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Immunohistochemical examination of lung cancers using monoclonal antibodies reacting with sialosylated Lewis^x and sialosylated Lewis^a

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Summary. In order to explore the relationship between the expression of cancer-associated glycolipids such as sialylated Le^x (SLEX) and sialylated Le^a (SLEA) and the histological subtypes of lung cancers, 30 cases of small cell carcinoma (SCC) and 47 cases of non-small cell carcinoma (non-SCC) were examined immunohistochemically using monoclonal antibodies reacting with SLEX and SLEA. The forty-seven cases of non-SCC included 20 cases of adenocarcinoma, 20 of squamous cell carcinoma and 7 of large cell carcinoma. Tumour cells of most non-SCCs expressed SLEX and SLEA. In adenocarcinomas, the number of tumour cells having SLEX and SLEA was more than that of squamous cell carcinomas, large cell carcinomas and SCC. In SCC, 14 of the 30 cases were found to be positive for both antigens. Although the cancer cells of 11 cases of 17 intermediate cell type SCC had both antigens, the cells of only 3 of 13 oat cell tumours expressed SLEX and SLEA. The present study shows that SLEX and SLEA are useful markers for lung adenocarcinomas, that most cases of intermediate cell type of SCCs have characteristics similar to non-SCC but that many oat cell tumours lack them.

Key words: Lung cancer – Sialylated Le^x – Sialylated Le^a – Monoclonal antibody – Immunohistochemistry

Introduction

Recently, a variety of monoclonal antibodies (MoAbs) raised against various kinds of cancers were found to be reactive with glycolipid antigens (Hakomori 1985). MoAbs reacting with Lacto-N-fucopentaose-III (LNF-III) were strongly reactive with cell lines derived from lung cancers and tumour tissues of lung cancers including small cell carcinoma (SCC) (Rosen et al. 1984). MoAbs reacting with the sialylated form of LNF-III, referred to as sialylated Lewis^x (SLEX), were reported (Fukushima et al. 1984). Sialylated fucolipids including sialylated Lewis^a (SLEA) and SLEX were highly expressed on a variety of cancers (Hakomori 1985). As those antigens were found in the sera of cancer patients as well as tumour tissues (Iguro et al. 1984), it is important to examine their expression on tumour tissue with respect to the diagnosis and monitoring of cancer patients. Although the expression of SLEX in lung and gastrointestinal cancers was much more frequent than in other kinds of cancer (Fukushima et al. 1984), lung cancers are histologically heterogenous and are classified into four major categories, which are biologically different from one another. Therefore, the neoplastic and non-neoplastic tissues of the lung were examined immunohistochemically employing MoAbs against SLEX and SLEA, and the expression of the antigens in histological subtypes of lung cancers was analysed.

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Materials and methods

Materials. Samples were collected from files of routine surgical and well preserved autopsy materials in Kitasato University Hospital. Tissues were fixed in 10% formalin and processed to obtain 3 μm paraffin sections. In a preliminary study fresh frozen tissue 4 μm-thick sections were cut serially on a cryostat and were postfixed in 4% buffered formalin, pH 7.2, for 5 min.

Antibodies: Two monoclonal antibodies, CSLEX1 and CSLEA1, were established in UCLA Tissue Typing Laboratory (Los Angeles, CA, USA) and have been described elsewhere in detail (Fukushima et al. 1984; Galton et al. 1985). Briefly, CSLEX1 reacts with sialylated Lewis^x epitope of Rauvala's ganglioside and CSLEA1 reacts with sialylated Lewis^a epitope. CSLEX1 and CSLEA1 were used at a concentration of 10 μg protein/ml.

Table 1. Comparison of immunohistochemical results between frozen sections and paraffin-embedded sections of the same tissues

No of case[a]	Histology	Expression of SLEX and SLEA in			
		frozen sections		paraffin sections	
		SLEX	SLEA	SLEX	SLEA
2	adenocarcinoma	+++	+	+++	+
4	adenocarcinoma	+	+	+	+
7	adenocarcinoma	+++	+++	+++	+++
9	adenocarcinoma	+++	+	+++	+
10	adenocarcinoma	+++	+	+++	+
12	adenocarcinoma	++	+	++	+
14	adenocarcinoma	+	+	+	+
16	adenocarcinoma	++	−	++	−
20	adenocarcinoma	+	++	+	++
2	squamous cell ca[b]	+	+	+	+
3	squamous cell ca	+	+	+	+
4	squamous cell ca	++	+	++	+
19	squamous cell ca	+	−	+	−
14	small cell ca[b] intermediate cell	−	−	−	−
1	small cell ca oat cell	+	++	+	++
3	small cell ca oat cell	+	+	+	+
5	small cell ca oat cell	−	−	−	−

[a] The number of case is same as that of adenocarcinoma in Table 3, squamous cell carcinoma in Table 4 and small cell carcinoma in Table 6

[b] ca; carcinoma

Immunoperoxidase procedure: The sections, deparaffinized with xylene were soaked in absolute methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. After washing in phosphate buffered saline (PBS) for 20 min, sections were incubated with CSLEX1 or CSLEA1 overnight at 4° C. Immunoperoxidase staining was then performed using the Avidin-biotin-complex (ABC) method (Hsu et al. 1981). Reagents for the ABC method were purchased from Vector Laboratory, CA, USA and the dilution and incubation time were as described in the staining procedure of the VECTASTAIN ABC KIT. The sections were colored with 0.05 M Tris-HCl buffer,

Table 2. Localization of sialylated Lewis^x and sialylated Lewis^a in non-neoplastic adult and fetal lung

	SLEX	SLEA
Adult lung		
Bronchial surface epithelium	+	−
Bronchial goblet cell	++	+
Bonchial gland	+++	+++
Bronchiolar surface epithelium		
ciliated	+	+
non-ciliated	−/+[a]	−/+[a]
Alveolar cell	−	−
Alveolar macrophage	+	−
Fetal lung		
Bronchial surface epithelium	+	+
Bronchial gland	++	++
Bronchiolar epithelium	+	+
Alveolar cell	−/+[b]	−

[a] SLEX and SLEA were expressed in a small number of the non-ciliated bronchiolar epithelium

[b] CSLEX1 reacted with the alveolar cells in a few cases, although the cells of most fetal lungs were not immunostained by CSLEX1

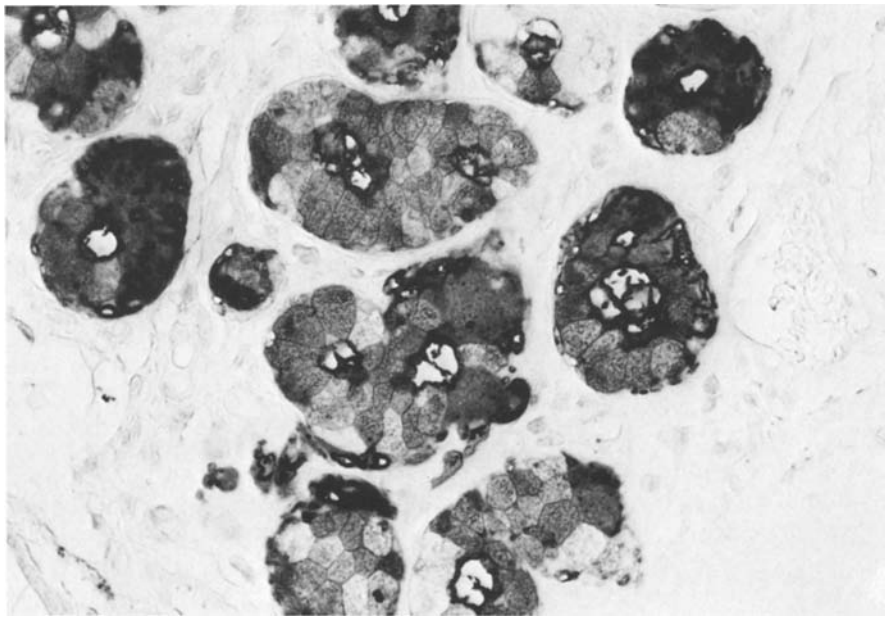

Fig. 1. Immunoperoxidase staining by CSLEX1 on a section of adult respiratory epithelium, showing cytoplasmic and membrane staining of bronchial glands. ×880

pH 7.6, containing 20 mg/100 ml of 3,3′-diaminobenzidine tetrahydrochloride and 0.05% H_2O_2 for about 5 min. After washing in PBS, the sections were counterstained with 0.2% methylgreen and mounted in plastic. As negative controls, sections were incubated with mouse myeloma IgM (10 μg/ml) or normal mouse IgG (10 μg/ml). Then the sections were stained by the ABC method. No enzyme reaction was found in these negative control sections.

Grading system: The degree of the number of cells positive for antigens was graded as negative (−), 1+ : <10%, 2+ : 10% to 50%, 3+ : >50% of antigen-positive cells in the sections.

Results

In view of preservation of SLEX and SLEA during preparation of sections from paraffin-embedded specimens, the immunohistochemical results of frozen sections and paraffin embedded sections of 17 lung cancers were compared initially (Table 1). There was little difference in immunoreactivity between frozen and paraffin-embedded sections of the same tissues. Paraffin embedded sections were immunostained in the present study to clarify the localization of SLEX and SLEA in the cell because morphology of the tissues in the sections was generally better than that in frozen sections.

In order to explore the expression of SLEX and SLEA on normal adult and fetal tissues of

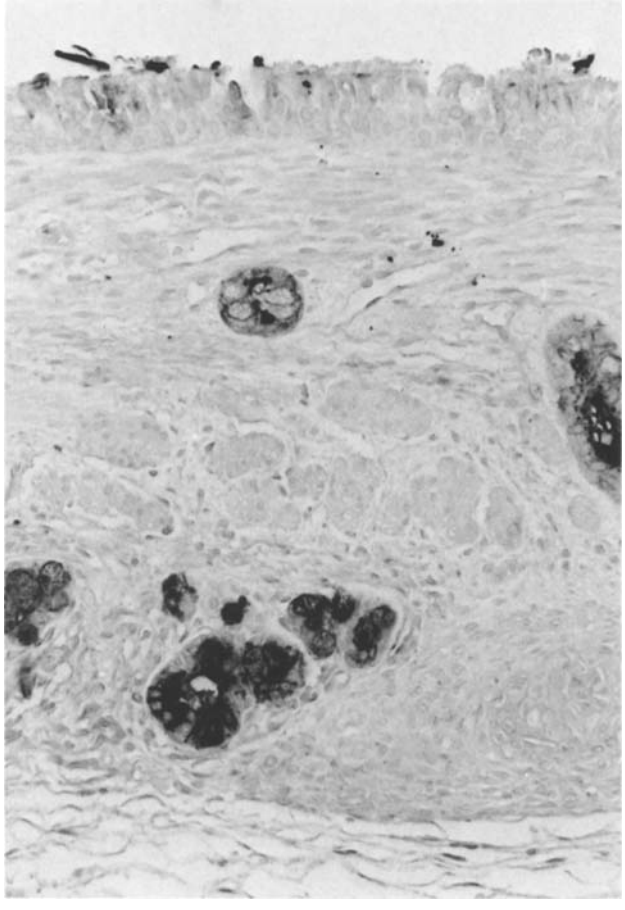

Fig. 2. Immunoperoxidase staining by CSLEX1 on a section of fetal lung, showing prominent cytoplasmic staining of bronchial gland and focal staining of bronchial epithelium. ×560

Table 3. Immunohistochemical results in adenocarcinomas

No of case	Histology	Subtype	SLEX	SLEA
1	papillary	Clara + BS	+ + +	+ +
2	bronchiolo-alveolar	goblet	+ + +	+
3	papillary	Clara	+ +	+ +
4	papillary	Clara	+	+
5	papillary	Clara + BS	+ + +	+ +
6	papillary	Clara	+ +	+ + +
7	bronchiolo-alveolar	goblet	+ + +	+ + +
8	acinar	BG	+ + +	+ +
9	papillary	Clara	+ + +	+
10	papillary	Clara	+ + +	+
11	bronchiolo-alveolar	goblet	+ + +	+ + +
12	acinar	BS	+ +	+
13	acinar		+ + +	+
14	papillary	Clara	+	+
15	poorly diff.,		+ + +	+ + +
16	papillary	Clara	+ +	−
17	papillary	Clara + goblet	+ + +	−
18	papillary	goblet	+ + +	−
19	poorly diff.,	BS	+ +	−
20	papillary	BS	+	+ +

papillary; papillary adenocarcinoma, acinar; acinar adenocarcinoma, bronchiolo-alveolar; bronchiolo-alveolar carcinoma, poorly diff.; poorly differentiated adenocarcinoma
BS; bronchial surface epithelial type, goblet; goblet cell type, BG; bronchial gland cell type, Clara; Clara cell type

Table 4. Immunohistochemical results in squamous cell carcinoma

No of case	Histology	SLEX	SLEA
1	well diff	+	+
2	moderately diff	+	+
3	moderately diff	+	+
4	moderately diff	+ +	+
5	moderately diff	+	+
6	moderately diff	+	+
7	moderately diff	+	+
8	moderately diff	+	+
9	moderately diff	+	+
10	well diff	+	+
11	moderately diff	+	+
12	moderately diff	+	+
13	moderately diff	+	−
14	moderately diff	+ +	−
15	moderately diff	+	−
16	moderately diff	+	−
17	moderately diff	+	+
18	moderately diff	+	+
19	moderately diff	+	−
20	poorly diff	−	+

well diff; well differentiated squamous cell carcinoma, moderately diff; moderately differentiated squamous cell carcinoma, poorly diff; poorly differentiated squamous cell carcinoma

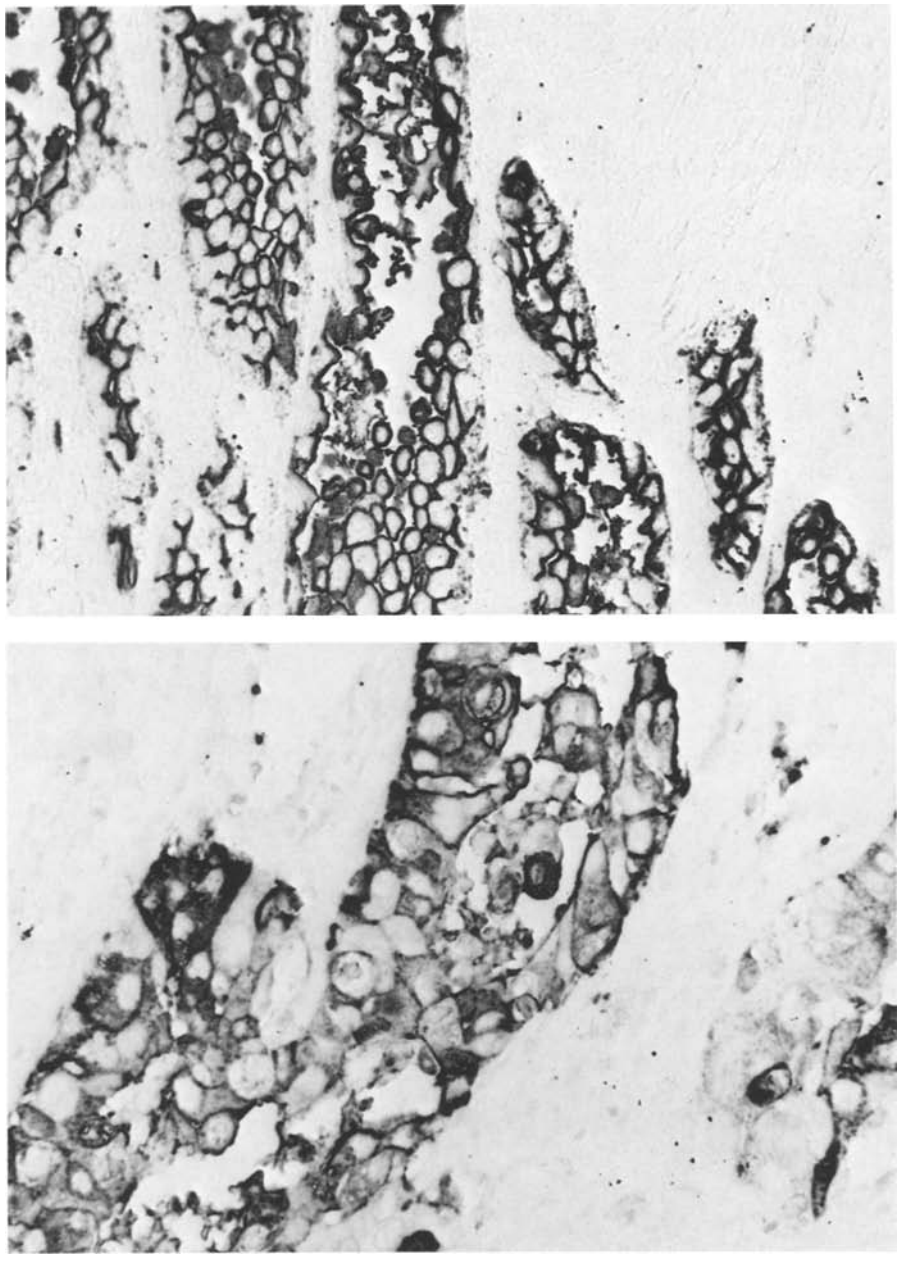

Fig. 3. Immunoperoxidase staining by CSLEX1 on a section of adenocarcinoma. Prominent immunoperoxidase reactivity is seen along the luminal surface of tumour cells of Clara cell type (**a**, ×560) and cytoplasmic staining by CSLEX1 is shown in tumour cells of bronchial gland type (**b**, ×880)

the lung, non-neoplastic tissues of the lung were examined immunohistochemically (Table 2). CSLEX1 and CSLEA1 reacted strongly with bronchial glands (Fig. 1) and goblet cells of the bronchial epithelium. The bronchial surface epithelium expressed SLEX but not SLEA. In non-ciliated bronchiolar epithelium, the luminal surface of a small number of the cells was stained by CSLEX1 and CSLEA1. SLEX and SLEA were not expressed on the alveolar epithelium, while alveolar macrophages had SLEX but not SLEA.

Sections of fetal lung tissues of 21 to 40 week gestation were immunostained. In the fetal lung, expression of SLEX and SLEA was observed from the 21st week of gestation onward. Strong cytoplasmic staining of SLEX and SLEA was observed in bronchial glands (Fig. 2). CSLEX1 and CSLEA1 reacted with the luminal surface of the bronchial epithelium. Although CSLEA1 was not reactive with alveolar cells, CSLEX1 reacted with them in a few cases. This type of reactivity of SLEX and SLEA was observed up to the 40th week of gestation.

Seventy-seven cases of lung cancer examined in the present study were classified histologically according to the WHO typing (WHO 1982), which

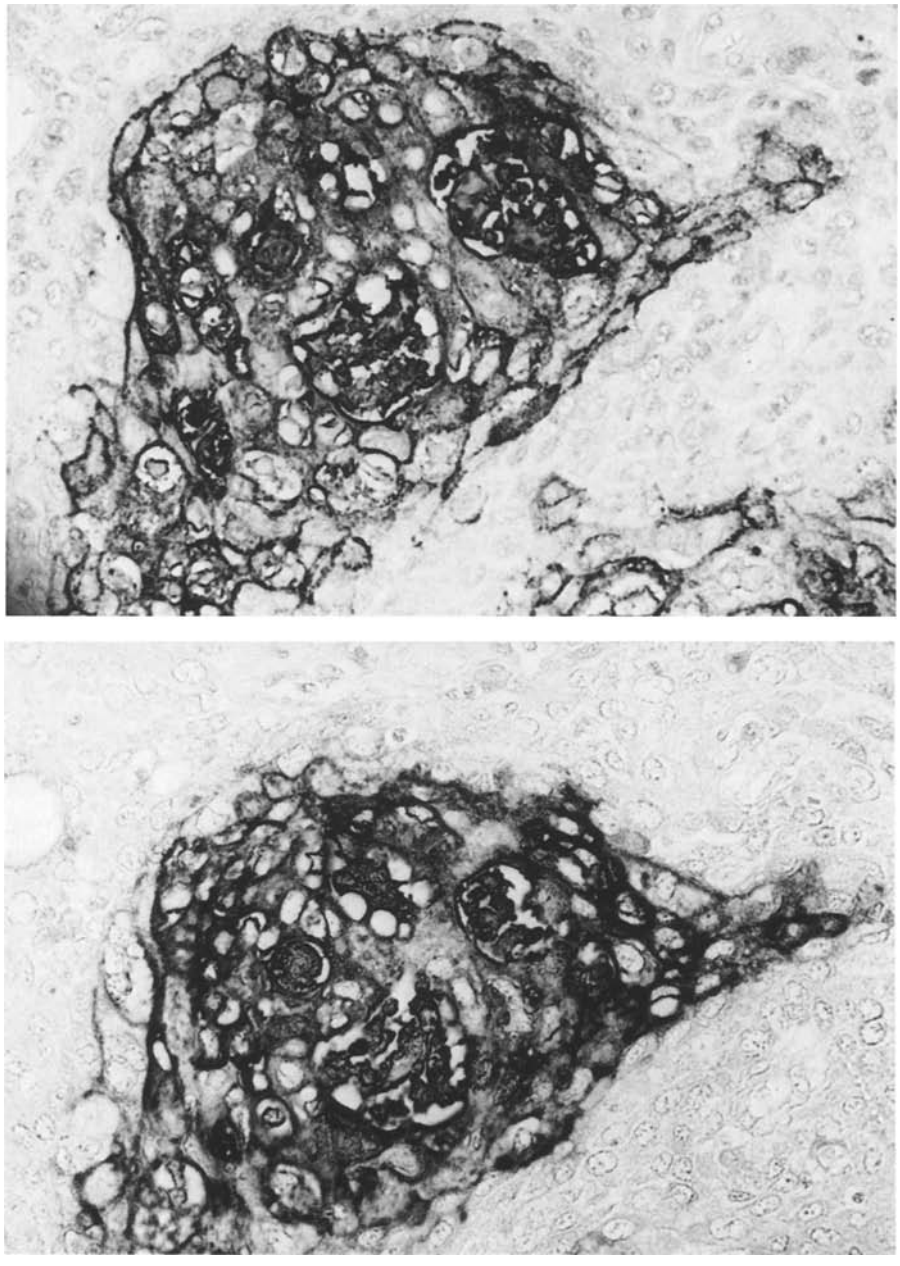

Fig. 4. Immunoperoxidase staining by CSLEX1 (**a**, ×670) and CSLEA1 (**b**, ×670) on sections of squamous cell carcinoma, showing cytoplasmic staining of the tumour cells near keratinization

included 20 cases of adenocarcinoma, 20 of squamous cell carcinoma, 7 of large cell carcinoma and 30 of small cell carcinoma. Further, adenocarcinomas were subclassified by morphological similarities to the normal bronchial, bronchiolar and alveolar epithelia according to Shimosato's classification (Shimosato et al. 1982).

Of 20 cases of adenocarcinoma, the cancer cells of 16 cases were found to be positive for SLEX and SLEA (Table 3). Tumour cells of 4 adenocarcinomas expressed SLEX but not SLEA. In most adenocarcinomas, a large number of carcinoma cells were shown to be positive for SLEX and SLEA. In cases of non-ciliated bronchiolar cell type (Clara cell type) and bronchial non-goblet cell type of adenocarcinomas, the luminal cell surfaces of tumour cells were heavily stained with CSLEX1 and CSLEA1, while the cytoplasm of the cells was not reactive with the antibodies (Fig. 3a). In cases of goblet cell type and in the bronchial gland type of adenocarcinoma, the cytoplasm of cancer cells was strongly reactive with CSLEX1 and CSLEA1 as well as the cell membrane of tumour cells (Fig. 3b). There was no relationship between 4 cases devoid of SLEA and 16 SLEA-positive cases in regard to the histological subclassification.

Table 5. Immunohistochemical results in large cell carcinoma

No of case	SLEX	SLEA
1	+	+
2	+	+
3	++	++
4	−	−
5	−	−
6	+	+
7	+	−

Table 6. Immunohistochemical results in small cell carcinomas

Intermediate cell type			Oat cell type		
No of case	SLEX	SLEA	No of case	SLEX	SLEA
1	+	+	1	+	++
2	++	+	2	++	++
3	+	+	3	+	+
4	+	+	4	−	+
5	+	++	5	−	−
6	+	+	6	−	−
7	+	+	7	−	−
8	++	+	8	−	−
9	+	++	9	−	−
10	+	+	10	−	−
11	+	++	11	−	−
12	−	+	12	−	−
13	+	−	13	−	−
14	−	−			
15	−	−			
16	−	−			
17	−	−			

Of 20 cases of squamous cell carcinoma, the tumour cells of 14 cases expressed both SLEX and SLEA, and the cells of the remaining 6 cases had either SLEX or SLEA (Table 4). The cell membrane and cytoplasm of a small number of tumour cells around keratinizing cells were stained with CSLEX1 and CSLEA1 (Fig. 4). The staining grades were generally lower than those of adenocarcinomas. There was no relationship between the differentiation grade or cell atypia and the expression of the antigens.

Of 7 large cell carcinomas, tumour cells of 4 cases had SLEX and SLEA (Table 5). Tumour cells of one case expressed SLEX but not SLEA, while the cells of 2 cases were devoid of both antigens. In large cell carcinomas, SLEX and SLEA were expressed on the cell membrane and cytoplasm of a small number of tumour cells.

In 30 cases of SCC, the cancer cells of 14 cases were found to be positive for SLEX and SLEA, and the cells of 3 cases had one of the antigens (Table 6). The cancer cells of 13 cases did not express either of the antigens. In most tumours having either SLEX or SLEA, the antigens were expressed on the cell membrane of a small number of cells (Fig. 5). The tumour cells of 14 of the 17 intermediate cell tumours (82%) expressed SLEX or SLEA, while the cells of only 4 of the 13 oat cell tumours (31%) had SLEX or SLEA.

Discussion

In the present paper the expression of SLEX and SLEA on the tissues of lung cancers, normal adult and fetal lungs were examined using formalin-fixed paraffin embedded sections because there was little difference in immunoreactivity between frozen and paraffin-embedded sections of the same tissues in a preliminary experiment. The antigenicity of SLEX as well as SLEA was found to be maintained in the sections, indicating that SLEX and SLEA are useful for immunopathological diagnosis and studies using routinely processed surgical pathology sections.

Although SLEA is related to Lewis (Le) blood group antigens, Le blood group antigens of the patients were not examined in the present study because it was retrospective. SLEA is expressed in tissues and saliva from most individuals belonging to the Le (a+b−) or Le (a−b+) blood group and is not found in those of individuals belonging to Le (a−b−) blood group. Therefore, cancer patients belonging to the latter blood group cannot synthesize SLEA (Koprowski 1983; Pak et al. 1984). However, it is thought that SLEA belongs to a class of oncodevelopmental antigens because a small percentage of the population belongs to the Le (a−b−) blood group and SLEA was detected in not only tumour tissues but also in fetal tissues and meconium (Atkinson et al. 1982; Koprowski 1983). In contrast to SLEA, SLEX is not associated with Le blood group antigens because Le^x does not have their activity and is not controlled by a gene at the Lewis locus (Watkins 1980), although Le^x was thought to be one of precursors of SLEX in tumour cells (Hakomori 1985).

In the respiratory epithelium of normal adult and fetal lung, both SLEX and SLEA were expressed strongly in bronchial glands, although the expression of only SLEA in bronchial gland was reported previously (Atkinson et al. 1982). CSLEX1 was also reactive with the surface epithelium of bronchi and bronchioles. Fukushima et al. reported that SLEX was not expressed in bronchi using an indirect immunoperoxidase procedure on frozen sections of the lung (Fukushima et al. 1984). It is likely that the discrepancy between our results

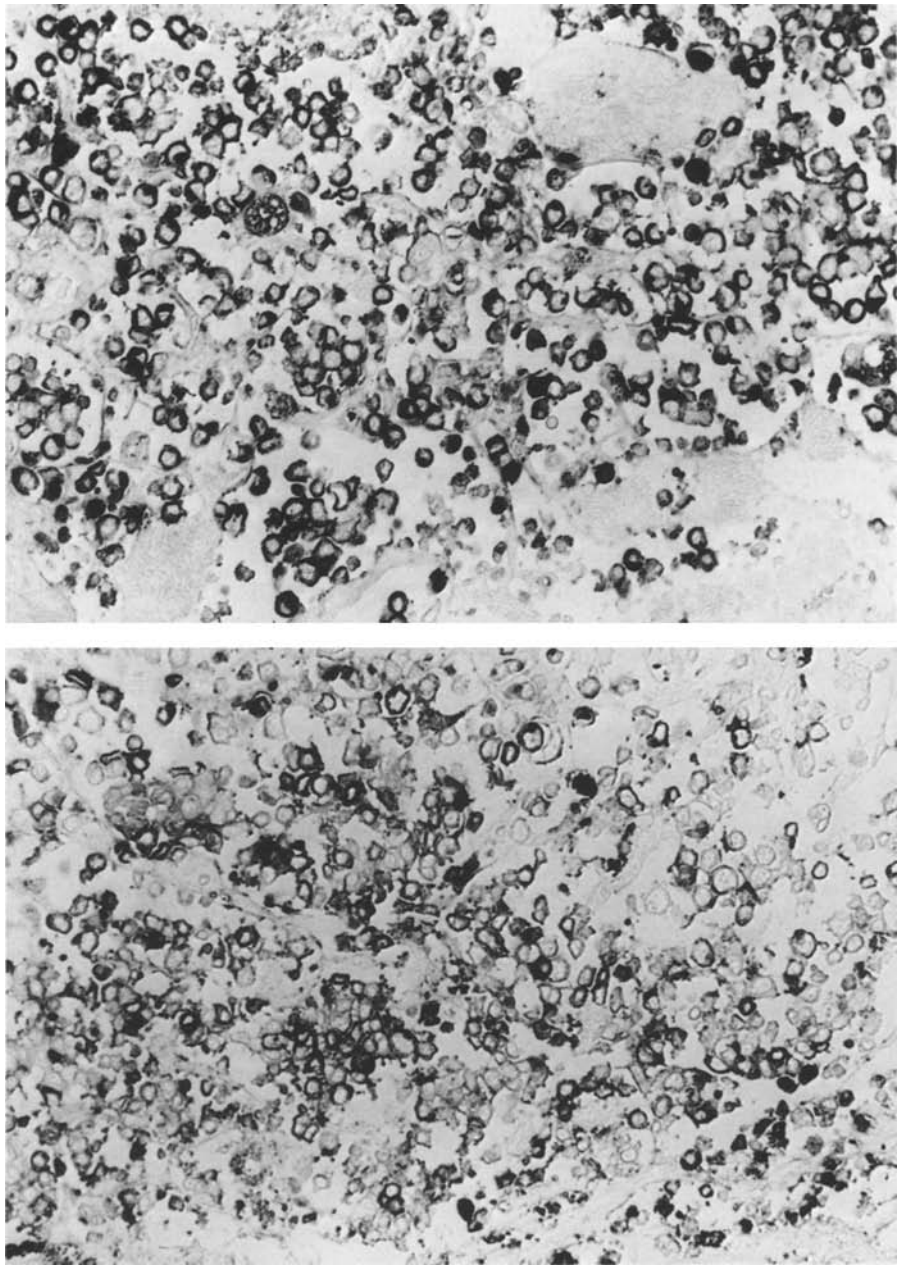

Fig. 5. Immunoperoxidase staining by CSLEX1 (**a**, ×880) and CSLEA1 (**b**, ×880) on sections of small cell carcinoma. Prominent immunoperoxidase reactivity is seen along the membrane of tumour cells

and theirs is due to the higher sensitivity of the ABC method. Therefore, the percentage of lung cancers with SLEX in the present study was higher than that of their cases. SLEA was also expressed in the surface epithelium of bronchioles in normal adult lung, in which it was described as being previously absent (Atkinson et al. 1982). One possible explanation for the discrepancy between our results and theirs is that CA19-9, which was used by them for SLEA staining, and CSLEA1, recognize different structural components of the sialosyl Le^a molecule (Galton et al. 1985).

The antigen detected by CSLEX1 was found in sera of patients with various cancers (Iguro et al. 1984). The sugar sequence of SLEX may be released into the serum by tumour cells and be present in glycoproteins in the cells, while CSLEX1 was reported to react with gangliosides. Kawahara et al. reported that SLEX-positive percentages in the sera of patients with lung adenocarcinomas were higher than those of squamous cell carcinomas and SCC (Kawahara et al. 1985). As SLEX as well as SLEA was strongly expressed in adenocarcinomas, it can be concluded that SLEX and

SLEA are useful markers for adenocarcinomas. Further, CSLEX1 was strongly reactive with the cytoplasm and cell membrane of goblet cell type and bronchial gland type of adenocarcinomas, in contrast to bronchial surface epithelium type and Clara cell type, suggesting that SLEX might be present in glycoproteins such as mucin in those cells. It would be interesting to study the relationship between subtypes of adenocarcinoma in regard to expression of SLEX by examining more cases.

SCC has been regarded as a tumour possessing neuroendocrine properties (Matthews and Gazdar 1982) and SCC behaves differently from other lung cancers. A variety of phenotypic characteristics that distinguish SCC from non-SCC have been reported; the tumour has neuron-specific enolase (Bergh et al. 1985), L-dopa decarboxylase (Nagatsu et al. 1985), peptide hormones (Abe et al. 1982, Yamaguchi et al. 1983), neurofilament (Bergh et al. 1984) and Leu-7 (Bunn et al. 1985), which are markers of neuroendocrine cells. Based on ultrastructual features such as dense-core secretory granules, cytoplasmic elongated processes, desmosomes, tonofibrils, tubular structures and cilia, Kameya et al. reported that oat cells were more akin to neuroendocrine cells and intermediate anaplastic cells to epithelial cells, although it was difficult to define an essential qualitative differences in ultrastructural features between both (Kameya et al. 1982). In this context, it is interesting to recognize that intermediate cell and oat cell types of the SCCs differ distinctly in their expression of SLEX and SLEA. As a variety of sialylated fucolipids such as SLEX and SLEA have been found in various human cancers, which originate from endodermal epithelium, they were considered biochemically to characterize endodermal epithelial cells (Hakomori 1985). Therefore, it is likely that the intermediate cell shares characteristics similar to those of adenocarcinoma and squamous cell carcinoma biochemically as well as histologically.

Recently, it was found that a variety of neuroendocrine tumours have mixed neuroendocrine and non-neuroendocrine characteristics (Kameya et al. 1983; DeLellis et al. 1984). Keratin filaments, which consist of a variable number of closely related peptides, are known to be expressed in non-neuroendocrine epithelial cells. In lung cancers, squamous cell carcinoma and adenocarcinoma expressed cytokeratin proteins in accordance with the epithelial nature of these tumours (Blobel et al. 1984). It was also reported that SCC and carcinoid tumours of the lung coexpressed cytokeratin proteins and neuroendocrine markers such as bombesin and neuron-specific enolase (Blobel et al. 1985). In the present study, SLEX and SLEA were expressed in cancer cells of about half the number of SCC cases, suggesting that SCC cases positive for SLEX and SLEA express a non-neuroendocrine epithelial phenotype. Further, it appears that SCC has the capacity for multidirectional differentiation, as manifested in the morphology and immunohistochemistry of common types of carcinoma as well as other neuroendocrine tumours.

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