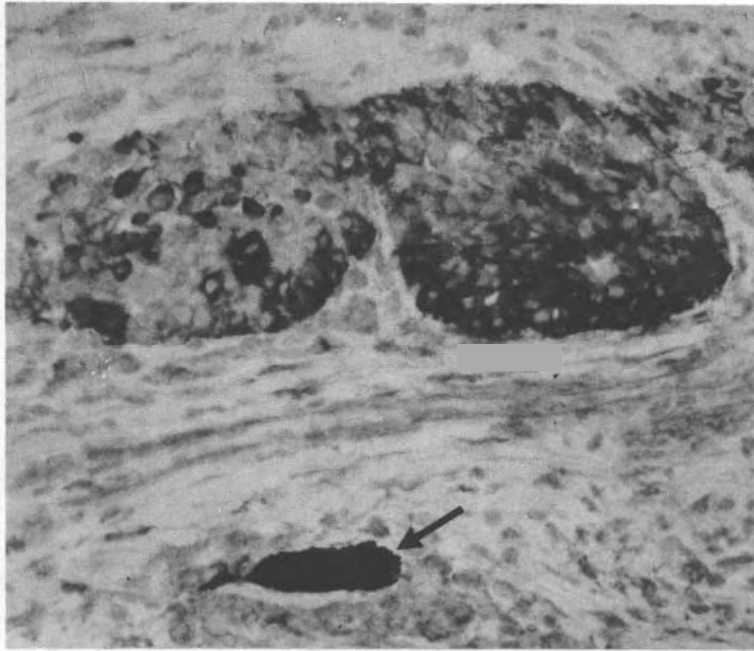


FIG. 6: Squamous carcinoma exhibiting some focal staining with antineural antibody UJ13A. A nerve (arrowed) is also present. Frozen section x 300.

bodies and with antibodies to epithelial membrane antigen.

Squamous carcinomas were positive in keratinous areas for the prekeratin antibodies K20 and K92 and also with antiCEA and there was reciprocal positive staining with anticytokeratin antibodies. Oat cell carcinomas exhibit uniform staining with the antineural antibody UJ13A as did carcinoid tumours but the latter were negative with antiCEA and with the anticytokeratin Hep54. Primary adenocarcinomas on the other hand stained with antibodies against epithelial membrane antigen, cytokeratin and CEA but were negative with prekeratin antibodies and UJ13A.

The general pattern of immunocytochemical staining is not however quite as clear cut. Firstly it is notable that cytokeratins and EMA were present in all tumours. Secondly it is apparent that in individual tumours there are often, indeed some would say always, localised areas which differ in their morphological and phenotypic patterns from the rest of the tumour.

Classes of intermediate filaments in lung tumours

At this point it would be as well to consider the intermediate filaments present in lung tumours. In the cytoskeleton of mammalian cells there are five main classes of intermediate

filaments. These are shown in Table 4. The other elements of the cytoskeleton, namely microfilaments or **actin** and microtubules, are not considered here. In analysing any tumour a word of caution is needed in interpreting preparations stained for these intermediate filament proteins by monoclonal antibodies in order to indicate tumour cell origin. In normal tissues there appears to be no overlap in their patterns of distribution. Yet following neoplastic transformation there is evidence in lung tumours of inappropriate co-expression.¹⁹ In a series of 94 lung tumours it was found that 37 specimens (40 per cent) reacted with monoclonal antibodies against at least one other class of intermediate filament (glial fibrillary acid protein was excluded from this study) and one small cell carcinoma was positive for all four. In most instances staining was focal. This co-expression is particularly marked in the case of vimentin and cytokeratins. It is possible that this is due to epitope sharing but this appears unlikely as evidence for multiple gene coding for each intermediate filament, other than cytokeratins, is lacking. This is not to say that antibodies to the intermediate filaments are of no value in diagnosis of anaplastic tumours but they must be employed in conjunction with other reagents detecting leucocyte common antigen, S100 protein or epithelial membrane antigen.

Ultrastructure of lung tumours

Careful electron microscopic examination of lung tumours reveals similar and corroborative evidence of cellular heterogeneity (Table 5) in spite of the considerable sampling problems involved. In squamous carcinoma Alcian blue/PAS preparations frequently reveal intracellular vacuoles containing mucin (Fig. 7) and electron

microscopy in our series similarly demonstrates intracytoplasmic secretory vacuoles in 17 out of 37 tumours labelled as squamous carcinomas by light microscopy.

Dense core granules are often difficult to detect in oat cell carcinoma but a few are present. They may also be seen in tumours showing either squamous or adenocarcinoma predominance and were present in individual cells containing tonofilaments (Fig. 8) or secretory vacuoles in many cases in our series (Table 5). Precise identification of these granules as neurosecretory is open to some debate especially as they tend to vary more in diameter than those found in typical carcinoid tumours. In some cases the uranaffin reaction described by Payne, Nagle and Borden²⁰ may prove a helpful method of labelling them. Yet there is strong circumstantial evidence that they do in fact represent true dense core granules rather than abnormal mucus-secreting vacuoles or lysosomes in that immunocytochemical investigation reveals neural-associated antigens in many of these tumours.

TABLE 4
INTERMEDIATE FILAMENTS

Neurofilaments	Neurons
*Keratin filaments	Epithelial cells
Vimentin	Connective tissue cells
Desmin	Muscle cells
Glial fibrillary acid protein	Astrocytes

*At least 19 different types of cytokeratin identified

TABLE 5*
CELL TYPES IDENTIFIED IN TUMOURS
USING ELECTRON MICROSCOPY IN
ADDITION TO LIGHT MICROSCOPY AND
HISTOCHEMISTRY

Squamous carcinoma alone	9
Squamous plus adenocarcinoma	6
Squamous plus small cell carcinoma	11
Squamous, adeno- and small cell carcinoma	11
Total	37

Adenocarcinoma alone	7
Adeno- plus squamous carcinoma	5
Adeno- plus small cell carcinoma	8
Adeno- small cell and squamous carcinoma	6
Total	26

Small cell carcinoma alone	2
Small cell, squamous and adenocarcinoma	1
Total	3

*From Dunnill et al¹⁴

Grading of tumours

The practice of grading tumours on simple histological appearances and of regarding squamous tumours as having a better outlook than others is one that must be called in question. Indeed results of treatment in lung cancer are so generally unsatisfactory and prognosis so unpredictable that any new approach may be welcomed. One possible advance has been opened up by use of the monoclonal antibody Ki 67, developed by Cerdes *et al*,²¹ which reacts with a nuclear antigen expressed by proliferating cells. In a recent investigation²² of 104 surgically resected tumours it has been shown that tumours that are predominantly small cell carcinoma have a high proliferation rate whereas carcinoids have a low proliferation rate. In contrast to this adenocarcinomas and squamous carcinomas vary widely in keeping with their heterogeneous morphological and clinical behaviour (Table 6). It remains to be seen whether there is a correlation between a low proliferation status and long term survival.

DISCUSSION

It has become apparent with increasing use of electron microscopy, conventional histochemistry, and immunocytochemistry employing monoclonal antibodies that many, some would say the majority, of lung tumours

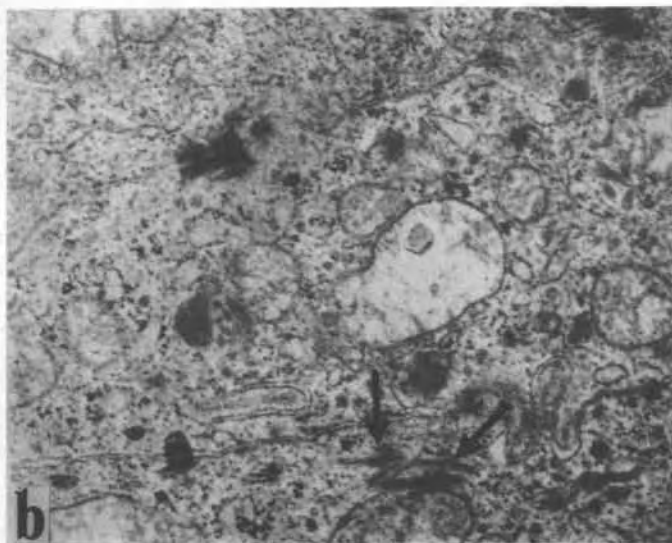
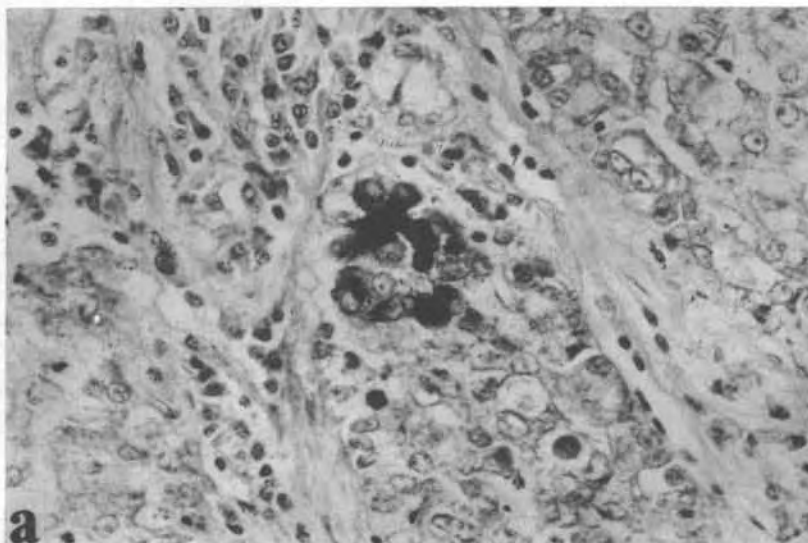


FIG. 7: Squamous carcinoma (a) Alcian blue preparation showing focus of mucus production x 300. (b) electron micrograph showing mucous vacuole, desmosomes and tonofilaments (arrowed) x 29,700.

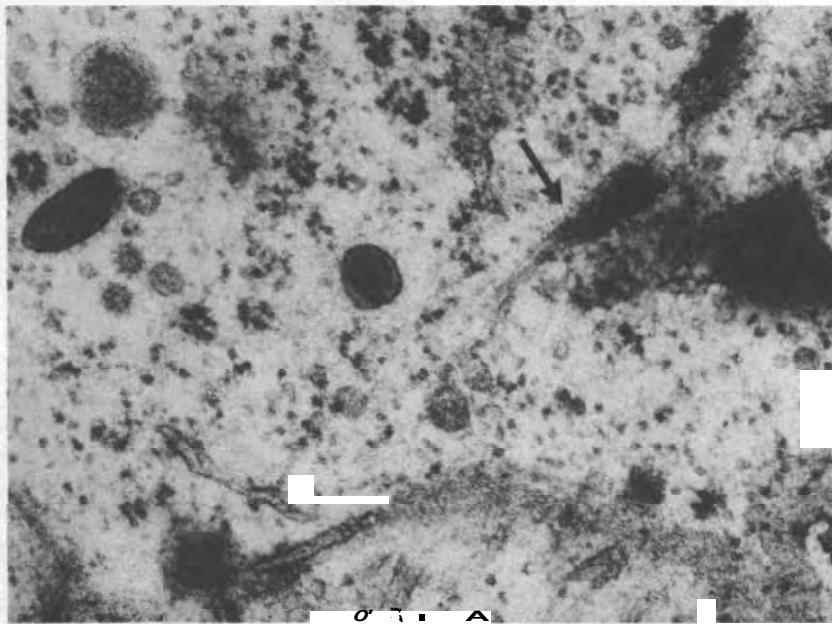


FIG. 8. Electron micrograph of squamous carcinoma showing dense core granules and tonofilaments (arrowed) x 78,000.

TABLE 6*

IMMUNOCYTOCHEMICAL LABELLING OF CELL PROLIFERATION IN LUNG TUMOURS USING MONOCLONAL ANTIBODY Ki67

WHO Type	Percentage of cells labelled				Total
	0-11	11-25	26-50	51-75	
Squamous carcinoma	17	20	20	6	63
Adenocarcinoma	14	5	8	0	27
Small (oat) cell carcinoma	0	0	2	3	5
Carcinoid	9	0	0	0	9
Total	40	25	30	9	104

*Modified from Gatter et al³⁷

do not differentiate along one pure cell line. This makes allocation of any given tumour into the somewhat rigid WHO classification difficult. Although pathologists may make a firm diagnosis of a category such as squamous carcinoma, ignoring the presence of small numbers of mucus-secreting cells, in order to satisfy clinical colleagues this is essentially an unsatisfactory procedure and gives a misleading idea with respect to histogenesis.

There are several reasons for difficulties experienced in making an adequate histological diagnosis in cases of lung cancer. One major problem is sample size. In many instances only a very small quantity of tissue is available, often obtained by fiberoptic bronchoscopy, which in addition to exhibiting crush artefacts only represents a very superficial portion of the neoplasm. Examination of the entire resected lung or lobe may reveal a very different picture.

Secondly the use of newer methods of investigation brings out features not always apparent on routine light microscopy.

These factors are particularly relevant in tumours labelled as small cell or large cell carcinoma, terms implying a knowledge of comparative measurements which all too often are not justified. Indeed when measurement is employed the results are quite surprising. The spurious nature of these terms was dramatically revealed by Vollmer^{2,3} who measured tumour cell and nuclear diameter in lung cancers from 197 patients and showed a continuous distribution from small to large cell undifferentiated cancer. Similarly Begin, Sahai and Wang^{2,4} demonstrated that giant cells could be found in all forms of lung carcinoma including small cell tumours. Others have shown that a separate category of clear cell carcinoma is insupportable as such tumours contain cells from all other categories.^{1,7,25}

If just one method other than light microscopy is employed, let alone more, obvious cellular heterogeneity becomes apparent. In one investigation electron microscopy revealed a second cell type in nine out of thirty-five cases;^{2,6} in another where sixty-one tumours were examined, mucus histochemistry revealed a second cell type in 18 per cent.^{2,7} Saba *et al*^{2,8} using a simple immunoperoxidase method for detecting keratin, as well as electron microscopy, found both glandular and squamous differentiation in 17 out of 52 cancers. Using electron microscopy alone two groups of investigators^{2,9,30} described carcinoma with dense core granules together with foci of squamous and glandular differentiation. In our investigations we have used all three methods and have revealed an even more extensive overlap in cell type. This was expected as the great advantage of immunocytochemistry is that a much larger sample can be examined than by electron microscopy. The fact that evidence of cellular heterogeneity is not found in every case may well be due to the necessary restriction in size of the sample examined in any individual.

It is of interest that biochemists approaching lung cancer from an entirely different viewpoint have come to very similar conclusions. Thus vasopressin has been described in both small cell carcinoma and adenocarcinoma, and calcitonin in all varieties.^{3,1} Berger *et al*^{3,2} in a most interesting study of 50 different lung carcinomas estimated a variety of biochemical markers including dopa decarboxylase, histaminase, beta-endorphin and calcitonin.

They were unable to detect any single marker that separated small cell carcinoma from the rest and concluded that though there were quantitative differences between various histological types of lung cancer all major forms of the disease 'represented a continuum of differentiation with a common cell lineage'. Much of the difficulty with regard to histogenesis and classification of lung cancer has been as a result of the acceptance of the theory that small (oat) cell carcinoma was separate from the other forms and that its cell of origin was part of the APUD system derived from the neural crest by migration during early foetal life. This view is now discredited mainly due to the experimental work of Andrew^{3,3,3,4} which failed to show that migration of neural crest cells to endoderm occurred. Furthermore there is strong epidemiological evidence linking small cell carcinoma with other forms of lung cancer. All are related to smoking and to uranium exposure.

There are thus three forms of evidence pointing to the fact that lung cancer is a single entity — cytological heterogeneity, epidemiology and the fact that ectopic hormone production, thought at one time to be unique to small cell carcinoma, may occur in tumours that are predominantly squamous or adenocarcinoma. If this view is conceded it seems likely that the initial carcinogenic stimulus affects pleuripotential basal or reserve cells of the bronchial mucosa. These cells are known to proliferate along one of several pathways as evidenced by the regenerative capacity of bronchial mucosa following trauma or inflammation. Simple squamous epithelium is first formed which is later replaced by mucus-secreting columnar ciliated epithelium. In lung cancer differentiation of proliferating basal cells may give rise to either a squamous, glandular or small cell predominance. It is implicit in this argument that the cells in such tumours containing dense core granules are also derived from basal cells. This view of a common histogenesis for lung cancer was one favoured by Willis^{3,5} who stated that 'while many pulmonary cancers consist predominantly, sometimes exclusively of growth of a particular type, it must be emphasised that there is only one entity carcinoma of the lung, that individual tumours show various structural combinations, and that great pleomorphism is possible in one tumour'. This has also been suggested more recently by Yesner^{3,6} when he declared that 'all lung

cancers are part of a spectrum of differentiation'.

The importance of these opinions is that they have some implications in relation to prognosis and possible treatment. It is of interest that one study by Sappino, Ellison and Gusterson³⁷ of immunohistochemical investigation of keratin in small cell carcinomas in its relation to response to chemotherapy has shown that, contrary to popular belief, the response of these small cell carcinomas containing focal areas of keratin is as good if not better than keratin negative tumours.

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