INTERMEDIATE FILAMENT PROTEIN EXPRESSION IN LUNG CANCER

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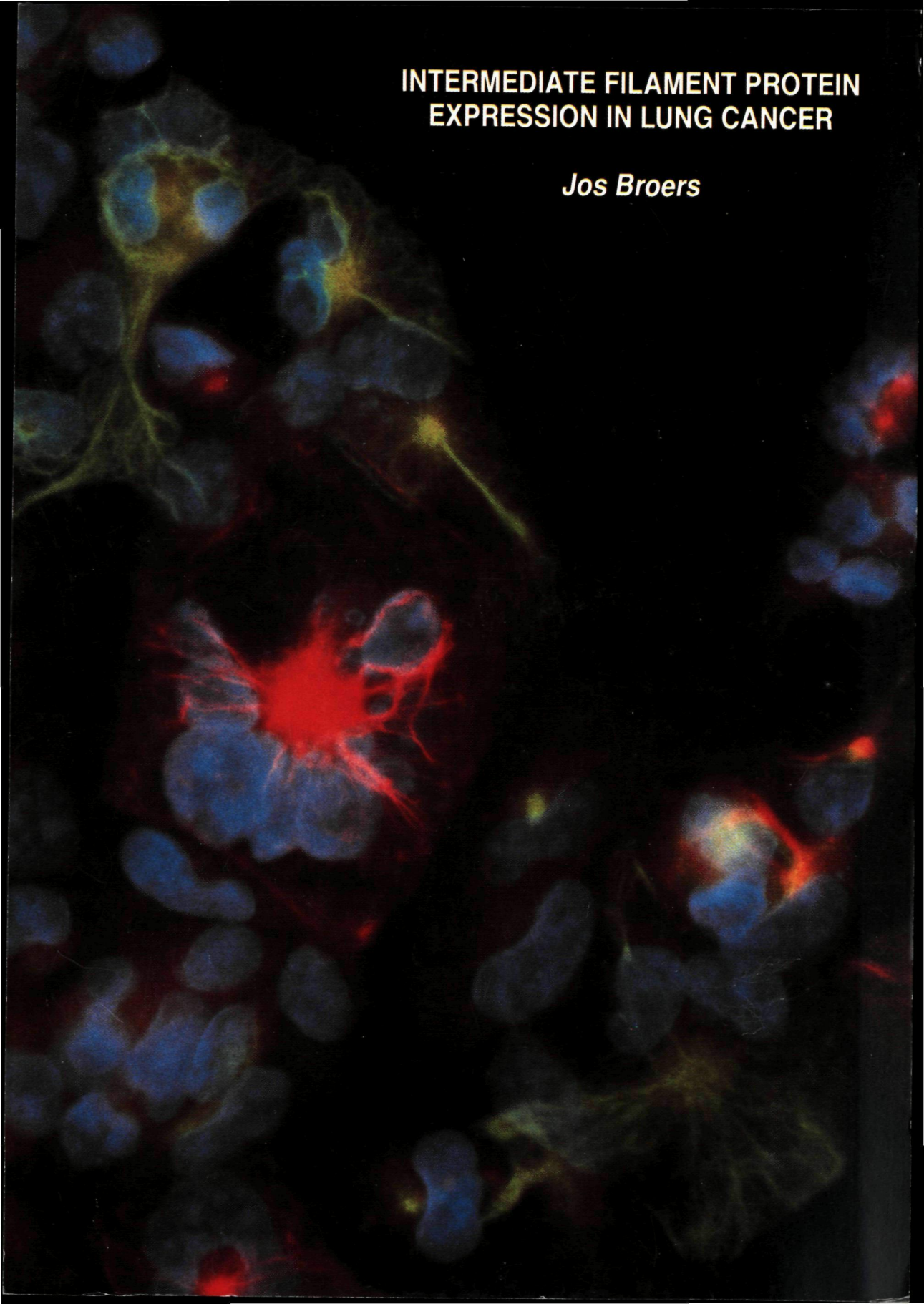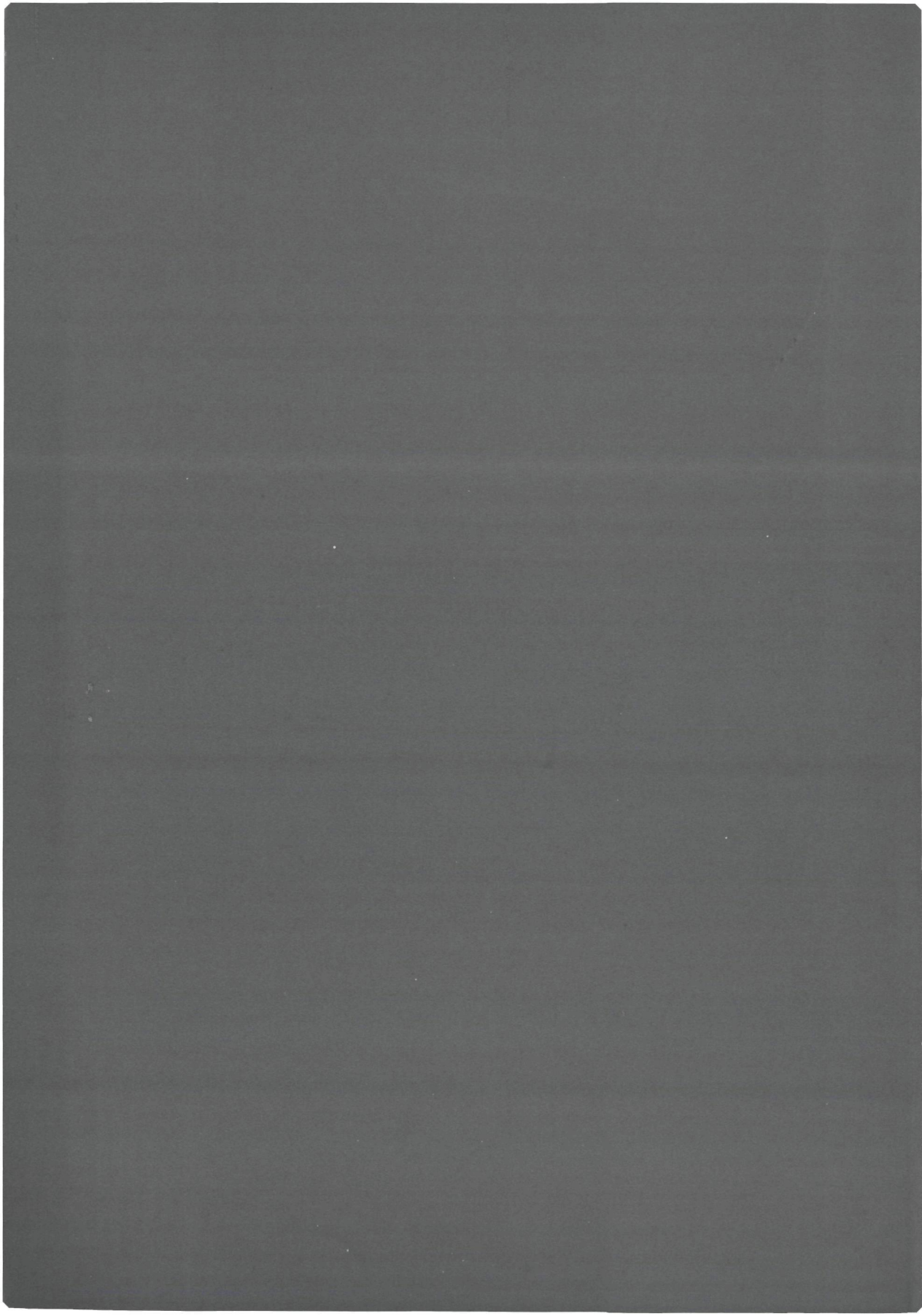
INTERMEDIATE FILAMENT PROTEIN

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INTERMEDIATE FILAMENT PROTEIN EXPRESSION
IN LUNG CANCER

een wetenschappelijke proeve op het gebied van
de Wiskunde en Natuurwetenschappen

Proefschrift

ter verkrijging van de graad van doctor **aan**
de Katholieke Universiteit van Nijmegen,
volgens het besluit van het college van decanen
in het openbaar te verdedigen
op vrijdag 4 november 1988
des namiddags te 1.30 uur precies

door

Joseph Leonardus Victor Broers
geboren op 6 januari 1957
te Gronsveld.

Aan mijn ouders

Aan Mariet

COVER:
Triple exposure of the lung carcinoma cell line GLC-A1 after incubation with RCK102 visualized with sheep anti-mouse Texas Red, pNF160 visualized with goat anti-rabbit FITC, and a nuclear DNA staining with Hoechst.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

Lung cancer is the leading cause of cancer deaths among men in western countries (Parkin et al., 1984). While the age-adjusted lung cancer death rates for men seem to stabilize, its rapidly growing incidence among women will make lung cancer the leading cause of cancer deaths among women also in most western countries within the next 5 - 10 years (Andrews et al., 1985; Silverberg and Lubera, 1988). For instance, it is estimated that in the USA in the course of 1988 46,000 women will die of lung cancer, as compared to 42,000 for breast cancer, the hitherto leading cause of cancer deaths among the female population (Silverberg and Lubera, 1988).

One of the major causes of lung cancer is evidently cigarette smoking. Already thirty years ago Hammond and Horn (1958) have investigated the correlation between death rates and smoking habits, and have shown that the lung cancer mortality ratio of smokers as compared to non-smokers was as high as 10 : 1. In light of these data the growing incidence of lung cancer among women, who in the last decades have adopted the smoking habits of men (Holleb, 1988), can be explained. This can also account for the rapidly growing incidence of lung cancer in developing countries (Parkin et al., 1984).

In the Netherlands in 1986 about 34,000 men and women have died of neoplasias. Of these, in about 9000 cases (26%) lung cancer was the cause of death, divided among men and women in a ratio 8:1 (CBS, 1988). In 1975 this ratio was 14:1 (CBS, 1986), indicating a rapid change to a more equal distribution between male and female death rates for lung cancer.

LUNG CANCER HISTOLOGY

The major types of lung cancer, as classified according to the W.H.O.-system (WHO, 1982), include a) epidermoid (squamous cell) carcinoma, occurring in about 33 to 64% of all cases (Minna et al., 1985), b) adenocarcinoma (16-26%), c) small cell lung cancer (20-25%), d) large cell carcinoma (9-20%), and e) lung carcinoids (less than 5%). The estimates of incidence of each subtype depend on the source of pathological material reviewed (Minna et al., 1985). Histologically the main types of lung cancer can be characterized as follows:
a) Squamous cell carcinoma (SQC). Three histological degrees of differentiation are discerned:
    1. Well differentiated SQC: Tumors showing orderly stratification, obvious intercellular bridges and keratinization with pearl formation.
    2. Moderately differentiated SQC: Carcinomas with features intermediate between well differentiated and poorly differentiated SQC.
    3. Poorly differentiated SQC: Carcinomas fulfilling the criteria for SQC, but only in part of the tumor, while the bulk of the tumor cells has an undifferentiated character. Also tumors, in which these criteria are discerned with difficulty, are included within this diagnosis.

b) Small cell lung carcinoma (SCLC). According to the WHO-classification a subdivision can be made into oat cell carcinoma, small cell carcinoma of the intermediate cell type, and combined oat cell carcinoma. In 1985, however, the pathology panel of the International Association for the Study of Lung Cancer (IASLC) has proposed an alternative classification (Yesner, 1985), which has been adopted in this thesis:

1. Classic small cell carcinoma: This group includes the former oat cell carcinomas and the intermediate cell type carcinomas, since no significant clinical, biological, or ultrastructural differences were found between these two subtypes (Radice et al., 1982; Hirsch et al., 1983; Yesner, 1985). These carcinomas are composed of more or less regular small cells, generally larger than lymphocytes. They have dense round and oval nuclei (oat cell type), or polygonal to fusiform nuclei (intermediate cell type), the chromatin is finely granular and distributed throughout the nucleus, nucleoli are inconspicuous, and cells have very sparse (oat cell type) to more abundant (intermediate cell type) cytoplasm.
2. Small cell/large cell carcinoma (Variant SCLC): Carcinomas in which an admixture of classic small cell carcinoma cells and large cells with open nuclei and prominent (eosinophilic) nucleoli is observed.
3. Combined small cell carcinoma: A small cell carcinoma with a substantial component of SQC or adenocarcinoma cells or both.

c) Adenocarcinoma. A malignant epithelial tumor with tubular, acinar, or papillary growth patterns, and/or mucus production by the tumor cells.

d) Large cell carcinoma. A malignant epithelial tumor with large nuclei, prominent nucleoli, abundant cytoplasm, and usually well defined cell borders, without the characteristic features of SQC, SCLC, or adenocarcinomas.

e) Carcinoid tumor. A tumor of the diffuse endocrine system derived from Kulschitsky-type cells (WHO, 1982), showing a mosaic or trabecular arrangement of polygonal cells with granular eosinophilic or clear cytoplasm and regular oval nuclei. Nucleoli are typically prominent, while mitoses are rare.

TREATMENT OF LUNG CANCER

In lung cancer treatment, in contrast to many other types of cancer, no great improvements have been achieved during the last decade, showing an overall 5-year survival of only 10% (Minna et al., 1985). Therefore, a better understanding of the cell-biological behaviour of the different types of lung cancer is needed, which might serve as a basis for new and more promising treatment regimens.

To decide upon a treatment protocol, especially the subdivision into SCLC and non-SCLC is important. Unlike the other types of lung cancer, SCLC is sensitive to chemotherapy. In about 75% of the cases of SCLC an initial response to chemotherapy can be noticed, with a clinically complete response in about 35% of all cases (Johnson and Greco, 1986,1987). Unfortunately, however, in most cases relapse occurs, resulting in a three-year survival rate of only 5-10%, and a five-year survival rate of about 1% (Minna et al., 1985). Within SCLC a clinically relevant subdivision can be made between classic and variant SCLC (Carney et al., 1985; Gazdar et al., 1985; Yesner, 1985). The variant-type of SCLC appears to be less sensitive to chemotherapy and radiotherapy. As a result the median survival time of patients suffering from the variant-type of SCLC is significantly shorter than of those with a classic type of SCLC (Radice et al., 1982; Hirsch et al., 1983). Also for patients with a combined SCLC a poorer prognosis than for patients with classic SCLC is observed (Sehested et al., 1986).

In non-SCLC treatment with chemotherapy is in general unsuccesful (Minna et al., 1985). Therefore, with the exception of high cure rates for surgical treatment of truly localized disease, the prognosis for patients with non-SCLC is grim (Mulshine et al., 1986). In a small subset of patients, however, a response to chemotherapy can be observed (for a review see Mulshine et al., 1986). In part, these cases might represent non-SCLC, in which SCLC-components occur. A heterogeneous composition is quite common in lung cancer. At the cytological (Hess et al., 1981), electron-microscopical (McDowell and Trump, 1981; Trump et al., 1982; Dingemans and Mooi, 1985; Hammond and Sause, 1985), immunocytochemical (Hammar et al. 1985; Dunnill and Gatter, 1986; Mooi et al. 1988), and even at the histological level (Roggli et al., 1985) a heterogeneity in type as well as degree of differentiation can be noticed in more than 50% of all cases.

In our studies we have tried to determine some of the biological properties of the different types of lung cancer. We have focussed on lung cancer heterogeneity, with special emphasis on the degree and type of differentiation. We have used surgically resected tumor samples, autopsy material, and cell lines, derived from the main subtypes of lung cancer. Immunocytochemical as well as biochemical methods, next to conventional histological staining procedures have been applied. Most immunocytochemical investigations have been performed using monoclonal antibodies to intermediate filament proteins, a group of cytoskeletal proteins with a tissue- and differentiation-related distribution.

INTERMEDIATE FILAMENT PROTEINS

Intermediate filaments are proteinaceous fibrillar structures, measuring 8-11 nm in diameter, which is between that of the two other major constituents of the cytoskeleton, the microfilaments (5 nm) and the microtubules (25 nm). About two decades ago intermediate filaments were firstly defined as a distinct class of organelles by Ishikawa and coworkers (1968). Thereafter, it took about ten years until a thorough ultrastructural and biochemical investigation of intermediate filaments was undertaken. Since that time, however, an increasing number of papers has been published on the expression of the different intermediate filament types (For reviews see Osborn and Weber, 1983; Traub, 1985; Wang et al., 1985). Interest in intermediate filament expression, which is ubiquitous to virtually all vertebrate cells (Traub, 1985), arose after the finding, that the five different types of intermediate filaments are not randomly distributed among vertebrate cells, but that a tissue-specific distribution appears to exist (Bennet et al., 1978a,b; Franke et al., 1978; Lazarides, 1980, 1982; Anderton et al., 1980). Cytokeratins are present in virtually all epithelial cells, vimentin occurs in mesenchymal cells, neurofilaments occur in neuronal cells, glial fibrillary acidic protein (GFAP) is found in astrocytes, and desmin can be found in muscle cells. Since this distribution corresponds strikingly well with the major tissue classification system based on histological principles, a large number of applications has become available, especially since (monoclonal) antibodies to the different types of intermediate filaments have been developed. Immunocytochemical application of these antibodies allows the in situ identification of intermediate filament proteins in different tissues.

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Especially, monoclonal antibodies to the different cytokeratin polypeptides appear to be very useful. Cytokeratins are a family of polypeptides, which are characterized by a remarkable biochemical diversity, represented in human epithelial tissues by at least 19 different polypeptides. They range in molecular weight from 40 to 68 kDa and are designated 1 to 19, cytokeratin 1 having the highest molecular weight and the highest isoelectric pH, and cytokeratin 19 having the lowest molecular weight and a low isoelectric pH (Moll et al., 1982). These different cytokeratins are not expressed randomly throughout epithelia but occur in cell-specific combinations. These combinations consist of at least one type I (acidic) cytokeratin polypeptide (Quinlan et al., 1985) and at least one type II (basic) cytokeratin polypeptide. In this way the cytokeratin pattern of a particular epithelium can be used for its identification (Quinlan et al., 1985).

The function of intermediate filaments is still a matter of debate. It has been suggested that these cytoskeletal structures are involved in forming a scaffold that retains cell structure. Recent investigations have shown that vimentin is linked to the lamins of the nuclear envelope through the nuclear pores, while at the cell periphery these intermediate filaments associate with the membrane skeleton. In this way an elaborate system for nucleolemmal/plasmolemmal anchorage and communication might be provided (Geiger, 1987).

INTERMEDIATE FILAMENT PROTEINS IN LUNG CANCER

In the early eigthies investigations on the expression of intermediate filaments in neoplasms (Gabbiani et al., 1981; Ramaekers et al., 1982) showed that when cells of a certain tissue become malignant, they retain their original intermediate filament expression pattern without expressing additional intermediate filament proteins. Therefore, carcinomas express cytokeratins, mesenchymal tumors such as sarcomas and lymphomas express vimentin, neuronal tumors can express neurofilaments, and myosarcomas express desmin. Although since then a considerable number of exceptions have been reported, this general rule has proven to be very useful in tumor diagnosis. This also holds true for the expression of intermediate filament proteins in the different types of lung cancer.

Early immunocytochemical investigations (Schlegel et al., 1980; Bejui-Thivolet et al., 1982; Gusterson et al., 1982; Nagle et al., 1983; Said et al., 1983a,1983b; Sappino et al., 1983; Wilson et al., 1985) reported on the expression of cytokeratins in pulmonary squamous cell carcinomas, a highly variable expression of cytokeratins in adenocarcinomas, and the absence of cytokeratins in most small cell lung cancers. Later investigations, however (Saba et al., 1983; Van Muijen et al., 1984, Blobel et al., 1985; Gatter et al., 1985; Hammar et al., 1985), have shown the presence of cytokeratins in all types of lung cancer. One of the shortcomings of the earlier investigations, resulting in these conflicting reports, was the limited cross-reactivity of the (polyclonal) antibodies used with each of the 19 different cytokeratin polypeptides (Moll et al., 1982), especially since most of the early cytokeratin antibodies were raised against epidermal cytokeratins. In addition, the distribution of the cytokeratin polypeptides is not random, but depending on degree and type of differentiation of the tissue investigated (Moll et al. 1982; Quinlan et al., 1985). Therefore, when investigating

cytokeratin expression in tumors we now use a panel of chain-specific cytokeratin antibodies covering a broad spectrum of cytokeratin polypeptides.

Another factor which might influence immunocytochemical results using intermediate filament antibodies is the type of tissue fixation used. Apparently, certain intermediate filament protein epitopes are sensitive to different fixation reagents. For instance, most cytokeratin antigens (epitopes) are sensitive to routine formaline fixation procedures and paraffin embedding, often resulting in a negative reaction in immunocytochemical staining assays.

A third factor influencing the reactivity of antibodies is selective masking of epitopes as, for example, described for cytokeratin 18. Although cytokeratin 18 is present in basal cells of normal bronchial epithelium, one of the cytokeratin 18 antibodies developed in our laboratory (RGE53) does not stain these cells, while another cytokeratin 18 antibody (RCK106) clearly shows a reaction with the same cell type (Broers et al., 1988).

We believe that, in using a large panel of different polyclonal and monoclonal antibodies from different institutes on (whenever possible) freshly frozen, unfixed tissue material most of these problems have been overcome. Therefore, we are able to present a reliable portrait of the intermediate filament expression patterns in normal bronchial tissues and in bronchial carcinomas.

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CHAPTER TWO

EXPRESSION OF INTERMEDIATE FILAMENT PROTEINS IN FETAL AND ADULT
HUMAN LUNG TISSUES

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ABSTRACT

    The expression patterns of intermediate filament proteins in fetal and
normal or non-pathological adult human lung tissues are described. In
early stages of development (9-10 weeks and 25 weeks of gestation) only
so-called simple cytokeratins such as cytokeratin 7 (minor amounts), 18 and
19 are detected in bronchial epithelial cells. At later stages of
development, the cytokeratin expression patterns become more complex. The
number of bronchial positive for cytokeratin 7 increases, but basal cells
in the bronchial epithelium remain negative. These latter cells show,
however, expression of cytokeratin 14 in the third trimester of gestation.
Developing alveolar epithelial cells express cytokeratins 7 and 18. In
adult human bronchial epithelium cytokeratins 4, 7, 13, 14, 18 and 19 can
be detected by chain-specific monoclonal antibodies.
    Vimentin is present in all mesenchymal tissues. In addition, fetal lung
expresses vimentin in bronchial epithelium, however, to a less extent with
increasing age, resulting in the expression of vimentin in only few
scattered bronchial cells at birth. Also in adult bronchial epithelium the
expression of vimentin is noticed in part of the basal and columnar
epithelial cells.
    Desmin filaments, present in smooth muscle cells of the lung, appear to
alter their protein structure with age. In early stages of development
smooth muscle cells surrounding blood vessels are partly reactive with
cytokeratin antibodies and with a polyclonal desmin antibody. At week 9-10
and week 25 of gestation a monoclonal antibody to desmin, however, is not
reactive with blood vessel smooth muscle cells but is only reactive with
smooth muscle cells surrounding bronchi. With increasing age the
reactivity of cytokeratin antibodies with smooth muscle cells in blood
vessels decreases, while the reactivity with the monoclonal desmin antibody
increases.

INTRODUCTION

    Since intermediate filament proteins (IFPs) exhibit a highly specific
distribution pattern within adult normal and neoplastic tissues [7, 19]
antibodies to the different types of IFPs have been used to study the
embryogenesis of specific cells and organs (for a review, see [27]. In
mammals virtually all epithelial tissues, including those of the upper and
lower respiratory tract, contain cytokeratins (CKs, [2, 18]) as their IFP
components. The family of CKs consists of at least 19 different proteins
[16], which are not randomly distributed among epithelial tissues. For
instance, CKs 7, 8, 18, and 19 are highly characteristic for many simple
epithelia. On the other hand, stratified, squamous epithelia are
characterized by the presence of CKs 4, 10, and 13. [5, 16, 23, 30].
Earlier studies of Jackson et al. [9] have shown, that CK 8- and
18-related proteins are the first IFPs to be expressed in pre-implantation
mouse embryos. More recent studies have shown that at later stages of
epithelial differentiation in embryonic life the synthesis of other CKs is
initiated. Also a transient vimentin expression has been found in several
embryonal epithelia [8, 11, 12, 13, 21, 22, 24, 31, 32, 33]. In this
immunohistochemical study we have applied a panel of monoclonal and
polyclonal IFP antibodies to developing lung specimens, with special
emphasis on CK antibodies. Most of these CK antibodies are chain-specific,

and thus enabled us to detect individual CK polypeptides at the cellular level.

The development of the lower respiratory system starts with the formation of the laryngotracheal groove in the ventral wall of the pharynx, at about 26 days of gestation. The endodermal lining of this groove gives rise to the epithelium and glands of the larynx, trachea, bronchi and the pulmonary lining epithelium [17]. First, the laryngotracheal tube develops, followed by the development of lung buds at about 4 weeks of gestation. Further lung development may be divided into four stages [3], including:
1) the pseudoglandular period (5 to 17 weeks of gestation), during which the bronchial divisions differentiate into the air-conducting system, and as a result bronchi branch into many small bronchial tubes. Until week 10 the bronchial buds are lined by unspecialized columnar epithelial cells. At week 10 the bronchial pseudostratified columnar epithelium starts to develop. At the end of this period all major elements of the lung have been formed except those involved with gas exchange;
2) the canalicular period (13 to 25 weeks of gestation), during which the lumina of the bronchi and bronchioli become much larger and the lung tissue becomes highly vascular. Terminal bronchioli give rise to respiratory bronchioli, while at the end of this period terminal sacs start to develop;
3) the terminal sac period (24 weeks of gestation to birth), during which many terminal sacs develop, initially lined with cuboidal epithelium, developing into type I alveolar epithelial cells, while also the type II alveolar cells start to develop;
4) the alveolar period (late fetal period to about two years of age, [26]) during which the epithelial lining of the terminal sacs attenuates to an extremely thin squamous epithelial layer, and the alveolar-capillary (respiratory) membrane becomes sufficiently thin to allow optimum gas exchange.
To study the expression of the individual CK proteins during human lung development, we have examined fetuses showing the above mentioned four stages of lung development, as well as normal adult lung tissues.

METHODS

LUNG TISSUES

Lung tissue material was obtained from fetuses at week 9-10, at week 25, a case of premature death (37 weeks of age), and from autopsy of a newborn (43 weeks), who died at birth.

Normal bronchi (15 cases) and normal lung tissue (5 cases) were obtained after resection of lungs or lung lobes for different medical reasons. In cases of lung carcinomas parts of lung tissues, uninvolved in neoplastic processes, were resected. As previously observed by others [14, 34] most of these cases show some degree of abnormality such as basal cell hyperplasia, secretory cell hyperplasia, stratification and/or epidermoid metaplasia.

IMMUNOPEROXIDASE TECHNIQUE.

The indirect immunoperoxidase assays were carried out on cryostat sections (about 5 μm, fixed in acetone for 2 min at -20°C) as described previously [4, 5]. Also the antibodies used in this study were described in these references. Their specificity is indicated in Table 1. Antibody LL001, specific for CK 14, will be described elsewhere [Lane et al., in preparation].

RESULTS

The IFP expression patterns in lung tissues at different stages of development, as detected immunohistochemically, are summarized in Table 1 and depicted in Figures 1 and 2.

9-10 weeks (Figs. 1A1-6, and 2A1-6)
At this stage of lung development epithelial cells show a strong and homogeneous reaction pattern is seen with antibodies RCK102 (Figs. 1A1 and 2A1) , RGE53, RCK106, and LP2K. These CK antibodies are also reactive with smooth muscle cells surrounding blood vessels (Fig. 2A6). No reaction with the CK antibodies is seen in stromal elements or developing cartilage. Monoclonal antibody RCK105 was found to react only weakly with few epithelial cells in a developing bronchiolus (Fig. 1A2), while no reaction is seen with other epithelial cells (Fig. 2A2), nor with smooth muscle cells surrounding blood vessels. Also the other CK antibodies did not react with lung cells at this stage (Figs. 1A3,4 and Fig. 2A3). The polyclonal desmin antibody pDes recognized all smooth muscle cells, while the monoclonal antibody RD301 reacted only with smooth muscle cells surrounding bronchi (Fig. 1A5), with muscle fibers surrounding epithelial clusters of cells (Fig. 2A5), but, surprisingly, not with most smooth muscle cells surrounding blood vessels. The monoclonal antibody to vimentin RV202 did not only react with stromal elements and cartilage, but also showed a clear reaction with all types of epithelial cells (Figs. 1A6 and 2A4). Also smooth muscle cells surrounding bronchi and blood vessels were clearly positive with this antibody. Comparable reaction patterns were seen with the pVim antibody, although less epithelial cells were reactive. At this stage and at all following stages the neurofilament antibody MNF was only reactive with nerve fibers.

25 weeks (Figs. 1B1-6 and 2B1-6)
Again all epithelial cells express CKs, as detected by RCK102 (Fig. 1B1), RGE53, RCK106 (Fig. 2B1), and LP2K. In addition, these antibodies reacted with smooth muscle cells surrounding blood vessels (Fig. 2B6). RCK105 now reacts with a significant part of the bronchial epithelial cells (Fig. 1B2). Basally located cells, which are occasionally visible in hematoxylin and eosin stained cryosections of the larger bronchi, did not react with this antibody. Also a positive reaction was seen in part of the epithelial cells of the respiratory bronchioli (Fig. 2B2). RCK105 did not react with smooth muscle cells (Fig. 2B5). No reactions were seen with the other CK antibodies in any of the cell types (Figs. 1B3,4 and Fig. 2B3).
RD301 reacted with the muscularis mucosae surrounding bronchi (Fig. 1B5) and did not stain smooth muscle cells surrounding blood vessels. pDes,

Table 1: Reactivity of intermediate filament antibodies in fetal, neonatal and adult human bronchial and lung tissues.

Antibody (reactivity)	Age	Epithelial cells Columnar or cuboidal	Basal cells	Term. sacs/ alveoli	(Sero-) mucous glands	Smooth muscle	Other stromal elements	Nerve fibers
RCK102 (CK5+8)	9-10	++				-/+	-	-
	25	++	+			-/+	-	-
	37	++	+	+	-/+	-/+	-	-
	newborn	++	+	+	-/+	-/+	-	-
	adult	++	++	+	-/+	-	-	-
RCK105 (CK7)	9-10	some c.				-	-	-
	25	-/+	-/+			-	-	-
	37	-/+	-/+	+	-/+	-	-	-
	newborn	++	-/+	+	-/+	-	-	-
	adult	++	-	+	-/+	-	-	-
RGE53 (CK18)	9-10	++				-/+	-	-
	25	++	+			-/+	-	-
	37	++	+	+	-/+	-/+	-	-
	newborn	++	+	+	-/+	-/+	-	-
	adult	++	-	+	-/+	-	-	-
RCK106 (CK18)	9-10	++				-/+	-	-
	25	++	+			-/+	-	-
	37	++	+	+	-/+	-/+	-	-
	newborn	++	+	+	-/+	-/+	-	-
	adult	++	some c.	+	-/+	-	-	-
LP2K (CK19)	9-10	+				-	-	-
	25	+	+			-	-	-
	37	+	+	+	-/+	-	-	-
	newborn	+	+	+	-/+	-	-	-
	adult	++	+	+	-/+	-	-	-
6B10 (CK4)	9-10	-				-	-	-
	25	-	-			-	-	-
	37	-	-	some c.	-	-	-	-
	newborn	-	-	-/+	-	-	-	-
	adult	-/+	-/+	-/+	-	-	-	-
1C7 (CK13)	9-10	-				-	-	-
	25	-	-			-	-	-
	37	-	-	some c.	-	-	-	-
	newborn	-	-	-/+	-	-	-	-
	adult	-/+	-	-	-	-	-	-
LL001 (CK14)	9-10	-				-	-	-
	25	-	--			-	-	-
	37	-	some c.	-	-	-/+	-	-
	newborn	-	-/+	-	-/+	-/+	-	-
	adult	-	-/+	-	-/+	-	-	-

Table 1 (cont.)

| Anti-body (reactivity) | Age | Epithelial cells | | | (Sero-) mucous glands | Smooth muscle | Other stromal elements | Nerve fibers |
		Columnar or cuboidal	Basal cells	Term. sacs/ alveoli				
RD301	9-10	-				-/+	-	-
(desmin)	25	-	-			-/+	-	-
	37	-	-	-	-	-/+	-	~
	newborn	-	-	-/+	-	+	-	-
	adult	-	-	-	-	+	-	-
pDes*	9-10	-				+	-/+	-
(desmin)	25	-	-			+	-/+	-
	37	-	-	-	-	++	-/+	-
	newborn	-	-	-	-	++	-/+	-
	adult	-	-	-	-	++	-	-
RV202	9-10	+				+	++	-
(vimen-tin)	25	-/+	-/+			+	++	-
	37	-/+	-/+	-	-	-/+	++	-
	newborn	some c.	some c.	-	-	-	++	-
	adult	-/+	-/+	-	-	-	++	-
pVim*	9-10	-/+				+	++	-
(vimen-tin)	25	-/+	-/+			+	++	-
	37	some c.	some c.	-	-	-/+	++	-
	newborn	some c.	some c.	-	-	-	++	-
	adult	some c.	some c.	-	-	-	++	-
MNF	9-10	-				-	-	+
(NF68+	25	-	-			-	-	+
NF200)	37	-	-	~	-	-	-	++
	newborn	-	-	-	-	-	-	++
	adult	-	-	-	-	-	-	++

++	=	strong reaction in all cells
+	=	reaction in most cells
-/+	=	reaction in part of the cells
some c.	=	reaction in some scattered cells
-	=	no reaction
*	=	polyclonal (rabbit) antiserum

however, did stain these blood vessel muscle cells next to the muscle cells surrounding bronchi.
The vimentin antibody RV202 was reactive in part of the bronchial epithelial cells (Fig. 1B6), in all stromal elements, including cartilage and smooth muscle cells, but in general not in epithelial cells of the respiratory bronchioli (Fig. 2B4). pVim showed a comparable reaction pattern again, however, reacting with less epithelial cells than RV202.
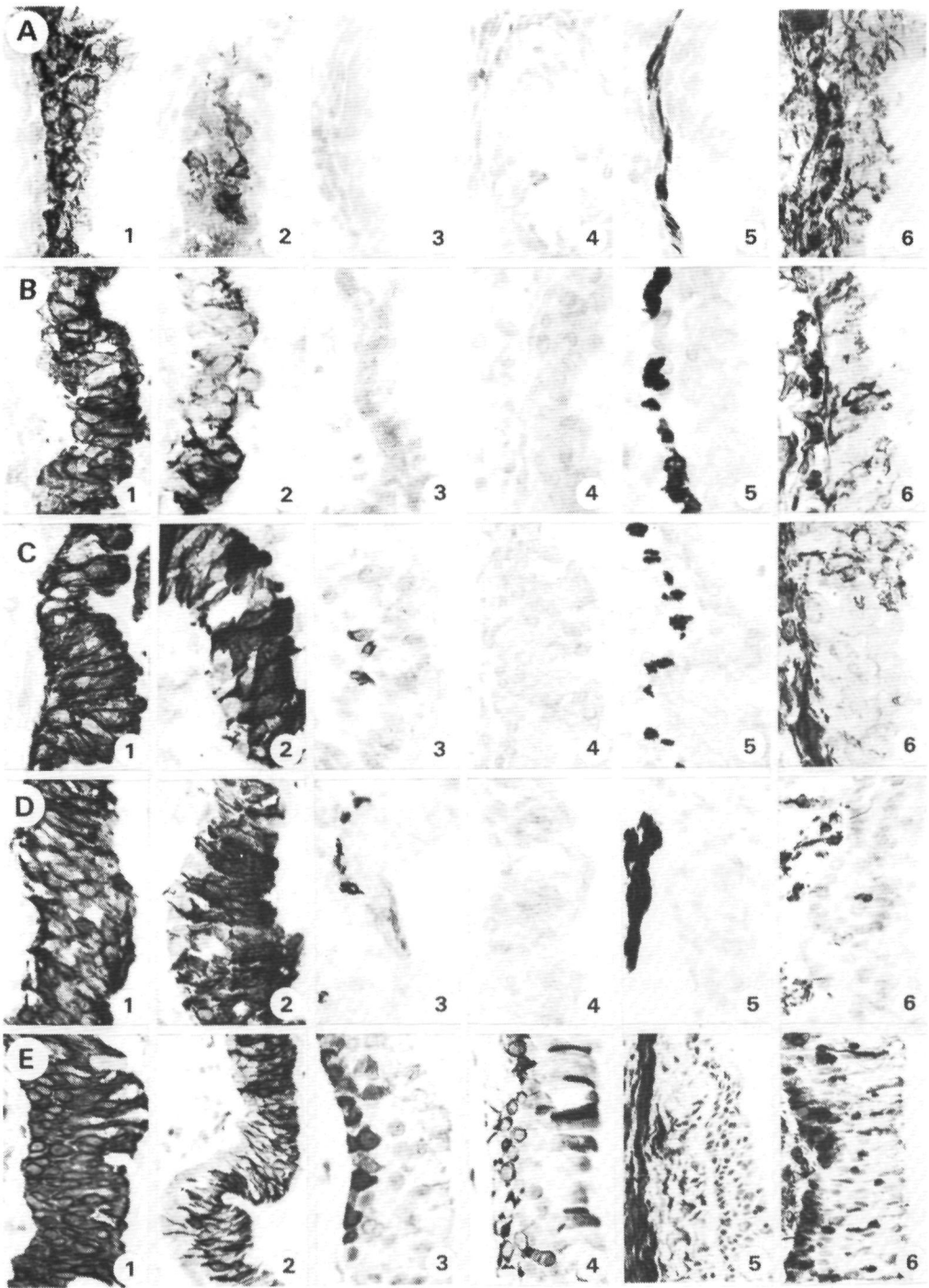
FIGURE 1

Reaction patterns of developing human lung bronchi with antibodies to intermediate filament proteins in the immunoperoxidase technique. Panel A, 9-10 weeks of gestation; panel B, 25 weeks of gestation; panel C, 37 weeks of gestation; panel D, newborn; and panel E, adult.

Panel A: The broadly cross-reacting CK antibody RCK102 reacted with all epithelial cells (A1, x300), while the CK 7 antibody RCK105 recognized only scattered cells (A2, x380). Other CK antibodies such as the CK 14 antibody LL001 (A3, x380) and the CK 13 antibody 1C7 (A4, x240) did not react with bronchial cells. The monoclonal desmin antibody RD301 reacted with smooth muscle cells surrounding bronchi (A5, x325). The vimentin antibody RV202 reacted not only with stromal elements, but also with epithelial cells of the bronchus (A6, x400).

Panel B: RCK102 reacted with all epithelial cells (B1, x420), while RCK105 reacted in a heterogeneous manner with these cells (B2, x420). LL001 (B3, x380) and 1C7 (B4, x330) did not react with these cells. RD301 again stained smooth muscle cells (B5, x380), and RV202 reacted with part of the epithelial cells and all stromal cells (B6, x300)

Panel C: The CK 18 antibody RCK106 reacted with basal as well as columnar cells (C1, x380), while RCK105 reacted with columnar epithelium cells, but not with basal cell (C2, x400). LL001 showed a reaction with scattered cells in the lower epithelial layer of the bronchus (C3, x350), while no reaction was seen with 1C7 (C4, x240). RD301 reacted with smooth muscle cells (C5, x220), and RV202 reacted with stromal cells, and with some bronchial epithelial cells (C6, x370).

Panel D: The CK 18 antibody RGE53 recognized all bronchial epithelial cells (D1, x240), while RCK105 reacted with epithelial cells except for the basal layer (D2, x330). LL001 reacted with some basal cells of the bronchus (D3, x300), while 1C7 was negative (D4, x250). RD301 reacted with groups of smooth muscle cells surrounding the bronchi (D5, x200), and RV202 reacted with stromal cells and few epithelial cells (D6, x230).

Panel E: RCK102 recognized all epithelial cells (E1, x160), while RCK105 recognized epithelial cells with the exception of the basal layer (E2, x160). LL001, in general, reacted with basal cells only (E3, x340). The CK 4 antibody 6B10 reacted in a heterogeneous manner with some basal and some columnar epithelial cell (E4, x370). RD301 reacted with the thick layer of smooth muscle cells (E5, x160), while RV202 reacted with all stromal cells, but sometimes also with epithelial cell (E6, x200).

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37 weeks (Figs. 1C1-6 and 2C1-6)

At this stage basal cells are clearly discernable in the bronchi, while alveoli start to develop from the terminal sacs, and sero-mucous glands are visible. Antibodies RCK102, RGE53, and RCK106 (Figs. 1C1 and 2C1) reacted with all bronchial and alveolar cells and with most of the sero-mucous glands. Part of the blood vessel smooth muscle cells reacted with these antibodies (Fig. 2C6).

RCK105 reacted with the bronchial epithelium, except for the basal cell compartment (Fig. 1C2), with all epithelial alveolar cells (Fig. 2C2), and with most of the sero-mucous glands. Again, no reaction was seen in smooth muscle cells with this antibody.

FIGURE 2
Reaction patterns of different lung tissues with antibodies to intermediate filaments using the immunoperoxidase technique. Panel A, 9-10 weeks of gestation; panel B, 25 weeks of gestation; panel C, 37 weeks of gestation; panel D, newborn; and panel E, adult.

Panel A: RCK102 recognized all epithelial cells of the bronchial tubes (A1, x250). RCK105 did not react with these cells (A2, x360), nor did LL001 (A3, x360). RV202 recognized not only all stromal cells, but also many epithelial cells (A4, x260). RD301 recognized smooth muscle fibers present in this bronchial tube tissue (A5, x300), but did not recognize blood vessel smooth muscle cells (not shown). RCK102 showed a clear reaction with developing blood vessels (A6, x360).

Panel B: RCK106 recognized all epithelial cells of the respiratory bronchioli (B1, x230), while RCK 105 recognized only few epithelial cells (B2, x360). The CK 4 antibody 6B10 did not react with the lung tissue (B3, x370), while RV202 reacted with stromal, but not with epithelial cells of these respiratory bronchioli (B4, x225). RCK105 did not react with blood vessels (B5, x330), while RGE53 clearly recognized these smooth muscle cells (B6, x330).

Panel C: RCK106 (C1, x350) and RCK105 (C2, x350) reacted with all epithelial cells of the terminal sacs, while antibody 1C7 occassionally reacted with scattered cells only (C3, x300). LL001 reacted with myo-epithelial cells of sero-mucous glands (C4, x320), but not with other epithelial cells in the terminal sacs. RD301 now started to react weakly with some smooth muscle cells surrounding blood vessels (C5, x300), while RCK106 stained more blood vessel smooth muscle cells (C6, x300) than RD301.

Panel D: RGE53 (D1, x330) and RCK105 (D2, x270) reacted with all epithelial cells of the early alveoli, while 6B10 reacted with isolated, scattered cells in these alveoli (D3, x400). Sero-mucous glands were partly positive with RCK105 (D4, x350). RD301 reacted weakly with smooth muscle cells in blood vessels (D5, x330), while the reactivity of RGE53 in blood vessels was lessened (D6, x300) as compared to earlier stages.

Panel E: RCK106 (E1, x250) and RCK105 (E2, x350) reacted with all alveolar epithelial cells. 1C7 did not react in alveoli (E3, x250). A cluster of sero-mucous glands reacted with RCK105 (E4, x160), while RD301 reacted strongly with part of the blood vessel muscle cells (E5, x 200). All CK antibodies used (E6, x175; RGE53), did not recognize blood vessel smooth muscle cells.

The CK 14 antibody LL001, which was negative in the earlier developmental stages, now reacted with some basal cells in the larger bronchi (Fig. 1C3), and with myoepithelial cells lining the sero-mucous glands (Fig. 2C4).

Antibodies 6B10 and 1C7 (Fig. 2C3), specific for CKs 4 and 13, respectively, and negative with all cells sofar, now showed a reaction with some scattered cells in the developing alveolar epithelium, while still no reaction was seen in larger bronchi (Fig.1C4).

RD301 reacted with smooth muscle cells surrounding bronchi (Fig. 1C5), and now also with some smooth muscle cells surrounding blood vessels (Fig. 2C5), while pDes reacted with all smooth muscle cells.

The expression of vimentin was mostly confined to stromal and cartilage cells. Only some cells of the bronchial epithelium reacted with RV202 (Fig 1C6), or with pVim.

Newborn (43 weeks, Figs. 1D1-6 and 2D1-6)

RCK102, RGE53 (Figs. 1D1 and 2D1), RCK106, and LP2K reacted with all bronchial and alveolar epithelial cells in this case, while also most sero-mucous glands were positive with these antisera. The reactivity with blood vessel smooth muscle cells was lessened as compared to the earlier stages of lung development (Fig 2D6).

RCK105 strongly reacted with all bronchial epithelial cells, again with the exception of the basal cells of larger bronchi (Fig. 1D2), while all alveolar epithelial cells (Fig. 2D2) and most sero-mucous glands were also positive (Fig 2D4). LL001 reacted with part of the basal cells of bronchi (Fig. 1D3), and also stained myoepithelial cells in sero-mucous glands.

6B10 and 1C7, which did not react with bronchial epithelial cells (Fig 1D4), did react with scattered cells in lung alveoli (fig. 2D3). These cells possibly belong to the group of pneumocytes (type I or type II), but the exact nature of these cells remains to be examined.

RD301 recognized smooth muscle cells surrounding bronchi (Fig. 1D5) and some smooth muscle cells in blood vessels (Fig. 2D5), while pDes recognized all smooth muscle cells.

RV202 reacted with few scattered cells in bronchi (Fig. 1D6) and with all stromal elements and cartilage structures. The number of bronchial cells reacting with RV202 was clearly reduced as compared to earlier stages. pVim reacted strongly with mesenchymal tissues, but only very weakly with few epithelial cells of the bronchial epithelium.

Normal adult bronchial tissues (Figs. 1E1-6 and 2E1-6).

A considerable heterogeneity was detected in the expression of the different CKs in normal adult bronchial tissues. Antibody RCK102 (Fig. 1E1) and LP2K reacted with all bronchial and alveolar epithelial cells. RCK106 (Fig. 2E1) reacted with all epithelial cells, except for part of the basal cells in bronchial epithelium, while RGE53 did not react with any of the basal cells in this type of epithelium. RCK102, RCK106, RGE53 and LP2K reacted with most sero-mucous glands, while no reaction with CK antibodies was noticed in smooth muscle cells of blood vessels (Fig. 2E6), as seen in the fetuses. RCK105 reacted with bronchial epithelial cells, except for the basal layer (Fig. 1E2), with epithelial alveolar cells (Fig. 2E2), and with some of the sero-mucous glands (Fig. 2E4). Antibody LL001 showed a considerable heterogeneity in its reaction with bronchial epithelium, varying from a negative reaction to a positive staining reaction with all bronchial epithelium cells. In general, however, basal cells of bronchial epithelium were preferently stained (Fig. 1E3), while also myoepithelial cells of the sero-mucous glands, but not alveolar cells were positive. Antibodies 6B10 and 1C7 reacted in a highly variable fashion in bronchial epithelium, ranging from a strong reaction in all bronchial epithelial cells, as seen in some cases with 6B10, to a completely negative reaction as seen in some cases with 1C7. In general, a heterogeneous reaction pattern was observed, as illustrated in Fig. 1E4, with more cells reactive for 6B10 than for 1C7. Note from Fig. 1E4 that also basal cells may be positive for 6B10. In alveolar epithelial cells as well as in sero-mucous glands no reaction was seen with 6B10 or with 1C7 (Fig. 2E3). The desmin antibody RD301 reacted with all smooth muscle cells surrounding bronchi (Fig. 1E5) and with a considerable part of the smooth muscle cells surrounding blood vessels (Fig. 2E5), while pDes recognized all smooth muscle cells. The vimentin antibodies RV202 and pVim in general did not react with epithelial cells. However, in some cases a clear reaction was seen with RV202 in basal cells and in some columnar epithelial cells of bronchi (Fig. 1E6).

DISCUSSION

    The development of chain-specific monoclonal antibodies to CKs and antibodies to other intermediate filament proteins has now provided a sensitive tool in the examination of differentiating cells in fetal and adult human lung tissues. Previous examinations on the expression of CKs in lung tissues were, in general, performed with broadly cross-reacting antibodies, which could differentiate between epithelial and non-epithelial cells [1, 15, 25, 34]. More recently polyclonal antibodies with a more narrow specificity and monoclonal antibodies were used to distinguish between different types of epithelial cells [2, 5, 18, 30]. The use of gel electrophoretic techniques has enabled the analysis of different CK polypeptides in cytoskeleton extracts of bronchial and lung epithelium [1, 2, 18]. Using this technique, however, detection at the single cell level is not possible. In a recent study we have reported on the use of monoclonal antibodies to different CK polypeptides in lung carcinomas [5]. From this study it has become clear that cells with a similar histologic appearance can express different CK polypeptides. This allows detection of subpopulations of cells which are histologically not distinguishable.

From our results it is clear that in all stages of human lung development a uniform staining reaction in all epithelial cells is seen with the CK 19 antibody, as well with a broadly cross-reacting CK antibody. CK 7 is expressed rather weakly and in only few cells at early stage, but develops towards a strong staining reaction in all epithelial cells (except the basal cells of the larger bronchi) at later stages.
CK 18 is found in all epithelial cells of the developing lung until birth, including the basal cells of the developing bronchus. In the adult bronchi, however, these basal cells are negative for RGE53 (Compare also [2]) and partly negative for RCK106. From these data we conclude that the structure of the CK 18 epitope recognized by RGE53 changes upon lung maturation after birth. Expression of CK 4 and 13 was not detected in the fetal lung tissues, with the exception of some scattered cells in early alveolar tissues. In adult bronchi CK 4 is found to be expressed heterogeneously in basal and columnar cells, while in some alveoli also CK 4 positive cells are found. CK 13 reacted with only few columnar epithelial cells.

    The expression of CK 14 occurs in the third trimester and is mainly confined to (part of) the basal cells of the bronchi(oli). This CK polypeptide, which is absent until at least week 25 of gestation, becomes more and more prominently expressed in basal cells of the bronchial epithelium upon maturation. However, when examining the fetal lung tissues more closely it becomes obvious that basal cells occur in the developing bronchus long before these cells become CK 14 positive. In fact, the fetal and newborn lung tissues that we have examined contained only few CK 14 positive cells, while in the adult human lung an extensive staining reaction is observed in the basal cells. In some cases of adult bronchus, areas were detected in which this antigen was also expressed in columnar epithelial cells, suggesting the presence of hyperplasia originating from basal cells of the bronchial epithelium. This observation is paralleled by our finding that most squamous cell carcinomas of the lung also contained CK 14 (unpublished data).

Our findings that only basal cells at later stages of development of the bronchus express CK 14 are in line with the hypothesis of McDowell et al. [14] that not the basal bronchial epithelial cells, but most probably undifferentiated secretory columnar cells are the progenitor cells of all other bronchial epithelial cells. Also our observations that most columnar cells do not contain CK 14, and that basal cells lose CK 7 and CK 18 upon maturation argues against the theory of basal cells as bronchial stem cells.

When comparing our immunohistochemical data with the gel electrophoretic patterns, described by Blobel et al. [2], it is clear that CK polypeptides occurring in low concentrations or in only a few bronchial epithelial cells, i.e. CKs 4, 13, and 14, may be missed in such integral biochemical procedures. We do feel, however, that the presence of CKs 4 and 13, which are characteristic of non-keratinizing squamous epithelia [23, 30], in areas of histologically normal bronchus points to some extent to an onset of abnormal differentiation. The same may hold true for bronchial regions in which CK 14 is detected in columnar epithelial cells. Future studies will have to reveal whether or not these CK antibodies may be used to detect 'preneoplastic' lesions in the bronchus, such as epidermoid metaplasia [14] at an early stage.

Examination of changes in IFP expression patterns in fetal tissues may have relevance regarding the histogenesis of pathologic lesions found in the adult epithelium. In the developing (mouse) embryo the first IFPs to appear are CK 8- and 18-related proteins [9, 13, 20], present in ectodermal and endodermal embryonal tissues. From our results it is clear that the expression of so-called simple CKs, i. e. CK 7 (minor amounts), 18 and 19 occurs in developing fetal bronchial tissues. Comparable CK expression patterns are observed in small cell lung cancer and adenocarcinomas of the lung [2, 5], while most squamous cell carcinomas express a more complex CK pattern. The finding that CK 7 is absent in many small cell lung cancers [5], and also almost completely absent in the early stage (9-10 weeks) of fetal lung development is in accord with earlier findings on the expression of neuroendocrine-related antigens in both small cell lung cancer and early fetal bronchial cells [6], suggesting a comparable degree of differentiation of both cell types

In some developing epithelia such as parietal endodermal cells a coexpression of CK and vimentin has been seen [12], while also in human amnion epithelial cells [24] and developing human embryonic kidney tubules [8] this coexpression was observed. Our investigations show that in the early stages of development next to the presence of CKs a clear expression of vimentin can be noticed in the bronchial epithelium. With increasing age of the fetus a decrease both in intensity and in number of vimentin positive cells is seen. At birth only few bronchial epithelial cells express vimentin. However, in some adult bronchial epithelia again the coexpression of vimentin was seen. Coexpression of cytokeratins and vimentin is quite common in carcinomas of the lung [10, 29]. Again, it remains to be examined whether this phenomenon points to a beginning transformation in the bronchus.

Van Muijen et al. [31] have already described coexpression of CKs, vimentin, and desmin in blood vessels of fetal lung. These data could be confirmed in this study. In addition, however, using two different

antibodies to desmin, differential staining patterns were seen. While the polyclonal antibody to desmin reacted with all smooth muscle cells, the monoclonal antibody RD301 reacted in early stages only with smooth muscle cells surrounding bronchi, and not with blood vessel smooth muscle cells. At these stages, these smooth muscle cells were positive with several CK antibodies. The reactivity of blood vessel smooth muscle cells with CK antibodies decreased with age, while the reactivity with RD301 increased. Recent data by van Muijen et al. [31] and Turley et al. [28] strongly suggest that this phenomenon has to be explained by true coexpression of these IFP, rather than by sharing of epitopes.

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CHAPTER THREE

SMALL CELL LUNG CANCERS CONTAIN INTERMEDIATE FILAMENTS OF
THE CYTOKERATIN TYPE.

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Small Cell Lung Cancers Contain Intermediate Filaments of the Cytokeratin Type

To the Editor: In view of the well-established neuroendocrine nature of the pulmonary carcinoids and small cell carcinomas (4) it was of interest to note recent reports describing the presence of neurofilaments in the cytoskeleton of these cells (2, 6). Another paper, however, has appeared challenging these observations and claiming that the pulmonary neuroendocrine tumors contain cytokeratin rather than neurofilament cytoskeletal proteins (3). Stimulated by these apparently contradictory findings we have examined eight small cell carcinomas of the lung and four carcinoids. Our data obtained by using a polyclonal antiserum raised against skin keratins and two monoclonal antibodies reacting with (cyto)keratins, as well as three monoclonal antibodies to the different neurofilament proteins, indicate that these tumors indeed contain cytokeratin proteins. No staining was seen with the antibodies to neurofilaments.

In brief, cryostat sections of freshly resected tumors were examined by the indirect immunoperoxidase technique. The relevant clinical and pathologic data of the small cell lung tumors and carcinoids used in this study are summarized in Table 1. Samples of human brain, peripheral nerves, and neural tumors were also used to test the specificity of the neurofilament antibodies. The following antibodies were used (1, 5, 7, 8): (a) an affinity-purified antiserum directed against human skin keratins (pK), which reacts with virtually all epithelial tissues but not with nonepithelial tissues (8); (b) the monoclonal antibody RGE 53, directed against cytokeratin 18, which specifically recognizes columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells (7); (c) the monoclonal antibody RKSE 60; directed against human skin keratins and specific for keratinizing squamous cells (8); (d) the monoclonal antibodies BF10 and RT97 directed against different neurofilament polypeptides. In immunoblotting assays BF10 was shown to react only with the 155-kilodalton (kd) neurofilament protein, whereas RT97 reacts mainly with the 210-kd neurofilament protein (1); (e) a monoclonal antibody to neurofilaments (MNF) purchased from Euro-Diagnostics B.V., Apeldoorn, The Netherlands. This antibody corresponds to the monoclonal antibody 2F11 described by Klück et al. (5). In immunoblotting assays we could show that this antibody reacts strongly with the 210-kd neurofilament polypeptide and to a somewhat lesser extent with the 70-kd neurofilament polypeptide.

Using frozen sections and paraffin sections from human brain, peripheral nerves, and some neural tumors, we found a positive reaction of the monoclonal antibodies to neurofilament proteins (BF10, RT 97, and MNF) in only the neural tissue.

In the normal lung the affinity-purified polyclonal antiserum to skin keratins shows a positive reaction with bronchial, broncholar, and alveolar epithelium. A relatively strong reaction is observed in basal cells of the bronchial epithelium. No reaction was seen in nonepithelial tissues. This antibody did strongly stain squamous cell carcinomas and adenocarcinomas of the lung. The antibody to cytokeratin 18 (RGE 53) strongly stained all epithelial components except the basal cells of the respiratory epithelium. Nearly all small cell carcinomas as well as the carcinoids were positively stained with the polyclonal keratin antiserum (Fig. 1a). The monoclonal antibody to cytokeratin 18 (RGE 53) gave a strong diffuse and homogeneous staining pattern in all of the examined small cell lung carcinomas and in the carcinoids (Fig. 1b). The monoclonal antibody specific for keratinizing epithelium (RKSE 60) did not react with the small cell lung carcinomas or carcinoids. None of the neurofilament monoclonal antibodies gave a positive reaction in the small cell lung carcinomas or in the carcinoids (Fig. 1c). They did, however, strongly stain peripheral nerves present in the lung tumor sections (Fig. 1d). Also, some isolated scattered cells in normal and tumor tissue were found to be positive with all three neurofilament antibodies.

In summary, our findings contradict some published papers (2, 6) but are in agreement with data reported by Blobel et al. (3). It is not clear whether these differences are due to technical problems related to the specificity of antibodies and fixation procedures or whether we indeed have used or selected neuroendocrine tumors that express cytokeratins and not neurofilament proteins.

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TABLE 1. CLINICOPATHOLOGIC AND IMMUNOHISTOCHEMICAL DATA OF SMALL CELL LUNG CARCINOMAS AND CARCINOIDS OF THE LUNG[a]

	Patient data						Reactivity pattern with intermediate filament antibodies					
No.	Sex	Age	Diagnosis	WHO 1982 Subtype classification	Clinical stage	Pathological stage	pK	RGE 53	RKSE 60	RT 97	BF 10	MNF
		yr										
1	F	70	SCLC	Intermediate	T2N0M0	T2N0M0	+	++	−	−	−	−
2	M	68	SCLC	Intermediate	T1N0M0	T2N1M0	+	++	−	−	−	−
3	M	58	SCLC	Intermediate	T3N0M0	T3N2M0	+	++	−	−	−	−
4	M	46	SCLC	Intermediate	T2N0M0	T2N1M0	++	+++	−	−	−	−
5	M	69	SCLC	Intermediate	T1N0M0	T1N2M0	++	+++	−	−	−	−
6	M	68	SCLC	Intermediate	T1N0M0	T1N2M0	++	+++	−	−	−	−
7	F	59	SCLC	Intermediate	T1N0M0	T1N0M0	−	++	−	−	−	−
8	M	64	SCLC	Intermediate	T2N0M0	T3N0M0	+	++	−	−	−	−
9	M	60	carcinoid		T1N0M0	T1N0M0	+	+++	−	−	−	−
10	F	58	carcinoid		T1N0M0	T1N0M0	++	+++	−	−	−	−
11	M	71	carcinoid		T1N0M0	T1N0M0	+/−	+++	−	−	−	−
12	F	62	carcinoid		T1N0M0	T1N0M0	−	+++	−	−	−	−

[a] Abbreviations: SCLC, small cell lung carcinoma; WHO, World Health Organization; pK, polyclonal rabbit antiserum to human skin keratins; T1N0M0, ≤3 cm and no invasion; T2N0M0, >3 cm and extension to hilar region; T3N0M0, gross extension, effusion, and atelectasis.
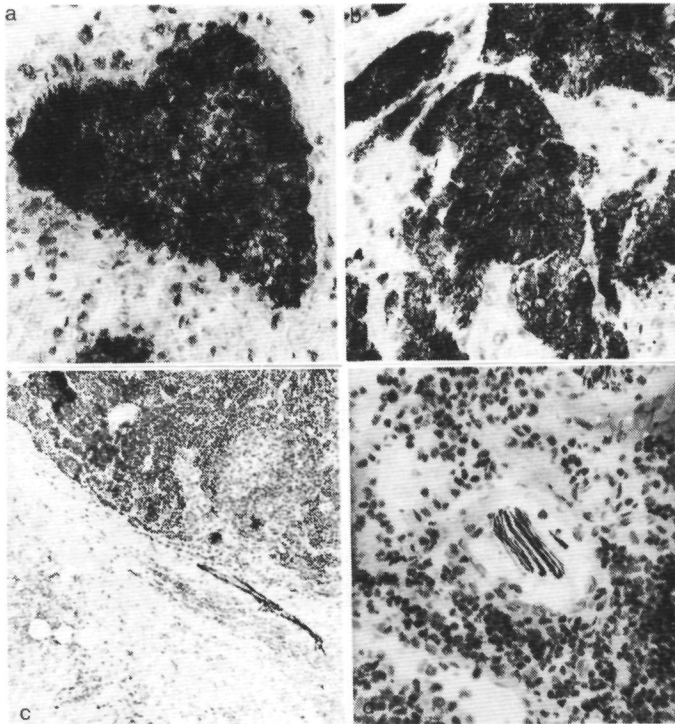

FIG. 1. Photomicrographs of cryostat sections of small cell lung carcinomas stained by the immunoperoxidase technique using the polyclonal keratin antiserum (a), the monoclonal antibody to cytokeratin 18 (b), and monoclonal antibody to neurofilaments (BF10; c and d). Note positive reaction of BF10 with peripheral nerves but not with the tumor. a and b, ×200; c, ×100; d, ×150.

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CHAPTER FOUR

IMMUNOCYTOCHEMICAL DETECTION OF LUNG CANCER HETEROGENEITY
USING ANTIBODIES TO EPITHELIAL, NEURONAL,
AND NEUROENDOCRINE ANTIGENS.

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Immunocytochemical Detection of Human Lung Cancer Heterogeneity Using Antibodies to Epithelial, Neuronal, and Neuroendocrine Antigens[1]

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ABSTRACT

Lung cancers were investigated for their heterogeneity as expressed by their immunoreactivity for cytokeratins and neurofilament proteins, as well as for the neuroendocrine differentiation antigen MOC-1. Using broadly cross-reacting antibodies, cytokeratins were detected in nearly all cases of lung carcinomas. Keratinization could be detected only in cases of moderately to well-differentiated squamous cell carcinoma (SQC) using a monoclonal antibody to cytokeratin 10, while a monoclonal antibody reactive with cytokeratin 18, and specific for glandular epithelia, reacted with adenocarcinomas, small cell lung carcinomas (SCLC), and lung carcinoids. In SQC this antibody could detect non-squamous cell differentiation, showing increasing numbers of positive cells with decrease of histologically detectable SQC differentiation. Cells positive for neurofilaments were demonstrated in some of the poorly differentiated SQCs and in some of the cases of SCLC, possibly representing the variant type of SCLC. Also in some of the lung carcinoids neurofilament proteins were present, colocalizing with cytokeratins. MOC-1 was present in all SCLC and lung carcinoids. This antibody could also detect neuroendocrine differentiation in all combined small cell carcinomas, in one poorly differentiated adenocarcinoma, and in about 30% of the poorly differentiated SQCs. Therefore, lung cancer heterogeneity can be detected using a panel of well-defined antibodies to intermediate filaments in combination with the MOC-1 antibody. The use of these antibodies in diagnosis can have prognostic significance and can lead to a more selective therapeutic approach.

INTRODUCTION

Human lung cancer is the main cause of cancer death in western countries and its occurrence is especially rapidly growing among women (1). Different subtypes of lung cancer can be classified according to their histological appearance. The four major subtypes are SCLC,[3] large cell carcinoma, SQC, and adenocarcinoma (2). The three latter subtypes are often referred to as non-SCLC. Individual lung tumors may show a profound heterogeneous composition. For instance, according to the work of Hess *et al.* (3) in cytological specimens about 50% of all lung tumors can be classified as admixtures of epidermoid and adenocarcinomatous subtypes, while also at the electron microscopic level up to 50% of lung tumors classified as SQC on the basis of routine histology appear to contain characteristics of adenocarcinomatous differentiation (4). Another admixture of different tumor subtypes can be found in combined small cell carcinoma (5), an entity of lung cancer in which SCLC and non-SCLC differentiation are found simultaneously. Also in tumors which are histologically indistinguishable from non-SCLC, (neuro)endocrine features, such as the production

of peptide hormones or the presence of marker enzymes, can be detected (6, 7). Because of differences in treatment and prognosis, differentiation between SCLC and non-SCLC is of utmost clinical importance (8). SCLC express neuroendocrine features, among which is the production of neurosecretory granules detectable in the electron microscope (9). Also the production of neurotransmitters, neuropeptides, amine precursor uptake and decarboxylation-related enzymes, and cell surface markers such as those detected by the monoclonal antibody specific for neural and neuroendocrine cells MOC-1 (10) indicate this type of differentiation. Another indicator of the neuroendocrine nature of tumor cells is the coexpression of both cytokeratins and neurofilament proteins, intermediate filament proteins generally specific for epithelial and neuronal cells, respectively (11–16).

SCLC can be subdivided into two different tumor types, namely the classic and the variant type of SCLC (5). The variant type of SCLC, occurring in about 6–15% of all SCLC at first diagnosis (17, 18), consists of a mixture of small cell and large cell components and occurs in up to 30% of all SCLC after chemotherapy and/or radiotherapy (19). Within SCLC cell lines the classic and variant subtypes can be distinguished from each other on the basis of the presence of cytokeratins and the absence of neurofilaments in the classic subtype and the absence of cytokeratins in the variant cell lines, which may contain neurofilaments (20, 21). Also coexpression of cytokeratins and neurofilaments in some SCLC cell lines has been suggested (22, 23).

In order to investigate the degree of heterogeneity in lung tumors and to detect the two different subtypes of SCLC we have applied immunocytochemical techniques in a large series of lung tumor specimens. We have tested for the presence of different subtypes of cytokeratins, the presence of neurofilaments, and the occurrence of the MOC-1 antigen (10). Our results are discussed in the light of lung cancer histogenesis and differentiation, and their significance for refinement of clinicopathological diagnosis is addressed.

MATERIALS AND METHODS

Normal Lung. Snap frozen specimens from normal human lung tissue from two different patients were stored in liquid nitrogen and 4–7-μm frozen sections were used in the immunoperoxidase technique. The frozen sections were fixed in acetone (−20°C, 5 min). Normal tissue material was resected with tumor material

Lung Tumors. Fresh surgical specimens of 218 cases of lung cancer were snap frozen and stored in liquid nitrogen. Next paraffin sections of 76 routinely processed lung cancers from the Pathology Departments in Nijmegen and Nieuwegein, partly overlapping with the frozen section material, were used. In addition, 93 cases of paraffin-embedded lung tumors were obtained from Dr. J. Wilde, Zentralklinik für Herz- und Lungenkrankheiten, Bad Berka, G.D.R. Autopsy material (61 specimens in total) from primary tumors and metastases of up to 12 different body sites from 6 patients with a histological diagnosis of SCLC were investigated for the presence of cytokeratins and neurofilaments.

All lung tumors were classified on the basis of routine histological

hematoxylin-eosin staining according to the latest WHO classification (2).

Antibodies Used in This Study. The antibodies used in this study are summarized in Table 1 and have been described extensively before (10, 14, 20, 21, 24–32) Antibodies pKer, RCK102, RGE53, RKSE60, MNF, and MOC-1 are available from Euro Diagnostics B. V. (Apeldoorn, The Netherlands), CAM5 2 was from Becton and Dickinson (Erembodegem, Belgium), and pNF160 was a generous gift from Dr. Y. Nakazato (Gunma, Japan)

Immunofluorescence and Immunoperoxidase Technique for Frozen Sections. The indirect immunofluorescence technique and immunoperoxidase technique for fresh frozen tissue sections, fixed in acetone (−20°C, 5 min), have been described extensively before (21). Dilutions of the primary antibodies were made in PBS (see Table 1).

As second antibodies in the indirect immunofluorescence technique either FITC-conjugated goat anti-rabbit IgG (1 25; Nordic, Tilburg, The Netherlands) or FITC-conjugated rabbit anti mouse IgG (1:25; Nordic) were used. In control experiments PBS was used instead of the primary antibody.

In the immunoperoxidase technique swine anti-rabbit immunoglobulin conjugated to peroxidase (DAKOpatts, Glostrup, Denmark) diluted 1 25 in PBS containing 10% normal goat serum or rabbit anti-mouse immunoglobulin conjugated to peroxidase (DAKOpatts) diluted 1 25 in PBS with 5% human AB serum were used in the second step.

In order to detect coexpression of different types of intermediate filament proteins we have used the double immunofluorescence technique as described before (33) using the mouse monoclonal cytokeratin antibody RGE53 or RCK102 in combination with the rabbit neurofilament antibody pNF160. As second antibodies a mixture of FITC-conjugated goat anti rabbit IgG (diluted 1 25; Nordic) and Texas Red-conjugated sheep anti mouse immunoglobulin (diluted 1:50; New England Nuclear, Boston, MA) was used

Paraffin-embedded Specimens Cytokeratin antibodies pKer, RCK 102, and CAM5 2 and the neurofilament antibodies MNF and pNF160 are the only antibodies of those summarized in Table 1 that can be applied successfully to formalin-fixed and paraffin-embedded material using the PAP method (34) or the avidin biotin labeling technique (35)

PAP Technique (34) Paraffin-embedded tumor material was cut into 6 μm-thick sections and fixed onto glass slides using Ortho tissue adhesive (Ortho Diagnostic, Raritan, NJ). After deparaffinizing in xylene and a descending alcohol series, sections were incubated for 30 min in 100% methanol containing 2% hydrogen peroxide

Sections used for the detection of cytokeratins were pretreated with 0 1% Pronase (Sigma Chemical Co., St. Louis, MO) in 0.05 M Tris-NaCl buffer, pH 7.6, for 15 min at 37°C Pronase activity was terminated by washing in Tris-NaCl at 4°C for 30 min Then, after a washing with PBS the sections were preincubated for 30 min with 10% normal swine serum in PBS and incubated overnight with the primary antiserum at 4°C Antibody dilutions were made in PBS containing 10% normal swine serum (see Table 1).

After washing in PBS, sections which were incubated with a monoclonal antibody in the first step were incubated with rabbit anti mouse immunoglobulin (DAKOpatts) diluted 1 200 in PBS containing 10% normal swine serum for 30 min After another washing step in PBS all sections were incubated with swine anti-rabbit immunoglobulin (DAKOpatts) diluted 1:30 in PBS with 10% normal swine serum for

30 min, washed again, and incubated with the rabbit PAP complex (DAKOpatts) diluted 1:100 in PBS with 10% normal swine serum for 30 min. Peroxidase activity was detected for 5 min using 500 mg/liter 3,3'-diaminobenzidine dissolved in 0 05 N Tris-NaCl containing 0 65% imidazole (Merck, Darmstadt, West Germany), and 0.015% hydrogen peroxide as a substrate. Cells were counterstained for 30 s with Harris' hematoxylin (Merck) and after dehydration were embedded with Permount (Fisher Scientific Co., Springfield, NJ).

ABC Technique (35). Labeling with the ABC technique was performed using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) After deparaffinizing and preincubation with 10% normal goat serum or 10% normal horse serum for 60 min, primary antibodies were applied overnight at 4°C using the same dilutions as for the PAP staining method. After a washing with PBS followed by a preincubation step with PBS containing 10% normal goat serum or 10% normal horse serum for 15 min the following conjugates were applied: biotinylated goat anti-rabbit IgG diluted 1:250 in PBS containing 10% normal goat serum for 30 min; or biotinylated horse anti-mouse IgG diluted 1:200 in PBS containing 10% normal horse serum for 30 min. After a second washing and preincubation step avidin-biotinylated peroxidase complex in PBS containing 10% normal horse serum or 10% normal goat serum was added for 30 min at an avidin-biotinylated horseradish peroxidase:PBS ratio of 1 1:100. Detection of peroxidase activity was performed as described for the PAP technique.

RESULTS

Normal Lung Tissue

The reactivity patterns of the antibodies described above in normal lung tissue are summarized in Table 2. Antibodies pKer, RCK102, and CAM5.2 react with all epithelial elements but not with nonepithelial components. RGE53 reacts with the columnar epithelial cells but not with basal cells of the bronchial lining. Also a positive staining reaction can be observed with this antibody in seromucous glands of the bronchi and in type I and type II pneumocytes within the alveoli. RKSE60 does not react with any of the epithelial cells of the normal lung. MNF and pNF160 react only with nerve fibers. The MOC-1 antibody reacts with nerve fibers and with bronchial cells of the dispersed neuroendocrine system, the so-called Kulchitsky cells (10). These Kulchitsky cells are also positive with the cytokeratin 18 antibody RGE53 (36).

Squamous Cell Carcinomas

All SQCs show a diffuse reaction pattern (meaning a reaction in virtually all tumor cells) with the broadly cross-reacting cytokeratin antibodies pKer (Fig. 1a), RCK102, and CAM5.2 in both frozen and paraffin sections (Tables 3 and 4) In well-differentiated SQC a focal staining reaction (a reaction in part of the tumor cells) is seen with the monoclonal antibody RGE53, which is reactive only in frozen sections. The percentage of cells positive for RGE53 varied from zero (in two cases)

Table 1 *Specificity of antibodies used in this study*

Antibody	Immunoglobulin subclass (species)	Antigen recognized	Tissue specificity	Reactivity in tissue sections		Ref
				Frozen (dilution)	Paraffin (dilution)	
pKer	Polyclonal (rabbit)	Several cytokeratins	Most epithelial tissues	+ (1 10)	+ (1 40)	24
RCK102	IgG1 (mouse)	Cytokeratins 5 and 8	Nearly all epithelial tissues	+ (1 5)	+ (1 10)	21
CAM5 2	IgG2a (mouse)	Several cytokeratins	Nearly all epithelial tissues	+ (undiluted)	+ (1 5)	25
RGE53	IgG1 (mouse)	Cytokeratin 18	Glandular epithelia, not with squamous epithelia	+ (undiluted)	−	26
RKSE60	IgG1 (mouse)	Cytokeratin 10	Keratinizing epithelial cells	+ (undiluted)	−	27–29
MNF	IgG1 (mouse)	Neurofilaments, *M*, 68,000 and 200,000	Nerve fibers and neuronal tissues	+ (1 5)	+ (1:10)	14, 20, 30, 31
pNF 160	Polyclonal (rabbit)	Neurofilaments, *M*, 160,000	Nerve fibers and neuronal tissues	+ (1 600)	+ (1 2000)	21, 32
MOC 1	IgG1 (mouse)	Cell surface	Neuronal and neuroendocrine tissues	+ (1 5)	−	10

to about one-half of the tumor cells. The staining reaction was mainly localized in the basal cells of the tumor nests (Fig. 1b). The overall percentage of tumor cells in well-differentiated SQCs reactive with RGE53 is low, however. In most cases of

well-differentiated SQCs tested in frozen sections [13 of 15 (Table 3)] keratinizing areas can be demonstrated using the monoclonal antibody RKSE60 [Fig. 1c], while no reaction was found with MOC-1 or with neurofilament antibodies. The moderately well-differentiated SQC contained keratinizing areas in about one-half of the cases of frozen sections as detected by RKSE60 [44 of 85 (Table 3)]. However, in frozen sections also many tumors [23 of 85] react diffusely with RGE53 [Table 3], while the presence of the MOC-1 antigen [13 of 77 (Table 3)] and neurofilaments could be demonstrated [5 of 77 (Table 3)] also, albeit in a lower number of cases as compared to poorly differentiated SQCs (17% *versus* 28% for MOC-1 and 6% *versus* 13% for neurofilaments; see below).

In contrast to the well-differentiated SQCs, in a great number of histologically poorly differentiated SQCs tested in frozen sections a diffuse staining reaction with RGE53 can be observed [14 of 27 (Table 3; Fig. 1d)], while in only a minor number of these latter tumors [8 of 28 (Table 3)] keratinizing areas or single keratinizing cells could be detected with RKSE60. Also the presence of the MOC-1 antigen was demonstrated in 7 of 25 cases of frozen sections of these poorly differentiated SQCs

Table 2 *Reactivity pattern of different antibodies to cytokeratins, neurofilaments, and surface protein in frozen sections of normal lung tissues*

Tissue type	pKer, RCK102 and CAM5.2	RGE53	RKSE60	MNF and pNF160	MOC-1
Bronchi and bronchioles					
Columnar–cuboidal epithelium	+	+	−	−	−
Basal cells	+	−	−	−	−
Kulchitsky cells	−	+[a]	−	−[a]	+[a]
Cartilage	−	−	−	−	−
Mucous glands	+	+	−	−	−
Alveoli					
Pneumocytes I and II	+	+	−	−	−
Macrophages	−	−	−	−	−
All stromal elements	−	−	−	−	−
Nerve fibers	−	−	−	+	+

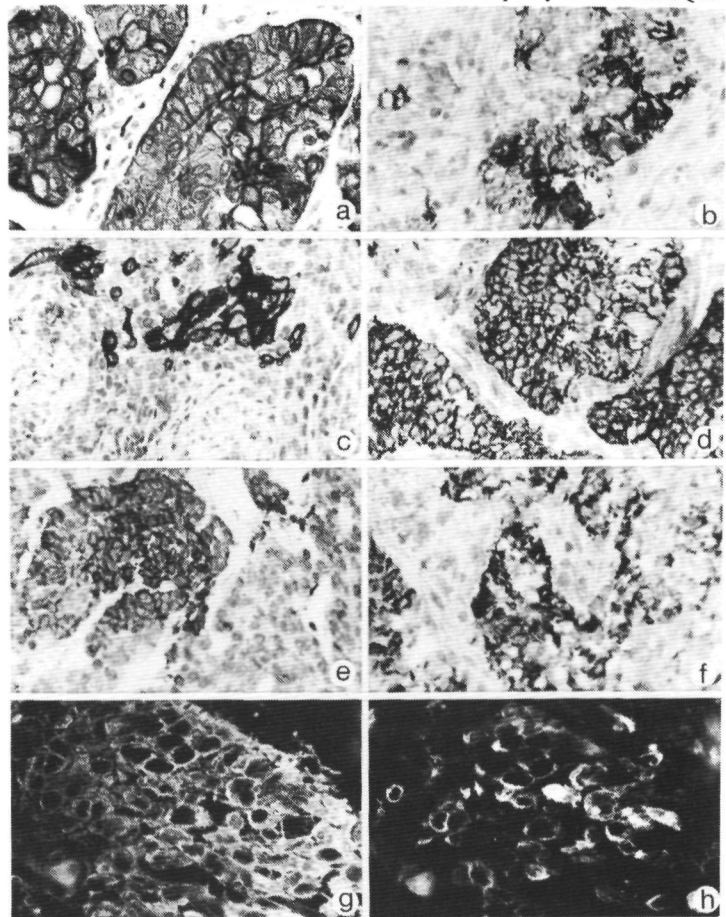[a] Data from report of De Leij *et al*. (36).

Fig. 1. Peroxidase anti-peroxidase staining on a paraffin section (a) and immunoperoxidase (b–f) and immunofluorescence (g, h) staining patterns in frozen sections of SQCs. a, cytokeratins detected in paraffin sections with the polyclonal cytokeratin antibody pKer. × 275. b, focal staining reaction of a small part of the tumor cells with the monoclonal antibody to cytokeratin 18, RGE53, in a well-differentiated SQC. × 290. c, RKSE60 stains only keratinizing cells within these well-differentiated SQC. × 170. In poorly differentiated SQC (nearly) all tumor cells react with RGE53 (d, × 220), while in some of these poorly differentiated tumors areas reacting with the MOC-1 antibody (e, × 175) or with the neurofilament antibody MNF (f, × 275) can be observed. g, h, double-label immunofluorescence technique in frozen sections demonstrating coexpression of cytokeratins (g, × 275) and neurofilaments (h, × 275) in some tumor cells of poorly differentiated SQC, while many tumor cells contain only cytokeratins and no neurofilaments.

Table 3 *Expression of cytokeratins neurofilaments and the MOC 1 antigen in frozen sections of human lung tumors*

	Squamous cell carcinoma			Adenosquamous cell carcinomas	Adeno-carcinoma	SCLC	SCLC squamous	Large cell carcinoma	Carcinoid
	Well differentiated	Moderately well differentiated	Poorly differentiated						
pKer									
F+[a]	0/15	5/85	2/28	2/8	6/43	4/20	3/4	0/2	2/10
D+	15/15	80/85	26/28	6/8	37/43	11/20	1/4	2/2	8/10
RCK102									
F+	0/1	0/25	0/14	0/5	0/29	1/16	0/3	0/2	0/9
D+	1/1	25/25	14/14	5/5	29/29	15/16	3/3	2/2	9/9
CAMS 2									
F+	2/2	0/2		0/1	0/4				
D+	0/2	2/2		1/1	4/4				
RGE53									
F+	13/15	57/85	11/27	2/8	4/45	3/20	3/4	0/2	2/10
D+	0/15	23/85	14/27	4/8	39/45	16/20	1/4	2/2	8/10
RKSE60									
F+	13/15	44/85	8/28	4/8	0/45	0/20	0/4	0/2	0/10
D+	0/15	0/85	0/28	0/8	0/45	0/20	0/4	0/2	0/10
MOC 1									
F+	0/10	9/77	7/25	1/6	1/39	3/19	4/4	0/2	9/9
D+	0/10	4/77	0/25	0/6	0/39	16/19	0/4	0/2	9/9
MNF									
F+	0/12	5/77	3/23	0/7	0/39	4/20	0/4	0/2	1/9
D+	0/12	0/77	0/23	0/7	0/39	0/20	0/4	1/2	3/9
pNF160									
F+	0/4	0/20	2/6		0/17	1/5	0/2		0/4
D+	0/4	0/20	0/6		0/17	0/5	0/2		1/4

[a] F+ focal reaction, D+ reaction in virtually all cells.

Table 4 *Patterns of intermediate filament protein expression of different types of lung cancer in paraffin sections*

	Non SCLC			Neuroendocrine tumors	
	Squamous cell carcinoma	Adeno-carcinoma	Undifferentiated carcinoma	SCLC	Carcinoid
pKer					
F+[a]	7/32	4/16	11/27	46/91	2/3
D+	25/32	11/16	15/27	32/91	0/3
RCK102					
F+	16/27			13/20	0/2
D+	10/27			4/20	2/2
CAMS 2					
F+	2/5	6/9	15/27	23/51	1/2
D+	3/5	3/9	8/27	24/51	1/2
MNF					
F+	2/27			1/20	0/2
D+	0/27			0/20	0/2
pNF160					
F+	6/14[b]	3/9[b]	1/23	3/72	2/3
D+	0/14[b]	0/9[b]	0/23	0/72	0/3

[a] F+, focal reaction D+ reaction in virtually all tumor cells.
[b] Only selected cases of poorly differentiated tumors were tested

(Fig 1e; Table 3) Strikingly, the presence of neurofilaments could also be observed in 3 of 23 cases (Table 3, Fig 1f), but no overlap was found between those cases staining for MOC-1 and neurofilaments, except for one case in which a coexpression of MOC 1 and neurofilaments in the same tumor occurred In most tumors only a focal reaction with these two antisera was observed Also in paraffin sections the presence of neurofilaments could be demonstrated in 2 of 27 cases using MNF and in 6 of 14 cases using pNF160 (Table 4)

Double immunofluorescence studies using antisera to cytokeratins and neurofilaments on frozen sections demonstrated that coexpression of cytokeratins and neurofilaments occurred in some cells within poorly differentiated SQCs, while other tumor cells expressed only cytokeratins No cells expressing only neurofilaments and no cytokeratins were seen (compare Fig 1, g and h)

Adenosquamous Cell Carcinomas

Lung tumors, histologically showing adenomatous and squamous cell differentiation also exhibited this bipartite nature immunohistochemically Most of these tumors are diffusely positive with pKer [6 of 8 (Table 3)] and RCK102 [5 of 5 (Table 3)] and have both keratinizing areas detectable with RKSE60 [4 of 8] and areas reacting diffusely with RGE53 [4 of 8] In one case also the presence of the MOC 1 antigen could be demonstrated, albeit in a small part of the tumor (see Table 3).

Adenocarcinomas

Most adenocarcinomas show a diffusely positive staining reaction with the antibodies pKer and RCK102 (Fig 2a), both in frozen and in paraffin sections (Tables 3 and 4) Also a diffuse staining reaction is seen in most frozen sections with the antibody RGE53 [39 of 45 (Table 3, Fig 2b)], although in some poorly differentiated adenocarcinomas [4 of 45 cases], only a focal positive reaction with RGE53 was found, while in two cases no reaction with RGE53 was observed Also no reaction was seen with RKSE60 [0 of 45 (Table 3)], indicating the absence of keratinization in these tumors The adenocarcinomas did not react with the antibody to MOC 1, except for one case, in which some scattered tumor cells were positive with this antibody or with the neurofilament antibodies in frozen sections In paraffin sections a focal staining reaction for neurofilaments was observed in 3 of 9 cases of poorly differentiated adenocarcinomas (Table 4) These selected cases were initially diagnosed as SCLC but upon revision appeared to be poorly differentiated adenocarcinomas

Small Cell Lung Cancer

Nearly all cases of SCLC examined showed the expression of cytokeratins. The polyclonal antibody pKer reacted in 15 of 20 cases on frozen sections while RCK102 reacted in all cases of frozen sections examined [16 of 16 (Table 3; Fig. 3a)]. In paraffin sections the presence of cytokeratins could be detected in 78 of 91 cases using pKer (Table 4; Fig. 3e), in 17 of 20

cases using RCK102 (Table 4), and in 47 of 51 cases using CAM5.2 (Table 4). RGE53 reacted in nearly all cases available as frozen sections [19 of 20 (Table 3; Fig. 3b)], while no reaction was seen with RKSE60 [0 of 20 (Table 3)]. In all cases of frozen sections [19 of 19] a staining reaction was observed with the MOC-1 antibody [Fig. 3c], although in 3 cases a reaction was seen in only part of the cells. In some cases of SCLC reacting only focally or not at all with cytokeratin antibodies a focal

Fig. 2. Immunoperoxidase staining on frozen sections of adenocarcinomas of the lung. Note the strong staining reaction of both the broadly cross-reacting monoclonal cytokeratin antibody RCK102 (a, × 250) and the monoclonal antibody RGE53 (b, × 275).
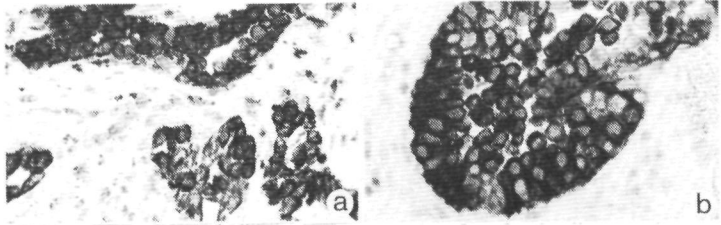

Fig. 3. Immunoperoxidase and immunofluorescence staining patterns in frozen sections (a–d, g, h) and peroxidase anti-peroxidase staining patterns in paraffin sections (e, f) of small cell lung cancer. Note the typical grainy staining reaction in all tumor cells with the broadly cross-reacting monoclonal cytokeratin antibody RCK102 (a, × 310) and with most tumor cells using the monoclonal antibody to cytokeratin 18, RGE53 (b, × 275). In all cases the presence of the MOC-1 antigen could be demonstrated (c, × 235), while in some cases neurofilament proteins were detected in part of the tumor cells, using the monoclonal antibody MNF (d, × 335). The presence of cytokeratins could also be demonstrated in paraffin sections of SCLC using the polyclonal cytokeratin antibody pKer (e, × 390), while additionally in some tumors a reaction with the neurofilament antibody pNF160 was observed (f, × 390). Double-label immunofluorescence studies using the monoclonal cytokeratin antibody RCK102 (g, × 235) in combination with the polyclonal neurofilament antibody pNF160 (h, × 235) show a coexpression of cytokeratins and neurofilaments in some cells (arrows in g and h), while most neurofilament-positive cells are negative for cytokeratins.

51
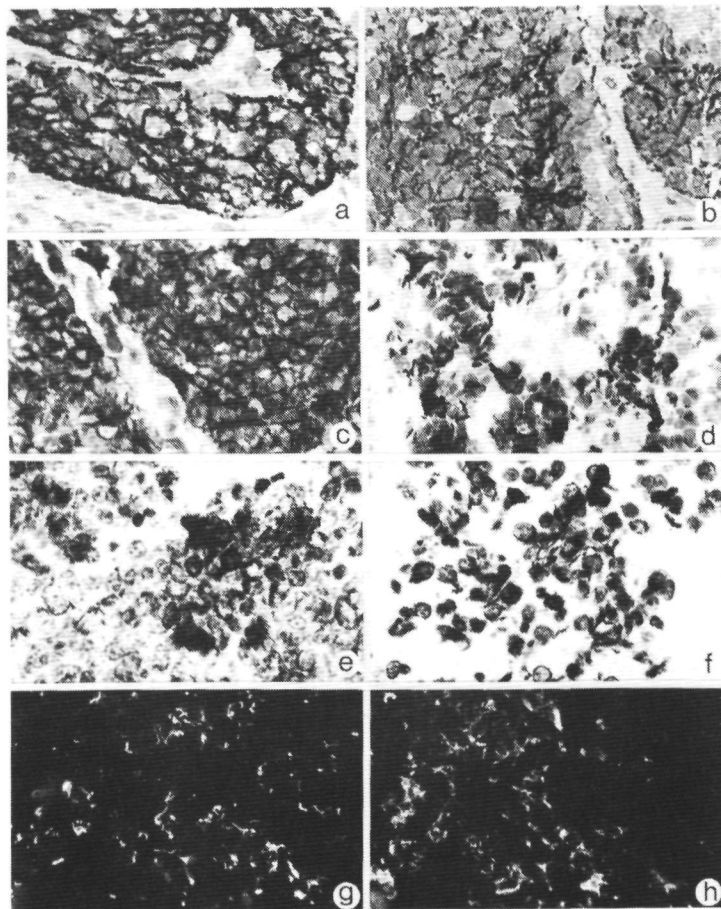
staining reaction with the neurofilament antibody MNF was seen [4 of 20 cases (Table 3; Fig. 3*d*)]. Also in paraffin sections neurofilaments could be demonstrated, reacting in 1 of 20 cases with MNF and in 3 of 72 cases with pNF160 (Table 4; Fig. 3*f*).

Using the double immunofluorescence technique in frozen sections of SCLC containing neurofilaments it could be demonstrated that coexpression of cytokeratins and neurofilaments seems to occur in only a few tumor cells (compare Fig. 3*g* with Fig. 3*h*).

Combined Small Cell Carcinomas

Mixed Small Cell-Squamous Cell Carcinomas. This tumor type with admixtures of SCLC- and non-SCLC (squamous cell) differentiation could be demonstrated histologically in four cases. Immunohistochemically their bipartite nature was characterized by a diffuse staining reaction with RGE53 in all tumor cells in one part of the tumor, similar to the staining reaction found in poorly differentiated SQC (Fig. 4*a*), while in other parts a speckled reaction pattern is seen, similar to the staining reaction of SCLC (Fig. 4*b*). In all cases a staining reaction with the MOC-1 antiserum was observed. As far as we can conclude from the 4 cases examined at least the histologically evident SCLC parts of the tumors showed a MOC-1 staining reaction (Fig. 4*c*), while in the poorly differentiated SQC areas only scattered MOC-1-positive cells were seen. The presence of neurofilaments could not be demonstrated.

Mixed Small Cell-Adenocarcinomas. In one case histologically this tumor type could be distinguished, showing a staining reaction with the cytokeratin antibodies pKer and RGE53. In part of this tumor a reaction with MOC-1 occurred, while neurofilaments could also be demonstrated in some tumor cells in frozen sections and in paraffin sections (results not shown).

Large Cell Undifferentiated Carcinomas

This group comprises all lung carcinomas in which at the light microscopic level no signs of differentiation could be observed. In frozen sections these types of tumors reacted diffusely with the cytokeratin antibodies pKer, RCK102, and RGE53, while in one case a reaction with MNF was also observed. In paraffin sections most cases reacted with pKer [13 of 16 (Table 4)] and all cases reacted with CAM5.2 [16 of 16]. In addition, one case in paraffin sections showed neurofilament-positive cells.

Lung Carcinoids

All lung carcinoids examined contained cytokeratins, as concluded from a positive reaction with pKer (Fig. 5*a*), RCK102, and RGE53 (Fig. 5*b*) in frozen sections. Also, in all cases of frozen sections (9 of 9) the MOC-1 antigen was present (Fig. 5*c*), while in 4 of 9 cases neurofilaments could be detected (Fig. 5*d*). Also in paraffin sections the three cases of lung carcinoids examined were positive for cytokeratins (Table 4; Fig. 5*e*), and two cases also reacted with the pNF160 neurofilament antibody (Fig. 5*f*). In contrast to SCLC, a clear coexpression of cytokeratins and neurofilaments was detected in virtually all cells of lung carcinoids using the double immunofluorescence technique (compare Fig. 5*g* and Fig. 5*h*). Both cytokeratin and neurofilament fluorescence patterns showed a dot-like concentration of these intermediate filament proteins in close proximity to the nucleus.

Comparison of Primary Lung Cancers with Their Metastases

In order to obtain an impression of the role of lung tumor heterogeneity in the metastatic process and to see if metastases showed a similar or different immunochemical reactivity pattern, we have examined 61 tumor specimens from 6 patients known with a primary SCLC. At all primary sites of the tumors as well as in all metastases the presence of cytokeratins could be demonstrated, although in variable amounts. In one case the presence of neurofilaments next to cytokeratins could be demonstrated at the metastatic site in the adrenal gland, while no neurofilaments were observed at other metastatic sites or at the primary tumor site of the same patient.

DISCUSSION

It has been suggested that in the future biomarkers will permit differentiation between different subtypes of major tumor groups (37).

The use of such markers on lung tumors, however, has occasionally led to confusing or contradictory results. In our studies we have used well-characterized biomarkers with known specificity for certain subtypes of lung tumors. As a general marker for epithelial differentiation broadly cross-reacting cytokeratin antibodies, which react with both simple (columnar) and squamous epithelia, were used. Keratinizing areas in SQCs can be detected by our monoclonal antibody to cytokeratin 10, RKSE60. Adenocarcinomas on the other hand contain merely simple epithelium-type cytokeratins such as cytokeratin 18,
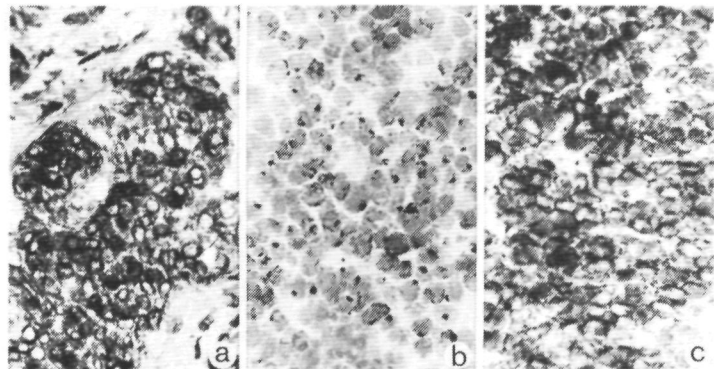

Fig. 4. Immunoperoxidase staining patterns in frozen sections of combined small cell-squamous cell carcinoma. Note the diffuse staining reaction with the monoclonal cytokeratin antibody RGE53 in one part of the tumor (*a*, × 280) and the grainy staining reaction, typical for SCLC with this antibody in another part of the tumor (*b*, × 360). In all cases of combined small cell-squamous cell carcinoma a positive staining with the MOC-1 antibody was observed (*c*, × 360).
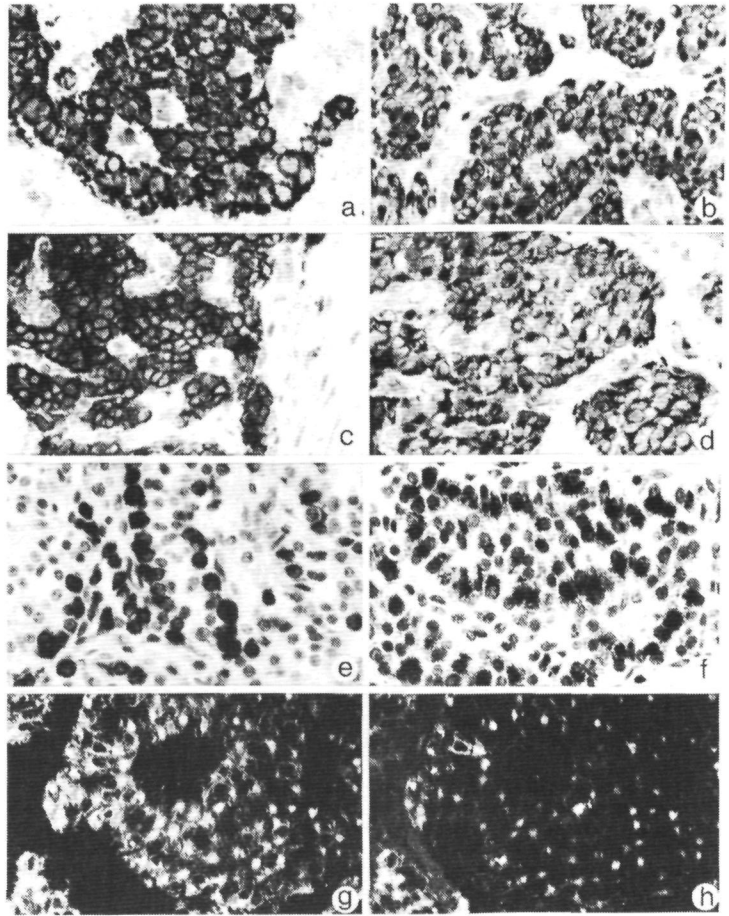
Fig. 5. Immunoperoxidase and immuno-fluorescence staining reactions in frozen sections (a–d, g, h) and peroxidase anti peroxidase staining reactions in paraffin sections (e, f) of lung carcinoids. Note the typical dot-like immunostaining patterns with the monoclonal cytokeratin antibodies RCK102 (a, × 335) and RGE53 (b, × 170). The membrane surface antigen MOC-1 reacts with all carcinoids in a characteristic manner (c, × 220). In some of these tumors also the monoclonal neurofilament antibody MNF reacts in a typical dot-like fashion (d, × 220). In paraffin sections the presence of cytokeratins can be demonstrated using the polyclonal antibody pKer (e, × 390), while in some tumors coexpression of neuro-filaments also can be detected with the poly-clonal pNF160 antibody (f, × 390). Double-label immunofluorescence studies clearly demonstrate this coexpression of cytokeratins and neurofilaments using RCK102 (g, × 235) and pNF160 (h, × 235) in the same tumor cells.

generally not occurring in squamous epithelia or in SQCs (27–29). Cytokeratin 18 is also found in neuroendocrine tumors such as SCLC and lung carcinoids (38).

Recently, several monoclonal antibodies have been developed against SCLC cell surface antigens such as SM-1 (39), TF1-4 (40), LAM8 (41), and MOC-1 (10), each of them suggested to be more or less specific for SCLC. One of these, the monoclonal MOC-1 antibody, used in this study, appears to be a marker for neuroendocrine differentiation and reacts with normal neuronal and neuroendocrine tissues, as well as with neuroendocrine lung tumors (10). In SCLC and SCLC cell lines also neurofilaments have been detected (42, 43). Cell culture studies suggest that these intermediate filament proteins occur in the variant type of SCLC (20, 21). Furthermore, in some lung carcinoids neurofilaments have been described to be coexpressed next to cytokeratins (11). Therefore neurofilament antibodies were also added to the panel of biomarkers used in this study. The fact that these latter antibodies as well as some of the cytokeratin antibodies are reactive in paraffin sections allowed us to perform a retrospective study using a large series

of paraffin blocks. In this way we could obtain an impression about the frequency of occurrence of cytokeratins and neurofilaments in lung cancer.

Our studies with these antibodies elucidate some of the confusion concerning intermediate filament protein expression in SCLC. Some authors demonstrated only neurofilaments in SCLC and in SCLC cell lines (42, 43), while others (38, 44, 45) have demonstrated only cytokeratin intermediate filaments and neurofilaments in SCLC. Also coexpression of both cytokeratins and neurofilaments in SCLC cell lines has been suggested (22, 23). These discrepancies have been partly explained by our recent findings (20, 21) that classic SCLC cell lines contain cytokeratins, while variant SCLC cell lines may contain neurofilaments and are negative for cytokeratins. Our results with tissue sections of SCLC, showing that about 5–10% of all SCLC examined contain focal areas positive for neurofilaments, seem to point to the variant-type differentiation of SCLC in these tumors. The frequency of the presence of neurofilaments matches quite well with the frequency of the variant type of SCLC as observed at first diagnosis by Radice et al. (17) and

Hirsch et al (18) One should keep in mind, however, that a cytokeratin negative reaction may be a fixation artifact (24), which may explain possible false negative findings (42, 43, 46, 47)

Our results in paraffin sections show that, when using the proper antibodies in most tumors cytokeratins can be demonstrated Some of the lung tumors investigated had been stored for up to 23 years in formalin, and yet in most cases cytokeratins and in some cases neurofilaments were demonstrable The pNF160 antibody reacts with more cells and lung tumors than the MN1 monoclonal antibody, probably due to the fact that both antibodies recognize different neurofilament proteins or that the polyclonal antibody pNF160 recognizes more antigenic determinants than the monoclonal antibody

Also with respect to the intermediate filament protein content of lung carcinoids discrepancies in the literature have arisen Some authors demonstrated neurofilaments in lung carcinoids (48), others only cytokeratins (38, 44, 49) while Lehto et al (11) reported the coexpression of neurofilaments and cytokeratins in three cases of such neoplasms Our results clearly demonstrate that indeed coexpression of cytokeratins and neurofilaments can occur, however, only in some of the cases

Coexpression of cytokeratins and neurofilament proteins seems to be restricted to (neuro)endocrine tumors and has been shown to occur in neuroendocrine skin carcinomas [Merkel cell tumors (12–15)], in some pancreatic islet tumors [insulinomas (16)] and in parathyroid adenomas (50) The possible cells or origin for these tumors, i e , Merkel cells in skin (51), pancreatic islet cells and parathyroid gland cells (50), respectively, express only cytokeratins and do not contain neurofilaments These findings are in parallel with the detection of only cytokeratins in the so called Kulchitsky cells of the lung (36, 52), a possible candidate for the cell type from which SCLC and lung carcinoids arise Apparently (neuro)endocrine cells have the potential to induce neurofilament expression as a result of malignant growth

Although not immediately obvious from histopathological examinations, lung cancers may show a profound heterogeneity when examined at the ultrastructural or immunocytochemical level Heterogeneity of lung cancer has been reported previously, however, also after histological examination of different sites of entire lung tumors by Roggli et al (53), who showed that only 34% of all lung carcinomas examined were homogeneous This large heterogeneity was also observed in cytological specimens (3) and after electron microscopic examinations (4) In some cases within the same tumor cell squamous cell differentiation, adenomatous and small cell differentiation can be observed at the ultrastructural level (54)

In our study, histological examination of lung cancers revealed a combined phenotype only in about 6% of the cases from which frozen sections were available After the immunohistochemical procedures, in about 66% (144 of 218) of all tumors, tested as frozen sections a more or less heterogeneous staining reaction was seen In 29% of histologically homogeneous SQC a nonsquamous differentiation (adenocarcinoma or SCLC) is observed in virtually all tumor cells using the RGE53 antibody, while nearly all cases of SQC showed at least a partial staining reaction with this antibody A neuroendocrine or neuronal differentiation was detected by MOC 1 and the neurofilament antibodies in 12 and 5% of the SQC cases, respectively The degree of heterogeneity seen with these immunohistochemical techniques in SQCs increased with decreasing degree of differentiation (see Table 3) The finding that in 28% of the cases of poorly differentiated squamous cell carcinoma and in

one case of poorly differentiated adenocarcinoma cells express ing MOC 1 are found suggests that these latter tumors contain cells with neuroendocrine differentiation The presence of neurofilaments in the moderately to poorly differentiated SQCs and in some poorly differentiated aednocarcinomas might point to the occurrence of the variant type of SCLC within poorly differentiated non SCLC

Also in histologically homogeneous adenocarcinomas heterogeneity could be detected, as concluded from focal staining reactions with RGE53 in some tumors indicating a less pronounced adenocarcinomatous differentiation in large parts of these tumors

Lung tumors, histologically recognizable as combined SCLC non SCLC, also showed a combined nature immunohistochemically Since normally combined small cell carcinomas are treated as SCLC (8) it is our belief that poorly differentiated carcinomas containing MOC 1, and therefore having features in common with the "large cell neuroendocrine tumors" as described before (55), should be treated as SCLC using combined chemotherapy and radiotherapy This opinion is supported by the findings by Hammond and Sause (55) who could demonstrate that histologically undifferentiated large cell carcinomas containing neuroendocrine elements (neurosecretory granules) at the electron microscopic level behaved clinically like SCLC Interestingly, coexpression of neurofilaments and MOC 1 in poorly differentiated lung carcinomas was quite rare This is in parallel with our findings in SCLC cell lines that the variant type of cell lines expressed only low amounts of MOC 1 as compared to the classic SCLC cell lines (21) Therefore it might well be that variant SCLC cells gradually lose their expression of MOC 1 with ongoing (de)differentiation

We have examined autopsy material of patients with SCLC to detect a possible differential expression of cytokeratins and/or neurofilaments in metastases as compared to the primary tumor site It is well known that in up to 30% of all cases which were first diagnosed as SCLC non SCLC components can be observed at autopsy, especially after chemotherapy or radio therapy (19) These mixed tumors respond less well to combined therapy and have a worse prognosis than pure SCLC (56)

The absence of neurofilaments in all but one case of metastases indicates that transition from classic SCLC (which are positive for cytokeratin) to the variant SCLC (positive for neurofilaments) is not a frequent event in the course of the disease, at least as far as we can conclude from our limited study

In summary, we can state that the main lung tumor types can be distinguished from each other on the basis of the panel of intermediate filament and surface protein antibodies used in this study A profound heterogeneity can be detected with these antibodies in lung tumors, especially in poorly differentiated (squamous cell) carcinomas Within SCLC the variant subtype may possibly be distinguished by the absence of cytokeratins in large parts of the tumor and the presence of neurofilaments in some cases The immunocytochemical detection of neuroendocrine components in histologically nonneuroendocrine tumors as well as subtyping within SCLC may become of clinicopathological significance

ACKNOWLEDGMENTS

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CHAPTER FIVE

CYTOKERATINS IN DIFFERENT TYPES OF HUMAN LUNG CANCER AS
MONITORED BY CHAIN-SPECIFIC MONOCLONAL ANTIBODIES.

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Cytokeratins in Different Types of Human Lung Cancer as Monitored by Chain-specific Monoclonal Antibodies[1]

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ABSTRACT

The expression of cytokeratins (CKs) in human lung cancer was studied using chain-specific monoclonal antibodies to CKs 4, 7, 8, 10, 13, 18, and 19. When applied to adenocarcinomas (ACs) of the lung, high levels of CKs 7, 8, 18, and 19 were detected in all tumors, while CK 4 was found in high concentrations in some ACs. CK 10 and 13 were completely absent, or only present in low numbers of cells. Small cell lung cancers (SCLCs) and lung carcinoids contained CK 18 and sometimes 8 and 19, but no CK 7 in most cases. Three out of four tumors, histologically classified as SCLC, and expressing CK 7 in a variable number of cells were found by electron microscopic studies to contain regions with AC and/or squamous cell carcinoma (SQC) differentiation. The monoclonal antibody specific for CK 7 can therefore possibly help to distinguish AC differentiation within SCLC. CKs 10 and 13 were completely absent in SCLCs and lung carcinoids, while few CK 4-positive cells were found in some SCLCs and in one lung carcinoid. Within SQCs the monoclonal antibodies revealed a pronounced heterogeneity in CK expression. CKs 4, 7, 8, 10, 13, 18, and 19 could be detected, although not evenly distributed among all tumor cells. Highly differentiated SQCs expressed high levels of the CKs specific for squamoid differentiation, i.e., CKs 4, 10, and 13 in variable numbers of cells. With decreasing histologically detectable SQC differentiation these markers were gradually lost, while the number of cells containing CKs 7, 8, 18, and 19 increased.

Application of this panel of monoclonal antibodies can therefore distinguish not only the main subtypes of lung cancer, but can also indicate the degree of differentiation and the degree of heterogeneity. These findings can be used as a diagnostic aid in lung tumor pathology, which may have an impact on treatment and prognosis.

INTRODUCTION

Lung cancer is now generally accepted to be a very heterogeneous type of cancer, in which a cooccurrence of the different subtypes is rather a rule than an exception. The three main subtypes are SCLC,[1] AC, and SQC. While after histological examinations heterogeneity is found in about 10–15%, this number increases to about 50% after cytological examinations (1), electron microscopy (2, 3), or when using refined immunohistochemical techniques (4, 5). Because of differences in treatment and prognosis of the different types of lung cancer, detection of each subtype, especially SCLC, within individual lung tumors is of clinical importance.

Virtually all epithelial tissues, both normal and malignant, contain CKs, which form the intermediate filament cytoskeleton within the epithelial cell. This family of human CKs consists of 19 different polypeptides, which have been numbered 1 through 19 by Moll *et al.* (6). These CKs are not randomly distributed in the different epithelia, but appear to be characteristic for certain types of epithelial differentiation. For instance, relatively high molecular weight CKs are characteristic for more complex, stratified epithelia and SQCs, while relatively low molecular weight CKs, such as CK 18, are specific for simple, columnar epithelia (6) and ACs (7). A possible method to detect CKs is the biochemical demonstration based on (two-dimensional) gel electrophoresis as performed previously for lung carcinomas (8–11). This integral biochemical approach, however, is limited to defining the total of CKs expressed by a carcinoma specimen as a whole. CKs which are only expressed by a small subset of cells may go undetected (12). Immunohistochemically, the presence of a certain CK, corresponding to a particular type of differentiation, is detectable at the cellular level with MoAbs to a specific subtype of CK, which have recently been developed (7, 12–22). Since the detection of type and degree of differentiation can be of clinical importance, the use of this panel of MoAbs can add to a more specific treatment for certain tumors.

MATERIALS AND METHODS

Lung Tissues. Fresh samples of normal bronchi (five cases), normal lung (three cases), and lung tumors were snap-frozen and stored in liquid nitrogen until use. Lung tumors examined in this study were classified on histological basis according to the WHO classification (23). From lung carcinoids, SCLC, AC, poorly differentiated SQC, moderately differentiated SQC, and well-differentiated SQC, 10 cases each were examined. In addition, five adenosquamous carcinomas, three large cell carcinomas, and four combined SCLC/SQC were examined. Frozen sections approximately 5 μm thick were cut on a cryostat, air dried, and stored at −20°C before use.

Cytokeratin Antibodies. The following mouse monoclonal antibodies were used in this study (see also Table 1): (a) RCK102 is a broadly cross-reacting CK antibody of the IgG₁ subclass, which recognizes CKs 5 and 8, and as a result stains virtually all epithelial tissues (13). (b) RCK105 (IgG₁; 13) in immunoblotting reacts only with CK 7 and stains a subgroup of glandular epithelia and their tumors, next to transitional bladder epithelium and bladder carcinomas. (c) LE41 (IgG₁; 14, 15) reacts with different types of epithelial tissues and in immunoblotting recognizes only CK 8. (d) RGE53 (IgG₁; 7, 16) and RCK106 (IgG₁, 13) are monospecific for CK 18 in immunoblotting. In general, these two antibodies recognize columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues and mesothelial cells, as well as their tumors. Generally no reaction is found in squamous epithelia or squamous cell carcinomas. (e) LP2K (15) and BA17 (IgG₁; 17, 18) stain most simple epithelia and basal cells in stratified squamous epithelia that are not keratinizing, and recognize only CK 19 in immunoblotting assays. (f) RKSE60 (IgG₁; 16, 19, 20) reacts only with keratinizing epithelial cells, and recognizes CK 10 in immunoblotting. (g) 6B10 (IgG₁; 21) reacts with noncornifying squamous epithelium, and with certain ciliated pseudostratified epithelia such as cylindrical epithelium of bronchi, and recognizes only CK 4 in immunoblotting. (h) Antibodies 1C7 (IgG₂ₐ; 21) and 2D7 (IgG₂ₐ; 21) both react with noncornifying squamous epithelia. These antibodies recognize only CK 13 in immunoblotting studies.

Table 1 *Specificity of monoclonal cytokeratin antibodies used in this study*

Antibody	Immunoglobulin subclass	Antigen recognized	Tissue specificity	Reference
RCK102	IgG₁	Cytokeratin 5 + 8	Nearly all epithelial tissues	13
RCK105	IgG₁	Cytokeratin 7	Some glandular epithelia	13
LE41	IgG₁	Cytokeratin 8	Different epithelial tissues	14 15
RGE53	IgG₁	Cytokeratin 18	Glandular epithelia not with squamous cell epithelia	7 16
RCK106	IgG₁	Cytokeratin 18	Glandular epithelia not with squamous cell epithelia	13
LP2K	IgG₁	Cytokeratin 19	Most simple epithelia	15
BA17	IgG₁	Cytokeratin 19	Most simple epithelia	17 18
RKSE60	IgG₁	Cytokeratin 10	Keratinizing epithelial cells	16 19 20
6B10	IgG₁	Cytokeratin 4	Noncornifying squamous epithelia, pseudo-stratified epithelia	21
1C7	IgG₂ₐ	Cytokeratin 13	Noncornifying squamous epithelia	21
2D7	IgG₂ᵦ	Cytokeratin 13	Noncornifying squamous epithelia	21

Immunoperoxidase Technique In the indirect immunoperoxidase technique cryostat sections were fixed in acetone at room temperature (3 times 5 s) and incubated with the primary monoclonal antibodies for 30-45 min at room temperature All primary antibodies were applied as undiluted culturing supernant. After repeated washing with 0 05 M phosphate buffered saline (pH 7 4) (PBS), sections were incubated with rabbit anti mouse serum conjugated to peroxidase (DAKOpatts, Den mark) diluted 1 25 in PBS with 5% human AB-serum, and incubated for 30-45 min

After washing in PBS, peroxidase activity was detected with 3 amino-9-ethylcarbazole (Aldrich Chemical Co) 40 mg of 3 amino-9-ethylcar bazole was dissolved in 10 ml N N dimethylformamide (Merck, Darm stadt FRG) and added to 190 ml of sodium acetate buffer (0 05 M, pH 4 85) Hydrogen peroxide was added to a final concentration of 0 01% After an incubation for 10 min cells were rinsed with tap water, counterstained with hematoxylin, and mounted with Kaiser's glycerol gelatin (Merck)

Gel Electrophoresis and Immunoblotting Assays Cytoskeleton prep arations from solid lung tumors were made as follows After microdis secting tumor areas 20 μm thick frozen sections were immediately extracted for 15 min at 0°C using a high salt buffer (1 5 M KCl 0 5% Triton X 100, 5 mM EDTA, 0 4 mM phenylmethylsulphonyl fluoride, and 10 mM Tris HCl (pH 7 2) centrifuged for 5 min at 3000 × g, and washed twice with cold (4°C) low salt buffer (5 mM FDTA, 0 4 mM phenylmethylsulphonyl fluoride and 10 mM Tris HCl pH 7 2), essen tially as described by Moll and Franke (10)

After centrifugation (3000 × g for 10 min) and washing in PBS, the cytoskeleton preparation was dissolved by boiling during 5 min in SDS sample buffer (24)

One dimensional gel electrophoresis was performed in 10% poly acrylamide slab gels containing 0 1% SDS as described by Laemmli (24) To compare the amounts of protein loaded on each lane, gels were stained with Coomassie Brilliant Blue R250 (Gurr Hopkin and Wil liams Chadwell Heath, Essex, U K) as described before (25) Two dimensional gel electrophoresis was performed essentially as described by O Farrell (26) In the first dimension, isoelectric focusing was performed in 4% polyacrylamide (Biorad, California, USA) rod gels containing 2% ampholines, pH 3 5-10 (LKB, Bromma, Sweden) For the second dimension, the rod gels were applied directly onto the stacking gel of SDS polyacrylamide gels and covered with a 1% agarose solution in running buffer

For immunoblotting experiments the electrophoretically separated polypeptides were transferred to a nitrocellulose sheet (Schleicher and Schull Membrane Filters BA 85 Dassel FRG) by overnight blotting at 250 mA using an electrophoresis buffer containing 25 mM Tris HCl, 192 mM glycine and 20% methanol (pH 8 3), essentially as described by Towbin et al (27) The nitrocellulose sheets were then immunohis tochemically stained essentially as described previously (25) In short, blots were preincubated for 90 min with PBS containing 0 05% Tween 20 (Sigma) All reagents were diluted in this solution which was also used for the washing steps After incubation overnight with undiluted culturing supernatants of the primary antibodies RCK106 1C7, RCK102, or RCK105, or with LP2K, diluted 1 10, blots were washed, incubated for 1 h with rabbit anti mouse peroxidase (DAKOpatts,

Denmark) diluted 1 200, washed again, and stained with 4-chloro-1-naphthol

RESULTS

Normal Bronchial and Lung Tissues The cytokeratin expres sion patterns of normal bronchial epithelia and lung alveolar epithelium are presented in Table 2 and illustrated in Fig 1 The cytokeratin antibodies are not reactive with other than epithelial tissues RCK102 (Fig 1, A and B), RCK106 (Fig 1E), LP2K and BA17 react with all bronchial and lung epithe lial tissues, while RCK105 (Fig 1C) and RGE53 (Fig 1, G and H) recognize all bronchial and lung epithelial tissues except the basal layer in the bronchial columnar epithelium LE41 recognizes only superficial cells of the bronchial columnar epithelium and (sero-)mucous glands, while again basal cells as well as lung alveoli are not recognized 6B10 (Fig 1D), 1C7 and 2D7 (Fig 1F) react with some cells of the bronchial epithelium, but are negative with most alveolar epithelial cells (Fig 1J) RKSE60 does not react with any type of epithelial cells in normal lung

Lung Tumors Results of the immunohistochemical staining procedures in lung tumor frozen sections with the different CK antibodies are summarized in Table 3 and illustrated in Figs 2, 3, and 4 On the basis of histological examinations, SQCs were subdivided into three groups, i e , well differentiated SQC, moderately differentiated SQC, and poorly differentiated SQC

In all SQCs examined, a reaction was seen with the CK 5+8 antibody (Fig 2, A and D) The CK 10 antibody, specific for keratinizing epithelial cells, reacts more with well differentiated SQC (Fig 2B) than with moderately and poorly differentiated SQC, which in general do not react with this antibody (Fig 2E) The same holds true for the two MoAbs to CK 13, which react in more cases and with more cells of well differentiated SQC as compared to poorly differentiated SQC (compare Figs

Table 2 *Reactivity pattern of different antibodies to cytokeratins in frozen sections of normal bronchial and normal lung tissues*

Cytokeratin antibody	Bronchi and bronchioli						See also Ref
	Columnar epithelium	Basal cells	Mixed glands	Lung alveoli	Stromal elements	Nerve fibers	
RCK102	+	+	+	+	−	−	4 13
RCK105	+	−	+	+	−	−	13
LE41	+	−	+	−	−	−	14 15
RGE53	+	−	+	+	−	−	4 7 16
RCK106	+	+	+	+	−	−	13
LP2K	+	+	+	+	−	−	15
BA17	+	+	+	±*	−	−	17
RKSE60	−	−	−	−	−	−	4 16
6B10	±	±	−	±	−	−	21
1C7/2D7	±	−	−	−	−	−	21

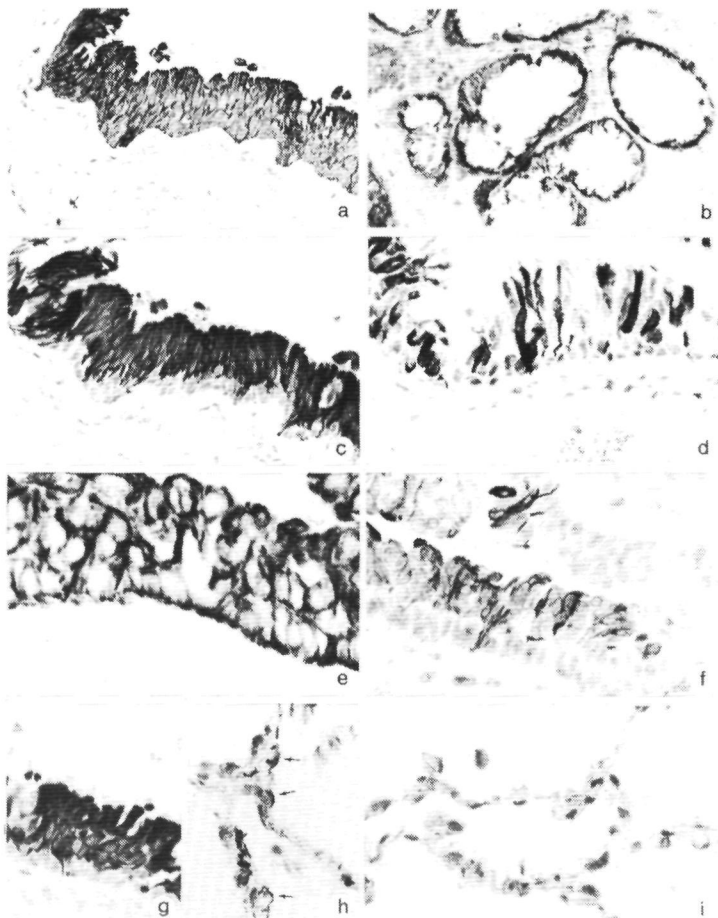* ± positive reaction in part of the cells

Fig. 1. Immunoperoxidase staining patterns in normal bronchial and lung epithelium. The broadly cross-reacting CK antibody RCK102 reacts with all bronchial epithelium cells (*A*, × 240), with seromucous glands (*B*, × 280), as well as with lung alveolar epithelial cells (not shown). In general, antibodies to cytokeratins typical for "simple" epithelia (*C, E, G,* and *H*) reacted with more cells of normal bronchus than the antibodies to cytokeratins characteristic of stratified epithelia (*D, F,* and *I*). The CK 7 antibody RCK105 reacts with the columnar cells but not with the basal cells of bronchial epithelium (*C*, × 280), while the CK 18 antibody RCK106 reacts with all bronchial epithelial cells (*E*, × 310). In contrast, the other CK 18 antibody, RGE 53, reacted similarly to RCK105, and was also negative in basal cells of normal bronchial epithelium (*G*, × 210). In lung alveolar tissues, antibodies such as RCK106 (*H*, × 330), but also RGE53, LP2K and RCK105 recognize pneumocytes of the alveolar epithelium (*arrows*). The CK 4 antibody, 6B10, recognizes some basal as well as some columnar cells of normal bronchus (*D*, × 330), while the CK 13 antibody 2D7 only occasionally recognized some columnar cells (*F*, × 350). In general, no cells were stained with 6B10 in alveoli (*I*, × 330).

2, *C* and *F*). In contrast, the antibodies to CK 19 react with more cases and with more cells within each tumor in poorly differentiated SQC (compare Figs. 2, *G* and *J*). The CK 8 antibody also seems to react with more cells in poorly differentiated SQC. However, the overall reaction of this antibody was quite weak in all cases. The CK 18 antibodies exhibited a very strong staining reaction in most, and sometimes in all tumor cells of poorly differentiated SQC, and staining of only a small percentage of the cells in well-differentiated SQC (compare Figs. 2, *H* and *K*; see also Reference 4). The antibody to CK 7, which reacts with scattered cells in only a few well-differentiated SQCs, however, stains a considerable percentage of most poorly differentiated SQCs (Figs. 2, *I* and *L*).

All ACs showed a staining reaction in virtually all tumor cells with the CK 5+8 antibody (Fig. 3*A*), while none of these tumors reacted with CK 10. Only some scattered cells of some ACs reacted with the MoAbs to CK 13 (Fig. 3*B*). Using the CK 4 antibody, a very heterogenous reaction pattern was observed in ACs, varying from a reaction in almost all tumor cells (Fig. 3*C*) to a completely negative reaction (not shown). The anti-

bodies directed against CK 19 (Fig. 3*D*), CK 18 (Fig. 3*E*), CK 8 (not shown), and CK 7 (Fig. 3*F*) reacted with all ACs in virtually all tumor cells, although with one MoAb to CK 19 (BA17) and with the antibody to CK 8, a relatively weak reaction in less tumor cells was seen.

The cancers which were histologically classified as adenosquamous carcinomas showed also immunohistochemically this bipartite nature, as expressed in the presence of areas with cornifying epithelium which were focally CK 10 positive (Fig. 3*G*), and other areas which were positive with the CK 7 antibody (Fig. 3*H*).

The SCLCs examined for this study all contained CKs, reacting in virtually all cells with the CK 5+8 antibody (Fig. 4*A*). The CK 10 and 13 antibodies were negative, while the CK 4 antibody showed in some isolated cells reactivity with these SCLCs (Fig. 4*B*). The antibodies to CK 19 reacted with varying numbers of cells (Fig. 4*C*), while the CK 8 antibody reacted only very weakly in some of these tumors. The antibodies to CK 18 reacted with nearly all SCLCs (Fig. 4*D*). Most SCLCs did not react with the antibody to CK 7 (Fig. 4*F*). However, in

Table 3 *Reactivity of cytokeratin antibodies in frozen sections of human lung cancers using the indirect immunoperoxidase technique*

| | Cytokeratin no | Squamous cell carcinoma differentiation | | | Adeno/ squamous (n = 5) | Adenocarcinoma (n = 10) | SCLC (n = 10) | SCLC/ squamous (n = 4) | Large cell carcinoma (n = 3) | Carcinoid (n = 10) |
		Well (n = 10)	Moderately (n = 10)	Poor (n = 10)						
RCK102	5 + 8									
–[a]		0	0	0	0	0	0	0	0	0
F+[b]		1	4	1	0	0	2	1	r	0
D+[c]		9	6	9	5	10	8	3	3	10
RKSE60	10									
–		1	5	7	3	10	10	4	3	10
F+		9	5	3	2	0	0	0	0	0
D+		0	0	0	0	0	0	0	0	0
1C7	13									
–		0	1	5	2	9	10	4	3	9
F+		7	9	5	3	1	0	0	0	1
D+		3	0	0	0	0	0	0	0	0
2D7	13									
–		2	0	5	2	8	10	4	3	9
F+		8	9	5	3	2	0	0	0	1
D+		0	1	0	0	0	0	0	0	0
6B10	4									
–		4	3	5	2	1	6	2	3	9
F+		6	6	5	3	9	4	2	0	1
D+		0	1	0	0	0	0	0	0	0
LP2K	19									
–		2	0	1	0	0	3	1	0	4
F+		7	1	1	2	1	6	1	0	5
D+		1	9	8	3	9	1	2	3	1
BA17	19									
–		7	0	2	1	0	5	1	0	8
F+		3	3	5	1	10	4	2	0	2
D+		0	7	3	3	0	1	1	3	0
LE41	8									
–		9	2	4	4	1	7	4	1	4
F+		1	8	6	1	7	3	0	2	6
D+		0	0	0	0	2	0	0	0	0
RGE53	18									
–		0	0	0	1	0	1	1	0	0
F+		10	8	7	3	0	2	2	2	3
D+		0	2	3	1	10	7	1	1	7
RCK106	18									
–		4	0	0	1	0	2	0	0	0
F+		5	7	5	1	3	4	1	0	0
D+		1	3	5	3	7	4	3	3	10
RCK105	7									
–		7	4	1	1	0	6	1	0	7
F+		3	6	7	3	0	3	1	0	1
D+		0	0	2	1	10	1	2	3	2

[a] –, no reaction in tumor cells
[b] F+, heterogeneous focal reaction
[c] D+, homogeneous diffuse reaction

four cases a reaction with a varying number of cells was seen (Fig. 4*E*). In three of these cases, electron microscopical examination revealed the presence of cells with AC and/or SQC differentiation, next to the typical neurosecretory granules.

Carcinomas classified as combined SCLC/SQC on histological grounds showed a heterogeneous reaction pattern with the different CK antibodies in accordance with their histological heterogeneity. Most strikingly, large areas were completely positive with the antibodies to CK 18 (Fig. 4*G*), while other parts did not react with these antibodies, but were focally positive with the antibody to CK 4 (Fig. 4*H*).

The CK expression of the three large cell carcinomas examined was characterized by a positive reaction with MoAbs to the "simple" CKs 7, 8, 18, and 19, and a negative reaction with the MoAbs to CKs 4, 10, and 13 (not shown).

All lung carcinoids examined reacted with the CK 5+8 anti-body (Fig. 4*I*), while in only one case a reaction was observed with the antibodies to CKs 4 and 13. Antibodies to CK 8 (Fig. 4*J*) and CK 19 (Fig. 4*K*) showed variable reaction patterns, while the antibodies to CK 18 were positive in all cases examined (Fig. 4*L*). CK 7 was detected in three lung carcinoids

Immunoblotting Studies. Results of the one-dimensional immunoblotting experiments on cytoskeleton preparations of tissue sections of SCLC, AC, and moderately to well-differentiated SQC are shown in Fig. 5. In the three SCLCs examined the presence of CK 18 was proven using RCK106 (Fig. 5*A, lanes 1–3*). The two ACs reacted strongly with this antibody (Fig. 5*A, lanes 4* and *5*), while the three SQCs showed a relative weak reaction (Fig 5*A, lanes 6–8*).

A subsequent incubation of this immunoblot with LP2K showed the presence of CK 19 in one SCLC (Fig. 5*B, lane 2*), in the two ACs (Fig. 5*B, lanes 4* and *5*), and in the three SQCs
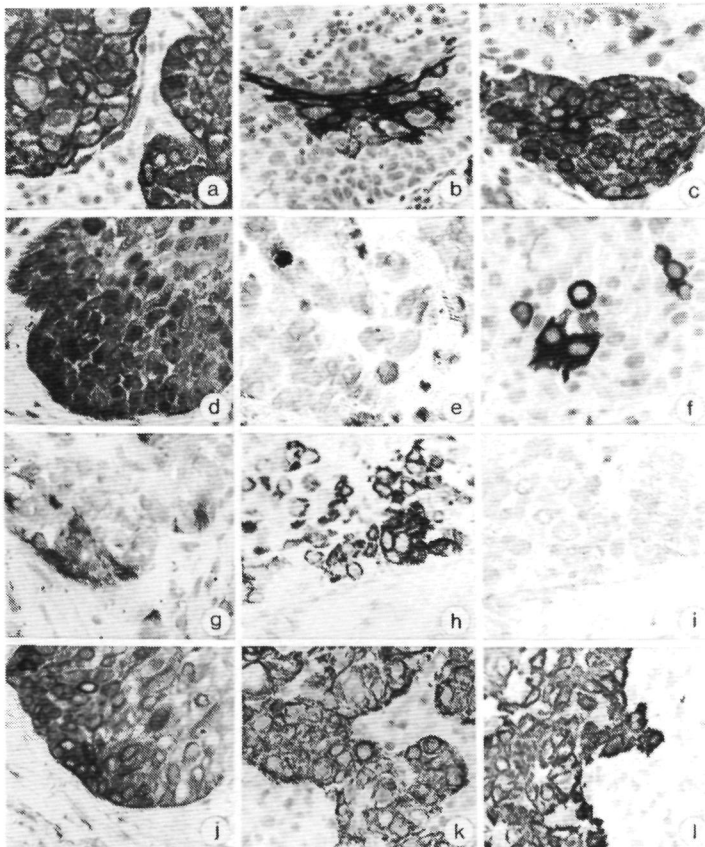
Fig. 2. Immunoperoxidase staining patterns in well-differentiated squamous cell carcinomas (SQCs; *A, B, C, G, H,* and *I*), and in poorly differentiated SQCs (*D, E, F, J, K,* and *L*) of the lung. The broadly cross-reacting CK antibody RCK102 reacts with all tumor cells in both well-differentiated (*A,* × 270) and poorly differentiated SQCs (*D,* × 290). Only in well-differentiated SQCs keratinizing areas, reacting with the CK 10 antibody RKSE60 are seen (*B,* × 250), while no reaction is seen with this antibody in poorly differentiated SQCs (*E,* × 360). Large areas of well-differentiated SQCs are stained with the CK 13 antibody 2D7 (*C,* × 340), while in poorly differentiated SQCs only scattered positive cells can be found (*F,* × 270). The CK 19 antibody LP2K reacts with only a few cells in well-differentiated SQCs (*G,* × 340), while large parts of poorly differentiated SQCs show a completely positive reaction (*J,* × 250). In most well-differentiated SQCs some cells react with the CK 18 antibody RGE53 (*H,* × 250). In poorly differentiated SQCs, however, the percentage of CK 18-positive tumor cells increases to almost 100% (*K,* × 250). The CK 7 antibody RCK105 does not react with most well-differentiated SQCs (*I,* × 250), but shows a completely positive reaction in some poorly differentiated SQCs (*L,* × 250). *H* and *I,* staining patterns with RGE53 and RCK105 in parallel sections of the same region of a well-differentiated SQC. Note the positive reaction of CK 18 and no staining for CK 7.

(Fig. 5*B, lanes 6–8*). Subsequent incubation of this immunoblot with 1C7 showed the presence of CK 13 in two SQCs (Fig. 5*B, lanes 6* and *7*), and the absence of this CK protein in the other SQC (Fig. 5*B, lane 8*). Also, the other lung tumors shown here were negative (Fig. 5*B, lanes 1–5*).

Incubation of a similar immunoblot with the antibody RCK105 showed a weak reaction for CK 7 in two SCLCs (Fig. 5*C, lanes 1* and *2*), a much stronger reaction in the two ACs (Fig. 5*C, lanes 4* and *5*), and only trace amounts in two of three SQCs (Fig. 5*C, lanes 7* and *8*). One SCLC and one SQC were negative (Fig. 5*C, lanes 3* and *6,* respectively). The findings of the immunoblotting match very well with the reactivity of the individual tumors with the CK antibodies in the immunoperoxidase staining.

Two-dimensional immunoblotting data on SCLC and AC are shown in Fig. 6. From these immunoblottings it is clear that this SCLC (showing AC differentiation on the basis of electron microscopy as well as on the basis of immunohistochemistry) contains small amounts of CK 19 (Fig. 6*A*), next to CK 18 (Fig. 6*B*) and CK 7 (Fig. 6*C*). Additional immunostaining with RCK102 (not shown) resulted in a spot in the CK 8 region. The AC contains CK 19 and CK 18 (Fig. 6*D*), while additional bands were stained at the CK 8 (Fig. 6*E*) and CK 7 (Fig. 6*F*)

level. Note that especially in the AC immunoblot breakdown products of CK 18 and CK 19 (more acidic spots with a lower molecular weight, see also Ref. 28) are also stained with their respective antibodies.

DISCUSSION

We have examined a selected group of lung carcinomas for their CK expression using a panel of MoAbs specific for one CK polypeptide. Until a few years ago, the presence of CKs in SCLCs and ACs of the lung was a matter of discussion. For instance, some investigators (29–31) could detect CKs in formalin-fixed, paraffin-embedded squamous cell carcinomas, but not in some ACs or in most SCLCs. Since then, the use of other fixation techniques such as cold methanol fixation, or even better, the examination of unfixed, frozen sections in immunohistochemistry, has improved the detection level for CKs in tissue sections considerably. In addition, better defined CK antibodies, not only recognizing the "larger" more acidic CK polypeptides, but also recognizing the CKs occurring in simple epithelia, have become available. Investigations using these techniques and antibodies have led to a general consensus that (nearly) all lung carcinomas express CKs (4, 5, 8–10, and 32–35). A biochemical examination by Blobel *et al.* (8, 9) and
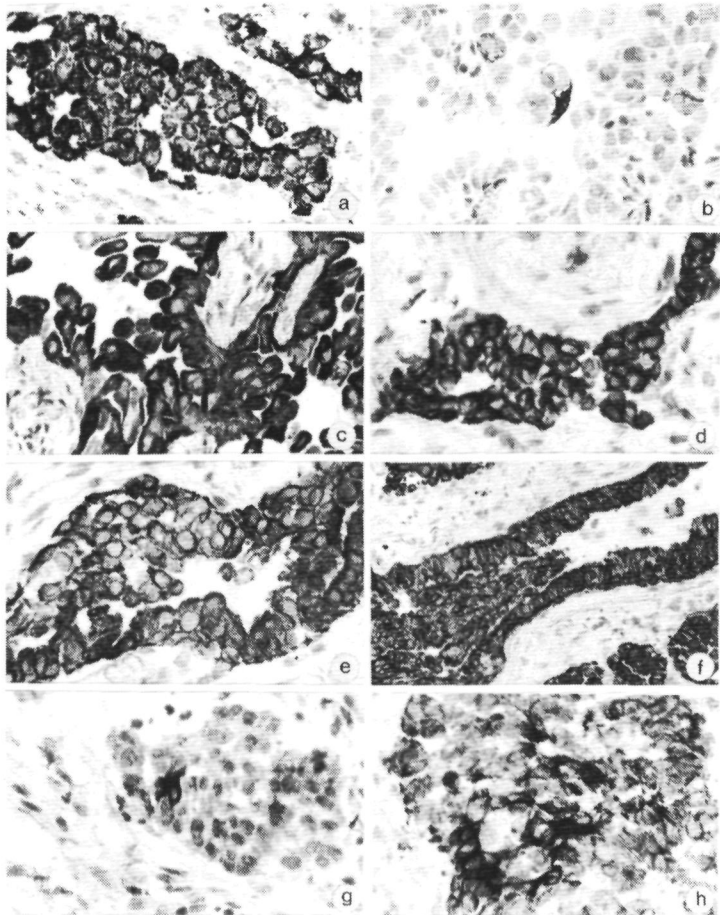
Fig. 3. Immunoperoxidase staining patterns of ACs (A–F) and adenosquamous cell carcinomas (G and H) of the lung. ACs are completely positive with the broadly cross-reacting CK antibody RCK102 (A, × 360). In some adenocarcinomas, some scattered cells react with the CK 13 antibody 2D7 (B, × 350). An extremely heterogeneous reaction is seen with the CK 4 antibody 6B10, varying from a positive reaction in all cells of some tumors (C, × 330) to a completely negative reaction in other cases (not shown). All ACs react strongly with the CK 19 antibody LP2K (D, × 360), with the CK 18 antibody RCK106 (E, × 350), and with the CK 7 antibody RCK105 (F, × 210). In adenosquamous cell carcinomas occasionally keratinizing areas are detectable with the CK 10 antibody RKSE60 (G, × 330), while other areas of the same tumors are positive with the CK 7 antibody RCK105 (H, × 330).

Moll and Franke (10) using two-dimensional gel electrophoresis suggested the presence of CKs 7, 8, 18, and 19 in ACs, the presence of CKs 4, 5, 6, 8, 13, 14, 15, 17, 18, and 19 in SQCs, the presence of CKs 7 (minor relative amounts), 8, 18 and sometimes 19 in SCLCs, and of CKs 7 (minor relative amounts), 8, 18, and 19 in lung carcinoids. The CK distribution within each tumor at the cellular level, however, could not be examined by this integral gel electrophoretic technique.

In this study we have used chain-specific MoAbs for CK polypeptides 4, 7, 8, 10, 13, 18, and 19, each of them recognizing only one single CK polypeptide. This panel of MoAbs enabled us to examine the presence of CKs within each type of lung tumor at a cellular level, and to detect a possible heterogeneous CK expression within each tumor. Such a heterogeneity has become apparent from earlier studies, which have shown that many, if not most, lung carcinomas contain more than one type of differentiation, when examined with other than conventional (histological) detection techniques.

When using MoAbs one should always be aware of false-negative reaction patterns as a result of epitope masking. For instance immunoblotting experiments, using RCK102, seem to prove the presence of CK 8 in all lung cancers. However, the reaction pattern with LE41, a monospecific antibody for CK 8, is in general very weak, while even in many cases no reaction is seen. Therefore it seems likely that in these tumors the CK 8 epitope recognized by LE41 is largely masked. Similarly, masking of epitopes recognized by one of the CK 19 antibodies, BA17, may explain why this antibody in general reacted with less tumors and with less tumor cells than LP2K, the other MoAb to CK 19. Epitope masking may also explain why the CK 18 antibody RGE 53 did not recognize basal cells of normal bronchial epithelium, while the other CK 18 antibody, RCK 106, showed a clear staining reaction in these cells.

From our results described here, it is obvious that a large heterogeneity in the expression of the different CK polypeptides within most lung carcinomas can occur. For instance the presence of CK 7, normally occurring in ACs, varied in SQC from no positive cells in well-differentiated SQCs, to a positive reaction in all tumor cells for poorly differentiated SQCs. This suggests the presence of varying degrees of AC differentiation within most SQCs.
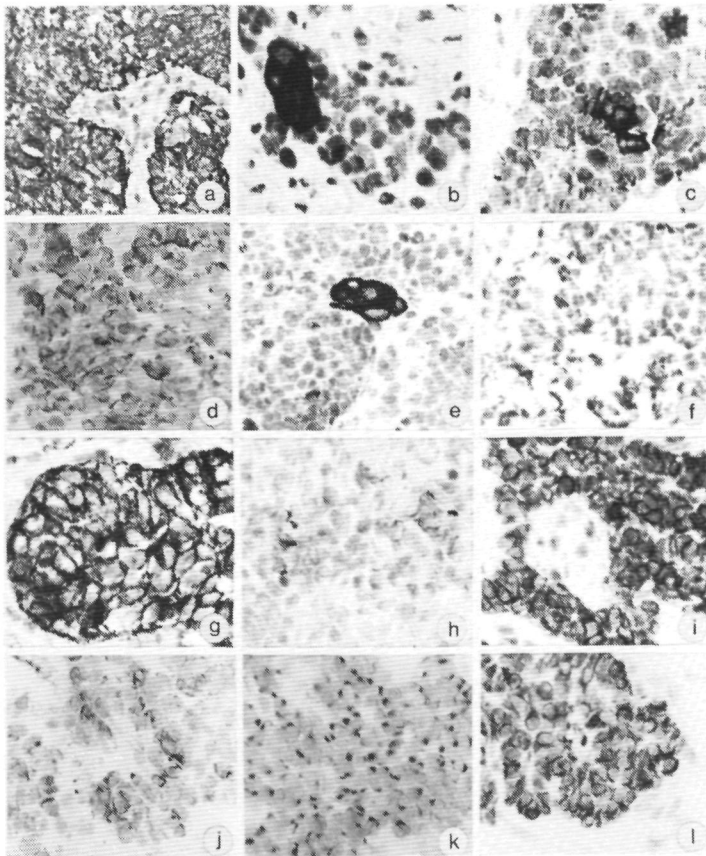
Fig. 4. Immunoperoxidase staining patterns of small cell lung cancers (SCLCs; *A–F*), combined small cell/squamous cell carcinomas (*G* and *H*), and lung carcinoids (*I–K*). Most SCLCs are completely positive with the broadly cross-reacting antibody RCK102 (*A*, × 290). In some cases a focal reaction is seen with the CK 4 antibody 6B10 (*B*, × 370). Varying numbers of cells are positive with the CK 19 antibody LP2K (*C*, × 370). Most SCLC react with the CK 18 antibody RCK106 (*D*, × 335), but do not react with the CK 7 antibody RCK105 (*F*, × 335). In some SCLC, in which at the electron-microscopic level a combined SCLC was demonstrated, focal areas reacting with CK 7 were present (*E*, × 250). Combined SCLC/SQC showed the presence of CK 18, as detected by RCK106, in large parts of the tumor (*G*, × 370), while also areas not reacting with this antibody were seen. These areas, on the other hand, contained CK 4 in some cells (*H*, × 370). All lung carcinoids are completely positive with RCK102 (*I*, × 370), while about half of the cases react with LE41 (*J*, × 250), and with LP2K (*K*, × 290). In addition, all lung carcinoids react with RCK106 (*L*, × 370). Note the typical dot-like appearance of CK 19 (*K*).

Another example of heterogeneous CK expression is the presence of CK 7 in some SCLCs, suggesting the presence of AC components in these malignancies. This finding is supported by the fact that in three of four SCLCs in which CK 7 is found, also at the electron microscopical level AC differentiation could be observed. On the basis of many other studies, comparable findings were reported. For instance, at the electron microscopic level in up to 50% of all histologically pure SQCs, AC differentiation can also be detected (2), while in some tumors even tripartite (*i.e.*, SQC, SCLC, and AC) differentiation can be found within the same tumor cell (36). This heterogeneity can also be found in the expression of peptide hormones produced by SCLCs and some non-SCLCs (37), the heterogeneity in expression of neuron-specific enolase and bombesin within SCLC (38), and even at the light microscopical level when whole tumors are examined (39).

In spite of the large heterogeneity, our results still indicate that SQC differentiation, AC differentiation, and SCLC differentiation can be distinguished on the basis of expression of different CK polypeptides. Histological squamous cell differentiation is found in parallel with the presence of CKs 10 and 13 and to a lesser extent CK 4, while pure ACs always express CKs 7, 18, and 19. SCLC differentiation is characterized by the presence of CK 18 and the absence of CKs 10 and 13, and CK 7 in most cases. These findings suggest that the presence of CK 7 may be used to detect AC differentiation. This hypothesis is supported by investigations on the cytokeratin expression in AC and SCLC cell lines, which showed that CK 7 was present in all five AC cell lines examined, while only one out of 17 SCLC cell lines showed a reaction in part of the tumor cells.[4]

In summary, the use of a panel of monospecific CK antibodies can immunohistochemically distinguish the main subtypes of lung carcinomas, but can also detect the degree and type of heterogeneity within each tumor. Therefore, application of this panel of CK antibodies is a useful aid in tumor diagnosis.

ACKNOWLEDGMENTS

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[4] J. L. V. Broers, unpublished results.

Fig. 5. One-dimensional immunoblotting assays using different chain-specific CK antibodies on cytoskeletal preparations from three small cell lung cancers (SCLCs; *lanes 1–3* of each panel), two ACs (*lanes 4* and *5* of each panel), and three moderately to well-differentiated squamous cell carcinomas (SQCs; *lanes 6–8* of each panel). Comparable amounts of cytoskeletal proteins from each sample were applied. In *A*, reaction patterns of these lung tumors with the CK 18 antibody RCK106. Note the very strong reaction of the ACs (*lanes 4* and *5*), the weaker reaction in the SCLCs (*lanes 1–3*), and the weak but evident reaction in the SQCs (*lanes 6–8*). The arrow denotes a breakdown product of CK 18. In *B*, the same immunoblot was subsequently incubated with the CK 13 antibody 1C7 and the CK 19 antibody LP2K. Note the presence of CK 13 in two SQCs (*lanes 6* and *7*), and the absence of this protein in the other SQC (*lane 8*), as well as in the SCLCs and ACs (*lanes 1–5*). In *lane 7* some degradation bands of CK 13 are clearly visible (*arrows*). CK 19 appears to be present in one SCLC (*lane 2*), in the two ACs (*lanes 4* and *5*), and in all three SQCs (*lanes 6–8*). Again, also breakdown products of CK 19 are detected (*arrow*). A parallel immunoblot was incubated with the CK 7 antibody RCK105, as shown in *C*. Two of three SCLCs show a weak reaction at the CK 7 level (*lanes 1* and *2*), while the third SCLC is negative (*lane 3*). Interestingly, the two SCLCs in which CK 7 is present, show, at the electron microscopic level, evidence for focal AC differentiation. The two ACs react very strongly with this antibody (*lanes 4* and *5*), while in two SQCs trace amounts of CK 7 can be detected (*lanes 7* and *8*). *Arrows*, breakdown products of CK 7.
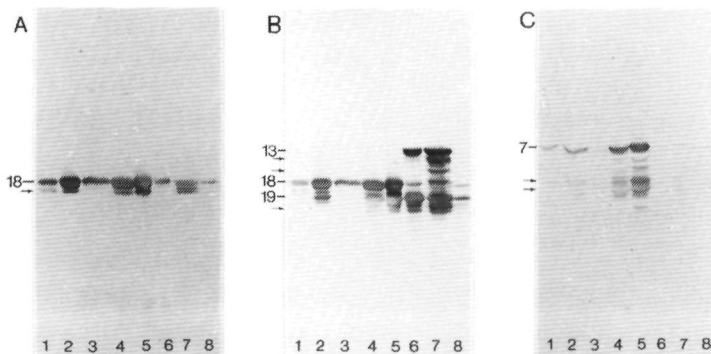

Fig. 6. Two-dimensional immunoblots of a cytoskeletal preparation from a small cell lung cancer (SCLC, *A–C*) with AC differentiation, and of a cytoskeletal preparation from an AC (*D–F*). The immunoblot of the SCLC preparation was first incubated with LP2K (*A*), resulting in a weak reaction with CK 19, followed by an incubation with RCK106, resulting in a clear recognition of CK 18 (*B*). Thereafter the same blot was incubated with 1C7, not reacting with this preparation, and with RCK105, resulting in a clear reaction at the CK 7 region (*C*). Additional staining with RCK102 resulted in a weak reaction at the CK 8 region (not shown). The immunoblot of the AC preparation was first incubated with LP2K, followed by an incubation with RCK106 resulting in clear CK 19 and CK 18 spots, respectively (*D*). Note that breakdown products of these two CKs, migrating towards the acidic side of the blots, are also immunostained. Subsequent incubation with RCK102 resulted in a weak CK 8 band (*E*), while additional staining with RCK105 resulted in a clear CK 7 spot (*F*). Incubation of this immunoblot with 1C7 did not result in additional spots.
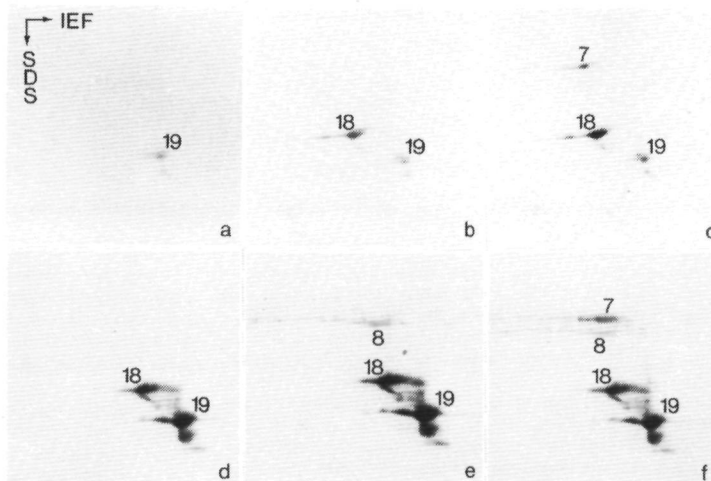
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CHAPTER SIX

DIFFERENTIAL EXPRESSION OF INTERMEDIATE FILAMENT PROTEINS
DISTINGUISHES CLASSIC FROM VARIANT SMALL-CELL LUNG CANCER
CELL LINES.

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Differential expression of intermediate filament proteins distinguishes classic from variant small-cell lung cancer cell lines

(cytokeratins/neurofilaments/vimentin/immunoblotting/immunofluorescence)

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ABSTRACT   The expression of intermediate filament proteins in classic and variant-type small-cell lung carcinoma (SCLC) cell lines was studied using immunocytochemical techniques, two-dimensional gel electrophoresis and immunoblotting assays. Classic SCLC cell lines contain cytokeratin proteins but no neurofilaments. In contrast, variant cell lines do not contain detectable amounts of cytokeratins but partly express neurofilaments and vimentin. These results explain apparent discrepancies on the intermediate filament content of SCLC described in the recent literature. The application of antibodies to fresh biopsy specimens of SCLC may in the future allow the identification of the variant type of cells in clinical SCLC specimens and may have a major impact on therapeutic strategy and prognosis in these patients.

Differential expression of intermediate filament proteins are characteristics of different tissues and tumors derived therefrom Antibodies to keratin, vimentin, desmin, glial fibrillary acidic protein, and the neurofilament proteins can distinguish between cells of epithelial, mesenchymal, and neural origin As malignant cells retain their tissue-specific intermediate filament proteins, it is possible to use these antibodies in tumor diagnosis Several studies have reported on the characteristics of intermediate filament proteins of small-cell lung carcinoma (SCLC) cells (1–8) Results so far have been inconsistent, probably because of the use of different antisera and detection methods Furthermore, it has been recognized that SCLC is often a very heterogenous tumor in which in a considerable number of cases a large-cell type can be distinguished (9–12)

SCLC accounts for 20–25% of all new cases of primary lung cancer, and unlike the other major forms of lung cancer, it is highly sensitive to both chemotherapy and radiation therapy (13) Recently, several laboratories have reported on their success in the establishment of continuous cell lines of SCLC (1, 14–16) In general, SCLC cell lines grow as floating aggregates of cells, show electron-microscopically detectable dense core vesicles, and express elevated levels of a variety of biomarkers including the key amine precursor uptake and decarboxylation (APUD) enzyme, L-3,4 dihydroxyphenylalanine decarboxylase (dopaDCase), bombesin-like immunoreactive substances (BLI), neuron-specific enolase (NSEase), and the BB isoenzyme of creatine kinase (CK-BB) With few exceptions these markers are not expressed in cell lines of non-SCLC (14, 17–19)

In a recent study of a large panel of SCLC cell lines, it has been shown that these cell lines can be subdivided into two major categories, i e , classic (70% of all lines) and variant (30%) cell lines (20) Variant cell lines do not express elevated

levels of dopaDCase or BLI but continue to express elevated levels of NSEase and CK-BB (11, 20) In addition and in contrast to classic cell lines, variant cell lines have a much looser morphology in vitro, a higher cloning efficiency, and faster doubling time and are radioresistant in vitro (11, 20, 21) Finally, amplification of the c-myc oncogene (20 to 25 fold) at the DNA level and increased c-myc mRNA expression have been observed in variant but not in classic SCLC cell cultures (22)

These data suggest a more malignant behavior for the variant SCLC cell lines, which may in part be related to c-myc amplification and expression in these cells As clinical correlates of this variant phenotype (most probably represented by the large-cell type within SCLC) may exist and be associated with a worse prognosis (12, 23), the development of assays that can distinguish classic and variant phenotypes of SCLC in biopsy specimens may be of therapeutic significance

Since different types of intermediate filament proteins have been shown to occur in different types of tissues and tumors (24–26), detection of such constituents in SCLC was likely to give an indication about the nature of the different cell types present in such malignancies In light of previously-published data (1–5), antibodies directed against cytokeratins and the neurofilament proteins are especially interesting in this respect

MATERIALS AND METHODS

Cell Lines. The establishment and characterization of the NCI cell lines have been described in detail elsewhere (10, 11, 14, 15, 17–22) NCI-H69, NCI-H128, NCI-H449, GLC-1-M13, GLC-7, and GLC-8 have been classified as classic SCLC cell lines, while NCI-H82, NCI N417, NCI-H524, GLC-1, GLC-2, and GLC-4 have been characterized as variant SCLC cell lines The characterization of GLC-1, GLC-2, GLC-4, GLC-1-M13, GLC-7, GLC-8, and GLC-12 will be described elsewhere GLC-1-M13 was obtained by limiting dilution of GLC 1 in serum-free medium

In these studies, cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 15% fetal bovine serum (GIBCO) and maintained in a humidified incubator at 37°C in 5% CO_2/95% air

Estimation of NSEase activity has been described (18) dopaDCase specific activity was calculated as nmol of ^{14}C released from L-[^{14}C]dopa per hr per mg of protein as described (27) c-myc expression was determined as described (22)

Abbreviations SCLC, small-cell lung carcinoma, NSEase, neuron-specific enolase, dopaDCase, L-3,4-dihydroxyphenylalanine decarboxylase
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Antisera. Antisera used in this study were as follows (*i*) An affinity purified polyclonal antiserum directed against human skin keratins (pK) This antiserum reacts with virtually all epithelial tissues but not with nonepithelial tissue (25) In most cases of SCLC frozen sections, this antiserum stains the tumor cells when used in immunocytochemical procedures (3) (*ii*) Monoclonal antibodies RGI 53 and CK18 2, both directed against cytokeratin 18, which specifically recognize columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells No reaction is found in squamous epithelial or nonepithelial tissues (28) The antibodies react with SCLC in frozen sections (3) (*iii*) Monoclonal antibodies directed against different neurofilament polypeptides, BF10 and R197 were provided by Brian Anderton (London) In immunoblotting assays BF10 was shown to react only with the 155-kDa neurofilament protein, while R197 reacts mainly with the 210 kDa neurofilament protein (29) A third monoclonal antibody to neurofilaments (MNF) was purchased from Euro Diagnostics (Apeldoorn The Netherlands) In immunoblotting assays, we could show that this antibody reacts strongly with the 210-kDa neurofilament polypeptide and to a somewhat lesser extent with the 68 kDa neurofilament polypeptide No reaction was found with vimentin, cytokeratins or with human callus keratins When frozen sections and paraffin sections from human brain, peripheral nerves and some neural tumors were used a positive reaction of monoclonal antibodies to neurofilament proteins (BF10, RT97, and MNF) was found only in the neural tissue No positive reaction of these neurofilament antibodies was seen in frozen sections of SCLC (*iv*) A rabbit antiserum to bovine lens vimentin Preparation and specificity of this serum have been described (25 28) (*v*) Antisera to chicken gizzard desmin and human glial fibrillary acidic protein were prepared as described before (25)

Indirect Immunofluorescence Technique. The cell lines, which grow as loose or tight floating aggregates, were either spun down in growth medium using a cytospin centrifuge (Shandon Southern Instruments, Sewickley, PA, 450 rpm for 5 min) or brought onto microscope slides by using the Cytopress system Cells were fixed by dipping in methanol (20°C) and acetone (three times), air dried and incubated with the primary antiserum for 30 min After repeated washing in phosphate buffered saline (P₁/NaCl), either goat anti rabbit antibody coupled to fluorescein isothiocyanate (FITC) (Nordic, Tilburg The Netherlands) diluted 1 25 in P₁/NaCl or rabbit anti mouse antibody coupled to FITC (Nordic, Tilburg The Netherlands) was used as a secondary antibody After incubation for 30 min, the cells were washed again in P₁/NaCl mounted in Gelvatol (Monsanto) containing 1,4 diazobicyclo[2 2 2]octane at 100 mg/ml (DABCO, Janssen Pharmaceutica Beerse Belgium)

Slides were viewed with a Leitz Dialux 20 FB microscope equipped with epifluorescent illumination Pictures were taken on 400 ASA Tri-X film (Kodak) with an automatic camera

Gel Electrophoresis and Immunoblotting Assays. Cytoskeletal preparations from the cultured cell lines were made by extracting cell pellets with 1% Triton X 100 in P₁/NaCl One dimensional gel electrophoresis was performed in 10% NaDodSO₄/polyacrylamide slab gels as described by Laemmli (30) Two dimensional gel electrophoresis (31) was performed by applying the 4% polyacrylamide rod gels after isoelectric focusing directly onto the stacking gel of the 10% NaDodSO₄/polyacrylamide gel Gels were stained with Coomassie brilliant blue R250

Immunoblotting assays were performed essentially as described by Towbin *et al* (32) All washing steps between incubations were performed with P₁/NaCl containing 0 5% Triton X 100 The immunoblots were incubated overnight

with the primary antibodies, CK 18 2, as undiluted culture supernatant, or MNF, diluted 1 20 in Tris HCl buffer (pH 7 6) containing 0 3% gelatin and 0 5% Triton X 100 They were then incubated 1 hr in rabbit anti mouse antibody coupled to peroxidase (DAKO patts, Denmark) diluted 1 200 in P₁/NaCl containing 0 3% gelatin and 0 5% Triton X 100 Peroxidase activity was detected by its reaction with 0 01% 4 chloro-1-naphtol (Merck), 0 06% H₂O₂ in P₁/NaCl for 10–30 min

RESULTS

Immunofluorescence. The intermediate filament characteristics of seven classic and six variant SCLC cell lines and two adenocarcinoma (non SCLC) cell lines were studied by use of a panel of monoclonal and polyclonal antibodies The results of these studies are shown in Table 1 and Fig 1 All seven classic SCLC cell lines contained cytokeratins, as demonstrated by a polyclonal antiserum to human skin keratins and a monoclonal antibody to human cytokeratin 18 (RGE53) Both these antisera reacted with intracellular filaments In most cases not all cells of the classic SCLC cell lines reacted with the cytokeratin antibodies used and no neurofilament proteins were detected (Fig 1 *A* and *B*) In addition, desmin, glial fibrillary acidic protein, and vimentin could not be demonstrated in classic cell lines

The intermediate filament characteristics of variant SCLC cell lines were different from those of classic SCLC cell lines in that none of them were positive for cytokeratins (Table 1 and Fig 1*C*) while several were positive for vimentin The staining pattern with the vimentin antiserum varied considerably within the variant SCLC cell lines In NCI-H82 and NCI H524, only a few cells reacted weakly positive In NCI N417, some cells reacted strongly with the vimentin antiserum while most of the other cells were only weakly stained or were negative In contrast, GLC-1 and GLC-2 exhibited a strong filamentous staining pattern in all cells with the vimentin antiserum The variant SCLC cell lines showed considerable heterogeneity when stained with antibodies to neurofilaments Of the six variant SCLC cell lines evaluated, three cell lines, NCI-H82, NCI H524, and GLC-2, were found to give a filamentous staining reaction with the neurofilament antibodies in a considerable percentage of the cells (Fig 1*D*) but the number of cells stained with the different monoclonal neurofilament antibodies varied considerably For example, the antibody MNF stained almost all cells in NCI-H82 and GLC 2 while the antibody BF10 stained only a small number of cells in these cell lines The remaining three cell lines, NCI N417, GLC-1, and GLC-4, were negative for both cytokeratin and neurofilaments

In both adenocarcinoma cell lines a strong filamentous staining reaction with the antisera to cytokeratins and the antiserum against vimentin was observed No reaction was seen with the antisera to neurofilament proteins, glial fibrillary acidic protein, or desmin

Gel Electrophoresis and Immunoblotting. In two-dimensional gel electrophoresis, all cell lines appeared to contain the microfilament protein actin (indicated as A in Fig 2 *a*, *b*, and *c*) In classic SCLC cell lines, variable but always low amounts of cytokeratins 7, 8, 18, and 19 could be demonstrated (Fig 2 *a* and *b*) In contrast, no cytokeratin spots were observed in two dimensional gels of the variant SCLC cell lines (Fig 2*c*) In classic and variant SCLC cell lines, two striking protein spots occurred at the level of vimentin (57 kDa, pI 5 7), however the presence of vimentin could not be confirmed by immunoblotting assays of cytoskeletal preparations of these cells (results not shown) When taking into account the negative reaction of classic SCLC cell lines with antibodies to vimentin in the immunofluorescence technique, it is very unlikely that these protein spots represent vimentin The two protein spots do not occur in
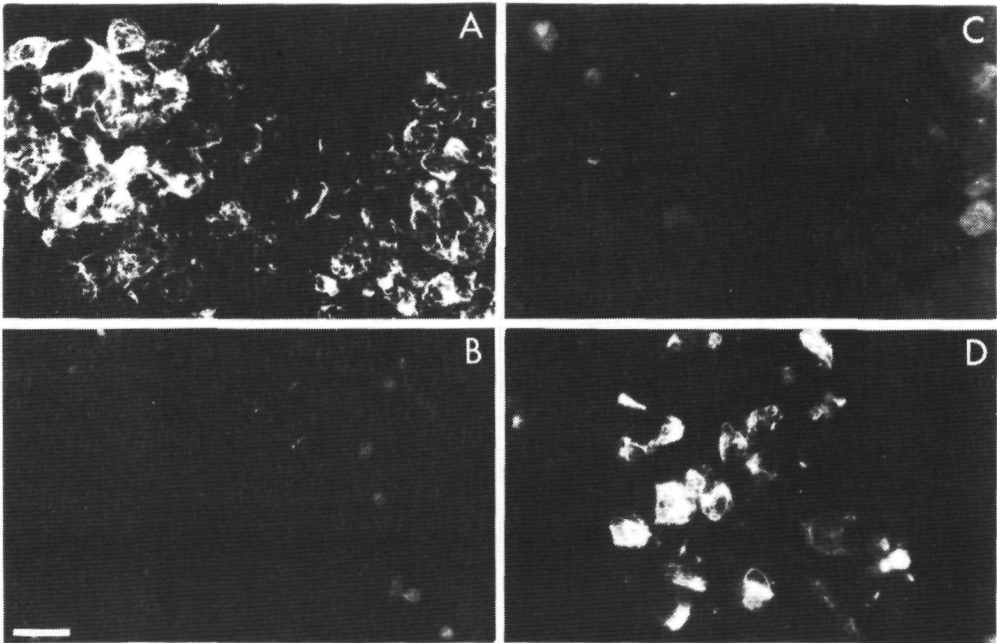
FIG. 1.   Immunofluorescence micrographs of classic SCLC cell lines (*A* and *B*) and variant SCLC cell lines (*C* and *D*) incubated with antibodies to cytokeratin (*A* and *C*) and to neurofilament protein (*B* and *D*). Note the strong filamentous reaction of classic SCLC with cytokeratin antibodies (*A*, polyclonal keratin antibody on NCI-H449) and no reaction with neurofilament antibodies (*B*, MNF on NCI-H69). On the contrary, variant SCLC cell lines are negative for the cytokeratin antibodies (*C*, RGE53 on NCI-N417) but can be stained with neurofilament antibodies (*D*, MNF on NCI-H82). (Bar = 20 μm.)

two-dimensional gels of non-SCLC cell lines (results not shown).

Immunoblotting assays could confirm our findings with the immunofluorescence technique. The presence of cytokeratin 18

Table 1.   Intermediate filament protein expression by immunofluorescence, presence of NSEase and dopaDCase activity, and c-*myc* oncogene expression in classic and variant SCLC cultures and in adenocarcinoma cultures

Cell line	NSEase	dopaDCase activity*	c-*myc* expression	Intermediate filament expression								
				Cytokeratin		Neurofilaments			Vimentin	Desmin	GFAP
				pK	RGE53	BF10	RT97	MNF			
Classic SCLC											
NCI-H69	+	264	−	+	+	−	−	−	−	−	−
NCI-H128	+	174	−	+	+	−	−	−	−	−	−
NCI-H449	+	98	−	+ +	+ +	−	−	−	−	−	−
GLC-1-M13	+			+ +	+ +	−	−	−	−	−	−
GLC-7	+			+	+	−	−	−			
GLC-8	+			+	+	−	−	−			
GLC-12	+			+	+	−	−	−			
Variant SCLC											
NCI-H82	+	0.6	+	−	−	+	+	+ +	+	−	−
NCI-N417	+	<0.1	+	−	−	−	−	−	+	−	−
NCI-H524	+	<0.1	+	−	−	+	+	+	+	−	−
GLC-1	+			−	−	−	−	−	+ +	−	−
GLC-2	+			−	−	+	+	+	+ +	−	−
GLC-4	+			−	−	−	−	−			
Non-SCLC											
NCI-H23	−	<0.1		+	+	−	−	−	+ +	−	−
NCI-H125	−	<0.1	−	+ +	+ +	−	−	−	+	−	−

−, no reaction in immunofluorescence test; +, filament staining in a varying number of cells; + +, strong filament staining in virtually all cells. GFAP, glial fibrillary acidic protein.
*Expressed as nmol of ^{14}C released from L-[^{14}C]dopa per hr per mg of protein assayed.
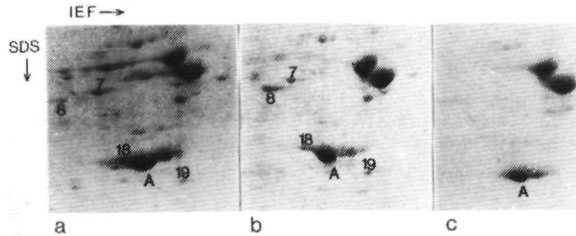
FIG. 2. Details of two-dimensional gels showing cytoskeletal proteins from classic SCLC cell lines (*a* and *b*) and a variant SCLC cell line (*c*). Note the presence of cytokeratins 7, 8, 18, and 19 (indicated as such) in the two classic SCLC cell lines (*a*, NCI-H128; *b*, NCI-H69) and the absence of cytokeratins in the variant SCLC cell line (*c*, NCI-H82). IEF, isoelectric focusing; A, actin.

could be demonstrated by using monoclonal antibody CK18-2 but only after heavily overloading the gels with cytoskeletal proteins (Fig. 3, lanes 1, 2, and 3). No reaction was seen with CK18-2 in immunoblotting of the variant SCLC cell lines (results not shown). Immunoblotting assays with the variant SCLC cell lines using the antibody MNF resulted in a clearly positive reaction with NCI-H82 at the 68-kDa level (Fig. 3, lane 5), while a very faint reaction was observed with NCI-H524 (Fig. 3, lane 6), and no reaction could be seen with NCI-N417 (Fig. 3, lane 4). These immunoblotting data agree with the results of the immunofluorescence experiments in which NCI-H82 was completely positive for antibody MNF, NCI-N417 was completely negative, and in NCI-H524 only a small number of cells were stained with this antibody. Classic SCLC cell lines did not react with antibody MNF (results not shown).

DISCUSSION

In the past few years, several investigators have tried to elucidate the origin of SCLCs. After the demonstration of neuroendocrine granules in these neoplasms by Bensch *et al.* (33), the presence of other neuroendocrine markers was described (34). The nature of the intermediate filament proteins in these tumors is still a matter of discussion. Antibodies to intermediate filament proteins have become
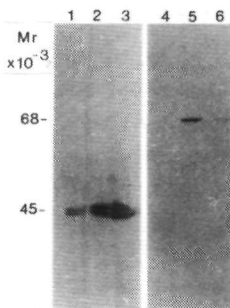

FIG. 3. Immunoblots of a cytokeratin antibody and a neurofilament antibody on cytoskeletal preparations from classic and variant SCLC cell lines. Note the positive reaction of NCI-H449 (lane 1), NCI-H128 (lane 2), and NCI-H69 (lane 3) with the monoclonal antibody to cytokeratin 18 (CK18-2). This antibody was negative on heavily overloaded gel blots from variant SCLC cell lines. With the monoclonal neurofilament antibody MNF a protein band from the variant SCLC cell line NCI-H82 (lane 5) migrating in the 68-kDa region is immunostained. A very faint reaction was also seen with NCI-H524 (lane 6) while no reaction is seen with MNF and NCI-N417 (lane 4).

available that react in a tissue-specific manner (24); for example, antibodies to cytokeratins react only with epithelial tissues and carcinomas while antibodies to neurofilament proteins react with tissues of neural origin and some neural tumors (25, 26). Application of such antibodies to SCLCs has resulted in contradictory findings (1–8). Lehto *et al.* (4) have reported the expression of neurofilament protein and no cytokeratin in such neoplasms while others (2, 3, 5) have obtained the opposite result; i.e., only cytokeratin intermediate filaments and not neurofilaments are identified.

Our findings explain these discrepancies and have led us to suggest the following hypothesis. The SCLC solid tumors and SCLC cell lines that have been described as containing neurofilament but not cytokeratin (1, 4) most probably represent the variant type of SCLC, while those tumors and cell lines described as having cytokeratin and no neurofilaments (2, 3, 5) most probably represent the classic type of this neoplasm. However, the inability to detect the presence of cytokeratins in SCLCs (1, 4, 6, 8) may be due in part to the low amount of cytokeratins present in SCLCs as demonstrated by our immunoblotting assays and two-dimensional gel electrophoresis. Furthermore, fixation procedures can result in the destruction of cytokeratin antigenic sites. Finally, the type of cytokeratin antiserum used is very critical in the detection of these intermediate filaments.

Vimentin intermediate filaments could be detected only in the non-SCLC and the variant SCLC cell lines, not in the classic SCLC cell lines. It should be stressed, however, that in three of the variant SCLC cell lines, only a few cells contained these filaments. Coexpression of vimentin filaments with other types of intermediate filaments is often observed in cultured cells (35) and it seems to be the result of adaptation of the cells to the environment or to the survival of cells as individuals that are not part of a cohesive tissue or cell cluster (36). Our findings with the anti-vimentin antibody suggest a correlation between growth pattern and morphology of the cells *in vitro* and the expression of this intermediate filament type. Cells growing attached to the surface of the culture dish (such as the non-SCLC H23 and GLC-1 and GLC-2 lines) express vimentin, while those growing as floating aggregates express much less or no vimentin at all. Within this latter group, a subdivision can be made even between cells growing as loose clusters (most variant cell lines) that contain small amounts of vimentin in a few cells and cells growing as tightly packed floating aggregates (the classic cell lines) that are negative for vimentin.

The coincidence of the cell and tissue specificity of intermediate filament proteins with the current concepts of embryologic derivation of the different cell lineages allows us to speculate about some aspects of the ongoing discussion dealing with the histogenetic origin of SCLCs. Since primary SCLC and all classic SCLC cell lines contain cytokeratins (refs. 2, 3, and 5 and this paper), it is obvious that these cells

are epithelial in nature Therefore, the origin of SCLC from a hematopoietic stem cell as suggested by Ruff and Pert (37) seems to be unlikely Furthermore, the presence of cytokeratin filaments and absence of neurofilaments within a tissue or tumor does not deny its neuroendocrine nature (compare refs 1 and 7) since several neuroendocrine tissues and tumors have been shown to contain cytokeratin intermediate filaments (38 39) Other neuroendocrine tumors, such as Merkel cell tumors, may contain both cytokeratin and neurofilament types of intermediate filament proteins (40, 41) Van Muijen *et al* (5) have reported the coexpression of cytokeratins and neurofilament proteins in an epidermoid cell carcinoma of the lung, while Bergh *et al* (1) have described a cell line derived from a large-cell carcinoma of the lung that also shows this coexpression

Therefore, the presence of neurofilaments and absence of cytokeratins in the variant SCLC cell lines raises several questions with respect to the origin and differentiated state of these cells Future studies with other markers will have to show whether these cells are derived from cells that originally contain both cytokeratins and neurofilaments, as suggested by Bergh *et al* (1) or whether they have (de)differentiated from cells positive for cytokeratin only via an intermediate cell type that is negative for both cytokeratins and neurofilaments The fact that the variant SCLC shows a particularly low sensitivity to chemo- and radiotherapy (12, 21, 23) and that patients with such tumors have a significantly worse prognosis than patients with classic SCLC makes it particularly important to recognize the variant phenotype in diagnostic biopsies The application of antibodies to cytokeratin and neurofilament proteins and other intermediate filament proteins in a rapid immunohistochemical technique may therefore become a valuable tool in the pathological diagnosis of these tumors

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CHAPTER SEVEN

INTERMEDIATE FILAMENT PROTEINS IN CLASSIC- AND VARIANT-TYPE
SMALL CELL LUNG CARCINOMA CELL LINES.
A BIOCHEMICAL AND IMMUNOCHEMICAL ANALYSIS USING A PANEL
OF MONOCLONAL AND POLYCLONAL ANTIBODIES.

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INTERMEDIATE FILAMENT PROTEINS IN CLASSIC AND VARIANT TYPES OF SMALL CELL LUNG CARCINOMA CELL LINES: A BIOCHEMICAL AND IMMUNOCHEMICAL ANALYSIS USING A PANEL OF MONOCLONAL AND POLYCLONAL ANTIBODIES

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SUMMARY

The intermediate filament protein (IFP) characteristics of a panel of lung cancer cell lines including adenocarcinoma (two cell lines) and small cell lung cancer (SCLC, three classic and three variant cell lines) were examined using one- and two dimensional gel electrophoretic techniques, immunocytochemical techniques and immunoblotting assays A panel of 28 monoclonal and polyclonal antibodies to the five different types of IFP were used The results of our studies indicate that these human lung adenocarcinoma, classic SCLC and variant SCLC cell lines can be differentiated on the basis of their pattern of IFP

The main conclusions from this study can be summarized as follows (1) The two adeno-carcinoma cell lines contain cytokeratins 7, 8, 18, and sometimes 19, next to vimentin intermediate filament (IF) (2) The three classic type SCLC cell lines contain only cytokeratin IFs but not vimentin IF or neurofilaments (NFs) Cytokeratin polypeptides 7, 8, 18 and 19 could be detected (3) All three variant type SCLC cell lines do not contain detectable amounts of cytokeratins In contrast, two out of three variant SCLC cell lines contain neurofilament proteins All three variant-type SCLC cell lines contain vimentin IF (4) Using immunoblotting assays with monoclonal and polyclonal antibodies to defined NF proteins the presence of the $68 \times 10^3 M_r$ and the $160 \times 10^3 M_r$ NF polypeptide could be demonstrated in two variant SCLC cell lines

As patients with SCLC variant phenotype have a poorer prognosis after cytotoxic therapy than patients with 'pure' SCLC, the use of antibodies to IFP in staining fresh lung tumours, especially anaplastic ones, may differentiate the two subtypes of SCLC Such a distinction would have a major impact on therapy selections and may be of prognostic importance

INTRODUCTION

Small cell lung cancer (SCLC) accounts for 20–25 % of all new cases of primary lung cancer Unlike the other major histological types of lung cancer (collectively referred to as non-SCLC), SCLC is highly sensitive to both chemotherapeutic agents and radiation therapy, so that up to 90 % of patients with SCLC will achieve a

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clinical remission after cytotoxic therapy. Although several histological subtypes of SCLC have long been recognized, including the oat-cell or lymphocyte-like variety and the intermediate cell type (World Health Organization, 1982), in several studies no significant differences have been observed among these subtypes in clinical presentation, response to therapy or overall survival (Radice *et al.* 1982; Hirsch *et al.* 1983). However, in up to 10% of all newly diagnosed patients with SCLC histological examination reveals features of a mixed small cell/large cell (SC/LC) carcinoma (Radice *et al.* 1982; Hirsch *et al.* 1983). Patients with this histological appearance have a significantly poorer response rate and shorter survival than patients with 'pure' SCLC.

Recently, investigators have reported on the growth *in vitro* and detailed biological characterization of 50 cell lines derived from patients with SCLC (Carney *et al.* 1985). On the basis of their expression of the key amine precursor uptake and decarboxylation (APUD) enzyme L-dopa decarboxylase (DDC) these cell lines could be subdivided into two main groups: namely, classic and variant SCLC cell lines. Classic SCLC cell lines express elevated levels of DDC, bombesin-like immunoreactive (BLI), neurone-specific enolase (NSE) and the BB isoenzyme of creatine kinase (CK-BB). These cell lines have typical SCLC morphology, have a relatively low cloning efficiency and prolonged doubling time, and are radiosensitive. Variant SCLC cell lines lack DDC and BLI but (unlike non-SCLC) continue to express elevated levels of NSE and CK-BB. Variant cell lines have a morphology similar to large cell undifferentiated carcinoma. Moreover, these cell lines have a more malignant growth behaviour *in vitro* and *in vivo*, are radioresistant and are amplified for the c-*myc* oncogene (Carney *et al.* 1985, and references therein). As some of these variant SCLC cell lines arose from biopsy specimens with a mixed SC/LC morphology, it is likely that these variant cell lines are the *in vitro* counterparts of the *in vivo* SC/LC carcinoma. As patients with this phenotype have a worse prognosis, and as difficulties may arise in their recognition on histological evaluations, it is clear that other assay systems, including immunohistochemical techniques that may distinguish the different phenotypes (classic *versus* variant) of SCLC, may be of clinical and prognostic importance.

Intermediate filaments (IF) occur in almost all cell types and appear to have a tissue-specific protein composition (Osborn & Weber, 1983). On the basis of their IF protein pattern the tissue origin of a given cell type can be determined, using biochemical and immunocytochemical techniques. For example, epithelial cells contain IFs of the cytokeratin type while cells of mesenchymal origin contain vimentin as their IF protein. Vimentin is also expressed in many cell types when introduced into tissue culture (Franke *et al.* 1979). Muscle tissue contains IF of the desmin type, glial cells contain glial fibrillary acidic protein (GFAP) as their IF subunit, and the IFs in neural cells consist of the neurofilament (NF) protein triplet (Dahl & Bignami, 1977). Normally, tumours derived from these tissues retain their typical intermediate filament protein pattern (Osborn & Weber, 1983; Ramaekers *et al.* 1983*b*), although some exceptions have been described (Holthöfer *et al.* 1983, 1984). This also holds true for cell cultures obtained from such neoplasms.

Table 1. *Characteristics of the lung carcinoma cell lines used in this study*

	Adenocarcinoma		Classic SCLC			Variant SCLC		
	NCI H-23	NCI H-125	NCI H-69	NCI H-128	NCI H-449	NCI H-82	NCI N-417	NCI H-524
DDC-act	<0·1	<0·1	240	646	98	0·6	<0·1	<0·1
BLI	<0·01	<0·01	1·7	15	0·8	0·2	<0·01	<0·01
NSE	<100	<100	817	2045	1998	317	580	234
CK-BB	94	68	15 921	11 768	13 207	6340	8024	8631
c-*myc* expr.	n.t.	−	−	−	−	+	+	+
Rad. surv. (*n*)*	2·0	2·0	1·1	1·2	1·0	11·1	5·6	n.t.
MOC-1†	−	−	+	+	+	+	+	+

Data extracted from Carney *et al.* (1985). Activity of L-dopa decarboxylase (DDC-act) was expressed as units mg^{-1}. Production of bombesin-like immunoreactive (BLI) was expressed as pmol mg^{-1}. Presence of neurone-specific enolase (NSE) and the BB isoenzyme of creatine kinase (CK-BB) were expressed as ng mg^{-1} soluble protein. n.t., not tested.
* *n*, extrapolation number; for definition see Carney *et al.* (1983).
† Our data.

Therefore, immunohistochemical staining of intermediate filaments in tumours and cell lines has proved to be a useful tool to determine their origin.

Results in using antisera to intermediate filament proteins (IFPs) for typing the origin of SCLC have been contradictory (Altmannsberger *et al.* 1984; Banks-Schlegel *et al.* 1985; Bergh *et al.* 1984; Bernal *et al.* 1983; Blobel *et al.* 1985; Broers *et al.* 1985a,b; Gusterson *et al.* 1982; Lehto *et al.* 1983, 1984; de Leij *et al.* 1985b; van Muijen *et al.* 1984; Schlegel *et al.* 1980), probably due to the use of different (sub)types of tumours, different fixation methods and the use of different antisera. Recently (Broers *et al.* 1985a) we have shown that classic and variant SCLC cell lines can be distinguished from each other on the basis of a differential expression of IFP. Classic cell lines express cytokeratins and no neurofilaments, while variant cell lines do not contain detectable amounts of cytokeratins, but may express neurofilaments.

To exclude false-positive or false-negative reactions, we have now extended this study elaborately by testing well-characterized cell lines with different fixation methods using a large panel of monoclonal and polyclonal antibodies to IFP, especially to different types of cytokeratins and neurofilament proteins, and we have used biochemical and immunochemical techniques to confirm these findings. The cell biological significance of our findings is discussed and the application of these antibodies to future management of patients is suggested.

MATERIALS AND METHODS

Cell lines

Methods used for the establishment and characterization of the cell lines used in this study have been described (Carney *et al.* 1985; Gazdar *et al.* 1980; see also Table 1). Three cell lines, NCI-H69, NCI-H128 and NCI-H449, are classic SCLC cell lines, while NCI-H82, NCI-N417 and

NCI-H524 are variant SCLC cell lines. Cell lines NCI-H23 and NCI-H125 were derived from patients with adenocarcinoma of the lung and form typical adenocarcinomas when inoculated into nude mice (Carney *et al.* 1985).

All cell lines were maintained in Roswell Park Memorial Institute Medium 1640 (RPMI-1640, GIBCO, Paisley, UK) supplemented with 15 % foetal bovine serum (GIBCO, Paisley, UK) and maintained in a humidified incubator at 37°C in 5 % CO_2. All SCLC cell lines grow as floating aggregates of cells, while the two adenocarcinoma cell lines grow as attached monolayer cultures.

Intermediate filament antibodies

The following antibodies were used in this study.

A. A panel of antibodies directed against cytokeratins

(1) An affinity-purified polyclonal antibody directed against human skin keratins (pKer). This antibody reacts with virtually all epithelial tissues, but not with non-epithelial tissues (Ramaekers *et al.* 1983b). In most cases of SCLC frozen sections this antibody stains the tumour cells when used in immunocytochemical procedures (Broers *et al.* 1985b).

(2) A monoclonal antibody RCK102, directed against cytokeratins from a human lung cancer cell line (MR21, kindly provided by Dr C. Gropp, Marburg, FRG). This antibody stains virtually all epithelial tissues, but not non-epithelial tissues. When tested on cultured epithelial cells this antibody shows a fibrillary staining pattern identical to that seen with the polyclonal antibody pKer.

(3) The monoclonal antibody RGE53 directed against cytokeratin 18 specifically recognizes columnar epithelial cells from digestive, respiratory and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells. No reaction is found in squamous epithelial or non-epithelial tissues (Ramaekers *et al.* 1983a). This antibody reacts with SCLC in frozen sections (Broers *et al.* 1985b). In immunoblotting assays RGE53 was shown to react with a $45 \times 10^3 M_r$ cytokeratin protein (cytokeratin 18 according to the catalogue by Moll *et al.* 1982) only present in certain epithelial tissues or epithelial cell cultures. No reaction is seen with non-epithelial tissue samples or with other cytokeratin polypeptides (Fig. 1A).

(4) The monoclonal antibody CK18-2 raised against human mesothelial cells and also directed against cytokeratin 18. This antibody gives a similar staining pattern to RGE53 when tested on human tissues. In immunoblotting assays this antibody only recognizes a $45 \times 10^3 M_r$ cytokeratin polypeptide and no other cytokeratins, nor does it react with non-epithelial cytoskeletal proteins (Fig. 1B).

(5) The monoclonal antibody RKSE60, directed against human skin keratins and specific for keratinizing stratified squamous cells. No reaction is found with columnar epithelial cells, non-keratinizing squamous cells or non-epithelial cells (Ramaekers *et al.* 1983a).

(6) The monoclonal antibodies PKK1 and PKK2 (Holthöfer *et al.* 1983, 1984), kindly provided by Dr I. Virtanen (Department of Pathology, University of Helsinki, Finland), were obtained after immunization of mice with cytoskeletal polypeptides from a pig kidney epithelial cell line (LLC-PK). PKK1 antibodies do not react with epidermal cells, but strongly stain all glandular and simple epithelial cells in human tissues. In immunoblotting assays PKK1 reacts with several cytokeratin polypeptides, including cytokeratins 8 and 18. PKK2 antibodies bind to basal cells of the epidermis, myoepithelial cells, bile-duct epithelial cells of the liver, kidney collecting duct epithelial cells, as well as various cultured epithelial cells. In immunoblotting experiments this antibody recognizes cytokeratins 17 and 19, but does not seem to react with cytokeratins 8 and 18 (Dr I. Virtanen, personal communication). PKK1 and PKK2 do not react with non-epithelial tissue.

(7) The monoclonal antibodies AE1, AE2 and AE3 (Cooper *et al.* 1985; Eichner *et al.* 1984; Woodcock-Mitchell *et al.* 1982) were kindly provided by Dr T.-T. Sun (Department of Dermatology, New York University Medical Centre) and were obtained after immunization of mice with human epidermal keratins. The AE1 antibody reacts with cytokeratins 10, 14/15, 16 and 19 (Cooper *et al.* 1985). In immunofluorescence the AE2 antibody stains the suprabasal cell layers in the epidermis, but is negative in other epithelia (e.g. oesophagus, urinary bladder or pancreas). AE3 uniformly stains all epithelia tested and reacts with cytokeratins 1 to 8 (Cooper *et al.* 1985). Thus, the cytokeratins recognized by AE1 are the more acidic polypeptides (type I cytokeratins)

within this protein family, while those recognized by AE3 represent the more basic polypeptides (type II cytokeratins). Using the three different antibodies no reaction was found with fibroblasts, muscle cells, fat cells, neural tissues, endothelial cells and blood cells.

(8) The monoclonal antibodies LE41, LE61 and LE65 were obtained after injection of mice with cytoskeleton preparations of cultured PtK1 cells (Lane, 1982). The monoclonal antibody LE41 is monospecific for cytokeratin 8, while LE61 and LE65 are both monospecific for cytokeratin 18. In frozen sections, LE61 and LE65 give tissue-staining patterns comparable with
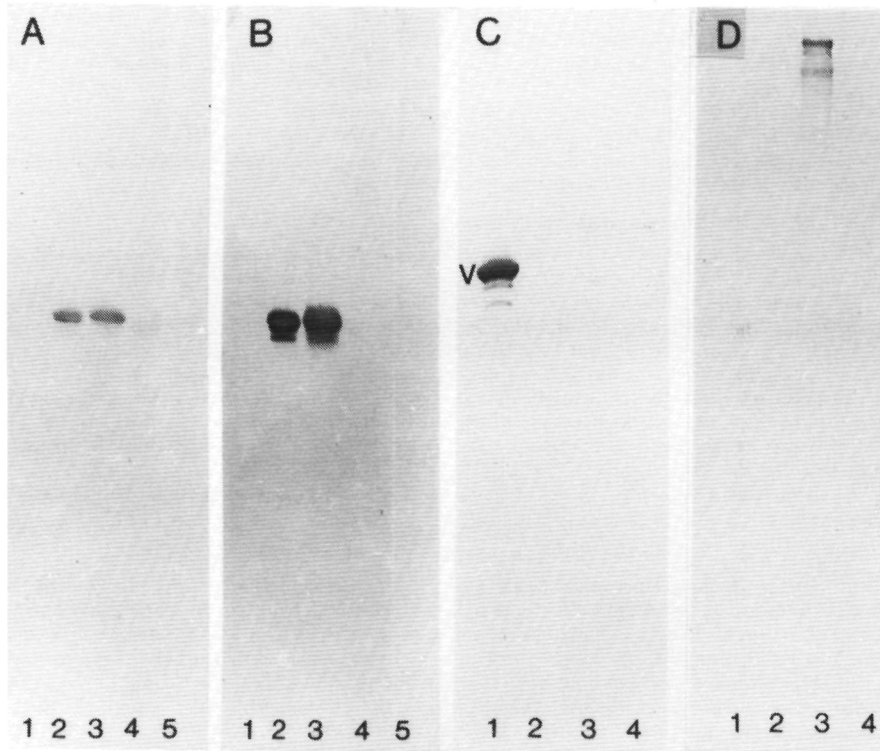

Fig. 1. Demonstration of antibody specificity by immunoblotting tests. A,B. Cytokeratin antibodies RGE53 (A) and CK18-2 (B) tested on proteins from callus keratins (lane 1), cytoskeletons from LLCMK-2 cells (lane 2), bovine liver cell cultures (lane 3), human neurofilament proteins (lane 4) and a preparation from uterine smooth muscle (lane 5). Note the strong reaction of these antibodies with cytokeratin 18 ($45 \times 10^3 M_r$) in lanes 2 and 3. C. The monoclonal antibody mVIM tested on a cytoskeletal extract from bovine lens (lane 1), callus keratins (lane 2), muscle desmin (lane 3) and a cytoskeletal preparation from BHK-21 cell cultures (lane 4). A strong reaction at the level of vimentin ($57 \times 10^3 M_r$) and its degradation products can be observed in the lens vimentin extract (lane 1). The negative reaction in BHK-21 cell extracts (known to contain vimentin) may be due to species specificity of the antibody. D. The neurofilament antibody RT97 tested on protein extracts from the lung squamous cell carcinoma cell line MR32 (lane 1), callus keratins (lane 2), a neurofilament preparation from human brain (lane 3) and bovine lens vimentin (lane 4). Note the reaction of RT97 with the neurofilament extract at the $200 \times 10^3 M_r$ level (top), and a weaker reaction with the $160 \times 10^3 M_r$ neurofilament polypeptide (lane 3).

biochemical data on the distribution of cytokeratin 18, while LE41 sees only some of the tissues known to contain cytokeratin 8, presumably recognizing a more variable aspect of the protein's conformation No reaction is seen with these three antibodies in any stratified squamous epithelia The antibodies LE41, LE61 and LE65 do not react with non epithelial cells

(9) The monoclonal antibodies LP1K, LP2K, LP3K and LP5K were raised against detergent-insoluble material from simian virus 40 (SV40) transformed human keratinocytes LP2K is monospecific for cytokeratin 19, and stains most simple epithelia and basal cells in stratified squamous epithelia that are not keratinizing (Lane & Perkis, unpublished data) LP1K recognizes many of the basic, type II cytokeratins, and stains simple epithelia but not epidermis in unfixed frozen sections LP3K and LP5K also recognize predominantly simple epithelia, and react principally with cytokeratin 8

(10) The monoclonal antibody LP34 recognizes all human epithelia tested by immuno-peroxidase on unfixed frozen sections, and by immunoblotting binds to several cytokeratin polypeptides of both type I (acidic) and type II (basic) groups It was raised by immunizing mice with human stratum corneum material (Lane *et al* 1985)

B. *Antibodies directed against vimentin*

(1) A polyclonal antibody (pVIM) to bovine lens vimentin Purification and characterization of the polyclonal rabbit antibody have been described in detail (Ramaekers *et al* 1983*b*)

(2) A monoclonal antibody (mVIM) directed against bovine lens vimentin, purchased from Euro-Diagnostics B V (Apeldoorn, The Netherlands) This antibody only stains tissues of mesenchymal origin, such as fibroblasts, endothelial cells, some blood vessel smooth muscle cells, glomeruli in the kidney, etc , but does not stain epithelial tissues In cultured bovine lens cells and HeLa cells the monoclonal antibody shows a filamentous staining pattern, identical to the pattern obtained with polyclonal antibody to vimentin In immunoblotting assays this monoclonal antibody gives a specific positive reaction with the vimentin band in cytoskeletal extracts from a human leiomyosarcoma, bovine lens (Fig 1C), and LLCMK-2 cultures

(3) A monoclonal antibody (RV201) to bovine lens vimentin, used for immunoblotting studies only

C. *A panel of antibodies directed against the neurofilament proteins*

(1) Two monoclonal antibodies BF10 and RT97 directed against different neurofilament polypeptides (Anderton *et al* 1982), were kindly provided by Dr Brian Anderton (St Georges Hospital Medical School, London) In immunoblotting assays BF10 was shown to react only with the $160 \times 10^3 M_r$ neurofilament protein, while RT97 reacted mainly with the $200 \times 10^3 M_r$ protein (Fig 1D) Both antibodies were shown to react exclusively with neural tissues

(2) Three polyclonal antibodies raised in rabbits after immunization with purified neuro-filament extracts, each raised against one of the three different neurofilament polypeptides as described by Nakazato *et al* (1984) The antibody against the $68 \times 10^3 M_r$ polypeptide shows no cross-reactivity with the other NF polypeptides, while the antibodies directed against the $160 \times 10^3 M_r$ polypeptide and the $200 \times 10^3 M_r$ polypeptide do exhibit some cross-reactivity with both the 200 and the $160 \times 10^3 M_r$ proteins, respectively No staining reaction is seen with other than neural tissues The antibodies were kindly provided by Dr Nakazato (Maebashi, Japan)

D. *Antibodies directed against desmin*

(1) A rabbit antibody to chicken gizzard muscle desmin Preparation and specificity of this antibody have been described in detail elsewhere (Ramaekers *et al* 1983*c*)

(2) A monoclonal antibody to chicken gizzard desmin (RD301), which showed a staining pattern in human, rat and chicken tissues virtually identical to that of the polyclonal desmin antibody In BHK-21 cells this antibody stains a filamentous network, while cultured bovine lens cells, fibroblasts, HeLa cells and several other cell lines, known to contain cytokeratin and, or, vimentin IF but no desmin, were negative In immunoblotting assays this antibody could be shown to react exclusively with the desmin protein band in cytoskeletal preparations from BHK-21 cells or from a human leiomyosarcoma

E. *A rabbit antibody to glial fibrillary acidic protein (GFAP) from human spinal cord, as described (Ramaekers* et al *1983b)*

F *Other antibodies*

The mouse monoclonal antibody MOC 1 was purchased from Euro-Diagnostics B V (Apeldoorn, The Netherlands) and has been described as a marker for neuroendocrine differentiation (de Leij *et al* 1985*a*)

As second antibodies in the indirect immunofluorescence technique either fluorescein isothiocyanate(FITC)-conjugated goat anti rabbit immunoglobulin G (IgG) (1 25 (v/v), Nordic, Tilburg, The Netherlands) or FITC-conjugated rabbit anti mouse IgG (1 25, Nordic, Tilburg, The Netherlands) were used In control experiments phosphate buffered saline (PBS) was used instead of the primary antibody

Immunofluorescence and immunoperoxidase techniques

Indirect immunofluorescence Cell lines NCI-H23 and NCI H125 were grown on glass coverslips (18 mm × 18 mm) in plastic Petri dishes The other cell lines, which grow as loose or tight-floating aggregates, were spun down onto glass slides using a Cytospin centrifuge (Shandon Southern, 500 rev min^{-1} for 10 min)

In order to trace and prevent possible fixation artefacts we have tested different fixation times with methanol and assayed the binding capacity of monoclonal cytokeratin antibodies under these conditions Overnight fixation in methanol (−20°C) resulted in an almost complete loss of cytokeratin filament staining reaction in all cells and cell lines, when incubated with the monoclonal antibody RGE53, PKK1 or PKK2 Reducing fixation time to 20 min resulted in a weak filamentous reaction of the adenocarcinoma cell lines and in a weak to negative reaction in the other cell lines To retain a good preservation of the cell shape and to prevent possible denaturation of cytokeratins and other IFP upon fixation cells were routinely fixed by dipping in methanol for 5 s (−20°C) and acetone (3 times, 5 s), air dried and thereafter incubated with the primary antibody for 30–45 min at room temperature

After repeated washing in PBS the appropriate FITC-conjugated antisera were applied to the cells After incubation for 30–45 min the cells were washed again in PBS, and mounted in Gelvatol (Monsanto, USA) containing 1,4 diazobicyclo [2,2,2,] octane (DABCO, Janssen Pharmaceutica, Beerse, Belgium) as described before (Langanger *et al* 1983) Slides were viewed with a Leitz Dialux 20EB microscope equipped with epifluorescent illumination Pictures were taken using a 400 ASA Tri-X film (Kodak) with an automatic camera

Immunoperoxidase technique In the indirect immunoperoxidase technique fixation and incubation with the primary antibodies were performed in the same manner as described above for immunofluorescence

Swine anti rabbit conjugated to peroxidase (DAKOpatts, Denmark) diluted 1 25 (v/v) in PBS containing 10 % normal goat serum, or rabbit anti mouse conjugated to peroxidase (DAKOpatts, Denmark) diluted 1 25 (v/v) in PBS with 5 % human AB serum were used in the second step, and incubated for 30–45 min

After washing in PBS, peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC, Aldrich Chem Comp , USA), 40 mg of AEC was dissolved in 10 ml N,N-dimethylformamide (Merck, Darmstadt, FRG) and added to 190 ml of sodium acetate buffer (0 05 M, pH 4 85) Hydrogen peroxide was added to a final concentration of 0 01 % (v/v) After incubation for 10 min cells were rinsed with tap water, counterstained with haematoxylin and mounted with Aquamount (Gurr, BDH chemicals Ltd, Poole, UK)

Gel electrophoresis and immunoblotting assays

Cells were harvested from 75 cm^2 culture flasks, spun down using a MSE Minor centrifuge at 1000 rev min^{-1} for 5 min, washed once with PBS and stored at −20°C until use

Cytoskeleton preparations were made essentially as follows after pelleting, the cells were resuspended in PBS containing 1 % Triton X 100 (BDH Chemicals, Poole, UK) and 1 mM-phenylmethylsulphonyl fluoride (PMSF, Merck, Darmstadt, FRG) and extracted for 10 min at 0°C After centrifugation (5000 g for 10 min) and washing in PBS the cytoskeletal preparation was dissolved by boiling during 5 min in sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970)

One-dimensional gel electrophoresis was performed in 10% polyacrylamide slab gels containing 0·1% SDS, as described by Laemmli (1970). One strip of the gels was stained with Coomassie Brillant Blue R250 (Gurr, Hopkin and Williams, Chadwell Heath, Essex, UK; 30 min, 60°C), while the remaining part was used for immunoblotting studies.

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975). In the first dimension isoelectric focusing (IEF) was performed in 4% polyacrylamide (Biorad, California, USA) rod gels containing 2% ampholines, pH 3·5–10 (LKB, Bromma, Sweden). For the second dimension the rod gels were applied directly onto the stacking gel of SDS–polyacrylamide gels and covered with a 1% agarose solution in running buffer.

For immunoblotting experiments the electrophoretically separated polypeptides were transferred to a nitrocellulose sheet (Schleicher and Schull Membrane Filters, BA 85, Dassel, FRG) by overnight blotting at 250 mA using an electrophoresis buffer containing 25 mM-Tris · HCl, 192 mM-glycine and 20% methanol (pH 8·3), essentially as described by Towbin *et al.* (1979). The sheets were then exposed to the following incubation steps at room temperature and under gentle shaking: (a) Preincubation (3 h) with a solution containing 350 mM-NaCl, 0·3% gelatin, 10 mM-Tris · HCl (pH 7·6) and 0 5 mM-PMSF. (b) Overnight incubation with the primary antibody either as undiluted culture supernatant or diluted in a buffer containing 150 mM-NaCl, 0·3% gelatin, 10 mM-Tris · HCl (pH 7·6), 0·1 mM-PMSF, 1% Triton X-100, 0·5% sodium deoxycholate (Merck, Darmstadt, FRG) and 0·1% SDS. Dilutions of the antibodies used for immunoblotting were as follows: RCK102, RGE53, CK18-2 and RV201 were used as undiluted culture supernatant; mVIM stock solution was diluted 1:20; RT97 culturing supernatant was diluted 1:20; anti-68×10^1M_r NF antibody was diluted 1:4000; anti-160×10^3M_r NF antibody was diluted 1:6000; and anti-200×10^3 M_r antibody was diluted 1:4000. (c) Three washing steps in PBS containing 0·5% Triton, for 10 min each. (d) Incubation for 60 min with rabbit anti-mouse peroxidase (DAKOpatts, Denmark) or with goat anti-rabbit peroxidase (DAKOpatts, Denmark) both diluted 1·200 in PBS containing 0 5% Triton and 0·3% gelatin. (e) Three washing steps in PBS containing 0·5% Triton, 10 min each. (f) Detection of the peroxidase activity on the immunoblot using 4-chloro-1-naphthol (Merck, Darmstadt, FRG) as a substrate; 50 mg of 4-chloro-1-naphthol was dissolved in 5 ml 96% ethanol, added to 95 ml PBS and stirred for 5 min. After filtration, hydrogen peroxide was added to the filtrate to a final concentration of 0·06%. The peroxidase reaction was developed for 10–30 min. (g) Rinsing of the blots with tap water for 30 min and drying between sheets of filter paper.

RESULTS

Characterization of the cell lines

In addition to the characteristics summarized in Table 1, the cell lines were investigated for the presence of the MOC-1 antigen, a cell surface antigen specific for SCLC and cells of neural and neuroendocrine nature. Using the indirect immunofluorescence technique, the MOC-1 antigen could be demonstrated to be present at the cell membrane in classic and variant cell lines, but not in the adenocarcinoma cell lines (Table 1, Fig. 2A,B).

Immunofluorescence studies with a panel of cytokeratin antibodies (Table 2, Fig. 3)

According to their different reaction profiles in human epithelial tissues the monoclonal and polyclonal cytokeratin antibodies can be subdivided into five groups, each with a distinct pattern of staining reactions.

(1) A group of antibodies with a broad cross-reactivity recognizing epidermal as well as non-epidermal epithelial cells. In general terms, these antibodies do not discriminate between any of the epithelial tissues. This group includes the monoclonal antibodies RCK102, AE1, PKK1, LP34 and the polyclonal cytokeratin

antibody (pKer). The classic SCLC as well as the adenocarcinoma cell lines reacted strongly with these antibodies (Fig. 3A,C) while no reaction was seen in variant SCLC cell lines (Fig. 3B).

(2) A group of antibodies recognizing stratified squamous (keratinizing) epithelia only, including the monoclonal antibodies RKSE60 and AE2. RKSE60 did not react with any of the cell lines examined, while AE2 reacted weakly with some cells in the adenocarcinoma cell lines.

(3) A group of antisera recognizing stratified squamous epithelia, but also other epithelia. This group includes the monoclonal antibodies PKK2 and AE3. AE3 showed a weak reaction with the classic SCLC and a strong reaction with the adenocarcinoma cell lines, while PKK2 reacted positive with the classic SCLC cell lines and with the adenocarcinoma cell line NCI-H125 (Fig. 3D,E). No reaction was seen with PKK2 in NCI-H23 (not shown).

(4) A group of antibodies reacting with simple epithelia and not with squamous epithelia, recognizing only cytokeratin 18 by immunoblotting analysis. This group includes the monoclonal antibodies RGE53, CK18-2, LE61 and LE65. These antibodies show a positive reaction with most classic SCLC and a strongly positive filamentous staining reaction with the adenocarcioma cell lines (Fig. 3F,H), but no significant positive reaction with the variant SCLC cell lines (Fig. 3G).

(5) A group of antibodies recognizing cytokeratin 8 as their major or exclusive target, i.e. the monoclonal antibodies LE41, LP1K, LP3K and LP5K. These stain the classic SCLC cell lines albeit more weakly than the cytokeratin 18 antibodies (Fig. 3I), do not stain the variant SCLC cell lines (not shown) and stain adeno-carcinoma cell lines strongly (Fig. 3J).

(6) An antibody to cytokeratin 19 (LP2K) whose tissue distribution appears to be distinct from the other cytokeratins since it occurs in most simple and in basal cells of stratified squamous epithelia (Lane *et al.* 1985). LP2K stained the adenocarcinoma cell line NCI-H125 strongly, the adenocarcinoma cell line NCI-H23 in part of the
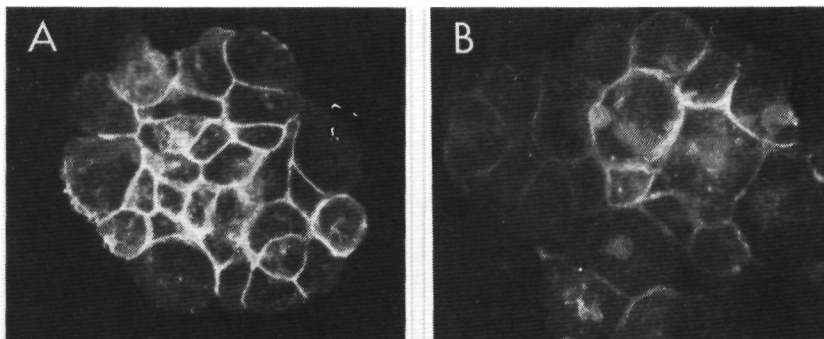

Fig. 2. Reaction pattern of MOC-1, a membrane surface antigen monoclonal antibody, specific for neuroendocrine cells and neural derivates, with a classic (NCI-H69; A, ×400) and a variant (NCI-H82; B, ×400) SCLC cell line in immunofluorescence. Note the characteristic staining pattern of the cell membranes with this antibody.

Table 2. *Intermediate filament expression in human lung cancer cell lines as detected by immunocytochemical techniques*

	Adenocarcinoma		Classic SCLC			Variant SCLC		
	NCI H-23	NCI H-125	NCI H-69	NCI H-128	NCI H-449	NCI H-82	NCI N-417	NCI H-524
Cytokeratins								
RCK102	+	++	+	+	+	−	−	−
AE1	Some c	++	+	++	++	−	−	−
PKK1	+	++	++	+	++	−	−	−
LP34	+	++	+	Some c	Some c.	−	−	−
pKer	+	++	++	++	++	−	−	−
RKSE60	−	−	−	−	−	−	−	−
AE2	Some c.	Some c.	−	−	−	−	−	−
AE3	+	+	+	−	Some c.	−	−	−
PKK2	−	++	+	+	+	−	−	−
RGE53	+	++	+	++	+	−	−	−
CK18-2	+	++	+	++	+	−	−	−
LE61	+	++	+	+	+	−	−	−
LE65	+	++	+	Some c.	Some c.	−	−	−
LE41	+	++	−	−	Some c.	−	−	−
LP1K	+	++	Some c.	−	+	−	−	−
LP3K	+	++	+	Some c.	+	−	−	−
LP5K	+	++	+	−	+	−	−	−
LP2K	+	++	Some c.	Some c	+	−	−	−
Neurofil								
RT97	−	−	−	−	−	+	−	−
BF10	−	−	−	−	−	+	−	+
p68kD	−	−	−	−	−	++	−	++
p160kD	−	−	−	−	−	++	−	++
p200kD	−	−	−	−	−	+	−	−
Vimentin								
pVim	++	+	−	−	−	Some c.	+	+
mVim	++	+	−	−	−	Some c	+	+
Desmin								
pDes	−	−	−	−	−	−	−	−
RD301	−	−	−	−	−	−	−	−
GFAP	−	−	−	−	−	−	−	−

−, no staining reaction; Some c., weak to strong staining reaction in some cells; +, positive reaction in most cells; ++, positive staining reaction in all cells; GFAP, glial fibrillary acidic protein.

cells, and the classic SCLC cell lines variably, while the variant SCLC cell lines were all negative (not shown).

Immunofluorescence studies with a panel of neurofilament antibodies (Table 2, Fig. 4)

The adenocarcinoma and classic SCLC cell lines do not show a significant reaction with any of the monoclonal or polyclonal antibodies to neurofilaments. A similar

observation is made with the variant SCLC cell line NCI-N417. However, the variant SCLC cell line NCI-H524 shows clearly positive staining reaction in a number of cells with the monoclonal antibody BF10 (Fig. 4A), while some cells of NCI-H82 showed a weak reaction with this antibody (not shown). Most cells of NCI-H82 show a staining reaction with RT97 (Fig. 4B), although background staining is relatively high; no reaction is seen in NCI-H524 with this antibody (not shown).

Using the polyclonal neurofilament antibodies a strong filamentous staining reaction is observed with the anti-$68 \times 10^3 M_r$ and the anti-$160 \times 10^3 M_r$ antibody in virtually all cells of the cell lines NCI-H82 and NCI-H524 (Fig. 4C,D,F,G). Using the polyclonal antibody to the $200 \times 10^3 M_r$ neurofilament protein a positive reaction is seen in NCI-H82 (Fig 4E). In NCI-H524 (Fig. 4H) a very weak to negative staining reaction is seen. Taking into account the relatively high background of this antiserum one might consider this to be a negative reaction.

Other intermediate filament antibodies (Table 2, Fig. 5)

The classic SCLC cell lines exhibited a very weak to negative reaction with the vimentin antibodies (Fig. 5A). In the variant SCLC cell lines only a fraction of the cells gave a positive reaction for vimentin (Fig 5B). Both adenocarcinoma cell lines are positive for the antibodies to vimentin, with a much stronger positive reaction in NCI-H23 (Fig 5C) than in NCI-H125. No difference was observed between the immunofluorescence staining patterns of the polyclonal and the monoclonal antibodies to vimentin In general, no significant reaction can be found in cells growing in floating tight aggregates, while few cells seem to express vimentin when growing in floating loose clusters. Cells growing as a monolayer or attached to the bottom of the Petri dish exhibit a stronger vimentin staining with more pronounced filamentous arrangements. No reaction was found in any of the cell lines tested with the antibodies to desmin or GFAP.

The results obtained by the indirect immunofluorescence technique with the antibodies described above could be confirmed using the peroxidase technique (results not shown). In some cases, however, some differences in intensity between the two methods were noticed. For example, the intensity of the staining reaction with monoclonal antibody CK18-2 was clearly stronger when assayed by the peroxidase technique than by the immunofluorescence technique.

Gel electrophoretic identification of IF polypeptides

The results of the one-dimensional gel electrophoretic separations of cytoskeletal proteins from lung cancer cell lines are shown in Fig. 6. In all cell lines the microfilament protein actin is demonstrable (denoted as a; $42 \times 10^3 M_r$), while in cell line NCI-H23 a distinct protein band at the vimentin position can be seen (denoted as v; $57 \times 10^3 M_r$; Fig 6, lane B,E), which cannot be seen in the other adenocarcinoma cell line NCI-H125 (Fig. 6, lane C) or in the classic SCLC cell line patterns (compare Fig. 6, lanes E,F). In the variant cell lines only a very faint

protein band migrating at the position of vimentin is observed in the one-dimensional gels (not shown).

In two-dimensional gel electrophoretic patterns all three classic SCLC cell lines can be shown to contain proteins migrating at positions of cytokeratins 7, 8, 18 and 19 albeit in minor amounts (Fig. 7A,B,C). In the three variant SCLC cell lines no
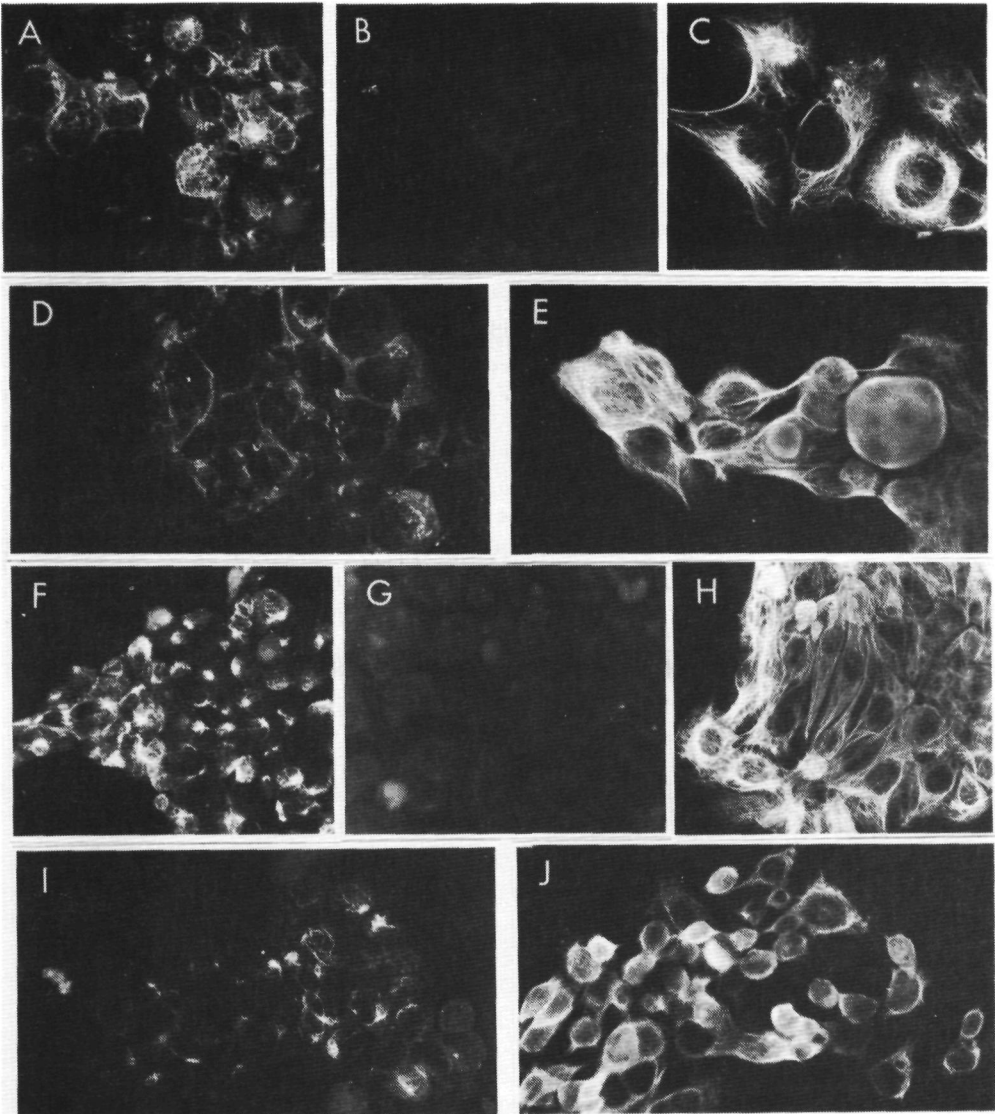
cytokeratins could be demonstrated using this technique (Fig. 7D,E,F), neither after silver staining procedures nor after heavily overloading the gels (not shown). The adenocarcinoma cell line NCI-H23 could be shown to contain at least cytokeratins 7, 8 and 18, next to vimentin with its characteristic degradation products (Fig. 7H). The adenocarcinoma cell line NCI-H125 contains also cytokeratin 19 next to cytokeratins 7, 8 and 18. Vimentin is present only in a minute amount in this cell line (Fig. 7G).

Both classic and variant SCLC cell lines contain two characteristic protein spots migrating close to vimentin in two-dimensional gels (pH 5·3, 55–57 $(\times 10^3) M_r$). Comigration of cytoskeletal preparations from NCI-H23 and NCI-H449 showed that the two protein spots of the classic SCLC cell line NCI-H449 (55–57 $(\times 10^3) M_r$) migrate to the same position as vimentin (Fig. 7I). Immunoblotting experiments (see below) using both one- and two-dimensional gels, however, failed to reveal significant amounts of vimentin in cytoskeletal preparations of these cells, indicating that these protein spots are not vimentin. Another indication is that no typical vimentin breakdown products are seen (compare Fig. 7D–F with H).

Immunoblotting experiments

The results obtained with the immunocytochemical and gel electrophoretic techniques could be confirmed using one- and two-dimensional immunoblotting. The presence of cytokeratin 18 could clearly be demonstrated in one-dimensional blots of the adenocarcinoma cell line NCI-H125 using the monoclonal antibodies RGE53 and CK18-2 (Fig. 8A, lane 1). In the other adenocarcinoma cell line NCI-H23 and in the classic SCLC cell lines this protein could only be detected when heavily overloaded gels were used, indicating that cytokeratin 18 is present in only small amounts in these cell lines (Fig. 8A, lanes 2–5). In all cases the variant SCLC cell lines were negative in immunoblotting with the cytokeratin antibodies, and when heavily overloaded gels were used.

In immunoblotting assays with the monoclonal antibodies to vimentin (RV201 and mVIM) only the adenocarcinoma cell line NCI-H23 exhibited a strong staining

Fig. 3. Indirect immunofluorescence staining patterns of classic SCLC, variant SCLC and andenocarcinoma cell lines incubated with various antibodies to cytokeratins. A,B,C. Reaction pattern of antibodies recognizing all types of epithelia. Note the positive reaction of the classic SCLC cell line NCI-H449 with PKK1 (A, ×350), the absence of staining in the variant SCLC cell line NCI-H524 with pKer (B, ×350) and the strong filamentous staining pattern in the adenocarcinoma cell line NCI-H23 with the pKer antibody (C, ×275). D,E. Reaction patterns with antibodies directed against stratified squamous epithelia. Note the weak reaction of the classic SCLC cell line NCI-H449 with PKK2 (D, ×375) and the strong filamentous staining of the adenocarcinoma cell line NCI-H125 also with PKK2 (E, ×450). F,G,H. Reaction patterns of antibodies directed against cytokeratin 18. Note the strong reaction of RGE53 with the classic SCLC cell line NCI-H449 (F, ×250), the absence of filament staining in the variant SCLC cell line NCI-N417 with RGE53 (G, ×250), and the very strong reaction of the adenocarcinoma cell line NCI-H125 with RGE53 (H, ×200). I,J. Reaction pattern of antibodies with cytokeratin 8 as their main antigen. Note the positive reaction of LP5K with the classic SCLC cell line NCI-H69 (I, ×300) and the strong filamentous staining pattern of the adenocarcinoma cell line NCI-H125 also with LP5K (J, ×250).

reaction (Fig. 8B, lane 1, RV 201; lane 2 mVIM) while in the other cell lines the amounts of vimentin were apparently too low to be detected (Fig. 8B, lanes 3–6).

Using the polyclonal antibodies to the three neurofilament polypeptides both the $68\times10^3 M_r$ neurofilament protein and the $160\times10^3 M_r$ neurofilament polypeptide could be demonstrated in the variant SCLC cell lines NCI-H82 and NCI-H524
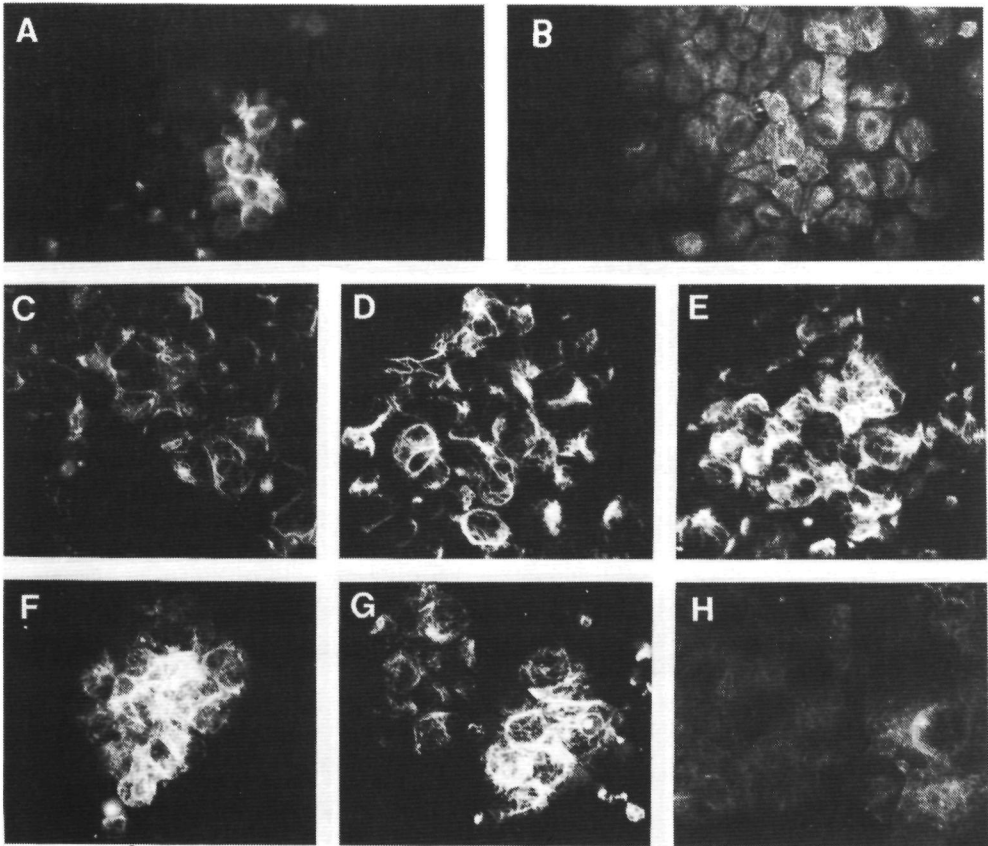

Fig. 4. Indirect immunofluorescence staining patterns of monoclonal and polyclonal antibodies to neurofilament proteins in variant SCLC cell lines. A,B. Reaction pattern with the monoclonal antibody BF10 in NCI-H524 and RT97 in NCI-H82, respectively. Note the strong positive reaction in some cells of NCI-H524 with BF10 (A, ×240) and the positive reaction with RT97 in NCI-H82 (B, ×240), which does, however, perform a higher background staining than BF10. C,F. Reaction pattern of the polyclonal anti-$68\times10^3 M_r$ neurofilament antibody in NCI-H82 (C, ×240) and NCI-H524 (F, ×240). D,G. Reaction pattern of the polyclonal anti-$160\times10^3 M_r$ neurofilament antibody in NCI-H82 (D, ×240) and NCI-H524 (G, ×240). E,H. Reaction pattern of the polyclonal anti-$200\times10^3 M_r$ neurofilament antibody with NCI-H82 (E, ×240) and NCI-H524 (H, ×300). Note the strong specific staining in NCI-H82 and the absence of a filamentous staining pattern in NCI-H524, although some diffuse (background) staining is observed.
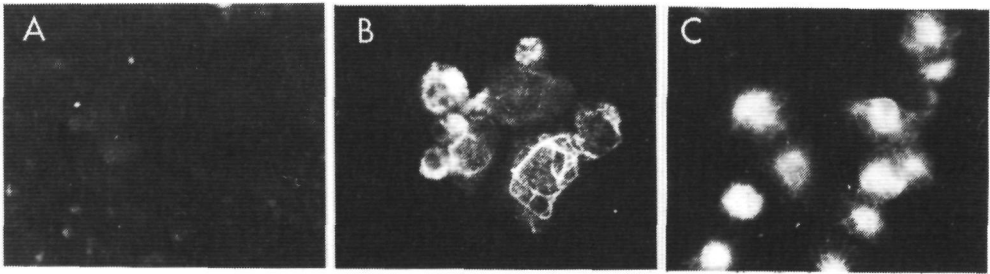
Fig. 5. Indirect immunofluorescence staining reaction of the vimentin antibody pVIM with the classic SCLC cell line NCI-H449 (A, ×225), the variant SCLC cell line NCI-H524 (B, ×425) and the adenocarcinoma cell line NCI-H23 (C, ×275). Note the absence of filamentous staining in the classic SCLC cell line, the presence of some vimentin filaments in the variant SCLC cell line and the well-developed filament pattern in the adenocarcinoma cell line.
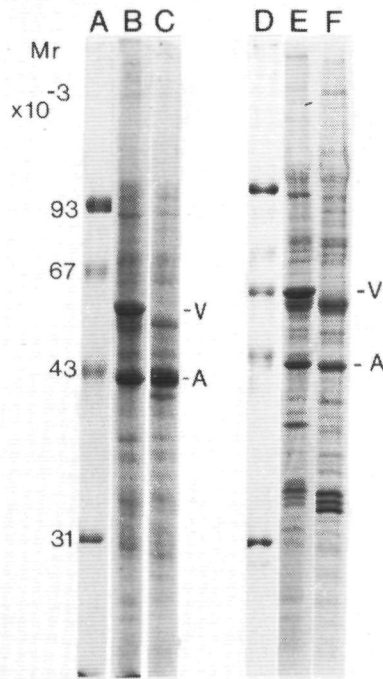

Fig. 6. One-dimensional SDS–polyacrylamide gel electrophoretic patterns of cytoskeletal preparations from the two adenocarcinoma cell lines NCI-H23 (lanes B,E) and NCI-H125 (lane C), and from the classic SCLC cell line NCI-H128 (lane F). Lanes A,D contain marker proteins as indicated. *v* and *a* indicate the positions of vimentin and actin, respectively. Note the presence of large amounts of vimentin in NCI-H23 and the absence of vimentin bands in NCI-H125 and NCI-H128.

(Fig. 8C,D). Two-dimensional immunoblotting experiments with the neurofilament antibodies anti-$68 \times 10^3 M_r$ and anti-$160 \times 10^3 M_r$ showed the acidic nature of these proteins (Fig. 9A,B). No reaction could be seen with the neurofilament
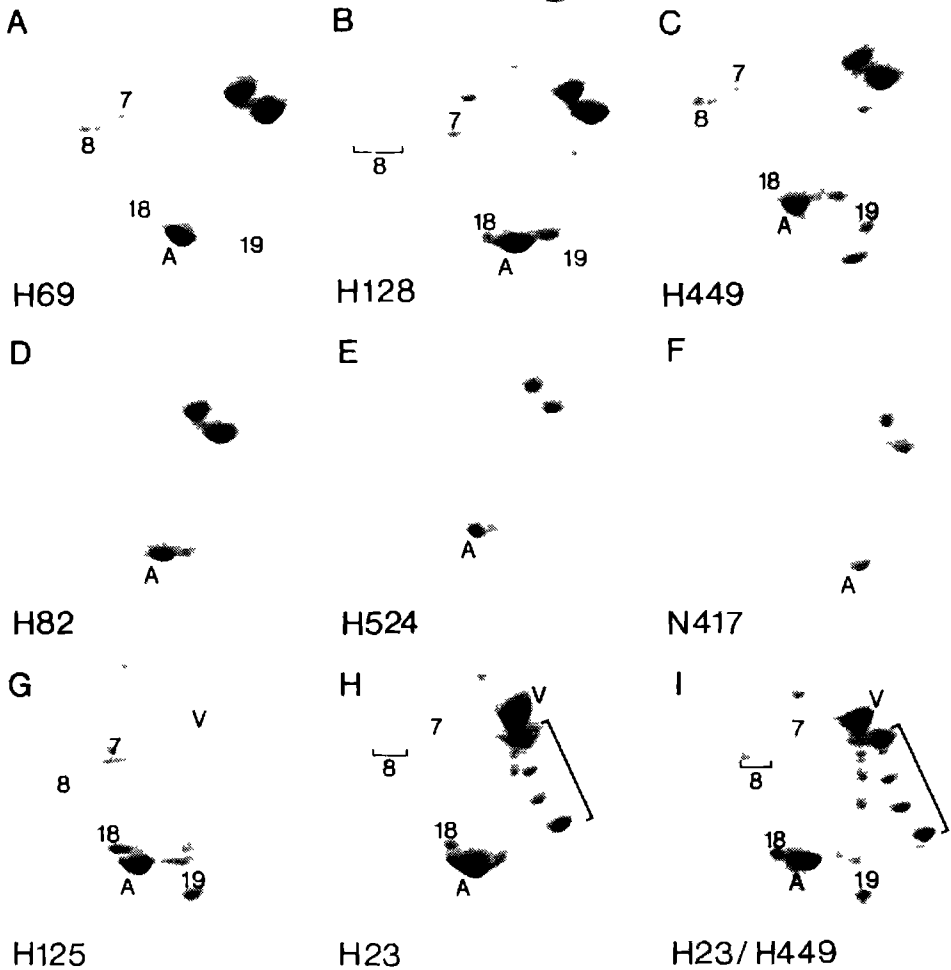

Fig. 7. Details of two-dimensional gel electrophoretic patterns of cytoskeletal preparations from the classic SCLC cell lines (A,B,C), the variant SCLC cell lines (D,E,F) and the adenocarcinoma cell lines (G,H). The different cytokeratin polypeptides are indicated by italic numerals corresponding to the cytokeratin nomenclature as proposed by Moll *et al* (1982). *v* and *a* indicate vimentin and actin, respectively, while the bracket embraces the typical degradation products of vimentin. I. Comigration of NCI-H23 and NCI-H449 cytoskeletal preparations.

antibodies described above in either one- or two-dimensional immunoblotting with other than the variant SCLC cell lines (not shown).

DISCUSSION

In this study we have examined the IFPs in classic and variant SCLC cell lines using immuno(cyto)chemical and gel electrophoretic techniques. The results show
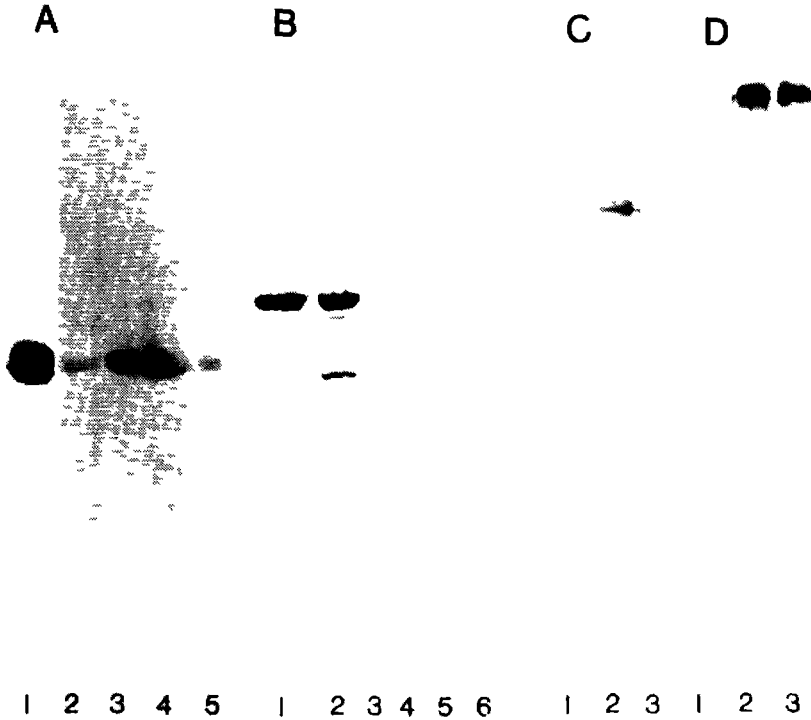

Fig 8 One dimensional immunoblotting data with cytoskeletal extracts from lung cancer cell lines using different intermediate filament antibodies A Monoclonal antibody CK18-2 reacting strongly with cytokeratin 18 from adenocarcinoma cell line NCI H125 (lane 1), but weaker with the adenocarcinoma cell line NCI H23 (lane 2) and the three classic SCLC cell lines (NCI H69, lane 3, NCI H128, lane 4, NCI-H449, lane 5) B Monoclonal vimentin antibody RV201 with the adenocarcinoma cell line NCI H23 (lane 1), and monoclonal antibody mVIM with NCI H23 (lane 2), the adenocarcinoma cell line NCI-H125 (lane 4), NCI H128 (lane 5) and NCI H449 (Lane 6) Note the positive reaction of RV201 and mVIM with vimentin and its typical degradation products in NCI H23 (lanes 1, 2) No reaction is found in the other adenocarcinoma cell line NCI-H125 (lane 3), or in the classic SCLC cell lines (lanes 4–6) C Polyclonal anti-$68 \times 10^3 M_r$ neurofilament protein with the variant SCLC cell lines NCI-N417 (lane 1), NCI-H82 (lane 2) and NCI-H524 (lane 3) Note the reaction at the $68 \times 10^3 M_r$ level in lanes 2 and 3 D Polyclonal anti-$160 \times 10^3 M_r$ neurofilament protein with the three variant SCLC cell lines NCI-N417 (lane 1), NCI-H82 (lane 2) and NCI-H524 (lane 3) Note the strong reaction at the $160 \times 10^3 M_r$ level (top)

A　　　　　　　　　　　　　　　**B**

NF 160 – ▮

NF 160 – ⸱

NF 68 – •　　　　　　　　　　　　　NF 68 – ✴

Fig 9 Two-dimensional immunoblotting of cytoskeletal preparations from NCI-H82 (A) and NCI H524 (B) with the polyclonal neurofilament antibodies against the 68 and the $160 \times 10^3 M_r$ polypeptides incubated subsequently on the same immunoblots Note the positive staining reaction with spots at the 68 and $160 \times 10^3 M_r$ levels

that these cell types differ in IFP content, insofar as classic SCLC contain cytokeratins and variant SCLC contain neurofilaments and, or, vimentin.

Small cell lung cancer is an aggressive type of lung tumour with a bad prognosis (5–10 % survival rates; Minna *et al* 1982) due to early and widespread metastasis and rapid progession of the disease. It occurs in about 20–25 % of all cases of primary lung cancer (Minna *et al* 1982). Unlike most other forms of lung cancer, SCLC is at first sensitive to chemotherapy and, or, radiotherapy, in some cases resulting in an almost complete response. Unfortunately, during therapy most tumours become resistant to further treatment and patients die within 8–10 months after discovery of the disease Amongst the patients with SCLC those with a 'mixed' SC/LC tumour have a lower response rate and a shorter survival period upon cytotoxic therapy (Hirsch *et al* 1983; Radice *et al* 1982; Vollmer *et al.* 1985). It is striking to notice that this mixed type of SCLC, which occurs in 6–15 % of all SCLC at first diagnosis (Gazdar *et al* 1981; Hirsch *et al* 1983; Radice *et al.* 1982), is seen more frequently (up to 35 %) after treatment with combined chemotherapy (Abeloff *et al.* 1979; Gazdar *et al.* 1981). Therefore, early histopathological subtyping of classic SCLC and variant SCLC (the mixed SC/LC carcinoma) is of prognostic significance.

The origin of classic and variant SCLC is yet unclear. Since Bensch *et al.* (1968) demonstrated the presence of neurosecretory granules in oat-cell carcinoma and suggested a neuroendocrine origin for this type of tumour, many authors have

demonstrated other neuroendocrine characteristics in these neoplasms (for a review, see Gould *et al* 1983). On the other hand, however, other authors have suggested an epithelial origin for SCLC (Gazdar *et al.* 1981; McDowell & Trump, 1983; Sidhu, 1979). Even the derivation of SCLC from haemopoietic stem cells has recently been proposed (Ruff & Pert, 1984)

Intermediate filaments consist of tissue-specific proteins (Osborn & Weber, 1982, 1983), which can be used to study the origin of cells and tissues. In general, neoplasms derived from certain tissues retain their IF characteristics, e.g. carcinomas express cytokeratins, sarcomas express vimentin or desmin, malignant gliomas contain glial fibrillary acidic protein (GFAP) and tumours derived from neural tissues can express neurofilaments. Conflicting results have been obtained using antisera to IFP on solid SCLC. Several authors have been able to demonstrate the presence of cytokeratins in SCLC, albeit in some instances in only a minor proportion of the cases examined (Altmannsberger *et al* 1984; Bernal *et al.* 1983; Blobel *et al* 1985; Broers *et al.* 1985*a,b*; Gusterson *et al.* 1982; van Muijen *et al.* 1984; Schlegel *et al* 1980) while others (Lehto *et al.* 1983, 1984; Nagle *et al.* 1983) could not demonstrate cytokeratins at all. Lehto *et al.* (1983, 1984) have shown a positive staining reaction in SCLC and carcinoids with a neurofilament antiserum but not with cytokeratin antisera Bergh *et al* (1984) have demonstrated only neurofilaments and not cytokeratins in cultured SCLC cell lines. Banks-Schlegel *et al* (1985) have suggested that SCLC cell lines contain both cytokeratins and neurofilaments These apparent discrepancies in the literature may be explained by our recent findings (Broers *et al* 1985*a*), and those described here. The main reason that we have used such a large panel of monoclonal and polyclonal antibodies to IFP on well-characterized cell lines was to exclude false negative or false positive reactions False negative results with cytokeratin antibodies may occur as a result of lack of cross-reactivity of a certain antibody with some specific cytokeratin polypeptides. It is well known that some antisera to skin keratins do not cross-react with epithelial tissues of certain internal organs, although these tissues are known to contain cytokeratins as detected by biochemical methods (Krepler *et al.* 1980). Furthermore, antigenic determinants recognized by specific monoclonal antibodies may be masked under certain conditions (Franke *et al.* 1983). False negative results may also occur as a result of tissue or cell-fixation. For example, the negative results of Bergh *et al* (1984) in several methanol-fixed SCLC cell lines with antibodies PKK1 and PKK2 can be explained by our findings that a considerable decrease or a total loss of cytokeratin reactivity in the classic SCLC cell lines occurs when these cells are treated with methanol for longer than a few minutes (see Materials and Methods) Fixation procedures should therefore be tested extensively before drawing any conclusions with respect to negative reaction patterns with monoclonal antibodies. This is especially true for cells that contain low amounts only of a certain antigen As can be concluded from our immunoblotting experiments and two-dimensional gel electrophoretic data, the concentration of cytokeratins in the classic SCLC cell lines is low Therefore, false negative results with these cell lines in immunofluorescence and immunoblotting assays are likely to occur.

We conclude from our studies with the large panel of antibodies to cytokeratins that the negative results with the variant SCLC cell lines in both immunoperoxidase and immunofluorescence indicate the complete absence of cytokeratins in these cell lines. Furthermore, this conclusion is supported by our two-dimensional gel electrophoretic data and our immunoblotting experiments.

The cytokeratin polypeptide patterns detected biochemically in the classic SCLC cell lines correspond relatively well to the cytokeratins found in solid SCLC by Blobel *et al.* (1985). As well as cytokeratins 7, 8 and 18 varying amounts of cytokeratin 19 are found. The presence of cytokeratins 8, 18 and 19 is also confirmed by the immunocytochemistry with monospecific antibodies. Discrepancies, such as that observed with LP2K (an antibody specifically directed against cytokeratin 19) staining NCI-H23 in some cells, while cytokeratin 19 was not detected on two-dimensional gels, indicate the greater discriminating powers of immunocytochemistry.

The positive reaction of some, but not all, variant SCLC cell lines with the NF antibodies points to a relation between at least some of the variant SCLC and neural cells. Whether this is caused by a neural origin or a neural differentiation is not clear. The fact that not all three NF proteins seem to be expressed in all NF-positive variant SCLC cell lines is an interesting phenomenon that deserves further elucidation. Also in the case of neurofilaments one should keep in mind that false negative results may occur because of masking, fixation and lack of cross-reactivity. The finding that monoclonal antibodies to neurofilaments react weaker and with less-variant SCLC cells than the polyclonal neurofilament antibodies may be explained by the fact, described by Goldstein *et al.* (1983) and Sternberger & Sternberger (1983), that such monoclonal antibodies may recognize specifically phosphorylated neurofilament proteins. Future studies will have to show whether or not neuroendocrine tumours, including variant SCLC and Merkel cell tumours (Gould *et al.* 1985; van Muijen *et al.* 1985), regulate their NF protein expression in a different way from neural tissues. The fact that both classic and variant SCLC cell lines express the MOC-1 antigen, a membrane marker also present in neural tissues, suggests a close relationship between these cell types, at least with respect to cell surface proteins. Furthermore, the fact that neurone-specific enolase and the MOC-1 antigen are found in classic SCLC cell lines illustrates that co-expression of epithelial markers (cytokeratins) and neural markers may occur (see also Blobel *et al.* 1985).

The suggestion that SCLC might be derived from stem cells of the haemopoietic system (Ruff & Pert, 1984) cannot be supported by our results. We have shown SCLC cell lines to contain cytokeratins or neurofilaments, intermediate filaments that do not occur in human cells of haemopoietic origin. The significance of the presence of vimentin in variant cell lines has been discussed before (Broers *et al.* 1985a). Taking together the suggestion that variant SCLC might originate from classic SCLC (Carney *et al.* 1985) with the fact that some variant SCLC cell lines have been found to be NF-negative and cytokeratin-negative, and the recent findings that solid SCLCs contain cytokeratins and not neurofilaments in most cases (Blobel *et al.* 1985; Broers *et al.* 1985a; van Muijen *et al.* 1984) strongly suggests that NF-

containing SCLC cells originate from cytokeratin-containing SCLC cells possibly *via* an undifferentiated cell type containing no intermediate filaments or *via* a cell type containing both cytokeratins and neurofilaments. The hypothetical model that we propose for the interrelationship between these lung cancers starts with a stem cell that contains cytokeratin IF only. Such a cell would be multipotential, so that it could differentiate into neuroendocrine as well as into true epithelial progenitor cells. The neuroendocrine progenitor cell may then give rise to classic SCLC and carcinoids, while the epithelial progenitor cells may give rise to pulmonary adeno-carcinomas and squamous cell carcinomas. Such a capacity of multidirectional differentiation may also explain the emerging body of evidence that lung cancers cover a spectrum of differentiated stages that are interchangeable and subject to various environmental influences, including therapy (Abeloff *et al.* 1979).

In summary, the results described here provide further proof for the presence of cytokeratins in classic SCLC cell lines, suggesting an epithelial character for these cells, the absence of cytokeratins in all variant SCLC cell lines and the presence of neurofilaments in some variant SCLC cell lines, suggesting a neural character for these cells. The distinction between these two types of neuroendocrine cells within solid SCLC using intermediate filament antibodies may become of clinicopathological significance in view of the differences in prognosis and treatment of classic and variant SCLC.

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Note added in proof

Recently we have developed a monoclonal antibody directed against cytokeratin 7 (RCK105). This antibody does not react with the classic SCLC cell lines, although in two-dimensional gels a protein spot migrating at the M_r and isoelectric pH level of cytokeratin 7 could be demonstrated (see Fig 7A–C) The adenocarcinoma cell lines, on the other hand, give a strong filamentous staining pattern with this antibody Future studies will have to elucidate the molecular basis of this discrepancy between immunocytochemistry and the gel electrophoretic analyses.

CHAPTER EIGHT

SPONTANEOUS CHANGES IN INTERMEDIATE FILAMENT PROTEIN EXPRESSION
PATTERNS IN LUNG CANCER CELL LINES.

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SUMMARY

The usefulness of cell lines in the study and prediction of the clinical behaviour of lung cancer is still a matter of debate. However, lung tumour cell cultures have been of value in investigations concerning molecular and cell biological aspects of these neoplasms. Especially in the examination of characteristics specific for the main types of differentiation (squamous cell carcinoma, adenocarcinoma, small cell carcinoma), in vitro studies have been most important.

Twenty eight lung cancer cell lines were cultured for up to four years, and were examined at regular intervals for their intermediate filament protein (IFP) expression patterns using a panel of cytokeratin (CK) and neurofilament (NF) antibodies. These studies showed that the classic type of small cell lung cancer (SCLC) cell lines contain CKs 8, 18, and occasionally CK 19, while the variant-type SCLC cell lines generally express no CKs but can contain NFs. Non-SCLC cell lines, such as squamous cell carcinoma and adenocarcinoma cell lines contain CKs 7 (in most cases), 8, 18 and 19. In one variant SCLC cell line and in one adenocarcinoma cell line CKs 4, 10 and 13, characteristic of squamous cell differentiation, were found. Although most cell lines have remained stable with respect to growth characteristics and IFP expression patterns, five lung cancer cultures exhibited a transition from one cell type to another, paralleled by changes in IFP expression. Progressions from classic to variant SCLC cell lines have been observed, next to conversions from variant SCLC to cell lines re-expressing cytokeratins. In some cases this resulted in a coexpression of CKs and NFs within a cell line and even within individual tumour cells. These results strongly support the earlier finding that CK expression in SCLC cell lines is a reliable marker for the classic type of differentiation, while the absence of CKs and the presence of NFs marks the variant type of differentiation. Our results are discussed in view of previous histological findings.

INTRODUCTION

Intermediate filament proteins (IFPs) are cytoskeletal components, which are expressed in a more or less tissue-specific fashion and are, in most cases, retained during neoplasia (Osborn & Weber, 1983; Ramaekers et al. 1983a, 1987c). In general, only one type of intermediate filament protein is expressed in a certain tissue or tumour. For instance, epithelial cells and carcinomas normally express only IFPs of the cytokeratin (CK) type (e.g., Ramaekers et al. 1983b), while neuronal cells and some neuronal tumours express only neurofilament (NF) proteins (Altmannsberger et al. 1984). In normal tissues, vimentin is in general found only in mesenchymal tissues.

However, in the last few years a growing number of cases has been reported, in which a coexpression of two or even three types of IFPs within the same type of (tumour) cells was demonstrated. Most frequently, if coexpression occurs, vimentin is one of the IFPs present. Expression of vimentin next to CK has been found in some normal epithelial cells, such as human mesothelium and epithelia from ovarium, amnion, endometrium, thyroid gland and foetal kidney (Moll et al. 1983; Holthöfer et al. 1984; LaRocca

& Rheinwald, 1984; Rheinwald et al. 1984; Czernobilsky et al. 1985; Regauer et al. 1985; Achtstätter et al. 1986). Also, in some types of primary carcinomas coexpression of CKs and vimentin is seen (Caselitz et al. 1981, 1984; Herman et al. 1983; Churg 1985; Blobel et al. 1985b; Gould, 1985; Jasani et al. 1985; Upton et al. 1986), and this coexpression may also be seen in metastatic carcinoma cells in body cavity fluids (Ramaekers et al. 1983c). In carcinoma-derived cell lines the presence of vimentin is a rule rather than an exception (cf. Franke et al. 1979; Broers et al. 1985). Vimentin is expressed next to desmin in muscle cell tumours (Molenaar et al. 1985).

More recently, coexpression of other types of intermediate filaments has been reported. For instance, Brown et al. (1987), Norton et al. (1987), van Muijen et al. (1987) and Ramaekers et al. (1988) reported on the coexpression of CKs and desmin in human foetal and adult muscle tissue, and in smooth muscle tumours. Kasper et al. (1986) showed the coexistence of CK, vimentin and NF protein in human choroid plexus, while Budka (1986) demonstrated a triple expression of glial fibrillary acidic protein, vimentin and CKs in papillary meningioma and renal carcinoma. Gatter et al. (1986) showed a double and triple expression of different IFP in many lung carcinomas. Coexpression of CKs and NFs seems to be characteristic of some neuroendocrine tumours, such as neuroendocrine skin carcinomas (Merkel cell tumours; van Muijen et al. 1985; Gould et al. 1985; Moll et al. 1986) in parathyroid tumours (Miettinen et al. 1985), in lung carcinoids (Lehto et al. 1985; Blobel et al. 1985a; Broers et al. 1987; Ramaekers et al. 1987b), in some small cell lung cancers (SCLCs; Gatter et al. 1986; Broers et al. 1987) and (poorly differentiated) squamous cell carcinomas (SQCs) of the lung (van Muijen et al. 1984; Broers et al. 1987). Also in a cell culture of a large cell carcinoma of the lung (Bergh et al. 1984), in a neuroendocrine skin carcinoma cell line (Rosen et al. 1987), in the neuroendocrine rat cell line PC12 (Franke et al. 1986) and in some SCLC cell lines (Bernal et al. 1983; Banks-Schlegel et al. 1985; Gupta et al. 1986) coexpression of CKs and NFs has been reported.

The three major types of lung cancer comprise SCLC, adenocarcinoma (AC) and SQC (W.H.O. 1982). In addition, according to the latest IASLC classification, SCLC can be subdivided into classic SCLC, variant SCLC and combined SCLC (Yesner, 1985). It is now generally accepted that lung cancer is an extremely heterogeneous type of malignancy. On the basis of detailed histological (Roggli et al. 1985), immunohistochemical (Hammar et al. 1985; Dunnill & Gatter, 1986; Broers et al. 1987, 1988) and electron-microscopic (McDowell & Trump, 1981; Trump et al. 1982; Dingemans & Mooi 1984; Mooi et al. 1988) studies, more than one type of differentiation can be detected in the majority of lung cancers. Occasionally more than one type of differentiation can be detected in cell lines, for example in the so-called multipotent lung tumour cell cultures (Carney et al. 1984; de Leij et al. 1985). Cloned cells of these cell lines are apparently able to differentiate again into the three major types of lung cancer within the same culture. In addition, sometimes a conversion of one cell type to another has been noticed. For example, progressions of classic SCLC cell lines into the variant type of SCLC cell line have been described (Bepler et al. 1987b, Carney et al. 1984, 1985a,b; Gazdar et al. 1981, 1985; Graziano et al. 1987). Bepler et al. (1987c, 1988a) described a transitional type of SCLC with features between the classic and variant types.

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Previous investigations in our laboratory on the intermediate filament expression of classic and variant SCLC cell lines have demonstrated the occurrence of CKs only in classic SCLC cell lines, while in variant SCLC cell lines no IFPs or only NFs could be demonstrated (Broers et al. 1985, 1986). In recent years, cell lines in which a morphological change from one cell type to another could be seen were examined for their IFP expression patterns.

Since different CK polypeptides are expresssed in different types of lung cancer (cf. Blobel et al. 1984, 1985a; Broers et al. 1988), we paid special attention to the CK polypeptide expression of these cell lines. Using a panel of monoclonal CK antibodies, most of which are specific for one CK polypeptide, it is possible to detect SQC differentiation, AC differentiation and SCLC differentiation at the cellular level (Broers et al. 1988).

In this paper we describe spontaneous changes in intermediate filament patterns that can occur in lung cancer cell lines, especially in SCLC cell lines. In part, these alterations follow the phenotypic changes in the tumour cells, and may be correlated with recent data obtained with lung tumour tissues.

MATERIALS AND METHODS

CELL LINES.

Establishment and characterization of the cell lines used in this study have been described (Carney et al. 1985a,b; de Leij et al. 1985; Gazdar et al. 1985; Bepler et al. 1987a,b, 1988b) or will be described elsewhere. In all, 19 SCLC cell lines were investigated (NCI-H69, NCI-H128, NCI-H449, NCI-N417, NCI-H524 and NCI-H82; GLC1-M13, GLC-1, GLC-2, GLC-3, and GLC-5; SCLC-16HC, SCLC-16HV, SCLC-21H, SCLC-22H, SCLC-24H and SCLC-86M1; NL-SCLC2 and NL-SCLC3). Next to these, one LCLC cell line (LCLC-103H), six AC cell lines, (NCI-H23, NCI-H125, GLC-A1, GLC-A2, GLC-A3, NL-AC1) and two epithelial cell lines derived from SQCs (EPLC-32M1 and EPLC-65M2) were used.

All cell lines were maintained in a humidified incubator at 37°C in 5% CO_2 and were grown in Roswell Park Memorial Institute Medium 1640 (RPMI-1640, Flow, Irvine, UK) containing Hepes, supplemented with 15% newborn calf serum (Flow). At regular intervals cells were passaged by splitting cells 1:2 to 1:3. If cells tended to attach to the culture flasks, the adhering cells were selected by removing the floating cells. As a result sublines NCI-N417-A1, NCI-N417-A2, NCI-H524-A and NCI-H82-A were obtained.

INDIRECT IMMUNOFLUORESCENCE TECHNIQUE.

The procedures for the application of the indirect immunofluorescence technique have been described (Broers et al. 1986). In brief, floating cells were placed on glass slides using a Cytospin centrifuge (500 revs min-1 for 10 min; Shandon Southern Instruments, Astmoor, Cheshire, UK), while cells growing attached to the culture flasks were treated with 0.05%

trypsin/0.02% EDTA in 0.05 M phosphate buffered saline (PBS) pH 7.4, and allowed to grow on glass coverslips for 1-2 days. In the first step, cells were incubated with the primary antibody (see below) for 30-45 min.
As second antibody either fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:25, Nordic, Tilburg, The Netherlands) or FITC-conjugated goat anti-rabbit IgG (1:25, Nordic) was applied to the cells for 30-45 min.

In the double-label immunofluorescence technique a mixture of two primary antibodies (one raised in mouse and one in rabbit) was applied in the first step. Cells were incubated for 30-45 min and, after washing with PBS, a mixture of FITC-conjugated goat anti-rabbit IgG and Texas Red-conjugated sheep F(ab')2 anti mouse IgG (Amersham, Little Chalfont, Bucks, UK)) with final dilutions of 1:25 and 1:50, respectively, was added. Cells were incubated for 30-45 min, and washed again. DNA was then stained by incubating the cells for 15 min with Hoechst 33258 (0.1 µg·ml^{-1} in 22 mM-citric acid, 56 mM-disodium hydrogen phosphate). After washing with PBS the cells were mounted as described above. Pictures were taken with an automatic camera as described above, with either a 400 ASA Tri-X film (Kodak) or with a 400 ASA Ektachrome film (Kodak) for triple exposures at three different wavelengths. Overlap between the FITC and Texas Red fluorescence at the respective wavelengths was checked using preparations labelled with a single second antibody. Incubations with the second antibodies alone were used as negative controls.

ANTIBODIES.

Specificity and references of the primary antibodies used in this study are summarized in Table 1. The following primary antibodies were used.
A) Mouse monoclonal CK antibodies (undiluted).
1. RCK102 is a broadly cross-reacting CK antibody of the IgG1 subclass, which recognizes CKs 5 and 8, and as a result stains virtually all epithelial tissues (Ramaekers et al. 1987a).
2. RCK105 (IgG1; Ramaekers et al. 1987a) reacts only with CK 7 in immunoblotting and stains a subgroup of glandular epithelia and their tumours, next to transitional bladder epithelium and bladder carcinomas.
3. RGE53 (IgG1; Ramaekers et al. 1983d, 1985) and RCK106 (IgG1; Ramaekers et al. 1987a) are monospecific for CK 18 in immunoblotting. In general these two antibodies recognize columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues and mesothelial cells, as well as their tumours. Generally, no reaction is found in squamous epithelia or SQCs.
4. LP2K (Lane et al. 1985) and BA17 (IgG1; Bartek et al. 1986a,b) stain most simple epithelia and basal cells in stratified squamous epithelia that are not keratinizing. Both antibodies recognize only CK 19 in immunoblotting assays.
5. RKSE60 (IgG1; Leigh et al. 1985; Puts et al. 1985) reacts only with keratinizing epithelial cells, and recognizes CK 10 in immunoblotting.
6. 6B10 (IgG1; van Muijen et al. 1986) reacts with non-cornifying squamous epithelium and with certain ciliated pseudostratified epithelia such as cylindrical epithelium of bronchi, and recognizes only CK 4 in immunoblotting.
7. Antibodies 1C7 (IgG2a; van Muijen et al. 1986) and 2D7 (IgG2b; van Muijen et al. 1986) react both with non-cornifying squamous epithelia.

Table 1: Specificity of antibodies used in this study.

Antibody	Mouse Ig subclass (dilution for immunofl.)	Antigen recognized	Tissue specificity	Reference
RCK102 al.	IgG1 (undil.)	Cytoker. 5+8	Nearly all epithelial tissues	Ramaekers et 1987a
RCK105 al.	IgG1 (undil.)	Cytoker. 7	Some glandular epithelia	Ramaekers et 1987a
RGE53 al.	IgG1 (undil.)	Cytoker. 18	Glandular epithelia, not with squamous cell epithelia	Ramaekers et 1983d, 1985
RCK106 al.	IgG1 (undil.)	Cytoker. 18	Glandular epithelia, not with squamous cell epithelia	Ramaekers et 1987a
LP2K 1985	(undil.)	Cytoker. 19	Most simple epithelia	Lane et al.
BA17	IgG1 (undil.)	Cytoker. 19	Most simple epithelia	Bartek et al. 1986a,b;
RKSE60 1985; 1985	IgG1 (undil.)	Cytoker. 10	Keratinizing epithelial cells	Leigh et al. Puts et al.
6B10 al.	IgG1 (undil.)	Cytoker. 4	Non-cornifying squamous epithelia, pseudostratified epithelia	van Muijen et 1986
1C7 al.	IgG2a (undil.)	Cytoker. 13	Non-cornifying squamous epithelia	van Muijen et 1986
2D7 al.	IgG2b (undil.)	Cytoker. 13	Non-cornifying squamous epithelia	van Muijen et 1986
MNF 1984	IgG1 (1:5)	NF 68, 200kD	Neuronal tissues	Klück et al.
pNF68	rabbit (1:400)	NF 68 kD	Neuronal tissues	Nakazato et al. 1984
pNF160	rabbit (1:600)	NF 160 kD	Neuronal tissues	Nakazato et al. 1984
pNF200	rabbit (1:400)	NF 200 kD	Neuronal tissues	Nakazato et al. 1984.

These antibodies recognize only CK 13 in immunoblotting studies.

B) NF antibodies
1. The mouse monoclonal antibody MNF (IgG1, diluted 1:5 in PBS), is reactive with the 68 kD and 200 kD NF subunits and stains neuronal tissues and tumours (Klück et al. 1984).
2. The polyclonal rabbit NF antibodies pNF68 (diluted 1:400), pNF160 (diluted 1:600) and pNF200 (diluted 1:400) are specific for the three NF subunits of 68kD, 160kD and 200kD, respectively (Nakazato et al. 1984), and react in neuronal tissues.

C) Vimentin antibodies.
1. The mononclonal antibody RV202 (IgG1; Ramaekers et al. 1987a) reacts with mesenchymal cells.
2. The polyclonal antibody pVIM prepared against bovine lens vimentin (Ramaekers et al. 1983a) also reacts with mesenchymal cells.

Antibodies RCK102, RCK105, RGE53, RKSE60, MNF, 6B10, 1C7, 2D7, and pVIM are available from Euro-Diagnostics B.V. (Apeldoorn, The Netherlands).

GEL ELECTROPHORESIS AND IMMUNOBLOTTING.

For gel electrophoresis and immunoblotting experiments the same procedures were followed as described (Broers et al. 1986, 1988). For immunoblotting the following antibodies were used: RCK102 (undiluted supernatant), RCK105 (undiluted supernatant), RCK106 (undiluted supernatant), LP2K (diluted 1:10) and pNF68 (diluted 1:5000).

FLOW CYTOMETRY.

The preparation of cell samples for flow cytometric analyses has been described (Ramaekers et al. 1986). Briefly, cells were centrifuged at room temperature (5 min., x500g), and the supernatant was discarded, but fluid sticking to the wall of the tubes was allowed to cover the pellet. Cells were resuspended in this fluid by vigorously vortexing, cold (-20°C) 70% ethanol was added, and the cells were kept in this solution until staining. The final cell concentration was about 1 million cells per ml of 70% ethanol. Approximately 1 million cells were taken from this suspension and stained for DNA with propidium iodide according to Feitz et al. (1985), and for CKs using the antibody RCK102 as described (Ramaekers et al. 1986).

Cell analysis was performed using a Cytofluorograph 50H (Ortho Instruments, Westwood, Ma, USA). Propidium iodide was excited at 488 nm and fluorescence was measured using a 630 nm longpass filter. For determination of the DNA index chicken red blood cells served as an internal standard and human lymphocytes as an external standard (Jakobsen, 1983).

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RESULTS

EXPRESSION OF CYTOKERATINS, NEUROFILAMENTS, AND VIMENTIN.

The expression patterns of CKs, NFs and vimentin in lung carcinoma cell lines as detected by the indirect immunofluorescence technique are shown in Table 2. The CK expression patterns are depicted in Fig. 1. Within the SCLC cell lines a subdivision can be made between the classic SCLC cell lines, which contain CKs but not vimentin or neurofilaments, transitional SCLC cell lines with a variable CK expression pattern and the expression of vimentin, and the variant SCLC cell lines, which in general do not contain CKs but contain vimentin and have a variable expression of neurofilaments. In all classic SCLC cell and in most transitional SCLC cell lines a positive staining reaction was observed with the broadly cross-reacting RCK102 antibody (CK. 5+8; Fig. 1A,B,C). Also in the large cell lung cancer (LCLC), the AC (Fig. 1D) and the SQC cell lines a strong staining reaction was seen with this antibody. Antibodies to stratified squamous cell epithelia, reacting with CK 4, 10 or 13, were reactive with two cell lines. One of these cell lines (GLC-3) was derived from a SCLC, which at the electron-microscopic level showed next to SCLC also AC differentiation, while the CK expression patterns indicate squamous cell differentiation. The other cell line (GLC-A2) was derived from an AC of the lung. The expression of CKs 4, 10 and 13 in this cell line clearly shows squamous cell differentiation, next to AC differentiation (a positive reaction with the CK 7 antibody). The classic SCLC cell lines reacted in general in most cells with the antibodies to CK 19 (Fig. 1E,G), while the transitional SCLC cell lines reacted only in part of the cells or not at all (Fig. 1F) with these antibodies. In non-SCLC cell lines a reaction pattern varying from only a weak reaction in some cells to a very strong reaction in all cells (Fig. 1H) was seen with the CK 19 antibodies. The monoclonal antibodies to CK 18 reacted with all classic (Fig. 1I,K), three out of four transitional (Fig 1J) and three variant SCLC cell lines, and a strong reaction with all non-SCLC cell lines could be observed (Fig. 1L). The antibody to CK 7 reacted with only one out of 19 SCLC cell lines in some of the tumour cells (Fig. 1M,N,O), while in contrast all but one of the non-SCLC cell lines were positive with this antibody (Fig. 1P).

Neurofilaments were present in seven out of ten variant SCLC cell lines. A filamentous staining reaction was not seen in the other types of SCLC cell lines with the NF antibodies. In only one out of nine non-SCLC cell lines was a reaction seen with the NF antibodies.

Vimentin was expressed in variable amounts, ranging from no reaction in the classic SCLC cell lines (see also Broers et al. 1985, 1986) to a strong filamentous staining reacting in adherent growing cells of most AC cell lines.

Our immunofluorescence findings on the CK expression in lung cancer cell lines were confirmed using the immunoblotting technique. Fig. 2 shows the results of one-dimensional immunoblotting assays of an AC cell line (A) and two classic SCLC cell lines (B). For comparison, the same nitrocellulose blots were subsequently incubated with different CK antibodies. Using LP2K, the presence of CK 19 could be demonstrated in the AC cell line (A, lane 1) and in one classic SCLC cell line (B, lane 2). CKs 8 and 18 were present in all three cell lines as detected by antibodies RCK102 and RCK106, respectively (A, lane 2; B, lanes 3,4). Application of

Table 2: Reaction patterns of lung carcinoma cell lines with antibodies to intermediate filaments.

Cell line	RCK102 (5+8)*	RCK105 (7)	RGE53/RCK106 (18)	LP2K/BA17 (19)	6B10 (4)	1C7/2D7 (13)	RKSE60 (10)	MNF	pNF68	pNF160	pNF200	RV202	pVim
SCLC													
Classic													
NCI-H128	++	-	++	+	-	-	-	-	-	-	-	-	-
NCI-H449	+	+/-	+	++	-	-	-	-	-	-	-	-	-
NCI-H69	+	-	+/-	+	-	-	-	-	-	-	-	-	-
GLC1-M13	+	-	+/-	+/-	-	-	-	-	-	-	-	-	-
NL-SCLC2	++	-	++	+/-	-	-	-	-	-	-	-	-	-
Transitional													
SCLC-16HC	+/-	-	+/-	+/-	-	-	-	-	dot-like	-	-	+/-	+/-
SCLC-22H	++	-	+	+	-	-	-	-	-	-	-	+/-	+/-
SCLC-24H	++	-	++	-	-	-	-	-	-	-	-	+/-	+/-
SCLC-86M1	-	-	-	-	-	-	-	-	-	-	-	+/-	+/-
Variant													
NCI-N417	-	-	-	-	-	-	-	-	-	-	-	+/-	+/-
NCI-H524	-	-	-	-	-	-	-	-	++	++	-	+/-	+/-
NCI-H82	-	-	-	-	-	-	-	++	++	++	++	+/-	+/-
GLC-1	-	-	-	-	-	-	-	-	-	-	-	+	+
GLC-2	-	-	-	-	-	-	-	+/-	++	++	-	+	+
GLC-3	+/-	-	+/-	+/-	some c.	some c.	some c.	-	-	-	-	+/-	+/-
GLC-5	some c.	-	some c.	-	-	-	-	+/-	++	++	+/-	+	+
NL-SCLC3p10	-	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
SCLC-21H	+/-	-	+/-	-	-	-	-	-	dot-like	-	-	+/-	+/-
SCLC-16HV	-	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
Large Cell													
LCLC-103H	++	-	+/-	+/-	-	-	-	-	-	-	-	+	+
Squamous Cell													
EPLC-32M1	++	+/-	++	+/-	-	-	-	-	-	-	-	+	+
EPLC-65M2	++	++	++	+/-	-	-	-	-	-	-	-	+	+
Adenocarc.													
NCI-H23	+/-	some c.	+/-	-	-	-	-	-	-	-	-	++	++
NCI-H125	++	++	++	++	-	-	-	-	-	-	-	+	+
NL-AC1	++	++	++	++	-	-	-	-	-	-	-	++	++
GLC-A1	+/-	some c.	+/-	+/-	-	-	-	+/-	+/-	+/-	+/-	+	+
GLC-A2	++	++	+/-	++	+/-	+/-	+/-	-	-	-	-	+	+
GLC-A3	++	++	++	++	-	-	-	-	-	-	-	+	+

Table 3: Reaction pattern of cell lines exhibiting transitions in intermediate filament expression patterns.

Cell line	RCK102 (5+8)*	RCK105 (7)	RGE53/ RCK106 (18)	LP2K/ BA17 (19)	6B10 (4)	1C7/ 2D7 (13)	RRSE60 (10)	MNF	pNF68	pNF160	pNF200	RV202	pVim
GLC-1	-	-	-	-	-	-	-	-	-	-	-	+	+
GLC-1-M13	+	-	+/-	+/-	-	-	-	-	-	-	-	-	-
SCLC-16HC	+/-	-	+/-	+/-	-	-	-	-	dot-like	-	-	+/-	+/-
SCLC-16HV	-	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
NCI-N417	-	-	dot-like	-	-	-	-	-	-	-	-	+/-	+/-
NCI-N417-A1	++	-	++	++	-	-	-	-	-	-	-	++	++
NCI-N417-A2	-	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
NL-SCLC3p1	-	-	-	-	-	-	-	-	-	-	-	-	-
NL-SCLC3p10	-	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
NCI-H524	-	-	-	-	-	-	-	-	++	++	-	+/-	+/-
NCI-H524-A	+/-	-	+/-	-	-	-	-	-	+/-	+/-	-	+/-	+/-
NCI-H82	-	-	-	-	-	-	-	++	++	++	++	+/-	+/-
NCI-H82-A	++	-	++	-	-	-	-	+/-	+/-	+/-	+/-	+	+

++   = strong reactivity in all cells
+   = reactivity in more than 90% of all cells
+/-   = reactivity in part of the cells (10-90%)
some c.   = reactivity in less than 10% of all cells
dot-like = a dot-like reaction in a small part of the cells
-      = no reaction in any cells

*(5+8) = CK polypeptide(s) recognized according to the catalog of Moll et al. (1982)
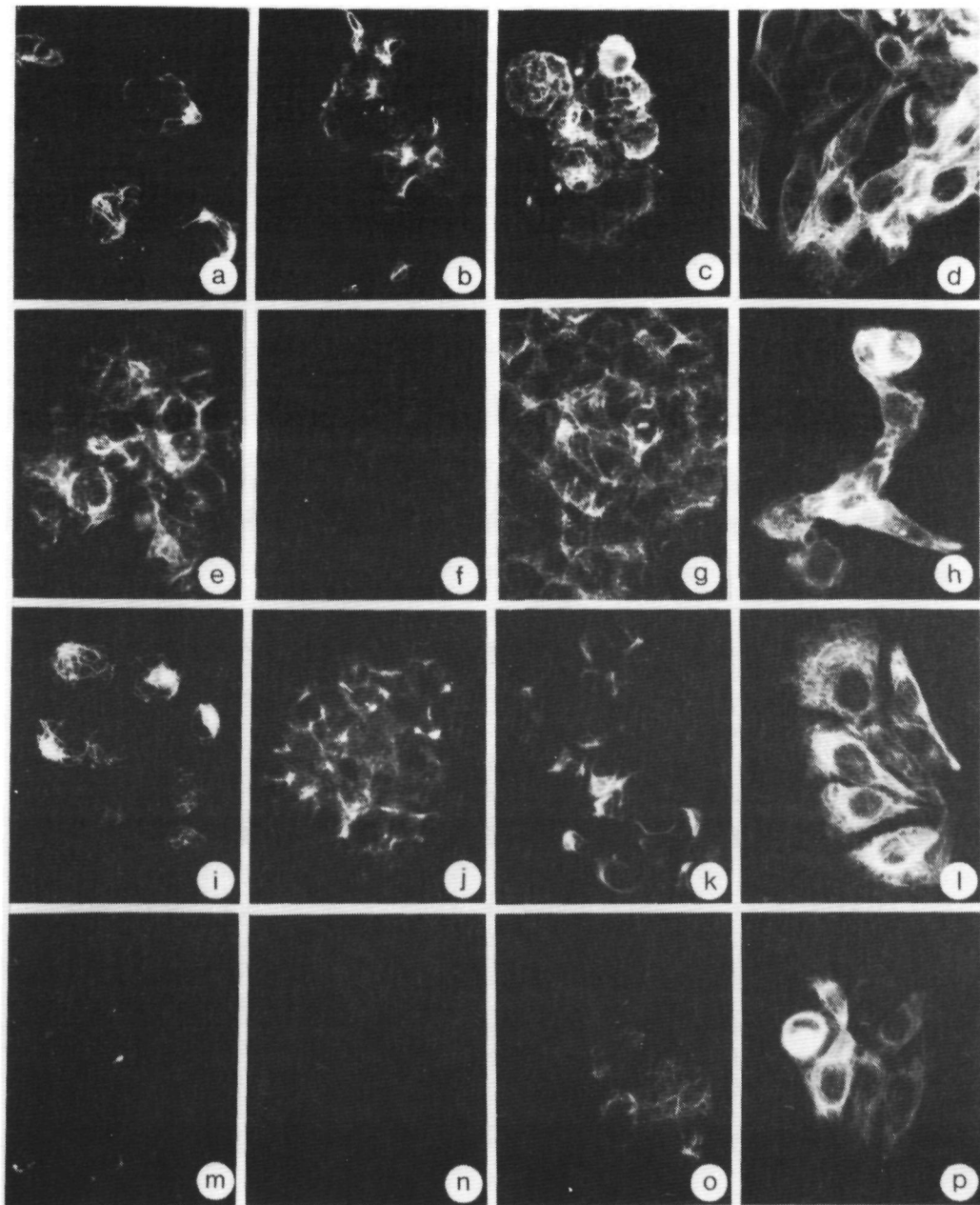
Expression of cytokeratins (CKs) in classic small cell lung cancer (SCLC) cell line GLC1-M13 (A,E,I, M), transitional SCLC cell line SCLC-24H (B, F, J, N), classic SCLC cell line NCI-H449 (C, G, K, O), and in adenocarcinoma (AC) cell line NCI-H125 (D, H, L, P) as monitored by (chain-specific) monoclonal antibodies in the indirect immunofluorescence technique. A strong filamentous reaction pattern is seen with the broadly cross-reacting antibody RCK102 in all classic and most transitional SCLC cell lines (A, x460; B, x400; C, x460), and in all AC cell lines (D, x300). A variable reaction pattern is seen in SCLC cell lines using LP2K, specific for CK 19, showing a clearly filamentous staining reaction in the classic SCLC cell lines (E, x360; G, x410), while no reaction is seen in the transitional SCLC cell line (F, x460). A strong reaction is seen with LP2K in the AC cell line (H, x300). All CK positive SCLC cell lines contained CK 18 as demonstrated with RCK106 (I, x460; J, x410; K, x460), while also the AC cell line showed a strong reaction with this antibody (L, x300). In SCLC cell lines no reaction was seen with the CK 7 antibody RCK105 (M, x460; N, x410), except for cell line NCI-H449, in which part of the cells showed a positive reaction (O, x460). Most cells of the AC cell line showed a strong staining reaction with RCK 105 (P, x300).

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the CK 18 antibody RCK106 alone showed the presence of large amounts of CK 18 in the AC cell line (A, lane 3) as compared to a weak reaction in the two SCLC cell lines (B, lanes 5 and 6). When applying the CK 7 antibody on these blots, an additional band was seen in the AC cell line (A, lane 4) but not in the SCLC cell lines (B, lanes 7,8). Probably owing to breakdown of CK polypeptides, a phenomenon often occurring as a result of (limited) proteolysis by an endogenous protease (see also Schiller & Franke, 1983), additional bands can be seen, which are denoted by arrows. In immunoblotting studies of variant SCLC cell lines no CKs could be demonstrated (results not shown).

COEXPRESSION OF CYTOKERATINS AND NEUROFILAMENTS
Cell line GLC-A1 was derived from an AC of the lung. In contrast to other AC cell lines (see Table 2), very few of its cells react with the CK 7 antibody, and many cells do not seem to contain any CKs at all. Fig. 3A shows that some cells of GLC-A1 react with RCK102, while others do not. In addition, an expression of NF proteins can be seen in part of the tumour cells (Fig. 3B). Quantification of labelled cells showed a reaction with RCK102 in about 7% of all cells, and a reaction with the pNF160 antibody in about 30%. Approximately 5% of all cells showed a coexpression of CKs and NFs, i.e. reacting with RCK102 and with pNF160 in the same cells. Double immunofluorescence staining showed that some cells clearly coexpress CKs and NFs, (compare Fig. 3C and D, E and F), while other cells express only NFs and no CKs (Fig. 3C, D). Staining of cell nuclei in the immunolabelled preparations (Fig. 3G) showed that most cells in the GLC-A1 culture do not express IFPs of the CK or NF type.

GLC-5 was initiated from a lymph node metastasis of a SCLC expressing NFs next to CKs (de Leij et al. 1986). In early passages of this cell line only NFs were found, while in later passages a coexpression of CKs and NFs was seen again. Subcloning of GLC-5 did not result in a separation of CK or NF positive cells, but after some time each cloned population contained cells positive for NFs and few cells positive for CKs. Cells
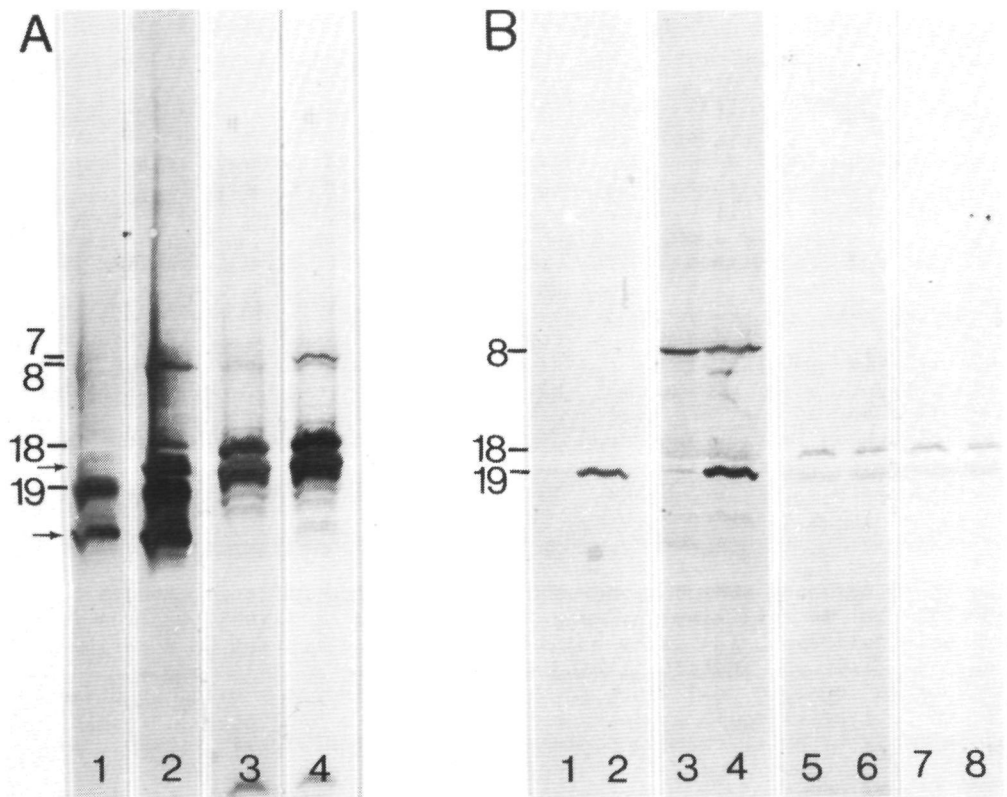
FIGURE 2

One-dimensional immunoblotting assays using different chain-specific CK antibodies on cytoskeleton preparations from an AC cell line (NCI-H125; A), and from the two classic SCLC cell lines NL-SCLC2 (B, lanes 1, 3, 5, 7) and NCI-H69 (B, lanes 2, 4, 6, 8). On the basis of Coomassie Brillant Blue staining patterns, comparable amounts of cytoskeletal proteins from each sample were applied. Immunoblotting with the antibody LP2K gave a band at the CK 19 level in the AC cell line (A, lane 1), and in the SCLC cell line NCI-H69 (B, lane 2), Subsequent incubation of the same immunoblots with a CK 18 antibody RCK106 and with RCK102, recognizing CKs 5 and 8, resulted in the appearance of bands at the level of CKs 18 and 8, respectively, in the AC cell line (A, lane 2), whereas in the SCLC cell lines a relatively strong band was detected in both cell lines at the CK 8 level. A much weaker reaction was seen at the CK 18 level (B, lanes 3 and 4). Incubation with RCK106 alone resulted in a strong reaction in the AC cell line (A, lane 3) and a weak, but evident reaction in the two SCLC cell lines at the CK 18 level (B, lanes 5 and 6). A subsequent incubation of these immunoblots with the antibody RCK105 gave an additional band at the CK 7 level in the AC cell line (A, lane 4), but not in the classic SCLC cell lines (B, lanes 7 and 8). Note the prominent bands in the AC cell line (denoted by arrows) representing breakdown products of CKs 18 and 19.

expressing CKs had the morphological appearance of classic SCLC cells, i.e. growing in very tight clusters of floating cells, while most of the cells expressing NFs grew as loose clusters, which tended to attach to the surface of culturing flasks.

TRANSITIONS OF IFP PATTERNS WITHIN SCLC CELL LINES

Subcloning of the variant SCLC cell line GLC-1 resulted in cell line GLC1-M13, having biochemically classic SCLC properties (de Leij et al. 1985) and expressing CKs in nearly all tumour cells (Fig. 1). The variant SCLC cell line GLC-1, however, does not express any CKs (Broers et al. 1985; de Leij et al. 1985; Tables 2, 3).

One of the SCLC cell lines, i.e. SCLC-16H, was initially characterized as a classic SCLC cell line (Bepler et al. 1987a,b) and later reclassified as transitional SCLC cell line (Bepler et al. 1988a). After about one year of culturing (approximately 65 passages) this cell line had lost its transitional SCLC morphology and expression of L-Dopa decarboxylase activity (Bepler et al. 1987b). An early passage of this cell line (SCLC-16HCp39, Table 3), with typical transitional SCLC properties, showed the expression of CKs in most cells (Fig. 4A) and the absence of NFs (Fig. 4B). Some cells, however, showed a dot-like reaction pattern with the pNF68 antibody (Fig. 4C). A late passage of this cell line (SCLC-16HVp93), which was biochemically characterized as a variant SCLC cell line (Bepler et al. 1987b) did not express any CKs (Fig. 4D), but showed the presence of NFs in about 10% of the cells (Fig. 4E).

Cell line NCI-N417 did not seem to express CKs or NFs when introduced in our laboratory (Fig. 5A,B; see also Broers et al. 1985, 1986). However, when grown for more than 6 months in the same culture flask with regular changes of the culture medium, a subset of cells arose (NCI-N417-A1), that grew firmly attached to the plastic. These cells appeared to express CKs (Fig. 5D,E), while some of the remaining floating cells of NCI-N417 showed a dot-like reaction with the CK 18 antibody RCK106 (Fig. 5C), but not with other CK antibodies. After several passages the formerly adhering cells were no longer attached, and the CK-positive subset of cells was lost. After another 6 months again some cells attached to the surface of the culture flasks (NCI-N417-A2). Both the floating and the attached cells now revealed a positive staining reaction with NF antibodies in about 10% of all cells (Fig. 5F,G).

A comparable switch in IFP expression was noticed in cell line NL-SCLC3. In frozen sections of the SCLC from which this cell line was derived, no CKs or NFs could be detected in the tumour cells. The same holds true for the cell line up to passage 7. However, at passage 10 a small subset of cells was found, that expressed NF proteins. With each subsequent passage the percentage of cells positive for NFs increased to about 50% at passage 20.

Another type of change in IFP expression was seen in the variant SCLC cell lines NCI-H524 (Fig. 6) and NCI-H82 (Fig. 7). These cell lines normally grow as loosely packed floating aggregates, which do not attach to the bottom of culturing flasks, and express NFs in all cells (Figs. 6D, 7A). CKs can not be detected in these floating cells (Figs. 6E, 7B). After a prolonged period of growing in the same culture flask, however, an
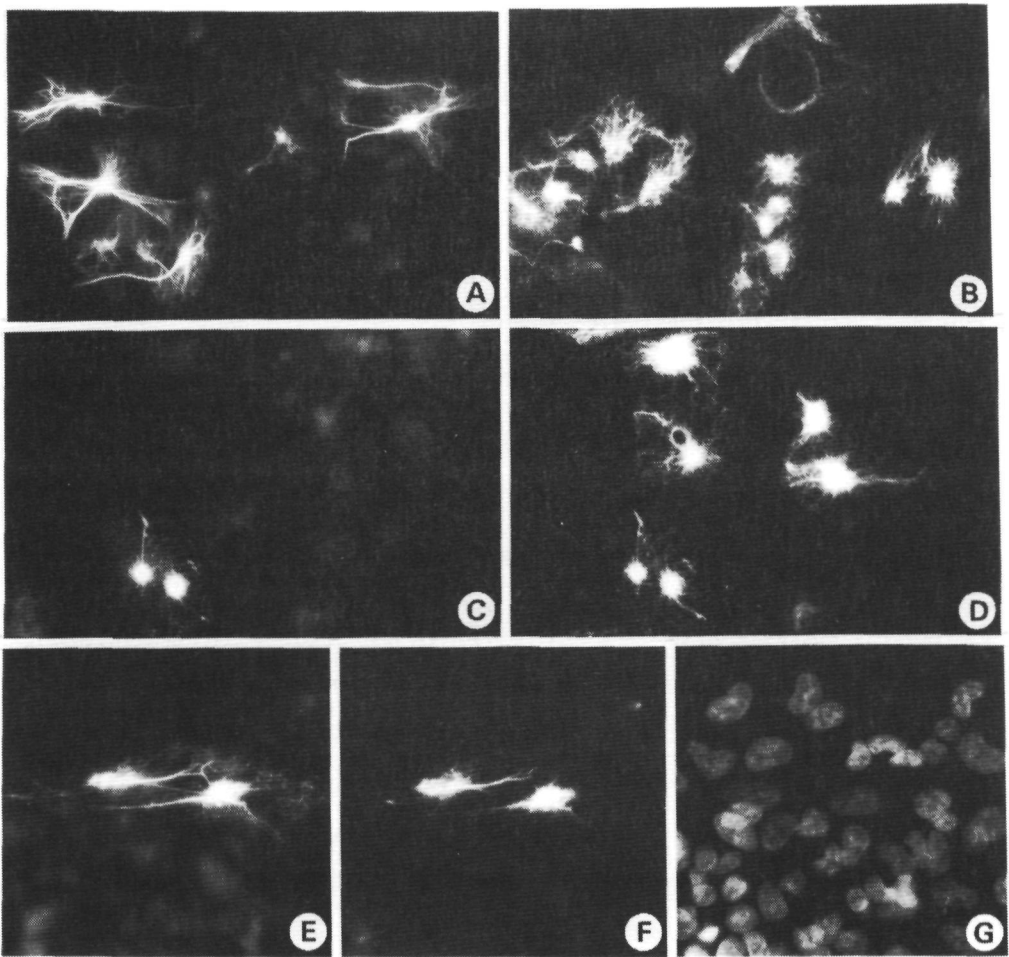
FIGURE 3.
Immunofluorescence staining patterns of cell line GLC–A1 derived from an AC of the lung. Part of the cells react strongly with RCK102 (A, x385), while also a strong reactivity is seen with the pNF160 antibody (B, x450). Double-label immunofluorescence studies (C-F) show that cells that are reactive with RCK102 (C, asterisk; x450) are also stained by the pNF160 antibody (D, x450). In addition, some cells that do not react with RCK102 are positive with the pNF160 antibody (D).
Double-label immunofluorescence in combination with nuclear DNA staining with Hoechst (E, F, G) shows that cells reactive with RCK106 (E, x350), may also react with the pNF 160 antibody (F, x350), while the distribution of fluorescence suggest colocalization of the two filament systems. The nuclear DNA staining (G, x350), however, shows that most cells of this sample do not contain CKs or NFs.

increasing number of cells attached to the bottom (see e.g. Fig. 6A,B,C). Immunofluorescence studies showed that these adhering cells not only lost their NF expression in part of the cells, but also that some of these cells initiated the (co-)expression of CKs (Fig. 6F,G and 7C,D). Double immunofluoresence studies showed that in NCI-H524 some cells coexpress NFs and CKs, while other cells express only NFs (cf. Fig. 6H and I). The same phenomenon can be observed in NCI-H82, where some cells express CKs (Fig. 7E and H) and other cells express NFs (Figs. 7F and I), while occasionally a clear coexpression of CKs and NFs can be observed (cf. Fig. 7H and I, asterisk). Counterstaining of the nuclear DNA with Hoechst shows that most cells in NCI-H82 express either CKs or NFs (Fig. 7G and J).
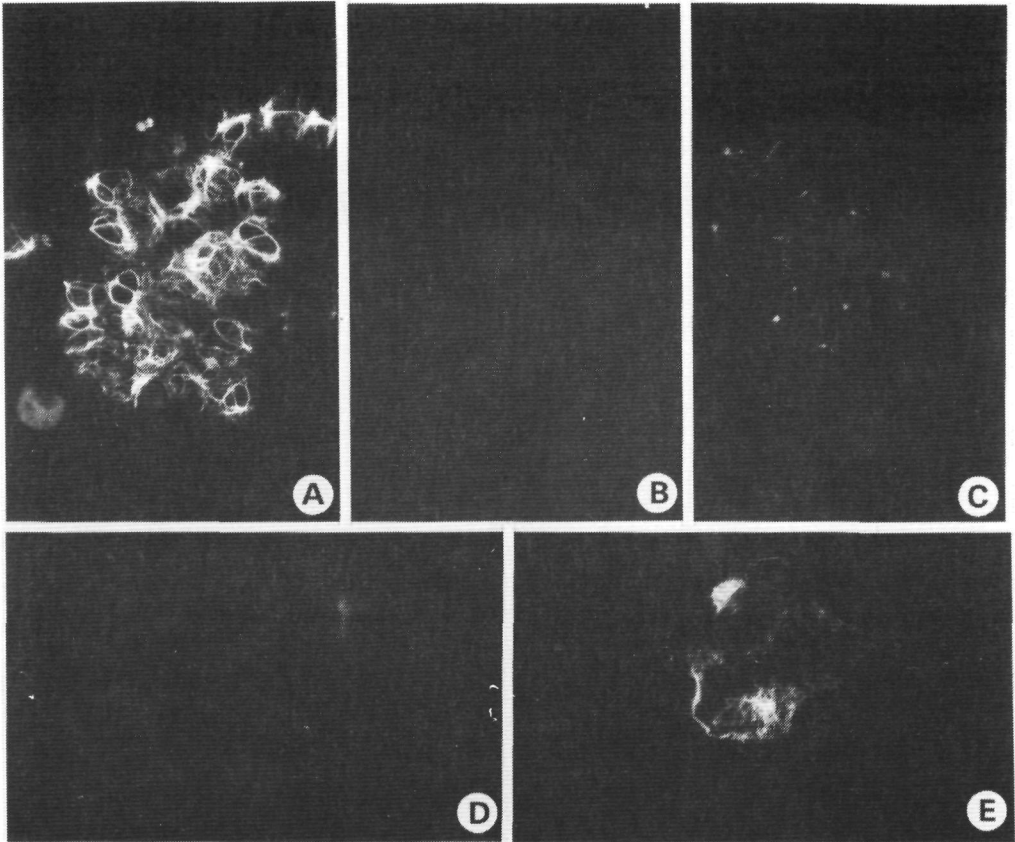
FIGURE 4
Immunofluorescence staining patterns of SCLC-16H at an early passage number (p39; A, B, C) and at a late passage number (p93; D, E). During early passages most cells reacted strongly with the CK antibody RCK102 (A, x450). Most cells did not react with the pNF68 antibody (B, x475), while in some cells a dot-like reaction pattern with this antibody was seen (C, x450). In the late passages all cells have lost CK expression (D, RCK102; x450), while in about 10% of all cells a reaction with NF antibodies was found (E, pNF200; x550).
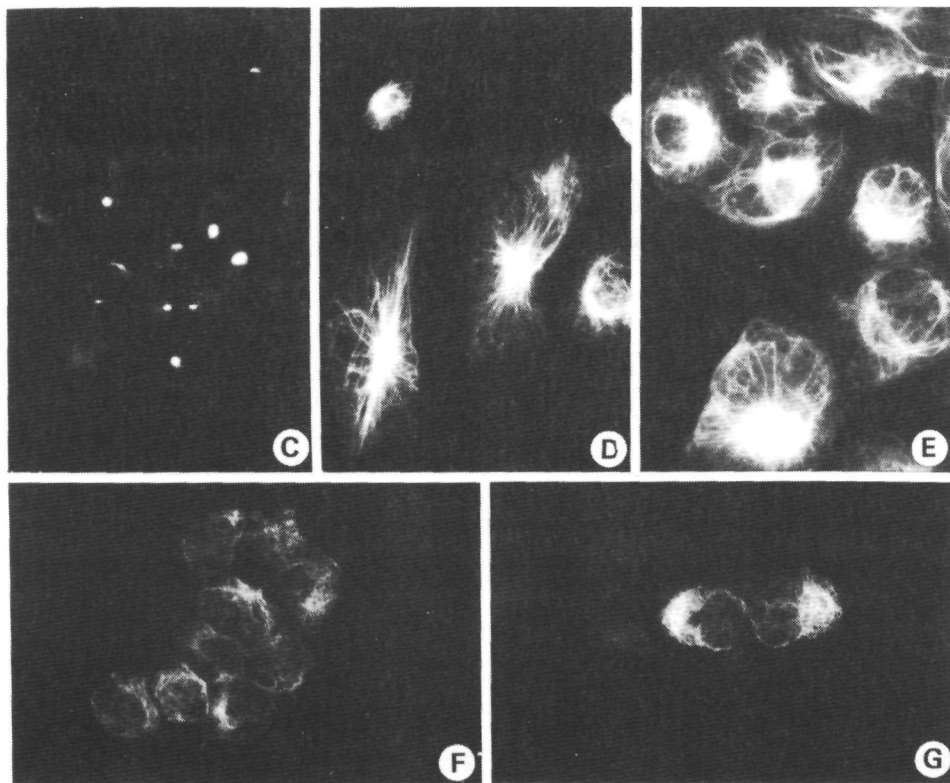
FIGURE 5.
Immunofluorescence staining patterns of NCI-N417 with (apparently) three different types of differentiation. Initially, this cell line did not express CKs, showing no reaction with RGE53 (A, x360). Also no NFs were detectable with pNF 200 (B, x175). After prolonged culturing some cells started to attach to the culturing flask (C, D, E). At this stage some floating cells started to express CKs, a reaction with RCK106, in a dot-like manner (C, x475). Cells that grew firmly adherent all contained CKs, reacting with RCK106 (D, x 410) and with RCK102 (E, x435). Upon culture, CK positive cells were lost, while some cells started to express NFs, which were reactive with the pNF68 antibody (F, x475) and with the pNF200 antibody (G, x475).

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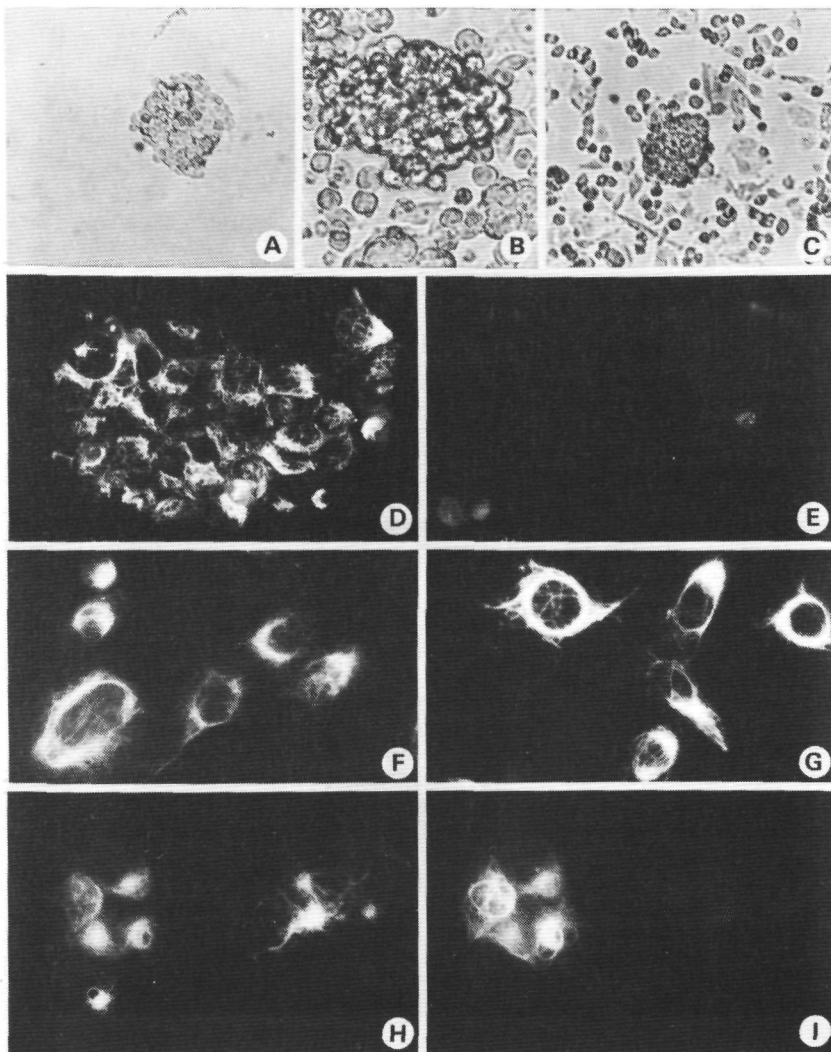
FIGURE 6.
Phase-contrast (A-C) and immunofluorescence (D-G) micrographs of the variant SCLC cell line NCI-H524. At first, this cell line grew as floating aggregates (A, x105) with cells expressing NFs, showing a reaction in all cells with, for example, the pNF160 antibody (D, x475). No cells could be detected to be positive for CKs (E, RCK106; x360). Upon culturing, an increasing number of cells started to grow adherent (B, x235; C, x105). These adherent cells clearly expressed CKs, showing a reaction with RCK102 (F, x435) and with RCK106 (G, x435). Double-label immunofluorescence experiments (H, I) show that some cells that were reactive with the pNF160 antibody (H, x475) also reacted with RCK102 (G, x475).
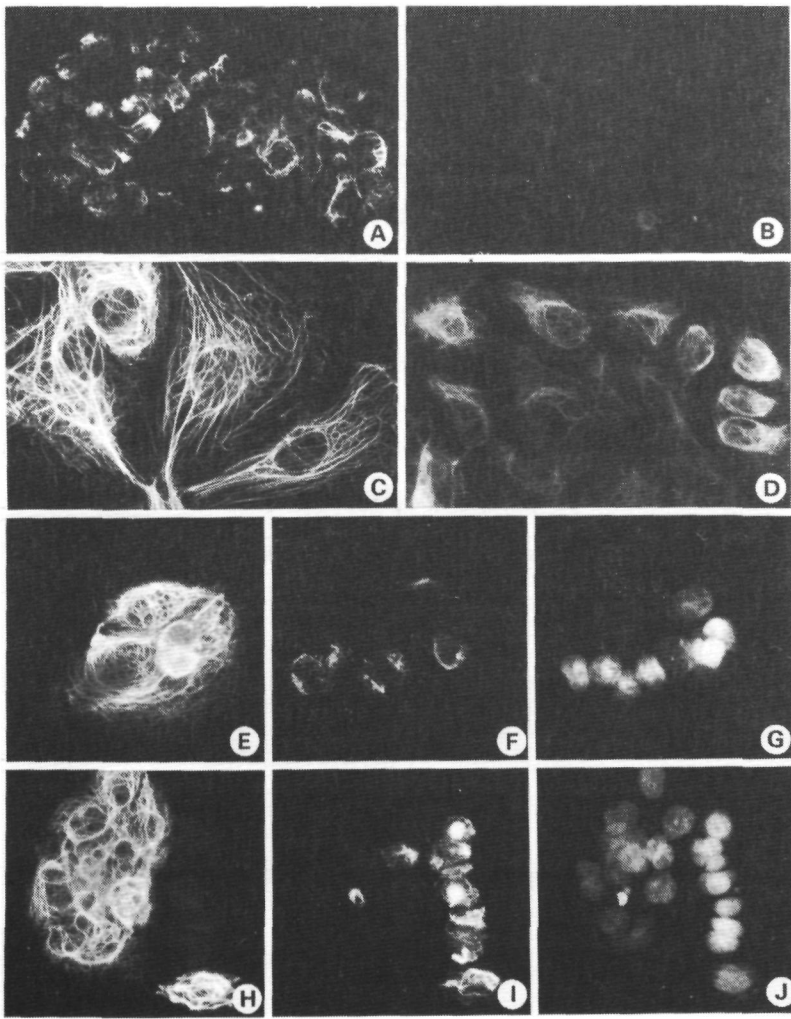
FIGURE 7.
Immunofluorescence staining patterns of the variant SCLC cell line NCI-H82.
This cell line, growing as floating aggregates, expressed NFs, (A, pNF160;
x310) and did not express CKs (B, RCK106; x420). However, after prolonged
culturing some cells started to grow adherent, and selection of these cells
resulted in a cell culture that was clearly positive with different CK
antibodies, such as RCK102 (C, x460) and RCK106 (D, x410). Double-label
immunofluorescence studies (compare E, F, G, with H, I, J) showed that
within this cell culture some cells expressed CKs, reactive with RCK102 (E,
x410; H, x345), while other cells were reactive with the pNF200 antibody
(F, x410) or with the pNF68 antibody (I, x345). Nuclear DNA staining (G,
x410; J, x345) showed that most cells were reactive with either CK or NF
antibodies, while in one cell probably a coexpression of CKs and NFs was
seen (compare H, I, J; asterisk).

Our immunofluorescence data were confirmed by immunoblotting experiments using RCK106 as a marker for CK expression and pNF68 as a NF marker (Fig. 8). A shows two weakly stained protein bands at the level of CK 18 (lane 1) and the somewhat stronger reaction with the 68 kD NF protein (lane 2) in GLC-A1, and in B the early passage (p39) of SCLC-16HC was compared with a late passage (p93) of this cell line (SCLC-16HV). Lane 1 shows the presence of CK 18 in SCLC-16HC, while SCLC-16HV (lane 2) does not show a reaction with RCK106. Subsequent incubation with the pNF 68 antibody (lanes 3, 4) does not result in extra bands, probably owing to the low number of cells expressing NFs in SCLC-16HV. Fig. 8C shows the presence of CK· 18 (lane 1) and of the 68 kD NF protein (lane 2) in the subset of cells from NCI-H524 that grows as an attached monolayer. Fig. 8D shows the differences between CK and NF expression in the floating and the attached sublines of NCI-H82. Incubation with the CK 18 antibody shows the presence of this protein in the adherent cells (lane 2) and no reaction in the floating cells (lane 1). Subsequent incubation with the NF antibody shows that both cell types express the 68 kD NF protein (lanes 3, 4).

MONITORING OF CROSS-CONTAMINATION.

To detect whether changes within cell lines might be due to possible cross-contamination with other cell lines simultaneously growing in our laboratory, flow cytometric analyses were performed at regular intervals. As an example, Fig. 9 shows the flow cytometric analysis of the floating fraction of NCI-H524, as compared with the adherent growing fraction of this cell line. No significant shift in DNA-index (DI), nor extra DNA peaks, indicating a possible contaminating cell line, were observed (cf. Fig. 9A, B). The DI value of both cell lines varied between 1.10 and 1.23. In addition, the attached fraction was labelled with the CK antibody RCK102 and analysed in a two-dimensional fashion, in which DNA was stained with propidium iodide. The results show that about 15% of the adherent cells contained CKs (Fig. 9C). Floating cells did not contain CKs (not shown).

The DI values of the two adherent growing variant SCLC cell lines (NCI-H82, DI=1.20-1.22; NCI-H524, DI=1.10-1.23) were compared with those for other adherent growing lung cancer cell lines such as EPLC-32M1 (DI=1.50) EPLC-65M2 (DI=1.55), LCLC-103 (DI=3.27) NCI-H23 (DI=1.60), NL-AC1 (DI=1.52), and non-lung cancer epithelial cell lines growing in our laboratory, such as T24 (DI=1.57), and HeLa (DI=1.48). Since the DI values for the G0/G1 cells of these cell lines are all significantly higher than 1.23, a cross-contamination with these cell lines can be excluded. In addition, the CK and NF expression patterns of the adherent growing variant SCLC cell lines appeared to be unique for these two cell lines.

FIGURE 8

One dimensional immunoblotting of different lung cancer cell lines. (A). Cell line GLC-A1 shows a faint (double) band at the 45 kD level with the CK 18 antibody RCK106 (lane 1). This weak reaction can be explained by the fact that CKs were detected in only 7% of all cells in immunofluorescence experiments. After subsequent incubation of the same immunoblot with the pNF68 antibody a stronger reaction is seen at the 68 kD level (lane 2).

(Panel B) Early passage of SCLC-16HC (passage 39; lanes 1 and 3) compared with a late passage (SCLC-16HV, passage 93; lanes 2 and 4) of this cell line. SCLC-16HC shows a clear reaction with RCK106 (lane 1), while no reaction is seen with RCK106 in SCLC-16HV (lane 2). Incubation of the same blot with pNF68 did not result in an additional band at the 68 KD level in either cell line, confirming the immunofluorescence data of SCLC-16HC with this antibody. A discrepancy is seen with the positive immunofluorescence reaction with pNF68 in SCLC-16HV, which might be explained by the low number of cells reacting with this antibody.

(C). Adherent growing culture of the variant SCLC cell line NCI-H524, incubated with RCK 106 (lane 1) and subsequently incubated with pNF68 (lane 2). Note the presence of the CK 18 band and the 68 kDa NF band in this cell line.

(D). Comparison of the floating (lanes 1, 3) and the adherent growing cells (lanes 2, 4) of the variant SCLC cell line H82. Incubation with RCK106 results in a reaction at the 45 kD level with the adherent growing cells only (compare lanes 1, 2), while subsequent incubation with pNF68 shows that both cell types contain the 68 KD NF polypeptide.
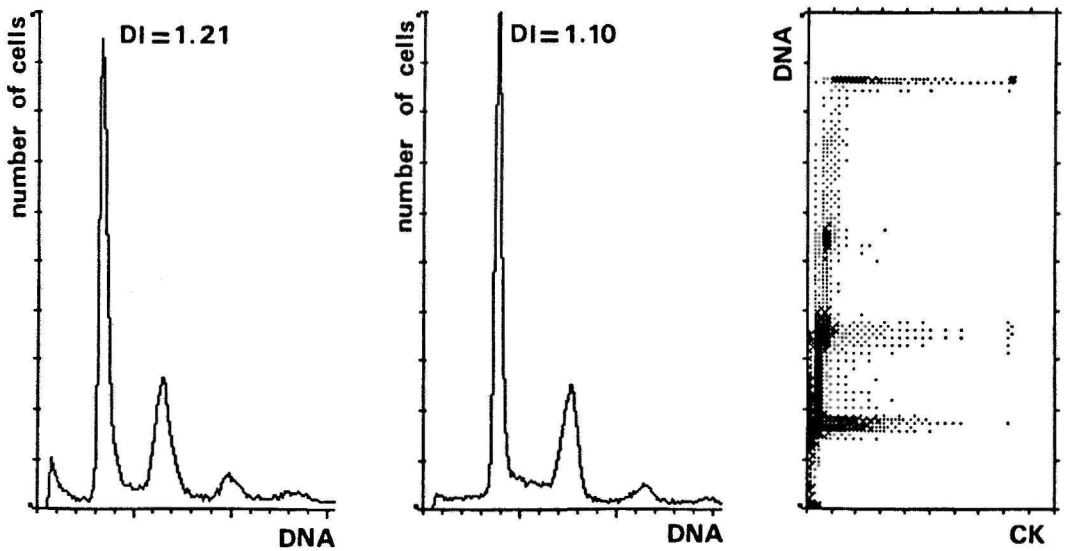
FIGURE 9.
Comparison of flow cytometric analyses of floating (A) and adherent growing
(B,C) cells of the variant SCLC cell line NCI-H524. The DNA indices (DI)
found for both cell types did not differ significantly
(C) Two-parameter flow cytometric analysis of cells from (B) separated
according to their cytokeratin (CK) content, as determined with RCK102
(labelled with rabbit anti-mouse FITC), and DNA content as determined by
labelling with propidium iodide. It was calculated that about 15% of the
adherent cells of NCI-H524 were positive with RCK102.

DISCUSSION

In this paper we describe changes in IFP expression in lung cancer
cell lines during long term culture. We have especially focussed on CKs
and NFs, since it is known that classic and variant SCLC cell lines exhibit
different expression patterns of these IFPs (Broers et al. 1985, 1986).

In the last decade lung carcinoma cell lines that have retained many
characteristics of the in vivo cancers have been established (Gazdar et al.
1981, 1985; Bergh et al. 1984; Carney et al. 1984, 1985a,b; Bepler et al.
1987a,b,1988b). Most of these cell lines form tumours when injected into
athymic nude mice with a histology similar to that of the original
malignancy. Banks-Schlegel et al. (1985) stated that cell lines derived
from different types of lung tumours continue to maintain a program of CK
expression reflective of the native tumour.

CYTOKERATINS AND NEUROFILAMENTS IN LUNG CANCER CELL LINES.

Our results on the IFP expression patterns in lung cancer cell lines are partly in accord with the findings in solid lung carcinomas with homogeneous morphology (Broers et al. 1988). In general, one can state that classic and transitional SCLCs and ACs contain CKs typical of simple epithelia, i.e. CKs 8, 18 and sometimes 19. In addition to these CKs, AC cell lines are characterized by the presence of CK 7, which is absent in SCLC. Variant SCLC cell lines in general do not contain CKs but may express NFs (see also Broers et al. 1985, 1986). Some exceptions, however, were seen.

1. Two cell lines derived from SQCs (EPLC-32M1 and EPLC-65M2) did not express the typical SQC CKs, i.e. CKs 10 and 13, but contained CKs 7, 8 and 18, occurring in simple epithelia, in this way resembling poorly differentiated SQC in their expression pattern (see also Broers et al. 1988). These changes are in accord with histological findings in mouse xenografts, in which these tumours were classified as undifferentiated large cell carcinomas (Bepler et al. 1988b). Furthermore, Blobel et al. (1984) have already shown that other cell lines derived from SQC mainly express CKs 7, 8 and 18.

2. On the other hand, a cell line derived from an AC (GLC-A2) did express CKs 10 and 13, characteristic of SQC as well as CKs 7, 8 and 18, characteristic of ACs.

3. Another cell line derived from an AC (GLC-A1) expressed NFs next to CKs, while CK 7, characteristic of ACs of the lung (see also Broers et al. 1988), was present in only a few cells of this culture. Therefore, on the basis of IFP expression patterns, this cell line resembles variant SCLC cell lines rather than AC cell lines.

4. One of the SCLC cell lines (GLC-3) exhibited SQC differentiation as concluded from its IFP expression pattern. Interestingly, this cell line was previously shown to express AC differentiation characteristics at the EM level (de Leij et al. 1985).

These data clearly demonstrate heterogeneity within some cell lines in both degree and type of differentiation. However, results from most other cell lines show that cultured SCLC lines may serve as suitable models with which to study the biology of lung carcinoma subtypes (see also Gazdar & Minna, 1986).

TRANSITIONS IN IFP EXPRESSION PATTERNS.

In earlier studies, changes in growth pattern were observed during prolonged culturing of lung cancer cell lines. For instance, progression of classic to variant SCLC cell lines has been reported by several authors (Gazdar et al. 1981, 1985; Carney et al. 1985b; Graziano et al. 1987; Bepler et al. 1987b,c). Since classic SCLCs express CKs, while variant SCLCs may express NFs (Broers et al. 1985, 1986), we wondered whether such a progression would be accompanied by a change or loss of IFPs. In one of our cell lines (SCLC-16H), showing a progression towards variant SCLC, a transition from CK to NF expression was indeed seen.

Two cell lines (NL-SCLC3 and NCI-N417) which at first did not express CKs or NFs, initiated the expression of NFs upon prolonged culturing, suggesting that the absence of CKs and NFs in some SCLC cell lines as well as in some solid SCLC tumour parts (see also Broers et al. 1987) may be a

temporary event occurring in some SCLC with a type of differentiation between classic and variant, the so-called transitional type of SCLC (Bepler et al. 1987c). In vitro the transitional cell type, in contrast to the classic cell type, expresses vimentin. Cytokeratin expression is found in varying degrees, and neurofilaments are absent or barely demonstrable. It remains to be determined to what extent the transitional type of SCLC can be compared with the so-called biochemical variant type of SCLC as defined by Gazdar et al. (1985). Examination of IFP expression patterns in these biochemical variant SCLC cell lines might elucidate this issue.

Another conversion that seems to occur within our cell lines is that from variant SCLC to non-SCLC differentiation, as described by Gazdar et al. (1981). In vitro non-SCLC cell lines, which do not express the neuroendocrine-related biomarkers present in SCLC (Carney et al. 1985a,b; Gazdar et al. 1985), grow as monolayers, firmly attached to the surface of culturing flasks. By selecting cells within the variant SCLC cell lines that tended to attach, we were able to grow sublines consisting of predominantly attached cells. Interestingly, these cells lose the expression of the neuroendocrine marker MOC-1 (results not shown), and start to express CKs next to NFs, resulting in a coexpression of CKs and NFs within these cell lines or within individual cells. Whether these cells lose the expression of other biomarkers, characteristic of (variant) SCLC, remains to be examined.

As far as the implications of these findings can be extrapolated to patient tumours it is important to note that there is growing evidence of heterogeneity of lung carcinomas. This heterogeneity can apparently also be found in (some) lung cancer cell lines and, even more interestingly, in cloned cell lines. Progression from classic to variant SCLC has been reported in vivo (Abeloff et al. 1979), especially after chemotherapy and/or irradiation of patients. Also conversions from SCLC to non-SCLC (Abeloff et al. 1979; Sehested et al. 1986) are often seen. In general, we can state that conversions from one cell type to another within cell lines are accompanied by a change in IFP expression patterns. The conversions observed in the cell lines closely resemble those occurring in vivo. Therefore, our in vitro findings strongly support the hypothesis that different types of lung carcinomas have a common origin (see also Gazdar et al. 1981; McDowell & Trump, 1983) and as a result heterogeneity in lung tumour types as well as conversions from one tumour type to another are likely to occur as a result of (trans)differentiation rather than derivation (Gould, 1986).

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SUMMARY AND CONCLUSIONS

In this thesis the expression of intermediate filament proteins in lung tissues, lung carcinomas and lung cancer cell lines has been examined. The picture of the expression of the different types of filaments in these tissues was hitherto rather incomplete and often contradictory.

In chapter two we describe the expression of intermediate filament proteins in fetal and adult human lung tissues. In early stages of development only so-called simple cytokeratins are present in bronchial epithelial cells. With increasing age, the cytokeratin expression pattern becomes more complex. In adult lung a heterogenous cytokeratin expression pattern is observed, showing a large diversity within histologically similar epithelium. Vimentin is present in all mesenchymal tissues. In addition, however, in fetal lung expression of vimentin is seen in developing bronchial epithelium, which decreases with age. In adult bronchus a staining reaction with scattered columnar and basal cells was seen with the vimentin antibodies. Desmin filaments appear to alter their antigenic determinants upon maturation. In early stages of development smooth muscle cells of blood vessels are reactive with cytokeratin and desmin antibodies. With increasing age the reactivity of the cytokeratin antibodies decreases, while the reactivity with desmin antibodies increases.

In chapter three the occurrence of cytokeratins in small cell lung cancer and in lung carcinoids is described. A previous investigation had demonstrated the presence of neurofilaments and the absence of cytokeratins in these tumors. Our results clearly indicate that the inability to demonstrate the presence of cytokeratins was a matter of fixation problems or lack of cross-reactivity of the antibodies used. Compared with other types of lung carcinomas, however, the amounts of cytokeratins, present in small cell lung cancer are low.

In chapter four we report on the expression of cytokeratins, neurofilaments, and a neuroendocrine differentiation antigen in an extended series of freshly frozen, as well as paraffin-embedded cases of lung cancer. A profound heterogeneity within most tumors is seen, as demonstrated a) by the presence of the neuroendocrine differentiation marker in many non-neuroendocrine tumors, b) the presence of neurofilaments additional to cytokeratins in several neuroendocrine and non-neuroendocrine tumors, and c) the reactivity of a cytokeratin antibody, characteristic for columnar epithelia, in squamous cell carcinomas.

In chapter five a number of cases from the series of tumors, investigated in chapter four were selected. We have examined the cytokeratin polypeptide expression patterns by immunoperoxidase as well as immunoblotting techniques, using chain-specific monoclonal cytokeratin antibodies, which recently have become available. On basis of cytokeratin expression patterns alone, a distinction between the main subtypes of lung carcinomas can be made. Meanwhile, however, again the profound heterogeneity in expression patterns within most tumors is striking.

Chapter six describes the expression patterns of two different types of small cell lung cancer (SCLC) cell lines, i.e. the classic type and the variant type of SCLC. Since in general cell lines derived from a certain

type of tumor retain a number of biological and biochemical properties, characteristic for the original tumor, cell lines are a powerful tool to study the biology of a certain tumor subtype. Our results demonstrate differences in the expression of intermediate filament proteins between the two types of cell lines. Classic SCLC cell lines express only cytokeratins, while variant SCLC cell lines do not express cytokeratins, but occasionally express neurofilaments. These findings partly explain why some investigators could detect neurofilaments in SCLC, while others could find only cytokeratins.

In chapter seven we have extended our examinations on some of the cell lines, investigated in chapter six, to determine their exact cytokeratin and neurofilament profile. For this purpose we have used a large panel of intermediate filament protein antibodies in immunocytochemical techniques. The immunocytochemical results were confirmed using one- and two-dimensional gel electrophoresis and one- and two-dimensional immunoblotting assays. Using these methods the absence of neurofilaments and presence of cytokeratins in classic, and the absence of cytokeratins and presence of neurofilaments in variant SCLC, was biochemically and immunochemically substantiated.

In chapter eight we have examined the intermediate filament protein expression patterns in a series of twenty-eight lung carcinoma cell lines, obtained from four different institutes. We have paid special attention to classic, variant, and so-called transitional SCLC cell lines. These transitional SCLC cell lines have characteristics between the classic and variant type of SCLC. During long-term culture in some cell lines changes in morphology and in intermediate filament expression patterns can be observed. Changes from classic to variant SCLC, possibly via transitional SCLC, and from variant SCLC towards non-SCLC are seen. During these changes a clear coexpression of cytokeratins and neurofilaments within cell lines and even within individual tumor cells is noticed.

The composition of an intermediate filament expression profile of lung and lung cancer goes beyond a merely descriptive investigation on the expression of a group of proteins of which the function in not even known. The investigations described in this thesis have proven to be of help in lung cancer diagnosis. For instance, histologic squamous cell carcinomas showing reaction with the antibody to cytokeratin 7 are diagnosed as adenosquamous carcinoma. Furthermore, antibodies indicating the degree of differentiation can be of help, since patients with well-differentiated carcinomas have a better prognosis than patients with poorly differentiated lung tumors. The presence of neuroendocrine differentiation markers in non-SCLC can indicate the presence of a small cell component within such cancers, which changes diagnosis and as a result the treatment regimen. Within most carcinomas certain tumor cells are sensitive to treatment, while other cells are not. These latter cells, which manage to escape from treatment, cause recurrence of the tumor, in many cases with a different histology as compared to the original tumor. Unfortunately, at this moment the tools to detect lung cancer at an early stage are not refined enough to benefit from the knowledge of the degree and type of differentiation. Therefore, to cope with the leading cancer killer in the Western world, attempts must be made to find new treatment regimens, based on our knowledge of lung cancer heterogeneity and its cell biological behaviour.

SAMENVATTING EN CONCLUSIES

In dit proefschrift werd een onderzoek verricht naar de expressie van intermediaire filament-eiwitten in normale longweefsels en longcarcinomen. Doordat eerdere onderzoeken tot tegenstrijdige conclusies met betrekking tot intermediaire filament-expressie waren gekomen, hebben we getracht deze problemen systematisch op te sporen en uit te bannen.

In hoofdstuk twee beschrijven we de expressie van intermediaire filament-eiwitten in foetaal en adult longweefsel. De vroege stadia van foetale ontwikkeling worden gekenmerkt door een expressie van uitsluitend zo genaamde "simpele" cytokeratines in bronchiaal epitheel. Met toenemende leeftijd wordt het cytokeratine-patroon steeds complexer, resulterend in een zeer heterogene reactie in adulte bronchi. Vimentine is niet alleen aanwezig in mesenchymale, maar ook in epitheliale cellen, vooral in de vroege stadia van ontwikkeling. Met leeftijdstoename neemt deze reactie in epitheliale cellen af. In adulte bronchi blijkt echter dat een aantal epitheliale cellen weer een expressie van vimentine vertonen. De reactiviteit van desmine antilichamen in gladde spiercellen van bloedvaten blijkt te veranderen met leeftijd. In vroege stadia reageert een desmine antilichaam in het geheel niet met deze gladde spiercellen, die in eerste instantie wel positief zijn met cytokeratine antilichamen. Met toenemende leeftijd neemt de reactiviteit van het desmine antilichaam toe, terwijl de reactiviteit van de cytokeratine antilichamen afneemt.

In hoofdstuk drie beschrijven we het voorkomen van cytokeratines in kleincellige longcarcinomen en in long-carcinoïden. Eerdere onderzoeken hadden gesuggereerd dat deze carcinomen neurofilamenten en geen cytokeratines zouden bevatten. Op basis van onze resultaten lijken in ieder geval de negatieve resultaten met de cytokeratine antilichamen op een fixatie-artefact of gebrek aan kruisreactiviteit van de betreffende cytokeratine antilichamen te duiden. Dit lijkt aannemelijk, gezien het feit dat de hoeveelheid cytokeratines, aanwezig in deze tumoren, relatief gering is.

In hoofdstuk vier beschrijven we een onderzoek naar de expressie van cytokeratines, neurofilamenten, en een neuro-endocrien differentiatie-antigeen in een uitgebreide serie van longcarcinomen. Opvallend aspect van dit onderzoek was de enorme heterogeniteit in expressie van deze markers binnen de verschillende typen longkanker. Zo kan in niet-kleincellige longcarcinomen vaak de expressie van neurale of neuro-endocriene componenten aangetoond worden, terwijl een cytokeratine antilichaam, dat normaal kenmerkend is voor cylindrisch epitheel, vaak wordt aangetroffen in plaveiselcelcarcinomen.

In hoofdstuk vijf wordt een geselecteerde groep longcarcinomen verder onderzocht op de expressie van verschillende cytokeratine polypeptiden. Doordat we de beschikking hebben over een panel van keten-specifieke monoclonale antilichamen, kunnen we op cellulair niveau de expressie van deze cytokeratine polypeptiden onderzoeken. Op basis van deze expressie-patronen kunnen de belangrijkste groepen longcarcinomen onderscheiden worden, waarbij de heterogeniteit binnen de verschillende groepen opnieuw zeer opvallend is.

Hoofdstuk zes beschrijft de reactiepatronen van twee groepen cellijnen, de klassiek- en de variant-kleincelligen. Aangezien in het algemeen blijkt dat cellijnen, afkomstig van een bepaald type tumor, een

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groot aantal biologische en biochemische eigenschappen van de oorspronkeljke tumor behouden, zijn deze uitermate geschikt voor onderzoek naar het biologisch gedrag van de verschillende typen tumor. Op basis van de expressie van intermediaire filamenten kan een onderscheid gemaakt worden tussen deze twee groepen. Klassiek-kleincellige cellijnen brengen cytokeratines tot expressie, terwijl variant-kleincellige cellijnen geen cytokeratines, maar in een aantal gevallen neurofilamenten tot expressie brengen. Deze resultaten kunnen voor een deel verklaren waarom eerder door sommige onderzoekers uitsluitend cytokeratines en door anderen uitsluitend neurofilamenten werden gevonden.

In hoofdstuk zeven hebben we ons onderzoek aan een groep cellijnen uitgediept met een groot aantal extra intermediaire filament antisera, om een zo volledig mogelijk beeld van de expressie van deze intermediaire filamenten te verkrijgen. Uit verder onderzoek met behulp van een- en twee-dimensionale gel-electroforese en immunoblotting werden onze eerdere resultaten biochemisch en immunochemisch bevestigd.

In hoofdstuk acht beschrijven we expressie-patronen van intermediaire filamenten in 28 cellijnen, afkomstig van vier verschillende instituten. Onze belangstelling ging hierbij met name uit naar klassiek-, transitionele, en variant-kleincellige cellijnen. Overgangen van klassiek, waarschijnlijk via transitioneel, naar variant kleincellig kunnen waargenomen worden, maar ook overgangen van variant in de richting van niet-kleincellig. Deze veranderingen blijken gepaard te gaan met een verandering in het expressie-patroon van intermediaire filamenten. Tijdens deze veranderingen treedt een co-expressie van cytokeratines en neurofilamenten op, zowel op cellijn-niveau, alsmede binnen individuele cellen.

Het samenstellen van een zo exact mogelijk profiel van de expressie van intermediaire filamenten in longcarcinomen heeft niet een louter beschrijvende betekenis, maar blijkt een goed hulpmiddel te zijn bij het diagnostiseren van longkanker. Bij voorbeeld de reactiviteit van een (histologisch) plaveiselcelcarcinoom met ons cytokeratine 7 antiserum wijzigt de diagnose in adeno-squameus carcinoom. Andere antilichamen kunnen de mate van differentiatie van een tumor nauwkeurig aangeven. Aangezien goed gedifferentieerde carcinomen in het algemeen een betere prognose hebben, is dit van klinische betekenis. De aanwezigheid van neuro-endocriene of neurale antigenen in een niet-kleincellig longcarcinoom duidt op de aanwezigheid van een kleincellige component binnen zo een tumor. Deze wijziging van de diagnose heeft tevens therapeutische gevolgen.

Bij de behandeling van een longcarcinoom reageren vaak uitsluitend cellen met een bepaalde graad of type differentiatie, terwijl andere cellen zich aan deze behandeling kunnen onttrekken. Het gevolg hiervan is, dat na een in een eerste instantie goede respons, zoals deze vaak bij kleincellige carcinomen optreedt bij chemotherapie, een tumor manifest wordt, die niet gevoelig is voor deze behandeling. Deze tumoren hebben vaak een andere morfologie dan de oorspronkelijke. Helaas zijn er op dit moment geen methoden bekend voor de detectie van longcarcinomen in een vroeg stadium, waarbij optimaal gebruik gemaakt kan worden van onze kennis van de heterogeniteit van de longcarcinomen. Voor het bestrijden van longkanker zal derhalve gezocht moeten worden naar totaal nieuwe methodes die tumoren kunnen bestrijden gebaseerd op deze kennis van heterogeniteit en cel-biologisch gedrag van longcarcinomen.

DANKWOORD

CURRICULUM VITAE

CURRICULUM VITAE

Jos Broers werd op 6 januari 1957 geboren in het kerkdorp Eckelrade, dat indertijd door ambtelijke willekeur uit de 19e eeuw voor een gedeelte ressorteerde onder de gemeente Gronsveld.
Na het doorlopen van de lagere school aldaar, volgde hij in de grote stad Maastricht het Gymnasium-B van 1969 tot 1975 aan het Henric van Veldeke College.
In 1975 vertrok hij naar Nijmegen,waar hij het Kandidaatsexamen Biologie behaalde in 1978 aan de Katholieke Universiteit.

Het Doctoraalexamen Biologie behaalde hij in 1982 aan de Katholieke Universiteit te Nijmegen.
Zijn doctoraalstudie bestond uit de volgende vakken:
Hoofdrichting Dieroecologie (Hoofd Dr. Oomen): Populatie-dynamisch onderzoek aan reptielen in Nederland en in de Oostenrijkse Alpen.
Bijvak Botanie Algemeen (Hoofd Prof. Dr. H.F. Linskens): Onderzoek naar allergeen-produktie bij xerofiele huisstofschimmel.
Bijvak Ontwikkelingsbiologie der dieren (Hoofd Prof. Dr. J.M. Denucé): Immunocytochemisch onderzoek naar het voorkomen van neuropeptiden in de oogsteel van Palaemon serratus.
Zijn lesbevoegdheid 1e graads Biologie behaalde hij in 1980.

Na een korte periode van werken op de boerderij in Eckelrade besloot hij tot het verrichten van vrijwilligerswerk bij Dr. L.G. Poels op de afdeling Cyto/Histologie (Hoofd Prof. Dr. C. Jeruzalem) van december 1982 tot februari 1984, waar hij zich verder specialiseerde in biochemische en immunologische technieken, zoals de bereiding van monoclonale antilichamen tegen celoppervlakte-antigenen.

Vanaf februari 1984 is hij werkzaam op de afdeling Pathologische Anatomie van het Academisch Ziekenhuis Nijmegen (Hoofd Prof. Dr. G.P. Vooijs) in het kader van een KWF-project (NUKC-1984-13), waar dit proefschrift tot stand kwam.

In september 1985 is hij getrouwd met Mariet Roodink.

LIST OF PUBLICATIONS

1. Rijckaert G, Broers JLV. 1980. Time dependent release of allergens from some xerophilic fungi. Allergy 35: 679-682.

2. Broers J, Huysmans A, Moesker O, Vooijs P, Ramaekers F, Wagenaar S. 1985. Small cell lung cancers contain intermediate filaments of the cytokeratin type. Lab Invest 52: 113-115.

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4. Broers JLV, Carney DN, Klein Rot M, Schaart G, Lane EB, Vooijs GP, and Ramaekers FCS. 1986. Intermediate filament proteins in classic and variant types of small cell lung carcinoma cell lines: A biochemical and immunochemical analysis using a panel of monoclonal and polyclonal antibodies. J Cell Sci 83: 37-60.

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6. Oud PS, Haag DJ, Zahniser DJ, Ramaekers FCS, Huysmans ACLM, Veldhuizen JAM, Verheyen RHM, Verrijp K, Broers JLV, Herman CJ, Vooijs GP. 1986. Cytopress: automatic slide preparation of cytologic material from suspensions. Cytometry 7: 8-17.

7. De Leij L, Broers J, Ramaekers F, Berendsen H, and Wagenaar Sj. 1987. Monoclonal antibodies in clinical and experimental pathology of lung cancer. In: D.J. Ruiter, G.J. Fleuren, S.O. Warnaar (eds), Application of monoclonal antibodies in tumor pathology, pp. 191-210. Dordrecht: Martinus Nijhoff.

8. Broers JLV, Klein Rot M, de Leij L, Wagenaar SjSc, Carney DN, Vooijs GP, Ramaekers FCS. 1987. Monoclonal antibodies in lung cancer pathology. Eur J Resp Dis (suppl.) 149: 11-18.

9. Broers JLV, Gropp C, Bepler G, Klein Rot M, Beck J, Schaart G, Vooijs GP, and Ramaekers FCS. 1987. Identification of cytoskeletal structures in hormone producing lung cancer cell cultures. Acta Histochem (suppl.) 34: 57-75.

10. Ramaekers F, Broers J, Klein Rot M, Oostendorp T, Wagenaar Sj, and Vooijs P. 1987. Detection of epithelial- and neural type of intermediate filament proteins in human lung tumors. Acta Histochem (suppl.) 34: 45-56.

11. Broers JLV, Klein Rot M, Oostendorp T, Huysmans A, Wagenaar SjSc, Wiersma-van Tilburg AJM, Vooijs GP, Ramaekers FCS. 1987. Immunocytochemical detection of lung cancer heterogeneity using antibodies to epithelial-, neuronal-, and neuroendocrine antigens. Cancer Res 47: 3225-3234.

12. Broers JLV, Ramaekers FCS, Klein Rot M, Oostendorp T, Huysmans A, van Muijen GNP, Wagenaar SjSc, Vooijs GP. 1988. Cytokeratins in different types of human lung cancer as monitored by chain-specific monoclonal antibodies. Cancer Res 48: 3221-3228.

13. Broers JLV, Pahlplatz MMM, Katzko MW, Ouds PS, Beck JLM, Ramaekers FCS, Carney DN, Vooijs GP. 1988. Quantitative description of classic and variant small cell lung cancer cell lines by nuclear image cytometry. Cytometry, in press.

14. Broers JLV, Klein Rot M, Oostendorp T, Bepler G, De Leij L, Carney DN, Vooijs GP, Ramaekers FCS. 1988. Spontaneous change of intermediate filament protein expression patterns in lung cancer cell lines. J Cell Sci 91 (1), in press.

15. Broers JLV, de Leij L, Klein Rot M, ter Haar A, Lane EB, Leight IM, Wagenaar SS, Vooijs GP, Ramaekers FCS. 1988. Expression of intermediate filament proteins in fetal and adult human lung tissues. Submitted for publication.

STELLINGEN

1. De uitspraak van Gatter en co-auteurs: "The dream of a panel of anti-intermediate filament antibodies solving all the problem areas of histopathology is over", suggereert ten onrechte dat zulk een droom ooit bestaan zou hebben.
    Gatter KC et al. 1986. J Clin Pathol 39: 950-954.

2. Aangezien kleincellige longtumor cellijnen spontaan kunnen veranderen qua differentiatie-kenmerken verdient het aanbeveling om deze cellijnen periodiek hierop te controleren.
    Gazdar AF et al. 1985. Cancer Res 45: 2924-2930.
    Dit proefschrift.

3. De detectie van neuro-endocriene differentiatie-kenmerken in niet-kleincellige longcarcinomen met behulp van monoclonale antilichamen kan een indicatie zijn om deze tumoren als kleincellige longcarcinomen te behandelen.
    Hammond ME, Sause WT. 1985. Cancer 56: 1624-1629.
    Dit proefschrift.

4. Gezien de beperkte vooruitgang bij de behandeling van longkanker in het afgelopen decennium, verdient het aanbeveling om voorrang te verlenen aan de ontwikkeling van nieuwe, experimentele, behandelingstechnieken.

5. Het feit, dat de Levendbarende Hagedis Lacerta vivipara in staat is zich te handhaven in het oostenrijks hooggebergte, moet worden toegeschreven aan de flexibele voortplantingscyclus en de relatief lage predatiedruk.

6. Toen de eerste suffragettes demonstratief een sigaar opstaken konden ze niet vermoeden dat, wellicht door dit gebaar, de vrouwenemancipatie later letterlijk over lijken zou gaan.
    Andrews JL et al. 1985. Lung cancer in women. Cancer 55: 2894-2898.
    Holleb AI. 1985. Lung cancer: a feminist issue. CA 35: 125-126.

7. Een efficiënte bestrijding van longkanker is alleen mogelijk als de politieke bereidheid ontstaat om de volksgezondheid belangrijker te achten dan de financiële voordelen van accijns-inkomsten uit de sigarettenverkoop.

8. De volksgezondheid zou gediend zijn als werkers in de gezondheidszorg in openbare ruimten niet zouden roken en er geen werkkleding zouden dragen.

9. De alfabetische rangschikking van de nederlandse letter ij onder y in telefoonboeken is niet alleen een miskenning van deze taaleigen letter, maar ook gewoon knap lastig voor mensen die vaker woordenboeken dan telefoonboeken raadplegen.

Nijmegen, 4 november 1988   J.L.V. Broers