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A Novel Human Monoclonal Antibody Directed to a Tumor-associated Antigen

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Twelve human monoclonal antibodies (HuMAb) were established by the fusion of (mouse × human) heteromyeloma cells with B-lymphoblastoid cells derived from the regional lymph nodes of three patients with squamous cell carcinoma of the lung. They were tested for reactivity to two kinds of proteins (purified protein derivatives and bovine serum albumin) by ELISA, Sq-19 (squamous cell carcinoma) culture cells by indirect membrane immunofluorescence tests, and Sq-19 tumor xenograft by immunohistological study. Among them, one HuMAb 904F (IgM, λ) was selected. In indirect membrane immunofluorescence tests, this 904F antibody reacted with various kinds of cell lines, e.g. lung cancer, esophageal cancer, endometrial cancer, and stomach cancer. It did not react with malignant hematopoietic and diploid fibroblast cell lines. Immunohistologically, it stained the tumor nests of squamous cell carcinoma, adenocarcinoma, and large cell carcinoma of the lung. It also stained those of esophagus and colon, but not those of small cell carcinoma of lung, or stomach. On frozen-section specimens of normal tissues from various organs, it showed only limited areas of positive staining. Limited positive findings were observed at a reticular zone of the adrenal gland, at the esophagus as weak staining, and at islets of the pancreas as very weak staining. Western blotting analysis demonstrated that it recognized a 54 kDa trypsin-sensitive molecule which is expressed on the surface of tumor cells. These results suggest the 904F monoclonal antibody detects a novel tumor-associated antigen which is recognized by the human immune system.

Key words: Human monoclonal antibody — Membrane antigen — Tumor-associated antigen

Recent advances in monoclonal antibody technology have led to improved analysis of human tumor-associated antigens. So far, most monoclonal antibodies raised against tumor-associated antigens have been of murine origin. The antigens recognized by murine monoclonal antibodies have been determined by their immunogenicity in the mouse. They do not necessarily correspond to antigens that are important for humans. Murine monoclonal antibodies have been of limited value in clinical medicine, because they are recognized as foreign proteins by humans, so they are often neutralized and cause allergic reactions.[1-3]

On the other hand, human monoclonal antibodies to tumor-associated antigens would be much more effective when administered to patients with cancer. This paper describes a study of the human monoclonal antibody 904F, which is directed to the surface antigen of various malignant tumor cells and is reactive only to a limited extent with normal tissues.

MATERIALS AND METHODS

Human B cell source The regional lymph nodes of lung cancer (squamous cell carcinoma) were resected at surgery from three patients. A single cell suspension was obtained by mincing each of them. Cell suspension enriched in B lymphocytes was obtained by the removal of the SRBC[6] rosette-forming T cells by Lymphoprep gradient centrifugation.[4)]

EBV-induced lymphoblastoid cells To establish EBV-transformed cell lines (EBV-LCL), the B-enriched cells were suspended at a concentration of 1×10^7/ml in RPMI 1640 plus 10% FBS, and an EBV suspension (1 ml) was added to this cell suspension. The cells were incubated for 90 min at 37°C and then washed once with this

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[6] The abbreviations used are: SRBC, sheep red blood cell; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HAT, hypoxanthine, aminopterine and thymidine; PPD, purified protein derivatives; DMSO, dimethylsulfoxide; LCL, lymphoblastoid cell line; FBS, fetal bovine surum; EBV, Epstein-Barr virus; IMIF, indirect membrane immunofluorescence; PBS, phosphate-buffered saline; PLP, periodate-lysine-paraformaldehyde.

medium. The number of cells was adjusted to 1×10^5/ml with the same medium and 0.2 ml of the cell suspension was put in each well of a 96-well plate (MS-8096F, Sumitomo Bakelite Co., Ltd., Tokyo). The cells were incubated at 37°C for about three weeks in a 5% CO_2 incubator with the medium being changed twice a week.

Antibody screening The culture supernatant in each well was tested for antibody activity to lung cancer cells by IMIF tests and cell-ELISA using Sq-19 and 1-87 cells. Sq-19 is a lung squamous carcinoma cell line and 1-87 is a lung adenocarcinoma cell line.

Cell fusion and hybridoma production Approximately 7×10^5 EBV-LCL cells and an equal number of heteromyeloma cells (SHM-D33)[5] were washed separately with serum-free RPMI 1640 medium three times then mixed together in a 15 ml test tube. The cell fusion was done as described previously using polyethyleneglycol 1000 (cat. #165-09085, Wako Pure Chemical Ind., Ltd., Osaka). The fused cells were suspended in GIT medium (Wako)[6,7] at a concentration of 2.4×10^5 cells/ml and 0.2 ml of the cell suspension was put in each well of a 96-well plate. After overnight cultivation, half of the medium was replaced with GIT-HAT-O selective medium which consisted of GIT medium supplemented with 1/50 volume of concentrated HAT solution (Flow Lab., Irvine, Scotland), ouabain (2×10^{-6} *M*), 2-mercaptoethanol (5×10^{-5}*M*), L-glutamine (2 m*M*) and dihydroxyethylglycine (1.8 mg/ml). The cells were cultured for 5 days in GIT-HAT-O medium and then cultured for hybridoma selection. The hybridoma cells producing antibodies directed to lung cancer cells were cloned under standard conditions by limiting dilution at densities of 1 cell/well three times. Thymocytes from BALB/c mice were used as feeder cells.

IMIF tests Target cells were collected by treatment with 0.02% EDTA-PBS from monolayer cultures. The cells (3×10^5/tube) were incubated with 50 μl of the culture supernatant from either EBV-LCL cells or hybridoma cells for 30 min at 37°C. They were washed three times with PBS plus 0.1% NaN_3, incubated with 50 μl of FITC-conjugated goat anti-human IgG, IgM, and IgA, then diluted with PBS containing 2% BSA and 0.1% NaN_3. The cells were again washed three times and analyzed with either an epi-illumination fluorescence microscope (Zeiss standard 18) or a cytofluorometer (Epics V type).

ELISA The antibody activity to PPD was examined by ELISA as reported previously.[6] For the measurement of the specific antibody activity to BSA, almost the same procedures were followed as in the measurement of anti-PPD antibody activity. The only difference in the two assay systems was that addition of PPD to the plate was omitted in the measurement of anti-BSA activity. Namely, 2% BSA-PBS was used as an antigen for ELISA and as a blocker for the plate. The BSA used in this assay was the Cohn fraction V of bovine serum (A-4503, Sigma, St. Louis, MO).

Cell-ELISA In order to improve the attachment of lung cancer cells to the plate, a 96-well polyvinylchloride plate (soft tray A-221, Biotech Co., Ind., Tokyo) was coated with 50 μg/ml Cell-Tak (BioPolymers, Inc., Farmington, CT). Then, 2×10^4 1-87 cells or Sq-19 cells were put in each well of the plate and incubated at 37°C for 60 min. After blocking of the wells with 2% BSA-PBS, as a test sample, 25 μl of culture supernatant was added to each well and incubation at 37°C was continued for 60 min. Fifty μl of HRP-conjugated goat anti-human IgG, IgM, and IgA (Dako Immunoglobulins a/s) was used as a second antibody. The plate was developed with a substrate of *o*-phenylenediamine and H_2O_2. The reaction was stopped by addition of 4 *N* sulfuric acid as reported previously.[6]

Xenograft Human lung cancer tumor xenografts were developed in female 8-week BALB/c-nu/nu mice by subcutaneous injection of 3×10^6 Sq-19 cells (squamous carcinoma of lung). The resected tumor was processed for either paraffin-section specimens or frozen-section specimens.

Immunohistological study Human tissues from various organs were fixed with neutral formalin, embedded in paraffin and sliced by a microtome. The tissue sections (4 μm thick) were deparaffinized by soaking in xylene and dehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in methanol for 5 min. The sections were washed with PBS and incubated with 0.1% trypsin (Sigma type 1) in 0.05 *M* Tris-HCl (pH 7.6) with 0.1% $CaCl_2$ at room temperature for 60 min. Then the sections were treated with 1% normal goat serum in PBS for 60 min. After washing with PBS, the sections were incubated with biotinylated 904F antibody at 4°C overnight. After further washing with PBS, the sections were incubated with ABC complex solution (Elite Vectastain ABC kit) at room temperature for 30 min. Washing was again done with PBS, and the sections were developed with substrate solution which contained 3,3′-diaminobenzidine (Dotite, Tokyo) and H_2O_2 for about 5 min. Finally, the sections were weakly counterstained with hematoxylin.

In the case of a xenografted tumor, the culture supernatant from different hybridomas was used as the first antibody and biotinylated goat anti-human μ (IgM) was used as the second antibody.

For an immunohistological study on the frozen sections, small pieces of fresh tissues were fixed with PLP solution[8] and rapidly frozen. The frozen tissues were sliced by a cryostat (Bright, Cambridgeshire) and subjected to immunostaining.

Purification of monoclonal antibody and biotin labeling For the concentration of monoclonal antibody 904F (IgM, λ type), the culture supernatant was fractionated by the 70% ammonium sulfate method. Then, the antibody was purified by affinity chromatography using goat anti-human μ antibody bound-FMP activated Cellulofine (Seikagaku Corporation, Tokyo) or a Chromatop protamine column (Nihon Gaishi, Nagoya). The purified 904F antibody was conjugated with biotin by O'Shannessy's method.[9]

Immunoblotting analysis Sq-19 cells (1×10^7 cells) or WI-38 cells (1×10^7 cells, human diploid fibroblast cells from embryonic lung tissue)[10] were suspended in 2 ml of 25 mM Tris-HCl (pH 7.6) containing 1 mM PMSF and sonicated at 30 W for 5 s, 5 times. Then, the cell suspension was centrifuged at 1300 rpm for 10 min. The cell pellet was suspended in 0.5 ml of 8 M urea and kept at room temperature for 30 min. After centrifugation at 1300 rpm for 10 min, the supernatant was subjected to SDS-PAGE (7.5%) under nonreducing conditions. The proteins in the gel were electroblotted to a nitrocellulose membrane and the membrane was blocked with 20 mM Tris-HCl-buffered saline (pH 7.6) (TBS) containing 5% normal goat serum and 3% BSA for 60 min. The membrane strips were washed with TBS containing 0.1% Tween 20 (TBS-T), and incubated with 904F antibody solution for 60 min with constant shaking. After washing with TBS-T, the strips were incubated with biotin-conjugated goat anti-human μ for 30 min. After further washing with TBS-T, the strips were incubated with avidin-biotinylated peroxidase complex solution (Elite Vectastain ABC kit) for 90 min at room temperature. After washing again with TBS-T, the strips were developed with a substrate solution containing 3,3′-diaminobenzidine and H_2O_2 for about 10 min.

Enzymatic treatment of membrane antigens Sq-19 cells (1×10^7) in 0.02% EDTA-containing PBS were treated with either 500 μg/ml trypsin (GIBCO) in PBS containing 2 mM $CaCl_2$ at 37°C for 30 min, or 10 mg/ml mixed glycosidases from *Turbo cornutus* (Seikagaku Corporation) in PBS at 37°C for 30 min with frequent shaking. After washing of the cells with PBS, they were suspended in 200 μl of sample buffer made of 125 mM Tris-HCl (pH 6.8) with 1% SDS, 6 M urea and 10% sucrose, then boiled for 3 min. After centrifugation, the extracted proteins in the supernatant were subjected to SDS-PAGE (7.5%) and electroblotted onto a nitrocellulose membrane. The membrane was immunostained by 904F antibody in the same way as in the previous immunoblotting analysis.

RESULTS

EBV-induced transformation, fusion frequency and initial screening The B-enriched cells infected with EBV, 2×10^4, were put in each well of a 96-well microplate. EBV-induced transformation was observed in 68.4% (242 wells/seeded 354 wells, #1214 lymph node), in 81.2% (353/410, #1208 lymph node), and in 100% (104/104, #904 lymph node) of the wells. The difference in transformation rate among the three lymph nodes depended mainly upon the viability of the lymph node cells. Antibody activity to lung cancer cells tested by IMIF and cell-ELISA was detected in 1.1–2.3% of the transformation-positive wells. Fifteen fusions between SHM-D33 myeloma cells and EBV-lymphoblastoid cells resulted in production of hybridomas in 523 of 566 seeded wells. The antibody activity to lung cancer cells was detected in 112 wells. From 19 wells, after cloning by limiting dilution, twelve monoclonal hybridomas were obtained.

Reactivity pattern of the 12 monoclonal antibodies The human monoclonal antibodies listed in Table I were all of IgM class. The culture supernatants from twelve monoclonal hybridomas were tested for their reactivity to two kinds of proteins (PPD and BSA) by ELISA, Sq-19 culture cells by IMIF tests and Sq-19 xenograft tumor by immunohistological study. Among 12 antibodies, eight antibodies reacted positively in all four tests (Table I). Four antibodies showed different reactivities depending on the tests. 1214C3-HE antibody reacted with PPD, BSA and Sq-19 culture cells, but did not react with Sq-19 xenograft tumor. 1214C3-TO reacted positively with PPD and Sq-19 xenograft tumor, but failed to react with

Table I. Reactivity of Human Monoclonal Antibodies

Antibody	PPD[a]	BSA[a]	Sq-19[b] (culture cells)	Sq-19[c] (nude mouse tumor)
1214 C3-RO	0.460	0.433	35%	+
1214 C3-HA	0.490	0.469	30	+
1214 C3-NI	0.206	0.184	15	+
1214 C3-HO	1.112	0.996	50	+
1214 C3–HE	0.752	0.165	80	−
1214 C3-TO	0.564	0.023	2	+
1214 C3-CHI	0.845	0.745	10	+
1214 C3-RI	1.010	0.665	5	+
1204 C2-I	0.112	0.133	10	+
1204 C2-RO	0.107	0.117	10	+
1204 C2-HO	0.168	0.184	20	+
904F	0.046	0.023	30	+

a) Determined by ELISA (results are expressed as A_{492}).
b) Determined by membrane immunofluorescence test using Sq-19 cells (cultured) as target cells.
c) Tested by immunohistological method using Sq-19 tumor (xenografted to a nude mouse).

BSA or Sq-19 culture cells. 1214C3-RI antibody reacted with PPD, BSA and Sq-19 xenograft tumor, but it reacted very weakly with Sq-19 culture cells. In contrast to these eleven antibodies, 904F antibody reacted with both Sq-19 culture cells and Sq-19 xenograft tumor, but did not react with PPD or BSA. The reactivity pattern of 904F antibody led us to do a further analysis of the antibody specificity. 904F antibody was of IgM, λ type. The hybridoma clone (904F) is stable and continuously secreted more than 5 μg/ml of 904F antibody for >12 months after cloning.

Table II. Reactivity of 904F Human Monoclonal Antibody on Various Cell Lines

Cell lines		Reactivity[a]
Malignant cell lines		
1-87	Adenocarcinoma of lung	+
11-18	Adenocarcinoma of lung	+
A549	Adenocarcinoma of lung	−
Sq-19	Squamous cell ca. of lung	++
86-2	Large cell ca. of lung	−
LK79	Small cell ca. of lung	−
S-1	Small cell ca. of lung	−
TE-1	Squamous cell ca. of esophagus	++
TE-7	Adenocarcinoma of esophagus	+
TE-12	Squamous cell ca.of esophagus	++
HEC-1	Adenocarcinoma of endometrium	+
Kato-III	Signet ring ca. of stomach	−
Az-521	Stomach ca.	+
Mia-paca-2	Pancreas ca.	±
VMRC-MELG	Melanoma	−
HMV-1	Melanoma	−
G361	Melanoma	−
Mewo	Melanoma	−
HT1080	Fibrosarcoma	±
Jurkat	T cell leukemia	−
MOLT-4F	T cell leukemia	−
Raji	Burkitt's lymphoma	−
LICR-LON-HMy2	Myeloma	−
CRL8062	B lymphoblastoid line	−
THP-1	Monocytic leukemia	−
Fibroblast cell lines		
Flow 2000	Embryonal fibroblast	−
WI-38	Embryonal fibroblast	−

a) Reactivity on these cell lines except for LICR-LON-HMy2 and CRL8062 was tested by indirect membrane IF test. LICR-LON-HMy2 and CRL8062 cell lines were tested by direct membrane IF test. Approximately 200 cells were scored for IF reactivity. Generally 1–3% of positive cells was observed as background staining. The reactivity was scored as ++ when more than 30% of cells was stained; + means 10–29% positive cells; ± means 4–9% positive cells.

Reactivity of 904F antibody with various cell lines The reactivity of 904F antibody was examined by IMIF tests (Table II). Two pulmonary adenocarcinoma cell lines (1-87 and 11-18) were reactive with 904F antibody, while another adenocarcinoma, A549, was negative. Pulmonary squamous cell carcinoma, Sq-19, was strongly positive. Pulmonary large cell carcinoma (86-2) and two small cell carcinoma lines (LK79 and S-1) were negative. Two squamous carcinoma cell lines of the esophagus (TE-1 and TE-12) were strongly positive. Adenocarcinoma cell line of the esophagus (TE-7) was moderately positive. Adenocarcinoma of endometrium (HEC-1) and stomach adenocarcinoma (Az-521) were also positive. Fibrosarcoma (HT-1080) and pancreatic carcinoma (Mia-paca-2) were borderline. Signet ring carcinoma cell of the stomach (Kato-III), four melanoma, leukemia, lymphoma, myeloma and B lymphoblast cell lines were all negative. Two diploid fibroblast cell lines were negative. Representative cytofluorographs are shown in Fig. 1.

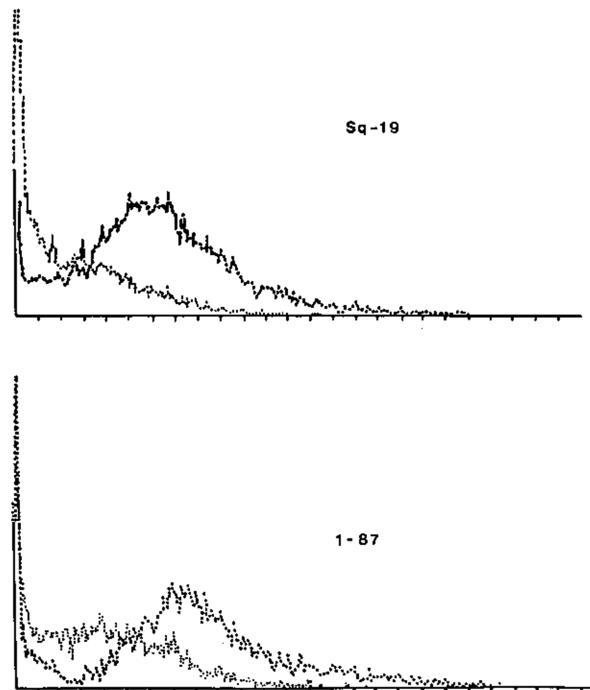

Fig. 1. Representative cytofluorographic determinations of indirect fluorescent cell surface reactivity of human monoclonal antibody 904F on the cell lines Sq-19 and 1-87. Upper panel: The thick line indicates Sq-19 cells stained with 904F antibody and the thin line, Sq-19 cells stained with an isotype-matched control antibody. Lower panel: The thick line indicates 1-87 cells stained with 904F antibody and the thin line, 1-87 cells stained with an isotype-matched control antibody.

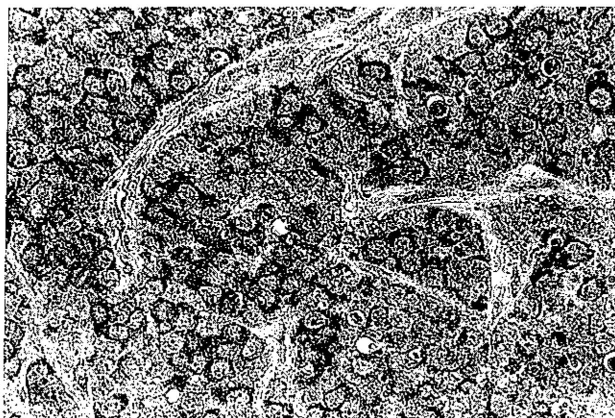

Fig. 2. Immunohistological study of 904F antibody on paraffin section of Sq-19 tumor obtained from nude mouse xenograft.

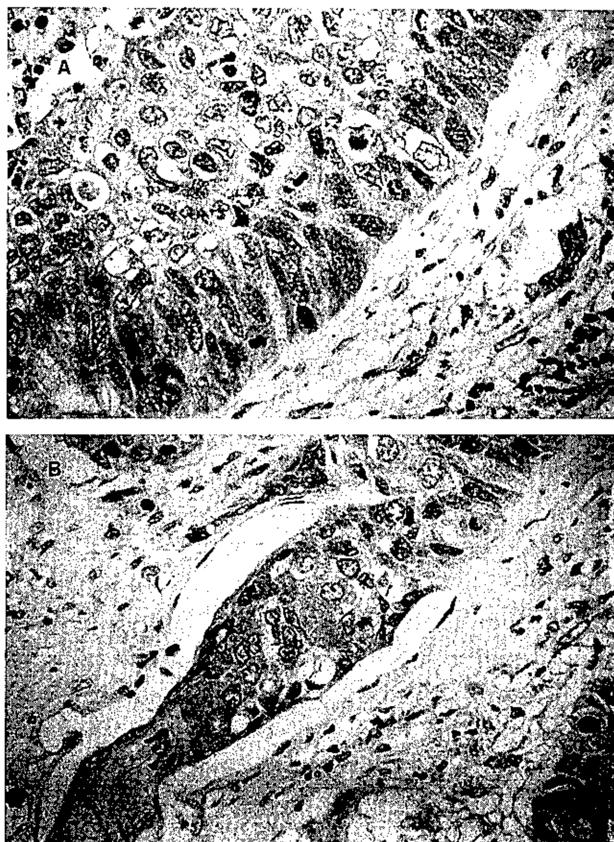

Fig. 3. Immunohistological staining patterns obtained with biotinylated 904F antibody using different (A and B) specimens of squamous cell carcinoma of lung. Positive staining in the immunoperoxidase reaction appears as a diffuse brown coloration in the tumor nests, whereas surrounding normal tissues appear negative.

Table III. Reactivity of 904F Antibody on Paraffin Sections of Normal Tissues

Organs	Immunohistological findings	
	Case 1 (70 years old)	Case 2 (64 years old)
Thyroid gland	NT	Positive granules on follicular epithelium
Lung	Negative	Negative
Esophagus	Negative	Negative
Heart	Negative	NT
Liver	Positive granules on hepatic cells	Positive granules on hepatic cells
Pancreas	Positive granules on pancreatic islets	Positive granules on pancreatic islets
Kidney	Negative	NT
Adrenal gland	Positive granules on reticular zone	NT
Spleen	Negative	Negative
Small intestine	NT	Negative
Large intestine	Negative	NT
Bladder	NT	Trace staining at epithelial cells
Lymph nodes	NT	Trace staining at macrophages

Normal tissues were fixed with neutral formalin and embedded in paraffin. Tissue sections were deparaffinized for immunohistological study as described in "Materials and Methods." NT, not tested.

Normal peripheral blood cells did not react with 904F antibody Reactivity of 904F antibody to peripheral blood cells from a healthy donor was examined by direct membrane IF tests using FITC-conjugated 904F antibody. There was no positive binding of the antibody with mononuclear cells or polymorphonuclear cells. The reactivity of 904F antibody to red blood cells was tested by hemagglutination assay using 904F antibody as a first antibody and goat anti-human μ as a second antibody. However, no hemagglutination was found in the test.

Immunohistological study of 904F antibody Paraffin sections of Sq-19 xenograft tumor were stained with 904F antibody. The antibody apparently stained the tumor region but did not react with the surrounding nude mouse tissues (Fig. 2). A similar staining pattern was obtained with 904F antibody on the frozen sections of Sq-19 xenograft tumor.

The reactivity of 904F antibody was also examined on paraffin sections of various carcinoma tissues which were obtained at autopsy. Fig. 3A and 3B show the immunohistological patterns of two specimens obtained from different patients with squamous cell carcinoma of the lung. Positive stainings were exclusively localized at tumor nests, and no staining was found at the surround-

Table IV. Reactivity of Human Monoclonal Antibody 904F on Frozen Sections of Normal Tissues

Organs	Findings
Adrenal gland	Positive fine granular staining at reticular zone
Liver	Negative
Lung	Negative
Kidney	Negative
Esophagus	Weakly diffuse staining at muscularis mucosa and lamina propria
Pancreas	Trace staining only at islets Other part negative

Normal tissues were cut into small pieces, fixed with PLP solution and rapidly frozen. The frozen tissues were sliced with a cryostat and tested for reactivity with 904F antibody.

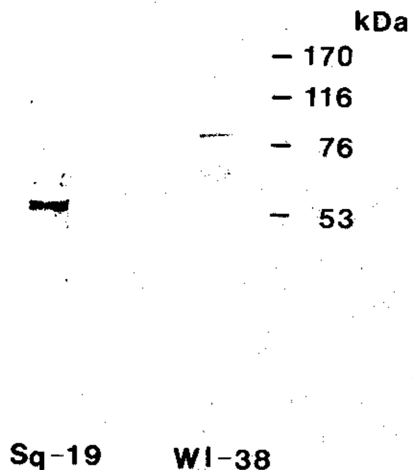

Fig. 5. Western blotting analysis of 904F antigen. Membrane proteins were extracted from either Sq-19 or WI-38 cells by detergent solution, subjected to SDS-PAGE (7.5%) and electroblotted onto a nitrocellulose membrane. The blotted membrane was incubated with 904F antibody, biotin-conjugated anti-human μ, and ABC complex solution, successively. The membrane was developed with 3,3′-diaminobenzidine and H_2O_2 solution.

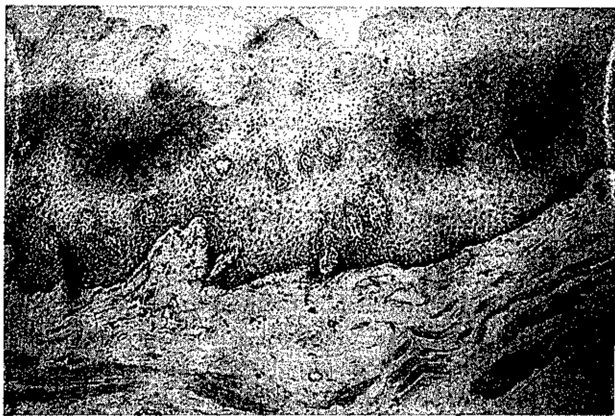

Fig. 4. Immunohistological staining pattern obtained with biotinylated 904F antibody using a frozen-section specimen of normal esophageal tissue. Weak but diffuse positive staining was found at the muscularis mucosa and lamina propria of the esophagus.

ing normal tissues. Similarly, 904F antibody stained the tumor nests of adenocarcinoma (2/2), and large cell carcinoma (1/2) of lung. It also stained those of squamous cell carcinoma of esophagus (2/2) and adenocarcinoma of colon (2/2). It did not stain small cell carcinoma of lung (0/2) or adenocarcinoma of stomach (0/2).

Table III shows the reactivity of 904F antibody on the paraffin sections of normal tissues from various organs. This antibody did not react with normal tissues of the lung. It did not react with normal esophagus, heart, kidney, spleen, small intestine or large intestine tissues. Trace staining was found on epithelial cells of the bladder, and macrophage cells of the lymph nodes. Fine granular stainings were observed at follicular epithelium of the thyroid gland. Similar fine granules were observed on hepatic cells, pancreatic islets and the reticular zone of the adrenal gland. The reactivity of 904F antibody on the frozen sections of normal tissues was examined (Table IV). No positive staining pattern was observed on the specimens from normal lung, and kidney, as was the case on the paraffin sections. No positive staining was observed on a frozen-section specimen from normal liver, though a granular staining pattern was observed on paraffin sections of normal liver.

On the frozen sections of adrenal gland, fine granular staining was observed at the reticular zone, as seen on the paraffin sections. A diffuse and weak staining pattern was found at the muscularis mucosa and lamina propria on the frozen-section specimen from normal esophagus (Fig. 4), while no positive staining was found on the paraffin section of normal esophagus.

Biochemical characterization of 904F antigen molecule In order to determine the molecular weight of 904F antigen, 904F-positive Sq-19 cells and 904F-negative WI-38 cells (human diploid fibroblast) were solubilized. The extracted proteins were subjected to SDS-PAGE and electroblotted onto a membrane. The membrane was

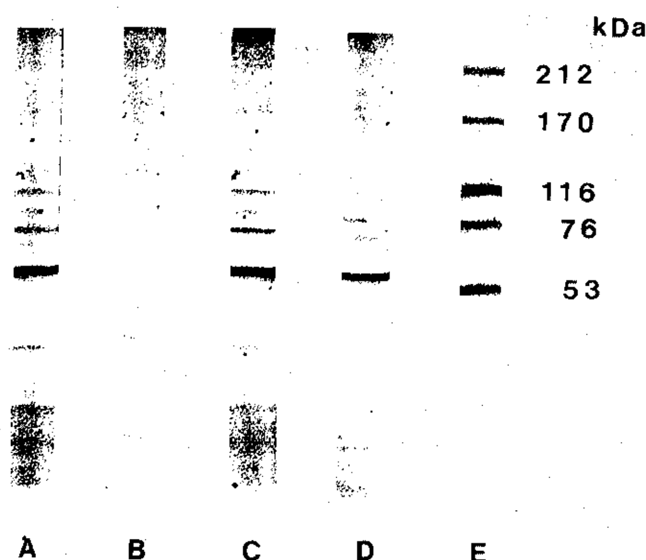

Fig. 6. Effect of enzymatic treatment on 904F antigen. Sq-19 cells were treated with either (A) mixed glycosidases or (B) trypsin. Then, membrane proteins were extracted, subjected to SDS-PAGE, and electroblotted. The blotted membrane was immunostained with 904F antibody in the same way as in Fig. 5. Lanes (C) and (D) show the Western blotting pattern of control cells (not treated with enzymes). Lane (E); molecular weight standards.

immunostained with 904F antibody. An apparent 54 kDa band was found on the lane of Sq-19 cells, while no clear band was found on the lane of WI-38 cells (Fig. 5). Next, Sq-19 cells were treated with different kinds of enzymes for analysis of the 904F molecule. Three kinds of proteins from 1) Sq-19 cells without enzymatic treatment, 2) Sq-19 cells treated with trypsin, and 3) Sq-19 cells treated with mixed glycosidases were subjected to Western blotting using 904F antibody. A clear 54 kDa band was observed on the lanes (C and D) of control Sq-19 cells, and on the lane (A) of Sq-19 cells treated with mixed glycosidases (Fig. 6). On the contrary, no clear band was found in lane (B) of Sq-19 cells treated with trypsin. These results indicate that 904F antigen is a trypsin-sensitive 54 kDa molecule expressed on the surface of tumor cells.

DISCUSSION

In this paper, we report the successful establishment of a human monoclonal antibody 904F, which reacts with surface antigen of tumor cells. The hybridoma 904F was established by cell fusion between EBV-transformed cells derived from a regional lymph node of a lung cancer patient (highly differentiated squamous cell carcinoma) and heteromyeloma cells. This strategy was employed on the assumption that the B lymphocytes of regional lymph nodes were already primed to tumor-associated antigens shed from lung cancer cells. Indeed, we could detect antibody activity to lung cancer cells in the culture supernatant of the lymphoblastoid cells (1.1–2.3% wells) from the regional lymph nodes of lung cancer while we could not detect any antibody activity to lung cancer cells in the culture supernatant of those from normal peripheral blood lymphocytes. Since the cloning of the lymphoblastoid cells by the limiting dilution method is very difficult,[11] we tried to produce human hybridomas. In this respect, our previous efforts[6, 12] to improve the fusion frequency contributed greatly to the success achieved here. In our laboratory, by the same method of cell fusion between EBV-lymphoblastoid cells from an SLE patient and myeloma cells as described in this paper, we have established a very interesting human monoclonal antibody which reacts specifically with immature leukemic T cell lines (Numasaki *et al.*, manuscript in preparation).

Initially, we obtained 12 human monoclonal antibodies by screening for antibodies reactive with lung cancer cells. Next, we constructed an antibody reactivity panel by using 4 antigens to obtain a specific antibody to the lung cancer cells. PPD was chosen as a screening antigen because we had previously found that some lung cancer cells had a common antigen with PPD (unpublished observation). BSA was chosen as a nonspecific control antigen. Sq-19 culture cells were used for the detection of antibody to membrane antigens, and a Sq-19 xenograft tumor was used for the study of immunohistological reactivity. By the use of these 4 tests, we found that most of our monoclonal antibodies were reactive with the 4 antigens (Table I). Among them, the 1214C3-HA antibody reacted with both PPD and Sq-19 cells. When this antibody was absorbed with BCG, the absorbed antibody did not show any reactivity to the Sq-19 xenograft tumor, suggesting the presence of a common antigen between BCG and Sq-19 cells (data not shown). Considering that the main components of PPD and BCG are heat-shock proteins, this preliminary result supports the recent finding that heat shock proteins have a significant biological meaning as a tumor-associated antigen.[13]

Among the 12 antibodies, one antibody (904F) showed specific antibody activity to Sq-19 culture cells in IMIF tests and immunohistological studies using xenografted tumor. In addition to the results listed in Table I, the immunohistological staining patterns obtained with 904F antibody on specimens from lung cancer tissues showed that it reacted with tumor nests, but not with normal tissues (Fig. 3A and 3B). These results prompted us to study intensively the specificity of 904F human monoclonal antibody.

By IMIF tests, we found that 904F antibody was reactive mainly with lung cancer cells. Immunohistologically we found that 904F antibody was also reactive with lung cancer tissues (squamous cell carcinoma, adenocarcinoma, large cell carcinoma). These findings are reasonable, because the B cells used for the monoclonal antibody production were derived from the regional lymph node of a lung cancer patient, and this hybridoma was selected by screening using both 1-87 and Sq-19 lung cancer cells as target cells. It is also interesting that two esophageal cell lines (squamous cell carcinoma) reacted with 904F antibody. Immunohistologically, 904F antibody stained the specimens from esophageal cancer tissues. The reactivity of 904F antibody with tissues from various tumors indicates that the antigenic determinant is expressed on a broad range of cancer cells.

When reactivity of 904F antibody on the normal tissue specimens was examined immunohistologically, we obtained different results depending on the method used for preparation of tissue sections, namely, paraffin sections and frozen sections. The antibody reacted positively with normal liver on paraffin sections, while it did not react with liver on frozen sections. The antibody definitely reacted with islets of pancreas on paraffin sections, but it reacted very weakly with islets of pancreas on frozen sections. On the contrary, weak and diffuse stainings were observed at the muscularis mucosa and lamina propria of normal esophagus on frozen sections, while no positive stainings were obtained at the esophagus on the paraffin sections. In paraffin-embedded tissues fixed with formalin, denaturation of proteins is more or less inevitable. However, in frozen sections treated with PLP-solution, denaturation of proteins is almost negligible. Since the 904F antigen molecule is trypsin-sensitive (Fig. 6), immunohistological results obtained on the frozen sections are considered more reliable than those obtained on the paraffin sections. It follows that 904F antibody has broad reactivity to malignant tumor cells and reacts with a very limited number of normal tissues.

We consider that 904F antibody is a new human monoclonal antibody different from any reported previously[14-38] on the basis of immunohistological reactivity, characteristics of the antigenic molecule and cell surface expression of the antigen. The reactivity of 4G12 antibody[27] which was made by fusion with mediastinal lymphocytes from a patient with squamous cell carcinoma is quite interesting. The 4G12 antibody reacted with various lung tumor tissues. Among 72 malignant tumor tissues, 100% of squamous cell carcinoma (29/29), 70% of adenocarcinoma (14/20) and 38% of large cell carcinoma (3/8) were reactive with 4G12 antibody, while no small cell carcinoma was stained by it (0/8). This reactivity pattern on lung tumor tissues is very similar to that of our 904F antibody. Comparative study between 4G12 antigen and 904F antigen is desirable for analysis of immune recognition in cancer patients.

In conclusion, we have succeeded in producing a new human monoclonal antibody (904F) directed to a novel tumor-associated antigen. Though more detailed immunohistological examinations are necessary, this antibody might be a candidate for clinical application by local administration in the diagnosis and therapy of malignant tumors.

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REFERENCES

1) Levy, R. J. and Miller, R. A. Tumor therapy with monoclonal antibodies. *Fed. Proc.*, **42**, 2650–2656 (1983).
2) Hoover, H. C., Jr. and Hanna, M. G., Jr. Immunotherapy by active specific immunization: clinical applications. *In* "Biologic Therapy of Cancer," ed. V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg, pp. 670–682 (1991). J. B. Lippincott Company, Philadelphia.
3) Schlom, J. Antibodies in cancer therapy: basic principles of monoclonal antibodies. *In* "Biologic Therapy of Cancer," ed. V. T. DeVita, Jr., S. Hellman and S. A. Rosenberg, pp. 464–481 (1991). J. B. Lippincott Company, Philadelphia.
4) Kudo, T. and Tachibana, T. Phenotype characteristics of human B cells studied by Epstein-Barr virus infection. I. Immunoglobulin expression on human lymphoblastoid cells established from various lymphoid cells. *Immunol. Lett.*, **8**, 335–341 (1984).
5) Teng, N. N. H., Lam, K. S., Riera, F. C. and Kaplan, H. S. Construction and testing of mouse-human heteromyelomas for human monoclonal antibody production. *Proc. Natl. Acad. Sci. USA*, **80**, 7308–7312 (1983).
6) Kudo, T., Asao, A. and Tachibana, T. Highly efficient procedure for production of human monoclonal antibodies: establishment of hybrids between Epstein-Barr virus-transformed B lymphocytes and heteromyeloma cells by use of GIT culture medium. *Tohoku J. Exp. Med.*, **154**, 345–355 (1988).
7) Kudo, T., Morishita, R., Suzuki (Seno), R. and Tachibana, T. A great improvement of fusion efficiency in mouse B cell hybridoma production by use of new culture medium, GIT. *Tohoku J. Exp. Med.*, **153**, 55–56 (1987).
8) McLean, I. M. and Nakane, P. K. Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.*, **22**, 1077–1083 (1974).
9) O'Shannessy, D. J., Dobersoen, M. J. and Quarles, R. H.

A novel procedure for labeling immunoglobulins by conjugation to oligosaccharide moieties. *Immunol. Lett.*, **8**, 273–277 (1984).
10) Hayflick, L. and Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.*, **25**, 585–621 (1961).
11) Kozbor, D. and Roder, J. C. The production of monoclonal antibodies from human lymphocytes. *Immunol. Today*, **4**, 72–79 (1983).
12) Kudo, T., Saeki, H. and Tachibana, T. A simple and improved method to generate human hybridomas. *J. Immunol. Methods*, **145**, 119–125 (1991).
13) Konno, A., Sato, N., Yagihashi, A., Torigoe, T., Cho, J., Torimoto, K., Hara, I., Wada, Y., Okubo, M., Takahashi, N. and Kikuchi, K. Heat or stress inducible transformation-associated cell surface antigen on the activated H-*ras* oncogene-transfected rat fibroblast. *Cancer Res.*, **49**, 6578–6582 (1989).
14) Schlom, J., Wunderlich, D. and Teramoto, Y. A. Generation of human monoclonal antibodies reactive with human mammary carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **77**, 6841–6845 (1980).
15) Irie, R., Sze, L. L. and Saxton, R. E. Human antibody to OFA-1, a tumor antigen, produced *in vitro* by Epstein-Barr virus-transformed human B-lymphoid cell lines. *Proc. Natl. Acad. Sci. USA*, **79**, 5666–5670 (1982).
16) Katano, M. and Irie, R. F. Human monoclonal antibody to tumor-associated ganglioside GD2: suppressed growth of human melanoma in nude mice. *Immunol. Lett.*, **8**, 169–174 (1984).
17) Koprowski, H., Herlyn, D., Lubeck, M., DeFreitas, E. and Sears, H. F. Human anti-idiotype antibodies in cancer patients: Is the modulation of the immune response beneficial for the patient? *Proc. Natl. Acad. Sci. USA*, **81**, 216–219 (1984).
18) Haspel, M. V., McCabe, R. P., Pomato, N., Janesch, N. J., Knowlton, J. V., Peters, L. C., Hoover, H. C., Jr. and Hanna, M. G., Jr. Generation of tumor cell-reactive human monoclonal antibodies using peripheral blood lymphocytes from actively immunized colorectal carcinoma patients. *Cancer Res.*, **45**, 3951–3961 (1985).
19) Imam, A., Drushella, M. M., Taylor, C. R. and Tokes, Z. A. Generation and immunohistological characterization of human monoclonal antibodies to mammary carcinoma cells. *Cancer Res.*, **45**, 263–271 (1985).
20) Cote, R. J., Morrissey, D. M., Houghton, A. M., Thomson, T. T., Daly, M. E., Oettgen, H. F. and Old, L. J. Specificity analysis of human monoclonal antibodies reactive with cell surface and intracellular antigens. *Proc. Natl. Acad. Sci. USA*, **83**, 2959–2963 (1986).
21) Hirohashi, S., Clausen, H., Nudelman, E. D., Inoue, H., Shimosato, Y. and Hakomori, S. A human monoclonal antibody directed to blood group i antigen: heterohybridoma between human lymphocytes from regional lymph nodes of a lung cancer patient and mouse myeloma. *J. Immunol.*, **136**, 4163–4168 (1986).
22) Kan-Mitchell, J., Imam, A., Kempf, R. A., Kempf, R. A., Taylor, C. R. and Mitchell, M. S. Human monoclonal antibodies directed against melanoma tumor-associated antigens. *Cancer Res.*, **46**, 2490–2496 (1986).
23) Yoshikawa, K., Ueda, R., Obata, Y., Utsumi, K. R., Notake, K. and Takahashi, T. Human monoclonal antibody reactive to stomach cancer produced by mouse-human hybridoma technique. *Jpn. J. Cancer Res.*, **77**, 1122–1133 (1986).
24) Smith, L. H., Yin, A., Bieber, M. and Teng, N. N. H. Generation of human monoclonal antibodies to cancer-associated antigens using limited numbers of patient lymphocytes. *J. Immunol. Methods*, **105**, 263–273 (1987).
25) Ho, M. K., Geist, C., Murray, J., Kato, K., DeLellis., R., Wolfe, H., Carney, W., Rabin, H. and Durda, P. Distribution and immunochemical characterization of a keratin-like antigen in epithelial tumors using mouse and human monoclonal antibodies. *Cancer Res.*, **48**, 4969–4975 (1988).
26) Kjeldsen, T. B., Rasmussen, B. B., Rose, C. and Zeuthen, J. Human-human hybridomas and human monoclonal antibodies obtained by fusion of lymph node lymphocytes from breast cancer patients. *Cancer Res.*, **48**, 3208–3214 (1988).
27) Saito, H., Uchiyama, K., Nakamura, I., Hiraoka, H., Yamaguchi, Y. and Taniguchi, M. Characterization of a human monoclonal antibody with broad reactivity to malignant tumor cells. *J. Natl. Cancer Inst.*, **80**, 728–734 (1988).
28) Skeletsky, E., Oh, E., Rulot, C., Baird, S. M., Burnett, K. G., Masuho, Y., Astarita, R. W., Haghighi, P., Wolf, P., Collins, H., Starr, R., Carlo, D. J., Rayston, I. and Sobol, R. E. A human monoclonal antibody to cytokeratin intermediate filament antigens derived from a tumor draining lymph node. *Hybridoma*, **7**, 367–376 (1988).
29) Furukawa, K., Yamaguchi, H., Oettgen, H. F., Old, L. J. and Lloyd, K. O. Two human monoclonal antibodies reacting with the major gangliosides of human melanomas and comparison with corresponding mouse monoclonal antibodies. *Cancer Res.*, **49**, 191–196 (1989).
30) Lloyd, K. O. and Old, L. J. Human monoclonal antibodies to glycolipids and other carbohydrate antigens: dissection of the humoral immune response in cancer patients. *Cancer Res.*, **49**, 3445–3451 (1989).
31) Miyake, M., Kohno, N., Nudelman, E. D. and Hakomori, S. Human IgG_3 monoclonal antibody directed to an unbranched repeating type 2 chain (Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→R) which is highly expressed in colonic and hepatocellular carcinoma. *Cancer Res.*, **49**, 5689–5695 (1989).
32) Nozawa, S., Narisawa, S., Kojima, K., Sakayori, M., Iizuka, R., Mochizuki, H., Yamauchi, T., Iwamori, M. and Nagai, Y. Human monoclonal antibody (HMST-1) against lacto-series type 1 chain and expression of the chain in uterine endometrial cancers. *Cancer Res.*, **49**, 6401–6406 (1989).

33) Posner, M. R., Sandos, D. J., Elboim, H. S., Tumber, M. B. and Frackelton, A. R., Jr. Human monoclonal antibodies reactive with human myelomonocytic leukemia cells. *Cancer Res.*, **49**, 1665–1670 (1989).
34) Iizasa, T., Yamaguchi, Y., Tagawa, M., Saito, H., Fujisawa, T., Kato, K. and Taniguchi, M. Establishment of human monoclonal antibody recognizing a new tumor-associated antigen from a patient with small cell lung carcinoma. *Hybridoma*, **9**, 211–219 (1990).
35) Iizasa, T., Yamaguchi, Y., Tagawa, M., Fujisawa, T., Saito, H., Kondo, H., Matsuo, Y., Minowada, J. and Taniguchi, M. Human monoclonal antibody detects a cell surface antigen expressed on hematopoietic malignant cells of lymphoid lineage. *Jpn. J. Cancer Res.*, **82**, 213–218 (1991).
36) Baba, M., Kobayashi, T., Tamaki, Y., Mishima, H., Yagyu, T., Morimoto, H., Monden, T., Shimano, T., Tsuji, Y., Murakami, H. and Mori, T. A human monoclonal antibody derived from axillary lymph nodes of a breast cancer patient reactive to a sulfated glycolipid. *Hybridoma*, **11**, 107–119 (1992).
37) Miyake, M., Taki, T., Kannagi, R. and Hitomi, S. First establishment of a human monoclonal antibody directed to sulfated glycosphingolipids Sm_{4S}-Gal and Sm_{4g}, from a patient with lung cancer. *Cancer Res.*, **52**, 2292–2297 (1992).
38) Yagyu, T., Monden, T., Tamaki, Y., Morimoto, H., Takeda, T., Kobayashi, T., Shimano, T., Murakami, H. and Mori, T. Use of a local immunotherapy as an adjunctive tool for the generation of human monoclonal antibodies from regional lymph nodes of colonic cancer patients. *Jpn. J. Cancer Res.*, **83**, 20–23 (1992).