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Quantitative Distribution of Cluster 1 Small Cell Lung Cancer Antigen in Cancerous and Non-cancerous Tissues, Cultured Cells and Sera

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Three monoclonal antibodies (mAbs), NCC-LU-243, -244 and -246, detected three different epitopes on a 145-kDa cell membrane antigen, which had been designated as the cluster 1 antigen at the First International Workshop on Small Cell Lung Cancer (SCLC) Antigens. The distribution of the antigen in various tissues, cultured cells and sera was examined by immunohistochemistry and sandwich radioimmunoassay using these mAbs. The antigen is a normal differentiation antigen and is present in neuronal, neuroendocrine and cardiac muscle cells. The level of the antigen was highest in central nervous tissues, while it was undetectable in the liver, kidney and peripheral lung. Among tumor tissues, the antigen was detected only in SCLC, carcinoid tumor and neuroblastoma, indicating its usefulness as a marker for discriminating SCLC from non-SCLC. The level of the antigen varied among SCLC tissues and tended to be lower in variant-type cultured SCLC cells. Although an increase in the antigen level was observed in sera of some patients with advanced SCLC, the antigen did not possess any additional value over neuron-specific enolase as a serum tumor marker for monitoring SCLC patients.

Key words: Lung cancer — Small cell carcinoma — Monoclonal antibody — Cluster 1 antigen — Radioimmunoassay

Small cell lung carcinoma (SCLC)[4] accounts for about 25% of all pulmonary carcinomas in western countries, and 15% in Japan.[1)] SCLC cells show features of differentiation similar to those of Kulchitsky's cells distributed in bronchi, terminal bronchioli and bronchial glands.[1,2)] SCLC cells often produce various peptide hormones,[3)] L-dopa-decarboxylase,[4)] neuron-specific enolase (NSE)[5)] and creatine kinase BB,[6)] which are markers of neuroendocrine cells, and thus illustrate that SCLC is biologically different from other histological types of pulmonary carcinoma.

Clinically, SCLCs grow very rapidly, so that more than 50% of cases are in an advanced state with metastasis to mediastinal lymph nodes or distant organs at the time of initial diagnosis. On the other hand, even when the tumors are very advanced, they show good responsiveness to chemotherapy and radiotherapy, and complete remission of the tumors can sometimes be obtained using recently developed anti-cancer agents.[7)] However, patients with SCLC still have the worst prognosis among all pulmonary carcinoma patients. For this reason, correct histological typing is required for the selection of appropriate treatment and for estimation of prognosis.

Monoclonal antibodies (mAbs) specific to SCLC would be helpful for histological typing. In addition, measurement of the antigens defined by such mAbs in sera might be useful for the monitoring of cancer patients. Many mAbs against SCLC have been reported from different laboratories. These can be roughly classified into two groups; those cross-reacting with neuroendocrine cells and those cross-reacting with epithelial cells. Some mAbs of the former group have shown the highest reactivity to SCLC, and have almost identical immunohistochemical reactivities. At the First International Workshop on SCLC Antigens held in London in 1987, these mAbs were characterized as forming a cluster, and the antigen recognized was named the cluster 1 SCLC antigen.[8)]

We have obtained three mAbs, each recognizing different epitopes on the cluster 1 antigen, and have developed a sandwich radioimmunoassay (RIA) for measuring the antigen level in various cancerous and non-cancerous tissues and cultured cells. In addition, we have studied the potential use of the cluster 1 antigen as a serological tumor marker for the diagnosis and monitoring of SCLC patients.

MATERIALS AND METHODS

Human tumor xenografts, cultured cell lines and human tissues Human cancer xenografts Lu-24, Lu-134 (SCLC), Lu-27, Lu-90 (pulmonary adenocarcinoma)

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[4] The abbreviations used are: SCLC, small cell lung cancer; NSE, neuron-specific enolase; RIA, radioimmunoassay; mAb, monoclonal antibody; FCS, fetal calf serum; PBS, phosphate-buffered saline, pH 7.4; BSA, bovine serum albumin; NP-40, Nonidet P-40.

and NB-1 (neuroblastoma) were maintained by serial transplantation into the subcutaneous tissue of athymic mice (BALB/c, *nu/nu*, purchased from Nihon CLEA, Tokyo).[9]

Human tumor cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Cell lines c-Lu-134, c-Lu-135, c-Lu-138 (SCLC, classic type); PC-1, PC-10 (pulmonary squamous cell carcinoma); c-Lu-90, c-Lu-27, Okada, PC-3, PC-9 (pulmonary adenocarcinoma); c-Lu-65, PC-13 (pulmonary large cell carcinoma); C-1 (colon carcinoma) had all been previously established in our laboratories.[10,11] SCLC cell lines NCI-H-69, NCI-H-128, NCI-N-231, NCI-N-857/230 (classic type), NCI-N-417 and NCI-H-82 (variant type) were kindly provided by A. F. Gazdar (NCI-Navy Medical Oncology Branch, Bethesda, USA).[12,13] Neuroblastoma cell line NB-1 and stomach carcinoma cell lines MKN-45, -74 and Kato-III were kindly provided by T. Suzuki (Niigata University, Niigata). HL-60, K-562 (leukemia), and HeLa S3 (uterine cervical carcinoma) were obtained from the Japanese Cancer Research Resources Bank.

Human tumor tissues and normal tissues were obtained either at surgery or at autopsy performed within 3 h *post mortem*. They were kept frozen at −80°C until use.

Monoclonal antibodies (mAbs) Details of the methods used for immunization and hybridoma production have been described previously.[14] Briefly, a nude mouse (BALB/c *nu/nu*) bearing Lu-24 was intraperitoneally injected with spleen cells from an immunocompetent mouse (BALB/c), which caused gradual regression of the tumor. Spleen cells from the nude mouse which had rejected the tumor were hybridized with P3-X63-Ag8-U1 cells using polyethylene glycol 4000. Hybridomas, which produced mAbs positive in membrane immunofluorescence analysis of NCI-H-69 cells and reactive with neuroendocrine cells in immunohistochemical analysis (described later), were selected and cloned using limiting dilution method. Three mAbs, named NCC-LU-243 (G2a), -244 (G1) and -246 (G1), were obtained from three different fusions, respectively. Isotypes of immunoglobulins were determined using the Ouchterlony double diffusion test.

Purification and iodination of mAbs Ascitic fluid was produced by injecting hybridoma cells intraperitoneally into pristane-primed mice (BALB/c), and the mAbs NCC-LU-243, -244 and -246 were purified from each ascitic fluid sample using an Affigel 10 Protein A column (Bio Rad Laboratories, Richmond, CA).[15] Iodination of mAbs was carried out using iodine 125 and IODO-BEADS™ (Pierce Chemical Company, Rockford, IL) according to the manufacturer's recommendation,[16] and unbound iodine 125 was removed by passage through a PD-10 column (Pharmacia Fine Chemicals Co., Uppsala).

Determination of antigen molecular weight Cultured cells were cell-surface iodinated using the Iodogen method,[17] and membrane antigens were solubilized with phosphate-buffered saline, pH 7.4, (PBS) containing 0.5% Nonidet P-40 (NP-40). The antigen was immunoprecipitated with Sepharose 4B conjugated with each mAb through the CN-Br residue.[18] Precipitated proteins were resolved by SDS-PAGE using 10% polyacrylamide gel under reducing conditions, as originally described by Laemmli,[19] and the antigen was detected by autoradiography.

Reactivities of three mAbs with the same antigen in solid-phase RIA The antigen was purified from SCLC tissues solubilized in PBS containing 0.5% NP-40 using an affinity column packed with Sepharose 4B conjugated with each mAb. The affinity-purified antigen was coated on wells of U-bottomed 96-well polyvinyl microtiter plates (Dynatech, Alexandria, VA). After incubation for 2 h with a blocking buffer composed of 2% bovine serum albumin (BSA) and 2% gamma-globulin-free horse serum in PBS, each of the iodinated mAbs was added to the wells in serial dilutions and incubated for 2 h at room temperature. After washing ten times with PBS, each well was cut out and the radioactivity was counted in a gamma-counter.

Competitive binding inhibition assay The affinity-purified antigen prepared with NCC-LU-243 was coated on wells of a 96-well microtiter plate. After incubation with the blocking buffer, 25 μl of iodinated mAb mixed with 25 μl of serially diluted cold competitor mAbs was added, and the plate was incubated for 2 h. After washing ten times with PBS, each well was cut out and the radioactivity was counted in a gamma-counter.

Immunohistochemical analysis Reactivities of the mAbs with cultured cells and various tissues were examined on acetone-fixed paraffin-embedded sections ("AMeX" sections) using the avidin-biotin-peroxidase complex method as previously described.[20]

Sandwich RIA of the antigen Experimental samples were assayed in duplicate using a "forward sandwich" RIA. Wells of U-bottomed 96-well polyvinyl microtiter plates were coated with NCC-LU-246 by incubation with 50 μl of the mAb in PBS (0.2 mg/ml) at 4°C overnight, and further incubated with the blocking buffer at room temperature for 2 h in order to block non-specific protein binding. Then, 100-μl aliquots of various samples were applied to the wells, and incubated for 3 h at room temperature. After washing ten times with PBS, the antigen trapped by the wells was detected by incubation with 50 μl of ^{125}I-labeled NCC-LU-243 diluted in the blocking buffer (5×10^5 cpm/well in the case of tissue and cultured cell extracts, 8×10^5 cpm/well in the case of

sera) for 3 h. After washing ten times with PBS, each well was cut out and radioactivity was counted in a gamma-counter. Serial dilution analysis was performed in most cases in order to confirm the reliability of this assay, diluting the samples with the blocking buffer. Samples for sandwich radioimmunoassay were prepared as follows.

a) Tissue extracts: One gram of minced tissue was mixed with 3 ml of PBS containing 0.25 *M* sucrose, 0.01% ethylenediaminetetraacetic acid disodium salt, 2 m*M* phenylmethylsulfonyl fluoride, 0.02% NaN_3, and 0.5% NP-40 (homogenization buffer) and ultrasonicated for 3 min. The solution was centrifuged at 10,000 rpm for 60 min, the supernatant was collected and the content adjusted to 6 mg/ml protein by addition of PBS. The protein concentration was determined using a Bio Rad protein assay kit (Richmond, CA).

b) Cultured cell extracts and spent media: To 5.0×10^7 cultured cells, 0.5 ml of the homogenization buffer was added and ultrasonication was performed for 40 s. The supernatant content was adjusted to 4 mg/ml protein after centrifugation. The spent medium was collected from confluent cultures and examined after centrifugation.

c) Sera of mice: Blood was collected from the subclavian vein of normal nude mice and nude mice transplanted with human pulmonary carcinoma (LU-24, LU-134, H-69 (SCLC); LU-90, LU-27 (pulmonary adenocarcinoma)), when the largest diameter of the tumor exceeded 1.5 cm. Sera were kept frozen at −20°C, and diluted with equal amounts of the blocking buffer before the assay.

d) Human sera: Sera from patients with SCLC were obtained at the Department of Surgery, Tokyo Medical College, and the National Cancer Center Hospital. Sera from healthy volunteers were also collected. These samples were also kept frozen at −20°C, and diluted with an equal amount of the blocking buffer before use. The levels of NSE in these sera were examined by RIA using an Eiken kit (Tokyo).

RESULTS

Antigen and epitopes recognized by the three mAbs

MAbs NCC-LU-243 and -246 immunoprecipitated an antigen with a molecular weight of 145 kDa from several SCLC cell lines and the neuroblastoma cell line NB-1 (Fig. 1), and mAb NCC-LU-244 also immunoprecipitated an antigen of the same molecular weight (data not shown). In addition, all three mAbs were reactive with the same antigen, which was affinity-purified using one of the mAbs (Fig. 2). The reactivity of one mAb with the antigen was not inhibited by the presence of the other two mAbs in the competitive bind-

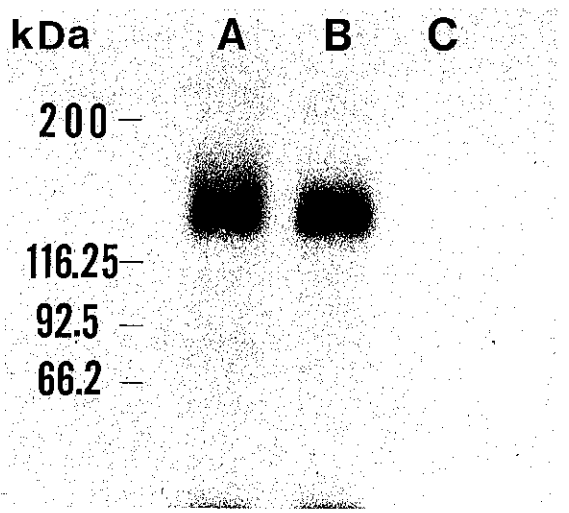

Fig. 1. Analysis of the molecular weight of the cluster 1 SCLC antigen in cultured NCI-H-69 SCLC cells by means of immunoprecipitation and SDS-PAGE. Iodinated cell-membrane proteins were immunoprecipitated with Sepharose 4B conjugated with mAbs and normal mouse IgG (as a negative control), respectively. Precipitated antigen was resolved by 10% SDS-PAGE under reducing conditions, and the antigen was detected by autoradiography. Lane A, NCC-LU-246; lane B, NCC-LU-243; lane C, normal mouse IgG.

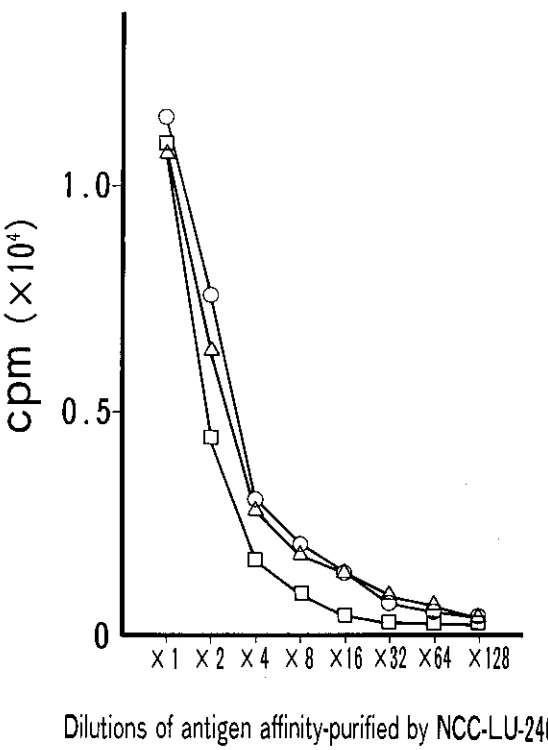

Fig. 2. Reactivities of three mAbs (NCC-LU-243 (○), -244 (□) and -246 (△)) with the antigen affinity-purified using mAb NCC-LU-246 in solid-phase RIA.

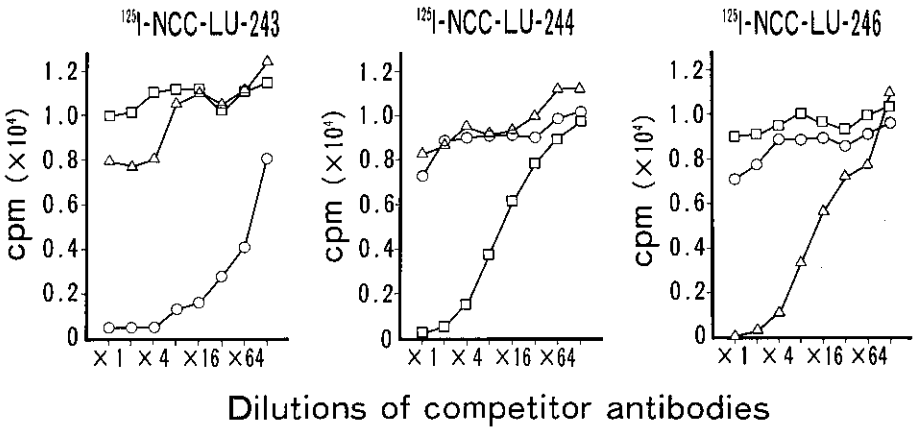

Fig. 3. Analysis of epitopes on the cluster 1 antigen defined by the three mAbs. Bindings of the three ^{125}I-labeled mAbs to the antigen in solid-phase RIA in the presence of non-labeled competitor antibodies are shown. Abscissae indicate serial dilutions of competitor antibodies, NCC-LU-243 (○), -244 (□) and -246 (△).

Table I. Reactivities of mAbs NCC-LU-243, -244 and -246 with Normal Tissues

Normal tissue	Immunohistochemical reactivities[a]	Sandwich RIA[b] (cpm)
Human		
Brain	+	18,393
Spinal cord	+	12,689
Peripheral nerve	+	NE
Cardiac muscle	+	3,412
Thyroid	+	1,405
Adrenal gland	+	3,923
Lung	−	243
Stomach	−[c]	719
Liver	−	198
Spleen	−	328
Kidney	−	238
Pancreas	+[d]	198
Testis	+[e]	NE
Ovary	+[f]	NE
Skin	−	NE
Skeletal muscle	−	478
Lymph node	−	NE
Bone marrow	−	NE
Mouse		
Brain	NE[g]	105
Liver	NE	77

a) Immunohistochemical reactivities of the three mAbs were identical.
b) The mean of three cases is shown.
c) Fundic gland and smooth muscle: faintly positive.
d) Islet of Langerhans.
e) Leydig cells.
f) Stromal cells.
g) NE: not examined.

Table II. Immunohistochemical Reactivities of mAbs NCC-LU-243, -244 and -246 with Various Human Cancer Tissues[a]

Human cancer tissues	No. of cases tested	No. of positive cases (%)
Pulmonary carcinoma		
Small cell carcinoma	14	14 (100)
Adenocarcinoma	11	0
Squamous cell carcinoma	10	0
Large cell carcinoma	5	0
Carcinoid tumor	1	1 (100)
Neuroblastoma	1	1 (100)
Gastric carcinoma	10	0
Colorectal carcinoma	14	0
Liver cell carcinoma	9	0
Breast carcinoma	2	0
Thymoma	3	0
Lymphoma	10	0
Melanoma	3	0

a) Immunohistochemical reactivities of the three mAbs were identical.

ing inhibition assay (Fig. 3). These results indicate that the three mAbs recognize three different epitopes on the same 145-kDa molecule.

Cluster 1 SCLC antigen in tissues The distribution of the antigen in normal tissues, as examined by immunohistochemistry and sandwich RIA, is shown in Table I. The largest amount of the antigen was detected in the human brain and spinal cord, and the antigen was also detected in human endocrine organs and cardiac muscle

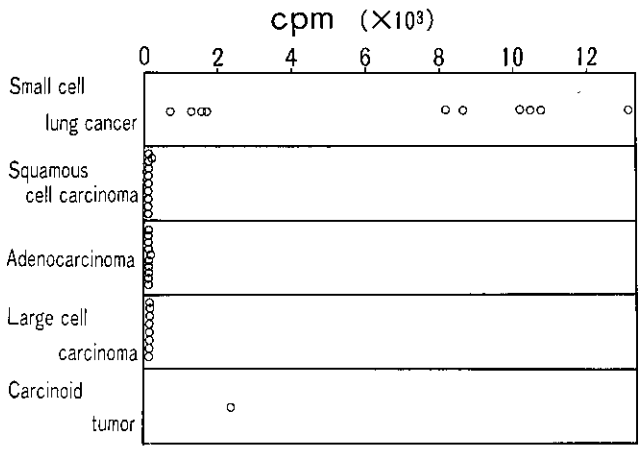

Fig. 4. The level of the cluster 1 antigen in tissue extracts of various pulmonary tumors examined by sandwich RIA. Small cell lung cancer (n=10), squamous cell carcinoma (n=10), adenocarcinoma (n = 10), large cell carcinoma (n = 8), carcinoid tumor (n=1). Tissue extract of brain gave a value of 18,975 cpm in the same assay.

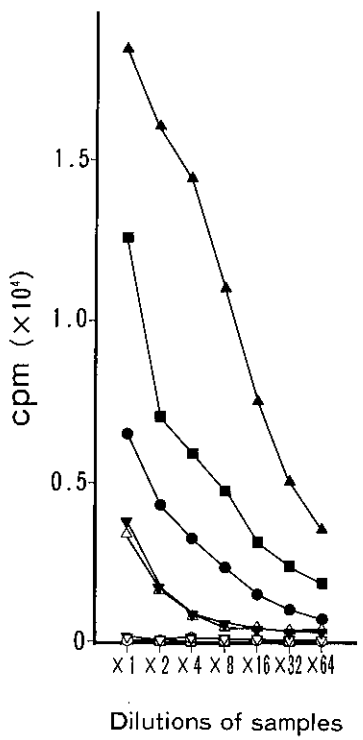

Fig. 5. Serial dilution analysis in sandwich RIA of various kinds of cancerous and non-cancerous tissue extracts. Mean values of samples examined are shown. ●, SCLC (n=10); ○, pulmonary adenocarcinoma (n=10); ▲, brain (n=3); ■, spinal cord (n=3); ▼, cardiac muscle (n=3); △, adrenal gland (n=3); □, liver (n=3); ▽, lung (n=3).

by sandwich RIA. The results of RIA corresponded very well with those of immunohistochemistry. The antigen was not detected in mouse tissues, including brain.

The distributions of the antigen in various cancer tissues examined by immunohistochemistry and sandwich RIA are shown in Table II and Fig. 4, respectively. The antigen was detected only in tissues of SCLC and carcinoid tumor among the pulmonary tumors examined. The level of the antigen showed a wide range of variation among SCLC cases. The antigen was detected in neuroblastoma, but not in gastrointestinal carcinoma, liver cell carcinoma, breast carcinoma, thymoma, lymphoma or melanoma.

The reliability of the RIA of cancerous and non-cancerous tissue extracts was confirmed by serial dilution analysis in all the cases examined, and representative data are shown in Fig. 5.

Cluster 1 SCLC antigen in cultured cells and spent media

Immunohistochemically, the antigen was detected in all of the SCLC cell lines examined (NCI-H-69, NCI-H-128, NCI-N-857/230, NCI-N-231, NCI-N-417, NCI-H-82, c-Lu-134, c-Lu-135 and c-Lu-138), and it was also detected in a neuroblastoma cell line (NB-1). On the contrary, cultured cells of non-SCLC (c-Lu-27, c-Lu-90, Okada, PC-3, PC-9, PC-1, PC-10, c-Lu-65 and PC-13), stomach carcinoma (KATO-III, MKN-45 and MKN-74), colon carcinoma (C-1), uterine cervical carcinoma (Hela S3) and leukemia (HL-60 and K-562) showed negative reactivities.

The sandwich RIA of cultured cell extracts confirmed the immunohistochemical reactivities, and demonstrated the presence of the antigen in all of the SCLC cell lines. In addition, the level of the antigen tended to be lower in SCLC cells of variant type (NCI-N-417 and NCI-H-82) (Table III). A small amount of the antigen was also

Table III. Sandwich RIA of Cultured Cells[a)]

Cultured pulmonary carcinoma cells		
Small cell carcinoma	NCI-H-69	657 cpm
	NCI-H-128	3,297
	NCI-N-231	1,965
	NCI-N-857/230	1,643
	c-Lu-135	1,773
	c-Lu-138	3,803
	NCI-H-82	571
	NCI-N-417	708
Adenocarcinoma	c-Lu-27	149
	c-Lu-90	81
	PC-9	252

a) Representative data of three assays. Tissue extract of brain gave a value of 14,504 cpm in the same assay.

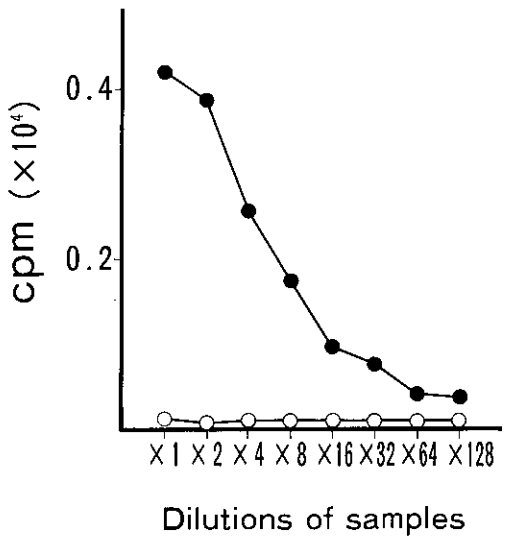

Fig. 6. Serial dilution analysis of the cluster 1 SCLC antigen in normal human and mouse sera. ●, normal human sera (n=3); ○, normal nude mouse sera (n=3). Tissue extract of brain gave a value of 28,013 cpm in the same assay.

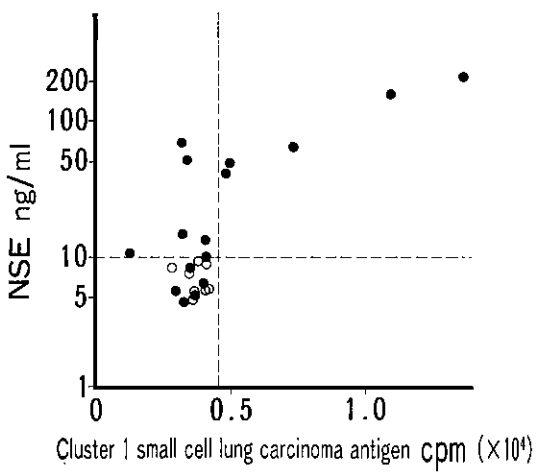

Fig. 7. Distribution of the cluster 1 SCLC antigen in sera of patients with SCLC and healthy donors in comparison with that of NSE. ●, sera from patients with SCLC (n=16); ○, sera from healthy donors (n=8). Cut-off values (mean+2SD of the results obtained with healthy donors) are indicated by dotted lines. Tissue extract of brain gave a value of 28,013 cpm in the same assay.

detected in spent media of cultured SCLC cells (data not shown).

Cluster 1 SCLC antigen in sera Figure 6 shows the results of serial dilution analysis of the antigen in normal human sera and mouse sera, indicating the presence of small amounts of the antigen in normal human sera. A slight increase in the antigen was detected in one of three serum samples obtained from nude mice bearing SCLC, while no increase was observed in sera of three nude mice bearing pulmonary adenocarcinoma. The levels of the cluster 1 SCLC antigen in comparison with those of NSE in sera of SCLC patients and normal healthy donors are shown in Fig. 7. The levels of the two different antigens were correlated and all cases positive for serum cluster 1 SCLC antigen were positive for serum NSE.

DISCUSSION

Three mAbs, produced with SCLC xenograft Lu-24 as an immunogen, recognized three different epitopes on the same cell-membrane antigen. These antibodies showed identical immunohistochemical reactivities, and were characterized as being grouped in cluster 1 together with some other mAbs including TFS-4,[18,21] MOC-1[22] and NE-25[23,24] according to the First International Workshop for SCLC Antigens.[8] These antibodies are highly reactive and specific to SCLC among pulmonary carcinomas, and are cross-reactive with neuronal, neuroendocrine and cardiac muscle cells.

In this study, the molecular weight of the cluster 1 antigen was shown to be 145 kDa by immunoprecipitation analysis with cultured cancer cells. Previous estimates of the molecular weight of this antigen have been rather variable, including 60 kDa,[22] 124 kDa,[20] 25 kDa[23] and 150 kDa.[24] The differences between these molecular weights and that obtained in our study may have been due to technical problems. All the mAbs of cluster 1 were shown to be reactive with the antigen affinity-purified using our NCC-LU-243.[25] The molecular weight of the cluster 1 antigen in normal tissues remains to be clarified.

Normal human tissues were classified into three groups according to their levels of cluster 1 SCLC antigen: tissues with a large amount of cluster 1 antigen (central nervous tissues), tissues with a small amount of cluster 1 antigen (adrenal gland, thyroid gland and heart muscle), and tissues without any detectable cluster 1 antigen (liver, kidney, peripheral lung, etc.). The results of RIA were dose-responsive, indicating the reliability of this assay system. In addition, the RIA results for tissues corresponded closely with those of immunohistochemistry.

In the RIA analysis of tissue extracts of pulmonary tumors, the antigen was detected only in SCLC and carcinoid tumor, whereas it was not detectable in non-SCLC by either RIA or immunohistochemistry. Therefore, the cluster 1 antigen is considered to be a marker capable of discriminating SCLC from non-SCLC. One of

the mAbs reported in this study, NCC-LU-243, can be applied to formalin-fixed paraffin-embedded biopsy specimens, and is useful for differential diagnosis between SCLC and non-SCLC in routine pathology practice (data not shown). On the other hand, de Leij *et al.* previously reported that a few cases of adenocarcinoma and adenosquamous carcinoma showed an immunohistochemically positive response to their mAb MOC-1, which was subsequently proved to detect the cluster 1 antigen.[22] The possibility remains that a small proportion of non-SCLCs show some features of SCLC, including the cell membrane phenotype. Further studies on a large number of cases are therefore required. Once such non-SCLCs with neuroendocrine differentiation have been identified, it would be very interesting and also important clinically to study how these tumors behave biologically.

The cluster 1 antigen was detected in tissue extracts from all SCLC cases, but the level of the antigen varied from case to case. The results obtained using cultured SCLC cell extracts were similar and the antigen level tended to be lower in the variant type.[12, 13] It is suggested that the histological subtype of SCLC may be correlated with the level of cluster 1 antigen expression.

Since it was possible to detect small amounts of the cluster 1 antigen in spent media of cultured SCLC and in one of three serum samples obtained from nude mice bearing SCLC, the possibility of the cluster 1 antigen being useful as a serum tumor marker in SCLC patients was studied. When the mean plus 2SD of the antigen level in sera of healthy donors was set as a tentative cut-off value, the positive rate for patients with SCLC was 31.3%, and such positive cases were all in the advanced stage. The presence of a small amount of the antigen in sera of normal healthy donors was confirmed by serial dilution analysis. In contrast, the antigen was not detected at all in sera of normal mice, which also lack the antigen in central nervous tissues. Recently, the serum level of NSE has been considered useful for the monitoring of SCLC patients.[5, 26, 27] Although the levels of these two antigens were well correlated, it is obvious that the cluster 1 SCLC antigen is different from NSE from the differences in molecular weight and subcellular distribution. More detailed comparison of the level of the cluster 1 antigen with that of NSE in the same serum samples clearly showed that all the cases positive for serum cluster 1 SCLC antigen were included among those cases positive for serum NSE. In addition, there were some cases negative for serum cluster 1 SCLC antigen and positive for serum NSE. Accordingly, it was concluded that the cluster 1 antigen was not useful as a serum tumor marker. The possibility of utilizing the cluster 1 antigen as a target for mAb-guided therapy for SCLC patients remains to be examined.

The distribution of the cluster 1 SCLC antigen resembles that of N-CAM, a calcium-independent cell-to-cell adhesion molecule of 140–200 kDa reported by McClain and Edelman.[28] However, our three mAbs and their Fab fragments did not inhibit cell adhesion of SCLC cells or induce any morphological change when they were added to culture media of SCLCs (data not shown), although anti-N-CAM Fab′ fragments have been reported to inhibit cell adhesion.[28] The functional roles of this molecule and its gene remain to be clarified.

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